“Development of Cell-based Therapeutic Approaches for the Treatment of Inherited Retinal Dystrophies” and “Experimental Myelination of the Lamina Cribrosa by Cell Transplantation.”

Gila Jung

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DISSERTATION

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Zu der vorliegenden Dissertation hat die Disputation
in Hamburg stattgefunden am: 09.11.2012.

Gutachter der Dissertation waren:
Professor Dr. Udo Bartsch
und
Priv.-Doz. Dr. Hartwig Lüthen

Gutachter der Disputation waren:
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The present work was developed in the Experimental Ophthalmology in the Department of Ophthalmology at the University Medical Center Hamburg-Eppendorf in the time of July 2008 until August 2012 under supervision of Prof. Dr. rer. nat. Udo Bartsch.

DEDICATION:

This work is dedictated to my family, who is all what I am.

QUOTATION:

The love of science, unbounded patience in reflecting over any subject, industry in observing and collecting facts and a fair share of invention as well as of common sense; I have steadily endeavoured to keep my mind free so as to give up any hypothesis, however much beloved (and I cannot resist forming one on every subject), as soon as facts are shown to be opposed to it.

(Life and Letters of Darwin, by Francis Darwin, 1887)

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Abstract

Impairment of vision constituted in inherited retinal degenerations is incurable and the major reason for the loss of visual acuity in industrialized countries until today. Genetic mutations lead to direct or through dysfunction mediated irreversible loss of the sensory cells of the retina, the photoreceptor cells. In the present thesis cell-based therapeutic approaches for progressive retinal dystrophies were addressed.

A cell-based replacement strategy that attempts to treat advanced retinal degenerations was addressed. The replacement of endogenous photoreceptor cells was employed in a transgenic mouse model of slowly progressing photoreceptor cell degeneration to investigate the influence of the dystrophic environment. Primary cell suspensions, containing high amounts of rod postmitotic precursors, were isolated from retinas of young postnatal EGFP-transgenic mice and subretinally transplanted into mice at the beginning and at advanced stage of degeneration. The impact of the pathology on engrafted donor cells was analysed. The transplanted cells integrated correctly with differentiation into fully mature photoreceptors at every post-transplantation interval analysed but with a significant decrease in quantities over time. The analysis showed a severe morphological alteration of the donor photoreceptors resembling the structure of the degenerate host retina. This suggests that the quantities of transplanted and integrated donor-derived cells are not sufficient to induce a beneficial impact on the degenerate retina.

For the early retinal degeneration neuroprotective strategies, that aim not to correct the gene defect but to treat its consequences, i.e. degeneration of photoreceptor cells and deterioration of visual function, offer the possibility of a therapy that is widely applicable across a range of conditions. For such approaches neurotrophic factors are promising candidates, if delivered continuously to the affected tissue. For the development of a stem-cell based intraocular delivery system for neurotrophic factors for preclinical investigations in mouse models, adherent cultivated neural stem cells have been genetically modified with a refined polycistronic lentiviral vector. The vector encoded for the neurotrophic factor ‘CNTF’, a reporter gene and a resistence gene. Pure genetically modified neural stem cells have been intravitreally transplanted into transgenic mouse models for progressive retinal degeneration. The intraocular and sustained delivery of ‘CNTF’ through the engineered neural stem cells resulted in highly significant preservation of photoreceptors compared to the control eye, which was treated with genetically modified cells lacking the expression of ‘CNTF’. Further, the cells evoked no adverse effects on the retinal structure and were trackable after analysis. All together these are arguments for the system to be applicable for the sustained delivery of therapeutical products in mouse models.

The method of intraocular neural stem-cell transplantation was used to address a basic neurobiological issue. The competence of the axonal segments of the retinal ganglion cells within the retina and the usually non-myelinated most retina proximal portion of the optic nerve to become myelinated was analysed. The proximal portion of the optic nerve inhibits the migration of oligodendrocyte progenitor cells from the optic nerve into the retina and the underlying mechanisms have to be further clarified. Retinal transplantations of oligodendrocyte progenitor cells to the optic disc demonstrated the competence of the retinal ganglion cell axonal segments within the lamina cribrosa to become myelinated and further confirmed the hypothesis that the prevented migration of oligodendrocyte progenitor cells into the proximal portion of the optic nerve and retina is controlled by non-neuronal factors located at the retinal end of the optic nerve to keep the retina free of myelin.

Kurzfassung

Die Verminderung der Sehfähigkeit durch hereditäre Netzhautdystrophien ist nach wie vor unheilbar und der Hauptgrund für Erblindung in industrialisierten Staaten. Aufgrund genetischer Mutationen gehen die Sehnervenzellen der Netzhaut, die Photorezeptoren, direkt oder durch Dysfunktion irreversibel verloren. In der vorliegenden Arbeit wurden zellbasierte Therapieansätze für Netzhautdystrophien mit progredientem Verlauf beschrieben.

Für fortgeschrittene Netzhautdystrophien wird der Zellersatz degenerierender Photorezeptoren durch die Verpflanzung primärer, retinaler Zellsuspensionen diskutiert. Für diese zellbasierte Strategie wurde hier der Einfluss der dystrophen Umgebung eines transgenen Mausmodels, charakterisiert durch progredienten Photorezeptorzellverlust, auf verpflanzte Spenderphotorezeptoren beschrieben. Die Analyse auf Integration, Reifung und Überlebensdauer der mit ‚postmitotischen Stäbchenvorläuferzellen' angereicherten Zellsuspension, aus neonatalen EGFP-Mäusen, nach Transplantation in die dystrophe Empfängernetzhaut zu verschiedenen Zeitpunkten, ergab, dass zu jedem analysierten Zeitpunkt ausgebildete Spenderphotorezeptoren zu finden waren. Jedoch nahm die Anzahl der integrierten Spenderphotorezeptoren mit der Länge des post-Transplantationsintervalls deutlich ab. Darüber hinaus konnte für die morphologische Beobachtung verzeichnet werden, dass die Spenderphotorezeptoren trotz Wildtyp-Charakters die Morphologie der endogenen, dystrophen Photorezeptoren annahmen.

Für frühe Stadien von Netzhautdystrophien wird die kontinuierliche, retinale Applikation von neuroprotektiven Faktoren diskutiert, da diese Strategie intendiert nicht den vorliegenden genetischen Defekt zu korrigieren, sondern die daraus folgenden Konsequenzen, wie die Degeneration der Photorezeptoren und die Verminderung der Sehfähigkeit, wird dieser Ansatz weitreichend einsetzbar. Hier wurde in einer Strategie der Einsatz von ‚zellulären Vektoren' als retinales Vehikel für therapeutische Genprodukte für das Mausauge beschrieben. Dafür wurde ein stammzellbasiertes, intraokulares Transportsystem für neurotrophe Faktoren zur prä-klinischen Untersuchung im Mausmodell entwickelt, für das adherent kultivierte neurale Stammzellen als ‚zellulärere Vektoren' für neurotrophe Faktoren verwendet wurden. Polycistronische lentivirale Vektoren wurden weiterentwickelt um genetisch modifizierten neuralen Stammzellen hervorzubringen, die sich durch die Expression des sezernierten neurotrophen Faktors ‚CNTF', eines fluoreszierenden Reporters sowie einer Antibiotikaresistenz auszeichnen. Neben Klon- und Differenzierungsanalysen konnte an zwei transgenen Mausmodellen für retinale Degeneration gezeigt werden, dass das etablierte System kontinuierlich ausreichend Faktor intraokular abgab, was durch den signifikanten Erhalt endogener Photorezeptoren belegt wurde. Eine weitere wichtige Beobachtung war die Abwesenheit einer morphologischen Beeinträchtigung der Empfängerretina, was das System einsetzbar macht für verschiedenste therapeutische Produkte in Mausmodellen.

Die Methodik der intraokularen neuralen Stammzelltransplantation wurde für die Untersuchung einer grundwissenschaftlichen Fragestellung herangezogen. Die Kompetenz der Myelinisierbarkeit axonaler Segmente der retinalen Ganglienzellen im retinanächsten Bereich des Sehnervs wurde analysiert. Dieser Bereich wirkt inhibitorisch auf die Migration oligodendrozytärer Vorläuferzellen vom Sehnerv in die Retina und der zugrunde liegende Mechanismus ist nicht gelöst. Über retinale Transplantation oligodendrozytärer Vorläuferzellen in den Blinden Fleck (Discus nervi optici), nahe der Lamina Cribrosa, wurden die axonalen Segmente retinaler Ganglienzellen in diesem Bereich myelinisiert, was die Hypothese einer Migrationshemmung oligodendrozytärer Vorläuferzellen vom Sehnerv in die Netzhaut durch ‚non-neuronal' Faktoren am retinalen Ende des Sehnervs bekräftig.

I. Introduction and Summary

1. Development of cell-based therapeutic approaches for the treatment of inherited retinal dystrophies

The thesis attends to evaluate the utilization of stem cells for cell-based therapies in the central nervous system with a particular focus on the dystrophic retina. The summary intends to provide a brief overview on how the scientific achievements in stem cell biology in the past decades have contributed towards developing concepts that have generated strategies to allow the first translational approaches for cell-based therapies in clinical practice.

The idea that a cell is capable of generating another cell was born as early as in the mid 1800s when Ernst Haeckel suggested the term *stem cell* in 1868 (Haeckel, 1868), for the developing fertilized egg. About 40 years later A. A. Maximow introduced the term *stem cell* for lymphocytes generating different blood elements (Maximow, 1909), and thus generating the modern concept of *stem cells*. The study of haematopoiesis represented one of the first fields that discussed the existence of a cell population that is capable of 'self-renewal', i.e. reproducing itself identically (by symmetric cell division), and to produce progeny with a finite proliferative lifespan and a more differentiated phenotype (by asymmetric cell division) (Smith, 1992). In parallel, experimental work began on embryonic stem cells when Walter Heape transferred a fertilized egg from one rabbit to another, in 1890 (Heape, 1890) and when Albert Brachet was able to culture a rabbit blastocyst for one day, 20 years later (Brachet, 1912). These experiments were the beginning of the research field 'experimental ontogeny' (originating in 'experimental embryology' founded by Wilhelm Roux and, e.g., Hans Driesch) (Alexandre, 2001). Based on these early works, cell therapy evolved as another area of research. The use of cells to treat diseases was started with the first transplantation experiments in the field of haematopoetic stem cells (Little and Storb, 2002). The existence of neurogenesis in adult mammals, first demonstrated by Altman and Das in the 1960s (but accepted only late in the 20th century; Altman, 1962; Gould and Gross, 2001), provided the foundation to think about cell therapy in the brain. The early results obtained by allogeneic

transplantations of bone marrow stem cells were unique as haematopoiesis is highly active throughout lifespan. But, the success of allogeneic transplantations of adult neural stem cells to treat neurological disorders could not be expected as neurogenesis in the adult mammalian brain is limited (Cameron and McKay, 2001; Ming and Song, 2011). However, transplantations of therapeutically relevant neural cell types derived from in vitro expanded stem cells, such as neural stem cells, embryonic stem cells or induced pluripotent stem cells, have achieved remarkable results in animal models of neurological disorders (reviewed in e.g.: Koch et al., 2009; Conti and Cattaneo, 2010; Breunig et al., 2011; Carney and Shah, 2011). In fact, the first stem cell-based therapeutic approaches for the treatment of neurodegenerative disorders are currently entering the clinic (reviewed in e.g.: Kim and de Vellis, 2009; Koch et al., 2009; Erceg et al., 2011; Meyer et al., 2010). The use of cells as a therapeutic tool to treat neurological disorders probably has been most successfully demonstrated for the inherited retinal dystrophies. The current progress in basic science and preclinical studies, is not only encouraging for a cure for one of the main causes of impaired visual acuity in industrialized countries but also for stimulating the development of stem-cell based therapies for other disorders, in particular the degenerative disorders of the brain (e.g. Müller et al., 2006, Breunig et al., 2011).

Before I go into some detail of the historical background and recent approaches of cell-based therapies in the retina, I briefly introduce the mechanisms of vision and retinal disorders and dystrophies leading to loss of vision.

Vision is a complex sensory process permitting visually guided living beings to detect objects in their environment. The primary visual pathway is processed within the retina, a thin sheet of neural tissue, lining in the posterior pole of the eye. Optical images formed on the retina are transformed into neural images according to three sequential stages: (i) Transduction of the light signals into electrical changes by photoreceptors in the outer retina; (ii) synaptic transmission of these electrical changes to neuronal cells (like bipolar and horizontal cells) in the inner retina where first image processing is conducted; and (iii) further synaptic transmission to retinal ganglion cells, the axons of which collect in the optic nerve, providing the only output to the brain (Gaillard and Sauvé, 2007; see also Fig. 1).

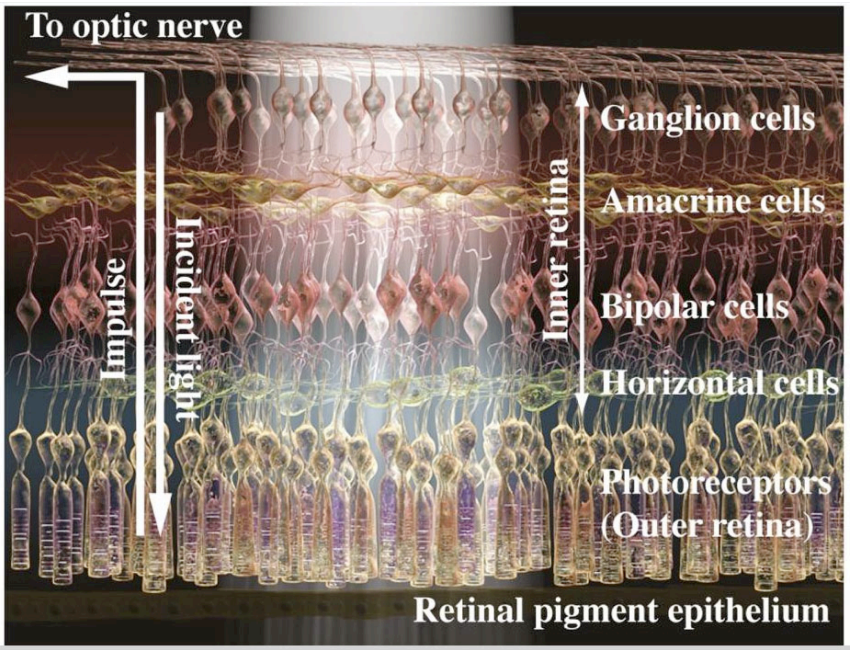

Figure 1: Cross-section of the human retina, showing its laminated structure, which consists of: (1) The ganglion cell layer, the axons of which form the optic nerve, which connects the retina to the brain; (2) the inner nuclear layer, which contains second-order neurons, such as bipolar, amacrine and horizontal cells; (3) the outer nuclear or photoreceptor (PR) layer, which contains the cell bodies and nuclei of the rod and cone PRs; (4) the PR outer segments, which are densely packed with opsin-containing discs and are separated from the inner segments and cell bodies by a narrow 200–500nm-long connecting cilium (not visible); and (5) the retinal pigment epithelium (RPE), a monolayer of cells containing tight junctions that separates the neural retina from the choroid, which supplies nutrients to the RPE and PRs (outer retina). Notice that light passes through the nerve fibre layers, inner retinal blood vessels and inner cell layers before reaching the light-sensitive PRs, which are located close to their blood supply. (from: Singh and MacLaren, 2011)

The work's focus was on retinal diseases characterised by a progressive photoreceptor cell loss. Photoreceptors are divided into rod photoreceptors, responsible for low light vision, and cone photoreceptors, enabling high acuity vision in bright light (see also caption of Fig. 1, in particular for retinal structure). A particular specialization of the human retina is the macula that is characterised by the highest density of cones, which additionally encloses the fovea centralis, the region of highest visual acuity that contains only cones. Photoreceptors depend on the retinal pigment epithelium (RPE) for metabolic activity and cones depend on rods for survival through their paracrine effects (Yang et al., 2009). The death or dysfunction of one of these cell types will lead to the irreversible death of photoreceptors. The progressive dysfunction and death of the photon-sensitive retinal cells is the major cause of adult blindness in industrialized countries. The etiology of photoreceptor degeneration is complex and multifactorial and caused by genetic or/and epigenetic factors (Jay, 1982). The genetic and mechanistic architecture of the photoreceptor degeneration is being successfully dissected and it encompasses a huge diversity of causes. The inherited forms of photoreceptor

degeneration[1], a common cause for visual impairment, are mainly subdivided into retinitis pigmentosa[2] (RP) and age related macular degeneration[3] (AMD). For instance, a majority of mutations, representing RP, affect photoreceptors' genes involved in intracellular transport mechanisms, movement of molecules between photoreceptors and RPE and the phototransduction machinery (Wright et al., 2010). Figure 2 demonstrates the dynamic of identified genes involved in inherited retinal diseases caused by (a) pathological mutation(s). For AMD, primary dysfunction occurs in RPE cells and Bruch's membrane, causing alterations in lipid disposition, protein cross-linking and reduced permeability to nutrients, leading to a secondary loss of photoreceptors in the macula. RP and AMD can have similar histopathalogical changes despite different aetiologies and in addition there is an overlap with RP-like diseases presenting with the characteristic symptoms of AMD and vice versa (Lund et al., 2001; Wright et al., 2010).

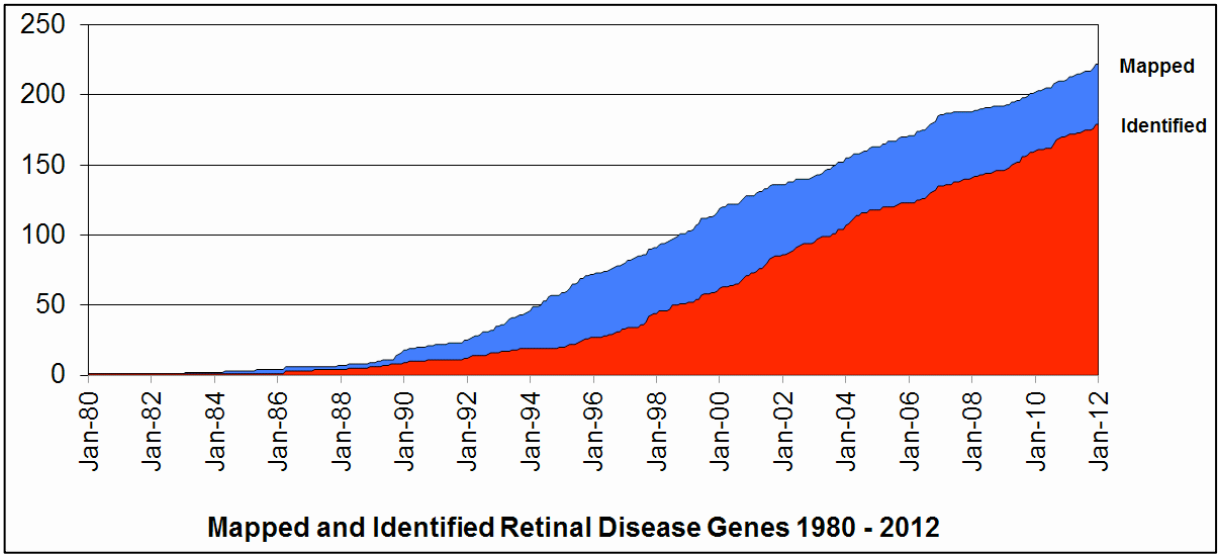

Figure 2: The total number of genes and loci involved in all categorised inherited retinal diseases is 225, whereas the number of identified genes counts 185. This graphic was taken from the official homepage of RetNet. RetNet provides a collection of (all) known pathological mutations based on scientific publications. Further on the website, each gene and mutation's locus is related to the disease(s).

Once photoreceptors are lost there is no endogenous replacement and once the casualties exceed the threshold the individual's eyesight is reduced augmenting to blindness. To treat retinal disorders characterised by the loss of photoreceptor cells, therapeutic approaches are being explored that take advantage of the technical

[1] This form of degeneration is predominantly monogenically inherited and causes in ~1 in 3,000 visual impairment (Wright et al., 2010)
[2] RP is the most common subtype and one of the two main causes of blindness in 20-64 year olds (Buch et al., 2004).
[3] AMD occurs in 30% of individuals over 75 years (Smith et al., 2001)

achievements in enabling work with materials in nanometre size range, in tools to manipulate DNA, or in stem cell technology. Concerning therapeutic approaches for late stages of the diseases, restoration of visual function is required. The exploration of electronic retinal devices has achieved remarkable results in restoring visual function in humans. A retinal electronic prosthetic device takes the place of dead or dysfunctional photoreceptor cells. It translates outside photic images into electrical signals in the retina that can ultimately be perceived by the brain as visual images. Technically, a small camera is mounted behind the patient's eyeglasses, which captures an image, and, in some models of the device, wirelessly sends the image to a microprocessor for conversion to an electronic signal. This signal moves to a specialized receiver and then to the intraocular prosthetic microelectrode implant. The implant transmits the signal to the retinal cells, which send the signal down the optic nerve for final processing (Chader et al., 2009). The devices can be classified according to the anatomical location of the implanted device (Weiland et al., 2005; NG, et al., 2009): on the retinal surface (epiretinal) (e.g.: Schwarz et al., 1999), in the subretinal space (e.g.: Zrenner et al., 2010), in the suprachoroidal space (Kanda et al., 2004) or even on the sclera (episcleral) (Siu and Morley, 2008), next to the intraocular implantations there are visual cortical prostheses, devices that can stimulate directly the brain (Brindley and Rushton, 1974; Chader et al., 2009). Trails on patients using electrical devices are providing hope for the restoration of vision based on such an approach. However, it became evident that even limited sight restoration was a slow learning process that took months for improvement to become apparent. Nevertheless, light perception was restored and discrete phosphenes were seen and simple visual spatial and motion tasks could be performed (Chader et al., 2009; Zrenner et al., 2010). Issues of such prostheses for real-time vision are a permanent power supply and data transfer. For instance, a wireless link for data telemetry and power supply is desirable as a tethered connection will degrade the device's performance, affect the mobility of the patient and be a potential site for infections (Ng et al., 2009). Of further concern is the material stability, which should be stable throughout the lifetime of the patient, as well as the available resolution of the device enabling e.g. face recognition and reading ability. Only this year new improvements on power supply and signal amplification were achieved. A photovoltaic retinal prosthesis contained per pixel three series-connected photodiodes, which photovoltaically converted pulsed near-infrared light into bi-phasic current to stimulate nearby retinal neurons without wired power connections (Mathieson et al., 2012).

As aforementioned, a large number of genetic mutations in diverse genes has been shown to cause visual impairment and blindness. Gene-based therapies target correcting the primary genetic defect or modulating the adverse effects associated with the presence of the genetic defect and encoded mutant protein (Farrar et al., 2012). Non-integrating viral vector systems, like recombinant adeno-associated viral[4] vectors (McCarty et al., 2004) or non-integrating lentiviral vectors (Yanez-Munoz et al., 2006), or non-viral vectors (Andrieu-Soler et al., 2006) were used for sustained expression of transgenes. In preclinical studies was one aim the amelioration of transduction efficiency of endogenous retinal cells and rapid onset of transgene expression what was achieved by pseudotyping the AAV2 genome with capsid proteins of other AAV serotypes (Auricchio, 2003; Alloca et al., 2007; Natkunarajah et al., 2008). In one pioneering study on human, the recombinant AAV2/2-vector expressing RPE65[5] cDNA under the control of a human RPE65 promoter was injected into the subretinal space of young adult patients affected by Leber's congenital amaurosis due to a mutation in the gene encoding RPE65[6]. The vector effectively transduced RPE cells and induced expression of a correct RPE65-form. Though, patients reported significant improvement in visual function and mobility, clinical measurements did not show any significant improvement (Bainbridge et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008). Gene-based therapies are also being developed for retinopathies with a dominant-negative inheritance, by supressing the mutated gene and simultaneously replacing the mutated gene with a suppression-resistant gene encoding a functional variant of the pathogenic protein (Farrar et al., 2012).

Strategies to restore or maintain vision also pursue cell-based therapeutic approaches such as cell-based retinal therapies to replace dysfunctional and/or degenerate retinal cells by cell transplantation or to genetically engineer cells as cellular vehicles to deliver therapeutic gene products to diseased retinal tissue. In the present thesis, both cell-based therapeutic approaches were evaluated in mouse models of inherited retinal dystrophies.

[4] AAV: an adeno-associated virus of the non-pathogenic human parvovirus.
[5] Retinal pigment epithelium-specific 65-kDa protein; involved in the processing of the chromophore cycle in RPE cells.
[6] Leber's congenital amaurosis describes a group of recessively inherited, severe, infantile-onset rod-cone dystrophies. Vision is impaired from birth and progresses to blindness in the third decade of life (Hanein et al., 2004; Lorenz et al., 2000).

Grafting into the mammalian central nervous system (CNS) (e.g. as early as Dunn, 1917 or more popular Das and Altman, 1971) has been of success because of the lack of a lymphatic system and the protection by a blood-brain- or blood-retina-barrier from immune cells, thus representing areas of immunologically privileged sites. Mammalian retinal transplantation was first performed from Katharine Tansley in 1946. She transplanted the eyes of embryonic rats onto the developing brain of 2-day-old rats and observed moths of survival of the donor tissue as well as the maturation of certain retinal structures. On the other hand, the mammalian eye has been a favourite site for the placement of a vast array of tissues for over 100 years. Possibly the earliest recorded attempt of an in oculo graft was that of Van Dooremal in 1873. The first attempt to transplant mammalian retina to the eye was performed from Royo and Quay, in 1959 (Turner et al., 1988; reviewed by Faldino, 1924). The authors' initial investigations established that the immature retina would indeed survive and continue to develop when placed in the anterior chamber of the maternal rat's eye. Retinal transplantation has been carried on only in the mid 1980s when del Cerro repeated Royo and Quay 's experiments, transplanting strips and cell aggregates of embryonic (postconceptual day 13-18) and perinatal retinas into the anterior chamber of different rat models to examine the importance of donor age and recipient strain in graft development (Del Cerro et al., 1985, 1987). The experiments indicated the possibility to transplant immature retinal tissue to adult hosts, also of different strains, and the growth of the transplant, which was more significant in damaged retinas but with rossettes formation. Thereupon, the transplantation of newborn rat retinal tissue into adult retinal lesion sites was examined (Turner and Blair, 1986). Grafting retinas from 1-day-old neonatal donors into lesioned adult retinas was conducted successfully with the observation of donor tissue survival and development, what was successfully repeated for embryonic retinal tissue (Turner and Blair, 1986; Aramant et al., 1988, 1990; Aramant and Seiler, 1991) (see for retinal neurogenesis: Fig. 3).

Heterochronic transplantations, as described above, also gave more indication of that retinal progenitors (see Fig. 3) are limited such that a particular progenitor can generate only a subset of cell types at a given time during development (Austin et al., 1995; Belliveau and Cepko, 1999). This limitation became a hurdle for the approaches to generate donor tissue from expandable cell cultures (see below). On the other hand, because of reports of rewiring of transplanted embryonic retinal sheets (Del Cerro et al., 1985 and 1987; Turner et al., 1988a, b; Turner and Blair, 1986), cell-based therapies for

retinal degenerative disorders were considered as feasible by (i) replacing lost retinal tissue or (ii) rescuing retinal cells and eliciting a wound-healing response through application of a neurotrophic stimulus (Turner et al., 1988a).

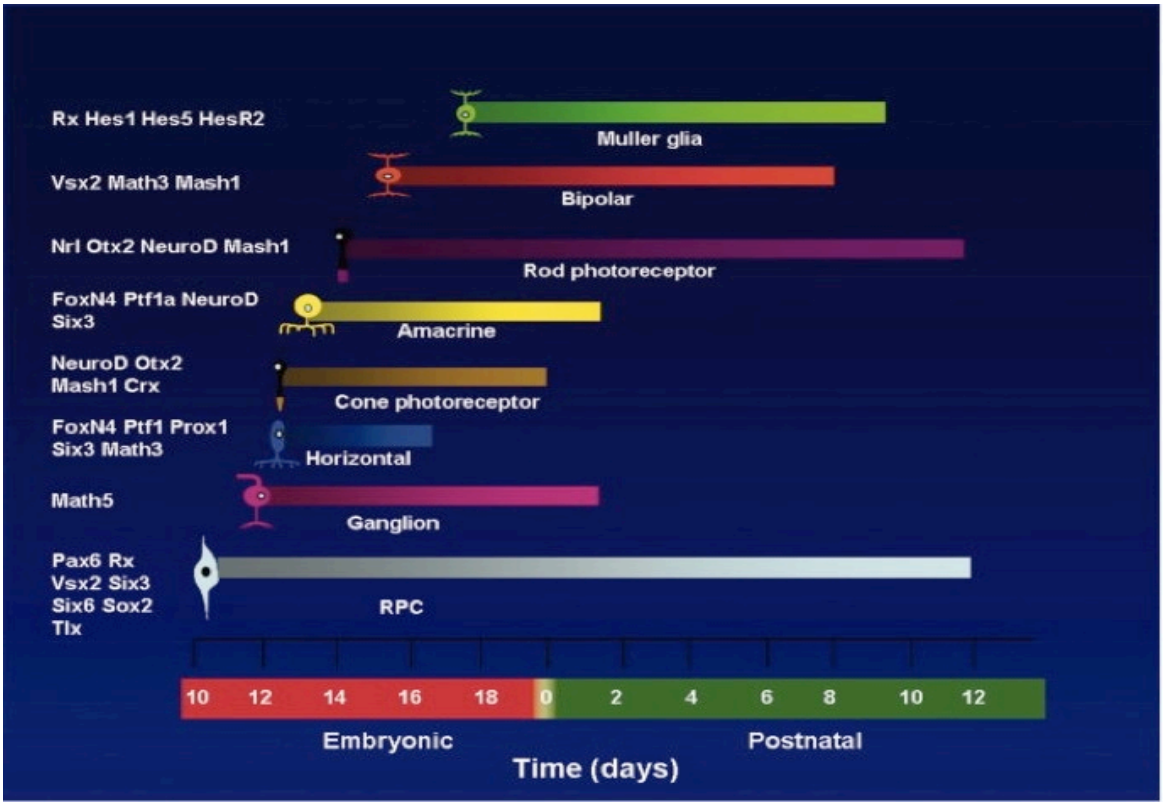

Figure 3: In the graphic the development of the single retinal cell types over time with involved transcription factors is shown. The retina develops from the diencephalon before neuronal differentiation, as two epithelial sheets of precursors continuous with the forebrain (Duke-Elder, 1970; McKay, 1989). This defines the retina as part of the central nervous system, with development from more specified neural stem cells called *multipotential retinal progenitor* cells, which reside in the inner neuroepithelium of the optic cup (Marquardt, 2003). So far it is understood, that the diversification of neurons in the mammalian retina is derived from one cell type, the multipotent retinal progenitor cell (Angevine et al., 1969; Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser 1988), that intrinsically change their potential to give rise to specific cell types over time (Gomes et al., 2011; summarized in Bassett and Wallace, 2012) in a conserved, but overlapping, chronological order (Rapaport et al., 2004; Young, 1985). The development of the (vertebrate) retina is called *retinogenesis* or, enhancing the affiliation of the retina to the CNS, *retinal neurogenesis*. Briefly, after ganglion cell development cones, horizontal cells and roughly half of the amacrine cells are generated prenatally. In a second postnatal wave, bipolar cells, Müller glia and the remaining amacrine cells develop. Rods are produced throughout the period of retinal histogenesis. Today it is assumed that the competence state of a progenitor cell is determined only intrinsically with temporal dependence, i.e. that cells both acquire and lose the ability to make various cell types (Livesey and Cepko, 2001; Ohsawa and Kageyama, 2008; Wallace, 2011). Changing its competent state in waves, the progenitor is able to generate different subsets of postmitotic cells at different time windows (Livesey and Cepko, 2001). From the environment it is receiving positive and negative feed-back information, signals send from committed cells to produce more of its cell type or inhibit its production (Morrison et al., 1997; Belliveau and Cepko, 1999; Reh and Tully, 1986; Waid and McLoon, 1998). Confronting the endogenous fine tuning of communication between intrinsic and extrinsic signals to turn an embryonic stem cell into a photoreceptor cell, what is until now barley explored in detail, elucidates the challenge in terms of cell therapy to find/generate the appropiate cell type for retinal therapies. (graphic taken from Wallace, 2011)

Regarding replacement of retinal tissue P. Gouras (1985) had conducted some remarkable transplantation studies. In 1985 he reported the successful

xenotransplantation of cultivated and radiolabeled human retinal pigment epithelial (RPE) cells into monkey eye (Gouras et al, 1985). Subsequently, the same group and Li and Turner transplanted wild-type rat RPE cells into the subretinal space, i.e. between RPE and outer nuclear layer (see Fig. 1), of the Royal College of Surgeons (RCS) rat[7] and observed a delay of the degeneration of host photoreceptors (Gouras et al., 1989; Li and Turner, 1988)[8]. In 1991, again Gouras, was able to isolate rod photoreceptors from adult wild-type rat retinas, identified by morphology, and subretinally transplanted the primary cell suspension into adult dystrophic albino rat retinas[8]. The donor rods survived for months and formed synaptic contacts with host retinal cells. Subsequent work for translational medicine, such as transplantation of single cell types, e.g. RPE cells to patients with macular degeneration (Algvere et al., 1994, 1997; Weisz et al., 1999) and neural retinal cells to patients with RP or macular degeneration (Kaplan et al., 1997), have passed the criteria for safety and survival (Berson and Jakobiec, 1999)(and see below). In fact, cell transplantation for photoreceptor replacement is one of the most feasible type of repair as photoreceptor degeneration initially leaves the inner retinal circuitry intact and thus new photoreceptors need only make single, short synaptic connections to retinal interneurons to contribute to the retinotopic map.

In more recent studies, new techniques and tools (e.g. confocal microscopy and transgenic mouse models) have been employed to analyse the efficacy of a cell-based photoreceptor replacement in wild-type mice and different transgenic mouse models for photoreceptor degeneration (MacLaren et al., 2006; Bartsch et al., 2008). From embryonic and postnatal EGFP[9] transgenic mice, retinas were isolated and dissociated to single cell suspension with subsequent subretinal transplantation into neonatal and adult mice. A subpopulation of the subretinally grafted retinal cell suspension was capable of integrating into the outer nuclear layer (ONL) and of differentiating into apparently fully mature EGFP-positive rod photoreceptor cells. The donor-derived cells developed normal morphology of rod photoreceptor cells as indicated by expression of the EGFP reporter and, visualised for the first time in detail with immunohistochemically staining, the expression of photoreceptor specific as well as synaptic connectivity markers. As earlier described by del Cerro et al. and Aramant et al.,

[7] Phagocytosis by RPE cells of photoreceptor outer segments, which is an important function of normal RPE cells, is deficient in the RCS rat and leads to photoreceptor cell death (Herron et al., 1969; LaVail et al., 1975).
[8] Del Cerro's group later analysed this effect published in 1998 (Little et al., 1998).
[9] EGFP: enhanced green fluorescent protein (Okabe et al., 1997) ubiquitously expressed in mouse.

integration, maturation and survival abilities of donor cells were not dependent on the age of the host retinas. In contrast, the state of maturity of the donor tissue had a significant impact on the integration capacity of the grafted cells (Del Cerro et al., 1985, Aramant et al., 1988). In Bartsch et al.'s study the appropriate age of primary cells for the highest capacity of integration and differentiation into rod photoreceptors was defined at postnatal day 4 and 5 (P4 and P5), arguing for an enrichment of cells committed towards a rod photoreceptor cell fate at that ontogenetic stage of retinogenesis. Experimental evidence strongly suggested that committed immature rod postmitotic photoreceptors and not proliferating progenitor or stem cells had the capacity to successfully integrate into host retinas and differentiate into mature rod photoreceptor cells. This was emphasised when pure immature donor-derived retinal cell populations with the expression of neural retinal leucin zipper expression[10] (Nrl), a transcription factor specific for rod postmitotic precursors that persists in adult rods (Akimoto et al., 2006; Swaroop et al., 1992; Mears et al., 2001; Swain et al., 2001), were used for transplantation and subsequently, integrated into the ONL of the host and differentiated into mature rod photoreceptors at similar numbers to unsorted immature retinal cell suspensions. Thereupon, Gust and Reh (2011) isolated an Nrl-GFP retinal cell population from adult mice and demonstrated that mature donor-derived retinal cell populations were also capable of integrating into adult recipient retinas displaying photoreceptor characteristics (Gust and Reh, 2011). The authors argued that mature donor-derived photoreceptor cells have the ability to regenerate their cellular structure after being isolated from their original microenvironment when subsequently implanted into a similar microenvironment. Subsequent studies were able to further improve donor cell integration by enriching primary retinal cell suspension with cells of the identified ontogenetic stage (Lakowski et al., 2011; Eberle et al., 2011; Pearson et al., 2012) and/or manipulating the recipient environment to a more developmental-like state facilitating outer nuclear layer integration (West et al., 2008, 2012b; Pearson et al., 2010). An important issue that further supports the cell-based replacement strategy is the evidence of (re-) generation of visual function by engrafted donor-cells as seen by the improved performance in behavioural tests of mice deficient for rod phototransduction following engrafted primary Nrl-gfp donor cells (Pearson et al., 2012).

[10] Transgenic mouse model expressing GFP under the Nrl promoter. Cells were isolated by fluorescence-activated cell sorting for GFP-positive rod precursor cells.

For potential clinical applications it is also important to understand the impact of the degenerating retina on the grafted donor-cells and vice versa. Therefore, it is necessary to examine the integration and survival capability of grafted donor-cells for an extended period of time, i.e. as the disease progresses. In one study, we addressed this question and subretinally transplanted primary EGFP-transgenic retinal cell suspensions (isolated from young mice of the age of P4 and P5) into the transgenic β2/β1 knock-in mutant mouse model, characterised by a slow and long-lasting apoptotic photoreceptor cell death. Qualitative analysis 4 weeks after transplantation into (i) adult wild-type, (ii) adult transgenic mice at the beginning and (iii) at advanced stage of the dystrophy, revealed no obvious difference in the number of integrated donor-derived cells. The donor cells, identified by EGFP expression, integrated correctly and differentiated into apparently fully mature photoreceptors. Furthermore we investigated the morphological appearance of grafted donor photoreceptors at different stages of degeneration of the host retinas. In the β2/β1 knock-in mouse model with progression of the dystrophy, the outer and inner segments shorten to a compressed structure over time (Weber et al., 1998). In transgenic mice with integrated mature donor-derived photoreceptors, the length of inner and outer segments of the engrafted photoreceptor cells was decreased to a similar extent as observed for dysfunctional endogenous photoreceptor cells. We also observed in experimental mutant mice of the same age, i.e. at a morphologically similar state, that the morphological appearance was independent of the retention time of the grafted donor-derived photoreceptor cells. Thus, the grafted photoreceptor cells adapted the state of morphology of the host retina implying that the pathology of the host retina had a significant impact on the morphology of grafted donor cells. Besides the morphological adaptation, the numbers of retrieved donor-derived photoreceptor cells declined with increase of post-transplantation intervals for wild-type and mutant mice. A loss of integrated mature donor-derived photoreceptor cells over time has been observed in a recent study, and was attributed to an immune response to the grafted cells (West et al., 2010).

Testing of the discussed protocols for increased integration of donor-derived photoreceptors could lead to preservation of the host retinal morphology. Sustaining or even rebuilding the retinal structure should be considered for clinical approaches. Many inherited retinal disorders encompass the loss of the outer retina characteristics what induces additional pathologies through strong metabolic changes, like the increase of oxygen level due to absence of the high energy requiring photoreceptor cells

(Samardzija et al., 2012). Though, it had been demonstrated that wild-type donor photoreceptors are functional even though they experienced morphological alterations (MacLaren et al., 2006), for clinical applications the preservation and construction of the outer retina must be considered. In several preclinical trails scaffolds of variable materials have been investigated. Scaffolds of naturally occurring membranes (Bruch's membrane), mimic natural mechanical properties and are biocompatible, but comprise ethical concerns. Polymers of natural structure, like gelatine, have been considered with promising results but the material has uncontrollable characteristics. Synthetic polymers like poly lactic-*co*-glycolic acid, polycaprolactone, poly glycerol sebacate, polyhydroxyalkanoates, or, only recently postulated, copolymers of N-isopropylacrylamid and acrylic acid N-hydroxysuccinimide (Mazumder et al., 2012) were characterised by: i) Biodegradation in diverse velocities and with different degradation products; ii) little to non-invasive delivery of the scaffold; iii) as well as a high flexibility; iv) and the opportunity of determining the design, accomplishing the requirements (Hynes and Lavik, 2010). Thereby, scaffolds have been identified as supporters for structure (Ge et al., 2008; Khan et al., 2008), for cell delivery (Tomita et al., 2005; Nguyen and West, 2002), for directing cell behaviour (Tao et al., 2007; Burman et al., 2004) and for delivering drugs or trophic molecules (Langer, 2000; Kobsa and Saltzman, 2008). An insight of the advances, progress and requirements of natural and synthetic scaffold was given in the review of Hynes and Lavik (2010). Progress in photoreceptor replacement for patients might be expected from the concept of designing scaffolds enabling the construction of photoreceptor layer accompanied with retinal pigment epithelium layer. It is in general accepted that photoreceptors and RPE cells form a functional unit and this principle should be considered when handling attempts to rescue or replace either cell type.

All transplantation experiments introduced above relied on the availability of primary donor tissue and thus, are of limited relevance for clinical applications due to ethical concerns. In vitro expansion of relevant cell types might offer the opportunity to generate large quantities of cells for therapeutic approaches. While some cell types of the adult mammalian eye retain the capacity to divide for some passages in culture, cells within the retina lose their ability to proliferate early during life.

Retinal cell culture finds its origin in primary cell culture as explanted, reaggregated and dispersed cultures, but all three forms cannot or only limited be

propagated in vitro. Alternative cultivation of retinoblastoma cells (Reid et al., 1974) or immortalized retinal cell types (Gallimore et al., 1986) enabled unlimited expansion of cells, but for in vivo studies these cells were potentially hazardous (McFall et al., 1977; Del Cerro et al., 1993; Xu et al., 1995) and for in vitro studies their retinal character was suspected (Jiang et al., 1984; Kyritsis et al., 1984; Terenghi et al., 1984). The possibility of identifying and isolating multipotent retinal progenitor cells (RPC) (Barnstable et al., 1985; Akagawa and Barnstable, 1986; Lemmon, 1985, 1988; Kelley et al., 1995), studies investigating the effects of growth factors as well as timing and signalling mechanism of fate determination (Lillien and Cepko, 1992; Reh, 1992; Adler, 1996; Cepko et al., 1996; Jensen and Raff, 1997) and the potential to re-specify cell fate (James et al., 2003) paved the way of cultivating and expanding RPCs with subsequent differentiation into specific retinal cell types. However, the cultivation of RPCs continues to be challenging, as, although they meet the established stem cell criteria of self-renewal and multipotency, they survive very poorly as isolated single cells (Klassen et al., 2004a; Gamm et al., 2008; Schmitt et al., 2009). Nevertheless, differentiation into rod photoreceptors and bipolar cells was observed (Angenieux et al., 2005; Merhi-Soussi et al., 2006; Canola and Arsenijevic, 2007) for retinal progenitor cells isolated from early postnatal mice, but on the other hand it was reported that these cells rapidly lose retinal marker expression in culture (Mansergh et al., 2010). As recently demonstrated further, RPCs isolated from neonatal mice and in vitro propagated as single monolayers lost their capacity to differentiate down the retinal lineage (Czekaj et al. 2012). The in vitro expanded retinal progenitor cells, down regulated the expression of genes important for retinal development like Chx10, Lhx2, Rax6, Six6 and Six3 (see Fig. 3) and up-regulated neural stem/progenitor markers like nestin, Sox2 and Pax6. The in vitro expanded RPCs acquired the ability to differentiate into oligodendrocytes, a glial cell type usually not generated within the retina (Czekaj et al., 2012). This implies that in vitro expanded RPCs lose their regional identity and acquire features similar to in vitro expanded NSCs (Gamm et al., 2008; Schmitt et al., 2009; Czekaj et al., 2012). Clonal analysis of cortical and retinal multipotent progenitors (Holt et al., 1988; Price et al., 1991; Reid et al., 1997; Turner and Cepko, 1987; Turner et al., 1990; Walsh and Cepko, 1990; Wetts and Fraser, 1988) indicates that the distribution of lineage tree sizes is consistent with a stochastic model in which the probabilities of undergoing a division are weighted according to cell generation (Slater et al., 2009; see Fig. 3). Generating photoreceptors in vitro in general from multicellular cultures (Shatos et al., 2001; Coles et al., 2004; Klassen et al., 2007),

has been impressively demonstrated by Eiraku and colleagues (Eiraku et al., 2011) who have shown the dynamic, autonomous formation of the optic cup structure from a three-dimensional culture of mouse embryonic stem cell aggregates (http://www.nature.com/nature/journal/v472/n7341/extref/nature09941-s3.mov). The optic cup developed to distinct layers, the RPE and the neural retina. The retinal layer contained all the major neural components, that is, photoreceptors, ganglion cells, bipolar cells, horizontal cells, amacrine cells and Müller glia. Within the layers the cells were arranged in a spatially correct order, comparable to the early neonatal eye, when analysed after a cultivation period of 24 days (Eiraku et al., 2011). Furthermore, the authors observed improved retinal induction when the formation of rigid continuous epithelial structure was promoted, thus emphasising the interdependence of pigmented and neurosensory layers. While this approach circumvents the identification of intrinsically and extrinsically signalling direct non-committed cells into photoreceptor-fate, it also accomplishes an in vitro opportunity to identify signalling cues involved in cell-competence and cell-fate decisions.

Thomas Reh's group developed a protocol, efficient to selectively direct human embryonic stem cells, cultivated adherently from the third day, to a neural retinal cell fate with preferential differentiation into inner retinal neurons (ganglion and amacrine cells) (Lamba et al., 2006). Using the same protocol the group was able to identify photoreceptor specific gene expression in the human embryonic stem cell culture after a 'three-week retinal determination'. The cells, defined as retinal progenitor cells, were then tested for their potential to integrate and maturate following subretinal transplantation into adult mice. When subretinally grafted into mice, the cells gave rise to photoreceptor cells and improved the electroretinogram recordings in dystrophic mice (Lamba et al., 2009). Just ahead of this study, Osakada and colleagues demonstrated the in vitro generation of rods and cones from mouse, monkey and human embryonic stem cells. Cells, expressing retinal progenitor marker, were isolated and cultivated as cell suspensions under defined culture conditions, in the absence of retinal tissues. Stepwise treatments with a modified differentiation protocol induced photoreceptor differentiation of all three embryonic stem cell types (Osakada et al., 2008). When this protocol was applied to induced pluripotent stem cells derived from mouse embryonic fibroblasts and human dermal fibroblasts, induction of retinal progenitor marker and, with additional adjustments, photoreceptor marker expression was observed (Hirami et al., 2009; also Meyer et al., 2009). Recently, Osakada's protocol

was used to direct mouse embryonic stem cells into photoreceptor cells for testing the potential of in vitro-derived photoreceptors to integrate subsequent to subretinal transplantation into mice. However, no correctly integrated donor-derived photoreceptor cells were detected, suggesting that the cell suspension contained insufficient photoreceptors at the correct ontogenetic stage (West et al., 2012a). In 2011 Zhou et al., showed the differentiation of induced pluripotent stem cells of swine into rods and their integration into the retina, applying the postulated protocol from Thomas Reh with some modifications (Zhou et al., 2011). Finally, this year an additional protocol for the differentiation of photoreceptors from human induced pluripotent stem cells was reported to induce a more stage-specific differentiation toward retinal photoreceptor cells (Mellough et al., 2012). Which protocol prevails for the derivation of retinal cells from embryonic stem cells or induced pluripotent stem cells, remains to be seen in the future. Still, this rapid progress in the derivation of specific retinal cell types from expandable pluripotent stem cells provides hope to translational medicine, as highlighted by a recent report of the successful implantation of human embryonic stem cell-derived RPE cells into patients suffering from Stargardt's macular dystrophy[11] and AMD (Schwartz et al., 2012). A human embryonic stem cell line was differentiated to a 99% pure RPE cell culture with typical behaviour, i.e. they integrated into the host RPE layer forming mature quiescent monolayers after transplantation into animals. Also here, the ontogenetic stage of the donor cells had obvious effects on how they attached and continued to persist during the study in the host. The clinical study was initiated to evaluate the safety and tolerability of subretinal transplantations of human embryonic stem cell-derived RPE cells in patients with Stargardt's macular dystrophy and dry AMD. Cells had been injected submacularly in a region of the pericentral macula that was not completely lost to the disease. Four months after surgery no signs of hyperproliferation, tumorigenicity, ectopic tissue formation or apparent rejection were observed in either of the patients and neither lost vision and in addition some improvement of visual acuity was observed in the eye that had undergone transplantation (Schwartz et al., 2012).

Cell replacement of either photoreceptors or RPE cells presents an elegant treatment option for retinal dystrophies at an advanced stage, when the endogenous cells are lost. Especially for disorders where photoreceptor cell loss is caused through

[11] Stargardt's disease is the most common form of inherited juvenile macular degeneration with visual loss in the macular with involvement of the RPE. It is an autosomal recessive trait with mutation in the ABCA4 (ATP-binding cassette transporter) gene (Foundation Fighting Blindness).

the dysfunction of RPE cells, like in AMD, engraftment of healthy RPE cells might prolong the survival of the remaining endogenous photoreceptors. However, this strategy still has to be evaluated more precisely in terms of survival of donor cells due to immune response or the impact on endogenously released apoptotic factors (see above in the section of retinal scaffolds). In addition, for photoreceptor transplantations, the correct integration of grafted cells has to be controlled, as it has been observed that donor cells also tend to remain in the subretinal space (Bartsch et al., 2008) Here, they differentiate into photoreceptor cells, neurons or glial cells and might cause adverse effects on visual acuity.

Neuroprotection is a treatment strategy for retinal dystrophies that is discussed as being closest to clinical applications. Generic gene therapies that aim not to correct the gene defect but to treat its consequences, i.e. the loss of photoreceptor cells, offer the possibility of therapies that are widely applicable across a range of conditions characterised by degeneration of photoreceptor cells (Buch et al., 2007). Indeed, first clinical studies of phase I and II (Sieving et al., 2006; Talcott et al., 2011; Zhang et al., 2011) have been published for a therapy based on the attenuation of photoreceptor cell loss in patients with inherited retinal degenerations, RP and Usher syndrome (Talcott et al., 2011) as well as for geographic atrophy and AMD (Zhang et al., 2011). The patients have been transplanted with an intraocular device that encapsulated a genetically modified human RPE cell line, which secreted a neurotrophic factor that is known to protect photoreceptors from apoptotic cell death. The device, 6mm in length, was surgically placed in the vitreous body of the eye and sutured in a manner that allowed modifications, like retrieval. This ‘encapsulated cell technology’ allows a sustained intraocular delivery of therapeutic products, and represents so far the only therapeutic approach that is evaluated in a broad clinical study on inherited retinal degenerations.

The survival of neurons in the eye and other parts of the central nervous system is known to depend upon several neuroprotective factors. These are generally defined as soluble proteins that are secreted by the target that will be innervated by the neurons. Binding of a neurotrophic factor to its cognate receptor usually leads to signalling that ultimately prevents apoptosis in the neuron (Purves et al., 2001). Following the description of nerve growth factor (NGF), several such protein growth factors have been shown to promote neuronal survival in the CNS. Neurotrophic factors that protect

diverse CNS nerve cell types and additionally act on photoreceptor cells either directly or via activation of other retinal cell types, are NGF (Hamburger et al., 1981), brain-derived neurotrophic factor (BDNF) (Hofer et al., 1988), basic fibroblast growth factor (bFGF) (Faktorovich et al., 1990), glial cell line-derived neurotrophic factor (GDNF) (Henderson et al., 1994), pigment epithelium-derived factor (PEDF) (Tombran-Tink et al., 1991; Cayouette et al., 1999), rod derived cone viability factor (RdCVF) (Leveillard et al., 2004), Osteopontin (Del Rìo et al., 2011), and ciliary neurotrophic factor (CNTF) (Sendtner et al., 1990). The binding of the neurotrophic factor to its receptor initiates various intracellular pathways, also depending on the developmental stage (Dechan et al., 2005). The neurotrophic factor receptors fall into the following main categories: (i) receptor tyrosine kinase (including tropomysosin receptor kinase proteins (A-C)) (Buch et al., 2007); (ii) p75 neurotrophin receptor (Dechan et al., 2005); (iii) GDNF family receptor forming heterotrimers with the receptor tyrosine kinase (RET) (Airaksinen et al., 2002; Sariola et al., 2003); (iv) CNTF receptor is similar to the Interleukin-6 receptor and upon CNTF binding forms a heterotrimer with two co-receptor molecules, gp130 and leukaemia inhibitor factor receptor-β (Sleeman et al., 2000). After binding of the ligand, downstream signalling is initiated for (iii) and (iv) by autophosphorylation at specific tyrosine residues resulting in inhibiting pro-apoptotic signalling pathways or up-regulating anti-apoptotic signalling. Binding of CNTF - a cytokine with potent neuroprotective activity on photoreceptor cells – to its receptor is followed by a transient complex formation with the co-receptors, and induces the extracellular signal-related kinase kinase (MEK)/extracellular signal-related kinase (ERK) pathway which modulates the signal transducer and activator of transcription 3 (STAT3) signalling (Chaum et al., 2003). The neuroprotective effect on photoreceptors is thought to be mediated by inner retinal neurons that primarily respond to the factor and secrete secondary messengers that directly act on photoreceptors to promote their survival. Though, the CNTF-receptor has also been identified on rod and cone photoreceptors in non-rodent mammalian species (Beltran et al., 2005).

Neuroprotection of retinal cell types was first attempted delivering recombinant neurotrophic factors to the vitreous compartment through intravitreal injection. The evaluation of the potential to protect photoreceptor cells from degeneration in a variety of animal models of inherited retinal degeneration was tested for several factors using this method, including bFGF in the RCS rat (Faktorovich et al., 1990); CNTF in the

Pde6b^{rd1} [12] and Q344ter[13] mouse (LaVail et al., 1998); and PEDF in Pde6b^{rd1} and rd2[14] mouse model (Cayouette et al., 1999). The inability of these and other neurotrophic factors to pass the blood-retina barrier and their short ocular half life meant that repeated intraocular injections of recombinant protein would be required to achieve long-term therapeutic effects in patients. Hence therapeutic strategies for sustained intraocular delivery of neurotrophic factors have been developed using either viral gene therapy vectors that deliver genes encoding neurotrophic factors, or encapsulated cell technology (ECT) devices made of genetically engineered cells secreting recombinant protein.

CNTF is one of the most extensively studied neurotrophic factor that demonstrates potential for preservation of photoreceptors from apoptotic cell death at high efficacy. Administration of CNTF via AAV-vectors to the intravitreal space was the first analysis of sustained delivery of CNTF that confirmed the neuroprotective potential. However, the evaluation of maintaining retinal function revealed impaired photoreceptors physiology as a result of prolonged CNTF administration. Several studies aimed at clarifying the deleterious effects of CNTF using AAV- or ECT-mediated sustained delivery. The observations can be summarized as such: the nuclei of cells incorporated in all three retinal layers increased in size, the outer segments of photoreceptor cells decreased in length, the electro retinograms (ERGs) for rods and cones were lost, expression levels of proteins of the phototransduction cascade were reduced. All these adverse effects were dose-dependent and reversible (summarized in Buch et al., 2007; McGill et al., 2007; Wen et al., 2008). Explanations for these detrimental effects of sustained CNTF delivery were: (i) an increase of the nuclear size was correlated to uncondensed euchromatin indicative of changes in gene expression patterns in these cells (Buch et al., 2007). (ii) Down-regulation of the phototransduction proteins, cone opsin and rhodopsin, resulting in greatly reduced photon catching, which was detected as reduced ERGs. Further, less amounts of rhodopsin and shorter rod outer segments are mutually dependent. Shorter outer segments save energy and resources, as the metabolic load associated with the dark current and the continuous renewal of outer segments is reduced (Wen et al., 2011). This effect is known to be associated to the

[12] rd1: a b-PDE null mutant, see below (pg. 28 or in project 2).
[13] Q344ter: mutation that removes the last five amino acids of rhodopsin, showing a loss of photoreceptors beginning at P10 and terminated at P21 (Sung et al., 1994).
[14] rd2: null mutation in peripherin2, a protein essential for outer segment disk morphogenesis, resulting in no photoreceptor outer segments (LaVail, 1981; Boesze-Battaglia and Goldberg, 2002).

elevated STAT3 expression in Müller cells what leads to a reduced expression levels of photoreceptor-specific genes such as rhodopsin (Buch et al., 2007). Therefore, CNTF mediated down-regulation of proteins of the phototransduction cascade could be beneficial to photoreceptors under degenerative pressure. (iii) In a study on normal rats it was shown that the adverse of CNTF on retinal function were reversible, being detectable 5 days after intravitreal injection of recombinant CNTF, but recovering to normal 21 days after injection. Postulated findings in animal models have revealed that administration of a defined dose of CNTF adjusted to non-detrimental levels did not preserve photoreceptors from death (Buch et al., 2006; Zeiss et al., 2005). Sustained delivery of CNTF through ECT in normal rabbits enabled to relate morphological and functional changes directly to a defined secreted dose. Doses up to 22ng/day were non-deleterious in terms of rod ERG recordings but reduced cone-ERGs at dim flash intensities, implying a reduced sensitivity of cones to photons probably due to reduced expression levels of opsins. Nuclear morphological changes were apparent at this dose, indicating potent bioreactivity of CNTF. Doses below 5ng/day neither induced alterations in retinal morphology nor in ERG recordings (Bush et al., 2004). In a different study using also the ECT device the CNTF secretion levels were related to the extent of photoreceptor cell rescue in a dog model for inherited retinal dystrophy. Major protection was achieved at doses of 5 to 15ng CNTF per day over the course of 7 weeks. These results suggest that it could be possible to induce neuroprotection without affecting photoreceptor's function, when doses are compared to the aforementioned study of Bush et al. However, the photoreceptors in the dog model showed nuclear morphological changes and reduced rhodopsin expression and photoreceptor function was not assessed (Zeiss et al., 2005). As aforementioned, the first clinical trails of phase I and II have been exploited with reported improvement of visual acuity of the affected participants, indicating that a therapeutically effective CNTF dose was administered that might be below the threshold for suppression of retinal function (Sieving et al., 2006; Talcott et al., 2011; Zhang et al., 2011).

As illustrated, the sustained delivery of neurotrophic factors through encapsulated cell devices is a highly attractive therapeutic option to treat degenerative retinal disorders characterised by the loss of photoreceptor cells and to be translated into clinic, particular caused in its broad and quite simple application. In view of the large number of known factors with neuroprotective activities on photoreceptor cells as

well as the ongoing investigations on up-regulated signals during neurotrophic stimulation or retinal degeneration attempting to identify new factors (Del Rìo et al., 2011; Samardzija et al., 2012; Karl and Reh, 2012), we argued that it is mandatory to develop a cell-based intraocular delivery system for the analysis of therapeutic gene products for mouse models of inherited retinal dystrophies. The mouse represents an excellent species with its numerous genetic and acutely induced models for degenerative retinal disorders to evaluate therapeutic approaches in preclinical studies (Hafezi et al., 2000). Hitherto, the methods used for a sustained delivery of neurotrophic factors for photoreceptor protection in mice were achieved mainly by invasive applications like the genetic modifications of endogenous retinal cells using subretinal or intravitreal injections of viral vectors encoding these factors, or the transplantation of engineered cells often showed adverse impacts on the host retina. Our aim was to develop a cell-based delivery system that is highly controllable and easy to handle in terms of: (i) Genetic modifications of cellular vectors; (ii) expression levels of therapeutic gene products; (iii) in vivo application of the engineered cells and (iv) intraocular tracking of the grafted cells. The encapsulated cell device usually used in large animal models of retinal dystrophies and human patients had a length of 6mm, exceeding significantly the size of a mouse eye. Other encapsulation devices like microspheres with a minimum diameter of 80µm exceed the capacity of the mouse's intravitreal space (a volume of ~5µl) (Andrieu-Soler et al, 2005; own observations). Therefore, to date a cell-based sustained delivery of proteins to the mouse eye can be only obtained by the injection of non-encapsulated cells. The achievement of such a cell-based delivery system requires the identification of a cell type that is greatly expandable, long-term cultivatable, genetically modifiable, tolerated in the eye without exerting adverse effects and that survives for long-time intraocular.

In earlier studies the injection of non-modified cells was observed to confer some neuroprotection in the CNS (e.g.: Pluchino et al., 2005; Crigler et al., 2006 (summarizes autocrinal support of MSCs)). Similarly, the transplantation of embryonic retinal cells promoted the survival of damaged ganglion cells in the chick retina (Stanke and Fischer, 2010). Subretinal transplantation experiments in the RCS rat identified several cell types as being neuroprotective for the degenerate photoreceptors including human neural progenitor cells (Gamm et al., 2007; Wang et al., 2008), rat Schwann cells (McGill et al., 2007) or human embryonic stem cell-derived RPE (Lu et al., 2009). Neuroprotective activity was also observed after subretinal transplantation of unmodified murine

neurosphere cells into a mouse model of retinal dystrophy (Lu et al., 2002). The protective effect on photoreceptors, however, rapidly diminished away in all studied models (Gaillard and Sauvé, 2007). In other studies the neuroprotective potential of the grafted cells was increased with genetically modification of cells to overexpress neuroprotective factors. For instance, mouse embryonic stem cells were engineered to secrete GDNF, engraftment into the intravitreal space in the RCS dystrophic retina attenuated photoreceptors degeneration (Gregory-Evans, et al., 2009). Immortalized neural progenitor cells expressing BDNF were capable of rescuing damaged ganglion cells when applied intravitreally and subretinally (Wang et al., 2002). Genetically modified mesenchymal stem cells secreting also BDNF and placed intravitreally, preserved damaged retinal ganglion cells (Harper et al., 2011). Nevertheless, all these cells comprise of features that make them less attractive to serve as cellular vehicles for the eye. Embryonic stem cells have not experienced normal developmental cues that may result in tumour formation in vivo (Arnhold et al., 2004; Müller et al., 2006) and indeed, have shown invasive traits after intraocular transplantation (Gregory-Evens et al., 2009; Chaudhry et al., 2009). Immortalized neural stem cells might show changed cellular characteristics such as extraordinary migratory abilities in vivo as well as a higher degree of multipotency and hence are associated with a higher risk of tumour formation (Müller et al., 2006; own observations). Mesenchymal stem cells are widely discussed in the literature for several reasons that display uncomplicated handling in the clinic. Nevertheless, these cells appear to be inappropriate for the development of an intraocular cell delivery system, given that homogeneous cultivation is contested and they normally differentiate down the chondrogenic, osteogenic and adipogentic lineages and not down the neural lineages (Yu and Silva, 2008; own observations). Our group investigated several cell types to identify an appropriate cell population complying with the above listed features. As indicated above, mesenchymal stem cells and immortalized neural stem cells (C17.2 cell-line) met the requirements of straightforward cultivation method for massive expansion, long-term cultivation, and efficient genetic modification. After intravitreal engraftment of mesenchymal stem cells into mice eyes, they induced severe adverse effects on the general morphology of the host retinas even within one week following transplantation, including a detachment of the retina from the RPE layer with subsequent degeneration of photoreceptor cells. Immortalized neural stem cells also had adverse effects on host retinas. In addition, these cells were still present in the vitreous one week after transplantation, but could no longer be detected two weeks

after transplantation, possibly as a result of an immunoreaction of the host. For our approach we identified another cell type that compromised all requisite features to serve as an intraocular vehicle, the adherently cultivated neural stem cells.

The concept of a neural stem (NS) cell, as today defined (Boulder Committee, 1970) (Breunig et al., 2011), was first conceptually described by the neurologist Wilhelm His in 1874 and 1904 (Breunig et al., 2011). At that time the analysis of tissue material was restricted to morphological methods, nevertheless many ideas and concepts about the brain's structure and development were created (see Fig. 1), which were later verified with more sophisticated methods.

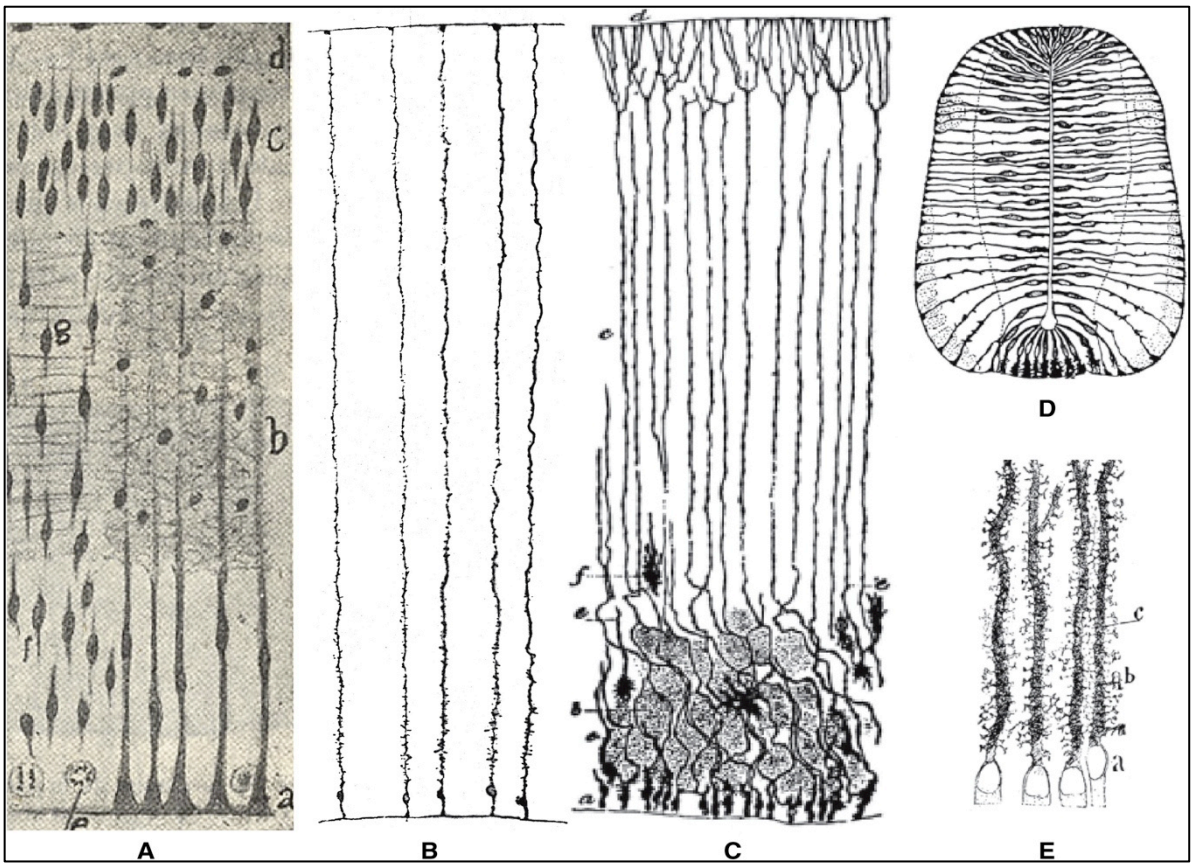

Figure 4: A Potpourri of Classical Depiction of Neural Glial Stem Cells(A) Illustration taken from the work of His (1904) on the human embryonic forebrain. Notice the incredible detail and fidelity with which cell types (mitotic figures, "pongiobasts" [radial glia], migrating neurons, etc.) were depicted without the use of modern methods, including the horizontal and vertical (asymmetric) division of the mitotic cells.(B) The drawings of the "ependymal glial cells" in the human fetal cerebrum at 10 weeks old stand with the Golgi method (Retzius, 1893).(C) Epithelial (radial glial) and neuroglial cells of the cerebral cortex at later stage of development in the neonatal rabbit stained with the Golgi method depicted by Ramón y Cajal (Ramón y Cajal, 1909).(D) Primordial epithelium including transition to glial cell morphology in the spinal cord of the chick embryo at the third day of incubation when, according to Ramón y Cajal, they become stainable by the Golgi method.(E) Characteristic lamellate expansion on the radial shafts of epithelial (radial glial) cells. Graphic from Breunig et al., 2011)

The term *neural stem cell*, as it is used now, was defined only in the 1990s. First conclusions had been made from studies on neuroepithelial stem cells in the developing

brain, fate mapping, tissue culture and transplantation studies. A neural stem cell was defined by two cardinal features: (i) the ability to self-renew by either symmetric division, giving rise to two identical novel stem cells, or asymmetric division, giving rise to a novel stem cell and a more committed sister cell (ii) giving rise to neurons, astrocytes and oligodendrocytes – all the principle cell types of the tissue of origin – defined as multipotency (McKay, 1997; Breunig et al., 2011). Since it had been accepted that neurogenesis continues postnatally in virtually all regions of the brain (Gage, 2000), the existence of postnatal NS cells has been accepted (Reynolds and Weiss, 1997; Eriksson et al., 1998), although its detailed characterisation is yet to be fully determined (Breunig et al., 2011). With the advances of cell culture techniques and protocols, it became possible to cultivate and expand primary NS cells from the embryonic or adult brain as well as NS cells derived from embryonic stem cells or induced pluripotent stem cells (Temple, 1989; Reynolds and Weiss et al., 1992; Okabe et al., 1996; Thomson et al., 1998; Wernig et al., 2008).

Primary NS cells were classically cultivated as neurospheres, a term describing a clonally derived free-floating cell aggregate in a mitogen-supplemented culture medium, introduced by Reynolds and Weiss (1992). They found that cells isolated from the CNS from embryonic as well as from adult mice were sensitive to epidermal growth factor (EGF) and in vitro expandable with the cardinal characteristics of stem cells. Firstly, these cells could be differentiated only into neurons and astrocytes questioning the multipotent character of the isolated cells (Altman and Das, 1965; Morshead and van der Kooy 1992; Lois and Alvarez-Buylla, 1993, 1994). A tripotent differentiation potential of these cells was demonstrated when the growth factor basic fibroblast growth factor-2 (FGF-2 or bFGF) was added to the medium to expand the cells, an efficient derivation of oligodendrocytes was possible (Weiss et al., 1996). Finally it was proven that tripotent NS cells from different human adult brain regions could be cultivated and expanded in vitro. However, neurosphere cultures encompass several disadvantages exacerbating experimental work. For instance, neurosphere cultures compromise only 10% of self-renewable and multipotent neural stem cells, whereas part of the remaining cells are committed progenitors mixed with differentiated astrocytes and neurons, representing dependence on a heterogenic population for a niche supplying a specialised cellular microenvironment (Gritti et al., 1999; Tropepe et al., 1999).

A novel method, published by Smith and colleagues (Conti et al., 2005), allowed cultivation and expansion of neural stem cells under niche-independent conditions. NS

cells from mouse and human embryonic stem cells and from embryonic mouse forebrain could be cultivated under adherent conditions in the presence of mitogens and gave the opportunity to generate pure and homogeneous tripotent neural stem cell cultures. Origin was a neurosphere culture derived from embryonic mouse brains, that was expanded for a short-term cultivation period on low-adhesion tissue culture flasks with subsequent enzymatic dissociation to single cell suspensions and plating onto gelatine coated tissue culture flasks in expansion medium containing EGF, FGF-2 and modified N2. The presence of EGF and FGF-2 was identified as sufficient for derivation and continuous expansion of pure symmetrically dividing NS cell cultures. The diploid and clonogenic NS cells, showing similarities to neurogenic radial glia, remained capable of differentiating down the neural-linage after prolonged expansion in vitro and transplantation into brain (Conti et al, 2005; Glaser et al., 2007).[15]

We argued, that cells with a neural differentiation potential, such as adherently propagated neural stem cells, would represent the optimal cell type for the development of a cell-based intraocular delivery system. These cells give rise to neurons and glial cells, in highly specified form as the normal cellular constituents of the retina. In fact, McGill et al. reported the absence of adverse effects on host retinas after subretinal neurosphere transplantation (McGill et al., 2012). Also, long-term survival of neural stem/progenitor cells after intraocular transplantation has been reported as well as in some studies the differentiation down the neural lineage (Ader et al., 2000; Pressmar et al., 2001; Wang et al., 2008; Meyer et al., 2006; Chaudhry et al., 2009; Francis et al., 2009). Based on all these observations, the aim of the study was to present a tool for preclinical testing of potential therapeutic products in the murine retina. The approach's claim was to develop a cell-based system capable to deliver therapeutic molecules, e.g. neurotrophic factors, in sufficient quantities to the murine retina by intraocular transplantation of genetically engineered neural stem cells. Consequently, we isolated NS cells from the cerebral cortex of the embryonic mice and propagated these cells under adherent conditions, establishing a pure tripotent NS cell culture. This cultivation

[15] This protocol was successfully applied to NS cells from the cortex of adult mice (Pollard et al., 2006). The transplantation of these cells, embryonic- or fetal-derived, into embryonic mice brain demonstrated widely integration and differentiation, even after directed differentiation into oligodendrocytic myelin formation in a myelin deficient animal model was observed (Conti et al., 2005; Glaser et al., 2007). The contiguity of multipotent retinal progenitor cells to neural stem cells (see Fig. 3) has raised experiments to use neural stem cells to replace retinal cells (reviewed in Lund et al., 2001 and Klassen et al., 2004a, see project 2 'Discussion'). However, the transplanted NS cells differentiated into astrocytes and neurons lacking retina-specific marker expression (Nishida et al., 2000; Kurimoto et al., 2001).

method facilitated the genetically modification of NS cell cultures, as here conducted by transduction. The design of the used lentiviral vector further increased/facilitated the formation of pure genetically engineered undifferentiated and differentiated NS cell cultures. We used a polycistronic vector based on the described lentiviral "gene ontology" (LeGO) vectors (Weber et al., 2008, 2010). The building block-principle not only enables easy exchange of the introduced DNA but the co-expression of a gene of interest to a reporter and resistance gene, separated by an internal ribosomal entry site (IRES; from the encephalomyocarditis virus) and by a P2A sequence (from the porcine teschovirus-1). To guarantee a strong and sustained expression of transgenes in adherently propagated neural stem cells and their differentiated progeny, transgenes were placed under regulatory control of the cytomegalovirus (CMV) enhancer/chicken β–actin (CAG) hybrid promoter.

To test whether lentivirally transduced neural stem cells might serve as cellular vectors to deliver functionally relevant quantities of therapeutic gene products to the murine retina, a secretable variant of the ciliary neurotrophic factor (CNTF) was cloned into these vectors and a 'proof of principle' study was designed. CNTF has been shown to be highly potential to attenuate photoreceptor degeneration in a variety of genetically and acutely induced animal models of retinal dystrophies, and was currently evaluated in clinical studies as a therapeutical to treat degenerative retinal diseases in human patients (Sieving et al., 2006; Talcott et al., 2011; Zhang et al., 2011). In addition to CNTF, vectors additionally encoded Venus as a reporter gene and zeocin as a resistance gene. Co-expression of the reporter gene allowed the identification of successfully transduced neural stem cells using fluorescence microscopy, and co-expression of the resistance gene allowed efficient derivation of pure CNTF-expressing neural stem cell cultures by application of antibiotics. Because adherently propagated neural stem cells are clonogenic cells (see above), the system additionally provides the possibility to establish genetically modified and clonally derived neural stem cell lines with defined expression levels of therapeutic transgenes. Furthermore, the system also allowed the efficient derivation of genetically engineered neural stem cell clones with low or high expression levels of the therapeutic gene products by using fluorescence-activated cell sorting (FACS) for cloning of modified cells and assuming a correlation between expression levels of the reporter genes and the co-expressed genes of interest. All the established genetically modified neural stem cell cultures and neural stem cell clones maintained

their stem cell character, and were capable of differentiating into all the principal neural cell types even after prolonged cultivation.

In our opinion, we have established a tool that fulfils the claim of an uncomplicated and highly controllable genetically engineering of a suitable cell type, the NS cell, for CNS applications, particularly for the retina. The basic concept of drug delivery is to transport therapeutic products near the affected area to restore the metabolic pathway. To test whether lentivirally modified neural stem cells allow to deliver functionally relevant quantities to the diseased retina of the mouse, we intravitreally injected CNTF-expressing neural stem cell bulk cultures and neural stem cell clones into Pde6b^{rd1} [16] and Pde6b^{rd10} [17] mice, two mouse models of retinitis pigmentosa. Both mouse lines are characterised by an early onset and rapid degeneration of photoreceptor cells (Pittler and Baehr, 1991; Chang et al., 2000, 2002). In both mouse strains, we observed significant attenuation of photoreceptor degeneration in eyes that received grafts of CNTF-expressing bulk cultures when compared to control eyes that received grafts of cells transduced with the same vector but lacking the CNTF cDNA. Furthermore, transplantations of a CNTF expressing clonal cell line, selected for high expression levels of CNTF, resulted in increased preservation of photoreceptors in the Pde6b^{rd1} when compared to animals treated with the CNTF-expressing cell culture the clone was derived from. Another very important result of these experiments was the fact that the grafted cells exerted no significant adverse effects on the host eyes. In fact, the intravitreally injected NS cells attached to the lens and vitread margin of the retina, where they differentiated into astrocytes and neurons. Importantly, continued proliferation of grafted cells and integration of intravitreally transplanted cells into host retinas was not observed. Finally, we detected donor cells in the vitreous of virtually every experimental animal that received intravitreal cell grafts, even after a post-transplantation interval of 4 months. All together, these results demonstrate that intravitreal transplantations of lentivirally modified neural stem cells allow the delivery of functionally relevant quantities of secreted gene products to the murine retina in controllable manner. We conclude that this cell-based intraocular

[16] Pde6brd1: also known as rd1; carries a spontaneous occuring mutation on the Pde6b, rod cGMP-specific 3',5'-cyclic phosphodiesterase subunit beta. Two mutations have been identified in rd1 mice. A murine leukimia virus (Xmv-28) insertion in reverse orientation in intron 1 is found in all mouse strains with the rd1 phenotype. Further, a nonsense mutation (C to A transversion) in codon 347 that results in a truncation eliminating more than half of the predicted encoded protein, including the catalytic domain has also been identified in all rd1 strains of mice.

[17] Pde6brd10: also known as rd10; transgenic mouse model with a missense mutation in exon 13 of the rod cGMP-specific 3',5'-cyclic phosphodiesterase subunit beta gene.

delivery system will be of use for preclinical studies aimed at evaluating the in vivo effects of therapeutic gene products in mouse models of retinal disorders.

Ongoing work in our lab is currently using this neural stem cell-based intraocular delivery system to evaluate the protective effects of other neurotrophic factors on photoreceptor cells, including glial cell-line-derived neurotrophic factor (GDNF) (Frasson et al., 1999; Gregory-Evans et al., 2009; Dalkara et al., 2011), pigment epithelium-derived factor (PEDF) (Cayouette et al., 1999; Semkova et al., 2002; Imai et al., 2005) and the recently identified neuroprotective factor for photoreceptors, osteopontin (Del Rio et al., 2011) in appropriate mouse models. Further, the delivery system is currently being used to identify potential synergistic protective effects of neuroprotective factors on photoreceptor cells, by co-expressing two or more different neurotrophic factors in neural stem cells, e.g. the indeed potent neuroprotection but functional deterioration on dystrophic photoreceptors of CNTF could be possibly counteracted by co-expression of the neurotrophic factor GDNF which preserves function in dystrophic photoreceptors (Buch et al., 2006). Another issue that is currently being investigated is the evaluation of long-term survival of the engineered cells and their long-term rescue effects on dystrophic photoreceptors. Finally, recent reports of a CNTF-induced regeneration of cone outer segments in dystrophic rat and human retinas (Li et al., 2010; Lipinski et al., 2011; Talcott et al., 2011) have initiated experiments to reassess these results in our mouse models of retinal dystrophies, including a detailed functional analysis of host retinas using electroretinogram (ERG) recordings.

2. The evaluation of the myelination of the lamina cribrosa.

The retina is part of the central nervous system and composed of all the characteristic cellular elements of the brain. For instance, retinal ganglion cells comprise the projection neurons and their axons form the optic nerve, which connects the retina to the brain. Interneurons, including horizontal cells, amacrine cells and bipolar cells are involved in the processing of visual information within the retina (see Fig. 1). Retinal astrocytes and Müller cells encompass the glial cells of the retina, which, like in other brain regions, are critically involved in retinal development and in structural and functional support of retinal neurons (see Fig. 3). In contrast to most other brain regions the retina is completely devoid of oligodendrocytes and, as a consequence, lacks myelinated cell processes (ffrench-Constant et al., 1988; Bartsch et al., 1989). Light has to pass all retinal layers to reach the outer segments of the photoreceptor cells, therefore, presence of myelin in the retina would be likely to impair vision. The absence of oligodendrocytes in the retina can thus be considered as a structural adaptation to the particular function of this brain region.

However, visual information has to be propagated rapidly to the brain, and rapid propagation of information in the mammalian brain is normally achieved by a saltatory propagation of action potentials along axons. The structural basis of a saltatory propagation of action potentials are myelin internodes which electrically insulate the axons, restricting the generation of actions potentials to myelin-free gaps between the internodes, the nodes of Ranvier (Peters et al., 1991; Baumann and Pham-Dinh, 2001). The retina is connected with the brain via the axons of retinal ganglion cells, which form the optic nerve and optic tract. The distribution of oligodendrocytes and myelin along these axons is unique in the CNS and reflects the functional needs discussed above. The most proximal segments of retinal ganglion cell axons within the retina and in the most retina-near part of the optic nerve remain non-myelinated, thus, vision is not impaired by a myelinated nerve fiber layer. In the optic nerve and optic tract virtually all retinal ganglion cell axons become myelinated by oligodendrocytes to assure rapid propagation of visual information to the brain. Such a differential distribution of oligodendrocytes and myelin along the same axon is unique for the primary visual pathway, and usually not found in other CNS regions. Normally, myelination is initiated by axonal signals (Weinberg and Spencer, 1975; Aguayo et al., 1976a, 1976b) and axons either become

myelinated along their entire length or remain non-myelinated along their entire length (Peters et al., 1991; Baumann and Pham-Dinh, 2001).

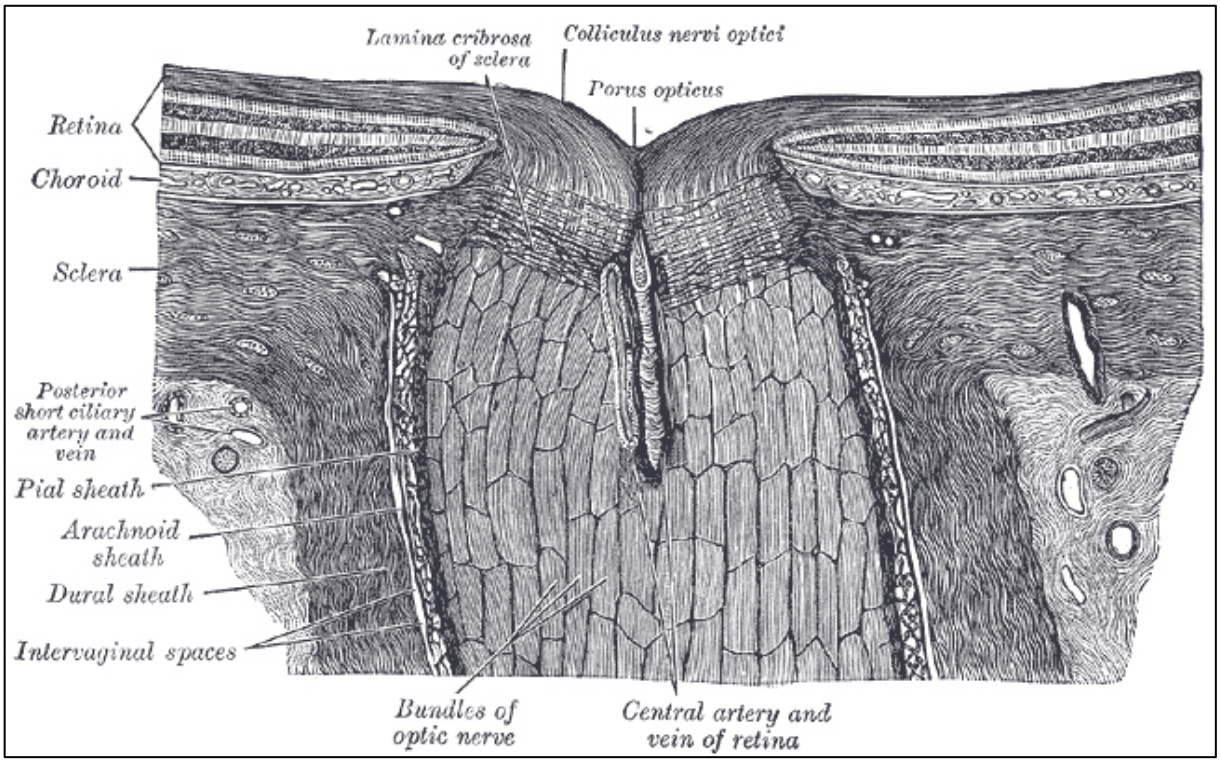

Figure 5: The retinal part of the optic nerve is schematically depicted. The unmyelinated axonal segments of the retinal ganglion cells are shown in the intrabulbar and retrobulbar part of the optic nerve. The optic disc is defined here as the entry of the RGC axons into/through the retina forming the optic nerve. The lamina cribrosa is distinctly drawn as a mesh-like structure that is formed by a multilayered network of collagen fibers each of which inserting the scleral canal wall. (graphic taken from Gray's Anatomy)

Oligodendrocytes in the primary visual pathway are neither generated in the retina, nor in the optic nerve or optic tract. This type of macroglia cell is generated in the brain from where oligodendrocyte progenitor cells migrate towards the retinal end of the optic nerve (Small et al., 1987). Here, they stop migrating shortly before the optic nerve enters the retina and starts to differentiate into myelinating oligodendrocytes, ultimately resulting in the characteristic differential distribution of oligodendrocytes and myelin along retinal ganglion cell axons (Bartsch et al., 1994; Fulton et al, 1992; Mudhar et al., 1993; Small et al., 1987). Several studies have attempted to elucidate whether neuronal or non-neuronal factors stop the migration of oligodendrocyte progenitor cells at the retinal end of the optic nerve. Most of these studies have suggested that non-neuronal factors at the retinal end of the optic nerve prevent the immigration of oligodendrocyte progenitor cells into the retina. For instance, a dense network of astrocytes and astrocytic processes at the retinal end of the optic nerve, the so-called lamina cribrosa, has been implicated in preventing migration of oligodendrocytes into the retina (Morcos and Chan-Ling, 2000; see Fig. 5). In addition, a

leaky blood-brain barrier and infiltration of serum components into the lamina cribrosa has been suggested to induce premature differentiation of oligodendrocyte progenitor cells in the region of the optic nerve, thereby preventing oligodendrocytes from entering the retina. Elevated expression levels of tenascin-C, a constituent of the extracellular matrix with inhibitory effects on oligodendrocyte adhesion and oligodendrocyte progenitor cell migration, have also been discussed to control the differential distribution of oligodendrocytes in the primary visual pathway (Bartsch et al., 1994; Garcion et al., 2001). However, direct experimental evidence for these suggestions was not provided.

As outlined above, myelination in the CNS is usually controlled by neuronal signals. In the nervous system a control of the distribution of oligodendrocytes and myelin not being associated with neurons would thus be unique. A non-neuronal control of the distribution of oligodendrocytes along retinal ganglion cell axons implies that these axons are, in principal, myelination-competent along their entire length. Intraretinal transplantations of myelinogenic cells provide the possibility to experimentally test the latter assumption. In fact, our group (Laeng et al., 1996; Ader et al., 2000; Pressmar et al., 2001; Stolt et al., 2002; Schmucker et al., 2003; Czekaj et al., 2012) and others (Setzu et al., 2004, 2006) have observed extensive myelination of the retinal nerve fiber layer after intraretinal injections of oligodendrocyte progenitor cells or neural stem cells. While these data strongly support the hypothesis of a non-neuronal control of the distribution of oligodendrocytes and myelin in the primary visual pathway, they do not completely preclude a neuronal involvement. In fact, myelination of the axonal segments of retinal ganglion cells located within the lamina cribrosa -the short non-myelinated part of the optic nerve close to the retina- has not been observed in these studies. However, these short axon segments in the lamina cribrosa might be refractory to myelination, thereby controlling the differential distribution of oligodendrocytes and myelin in retina and optic nerve.

The competence of the segments of retinal ganglion cell axons to become myelinated within the lamina cribrosa has been tested. Neurosphere cultures were exposed for a short time period to platelet-derived growth factor (PDGF) what led to an increase of the number of oligodendrocytes progenitor cells in these cultures. Small cell aggregates of these PDGF-primed neurosphere cultures were injected into the retina of young postnatal mice. To increase the probability that grafted cells would enter the lamina cribrosa, all aggregates were injected close to the optic disc. This methodological

approach induced i) a massive integration of grafted cells into host retinas; ii) differentiation of a significant fraction of donor cells into oligodendrocytes; iii) and massive myelination of the nerve fiber layer of host retinas in virtually all experimental animals. Importantly, axon segments of retinal ganglion cells located within the lamina cribrosa have been extensively myelinated. Thus, the experiments unequivocally demonstrated that retinal ganglion cell axons are, in principal, competent to become myelinated along their entire length. We therefore concluded that the differential distribution of oligodendrocytes and myelin in the primary visual pathway of mice is indeed controlled by non-neuronal factors, which are located at the retinal end of the optic nerve and prevent oligodendrocyte progenitor cells from entering the lamina cribrosa and, as consequence, the retina.

3. Summary of the results

The present thesis closer examined two promising strategies for the treatment of inherited retinal dystrophies causing an irreversible photoreceptor cell death. In general, the treatments for inherited retinal disorders include the replacement of lost endogenous retinal cells, the amendment of the genetic mutation or the application of pharmacological products. We focused on the analysis of cell-based strategies that once aim at the replacement of photoreceptor cells as well as on a cell-based sustained delivery of pharmacological products, such as neurotrophic factors. Both approaches have been studied in mouse models of inherited retinal disorders.

1) The cell-based photoreceptor cell replacement was explored in a mouse model characterised by slowly progressing photoreceptor degeneration to determine the impact of the degenerating environment on the engrafted donor-derived photoreceptors. The results demonstrate that:
   a) Primary cell suspensions isolated from young postnatal EGFP-transgenic mice with subsequent subretinal transplantation integrated properly into the dystrophic host retina. They differentiated into mature photoreceptor cells and formed presynaptic connections. Therefore, we argue that a cell-based replacement of photoreceptor cells in adult dystrophic retinas is feasible.
   b) The examinations also elucidated a strong influence of the dystrophic environment on the morphology of the engrafted donor-derived photoreceptor cells at a late stage of the disease. The outer segments of the endogenous photoreceptors shorten in length and the donor cells resembled similar morphological appearance. We assume, that the number of integrated donor cells must be significant higher to preserve the retinal structure, what requires an adjustment of the present published protocols.
   c) Another observation is the decreased number of retrieved donor-derived photoreceptor cells after long-term compared to short-term post-transplantation intervals. This surely has an influence in terms of possible clinical applications and for a possible treatment to be long-term beneficial the significant amount of integrated donor-derived photoreceptors has to be determined.
2) The incipient clinical presence of cell-based delivery of a neurotrophic factor via encapsulated cell device technology, an installation, that is independent of the

etiology, and the alleged simple application are profound arguments to reason the in vivo examination of further neurotrophic factors in mouse models of inherited retinal degeneration. Our purpose was to establish a highly controlled cell-based intraocular delivery system for therapeutic gene products in the mouse model. The results demonstrate that adherently cultivated neural stem cells represent a proper cell type for transporting neurotrophic factors in relevant quantities to the degenerate retina in mouse models of inherited retinal degeneration.

a) Propagating neural stem cells as adherent and homogeneous cultures enabled efficient genetic modification what increased the transduction efficiency with lentiviral vectors distinctly.
b) After transduction, cells could be selected and visually traced in vitro and in vivo because of the expression of reporter and resistant gene. They could be long-term propagated in vitro and directed into the principle neural cell types.
c) The secretion of relevant quantities of a neurotrophic factor through intravitreally grafted genetically engineered neural stem cell was demonstrated by the high significant preservation of photoreceptors in mouse models of inherited photoreceptor degeneration.
d) In regard to further demonstrate the controllability of the system, clonal neural stem cell lines were derived from cells expressing high levels of reporter protein. This resulted in cell clones of increased secretion of the neurotrophic factor and, when intravitreally transplanted, in the preservation of more endogenous photoreceptors when compared to in vivo studies with the original cell culture.
e) Another important feature of the delivery system was the differentiation of the used cell type into neural cell types, astrocytes and neurons. It was documented that grafted neural stem cells attached either to the inner retina or the lens, where they differentiated into neurons and astrocytes. Both qualities of neural stem cell, being non-invasive and closely related to the retina, are argumentative preconditions and part for the absence of adverse effects in the experimental mouse retinas.

3) The method to transplant neural stem cells into the retina has been used to closer analyse the underlying mechanisms that control the differential distribution of myelin along retinal ganglion cell axons. Previous experiments suggested that non-neuronal factors located at the retinal end of the optic nerve prevent the migration of oligodendrocyte progenitor cells into the retina whereupon myelination of the

retinal ganglion cell axons in the retina and the most proximal portion of the optic nerve does not occur. In this study the hypothesis of the presence of non-neuronal factors at the retinal end of the optic nerve was closer examined by the analysis of myelination capacity of this region of the nerve. Neural stem cells have been propagated as aggregate cultures and primed to oligodendrocyte progenitor cells before intraretinal transplantations close to the optic disc. The observed myelination of axonal segments of the retinal ganglion cells within the retina and the most proximal portion of the optic nerve by the grafted cells demonstrated that the entire length of the axon is competent to be myelinated. These results further confirm the hypothesis that the prevented migration of oligodendrocyte progenitor cells into the proximal portion of the optic nerve and the retina is controlled by non-neuronal factors located at the retinal end of the optic nerve to keep the retina free of myelin. They thus demonstrate that intraocular cell transplantations provide a useful methodological tool to experimentally address basic neurobiological issues.

II. Projects

1. Project: 'Replacement of photoreceptor cells in dystrophic adult retinas by cell transplantation'

ABSTRACT

Stem cell-based replacement strategies aiming at replacing lost retinal cell types might be an option to treat retinal dystrophies. We and others have recently demonstrated that primary retinal cell suspensions from young postnatal mouse retinas contain a cell population capable of integrating into the outer nuclear layer and of differentiating into apparently fully mature rod photoreceptor cells after transplantation into the subretinal space of adult wild-type mice. Here, we have analysed the potential of these cells to integrate, survive and differentiate in the subretinal space of adult mice displaying a slowly progressing apoptotic degeneration of photoreceptor cells. Therefore, retinal cell suspensions were isolated from four to five days old transgenic EGFP-mice. Cells were injected into the subretinal space of adult mice characterised by a slowly progressing degeneration of photoreceptor cells. Host retinas were screened for the presence of EGFP-positive donor-derived photoreceptor cells in the outer nuclear layer. Donor-derived cells were analysed morphologically and their antigen profile was studied using immunohistochemistry. A fraction of the subretinally grafted cells integrated into the outer nuclear layer of the dystrophic host retinas irrespectively of transplantation into retinas at initial or advanced stages of degeneration. Here, they differentiated into cells displaying the characteristic morphology of photoreceptor cells. Immunohistochemical analysis revealed that the donor cells expressed photoreceptor-specific markers and extended a synaptic terminal into the outer plexiform layer of the host retinas. However, donor-derived photoreceptor cells displayed degenerative alterations that increased with increasing severity of the retinal dystrophy of the host. Results of the study demonstrate that degenerated photoreceptor cells in adult dystrophic retinas can be replaced by cell transplantation. However, compared to the number of endogenous photoreceptor cells, only a small number of donor-derived photoreceptor cells was detectable in the outer nuclear layer of host retinas. Furthermore, grafted cells displayed morphological abnormalities in response to the progressive degenerative alterations of the dystrophic host retinas.

INTRODUCTION

Retinal degeneration due to photoreceptor cell loss, the photon-sensitive retinal cell type, occurs in most of the retinal disorders which can be either monogenic, like the inherited retinal disorder retinitis pigmentosa (RP), or a genetically complex multifactorial form, like age-related macular degeneration (AMD). The loss of photoreceptor cells is a genetically heterogeneous disorder that affects protein degradation, lipid metabolism, ciliary trafficking, the visual cycle, or phototransduction, to mention just the major alterations due to mutation(s). In fact, in the industrialized countries the progressive dysfunction and death of retinal photoreceptors is the major cause for blindness (Wright et al. 2010).

Until today the irreversible death of photoreceptors can neither be stopped nor reversed. Approaches for a treatment have been developing for several decades and are advancing towards improving the quality of life of the patients. Basically the therapeutic strategies aiming at a cure can be categorised into three attempts, that would be (i) the correction of the genetic disorder (gene therapy (see special issue of 'Gene Therapy' 19 (2); 2012)); (ii) slowing down or even stopping the process of photoreceptor degeneration applying pharmacological trophic molecules (for reviews, see: Chaum, 2003; Wenzel et al., 2005; Buch et al., 2007; Wen et al., 2011) and (iii) the replacement of the lost endogenous retinal cells using stem or progenitor cells (recently reviewed by Stern and Temple, 2012). All approaches are being tested at present either in first clinical trials or at least on single patients. On that note, it has been demonstrated that (i) vision improved in patients of Leber's congenital amaurosis by gene therapy (e.g. Bainbridge et al., 2008); (ii) photoreceptor degeneration resulting in visual impairment was slowed down in patients of geographic atrophy, RP and Usher syndrome by cell-based sustained delivery of a neurotrophic pharmatheutical (CNTF) (Talcott et al., 2011; Zhang et al., 2011); and (iii) one very advanced approach was the successful engraftment of human embryonic stem cell-derived retinal pigment epithelium (RPE) cells into patients of Stargardt's disease (Schwartz et al., 2012).

The strategies to replace lost or dysfunctional photoreceptors involve electronic devices, retinal sheets, retinal scaffolds, or by the subretinal transplantation of primary retinal cell suspensions (Chader et al., 2009; Zrenner et al., 2010; Seiler and Aramant, 2012; Hynes and Lavik, 2010; MacLaren et al., 2006; Bartsch et al., 2008, respectively). The cell-based replacement of lost photoreceptors was demonstrated in an impressive manner when EGFP-positive photoreceptors derived from young postnatal mice were

found integrated in age-matched wild-type or degenerate adult host retinas (MacLaren et al., 2006; Bartsch et al., 2008). The donor-derived photoreceptors integrated into the outer nuclear layer with correct orientation and adequate morphology and matured into functional rod photoreceptors with expression of key factors involved in the phototransduction process and in synaptic connectivity (MacLaren et al., 2006; Bartsch et al., 2008). Highest potential of integration was identified for postnatal day (P) 4-5. Within this narrow developmental time window, the retina contains high quantities of postmitotic immature precursors engaged in a rod fate as indicated by the expression of Nrl-transcription factor. Further, in a very recent study it was demonstrated that visual function could be authored through engrafted donor-photoreceptor cells. In behavioural testing of Gnat1$^{-/-}$ mice, deficient for rod α-transducin, a protein essential for phototransduction, an improvement of rod vision was assessed. The subretinal engraftment of primary wild-type Nrl-GFP postmitotic photoreceptor cells resulted in improved visual guided behaviour when compared to control mice, that received Gnat1$^{-/-}$ Nrl-GFP postmitotic photoreceptor cells (Pearson et al., 2012). This achievement and the first clinical trials using embryonic-derived RPE cells emphasise the promise of relying on cell-based therapies to treat degenerating retinas in humans.

For cell transplantation to be a viable therapeutic strategy, donor cells must integrate and survive in dystrophic retinas characterised by the degeneration of photoreceptors and restore visual function. It is also important to understand the influence of the degenerating retina on the grafted donor photoreceptor cells and vice versa. In several studies transgenic animals for a dystrophic retina have been analysed after transplantation of different donor-cell types. For retinal and neural stem cells derived from CNS or from embryonic stem cells, the degenerating retinal environment was described as advantageous for migration and/or integration of donor cells (e.g. reviewed in Klassen et al., 2004), whereas for primary retinal cells this fact was not observed (MacLaren et al., 2006, in rd1, rd2, or rhodopsin knock-out mouse line). Morphological appearance was described as variable owned to the collapse of the surrounding tissue when analysed 3 weeks after transplantation into P1 rd mouse retina (MacLaren et al., 2006). To further confirm a therapeutic application of this cell-based replacement strategy in degenerative retinas, it was necessary to examine the integration and survival capability of these cells for an extended period of time, i.e. as the disease progresses. In a proof of principle study, primary retinal cell suspension was isolated from P4-5 old EGFP-mice and subretinally transplanted into the transgenic

β2/β1 knock-in mutant mouse model. This transgenic mouse is characterised by a slow and long-lasting apoptotic photoreceptor cell death, caused by a suboptimal or insufficient Na,K-ATPase activity in the photoreceptor cell (Weber et al., 1998). The photoreceptor cell loss starts at postnatal week 8 and progresses down to a few photoreceptor cells in retinas of 9-month-old β2/β1 knock-in mice.

In this study we tried to answer the questions (1) whether the substituted dysfunctional or lost photoreceptor cells remain replaced by grafted cells in degenerative retinas over the process of degeneration, and (2) whether a long-term replacement of lost photoreceptors is realisable in advanced degenerated retinas. Therefore, the primary retinal cell suspension was subretinally transplanted into the transgenic host retina at the onset and at an advanced stage of the photoreceptor degeneration. The analysis was accomplished shortly after the transplantation and at the end of the photoreceptor degeneration.

MATERIALS AND METHODS

Animals

Primary retinal cell suspensions were prepared from transgenic mice ubiquitously expressing enhanced green fluorescent protein (EGFP) under control of a chicken β-actin promoter (Okabe et al., 1997). The integration potential of the isolated cell suspension into a retina characterised by a slowly progressing dystrophy of the outer nuclear layer (ONL) was evaluated in the transgenic β2/β1 knock-in mutant mice kept on C57BL/6J-129/Ola genetic background (Weber et al., 1998). Age-matched wild-type mice on the same genetic background served as a control. The genotype of the EGFP-transgenic mice was determined by analysing tail biopsies for the presence or absence of EGFP-fluorescence. Genotyping of β2/β1 knock-in mutant mice was conducted analysing DNA from tail biopsies with PCR, as described elsewhere (Weber et al., 1998). All animal experiments were approved by the local ethic committee and were in accordance with ARVO (Statement for the Use of Animals in Ophthalmic and Vision Research).

Preparation of retinal cell suspensions

Retinas were isolated from EGFP reporter mice (Okabe et al., 1997) at postnatal day (P) 4 or P5 as described (Bartsch et al., 2008). Briefly, retinas were enzymatically dissociated with Accutase (PAA Laboratories, Coelbe, Germany) for 10 – 15 min at 37°C immediately after isolation, and then carefully triturated into single cells suspensions. Cells were collected by centrifugation (1,200g for 5 min) and resuspended in Ca^{2+}- and Mg^{2+}- free Hank's balanced salt solution (Sigma, St. Louis, MO) at a concentration of approximately 400,000 cells/µl and stored at 4°C until use.

Subretinal cell transplantations

For subretinal transplantations of retinal cell suspensions, adult (for exact ages see below) β2/β1 knock-in mutant mice (Weber et al., 1998) and age-matched wild-type mice were deeply anaesthetized by an intraperitonal injection of Ketanest/Rompun. Eyes were punctured with a 30 gauge needle, and a blunt-ended 33 gauge needle attached to a Hamilton syringe was inserted tangentially through the conjuctiva and sclera into the subretinal space. Subsequently, approximately 2µl of a retinal cell suspension was slowly injected between ONL and RPE under visual control to produce a bullous retinal detachment, which was considered as an indication for a successful

subretinal injection. Retinal cell suspensions were injected into 8- and 16-week-old β2/β1 knock-in mutant mice, and 8 weeks old wild-type mice. Wild-type mice and β2/β1 knock-in mice that received cell transplants at 8 weeks of age were allowed to survive for 4 or 28 weeks. β2/β1 knock-in mice that received cell transplants at 16 weeks of age were allowed to survive for 4 or 16 weeks after transplantation.

Immunohistochemical analysis of host retinas

The grafted cell suspensions were analysed for their morphological appearance as well as for the expression of retina-specific markers. For immunohistochemical analysis, the animals were killed at the defined post-transplantation intervals, the eyes were quickly removed, fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.3), cryoprotected in an ascending series of sucrose (7.5%, 15%, 30%), embedded in Tissue-Tek (Sakura Finetek, Zouterwoude, Netherlands), frozen and serially sectioned at 25µm using a cryostat (Leica CM1900). Sections were blocked and permeabilized in PBS containing 0.1% bovine serum albumin and 0.3% Triton X-100 (both from Sigma) for 1 h at room temperature and then incubated in primary antibodies over night at 4°C. The following primary antibodies were used (see also table 1): mouse monoclonal rhodopsin (1:100 Chemicon, Temecula, CA), mouse monoclonal bassoon (1:2000, Stressgen, Victoria, Canada), rabbit polyclonal recoverin (1:3000, Chemicon, Temecula, CA), mouse monoclonal glial fibrillary acidic protein (GFAP, 1:500, Sigma), rabbit polyclonal β-tubulin III (1:1000, Covance, Berkley, CA) and rabbit polyclonal protein kinase C α (PKCα, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were visualized with Cy3-conjugated secondary antibodies (1:200, Jackson ImmunoResearch, West Grove, PA). Sections were counter-stained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI, Sigma) and mounted on slides. As a negative control, sections were processed as described above with the only exception that incubation with primary antibodies was omitted. Retinal sections were examined using an Olympus FV 1000 confocal microscope (Olympus, Hamburg, Germany).

Table 1: Primary antibodies used for immunohistochemistry

Name	Cell type/structure	Species	Dilution	Supplier	Product code
Bassoon	Ribbon synapses	Mouse	1:2000	Stressgen	VAM-PS003
GFAP	Astrocytes/Müller glia	Rabbit	1:500	Sigma	G3893
PKC α	Bipolar cells	Rabbit	1:500	Santa -cruz	Sc-208
Recoverin	Photoreceptors/cone bipolar cells	Rabbit	1:3000	Chemicon	AB5585
Rhodopsin	Rod photoreceptors	Mouse	1:100	Chemicon	MAB5356
β-Tubulin III	Neurons	Rabbit	1:1000	Convance	PRP-435

RESULTS

Subretinally transplanted primary retinal cells integrate into the outer nuclear layer (ONL) of adult β2/β1 knock-in mutant mice

Retinal cell suspensions isolated from EGFP-transgenic mice at P4 or P5 were subretinally injected into adult wild-type and β2/β1 knock-in mutant mice. Experimental animals were sacrificed 4 to 28 weeks after transplantation. Analysis of host retinas of wild-type and β2/β1 knock-in mutant mice revealed the presence of viable EGFP-positive donor cells at all post-transplantation intervals (Fig. 1). Integrated donor cells were either found in clusters or as single cells throughout the outer nuclear layer (ONL) of the host retinas (Fig. 1). The grafted cells showed the characteristic morphology of mature rod photoreceptor cells in both wild-type and β2/β1 knock-in mutant mice. In the host retinas, the donor cells had small and round cell bodies located within the ONL. The apical processes extending above the external limiting membrane terminated in inner and outer segment-like structures and the apical processes approaching towards the inner retina reached into the outer plexiform layer. Furthermore the analysis of protein expression of the engrafted photoreceptors showed the expression of characteristic proteins involved in the phototransduction machinery and the synaptic connectivity. The donor cells expressed the phototransduction-specific antigens recoverin (Fig. 3) and rhodopsin (data not shown). Additionally, in the outer plexiform layer the bassoon-positive ribbon-like structure (a typical feature of a photoreceptor synaptic terminal) in basal processes of the donor cells was detectable and the processes terminated in close proximity of the dendrites of host PKCa-positive bipolar cells (Fig. 2). Together, these data demonstrate that a fraction of cells within the primary retinal cell suspensions is capable of integrating into healthy and dystrophic adult retinas where they differentiate into apparently fully mature rod photoreceptors, what is in line with previous reports (MacLaren et al., 2006; Bartsch et al., 2008).

Subretinal cell transplantations into adult β2/β1 knock-in mice at initial and advanced stages of retinal degeneration

Photoreceptor degeneration in 8 weeks old β2/β1 knock-in mice is in its initial stage and the ONL is only mildly affected, while photoreceptor degeneration in 16 weeks old mutant mice is significantly advanced and the ONL severely atrophied (Weber et al., 2002). To analyse the impact of the retinal phenotype on the integration and

differentiation potential of subretinally grafted primary retinal cells in the ONL, isolated retinal cell suspensions were subretinally injected into 8 weeks old wild-type mice and into β2/β1 knock-in mutants 8 and 16 weeks of age and 4 weeks after transplantation retinas were analysed (Fig. 1a-c). Qualitative observations suggested that no obvious differences in the number of integrated donor-derived photoreceptors in the ONL were present in wild-type and mutant mice that had received subretinal cell grafts at 8 weeks of age (Fig. 1a-c). In mutants transplanted at 16 weeks of age, we found similar quantities of integrated donor-derived photoreceptors in the ONL when compared to wild-type and mutants transplanted at 8 weeks. It should be noted, that the number of integrated photoreceptor cells in the ONL varied considerably between different animals of each experimental group, possibly due to variations during the injection procedure and/or differences in the viability of the grafted cell suspensions. A quantitative analysis of the experimental retinas was therefore not performed, and minor effects of the retinal phenotype on the integration potential thus cannot be completely excluded.

Evaluating the survival capacity of integrated mature donor-derived photoreceptor cells in the ONL of dystrophic retinas compared to wild-type retinas, both transplanted at the age of 8 weeks, was conducted 28 weeks after transplantation, when the ONL of the untreated mutant almost disappeared. Qualitatively, we found no obvious differences in the number of integrated mature donor-derived photoreceptor cells in the ONL, irrespective of whether the cells were grafted into 8 weeks old wild-type or mutant mice (Fig 1d, e, respectively). However, retinas analysed at this long-term post-transplantation interval clearly showed a significant reduction of the quantities of integrated mature donor-derived photoreceptor cells for both experimental groups compared to quantities of integrated mature donor-derived photoreceptor in ONL of retinas of both experimental groups analysed 4 weeks after transplantation (Fig. 1 compare a with d for wild-type, b with e for mutant mice). The reduction in the number of integrated mature donor-derived photoreceptor cells was also observed in retinas of mutant mice transplanted at the age of 16 weeks, when post-transplantation intervals of 16 weeks were compared to post-transplantation intervals of 4 weeks. Here it should be mentioned, that the reduction of the quantities of integrated mature donor-derived photoreceptors at 16 weeks after transplantation was qualitatively less pronounced then at post transplantation intervals of 28 weeks.

Integrated mature donor-derived photoreceptor cells display dystrophic morphologies in adult β2/β1 knock-in retinas

As described in the Weber et al. (1998) study the morphology of photoreceptors in β2/β1 knock-in transgenic mice alters with the progression of the dystrophy. Besides the initiation of the reduction in ONL thickness at 8 weeks of age to a few rows of photoreceptors at 9 months of age, the length of inner and outer segments reduces as well. To analyse the impact of the retinal phenotype of the β2/β1 knock-in transgenic host retina on the morphological appearance of the integrated mature apparently wild-type-derived photoreceptors, experimental groups were transplanted and analysed at the same age and post transplantation intervals as stated above for the evaluation of integration and survival capacity. At any time point of analysis, the integrated mature donor-derived photoreceptors in the mutant retinas displayed an altered morphological appearance compared to integrated mature photoreceptors in wild-type retinas. In the transgenic retinas the grafted cells revealed a reduced length of inner and outer segments and a denser EGFP-signal within the segments and appeared more compact (compare e.g.: Fig. 2d with e), comparable to the host photoreceptors of the β2/β1 knock-in mouse model (compare e.g:. Fig. 2b with a, respectively). Additionally, we noticed a shortened morphology of the rod bipolar cell dendrites of the dystrophic retinas.

The morphological differences for the integrated donor-derived photoreceptor cells in the dystrophic retinas of the β2/β1 knock-in mice compared to recipient wild-type mice were visible as early as in the earliest post-transplantation interval analysed. Four weeks after transplantation into 8-week-old animals (total age of 3 months), the length of inner and outer segments was already reduced (compare Fig. 1a (wild-type) with b (mutant)). The morphological adaptation of the integrated donor-derived photoreceptor cells advanced with the progression of the dystrophy until the age of 9 months of the recipient mutant mice (see Fig. 1b and e) what was not observed for grafted photoreceptor cells in wild-type mice (compare Fig. 1e with d, respectively). Besides the morphological differences of the integrated donor photoreceptors between wild-type and β2/β1 knock-in mice due to the phenotype of the recipient retinas, an alteration in the appearance of the PKCα-positive rod bipolar cells in β2/β1 knock-in was also observed. Comparing figure 2a with 2b, one will notice that the PKCα-positive rod bipolar cell dendrites in β2/β1 knock-in mice (Fig. 2b) regressed and led to a reduction

of the thickness of the outer plexiform layer. Further, when wild-type-derived photoreceptor cells grafted into mutant retinas of advanced stages of retinal degeneration (16 weeks of age) were analysed as early as 4 weeks after transplantation, the length of the inner and outer segments was more reduced when compared to mutant mice transplanted at the age of 8 weeks and analysed 4 weeks after transplantation (see Fig. 1c and b, respectively). A more distinct reduction for the thickness of the outer plexiform layer was observed in the retina of mutants transplanted at 16 weeks of age and analysed at the post transplantation interval of 4 weeks. Taking the age of the β2/β1 knock-in mutant mice into consideration for the morphological analysis of integrated mature donor-derived photoreceptor cells it was conveyed that the morphological alterations on grafted cells were identical in age-matched mutants and pronounced to the same extent with increasing post-transplantation intervals independent of the post transplantation interval (see Fig. 1). It is noteworthy that the cell-body as well as the spherule synapse (see insets in Fig. 2c and d) showed no apparent difference in appearance when compared to control.

Transplanted EGFP-positive cells remain as clusters in the subretinal space

A fraction of the retinal cell suspension subretinally transplanted into 8 weeks old wild-type and 8 weeks and 16 weeks old β2/β1 knock-in mutant mice did not integrate into the ONL and rather remained as aggregates in the subretinal space of the host retinas and survived for at least 28 weeks, which is the longest post-transplantation interval analysed. Analysis of subretinally located aggregates of donor-derived cells showed that most of these cells differentiated into GFAP-positive, β-tubulin III-positive or recoverin-positive cells. Interestingly, more donor-derived cells were found as aggregates in the subretinal space than as singly grafted photoreceptors at all time points analysed. Qualitative observations led to the impression that, like donor-derived photoreceptors, with increasing post-transplantation intervals the EGFP-positive cell aggregates decreased.

DISCUSSION

The replacement of dystrophic or lost photoreceptors by a cell-based therapeutic strategy to cure retinal dystrophies has been in discussion for more than two decades. At that time, Gouras et al. transplanted single retinal cell suspensions, isolated from normal adult rat retinas, into the subretinal space of adult dystrophic rat retinas (Gouras et al., 1991). Electronmicroscopic analysis revealed that the donor-derived cells displayed characteristics of photoreceptors, with outer and inner segments and rod spherules. Since then and until recently, cell-based therapeutic strategies focused on the identification of an appropriate cell type to derive photoreceptors from expandable cell cultures. Several studies have discussed the application of different stem cells for retinal cell replacement (for reviews see e.g.: Baker and Brown, 2009; West et al., 2009). The evidence of neurogenesis in adults and the embryonic development of the retina from neural stem cells (see Fig. 3) make neural stem cells an attractive donor cell type for strategies aimed at replacing photoreceptors (for reviews see e.g.: West et al., 2009; Stern and Temple, 2012). Indeed, in several studies when embryonic-derived or adult neural stem cells were injected intravitreally, intraretinally or subretinally, migration of the stem cells into the retina with subsequent incorporation and differentiation into neural cell types as well as the development of processes within the hosts' plexiform layers were described (Takahashi et al., 1998; Sakaguchi et al., 2003; Mizumoto et al., 2003; Banin et al., 2006; Meyer et al., 2006; Klassen et al., 2007; Mellough et al., 2007; Chaudhry et al., 2009). Transplantations of expandable neural stem/precursor cells into the retina of adult rodents have demonstrated the ability of the mature retina to incorporate grafted neural stem cells (Delyfer et al., 2004; Klassen et al., 2004a; Gaillard and Sauvé, 2007). However, in all cases neural stem cells had failed to differentiate into authentic retinal cell types (Young et al., 2000; Pressmar et al., 2001; Sakaguchi et al., 2005; for review see e.g.: West et al., 2009). Retinal stem cells from the developing neuroretina are considered as a more appropriate cell type for cell replacement strategies, particularly, because the discussion about the presence of adult retinogenesis in mammals is still ongoing. (for review see e.g.: Wohl et al., 2012). In fact, when rat retinal stem cells were transplanted into an adult rat host retina the donor cells survived and differentiated into cells expressing photoreceptor-specific markers (Chacko et al., 2000; Klassen et al. 2004b; Qiu et al., 2005, 2007; Canola et al., 2007). In a very recent study by Czekaj et al. it was demonstrated that, when retinal stem cells from the neuroretina were cultivated homogeneously and for long periods of time, they lost

retinal cell marker expression, probably due to permanent exposure to un-physiologically high concentrations of the mitogenic factors FGF and EGF, and acquired properties of neural stem cells (Czekaj et al., 2012; Liu et al., 2005; Inoue et al., 2005; Mac Neil et al., 2007).

Evidence that the adult retina represents a feasible target for stem cell-based therapies is supported by the successful engraftment of retinal pigment epithelium (RPE) cells. In several studies, it has been shown that RPE cells derived from embryonic stem cells can be directed into mature RPE cells in vitro (Lund et al., 2006; Lu et al., 2009). Recently, RPE cells derived from human embryonic stem cells have been transplanted into human patients, and first data from this clinical study indicated that these transplantations fulfilled safety conditions and without evidence for tumour formations of the grafted embryonic stem cell-derived cells (Schwartz et al., 2012).

The identification of rod postmitotic precursor cells as the appropriate cell type being capable of integrating and maturating into photoreceptors after subretinal transplantation provides an idea of the required ontogenetic state of donor-derived cells for cell-based photoreceptor replacement strategies. In addition to postmitotic rods, retinal cell suspensions isolated from earlier time windows of retinogenesis (embryonic day 16.5 – P1) were also shown to integrate into the host retina. It was suggested that this retinal cell suspension might represent a source to replace dysfunctional or degenerated cone photoreceptor cells (MacLaren et al., 2006; Gaillard et al., 2007, see also Fig. 3).

Though the first steps towards clinical translation of a stem cell-based replacement therapy have indeed been demonstrated for the retinal pigment epithelium and thus indicating a promising cure for disorders characterized by the primary dysfunction and/or loss of RPE cells, like AMD (Schwartz et al., 2012), the same strategy for disorders with primary loss/dysfunction of photoreceptors has to be further evaluated before being applied to humans. First questions concerning clinical requirements have been clarified in different studies, such as the purification of transplantable cells (Lakowski et al., 2011; Eberle et al., 2011). The expression of reporter genes to enrich the appropriate cell type for transplantations is not suitable for therapeutic applications in humans. Therefore, combinations of cell type-specific surface markers have been used to obtain by MACS or FACS an unmodified homogeneous cell population that is enriched in correctly staged rod precursor cells from mice. When these rod precursor-enriched cell populations were subretinally transplanted into mouse retinas, they gave rise to

significantly higher numbers of donor-derived photoreceptors than cell suspensions that had not been enriched in rod precursor cells (Lakowski et al., 2011; Eberle et al., 2011).

In the present study the influence of the retinal degenerative environment, characterized by a slowly progressing photoreceptor degeneration, on the integration and differentiation potential of engrafted donor-derived retinal cell suspensions was analysed. Further, the long-term influence on the morphology and survival of integrated mature donor photoreceptors was addressed. The transgenic β2/β1 knock-in mouse, resembling an animal model of retinitis pigmentosa with a photoreceptor degeneration beginning at 8 weeks of age and slowly progressing until the age of 9 months, was taken for the experimental evaluation (Weber et al., 1998). Integration capacity of grafted primary rod precursor cells was compared between different time points of transplantation: (1) Wild-type mice with the same genetic background as the β2/β1 knock-in mutants were 8 weeks of age at the time of transplantation, and served as control, (2) the mutant mice either received the grafts at initial stages of the dystrophy (i.e. at 8 weeks of age) or (3) at advanced stages of the dystrophy (i.e. at 16 weeks of age). Thus, the cells were grafted in mildly (8 weeks old mutants) and severely affected retinas (16 weeks old mutants). The qualitative analysis of all three experimental groups 4 weeks after transplantation revealed no significant effects of the retinal phenotype on the capacity of the grafted cells to integrate into the host retinas and to differentiate into rod photoreceptors. This observation is supported by other reports where transplantation of primary retinal cells into dystrophic and wild-type retinas have resulted in similar quantities of integrated mature donor-derived photoreceptors (MacLaren et al., 2006; Bartsch et al., 2008; Pearson et al., 2012). However, in other studies it had been documented, that neural, retinal or embryonic stem cell-derived cells integrate more efficiently into degenerated retinas as opposed to healthy retinas. Most of these observations were made after intravitral or intraretinal transplantations. It was argued that an altered expression level of trophic factors (Gao et al., 1995, 1996; Wen et al., 1995;) and/or loosening of endogenous retinal barriers like the inner or outer limiting membrane by alteration of Müller cells (Young et al., 2000) might facilitate the infiltration of grafted cells. However, it has also been argued that the processes of GFAP-expressing Müller cells, indicative for reactive gliosis in dystrophic retinas, grow into the subretinal space and might form an additional glial barrier (Fisher et al., 2005), thus rather exacerbating the integration of donor cells after subretinal transplantation (West

et al., 2009). Furthermore the morphological appearance of grafted donor cells at different stages of degeneration was investigated. In the β2/β1 knock-in mouse, the inner and outer segments of photoreceptor cells shorten and acquire a compressed structure over time (Weber et al., 1998). When 12-week-old (transplantation at 8 weeks and analysis 4 weeks afterwards) wild type and mutant mice were compared the thickness of the outer nuclear layer (ONL) was similar but the layer of the inner and outer segments of the mutants were reduced in thickness and had a condensed appearance. Four weeks after transplantation of primary retinal cell suspensions into mildly or severely affected host retinas, the integrated mature donor-derived photoreceptors in the severely affected host retinas revealed a more distinct alteration in morphology. Comparison of mutant mice of the same age but at different post-transplantation intervals suggested that the morphological appearance of donor-derived photoreceptor cells was not related to the length of the post-transplantation interval. Rather, the grafted photoreceptor cells adapted the morphology of the endogenous photoreceptor cells of the host retina. However, the post-transplantation interval had an influence on the number of integrated mature donor-derived photoreceptors in the host retinas. In fact, the number of integrated donor-derived photoreceptors decreased significantly with increasing post-transplantation intervals in both, wild-type and mutant mice. The loss of grafted cells over time had been examined and was attributed to an immune response to the grafted cells (West et al., 2010).

A significant increase in the number of integrated rod photoreceptor cells might result in a better preservation of the morphology of the host retina, and as a consequence might also preserve the morphology of the donor-derived rods. Attempts to improve the integration of primary rod postmitotic precursor cells included, for instance, a pharmacological (West et al., 2008) or a transgenic (Pearson et al., 2010) disruption of the outer limiting membrane. An improved ability of the grafted cells to integrate into the outer nuclear cells was detectable but the studies also suggested that the capacity for integration from the subretinal space into the ONL is influenced by additional yet unknown factors (West et al., 2008; Pearson et al., 2010). The hypothesis that elevated expression levels of one or more neurotrophic factors promote the integration capacity of grafted rod precursor cells was also examined. To this aim, endogenous retinal cells were transduced with a viral vector encoding IGF1, FGF-2 or CNTF and rod precursor cells were subsequently grafted into the subretinal space (West et al., 2012b). Analysis of experimental retinas revealed that IGF1 promoted integration of grafted cells, while

FGF-2 had no and CNTF a negative effect (West et al., 2012b). Taking these results together it is suggestive that only a massive insult/alteration of the retinal structure could significantly increase the integration of subretinally grafted cells. Preclinical studies that focus on the amelioration of the integration capacity of grafted rod postmitotic precursor cells have so far achieved integration levels that correspond to less than 1% of the number of endogenous photoreceptors (West et al., 2008, 2010, 2012b; Pearson et al., 2010). Nevertheless, in a recent study a beneficial effect on the visual acuity has been achieved after transplantation of primary donor-derived rod postmitotic precursor cells into a mouse model deficient in functional rods (Pearson et al., 2012). In this study, the number of integrated donor-derived photoreceptors was increased by two separate subretinal transplantations of rod precursor cells. Although the number of donor-derived photoreceptor cells was below 1% of the number of endogenous photoreceptors, visual acuity as measured by visually guided behaviour was improved in these animals. Interestingly, in the same study, it was observed that a correction of about 1% of endogenous photoreceptor cells in the same mutant strain by corrective gene therapy is sufficient to induce recordable scotopic electroretinogram responses (Pearson et al., 2012). In a previous study, a light-induced pupil reflex was more easily induced in eyes of mutant mice that had received grafts of rod precursor cells as opposed to control eyes. Interestingly, and in line with the data of the present study, morphological analysis on the engrafted photoreceptors revealed a severe morphological alteration of the outer segments (MacLaren et al., 2006). Results thus indicate that a normal morphology of donor-derived photoreceptor cells is not necessarily a prerequisite for a functional rescue.

While the preclinical studies demonstrate the feasibility of cell replacement strategies in the retina, the source for appropriate donor tissue is ethically problematic and logistically difficult. Alternative promising sources for donor cells include pre-differentiated human embryonic stem cells or human induced pluripotent stem cells. Several groups have recently demonstrated that heterogeneous cultures of retinal stem cells can be derived from those stem cell cultures after application of differentiation protocols mimicking retinal development in vivo (Lamba et al., 2006; Osakada et al., 2008; Meyer et al., 2009). Retinal progenitor cells isolated from the fetal human retina have also been expanded and differentiated in vivo (Kelley et al., 1995; Aftab et al., 2009). In a very recent study the generation of Nrl-positive rod postmitotic precursors was impressively demonstrated on the dynamic, autonomous formation of the optic cup

structure from a three-dimensional culture of mouse embryonic stem cell aggregates (Eiraku et al., 2011, West et al., 2012a). However, the integration capacity and differentiation potential of these embryonic stem cell-derived rod precursor cells has still to be analysed in transplantation experiments. In earlier studies, the capacity of embryonic stem cell-derived photoreceptors or induced pluripotent stem cell-derived photoreceptors to integrate into the retina after transplantation was investigated. The integration potential observed for these cells was, however, very limited (Lamba et al., 2009, 2010). Nevertheless, the use of induced pluripotent stem cells is attractive, as this approach allows the generation of ethically unproblematic donor cells in high quantities with options for engineering the cells to improve integration and survival capacities, like incorporated expression of neurotrophic factors (West et al., 2012b, our data: manuscript in preparation). These results achieved in the photoreceptor replacement studies are comparable to those of stem cell-based strategies conducted with donor-derived RPE cells. As for donor-derived photoreceptors the retrieved quantities of engrafted RPE cells corresponded to only a fraction of the isolated and initially transplanted cell suspension (Schwartz et al., 2012). Nevertheless, the availability of donor cells that are free of ethic and juristic concerns for human application is encouraging and justifies further efforts to establish cell replacement strategies for the retina. It is particularly encouraging in this context, that already low numbers of functional photoreceptor cells are sufficient to rescue some visual abilities. The combined results therefore fortify arguments to initiate first clinical steps to evaluate the safety and security of stem cell-based strategies aimed to replace lost photoreceptor cells.

FIGURES AND FIGURE LEGENDS

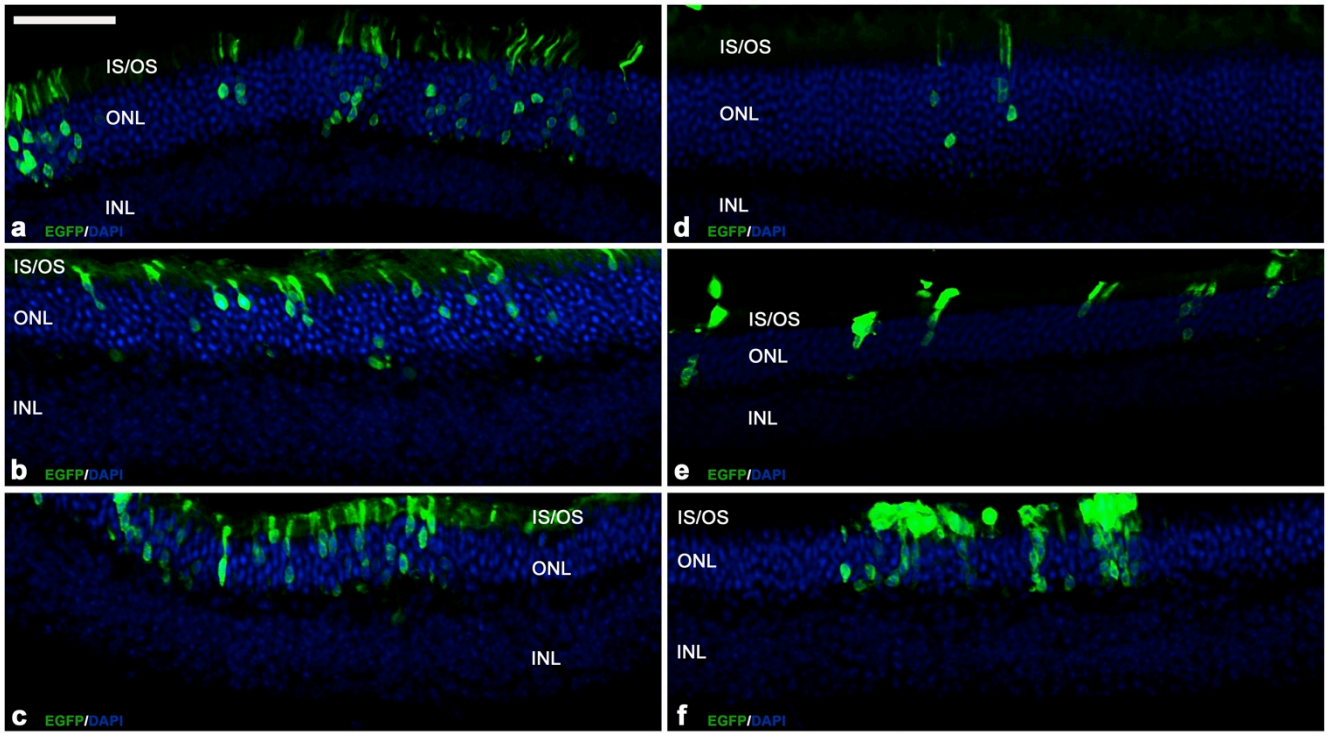

Figure 1: Integration, differentiation and survival of primary retinal cells after subretinal transplantation into adult wild-type and β2/β1 knock-in mutant mice. The donor-derived EGFP cells integrated into host retinas and differentiated into fully mature (rod) photoreceptor cells with cell bodies in the outer nuclear layer, basal processes reached into the outer plexiform layer/ towards the inner nuclear layer and apical processes as outer and inner segments were present, expressing EGFP in all segments. Cells were grafted into 8-week-old wild-type mice (a) and β2/β1 knock-in mutant mice (b) and 16-week-old β2/β1 knock-in mutant mice (c) and were allowed to survive for 4 weeks. Retinas for wild-type and β2/β1 knock-in mutant mice with 8 weeks of age at transplantation were additionally evaluated for a post-transplantational interval of 28 weeks (d and e, respectively) as well as 16-week-old β2/β1 knock-in mutant mice surviving 16 weeks after transplantation (f). IS/OS: Inner and outer segments; ONL: outer nuclear layer; INL: inner nuclear layer; EGFP: enhanced green fluorescent protein; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride hydrate. Bar in (a) for (a-f): 50µm.

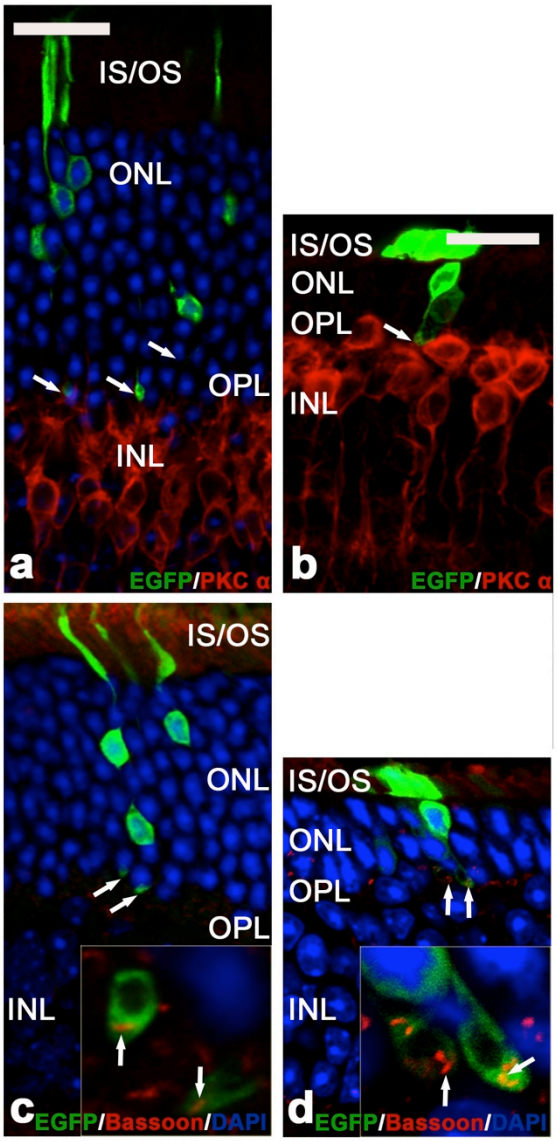

Figure 2: Axonal terminals of integrated donor-derived photoreceptor cells express synaptic connectivity marker. Immunohistochemical analysis of synaptic plasticity of the grafted EGFP-positive cells for wild-type (a, c) and β2/β1 knock-in mutant mice (b, d) 28 weeks after transplantation. The axonal terminals of integrated donor cells, identifiable by EGFP-expression in the segments, terminated in close proximity to the dendrites of endogenous PKCα-positive bipolar cells, both in wild-type (a, arrows) and mutant (b, arrows) mice. The terminals also contained a bassoon-positive ribbon-like structure for wild-type (c, arrows) and β2/β1 knock-in mutant mice (d, arrows). In this high magnification the morphological adaptation of the grafted cells to the advanced morphological alteration of the dystrophic retina of β2/β1 knock-in mutant mice was perceptible by the reduced length of inner segments as well as the regressed rod bipolar cell dendrites (compare b with a). In age matched wild-type mice the morphological appearance remained normal. IS/OS: Inner and outer segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; EGFP: enhanced green fluorescent protein; PKCα: protein kinase C α; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride hydrate. Bar in (a) for (a,c) and in (b) for (b,d): 20µm.

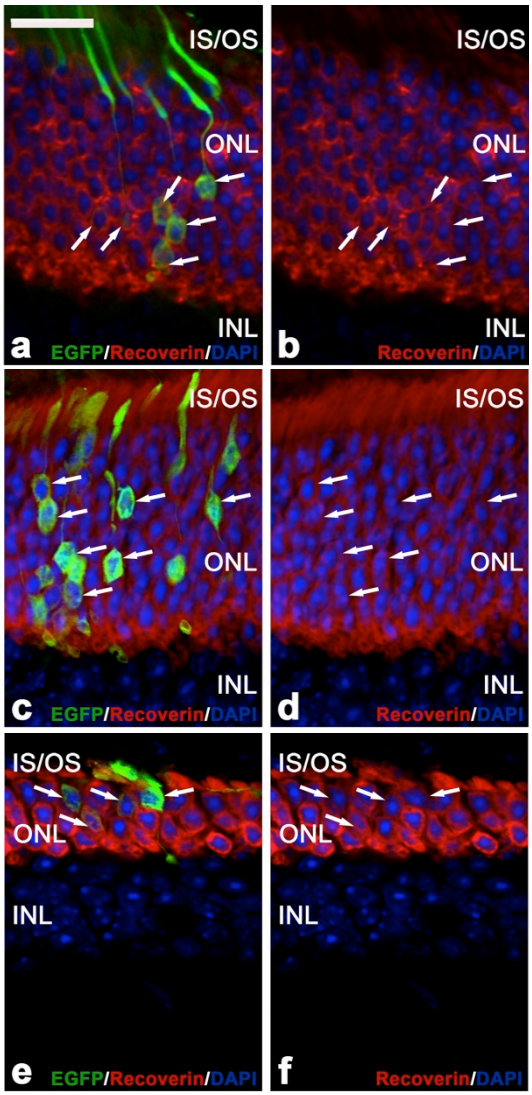

Figure 3: Integrated donor-derived photoreceptors express photoreceptor-specific markers. Here the expression of the photoreceptor-specific antigen recoverin is co-localized with EGFP-expression in the grafted donor-derived cells in adult wild-type (a, b) and age-matched β2/β1 knock-in mutant mice (c-f). Donor-derived cells were analysed 28 weeks (a, b; e, f) or 4 weeks (c, d) after transplantation. All donor cells were positive for recoverin indicated by arrows. For a massive morphologic alteration of grafted photoreceptors compare the inner segments in wild-type (a) with the mutant (e) mice at age of 36 weeks (stars) as well as the reduced ONL in mutant mice after prolonged survival period (e and f compare with c and d or a and b). IS/OS: Inner and outer segments; ONL: outer nuclear layer; INL: inner nuclear layer; EGFP: enhanced green fluorescent protein; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride hydrate. Bar in (a) for (a-f): 20µm.

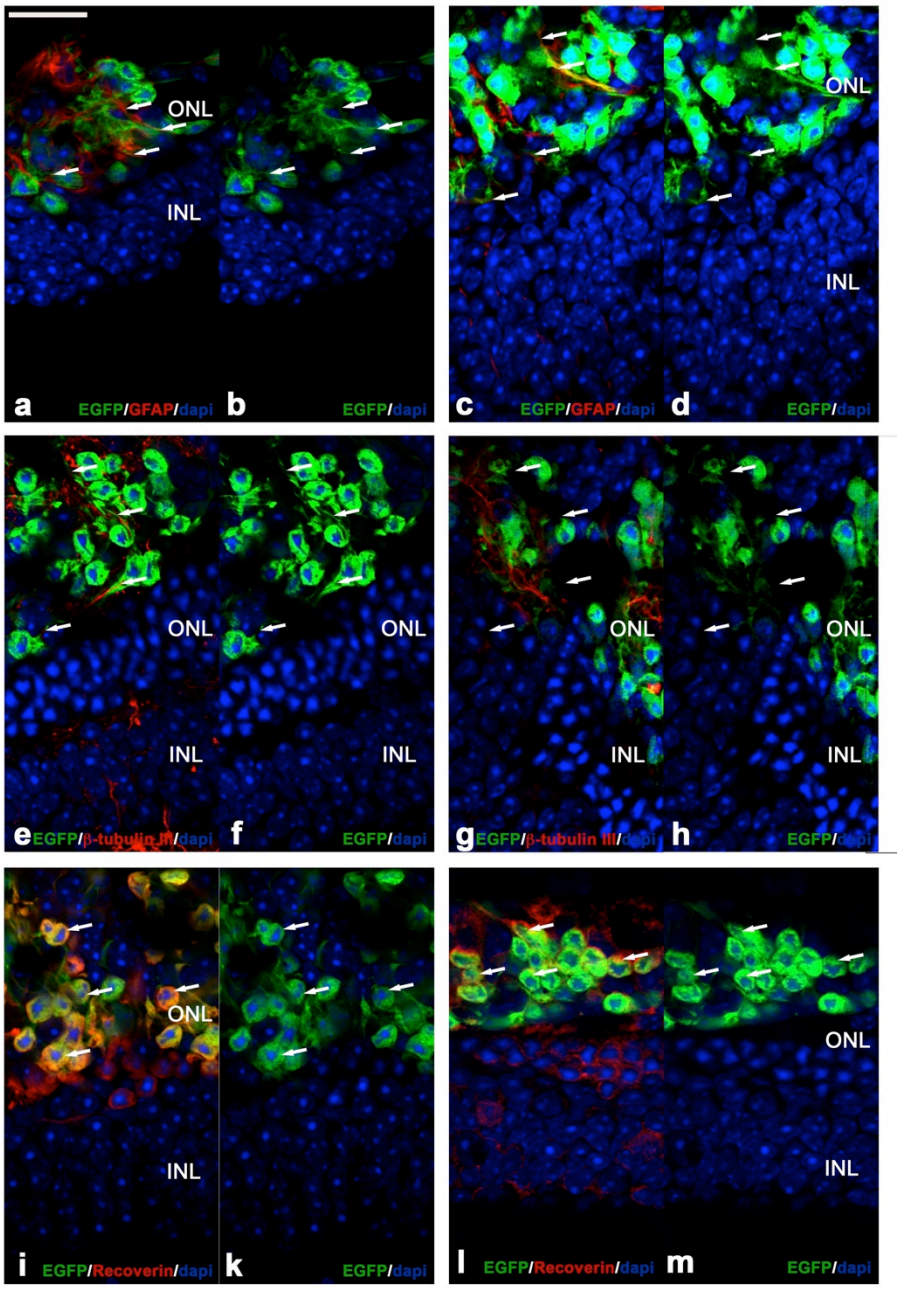

Figure 4: A fraction of the subretinally grafted cell suspension remains as clusters in the subretinal space without integration. Characterisation of transplanted primary cells found as clusters in the subretinal space of wild-type and β2/β1 knock-in mutant mice were present at all time points analysed. A fraction of aggregates remained in the subretinal space for at least 28 weeks post transplantation, longest period analysed. Here aggregates were analysed at 28 weeks post transplantation for wild-type (a-c; g-I; n-p) and β2/β1 knock-in mutant (d-f; k-m; q-s) mice. The characterisation of clusters of the grafted cells for neuronal markers showed grafted cells differentiated into GFAP (glial fibrillary acidic protein)-positive cells for wild-type (a-c) and mutant (d-e) mice, into β-tubulin III-positive cells for wild-type (g-i) and mutant (k-l) mice, and into recoverin-positive cells for wild-type (n-o) and mutant (q-s) mice. ONL: outer nuclear layer; INL: inner nuclear layer; EGFP: enhanced green fluorescent protein; dapi: 4',6-diamidino-2-phenylindole dihydrochloride hydrate. Bar in (a) for (a-m): 20µm.

2. Project: 'A neural stem cell-based intraocular delivery system to transport neuroprotective factors to the murine retina'

ABSTRACT

A cell-based continuous intraocular delivery of neuroprotective factors is among the strategies to attenuate photoreceptor degeneration in yet untreatable degenerative retinal disorders, irrespective of the etiology of the disease. To establish a cell-based system, which allows a controlled delivery of therapeutic gene products to the mouse retina, we used a polycistronic lentiviral vector to genetically modify murine neural stem (NS) cells. The vector concurrently encoded a gene of interest, a reporter gene and a resistance gene, and thus facilitated the selection, cloning and in vivo tracking of engineered NS cells. To functionally characterise the stem cell-based delivery system in vivo, we expressed a secretable variant of ciliary neurotrophic factor (CNTF) in NS cells and grafted the modified cells into the vitreous space of Pde6b^{rd1} and Pde6b^{rd10} mice, two animal models of retinitis pigmentosa. Here, the intravitreally grafted cells attached to the retina and lens where they differentiated into astrocytes and neurons. Importantly, intravitreal injections of CNTF-expressing NS cells resulted in significant attenuation of photoreceptor degeneration in both mutant mouse lines. The neuroprotective effect was significantly increased when clonally derived NS cell lines selected for high expression levels of CNTF were grafted into Pde6b^{rd1} mice. Obvious adverse effects of the transplanted cells on the general morphology of the host retinas were not observed. Together, we have established a neural stem cell-based delivery system that will be of use to evaluate the therapeutic potential of neuroprotective factors or other secreted and therapeutically relevant gene products in mouse models of retinal disorders.

INTRODUCTION

Progressive dysfunction and degeneration of photoreceptor cells, as it occurs in retinitis pigmentosa or age-related macular degeneration, results in currently incurable visual impairment and eventually blindness (Wright et al., 2010). Corrective gene therapy, optogenetic therapy, stem cell-based replacement strategies, electronic retinal implants and neuroprotective strategies are among the approaches aiming at establishing effective treatments for such conditions (for reviews, see: Chaum, 2003; Wenzel et al., 2005; Lamba et al., 2009b; Smith et al., 2009; West et al., 2009; Busskamp et al., 2012; Cepko, 2012; Dagnelie, 2012). All these therapeutic strategies have achieved remarkable results in animal models, and some have entered clinical trials (e.g. Cideciyan, 2010; Dagnelie, 2012; Wen et al., 2011; Schwartz et al., 2012).

Generic gene therapies, like neuroprotective strategies, that aim not to correct the gene defect but to treat its consequences, i.e. degeneration of photoreceptor cells and deterioration of visual function, offer the possibility of therapies that are widely applicable across a range of conditions (Buch et al., 2007). During the last two decades, a number of different neurotrophic factors have been shown to attenuate photoreceptor degeneration and to partly preserve retinal function in a variety of animal models of hereditary retinal degeneration and other pathologies involving photoreceptor death (for reviews, see: Chaum, 2003; Wenzel et al., 2005; Buch et al., 2007). Protection of photoreceptor cells has been observed, for instance, after sustained intraocular delivery of glial cell line-derived neurotrophic factor (GDNF) (Frasson et al., 1999; Dalkara et al., 2011), brain-derived neurotrophic factor (BDNF) (Okoye et al., 2003; Gauthier et al., 2005), or pigment epithelium derived growth factor (PEDF) (Semkova et al., 2002; Imai et al., 2005), to mention only a few. Because neurotrophic factors normally have relatively short half-life times, do not ordinarily cross the blood-retina barrier and bear the potential risk of unacceptable side effects when administered systemically, strategies are being developed that permit a local and sustained delivery of these factors to the retina. These include intravitreal injections of biodegradable factor-loaded microspheres, forced expression of neurotrophic factors in endogenous retinal cells using viral or non-viral vectors, or intraocular transplantations of cells genetically engineered to secrete such factors (e.g.: Lawrence et al., 2004; Andrieu-Soler et al., 2005; Gauthier et al., 2005; Gamm et al., 2007; Gregory-Evans et al., 2009; Read et al., 2010).

The intraocular implantation of encapsulated cells provides a straightforward strategy to use genetically engineered cells as vectors to deliver neurotrophic factors to

dystrophic retinas, as the encapsulation not only protects the grafted cells from the immune system of the host, but also the host retina from potential adverse effects of the grafted cells. Experiments with an encapsulated human retinal pigment epithelium (RPE) cell line engineered to secret CNTF have indeed demonstrated the feasibility of this approach in large animal models (Tao et al., 2002; Thanos et al., 2004; Li et al., 2010). However, the size of the encapsulated cell implants precludes their use for preclinical studies in small-sized animal models, including the mouse with its numerous genetic and acutely induced models of degenerative retinal disorders.

With the advances in stem cell and progenitor cell technology, much interest is focusing on cell replacement strategies as a possible option to develop treatments for degenerative retinal disorders (Klassen et al., 2004; Pellegrini et al., 2007; Lamba et al., 2009b). Transplantations of primary retinal cells into the subretinal space of normal adult or dystrophic mouse retinas have recently indeed provided evidence that replacement of photoreceptor cells by cell transplantation might be feasible (MacLaren et al., 2006; Bartsch et al., 2008; Pearson et al., 2012), and recent studies have successfully generated photoreceptor cells or RPE cells from embryonic stem cells and induced pluripotent stem cells (Comyn et al., 2010; Singh and MacLaren, 2011; Rowland et al., 2012). Given that stem cells are highly expandable cells and considering their unprecedented amenability to genetic modifications they not only comprise an interesting cell population for cell replacement strategies, but might also serve as cellular vehicles to achieve a local and sustained delivery of neuroprotective factors to diseased retinas. Neurally committed stem and progenitor cells have been shown to survive for long periods of time in host eyes after intraocular transplantations, and to confer some neuroprotective activity to degenerating photoreceptor cells even when grafted without prior genetic modifications (Meyer et al., 2006; Gamm et al., 2007; Wang et al., 2008; McGill et al., 2012). Neural stem or progenitor cells are thus among the candidate cell types that might serve as cellular vectors to target neuroprotective factors to the murine retina.

Neural stem/progenitor cells are usually expanded as free-floating cellular aggregates, so-called neurospheres (Reynolds and Weiss, 1992, 1996). However, only a small percentage of cells in these cultures display the cardinal features of stem cells, i.e. multipotentiality and the ability to self-renew. The majority of cells in these cultures instead corresponds to committed neural progenitor cells and terminally differentiated neural cell types (Gritti et al., 1999; Tropepe et al., 1999). However, recent studies have

shown that adherently cultivated neurosphere cells give rise to homogeneous populations of self-renewing, clonogenic neural stem cells (Conti et al., 2005; Glaser et al., 2007). These cells, which in analogy to continuously self-renewing embryonic stem (ES) cells have been termed neural stem (NS) cells, show similarities to neurogenic radial glia, undergo long-term symmetric cell division in vitro, and give rise to neurons, astrocytes and oligodendrocytes in vitro and after transplantation into the brain (Conti et al., 2005; Glaser et al., 2007) or the retina (our unpublished data). NS cells have also been derived from ES cells, induced pluripotent stem cells and the adult human brain (Conti et al., 2005; Sun et al., 2008; Onorati et al., 2010; for review, see: Conti and Cattaneo, 2010), and thus represent an interesting cell population for therapeutic applications.

Ciliary neurotrophic factor (CNTF) is a member of the interleucin-6 cytokine family, and has neuroprotective effects on a variety of neurons of the peripheral and central nervous system (Sleeman et al., 2000; Sendtner et al., 1994). In the retina, CNTF is neuroprotective for photoreceptor cells and ganglion cells, and is probably the most extensively studied neurotrophic factor in the context of degenerative retinal disorders (for reviews, see: Chaum, 2003; Mey and Thanos, 1993; Wenzel et al., 2005; Johnson et al., 2011; Wen et al., 2011). While CNTF potently protects photoreceptors from cell death in various animal models of inherited or acquired retinal disorders, it negatively affects retinal function in a dose-dependent and reversible manner (Liang et al., 2001; Schlichtenbrede et al., 2003; Bush et al., 2004; Wen et al., 2006; McGill et al., 2007). Of note, CNTF is currently being evaluated in clinical trials for the treatment of inherited retinal degenerations and geographic atrophy (Sieving et al., 2006; Talcott et al., 2011; Zhang et al., 2011).

The present study was performed to establish a controlled neural stem cell-based intraocular delivery system for experimental work in the mouse. To this aim, we took advantage of the strong protective effect of CNTF on retinal structure, and expressed this cytokine in NS cells. When CNTF-expressing NS cell cultures or clonally derived NS cell lines were intravitreally grafted into Pde6b^{rd1} or Pde6b^{rd10} mice, they preferentially differentiated into astrocytes and significantly attenuated photoreceptor degeneration in both mutant mouse lines. Obvious adverse effects of the grafted cells on the host retinas were not observed. We conclude that the NS cell-based intraocular delivery system provides a powerful tool for preclinical studies aimed at evaluating in vivo

effects of neuroprotective factors or other secreted and therapeutically relevant gene products in genetic or acutely induced mouse models of retinal disorders.

MATERIAL AND METHODS

Animals

Neural stem cells were isolated from the cerebral cortex of 14 days old wild-type C57BL/6J mouse embryos. The neuroprotective effect of genetically engineered NS cells on photoreceptor cells was evaluated in Pde6b^{rd1} and Pde6b^{rd10} mutant mice. Both transgenic mouse lines were maintained on a C57BL/6J genetic background and their genotype was determined by polymerase chain reaction (PCR) analysis of DNA from tail biopsies as described elsewhere (Gimenez and Montoliu, 2001; Chang et al., 2007). All animal experiments were approved by the local ethic committee and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation, cultivation and differentiation of neural stem cells

Adherently propagated NS cell cultures were derived from the cerebral cortex of 14 days old wild-type mouse embryos essentially as described elsewhere (Conti et al., 2005; Weber et al., 2010). In brief, we first expanded neurosphere cultures (Pressmar et al., 2001; Ader et al., 2004) in Dulbecco's modified Eagle's/F12 medium (Life Technologies, Darmstadt, Germany), containing 10ng/ml epidermal growth factor (EGF), 10ng/ml fibroblast growth factor-2 (FGF-2; both from TEBU, Offenbach, Germany) and 1% N2 (Life Technologies). After 2 to 3 passages, neurospheres were enzymatically dissociated with Accutase (PAA Laboratories, Coelbe, Germany), and cells were further cultivated under adherent conditions in tissue culture flasks coated with poly-L-ornithine (PLO; Sigma, St. Louis, MO) and 0.1% Matrigel (Becton Dickinson, Heidelberg, Germany) in NS-A medium (Euroclone, Pero, Italy) supplemented with 10ng/ml FGF-2, 10ng/ml EGF, and 1% modified N2 (Conti et al., 2005). Expansion media for genetically engineered NS cells additionally contained 1% B27 (Life Technologies).

Differentiation of NS cells into astrocytes was induced by maintaining cultures for 5 days in NS-A medium containing 1% fetal calf serum (FCS; Life Technologies) and 2% B27. Neuronal differentiation was induced by first cultivating NS cells for 5 days in NS-A medium supplemented with 5ng/ml FGF-2, 1% N2 and 2% B27, followed by a further cultivation period of 5 days in a 1:1 mixture of NS-A and Neurobasal (Life Technologies) medium containing 0.25% N2 and 2% B27.

Lentiviral vectors and virus production

The open reading frame of mouse CNTF was PCR amplified from mouse brain cDNA, and ligated in-frame with the Ig κ-chain leader sequence of pSecTag2 B (Life Technologies). The secretable variant of CNTF was then cloned into the lentiviral vector pCAG-IRES-Venus-2A-ZEO, giving rise to pCAG-CNTF-IRES-Venus-2A-ZEO. The vector is based on the recently described lentiviral "gene ontology" (LeGO) vectors (Weber et al., 2008; Weber et al., 2010), and contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus, and a Venus reporter gene separated from a zeocin resistance gene by a P2A sequence of porcine teschovirus-1 under regulatory control of the cytomegalovirus (CMV) enhancer/chicken β-actin (CAG) promoter (Fig. 1). Lentiviral particles, pseudotyped with the envelope G protein of the vesicular stomatitis virus (VSV-G), were produced by transient transfections of HEK 293T cells using standard protocols (see: http://www.LentiGO-Vectors.de).

Transduction and selection of neural stem cells

For transductions, NS cells were plated at a density of $2x10^4$ cells/cm^2 into 24-well plates coated with 0.1% Matrigel. Cells were spinoculated with pCAG-IRES-Venus-2A-ZEO (control-NS cells) or pCAG-CNTF-IRES-Venus-2A-ZEO (CNTF-NS cells) at 1,000g for 1 h at room temperature in the presence of 8µg/ml polybrene. Subsequent cultivation of cells was done in expansion medium containing 200µg/ml zeocin (Invivogen, San Diego, CA) to select for positive cells. To establish clonally derived control-NS and CNTF-NS cell lines, single cells with the highest expression levels of the reporter gene were plated into 96-well plates by fluorescence activated cell sorting (FACS; FACSAria, Becton Dickinson Bioscience, Heidelberg, Germany). Expression levels of CNTF in different CNTF-NS cell clones were compared by immunoblot analysis of culture supernatants, and the NS cell clone with the highest expression level of CNTF was selected for transplantation experiments.

Immunocytochemistry, immunoblot analysis and ELISA

For immunocytochemical analysis, non-differentiated and differentiated control-NS and CNTF-NS cells were fixed in 4% paraformaldehyde (PA) in phosphate-buffered saline (PBS; pH 7.3), blocked in PBS containing 0.1% bovine serum albumin (BSA; Sigma) and 0.3% Triton X-100 (Sigma), and incubated with a polyclonal rabbit anti-CNTF antibody (1:1000) overnight at room temperature. Astrocytes and neurons in differentiated NS

cell cultures were identified using a monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:50; Sigma) and a monoclonal mouse anti-microtubule associated protein 2 (MAP2) antibody (1:1000; Sigma), respectively. Primary antibodies were detected with anti-rabbit Cy3- and anti-mouse Cy5-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA, USA). All cultures were stained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma) to label cell nuclei.

To confirm secretion of CNTF into the culture medium of CNTF-NS cell bulk cultures and CNTF-NS cell clones, supernatants were concentrated using Amicon Ultra Centrifugal Filter devices (Millipore, Bedford, MA) and subjected to Western Blot analysis. CNTF was detected using a polyclonal rabbit anti-CNTF antibody (1:200; Santa Cruz Biotechnologies, Santa Cruz, CA) and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:15,000; Jackson ImmunoResearch), and immunoreactive bands were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instruction.

To quantify CNTF levels in the culture supernatants, $1x10^6$ control-NS or CNTF-NS cells were plated into 6-well plates and cultivated for 24 hrs in 2ml expansion medium. Subsequent analysis of supernatants was performed with an enzyme-linked immunosorbent assay (ELISA) kit for mouse CNTF (USCN Life Science Inc., Wuhan, China) according to the manufacturer's instructions. ELISA plates were analysed using a Sunrise microplate reader and Magellan software (Tecan, Männedorf, Switzerland).

Intravitreal cell transplantations and immunohistochemical analysis

Genetically modified NS cells were intravitreally grafted into 7 days old $Pde6b^{rd1}$ and 14 days old $Pde6b^{rd10}$ mice. Animals were deeply anaesthetized, a glass micropipette was inserted into the vitreous space at the junction between sclera and cornea, and 1µl and 2µl of vitreous fluid was removed from the eyes of $Pde6b^{rd1}$ and $Pde6b^{rd10}$ mice, respectively. $Pde6b^{rd1}$ mice received intravitreal injections of $8x10^5$ CNTF-NS cells in 1µl PBS into one eye, and $8x10^5$ control-NS cells in 1µl PBS into the contralateral eye. $Pde6b^{rd10}$ mice received intravitreal transplantations of $1.6x10^6$ CNTF-NS cells in 2µl PBS into one eye, and the same number of control-NS cells in the same volume of PBS into the contralateral eye. Particular care was taken not to damage the lens during the removal of vitreous fluid or the injections of cells. $Pde6b^{rd1}$ mutants were analysed at

postnatal day 15 (P15). Pde6b^{rd10} mice were kept in a 12-hour light/12-hour dark cycle with illumination levels of about 200 lux, and were analysed at P30.

Animals were killed by cervical dislocation, eyes were marked in situ at their dorsal poles, and the temporal half of the corneas was removed to assure later identification of the nasal and temporal retina in histological sections. Eyes were fixed in 4% PA in PBS (pH 7.3), cryoprotected in an ascending series of sucrose, embedded in Tissue-Tek (Sakura Finetek, Zouterwoude, Netherlands), frozen, and serially sectioned with a cryostat at a thickness of 25µm. For immunohistochemistry, sections were incubated in PBS containing 0.1% BSA and 0.3% Triton X-100 for 1 h at room temperature. Sections were then incubated with polyclonal rabbit anti-recoverin antibodies (1:3000; Chemicon, Temecula, CA) overnight at room temperature, washed with PBS, incubated with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch), stained with DAPI, washed again and mounted onto slides. Analysis of retina sections was performed with an Olympus FV 1000 confocal microscope (Olympus, Hamburg, Germany).

Quantitative analysis of photoreceptor survival

Central retina sections (i.e. in the plane of the optic disc) stained with anti-recoverin antibodies and DAPI were photographed with an Olympus FV 1000 confocal microscope at a magnification of x600. Photomicrographs were merged to generate an image of the entire nasal half of the retina using Photoshop CS3 software (Adobe Systems Inc., San Jose, CA). Photoreceptor cells were counted in three areas located at defined distances from the optic nerve, corresponding to 25%, 50% and 75% of the entire length of the nasal retina. Areas defined for cell countings covered the outer nuclear layer over a length of 220µm. Individual photoreceptor nuclei or nuclear fragments were identified in overlays of DAPI and recoverin stainings. Eyes with grafted CNTF-NS cells and contralateral eyes with grafted control-NS cells of six animals from three independent transplantation experiments were analysed for each experimental group (i.e. Pde6b^{rd10} mice with grafted NS cell bulk cultures, Pde6b^{rd1} mice with grafted NS cell bulk cultures, Pde6b^{rd1} mice with a grafted NS cell clone). Statistical analysis of data was performed with the two-way ANOVA test using Statistica software (StatSoft Inc., Tulsa, OK).

RESULTS

Neural stem cell cultures

To establish NS cell cultures from the cerebral cortex of embryonic mice, we first generated neurosphere cultures according to standard protocols. After two to three passages, neurosphere cultures were converted into adherently growing cultures and further propagated on Matrigel-coated tissue culture plastic in the presence of EGF, FGF-2 and N2. Under these conditions, cultures consist of a morphologically homogeneous population of symmetrically dividing tripotent NS cells with molecular features reminiscent of neurogenic radial glia (Conti et al., 2005; Glaser et al., 2007; M. Gebhardt, C. Rinn, V. Wörpel, G. Jung, M. Czekaj, G. Richard, M. Ader and U. Bartsch, manuscript in preparation).

Lentiviral expression of CNTF in neural stem cells

To genetically engineer NS cells, we generated a polycistronic lentiviral vector that is based on the recently described lentiviral "gene ontology" (LeGO) vectors (Weber et al., 2008; Weber et al., 2010). Under regulatory control of the ubiquitously active CAG promoter, the vector encodes a secretable variant of mouse CNTF, and a Venus reporter gene and a zeocin resistance gene, which are both located downstream of an IRES sequence and separated from each other by a P2A sequence of porcine teschovirus-1 (hereafter termed "pCAG-CNTF-IRES-Venus-2A-ZEO"; Fig. 1a). Control experiments were performed with the same vector with the only exception that it lacked the mouse CNTF cDNA (hereafter termed "pCAG-IRES-Venus-2A-ZEO"; Fig. 1b). Spinoculations of NS cells from passage 8 with either pCAG-CNTF-IRES-Venus-2A-ZEO or pCAG-IRES-Venus-2A-ZEO were performed to establish CNTF-NS cell cultures and control-NS cells, respectively. Further cultivation of the transduced cells in the presence of zeocin gave rise to CNTF-NS and control-NS cell cultures exclusively composed of Venus-positive cells (Fig. 2a, c). Immunocytochemical analysis of CNTF-NS cell cultures revealed strong CNTF-immunoreactivity in the perinuclear region of essentially every cell (Fig. 2d). Control NS cell cultures processed in parallel, in contrast, lacked detectable levels of CNTF expression (Fig. 2b).

Because intravitreally grafted NS cells differentiate into astrocytes and neurons (data not shown), we next analysed expression of CNTF in terminally differentiated NS cell cultures. To this aim, CNTF-NS and control-NS cultures were differentiated into astrocytes and neurons using directed differentiation protocols, and subsequently

analysed for CNTF expression using immunocytochemistry. Experiments revealed expression of CNTF in virtually all GFAP-positive astrocytes and MAP2-positive neurons derived from CNTF-NS cells (Fig. 3). Astrocytes and neurons derived from control-NS cells, in contrast, lacked detectable levels of CNTF (Fig. 3). As a next step, we analysed secretion of CNTF into the supernatant of engineered NS cell cultures. While immunoblot analysis confirmed secretion of CNTF into the culture supernatant of CNTF-NS cell bulk cultures and clonally derived CNTF-NS cell lines (see below), the protein was undetectable in supernatants of control-NS cell bulk cultures (Fig. 4) and control-NS cell clones (not shown). Expression of CNTF in CNTF-NS cell cultures remained detectable by immunocytochemistry and immunoblot analysis for at least 35 passages (higher passages were not analysed), corresponding to a total culture period of approximately 6 months.

CNTF-expressing neural stem cell clones

To establish NS cell cultures with elevated and defined expression levels of CNTF, we next generated clonally derived CNTF-NS cell lines. Assuming that expression levels of CNTF correlate with expression levels of the reporter gene, we isolated individual NS cells with the highest expression levels of Venus using FACS, and clonally expanded these cells. Immunocytochemistry (data not shown) and immunoblot analysis (Fig. 4) demonstrated expression and secretion of CNTF in all CNTF-NS cell clones established, while control-NS cell clones lacked expression of CNTF (not shown). The CNTF-NS cell clone with the highest expression level of CNTF was identified by immunoblot analysis and further used for intravitreal transplantation experiments in Pde6b^{rd1} mutant mice (see below). Analysis of the original CNTF-NS cell bulk culture and the CNTF-NS cell clone derived from this bulk culture by ELISA confirmed elevated expression levels of CNTF in the clonal cell line. While the CNTF-NS cell bulk culture expressed 4.0ng CNTF per 1x10^6 cells in 24 hrs, the clonal NS cell line expressed 5.4ng CNTF per 1x10^6 cells in 24 hrs, corresponding to an approximately 35% increase in the expression level of CNTF in the CNTF-NS cell clone.

Intravitreal transplantations of modified neural stem cells into Pde6b^{rd10} and Pde6b^{rd1} mice

To analyse whether intraocular transplantations of genetically engineered NS cells allow to deliver functionally relevant quantities of CNTF to the murine retina, we grafted

CNTF-NS cells into the vitreous space of Pde6b^{rd10} and Pde6b^{rd1} mice, two animal models of retinitis pigmentosa with an early onset and rapid degeneration of photoreceptor cells (Chang et al., 2002; Chang et al., 2007; Gargini et al., 2007). Intravitreal transplantations of control-NS cells into the contralateral eyes served as a control. In both mouse lines, cells were grafted at initial stages of photoreceptor degeneration (i.e. at postnatal day (P) 7 for Pde6b^{rd1} and P14 for Pde6b^{rd10} mice), and retinas were analysed at advanced stages of photoreceptor degeneration (i.e. at P15 for Pde6b^{rd1} and P30 for Pde6b^{rd10} mice).

Analysis of eyes that had received intravitreal injections of CNTF-NS or control-NS cells revealed the presence of numerous Venus-positive donor cells in both mouse lines (data not shown). Grafted cells were attached to the vitread margin of the retina and the posterior pole of the lens. Evidence for integration of donor cells into the host retinas was not observed. Immunohistochemical analysis revealed that both CNTF-NS and control-NS cells had differentiated into GFAP-positive astrocytes or β-tubulin III-positive nerve cells, but not into myelin basic protein- (MBP) positive oligodendrocytes (data not shown). Furthermore, we observed a robust co-expression of CNTF with Venus in grafted CNTF-NS cells, but not in grafted control-NS cells (data not shown). Finally and importantly, we found no obvious adverse effects of the grafted cells on the general morphology of Pde6b^{rd10} (Fig. 5) or Pde6b^{rd1} retinas (Fig. 6, 7), in contrast to results obtained with subretinal or intraretinal NS cell transplantations (G.J. and U.B., unpublished data).

Intravitreally grafted CNTF-expressing neural stem cells protect photoreceptors in Pde6b^{rd10} and Pde6b^{rd1} mice

The neuroprotective effect of intravitreally grafted CNTF-NS cells on photoreceptor cells in Pde6b^{rd10} retinas was analysed in central retinal sections (i.e. in the plane of the optic nerve) that were stained with anti-recoverin antibodies and DAPI (Fig. 5). A comparison of eyes that had received injections of CNTF-expressing NS cells with eyes that had received injections of control-NS cells consistently revealed a significantly increased thickness of the outer nuclear layer in the CNTF-treated eyes (Fig. 5). Importantly, this neuroprotective effect of the CNTF-NS cells on photoreceptor cells was evident over the entire length of all retinal sections analysed. To quantify the neuroprotective effect of the CNTF-expressing NS cells, photoreceptor numbers were counted in eyes with control-NS and CNTF-NS cell grafts of totally 6 Pde6b^{rd10} mice from 3 independent

transplantation experiments. Photoreceptor counts were performed on the nasal halves of the retinas in three areas, each covering the outer nuclear layer over a length of 220µm and located at defined positions from the optic disc, corresponding to 25%, 50% and 75% of the distance between optic disc and periphery of the retina. In CNTF-treated retinas, 274.8 ±31.6 (mean ±SEM) photoreceptors were present at the 25% position, 311.5 ±26.9 at the 50% position, and 339.8 ±23.5 at the 75% position. In control retinas, in comparison, we counted 101.7 ±18.9, 151.0 ±25.3 and 181.7 ±27.3 photoreceptors at the 25%, 50% and 75% position, respectively (Fig. 8a). Thus, the number of photoreceptor cells in CNTF-treated Pde6b^{rd10} retinas was increased by a factor of 1.9 to 2.7 when compared to control retinas, depending on the retinal region analysed. Statistical analysis of data revealed that this difference was significant ($p<0.01$ according to the two-way ANOVA test; Fig. 8a). Close inspection of CNTF-treated retinas additionally revealed significantly longer inner and outer photoreceptor segments than in retinas that received intravitreal injections of control-NS cells.

A significant neuroprotective effect of the CNTF-NS cells was also observed in the second mouse model of retinitis pigmentosa, the Pde6b^{rd1} mouse (Fig. 6). Similar to Pde6b^{rd10} mice, we observed a significantly increased thickness of the outer nuclear layer in all eyes that had received CNTF-NS grafts when compared to the contralateral eyes that had received control-NS grafts (Fig. 6). As in Pde6b^{rd10} mice, the neuroprotective effect of CNTF-expressing NS cells was evident over the entire length of all retina sections analysed. Quantitative analysis of 6 Pde6b^{rd1} mice from 3 independent transplantation experiments confirmed a pronounced protective effect of the CNTF-expressing NS cells on photoreceptor cells also in this mutant, with 253.7 ±11.4 (mean ±SEM) photoreceptor cells at the 25% position, 260.5 ±17.7 photoreceptors at the 50% position and 268.3 ±13.7 photoreceptors at the 75% position in CNTF-treated eyes, compared to 109.2 ±8.6, 110.5 ±5.6 and 130.7 ±8.9 photoreceptor cells at the 25%, 50% and 75% position in the control retinas, respectively (Fig. 8b). Depending on the retinal region analysed, the number of photoreceptor was thus increased 2.1-fold to 2.4-fold in CNTF-treated eyes when compared to control eyes, and this difference was statistically highly significant ($p<0.001$ according to the two-way ANOVA test; Fig. 8b). Finally and similar to the observations in Pde6b^{rd10} mice, we observed significantly longer inner and outer photoreceptor segments in CNTF-treated Pde6b^{rd1} retinas than in the corresponding control retinas.

Neuroprotective effect of a CNTF-expressing neural stem cell clone in Pde6b^{rd1} mice

The derivation of genetically modified clonal NS cell lines provides a strategy to establish cultures with elevated and defined expression levels of transgenes. Clonal expansion of FACS-sorted CNTF-expressing NS cells gave rise to a NS cell clone with an approximately 35% increase in the expression level of CNTF when compared to the original NS cell bulk culture (see above). The neuroprotective potential of this CNTF-NS cell clone was tested in Pde6b^{rd1} mice, and compared to the neuroprotective activity of the original CNTF-NS cell bulk culture. Intravitreal transplantations of the CNTF-NS cell clone resulted in a significant rescue of photoreceptor cells in Pde6b^{rd1} mice which was evident over the entire length of the retina sections (Fig. 7), and appeared more pronounced than the rescue observed for the original CNTF-NS cell bulk culture (compare Fig. 6 and 7). Quantitative analysis of 6 Pde6b^{rd1} mice from 3 independent transplantation experiments confirmed a massive protection of photoreceptor cells by the grafted CNTF-NS cell clone, with 283.5 ±13.3 (mean ±SEM), 288.7 ±11.4 and 314.5 ±11.5 photoreceptor cells at the 25%, 50% and 75% positions of CNTF-treated retinas, respectively. In comparison, 87.8 ±3.0, 92.7 ±8.9 and 110.0 ±6.7 photoreceptor cells were present at the 25%, 50% and 75% positions of control retinas, respectively (Fig. 8c). Thus, photoreceptor cell numbers were increased 2.9-fold to 3.2-fold in CNTF-treated Pde6b^{rd1} retinas when compared to control retinas, and this difference was statistically highly significant ($p<0.001$ according to the two-way ANOVA test; Fig. 8c).

To compare the neuroprotective activity of the original CNTF-NS cell bulk culture and the CNTF-NS cell clone in Pde6b^{rd1} mice, we calculated the quotients of photoreceptor numbers in CNTF-treated and control retinas for both experimental groups at the three retinal positions analysed. Values for eyes that received injections of CNTF-NS cell bulk cultures and eyes that received injections of the CNTF-NS cell clone were: 2.42 ±0.26 (mean ±SEM) and 3.25 ±0.2 for the 25% position, 2.38 ±0.17 and 3.24 ±0.31 for the 50% position, and 2.1 ±0.17 and 2.92 ±0.23 for the 75% position, respectively (Fig. 8d). Analysis of data revealed that this difference was statistically significant ($p<0.01$ according to the two-way ANOVA test; Fig. 8d).

DISCUSSION

The present study was aimed at establishing a cell-based intraocular delivery system that permits a controlled and sustained targeting of neurotrophic factors to the dystrophic mouse retina. The intraocular implantation of encapsulated and genetically engineered cells provides a strategy to achieve a sustained delivery of secreted gene products to the retina, and encapsulated cell technology has indeed been successfully used to target neurotrophic factors to the retina of large animal models of retinal disorders (Tao et al., 2002; Bush et al., 2004; Thanos et al., 2004; Li et al., 2010). However, the methodology is not easily applicable to the many genetic or acutely induced mouse model of retinal disorders, due to the small-sized eyes of this species. We therefore searched for a cell type that might serve as a cellular vector to deliver secreted gene products to the murine retina without exerting adverse effects on the host retina.

Neural stem or precursor cells are among the candidate cell types for the development of a cell-based intraocular drug delivery system. In fact, a number of studies has demonstrated that neural stem/progenitor cells isolated from the developing or adult brain, or derived from embryonic stem cells or induced pluripotent stem cells survive for extended periods of time after intraocular transplantations (Ader et al., 2000; Pressmar et al., 2001; Meyer et al., 2006; Wang et al., 2008; Chaudhry et al., 2009; Francis et al., 2009; McGill et al., 2012). Furthermore, recent studies have observed some neuroprotective effects of non-modified neural stem/precursor cells on photoreceptor cells (Lu et al., 2002; Gamm et al., 2007; Wang et al., 2008), or have successfully used genetically engineered neural stem/progenitor cells to target neurotrophic factors into dystrophic retinas (Gamm et al., 2007; McGill e al., 2012). In the present study, we have expanded neural stem cells from the cerebral cortex of mouse embryos under adherent conditions in the presence of EGF and FGF-2. Cells cultivated under such conditions gave rise to cultures consisting of a homogeneous population of symmetrically dividing neural stem cells which, in analogy to continuously proliferating ES cells have been termed NS (for neural stem) cells (Conti et al., 2005). NS cells show high similarities to neurogenic radial glia, can be expanded over many passages, are clonogenic, and give rise to neurons, astrocytes and oligodendrocytes in vitro and after transplantation into the brain, spinal cord or retina (Conti et al., 2005; Glaser et al., 2007; our unpublished observations). NS cell cultures have also been established from the adult mouse brain, the fetal human brain, ES cells and induced pluripotent stem cells (Pollard et al., 2006; Sun et al., 2008; Onorati et al., 2010).

In a series of pilot experiments, we analysed the fate of NS cells after intraocular transplantations into wild-type mice, and Pde6b^{rd1} and Pde6b^{rd10} mutants. When NS cells were grafted into the subretinal space, they separated the photoreceptor cell layer from the RPE cell layer, resulting in aggravated degeneration of the outer nuclear layer. However, when the cells were grafted into the vitreous cavity, they attached to the inner surface of the retina and to the lens and survived for up to three months, the latest post-transplantation interval analysed (G.J., K.K. and U.B., unpublished observations), and no adverse effects were observed. We also used murine mesenchymal stem cells and the immortalized murine neural progenitor cell line C17.2 (Snyder et al., 1992) in intraocular transplantation experiments. Mesenchymal stem cells have been shown to survive for extended periods of time after subretinal or intravitreal transplantation in various animal models of photoreceptor loss, and to exert some neuroprotective effect on photoreceptor cells even without prior genetic modification, possibly because these cells secrete endogenous neurotrophic and/or anti-inflammatory factors (Joe and Gregory-Evans, 2010). In our hands, however, subretinally or intravitreally grafted mouse mesenchymal stem cells frequently caused retinal detachments even after transplantation of low cell numbers and, as a consequence, aggravated degeneration of the outer nuclear layer (G.J. and U.B., unpublished observations). Intravitreally grafted C17.2 neural precursor cells also had adverse effects on host retinas which, although not specifically analysed, were at least in part the result of a continued proliferation of these cells after transplantation, causing mechanical damage of the retinas.

We next analysed whether intraocular transplantations of genetically engineered NS cells permit a sustained delivery of functionally relevant quantities of therapeutic gene products to the murine retina, and expressed a secretable variant of mouse CNTF in NS cells. CNTF was selected for these proof-of-concept experiments, because of its strong neuroprotective effect on photoreceptor cells. Initially demonstrated to attenuate light-induced degeneration of photoreceptor cells (LaVail et al., 1992), CNTF has subsequently been shown to rescue photoreceptors in a variety of animal models of inherited or acquired photoreceptor loss (for review, see: Chaum, 2003; Wenzel et al., 2005; Wen et al., 2011). Recent studies suggest that CNTF also promotes regeneration of cone outer segments in a rat model (Li et al., 2010; Lipinski et al., 2011). Of note, the therapeutic potential of CNTF for the treatment of inherited retinal degenerations and geographic atrophy is currently being explored in clinical studies using an encapsulated human RPE cell line genetically engineered to secrete this cytokine, and some positive

results have been reported (Sieving et al., 2006; Talcott et al., 2011; Zhang et al., 2011). However, while CNTF potently preserves retinal structure, it negatively affects retinal function in a reversible and dose-dependent manner (Liang et al., 2001; Schlichtenbrede et al., 2003; Wen et al., 2006; Bush et al., 2004; McGill et al., 2007). Negative effects of CNTF on electroretinogram (ERG) amplitudes are accompanied with a dysregulated expression of numerous genes including some that encode components of the phototransduction cascade. Sustained delivery of CNTF also results in morphological alterations in photoreceptor cells, including increase in nuclear size and reduced outer segment length (Zeiss et al., 2005; Wen et al., 2006; Rhee et al., 2007; for review, see: Buch et al., 2007; Wen et al., 2011).

In the present study, we selected CNTF to establish the NS cell-based intraocular delivery system in mouse models of retinitis pigmentosa, and used the potent protective effect of CNTF on retinal structure as a measure for the efficacy of this approach. To this aim, we engineered NS cells with a polycistronic lentiviral vector to express a secretable variant of mouse CNTF together with a resistance gene and a Venus reporter gene under regulatory control of the chicken β-actin/CMV enhancer promoter. Transduction and subsequent selection of positive cells gave rise to cultures with a co-expression of CNTF and Venus in essentially every cell, while control cells transduced with the same vector but lacking the CNTF cDNA, were positive for Venus but negative for CNTF. In line with these data, immunoblot analysis of supernatants revealed secretion of CNTF into the culture supernatant of CNTF-transduced NS cells, but not into the culture supernatants of control NS cells. Because NS cells start to differentiate into neural cell types after intraocular transplantation, we next employed directed differentiation protocols to analyse transgene expression in terminally differentiated astrocytes and neurons in vitro. Experiments revealed robust expression of CNTF in GFAP-positive astrocytes and β-tubulin III-positive neurons derived from CNTF-transduced NS cells, but not in astrocytes or neurons derived from control NS cells.

We next grafted the modified NS cells into the vitreous cavity of Pde6b^{rd1} and Pde6b^{rd10} mice, two mouse models of retinitis pigmentosa with an early onset and rapid degeneration of photoreceptor cells (Pittler and Baehr, 1991; Chang et al., 2002; Chang et al., 2007), to evaluate whether NS cells secrete sufficient amounts of CNTF to protect photoreceptor cells in vivo. CNTF-transduced or control NS cells were grafted at initial stages of retinal degeneration (i.e. at P7 into Pde6b^{rd1} and at P15 into Pde6b^{rd10} mice), and retinas were analysed at late stages of retinal degeneration (i.e. at P15 for Pde6b^{rd1}

and at P30 for Pde6b^{rd10} mice). Intravitreal transplantations of 8x10^5 NS cells from a bulk culture producing 4.0ng CNTF/24hrs/1x10^6 cells resulted in significant protection of photoreceptor cells in Pde6b^{rd1} mice. In fact, the number of photoreceptor cells was increased up to 2.4-fold in CNTF-treated eyes when compared to eyes that received transplants of control NS cells, depending on the retinal region analysed. Intravitreal transplantations of 1.6x10^6 CNTF expressing NS cells from the same CNTF secreting bulk culture significantly attenuated photoreceptor degeneration also in Pde6b^{rd10} mice, with an up to 2.7-fold increase in the number of photoreceptor cells when compared to control eyes. In both mutant mouse lines, we additionally observed that the length of the inner and outer segments of photoreceptor cells was increased in CNTF-treated retinas when compared to control retinas, in line with observations in other animal models of retinal degeneration (Cayouette et al., 1998; Schlichtenbrede et al., 2003). Importantly, the neuroprotective effect of the grafted CNTF-NS cells was evident over the entire length of the outer nuclear layer. Finally, the grafted cells had no obvious adverse effects on the general morphology of host retinas of neither mouse strain this is in line with observation of other studies (Francis et al., 2009; McGill et al., 2012).

The expression of the reporter gene in CNTF-NS cells and control-NS cells greatly facilitated in vivo tracking of the modified cells after transplantation. In both Pde6b^{rd1} and Pde6b^{rd10} mice, CNTF-NS cells and control-NS cells formed dense cell layers, which were attached to the inner surface of the retina and the posterior pole of the lens. The majority of these cells were identified as GFAP-positive astrocytes, and a few as β-tubulin III-positive nerve cells. Differentiation of grafted cells into oligodendrocytes was not observed. Despite the presence of numerous donor cells in the vitreous cavity, significant integration of grafted cells into the host retinas was not observed. This observation is in contrast to other studies that have shown extensive integration of intravitreally transplanted neural stem/progenitor cells into developing or dystrophic retinas (Takahashi et al., 1998; Young et al., 2000; Sakaguchi et al., 2004). Intravitreal transplantation of NS cells into Pde6b^{rd1} or Pde6b^{rd10} mice at initial stages of the retinal dystrophy and/or intrinsic differences between NS cells and the neural stem/progenitor cell populations used in the other studies are among the possible explanations for these discrepant results. Finally, we also identified the grafted cells for expression of CNTF, and found detectable levels of CNTF only in cells derived from CNTF-NS cells but not in cells derived from control NS cells.

Expression levels of transgenes in engineered bulk cultures might differ between different cultures and between different passages of the same culture, impeding the delivery of defined quantities of transgenes and analysis of dose effects of secreted gene products using a cell-based system. We took advantage from the fact that NS cell cultures consist of a homogeneous population of clonogenic neural stem cells (Conti et al., 2005), and established clonally derived CNTF-expressing NS cell lines from engineered bulk cultures using FACS. This approach indeed allowed efficient derivation of NS cell clones that produced defined amounts of CNTF over many passages, as judged from the intensity of reporter gene expression and semi-quantitative immunoblot analysis of CNTF expression. Assuming that expression levels of CNTF from the polycistronic lentiviral vector correlate with expression levels of the reporter gene, we next used FACS to establish clonal cell lines from NS cell with the highest reporter gene expression, thereby expecting to generate NS cell clones with high CNTF expression levels. Analysis of such NS cell clones confirmed elevated expression levels of CNTF when compared to the original bulk culture, and one NS cell clone with a 35% increase in CNTF expression was selected for transplantation experiments. When this clone was intravitreally grafted into Pde6b^{rd1} mice, protection of photoreceptor cells was indeed significantly more pronounced than observed with the original bulk culture.

In summary, we have used genetically engineered NS cells to deliver CNTF to the dystrophic retina of two mouse models of retinitis pigmentosa. Intravitreal transplantations of the modified NS cells resulted in significant attenuation of photoreceptor degeneration in both mouse strains. Adverse effects of grafted cells on host retinas were not observed. We conclude that this NS-cell based intraocular delivery system provides a powerful tool for preclinical studies aimed at characterising the in vivo effects of secreted and therapeutically relevant gene products in mouse models of retinal disorders.

FIGURES AND FIGURE LEGENDS

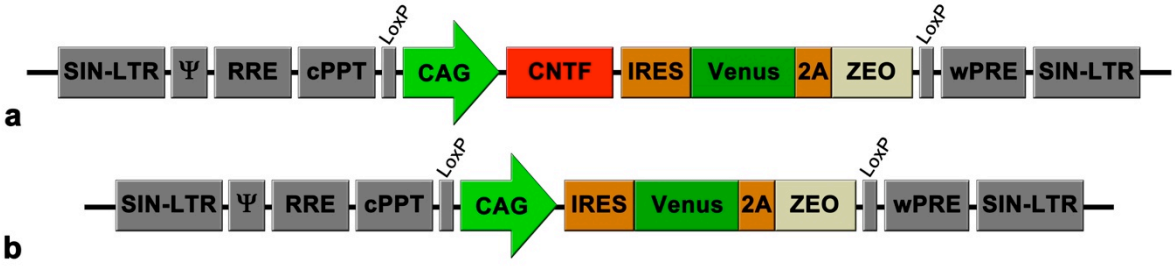

Figure 1: Schematic presentation of the lentiviral vectors used in this study. To express CNTF in NS cells, cells were transduced with a vector encoding a secretable variant of mouse CNTF under regulatory control of the human CMV enhancer/chicken β-actin (CAG) promoter (a). The vector additionally encodes a Venus reporter gene and a zeocine (ZEO) resistance gene, both being located downstream of an internal ribosome entry site (IRES) of the encephalomyocarditis virus and separated from each other by a P2A sequence (a). For control experiments, NS cells were transduced with the same vector with the only exception that it lacked the CNTF cDNA (b). Ψ: packaging signal; CNTF: ciliary 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.

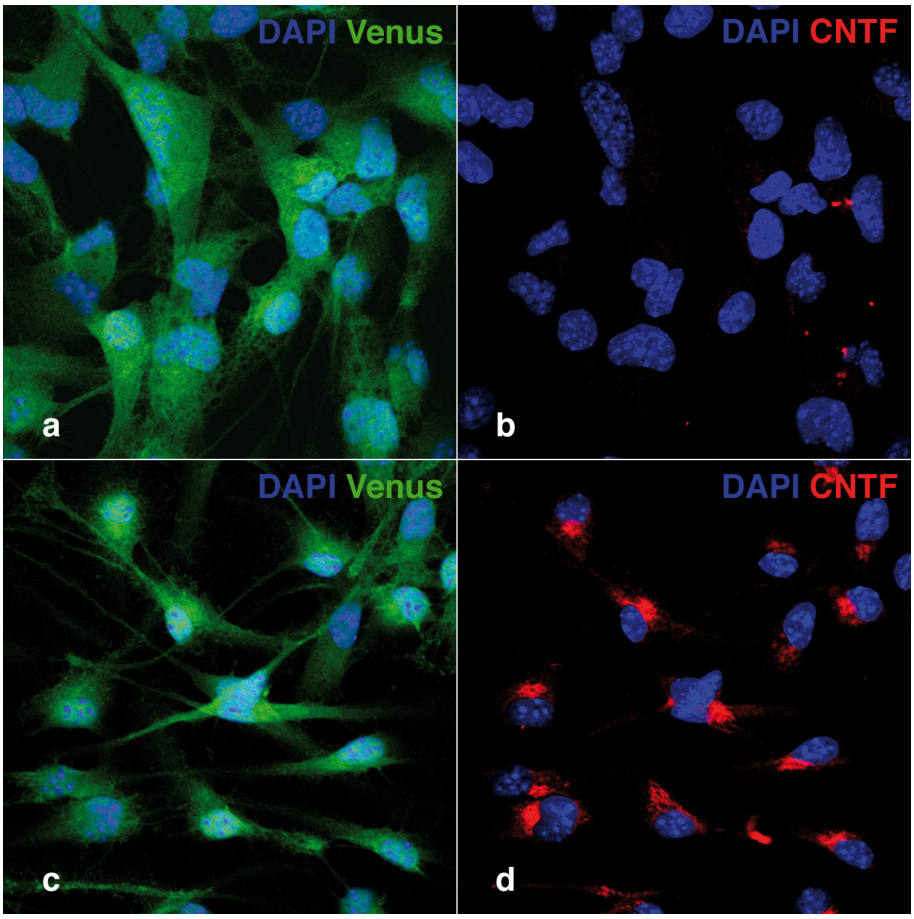

Figure 2: NS cells with an ectopic expression of CNTF. NS cells were transduced with pCAG-CNTF-IRES-Venus-2A-ZEO, positive cells were selected using zeocin and subsequently immunostained with anti-CNTF antibodies (c, d). Note that all cells were positive for Venus (c), and showed strong CNTF immunoreactivity in a perinuclear location (d). NS cells transduced with the control vector pCAG-IRES-Venus-2A-ZEO, in comparison, showed strong expression of Venus (a) but no detectable levels of CNTF (b). All cultures were counterstained with DAPI to visualize cell nuclei (a-d). DAPI: 4',6-diamidino-2-phenylindole. Bar in (d) for (a-d): 20µm.

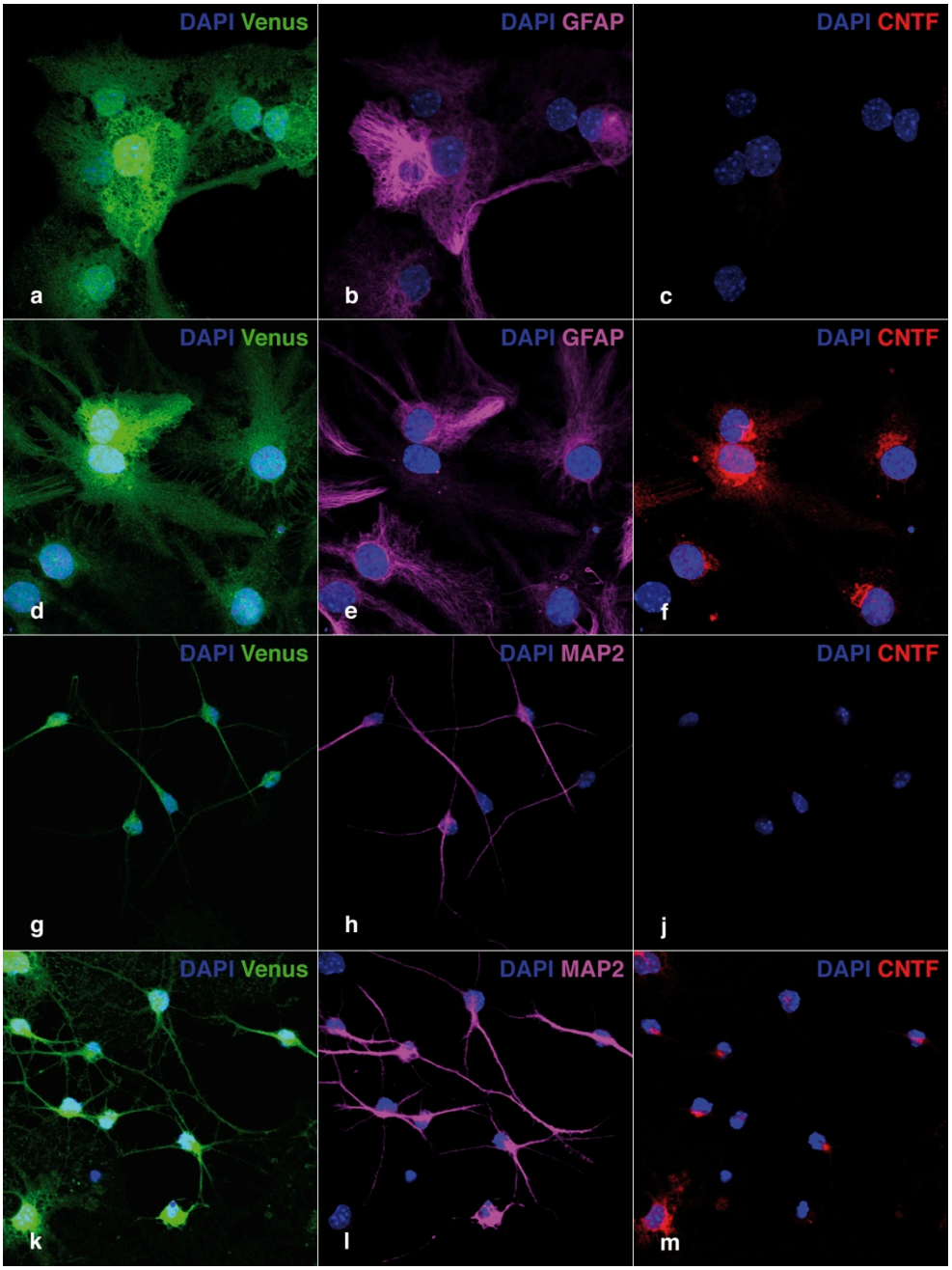

Figure 3: Ectopic expression of CNTF in terminally differentiated neural cell types derived from genetically engineered NS cells. NS cells were transduced with pCAG-CNTF-IRES-Venus-2A-ZEO (d-f, j-l) or the control vector pCAG-IRES-Venus-2A-ZEO (a-c, g-i), positive cells were selected with zeocin, and cultures were differentiated into either astrocytes (a-f) or neurons (g-m). Note that essentially all GFAP-positive astrocytes (d-f) and MAP2-positive neurons (j-l) derived from CNTF-NS cells co-expressed the reporter gene Venus and CNTF. Astrocytes (a-c) and neurons (g-i) derived from control-NS cells, in comparison, expressed Venus but no detectable levels of CNTF. All cultures were counterstained with DAPI to visualize cell nuclei (a-l). DAPI: 4',6-diamidino-2-phenylindole; GFAP: glial fibrillary acidic protein; MAP2: microtubule-associated protein 2. Bar in (l) (for a-l): 20µm.

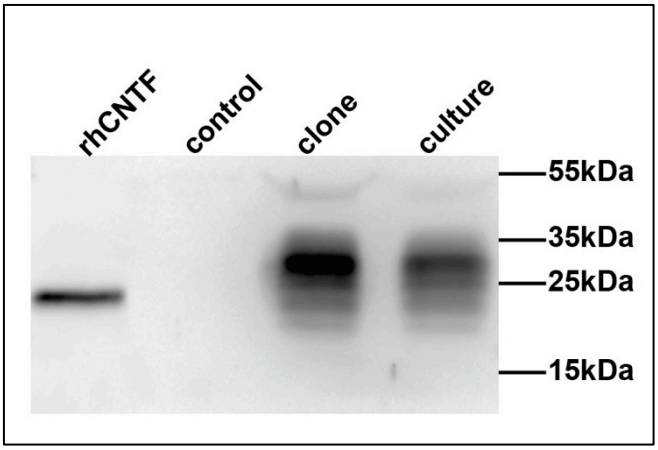

Figure 4: Immunoblot analysis of culture supernatants from CNTF-NS and control-NS cell cultures. CNTF was detected in the culture medium of CNTF-NS cell bulk cultures (bulk) and CNTF-NS cell clones (clone), but not in control-NS cell cultures (control). Recombinant human CNTF (rhCNTF) was loaded as a control.

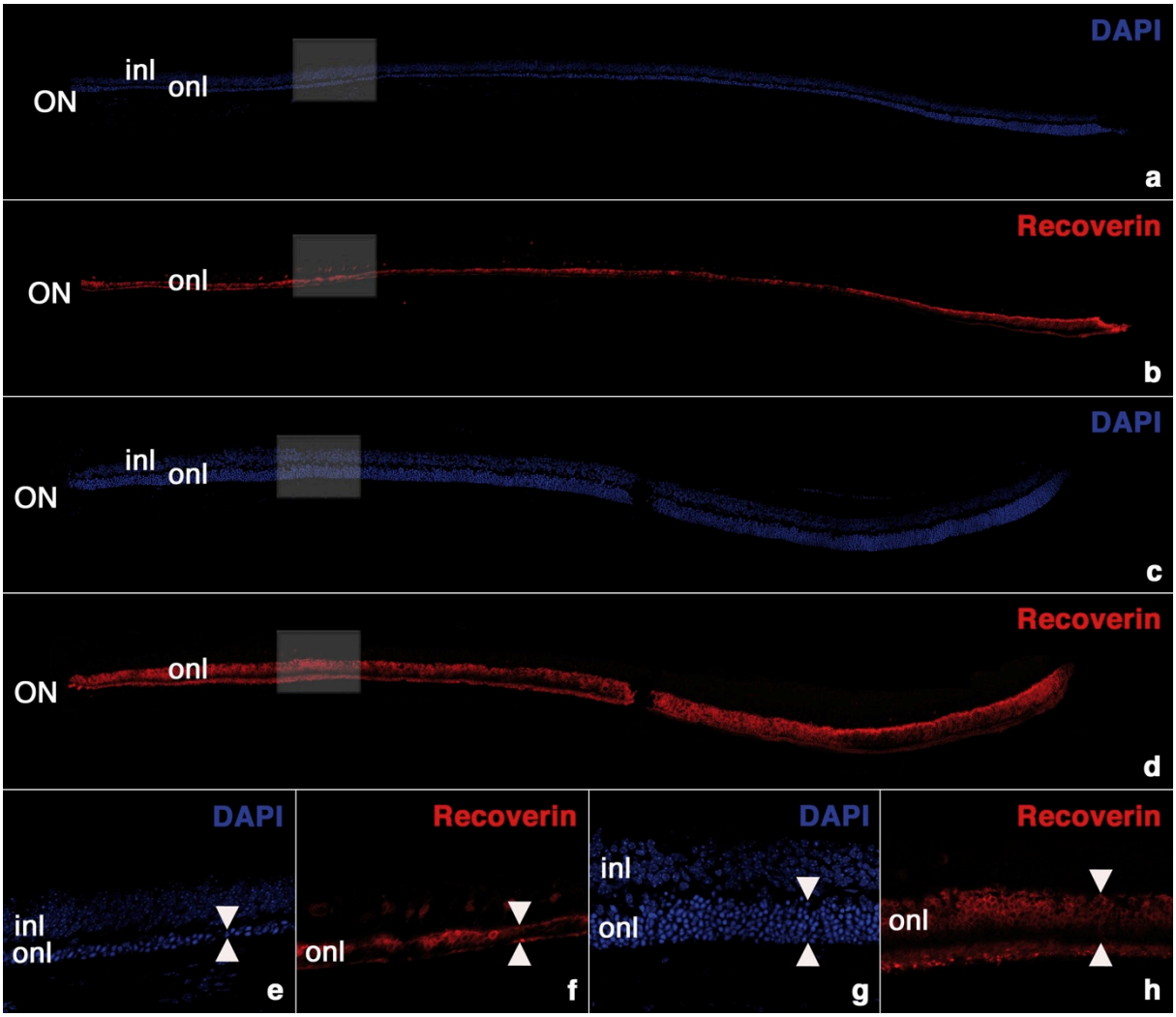

Figure 5: Rescue of photoreceptor cells in Pde6b^{rd10} mice by intravitreally grafted CNTF-NS cells. Control-NS cell bulk cultures were grafted into one eye (a, b, e, f) and CNTF-NS cell bulk cultures into the contralateral eye (c, d, g, h) of 14 days old Pde6b^{rd10} mice, and animals were analysed 16 days later. Stainings of central retinal sections with DAPI or anti-recoverin antibodies revealed a significantly increased thickness of the outer nuclear layer (onl) in eyes that received CNTF-NS cells (c, d, arrowheads in g, h) when compared to eyes that received control-NS cells (a, b, arrowheads in e, f). Note that the increased thickness of the onl is evident along the entire length of the section (c, d). Obvious adverse effects of the grafted cells on the general morphology of the host retinas are not detectable (a-h). (e, f) and (g, h) are higher magnifications of the boxed areas in (a, b) and (c, d), respectively. DAPI: 4',6-diamidino-2-phenylindole; inl: inner nuclear layer; ON: optic nerve. Bar in (d) for (a-d): 100µm; in (h) for (e-h): 50µm.

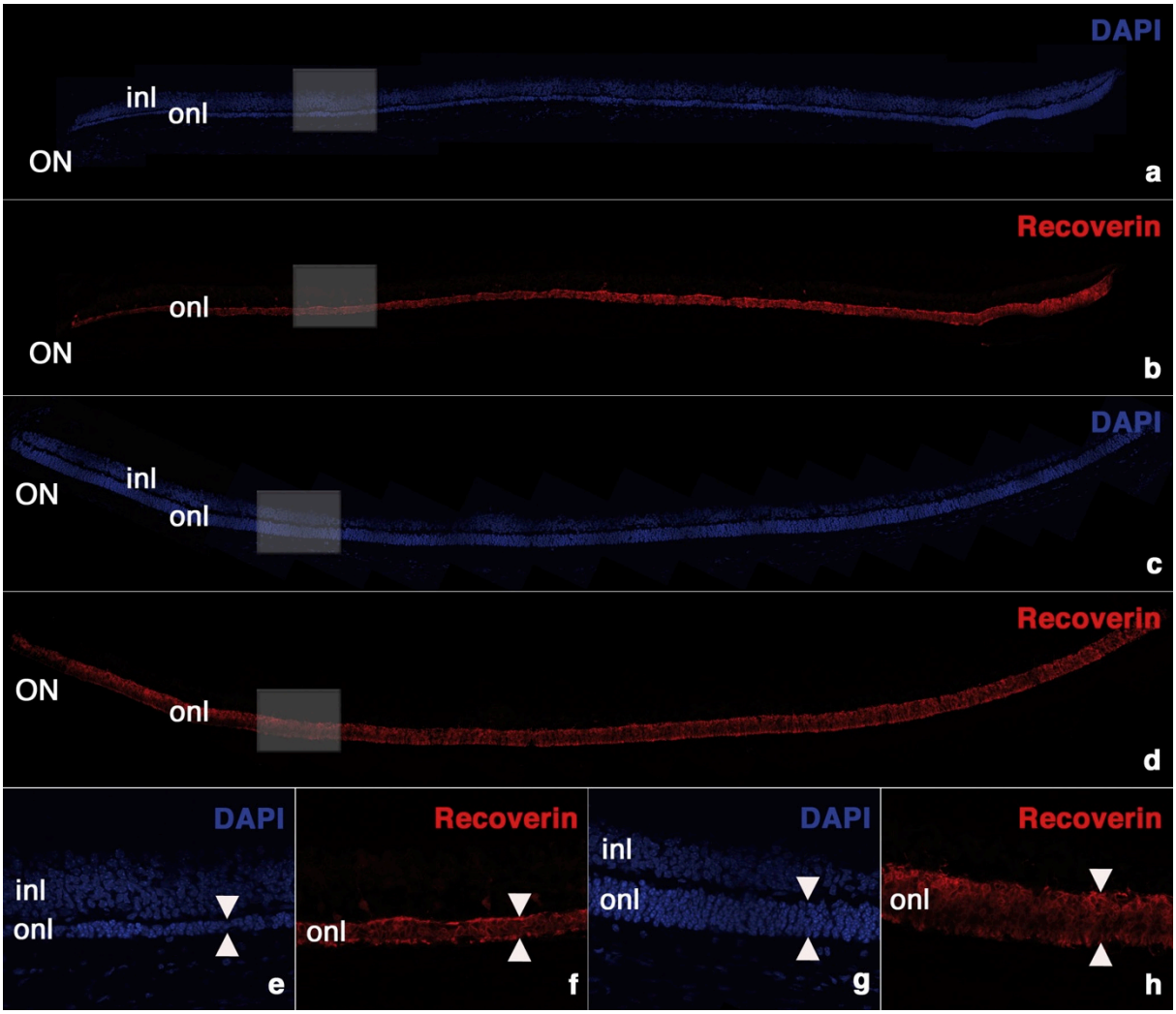

Figure 6: Intravitreally transplanted CNTF-NS cell cultures attenuate photoreceptor degeneration in Pde6b^{rd1} mice. Intravitreal transplantations of control-NS (a, b, e, f) and CNTF-NS cell bulk cultures (c, d, g, h) were performed on 7 days old animals, and mice were analysed at postnatal day 15. Note the increased thickness of the outer nuclear layer (onl) in eyes with grafted CNTF-NS cells (c, d, arrowheads in g, h) when compared to contralateral eyes with grafted control-NS cells (a, b, arrowheads in e, f). Note also that the neuroprotective effect is evident over the entire length of the nasal retina (c, d). Adverse effects of the transplanted cells on the general morphology of the recipient retinas are not detectable (a-d). The boxed areas in (a, b) and (c, d) are shown at higher magnification in (e, f) and (g, h), respectively. DAPI: 4',6-diamidino-2-phenylindole; inl: inner nuclear layer; ON,:optic nerve. Bar in (d) for (a-d): 100µm; in (h) for (e-h): 50µm.

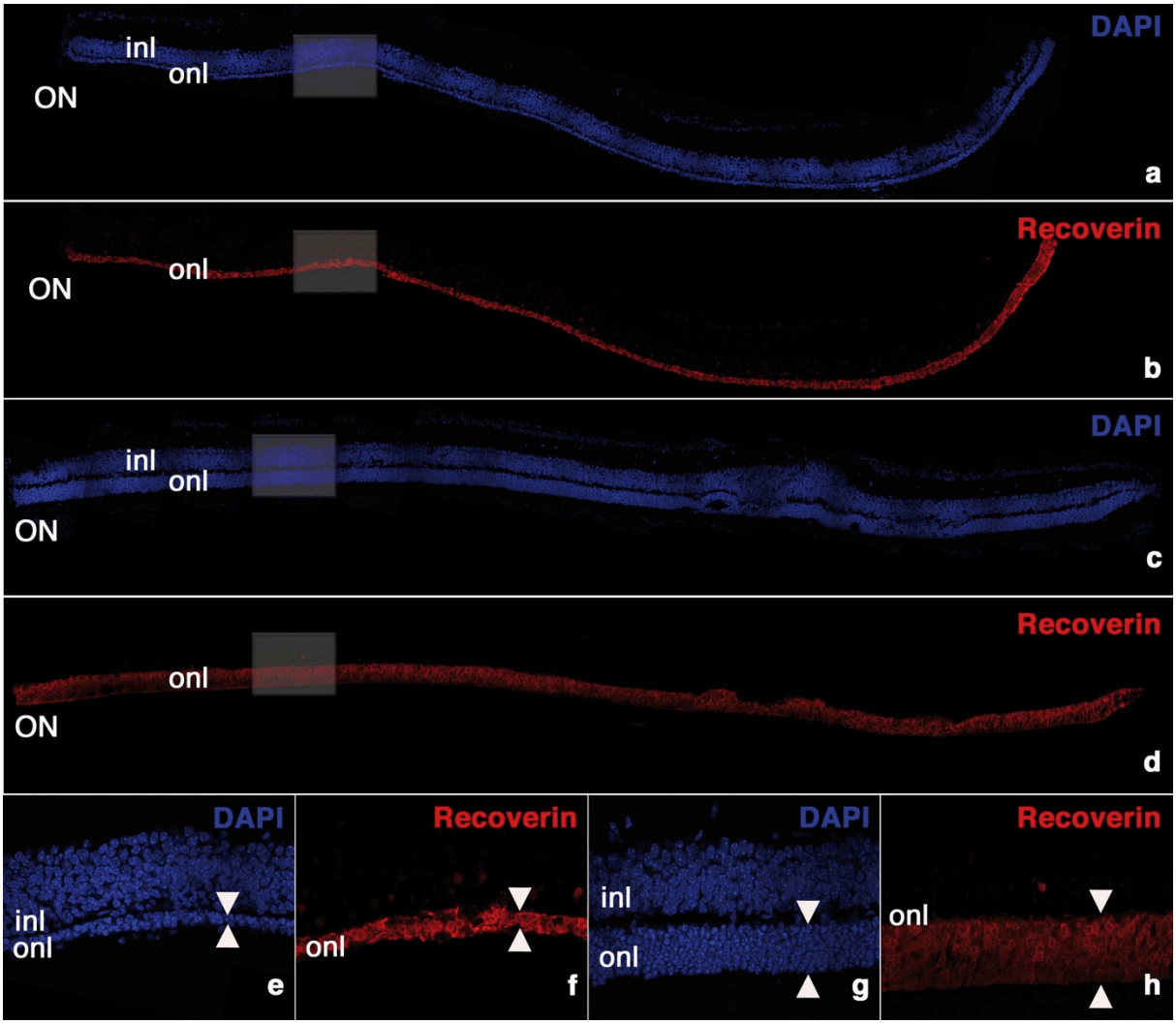

Figure 7: Rescue of photoreceptor cells in Pde6b^{rd1} mice after intravitreal transplantation of a clonally derived CNTF-NS cell line. A control-NS cell clone was intravitreally injected into one eye (a, b, e, f) and a CNTF-NS cell clone into the contralateral eye (c, d, g, h) of 7 days old Pde6b^{rd1} mice. DAPI stainings and recoverin immunohistochemistry was performed on central retina sections of 15 days old animals. Note the significantly increased thickness of the outer nuclear layer (onl) in eyes with a grafted CNTF-NS cell clone (c, d, arrowheads in g, h) when compared to eyes with a grafted control-NS cell clone (a, b, arrowhead in e, f). Note also that the increased thickness of the onl is evident over the entire length of the retina section, and that transplanted cells had no obvious adverse effects on the general morphology of the host retinas. (e, f) and (g, h) are higher magnifications of the boxed areas in (a, b) and (c, d), respectively. DAPI: 4',6-diamidino-2-phenylindole; inl: inner nuclear layer; ON: optic nerve. Bar in (d) for (a-d): 100µm; in (h) for (e-h): 50µm.

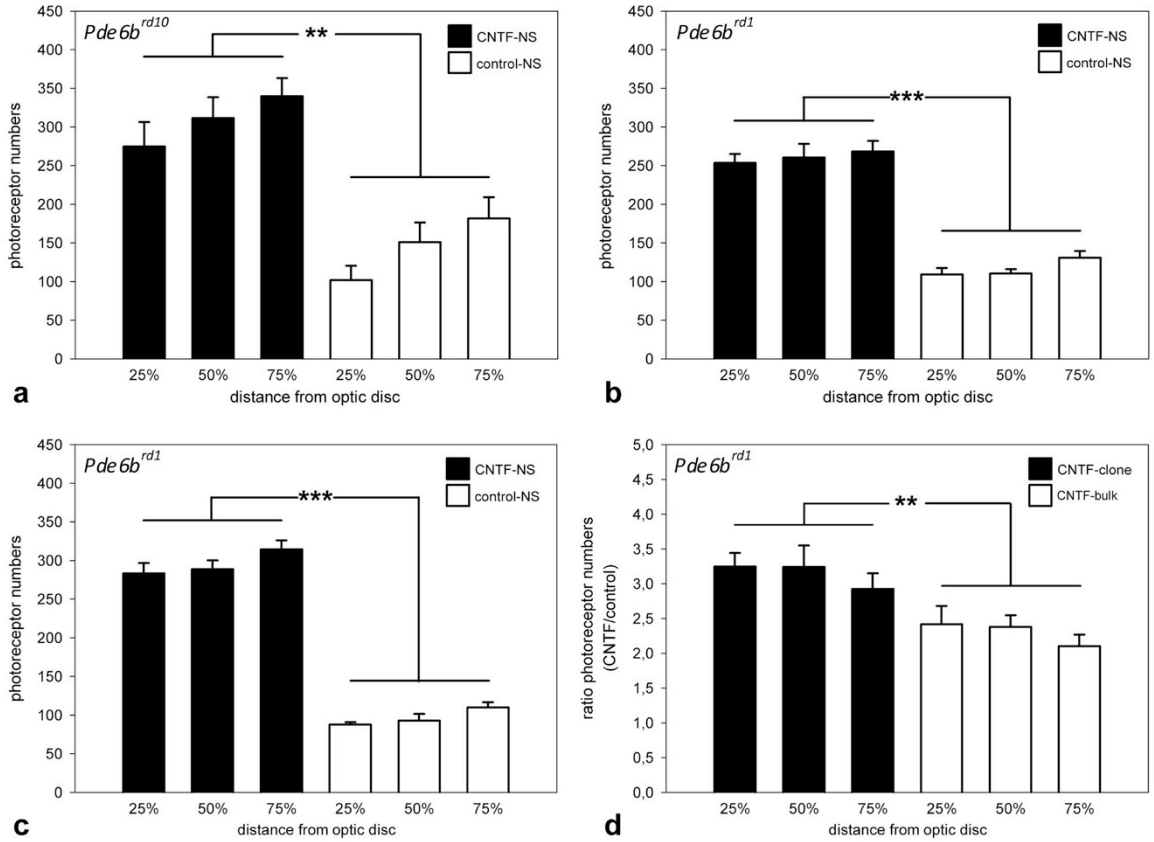

Figure 8: Quantitative analysis of the neuroprotective effect of intravitreally grafted CNTF-NS cells on photoreceptor cells in Pde6b^{rd10} and Pde6b^{rd1} mice. Numbers of photoreceptor cells in Pde6b^{rd10} (a) and Pde6b^{rd1} (b, c) mice were significantly increased after intravitreal transplantations of CNTF-NS cell bulk cultures (a, b) or clonally derived CNTF-NS cell lines (c) when compared to intravitreal transplantations of the corresponding control cultures. In addition, CNTF-NS clonal cell lines selected for high levels of CNTF expression had a significantly more pronounced neuroprotective effect on photoreceptor cells in Pde6b^{rd1} mice than the original CNTF-NS cell bulk culture (d; bars in (d) indicate the quotient of photoreceptor numbers in CNTF-NS cell treated versus control-NS cell treated eyes). Photoreceptor cells in each animal were counted in three areas of the nasal retina at defined distances from the optic disc corresponding to 25%, 50% and 75% of the entire length of the nasal retina. Each bar in (a-d) represents the mean value ±SEM from six retinas. **, $p<0.01$; ***, $p<0.001$ according to the two-way ANOVA test.

3. Project: 'Non-neuronal control of the differential distribution of myelin along retinal ganglion cell axons in the mouse'

ABSTRACT

In most mammalian species, oligodendrocytes and myelin are differentially distributed along retinal ganglion cell (RGC) axons. In mice, for instance, almost all RGC axons are myelinated in the optic nerve, whereas the same axons remain non-myelinated in the retinal nerve fiber layer and the most proximal (i.e. retina-near) portion of the optic nerve. However, when myelinogenic cells are grafted into the retina of mice or rats, they effectively myelinate the intraretinal segments of RGC axons. It was therefore concluded that the differential distribution of myelin along RGC axons is controlled by non-neuronal factors that are located at the retinal end of the optic nerve and prevent migration of oligodendrocyte progenitor cells (OPCs) into the retina. Alternatively, RGC axon segments within the most proximal region of the optic nerve might be refractory to myelination, thereby restricting oligodendrocytes and myelin to the distal portion of the nerve. To experimentally address this possibility, we enriched neurosphere cultures in OPCs by short-term cultivation in a medium supplemented with platelet-derived growth factor (PDGF). Intraretinal transplantations of small-sized PDGF-treated neurospheres close to the optic disc resulted in massive integration of grafted cells into host retinas, and extensive myelination of the nerve fiber layer. Importantly, RGC axon segments in the normally non-myelinated intrabulbar and most proximal retrobulbar portion of the optic nerve were also myelinated in a fraction of animals, unequivocally demonstrating that the entire length of RGC axons is competent to become myelinated. These results further support the view that the differential distribution of myelin in the primary visual pathway is controlled by non-neuronal factors that are located at the retinal end of the optic nerve where they prevent OPCs from entering the most proximal region of the optic nerve and the retina.

INTRODUCTION

During development of the central nervous system, oligodendrocyte progenitor cells (OPCs) migrate into the optic nerve from the chiasmal end towards the retinal end (Small et al., 1987; Fulton et al., 1992; Pringle et al., 1992). Shortly before the optic nerve enters the retina, OPCs stop migrating and differentiate into myelinating oligodendrocytes (Bartsch et al., 1994). As a result, the retina of most mammalian species, including rats and mice, is devoid of oligodendrocytes, and the retinal nerve fiber layer thus remains non-myelinated (Hildebrand et al., 1985; Ffrench-Constant et al., 1988; Bartsch et al., 1989; Perry and Lund, 1990).

Abnormal myelination of the retinal nerve fiber layer has occasionally been observed in various mammalian species that normally lack intraretinal myelination, including the mouse, rat, guinea pig, cat, rhesus monkey (Büssow, 1978; Jung et al., 1978; Bellhorn et al., 1979; May, 2009) and human (Straatsma et al., 1981; FitzGibbon and Nestorovski, 1997; Hunter et al., 1997). Furthermore, intraretinal myelination of the nerve fiber layer by Schwann cells was frequently seen in the Browman-Wyse rat, an inbred strain selected for microphthalmos and displaying a variety of ocular abnormalities (Wyse, 1980; Berry et al., 1989). Myelination of the nerve fiber layer by infiltrating Schwann cells also occured in normal rats after lesioning the retina via the sclera and choroid (Perry and Hayes 1985). Finally, transplantation studies have reported extensive intraretinal myelination after intraocular injections of Schwann cells, oligodendrocyte lineage cells or neural progenitor cells into developing or adult mice or rats (Huang 1991; Laeng et al., 1996; Ader 2000; Pressmar et al., 2001; Setzu et al., 2004; Woodhoo et al., 2007; Gibney and McDermott 2009). Based on all these results, it has been concluded that non-neuronal factors at the retinal end of the optic nerve, rather than the retinal ganglion cells (RGCs) themselves, control the differential distribution of oligodendrocytes and myelin in the primary visual pathway by preventing OPCs from entering the retina (Ffrench-Constant et al., 1988; Perry and Lund, 1990; Bartsch et al., 1994; Laeng et al., 1996). A well-developed lamina cribrosa (Berliner, 1931) or a dense astrocytic network at the retinal end of the optic nerve (Morcos, 2000) are among the non-neuronal factors that have been suggested to act as a barrier for migrating OPCs. Furthermore, the extracellular matrix glycoprotein tenascin-C and netrin-1 have been implicated in preventing migration of OPCs into the retina at the molecular level. Tenascin-C is expressed at elevated levels by astrocytes in the most proximal portion of the optic nerve and has been demonstrated to inhibit OPC adhesion and migration in

vitro (Bartsch et al., 1994; Kiernan et al., 1996). Netrin-1 is also expressed at the retinal end of the optic nerve and has been shown to act as a chemorepellent for migrating OPCs (Sugimoto et al., 2001; Spassky et al., 2002; Tsai et al., 2003). However, direct in vivo evidence for a critical role of any of these factors in restricting oligodendrocytes and myelin to the distal portions of the optic nerve has not been provided.

While the available data are in line with the view that non-neuronal factors in the most proximal portion of the optic nerve prevent migration of OPCs into the retina and thus intraretinal myelination, they do not exclude a neuronal control of the differential distribution of oligodendrocytes and myelin along RGC axons. In fact, axon segments of RGCs within the intrabulbar and most proximal retrobulbar portion of the optic nerve might be refractory to myelination, thereby restricting oligodendrocytes and myelin to the distal part of the nerve. To experimentally address this possibility, we have established neurosphere cultures from the cerebral cortex of EGFP-transgenic mice, and have enriched these cultures in oligodendrocyte lineage cells by short-term cultivation in a medium supplemented with platelet-derived growth factor (PDGF). Small-sized PDGF-treated spheres were grafted into the retina of young postnatal mice close to the optic disc, and the retina and the most proximal portion of the optic nerve were analysed for the presence of donor-derived myelin. We found wide-spread myelination of the retinal nerve fiber layer in virtually all experimental retinas. Of note, we additionally observed in a fraction of animals that the donor-derived myelin in the retina was in direct continuity with the endogenous myelin in the optic nerve.

MATERIAL AND METHODS

Animals

Neural precursor cells were isolated from the cerebral cortex of 14 days old transgenic mouse embryos ubiquitously expressing enhanced green fluorescent protein (EGFP) under control of a CMV early enhancer/chicken β-actin promoter (Okabe et al., 1997). The genotype of embryos was determined by analysing tail biopsies for the presence of EGFP fluorescence using an Olympus IX51 microscope (Olympus, Hamburg, Germany). Intraretinal transplantation experiments were performed on three to seven days old C57BL/6J wild-type mice. Six weeks old C57BL/6J mice were used for immunohistochemical analysis of untreated retinas and optic nerves. All animal experiments were approved by the local ethics committee and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Every effort was made to minimize the number of animals used and their suffering.

Neurosphere cultures and oligodendrocytic differentiation

Cerebral cortices were quickly removed from EGFP-transgenic mouse embryos and carefully freed from the meninges. Subsequently, tissue was incubated in Accutase (PAA Laboratories, Coelbe, Germany) for 10 min at 37°C, and then dissociated into single cells by tituration with a fire-polished Pasteur pipette. Cells were plated into uncoated tissue culture flasks at a density of 200,000 cells/ml in a medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 (DMEM/F12; Life Technologies, Darmstadt, Germany) containing 2mM glutamine, 5mM HEPES, 3mM sodium bicarbonate, 0.3% glucose, (all from Sigma, St. Louis, MO), 1% N2 and 1% B27 (both from Life Technologies), and 20ng/ml epidermal growth factor (EGF) and 20ng/ml fibroblast growth factor-2 (FGF-2; both from TEBU, Offenbach, Germany). After approximately seven days of culture, cells had grown to free-floating neurospheres. For further expansion, neurosphere cultures were passaged at least once a week and further cultivated in the same medium but lacking B27 (”expansion medium”). After three or more passages, neurospheres were dissociated and cells were transferred to a medium lacking EGF and containing 10ng/ml FGF-2, 10ng/ml platelet-derived growth factor (PDGF; R&D Systems, Minneapolis, MN) and 10μM forskolin (Sigma; “priming medium”) to increase the number of oligodendrocyte progenitor cells in the cultures (in the following termed “PDGF-neurospheres”). After a culture period of four days in priming medium, PDGF-neurospheres consisted of small cellular aggregates that were either

floating in the medium or attached to the tissue culture plastic. Spheres were collected and used for intraretinal transplantation experiments (see below) or in vitro differentiation experiments.

For in vitro differentiation experiments, dissociated neurosphere cells were cultivated for four days in either expansion medium to derive neurospheres, or in priming medium to derive PDGF-neurospheres. Subsequently, neurospheres and PDGF-neurospheres were plated onto coverslips coated with poly-D-lysine (Sigma) and 1% Matrigel (Becton Dickinson, Heidelberg, Germany), and differentiation of cells was induced by cultivation for three days in a medium consisting of DMEM/F12 supplemented with 2mM glutamine, 5mM HEPES, 3mM sodium bicarbonate, 0.3% glucose, 1% N2, and 30ng/ml 3,3´,5-triiodothyronine (T3) and 200µM ascorbic acid (both from Sigma).

Immunocytochemistry and oligodendrocyte quantification in vitro

To compare the numbers of oligodendrocytes in differentiated neurosphere and PDGF-neurosphere cultures, cells were fixed in phosphate-buffered saline (PBS; pH 7.3) containing 4% paraformaldehyde (PA) for 10 min, and then immersed in PBS containing 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 (both from Sigma) for 30 min. Subsequently, cultures were incubated with rat monoclonal antibodies to myelin basic protein (MBP; Millipore, Billerica, MA) to visualize oligodendrocytes. Oligodendrocytes were additionally identified by incubating live cultures with mouse monoclonal O4 or O1 antibodies (Sommer and Schachner 1981) for 45 min at 4°C, followed by fixation in 4% PA. After several washes with PBS, cultures were incubated with Cy3-conjugated secondary anti-rat or anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA) for 4 hrs. Finally, cultures were stained with 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma) for 10 min to visualize cell nuclei, washed again, and mounted onto slides. For each coverslip, photomicrographs of 5 randomly selected areas were taken using a fluorescence microscope, and the percentage of O4-, O1- or MBP-positive cells from the total number of cells in these areas was calculated. Between 510 and 752 cells from at least 3 independent experiments were analysed for each culture condition and antigen. Statistical analysis of data was performed with the independent t-sample test using SPSS 12.0 software (SPSS Inc., Chicago, IL).

Intraretinal transplantation experiments

After a culture period of four days in priming medium, PDGF-neurospheres were collected to prepare a dense suspension of small-sized spheres in PBS. Three to seven days old C57BL/6J mice were deeply anaesthetized using isoflurane (Abbott, Wiesbaden, Germany). A glass micropipette was inserted into the vitreous space at the junction between sclera and cornea under visual control using a surgical microscope, and 1µl of vitreous fluid was slowly removed from the eye. Subsequently, the same volume of a dense suspension of PDGF-neurospheres was injected into the vitreous. To increase integration of grafted cells into host retinas, retinas were gently lesioned close to the optic disc at the time of the injections using the tip of the micropipette (Laeng et al., 1996; Ader et al., 2000; Pressmar et al., 2001). Mice were allowed to survive for at least one month before eyes with attached optic nerves were subjected to immunohistochemical analysis.

Immunohistochemistry

Eyes with attached optic nerves were quickly removed, immersion-fixed in PBS containing 4% PA, dehydrated in an ascending series of sucrose, embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, Netherlands) and frozen. Longitudinal cryostat sections from optic nerves with attached retinas, 25µm in thickness, were incubated in 0.1% BSA and 0.3%Triton X-100 for 1 h, followed by incubation with primary antibodies overnight at 4°C. The following primary antibodies were used: rabbit polyclonal antibodies to glial fibrillary acidic protein (GFAP; DAKO, Glostrup, Denmark), rabbit polyclonal antibodies to β-tubulin III (Sigma), rat monoclonal antibodies to MBP, and goat polyclonal antibodies to myelin-associated glycoprotein (MAG; R&D Systems). Some sections were additionally incubated with polyclonal chicken anti-EGFP antibodies (Millipore) to increase the EGFP fluorescence of the grafted cells. Primary antibodies were detected using the appropriate Cy2-, Cy3- or Cy5-conjugated secondary antibodies. Finally, sections were immersed in DAPI for 10 min at room temperature to stain cell nuclei, washed with PBS and mounted onto slides. Some retinas with grafted cells were flat-mounted, fixed in PBS containing 4% PA, and incubated with goat polyclonal anti-MAG antibodies followed by Cy3-conjugated secondary anti-goat antibodies and DAPI. Tissue sections and flat-mounted retinas were analysed with a confocal fluorescence microscope (Fluoview FV1000, Olympus).

RESULTS

Oligodendrocytic differentiation of neurosphere cultures

In previous studies, we have expanded neurosphere cultures in the presence of the mitogens EGF and FGF-2. When these cells were intraretinally grafted into young postnatal or adult mice, they integrated into the inner retinal layers where a fraction of donor cells differentiated into oligodendrocytes that myelinated the normally non-myelinated retinal nerve fiber layer (see e.g.:Ader et al., 2000; Pressmar et al., 2001; Stolt et al., 2002; Schmucker et al., 2003). In the present study, cells were maintained for four days in a medium containing FGF-2 and PDGF, a mitogen for oligodendrocyte progenitor cells (Noble et al., 1988; Richardson et al., 1988), to further increase the myelinogenic potential of these cultures. After a culture period of four days in expansion medium or priming medium, cells had formed small-sized neurospheres or PDGF-neurospheres, respectively. To analyse the effect of a short-term exposure to PDGF on the number of oligodendrocytes, neurospheres and PDGF-neurospheres were differentiated in a medium containing T3 and forskolin, and the percentage of O4-, O1- and MBP-positive oligodendrocytes from the total cell population was calculated (Fig. 1, 2). While PDGF-neurosphere cultures contained 19.3% ±1.1% (mean ±SEM) O4-positive cells, neurosphere cultures contained 7.7% ±1.1% O4-positive cells. This difference between PDGF-neurospheres and neurospheres was statistically significant ($p<0.001$). PDGF-neurosphere cultures also contained significantly more O1-positive oligodendrocytes (11.6% ±1.3%) when compared to neurosphere cultures (7.7% ±0.3%; $p<0.05$). The percentage of MBP-positive oligodendrocytes was also significantly increased in PDGF-neurosphere cultures (8.4% ±0.5%) when compared to neurosphere cultures (3.7% ±1.2%; $p<0.05$). Thus, a short time exposure to PDGF significantly increased the number of oligodendrocytes in neurosphere cultures (Fig. 1, 2), and all intraretinal transplantation experiments were therefore performed with PDGF-neurospheres (see below).

Integration and oligodendrocytic differentiation of intraretinally grafted PDGF-neurosphere cells

In adult mice, fascicles of retinal ganglion cell axons extend through the retinal nerve fiber layer towards the optic disc where they leave the retina to form the optic nerve (Fig. 3). While GFAP-positive astrocytes are associated with RGC axons along their entire length, MBP-positive myelin is restricted to the distal part of the optic nerve, but

is absent from the retinal nerve fiber layer, the intrabulbar portion and the most retina-near retrobulbar portion of the optic nerve (Fig. 3; Bartsch et al., 1989; (May and Lutjen-Drecoll, 2002)). To test the myelinogenic potential of PDGF-neurospheres in vivo, neurospheres were dissociated into single cell suspensions and intraretinally grafted into young postnatal mice. Analysis of animals one month after transplantation revealed that only a limited number of EGFP-positive donor cells in only a fraction of animals had integrated into the host retinas. Furthermore, evidence for the presence of oligodendrocytes and myelinated intraretinal RGC axon segments was found in only some experimental animals (data not shown). However, when small-sized PDGF-neurospheres instead of single cell suspensions were grafted, numerous EGFP-positive donor cells were present in virtually all host retinas (Fig. 4-6). Almost all EGFP-positive donor cells were located in the inner retina (i.e. inner plexiform layer, ganglion cell layer and nerve fiber layer), where they had developed a complex morphology with processes extending mainly through the inner plexiform layer (Fig. 4-6). Of note, EGFP-fluorescence was also associated with fascicles of RGC axons in the nerve fiber layer (Fig. 4).

Immunohistochemical analysis of retina sections (Figs. 4a, b; 5, 6) and flat-mounted retinas (Fig. 4c-e) revealed strong MBP- or MAG-immunoreactivity in the nerve fiber layer of the vast majority of animals with grafted PDGF-neurospheres. Here, both MBP- and MAG-immunoreactivity were found in close association with axon fascicles of RGCs (Fig. 4-6). Double-immunostainings with antibodies to β-tubulin III confirmed the association of both myelin-specific markers with fascicles of RGC axons (Fig. 5a-c and data not shown), and strongly suggested extensive myelination of RGC axons by donor-derived oligodendrocytes. Electron microscopic analysis of host retinas indeed revealed the presence of many RGC axons that were surrounded by ultrastructurally intact central nervous system myelin sheaths (data not shown, but see: Ader et al., 2000; Schmucker et al., 2003).

Expression of MAG was additionally detected in the cell bodies of a large number of EGFP-transgenic cells (Fig. 4c-e; Fig. 5a-c), demonstrating oligodendrocytic differentiation of a significant fraction of grafted cells. All MAG-positive donor-derived oligodendrocytes extended processes exclusively towards myelinated RGC axons (Fig. 4c-e), even when their cell bodies were located in the inner plexiform layer at some distance from the nerve fiber layer (Fig. 5a-c). Not all EGFP-positive cells were either positive for MAG, suggesting differentiation of grafted cells into neural cell types others

then oligodendrocytes. Immunohistochemical analysis of host retinas with cell type-specific antibodies revealed the presence of numerous donor-derived GFAP-positive astrocytes in the inner retina (Fig. 4d-f). Evidence for differentiation of grafted PDGF-neurosphere cells into neurons was, however, not observed.

In 90 of the 139 eyes (64.7%) with a myelinated nerve fiber layer, we found myelinated RGC axons in close neighborhood to the optic disc. To analyse these eyes for the presence of donor-derived myelin in the normally non-myelinated intrabulbar and most proximal retrobulbar portions of the optic nerve, we stained longitudinal sections of optic nerves with attached retinas with anti-MBP or anti-MAG antibodies. The presence of myelinated RGC axons at the optic disc correlated with the presence of numerous EGFP-positive donor cells in all retinas analysed (Fig. 6). In some eyes, RGC axons were myelinated in the nerve fiber layer and the normally non-myelinated intrabulbar portion of the optic nerve, but not in the most proximal retrobulbar portion of the optic nerve, resulting in a myelin-free gap between donor-derived myelin in the retina and endogenous myelin in the optic nerve (Fig. 6a, b). In other eyes, myelinated RGC axons were also found in the normally non-myelinated retrobulbar region of the nerve, with the donor-derived myelin being separated by a narrow myelin-free gap from the endogenous myelin in the optic nerve (Fig. 6c, d). Finally and most interestingly, we found in totally 30 eyes (33.3%) that the donor-derived myelin in the retinal nerve fiber layer was in direct continuity with endogenous myelin in the optic nerve (Fig. 6e-h). In 19 of these eyes, only a few myelinated RGC axons extended from the retinal nerve fiber layer towards the endogenous myelin of the optic nerve. However, in 11 eyes both the intrabulbar and most retrobulbar portion of the nerve was massively myelinated, making it impossible to define the transition zone between donor-derived and endogenous myelin (Fig. 6g, h). These observations demonstrate that RGC axons within the primary visual pathway of the mouse, at least at these early developmental ages, are competent to become myelinated along their entire length.

DISCUSSION

Oligodendrocyte progenitor cells are highly motile cells that migrate over long distances in the developing brain to colonize prospective white matter tracts where they ultimately differentiate into mature myelin-forming oligodendrocytes (Baumann and Pham-Dinh, 2001; Miller, 2002; Richardson et al., 2006). In the optic nerve of rodents, for example, oligodendrocyte progenitors originate in the floor of the third ventricle from where they populate the nerve from its chiasmal end during the first postnatal week (Small et al., 1987; Pringle et al., 1992; Bartsch et al., 1994; Garcion et al., 2001). At the retinal end of the nerve, however, oligodendrocyte progenitor cells stop migrating and differentiate into myelinating oligodendrocytes. In fact, oligodendrocytes are normally not detectable in freshly isolated retinal cultures from rats or mice, even under culture conditions that have been optimized for oligodendrocyte lineage cells (ffrench-Constant et al., 1988; Gao et al., 2006; Czekaj et al., 2012). As a result, the proximal segments of RGC axons within the retina, and the intrabulbar portion and most proximal retrobulbar portion of the optic nerve remain non-myelinated throughout life (ffrench-Constant, 1988; Bartsch et al., 1989; Perry and Lund 1990; Bartsch et al., 1994; Morcos and Chan-Ling, 2000; May and Lutjen-Drecoll, 2002).

Various investigations demonstrated that the normally non-myelinated retinal nerve fiber layer is competent to become myelinated. For instance, myelination of the nerve fiber layer by Schwann cells has sporadically been observed in various species that normally lack intraretinal myelin (Büssow, 1978; Jung et al., 1978; Bellhorn et al., 1979; May, 2009), has been frequently seen in the Browman-Wyse rat strain (Wyse, 1980; Berry et al., 1989), and has occured in response to retinal lesions via sclera and choroid in normal rats (Perry and Hayes, 1985). Intraretinal myelination also occurs in < 1% of human eyes (Straatsma et al., 1981; Kodama et al., 1990). Furthermore, extensive myelination of the nerve fiber layer of mice or rats has been observed after intraocular transplantations of Schwann cells, oligodendrocyte lineage cells and neural progenitor cells (Huang et al., 1991; Laeng et al., 1996; Ader et al., 2000; Setzu et al., 2004; Woodhoo et al., 2007; Gibney and McDermott, 2009), thus providing direct experimental evidence that the intraretinal segments of RGC axons are in principal competent to become myelinated, when myelinogenic cells have access to the nerve fiber layer.

The observations outlined above suggest the presence of a non-neuronal barrier at the retinal end of the optic nerve that prevents oligodendrocyte progenitor cells to populate the most proximal portion of the nerve and the retina. The presence of a lamina cribrosa,

a distinct band of connective tissue formed by collagen fibers of the sclera, is among the factors that have been implicated in preventing intraretinal myelination (Berliner, 1931). In the human optic nerve, myelination of RGC axons stops at the lamina cribrosa. The rabbit, in comparison, lacks a well-developed lamina cribrosa and RGC axons in this species are myelinated also intraretinally (Berliner, 1931; Reichenbach et al., 1988). However, rats and mice also lack a well-developed lamina cribrosa but have a non-myelinated retina. A dense astrocytic network is present at the proximal end of the optic nerve of several species that lack intraretinal myelin, while a similar concentration of astrocytes is absent from the optic nerve of rabbits and chicken, two species with intraretinal myelination (Morcos and Chan-Ling, 2000). In the Browman-Wyse rat, however, the proximal part of the optic nerve remains non-myelinated over an abnormally long distance, and lacks a concentration of astrocytes at the transition zone between the myelinated and non-myelinated portion of the optic nerve (Berry et al., 1989).

To futher study the mechanisms that control the differential distribution of oligodendrocytes and myelin in the primary visual pathway, we extended our previous work (Laeng et al., 1996; Ader et al., 2000), and grafted neurosphere cells into the developing mouse retina close to the optic disc. Prior to transplantation, neurosphere cells were cultivated for a short time period in a medium supplemented with PDGF and FGF-2 to increase the percentage of oligodendrocyte lineage cells (Gibney and McDermott, 2007; Pedraza et al., 2008). Small-sized PDGF-treated neurospheres were then grafted into young postnatal mice close to the optic disc to analyse whether these cells are capable to myelinate the most proximal portion of the optic nerve. Comparing neurospheres expanded in the presence of EGF (Ader et al., 2000) or FGF-2 and EGF (Stolt et al., 2002; Schmucker et al., 2003) to dissociated neurosphere cells that were exposed for a short time period to PDGF and FGF-2 (see “Results”), we observed a markedly increased capability of PDGF-neurospheres to integrate into host retinas and to differentiate into myelinating oligodendrocytes. In fact, we observed intraretinal myelin in more than 80% of the experimental retinas. We suggest that this high myelinogenic potential of grafted PDGF-neurospheres is the result of both, an increased number of oligodendrocyte lineage cells present in these cultures and a better survival of cells when grafted as “minispheres” as opposed to single cells.

Differentiation of a significant fraction of grafted PDGF-neurosphere cells into mature oligodendrocytes was confirmed by the co-localization of EGFP-fluorescence and MAG-

immunoreactivity in their cell bodies and processes. EGFP-fluorescence was also associated with myelinated RGC axons, but decreased to very low levels as a result of myelin compaction. However, intraretinal myelin was never observed after intraretinal injections of the vehicle only, indicating that the myelin was donor-derived. Furthermore, ultrastructural studies exclusively revealed RGC axons that were surrounded by central nervous system myelin sheaths (data not shown), thus excluding the possibility that infiltrating Schwann cells had myelinated the nerve fiber layer.

In totally 90 of the 139 eyes with intraretinal myelin, we found a myelinated nerve fiber layer extending towards the optic disc. Further analysis of the proximal optic nerves revealed the presence of myelinated RGC axons in the intrabulbar portion of the nerves in about 72%, and in the retrobulbar portion of the nerves in about 50% of these eyes. Of note, in 30 eyes myelinated RGC axons extended from the nerve fiber layer throughout the entire length of the intrabulbar and retrobulbar portions of the optic nerve to the endogenous myelin in the distal nerve. In 19 of these eyes, only a few myelinated RGC axons were seen to extend from the retina to the normally myelinated distal region of the nerve. However, in 11 eyes myelination of the entire proximal part of the nerve was extensive, precluding a clear identification of the normal transition zone between the myelinated and non-myelinated portion of the optic nerve. Results thus unequivocally demonstrated that the entire length of retinal ganglion cell axons is competent to become myelinated, at least during these early developmental ages. Our observations thus further support the view that the differential distribution of oligodendrocytes and myelin in the primary visual pathway is controlled by a non-neuronal barrier for migratory oligodendrocyte progenitor cells at the retinal end of the nerve, rather than by the RGCs themselves.

We also attempted to clarify whether or not myelination of the intra- and retrobulbar portions of the optic nerve correlates with the presence of oligodendrocyte cell bodies in these regions of the nerve. Although we found in some rare cases EGFP-positive cell bodies and cell bodies containing proteolipid protein-specific transcripts in the experimentally myelinated proximal region of some nerves (data not shown), such cells were absent from the vast majority of nerves. Given that a few oligodendrocytes are capable to myelinate a significant number or RGC axons, it is possible that we missed these cells in our analysis. In fact, due to the technical problems associated with the histological analysis of the small proximal portion of the optic nerve, we were only rarely able to evaluate a complete series of optic nerve sections from one eye. Therefore,

we cannot exclude the possibility that processes of oligodendrocytes located at the optic disc myelinated the intra- and retrobulbar portions of optic nerves.

One might argue that the grafted oligodendrocyte lineage cells have access to the most proximal portion of the optic nerve before the hypothetical barrier for oligodendrocytes has formed, and are therefore capable to myelinate the entire proximal segment of RGC axons. Myelination of the proximal region of the optic nerve was indeed most frequently observed after intraretinal transplantations of PDGF-neurospheres into three days old animals. However, myelination of the intra- and retrobulbar region of the optic nerve was also found in about 15% of all eyes that received PDGF-neurosphere grafts between postnatal day 5 and 7, developmental ages at which oligodendrocyte progenitor cells in the optic nerve have reached the retinal end of the nerve (Bartsch et al., 1994; Garcion et al., 2001). These observations thus imply that the most retrobulbar region of the optic nerve can be myelinated by oligodendrocytes from the retinal side, but not by oligodendrocytes located in the optic nerve. A barrier with such properties is most easily explained with the presence of one or more factors which are expressed in a graded pattern at the retinal end of the optic nerve where they either allow or prevent myelination of RGC axons, depending on the slope oligodendrocyte lineage cells encounter this gradient.

In summary, we have shown that intraretinal transplantations of PDGF-treated small-sized neurospheres into three to seven days old mice result in extensive myelination of the retinal nerve fiber layer. The normally non-myelinated intrabulbar and most proximal retrobulbar portions of the optic nerve were also myelinated in a fraction of animals. Results thus unequivocally demonstrate that the entire proximal and normally non-myelinated segments of RGC axons are competent to become myelinated. We therefore conclude that RGCs are not involved in the control of the differential distribution of oligodendrocytes and myelin in retina and optic nerve. Instead, results further support the view that non-neuronal factors located at the retinal end of the optic nerve prevent oligodendrocytes from myelinating the most proximal portion of the nerve and the retina.

FIGURES AND FIGURE LEGENDS

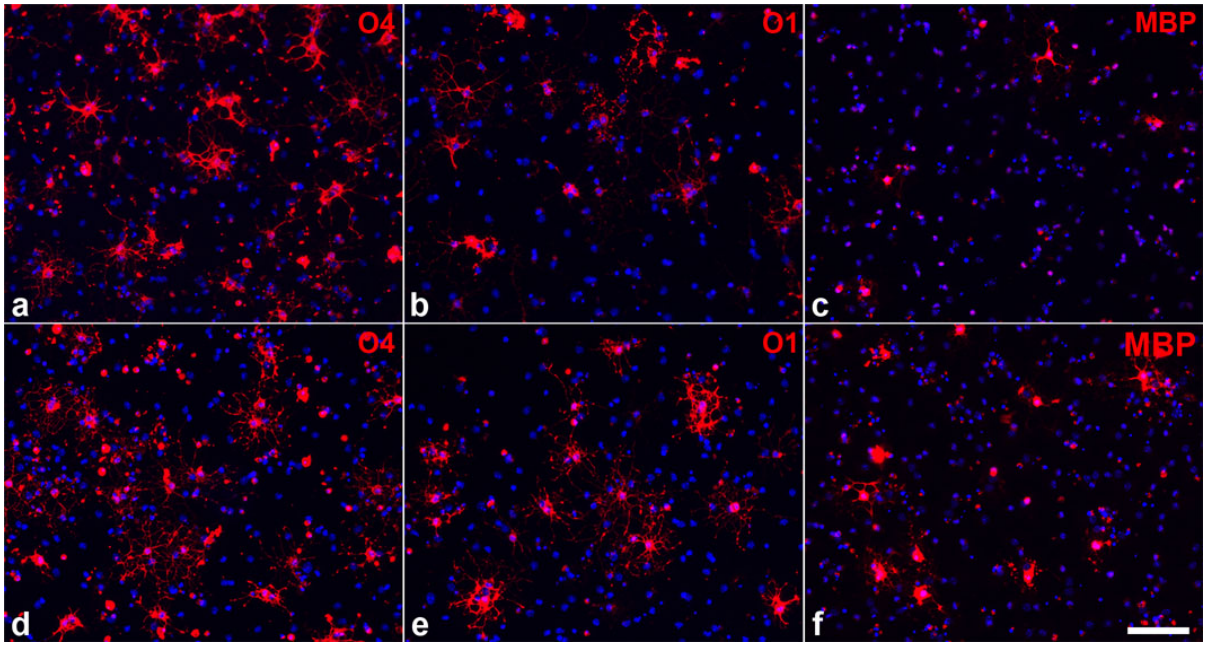

Figure 1: Increased numbers of oligodendrocytes in neurosphere cultures after short-term cultivation in a medium containing PDGF. Neurosphere cultures were maintained for four days in a medium containing either EGF and FGF-2 (a-c) or PDGF and FGF-2 (d-f) as mitogens, induced to differentiate, and analysed for the presence of oligodendrocytes using O4 (a, d), O1 (b, e) or anti-myelin basic protein (MBP) antibodies (c, f). Note the increased number of oligodendrocytes in neurosphere cultures exposed to PDGF and FGF-2 as opposed to cultures exposed to EGF and FGF-2. All cultures were counterstained with DAPI to visualize cell nuclei. EGF: epidermal growth factor; FGF-2: fibroblast growth factor-2; PDGF: platelet-derived growth factor; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride hydrate. Bar in (f) for (a-f): 50µm.

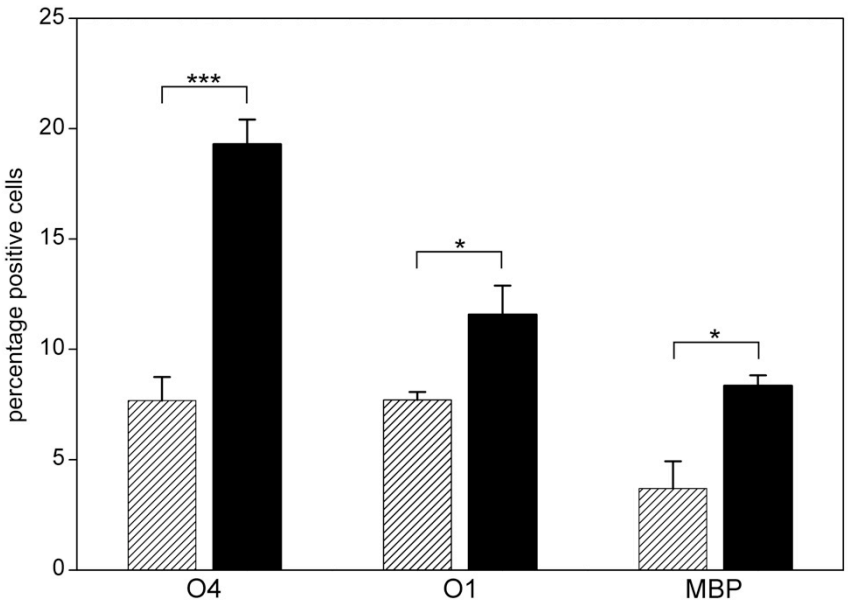

Figure 2: Oligodendrocytic differentiation of neurosphere and PDGF-neurosphere cultures. Neurosphere and PDGF-neurosphere cultures were differentiated for three days, and the percentage of O4-, O1- and MBP-positive cells was determined. Note the significantly higher number of oligodendrocyte lineage cells in differentiated PDGF-neurosphere cultures (filled bars) when compared to differentiated neurosphere cultures (shaded bars). Bars represent the mean values (±SEM) from at least three independent experiments. MBP: myelin basic protein; *, $p<0.05$; ***, $p<0.001$.

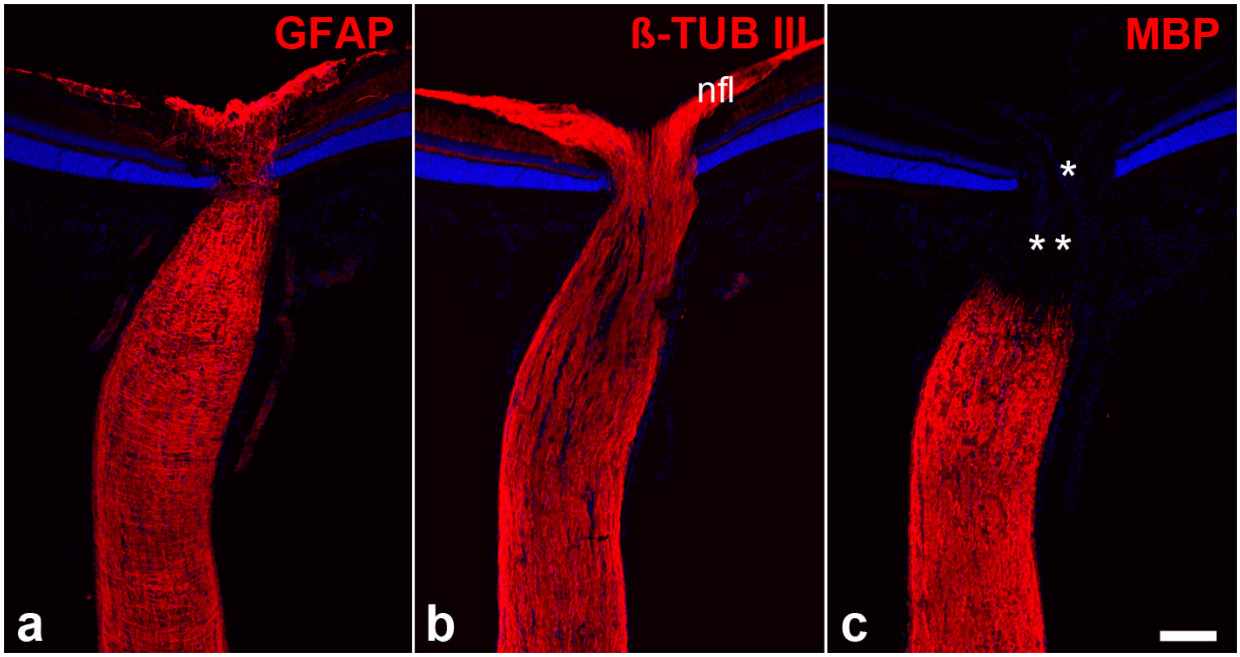

Figure 3: Visualization of astrocytes, retinal ganglion cell axons and myelin in the retina and optic nerve of the adult mouse. Consecutive longitudinal sections of an adult mouse optic nerve with attached retina were stained with anti-glial fibrillary acidic protein (GFAP; a), anti-β-tubulin III (β-TUB III; b) and anti-myelin basic protein (MBP; c) antibodies. GFAP-positive astrocytes are associated with β-TUB III-positive retinal ganglion cell axons throughout the optic nerve and in the retinal nerve fiber layer (compare a and b). MBP-positive myelin, in contrast, is restricted to distal regions of the optic nerve but is absent from the nerve fiber layer (nfl in b) and the intrabulbar (one asterisk in c) and retrobulbar retina-near portion (two asterisks in c) of the optic nerve. All sections were counterstained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride hydrate) to visualize cell nuclei. Bar in (c) for (a-c): 100µm.

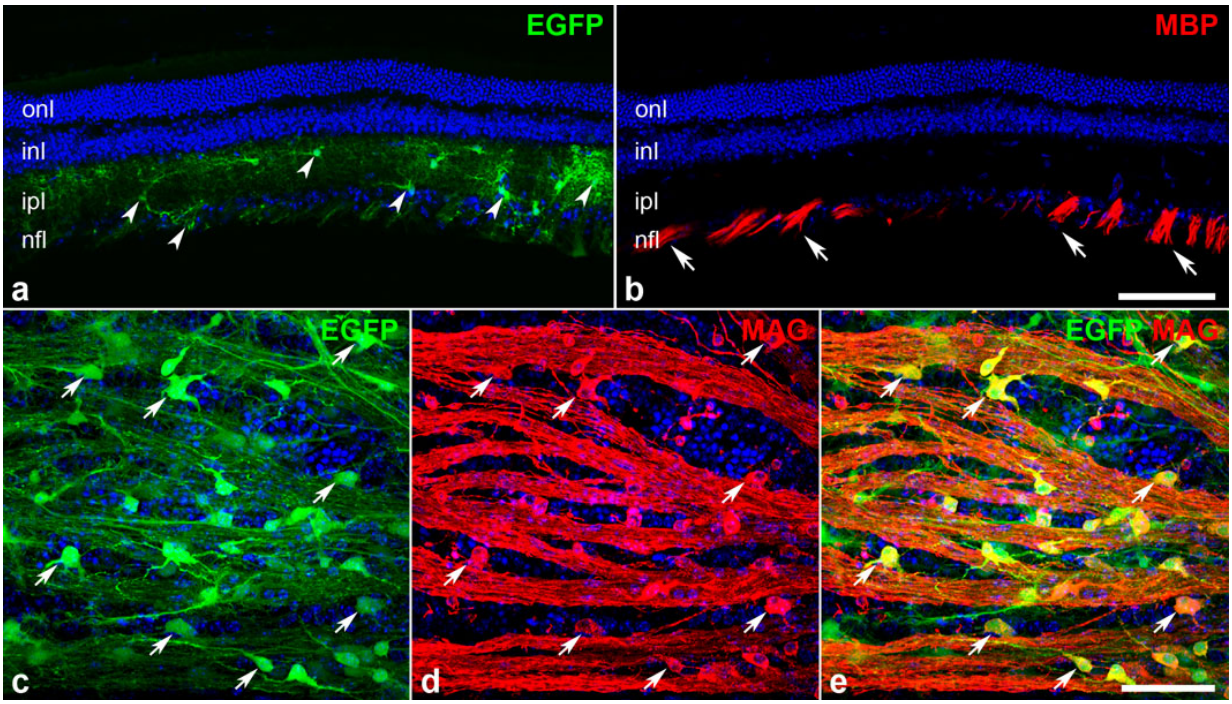

Figure 4: Intraretinally grafted PDGF-neurosphere cells integrate into the retina and differentiate into myelinating oligodendrocytes. Analysis of retinas six weeks after transplantation of small-sized PDGF-neurospheres reveals the presence of EGFP-positive donor cells (some cell bodies are labeled with arrowheads in a) in the inner plexiform layer (ipl), ganglion cell layer and nerve fiber layer (nfl). EGFP-fluorescence is also associated with axon fascicles in the nerve fiber layer (a). Immunostaining of the same section with antibodies to myelin basic protein (MBP) demonstrates the presence of myelinated axon fascicles in the nfl (b). Numerous EGFP-positive donor cells and EGFP-fluorescence in association with axon fascicles are also detectable in flat-mounted retinas four weeks after transplantation of PDGF-neurospheres (c). Co-localization of EGFP-fluorescence with MAG-immunoreactivity (compare c and d) identifies a fraction of donor cells as oligodendrocytes (some labeled with arrows in (c–e)), and additionally demonstrates the presence of donor-derived myelin in the nfl. (e) is the overlay of (c) and (d). EGFP: enhanced green fluorescent protein; inl: inner nuclear layer; MAG: myelin-associated glycoprotein; onl: outer nuclear layer; PDGF: platelet-derived growth factor. Bar in (b) for (a, b): 100µm; in (e) for (c-e): 50µm.

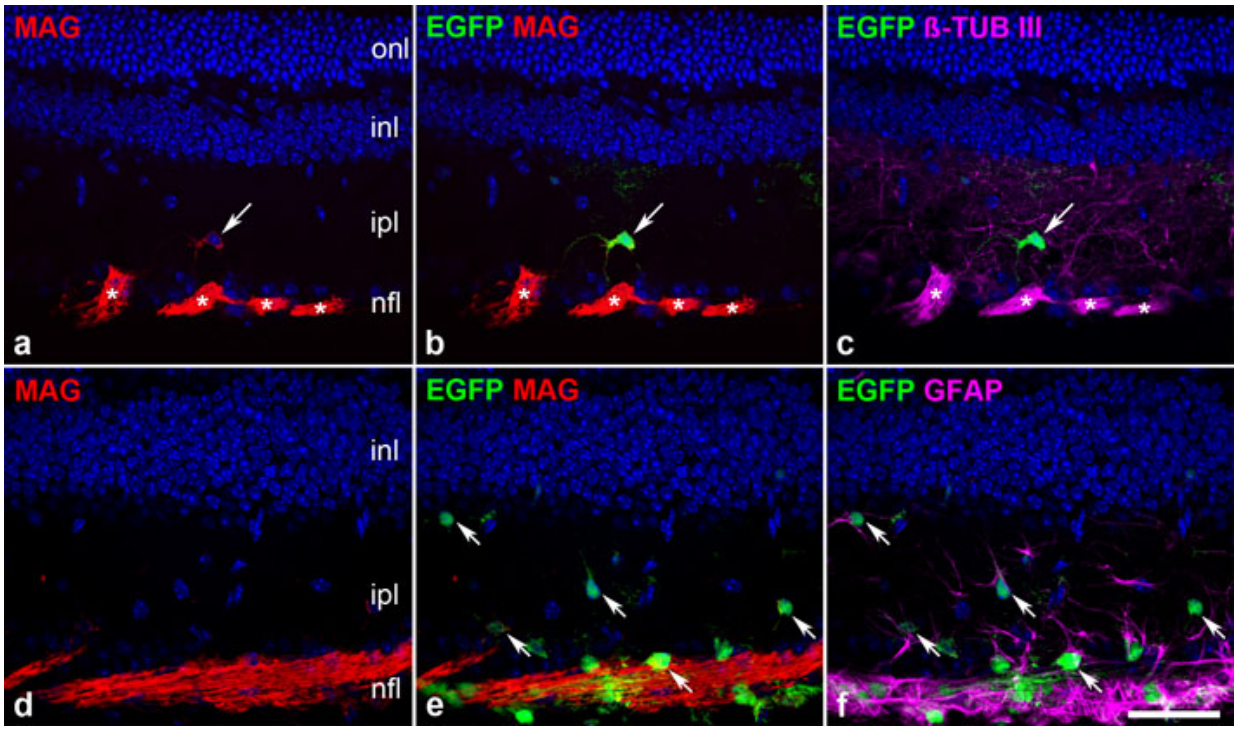

Figure 5: Intraretinally grafted PDGF-neurosphere cells differentiate into oligodendrocytes and astrocytes. Myelin-associated glycoprotein- (MAG) immunoreactivity (a, b) is associated with β-tubulin III- (β -TUB III) positive (c) fascicles of ganglion cell axons (marked with asterisks in (a-c)) in the nerve fiber layer (nfl). Co-localization of MAG and EGFP identifies a donor-derived oligodendrocyte located in the inner plexiform layer (ipl; arrows in (a-c)) with processes extending towards the nerve fiber layer. Other EGFP-positive cells in myelinated areas of host retinas (d, e) correspond to glial fibrillary acidic- (GFAP) positive astrocytes (f; some marked with arrows in e and f). Animals were analysed 5 weeks after transplantation of PDGF-neurospheres. EGFP: enhanced green fluorescent protein; inl: inner nuclear layer; onl: outer nuclear layer; PDGF: platelet-derived growth factor. Bar in (f) for (a-f): 50µm.

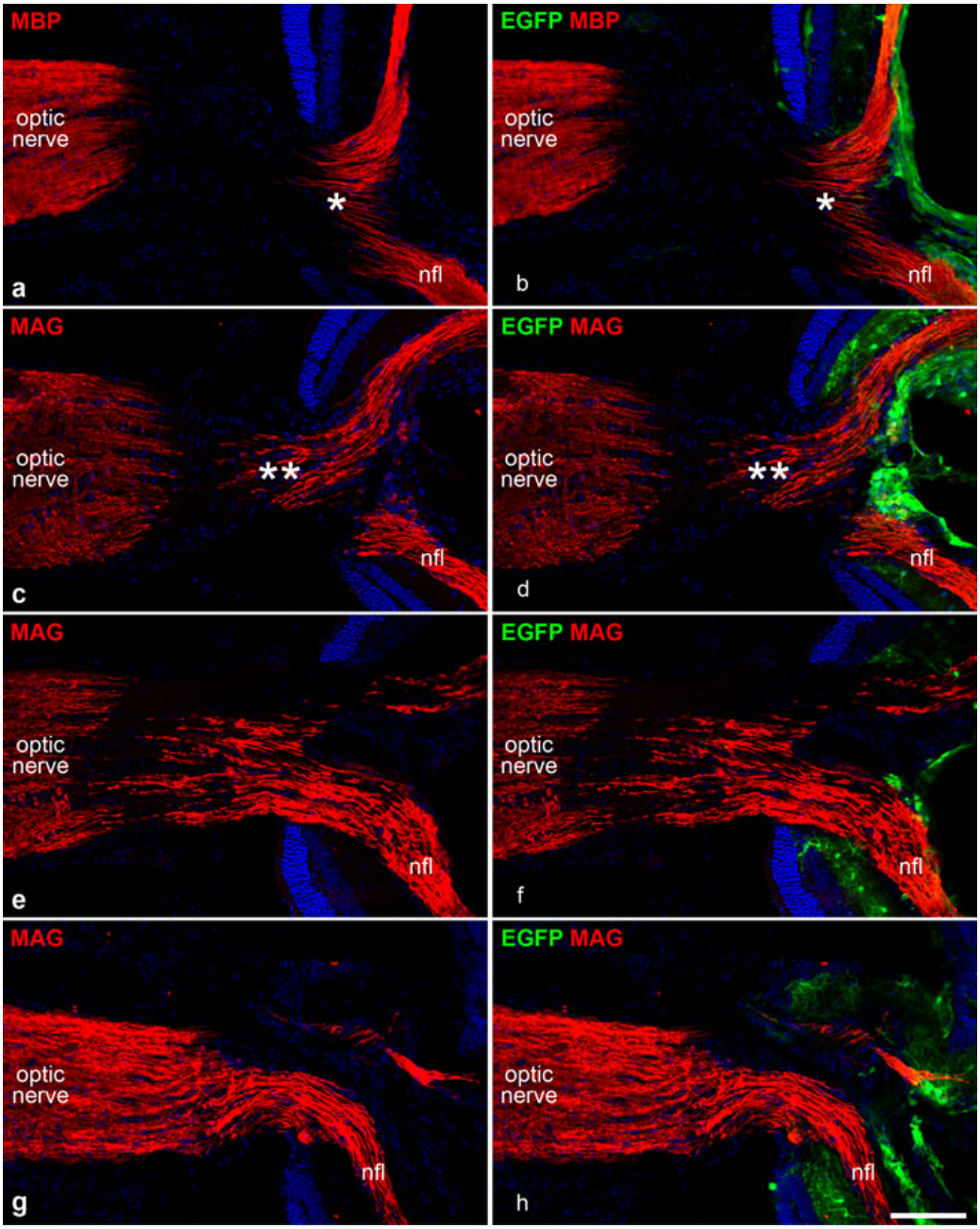

Figure 6: Myelination of retinal ganglion cell axons in the normally non-myelinated nerve fiber layer and proximal portion of the optic nerve. Longitudinal sections of optic nerves with attached retinas were stained with antibodies to myelin basic protein (MBP; a, b) or myelin-associated glycoprotein (MAG; c-h) to visualize myelinated retinal ganglion cell axons. Representative examples of animals with a myelinated nerve fiber layer (nfl; a-h) and numerous EGFP-positive donor cells (b, d, f, h) at the optic disc are shown. In some animals, myelination of the nerve fiber layer extends into the normally non-myelinated intrabulbar portion of the optic nerve (asterisk in a and b). In other animals, a few myelinated RGC axons are present in the most proximal retrobulbar portion of the optic nerve (two asterisks in c and d). In a fraction of animals donor-derived myelin in the retina is in direct continuity with endogenous myelin in the optic nerve (e-h). Animals were analysed 4 (a, b), 5 (c-f) and 7 (g, h) weeks after transplantation of PDGF-neurospheres. EGFP: enhanced green fluorescent protein; PDGF: platelet-derived growth factor. Bar in (h) for (a-h): 100 µm.

IV. Abbreviations

AAV:	adeno-associated virus
ABCA4*:*	ATP-binding cassette, sub-family A, member 4
AMD:	age-related macular degeneration
AP2a:	activating enhancer binding protein 2 alpha
BDNF:	brain-derived neurotrophic factor
BSA:	bovine serum albumin
bFGF/FGF-2:	basic fibroblast growth factor
C17.2:	name of a cell line
CAG:	combination of cytomegalovirus enhancer and chicken β-actin promoter
cDNA:	complementary DNA
Chx10:	ceh-10 homeo domain containing homolog, or visual system homeobox (VSX2)
CMV:	cytomegalovirus
CNS:	central nervous system
CNTF:	ciliary neurotrophic factor
Cy:	cyanine
DAPI:	4´,6-diamidino-2-phenylindole dihydrochloride hydrate
DMEM/F12:	Dulbecco's modified Eagle's medium and F-12
e.g.:	exempli gratia
ECT:	encapsulated cell technology
EGF:	epidermal growth factor
EGFP:	enhanced green fluorescent protein
ELISA:	enzyme-linked immunosorbent assay
ERK:	extracellular signal-related kinase
ES:	embryonic stem
FACS:	fluorescence activated cell sorting
GDNF:	glial cell line-derived factor
GFAP:	glial fibrillary acidic protein
Gp130:	glycoprotein 130
i.e.:	id est
IRES:	internal ribosome entry site
ipS:	induced pluripotent stem
IRES:	internal ribosomal entry site
kDa:	Kilodalton
LeGO:	lentiviral „gene ontology“
Lhx2	LIM homeobox protein 2
MAG:	myelin-associated glycoprotein
Map2:	Microtubule-associated protein 2

MBP:	myelin basic protein
MEK:	mitogen-activated protein kinase or extracellular signal-regulated kinase kinase
N2:	supplement
NGF:	nerve growth factor
Nrl:	neural retinal leucin zipper
NS:	neural stem
O1:	oligodendrocyte marker
O4:	pro-oligodendrocyte marker
onl:	outer nuclear layer
OPC:	oligodendrocyte progenitor cell
P:	postnatal day
PA:	paraformaldehyde
Pax6:	paired box protein 6
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
Pde6b:	rod cGMP-specific 3',5'-cyclic phosphodiesterase subunit beta
PDGF:	platelet-derived growth factor
PEDF:	pigment epithelium-derived factor
PKCα:	polyclonal protein kinase C α
Q344ter:	glutamine-344-to-ter mutation
Rax6:	retina and anterior neural fold homeobox protein
RCS:	Royal College Surgeons rat
Rd1:	retinal degeneration 1
Rd10:	retinal degeneration 10
Rd2:	retinal degeneration 2
RdCVF:	rod derived cone viability factor
RET:	receptor tyrosine kinase
RGC:	retinal ganglion cell
RP:	retinitis pigmentosa
RPC:	retinal progenitor cell
RPE:	retinal pigment epithelium
RPE65:	retinal pigment epithelium-specific 65 kDa protein
SEM:	standard error of the mean
Six3:	homebox protein (‚sine oculis')
Six6:	homebox protein (‚sine oculis')
Sox2:	sex determining region Y-box 2
STAT3:	signal transducer and activator of transcription 3
T3:	3,3´,5-triiodothyronine
VSV-G:	envelope G protein of the vesicular stomatitis virus

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