“Development of a cell-based intraocular delivery system for neurotrophic factors to attenuate retinal ganglion cell loss in a mouse model of glaucoma”

Kai Flachsbarth

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Dissertation

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Dedication:

This work is dedicated to my family and friends, who made me the person I am today.

Publications:

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Jankowiak W., Kruszewski K., Flachsbarth K., Skevas C., Richard G., Rüther K., Braulke T., Bartsch U. Sustained neural stem cell-based intraocular delivery of CNTF attenuates photoreceptor loss in the *nclf* mouse model of neuronal ceroid lipofuscinosis. *Invest Ophthalmol Vis Sci., in revision.*

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Quotation:

Imagination will often carry us to worlds that never were. But without it we go nowhere.

(Cosmos, by Carl Sagan, 1980)

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Abstract

Loss of vison as a result of glaucomatous optic neuropathies is the second leading cause for blindness in industrialized countries. Glaucomatous optic neuropathies are characterized by a progressive degeneration of retinal ganglion cell (RGCs) bodies in the retina and their axons in the optic nerve, ultimately resulting in a disruption of signal transduction from the eye to the brain. The progressive loss of retinal ganglion cells results in localized visual field defects, and eventually in complete blindness. Clinically, the major risk factor for glaucoma is elevated intraocular pressure (IOP), and lowering IOP is currently the only proven treatment for glaucoma. However, the diseases progresses in a significant proportion of patients despite successful lowering of IOP. Furthermore, glaucoma may develop in patients despite a normal IOP. Thus, there is a need for alternative treatments of this neurodegenerative disorder of the inner retina. The aim of the present thesis was to establish a neural stem cell-based delivery system that allows a continuous and long-lasting intraocular supply of neurotrophic factors, with the ultimate aim to attenuate the loss of RGCs in a mouse model of glaucoma.

In the present thesis, adherently cultivated neural stem (NS) cells from the cerebral cortex of embryonic mice were used as cellular vectors to administer neurotrophic factors to the murine retina. NS cells maintained under adherent culture conditions comprise a homeogenous population of clonogenic, symmetrically dividing tripotent stem cells. We then used polycistronic lentiviral vectors to stably co-express different neurotrophic factors together with a fluorescent reporter protein and a resistance gene in NS cells. To establish and evaluate this cell-based neuroprotective approach, we expressed three different neurotrophic factors with a known neuroprotective activity on RGCs, ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) in NS cells. We then took advantage from the fact that expression levels of the neurotrophic factors and the fluorescent reporter proteins from the polycistronic lentiviral vectors are proportional to each other, and selected single cells with high expression levels of the reporter gene using fluorescent activated cell

sorting. Subsequent clonal expansion of these cells resulted in clonal NS cell lines with high expression levels of CNTF, GDNF or BDNF.

To analyze the neuroprotective potential of the modified NS cell lines *in vivo*, degeneration of RGCs was induced in adult mice by an intraorbital crush of the optic nerve and cells were intravitreally grafted one day after the lesion. All three clonal cell lines attached to the posterior pole of the lenses and the vitreal surface of the retinas, preferentially differentiated into astrocytes and survived for up to four months in the host eyes. Furthermore, the donor cells stably expressed the neurotrophic factors and fluorescent reporter proteins. Adverse effects of the donor cells on the morphology of the host retinas were not observed. Importantly, all three NS cell lines significantly attenuated the lesion-induced degeneration of RGCs over a time period of at least two (for the GDNF- and BDNF-expressing cell line) or four months (for the CNTF-expressing cell line; longer post-lesion intervals were not analyzed). The CNTF-expressing NS cell line additionally stimulated long distance regeneration of the lesioned RGC axons. Of note, neuroprotection of axotomized RGCs was markedly enhanced after transplantation of a mixture of the GDNF- and CNTF-expressing cell line when compared to transplantations of each individual clonal cell lines. Quantitative analyses of these data revealed a significant synergistic neuroprotective activity of GDNF and CNTF on axotomized RGCs.

Together, data of the present thesis indicate that genetically modified NS cell lines may serve as valuable tools to evaluate the therapeutic potential of a sustained cell-based intraocular administration of neurotrophic factors in animal models of glaucoma. Clonal NS cell lines with a forced expression of neurotrophic factors may also be a useful tool for combinatorial neuroprotective approaches aimed at identifying combinations of neurotrophic factors with additive or synergistic neuroprotective effects on RGCs.

Kurzfassung

Das Glaukom ist die zweithäufigste Ursache für Blindheit in industrialisierten Ländern. Charakteristisch für glaukomatöse Optikusatrophie ist die progressive Degeneration der Zelkörper retinaler Ganglienzellen (RGC) in der Retina und ihrer Axone im optischen Nerven, was zu einer Unterbrechung der Signaltransduktion zwischen Auge und Gehirn führt. Der fortschreitende Verlust der RGC resultiert in lokalen Gesichtsfeld-Einschränkungen, welche schließlich zu vollständiger Blindheit führen. Der, aus klinischer Sicht, wichtigste Risikofaktor für eine Glaukomerkrankung ist ein erhöhter Augeninnendruck und eine Reduktion des Augeninnendrucks ist die zurzeit einzig bekannte Therapieoption. In einem signifikanten Teil der Patienten schreitet die Krankheit jedoch trotz einer erfolgreichen Senkung des Augeninnendrucks weiter fort, während andere Patienten auch ohne erhöhten Augeninnendruck an einem Glaukom erkranken. Es ist also notwendig für diese neurodegenerative Erkrankung der inneren Retina alternative Therapiemethoden zu entwickeln. Das Ziel der vorliegenden Arbeit war die Etablierung eines auf neuralen Stammzellen (NSC) basierenden Applikationssystems, welches eine kontinuierliche und dauerhafte Versorgung mit neurotrophen Faktoren ermöglicht, mit dem Ziel dem Verlust von RGC in einem Glaukom-Mausmodell entgegen zu wirken. In der vorliegenden Arbeit wurden adhärent kultivierte NSC aus dem zerebralen Kortex von Mausembryonen als zelluläre Vektoren genutzt um neurotrophe Faktoren der murinen Retina zuzuführen. NSC die unter adhärenten Kulturbedingungen gehalten wurden, bildeten eine homogene Population aus klonogenen, tripotenten Stammzellen, welche sich symmetrisch teilen. Diese NSC wurden dann mit einem polycistronischen lentiviralen Vektor transduziert um eine stabile Ko-Expression verschiedener neurotropher Faktoren zusammen mit fluoreszierenden Reporter-Proteinen in diesen Zellen zu erreichen. Um dieses Zell-basierte System zu etablieren und zu untersuchen wurden drei verschiedene neurotrophe Faktoren verwendet, welche bereits bekannt waren für ihre neuroprotektive Aktivität im Zusammenhang mit RGC, um diese in unseren NSC zu exprimieren. Diese drei Faktoren waren der „ciliary neurotrophic factor“ (CNTF), der „glial cell line-derived neurotrophic

factor“ (GDNF) und der „brain-derived neurotrophic factor“ (BDNF). Da die Expressionsstärke der neurotrophen Faktoren und der fluoreszierenden Reporterproteine proportional zueinander stehen, konnten anschließend Zellen mit hoher Expression des Reportergens durch „fluorescent activated cell sorting“ (FACS) ausgewählt und vereinzelt werden. Diese Einzelzellen wurden dann genutzt um durch klonale Expansion klonale NSC-Linien mit hoher Expression von CNTF, GDNF und BDNF zu produzieren. Um das neuroprotektive Potential der modifizierten NSC *in vivo* zu analysieren, wurde die Degeneration von RGC in adulten Mäusen durch eine Quetschung des optischen Nervens induziert und die Zellen einen Tag nach der Läsion intravitreal transplantiert. Alle drei klonalen Zell-Linien adhärierten an der posterioren Seite der Linse oder an der vitrealen Seite der Retina, wo sie präferentiell in Astrozyten differenzierten und bis zu vier Monate überlebten. Weiterhin zeigten die transplantierten Zellen eine stabile Expression der neurotrophen Faktoren und der Reporterproteine über die gesamte Versuchsdauer hinweg. Negative Effekte der transplantierten Zellen auf die Morphologie der Empfängerretinas wurden nicht beobachtet. Alle drei Zell-Linien waren in der Lage die induzierte Degeneration von RGC über einen Zeitraum von zwei (im Fall der GDNF- und BDNF-Linie) oder vier Monaten (im Falle der CNTF-Linie; längere Versuchszeiträume wurde nicht untersucht) signifikant zu reduzieren. Die CNTF-exprimierende NSC-Linie stimulierte überdies eine weitreichende Regeneration der lädierten Axone. Die Neuroprotektion axotomierter RGC erhöhte sich merklich nach der Transplantation einer Mischung der GDNF- und CNTF-Linie im Vergleich zu Transplantationen der einzelnen klonalen Zell-Linien. Die quantitative Analyse dieser Daten zeigte eine signifikante synergistische neuroprotektive Aktivität von GDNF und CNTF auf axotomierte RGC. Zusammengefasst deuten die Daten der vorliegenden Arbeit darauf hin, dass genetisch modifizierte NSC-Linien als hilfreiches Werkzeug fungieren können um das therapeutische Potential einer dauerhaften Zell-basierten intraokulären Applikation von neurotrophen Faktoren in Glaukom-Tiermodellen zu untersuchen. Klonale NSC-Linien mit einer Überexpression neurotropher Faktoren könnte weiterhin nützlich sein um Kombinationen von neurotrophen Faktoren zu identifizieren, welche additive oder synergistische neuroprotektive Effecte auf RGC zeigen.

I. Introduction and Summary

1. Establishment of a stem cell-based delivery system of neurotrophic factors to protect retinal ganglion cells from apoptosis in glaucoma

The thesis was aimed at developing a sustained neural stem cell-based intraocular delivery system for neurotrophic factors to protect retinal ganglion cells from apoptotic degeneration in a mouse model of glaucoma. The following introduction is intended to summarize the scientific progress in stem cell biology throughout recent years, which ultimately may lead to the development of clinically relevant cell-based therapies for degenerative retinal disorders.

To better understand the mechanisms that may lead to vision loss in glaucomatous optic neuropathies, one has to understand the processes involved in normal vision and the different cell types that are participants in these processes. The retinal tissue, a part of the central nervous system, makes up the part of our eyes that enables us to perceive light and consequently to see our environment. The mammalian retina contains six different neuronal cell types: the ganglion cells located on the vitreal side of the retina in the ganglion cell layer, the amacrine, bipolar and horizontal cells which are co-located with the supporting cells of the retina, the müller glia, in the inner nuclear layer of the retina, and finally the two types of photoreceptor cells located in the outer nuclear layer, the rods (responsible for scotopic vision) and the cones (responsible for photopic and color vision) (Karl 2013). If light hits the eye, it has to pass through the retinal tissue, until it reaches the photoreceptor cells. Photoreceptor cells then transduce the visual stimulus into an electrical signal, which is then transmitted back through the inner retina via the synaptically interconnected amacrine, bipolar and horizontal cells until it finally reaches the ganglion cell layer. The ganglion cells transmit the signal through their axons, which form the optic nerve, towards the visual centers of the brain. It is therefore obvious that damage to one of the mentioned cells will result in disruption of the signal cascade and as a consequence in visual impairment or even blindness (Gaillard & Sauve 2007) (Fig. 1).

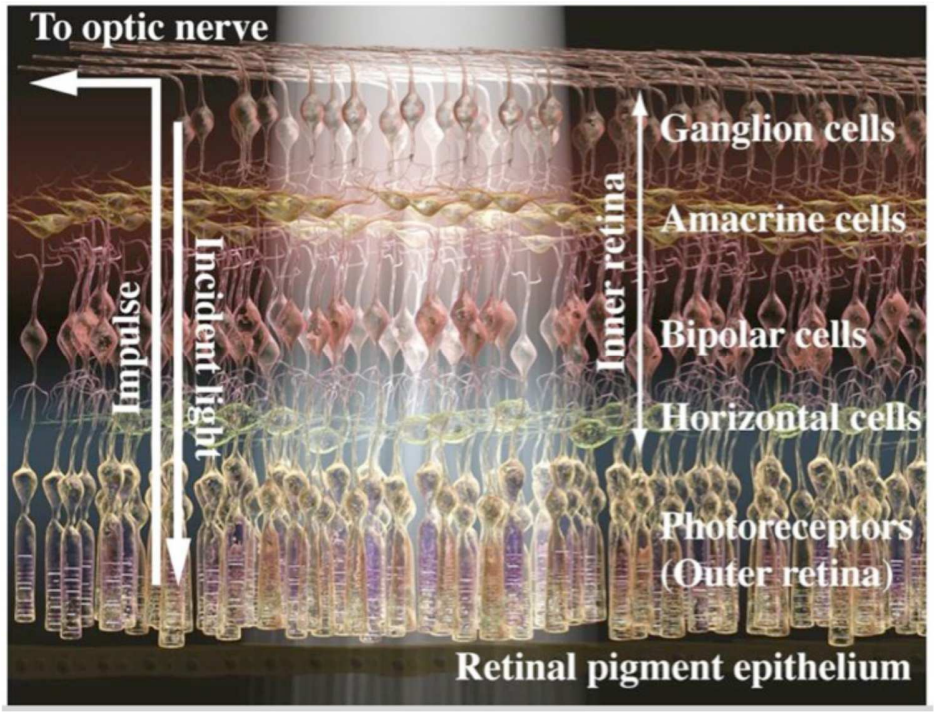

Figure 1: Section of a human retina, displaying all relevant cell types. The retinal ganglion cells are located in the ganglion cell layer at the vitreal side of the retina. The ganglion cell axons form the optic nerve which connects the retina with the brain. Ganglion cells extend dendrites into the inner plexiform layer, in which they interlace with dendrites from the cells of the inner nuclear layer, the amacrine, bipolar and horizontal cells. These cells in return extend dendrites into the outer plexiform layer, where they connect to the rod and cone photoreceptor (PR) cells, which form the outer nuclear layer and are closely associated with the retinal pigment epithelium (RPE). The RPE consists of a mononlayer of cells that is interconnected through tight junctions and separates the retina from the choroid, which provides nutrition to the RPE and PRs. Note that light has to pass through the entire laminated structure of the retina to reach the PR cells, where the visual stimulus is transduced into an electric signal, which is then propagated through the whole retina and the optic nerve towards the visual centers of the brain (from Singh & MacLaren 2011).

Glaucomatous optic neuropathies are characterized by the progressive degeneration of retinal ganglion cells in the retina and their axons in the optic nerve, and are among the retinal disorders that may result in visual impairment or complete blindness. Glaucoma or open angle glaucoma (OAG) affects about 45 million people worldwide, with about 4.47 million patients becoming blind as a result of this neurodegenerative disease of the inner retina. Recent calculations predict an increase in the number of glaucoma patients to about 59 million in 2020, which would be equivalent to 2.1% of the world population aged above 40. If one additionally takes into account patients that suffer from angle closure glaucoma (ACG), this number further increases to 80 million affected patients, or 2.86% of the world population aged above 40. Furthermore, it is estimated that about 11.1 million patients with OAG or ACG will lose their sight bilaterally during the progression of

the disease. These numbers establish glaucoma as the second most frequent disorder causing vision impairment worldwide (Quigley & Broman 2006), and as one of the leading causes for blindness in industrialized countries (Gaillard & Sauve 2007).

While the pathomechanism leading to the progressive loss of ganglion cells in glaucomatous optic neuropathies are only poorly understood, several risk factors have been implicated in the development of this mulitfactorial and age-related neurodegenerative disease. The major and clinically proven risk factor of glaucoma is an elevated intraocular pressure (IOP) (Agarwal et al 2009). Other studies have shown that a decreased thickness of the central cornea correlates with a higher probability to develop visual field defects (Brandt 2004, Brandt et al 2008, Medeiros et al 2003). Reduced ocular blood flow velocities have been identified as another risk factor to develop glaucoma (Butt et al 1995, Kaiser et al 1997, Zeitz et al 2006). Increased levels of oxygen free radicals (OFR) found in glaucomatous eyes have also been implicated in RGC degeneration, since OFR are known to induce the release of cytochrome c, which in turn plays an important role in pro-apoptotic cascades (Raha & Robinson 2001). Other factors implicated in glaucoma include mitochondrial dysfunction, glutamate mediated excitotoxic damage or excitotoxic damage caused by activated glial cells, and nitric oxide mediated toxicity or oxidative stress (Agarwal et al 2009, Almasieh et al 2012, Qu et al 2010). Deprivation from target-derived neurotrophic factors as a result of an impaired axonal transport in retinal ganglion cells is discussed as another pathomechanism resulting in progressive apoptotic degeneration of ganglion cells (Fig. 2).

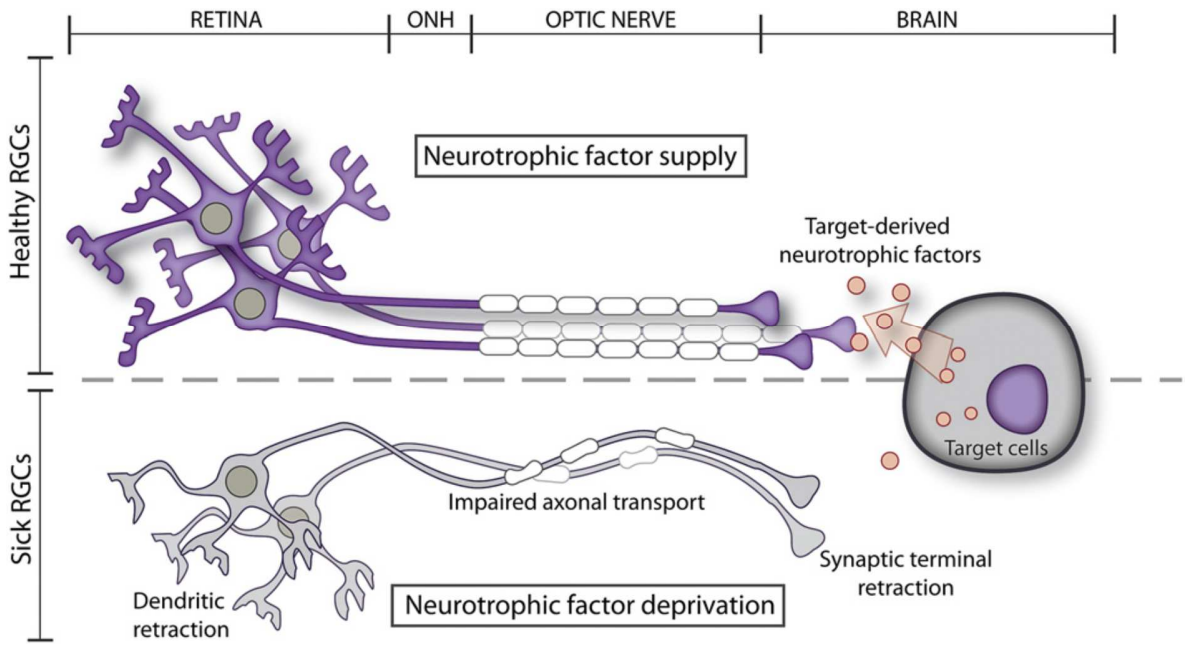

Figure 2: Neurotrophic factor deprivation due to axonal transport failure leads to apoptotic RGC death. Healthy neurons with functioning axonal transport are able to obtain their needed amounts of target-derived neurotrophic factors, whereas RGCs in glaucomatous eyes show an obstructed axonal transport, which leads to neurotrophic deprivation and ultimately apoptotic cell death (from Almasieh et al 2012).

Neurotrophic factor deprivation in ganglion cells may be either the result of an impaired retrograde transport of target-derived neurotrophic factors through the optic nerve to the ganglion cell bodies (Anderson & Hendrickson 1974, Pease et al 2000, Quigley et al 2000), or of reduced expression levels of these factors in glaucomatous retinas as a result of ocular hypertension (Rudzinski et al 2004). Regardless of the reason for the slowly progressing degeneration of RGCs during glaucoma, the cells ultimately die through apoptosis, which in turn disrupts the propagation of visual information from the retina towards the visual centers of the brain, resulting first in visual field defects and ultimately eventually in complete blindness. A problem associated with this slowly progressing neurodegenerative disorder is the fact, that glaucoma is often diagnosed at advanced stages of the disease when a large proportion of ganglion cells is irreversibly lost (Almasieh et al 2012, Qu et al 2010).

Degeneration of ganglion cells in glaucoma may be induced through the intrinsic or the extrinsic apoptotic pathway. The intrinsic apoptotic pathway is activated by a variety of stress signals such as neurotrophin deprivation or oxidative stress. These stress signals activate the apoptosis signal regulating kinase 1 (ASK1), which in turn

activates the pro-apoptotic MAPKs c-jun n-terminal kinase (JNK) and p38. The JNK kinase can then phosphorylate the transcription factor c-jun, which in turn mediates the expression of pro-apoptotic genes, such as the pro-apoptotic member of the Bcl-2 family, Bax. When the concentration of Bax protein inside the mitochondria is higher than the protein concentrations of anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-$_{XL}$, the mitochondrial membrane becomes more permeable and a variety of cell death mediators are released. Of these cell death mediators, cytochrome c is among the first being released, and it binds to the apoptotic protease-activating factor-1 (Apaf-1) as soon as it reaches the cytoplasm. The Cytochrome c and Apaf-1 complex recruits procaspase-9 to form the apoptosome. Inside the apoptosome, procaspase-9 is processed into activated caspase-9, which then facilitates the activation of caspase-3 and -7, which in turn activate other caspases that digest the cellular contents and cause the death of the apoptotic cell (Almasieh et al 2012, Qu et al 2010). The extrinsic pathway is mediated by the death receptors Fas receptor (FasR) or tumor-necrosis factor receptor (TNFR) and their ligands Fas ligand (FasL) and tumor-necrosis factor-α (TNFα). When the ligands bind their respective receptors located on the cytoplasmic membrane, the receptors are activated which results in the recruitment of the intracellular adaptor Fas-associated death domain (FADD). FADD in turn recruits and forms a cluster with procaspase-8 in which procaspase-8 is cleaved and activated. The activated caspase-8 can then activate capase-3 and -7, converging in the same cell death cascade as the intrinsic apoptotic pathway (Almasieh et al 2012, Qu et al 2010).

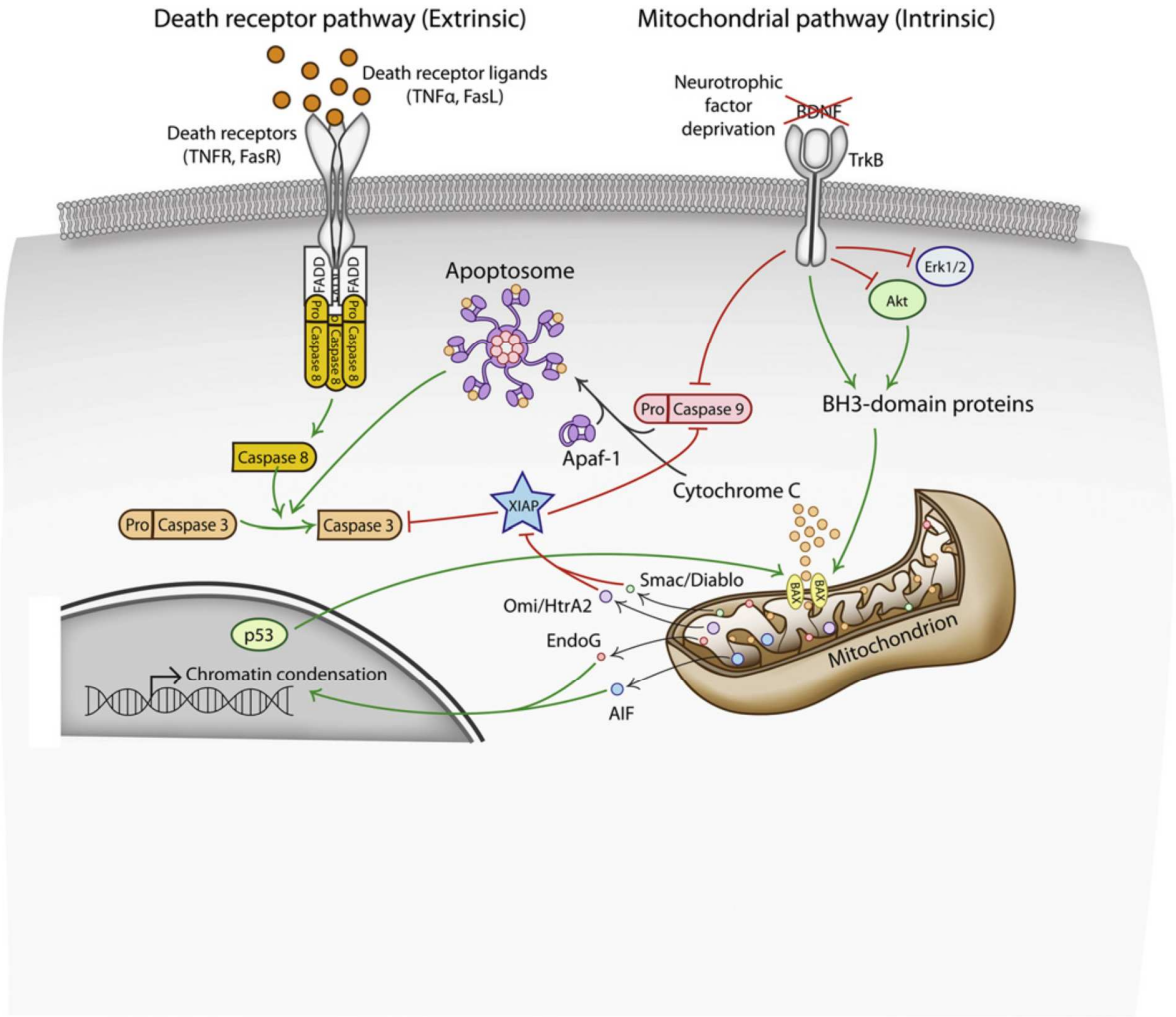

Figure 3: Schematic presentation of the intrinsic and extrinsic apoptotic pathways. The intrinsic pathway may be triggered by stress signals like neurotrophic factor deprivation, after wich proapoptotic members of the Bcl-2 family, such as Bax are activated, resulting in the release of mitochondrial cytochrome C. Cytochrome C forms the apoptosome together with Apaf-1 and procaspase-9, which triggers a cascade that results in the activation of caspase-3. The extrinsic pathway is activated by death receptors such as TNFR or FasR, which recruit procaspase-8 with the help of FADD to generate active caspase-8 which in turn activates caspase-3 (from Almasieh et al 2012).

To investigate the pathomechanisms of glaucoma and to develop novel therapeutic treatment for the disease, several different animal models that differ in the severity of RGC degeneration, onset of degeneration and the speed of ganglion cell loss have been established. One of the most frequently used experimental models of glaucoma is the optic nerve crush (ONC) model. An optic nerve crush results in an axotomy of all RGCs by applying pressure over a short period of time via small watchmaker's forceps to the optic nerve (Allcutt et al 1984, Parrilla-Reverter et al 2009). The ONC approach has several advantages compared to other experimentally induced glaucoma models, as it is relatively easy to perform without the need of special equipment. To avoid damage to

ocular blood vessels during the nerve crush, some studies perform a complex operation before the ONC, in which the conjunctiva is incised and extraocular muscles are separated from the eye to better visualize the optic nerve and control the crush (Gabriele et al 2011, Miotke et al 2007). The RGC degeneration induced by an optic nerve crush is extremely fast, with about 80 – 90% of the cells being lost 14 days after the lesion (Berkelaar et al 1994). Although this rapidly progressing lesion-induced ganglion cell degeneration does not mimic the slowly progressing pathology in glaucoma patients, the ONC approach is widely accepted as a useful model for basic research. A similar approach to induced ganglion cell degeneration in animal models is the optic nerve transection (ONT) model. The ONT differs from the ONC in that the nerve is exposed and completely transected, ruling out that some RGC axons might be spared by the lesion as it may occur in the ONC approach. One disadvantage of this method is the complex and time-consuming operation that has to be performed (Koeberle & Ball 1998, Koeberle & Ball 2002, Mey & Thanos 1993, Nadal-Nicolas et al 2009). Compared to animal models with a lesion-induced degeneration of ganglion cells, the pathology of the disease is more closely mimicked in animal models with an elevated IOP. An elevated IOP can be acutely induced by an obstruction of the ocular outflow of vitreous fluid through laser-photocoagulation of the episcleral and limbal veins and the trabecular meshwork (Aihara et al 2003, Ji et al 2005, Levkovitch-Verbin et al 2002). Other methods to disrupt the outflow of vitreous fluid include the occlusion of the veins and the trabecular meshwork through injections of hyperosmotic saline solutions (Johnson et al 1996, Morrison et al 1997), polystyrene (Frankfort et al 2013), or ferromagnetic microbeads (Samsel et al 2011). Another animal model of glaucoma in which the degeneration of RGCs progresses at a slower rate than in the ONC or ONT model is the myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis, in which recombinant MOG together with heat-inactivated mycobacterium tuberculosis induces a optic neuritis, that leads to axonal degeneration and apoptotic death of ganglion cells (Maier et al 2004). Finally there are several genetic mouse models of glaucoma which differ in the onset and speed of ganglion cell loss (Jakobs et al 2005, John et al 1998, Senatorov et al 2006).

The only clinically proven treatment of glaucoma available today is a lowering of the elevated IOP which might prevent disease progression (2000, Kass et al 2002). Lowering of elevated IOP can be achieved either by application of pharmaceutical agents or through surgery. The treatment of an enhanced intraocular pressure in glaucomatous eyes via pharmaceutical means allows for a wide variety of therapeutic approaches. For example, application of brinzolamide, a thienothiazine sulfonamide, leads to a significant decrease of IOP in a model of argon laser trabeculoplasty-induced hypertension in cynomolgus monkeys and in a spontaneous developing ocular hypertension model in Dutch-belted pigmented rabbits (DeSantis 2000). Latanoprost, an analogue of the prostaglandin $F_{2\alpha}$, is able to reduce IOP by increasing the uveoscleral outflow of vitreous fluid in monkey and human eyes (Nordmann et al 2000, Stjernschantz et al 1995, Toris et al 1993, Watson 1998), similar as another $F_{2\alpha}$ analogue, travoprost (Schallenberg et al 2012) or the selective α-adrenergic antagonist bunazosin hydrochloride (Zhan et al 1998). Surgical methods to reduce elevated IOP include the trabeculectomy, in which the sclera and conjunctiva are opened to excise portions of the trabecular meshwork and to enhance vitreous fluid outflow (Cairns 1968, Membrey et al 2001, Watson & Barnett 1975), or the implantation of aqueous drainage devices (Schwartz et al 2006). However, although elevated IOP is considered as the major risk factor for glaucoma, and lowering the IOP is the only proven treatment of this disease, glaucoma progresses in a significant proportion of patients despite successful IOP reduction. Furthermore, some patients develop glaucoma without ever showing a clinically significant elevated IOP (Caprioli 1997, Leske et al 2003).

Therapeutic approaches like electronic retinal devices (Chader et al 2009) or cell replacement therapies (MacLaren et al 2006b) envisioned for restoration of visual function after photoreceptor cell loss in patients with retinitis pigmentosa, age-related macular degeneration or diabetic retinopathy, are unlikely to be effective in glaucoma. Electronic retinal devices functionally substitute for lost photoreceptors by transforming light stimuli into electrical signals, which are then transferred to other retinal cells and finally propagated via the retinal ganglion cells towards the brain (Chader et al 2009, Humayun 2001). However, ganglion cells are lost in glaucoma. Intracortical microstimulation with electronic devices may circumvent the problem of the interrupted

connections between retina and visual brain centers (Schmidt et al 1996). Cell replacement strategies have shown promising results for the treatment of degenerative retinal disorders characterized by the loss of photoreceptor cells or retinal pigment epithelial cells. However, also this approach is unlikely effective in glaucoma for several reasons. The transplanted cells would not only have to integrate into the retina and to differentiate into functional ganglion cells, but additionally would have to regenerate their axons through the optic nerve to the visual centers in the brain where they would have to form synaptic connections in a topographically appropriate manner (Almasieh et al 2012, Harvey et al 2006, Pascale et al 2012, Qu et al 2010, Wilson & Di Polo 2012). Because of the reasons outlined above, neuroprotection of existing endogenous ganglion cells is currently probably the most promising treatment option for glaucoma. Among the neuroprotective approaches that have been demonstrated to be effective in animal models is the expression of anti-apoptotic factors in ganglion cells, such as BCL-2. Analyses of transgenic mice overexpressing BCL-2, for instance, have demontrated a significant and long-lasting protective effect on axotomized RGCs (Bonfanti et al 1996, Cenni et al 1996, Leaver et al 2006a). Similar results were obtained, when pro-apoptotic factors were functionally blocked through the administration of their respective antagonists. Application of antibodies against semaphorine, a pro-apoptotic factor (Shirvan et al 2002), or treatment with aurintricarboxylic acid (ATA), an antagonist of endonucleases which play a prominent role in the process of apoptosis, have been shown to result in attenuation of RGC loss (Heiduschka & Thanos 2000) in rats with transsected optic nerves. Experimental studies regarding pharmacological reagents no longer aim only at reducing the enhanced IOP of glaucomatous eyes, but try to actively prevent or attenuate the damage done during the disease progression (Foureaux et al 2013, Horsley & Kahook 2010, Moraczewski et al 2009). Contrary to cell replacement strategies, cell transplantation strategies try not to replace already lost cells, but to prevent or reduce cell degeneration by introducing autologous or allogenic cells into the afflicted eye. Several studies have demonstrated that intraocular transplantations of a variety of different cell types might also result in attenuation of ganglion cell loss in various animal models of glaucoma. Mesenchymal stem cells (MSCs) are the most frequently used cells in these experiments, and different types of MSCs have been shown to delay RGC degeneration. Bone-marrow MSCs, for instance, have been

reported to slow down RGC loss by approximately 25% in an ischemia/reperfusion rat model for up to 4 weeks. In a rat model of ocular hypertension, intravitreally injected bone-marrow derived MSCs enhanced RGC survival by 27% after 4 weeks when compared to control animals that received intravitreal injections of dead MSCs. These protective effects are probably mediated through the secretion of endogenously expressed neurotrophic factors in the grafted MSCs, or the induction of neurotrophic factor expression in ocular cells through the grafted cells (Johnson et al 2010, Li et al 2009). When rat or human bone marrow-derived MSCs were induced to secrete elevated levels of neurotrophic factors and then grafted into eyes of rats with an optic nerve transection, the number of surviving RGCs was between 13% (rat MSCs) and 26% (human MSC) higher than in control animals (Levkovitch-Verbin et al 2010). Another cell type exerting potent neuroprotective effects on RGCs is the olfactory ensheathing cell (OEC). *In vitro,* robust survival of RGCs was observed in retinal explant cultures that were co-cultured with either bulbar OECs or mucosal OECs for up to 14 days, whereas retinal explants in control experiments were already strongly depleted of RGCs after a 10 days culture period (Dai et al 2010). *In vivo* experiments in which OECs were grafted onto the optic nerve stump of rats after optic nerve transection revealed the presence of 24% more surviving RGCs than in control animals 7 days after the nerve transection. In close analogy to the experiments with MSCs, the protective effects of OECs were attributed to the secretion of endogenously expressed neurotrophic factors or the induction of neurotrophic factor expression in endogenous ocular cells, since the levels of BDNF mRNA were significantly higher in the optic nerve stump and retina from eyes transplanted with OECs than in control retinas (Wu et al 2010). Transplantations of OECs into rat eyes with an elevated IOP induced by the injection of magnetic microspheres also resulted in a significant rescue of RGCs (Dai et al 2012). Also noteworthy as a neuroprotective cell type is the Schwann cell of the peripheral nervous system. Purified RGCs that have been cultured in Schwann cell-conditioned medium showed an enhanced neuritogenic response, which has been related to the secretion of osteonectin from the Schwann cells (Bampton et al 2005). Furthermore, when transplanted into rats with a transected optic nerve, Schwann cells were able to rescue about 3 times as many RGCs as were found in control animals (Li et al 2004). Other cell types that confer neuroprotection to RGCs include oligodendrocyte precursor cells and dental pulp stem

cells. Oligodendrocytes precursor cells protected RGCs after being stimulated by the application of zymosan in a rat glaucoma model of ocular hypertension, leading to 20% higher survival in experimental eyes than in control eyes 4 weeks after disease induction (Bull et al 2009). Dental pulp stem cells were able to protect RGCs in an *in vitro* co-culture experiment over 4 days, with about 3 times as many surviving RGCs then in the corresponding control experiment. Transplantations of dental pulp stem cells into a rat optic nerve crush model also resulted in protection of RGCs, as indicated by a significantly thicker retinal nerve fibre layer (RNFL) compared to untreated control animals 14 days after the lesion (Mead et al 2013).

As already mentioned, the neuroprotective effects of intraocularly grafted cells have usually been attributed to the secretion of endogenously expressed neurotrophic factors from the transplanted cells. This view is in line with the neuroprotective effects that have been observed after intraocular administration of a number of different defined neurotrophic factors. Intravitreal injections of CNTF, for instance, have been shown to rescue RGCs in the ONC (Chen & Weber 2001, Lingor et al 2008, Parrilla-Reverter et al 2009), ONT (van Adel et al 2003, Zhang et al 2005) and in the MOG induced experimental autoimmune encephalomyelitis model (Maier et al 2004). RGC protection was significantly more pronounced when the application of CNTF was combined with the transplantation of an autologous peripheral nerve graft to the damaged site of the optic nerve (Lingor et al 2008). Intravitreal BDNF injections were also effective in preventing RGC death after ONC (Chen & Weber 2001, Weibel et al 1995) or ONT (Peinado-Ramon et al 1996) and, similar to CNTF, were more effective when combined with peripheral nerve grafts (Koeberle & Ball 2002, Mansour-Robaey et al 1994, Peinado-Ramon et al 1996). GDNF also attenuated RGC loss in animal models of optic nerve injury (Klocker et al 1997, Koeberle & Ball 1998) or an animal model of age-related RGC degeneration (Ward et al 2007). Intraocular injections of recombinant neurotrophic factors result in only short-term neuroprotection due to the short half-life of these proteins. To achieve significant long-term rescue effects, application systems are currently under development that allow a continuous intraocular supply of neurotrophic factors over an extended period of time, such as implantable miniature pumps (Saati et al 2009) or biodegradable factor-loaded slow release devices (Aburahma & Mahmoud 2011). Another strategy to

continuously supply neurotrophic factors to RGCs in glaucoma is the forced expression of these factors in the affected cells or other endogenous ocular cell types with adeno-associated or lentiviral vectors. This kind of approach showed promising results with a variety of neurotrophic factors in a wide spectrum of different animal models of glaucoma including the ONC (MacLaren et al 2006a, Weise et al 2000), ONT (Leaver et al 2006b, van Adel et al 2003) or laser-induced ocular hypertension models of glaucoma (Pease et al 2009). Intraocular transplantations of cells genetically modified to stably secrete neurotrophic factors represent another strategy to continuously deliver these proteins to the diseased retina. Most studies that have explored the efficacy of a cell-based delivery of neurotrophic factors to rescue RGCs have focused on either CNTF or BDNF, using a variety of cell types as cellular vectors. For instance, purified astrocytes retrovirally infected with BDNF cDNA have been studied for their ability to protect isolated RGCs *in vitro* (Castillo et al 1994). Furthermore, mesenchymal stem cells lentivirally modified to express BDNF have been shown to confer protection to isolated RGCs in *in vitro* models of glutamate- and hydrogen peroxide-mediated cell death, or to RGCs in a rat model of laser-induced chronic ocular hypertension (Harper et al 2009, Harper et al 2011). BDNF has also been expressed in neural progenitor cells, which significantly delayed apoptosis of axotomized RGCs over a 30 days time period in a partial optic nerve crush model (Wang et al 2002). Furthermore, Schwann cells retrovirally modified to express BDNF stimulated axotomized RGCs to regenerate their axons across the lesion site in adult rats (Menei et al 1998). Similarly, Schwann cells modified with lentiviral vectors to overexpress CNTF promoted axonal regeneration of injured RGC axons into a nerve graft and protected the axotomised RGC from apoptosis (Hu et al 2005). Finally, a recent study demonstrated that neural stem (NS) cells transduced with a polycistronic lentiviral vector to express CNTF effectively attenuated the loss of photoreceptor cells in two mouse models of retinitis pigmentosa (Jung et al 2013). Importantly, the genetically modified cells may be encapsulated in devices with semipermeable membranes, making this cell-based approach interesting for potential clinical applications. When implanted into the vitreous cavity, the devices ensure nutrition of the encapsulated cells from the vitreous fluid and allow diffusion of the secreted factors from the cells to the diseased retina. The encapsulation further protects the grafted cells from the immune system of the host, and vice versa the host eye from potential adverse effects of the grafted cells.

Finally, in case of complications, the cell implant can simply be retrieved from the vitreous cavity, adding another important safety aspect to this approach. Of note, the efficacy of a neuroprotective approach using modified and encapsulated cells is currently being evaluated in patients with retinitis pigmentosa or geographic atrophy, using human retinal pigment epithelial cells with an ectopic expression of CNTF (Birch et al 2013, Kauper et al 2012, Sieving et al 2006, Talcott et al 2011, Zhang et al 2011a).

A variety of neurotrophic factors have been identified during the last few decades that can be grouped into four major classes. The neurotrophin family is composed of the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (NT-6). The glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs) consists of GDNF, neurturin, artemin and persephin (Maruyama & Naoi 2013, Noble et al 2011). A third group of neuroprotective factors are the neurotrophic cytokines with ciliary neurotropic factor (CNTF), interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF) as the best known members of this family (Bauer et al 2007). A novel group of factors comprises cerebral dopamine neurotrophic factor and mesencephalic astrocyte-derived neurotrophic factor (Maruyama & Naoi 2013). In the present thesis, members of three of these protein families have been expressed in neural stem cells with the aim to analyze the therapeutic potential of a cell-based administration of neurotrophic factors to glaucomatous retinas, namely the cytokine CNTF, the neurotrophin BDNF and the GFLs family member GDNF.

Ciliary neurotrophic factor (CNTF) was purified from chick embryos and shown to exert potent neuroprotective activities on ciliary ganglion neurons (Adler et al 1979, Barbin et al 1984, Helfand et al 1976). The mature protein consists of 200 amino acids with a molecular weight of 22.7 kDa. CNTF shares common features of cytosolic proteins, as it lacks a signal peptide for secretion and consensus sequences for glycosylation. Even though the cytokine lacks a signal peptide, it may be secreted via unconventional release mechanisms (Stockli et al 1989). CNTF is a member of the IL-6 family of cytokines based on structural and functional similarities as well as the use of the gp130 protein as a critical component of the receptor complex responsible for signal transduction (Bauer et al 2007, Taga & Kishimoto 1997). The receptor complex of CNTF additionally consists of the CNTF-specific receptor CNTFRα and the leukaemia inhibitory

factor receptor ß (LIFRß) (Thanos & Emerich 2005). Gp130 and LIFRß comprise the critical receptor subunits for signal transduction, since CNTFRα lacks transmembrane or intracellular domains. CNTF first forms a complex with CNTFRα, after which gp130 is recruited. Subsequently gp130 forms hetero-dimers with LIFRß, which in turn leads to the activation of Jak/Tyk kinases. Activation of Jak/Tyk is followed by recruitment and phosphorylation of the signal transducer and activator of transcription 3 (STAT3), which forms homo- or hetero-dimers with phosphorylated STAT1, ultimately modifying gene transcription and confering neuroprotection to the cell (Thanos & Emerich 2005, Wen et al 2012). Even though the CNTFRα receptor is essential for CNTF-mediated effects, cells that lack CNTFRα expression may also be responsive to CNTF provided they express LIFRß and gp130. CNTFRα is attached to the cell membrane via a glycosylphosphatidylinositol anchor and can thus be cleaved by phospholipase C, releasing CNTFRα from the membrane as a soluble receptor (Davis et al 1991).

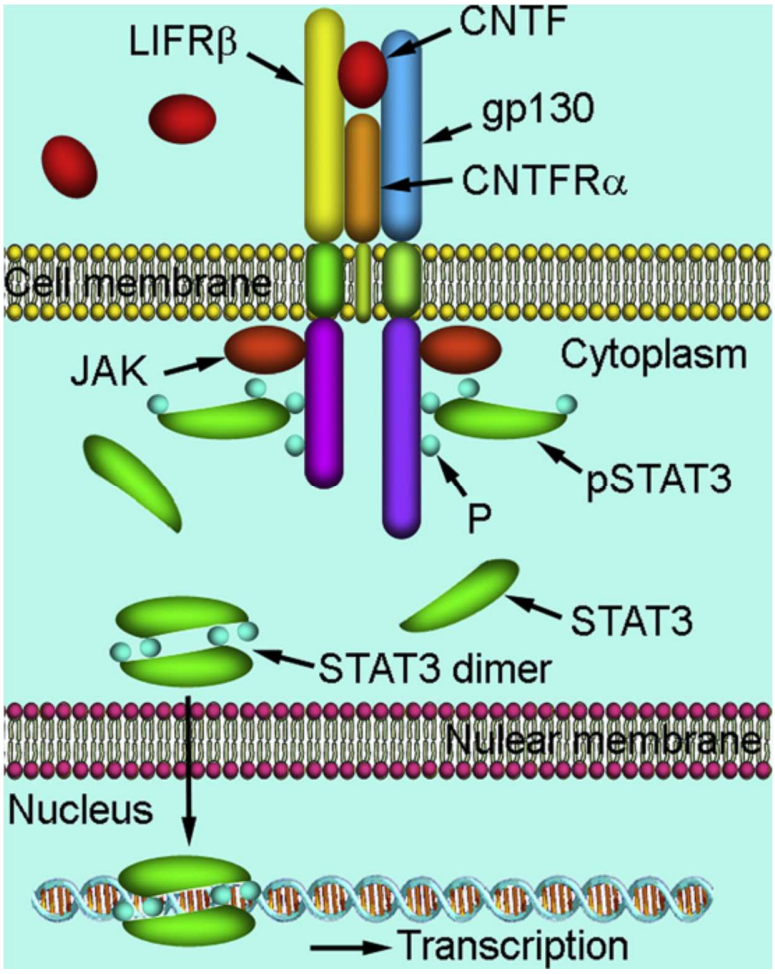

Figure 4: Schematic presentation of CNTF signaling. CNTF binds to CNTFRα, which forms a complex with gp130 and LIFRβ, resulting in activation of JAK kinase. After activation of JAK kinase, the intracellular domains of gp130 and LIFß are phosphorylated at their tyrosine residues (P), which are then able to recruit STAT3. STAT3 is phosphorylated by JAK kinase, and the synthetized phospho-STAT3 (pSTAT3) dimerizes and translocates to the nucleus to modify gene transcription (from Wen et al 2012).

Glial cell line-derived neurotrophic factor (GDNF) was purified in 1993 from glial cells of the embryonic rat midbrain, and showed protective effects on dopaminergic neurons. The protein exists as a disulfide-bonded dimer, with the monomer having a molecular weight of 18 to 22 kDa. The GDNF gene encodes a precursor protein, which is secreted from the cell. The signal peptide of the precursor is cleaved during maturation and secretion, resulting in a mature protein consisting of 134 amino acids (Lin et al 1993). Like the other three proteins in the glial cell line-derived neurotrophic factor family of ligands subfamily, GDNF signals through the transmembrane receptor tyrosine kinase (RTK) rearranged during transfection (RET). RET differs significantly from other RTKs with regard to its

extracellular domain and the way it interacts with its ligands. The extracellular domain consists of four cadherin-like domains, which can only bind ligands in the presence of a co-receptor, the cell surface-bound GDNF family receptor α (GFRα). Four different GFRα can be distinguished, GFRα1 to GFRα4, with GDNF binding preferentially to GFRα1 and with lower affinity also able to GFRα2 and GFRα3 (Bespalov & Saarma 2007). Activation of downstream signaling pathways occurs after a GDNF dimer binds to either a monomeric or dimeric GFRα1 to form a GDNF-GFRα1 complex, which then binds two RET molecules. Both RET proteins homodimerize after binding to the GDNF-GFRα1 complex and autophosphorylate, leading to the activation of the intracellular domain, which then serves as binding site for various intracellular signaling proteins that in turn activate pro-survival pathways including the phosphatidylinositol 3-kinase (PI3-kinase), phospholipase Cγ (PLC-γ) or mitogen-activated protein kinase (MAPK) pathway. GDNF is furthermore able of intracellular signaling independent of RET, by activating Src-family kinases (SFKs), which also induce the phosphorylation of MAPK or PLC-γ (Airaksinen & Saarma 2002, Maruyama & Naoi 2013).

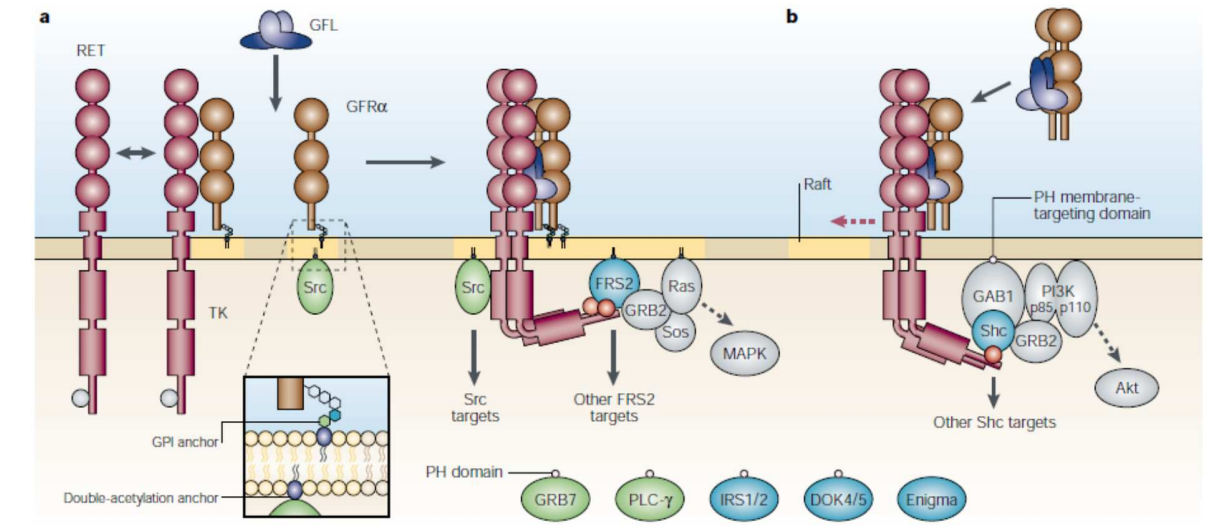

Figure 5: Schematic presentation of GDNF-family ligand signaling. The figure shows different RET-binding proteins (blue) and downstream signaling pathways (grey) that are activated through RET TK stimulation, either in rafts (yellow) by GFL in a complex with glycosylphosphatidylinositol (GPI)-anchored GDNF-family receptor-α (GFRα) or **b** outside rafts by a soluble GFL–GFRα complex. GFL are able to induce the dimerization of GFRα molecules. The GFL–GFRα complex shows an enhanced affinity to RET and drags the protein to the rafts and facilitates its dimerization. The stimulation of RET by the soluble GFL-GFRα outside the rafts initially activates signaling pathways mediated by soluble adaptors such as Src-homologous and collagen-like protein (Shc) (from Airaksinen & Saarma 2002).

Brain-derived neurotrophic factor (BDNF) was first purified from pig brain in 1982 (Barde et al 1982). Similar to the other members of the neurotrophin family, BDNF is expressed as a 32 kDa immature form and later processed to its 13.5 kDa mature form (Leibrock et al 1989, Noble et al 2011). BDNF is the ligand of two different classes of receptors, the tropomyosin receptor kinase receptor (TRKR) which belongs to the RTK family, and the p75 neurotrophin receptor (p75NTR) (Hennigan et al 2007). The TRK receptor family is composed of three members, TrkA, TrkB and TrkC, with TrkB showing the highest affinity for BDNF. The receptor dimerizes after binding of BDNF, which then results in autophosphorylation of the receptor and subsequent activation of different signaling cascades including the phosphatidylinositol-3 kinase (PI3K)/Akt, the PLC-γ and the Ras/Raf/MAPK pathways, all of which can also be activated through GDNF and the extracellular signal-regulated kinases 1/2 (Erk1/2) pathway (Almasieh et al 2012, Hennigan et al 2007). The neurotrophic effects observed after BDNF application most likely result from activation of the Erk1/2 pathway, as stimulation of this pathway leads to an enhanced RGC survival rate in different *in vivo* models (Almasieh et al 2012). The p75NTR has a special role in BDNF signaling, as it activates signaling pathways that may lead to neuroprotection as well as cell death. P75NTR has a low affinity for the mature form of BDNF, but a high affinity for the secreted proform of the protein, and binding of proBDNF to a p75NTR-sortilin complex initiates pathways that lead to apoptosis. However, when p75NRT is co-expressed with TrkB, it can enhance the activation of TrkB by BDNF, thus promoting neuroprotection (Almasieh et al 2012, Hennigan et al 2007).

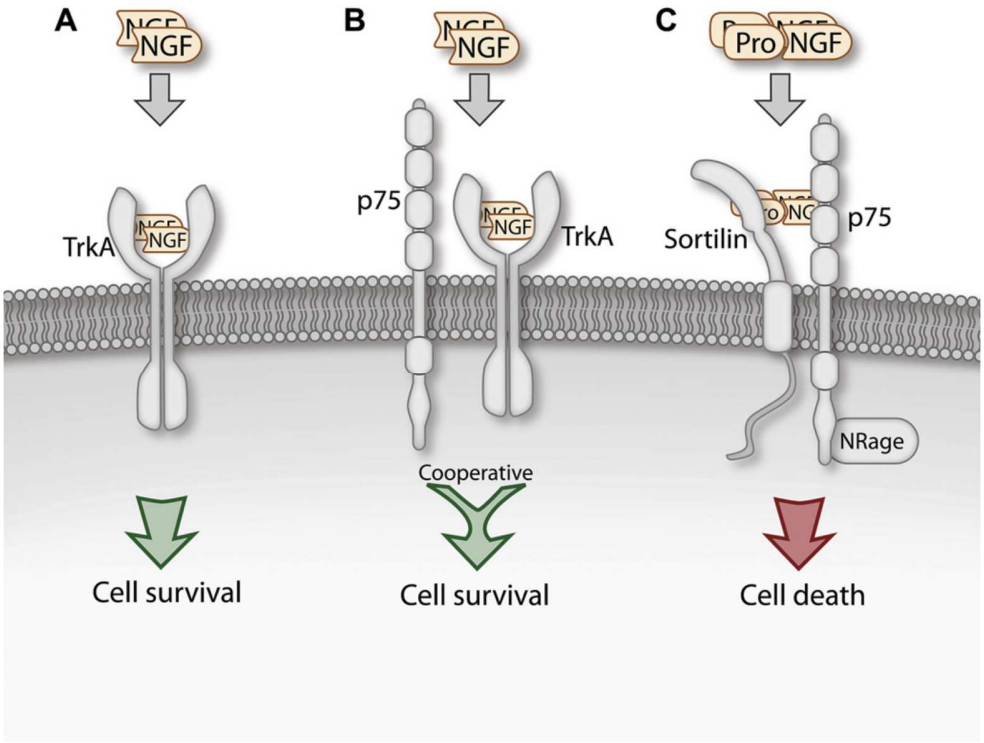

Figure 6: Schematic presentation of cell survival or cell death initiation by binding of neurotrophins to Trk or p75. A) When BDNF or another neurotrophin binds to its high affinity Trk receptor, the activated pathways lead to promotion of cell survival. B) When p75 and the Trk receptor are co-expressed, binding of a neurotrophin increases Trk activation and subsequently cell survival. However, should the Trk/p75 ratio tend to be in favor of p75, the activation of p75 can lead to neuronal death. C) Pro-neurotrophins, like proBDNF bind to p75, which has a high affinity for these forms of neurotrophins. The binding of pro-neurotrophins to the p75-sortilin receptor complex initiates apoptosis of the respective cell (from Almasieh et al 2012).

All three factors used in the present thesis are well known to confer neuroprotective activities on different retinal cell types in different various *in vitro* and *in vivo* models. For example, CNTF exerts strong neuroprotective effects in models of photoreceptor as well as RGC degeneration. Specifically, the cytokine protects photoreceptor cells in light-induced as well as in genetic models of photoreceptor degeneration (LaVail et al 1998, Wenzel et al 2005). It also protects RGCs under different pathological conditions including ONC (Leaver et al 2006a, Parrilla-Reverter et al 2009), ONT (van Adel et al 2005, Zhang et al 2005) or laser-induced ocular hypertension (Pease et al 2009). Furthermore CNTF promotes long distance axonal regeneration *in vivo* (Jo et al 1999, Leaver et al 2006b). GDNF attenuates the lesion-induced loss of RGCs in rats (Koeberle & Ball 2002, Ozden & Isenmann 2004, Yan et al 1999) or the age-related loss of RGCs in mice (Ward et al 2007), the degeneration of photoreceptor cells *in vitro* (Carwile et al 1998) or *in vivo* in mouse (Frasson et al 1999)

or rat (McGee Sanftner et al 2001) models of retinitis pigmentosa or retinal detachment (Wu et al 2002). Finally, BDNF protects axotomized RGCs in rats (Koeberle & Ball 2002, Mansour-Robaey et al 1994, Weibel et al 1995) and cats (Chen & Weber 2001), as well as in a model of laser-induced ocular hypertension (Harper et al 2011, Pease et al 2009), and stimulates axon regrowth *in vitro* (Takano et al 2002) and *in vivo* (Mansour-Robaey et al 1994). Furthermore, BDNF rescues photoreceptor cells from light-induced damage in rats (LaVail et al 1992), preserves the retinal structure in the *Pde6b*^{rd1} mutant mouse (Chen et al 2012), or protects photoreceptors from oxidative stress-induced cell death (Okoye et al 2003).

2. Summary of discussion

Glaucomatous optic neuropathies are degenerative retinal disorders that are characterized by a progressive degeneration of retinal ganglion cells (RGCs). Elevated intraocular pressure (IOP) is the major risk factor for this disease, and lowering IOP is the only proven clinical treatment. However, in a significant number of glaucoma patients, degeneration of RGCs progresses despite successful lowering of the IOP, indicating the need for additional treatment options. Neuroprotection is among the alternative strategies to develop effective treatments for this degenerative retinal disorder.

The present thesis was aimed at developing a neural stem cell-based intraocular delivery system for neurotrophic factors as a potential treatment option for glaucomatous optic neuropathies. The efficacy of this neuroprotective approach to rescue RGCs from cell death was evaluated in a mouse model of glaucoma.

The present work demonstrates that intraretinal transplantations of clonal neural stem cell lines or mixtures of clonal cell lines, with a forced expression of neurotrophic factors significantly attenuated a lesion-induced loss of RGCs over an extended period of time. The most important results of this thesis include:

a) Transductions of adherently cultivated neural stem cells with polycistronic lentiviral vectors robustly led to the derivation of modified stem cells with a forced co-expression of a neurotrophic factor, a fluorescent reporter protein, and a resistance gene.

b) Because expression levels of the neurotrophic factors and fluorescent reporter proteins are proportional to each other, clonal neural stem cell lines with high expression levels of neurotrophic factors could be generated by multiple rounds of transductions, and subsequent selections and clonal expansions of single cells with the highest expression levels of the reporter genes.

c) Intravitreally grafted NS cells attached to the posterior poles of the lenses and the vitreal surface of the retinas, where they preferentially differentiated into astrocytes. The grafted cells survived for up to four months in the vitreous cavity. Tumor formation,

integration of the grafted cells into the host retinas, or other adverse effects of the donor cells on the host retinas were not observed.

d) When the modified neural stem cells were differentiated into neural cell types *in vitro* or intravitreally transplanted into a mouse model of glaucoma, the differentiated neural cell types stably expressed the neurotrophic factors and fluorescent reporter proteins for at least two and four months, respectively.

e) Intravitreally grafted CNTF-, GDNF- or BDNF-expressing neural stem cell lines significantly attenuated the degeneration of retinal ganglion cells in a mouse optic nerve crush model. Neuroprotective effects of the grafted cell lines were detectable for at least two months (GDNF- and BDNF-expressing cell lines) and four months (CNTF-expressing cell line), the longest post-lesion intervals analyzed. Grafted CNTF-expressing neural stem cells additionally stimulated long distance regeneration of the axotomized ganglion cell axons.

f) Co-transplantations of a GDNF- and a CNTF-expressing neural stem cell line resulted in a markedly enhanced neuroprotection of axotomized ganglion cells when compared to the neuroprotective effects exerted by each individual cell lines. The number of surviving ganglion cells in eyes with grafted GDNF- and CNTF-expressing neural stem cells was significantly higher than expected if both neurotrophic factors would have exerted their neuroprotective activities in and additive manner. Results indicate that combinatorial neuroprotective approaches may result in significant neuroprotection of ganglion cells in an animal model of glaucoma.

Together, results of the present work demonstrate that genetically modified and clonally derived neural stem cell lines represent a valuable tool to evaluate the therapeutic potential of a sustained cell-based intraocular administration of neurotrophic factors in mouse models of glaucoma. Furthermore, neural stem cell lines with a forced expression of different neurotrophic factors with known neuroprotective activities on retinal ganglion cells represent a useful tool for combinatorial neuroprotective approaches aimed at identifying factor combinations that confer additive or synergistic neuroprotective effects to ganglion cells in glaucomatous retinas.

II. Projects

1. Project: Neural stem cell-based intraocular administration of ciliary neurotrophic factor attenuates the loss of axotomized ganglion cells in adult mice

Abstract

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 one day after an intraorbital crush of the optic nerve. Brn-3a-positive RGCs were counted in flat-mounted retinas at different post-lesion 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 four months, the latest post-lesion time point analyzed. Depending on the post-lesion 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.

Introduction

Glaucoma is among the leading causes of blindness with an estimated 80 million people being affected worldwide in 2020 (Quigley & Broman 2006). 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 (Almasieh et al 2012, Qu et al 2010). The pathomechanisms leading to the apoptotic death of RGCs in this age-related and multifactorial disease are not fully understood. Clinically, an increased intraocular pressure (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 (Agarwal et al 2009, Almasieh et al 2012, Pascale et al 2012, Qu et al 2010). 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 (Caprioli 1997, Leske et al 2003), indicating the need for alternative treatments.

It has been proposed that impaired axonal transport as a result of elevated intraocular pressure leads to a scarcity of target-derived neurotrophic factors and subsequent apoptotic degeneration of RGCs (Pease et al 2000, Quigley et al 2000). Stimulation of pro-survival 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 (Almasieh et al 2012, Harvey et al 2006, Johnson et al 2011, Lebrun-Julien & Di Polo 2008, Wilson & Di Polo 2012). For instance, intraocular administration of brain-derived neurotrophic factor (BDNF) or glial cell line-derived neurotrophic factor (GDNF) has been demonstrated to significantly delay the degeneration of RGCs in animal models of optic nerve injury (Di Polo et al 1998, Isenmann et al 1998, Klocker et al 1997, Koeberle & Ball 2002, Mansour-Robaey et al 1994, Mey & Thanos 1993, Yan et al 1999) or ocular hypertension (Fu et al 2010, Jiang et al 2007, Ko et al 2000, Martin et al 2003).

Ciliary neurotrophic factor (CNTF), a member of the interleukin- (IL) 6 family of cytokines (Bauer et al 2007), is another neurotrophic factor that has been shown to potently rescue RGCs in various pathological conditions (Hellstrom et al 2011, Ji et al 2004, MacLaren et al 2006a, Maier et al 2004, Mey & Thanos 1993, Parrilla-Reverter et al 2009, Pease et al 2009, van Adel et al 2003, Weise et al 2000). 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 (Cui et al 2003, Hellstrom et al 2011, Leaver et al 2006b, Lingor et al 2008, Muller et al 2009, Pernet et al 2013). 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 (Dittrich et al 1994). In comparison, more robust 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 (Almasieh et al 2012, Harvey et al 2006, Wilson & Di Polo 2012).

Intraocular transplantations of genetically modified cells represent another strategy to continuously deliver neuroprotective factors to the retina (Johnson & Martin 2013, Wen et al 2012). Importantly, the use of *ex vivo* modified cells offers the possibility to adjust the amount of neurotrophic factors administered to the retina prior to 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 (Tao 2006, Thanos & Emerich 2005, Wen et al 2012). In fact, the therapeutic potential of a cell-based intraocular administration of a neurotrophic factor is currently being evaluated in patients with retinitis pigmentosa or geographic atrophy, using intravitreal implants of an encapsulated retinal pigment epithelium (RPE) cell line genetically modified to secrete CNTF (Birch et al 2013, Kauper et al 2012, Sieving et al 2006, Talcott et al 2011, Zhang et al 2011a).

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 one 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 days old C57BL/6J mouse embryos. Intraorbital optic nerve lesions and intravitreal NS cell transplantations 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.

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 ß-actin promoter, giving rise to pCAG-CNTF-IRES-Venus-2A-ZEO (Jung et al 2013). Lentiviral particles were pseudotyped with the envelope G protein of the vesicular stomatitis virus (VSV-G) and produced by transient transfection of HEK 293T cells as described elsewhere (http://www.LentiGo-Vectors.de) (Weber et al 2010).

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) (Jung et al 2013) 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 DMEM/F12 (Life Technologies) supplemented with 2 mM glutamine, 5 mM HEPES, 3 mM sodium bicarbonate, 0.3% glucose (all from Sigma, 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), 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). Positive cells were selected by further cultivating the cells under adherent conditions (Conti et al 2005, Jung et al 2013, Weber et al 2010) in culture flasks coated with poly-L-ornithine (Sigma) and 0.1% Matrigel in the same medium but additionally containing 200 µg/ml zeocin (Invivogen, San Diego, CA). 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) 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) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). 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/).

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 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), 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) or monoclonal mouse anti-microtubule associated protein 2 (MAP2)

antibodies (Sigma) 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).

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 sec at a distance of 0.5 – 1 mm from the eye (Bartsch et al 1995, Bartsch et al 1992). 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 of 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 of PBS containing either $7.6x10^5$ CNTF-NS cells or control-NS cells were slowly injected into the vitreous cavity (Jung et al 2013, Pressmar et al 2001). 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 sacrificed 4 months after transplantation, and eyes were immersion-fixed for 1 h 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) or rat anti-myelin basic protein (MBP) antibodies (Millipore, Bedford, MA) 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 four 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 25µm, and stained with DAPI.

Anterograde axonal tracing

To anterogradely label RGC axons, a saturated solution of biotin-N-hydroxysuccinimidester (Sigma) in dimethylformamide (Carl Roth GmbH) was diluted 1:1 with ethanol, and intravitreally injected into the eyes with crushed optic nerves (Bartsch et al 1995, Halfter 1987). After 24 hrs, animals were sacrificed 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 one month after the crush. Analysis of sections was done with an Olympus IX51 fluorescence microscope (Olympus).

Analysis of retinal ganglion cell survival

Animals were sacrificed one, two, three or four 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 in 4% PA for 1 h, 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 5 photomicrographs from the center to the periphery of the superior, inferior, nasal and temporal retinal quadrant were taken, covering a total retinal area of ~1.9 mm^2. All Brn-3a-positive RGCs visible on these 20 photomicrographs were counted using Adobe

Photoshop CS3 software (Adobe Systems Incorporated, San Jose, CA), 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 post-lesion interval (i.e. one, two, three and four months after the optic nerve crush). RGC densities were additionally determined in eyes with intravitreally injected PBS one 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 (NS) cells and lentiviral vectors

To express CNTF in adherently cultivated neural stem (NS) cells (Conti et al 2005, Pollard et al 2006) 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 (Weber et al 2008, Weber et al 2010).

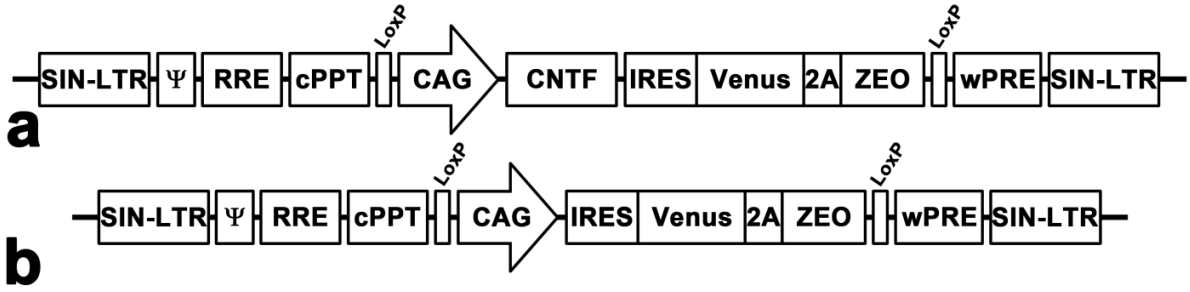

Figure 1: *Lentiviral vectors for the generation of modified neural stem cell lines.*
Neural stem (NS) cells were transduced with a polycistronic lentiviral vector encoding a secretable variant of mouse CNTF under regulatory control of the human CMV enhancer/chicken ß-actin (CAG) promoter. The vector 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 (a). The same vector but lacking the CNTF cDNA was used to transduce NS cell for control experiments (Ab). Ψ, packaging signal; CNTF, ciliary neurotrophic factor; cPPT, central polypurine tract; IRES, internal ribosome entry site; LoxP, recognition site of Cre recombinase; RRE, rev-responsive element; SIN-LTR, self-inactivating long-terminal repeat; wPRE, Woodchuck hepatitis virus posttranscriptional regulatory element.

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 ß-actin CAG promoter (pCAG-CNTF-IRES-Venus-2A-ZEO; Fig. 1a) to assure robust transgene expression in undifferentiated NS cells and their differentiated progeny. NS 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. 1b) (Jung et al 2013).

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 (Jung et al 2013), 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.

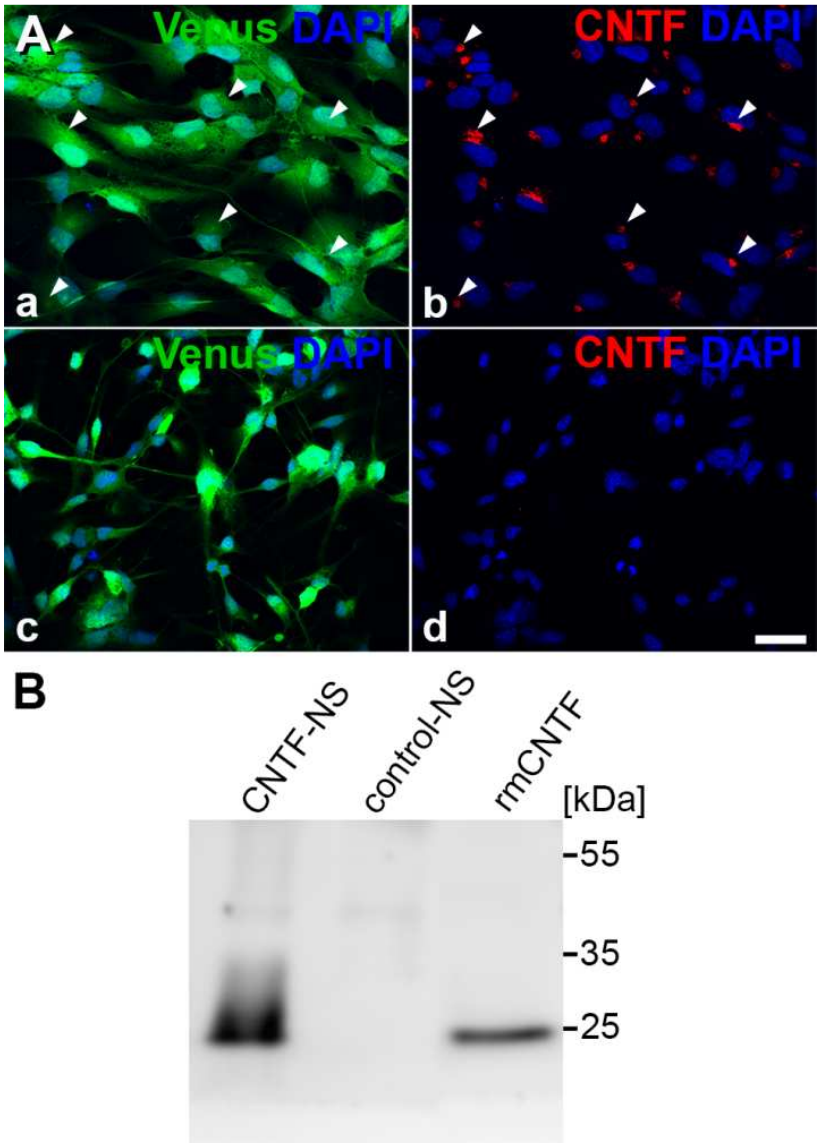

Figure 2: *Expression of CNTF and Venus in genetically modified clonal NS cell lines.*
(A) Clonally derived CNTF-NS (a, b) and control-NS cell lines (c, d) were generated using fluorescence activated cell sorting and immunostained with anti-CNTF antibodies. While all cells in the CNTF-NS (a) and control-NS cell line (c) expressed Venus, expression of CNTF was only detectable in the CNTF-NS cell clone (b; some cells labeled with arrowheads in a and b), but not in the control-NS cell clone (d). (B) 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. CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole; rmCNTF, recombinant mouse ciliary neurotrophic factor. Bar in (d) for (a-d): 25 µm.

Immunocytochemical analyses revealed that all cells in the CNTF-NS cell line co-expressed Venus and CNTF (Fig. 2A a, b). Control-NS cells were also Venus-positive, but lacked detectable levels of CNTF immunoreactivity (Fig. 2A c, d). 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.

2B). Expression of CNTF in the clonal CNTF-NS cell line was detectable by immunocytochemical and immunoblot analyses for more than 30 passages, corresponding to a culture period of about six months. Furthermore, immunoblot analyses (n=3) of culture supernatants from this cell line revealed secretion of 87.2 ±10.1ng (mean ±SEM) CNTF per 10^5 cells in 24hrs 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. 3).

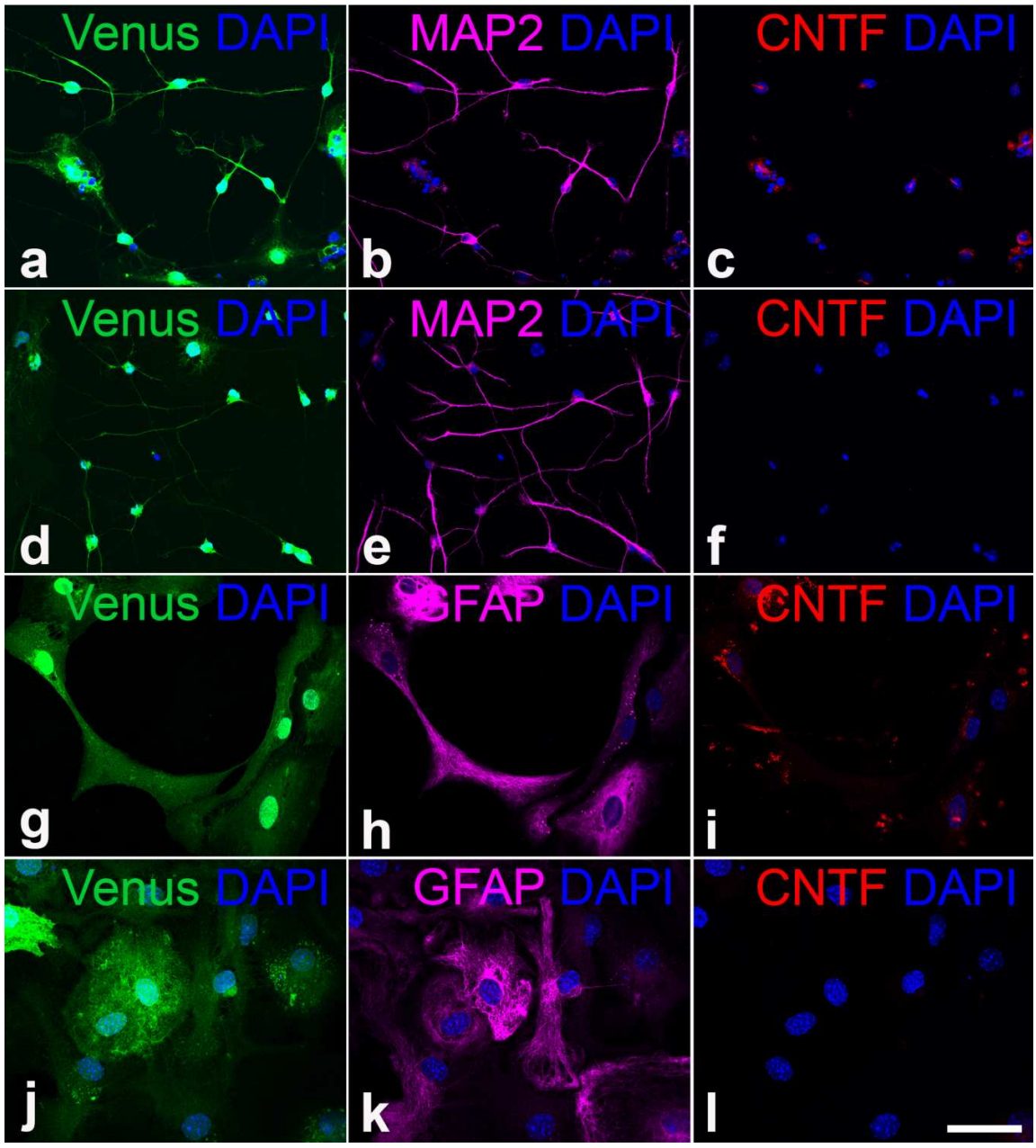

Figure 3: *Expression of CNTF and Venus in differentiated clonal NS cell lines.*
CNTF-NS (a-c, g-i) and control-NS cell clones (d-f, j-l) were differentiated into MAP2-positive neurons (a-f) or GFAP-positive astrocytes (g-l). The reporter gene Venus was expressed in all neurons (a, d) and astrocytes (g, j) derived from the CNTF-NS (a, g) and control-NS cell line (d, j). CNTF was only detectable 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 one week and two months after induction of differentiation, respectively. 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.

While all MAP2-positive neurons derived from CNTF-NS and control-NS cells were positive for Venus, expression of CNTF was only detectable in neurons derived from CNTF-NS cells, but not in neurons derived from control-NS cells (Fig. 3 a-f). Similarly, GFAP-positive

astrocytes from both CNTF-NS and control-NS cells were positive for Venus, while expression of CNTF was only detectable in astrocytes derived from CNTF-NS cells but not in astrocytes derived from control-NS cells (Fig. 3 g-l). Of note, expression of CNTF in cultured astrocytes derived from the CNTF-NS cell line remained detectable for at least two months (Fig. 3 i), the longest cultivation period analyzed.

Intravitreal transplantations of CNTF-NS and control-NS cells

Intravitreally grafted CNTF-NS (Fig. 3 a-d; Fig. 4) and control-NS cells (Fig. 3 e-h) were identified in the host eyes by their expression of the reporter gene Venus (Fig. 3 a, e; Fig. 4 a-e).

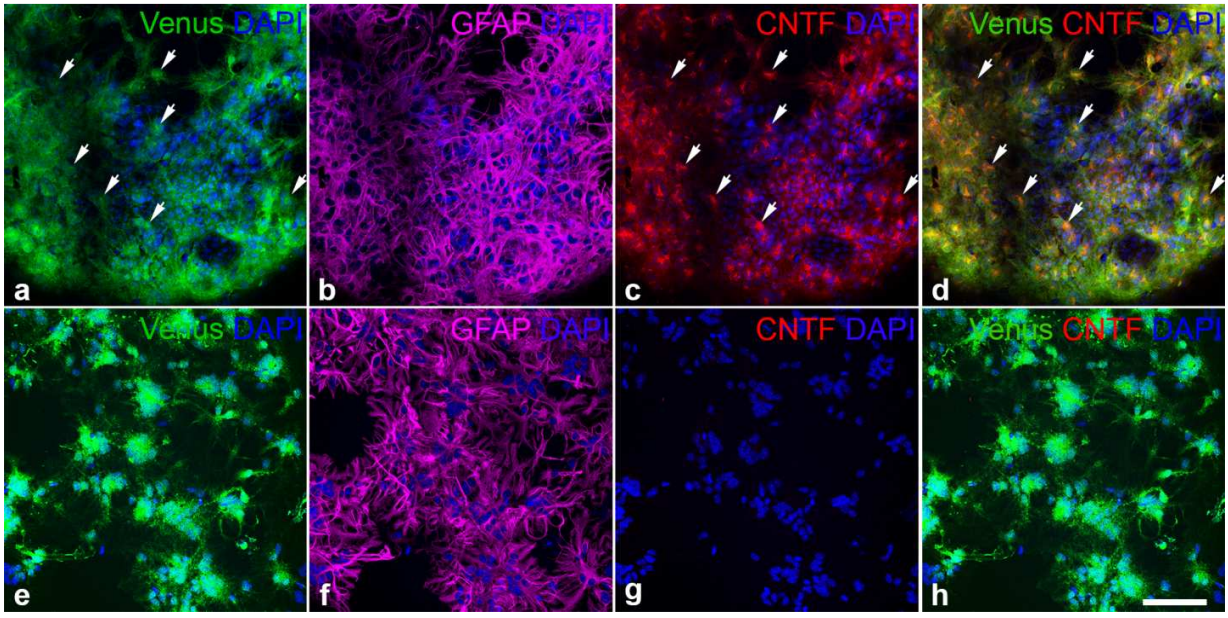

Figure 3: *Neural differentiation and expression of CNTF in intravitreally grafted NS cell lines.*
Analyses of eyes four months after intravitreal transplantations of CNTF-NS (a-d) and control-NS cell lines (e-h) revealed the presence of Venus-positive donor cells (a, e) that were attached to the posterior 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 co-expressing Venus and CNTF are marked with white arrows in (a, c and d). CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein. Bar in h (for a-h): 100 µm.

Of note, both cell populations survived in the vitreous cavity of the recipient eyes for at least four months, the longest post-transplantation 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; Fig. 4 a) or to the vitreal surface of the retinas (Fig. 4 b-

e). Evidence for the formation of tumors by the grafted cells, or for integration of Venus-positive donor cells into the host retinas (Fig. 4 b, d, e) was not observed.

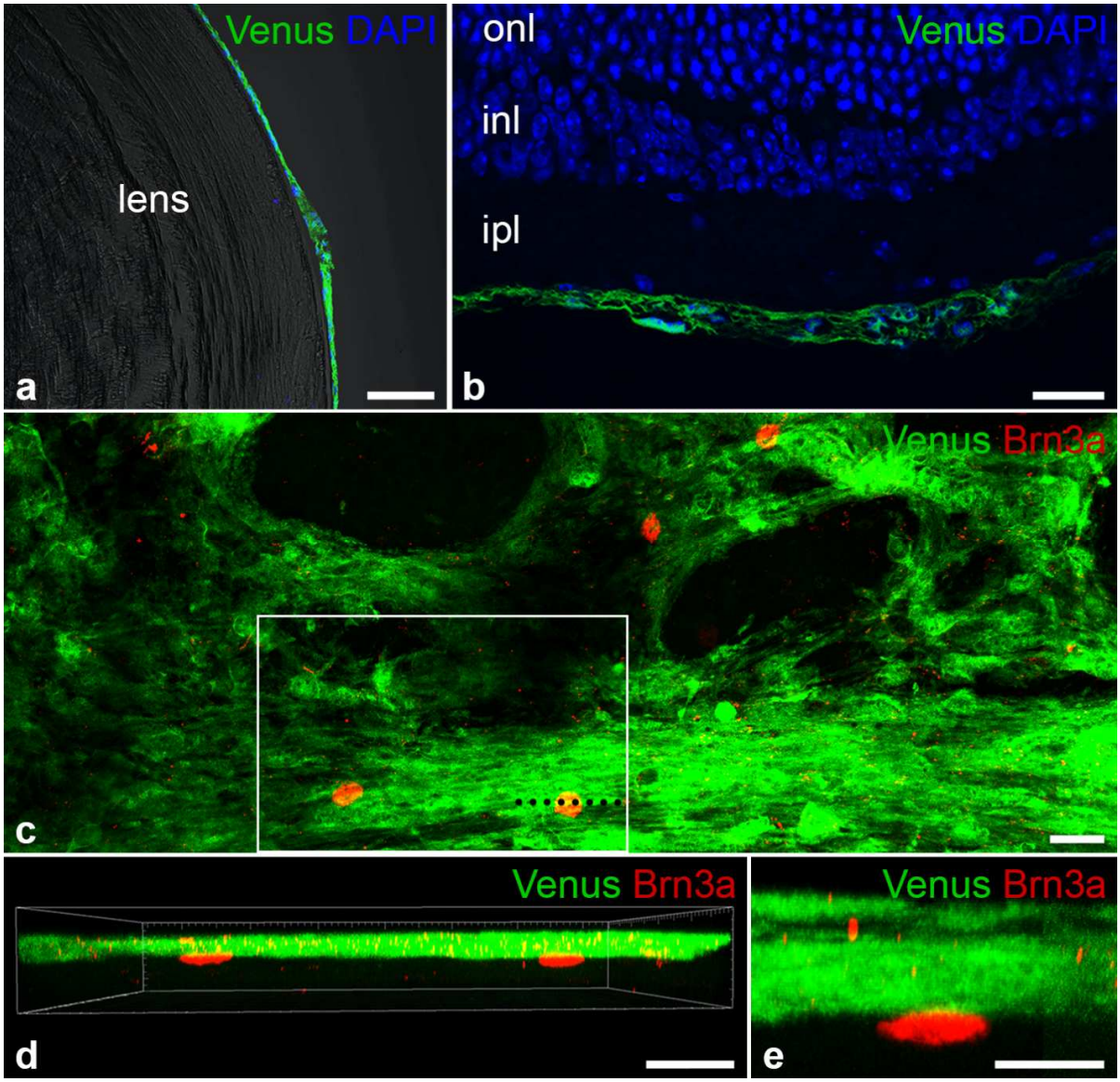

Figure 4: *Intraocular localization of intravitreally grafted CNTF-NS cells.*
Four months after intravitreal transplantations of CNTF-NS cells, donor cells were identified in the host eyes by their expression of the Venus reporter gene. Analysis of tissue sections revealed the presence of dense layers of Venus-positive donor cells that were attached to the posterior pole of the lenses (a) and the vitreal surface of the host retinas (b). (c) is a merged z-stack of confocal images showing Venus-positive donor cells and Brn-3a-positive retinal ganglion cells in a flat-mounted retina four months after an intravitreal injection of CNTF-NS cells. Lateral views of the boxed area in (c) and the Brn-3a-positive cell at the level of the dashed line in (c) demonstrate that the Venus-positive donor cells are located above the Brn-3a-positive ganglion cells (d and e, respectively). Integration of donor cells into the host retinas was not observed (b, d, e). DAPI, 4',6-diamidino-2-phenylindole; inl, inner nuclear layer; ipl, inner plexiform layer; onl, outer nuclear layer. Bar in (a): 100 µm; in (b-d): 20 µm; ín (e): 10 µm.

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 the majority of the CNTF-NS and control-NS cells were differentiated into GFAP-positive astrocytes (Fig. 3 b, f). 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 four months after transplantation, the latest post-transplantation time point investigated (Fig. 3 c, d). Astrocytes derived from the control-NS cell clone, in contrast, lacked detectable expression of the cytokine (Fig. 3 g, h).

Intravitreally grafted CNTF-NS cells attenuate the degeneration of axotomized retinal ganglion cells

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 (Nadal-Nicolas et al 2009). Quantitative analysis of retinas from untreated animals (n=6) revealed the presence of 3,993.8 ±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 one, two, three and four months after the lesion, respectively (n=6 for each post-lesion interval; Fig. 5 e-h, Fig. 6). In animals with intravitreally grafted CNTF-NS cells, in comparison, we detected 529.0 ±20.9 RGCs/mm^2 one month after the lesion, 429.8 ±30.7 RGCs/mm^2 two months after the lesion, 320.5 ±11.5 RGCs/mm^2 three months after the lesion, and 302.7 ±4.3 RGCs/mm^2 four months after the lesion (n=6 for each post-lesion interval; Fig. 5 a-d, Fig. 6).

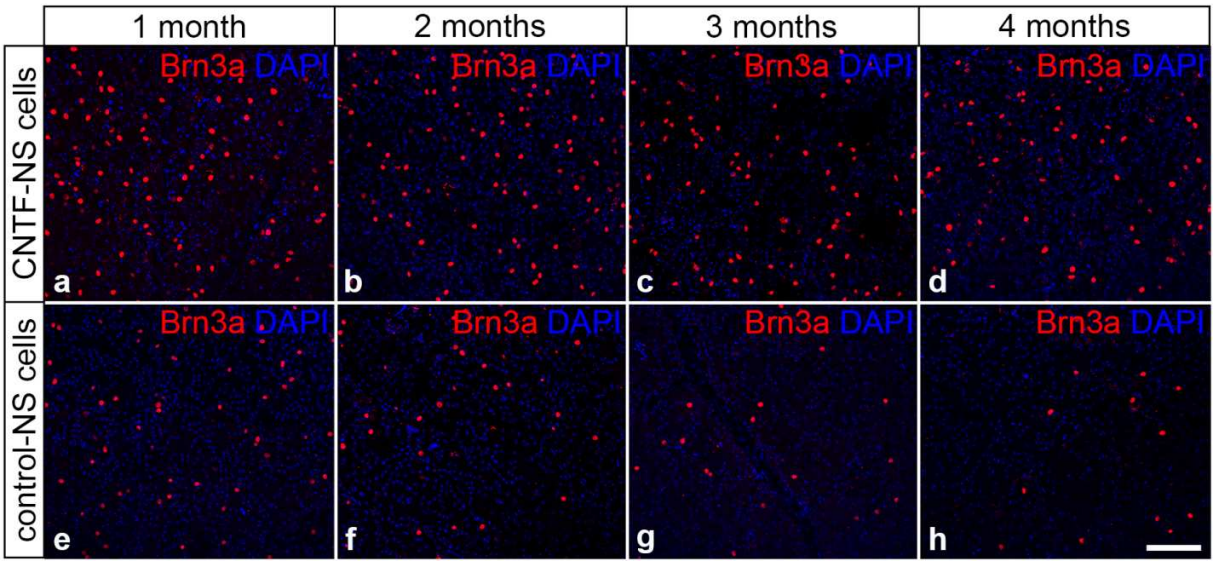

Figure 5: *Intravitreally grafted CNTF-NS cells attenuate degeneration of axotomized retinal ganglion cells in adult mice.* Adult mice received intravitreal injections of a CNTF-NS cell clone (a-d) or a control-NS cell clone (e-h) one day after an intraorbital optic nerve crush. Analysis of flat-mounted retinas one (a, e), two (b, f), three (c, g) and four months (d, h) after the lesion revealed the presence of significantly more Brn-3a-positive retinal ganglion cells in eyes with grafted CNTF-NS cells (a-d) than in eyes with grafted control-NS cells (e-h) at all post-lesion intervals. DAPI, 4',6-diamidino-2-phenylindole. Bar in h (for a-h): 100 µm.

CNTF-treated retinas thus contained 2.8-, 4.4-, 6.4- and 4.8-fold more surviving RGCs than control retinas at the one, two, three and four months post-lesion interval, respectively. This difference between RGC numbers in CNTF-treated and control retinas was statistically significant at all post-lesion time points analyzed ($p<0.001$ according to the Student´s t-test; Fig. 6).

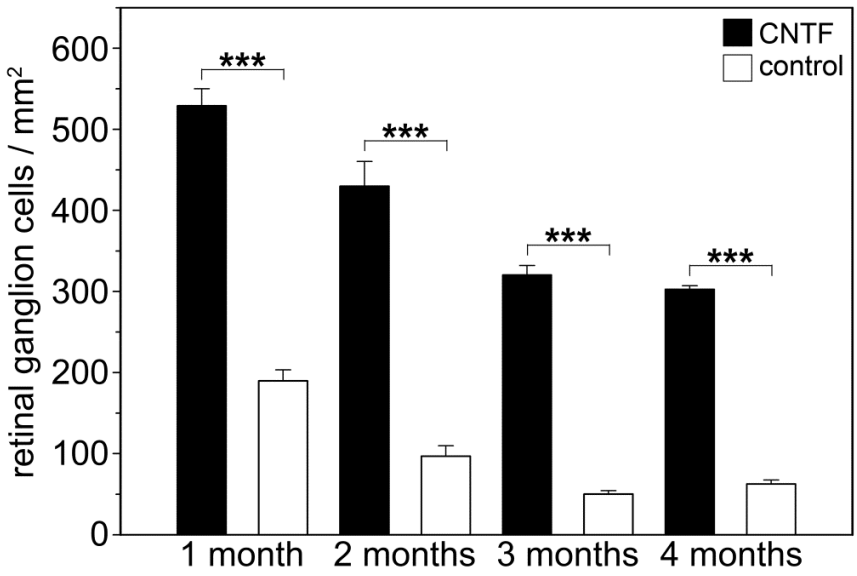

Figure 6: *Quantitative analysis of the neuroprotective effect of intravitreally grafted CNTF-NS cells on axotomized retinal ganglion cells.*
The number of Brn-3a-positive retinal ganglion cells was determined in eyes with intravitreally grafted CNTF-NS cells (filled bars) or control-NS cells (open bars) one, two, three and four months after an intraorbital optic nerve crush. Note that the CNTF-treated retinas contained significantly more retinal ganglion cells than the control retinas at all post-lesion intervals. Each bar represents the mean number (±SEM) of retinal ganglion cells per mm^2 from six retinas. ***: $p<0.001$ according to the Student´s t-test.

Anterograde axonal tracing experiments performed in a fraction of animals from the different experimental groups at the different post-lesion 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 control-NS cells and grafted CNTF-NS cells (n=6 for each experimental group) one month after an intraorbital nerve crush (Fig. 7). RGC axons in control animals extended for only short distances (459.3 ±47.0 µm (mean ±SEM); Fig. 7 a, b, e) across the lesion site into the distal optic nerve stump. In CNTF-treated animals, in comparison, axotomized RGC axons regrew for up to 2,800 µm across the lesion site into the distal optic nerve stump (2,302.7 ±162.8 µm; Fig. 7 c, d, e). Axons in the distal optic nerve stump of CNTF-treated animals followed an irregular course (Fig. 7 c, d), indicating that they corresponded to regrown axons and not to axons that had escaped the nerve crush.

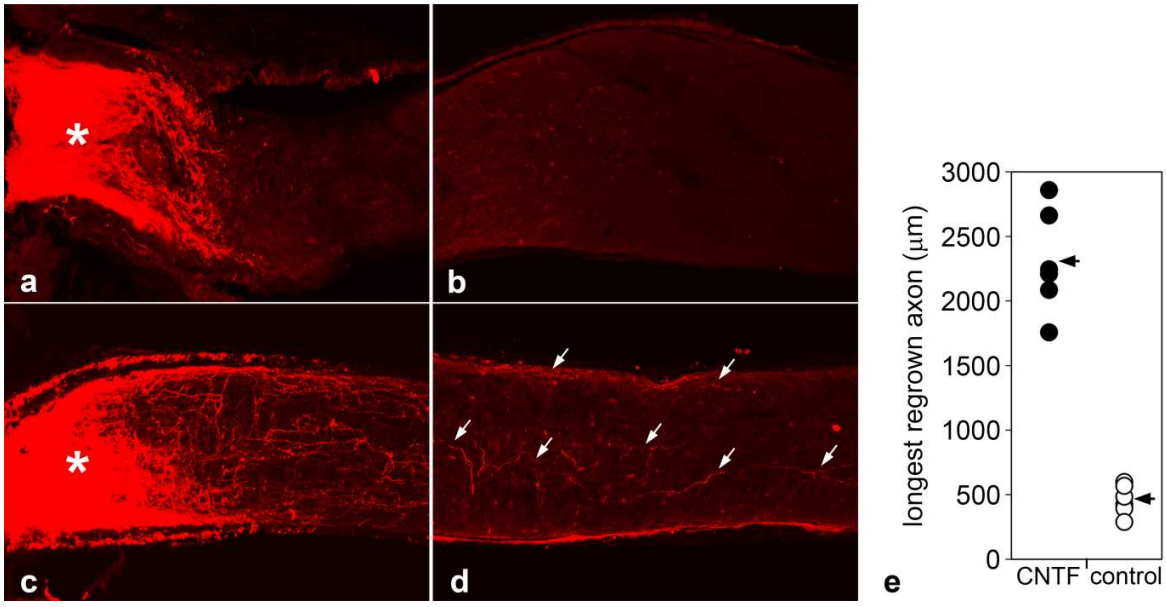

Figure 7: *Long-distance regrowth of axotomized retinal ganglion cell axons in CNTF-treated retinas.*
Retinal ganglion cell axons in eyes with intravitreally grafted control-NS cells (a, b) or CNTF-NS cells (c, d) were anterogradely labeled one month after an intraorbital optic nerve crush. RGC axons 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 1mm 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 1mm 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 to 2,302.7 ±162.8 µm in animals with grafted CNTF-NS cells (n=6 for each experimental group; e). Arrows in (e) indicate mean values.

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 (Borooah et al 2013, Ramsden et al 2013, Rowland et al 2012, West et al 2009). 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 non-glaucomatous optic neuropathies are therefore primarily aimed at delaying the loss of endogenous RGCs rather than at replacing degenerated RGCs (Almasieh et al 2012, Harvey et al 2006, Pascale et al 2012, Qu et al 2010, Wilson & Di Polo 2012).

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 non-viral gene transfer to retina cells (Almasieh et al 2012, Harvey et al 2006, Johnson et al 2011, Wilson & Di Polo 2012). 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 (Zhang et al 2011b). 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 (Harper et al 2011). 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 (Logan et al 2006).

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 these cells are cultivated under adherent conditions in the presence of EGF and FGF-2, they give rise to cultures consisting of homogenous populations of symmetrically dividing clonogenic stem cells which, in analogy to embryonic stem (ES) cells, have been termed NS cells (Conti et al 2005, Pollard et al 2006). To evaluate the efficacy of a 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 (Hellstrom et al 2011, Ji et al 2004, MacLaren et al 2006a, Maier et al 2004, Mey & Thanos 1993, Parrilla-Reverter et al 2009, Pease et al 2009, van Adel et al 2003, Weise et al 2000), and expressed the cytokine in NS cells using polycistronic lentiviral vectors. In a recent study, we have 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 (Jung et al 2013). 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 (Wen et al 2012, Wenzel et al 2005). However, retinas of *Pde6b*^{rd1} and *Pde6b*^{rd10} mice were analyzed already 18 and 16 days after transplantation of the CNTF-NS cells, respectively (Jung et al 2013). 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 (Jung et al 2013) 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 two months, the longest cultivation period evaluated. More importantly, grafted NS

cells survived for at least four 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 poles 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 (Jung et al 2013). Furthermore, we found some small-sized and locally restricted retinal folds in a fraction of eyes with grafted CNTF-NS cells. Retinal folds have also been described in dystrophic and normal retinas of cats that had received repeated intravitreal injections of the human CNTF analogue axokine (Chong et al 1999). Formation of retinal folds thus appears to be among the complications associated with the intraocular delivery of CNTF (Chong et al 1999, Liang et al 2001, Rhee et al 2007, Schlichtenbrede et al 2003, Wen et al 2006), 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 four 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 one, two, three and four months post-lesion interval, 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 of time. Given that intraorbital optic nerve lesions induce a rapid apoptotic degeneration of RGCs (Berkelaar et al 1994), it will be interesting to evaluate the neuroprotective potential of the clonal CNTF-NS cell line in animal models of ocular hypertension which more closely mimic the slowly progressing RGC loss in human glaucoma patients (Johnson & Tomarev 2010, McKinnon et al 2009, Pang & Clark 2007).

In addition to protecting RGCs from degeneration, CNTF has been shown to promote regrowth of injured RGC axons (Hellstrom et al 2011, Leaver et al 2006b, Muller et al 2009, Pernet et al 2013). 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, while axons in eyes with grafted control-NS cells extended for only ~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 (Bull & Martin 2011, Johnson & Martin 2013). While 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 endogenous neurotrophic factors and/or to immune modulatory effects of the grafted cell types. To analyze whether non-modified 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 one 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 time period of four months, the longest post-transplantation time period analyzed. Furthermore, we have 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.

2. Project: Neuroprotection of lesioned retinal ganglion cell axons by a sustained neural stem cell-based intraocular administration of brain derived neurotrophic factor in the adult mouse

ABSTRACT

Purpose: To analyze the neuroprotective effects of a neural stem (NS) cell-based sustained intraocular administration of brain-derived neurotrophic factor (BDNF) on axotomized retinal ganglion cells (RGCs) in the adult mouse.

Methods: A bicistronic lentiviral vector encoding the murine BDNF cDNA and a tdTomato reporter gene fused to a blasticidin resistance gene was used to establish clonal NS cell lines with an ectopic expression of the neurotrophin. The modified NS cell lines were intravitreally grafted into adult mice one day after an intraorbital crush of the optic nerve. The numbers of Brn3-a-positive RGCs in eyes with grafted BDNF-expressing NS cells or control NS cells were determined in flat-mounted retinas 28 and 56 days after the nerve lesion.

Results: The genetically modified NS cells preferentially differentiated into astrocytes that survived in the host eyes and stably expressed the transgenes for at least two months, the longest post-transplantation interval analyzed. Adverse effects of the grafted cells on the morphology of the host retinas were not observed. The number of Brn3-a-positive RGCs in eyes with grafted BDNF-expressing NS cells 28 days and 56 days after the lesion was 2.0-fold and 3.2-fold higher than in eyes with grafted control cells, respectively.

Conclusions: The study demonstrates significant neuroprotective effects of a sustained NS cell-based intraocular administration of BDNF on axotomized RGCs in the mouse. Data suggest that genetically modified clonal NS cell lines represent a valuable tool to evaluate the therapeutic potential of a cell-based intraocular delivery of neurotrophic factors in mouse models of glaucoma.

Introduction

Glaucoma is a neurodegenerative disorder of the inner retina and the second leading cause of blindness in industrialized countries (Quigley & Broman 2006). The disease is characterized by a progressive apoptotic degeneration of ganglion cells in the retina and their axons in the optic nerve, leading to visual field restrictions and eventually to complete loss of vision (Almasieh et al 2012, Qu et al 2010). The pathomechanism of glaucoma is only poorly understood. A variety of risk factors have been implicated in causing this disease, including a decreased thickness of the central cornea (Medeiros et al 2003) or vascular dysfunctions (Quaranta & Floriani 2011, Zeitz et al 2006). Other factors that have been implicated in the progressive degeneration of RGCs include mitochondrial dysfunction, excitotoxic damage, oxidative stress, inflammation, glial cell activation and neurotrophic factor deprivation (Agarwal et al 2009, Almasieh et al 2012, Pascale et al 2012, Qu et al 2010). Clinically, an elevated intraocular pressure (IOP) is considered as the major risk factor for this disease, and lowering IOP is the only proven treatment for glaucomatous optic neuropathies (Varma et al 2008). However, in a significant proportion of glaucoma patients the disease develops despite a clinically normal IOP, or progresses despite successful lowering of the IOP (Caprioli 1997, Leske et al 2003, Varma et al 2008).

The inhibition of pro-apoptotic and the stimulation of pro-survival signaling pathways are among the strategies to develop novel treatments for glaucoma. For instance, intravitreal injection of antibodies against semaphorine, a pro-apoptotic factor (Shirvan et al 2002), or the application of aurintricarboxylic acid (ATA), an antagonist of endonucleases which play a major role in apoptosis (Heiduschka & Thanos 2000), have been shown to prolong the survival of axotomized RGCs *in vivo*. Other studies have proposed that the elevated IOP often associated with glaucoma impairs the axonal transport in RGCs, thereby depriving the ganglion cells from target-derived neurotrophic factors and ultimately causing their apoptotic death (Pease et al 2000, Quigley et al 2000). Neurotrophic factor supplementation has therefore been extensively studied as a potential treatment option for glaucoma, and a variety of neurotrophic factors with neuroprotective activities on RGCs have been identified. For instance, interleukin-6 (IL-6) has been shown to support the survival of RGCs in a dose-dependent manner *in vitro* (Mendonca Torres & de Araujo 2001) and also rescued RGCs challenged by elevated pressure *in vitro* (Sappington et al 2006). Furthermore, ciliary

neurotrophic factor (CNTF) significantly improved survival rates of axotomized RGCs in rats (Lingor et al 2008, Parrilla-Reverter et al 2009) and hamsters (Zhang et al 2005) *in vivo*, and hepatocyte growth factor attenuated the degeneration of RGCs in rats, mice (Tonges et al 2011) and hamsters (Wong et al 2014). Brain derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors (Barde 1994, Barde et al 1982), has been identified as another potent neuroprotective factor for RGCs in a variety of animal models of glaucoma. For instance, intraocular application of BDNF by intravitreal injections of the recombinant protein or adeno-associated virus-mediated gene transfer has been shown to rescue axotomized RGCs in cats (Chen & Weber 2001, Weber & Harman 2013), rats (Di Polo et al 1998, Klocker et al 2000, Koeberle & Ball 2002, Mansour-Robaey et al 1994, Mey & Thanos 1993, Parrilla-Reverter et al 2009, Yan et al 1999), mice (Galindo-Romero et al 2013) or hamsters (Zhang et al 2005). The neurotrophin has also been shown to attenuate RGC degeneration in animal models of ocular hypertension (Fu et al 2009, Harper et al 2011, Martin et al 2003, Ren et al 2012).

Because neurotrophic factors have a short half-life, long-term protective effects require a continuous intraocular supply of these proteins. This aim may be achieved by repeated intraocular injections of the recombinant proteins, or by viral or non-viral gene transfer to endogenous ocular cells (Almasieh et al 2012, Harvey et al 2006, Lebrun-Julien & Di Polo 2008, Wilson & Di Polo 2012). Intraocular transplantations of genetically modified cells represent another strategy to continuously deliver neuroprotective factors to glaucomatous retinas, and has been successfully employed in animal models of optic nerve injury or ocular hypertension (Flachsbarth et al 2014, Harper et al 2011, Logan et al 2006, Wang et al 2002, Zhang et al 2011b). Of note, a sustained cell-based intraocular administration of neurotrophic factors may be of potential interest for clinical applications. In fact, this strategy is currently being evaluated in patients with retinitis pigmentosa or geographic atrophy using intravitreal implants of encapsulated retinal pigment epithelial cells genetically modified to secrete CNTF (MacDonald et al 2007, Tao 2006, Wen et al 2012).

We have recently demonstrated that intravitreal transplantations of clonally derived neural stem cell lines with a forced expression of CNTF or GDNF resulted in significant attenuation of RGC loss in a mouse optic nerve crush model (Flachsbarth et al 2014; our own results, manuscript in preparation). In the present study, we extended this work and established

clonal neural stem cell lines with an ectopic expression of BDNF to study the therapeutic potential of a sustained cell-based administration of the neurotrophin in a mouse model of glaucoma. The modified neural stem cells were intravitreally transplanted into adult mice one day after an intraorbital crush of the optic nerve, and the effect of the grafted stem cells on RGC survival was assessed one and two months after the lesion.

Materials and Methods

Animals

All mice used in this study were maintained on a C57BL/6J genetic background and housed in the animal facility of the University Medical Center Hamburg-Eppendorf according to the institutional guidelines with ad libitum access to food and water. Neural stem cells were isolated from 14-days-old embryos obtained from timed pregnancies. Optic nerve lesions, intravitreal cell transplantations and anterograde axonal tracing experiments were performed on adult (i.e. older than two months) mice. Animal experiments were approved by the University and State of Hamburg Animal Care Committee, and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cloning of the murine BDNF cDNA

For cloning of the murine BDNF cDNA, RNA was prepared from adult mouse brain by incubation in 1ml peqGOLD Trifast (PEQLAB, Erlangen, Germany) for 5 minutes at room temperature and subsequent isolation using the SV Total RNA Isolation System Kit (Promega, Madison, WI) according to the manufacturer's protocol. The RNA was used to generate cDNA in a RT-PCR reaction (table 1 - 4).

Table 1: Composition of the RT-PCR mix for cDNA synthesis

Volume	Ingredient
1 µl	Oligo dt Primer (10 µM; Eurofins Genomics, Ebersberg, Germany)
1 µl	dNTPs (2 mM; Thermo Scientific, Schwerte, Germany)
3 µl	RNA (2 µg)
8 µl	DEPC-H_2O
13 µl	RT-PCR Mix

Table 2: Conditions for the first RT-PCR reaction

Time	Temperature
5 minutes	65℃
1 minute	4℃

Table 3: Composition of final RT-PCR mix

Volume	Ingredient
13 µl	RT-PCR mix
4 µl	First Strand Buffer (5x; Life Technologies, Darmstadt, Germany)
1 µl	DTT (0.1 M; Life Technologies)
1 µl	RiboLock RNase Inhibitor (40U/µl; Thermo Scientific)
1 µl	Superskript III Polymerase (200U/µl; Life Technologies)
20 µl	Final RT-PCR mix

Table 4: Conditions for the final RT-PCR reaction

Time	Temperature
60 minutes	55℃
15 minutes	70℃
pause	4℃

The cDNA was then used in a standard PCR reaction to amplify the BDNF cDNA through the use of specific primers (table 5 – 7).

Table 5: Cloning primers for the neurotrophic factor mBDNF

mBDNF forward	CCTGGATCCGCCACCATGTTCCACCAGGTGAGAAGAGT
mBDNF reverse	CCTGCGGCCGCCTATCTTCCCCTTTTAATGGTCAGTGTACA

Table 6: Composition of PCR mix

Volume	Ingredient
5 µl	Accu Jump Taq Buffer (10x; Sigma-Aldrich, St. Louis, MO)
1,5 µl	dNTPs (2 mM; Thermo Scientific)
2 µl	primer fw (10µM; Eurofins Genomics)
2 µl	primer rev (10µM; Eurofins Genomics)
3 µl	mouse brain DNA (300 ng/50µl)
0,5 µl	Accu Jump Taq (2,5 U/50µl; Sigma-Aldrich)
36 µl	H_2O
50 µl	PCR-mix

Table 7: Conditions for the PCR reaction to amplify the BDNF cDNA

Step	Cycles	Time	Temperature
initial denaturation	1	30 seconds	96℃
denaturation	35	15 seconds	94℃
annealing	35	30 seconds	60℃
elongation	35	2 minutes	68℃
final elongation	1	20 minutes	68℃
pause		-	4℃

The correct size of the PCR product was verified by gel electrophoresis, digested with BamHI and Not I (see table 8 for exact mixture chart), and ligated into a lentiviral "gene ontology" (LeGO) vector (Weber et al 2008, Weber et al 2010) with the help of a T4 DNA Ligase ligation kit (Promega; Table 9).

Table 8: Digestion mix

Volume	Ingredient
3.5 µl	mBDNF cDNA (2 µg/µl) or pCAG-IRES-tdTomato/BSD vector (1,6µg/µl)
1 µl	buffer BamHI (10x; Thermo Scientific, Schwerte, Germany)
0,5 µl	BamHI (10U/µl; Thermo Scientific)
1 µl	Not I (10U/µl; Thermo Scientific)
6 µl	complete mixture

Table 9: Ligation mixture

Volume	Ingredient
3,5 µl	insert (6 µg/µl)
0,5 µl	pCAG-IRES-tdTomato/BSD vector (5 µg/µl)
5 µl	T4 Ligase buffer (2x; Promega)
1 µl	T4 Ligase (3 U/µl; Promega)
10 µl	complete mixture

Lentiviral vectors and production of lentiviral particles

To ectopically express BDNF in neural stem cells, the murine BDNF cDNA was cloned into pCAG-IRES-tdTomato/BSD, giving rise to the bicistronic lentiviral vector pCAG-IRES-tdTomato/BSD. In addition to the BDNF cDNA, this vector encoded the internal ribosome entry site of the encephalomyocarditis virus and a fusion gene composed of a tdTomato reporter gene and a blasticidin resistance gene under regulatory control of the human CMV enhancer/chicken ß-actin (CAG) promoter. The vector pCAG-IRES-tdTomato/BSD was used to generate neural stem cell cultures for control experiments. Lentiviral particles were pseudotyped with the envelope G protein of the vesicular stomatitis virus (VSV-G) and produced by transient transfection of HEK 293T cells using standard protocols as described in detail elsewhere (see: www.LentiGO-Vectors.de).

Isolation, cultivation and transduction of neural stem cells

Neurosphere cultures were established from the cerebral cortex of 14-days-old mouse embryos as described (Ader et al 2000, Pressmar et al 2001). After three passages, neurospheres were enzymatically dissociated using Accutase (PAA Laboratories, Pasching, Austria), and cells were further cultivated under adherent conditions on Matrigel- (BD Bioscience, Heidelberg, Germany) coated tissue culture plastic in DMEM/F12 (Life Technologies, Darmstadt, Germany) supplemented with 2 mM glutamine, 5 mM HEPES, 3

mM sodium bicarbonate, 0.3% glucose (all from Sigma, 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 (TEBU, Offenbach, Germany), and 1% N2 and 1% B27 (Life Technologies) to establish adherently growing NS cell cultures (Flachsbarth et al 2014, Jung et al 2013). NS cells were transduced by spinoculation with pCAG-BDNF-IRES-tdTomato/BSD and pCAG-IRES-tdTomato/BSD to derive BDNF-NS and control-NS cell cultures, respectively and cells were further cultivated in the presence of 4 µg/ml blasticidin (Life Technologies) to select for positive cells. Subsequently, single cells with the highest expression levels of tdTomato were isolated by fluorescence-activated cell sorting (FACS; FACSAriaIIIu, BD Bioscience, San Diego, CA) and clonally expanded to generate clonal BDNF-NS and control-NS cell lines with high expression levels of the transgenes. After five rounds of transductions and subsequent clonal expansions of single cells with highest expression levels of the reporter gene, a BDNF-NS and a control-NS cell line were selected for all further experiments.

Characterization of BDNF-NS and control-NS cell lines in vitro *and* in vivo

To examine expression of BDNF in undifferentiated BDNF-NS and control-NS cells, cultures were fixed in 4% paraformaldehyde (PA; Carl Roth GmbH, Karlsruhe, Germany) in phosphate buffered saline (PBS, pH 7.4), blocked for 1 hour in PBS containing 0.1% bovine serum albumin and 0.3% Triton X-100 (both from Sigma), and incubated with mouse anti-BDNF antibodies (Sigma) overnight at 4℃. Primary a ntibodies were detected with Cy2-conjugated donkey anti-mouse antibodies (Jackson Immunoresearch Laboratories Inc.), and cell nuclei were stained with 4',6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma). To analyze expression of BDNF in differentiated neural cell types *in vitro*, the BDNF-NS and control-NS cell lines were differentiated into neurons or astrocytes according to published protocols (Flachsbarth et al 2014, Jung et al 2013). One week after induction of differentiation, cultures were fixed in 4% PA, blocked, and simultaneously incubated with mouse anti-BDNF antibodies and either rabbit anti-glial fibrillary acidic protein (GFAP) antibodies (DAKO, Glostrup, Denmark) or rabbit anti-ß-tubulin III antibodies (Sigma). Primary antibodies were visualized with Cy2-conjugated donkey anti-mouse and Cy5-conjugated donkey anti-rabbit antibodies (Jackson Immunoresearch Laboratories Inc.),

cultures were stained with DAPI, mounted onto slides, and analyzed with an Olympus FV 1000 confocal microscope (Olympus, Hamburg, Germany). Secretion of the neurotrophin from the modified NS cell lines was evaluated in Western blot analyses of culture supernatants using polyclonal rabbit anti-BDNF antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories Inc.). To estimate the amount of secreted BDNF, 500.000 BDNF-NS cells or control-NS cells were cultivated for 24 hours in 500 µl medium. The culture supernatants and different concentrations of recombinant mouse BDNF (Peprotech, Hamburg, Germany) were subjected to Western blot analyses, and the amount of the mature 14 kDa form of BDNF was estimated by densiometric analyses of immunoreactive bands using ImageJ software (http://imagej.nih.gov/ij/).

Optic nerve lesions, NS cell transplantations and anterograde axonal tracings.

Intraorbital lesions of optic nerves and intravitreal transplantations of BDNF-NS or control-NS cells were performed as described elsewhere (Flachsbarth et al 2014, Jung et al 2013). In brief, animals were deeply anesthetized by intraperitoneal injection of Ketanest S (Pfizer Deutschland GmbH, Berlin, Germany) and Rompun (Bayer Vital GmbH, Leverkusen, Germany), and the optic nerves were intraorbitally crushed for 15 seconds using a watchmaker´s forceps (Bartsch et al 1995, Bartsch et al 1992). Loss of the pupillary light reflex and absence of retinal bleeding or a cataract were considered as criteria for a successful crush. One day after the lesion, animals were again deeply anesthetized, 2µl of vitreous fluid was removed from the eyes using a glass micropipette attached to a syringe, and the same volume of phosphate buffered saline (PBS, pH 7.4) containing 6.4 x 10^5 BDNF-NS or control-NS cells was slowly injected (Flachsbarth et al 2014, Jung et al 2013). In some animals, retinal ganglion cell axons were anterogradely labeled by intravitreal injections of biotin-N-hydroxysuccinimidester (Sigma) one month after the lesion as described elsewhere (Bartsch et al 1995, Flachsbarth et al 2014) to control the optic nerve crush.

Quantitative analysis of RGC survival

Animals were sacrificed one or two months after the optic nerve lesion, and eyes were enucleated and fixed in 4% PA in PBS for 15 minutes. The superior pole of the retinas was marked by a small incision, lenses were removed and retinas were flat-mounted onto nitrocellulose membranes (Sartorius AG, Göttingen, Germany) and fixed again together with the lenses in 4% PA for 1 hour. Retinas were then blocked in PBS containing 0.1% BSA and 1% Triton X-100 for 1 hour and incubated with goat anti-Brn3-a antibodies (Santa Cruz Biotechnology Inc.) overnight at room temperature. Primary antibodies were detected with Cy3-conjugated donkey anti-goat antibodies (Jackson ImmunoResearch Laboratories Inc.), and cell nuclei were stained with DAPI. Retinal flat-mounts were washed three times for 5 minutes each in PBS, mounted onto slides and number-coded. To determine the number of surviving RGCs in eyes grafted with BDNF-NS or control-NS cells, 5 photomicrographs were taken from each retinal quadrant from the optic disc to the periphery using an Olympus IX51 fluorescence microscope (Olympus, Hamburg, Germany), covering a total retinal area of ~1,9 mm^2. All Brn3-a-positive cells on these photomicrographs were counted at a final magnification of x7900 using Adobe Photoshop CS3 software (Adobe Systems Inc., San Jose, CA), and values were used to calculate the number of surviving RGCs per mm^2 retinal area. Six eyes with grafted BDNF-NS cells or control-NS cells from three independent experiments were analyzed for each post-lesion interval (i.e. 1 and 2 months after the crush). Statistical analysis of data was performed using the Student´s t-test. The fixated lenses were used to verify the survival of the transplanted cells in the vitreous chamber by localization of the expressed reporter gene under an Olympus F1000 confocal microscope.

Results

Cloning of murine BDNF from mouse tissue

The murine BDNF cDNA was cloned from adult mouse brain tissue by polymerase chain reaction (PCR). A 774 bp long fragment was detected by gel electrophoresis. Sequence analysis confirmed that the PCR product corresponded to the complete open reading frame of murine BDNF without any mutations.

Lentiviral expression of BDNF in clonally derived NS cell lines

Clonal BDNF-NS and control-NS cell lines with high expression levels of transgenes were established by five rounds of transductions with pCAG-BDNF-IRES-tdTomato/BSD (Fig. 1 a) and pCAG-IRES-tdTomato/BSD (Fig. 1 b) respectively, and subsequent selections of single cells with high expression levels of tdTomato using fluorescence activated cell sorting (FACS) and clonal expansions.

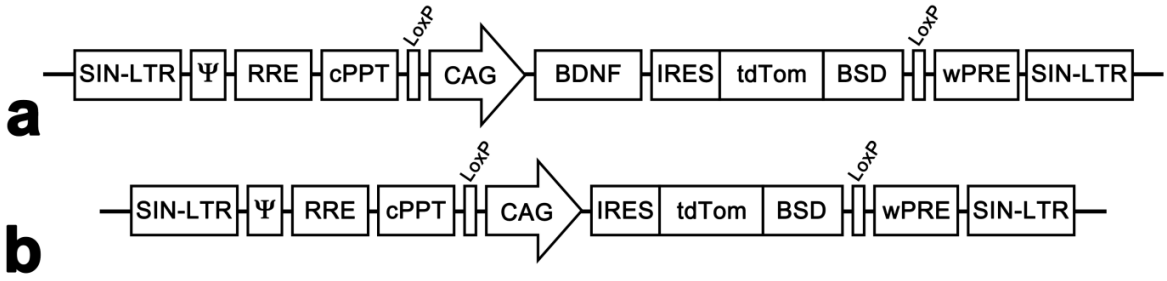

Figure 1: *Lentiviral vectors for genetic modifications of NS cells.*
To establish NS cell lines with an ectopic expression of BDNF, cells were transduced with a bicistronic lentiviral vector composed of the human CMV enhancer/chicken ß-actin (CAG) promoter, the murine BDNF cDNA, the internal ribosome entry site (IRES) of the encephalomyocarditis virus, and a tdTomato reporter gene fused to a blasticidin (BSD) resistance gene (a). The same vector but lacking the BDNF cDNA was used to establish NS cell lines for control experiments (b). Ψ, packaging signal; BDNF, brain-derived neurotrophic factor; 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.

Immunocytochemical analyses of undifferentiated clonal NS cell lines with anti-BDNF antibodies revealed co-expression of the reporter protein tdTomato and BDNF in all cells of the BDNF-NS cell line (Fig. 2 a, b).

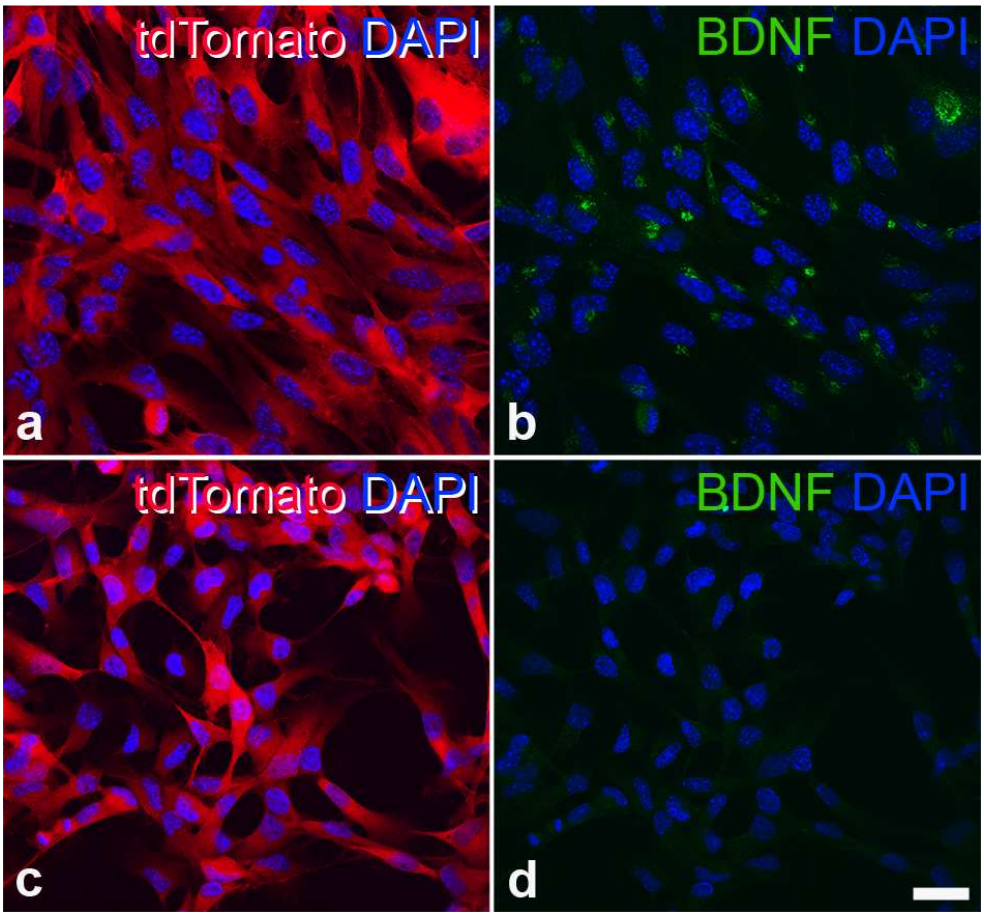

Figure 2: *Expression of BDNF in clonally derived NS cell lines.*
Clonal NS cell lines were established from cultures that were transduced with a lentiviral vector encoding mouse BDNF (a, b) or with a control vector (c, d) using fluorescence-activated cell sorting. Immunocytochemical analyses of these cell lines revealed co-expression of the reporter gene tdTomato (a) and BDNF (b) in all cells of the BDNF-NS cell line. Cells in the control-NS cell line expressed tdTomato (c), but no detectable levels of the neurotrophin (d). BDNF, brain-derived neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole. Bar in (d) for (a-d): 25 µm.

Cells in the control-NS cell line, in comparison, expressed tdTomato but no detectable levels of BDNF (Fig. 2 c, d). Expression of BDNF and tdTomato in the modified NS cell lines were detectable for at least 31 passages, corresponding to a culture period of more than five months, the longest cultivation period analyzed. Secretion of the neurotrophin from the modified NS cells was evaluated in immunoblots of cell culture supernatants. BDNF was detected in the supernatants of the BNDF-NS cell line, but not in the supernatants of the control-NS cell line (Fig. 3).

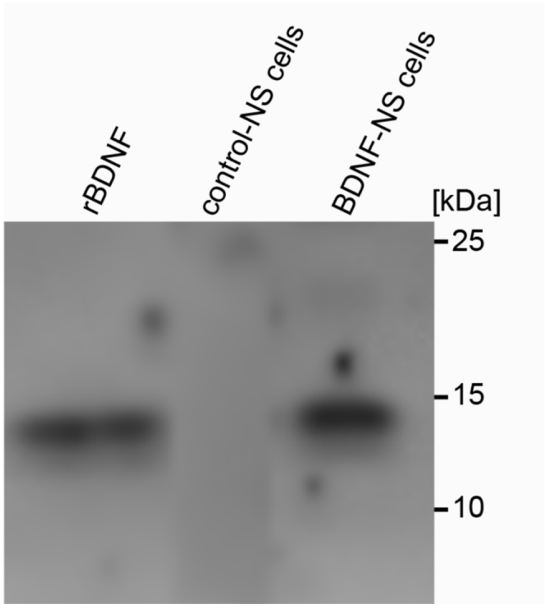

Figure 3: *Immunoblot analysis of culture supernatants from the modified NS cell lines.*
Western blot analysis of culture supernatants revealed secretion of the mature 14 kDa form of BDNF in the BDNF-NS cell line. Supernatants from the control-NS cell line contained no detectable levels of the neurotrophin. Recombinant mouse BDNF was loaded as a reference. BDNF, brain-derived neurotrophic factor; kDa, kilo Dalton; rBDNF, recombinant brain-derived neurotrophic factor

Densiometric analyses of the immunoreactive band of the mature 14 kDa form of BDNF was performed to estimate expression levels of the neurotrophin in the BDNF-NS cell line. Analyses of the cell line at passage four revealed secretion of 133.9 ± 18.8 ng BDNF per 10^5 cells in 24 hours (mean ± SEM from three experiments). BDNF-NS cells at passage 30, in comparison, expressed 136.9 ± 8.4 ng BDNF per 10^5 cells in 24 hours. Together with the immunocytochemical findings, these data indicate stable expression of the neurotrophin in the BDNF-NS cell line.

Expression of BDNF was additionally studied in differentiated neural cell types *in vitro*. To this aim, BDNF-NS and control-NS cells were differentiated into astrocytes or neurons, and cultures were stained with anti-BDNF antibodies. GFAP-positive astrocytes (Fig. 4 a-c) and MAP2-positive neurons (Fig. 4 g-i) derived from the BDNF-NS cell clone coexpressed tdTomato (Fig. 4 a, g) and the neurotrophin (Fig. 4 c, i). Astrocytes (Fig. 4 d-f) and neurons (Fig. 4 j-l) from the control-NS cell line, in comparison, were tdTomato-positive (Fig. 4 d, j), but BDNF-negative (Fig. 4 f, l).

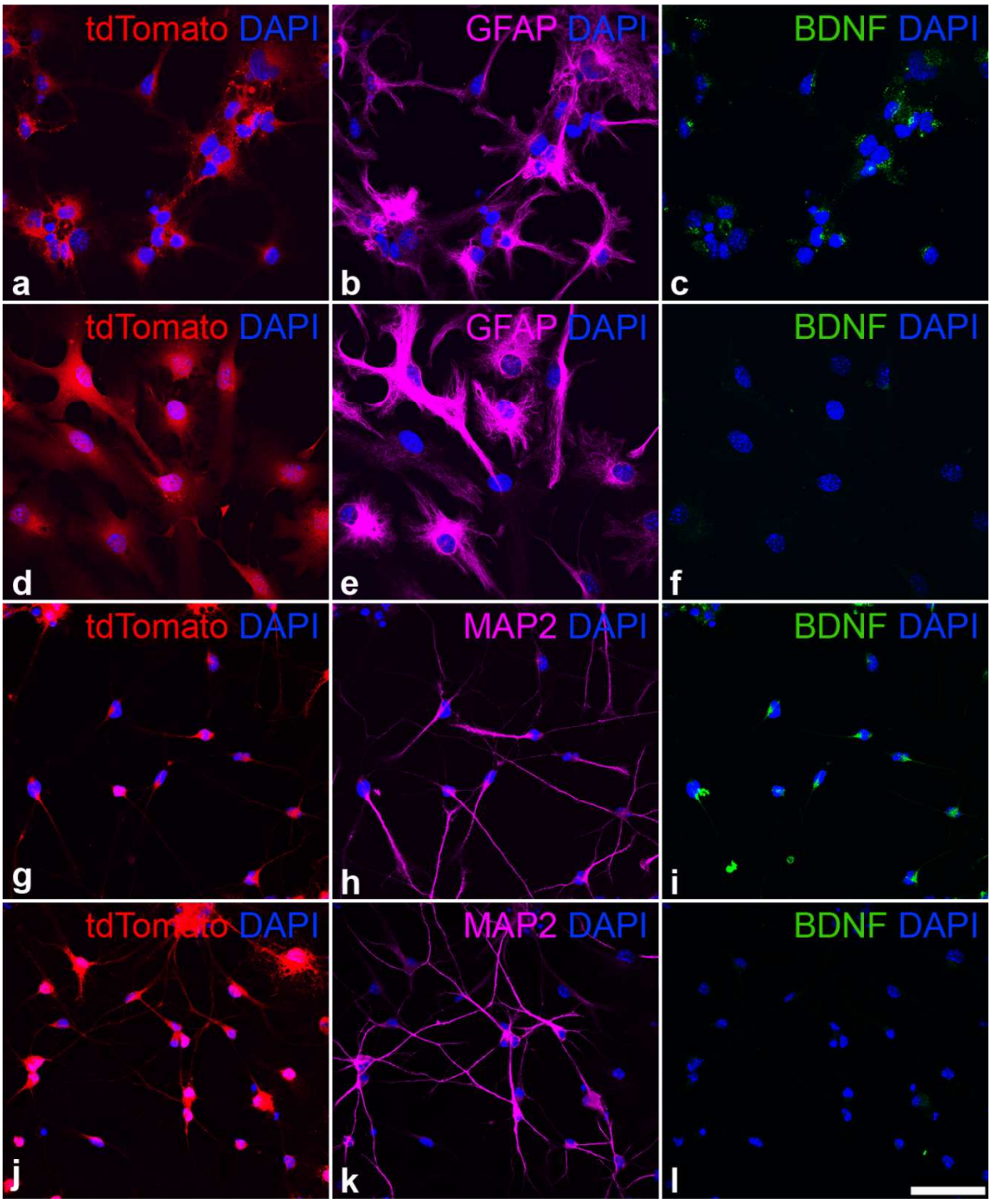

Figure 4: *Expression of BDNF and tdTomato in differentiated clonal NS cell lines.*
To analyze expression of BDNF and tdTomato in differentiated NS cells *in vitro*, the BDNF-NS cell line (a-c, g-i) and the control-NS cell line (d-f, j-l) was differentiated into astrocytes (a-f) or neurons (g-l). One week after induction of differentiation, expression of tdTomato was observed in all GFAP-positive astrocytes (b, e) and MAP2-positive neurons (h, k) derived from both cell lines. Expression of BDNF, in comparison, was only detectable in astrocytes (c) and neurons (i) derived from the BDNF-NS cell line but not in astrocytes (f) and neurons (l) derived from the control-NS cell line. BDNF, brain derived 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.

Intraocular localization and transgene expression in intravitreally grafted NS cell lines

The intraocular localization of the intravitreally grafted NS cells was analyzed two months after the transplantation. Donor cells were identified in the host eyes by their expression of the red fluorescent reporter protein tdTomato. The grafted cells formed a dense cell layer that was attached to the posterior pole of the lenses or the vitreal margin of the host retinas (data not shown). Analyses of retinal flat-mounts showed no adverse effects on any retina that had been transplanted with BDNF-NS or control-NS cells.

Neuroprotection of axotomized RGCs by transplanted BDNF-NS cells

To evaluate whether the BDNF-NS cell line exerted neuroprotective effects on the axotomized RGCs, the numbers of Brn3-a-positive ganglion cells were determined in flat-mounted retinas from eyes with grafted BDNF-NS cells and control-NS cells one and two months after the optic nerve lesion.

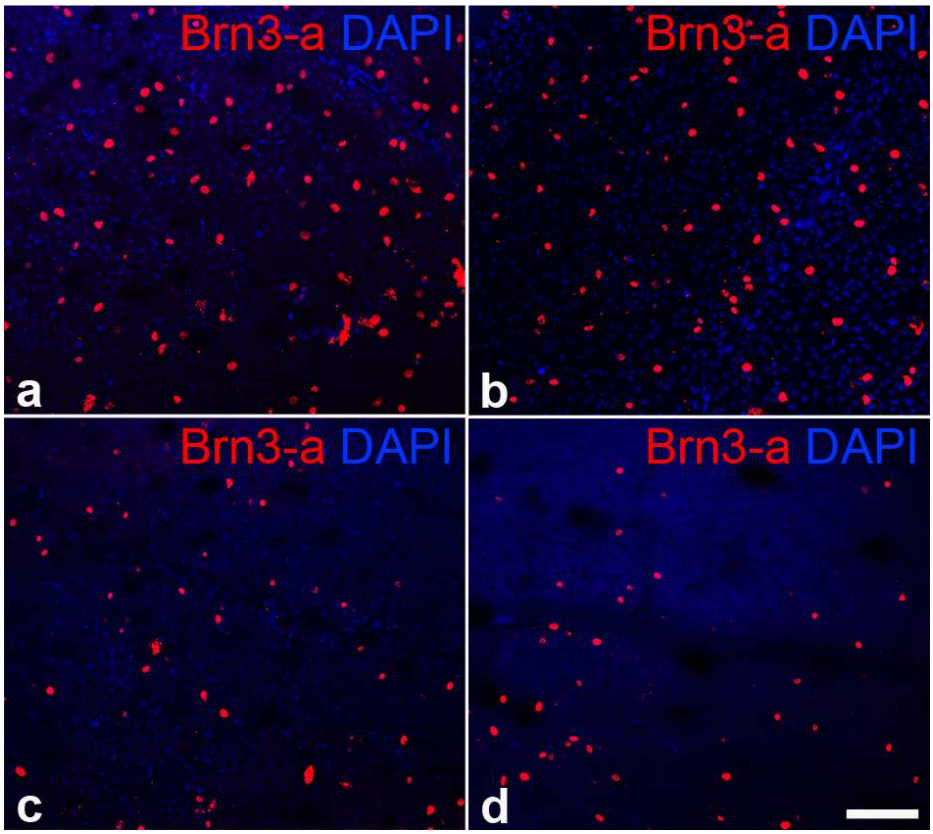

Figure 5: *Intravitreally grafted BDNF-NS cells delay the loss of intraorbitally lesioned retinal ganglion cells.*
BDNF-NS cells (a, b) or control-NS cells (c, d) were intravitreally grafted into adult mice one day after an intraorbital nerve crush, and surviving retinal ganglion cells were visualized one (a, c) and two months (b, d) after the lesion in retinal flat-mounts using anti-Brn3-a antibodies. Note the significantly higher numbers of ganglion cells in the BDNF-treated retinas (a, b) when compared to the control retinas (c, d) at both post-transplantation time points.. DAPI, 4',6-diamidino-2-phenylindole. Bar in d (for a-d): 100 µm.

Qualitative inspection of the retinal flat mounts revealed a higher density of Brn3-a-positive RGCs in BDNF-treated retinas than in control retinas at both, the one month (compare Fig. 5 a and c) and the two months (compare Fig. 5 b and d) post-lesion interval. Determination of RGC numbers in six flat-mounted retinas for each NS cell line and post-lesion time point confirmed a significant rescue of the injured RGCs by the BDNF-NS cells (Fig. 6).

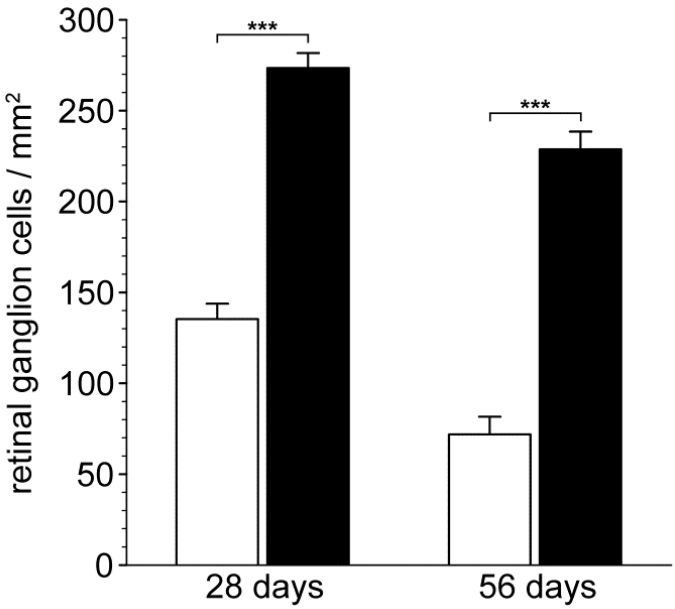

Figure 6: *NS cell-based intraocular delivery of BDNF attenuates the loss of axotomized retinal ganglion cells.*
The number of Brn-3a-positive retinal ganglion cells was determined in eyes with intravitreally grafted control-NS cells (open bars) or BDNF-NS cells (filled bars) one and two month after the optic nerve lesion. Note the significantly higher RGC numbers in BDNF-treated retinas when compared to control retinas at both post-lesion time points. Each bar represents the mean number (±SEM) of retinal ganglion cells per mm^2 from six retinas. ***: $p<0.001$ according to the Student´s t-test.

One month after the lesion, control retinas contained 135.3 ± 8.5 RGCs/mm^2 (mean ± SEM), whereas BDNF-treated retinas contained 273.5 ± 8.5 RGCs/mm^2. At the two months post-lesion time point, we detected 71.9 ± 9.7 Brn3-a-positive RGCs/mm^2 in eyes with grafted control-NS cells as opposed to 228.7 ± 9.7 Brn3-a-positive RGCs/mm^2 in eyes with grafted BDNF-NS cells. The difference in the number of RGCs between control and BDNF-treated retinas was statistically significant at both post-lesion time points ($p<0.001$ according to the Student´s t-test; Fig. 6). Anterograde axonal tracing experiments performed on a fraction of experimental mice confirmed complete transection of retinal ganglion cell axons in all animals.

Discussion

Neurotrophic factor deprivation is discussed as a potential reason for the loss of retinal ganglion cells (RGCs) during disease progression of glaucoma (Almasieh et al 2012, Johnson et al 2011), the second leading cause for blindness in industrialized countries (Quigley & Broman 2006, Varma et al 2008). The most probable cause for neurotrophic factor deprivation in glaucomatous eyes is an impaired axonal transport due to enhanced intraocular pressure, the main risk factor in glaucoma. Supplementation of glaucomatous retinas with exogenous neurotrophic factors is therefore among the strategies aimed at establishing effective treatments for this neurodegenerative retinal disease. Since neurotrophic factors usually have short half-life times and do not cross the blood-retina barrier, intraocular delivery systems have been established that allow a sustained local supply of neuroprotective reagents to the diseased retina. Delivery systems that have been successfully used to achieved a sustained intraocular administration of neurotrophic factors include factor-loaded slow-release devices that are implanted into the diseased eyes, or a viral or non-viral gene transfer to endogenous ocular cells (Almasieh et al 2012, Harvey et al 2006, Johnson et al 2011, Wilson & Di Polo 2012).

Transplantations of genetically modified cells that overexpress neurotrophic factors represent another strategy to continuously deliver these factors to glaucomatous retinas. For example, intravitreal injections of genetically modified neural progenitor cells with an ectopic expression of BDNF (Wang et al 2002), implantations of encapsulated cells that were modified to secrete glucagon-like peptide-1 (Zhang et al 2011b), intravitreal injections of mesenchymal stem cells modified to secrete BDNF (Harper et al 2011), or intravitral transplantations of fibroblasts with a forced expression of FGF-2, NT-3 and BDNF (Logan et al 2006) have all been demonstrated to confer neuroprotective effects on RGCs in animal models of glaucoma. Furthermore, we have recently demonstrated that intravitreal transplantations of a clonally derived neural stem (NS) cell line lentivirally modified to secrete CNTF resulted in long-term protection of axotomized RGCs, and stimulated long distance axonal regeneration in a mouse optic nerve crush model (Flachsbarth et al 2014). Using the same approach, we additionally have shown that a GDNF-expressing NS cell line also significantly attenuated RGC loss in a mouse optic nerve crush model (our own results, manuscript in preparation). More importantly, we observed a markedly enhanced synergistic

neuroprotective effect of GDNF and CNTF in animals that had received intravitreal injections of a 1:1 mixture of a GDNF- and a CNTF-expressing NS cell line (our own results, manuscript in preparation).

In the present study, we used a bicistronic lentiviral vector to generate a clonal NS cell line with a forced expression of BDNF, another neurotrophic factor that has been shown to confer potent neuroprotective effects on RGCs (Chen & Weber 2001, Harper et al 2011, Koeberle & Ball 2002, Peinado-Ramon et al 1996). To increase expression levels of the neurotrophin in NS cells, cells were subjected to five rounds of transductions and subsequent clonal expansions of single cells with the highest expression levels of BDNF, as previously described for the generation of CNTF- or GDNF-expressing NS cell clones (Flachsbarth et al 2014, Jung et al 2013). We then selected one clonal cell line to evaluate the efficacy of a sustained cell-based intraocular administration of BDNF to attenuate a lesion-induced degeneration of RGCs. When the BDNF-expressing NS cells were intravitreally grafted into adult mice one day after an intraorbital crush, cells attached to the posterior poles of the lenses and the vitreal surfaces to the host retinas where they preferentially differentiated into astrocytes. The donor-derived astrocytes survived in the vitreous cavity and expressed BDNF for at least two months (the longest post-lesion interval analyzed), in close analogy to our observations with CNTF- or GDNF-expressing NS cells (Flachsbarth et al 2014; our own results, manuscript in preparation). Integration of the grafted cells into the host retinas, or adverse effects of the donor cells or the sustained intraocular administration of BDNF on the morphology of the host retinas was not apparent. More importantly, the sustained secretion of BDNF from the transplanted BDNF-NS cells led to a significant protection of axotomized RGCs, with BDNF-treated retinas containing 2.0- and 3.2-fold more surviving RGCs than control retinas at the one and two months post-lesion time point, respectively. In addition to our results obtained with CNTF- and GDNF-expressing NS cell lines, these results thus add another example that lentivirally modified NS cells represent a valuable tool to continuously administer functionally relevant amounts of a neurotrophic factor to glaucomatous retinas. Given that an intraorbital optic nerve lesion induces a rapid apoptotic degeneration of RGCs (Berkelaar et al 1994), it will be interesting to evaluate the neuroprotective potential of the BDNF-secreting NS cell line in animal models that mimic more closely the slowly progressing RGC loss in human glaucoma

patients (Johnson & Tomarev 2010, McKinnon et al 2009, Pang & Clark 2007). The BDNF-NS cell line additionally represents an interesting tool for combinatorial neuroprotective approaches aimed at identifying factor combinations that exert additive or synergistic rescue effects on RGCs.

Previous studies have identified a variety of cell types that are capable to rescue RGCs under different pathological conditions without genetic modifications prior to transplantation, including such diverse cell types as oligodendrocyte precursor cells (Bull et al 2009), mesenchymal stem cells (Johnson et al 2010), olfactory ensheathing cells (Wu et al 2010), bone-marrow mononuclear cells (Zaverucha-do-Valle et al 2011), dental pulp cells (Mead et al 2013) and umbilical cord blood stem cells (Zhao et al 2011). To analyze whether non-modified NS cells also exert neuroprotective activities on RGCs, we analyzed retinas that had received transplantations of control NS cells and retinas that had received injections of PBS, and found no significant difference in the number of surviving RGCs.

Together, we have achieved a continuous administration of functionally relevant quantities of BDNF to glaucomatous retinas through intravitreal transplantations of a NS cell line lentivirally modified to express high levels of this neurotrophin. Significant protection of axotomized RGCs over an extended period of time by the grafted BDNF-secreting NS cells confirms and extends similar results obtained with CNTF- or GDNF-secreting NS cell lines. In future work, it will be interesting to analyze the neuroprotective potential of the BDNF-NS cell line in glaucoma models which more closely mimic the slowly progressing RGC loss in human glaucoma patients. The BDNF-NS cell line additionally represents an interesting tool for combinatorial neuroprotective approaches aimed at identifying factor combinations that exert additive or synergistic neuroprotective effects in animal models of glaucomatous optic neuropathies or other degenerative retinal disorders characterized by a progressive loss of RGCs.

3. Project: Glial cell line-derived neurotrophic factor and ciliary neurotrophic factor synergistically attenuate the loss of axotomized retinal ganglion cells in the adult mouse

Abstract

Purpose: To analyze the neuroprotective effects of a simultaneous cell-based intraocular administration of ciliary neurotrophic factor (CNTF) and glial cell line-derived neurotrophic factor (GDNF) on intraorbitally lesioned retinal ganglion cells (RGCs).

Methods: Clonally derived neural stem (NS) cell lines with an ectopic expression of CNTF or GDNF were established using polycistronic lentiviral vectors. The cell lines were intravitreally grafted either separately or as a mixture into adult mice one day after an intraorbital optic nerve crush. The number of Brn3a-positive RGCs was determined in flat-mounted retinas 2, 4 and 8 weeks after the optic nerve lesion to assess the neuroprotective effects of the grafted cells..

Results: The grafted NS cells preferentially differentiated into astrocytes that survived in the host eyes and stably expressed the transgenes for at least two months. The degeneration of the axotomized RGCs was significantly attenuated to a similar extent by the grafted CNTF- and GDNF-secreting NS cells at all postlesion intervals analyzed. Two months after the nerve crush, CNTF- and GDNF-treated retinas contained 4.1-fold and 3.8-fold more RGCs respectively, than retinas with grafted control NS cells. Of note, the neuroprotective effect on injured RGCs was markedly increased when both factors were administered simultaneously. Retinas from eyes with grafted CNTF- and GDNF-secreting NS cells contained 14.3-fold more RGCs than control retinas two months after the lesion.

Conclusions: A sustained cell-based intraocular administration of CNTF and GDNF results in synergistic neuroprotective effects on RGCs in a mouse optic nerve crush model.

Introduction

Glaucoma is one of the leading causes of blindness in the industrialized countries. It is a neurodegenerative disorder of the inner retina that is characterized by a progressive apoptotic degeneration of retinal ganglion cells (RGCs). The loss of the RGCs and their axons, which made up the optic nerve, results in a progressive loss of vision, and eventually in irreversible blindness (Almasieh et al 2012, Qu et al 2010, Quigley 1993, Quigley & Broman 2006). An elevated intraocular pressure (IOP) is widely considered as an important contributing factor to disease progression, and lowering of IOP is until now the only successful treatment of the disease. However, many patients suffer from glaucoma even after successful lowering of the IOP, indicating that the pathomechanism of this neurodegenerative disorder is complex (Caprioli 1997, Coleman & Miglior 2008, Leske et al 2003, Sommer et al 1991). In fact, a number of additional risk factors are discussed to contribute to the loss of RGCs in glaucoma, including mitochondrial or vascular dysfunction, inflammation and excitotoxic or oxidative stress (Agarwal et al 2009, Almasieh et al 2012, Qu et al 2010). Deprivation of neurotrophic factors as a result of a disturbed axonal transport is discussed as another potential cause of glaucoma (Anderson & Hendrickson 1974, Pease et al 2000, Quigley et al 2000). Accordingly, supplementation of neurotrophic factors has been studied extensively as a potential treatment option for this disease. Several preclinical studies have indeed demonstrated that supplementation of neurotrophic factors results in significant attenuation of RGC loss in a variety of animal models of glaucoma. Ciliary neurotrophic factor (CNTF) (Hellstrom et al 2011, van Adel et al 2005), glial cell line-derived neurotrophic factor (GDNF) (Ozden & Isenmann 2004, Ward et al 2007), brain-derived neurotrophic factor (BDNF) (Chen & Weber 2001, Harper et al 2011), erythropoietin (EPO) (King et al 2007) or vascular endothelial growth factor B (VEGF-B) (Li et al 2008) are among the neuroprotective factors that have been shown to delay RGC degeneration, to mention only a few. A variety of methods has been used to deliver these proteins to glaucomatous retinas, including intravitreal injections of recombinant proteins (Lingor et al 2008, Parrilla-Reverter et al 2009), or the ectopic expression of these factors in endogenous retinal cells through intraocular injections of adeno-associated viral vectors (Leaver et al 2006b, Ozden & Isenmann 2004, Schmeer et al 2002), lentiviral vectors (van Adel et al 2003) or non-viral vectors. A sustained intraocular administration of neuroprotective factors has also been achieved through intraocular transplantations of cells that had been genetically modified to

express these factors (Castillo et al 1994, Harper et al 2011, Hu et al 2005, Ningli et al 1997). Using these different delivery approaches, significant attenuation of RGC loss has been demonstrated in such diverse glaucoma models as the laser-induced hypertension model (Harper et al 2011, Pease et al 2009), the optic nerve crush model (Chen & Weber 2001, Leaver et al 2006a, MacLaren et al 2006a), the optic nerve transection model (Hellstrom et al 2011, Koeberle & Ball 2002, Ozden & Isenmann 2004) or the MOG-induced experimental autoimmune encephalomyelitis model (Maier et al 2004).

Studies that have evaluated the therapeutic potential of neurotrophic factor supplementation in animal models of glaucoma have usually tested the potential of one specific factor to rescue RGCs from degeneration. However, given the large number of proteins that exert neuroprotective effects on RGCs, it is reasonable to assume that the co-administration of two or more neurotrophic factors that activate different pro-survival signaling pathways in RGCs will result in more pronounced neuroprotection than the separate administration of each individual factor. Some studies have tested this hypothesis by simultaneously administrating two or more factors known to protect RGCs, including BDNF, CNTF, GDNF or Neurturin, and have indeed observed additive or synergistic neuroprotective effects in the optic nerve transection or laser-induced hypertension model (Koeberle & Ball 2002, Pease et al 2009, Yan et al 1999, Zhang et al 2005).

One of the most prolific neurotrophic factors in the context of glaucoma is ciliary neurotrophic factor (CNTF), which has been extensively studied in various animal models of RGC loss (Hellstrom et al 2011, Ji et al 2004, MacLaren et al 2006a, Maier et al 2004, Mey & Thanos 1993, Parrilla-Reverter et al 2009, Pease et al 2009, van Adel et al 2003, Weise et al 2000). The protein belongs to the interleukin- (IL) 6 family of cytokines (Bauer et al 2007) and is one of the first neurotrophic factors identified (Helfand et al 1976, Varon et al 1979). CNTF exerts its neuroprotective activity through binding to a receptor complex composed of CNTF receptor alpha (CNTFRα) and two signal-transducing transmembrane subunits, gp130 and leukaemia inhibitory factor receptor ß (LIFRß) (Boulton et al 1994, De Serio et al 1995, Stahl & Yancopoulos 1994, Thanos & Emerich 2005), which activate the signal transducer and activator of transcription 3 (STAT3), STAT1 and extracellular-signal-regulated kinase (ERK) signaling pathways (Heinrich et al 1998, Leonard & O'Shea 1998, Lutticken et al 1994, Meyer-Franke et al 1995, Raz et al 1994, Segal & Greenberg 1996). Another neurotrophic factor that exerts a potent neuroprotective activity on RGCs is glial cell

line-derived neurotrophic factor (GDNF). GDNF belongs to the glial cell line-derived neurotrophic factor family of ligands (GFL) and exerts its anti-apoptotic effects through binding to the receptor GFRα1-α4 and activation of the GFRα-Ret complex. Pro-survival signaling pathways that are activated through binding of GDNF to its receptor include the PI3K/Akt-, ERK- and PLC-γ-pathway (Maruyama & Naoi 2013). Similar to CNTF, GDNF has been demonstrated to effectively rescue RGCs in various animal models of glaucoma (Klocker et al 1997, Koeberle & Ball 2002, Ozden & Isenmann 2004, Schmeer et al 2002, Ward et al 2007).

Because neurotrophic factors have a short half-life and do not ordinarily cross the blood-retina barrier, they cannot be administered systemically. To achieve significant neuroprotective effects, methods have therefore been developed that allow a continuous intraocular supply of these proteins. In preclinical studies, this aim has been usually achieved through the forced expression of these proteins in endogenous retinal cells using viral or non-viral expression vectors (Di Polo et al 1998, MacLaren et al 2006a, Pease et al 2009, Schmeer et al 2002, van Adel et al 2005, van Adel et al 2003, Weise et al 2000). A sustained delivery of neuroprotective factors to the glaucomatous retina may also be achieved through intraocular transplantations of cells that have been genetically modified to ectopically express these proteins (Harper et al 2009, Harper et al 2011, Hu et al 2005, Ningli et al 1997, Yu et al 2006). A sustained cell-based intraocular administration has several advantages compared to other delivery strategies, and may therefore be of potential interest not only for preclinical research but also for clinical applications. For instance, this approach circumvents the need to genetically modify endogenous retinal cells. In addition, the amount of neurotrophic factors delivered to the retina can be controlled by determining expression levels in the modified cells prior to transplantation. Furthermore, the modified cells can be encapsulated in devices that then can be implanted into the vitreous cavity. The devices allow diffusion of the neurotrophic factors from the modified cells to the retina, and nutrition of the encapsulated cells from the vitreous fluid through semipermeable membranes. In addition, they protect the grafted cells from the immune system of the host, and *vice versa* the host eyes from potential adverse effects of the grafted cells. The use of encapsulated cell implants additionally adds an important safety aspect, in that the implant can simply be retrieved from the vitreous in case of complications (Tao 2006, Thanos & Emerich 2005, Wen et al 2012). Of note, a cell-based sustained administration of CNTF is

currently being evaluated in clinical trials in patients with retinitis pigmentosa or geographic atrophy (Birch et al 2013, Kauper et al 2012, Sieving et al 2006, Talcott et al 2011, Zhang et al 2011a).

In the present study we generated a series of clonal neural stem cell lines with an ectopic expression of either CNTF or GDNF. We then selected a CNTF- and a GDNF-expressing cell line exerting similar neuroprotective effects on axotomized RGCs, and grafted the cell lines either separately or as a mixture into the vitreous cavity of adult mice one day after an optic nerve crush. The numbers of surviving RGCs were determined 14, 28 and 56 days after the crush in retinal flat-mounts to analyze both neurotrophic factors for potential additive or synergistic neuroprotective activities on lesioned RGCs.

Materials and Methods

Animals

All experiments were performed on C57BL/6J mice that were obtained from the animal facility of the University Medical Center Hamburg-Eppendorf, and housed according to the institutional guidelines with ad libitum access to food and water. Neural stem (NS) cells were isolated from the cerebral cortex of 14-days-old embryos. Intraorbital optic nerve lesions and intravitreal cell transplantation experiments were performed on adult (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.

Lentiviral vectors

NS cells were genetically modified with polycistronic lentiviral vectors that are based on the lentiviral "gene ontology" (LeGO) vectors (Weber et al 2008, Weber et al 2010). To express CNTF in NS cells (CNTF-NS cells), a secretable variant of mouse CNTF was cloned into the vector pCAG-IRES-Venus-2A-ZEO encoding the internal ribosome entry site (IRES) 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 ß-actin (CAG) promoter, giving rise to pCAG-mCNTF-IRES-Venus-2A-ZEO (Flachsbarth et al 2014, Jung et al 2013). To express GDNF in NS cells (GDNF-NS cells), we generated the bicistronic lentiviral vector pCAG-mGDNF-IRES-eGFP-NEO, encoding the cDNA of mouse GDNF, an IRES sequence, and a fusion gene composed of an enhanced green fluorescent protein (eGFP) reporter gene and a neomycin resistance gene under regulatory control of the CAG promoter. NS cells for control experiments (control-NS cells) were transduced with pCAG-IRES-Venus-2a-ZEO. Lentiviral particles were pseudotyped with the envelope G protein of the vesicular stomatitis virus and produced according to standard protocols as described elsewhere (www.LentiGO-Vectors.de).

Cultivation and transduction of NS cells

NS cells were derived from neurosphere cultures of the embryonic cerebral cortex, and cultivated under adherent conditions on tissue culture plastic coated with 0.1% Matrigel (BD Bioscience, Heidelberg, Germany) in DMEM/F12 (Life Technologies, Darmstadt, Germany)

supplemented with 2 mM glutamine, 5 mM HEPES, 3 mM sodium bicarbonate, 0.3% glucose (all from Sigma, 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), and 1% N2 and 1% B27 (both from Life Technologies) (Conti et al 2005, Flachsbarth et al 2014, Jung et al 2013). Cells were transduced in 24 well plates by spinoculation in the presence of 8 µg/ml hexadimethrine bromide (Polybrene; Sigma). Positive cells were selected by further cultivation in NS cell medium supplemented with EGF, FGF-2, N2, B27 and 200 µg/ml zeocin (Invivogen, San Diego, CA) or 200 µg/ml G418 (Life Technologies). To establish clonally derived CNTF-NS, GDNF-NS or control-NS cell lines with high expression levels of the transgenes, single cells with the highest expression level of the reporter gene were isolated by fluorescence-activated cell sorting (FACS; FACSAriaIIIu, BD Bioscience, San Diego, CA), and clonally expanded.

Intraorbital optic nerve crush and intravitreal NS cell transplantations

Animals were deeply anesthetized by an intraperitoneal injection of Ketanest S (Pfizer Deutschland GmbH, Berlin, Germany) and Rompun (Bayer Vital GmbH, Leverkusen, Germany), and the optic nerves were intraorbitally crushed for 15 sec using a watchmaker´s forceps (Bartsch et al 1995, Bartsch et al 1992, Flachsbarth et al 2014). 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. One day after the lesion, animals were again deeply anesthetized, and 2µl of vitreous fluid was slowly removed from the eyes using a glass micropipette attached to a syringe. The same volume of phosphate buffered saline (PBS, pH 7.4) containing 7.6 x 10^5 CNTF-NS cells, GDNF-NS cells or control-NS cells, or 7.6 x 10^5 cells of a 1:1 mixture of CNTF-NS and GDNF-NS cells was slowly injected into the vitreous cavity (Flachsbarth et al 2014, Jung et al 2013). Care was taken to not damage the lens during removal of the vitreous fluid or injection of the cells. In some animals, RGC axons were anterogradely labeled with biotin-N-hydroxysuccinimidester (Sigma) to control the optic nerve crush. The tracer was dissolved in dimethylformamide (Carl Roth GmbH, Karlsruhe, Germany), diluted 1:1 with ethanol, and intravitreally injected into animals with lesioned optic nerves (Bartsch et al 1995, Flachsbarth et al 2014). One day later, eyes with attached optic nerves were immersion-fixed in PBS containing 4% paraformaldehyde (PA; Carl Roth GmbH), cryoprotected and frozen. Longitudinal sections of optic nerves were

prepared at a thickness of 25 µm, and stained with Cy3-conjugated Streptavidin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA).

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

Expression of GDNF and CNTF in undifferentiated NS cells was analyzed in cultures composed of a 1:1 mixture of GDNF-NS and CNTF NS cells. Cells were fixed in 4% PA, blocked in PBS containing 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 (both from Sigma), and simultaneously incubated with goat anti-GDNF antibodies (R&D Systems Inc., Minneapolis, MN) and rabbit anti-CNTF antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Primary antibodies were detected with Cy3-conjugated donkey anti-goat and Cy5-conjugated donkey anti-rabbit antibodies (both from Jackson ImmunoResearch Laboratories Inc.), and cell nuclei were stained with 4',6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma). To analyze expression of GDNF in differentiated neural cell types, GDNF-NS cells were differentiated into astrocytes or neurons as described (Flachsbarth et al 2014, Jung et al 2013). Differentiated cultures were fixed in 4% PA, blocked, and incubated with goat anti-GDNF antibodies and rabbit anti-glial fibrillary acidic protein (GFAP) antibodies (DAKO, Glostrup, Denmark) or mouse anti-microtubule associated protein 2 (MAP2) antibodies (Sigma). Primary antibodies were visualized with Cy3- and Cy5-conjugated secondary antibodies (all from Jackson Immunoresearch Laboratories Inc.), and cultures were stained with DAPI. Secretion of GDNF and CNTF was evaluated by Western blot analyses of culture supernatants from GDNF-NS or CNTF-NS cell lines using goat anti-GDNF and rabbit anti-CNTF antibodies, and horseradish peroxidase-conjugated donkey anti-goat or goat anti-rabbit secondary antibodies (both from Jackson ImmunoResearch Laboratories Inc.). To estimate the amount of secreted GDNF or CNTF, 500.000 GDNF-NS cells, CNTF-NS cells or control-NS cells from low and high passages were cultivated for 24 hours in 500 µl medium. Defined volumes of the supernatants and a series of different concentrations of recombinant mouse GDNF (Peprotech, Hamburg, Germany) or mouse CNTF (Biomol, Hamburg, Germany) were subjected to Western blot analysis. Densiometric analysis of immunoreactive bands from three different immunoblots for CNTF or GDNF was performed using ImageJ software (http://imagej.nih.gov/ij/).

For characterization of grafted NS cells *in vivo*, animals were sacrificed two months after transplantation, and eyes were enucleated and fixed for 1 hour in 4% PA. The lenses were

removed, blocked in PBS containing 0.1% BSA and 1% Triton X-100, and simultaneously incubated with goat anti-GDNF and rabbit anti-CNTF antibodies to monitor expression of the neurotrophic factors. Lenses were also stained with rabbit anti-GFAP antibodies or rabbit anti-ß-tubulin III antibodies (Sigma) to evaluate neural differentiation of the grafted cells. Primary antibodies were detected with donkey anti-goat Cy3- and donkey anti-rabbit Cy3- or Cy5-conjugated secondary antibodies, cell nuclei were stained with DAPI, and lenses with attached donor cells were analyzed with an Olympus FV 1000 confocal microscope (Olympus, Hamburg, Germany).

Quantitative analysis of RGC survival

Animals were sacrificed 14, 28 and 56 days after the optic nerve crush, and eyes were enucleated and immersion-fixed in 4% PA in PBS for 15 minutes. The superior pole of each retina was marked by a small incision before retinas were flat-mounted on nitrocellulose membranes (Sartorius AG, Göttingen, Germany). For immunohistochemical stainings, flat-mounted retinas were again fixed in 4% PA for 1 hour, blocked in PBS containing 0.1% BSA and 1% Triton X-100 for 1 hour, and incubated with goat anti-Brn3-a antibodies (Santa Cruz Biotechnology Inc.) overnight at room temperature. Primary antibodies were detected with C3-conjugated donkey anti-goat antibodies, retinal flat-mounts were stained with DAPI, washed with PBS and mounted onto slides. For determination of RGC numbers, retinal flat-mounts were number-coded and 5 photomicrographs were taken from each retinal quadrant from the optic disc to the retinal periphery using an Olympus IX51 fluorescence microscope (Olympus, Hamburg, Germany), corresponding to a retinal area of ~1.9 mm^2. All Brn3-a-positive RGCs on the 20 photomicrographs were counted at a final magnification of x7900 using Adobe Photoshop CS3 software (Adobe Systems Inc., San Jose, CA), and the number of RGCs per mm^2 was calculated. Six eyes with grafted CNTF-NS cells, GDNF-NS cells, CNTF- and GDNF-NS cells, or control-NS cells were analyzed for each post-lesion interval (i.e. 14, 28 and 56 days after the crush). RGC numbers in CNTF-treated eyes 28 and 56 days after the lesion were taken from a previous study (Flachsbarth et al 2014). Statistical analysis of data was performed using the Student´s t-test.

Results

Generation of GDNF- and CNTF-secreting clonal neural stem cell lines

Neurosphere cultures were established from the cerebral cortex of embryonic mice and further cultivated under adherent conditions on Matrigel-coated culture plastic in the presence of EGF and FGF-2 to generate neural stem (NS) cell cultures. Cultures maintained under these conditions have recently been shown to consist of a homogenous population of clonogenic and symmetrically dividing stem cells that retain a high neuronal and glial differentiation potential after prolonged passaging (Conti et al 2005). To express GDNF or CNTF in NS cells, cells were transduced with lentiviral vectors encoding either a secretable variant of mouse CNTF or mouse GDNF together with a reporter and a resistance gene. The generation of a NS cell line with high expression levels of CNTF (CNTF-NS cells) by several rounds of transductions, selection of single cells with high expression levels of the reporter gene Venus, and subsequent clonal expansion has been described in a recent study (Flachsbarth et al 2014). In the present study, we used the same strategy to establish a clonal NS cell line with high expression levels of mouse GDNF and the reporter gene eGFP (GDNF-NS cells). NS cells for control experiments were transduced with a lentiviral vector encoding a Venus reporter gene and a zeocin resistance gene.

Characterization of GDNF- or CNTF-secreting clonal NS cell lines

From several established clonal GDNF-NS cell lines, we selected one clone for further characterization that exerted a similar neuroprotective activity on axotomized RGCs (see below) as a previously established CNTF-NS cell line (Flachsbarth et al 2014). To confirm expression of GDNF and CNTF in the cell lines used in this study, both clones were mixed at a 1:1 ratio and immunostained with anti-GDNF and anti-CNTF antibodies (Fig. 1 a, b). All cells in these cultures either co-expressed GDNF and eGFP or CNTF and Venus (Fig. 1 a, b). Control-NS cells were stained with both antibodies in parallel and expressed Venus (Fig. 1 c) but no detectable levels of GDNF or CNTF (Fig. 1 d). Of note, immunocytochemical analyses revealed expression of GDNF and CNTF in both clonal cell lines for more than 30 passages, indicating stable expression of the transgenes. Western blot analyses of culture supernatants from the GDNF-NS cell line (Fig. 1 e) or the CNTF-NS cell line (Fig. 1 f) confirmed secretion of the neurotrophic factors from the modified NS cells. Cell culture

supernatants from control-NS cells, in comparison, contained no detectable levels of GDNF (Fig. 1 e) or the cytokine (Fig. 1 f).

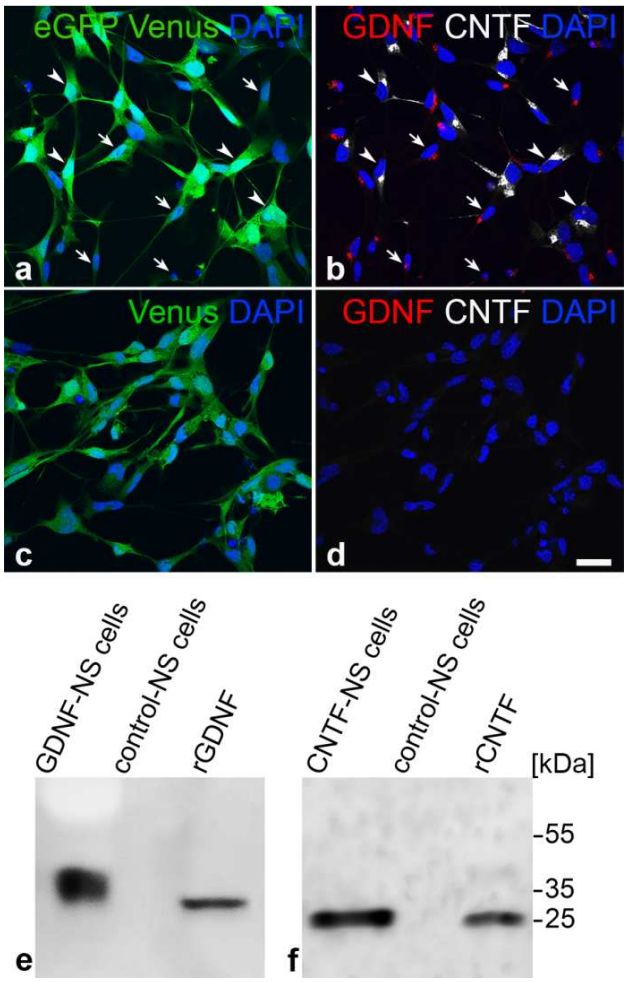

Figure 1: *Expression of GDNF and CNTF in undifferentiated clonal NS cell lines.*
A culture composed of a 1:1 mixture of GDNF-NS cells and CNTF-NS cells (a, b) and a control-NS cell culture (c, d) were stained with anti-GDNF and anti-CNTF antibodies. Whereas cells in the mixed GDNF-NS cell/CNTF-NS cell culture co-expressed either GDNF and eGFP or CNTF and Venus (a, b; some double positive cells are labeled with arrows and arrowheads, respectively), control-NS cells expressed Venus (c), but not detectable levels of the neurotrophic factors (d). Western blot analyses of culture supernatants from a GDNF-NS cell line (e) or a CNTF-NS cell line (f) confirmed secretion of the neurotrophic factors from the modified cells. Supernatants from control-NS cells contained neither detectable levels of GDNF (e) nor of CNTF (f). Recombinant mouse GDNF (rGDNF; e) and recombinant mouse CNTF (rCNTF; f) was loaded as a reference. CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole; GDNF, glial cell line derived neurotrophic factor; NS, neural stem. Bar in (d) for (a-d): 25 µm.

Immunocytochemical analyses revealed sustained expression of CNTF in astrocytes and neurons derived from the CNTF-NS cell line *in vitro* (Flachsbarth et al 2014). To analyze expression of GDNF in differentiated neural cell types derived from the GDNF-NS cell line, cells from passage 29 were differentiated into neurons or astrocytes and analyzed with anti-GDNF antibodies and the neuron-specific marker MAP2 (Fig. 2 a-c) or the astrocyte-specific marker GFAP (Fig. 2 g-i). One week after induction of differentiation, all neurons and astrocytes expressed the reporter gene eGFP (Fig. 2 a, g) and additionally showed GDNF-immunoreactivity in a perinuclear location (Fig. 2 c, i). Neurons and astrocytes derived from

the control-NS cell line analyzed in parallel expressed Venus (Fig. 2 d, j) but no detectable levels of GDNF (Fig. 2 f, l).

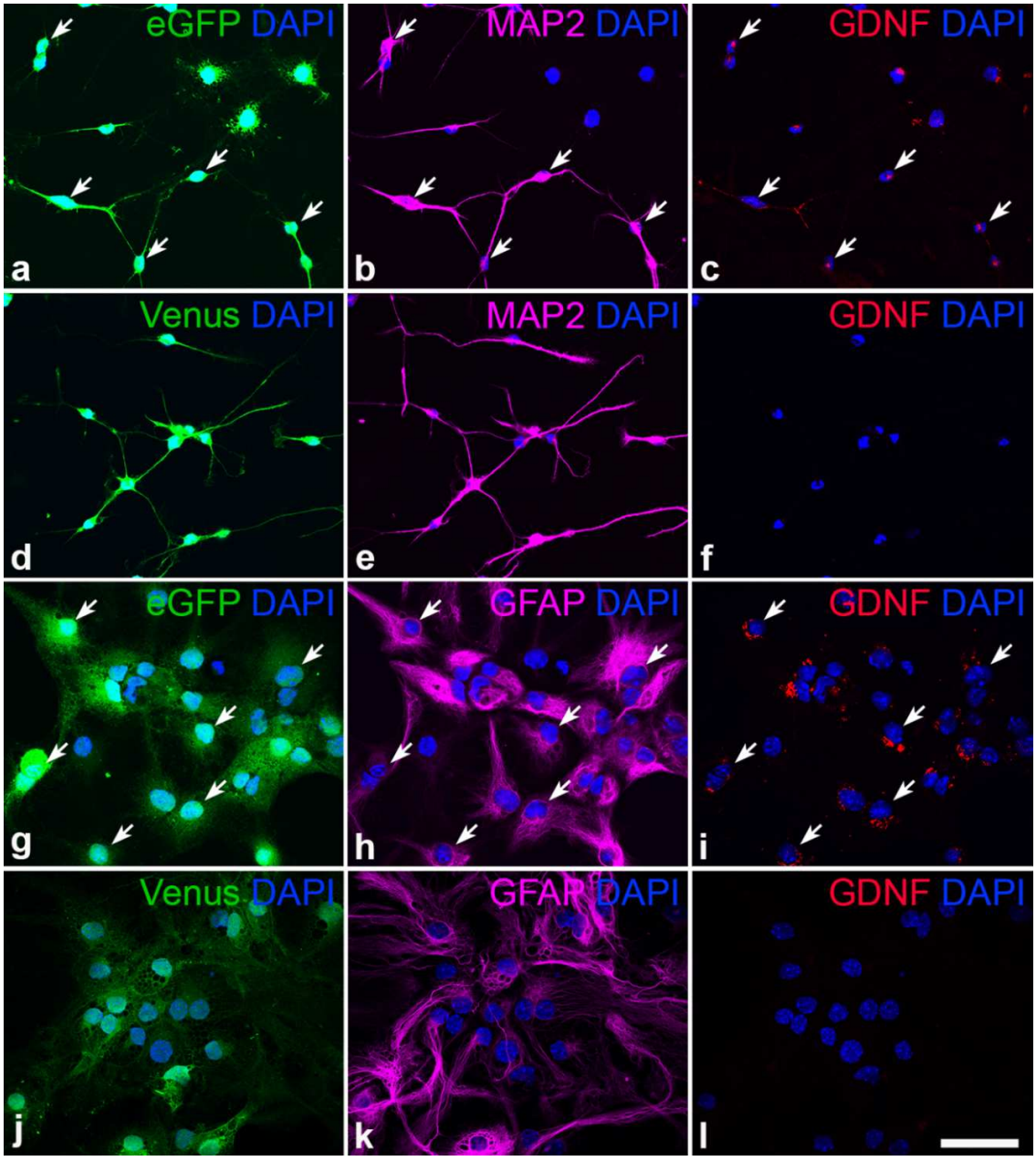

Figure 2: *Expression of GDNF in differentiated neural cell types in vitro.*
Neurons and astrocytes derived from a GDNF-NS cell line (a-c, g-i) or from a control-NS cell line (d-f, j-l) were stained with anti-GDNF antibodies (c, f, I, l) and anti-MAP2 (b, e) or anti-GFAP antibodies (h, k) one week after induction of differentiation. Note that all neurons (a-c) and astrocytes (g-i) derived from the GDNF-NS cell line co-expressed GDNF and eGFP (some double positive cells are labeled with white arrows in a-c and g-i). Neurons (d-f) and astrocytes (j-l) derived from a control-NS cell line expressed the reporter gene Venus, but no detectable levels of GDNF. DAPI, 4',6-diamidino-2-phenylindole; eGFP, enhanced green fluorescent protein; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2. Bar in l (for a-l): 50 µm.

Expression levels of the neurotrophic factors were estimated by densiometric analyses of immunoreactive bands in Western blots of culture supernatants. Recombinant mouse GDNF or recombinant mouse CNTF served as a reference. The CNTF-NS cell line expressed 87.2 ± 10.1 ng CNTF per 10^5 cells in 24 hours (mean ±SEM from three experiments) at passage 15 and similar amounts of the cytokine at passage 34, as reported previously (Flachsbarth et al 2014). Analysis of the GDNF-NS cell line revealed secretion of 156.4 ±4.8 ng GDNF per 10^5 cells in 24 hours at passage 10, and 143.4 ± 10.0 ng GDNF per 10^5 cells in 24 hours at passage 31, supporting stable expression of the neurotrophic factor in the modified NS cell lines.

Intraocular localization, neural differentiation and transgene expression in intravitreally grafted NS cells

The intraocular localization of the intravitreally grafted GDNF-NS cells, CNTF-NS cells and control-NS cells was analyzed two months after the transplantation. Donor cells were identified in the host eyes by their expression of the green fluorescent reporter proteins eGFP or Venus. All grafted cell lines had formed dense cell layers that were attached to the posterior pole of the lenses (Fig. 3) or the vitreal margin of the host retinas.

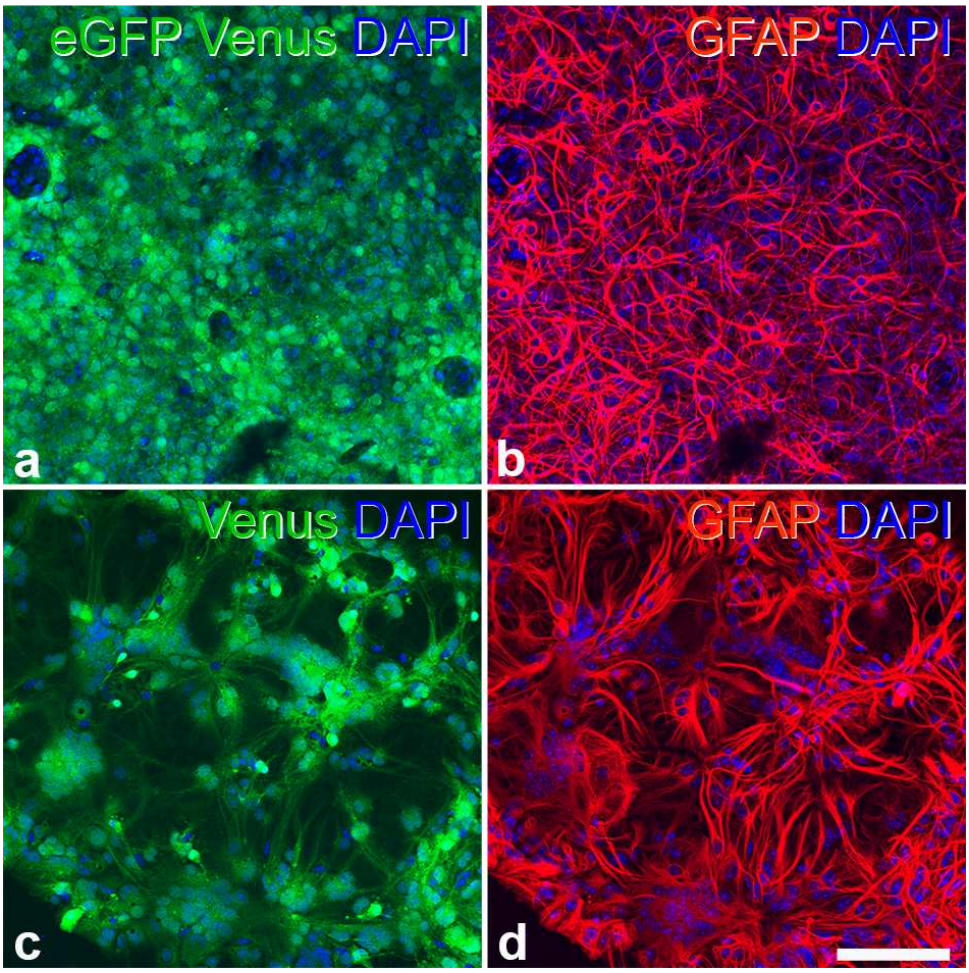

Figure 3: *Neural differentiation of intravitreally grafted GDNF-NS cells and CNTF-NS cells.*
Immunohistochemical analyses of lenses two months after intravitreal transplantations of a 1:1 mixture of GDNF-NS cells and CNTF-NS cells (a, b) or control-NS cells (c, d) revealed the presence of donor cells that were attached to the posterior poles of the lenses and expressed the green fluorescent reporter proteins eGFP or Venus (a) or Venus (c). Note that virtually all grafted NS cells were differentiated into GFAP-positive astrocytes (b, d). DAPI, 4',6-diamidino-2-phenylindole; eGFP, enhanced green fluorescent protein; GFAP, glial fibrillary acidic protein. Bar in d (for a-d): 100 µm.

Evidence for ongoing proliferation of the donor cells, or integration of the grafted NS cells into the host retinas was not observed. Analyses of retinal flat-mounts revealed the presence of some small-sized retinal folds in eyes that had received transplantations of CNTF-NS cells or a mixture of GDNF-NS cells and CNTF-NS cells. Similar retinal folds were not observed in eyes with grafted GDNF-NS cells or control-NS cells.

Neural differentiation and transgene expression in grafted NS cells was studied in animals that had received intravitreal transplantations of a 1:1 mixture of the GDNF-NS and CNTF-NS cell line. Analyses of lenses with attached donor cells revealed that virtually all eGFP- or Venus-positive cells were differentiated into GFAP-positive astrocytes (Fig. 3 a, b). Almost

exclusive astrocytic differentiation was also observed for the Venus-positive donor cells that were derived from grafted control-NS cells (Fig. 3 c, d). Differentiation of grafted NS cells into ß-tubulin III-positive neurons was only rarely observed.

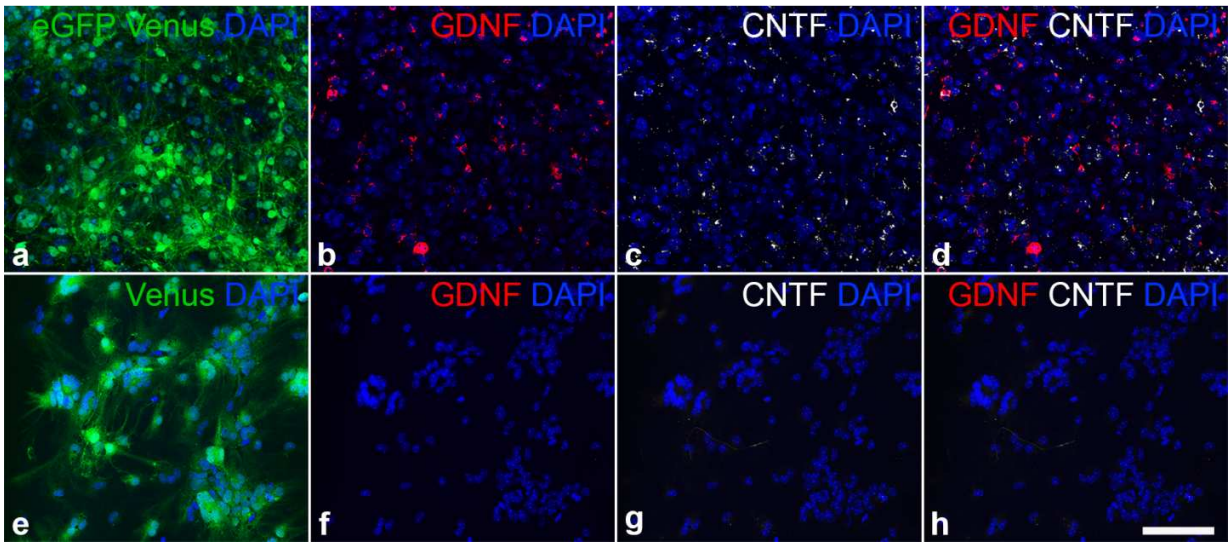

Figure 4: *Expression of GDNF and CNTF in intravitreally grafted NS cells.*
Two months after intravitreal transplantations of a 1:1 mixture of GDNF-NS cells and CNTF-NS cells (a-d) or control-NS cells (e-h), grafted cells were analyzed for expression of GDNF (b, f) and CNTF (c, g). Donor cells derived from GDNF-NS or CNTF-NS cells co-expressed eGFP and GDNF or Venus and CNTF, respectively (a-c). Donor cells derived from control-NS cells, in comparison, expressed Venus but no detectable levels of the neurotrophic factors (e-g). (d) and (h) are the merged overlays of (b and c) and (f and h), respectively. CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole; GDNF, glial cell line-derived neurotrophic factor. Bar in h (for a-h): 100 µm.

Immunostainings of a grafted 1:1 mixture of the GDNF-NS and CNTF-NS cell line two month after transplantation with anti-GDNF and anti-CNTF antibodies revealed co-expression of the green fluorescent reporter proteins with either GDNF or CNTF in all cells (Fig. 4 a-d). Grafted control-NS cells, in comparision, expressed Venus but no detectable levels of GDNF or the cytokine (Fig. 4 e-h).

Intravitreally grafted GDNF-NS or CNTF-NS cells attenuate the loss of axotomized RGCs

To determine the neuroprotective effects of the transplanted NS cell lines, the number of Brn3-a-positive RGCs was determined in number-coded retinal flat-mounts (Figs. 5, 6). RGC numbers in eyes with grafted GDNF-NS and control-NS cells were analyzed 14, 28 and 56 days after the lesion. RGC numbers in eyes with grafted CNTF-NS cells were analyzed 14 days after the lesion, while values for the 28 and 56 days post-lesion interval were taken from a previous study (Flachsbarth et al 2014). Six retinas were analyzed for each cell line and post-lesion interval. Eyes with grafted control-NS cells contained 305.7 ± 34.0

RGCs/mm^2, 178.8 ± 28.0 RGCs/mm^2 and 105.7 ±12.9 RGCs/mm^2 14, 28 and 56 days after the optic nerve crush, respectively (Fig. 5 g-l, Fig. 6). Eyes that received injections of the GDNF-NS cell line, in comparison, contained 737.2 ± 20.3 RGCs/mm^2, 447.5 ± 45.3 RGCs/mm^2 and 400.7 ± 24.2 RGCs/mm^2 at the 14, 28 and 56 days post-lesion time point, respectively (Fig. 5 a-c, Fig. 6). In the CNTF-treated retinas, we found 937.5 ± 45.4 RGCs/mm^2 14 days after the lesion, 529.0 ± 20.9 RGCs/mm^2 28 days after the lesion and 429.8 ± 30.7 RGCs/mm^2 56 days after the lesion (Fig. 5 d-f, Fig. 6). GDNF-treated retinas thus contained 2.4-, 2.5- and 3.8-fold more and CNTF-treated retinas 3.1-, 3.0- and 4.1-fold more RGCs than the eyes with grafted control-NS cells at the 14, 28 and 56 days post-lesion time points, respectively ($p<0.001$ for each factor and post-lesion interval). While the neuroprotective effect of the CNTF-NS cells was significantly stronger than that of the GDNF-NS cells 14 days after the lesion, both cell lines rescued RGCs to a similar extent 28 and 56 days after the lesion (Fig. 6).

Neuroprotection of axotomized RGCs by a simultaneous cell-based intraocular administration of GDNF and CNTF

To test whether a simultaneously cell-based intraocular administration of GDNF and CNTF results in a more pronounced rescue of axotomized RGCs, we additionally performed transplantation experiments with a 1:1 mixture of both cell lines. Analyses of Brn3-a labeled retinal flat-mounts from these animals (Fig. 5 j-l) revealed a remarkably increased number of RGCs 14, 28 and 56 days after the lesion when compared to GDNF-treated retinas, CNTF-treated retinas or control retinas (Fig. 5 a-i).

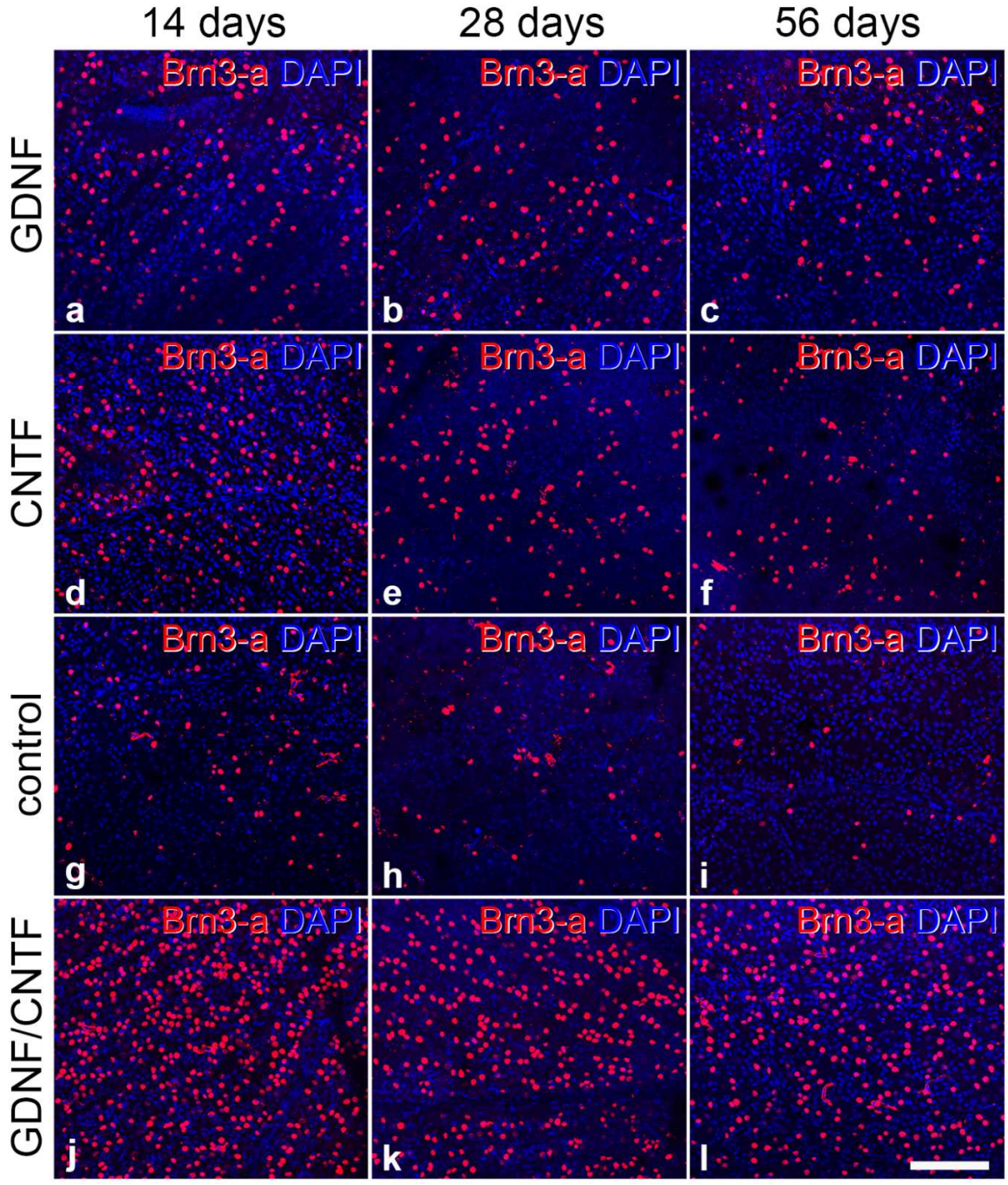

Figure 5: *Intravitreally grafted GDNF- and CNTF-secreting NS cell lines attenuate the degeneration of intraorbitally lesioned retinal ganglion cells.*
Adult mice received intravitreal injections of GDNF-NS cells (a-c), CNTF-NS cells (d-f), control-NS cells (g-i) or a 1:1 mixture of the GDNF-NS and CNTF-NS cells (j-l) one day after an intraorbital optic nerve lesion. Analyses of retinal flat-mounts 14, 28 and 56 days after the optic nerve crush revealed the presence of significantly more Brn3-a-positive retinal ganglion cells in eyes with grafted GDNF-NS cells (a-c) or CNTF-NS cells (d-f) than in eyes with grafted control-NS cells (g-i). Note the markedly increased number of ganglion cells in eyes that had received injections of a 1:1 mixture of GDNF- and CNTF-secreting NS cells when compared to eyes that had received injections of either the GDNF-NS or the CNTF-NS cell line. CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole; GDNF, glial cell line-derived neurotrophic factor. Bar in l (for a-l): 100 µm.

Of note, retinas simultaneously treated with GDNF and CNTF contained 2036.7 ± 54.7 RGCs/mm^2 14 days after the lesion, 1573.7 ± 51 RGCs/mm^2 28 days after the lesion, and 1506.3 ± 64.7 RGCs/mm^2 56 days after the lesion (n=6 for each post-lesion interval; Fig. 6). Eyes with a grafted mixture of GDNF-NS and CNTF-NS cells thus contained 2.8-, 3.5- and 3.8-fold more RGCs than eyes with grafted GDNF-NS cells, 2.2-, 3.0- and 3.5-fold more RGCs than eyes with grafted CNTF-NS cells, and 6.7-, 8.8-, and 14.3-fold more RGCs than eyes with grafted control-NS cells at the 14, 28 and 56 days post-lesion time points, respectively ($p<0.001$ for each NS cell population and post-lesion time point; Fig. 6).

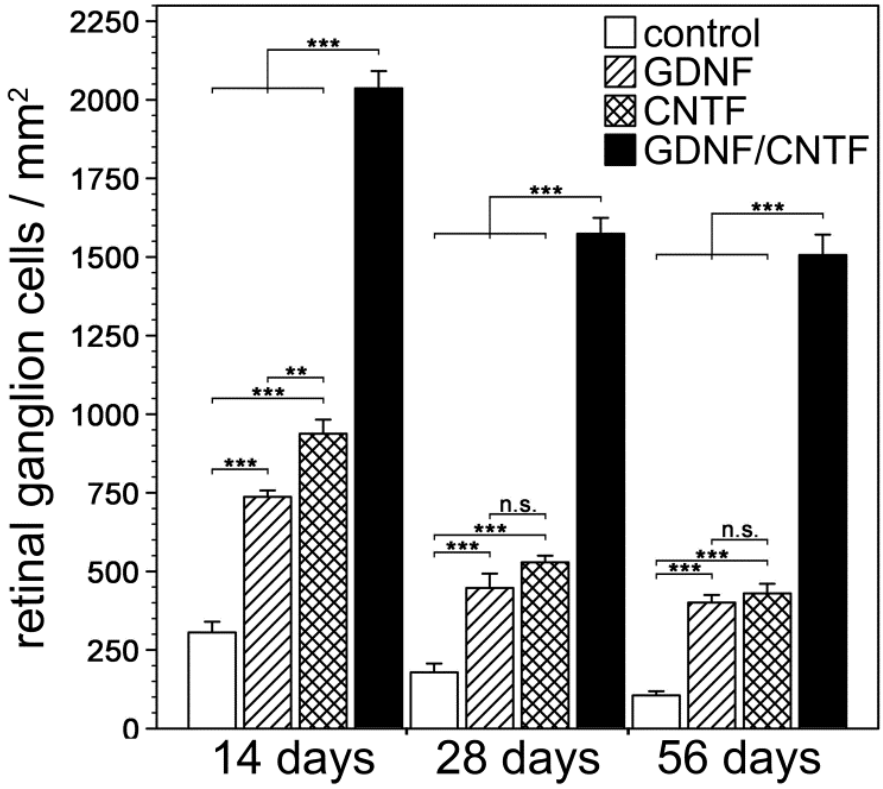

Figure 6: *Quantitative analysis of the neuroprotective effects of intravitreally grafted NS cells on axotomized retinal ganglion cells.*
The numbers of retinal ganglion cells were determined in flat-mounted retinas from eyes with intravitreally grafted control-NS cells (open bars), GDNF-NS cells (hatched bars), CNTF-NS cells (crossed-hatched bars) or GDNF-NS and CNTF-NS cells (filled bars) 14, 28 and 56 days after an intraorbital crush of the optic nerve. The GDNF-NS cell line and the CNTF-NS cell line significantly attenuated the loss of RGCs to a similar extent at all postlesion time points analyzed. Note the markedly higher number of RGCs in eyes that had received injections of a 1:1 mixture of GDNF-NS cells and CNTF-NS cells when compared to eyes that had received transplantations of the individual GDNF- and CNTF-expressing cell lines. Each bar represents the mean number (± SEM) of retinal ganglion cells per mm^2 from six retinas. CNTF, ciliary neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; n.s., not significant; **, $p<0.01$; ***, $p<0.001$ according to the Student's t-test.

RGC axons were anterogradely labeled in a fraction of experimental animals to control the optic nerve crush. Long distance regeneration was observed in animals that had received injections of CNTF-NS cells or a mixture of GDNF-NS cells and CNTF-cells, but not animals that had received transplantations of GDNF-NS cells or control-NS cells. Incomplete lesions of the optic nerve were observed in none of these animals.

Discussion

Retinal ganglion cells in glaucomatous optic neuropathies degenerate by apoptosis and are invariably lost (Almasieh et al 2012, Qu et al 2010). The only clinically successful treatment of glaucoma currently available is the reduction of the intraocular pressure (IOP) in the glaucomatous eyes. However, even after successful IOP reduction, degeneration of RGCs progresses in a significant proportion of patients (Caprioli 1997, Leske et al 2003). Therefore, additional therapies need to be established that effectively delay or even stop the progressive degeneration of RGCs. Cell replacement strategies based on pluripotent embryonic stem (ES) cells or induced pluripotent stem (iPS) cells represent promising treatment options for degenerative retinal disorders characterized by the dysfunction or loss of retinal pigment epithelial (RPE) cells or photoreceptor cells (Osakada et al 2010, Ramsden et al 2013, Rowland et al 2012, Westenskow et al 2014, Wright et al 2014). However, a similar approach aimed at replacing dysfunctional or degenerated RGCs is faced with the problem that the grafted cells not only have to integrate as functional ganglion cells into the glaucomatous retinas, but additionally have to extend their axons over long distances to the brain where they have to establish synapses with the correct postsynaptic neurons in a topographically appropriate manner. Strategies aimed at developing therapies for glaucomatous optic neuropathies therefore usually attempt to delay or prevent the degeneration of RGCs (Almasieh et al 2012, Harvey et al 2006, Pascale et al 2012, Qu et al 2010, Wilson & Di Polo 2012).

To this end several different treatment options for glaucoma have been evaluated in preclinical studies during the last years, including the application of pharmacological neuroprotective agents such as n-acetylcysteine (Yang et al 2012), brimonidine (Aburahma & Mahmoud 2011), aurintricarboxylic acid (Heiduschka & Thanos 2000), or inhibitors of pro-apoptotic proteins such as antibodies to semaphorins (Shirvan et al 2002). Because a deprivation in neurotrophic factors as a result of an impaired axonal transport is considered to contribute to the progressive loss of RGCs in glaucoma, supplementation of neurotrophic factors to glaucomatous retinas represents another extensively studied strategy to develop treatments for this condition (Almasieh et al 2012, Harvey et al 2006, Johnson et al 2011, Wilson & Di Polo 2012). In initial experiments, recombinant proteins were injected into the eyes of animal models of glaucoma to study potential protective effects of diverse neurotrophic factors on RGCs (Klocker et al 1997, Maier et al 2004, Weibel et al 1995, Yan et al 1999). However, since these proteins

usually have short half-life times (Saragovi & Gehring 2000), these experiments failed to achieve long-term protection of RGCs. To address this problem, delivery methods were developed that allow a sustained intraocular administration of pharmacological drugs or neurotrophic factors, such as micro-pumps (Saati et al 2009), biodegradable intraocular drug carrier (Aburahma & Mahmoud 2011, Mundada & Shrikhande 2008), or gene transfer using adeno-associated vectors (Di Polo et al 1998, Schmeer et al 2002, Weise et al 2000) or lentiviral vectors (van Adel et al 2003) to continuously express the therapeutic gene products in endogenous retinal cells. Intraocular transplantations of cells genetically modified to overexpress neurotrophic factors represent another approach to continuously deliver these proteins to glaucomatous retinas. In the context of glaucoma, a cell-based delivery approach has been successfully employed with an encapsulated cell implant to administer glucagon-like peptide-1 to a rat model of optic nerve crush (Zhang et al 2011b). Furthermore, attenuation of RGC degeneration has been achieved in a rat model of ocular hypertension by intravitreal injections of mesenchymal stem cells lentivirally modified to secrete BDNF (Harper et al 2011). In addition, Schwann cells modified to overexpress CNTF have been shown to recue RGCs and to promote axonal regeneration in a rat optic nerve transection model (Hu et al 2005). Protection of axtomized RGCs and stimulation of axonal regeneration has also been observed in adult rats after intravitreal transplantations of fibroblasts modified to secrete fibroblast growth factor-2 (FGF-2), neurotrophin-3 (NT-3) and BDNF (Logan et al 2006).

With the aim to achieve a more pronounced rescue of RGCs in animal models of glaucoma, some studies have evaluated whether a simultaneous administration of two or more neurotrophic factors might result in additive or synergistic neuroprotective effects. In hamsters, for instance, combined injections of low concentrations, but not of high concentrations, of recombinant BDNF and CNTF resulted in slightly better neuroprotection of axotomized RGCs than separate injections of each individual factor (Zhang et al 2005). Finally and importantly, the combined administration of FGF-2, NT-3 and BDNF through intravitreal transplantations of modified fibroblasts significantly and synergistically increased the number of regenerating RGCs after an optic nerve transection in adult rats (Logan et al 2006).

Although CNTF and GDNF are both potent neuroprotective factors for RGCs, they have not been studied yet for potential additive or synergistic neuroprotective activities. We

therefore generated a series of clonal NS cell lines expressing different levels of either factor using lentiviral vectors, and selected a CNTF- and a GDNF-expressing NS cell line that exerted similar neuroprotective effects on intraorbitally lesioned RGCs. Both the CNTF-NS cell line (Flachsbarth et al 2014) and the GDNF-NS cell line stably expressed the respective neurotrophic factor together with a fluorescent reporter protein in undifferentiated NS cells and differentiated neural cell types *in vitro*. When grafted into the vitreous cavity of adult mice one day after an intraorbital optic nerve crush, both cell lines survived in the host eyes for at least two months. Similar to our observations with intravitreally grafted CNTF-expressing NS cells in *Pde6b*^{rd1} and *Pde6b*^{rd10} (Jung et al 2013) mice or the optic nerve crush model (Flachsbarth et al 2014), virtually all GDNF-cells differentiated into astrocytes that were attached to the posterior poles of the lenses and the vitreal surfaces of the host retinas. Formation of tumors or integration of donor cells into the host retinas was not observed for either cell line. Importantly, both clonal cell lines stably expressed the transgenes over the two months post-lesion interval and significantly attenuated the lesion-induced loss of ganglion cells to a similar extent when compared to eyes that received transplantations of the control-NS cell line.

Having established that the CNTF-NS and GDNF-NS clones rescued similar numbers of axotomized RGCs from degeneration, a 1:1 mixture of both cell lines was used for intravitreal transplantation experiments. These experiments revealed markedly increased RGC numbers in retinas simultaneously treated with GDNF and CNTF, containing 2.8-, 3.6- and 3.8-fold more RGCs than GDNF-treated retinas, 2.2-, 3.0- and 3.5-fold more RGCs than CNTF-treated retinas, and 6.7-, 8.8- and 14.3-fold more RGCs than control retinas at the 14 days, 28 days and 56 days post-lesion interval, respectively. In fact, the numbers of RGCs in eyes simultaneously treated with GDNF and CNTF were significantly higher than expected if both factors would exert additive neuroprotective effects on RGCs. Results thus demonstrate that GDNF and CNTF synergistically rescue axotomized RGCs. They also indicate the therapeutic potential of combinatorial neuroprotective approaches for the treatment of degenerative retinal disorders.

In summary, we have demonstrated that intravitreally grafted CNTF-NS or GDNF-NS cells survived for up to two months in the vitreous cavity where both cell lines stably expressed the neurotrophic factors and significantly and to a similar extent attenuated the lesion-induced degeneration of RGCs at all post-lesion time points analyzed. Of note, transplantations of a mixture of the CNTF- and GDNF-NS cell lines resulted in a rescue

of axotomized RGCs that was significantly more pronounced than observed for each individual cell line or a theoretically calculated additive neuroprotective activity of both factors. To our best knowledge, this is the first demonstration of a synergistic neuroprotective activity of CNTF and GDNF on RGCs. Results demonstrate that significant neuroprotection may be achieved through combinatorial approaches, and that genetically modified and clonally derived NS cell lines may represent a valuable tool to analyze the therapeutic potential of a simultaneous intraocular administration of two or more neuroprotective factors in animal models of ganglion cell loss.

III. Abbreviations

AAV:	adeno-associated virus
ACG:	angle closure glaucoma
Apaf-1:	apoptotic protease-activating factor-1
ASK1:	apoptosis signal regulating kinase 1
ATA:	aurintricarboxylic acid
BDNF:	brain derived neurotrophic factor
BSA:	bovine serum albumin
bFGF / FGF-2:	basic fibroblast growth factor
CAG:	combination of cytomegalovirus enhancer and chicken ß-actin promoter
cDNA:	complementary deoxyribonucleic acid
CMV:	cytomegalovirus
CNTF:	ciliary neurotrophic factor
CNTFRα:	ciliary neurotrophic factor receptor α
Cy:	cyanine
DAPI:	4’,6-diamidino-2-phenylindole dihydrochloride hydrate
DMEM / F12:	Dulbecco’s modified Eagle’s medium and F-12
dpl:	days past lesion
EGF:	epidermal growth factor
EGFP:	enhanced green fluorescent protein
ERK:	extracellular signal-related kinase
FACS:	fluorescence activated cell sorting
FADD:	Fas-associated death domain
FasL:	Fas ligand

FasR:	Fas receptor
GDNF:	glial cell line derived neuroptrophic factor
GFAP:	glial fibrillary acidic protein
GFLs:	glial fibrillary acidic protein family of ligands
GFRα:	GDNF family receptor α
HEK:	human embryonic kidney
IL-6:	interleukin 6
IOP:	intraocular pressure
IRES:	internal ribosome entry site
JAK:	janus kinase
JNK:	c-jun n-terminal kinase
kDA:	kilo Dalton
LeGO:	lentiviral “gene ontology”
LIF:	leukaemia inhibitory factor
LIFRß:	leukemia inhibitory factor receptor ß
MAP-2:	microtubule-associated protein 2
MAPK:	mitogen-activated protein kinase
MBP:	myelin basic protein
mm^2:	square millimetre
MOG:	myelin oligodendrocyte glycoprotein
mRNA:	messenger ribonucleic acid
MSC:	mesenchymal stem cell
Neo:	neocin
NGF:	nerve growth factor
NS:	neural stem
NT:	neurotrophin

OAG:	open angle glaucoma
OEC:	olfactory ensheathing cell
OFR:	oxygen free radicals
ONC:	optic nerve crush
ONT:	optic nerve transsection
onl:	outer nuclear layer
p75NTR:	p75 neurotrophin receptor
PA:	paraformaldehyde
PBS:	phosphate-buffered saline
Pde6B:	rod cGMP-specific 3',5'-cyclic phosphodiesterase subunit beta
PI3-K:	phosphatidylinositol 3-kinase
PLC-γ:	phospholipase Cγ
Rd1:	retinal degeneration 1
Rd10:	retinal degeneration 10
RET:	receptor tyrosine kinase rearranged during transfection
RGC:	retinal ganglion cell
RNFL:	retinal nerve fibre layer
RP:	retinitis pigmentosa
RPE:	retinal pigment epithelium
RTK:	receptor tyrosine kinase
SEM:	standard error of the mean
SFK:	Src-family kinase
STAT:	signal transducer and activator of transcription
TNFα:	tumor-necrosis factor-α
TNFR:	tumor-necrosis factor receptor
TRKR:	tropomyosin receptor kinase receptor

Tyk:	tyrosin kinase
VSV-G:	envelope G protein of the vesicular stomatitis virus
Zeo:	zeocin

IV. References

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Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

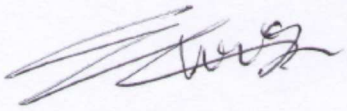
Hamburg , den 06.11.2015

Kai Flachsbarth

Declaration

As an English native speaker, I can confirm that the language used in the current thesis is correct in grammar and syntax.

Elizabeth Wolfe



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