Ai miei nonni , alla mia famiglia

*Quando tutte le più belle sinfonie sono state già scritte per un musicista l'ambizione più grande
è quella di cambiare una sola nota.
(Bocelli A.)*

UNIVERSITÀ DI PISA

Corso di Dottorato in Neuroscienze di Base e dello Sviluppo

Presidente: Prof. Giovanni Cioni

"Environmental Enrichment: effects on the visual system of an animal model of Retinitis pigmentosa (RP)"

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Consiglio
Nazionale delle
Ricerche

XXIV CICLO (2009-2011)
SSD BIO/09

SUMMARY

1 RIASSUNTO

La Retinite Pigmentosa (RP) comprende una famiglia di degenerazioni ereditarie eterogenee (Kennan et al. 2005) (Paskowitz et al. 2006) caratterizzate dall'iniziale morte apoptotica dei bastoncelli, con sviluppo di insufficiente capacità visiva in condizioni di scarsa luminosità e progressivo restringimento concentrico del campo visivo, cui segue la degenerazione dei coni che comporta il declino dell'acuità visiva, fino alla cecità. Sebbene la causa primaria della RP sia tipicamente un difetto di un gene specifico dei bastoncelli, la degenerazione di queste cellule attiva una cascata di eventi che portano sia alla morte secondaria dei coni che al cosiddetto "remodeling" progressivo dei neuroni della retina interna (Marc et al. 2003). Nella patologia umana come nei suoi modelli animali, questi eventi seguono un pattern spazialmente irregolare sulla superficie retinica, rendendo difficile un'analisi quantitativa della sopravvivenza e della morte cellulare.

Lo scopo principale di questa tesi è quello di far luce sugli effetti precoci e tardivi dell'applicazione del paradigma sperimentale dell'Arricchimento Ambientale (AA) sulla degenerazione dei bastoncelli, sulla sopravvivenza dei coni e sulla morfologia dei neuroni interni della retina del mutante rd10, un modello murino di RP caratterizzato dalla progressiva degenerazione dei fotorecettori retinici secondo un pattern simile a quello che avviene nell'uomo affetto da analoghe mutazioni. L'AA è un paradigma di stimolazione motoria, sensoriale e sociale ampiamente usato come strategia non invasiva di neuroprotezione in diversi modelli di patologie neurologiche, incluse la malattia di Alzheimer e la malattia di Huntington. In questa tesi vengono riportati per la prima volta i risultati dell'esposizione prolungata di topi rd10 ad AA.

Attraverso studi di microscopia del tessuto retinico, registrazioni elettroretinografiche, test comportamentali e analisi molecolari, viene mostrato che l'AA dalla nascita è in grado di preservare in modo

considerevole la morfologia, la fisiologia dei fotorecettori e la percezione visiva negli animali rd10. Studi preliminari suggeriscono che l'effetto protettivo dell'AA possa essere mediato da fattori neurotrofici, tra i quali il CNTF.

Questi risultati possono incoraggiare lo sviluppo di nuove strategie applicative in cui la stimolazione ambientale, da sola o in combinazione con opportuni trattamenti farmacologici, può promuovere la conservazione e l'integrità funzionale dei neuroni retinici.

2 ABSTRACT

Retinitis pigmentosa (RP) is a family of inherited disorders leading to progressive photoreceptor death and is one of the major causes of genetic blindness in the world with no cure yet (Kennan et al. 2005) (Paskowitz et al. 2006). In typical RP, first rods die out, as usual for a mutation in rod-specific genes, and night blindness occurs. Later, secondary degeneration of cones follows until all the useful sight is lost. Both in human RP and in animal models of this disease, photoreceptor death is accompanied and followed by gradual remodeling of the inner retina. Both photoreceptor degeneration and secondary remodeling of the inner retina occur with an irregular pattern over the retinal surface, making quantitative analysis of survival and death difficult.

The general aim of this Thesis is investigate the early and late effects of an Environmental Enriched paradigm upon rod degeneration and viability of cones and inner retinal neurons in a mouse model of RP. Environmental enrichment (EE), a neuroprotective strategy based on enhanced motor, sensory and social stimulation, has already been shown to exert beneficial effects in animal models of various neurological disorders, including Alzheimer and Huntington disease. Here, I report the results of a prolonged exposure of rd10 mice, a mutant strain undergoing progressive photoreceptor degeneration and used to model human RP, to such an enriched environment from birth.

By means of microscopic examination of retinal tissue, electrophysiological recordings, visual behavior assessment and molecular analysis, I show that EE considerably preserves retinal morphology and physiology as well as visual perception over time in rd10 mutant mice. The protective effects of EE are likely mediated by increased production of endogenous protective molecules, including CNTF.

The therapeutic option I applied in this Thesis produced strikingly positive results, also in comparison to other strategies applied to the same animal model. This work opens the exciting possibility that non invasive manipulations of the outer environment can be used, alone or in combination with other treatemtns, to prolong the lifespan of retinal neurons otherwise doomed to death.

3   INTRODUCTION AND AIMS OF THE THESIS

The mature vertebrate retina consists of five neural cell types that have common specializations throughout species.

Photoreceptors are the neurons containing the light-sensitive photo pigment that captures light. These cells pass the information through the entire retina that encodes spatio-temporal and chromatic information transmitted to other parts of the visual processing centers. The basic retina anatomo-physiology involves two kinds of circuitry: the vertical or "through" pathway, which includes photoreceptors, bipolar cells and ganglion cells, and the horizontal or "lateral" pathway, which is made by horizontal cells and amacrine cells. In the mammalian rod pathway, some subtypes of amacrine cells have a function similar to bipolar cells.

At first look, the spatial arrangement of retinal layers seems counter-intuitive, because light rays have to pass through various non-light-sensitive elements of the retina as well as the retinal vasculature before reaching the outer segments of the photoreceptors, where photons are absorbed. The reason of this curious feature of retinal organization lies in the special relationship that exists among the outer segments of the photoreceptors, the pigment epithelium, and the underlying choroid. The disks present in the photoreceptor outer segments are formed near the inner segment of the photoreceptor and move toward the tip of the outer segment, where they are shed. The pigment epithelium plays an essential role in removing the exhausted receptor disks. In addition, the pigment epithelium contains the biochemical machinery that is required to regenerate photo pigment molecules after they have been exposed to light. Finally, the capillaries in the choroid underlying the pigment epithelium are the primary source of sustenance for retinal photoreceptors. These functional considerations explain why rods and cones are found in the outermost rather than in the innermost layer of the retina. They also explain why disruptions in the normal relationships between the choroids, pigment

epithelium and retinal photoreceptors, such as those that occur in some forms of Retinitis pigmentosa, have severe consequences for vision.

Retinitis pigmentosa (RP) refers to a heterogeneous group of hereditary retinal disorders characterized by progressive vision loss due to a gradual degeneration of photoreceptors. An estimated 100,000 people in the United States have RP. In spite of the name, inflammation is not a prominent part of the disease process; instead the photoreceptor cells appear to die by apoptosis (determined by the presence of DNA fragmentation). Multiple mutations have been found, and the heterogeneity of RP at all levels, from genetic mutations to clinical symptoms, has important implications for understanding the pathogenesis of the disease and designing therapies.

Given the complex molecular etiology of RP, it is unlikely that a single cellular mechanism will explain the disease in all cases. Despite the specific mutation or causal sequence, the major vision loss in RP patients is due to the gradual degeneration of cones. Typically, the mutated protein causing RP is not expressed in cones and the loss of these cells is an indirect result of a rod-specific mutation. Hence, understanding and treating this disease presents particularly difficult challenges. Recently, there is increasing excitement about the possibility of using gene therapy to substitute the rod defecting gene, and multiple groups are developing vectors encoding growth factors (Schlichtenbrede et al. 2003; Yang 2009) or antioxidant enzymes (Li et al. 2008; Koilkonda and Guy 2011) to retard the secondary loss of photoreceptors due to a lack of tropic factor or an excessive oxidative stress. There are also other therapeutic strategies under development, such as the optogenetic and the stem cell approaches, or the use of nanoparticles to deliver genes or proteins (Berson et al. 1993; Busskamp and Roska 2011). The endurance of these experimental designs and their effective applicability to RP will certainly be tested in the near future. It is important to recall that combinations of approaches are likely to be more powerful than any individual treatment (Yao et al. 2011).

The main aim of the present Thesis was to investigate the non invasive approach of Enviromental Enrichment (EE) as a strategy to retard the secondary loss of cones in a well known mouse model of RP, the r10 mutant mouse. The rationale is that it is well known that through sensory, cognitive and motor stimulation it is possible to enhance the capability of the central nervous system to respond to different pathological conditions (Nithianantharajah and Hannan 2006; Baroncelli et al. 2010).

The working hypothesis is that EE, a paradigm whereby the animals are raised and kept in large cages and social groups with additional toys specifically devoted to multi-sensory, cognitive and motor stimulation, can retard the secondary events due to the loss of rod photoreceptors in rd10 mutant mice, delaying the degeneration of cones. To test this hypothesis, rd10 mutant mice were kept and grown in either EE conditions or in Standard laboratory (ST) conditions from birth.

More specifically, in this Thesis we focused on two specific aims:

Aim 1: to asses morphological, molecular and functional effects of EE in adult rd10 mutant mice.

To achieve this aim we:

- Compared retinal morphology in ST mice and EE mice using quantitative immunostochemistry to analyze in detail photoreceptor morphology and number, as well as the morphology of the inner retina.

- Compared the expression of photoreceptor specific proteins by means of western blot analysis.

- Compared the viability of photoreceptors by means of electroretinogram (ERG) recordings.

- Compared the visual performance of mice by means of a visual behavioral test used to measure both visual acuity and contrast sensitivity.

- Compared the expression of different trophic factor mRNAs by means of Real Time RT-PCR in ST and EE retinas.

Aim 2: to assess the long run beneficial effects of EE in 1 year old rd10 mutant mice.

To achieve this aim we:

- Compared retinal morphology in ST and EE mice using immunostaining techniques to analyze photoreceptor morphology and number in the inner retina.

- Compared the visual performance of mice by means of a visual behavioral test used to measure both visual acuity and contrast sensitivity.

3.1 The retina

3.1.1 Anatomical organization of the retina

The eye is a sphere filled of fluid surrounded by three layers of tissue. The inner layer of the eye, the retina, contains neurons that are sensitive to light and are capable of transmitting visual signals to central targets. Despite its peripheral location, the retina is part of the central nervous system (CNS). During the embryonic development, the vertebrate retina and the optic nerve form as an out pocketing of the diencephalon, called the optic vesicle, which invaginates forming the optic cup. The inner coat of the optic cup gives rise to the retina, while the outer one gives rise to the retinal pigment epithelium. This is a thin melanin-containing layer that reduces backscattering of light that enters in the eye; it also plays a critical role in the maintenance of photoreceptors, renewing photo pigments and phagocyting the photoreceptor disks.

Compared to other portions of the CNS, the retina comprises fewer classes of neurons, and these are arranged in a manner that has been less difficult to unravel than the circuits in other areas of the brain. There are, at

all, five major classes of retinal neurons: photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells. In the adult retina, these are organized into three main cell body layers that are separated by synaptic terminals (plexiform layers) (**Fig. 1**). Light passes through the lens and across the whole retinal thickness to stimulate photopigments in the outer segments of rod and cone photoreceptors, which are located adjacent to the Retinal Pigment Epitelium (RPE). The photoreceptors, whose cell bodies costitute the outer nuclear layer (ONL), form synapses in the outer plexiform layer (OPL) with horizontal cells and bipolar neurons, whose cell bodies are located in the inner nuclear layer (INL). Bipolar cells synapse directly or indirectly via amacrine neurons in the inner plexiform (IPL) layer to ganglion cells (GCs) located in the GC layer. GCs are the projection neurons of the retina and transmit visual information via their axons through the optic nerve to visual processing centers in the brain. Amacrine cell bodies are located in the inner part of the INL and comprise roughly half of the cells in the GC layer. The retina also contains a population of radial glial cells called Muller glia, whose cell bodies are located in the INL. Their apical processes form adherents junctions with photoreceptors at the outer limiting membrane and their end feets attach to the basal lamina on the vitreal surface of the retina at the inner limiting membrane. Retinal astrocytes, which develop from a separate cell lineage at the optic disc, form a dense network on the vitreal surface of the retina and are closely associated with the vasculature. Microglial cells, of mesodermal origin, also reside permanently in the retina.

The combination of highly specialized cell types in well-organized networks performing complex modulatory activities results in an amazing and flexible sensory processing system and makes the retina a unique model for the comprehension of the relationship between structure and function inside the CNS.

(A) Section of retina   (B)

Pigment epithelium
Photo-receptor outer segments
Cone   Cone   Cone
Rod   Rod   Rod
Outer nuclear layer
Outer plexiform layer
Distal
Horizontal cell
Lateral information flow
Bipolar cell
Inner nuclear layer
Amacrine cell
Vertical information flow
Proximal
Inner plexiform layer
Ganglion cell
Ganglion cell layer
To optic nerve
Nerve fiber layer
Light
Light

Fig. 1. Structure of the retina. (**A**) Section of the retina showing the overall arrangement of retinal layers. (**B**) Diagram of the basic circuitry of the retina. A three-neuron chain photoreceptor, bipolar cell, and ganglion cell provides the most direct route for transmitting visual information to the brain. Horizontal cells and amacrine cells mediate lateral interactions in the outer and inner plexiform layers, respectively. The terms inner and outer designate relative distances from the center of the eye (inner, near the center of the eye; outer, away from the center, or toward the pigment epithelium). From Purves 3ed 2004.

3.2   The architecture of the mouse retina

The mouse retina resembles that of other vertebrates, so that in a vertical section the following layers and cells can be recognized:

- ➢ Photoreceptor layer: formed by the outer and inner segments of rods and cones;
- ➢ Outer Limiting Membrane (OLM);
- ➢ Outer Nuclear Layer (ONL): constituted by photoreceptor cell bodies;
- ➢ Outer Plexiform Layer (OPL) where processes of photoreceptors, bipolar and horizontal cells are synaptically connected;
- ➢ Inner Nuclear Layer (INL): formed by cell bodies of bipolar, horizontal, amacrine, and Müller glial cells;
- ➢ Inner Plexiform Layer (IPL) where processes of bipolar, amacrine and ganglion cells synaptically connect;
- ➢ Ganglion Cell Layer (GCL), containing the cell bodies of ganglion cells and displaced amacrine cells;
- ➢ Optic nerve Fiber Layer (OFL), constituted by axonal bundles of ganglion cells and supporting astrocytes;
- ➢ Inner Limiting Membrane (ILM).

The fundamental plan of the mouse retina follows the blue-print common to all mammalians: rods represent the majority of the photoreceptor population; however, the mouse retina is particularly rod-dominated and rods sum up to 97% of all the photoreceptors (Jeon et al. 1998). In the C57B16/J strain of mouse, used for the experiments described thereafter, there are approximately 6.4 million rods and 180,000 cones (Eye, Retina, and visual system of the mouse; chapter 12).
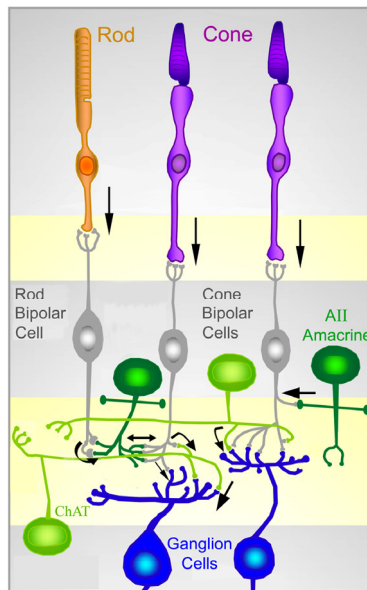
Fig. 2. Schematic representation of the main connections among retinal neurons. The physiology of the retina can be simplified into 4 main processing stages: photoreception, transmission to bipolar cells, transmission to ganglion cells and transmission along the optic nerve. At each synaptic stage there are also laterally connecting horizontal and amacrine cells.

Figure 2 summarizes the main connections among retinal neurons. Photoreceptors - rods and cones - convert light information into electrical and then chemical signals relayed to inter-neurons in the outer retina. At low light levels, only rods have sufficient sensitivity to capture the few photons that are available. Cones permit day and color vision. Photoreceptors are connected to each other by gap junctions (electrical synapses) and make chemical synapses onto bipolar and horizontal cells. Two types of bipolar cells, rod and cone bipolars, are postsynaptic to the homologous photoreceptor populations. Cone bipolar cells are further divided in two functional classes, called "ON" and "OFF" that respectively depolarize and hyperpolarize in response to the illumination of their receptive field centers. Rod bipolar cells belong to the single functional category of ON cells. Bipolar cells are then connected to RGCs to the same type (ON or OFF): this connection can be direct as in the

case of cone bipolar cells, or through a chain of neurons comprising dedicated amacrine cells, as for rod bipolar cells.

Differently from most mammals that have at least two horizontal cell types, mice have only one type of horizontal cells (Peichl and Gonzalez-Soriano 1994). Horizontal cells contribute to enhance contrast between adjacent light and dark regions. This is because they are constituted by two different, functionally distinct portions, by which they can control bipolar cell sensitivity: the dendritic arborization makes synapses with cones, while the axonal arborization is linked to rods. The connection horizontal cells - photoreceptors is bidirectional: not only horizontal cells are post- synaptic to rods, but also pre-synaptic to cones on which they exert a negative feed-back. Amacrine cells (more than 30 types) modulate signals from bipolar cells by supplying inhibition directly onto ganglion cells; moreover, they modulate transmitter release from bipolar cells. Light information leaves the retina and reaches other stations in the brain via axons of RGCs (the only exit neurons of the retina) that collectively form the optic nerve. The commonly employed C57Bl6/J mouse retina contains approximately 50,000 ganglion cells. Most of them project contralaterally, since their axons cross at the optic chiasm; only less than 5% of all ganglion cells project ipsilaterally. Beside these above mentioned five classes of neurons, the retina contains one type of macroglial cells, the Müller cells. These have a radial arrangement spanning the depth of the retina and provide important structural and functional support to retinal neurons.

Within the above described basic organization, many specialized sub-circuits are present in the vertebrate retina: they work both in sequence and in parallel to process different features of the visual image such as luminosity, contrast, chromatic composition and direction of motion (Weng et al. 2005) .

3.2.1 Photoreceptors

The two types of photoreceptors, rods and cones, are distinguished by shape (from which they derive their names), the type of photo pigment they contain, the distribution across the retina, and the pattern of synaptic connections. These properties reflect the fact that the rod and cone systems (both the receptors and their connections within the retina) are specialized for different aspects of vision. The rod system has very low spatial resolution but is extremely sensitive to light; it is therefore specialized for sensitivity at the expense of resolution. Conversely, the cone system has very high spatial resolution but is relatively insensitive to light and it is specialized for acuity at the expense of sensitivity. The properties of the cone system allow humans and many other animals to see colors. The rod-mediated perception is called scotopic vision because at the lowest levels of light, only the rods are activated.

Although cones begin to contribute to visual perception at about the level of starlight, spatial discrimination at this light level is still very poor. As illumination increases, vision becomes more and more dominated by cones and they are the major determinant of perception under relatively bright conditions such as normal indoor lighting or sunlight. The contribution of rods to vision drops out nearly entirely in the so called photopic vision, because their response to light saturates. This means that the membrane potential of individual rods no longer varies as a function of illumination because all of the light-sensitive channels are closed. Mesopic vision occurs in levels of light at which both rods and cones contribute, for example at twilight.

The ability of rods and cones to respond to different ranges of the light absorption spectrum is due to differences in their photo pigments. The spectral sensitivity of mouse rod photoreceptors peaks at 497-500nm (Soucy

et al. 1998; Toda et al. 1999; Fan et al. 2005). In the mouse retina, there are two types of cone photoreceptors: one has peak sensitivity at 360nm (UV light), while the other has peak sensitivity at 508 nm ((M)-wavelength). Most cones express both UV and M photopigments but their maximum sensitivity is at 508 nm (Nikonov et al. 2006). Another difference between the two types of photoreceptors is their sensitivity to light intensity. Rods produce a reliable response to a single photon of light, whereas more than 100 photons are required to produce a comparable response in a cone (Eye, Retina and visual system of the mouse; chapter 12). Also, the response of an individual cone does not saturate at high levels of steady illumination, as does the rod response. Although both rods and cones adapt to operate over a range of luminance values, the adaptation mechanisms of cones are more effective. This difference is apparent in the time course of the response of rods and cones to light flashes. The response of a cone, even to a bright light flash that produces the maximum change in photoreceptor current, recovers in about 500 milliseconds, more than four times faster than rod recovery.

The arrangement of the circuits that transmit rod and cone information to retinal ganglion cells also contributes to the different characteristics of scotopic and photopic vision. In most parts of the retina, rod and cone signals converge on the same ganglion cells; i.e., individual ganglion cells respond to both rod and cone inputs, depending on the level of illumination. The early stages of the pathways that link rods and cones to ganglion cells, however, are largely independent. The pathway from rods to ganglion cells involves a distinct class of bipolar cell (rod bipolar) that, unlike cone bipolar cells, does not contact retinal ganglion cells. Rod bipolar cells synapse with the dendritic processes of a specific type of amacrine cell (the AII amacrine) that makes gap junctions and chemical synapses with the terminals of cone bipolars; these processes, in turn, make synaptic contacts on the dendrites of ganglion cells in the IPL. As a consequence, the circuits linking the rods and cones to retinal ganglion cells differ a) in the number of interposed neurons and b) in

their degree of convergence. Each rod bipolar cell is contacted by a large number of rods, and many rod bipolar cells contact a given amacrine cell. In contrast, the cone system is much less convergent and thus less sensitive.

3.3 Genetics

3.3.1 Genetic mutations affecting photoreceptors

A very high number of genetic mutations affect the eye. Those occurring in photoreceptor- or pigment epithelium -specific genes often cause Retinal Degenerations (RDs), a family of inherited dystrophies characterized by photoreceptor dysfunction and death. It is estimated that more than 15 million people worldwide have vision loss due to inherited forms of RD. These include patients suffering from Retinitis pigmentosa (RP), a disease for which there is no cure yet (Chader 2002).

3.3.2 Retinitis pigmentosa (RP)

One of the common subtypes of inherited forms of photoreceptors degeneration is Retinitis pigmentosa (RP), which is the second main cause of blindness in 20-64 years olds (Wright et al. 2010). RP represents an heterogeneous group of genetic disorders that lead to loss of vision. Typically, rods, the first cells affected, start to die (as a result of a genetic abnormality) and patients experience night-vision limitations and develop visual field constriction (or tunnel vision) (**Fig. 3d**). Secondary cone degeneration eventually affects central vision, leading to blindness. RP cause severe visual impairment in as many as 1.5 million patients worldwide (Kennan et al. 2005; Mendes et al. 2005) (Paskowitz et al. 2006).

Fig. 3. Typical examples of night blindness (**b**) and tunnel vision (**d**). This is a typical example of eye field degradation in RP patients. Normal night vision (**a**) and a normal day vision (**c**) in human.

The progressive demise of photoreceptors also precipitates other pathological symptoms in the retina, including the attenuation of the retinal vasculature and the accumulation of intra-retinal pigment deposits, from which the disease derives its name (**Fig. 4**).
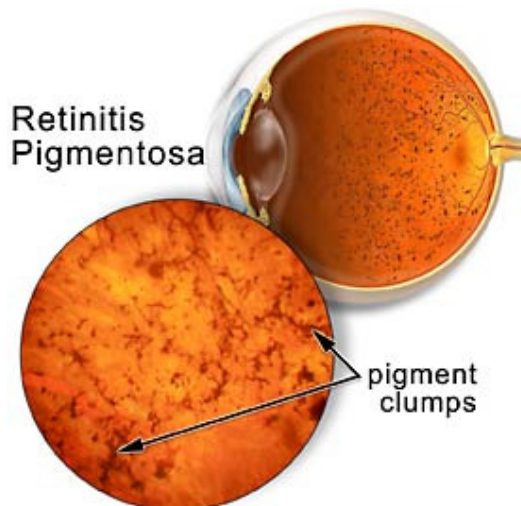

Fig. 4. Example of fundus views of the retina of a RP patient. A typical brown pigmentation is present in the pathological condition. Pigment deposits, named bone spicules for their typical shape, are responsible for the name of the disease. A clear attenuation of retinal blood vessels is also visible. From:Web Vision.

There is considerable variation in the severity of the conditions of the RP patients: it is unusual for them to become totally blind, while most of them retain some useful vision well into old ages. Classic clinical findings of RP include: bone spicule pigmentation or pigment clumping, retinal arteriolar narrowing, waxy pallor of the optic nerve, epiretinal membrane formation, atrophy of the RPE and choriocapillaris (starting at the mid periphery of the retina with preservation of the RPE in the macula until late in the disease), posterior subcapsular cataract, epiretinal membrane formation, and cystoid macular edema (CME) (Hamel 2006).

3.3.3 Genetics and molecular mechanisms of RP

The genetics of RP is complex: approximately 180 mutations in more than 40 genes have been identified as the cause of different forms of RP. In a segregation analysis of RP, families were divided as autosomal dominant (24%), autosomal recessive (41%) and X-linked (22%), and the remaining 12% of cases were supposed to result from non-genetic factors, non-Mendelian inheritance (for example, mitochondrial or de novo mutations) or complex inheritance (Wright et al. 2010). The large number of mutations causing RP comprise insertions, deletions, or substitutions, in turn producing missense mutations or truncations (Wang et al. 2005). RP is caused preferentially by different single gene mutations including the following: GTPase regulator (RPGR: 10-20% of cases) rodopsin (RHO: 8-10% of cases) and usherin (USH2A: ≈3% of cases) (Jos´e M. Mill´an and Ascensi ´on Gimenez-Pardo 2011). Some of these genes encode for proteins of the phototransduction pathway, or play a role in the maintenance of cellular structure or in the transcriptional control of other photoreceptor genes (Kennan et al. 2005; Mendes et al. 2005).

Here I will describe one mutation of the phototransduction cascade: the *Pdeb* mutation that might cause autosomal recessive RP (arRP) and is also carried by the mouse model used in this study.
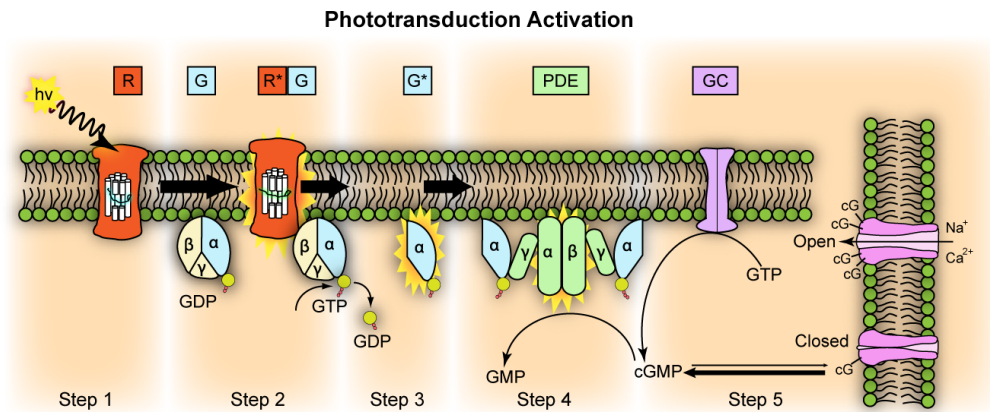

Fig. 5. Phototransduction activation. The enzyme phosphodiesterase (Pde) comprises different subunits (alpha, beta, gamma and delta). Mutations in each of these subunits can lead to RP. From: Wikipedia.

3.3.4 Pdeb mutations and the rd10 mutant mouse

For over 30 years, the retina of rodents has provided an invaluable tool to study the dynamics and mechanisms of inherited RD, as mouse photoreceptors undergo dystrophies caused by spontaneous DNA mutations, closely related to those of humans. The so-called rod-less mice (Keeler 1924) expresses a nonsense mutation in the *Pdeb* gene coding for the β-subunit of cGMP phosphodiesterase of rods. This spontaneous mutation later named rd1 (rd1: *Pde6brd1*) (Pittler et al. 1993) was subsequently found in humans with arRP (McLaughlin et al. 1993; McLaughlin et al. 1995) and had forms of night blindness (Gal et al. 1994).

There is a surprisingly high degree of evolutionary stability for mutations that cause loss of vision in mice, in fact many of the commonly used inbred strains, including C3H and its derivatives and CBA/J, are homozygous for rd1. In particular, two mutations in the *Pdeb* gene of rd1 mice have been identified: besides the nonsense mutation that causes truncation of the protein (Bowes et al. 1990), the intronic insertion of an endogenous mouse leukemia virus *(Xmv28)* causes an incorrect splicing. *Xmv28* has also been found in strains derived from wild type mice, as well as from inbred laboratory strains not known to have recent common origins (Bowes et al. 1993). Evidently, there is little or no selective pressure to maintain a functional β-PDE gene, and therefore vision, in these nocturnal animals.

Biochemical studies comparing retinas from normal and rd1 mice have show that the lack of cGMP-PDE activity causes a dramatic increase in cytoplasmic cGMP concentration (Farber and Lolley 1974). The *Pdeb* gene-triggered cell death alters the expression of genes involved in diverse cellular pathways including Ca^{2+} homeostasis, catabolism, neuro-inflammation and tissue remodeling (as the blood-retina barrier undergoes a breakdown). In mouse photoreceptors, normal Ca^{2+} levels range between ~250 nmol/liter (in

complete darkness) and ~60 nmol/liter (in light) (Woodruff et al. 2002). However, in the rd1 photoreceptors, Ca^{2+} levels are increased up to ~190% over wt levels (Fox et al. 1999). Consequently, messages for Ca^{2+}-binding proteins, and in particular Ca^{2+} sensors such as calbindin, are up-regulated in the rd1 photoreceptors. So far, the focus has been on Ca^{2+} as major trigger of the apoptosis cascade, while the role of high cGMP levels remains unanswered yet.

The animal model we used for our studies is another example of autosomic recessive RP, isolated at the Jackson Laboratories in the USA (Chang et al. 2002), and namely the rd10 (retinal degeneration 10) mouse. Genetic analysis shows that this strain carries an autosomal recessive mutation that maps to mouse chromosome (Chr) 5. Sequence analysis shows that the retinal degeneration is caused by a missense point mutation in exon 13 in the *Pde6b* gene of the β-subunit of the rod cGMP phosphodiesterase (β-PDE) gene (*Pde6b*). The mutation changes codon 560 from CGC to TGC resulting into an arginine to a cysteine change and in the loss of a *Cfo*I site. The exon 13 missense mutation is the first known occurrence of a re-mutation in the *Pde6b* gene in mice and represents an additional model for studying the pathogenesis of autosomal recessive RP in humans (arRP). It may also provide a better experimental model for RP because of its later onset and milder phenotype compared to the more common rd1 mutant (Otani et al. 2004). In fact, in rd10 mutant mice, photoreceptor degeneration starts around P18 (about 10 days after the rd1 mutation), when retinal development is far more advanced. Rod death follows a central to periphery gradient; then a slower degeneration of cones occurs. A scattered population of aberrant cones persists up to at least 9 months of age. Atrophic retinal vessels are found at four weeks of age, consistent with retinal degeneration (Chang et al. 2007; Gargini et al. 2007). Electroretinograms (ERGs) of rd10 mice are never normal, but rod and cone ERG a- and b-waves can be measured at P18 while there is no more response at three months of age and later. Interestingly,

rearing rd10 mice in total darkness delays degeneration for at least a week, after which morphological and functional loss progresses irregularly (Chang et al. 2002).

As in other models of retinal degeneration (Chang et al. 1993; Reme et al. 1998), death of photoreceptors in rd10 mice shows the hallmarks of apoptosis: nuclei in the ONL are picnotic and some of them express a form of activated Caspase 3. Apoptotic markers appear in the retina in a centre to periphery gradient.

Secondary changes also affect inner retinal cells. Modifications in their morphology become detectable at around one month of age, and are more widespread at 2 months. Mostly, changes affect rod bipolar and horizontal cells. Their dendritic complement becomes progressively more scant and disorganized ultimately regressing completely. Cone bipolar cells follow, at a slower rate. Dendritic retraction in bipolar cells which carry glutamate mGluR6 receptors is preceded by a decrease in the immunoreactivity for this marker, and by its displacement to the cell bodies and bipolar cell axons in the INL. Morphological changes in bipolar and horizontal cells occurring in the rd10 retina are followed by their death partial death. Other cell types, such as AII and cholinergic amacrine cells, do not seem to be affected in the first 5 months of life (Gargini et al. 2007).

3.4 Therapeutic Approaches

3.4.1 Therapeutic strategies for RP

At present, there is no therapy able to stop the evolution of RP or to restore vision loss in this disease. However, there are several therapeutic strategies aimed at slowing down retinal degeneration or at supplying the loss of cells in this tissue.

In general, prenatal diagnosis can be useful in families in which the responsible gene has been identified, particularly for early onset and severe forms of RP. For other cases, clinical evidence and data from animal studies indicate that some genetic types of RP are partly light-dependent (Wang et al. 1997). Thus, patients with RP are recommended to wear dark glasses outdoor, or yellow-orange spectacles minimizing photophobia. Beside therapies aimed at preserving visual function and preventing cell death, researchers experience the tremendous challenge of trying to restore visual function. However, because mammalian photoreceptors are neurons and do not divide after birth, their loss is irreversible. In addition, the loss of photoreceptors leads to a dramatic remodeling of the inner retina which would probably modify the visual information process. Nevertheless, numerous groups are now working to achieve visual restoration either by photoreceptor replacement or by using artificial prosthesis that substitute their function. A synthesis of the most representative clinical or experimental strategies to treat RP is given below.

3.4.2 Vitaminotherapy

Vitamins A and E seem to protect the photoreceptors because of their trophic and anti-oxidant effects, respectively. It has been shown that long term (5–12 years) vitamin A supplementation slightly slows down the loss in ERG amplitude, while vitamin E seems to have adverse effects. Indeed, based on a study of the natural course of RP, patients who happen to be taking vitamin A, vitamin E, or both were recorded to have slower declines in ERG amplitudes than those not taking such supplements (Berson et al. 1985; Berson et al. 1993). This observation prompted a randomized clinical trial of oral vitamin A and E supplements in 601 patients with dominant, recessive, and X-linked non-syndromic Retinitis pigmentosa and Usher's syndrome type II. Participants were randomly assigned either daily vitamin A as retinyl palmitate15 000 IU, vitamin E 400 IU as dl-α-tocopherol, the combination, or trace amounts of both vitamins; follow-up was for 4–6 years. In this study patients assigned high-dose of vitamin A showed a significantly slowed decline in cone ERG amplitudes than the other groups; however, in a subgroup in which the effect was higher there was also a significant negative effect of vitamin E. These studies are still debated: vitamin A should not be given to RP patients with mutations in ABCA4 (Berson et al. 1993; Berson et al. 1993), a gene responsible for a subset of recessive Retinitis pigmentosa. Other mutant alleles of ABCA4 cause related diseases, recessive cone-rod dystrophy, and recessive Stargardt macular degeneration. A study on abca4 (-/-) mice indicates that vitamin A supplementation should be avoided in patients with ABCA4 mutations or other retinal or macular dystrophies associated with lipofuscin accumulation in the retinal pigment epithelium because it enhances this accumulation.

Another nutritional treatment assessed for patients with RP is based on docosahexaenoic acid (DHA), an omega-3 fatty acid found in high

concentrations in oily fish such as salmon, tuna, mackerel, herring, and sardines. DHA is apparently important for photoreceptor function, since membranes containing rodopsin and cone opsins in photoreceptor cells have very high concentrations of this fatty acid. In a recent study, patients were given docosahexaenoic acid (DHA) supplementation, in addition to vitamin A. It was shown that the RP degeneration of photoreceptors initially slowed down by the addition of DHA, but the beneficial effect did not last over 2 years (Berson et al. 2010).

Recently, Clemson et al. 2011 demonstrated that the use of retinoids and other small molecules as pharmacological chaperones increases the yield of properly folded RP mutant rhodopsins in heterologous cell culture. Also they tested whether other known small molecules can provide similar effects. Valproic acid (VPA) was identified as a potent inhibitor of histone deacetylase (HDAC) also effective increasing the levels of various neurotrophic factors. VPA and its derivative, divalproex sodium, is used off-label for a variety of indications including chronic pain syndromes, cancer therapy and schizophrenia. In their work Clemson et al. suggested that VPA may be an appropriate agent to treat patients with retinal dystrophies such as RP (Clemson et al. 2011).

3.4.3 Pharmacological treatments

Pharmacological treatments may be a good choice in those cases where some aspects of the pathophysiological mechanisms of RP are known. Pharmacological agents can compensate for a biochemical defect, and enhance or inhibit the activity of various effectors. Calcium-channel blockers have been tried in different animal models of RP (Nakazawa 2011) suggesting that calcium channel antagonists may inhibit photoreceptor apoptosis triggered by abnormally high levels of calcium. Recently an in vivo study on

rd10 mutant mice has demonstrated that photoreceptor loss can be slowed by pharmacologically inhibiting the rate limiting enzyme of the synthesis of ceramide, a known pro-apoptotic messenger (Strettoi et al. 2010).

3.4.4 Artificial devices

The development of artificial implants inserted in substitution of an entire part of the pathological retina represents an active line of investigation to treat both RP and other retinal diseases, such as macular degeneration. Micro-photodiodes arrays replacing degenerated photoreceptors or more sophisticated devices capturing light and stimulating retina, optic nerve or visual cortex have been developed. Several clinical trials have essentially demonstrated the tolerance of these electronic implants (Zrenner 2002). Some of these approaches have already improved the eyesight of patients with major visual impairments.
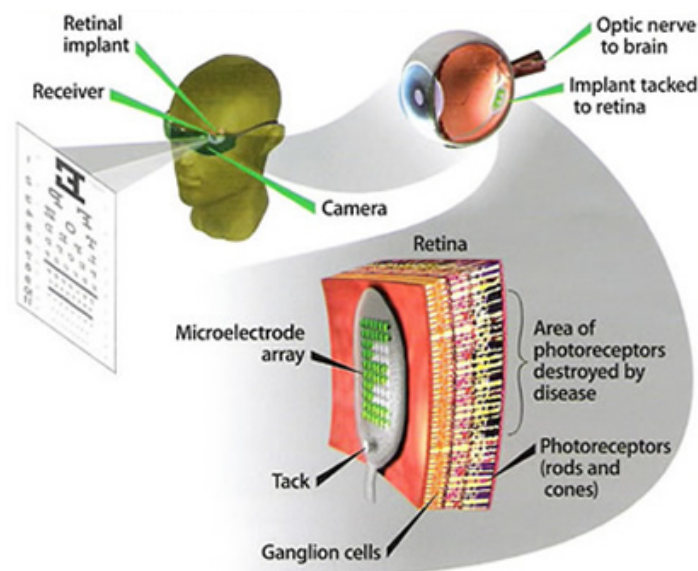

Fig. 6. Epiretinic implant: on a normal glasses is mounted a microcamera that supplies the signals, this signals are transmitted to the chip through microcable in the eyes. The chip gives energy to the electrodes in epiretinic space.
From:www.forumsalute.it/community/forum_65_oculistica/thrd_149058_un_microchip_i mpiantato_nella_retina_rida_la_speranza_chi_non_vede_1.html.

Of course, retinal prostheses are only effective if the visual pathway distal to the retinal implant is still intact and functional (**Fig. 6**). Two types of prostheses have been developed according to the site of retinal implant: epiretinal (on the surface of the retina) and subretinal (between the retina and the RPE) prostheses (Chow et al. 2004) (Hossain et al. 2005), briefly illustrated below subretinal prostheses contain microphotodiodes attached to microelectrodes. These implants, such as the artificial silicon retina (5000 microelectrodes), are placed in the subretinal space between the outer retina and retinal pigment epithelium (**Fig. 6**). The photodiodes are stimulated by light passing through the retina, and the resulting electric current excites adjacent retinal sensory neurons. The specifications for subretinal implants vary. For example, a typical device measures 50-100 μm wide, has a diameter of 2-3 mm, and carries microphotodiodes on a microelectrode array (Chow et al. 2004). These implants do not require an external electrical source as incident light is sufficient for stimulation (Zrenner 2002). Subretinal devices have been tested in animal experiments (Butterwick et al. 2009). Several species have shown tolerance to the implants, for up to 30 months of time. Histological evidence has shown no relevant changes in the architecture of the retina. Visual perception is improved in six human patients with RP who received an artificial silicon retina, including subjective improvement in appreciation of brightness, contrast color, movement, shape and visual field. Some patients show an improvement in visual acuity.

Two major advantages of subretinal prostheses are the utilization of existing forces between the neural retina and retinal pigment epithelium to maintain their position and the potential of a high spatial resolution, as they are positioned close to retinal nerve cells and can stimulate neurons by means of low electrical currents. The main disadvantages of subretinal implants include impaired nourishment of the inner retina due to the creation of a mechanical barrier between the outer retina and the choroid and the occurrence of trauma to the retina during implanting. These prostheses also

show poor dissipation of heat and therefore could damage the retina. The mechanism of action of these implants may be by direct stimulation of retinal neurons. A specific neurotrophic effect elicited locally onto photoreceptors by means of the surgical procedure cannot be excluded: a study of subretinal artificial silicon implants in rats showed a temporary protective effect on the retina, resulting in decreased degeneration of photoreceptors (Zrenner 2002).

Epiretinal prostheses are composed of an array of electrodes implanted on the surface of the retina between the vitreous and the inner limiting membrane. The implants receive electrical signals from a camera positioned outside the body (**Fig. 6** for more details; see also **Fig. 7**, for *in vivo* application). In one such device, the camera transmits light signals to a microchip within the camera. This microchip deciphers the signal and relays it, using wireless transmission, to a microchip in the epiretinal implant which in turn stimulates the RGCs (Zrenner 2002). Early studies in dogs and rabbits showed the flexibility of using epiretinal prostheses. A few clinical trials in humans have reported simple visual perception as phosphenes. Perception of light has been only reported by three "completely blind" patients with RP who received the so-called "second sight" model: these implants have survived for up to two years.
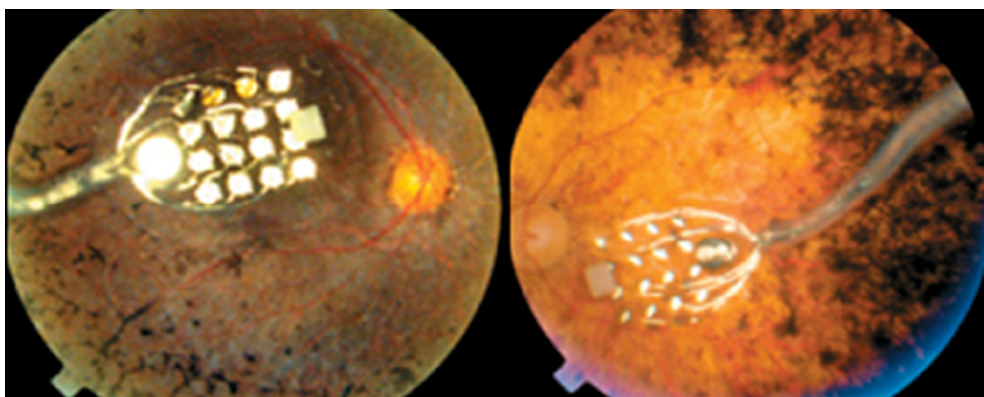

Fig. 7. Fundus photography showing an epiretinal electronic implant.
From: www.ophthalmologytimes.com/ophthalmologytimes/articleDetail.jsp?id=140725.

One advantage of epiretinal implants over subretinal devices is that the camera can process signals before they reach the implant: this allows optimization of the signal quality, which may lead to improved visual perception. Epiretinal implants can also use the heat-dissipating properties of the vitreous and are therefore less likely than subretinal implants to damage the retina. The superficial location of the implant reduces risks of trauma during implantation and allows for the implant to be replaced. The main disadvantages of epiretinal implants are the need for complicated microtechnology and surgical techniques that ensure secure fixation of the implant on the retina. The device also requires a higher electrical current than the subretinal implant (Lakhanpal et al. 2003). Implants are being developed to generate their own electrical currents on stimulation.

Some epiretinal prostheses used to attempt restoring sight in RP patients are designed to produce direct or near stimulation of RGCs. The success of such electronic devices is partly based upon the hope that RGCs are still viable after photoreceptors death. Yet, recent data show that some RP subjects lack phosphenes in respond to epiretinal stimulation (Delbeke et al. 2001). In addition, it has been reported that perceptual thresholds for electrical activation of the retina are surprisingly high in RP patients (Rizzo 2011). This is partially confirmed by the work of Stasheff with microelectrode arrays on RGCs in the rd1 mouse (Stasheff 2008). Here, RGCs have a much higher spontaneous frequency than normal, sometimes in rhythmic bursts reminiscent of epileptic discharges. A probable explanation could be that a strong remodeling of RGCs has occurred, and their membrane has become relatively less excitable to exogenous stimulation.

According to the group of Fishman (Stanford Nanofabrication Facility), all the present types of prosthetic chips (either sub or epiretinal) stimulate neurons electrically with limited spatial control and without cell specificity. For example, extracellular electrodes can excite retinal cells but cannot inhibit them (unlike physiological neurotransmitters such as GABA and glycine). An

ideal chip should deliver a chemical stimulation so that different transmitters are recognized by different cells and produce different, and specific, effects. To overcome these limits, researchers are working on the ''artificial synapse chip'', in which the advantages of two technologies, electronic engineering and cell biotechnology, are combined. The prototype chip should drive growth of retinal cell dendrites and axons directly into the chip, essentially mimicking a synapse, by controlled, repeatable release of neurotransmitters, as occurs in natural vision (Peterman et al. 2003; Peterman et al. 2004).

3.4.5 Gene therapy

This therapeutic approach consists in the introduction into the retina of adeno-associated viral vectors carrying correct copies of the gene whose mutation causes the disease. This approach obviously requires the implicated genes to be identified and therefore the availability of efficient genotyping methods. Gene replacement therapy is relatively simple for RP due to loss-of-function mutations (usually recessively inherited). In this case, it is predicted that the expression of wild-type cDNA in the appropriate cell (photoreceptor or RPE) will avoid cell death. The most advanced experimental studies in this direction have been performed in blind dogs mimicking Leber's congenital amaurosis (LCA). The group of J. Bennett succeeded in surgical administration in the subretinal space of AAV vectors carrying the RPE65 cDNA, allowing restoration of vision in four month-old dogs in USA (Acland et al. 2001). Five years later, the dog vision was still stable, and at present the long-term efficiency of the cure seems ascertained.

Several clinical trials are currently ongoing in RPE65 patients, and more than 30 patients have been treated to date. For example in the Jacobson Study, which employed an adeno-associated virus 2 carrying the RPE65 gene on 15 patients, no ocular adverse events or systemic toxicity were detected

related to surgery or to the viral vector itself. Visual function improved in all patients to different degrees and improvements were localized to treated retinal areas. Cone and rod sensitivities increased significantly in the treated eyes but not in the control eyes. Yet a group of patients with better foveal structure lost retinal thickness and acuity after sub-foveal injections. Even though only a very limited number of patients will greatly benefit from this still experimental treatment protocol, the technique itself has been shown to be safe and will likely be used in other retinal disorders in the near future. At date a canine model for achromatopsia has been treated successfully as well as mouse models for different forms of LCA.

Gene therapy is more complicated for RP due to dominant negative pathogenic mechanisms in which the expression of the mutated gene should be both inhibited and replaced. Experimentally, inhibition has been achieved by use of ribozymes or siRNA. In the last 10 years, studies have been carried out in several animal models with gain-of-function mutations. Although all studies showed a significant rescue of photoreceptors upon gene therapy, there was still progressing photoreceptor death, which could be due to an inappropriate expression level of the therapeutic gene and to an insufficient percentage of transduced photoreceptors. Thus, for patients with autosomal dominant Retinitis pigmentosa (adRP), a combined gene knockdown and gene addition therapy is being developed using RNA interference to block mRNA of the mutant allele (Stieger and Lorenz 2010).

3.4.6 Optogenetic approach

Based on the assumption that photoreceptors work differently than most neurons, since they hyperpolarize when stimulated, Busskamp et al. used an adeno-associated vector (AAV) to deliver halorhodopsin, a light-activated chloride pump of archaebacteria (Nagel et al. 2003), to cone photoreceptors in two mouse models of RP. The aim of this approach was to bypass the need for the normal light sensor (opsin) in cones and the normal phototransduction process. The authors detected light-induced electrical currents in the vector-infected photoreceptor cells, not unlike those measured in normal cones, in which light stimulates cone opsin (Sieving et al. 2006). For those patients suffering from RP with unknown mutations, an AAV-based transfer of bacterial forms of rhodopsin in the central retina might be an option to reactivate residual photoreceptors in the future. In perspective, cones should be treated with a dual gene therapy, a viral vector that delivers a gene to combat the underlying cause of death, along with the halorhodopsin gene. Alternatively, a combination therapy of antioxidants, and/or growth factors, and AAV-halorodopsin might prolong cone survival and function.

3.4.7 Cell or tissue transplantation

The first successful transplant of a mammalian retina happened in 1959, when Royo and Quay transplanted fetal rat retinas into the eyes of adults of the same strain (Royo and Quay 1959). Although the transplanted retinas did not seem to connect with the host retinas, they survived for months. Since then, photoreceptor replacement has been shown to be feasible in RP animal models, but any cell-replacement strategy will require a source of new retinal cells. Retinal cells from fetal or adult retinas have been transplanted in

humans, and layers of photoreceptors or even entire retinas in animal models (rats and rabbits). Generally, the survival of transplanted photoreceptors is rarely observed; moreover, they do not organize in the retina (forming rosettes) and lack, with rare exceptions, functional synapses (Hamel 2006).

Researchers are also becoming interested in using embryonic or adult stem cells, since fetal or embryonic retinal progenitors can be grown in vitro (Anchan et al. 1991) and used for transplantation (Klassen et al. 2004). Neurospheres can be grown from the adult pigmented ciliary epithelium, and these cells can also be transplanted into the retina (Coles et al. 2006). Neural stem cells derived from the hippocampus show a good ability to integrate into the retinal layers and form morphologically normal-appearing retinal neurons (Suzuki et al. 2006).

The best evidence for functional photoreceptor replacement comes from the study of MacLaren et al. (2006), in which freshly dissociated, postmitotic rod photoreceptors were transplanted to the subretinal space; however, the limited number of implanted cells could not be expanded in vitro due to their postmitotic state.

Embryonic stem cells (ESCs) might be a good source for replacement of photoreceptors, but this therapeutic approach is still far from realistic use in a near future. In spite of the possibility that retinal transplantation might constitute a hope for the restoration of vision, getting the transplanted cells to establish right connections within the retina has remained a major problem by far. It appears that an early developmental stage of the retina of acceptor's represents a key feature of a successful transplant. Despite decades of experimental attempts, transplants have yet to produce better vision in mammals with retinal degeneration because the transplanted cells do not wire up properly.

It has been proven that the RPE grafts may rescue the photoreceptors in Royal College of Surgeons (RCS) rats, in which a mutation of c.Merk causes a retinal dystrophy by lack of outer segment phagocytosis of the RPE. A

similar mutation causes a rare form of RP in humans (Li and Turner 1988). In RP due to RPE defects, RPE transplantation is then theoretically possible, but the immunogenic reaction against allogenic, wild type RPE is a limitation to this approach.

Based on a different rationale, promising results for RP treatment have been obtained by Otani et al. (2004), still using a cell-based approach. These authors have shown that injections of hematopoietic stem cells (lineage-negative called Lin-HSCs) into the eye of mouse models of RP (rd1 and rd10 mice) result into a dramatic rescue of both blood vessels and photoreceptors, mainly cones. These mice show an improvement of the ERG at an age when usually it is completely extinct and the rescue effects are long lasting.

3.4.8 Neurotrophic factors

In humans, it would be sufficient to preserve 10% of all cones to maintain an independent life, while 50% of cone functionality would ensure a normal vision acuity (Hartong et al. 2006). Thus, neuroprotection appears as a promising and feasible approach. In addition, neuroprotection would work independently of the underlying genetic mutation causing RP, and therefore could bypass the tremendous genetic heterogeneity of this disease and the high incidence of sporadic cases.

Various studies tested different neurotrophic factors for their ability to slow retinal cell loss (LaVail et al. 1998). Some of them have been found to be effective when injected into the vitreous or in the subretinal space (Chong et al. 1999), or given as a supplement to transplants (Panni et al. 1994). It has been shown that four different neurotrophic factors, and namely ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) and one 'viability' factor (rod-derived cone viability factor) delay rod degeneration in some animal models of RP (Caffe et al. 2001). Among them, CNTF shows efficacy in 13 different animal models and has progressed to a phase II clinical trial in RP (Chaum 2003). Therefore, CNTF is currently considered the most effective neurotrophin to protect photoreceptors from degeneration (Sieving et al. 2006).

Exogenous administration methods for neurotrophic factor delivery to the retina have mostly relied upon intraocular injections (Whiteley et al. 2001). More recently, a delivery system based on the endovitreal implant of a special capsule (**Fig. 8.A**) containing engineered cells (**Fig. 8.B**) has been devised by Tao and co-workers (2002). The encapsulated cells produce CTNF. The results of a recent phase I trial of an encapsulated cell therapy delivering CNTF are presently under evaluation (MacDonald et al. 2007).
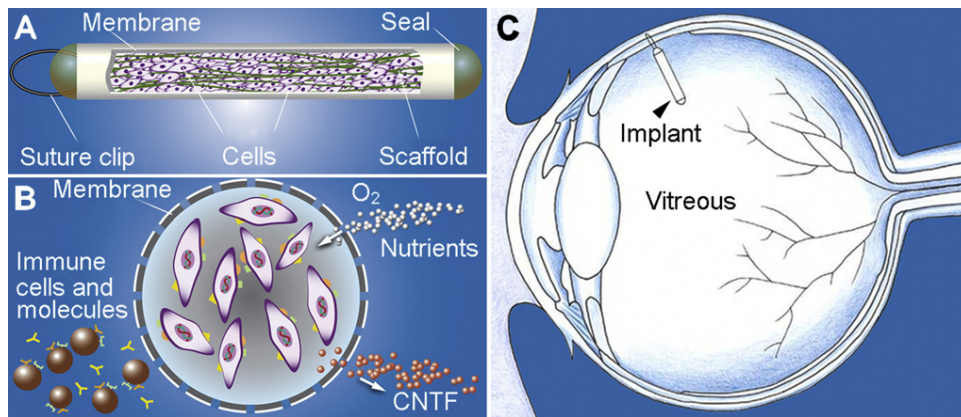
Fig. 8. Schematic illustration of CNTF secreting implant using Encapsulated Cell Technology. The implant is composed of a section of semi-permeable membrane capsule which contains CNTF-secreting cells and scaffold. The membrane capsule is sealed at both ends with a suture clip at one end for anchoring on the sclera (**A**). The membrane allows O2 and nutrients to diffuse in and therapeutic agent (CNTF in this case) to diffuse out. It also keeps components of the immune system out (**B**). The implant is 6 mm in length and 1 mm in diameter. It is outside the visual axis of the eye when anchored to the sclera (**C**).From R. Wen et al.2012.

A very important discovery is the existence of intraretinal viability factors. It is postulated that these molecules are normally released by rods (among other cells) and sustain the survival of cones. The latter would undergo secondary degeneration when the density of rods (and therefore the supply of factor) falls below a certain threshold. The French laboratory of Sahel and collaborators has been the first to isolate what has been called the rod-derived cone-viability factor (RdCVf) (Leveillard et al. 2004). These authors observed that in retinal cultures from chicken embryos cones degenerated after a few days. However, the degeneration could be considerably delayed by supplementing the culture with medium derived from wild-type mouse retinal explants. This early experiment led to the biochemical characterization of RdCVF, a secreted protein expressed in rods and necessary for the survival of cones. The injection of antibodies anti RdCVF in the subretinal space of wt mice causes a remarkable reduction in cone number. The administration of RdCVF in rd1 mice preserves about 40% of cones from degeneration. At present, the French group is working on the

mechanism of action of this molecule, also devising pre-clinical trial studies for its application on humans.

3.5   Enviromental Enrichment (EE)

We know that the quality and intensity of environmental stimulation can influence both neural development and plasticity in the adult brain. This concept was first clearly articulated by Hebb in 1949 (Hebb D.O., The organization of behavior; a neuropsychological theory. Wiley, New York, 1949). Hebb proposed that synapses are strengthened when presynaptic fibers repeatedly participate in activating the postsynaptic neuron. Subsequently, Hubel and Wiesel's classic experiments (Hubel and Wiesel 1970) demonstrated that the environment influences neural plasticity and neurodevelopment in the cat visual system. Rosensweig and colleagues in the 1960's (Rosenzweig et al. 1962; Rosenzweig 1966; Rosenzweig et al. 1969) systematically began to examine the influence of environmental enrichment on cognitive functions, brain weight, cortical thickness and the structure of dendrites. In these early studies, Environmental Enrichment was defined as a "combination of complex inanimate and social stimulation" (Rosenzweig et al. 1978). Obviously, this concept is relative: an environmental setting is enriched with respect to another conditions that may lack some stimuli. An essential component of a typical EE setting is the opportunity to perform high levels of voluntary physical activity on running wheels (Spolidoro et al. 2009). Therefore, living in an enriched environment provides the animals with optimal conditions for enhanced exploration, cognitive activity, social interaction and physical exercise.

It is know that EE is a way of rearing the animals in a setting more similar to the wildlife: a sort of naturalistic-like condition. However, the observation of rodents playing in an Enriched Environment, choosing when and how much

to run on the wheel and to explore the new objects show that EE is not only a way to reproduce more natural life conditions. Rather, EE implies a kind of challenge-free interaction with a stimulating surrounding, with few stressful experience then normal. We can also argue that, although the activity of mice in the wild is mostly driven by necessity, and can introduce some stressful experiences, in an EE it is usually prompted by a combination of curiosity and play, because it is carried on with pleasure.

3.5.1 Effects of EE on visual system development

In spite of the vast literature on the effects of EE on the CNS, it was only during the past decade that new light has been shed on the remarkable EE impact on the developmental plasticity of the visual system.

The most remarkable effect that an EE procedure started from birth produces on visual system development is an accelerated maturation of the visual system acuity (VA). This has been initially studied in mice both electrophysiologically, by visual-evoked potentials (VEPs) recordings, and behaviorally, by visual water box task, a visual discrimination task (Cancedda et al. 2004). EE influences also the possibility to induce long-term potentiation (LTP) of layer II-III field potentials after theta-burst stimulation of the white matter (WM/II-III LTP) in the visual cortex, a well established in vitro model of developmental plasticity not present in the adult cortex (Huang et al. 1999). The processes underlying cortical plasticity induced by EE are not well known but different studies agree that there are several molecules involved. One of the most important appears to be BDNF. Indeed, in mice reared from birth in enriched conditions the expression of this neurotrophic factor is significantly higher in their visual cortex at P7 with respect to mice of the same age reared under standard conditions. It is also important to know that such an increase does not persist after P10 (SC) (Sale et al. 2004). Huang

and colleagues in 1999 found, in transgenic animals over-expressing BDNF, that this molecule accelerates the development of the inhibitory GABAergic system. This affects the receptive field development and cortex synaptic plasticity, determining a faster maturation of VA and the accelerated decline of synaptic plasticity. This model is also confirmed in EE mice, in which an increased expression of the GABA biosynthetic enzymes GAD65 and GAD67 has been found in enriched pups at both P7 and P15 (Cancedda et al. 2004; Sale et al. 2004).

Another molecule that seems to be crucial for the effects elicited by EE at visual cortical level is IGF-I and his co-factor mTOR. IGF-I increases postnatally in the visual cortex of enriched rats and post-weaning administration of this molecule in the same structure mimics the EE effects on VA acceleration (Ciucci et al. 2007). Blocking IGF-I action in the visual cortex of developing enriched animals, completely prevents the EE effects on VA acceleration (Ciucci et al. 2007).

One of the targets of IGF-I and BDNF signaling is the activation of the transcription factor CREB. Indeed, it has been demonstrated by Cancedda et al. (2004) that animals enriched from birth display an accelerated time course of CRE/CREB induced gene expression and that treatment of non-EE mice with rolipram, (a specific inhibitor of the high affinity phosphodiesterase type IV that, in turn, activates cAMP system resulting in an increased phosphorilation of the transcription factor CREB), partially mimics EE effects on CREB pathway and on visual acuity development. Moreover, it is very likely that EE effects are not restricted to the visual cortex only, as suggested by the influence on CRE-mediated gene expression observed in the somatosensory cortex as well (Cancedda et al. 2004) .

3.5.2 Maternal enrichment and visual-system development

Experiences acquired between birth and weaning age are essential in promoting and regulating neural development and behavioral traits in the newborn of most mammalian species (Fleming et al. 1999). A key point of this assumption is that newborn animals reared in EE do not interact with the external environment for, at least, the first two weeks of life, spending most of their time in the nest, totally dependent on the mother and, in the case of mammalians that live in large social groups, on the mother helper, which are the most important source of sensory experience for the developing pups (Liu et al. 2000). The thesis that maternal behavior could be the most important source of environmental stimuli for the accelerated development of enriched pups has been directly confirmed by a detailed quantitative analysis of maternal care, which turned out to be higher in pups reared under enriched conditions compared to standard reared pups (Sale et al. 2004).

The enhanced tactile stimulation and the continuous physical contact that enriched pups receive from their mothers seem to greatly influence brain development, providing a source for the earliest changes observed under enriched conditions. All these results are in line with the findings that the offspring of mothers that express higher levels of pup licking and grooming and arched back nursing, show increased expression of NMDA receptor subunit and BDNF-mRNA, increased cholinergic innervation of the hippocampus and enhanced spatial learning and memory (Liu et al. 2000). Moreover, Meaney and co-workers (2001) (Meaney 2001; Meaney 2001) found that naturally occurring variations in maternal care alter the expression of genes that regulate behavioral and endocrine response to stress, as well as hippocampal synaptic development. These effects form the basis for the development of stable, individual differences in stress reactivity and certain forms of cognition. Strikingly, part of these different abilities to respond to

stress in individuals are due to epigenetic mechanisms occurring at the level of chromatin structure and function: higher demethylation occurs in the hippocampus of the offspring that receive more maternal cares, at the level of the glucocorticoid receptor (GR) gene promoter (Weaver et al. 2004), facilitating transcription and leading to higher hippocampal density of glucocorticoid receptor .

More recently, an essential role for maternal stimulation in driving the development of the visual system has been provided by the work of Guzzetta and colleagues (2009), who hypothesized that enriching the environment in terms of body massage accelerates brain development in infants. The results from this work indicate that enriching the environment in terms of multisensory stimulation "massage therapy" affects brain development and in particular visual system maturation in both human preterm babies and rat pups. This not only underlines the importance of the environment as a driving force in early postnatal development, but also suggests that the environment acts modulating the level of endogenous factors such as IGF-1(Landi et al. 2007; Landi et al. 2009).

3.5.3 EE influences retinal development

Most studies have investigated the numerous beneficial effects of EE on brain and behavior. Recently, the issue has been raised whether EE can also affect structures traditionally considered less plastic then the visual cortex, such as the retina. It is generally assumed that retinal development is independent from sensory and epigenetic inputs and that retinal acuity is not conditioned by visual deprivation, such as monocular deprivation (MD) or dark rearing (DR), which, on the contrary, have dramatic effects on visual-cortical acuity (Fagiolini et al. 1994; Fine et al. 2003). This traditional view has been challenged; indeed, it has been shown that dark rearing impairs retinal

development in mice, preventing the segregation of the ON and OFF pathways both at the electrophysiological and anatomical level (Tian and Copenhagen 2003). Moreover, Landi and colleagues in a 2007 study have shown that EE dramatically influences retinal development. By performing pattern electroretinogram (a sensitive measure of retinal ganglion cells function) recordings from enriched and standard reared rats, they found that retinal acuity development is sensitive to EE on the same time scale as cortical acuity. These authors also reported a significant acceleration of RGC dendritic segregation into ON and OFF retinal sublaminae in enriched animals. They also tested if and how BDNF is involved in these processes: when the neurotrophin expression was blocked in the retina of enriched pups, all the effects elicited by EE were completely prevented (Landi et al. 2007).

Strikingly, EE turned out to very effective also during fetal development. In a recent work by Sale and colleagues (Sale et al. 2007), it has been shown that in the embryos of pregnant rats housed in an enriched environment there was a marked acceleration in the development of retinal morphology, with faster dynamics of neural progenitor migration and natural cell death. Interestingly, all these effects found in enriched fetuses were under the control of IGF-I, whose levels were higher not only in the milk of pregnant rats, but also in the retina of the embryos. Silencing of this molecule in enriched mothers completely prevented the accelerating EE effects. Furthermore, the infusion of IGF-I in standard pregnant mothers mimicked the positive effects of EE found in enriched fetuses (Sale et al., 2007).

3.5.4 EE and pathology

It has been demonstrated by several studies (for a complete review see also Baroncelli et al. 2011) that exposure to an EE elicits positive effects on recovery from pathological conditions, stress and aging. Since 1956 Bilowit et al. (1956) provided evidence that sensory-motor stimulation played an important role in ameliorating Parkinson's disease symptoms.

More recent studies have reported also an effect of EE on increased hippocampal neurogenesis (Kempermann et al. 1997) and on the reduction of apoptotic cell death (Williamson et al. 2012).

Moreover rearing animal models (transgenic animals) of nervous system disorders, including neuorodegenerative diseases (for a complete review see also (Nithianantharajah and Hannan 2006) and brain injuries (Johansson 1996, Comeau et al 2008), in an enriched environment, leads to striking beneficial effects.

Recently, several studies on the mechanisms that limit plasticity to early life, indicate that the brain is not "hard-wired" whit fix and immutable neural circuits, but that genes and environment work in concert in shaping CNS connections and behavior, even in hereditary pathological conditions (for review see Rutter 2007).

In this Thesis we consider an inherited disease, Retinitis pigmentosa (RP), which affects neurons (photoreceptors in first place) in an area of the CNS shown to be sensitive to EE; survival of these neurons is prolonged by various neurothropic factors and cytokines and some of them (like BDNF) are increased in rodent paradigms of EE. For these reasons it is well-grounded to hypothesize EE as a strategy to delay RP symptoms in the mouse model employed here and, in perspective, in human patients. The Results obtained collectively support this view.

4   MATERIALS AND METHODS

4.1   Animals

All the experimental procedures were in accordance with Italian and International institutional guidelines. Mice of the rd10 strain (on a C57Bl6/J background) and wild-type (wt) C57Bl6/J mice (both from the Jackson Laboratories, Bar Harbor, ME) were kept in a local facility with water and food ad libitum, in a 12-hour light/dark cycle, with illumination levels below 60 lux.

4.2   Rearing environments

4.2.1   Standard environment

Control animals were rd10 mice born and raised in the same room, with the same illumination levels and administered the same diet of enriched mice. Control cages were standard laboratory cages of smaller size (42x26x 18 cm), without objects and hosting only the pregnant female and, later, her litter. The litter remained in the same cage until retinal examination, performed at the same age of EE mice.

4.2.2 Enriched Environment

Enriched environment consisted of a large size (60x38x20 cm) Plexiglass cage containing a running wheel, at least two Plexiglass tunnels, hard-pressed cardboard nesting material, and 6-8 small objects which were replaced once a week. An rd10 female was placed in an enrichment cage with a male. Five additional and older females, previously mothers (helpers), were also present. The rd10 progeny (usually, 6-8 pups) remained in the same enriched environment up to the time of final testing and retinal examination (performed at 24, 45, 60 and 360 days).

Fig. 9. Environmental Enrichment (EE) is a manipulation of the standard laboratory conditions that modify the quality and intensity of environmental stimulation, reaching an optimization of the rearing environment. The goal of EE is to provide animals with increased levels of multisensory stimulation, physical activity and social interactions, and by eliciting spontaneous explorative behaviors.

4.3 Fluorescence microscopy for pycnotic photoreceptors

Mice from EE and ST at P24 were deeply anaesthetized intraperitoneally with Avertine (tribromoethanol in amylene hydrate 20μl/gm of body weight). For retinal histology, after eye enucleation, mice were euthanized with an overdose of the same agent. Eyes were quickly enucleated making a reference on the dorsal pole with permanent ink, rinsed and then eye cups were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4), for 1 hour. Afterwards they were included in 30% sucrose in 0.1 M PB o/n and frozen in tissue-tek (Sakura, NL) at -20 °C . The retinas were detached and whole mounts pre-treated with RNAase A (1:500;Invitrogen) at 37°C in a solution with 0,5% Triton X-100 for 1 hour, washed extensively and then stained for 1 hr with Ethidium homodimer 2 Invitrogen. This is a fluorescent DNA-intercalating molecule to which fixed tissue is permeable, but that also stains RNA. Whole-mounted retinas were examined under a Leica TCS-SP confocal microscope for the presence of pycnotic (apoptotic) photoreceptor nuclei, brighter than others for their condensed DNA. The ONL, containing the nuclei of photoreceptors, was sampled along the whole z axis. Photoreceptor pycnotic nuclei were counted on projection images of the ONL in fields of 150.6×150.6 μm2 (32 fields/retina), spaced at 500-μm intervals along the dorsal-ventral and nasal-temporal retinal meridians. The total number of pycnotic photoreceptors for each retina was calculated by multiplying the average density of pycnotic cells in field images by the corresponding retinal area, measured by low-power light microscopy with an image analyzer.

4.4 Immunofluorescence: cone counting

Mice were deeply anaesthetized as described previously. For retinal histology, after eye enucleation, mice were euthanized with an overdose of the same agent (Avertine; 20μl/gm of body weight). Eyes were quickly enucleated making a reference on the dorsal pole with permanent ink, rinsed and then eye cups were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4), for 1 hour. Afterwards they were included in 30% sucrose in 0.1 M PB o/n and frozen in tissue-tek (Sakura, NL) at -20 °C. For whole mount staining, the eye cup were de-frosted, the retinas isolated, deprived of pigmented epithelium, and 4 incisures were cut to identify the main quadrants. The retinas were then washed in 0.1 M PB (3x 10 minutes) and left over night in a blocking solution with 0,5% Triton X-100, 5% BSA in phosphate-buffered saline (PBS; pH 7.4) at 4°C. The day after, the retinas were placed in a solution of rabbit anti-Red/Green Opsin and anti-Bue Opsin antibodies (3 days at 4°C, 1:500; SIGMA) revealed with Alexa-568 secondary antibodies (2 hours at RT. 1:1000; Invitrogen). All solutions contained 0,3% Triton X-100, 0,5% BSA in phosphate-buffered saline (PBS; pH 7.4).

To estimate the number of cones in EE, ST and wt mice at p45 and p60, we used 6 retinas (from 6 different mice) from both EE and ST mice and 2 retinas from wt mice. Each retina was systematically imaged with a Peltier-cooled Axiocam color camera interfaced with a Zeiss Axioscope fluorescence microscope, using a 5x objective. Images were assembled into single, high resolution montages of the whole retinal surface by means of Adobe PhotoMerge. Using differences in labeling brightness, displayed on the images as color gradients, cone isodensity curves were then traced digitally. Cone densities within each curve were estimated separately by counting cones on high resolution confocal images. For this purpose, serial optical sections were obtained at 0.5 μm intervals to cover the thickness of the entire layer

containing the outer segments. Scanning areas were 16 fields 375X375 sq. micrometers. Fields were regularly spaced along the dorso-ventral and naso-temporal retinal meridians. Counts of cone outer segments were performed on serial reconstructions covering the entire thickness of the outer segments by stacking a series of single focal planes (usually 3 -4 consecutive planes, each of them 0.5 μm thick), with the aid of Metamorph® software.

Locally estimated cone densities were assigned to the corresponding areas displayed on a retinal map. Total numbers of cells were obtained multiplying average cellular densities by corresponding retinal areas. Statistical analysis (t-test) was performed with OriginPro 7.0. Average density values at each retinal locations were used to generate 7x7 squared matrices that were then analyzed with Sigmaplot 8.0 software, to obtain retinal isodensity maps with a dedicated SigmaPlot application.

4.5   Fluorescence microscopy on retinal vertical sections

Retinas from three animals of rd10 EE, ST and wt mice at P45, P60 and P360 were harvested for morphological analysis. Photoreceptor survival was assessed by counting nuclei in the ONL. Photoreceptor morphology was assessed by immunohistochemistry.

Eyes were removed, fixed for 1 h in 4% paraformaldehyde in 0.1 M sodium PB buffer (pH 7.4), infiltrated with 30% sucrose in the same buffer, and frozen at −20 °C on a cryostat stage (Leica). Vertical sections of 14 μm were cut, collected on glass microscope slides. For the stain with Ethidium homodimer 2 (1:1000, Invitrogen) or YOYO (1:1000, Invitrogen) slice were treated with RNAase A (1:500) at 37°C in a solution with 0,5% Triton X-100 prior to examination by confocal microscopy. We estimated the number of surviving photoreceptors by counting the rows of nuclei in the outer nuclear layer on high-resolution images of vertical sections obtained from both central

and peripheral retinal areas (10 images/retina, each covering a linear retinal extension of 250 μm). Mean values per retina were averaged per group and expressed as mean and SE. At least 3 retinas/age group were examined.

For immunocytochemistry of photoreceptor morphology and retinal histology, retinal sections were treated as follows: Primary antibodies used were are reported in **Table 1**. Before the incubation with the primary antibodies, retinal sections were incubated with a blocking solution containing 0.5% Triton X-100, 5% BSA , PBS; pH 7.4, at RT for 2 hours. The antibodies were diluted in the same buffer with 0.1% Triton X-100, 0.5% BSA and revealed with anti-mouse Oregon Green 488,anti-mouse Alexa Fluor 488; and anti-rabbit Alexa 568 (all used as 1:400); or anti-mouse/anti-rabbit rodamine RedX-conjugated (1:500, Jackson Immunoresearch laboratories) secondary antibodies. Retinal sections were counterstained with Ethidium homodimer 2 or with YOYO to reveal nuclei.

Table 1: Primary antibodies used in indirect fluorescence immunocytochemistry

Primary Antibody	Working dilution	Host; source	Main target
Rhodopsin	1:1000	Mouse monoclonal; SIGMA	rods
S Opsin	1:500	Rabbit policlonal; CHEMICON	S-cones
M/L Opsin	1:500	Rabbit policlonal; CHEMICON	M/L cones
PSD-95	1:500	Mouse monoclonal; CHEMICON	Photoreceptor synaptic terminals
Protein Kinase C (PKC)	1:1000	Rabbit policlonal; SIGMA	Rod bipolar cells
Protein Kinase C (PKC) clone MC5	1:1000	Mouse monoclonal; SIGMA	Rod bipolar cells
Calbindin D 28K	1:500	Mouse monoclonal; SIGMA	Horizontal and amacrine cells
Bassoon	1:500	Mouse monoclonal; SIGMA	Photoreceptor synapses
cGMP-gated, rod specific channel	1:500	Mouse monoclonal; a kind gift of Prof. Joseph Beavo University of Washington Dep.of Pharmacology	Outer segments of rods

4.6 Western blot

To detect photoreceptor-specific proteins in retinal tissues of different experimental groups, we performed Western blot on corresponding retinal homogenates.

Three EE and ST mice aged P45 and P60 were anesthetized as described earlier, eyes removed rapidly, retinas detached and frozen on dry ice. Two retinas for each mouse were then homogenized in a lysis buffer containing (in mM): Tris (pH 7.4), 50; EDTA, 1; sodium pyrophosphate, 1; NaCl, 150 ;sodium orthovanadate, 1; NP40, 1%, Na-deoxicolate, 0,25% and phenylmethylsulfonylfluoride, 1; with aprotinin, 10 mg/mL; leupeptin (Sigma, Italy), 10 mg/mL. Samples were first homogenated and subjected to lysis for 30 minutes at 4°C, after the insoluble fraction was pelleted by centrifugation (12,000 rpm; 30 min; 4°C). The protein pellet was then recuperated and the protein concentration determined by Biorad assay (Biorad, Italy). Subsequently, 60 mg homogenate from each sample, for each line in duplicate, was loaded into 12% acrylamide gels using the Precast Gel System (Biorad, Italy). Samples were blotted onto PVDF membrane (Bio-Rad Laboratories), blocked in 10% nonfat dry milk in Tris buffered saline with 1% NP40 for 1 hr, and then probed with Monoclonal anti Rhodopsin (1:1000 Chemicon), Monoclonal anti β Actin Clone AC-74 (1:20.000 SIGMA), Monoclonal anti CGMP gated cannel (1:1000), Polyconal anti OPN1MW/LW (H-55) (1:1000 Santa Cruz biotechnology), Polyclonal anti Blue sensitive opsin (H-40) (1:1000 Santa Cruz biotechnology). All antibodies were diluted in NP-40 1% Tris Buffered Saline (TBS) and 5% milk incubated overnight at 4°C. Blots were then rinsed for 20 min in TBS NP-40, incubated in horseradish peroxidase-conjugated anti-mouse or anti-rabbit (1:3000; Biorad, Italy, in 5% milk and TBS NP-40), rinsed, incubated in enhanced chemiluminescent substrate (Biorad), and exposed to film (Hyperfilm;

Amersham Biosciences, Europe). Films were scanned, and densitometry was analyzed through ImageJ free software (http://rsb.info.nih.gov/ij/).

To minimize variability, each sample was loaded in parallel in two lanes and two gels were run simultaneously on the same apparatus. The densitometric quantification of the band corresponding to Rhodopsin, Cone specific Opsin and Ligth-sensitive channel was then normalized to the value obtained for the total amount of Actin from the same gel. Results are presented as a significative example of multiple experiments.

4.7 qPCR

The reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for the detection of low-abundance mRNA, often obtained from limited tissue samples. The recent introduction of fluorescence-based kinetic RT-PCR procedures significantly simplifies the process of producing reproducible quantification of mRNAs and promises to overcome these limitations. The figure below shows a schematic procedure of both conventional RT-PCR and qPCR.
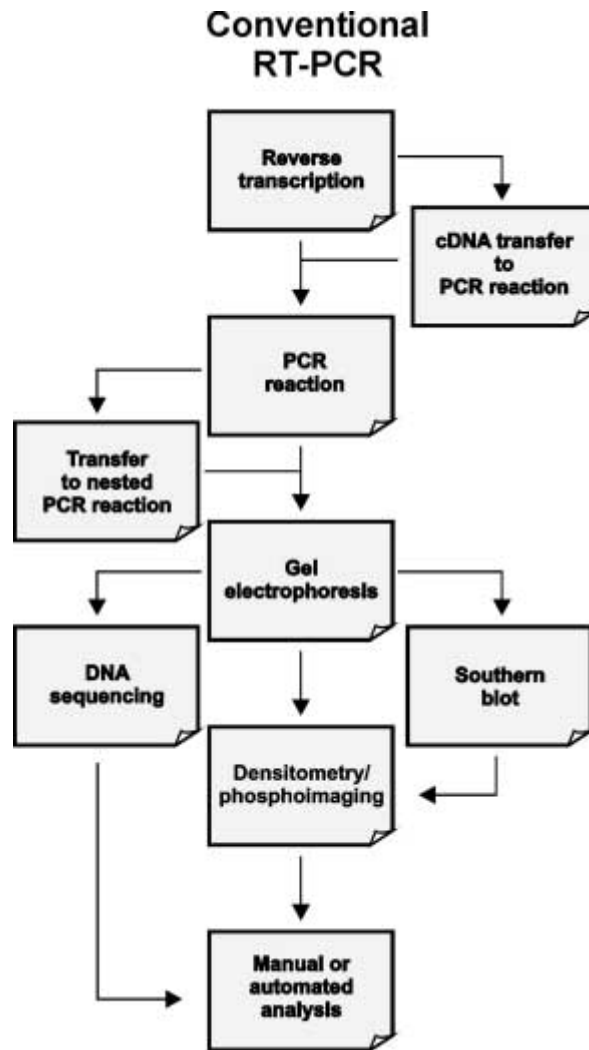
Conventional RT-PCR

Fig. 10. Conventional RT-PCR. The reaction can be carried out either with reverse transcriptase in a separate reaction, followed by transfer of some of the cDNA into a second reaction for the PCR, or as a combined one-tube reaction with a heat-stable DNA-dependent polymerase. The former may be more sensitive and has the advantage of generating a cDNA reservoir that can be re-used repeatedly to carry out further PCR reactions. The latter has the advantages of speed, simplicity and less chance of contamination. Maximum sensitivity is achieved using a nested PCR reaction; however, this is frequently at the cost of reduced specificity. Gel electrophoresis is common to all procedures, but Southern blotting and/or DNA sequencing may be required to confirm the identity of the amplicon. A final step involves some method for quantitating the bands on the gel. All subsequent steps are carried out by the thermal cycler/detector and a quantitative result is processed by computer. From: (Bustin 2000).

4.7.1 Template preparation

In the procedure we followed, retinas are isolated from mice anesthetized as above and the lysis and homogenization of frozen retinas performed according to the QIAzol protocol (QIAGEN).

4.7.2 Determining concentration and purity of nucleic acids

The concentration of RNA is determined by measuring the absorbance at 260 nm (A_{260}) and at 280 nm (A_{280}) in a spectrophotometer. The ratio between the absorbance values at 260 nm and 280 nm gives an estimate of the purity of RNA. For accuracy we perform a Acrylamide (1%) with formamide gel with 1 μg of RNA before and after the Reverse transcription reaction.
Purified RNA is stored at -20°C in RNase-free water. If the template is not degraded the experiment can be continued over time.

4.7.3 Removal of genomic DNA contamination and cDNA synthesis

For cDNA syntesis with integrated removal of genomic DNA contamination we use the QuantiTect Reverse Transcription Kit (QIAGEN) that is specific for Real time uses. For the retrotrascription reaction we use 0,5 μg of RNA.

4.7.4 qPCR

Quantitative real time PCR (qPCR) was performed using the TaqMan® Gene Expression Assays (20x). We used 10 μL of PCR reaction mix into each well of a 48 well reaction plate (Applied Biosystem).

The reference genes we use is glyceraldehyde-3-phosphate-dehydrogenase (GADPH):Mm99999915_g1*.

For CNTF mRNA amplification we use the Inventoried assay: Mn00446373_m1.

We observed that this assay does not amplify genomic DNA. After the reaction, we run Amplicons on Agarose gel (1%) to control the effective presence of one Amplicon only, or weather annealing between the two primers had caused the undesired formation of multiple Amplicons.

Initially, we used the Inventoried assay Mm01334043_m1 for BDNF mRNA amplification; since this assay detects also the Genomic DNA, we made a custom intron-spanning assay with the target sequence:

Fw: GTTCCTTCAACTGCCACCACT

Rw:GCCTTCATGCAACCGAAGTATGA

Estimed Amplicon Size: 108

In silico analysis gives an accuracy of 98% for this assay.

4.7.5 Detection of a correct amplicon on gel

To evaluate the correct procedure of amplification, the results of this step were loaded on a 1% Agarose gel. This method allows the direct observation of the amplicons used for the PCR and detects abnormal signaling (presence of Genomic DNA, incorrect annealing).

4.7.6 Data analysis

To perform calculations of real-time PCR results we used the Delta-Delta Ct method. Before that, we tested the efficiencies of all our primers, and found they have a similar efficiency. This means that the delta Ct remains

constant when we performed a series of 10-fold dilutions. This allows the use of the $2^{\text{delta-delta Ct}}$ to calculate the difference in the expression of our genes in our samples.

4.8 Behavioral test: visual acuity and contrast sensitivity

 Visual acuity is defined as the ability to detect small distance separating two points, and the threshold for visual acuity is reached when the observer can no longer determine a separation between two different points. Spatial frequency is a measure of how often sinusoidal components (as determined by the Fourier transform) of the structure repeat per unit of distance and is measured in cycles per degree (c/deg). For a grating stimulus, spatial frequency refers to the frequency with which the grating repeats per degree of visual angle. To determine the visual acuity in experimental mice we used the visual water box adapted by Prusky (Prusky et al. 2000). We used the same visual water box also for determining the contrast sensitivity. Contrast sensitivity is defined as the ability to perceive differences between an object and its background.
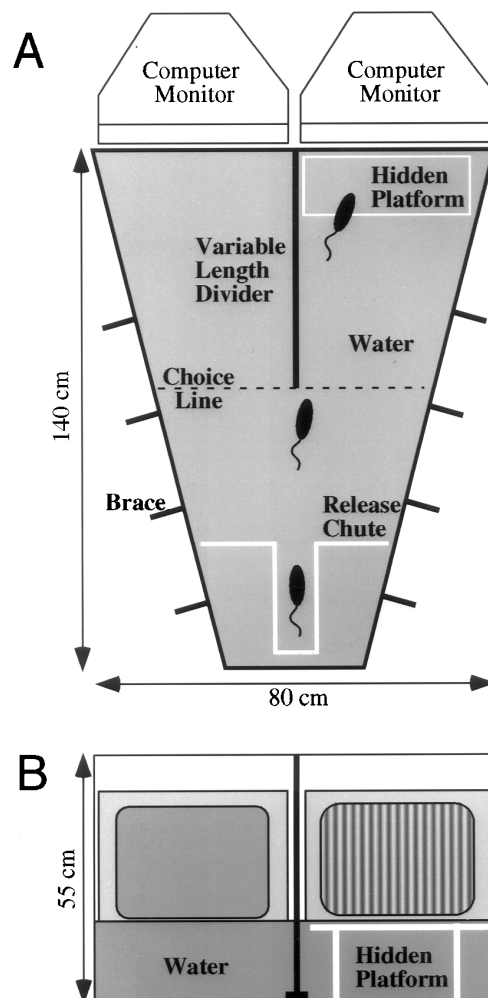
In both cases we trained P30 mice both from EE and from ST conditions to discriminate between vertical gratings whit different frequencies. Normally, mice with non-impaired vision were capable of pattern discrimination and they reached the criterion of 70% correct within the 8 days of testing. The rationale of the task described here is to use an animal's ability to associate a grating with escape from water, as an index of its acuity.

The basic apparatus consists of a trapezoidal-shaped pool with two computer-controlled monitors placed side-by-side at one end (**Fig. 11**). A flat divider is painted black on both sides to make it opaque and reduce reflections within the pool. The length of the divider sets the choice point and effective spatial frequency: it is the closest an animal can get to the monitors without entering one of the two arms. A portable escape platform is placed below one of the monitors. The pool is filled with tepid (22°C) water. Screen reflections on the surface of the water render the platform invisible from water level. Visual stimuli are displayed on two identical computer monitors that face into the wide end of the pool. The bottoms of the screens are at water level. The black levels and contrast of the monitors are equated and the mean luminance is set at 39.95 candels/m^2. The monitors are driven by a home-made software which presents visual stimuli in a left-right pseudo random order.

There are three phases in the task: 1) shaping; 2) task training and 3) acuity testing. In a shaping phase, animals are shaped gradually to locate a platform hidden below a screen displaying a low spatial frequency grating (0.117 cycles per degree). On the first trial, animals are removed from their holding cage and released, facing the screen, into the pool a few centimeters from the platform. On the next trial, the location of the grating and the platform are switched to the opposite side and another trial is run. After this routine is repeated a few times, the release distance from the platform is gradually increased until animals can reliably swim to the platform from the opposite end of the pool. In the training phase, animals are conditioned to distinguish between a low spatial frequency sine-wave grating (0.117 cpd)

and homogeneous gray. On all trials, animals are required to swim until they find the platform. After the animals have achieved near-perfect (80%) performance over 20–40 trials on a pseudorandom schedule in the training phase, the testing of visual acuity can begin. For the testing phase we used a method to minimize the number of incorrect responses by the animals (Prusky et al. 2000).small incremental changes in the spatial frequency of the stimulus were made between successive blocks of trials until the ability of animals to distinguish a grating from gray reached the same of the chance. If the animal made a correct choice, the spatial frequency of the stimulus was increased by adding one cycle on the screen, and another trial was executed. This procedure continued for the low spatial frequencies until an error was made. After the number of trials covering approximately half of the animal's postulated threshold was completed, the minimum number of trials was increased to three, and then again increased to four around three-quarters of the projected threshold. This method of consecutive testing utilizes about 15 different spatial frequencies.

We tested mice in groups of five to six in a session of ten interleaved trials, with each session lasting 45–60 min and no more than three sessions were performed in a single day. A preliminary threshold was attained when mice fail to achieve 70% accuracy at a spatial frequency. In order to ensure the accuracy of this estimate, spatial frequencies around the threshold were re-tested until a clear pattern of performance was generated.

A similar procedure was used to measure contrast sensitivity but, in this case, we used the spatial frequency as a fixed parameter and the stimulus contrast as the changing one.

The highest spatial frequency or contrast achieved were recorded as the acuity threshold or contrast threshold, respectively. The data around the estimate were averaged and a frequency-of-seeing curve or a contrast-of-seeing curve was built. The time necessary to estimate thresholds with accuracy varies between 2 and 3 days.

4.9 Electroretinogram recordings (ERG)

To gain insight into retinal function of EE mice and ST mice we collaborated with the group of Prof. Gargini at the University of Pisa, who performed Electroretinogram (ERG) recordings. Mice were dark-adapted overnight and the day after anesthetized with an i.p. injection of uretane 20% (0,1 ml/10 g body weight). Body temperature was maintained at 37 °C. Pupils were dilated by administration of tropicamide (1%) eye drops, and the cornea was kept moist with a methylcellulose solution. For ERG recordings, an electronic flash unit generated a light stimulus of 492 nm whose energy decayed with a τ of 1.7 ms. Full-field stimulation was achieved using a Ganzfeld sphere, and flash intensities were attenuated by neutral-density filters. Mice were subjected to 8 different flash intensities, each repeated five times, with an interstimulus interval that ranged from 30 s for dim light to 60 s for the brightest flashes. The flash luminance was measured at the corneal plane in photometric units (cd·s/m2) using a Minolta CS100 photometer with a scotopic filter. ERG signals were recorded using coiled gold corneal electrodes. Responses were differentially amplified, band-pass-filtered at 0.3–500 Hz, digitized at 0.25- to 0.5 ms intervals, and stored on disk for processing.

Five ERG traces at each flash luminance were averaged before measurements of b-wave amplitudes. The amplitude of the b-wave was measured from the trough of the a-wave to the peak of the b-wave. Isolated cone components (photopic ERG) were obtained by superimposing test flashes on a background of saturating intensity for rods (30 cd·s/m2).

5 RESULTS

5.1 Early EE retards the onset of rod and cone loss during weaning

In rd10 mice, rod death starts around postnatal day 18 (P18), peaks at around P24 and is almost complete by P40. A rod-driven ERG is extinguished at around P30. Cone death proceeds at a slower rate and a cone-mediated ERG can be recorded only up to P55-P60 (Gargini et al. 2007).

5.1.1 Photoreceptor survival in EE: pycnotic photoreceptors

We started our studies by estimating the death rate of rod photoreceptors, by counting pycnotic nuclei (positive for Ethidium homodimer 2 marker) in whole mount retinal preparations from rd10 mice aged P24 and reared either in EE or in ST condition. This age was chosen because, as already mentioned, it is around the peak of rod death for this mutant.

As shown in **Fig. 12** , we found a higher number of pycnotic nuclei in ST as compared to age-matched EE retinas. Total values were: ST, 17156,51 (SE±1619; n=6) pycnotic photoreceptor nuclei per retina; EE, 28698,29 (SE±4449,8; n=6). Thus, EE delays the death of rods related to the rd10 mutation.
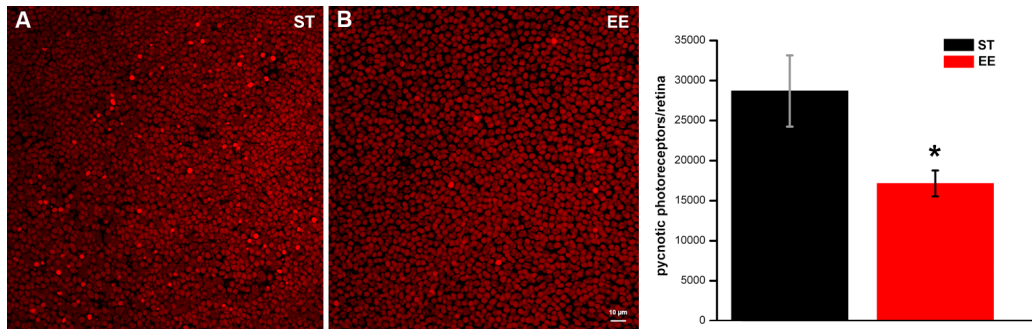
Fig. 12. Pycnotic profiles in the ONL are lower in EE retinas (B) than in age matched mutants exposed to standard conditions (ST) (A). Red signal: Ethidium homodimer 2. The graphic shows counts of pycnotic cell nuclei in retinas from ST (black bars) and EE (red bars) rd10 mice at 24 days of age (C). Counts are from 6 retinas from different animals for each column. Bars indicate SEM. Student t-test: *p= 0.036.

5.1.2 Photoreceptor survival in EE: quantitative analysis and morphology

Two groups of 6 mice aged P45 from either EE and ST conditions were enucleated and their retinas used for evaluate the persistence of surviving cells in the ONL.

We found that the number of surviving photoreceptors is consistently higher in retinas of rd10 P45 mice born and raised in EE than in those of rd10 age-matched controls maintained in ST conditions (**Fig.13**). The number of photoreceptor rows in EE retinas have a mean of 1,57 (SE±0,33) against a mean of 1,15 (SE±0,04).
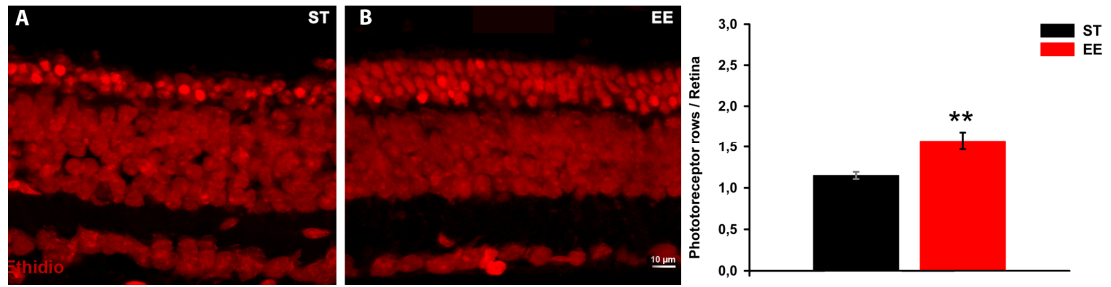
Fig. 13. Vertical retinal sections from 45-day-old rd10 mice raised in ST (**A**) and EE (**B**). Nuclear counterstaining in red is used for counting nuclear rows. EE is associated with a greater number of nuclear rows in the ONL. Counts are from 3 retinas from both groups (**C**). The number of photoreceptor rows in EE retinas is greater (mean 1,57± 0,10) than in ST retinas(1,15± 0,04) Student t-test. **: p=0.002.

We also assessed the preservation of single photoreceptor types by means of vertical sections from P45 EE and ST retinas. This study revealed that rods in retinas of EE mice have elongated outer segments and still express the light sensitive channel ("the" GMP channel specific for rods). a protein virtually missing in ST retinal samples (**Fig. 14**).

PSD95 (postsynaptic density 95 protein) is a well described marker of photoreceptor synaptic terminals in the OPL (Koulen et al. 1998). We found that this marker is still clearly identifiable in the OPL of EE retinas and almost undetectable in ST controls (**Fig. 14**).
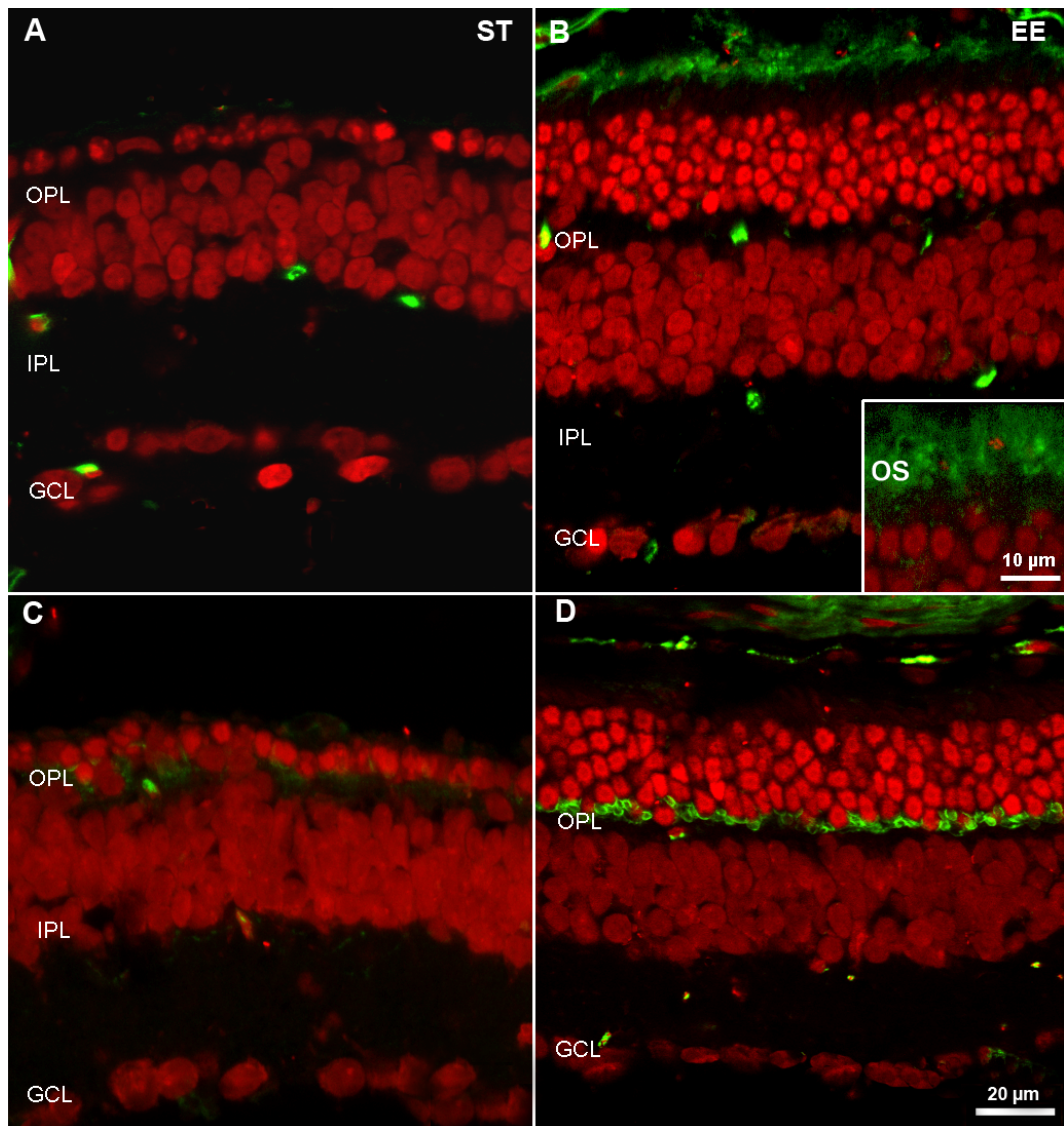
Fig. 14. Vertical retinal sections from 45-day-old rd10 mice raised respectively in environmental enrichment (EE) (**B**) or in standard conditions (ST) (**A**). EE is associated with a greater number of nuclear rows and preservation of rod outer segments. **A** and **B**: cGMP-gated light-sensitive channel staining of rods (green) and nuclear counterstaining (red). **C** and **D**: PSD-95 staining for synaptic terminals of photoreceptors in the OPL (green) show that in EE retinas these are much better preserved.

5.1.3 Photoreceptors survival in EE: cones

We found a higher preservation of cones in EE versus ST retinas aged 45 days. This is clearly visible by immunocitochemistry on retinas slides, as it is shown in **Fig. 15,** where the sections are immunostained with cone-Opsins specific antibodies.
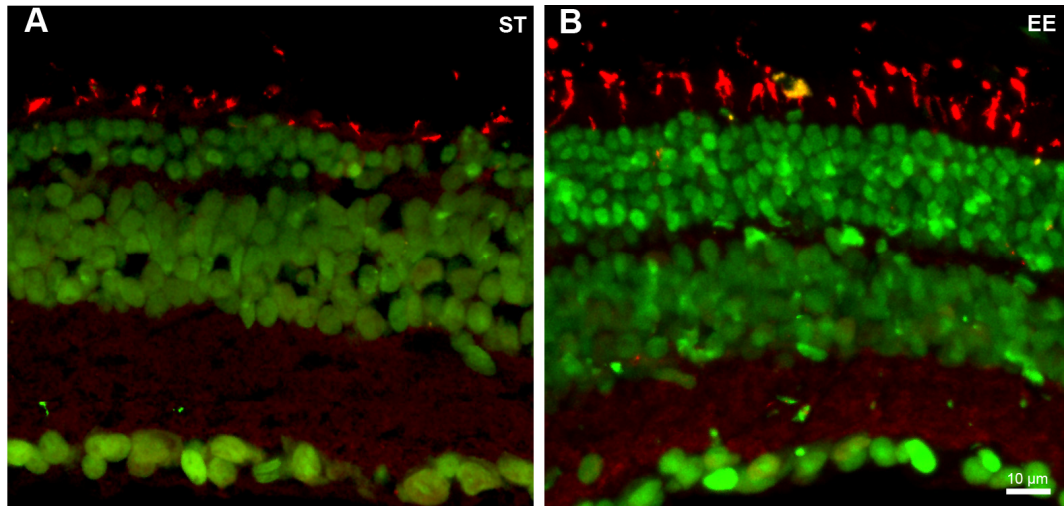

Fig. 15. Vertical retinal sections from ST mice (**A**) and EE mice (**B**) aged P45. Red signal shows immunostaining for cone outer segments that are better preserved than in ST mice. Nuclei are counterstained in green.

As described in Methods, we developed an *ad hoc* procedure for cone counting that allows to see local cell distributions in whole mount retinas. We validated this method counting two retinas from adult Wild Type (WT) mice of the same genetic background (C57BL/6) and obtaining a result similar to what previously shown in literature (**Fig. 16**) (Jeon et al. 1998). With this method, we found that at 45 days of age approximately 100,000 cones persist in retinas of EE rd10 mice, instead of a mean of 83,000 cones in ST rd10 mice. An area devoid of cones starts to become visible in the central part of ST retinas at this age (**Fig. 16**).
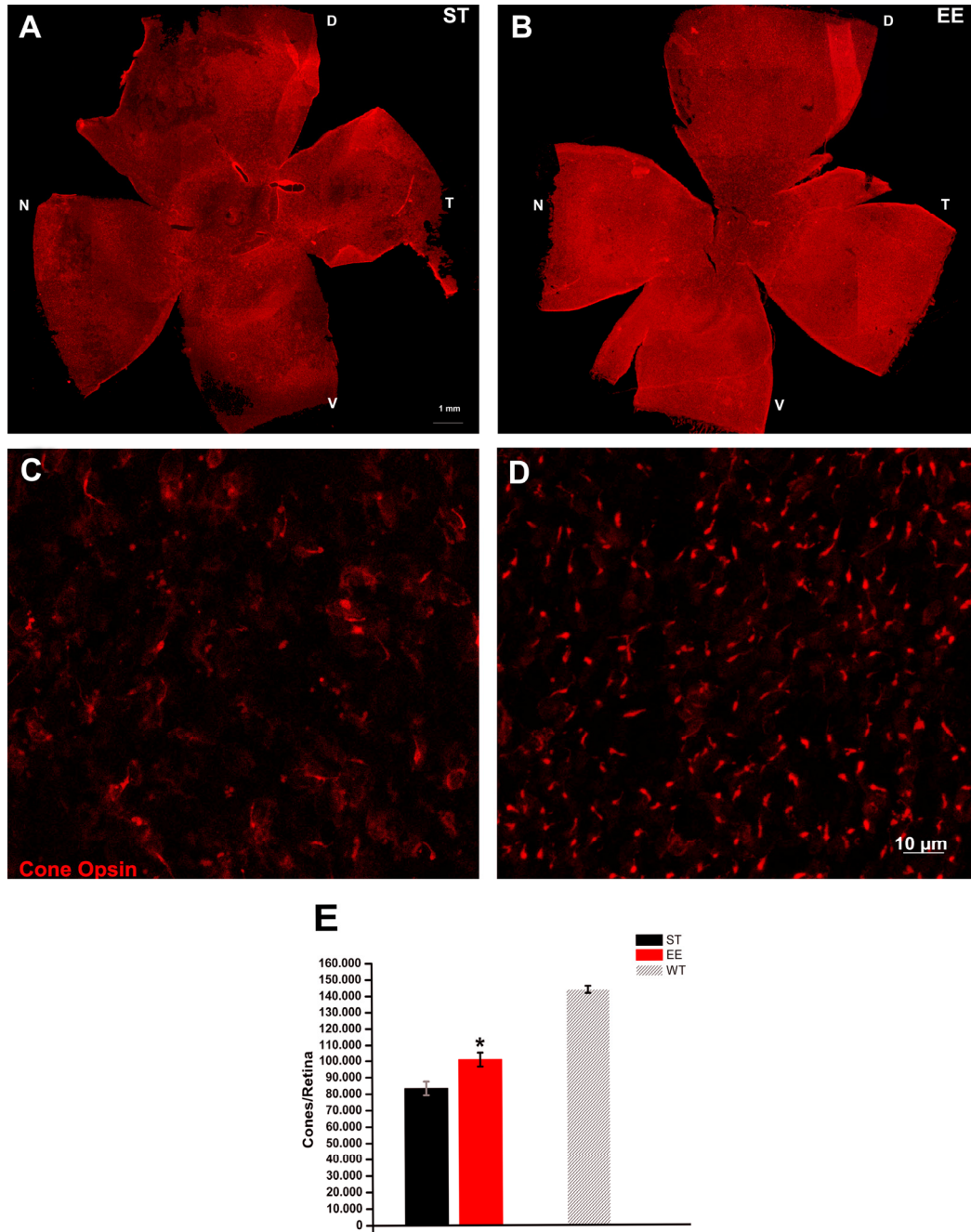
Fig. 16. Examples of whole mount retina immunostaining for specific cone-Opsins (red) from mice aged P45 raised in ST and EE condition (**A** and **B**).

High-magnification confocal images of retinas stained for cone-Opsins from rd10 mice raised in ST or in EE (**C** and **D**). Focus is on outer segments. Note that residual cones in ST start to lose outer segments while the latter are still considerably long in EE.

Counts of cones in retinas from rd10 mice (n=6 for condition) raised in ST (black bar) and EE (red bar) at 45 days of age and from wild type mice at P60 (gray bar) (**E**).

Values are mean of means and SEM. Student's t test. *p=0.018.

5.1.4 Inner retina in EE: morphology

Considerable literature shows that photoreceptors death is followed by a process of secondary remodeling that takes place in the inner retina and that includes dendritic retraction in bipolar and horizontal cells, displacement of rod bipolar cell bodies in the outer retina and progressive synaptic atrophy of the Outer Plexiform Layer (Marc et al. 2003). These processes also affect rd10 mice (Gargini et al., 2007).

We extended our observations to the inner retina of rd10 mutant mice born and raised in EE or born and raised in ST. Immunocitochemistry studies of P45 retinal sections revealed that the EE inner retina is better preserved than the ST inner retina. Indeed, rod bipolar cells still maintain their laminar position and their dendritic tree, and are still connected to photoreceptors as shown in **Fig. 17**.
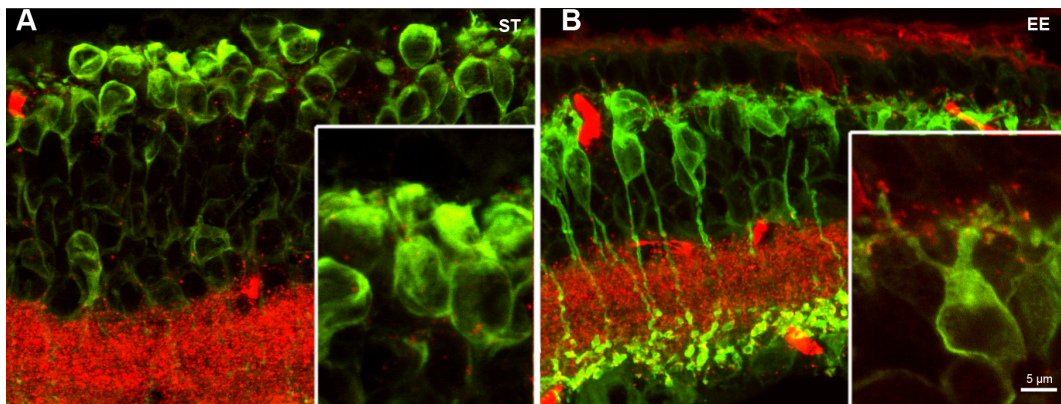

Fig. 17. Vertical retinal sections showing inner retina morphology in P45 mutant mice raised in ST condition (**A**) and EE conditions (**B**). Green signal: PKC immunostaining for rod bipolar cell shows how better preservation of their morphology in EE retinas. Red signal shows immunostaining for bassoon (a typical presynaptic protein).

The quantification method based on topography after opsin-immunostaining on whole mount retinas was used also on animals aged P60. Using 6 animals from EE and ST groups, we confirmed that twice as many cones survive in the EE compared to the ST retinas at this age. Approximately 95,000 cones (i.e. over 65% of the cone population of a normal mouse retina) persist in retinas of EE rd10 mice at this age. This is a remarkable observation considering that only 52,000 aberrant cones (36% of the total number) survive in retinas from age-matched ST control animals. Unlike what we observed in P45 retinas, most of the opsin immunoreactivity in cones from ST retinas is concentrated in cell bodies while outer segments are virtually undetectable (**Fig. 18**). Cones with semi-normal morphology and shorter outer segments are observed in EE but not in ST mice (**Fig. 18**).
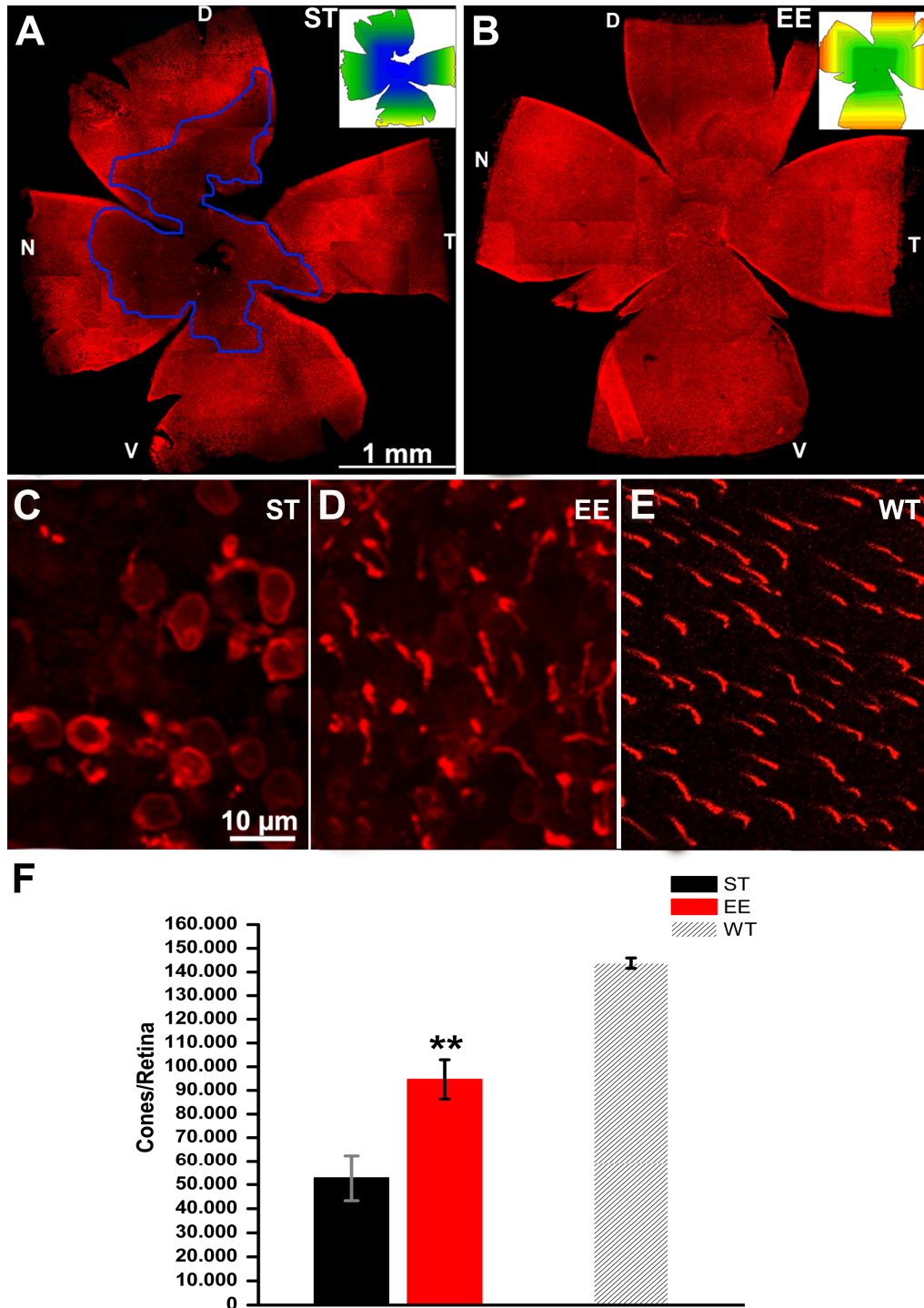
Fig. 18. Examples of retinal whole mounts from standard (**A**) and enriched (**B**) rd10 mice stained with antibodies against cone-specific opsins (red labeling). An area devoid of cones is clearly evident in the central part of the retina shown in A (blue line); a central-to-periphery pattern of degeneration is typical of rodent models of RP. High magnification confocal images of cones from WT (**E**), rd10 ST (**C**) and rd10 EE (**D**) retinas. Note that residual cones have lost outer segments in **C**, while they are still considerably long in **D**. **F**: counts of retinal cones in retinas from ST (black bars) and EE (red bars) rd10 mice at 60 days of age. The number of cones in the retina of normal, wt mice of the C57Bl6/J strain is also shown (shaded column). Bars indicate SEM. Student T test. ******: p<0.01. Counts are from 6 retinas from different animals for each column.

Morphological signs of retinal regressive events are significantly reduced in rd10 EE mice (**Fig. 19**), where rod bipolar cells, stained with PKCα antibodies, show intimate association between dendrites and photoreceptor endings stained whit PSD95 in the Outer Plexiform Layer. Moreover, bassoon antibodies, labeling synaptic ribbons in photoreceptor terminals, show that these are also more preserved in EE than in ST controls (**Fig. 19**).
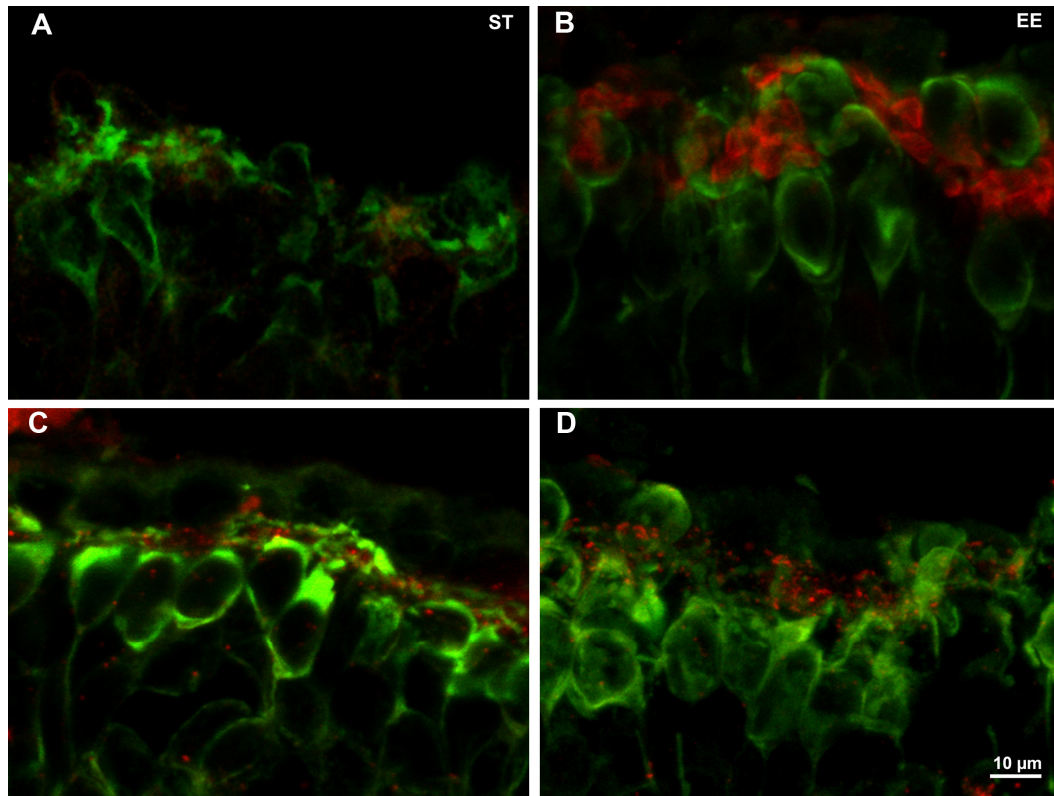

Fig. 19. Morphology of inner retinal cells in mice raised in ST (**A** and **C**)and EE (**B** and **D**). **A** and **B**: Vertical retinal sections stained with antibodies against PKCα (green), labeling rod bipolar cells, and with antibodies against PSD95 (red) that, in the outer plexiform layer (OPL), label synaptic contacts established by photoreceptors. Age is P60. In EE, bipolar cells dendrites are decorated by bassoon-positive puncta (red, bottom panel) (**D**) that are less numerous in ST (**C**).

5.1.5 Photoreceptor survival in EE: western blot.

We evaluated, by Western Blot analysis, the expression of photoreceptor specific proteins to validate the higher survival and preservation of rods and cones in EE rd10 mice. In line with what previously found by means of immunostaining techniques, we observed that retinal levels of the same photoreceptor-specific proteins (and namely, Light sensitive channel, Rhodopsin and cone-Opsins) are higher in retinas of EE than in ST age matched mice (**Fig. 20A**). Measurements performed on sets of 3 gels from different retinal samples show average values of cone-Opsins that are 20 % higher in EE as compared to ST samples. Similarly, the light sensitive channel has an expression of 54% higher than ST level, while rhodopsin is 10% higher in EE retinal preparations (**Fig. 20B**).
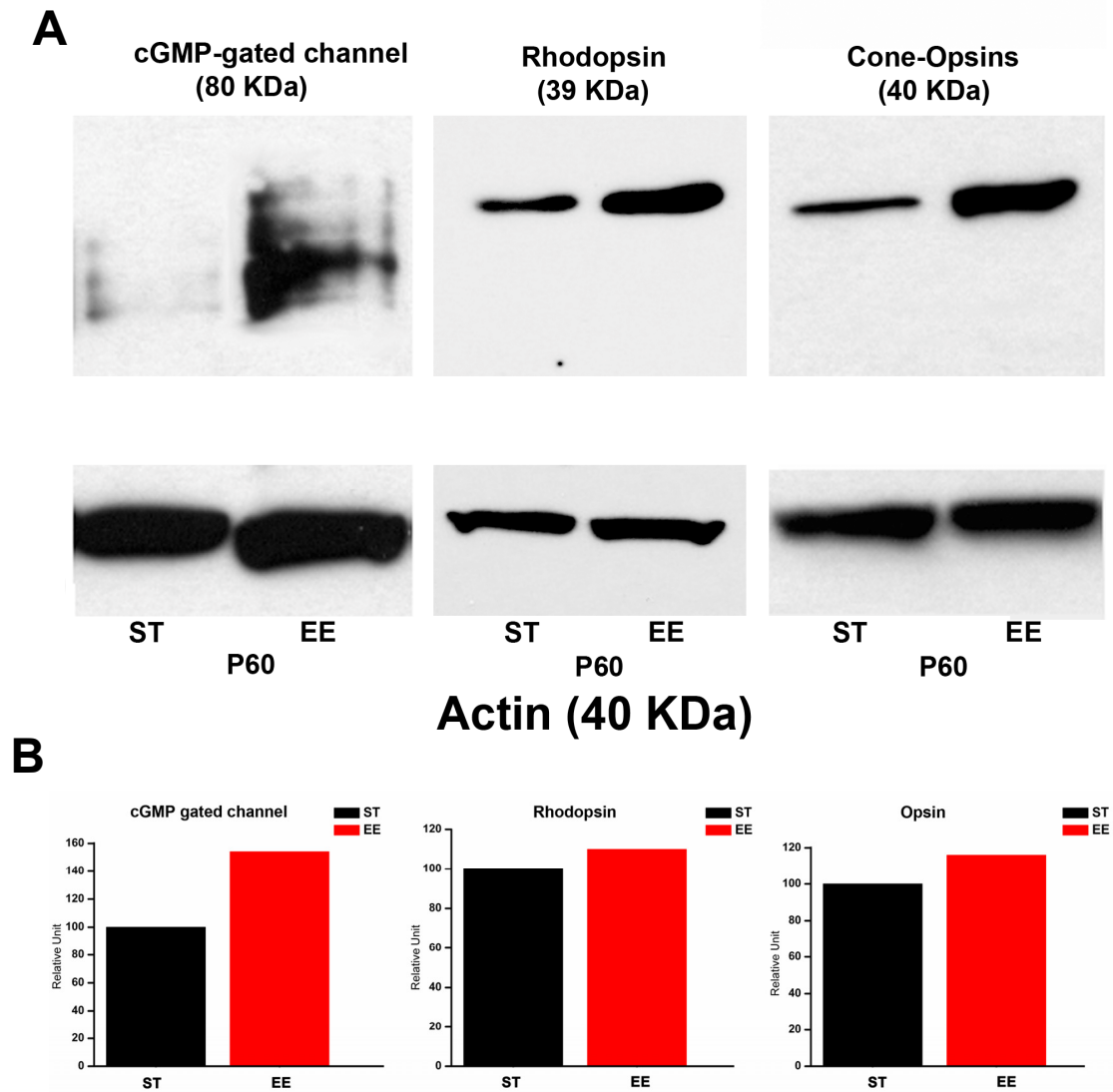
Fig. 20. (A) Western blots for photoreceptor specific proteins from retinal preparations obtained from ST and EE rd10 mice aged 60 days. The rod specific, cGMP-gated channel is 54% more express in EE retinas than ST retinas, also rhodopsin have an 10% higher expression in EE than in the ST samples, respectively. Cone-Opsins are 20% higher expressed in EE than in ST retinas. Homogenates were obtained from 3 different retinal preparations. Loading on gel and subsequent quantification were done in duplicate.**(B)** western blot densitometry results normalized against each ST actin level. Values represent the arbitrary mean value.

5.1.6 Retinal physiology in EE: electroretinogram recordings

Morphological preservation of cones does not imply automatically their ability to function properly. Hence, we used mice (ST and EE) to test the photoreceptor ability to respond to light by electrophysiological recording of the ERG in different age groups (P45, P60 and P75). Results from ERG measurements are illustrated in **Figure 21**. Individual photopic ERG responses from P60 EE and age-matched ST rd10 mice are compared in **A**. The photopic b-wave amplitude as a function of the flash luminance in P60 EE and age-matched ST mice is compared in **B**. The amplitude of the photopic b-wave at P45, P60 and P75 is reported in **C** respectively for ST and EE exposed mice. It is seen that EE exposure is associated to photopic (i.e. cone-driven) responses that at P60 and P75 are significantly larger than in ST mice.
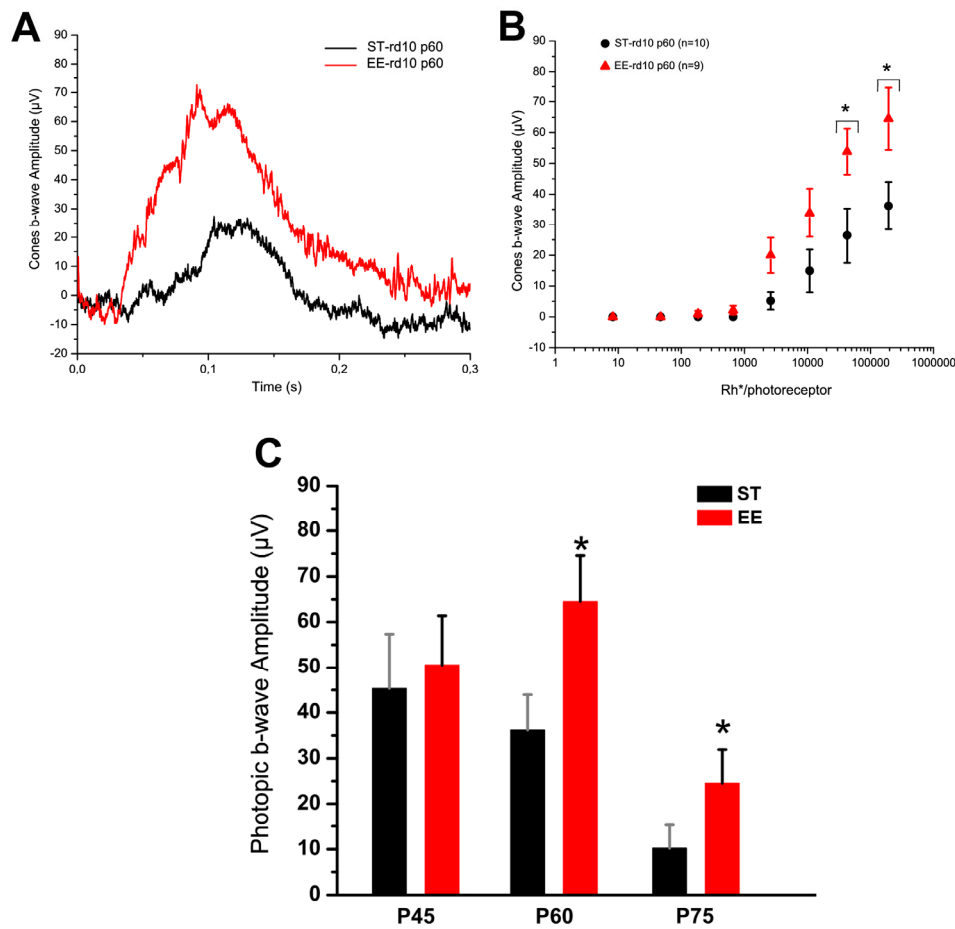
Fig. 21. (**A**) b-wave amplitudes of photopic ERG responses at high luminance
(192,600 Rh*/photoreceptor) are significantly more preserved in EE at P60 .
(**B**) Photopic ERG responses recorded at P60 and as a function of the flash intensity.At
high intensities, amplitudes are significantly greater in mice raised in EE. Schematic
representation of b-wave at P45, P60 and P75 is reported in (**C**) respectively for ST and EE
exposed mice. Bars indicate SEM. Student t-Test (*****p<0.05).

5.1.7 Visual system physiology in EE: behavioral tests

In a group of 10 EE and ST mice we assessed overall visual performance by
measuring visual acuity and contrast sensitivity in photopic conditions by
means of the visual water maze task. For both measurements, mice learn to
locate an escaping submerged platform whose position is indicated by a visual
stimulus consisting of a grating of increasing spatial frequency or decreasing
contrast (see Methods for further details). The highest spatial frequency

perceived defines the upper limit of the visual acuity which in turn is set by the cone density (Prusky et al. 2000). At all the age tested, EE mice performed significantly better than age-matched ST. Indeed, despite we have trained the two groups of animals (ST and EE) together at the age of P30, since the first age that we have tested EE mice performed an higher value of VA respect to ST age-matched mice. Starting from P45 (the first age analyzed) EE mice had amean of VA of 0,44 instead of a mean of 0,26 c/dg of age-matched ST mice, later the ST mice Visual Acuity decrease and remain constant on a mean value of 0,23 and the EE mice VA remain higher (EE VA mean=0,29 c/dg). Even at 8 months of age, a residual average acuity of 0.20 cycles/degree was still measured in EE animals. ST mice turned out unable to perform the test by the age of 6 months (**Fig. 22**).

In the same cohorts of animals we also measured contrast sensitivity using as stimuli black and white gratings of fixed frequency (0.117 cycles/degree) and progressively decreasing contrast. Contrast sensitivity is of paramount importance in assessing the performance of the visual system because it also reflects the integrity of the overall neuronal processing of visual signals (Histed et al. 2011). We tested contrast sensitivity in EE and ST mice aged P45, P60 and P90 (**Fig. 23**). Although higher than in normal wt mice (**Fig. 22**) at 45 days, a minimum 7% contrast is necessary for task discrimination in EE mice; this value rises to 9% at 60 days and to 16% at 90 days. In age-matched ST mice, minimum contrast values were several times higher, namely 25% and 35% at, respectively, 45 and 60 days, while they reached 55% at 90 days.
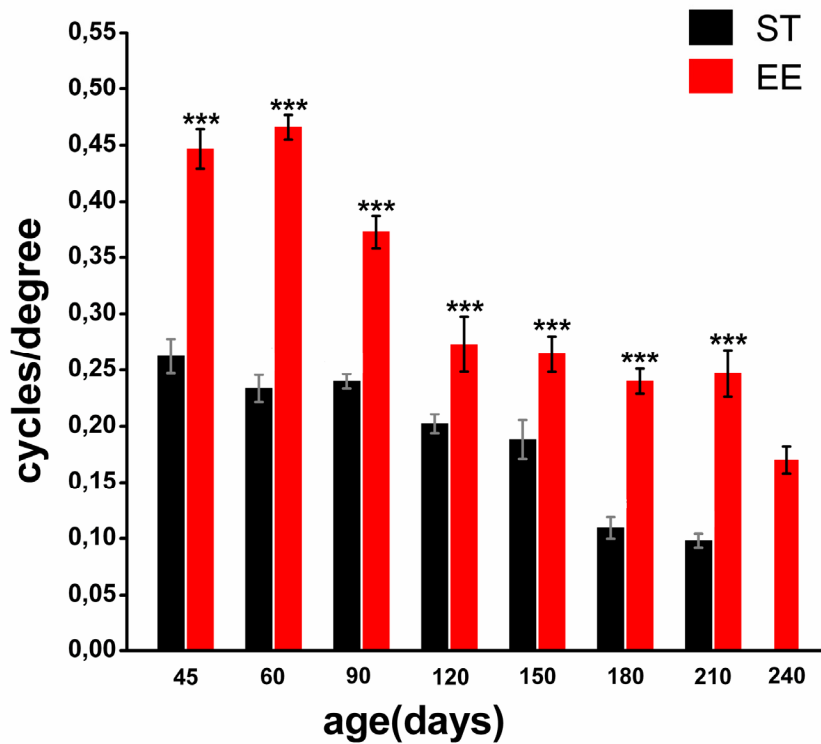
Fig. 22. Visual acuity measured at various ages in ST and EE mice with Prusky water maze.

Acuity is significantly higher in EE animals at all the ages tested. Bars indicate SEM. Student t test. *** $p < 0.001$. Each column is the average response from a cohort of 10 animals.
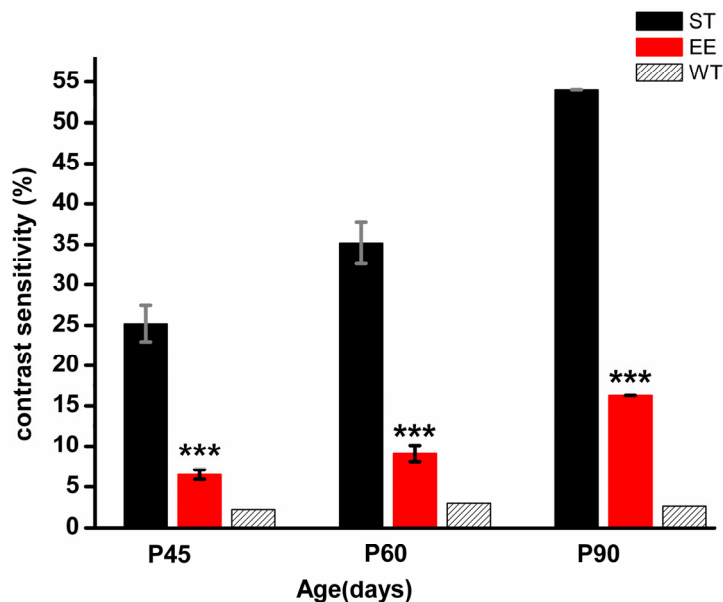

Fig. 23. Contrast sensitivity measured by means of a Prusky water maze. Stimuli were gratings of decreasing contrast, fixed frequency of 0.117 cycles/degree and fixed luminance of 39.95 candels/m2 . At P45, P60 and P90, contrast sensitivity was higher (and therefore the minimum contrast detected lower) in EE as compared to ST rd10 mice. The sensitivity of wt animals is obviously even higher. Student t test. ***: $p < 0.001$.

5.2 EE might change the expression of specific mRNAs in retinas of adult rd10 mice: preliminary data

Numerous studies suggest that retinal neurons have an endogenous mechanism of protection from chronic injury and genetic degeneration. For example, mild light stress has been shown to induce endogenous protection of photoreceptors from a subsequent light damage, and protection coincided with prolonged up-regulation of neurotrophic factors (Liu et al. 1998). Indeed, bFGF mRNA expression was up-regulated in mice homozygous for the *rd1* mutation in the PDE6b gene (a model of Retinitis pigmentosa whit a faster photoreceptor degeneration than our model) (Gao and Hollyfield 1995). Elevated levels of retinal bFGF or CNTF expression, but not of BDNF expression, were also found in light-induced as well as inherited models of photoreceptor degeneration in both mice and rats (citare). These studies report that contrary to the initial working hypothesis, a reduction in BDNF levels resulted in photoreceptor protection against light damage. Survival was paralleled by a reduction in oxidative stress and the preservation of neurotrophin levels, suggesting that chronic reduction of BDNF in the retina provides a level of preconditioning against stress (Gao and Hollyfield 1996; Wen et al. 1998). Collectively, these studies demonstrate that stress in the retina caused by light, mechanical injury, and genetic mutations can induce endogenous up-regulation or modification of neurotrophic factors. Regulation of these factors is likely functional, since a number of studies have shown that injection or viral-mediated expression of these factors can protect photoreceptors (LaVail et al. 1992; Sieving et al. 2006).

All together our results point to the effectiveness of EE in extending the time window during which rd10 mutant mice can effectively see. In order to test the hypothesis that EE promotes production and release of neurotrophic factors in the retina, we measured by qRT-PCR retinal mRNA for the neurotrophins already known to promote survival of photoreceptors.

In our preliminary studies we did not find any major change in gene expression for BDNF and NGF between EE and age-matched ST mice. Data appear variable from sample to sample and this might reflect a true biological difference among different animals, as well as the combination of factors running out at a given age (photoreceptor death, reactive gliosis, completion of of retinal development). However, we found a down regulation of CNTF in EE mice at P13. Such down regulation does not appear to extend to adult life, when CNTF mRNAs becomes similar in EE and ST retina mice (**Fig. 24**).
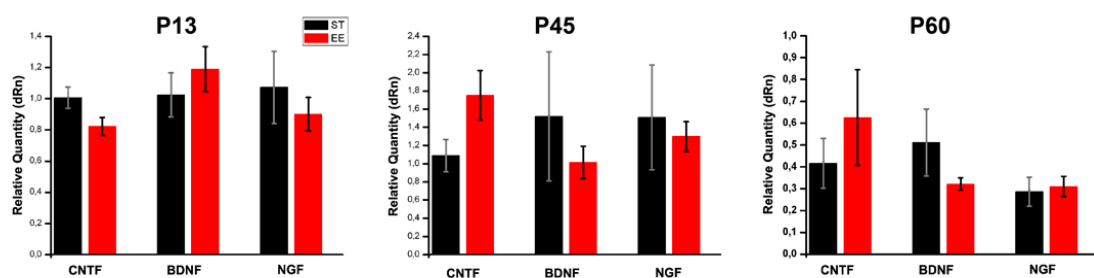

Fig. 24. Relative amounts of mRNA for CNTF, BDNF and NGF detected by qRT-PCR in retinal extracts from rd10 mice raised in ST (black bars) and EE (red bars) at 3 different ages. Rq values (referred to GADPH mRNA taken as reference value) in EE were normalized to those of ST. CNTF mRNA significantly higher at P45 and P60.

Building a time-course diagram of gene expression for BDNF, NGF and CNTF reveals an effect of EE on neurotrophin gene expression. Indeed, as it is shows in **Fig .25,** there is a clear and selective down regulation of BDNF mRNAs in EE retinas during the period P13-P45-P60, with respect to the expression in ST retinas. Comparing the two types of treatment (EE vs. ST) by means of ANOVA two way test, we found a statistically significant difference (P=0.036) between the mean of ST retina extracts and the mean of EE retina extracs. CNTF mRNAs expression in EE appears to move in a reverse way respect to ST **(Fig. 25).** At least NGF mRNAs expression does not seem to be affected by EE treatment during the time window considered.
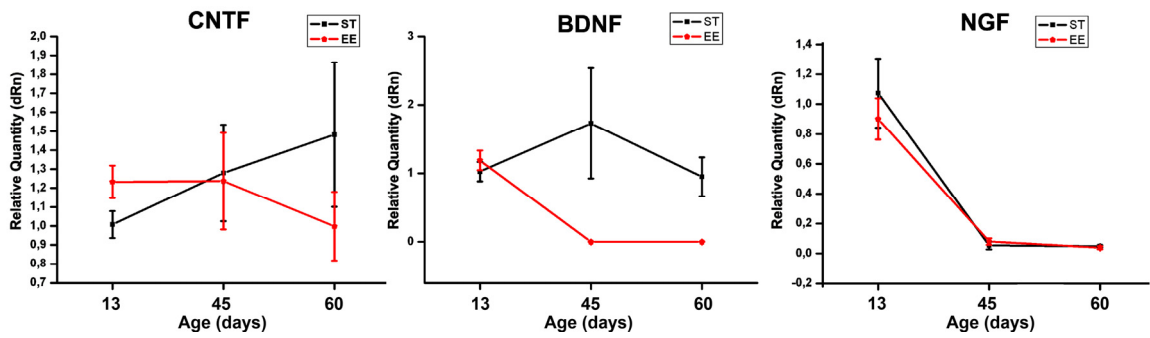
Fig. 25. Relative amounts of mRNA for CNTF, BDNF and NGF detected by qRT-PCR in retinal extracts from rd10 mice raised in ST (black) and EE (red) during 3 different ages. Rq values (referred to the mean of GADPH mRNA mean at 13 days of life taken as reference value) in EE were normalized to those of ST. There is a statistically significant difference in BDNF mRNAs expression between ST and EE treatments during the time point analyzed. ANOVA two way *p= 0,036.

5.3 Long term effects of EE

The results reported in this thesis reveal that early exposure to EE is a powerful tool able to expand the window of cone viability during the peak of rod degeneration rate and afterward. One main purpose of this thesis is to assess if EE can delay the degeneration, in particularly of cones, even in the long run, also in perspective of cures for human RP requiring cone maintenance. Thus, we allowed a group of rd10 mice born and raised in enriched conditions to stay under EE until reaching one year of age. Then, we started analyzing retinal morphology in these animals, comparing it to that of age-matched rd10 mice always kept in ST conditions. On the same groups of animals, we also performed behavioral experiments to determine visual acuity.

Low magnification fluorescence microscopy of whole mount retinas, again immunostained with cone-specific opsins for S and M/L cones, did not show apparent differences between retinas of EE and ST mice at one year of age. However, cone counting revealed that, even if a continued decline in the number of residual cones was present both in EE and in ST mice, rd10 EE mice of 1 year showed almost three times as many surviving cones (34000±4000) than ST control mice (12700±1800)t-test **p=0.003 (**Fig. 26**). Moreover, although cone remodeling proceeded in both groups of animals in the same way demonstrated in literature (Lin et al. 2009), in EE retinas residual cones retained a form more reminiscent of their original shape, with clear remnants of outer segments. Electron microscopy analysis is programmed to confirm this point.

To look through the morphology of the inner retina, we analyzed again the distribution of different markers such as PKC α (for rod bipolar cells) and Calbindin D (for horizontal and amacrine cells), comparing retinal sections of

rd10 mice kept in EE condition or in ST condition at 1 year of age. We did not detect any difference (**Fig. 27**).

And yet, at 1 years of age, EE mice are still capable to perform the visual water task. This demonstrated that EE mice at 1 years of age still conserv a residual visual acuity (0,2 c/dg) that is virtually absent in ST age-matched mice(0,06 c/dg) (**Fig.28**).
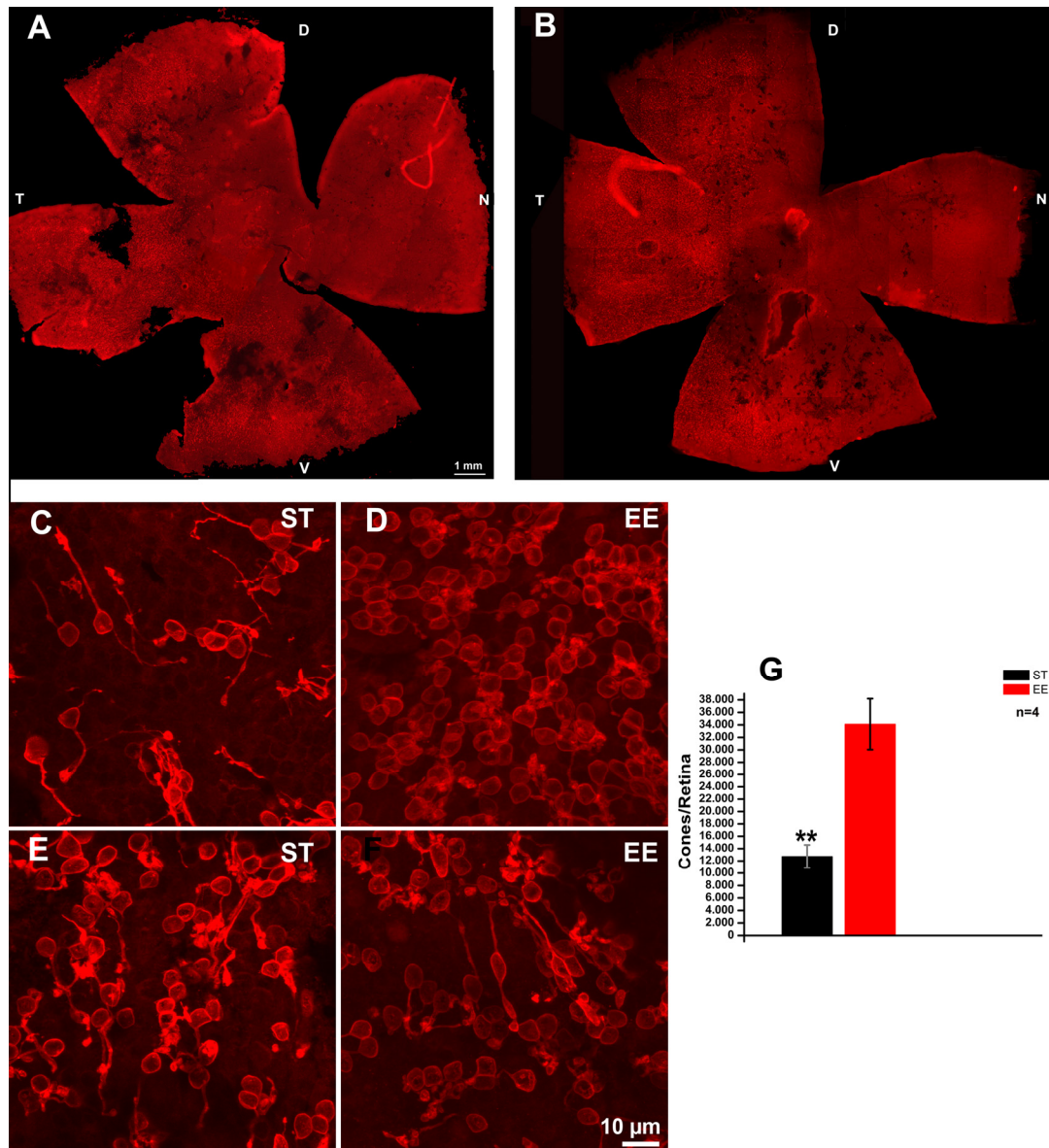
Fig. 26. Two examples of retinal morphology and survival of cones in mice raised in ST and EE. (**A** and **B**) Retinal whole mounts from 1 year old rd10 mice raised in ST (**A**) and EE (**B**), stained with antibodies against cone-specific opsins (red). A big area devoid of cones is evident in the central part of the retina, showing a nasal-temporal pattern of degeneration. Cones are still preserved in the periphery of both retinas. No major differences are evident.

High-magnification confocal images of retinas stained for cone opsin from rd10 mice raised in ST (**C:** central retina and **E:** peripheral retina) or in EE (**D:** central retina and **F:** peripheral retina) shows that 1) local density is higher in EE as compared to ST samples. 2) the cone remodeling typical of degeneration does occur in both cases, but 3) EE cones display a better morphology(both in centre and in periphery **D** and **F**) and more preserved outer segments in periphery (**F**) .

Counts from 4 retinas of each group show that rd10 EE mice of 1 year have almost three times as many surviving cones (34000±4000) than ST control mice (12700±1800) t-test **p=0.003.
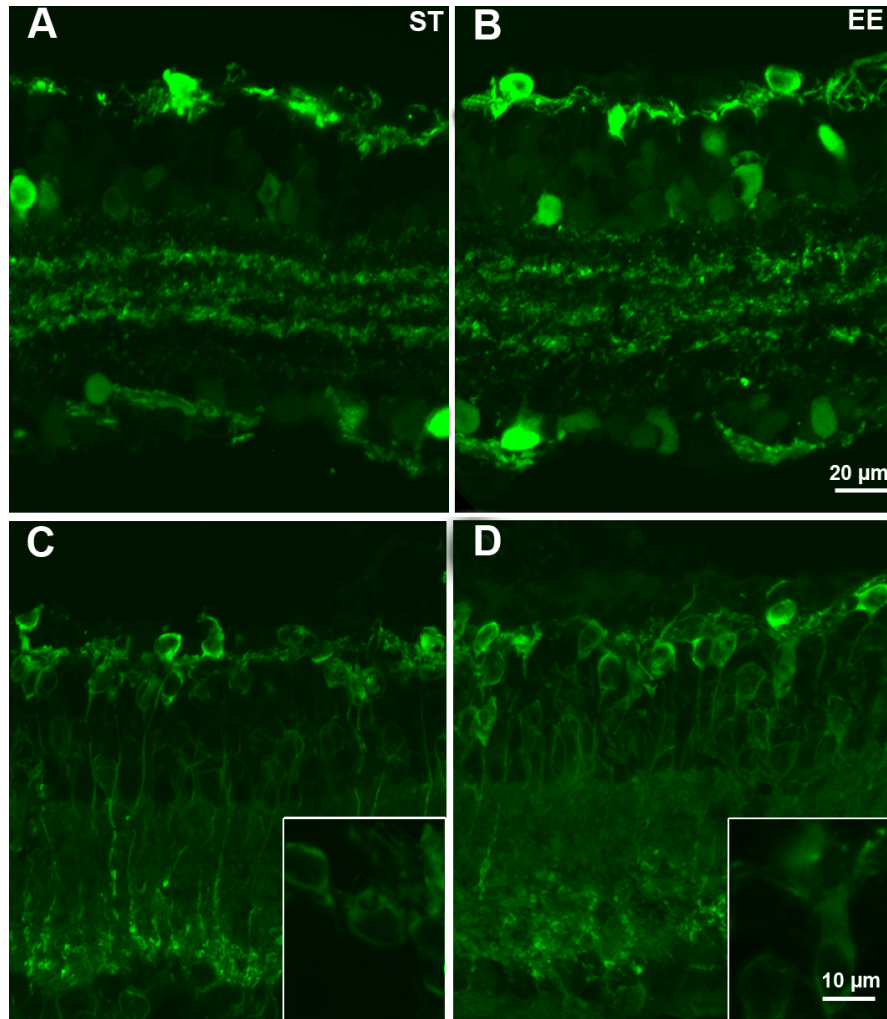
Fig. 27. Vertical retinal sections showing the inner retina at 1 year of age in ST mice (**A** and **C**) and EE mice (**B** and **D**). Starting from top: Calbindin-D-28 staining of horizontal cells and amacrine cells (green). Sections. Sections stained with antibodies against PKCα (green),labeling rod bipolar cells (rbc).

These images do not show a difference between the two groups of animals, but again high magnification shows better preservation of rod bipolar dendritic trees in EE samples.
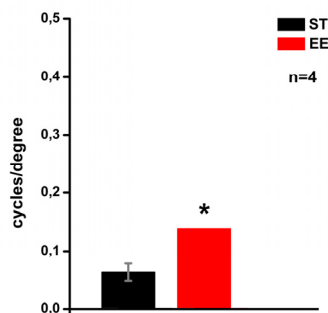

Fig. 28. Visual acuity of rd10 mice 1 year hold is significantly higher in EE than in age-matched ST tested. Student's *t* test, *p=0,029. Each column shows the mean of 4 animals for each groups (and SEM).

6   DISCUSSION

6.1   Early EE retards the onset of rod and cone loss during weaning

Retinitis pigmentosa (RP) is one of the most important subtypes of inhered forms of blindness, actually the second main causes of hereditary blindness in 20-64 years people (Wright et al. 2010). RP is an excellent model for diseases that lead to loss of vision. It has defined genetic causes and there are several animal models with mutations in the same genes leading to human RP.

In our study, we used a well know mouse model of RP, the rd10. It has already been shown that in the rd10 mouse, photoreceptors start to die past the late phases of retinal synaptogenesis (Sharma et al. 2003). Therefore, we analyzed the pycnotic photoreceptor profile at P24 in ST and EE retinas. We found that, while at this age, ST retinas go through a massive photoreceptor death, retinas of EE mice displayed fewer numbers of pycnotic photoreceptor nuclei.

6.2   EE promotes cone survival in RP adult mutant animals

One of the most important aspects of the RP pathology is that secondary degeneration of cones proceeds in parallel at a slower rate than rods and a scattered population of aberrant cones persists for up to at least 9 months of age causing a day blindness in human patients (Chang et al. 2002; Otani et al. 2004). Electrophisiologically, ERG responses cannot be recorded after P55-P60 (Gargini et al. 2007) (I. Piano et all. ERM 2011, Amsterdam Settember 22-24, 2011). Besides rod photoreceptors, that in this model carry the mutation and are doomed to die, our work focused on cone preservation, studying the effects of EE. Indeed, although no cure for RP is presently available, different therapeutic approaches, such as neuroprotection, promise

to delay the intrinsically slow degenerative process of RP by interfering with the death of rod and cone photoreceptors.

Applying from birth the EE paradigm to rd10 mice we found a higher number of cone survival both at P45 and at P60, age in which rod are already die out and also cone photoreceptor are dying. The rationale for this approach lies in the fact that increased rod survival extends cone viability as well (Leveillard et al. 2004; Lipinski et al. 2011). Given the importance that cones have for everyday life, and considering that as little as 10% of the total number of cones is sufficient for independent living in humans (Hartong et al. 2006), developing treatments finalized to extend the lifespan of these cells is particularly relevant. For that reason we decided to apply a neuroprotective approach that had already shown to promote plasticity in the adult nervous system, having remarkable effects in animal models of different CNS affections, the environmental enrichment (EE). EE could pave the way for novel therapeutic strategies for the treatment of brain injuries and neurological disorders in adulthood, when recovery and functional rehabilitation are very hard to achieve.

6.2.1 Photoreceptor survival in EE: morphology

Our data show that in retinas of rd10 mice born and raised in EE, the number of rows of surviving photoreceptors in the outer nuclear layer is higher than in rd10 mice maintained in ST at 45 post-natal days. At the same age in EE, but not ST, rod outer segments showed positive staining with antibodies against rhodopsin and the cGMP-gated light-sensitive channel, suggesting that these cells were still light responsive. Although our data show that in EE rd10 retina several morphological modifications were detectable at 45 days of age, these modifications were much more limited than in rd10 ST counterparts, in which they became widespread at 2 months.

6.2.2 Photoreceptors survival in EE: cones

It is well know that in many forms of retinal degeneration, including RP, the primary pathological event is rod death. In the rd10 mouse model, rods die because they express a missense mutation in *Pde6b* gene (Arg560Cys) (Chang et al. 2007). Afterwards, and for yet unknown reasons, cones cannot survive. The dynamics of this process have been already clearly explained for the rd1 mutant (Lin et al. 2009). These authors showed that after the primary death of rod photoreceptors, cones lose their outer segments and partially retract or lose their axon and synaptic pedicle. Until the end, they retain many fundamental features of the original cone phenotype, and for many weeks the cells also preserve their laminar position, forming a cell row just distal to a much thinned outer plexiform layer. The somata subsequently enlarge (Lin et al. 2009).

In our work, we assessed cone survival at 45 and 60 days of life and we found that at 45 days of age approximately 100,000 cones persisted in retinas of EE rd10 mice, instead of a mean of 83,000 cones in ST rd10 mice. The most exciting observation was that at P60 in control rd10 mice, an area devoid of cones was clearly evident in the central part of the retina, according to the centre-to-periphery pattern of degeneration typical of RP-like rodent retinas. Instead, in rd10 mice raised in EE this degeneration was much milder. At higher magnification, the cones in ST appeared aberrant and devoid of outer segments, with opsins immunoreactivity concentrated in cell bodies. In EE, surviving cones had normal morphology and long outer segments. We found approximately 95,000 cones persisting in retinas of EE at P60, corresponding to over 65% of the cone population of a normal mouse retina. This is a remarkable observation considering that only 53,000 aberrant cones (36% of that in wild type mice) survived in retinas from age matched control animals

in ST. Studying cone morphology in whole mount preparations, we observed that the persisting cones in EE retinas were better conserved that cones in ST retinas at all the age tested, strongly indicating a delay in their secondary remodeling and death.

6.2.3 Inner retina in EE: morphology

Retinal remodeling can be defined as the expression of molecular and cellular phenotypical changes in the neural retina in response to inherited or acquired degenerations of the sensory retina (Marc and Jones 2003). Degenerations in the mammalian retina generally progress through three phases. Phase 1 initiates with expression of a primary insult, followed by phase 2 photoreceptor death that ablates the sensory retina via initial photoreceptor stress, phenotype deconstruction, irreversible stress and cell death, including bystander effects or loss of trophic support. The loss of cones heralds phase 3: a protracted period of global remodeling of the remnant neural retina. Remodeling resembles the responses of many CNS assemblies to differentiation or trauma, and includes neuronal cell death, neuronal and glial migration, elaboration of new neurites and synapses, rewiring of retinal circuits, glial hypertrophy and the evolution of a fibrotic glial seal that isolates the remnant neural retina from the surviving RPE and choroid. It is important to remember that retinal remodeling is not a form of plasticity, but represents the invocation of mechanisms resembling developmental and CNS plasticity in order to preserve at least as possible neural function, involving changes on a set of inner retina cells such as bipolar cells and amacrine cells with a negative impact on visual recovery.

The effects of EE on inner retinal morphology were examined by immunostaining P45 and P60 retinal vertical sections with antigens specific to rod bipolar cells (PKCα) (Vaquero et al. 1996) and horizontal cells (Calbindin

D 28 K) (Lin et al. 2009). In ST retinas from both ages, regressive events were seen as pronounced dendritic retraction of rod bipolar cells and loss of bassoon immunoreactivity, a photoreceptor ribbon synapse marker (Dick et al. 2003). In EE retinas, rod bipolar cell dendrites were more extensively ramified and decorated by bassoon-positive puncta that label synaptic ribbons in photoreceptor terminals. Also the contours of photoreceptor endings in the outer plexiform layer (OPL), were more preserved in EE than in ST as it is show by anti-PSD95 immunolabelling. Thus, EE was effective in limiting the regressive remodeling of retinal neurons postsynaptic to photoreceptors that is typically associated with the death of these cells (Marc et al. 2003).

6.2.4 Photoreceptor survival in EE: western blot.

Increased survival and preservation of rods and cones in EE rd10 mice aged P60 was confirmed by western blot analysis. Retinal expression levels of the same photoreceptor specific proteins documented by immunostaining were higher in EE than in ST. Our experiments demonstrated that cone-Opsins were 20% more expressed in EE retina than in ST. Similarly, the cGMP-gated light-sensitive channel was 54% higher, while rhodopsin was 10% more expressed in EE respect to ST retinas. Thus, convergent results from different morphological and biochemical determinations indicate that exposure of rd10 mice to EE is effective in preserving retinal morphology and synaptic connections and in extending the lifetime of retinal rods and cones.

6.2.5 Retinal physiology in EE: electroretinogram recordings

Electroretinogram recordings of rd10 mutant mice reared in standard laboratory conditions reveal alterations in the physiology of the inner retina as early as P18 (before any obvious morphological change of inner neurons) and

cannot be recorded after P55-P60 (Gargini et al. 2007) (I. Piano et all. ERM 2011, Amsterdam September 22-24, 2011). In our studies at P45, the amplitude of the photopic response did not differ between EE and ST mice. However, at P60 and P75, photopic ERG responses at increasing flash luminance showed greater b-wave amplitude in EE, demonstrating a preservation of cone function. Thus, in EE, surviving cones were viable, functional and capable of generating light responses.

6.2.6 Visual system physiology in EE: behavioral tests

In recent studies carried out in rodents, EE turned out to be very effective for treating amblyopia in adulthood. A brief exposure (two to three weeks) of adult amblyopic rats to EE has been demonstrated to promote a complete recovery of visual acuity, an effect documented not only at the electrophysiological level, but also using behavioral assessments (Sale et al. 2007). Encouraged by this promising results obtained with EE in rodents, we assessed whether the morphological, biochemical and physiological preservation of the retina achieved by raising rd10 mice in EE translated into maintained visual perception. We found that rd10 mice raised in EE had significantly higher VA at all adult time points tested.

We also measured contrast sensitivity. At P45, mice raised in ST had poor contrast sensitivity (requiring a minimum of 25% contrast to locate the platform) and this deteriorated noticeably over time to 54% at P90. Instead, mice raised in EE had significantly better contrast sensitivity at all three time points (detecting gratings with only 7% contrast at P45 and 16% at P90), although here too there was a deterioration with age.

Altogether, these results indicate a great effectiveness of EE in considerably extending the time window during which rd10 mice can effectively see.

6.2.7 EE and neurotrophins mRNA expression

Retinal neurons, such as all different cells in the body, have the same genome, that is, exactly the same DNA nucleotide-sequence, with only a few exceptions in the reproductive and immune systems; thus, liver cells have the same DNA as neurons, but how can two cells have exactly the same DNA and be so different? And in which specific way neurons can be different?

One of the mechanisms that determine the amount and which gene have to be transcript is the regulation of gene expression.

Gene regulation is essential as it increases the versatility and adaptability of an organism by allowing the cell to express protein when needed. Regulation of transcription is a central part of the gene expression and is crucial because controls when transcription occurs and how much RNA is created.

A large part of this pathways in the CNS is influenced by the experience (Ossipow et al. 2004) and the long-term effects of EE are likely mediated by signaling cascades regulating experience-dependent gene transcription, and this has already been demonstrated in the visual system (Putignano et al. 2007). It has been also demonstrated that Environmental Enrichment strongly affects visual system maturation both at retinal and cortical levels, enhancing neurotropic factor expression (Landi et al. 2009). To determine whether the effects on cone survival in retina of EE mice were modulated by increased production of neurotrophins, we measured levels of BDNF, NGF and CNTF mRNAs. We did measurements on retinal extraction of EE and ST animals at P13, P45 and P60. In our preliminary studies, we did not find difference in relative levels of all mRNAs tested between mice raised in ST and EE at all ages tested. The only noticeable observation was that CNTF mRNAs are very variable over time, with a higher intrinsic variability is ST samples. Also,

there was a small decrement of CNTF mRNAs in EE retinas at P13 (day when ST mice open their eyes and two days after EE eye opening), this data have to be confirmed.

However, performing a time-course analysis of the relative gene expression using as a reference the mean of ST P13 housekeeping gene value, we noticed that there was a different modulation of BDNF and CNTF mRNAs expression in EE retinas during the time course of retina development. In particular, there was a down regulation of BDNF during youth in EE retinas with respect to ST retinas (P13); also, there was an inversion of CNTF relative levels of expression between EE and ST retinas in the same time window.

While also these data have to be confirmed, the very fact that only CNTF mRNA is highly variable between the animals from the same group supports the idea that CNTF could be one of the EE effectors and itself, or some molecules that could interact with, influence cone survival and that this factor, alone or in combination with other neurotrophins, might have beneficial effects on the survival of these cells (LaVail et al. 1992; LaVail et al. 1998; Sieving et al. 2006).

There is also evidence that (presumably through Muller cells) a feed-forward loop exists between low levels of BDNF (Wilson et al. 2007) and an increase of CNTF (Wilson et al. 2007) or bFGF (Wilson et al. 2007) in retinas exposed to light damage. Indeed, BDNF levels lower than normal mimic a protection of photoreceptors cells similar to a preconditioning in light damage retinas (Wilson et al. 2007). This may be what happens in EE retinas that are most durable in terms of secondary degeneration of cones. If confirmed, this could be another proof that BDNF is one of the executors of EE effects.

Other recent studies enhance the fact that CNTF during retinas development could change the fate of photoreceptors, promoting the development of S-cone opsins instead of rod-opsin (Rhee and Yang 2010). These findings are in line whit the hypothesis formulated by Landi et al. that

underlines the role of IGF1 in EE effects during retinal development (Landi et al. 2007; Landi et al. 2009). Both IGF1 and CNTF stimulate phospho Inositol 3 phosphate (PI3K), which, in turn, modulates different growth factor receptors and is downstream of the insulin receptor, but upstream of mTOR kinase activity (Punzo et al. 2009) (**Fig. 29).**
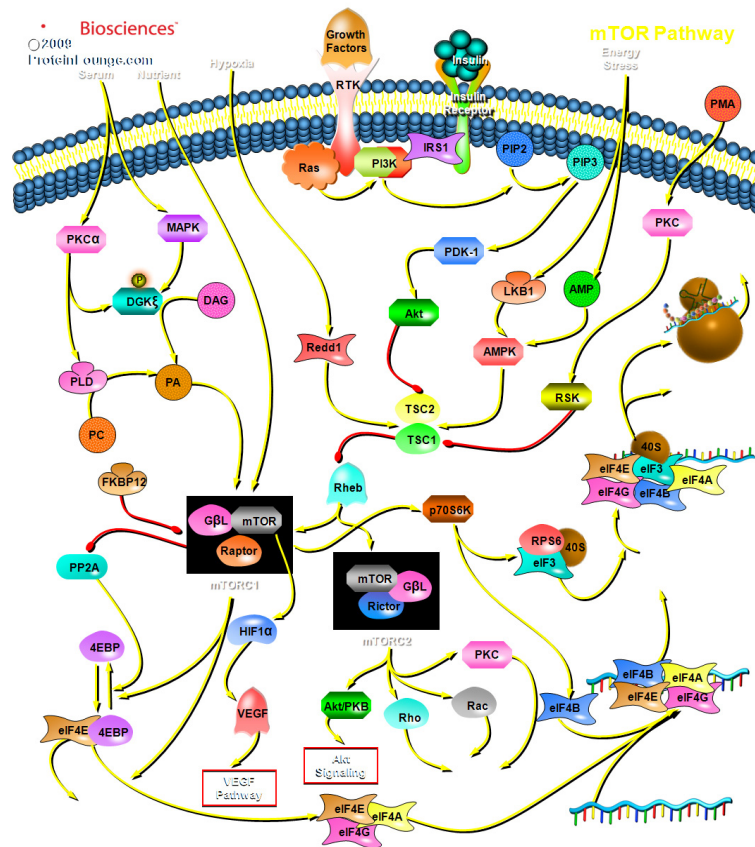
Fig. 29. The mTOR pathway is regulated by a wide variety of cellular signals, including Mitogenic Growth Factors, Hormones such as Insulin, Nutrients (Amino acids, Glucose), Cellular Energy Levels, and Stress conditions. A principal pathway that signals through mTOR is the PI3K/Akt (v-Akt Murine Thymoma Viral Oncogene Homolog-1) signal transduction pathway, which is critically involved in the mediation of cell survival and proliferation. Signaling through the PI3K/Akt pathway is initiated by mitogenic stimuli from growth factors that bind receptors in the cell membrane. These receptors include IGFR (Insulin-like Growth Factor Receptor), PDGFR (Platelet-Derived Growth Factor Receptor), EGFR (Epidermal Growth Factor Receptor). The signal from the activated receptors is transferred directly to the PI3K/Akt pathway, or, alternatively, it can be activated through activated Growth Factor Receptors that signal through oncogenic Ras. Ras is another central switch for signal transduction and has been shown to be a pivotal activator of the MAPK (Mitogen-Activated Protein Kinase) signal transduction pathway. PI3K/Akt pathway can also be activated by Insulin via IRS1/2 (Insulin Receptor Substrate-1/2). Insulin binding activates the IR (Insulin Receptor) tyrosine kinase, which phosphorylates IRS1 or IRS2. PI3K binds phosphorylated IRS by SH2 (Src-Homology-2) domains in the p85 regulatory subunit. This interaction activates the p110 catalytic subunit. PI3K then catalyzes the conversion of membrane-bound PIP2 (Phosphatidylinositol (4,5)-bisphosphate) to PIP3 (Phosphatidylinositol (3,4,5)-triphosphate). PIP3 then binds the pleckstrin homology domain of Akt, which results in Akt activation through dimerization and exposure of its catalytic site. Akt can also be phosphorylated and activated by PDK-1 (Phospholipid-Dependent Kinase-1). Akt phosphorylates mTOR directlyRHEB-GTP activates mTOR. PMA (Phorbol Myristate Acetate) can also lead to mTOR phosphorylation independently. From Sabioscience.

6.3 EE effects endure in the retinas of aged mutant animals

6.3.1 Photoreceptors survival in 1 year old EE mice: cones

There are many articles dedicated to study the effects of EE during different stages of life. Some of them highlight the fact that these effects are only transient both in healthy and diseased brains; others indicate that if the enrichment is prolonged it does ameliorate the outcome of some pathologies (van Praag et al. 2000; Pizzorusso et al. 2007; Spolidoro et al. 2009).

In our case, we found that, mice born and maintained in EE for a lifetime showed a larger number of surviving cones than mice that spent all their life in ST conditions. Furthermore, in EE retinas cones appeared less modified than in ST retinas, having a better preservation of shape and presumptive outer segment and showing a smaller cell body with respect to cones in ST retinas.

This observation point out the fact that EE have durable effects on cones avoiding that these cells die .

6.3.2 Inner retina in old EE: morphology

There is no evidence of an effect of EE on inner retinal morphology. Rod bipolar cell are remodeled and with scant dendrites in both EE and ST retina from 1 year old mice. No difference is shown also in immunostaining for horizontal cells. This lack of effects may be due in part to the fact that the inner retina is quite degeneration-resistant and that remodeling is brought to light only buy extensive cell characterization and counting. We used a limited number of markers as the study of the morphology of other retinal neurons (i.e. cone bipolar cells, ganglion cells) was beyond the scope of this thesis.

6.3.3 Visual system physiology in old EE: behavioural test

Visual Acuity (VA) is a parameter directly linked to the spatial resolution of the visual processing system and the decay of VA represents the first problem for RP patients since it is directly connected to a non autonomous life. Thus far there were few therapies able to slow down the loss of visual acuity in RP patience for that reason we consider a very important result the finding that 1 year old EE mice retain a minimum VA and that at this age they are still able to perform the task, unlike age matched ST mice.

7   CONCLUSIONS AND SPECULATIONS

In RP, photoreceptors are condemned to death and therefore blindness is the inevitable outcome. So far, no treatment other than replacing, when possible, the defective gene can definitely stop the progression of this disease. In the present Thesis, we performed a multidisciplinary study on a mouse model of RP, applying the paradigm of Environmental Enrichment (EE), in which mice are born and raised under conditions that stimulate voluntary motor activity, sensory experience and social interactions. This non-invasive physiological manipulation produced remarkable therapeutic effects on the visual system by significantly extending the time during which rd10 mice, doomed to early blindness, maintained good visual functions.

All measurements carried out to test the effects of EE were consistent in showing significant retinal preservation. Morphological studies documented an increased photoreceptor survival, long lasting maintenance of rod and mainly of cone integrity and preservation of their connectivity; ERG recordings confirmed retention of retinal capability to respond to light in adult age; visual acuity, which is set by the cone density and which we measured in a behavioral task, confirms preservation of retinal cones; finally, contrast sensitivity, which reflects the integrity of the overall neuronal processing of visual signals, demonstrates conserved visual function over time in EE mice. Thus, in agreement with the latest results obtained for other CNS pathologies, EE also seems to have substantial benefits on inherited retinal degeneration introducing a postponement in the unavoidable photoreceptor death.

The rescue effects of EE are both powerful and long lasting over animal life. We can speculate that EE acts by up regulating retinal self-defense mechanisms. Indeed in diseased retinas, several tropic factors (e.g. CNTF, FGF-2) are chronically up regulated (Wilson et al. 2007) (Gargini et al. 2004) and are also important for a better preservation of photoreceptors in culture (Lipinski et al. 2011). The major local sources of tropic molecules are Muller

cells, astrocytes, activated microglia, and the retinal pigment epithelium (Harada et al. 2002; Harada et al. 2003; Gargini et al. 2004; Ming et al. 2009). Regulation of trophic factors and the feed-forward between the different molecules is an homeostatic response probably enhanced by both the disease itself and EE, as suggested by the decreased levels of CNTF found here at P13, before the acute phase of photoreceptor degeneration. Indeed, it is reported that ERG detects signs of physiological alterations in rd10 retinas before manifest signs of major photoreceptor death (Gargini et al. 2007). Later, at P45, CNTF mRNA was relatively higher in EE versus ST mice. This could reflect a difference in the response to the pathology in EE individuals. CNTF is the most effective of all factors tested for the ability to protect photoreceptors in a large variety of retinal diseases (Sieving et al. 2006).

Certainly the change of expression in CNTF mRNAs is not the only downstream effect of EE. Another mechanism through which EE could exert its effects is the increased production of insulin-like growth factor 1 (IGF-1) in healthy pup retina. Sale and collaborators measured enhanced levels of IGF1 in the milk of enriched rodent mothers and hypothesized that this molecules mediates the effects of EE on retinal and cortical development. These and other studies report that EE outcome is mediated also by BDNF, which has different effect on the visual system in development versus adulthood (Landi et al. 2007; Sale et al. 2007; Landi et al. 2009). It has also been shown that insulin, which shares receptors and largely overlapping signaling pathways with IGF-1 (Denley et al. 2005), protects photoreceptors (and cones in particular) from degeneration, by activating the insulin-mTOR pathway and reducing the shortage of glucose that leads to the starvation of these cells (Punzo et al. 2009). In turn, it is known that the mTOR pathway is based on a classic survival kinase that links extracellular tropic/growth factors with intracellular antiapoptotic pathways named phosphoinositide 3-kinase (PI3K) (Ivanovic et al. 2011). For these reasons, it is possible that in EE both a direct action on cones of various up regulated molecules and an indirect

effect of rod-mediated survival contribute to increased cone lifespan. The beneficial effects of EE on vision are also likely to involve not only retinal changes, but other visual areas of the brain including the cortex. Indeed our results regarding the preservation of visual acuity in one year old EE mice could be mediated also by an increased neural response of visual cortex as is already demonstrated in other rodent models (Sale et al. 2007; Baroncelli et al. 2010).

The non-invasive nature of EE makes this therapeutic approach particularly eligible for human application. Given the efficacy of EE as a method to promote CNS plasticity and repair in general, and the results reported here on inherited retinal degeneration, manipulation of the external *milieu* could be considered a promising strategy to delay symptoms in patients with RP that are waiting for a decisive remedy. Translation of EE from the laboratory to the community could involve recommending healthy life styles, regular physical exercise and frequent social interactions, often sacrificed by limitations imposed or associated with visual impairment in these patients. Although most persons live in complex environments and commonly experience novelty, the exposure to cognitive, social and physical stimulation is highly variable among individuals and, for a single person, changes throughout the course of a lifetime. Moreover, it should be considered that EE favors stress-free and challenge-free interactions in a stimulating surrounding that might enhance self-defense responses of the body to disease (Cancedda et al. 2004; Sale et al. 2004). We hope that the results reported here should encourage the exploration of application of EE paradigms, alone or in combination with pharmacological approaches, for the therapy of RP.

8 BIBLIOGRAPHY

Acland, G. M., G. D. Aguirre, J. Ray, Q. Zhang, T. S. Aleman, A. V. Cideciyan, S. E. Pearce-Kelling, V. Anand, Y. Zeng, A. M. Maguire, S. G. Jacobson, W. W. Hauswirth and J. Bennett (2001). "Gene therapy restores vision in a canine model of childhood blindness." Nat Genet **28**(1): 92-5.

Anchan, R. M., T. A. Reh, J. Angello, A. Balliet and M. Walker (1991). "EGF and TGF-alpha stimulate retinal neuroepithelial cell proliferation in vitro." Neuron **6**(6): 923-36.

Baroncelli, L., C. Braschi, M. Spolidoro, T. Begenisic, L. Maffei and A. Sale (2011). "Brain plasticity and disease: a matter of inhibition." Neural Plast **2011**: 286073.

Baroncelli, L., C. Braschi, M. Spolidoro, T. Begenisic, A. Sale and L. Maffei (2010). "Nurturing brain plasticity: impact of environmental enrichment." Cell Death Differ **17**(7): 1092-103.

Baroncelli, L., A. Sale, A. Viegi, J. F. Maya Vetencourt, R. De Pasquale, S. Baldini and L. Maffei (2010). "Experience-dependent reactivation of ocular dominance plasticity in the adult visual cortex." Exp Neurol **226**(1): 100-9.

Berson, E. L., B. Rosner, M. A. Sandberg, K. C. Hayes, B. W. Nicholson, C. Weigel-DiFranco and W. Willett (1993). "A randomized trial of vitamin A and vitamin E supplementation for retinitis pigmentosa." Arch Ophthalmol **111**(6): 761-72.

Berson, E. L., B. Rosner, M. A. Sandberg, K. C. Hayes, B. W. Nicholson, C. Weigel-DiFrano and W. Willett (1993). "Vitamin A supplementation for retinitis pigmentosa." Arch Ophthalmol **111**(11): 1456-9.

Berson, E. L., B. Rosner, M. A. Sandberg, C. Weigel-DiFranco, R. J. Brockhurst, K. C. Hayes, E. J. Johnson, E. J. Anderson, C. A. Johnson, A. R. Gaudio, W. C. Willett and E. J. Schaefer (2010). "Clinical trial of lutein in patients with retinitis pigmentosa receiving vitamin A." Arch Ophthalmol **128**(4): 403-11.

Berson, E. L., M. A. Sandberg, B. Rosner, D. G. Birch and A. H. Hanson (1985). "Natural course of retinitis pigmentosa over a three-year interval." Am J Ophthalmol **99**(3): 240-51.

Bilowit, D. S. (1956). "Establishing physical objectives in the rehabilitation of patients with Parkinson's disease; gymnasium activities." Phys Ther Rev **36**(3): 176-8.

Bowes, C., T. Li, M. Danciger, L. C. Baxter, M. L. Applebury and D. B. Farber (1990). "Retinal degeneration in the rd mouse is caused by a defect in the beta subunit of rod cGMP-phosphodiesterase." Nature **347**(6294): 677-80.

Bowes, C., T. Li, W. N. Frankel, M. Danciger, J. M. Coffin, M. L. Applebury and D. B. Farber (1993). "Localization of a retroviral element within the rd gene coding for the beta subunit of cGMP phosphodiesterase." Proc Natl Acad Sci U S A **90**(7): 2955-9.

Busskamp, V. and B. Roska (2011). "Optogenetic approaches to restoring visual function in retinitis pigmentosa." Curr Opin Neurobiol.

Bustin, S. A. (2000). "Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays." J Mol Endocrinol **25**(2): 169-93.

Butterwick, A., P. Huie, B. W. Jones, R. E. Marc, M. Marmor and D. Palanker (2009). "Effect of shape and coating of a subretinal prosthesis on its integration with the retina." Exp Eye Res **88**(1): 22-9.

Caffe, A. R., P. Ahuja, B. Holmqvist, S. Azadi, J. Forsell, I. Holmqvist, A. K. Soderpalm and T. van Veen (2001). "Mouse retina explants after long-term culture in serum free medium." J Chem Neuroanat **22**(4): 263-73.

Cancedda, L., E. Putignano, A. Sale, A. Viegi, N. Berardi and L. Maffei (2004). "Acceleration of visual system development by environmental enrichment." J Neurosci **24**(20): 4840-8.

Chader, G. J. (2002). "Animal models in research on retinal degenerations: past progress and future hope." Vision Res **42**(4): 393-9.

Chang, B., N. L. Hawes, R. E. Hurd, M. T. Davisson, S. Nusinowitz and J. R. Heckenlively (2002). "Retinal degeneration mutants in the mouse." Vision Res **42**(4): 517-25.

Chang, B., N. L. Hawes, M. T. Pardue, A. M. German, R. E. Hurd, M. T. Davisson, S. Nusinowitz, K. Rengarajan, A. P. Boyd, S. S. Sidney, M. J. Phillips, R. E. Stewart, R. Chaudhury, J. M. Nickerson, J. R. Heckenlively and J. H. Boatright (2007). "Two mouse retinal degenerations caused by missense mutations in the beta-subunit of rod cGMP phosphodiesterase gene." Vision Res **47**(5): 624-33.

Chang, G. Q., Y. Hao and F. Wong (1993). "Apoptosis: final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice." Neuron **11**(4): 595-605.

Chaum, E. (2003). "Retinal neuroprotection by growth factors: a mechanistic perspective." J Cell Biochem **88**(1): 57-75.

Chong, N. H., R. A. Alexander, L. Waters, K. C. Barnett, A. C. Bird and P. J. Luthert (1999). "Repeated injections of a ciliary neurotrophic factor analogue leading to long-term photoreceptor survival in hereditary retinal degeneration." Invest Ophthalmol Vis Sci **40**(6): 1298-305.

Chow, A. Y., V. Y. Chow, K. H. Packo, J. S. Pollack, G. A. Peyman and R. Schuchard (2004). "The artificial silicon retina microchip for the treatment of vision loss from retinitis pigmentosa." Arch Ophthalmol **122**(4): 460-9.

Ciucci, F., E. Putignano, L. Baroncelli, S. Landi, N. Berardi and L. Maffei (2007). "Insulin-like growth factor 1 (IGF-1) mediates the effects of

enriched environment (EE) on visual cortical development." <u>PLoS One</u> **2**(5): e475.

Clemson, C. M., R. Tzekov, M. Krebs, J. M. Checchi, C. Bigelow and S. Kaushal (2011). "Therapeutic potential of valproic acid for retinitis pigmentosa." <u>Br J Ophthalmol</u> **95**(1): 89-93.

Coles, B. L., D. J. Horsford, R. R. McInnes and D. van der Kooy (2006). "Loss of retinal progenitor cells leads to an increase in the retinal stem cell population in vivo." <u>Eur J Neurosci</u> **23**(1): 75-82.

Delbeke, J., D. Pins, G. Michaux, M. C. Wanet-Defalque, S. Parrini and C. Veraart (2001). "Electrical stimulation of anterior visual pathways in retinitis pigmentosa." <u>Invest Ophthalmol Vis Sci</u> **42**(1): 291-7.

Denley, A., L. J. Cosgrove, G. W. Booker, J. C. Wallace and B. E. Forbes (2005). "Molecular interactions of the IGF system." <u>Cytokine Growth Factor Rev</u> **16**(4-5): 421-39.

Dick, O., S. tom Dieck, W. D. Altrock, J. Ammermuller, R. Weiler, C. C. Garner, E. D. Gundelfinger and J. H. Brandstatter (2003). "The presynaptic active zone protein bassoon is essential for photoreceptor ribbon synapse formation in the retina." <u>Neuron</u> **37**(5): 775-86.

Fagiolini, M., T. Pizzorusso, N. Berardi, L. Domenici and L. Maffei (1994). "Functional postnatal development of the rat primary visual cortex and the role of visual experience: dark rearing and monocular deprivation." <u>Vision Res</u> **34**(6): 709-20.

Fan, J., M. L. Woodruff, M. C. Cilluffo, R. K. Crouch and G. L. Fain (2005). "Opsin activation of transduction in the rods of dark-reared Rpe65 knockout mice." <u>J Physiol</u> **568**(Pt 1): 83-95.

Farber, D. B. and R. N. Lolley (1974). "Cyclic guanosine monophosphate: elevation in degenerating photoreceptor cells of the C3H mouse retina." <u>Science</u> **186**(4162): 449-51.

Fine, I., A. R. Wade, A. A. Brewer, M. G. May, D. F. Goodman, G. M. Boynton, B. A. Wandell and D. I. MacLeod (2003). "Long-term deprivation affects visual perception and cortex." <u>Nat Neurosci</u> **6**(9): 915-6.

Fleming, A. S., D. H. O'Day and G. W. Kraemer (1999). "Neurobiology of mother-infant interactions: experience and central nervous system plasticity across development and generations." <u>Neurosci Biobehav Rev</u> **23**(5): 673-85.

Fox, D. A., A. T. Poblenz and L. He (1999). "Calcium overload triggers rod photoreceptor apoptotic cell death in chemical-induced and inherited retinal degenerations." <u>Ann N Y Acad Sci</u> **893**: 282-5.

Gal, A., S. Xu, Y. Piczenik, H. Eiberg, C. Duvigneau, E. Schwinger and T. Rosenberg (1994). "Gene for autosomal dominant congenital stationary night blindness maps to the same region as the gene for the beta-subunit of the rod photoreceptor cGMP phosphodiesterase (PDEB) in chromosome 4p16.3." <u>Hum Mol Genet</u> **3**(2): 323-5.

Gao, H. and J. G. Hollyfield (1995). "Basic fibroblast growth factor in retinal development: differential levels of bFGF expression and content in normal and retinal degeneration (rd) mutant mice." Dev Biol **169**(1): 168-84.

Gao, H. and J. G. Hollyfield (1996). "Basic fibroblast growth factor: increased gene expression in inherited and light-induced photoreceptor degeneration." Exp Eye Res **62**(2): 181-9.

Gargini, C., S. Bisti, G. C. Demontis, K. Valter, J. Stone and L. Cervetto (2004). "Electroretinogram changes associated with retinal upregulation of trophic factors: observations following optic nerve section." Neuroscience **126**(3): 775-83.

Gargini, C., E. Terzibasi, F. Mazzoni and E. Strettoi (2007). "Retinal organization in the retinal degeneration 10 (rd10) mutant mouse: a morphological and ERG study." J Comp Neurol **500**(2): 222-38.

Hamel, C. (2006). "Retinitis pigmentosa." Orphanet J Rare Dis **1**: 40.

Harada, C., T. Harada, H. M. Quah, F. Maekawa, K. Yoshida, S. Ohno, K. Wada, L. F. Parada and K. Tanaka (2003). "Potential role of glial cell line-derived neurotrophic factor receptors in Muller glial cells during light-induced retinal degeneration." Neuroscience **122**(1): 229-35.

Harada, T., C. Harada, S. Kohsaka, E. Wada, K. Yoshida, S. Ohno, H. Mamada, K. Tanaka, L. F. Parada and K. Wada (2002). "Microglia-Muller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration." J Neurosci **22**(21): 9228-36.

Hartong, D. T., E. L. Berson and T. P. Dryja (2006). "Retinitis pigmentosa." Lancet **368**(9549): 1795-809.

Histed, M. H., L. A. Carvalho and J. H. Maunsell (2011). "Psychophysical measurement of contrast sensitivity in the behaving mouse." J Neurophysiol.

Hossain, P., I. W. Seetho, A. C. Browning and W. M. Amoaku (2005). "Artificial means for restoring vision." BMJ **330**(7481): 30-3.

Huang, Z. J., A. Kirkwood, T. Pizzorusso, V. Porciatti, B. Morales, M. F. Bear, L. Maffei and S. Tonegawa (1999). "BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex." Cell **98**(6): 739-55.

Hubel, D. H. and T. N. Wiesel (1970). "The period of susceptibility to the physiological effects of unilateral eye closure in kittens." J Physiol **206**(2): 419-36.

Ivanovic, I., R. E. Anderson, Y. Z. Le, S. J. Fliesler, D. M. Sherry and R. V. Rajala (2011). "Deletion of the p85alpha regulatory subunit of phosphoinositide 3-kinase in cone photoreceptor cells results in cone photoreceptor degeneration." Invest Ophthalmol Vis Sci **52**(6): 3775-83.

Jeon, C. J., E. Strettoi and R. H. Masland (1998). "The major cell populations of the mouse retina." J Neurosci **18**(21): 8936-46.

Jos´e M. Mill´an, E. A., Teresa Jaijo, Fiona Blanco-Kelly, and a. C. A. Ascensi ´on Gimenez-Pardo (2011). "An Update on the Genetics of Usher Syndrome." Journal of Ophthalmology **Volume 2011, Article ID 417217, 8 pages**.

Keeler, C. E. (1924). "The Inheritance of a Retinal Abnormality in White Mice." Proc Natl Acad Sci U S A **10**(7): 329-33.

Kempermann, G., H. G. Kuhn and F. H. Gage (1997). "More hippocampal neurons in adult mice living in an enriched environment." Nature **386**(6624): 493-5.

Kennan, A., A. Aherne and P. Humphries (2005). "Light in retinitis pigmentosa." Trends Genet **21**(2): 103-10.

Klassen, A., U. Bahner, K. Sebekova and A. Heidland (2004). "[The importance of overweight and obesity for the development and progression of renal diseases]." Dtsch Med Wochenschr **129**(11): 579-82.

Koilkonda, R. D. and J. Guy (2011). "Leber's Hereditary Optic Neuropathy-Gene Therapy: From Benchtop to Bedside." J Ophthalmol **2011**: 179412.

Koulen, P., E. L. Fletcher, S. E. Craven, D. S. Bredt and H. Wassle (1998). "Immunocytochemical localization of the postsynaptic density protein PSD-95 in the mammalian retina." J Neurosci **18**(23): 10136-49.

Lakhanpal, R. R., D. Yanai, J. D. Weiland, G. Y. Fujii, S. Caffey, R. J. Greenberg, E. de Juan, Jr. and M. S. Humayun (2003). "Advances in the development of visual prostheses." Curr Opin Ophthalmol **14**(3): 122-7.

Landi, S., M. C. Cenni, L. Maffei and N. Berardi (2007). "Environmental enrichment effects on development of retinal ganglion cell dendritic stratification require retinal BDNF." PLoS One **2**(4): e346.

Landi, S., F. Ciucci, L. Maffei, N. Berardi and M. C. Cenni (2009). "Setting the pace for retinal development: environmental enrichment acts through insulin-like growth factor 1 and brain-derived neurotrophic factor." J Neurosci **29**(35): 10809-19.

LaVail, M. M., K. Unoki, D. Yasumura, M. T. Matthes, G. D. Yancopoulos and R. H. Steinberg (1992). "Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light." Proc Natl Acad Sci U S A **89**(23): 11249-53.

LaVail, M. M., D. Yasumura, M. T. Matthes, C. Lau-Villacorta, K. Unoki, C. H. Sung and R. H. Steinberg (1998). "Protection of mouse photoreceptors by survival factors in retinal degenerations." Invest Ophthalmol Vis Sci **39**(3): 592-602.

Leveillard, T., S. Mohand-Said, A. C. Fintz, G. Lambrou and J. A. Sahel (2004). "The search for rod-dependent cone viability factors, secreted factors promoting cone viability." Novartis Found Symp **255**: 117-27; discussion 127-30, 177-8.

Li, L. X. and J. E. Turner (1988). "Inherited retinal dystrophy in the RCS rat: prevention of photoreceptor degeneration by pigment epithelial cell transplantation." Exp Eye Res **47**(6): 911-7.

Li, Q., A. M. Timmers, J. Guy, J. Pang and W. W. Hauswirth (2008). "Cone-specific expression using a human red opsin promoter in recombinant AAV." Vision Res **48**(3): 332-8.

Lin, B., R. H. Masland and E. Strettoi (2009). "Remodeling of cone photoreceptor cells after rod degeneration in rd mice." Exp Eye Res **88**(3): 589-99.

Lipinski, D. M., M. S. Singh and R. E. MacLaren (2011). "Assessment of cone survival in response to CNTF, GDNF, and VEGF165b in a novel ex vivo model of end-stage retinitis pigmentosa." Invest Ophthalmol Vis Sci **52**(10): 7340-6.

Liu, C., M. Peng, A. M. Laties and R. Wen (1998). "Preconditioning with bright light evokes a protective response against light damage in the rat retina." J Neurosci **18**(4): 1337-44.

Liu, D., J. Diorio, J. C. Day, D. D. Francis and M. J. Meaney (2000). "Maternal care, hippocampal synaptogenesis and cognitive development in rats." Nat Neurosci **3**(8): 799-806.

MacDonald, I. M., Y. Sauve and P. A. Sieving (2007). "Preventing blindness in retinal disease: ciliary neurotrophic factor intraocular implants." Can J Ophthalmol **42**(3): 399-402.

Marc, R. E. and B. W. Jones (2003). "Retinal remodeling in inherited photoreceptor degenerations." Mol Neurobiol **28**(2): 139-47.

Marc, R. E., B. W. Jones, C. B. Watt and E. Strettoi (2003). "Neural remodeling in retinal degeneration." Prog Retin Eye Res **22**(5): 607-55.

McLaughlin, M. E., T. L. Ehrhart, E. L. Berson and T. P. Dryja (1995). "Mutation spectrum of the gene encoding the beta subunit of rod phosphodiesterase among patients with autosomal recessive retinitis pigmentosa." Proc Natl Acad Sci U S A **92**(8): 3249-53.

McLaughlin, M. E., M. A. Sandberg, E. L. Berson and T. P. Dryja (1993). "Recessive mutations in the gene encoding the beta-subunit of rod phosphodiesterase in patients with retinitis pigmentosa." Nat Genet **4**(2): 130-4.

Meaney, M. J. (2001). "Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations." Annu Rev Neurosci **24**: 1161-92.

Meaney, M. J. (2001). "Nature, nurture, and the disunity of knowledge." Ann N Y Acad Sci **935**: 50-61.

Mendes, H. F., J. van der Spuy, J. P. Chapple and M. E. Cheetham (2005). "Mechanisms of cell death in rhodopsin retinitis pigmentosa: implications for therapy." Trends Mol Med **11**(4): 177-85.

Ming, M., X. Li, X. Fan, D. Yang, L. Li, S. Chen, Q. Gu and W. Le (2009). "Retinal pigment epithelial cells secrete neurotrophic factors and

synthesize dopamine: possible contribution to therapeutic effects of RPE cell transplantation in Parkinson's disease." J Transl Med **7**: 53.

Nagel, G., T. Szellas, W. Huhn, S. Kateriya, N. Adeishvili, P. Berthold, D. Ollig, P. Hegemann and E. Bamberg (2003). "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel." Proc Natl Acad Sci U S A **100**(24): 13940-5.

Nakazawa, M. (2011). "Effects of calcium ion, calpains, and calcium channel blockers on retinitis pigmentosa." J Ophthalmol **2011**: 292040.

Nikonov, S. S., R. Kholodenko, J. Lem and E. N. Pugh, Jr. (2006). "Physiological features of the S- and M-cone photoreceptors of wild-type mice from single-cell recordings." J Gen Physiol **127**(4): 359-74.

Nithianantharajah, J. and A. J. Hannan (2006). "Enriched environments, experience-dependent plasticity and disorders of the nervous system." Nat Rev Neurosci **7**(9): 697-709.

Ossipow, V., F. Pellissier, O. Schaad and M. Ballivet (2004). "Gene expression analysis of the critical period in the visual cortex." Mol Cell Neurosci **27**(1): 70-83.

Otani, A., M. I. Dorrell, K. Kinder, S. K. Moreno, S. Nusinowitz, E. Banin, J. Heckenlively and M. Friedlander (2004). "Rescue of retinal degeneration by intravitreally injected adult bone marrow-derived lineage-negative hematopoietic stem cells." J Clin Invest **114**(6): 765-74.

Panni, M. K., J. Atkinson and R. D. Lund (1994). "Evidence for a tropic role of brain-derived neurotrophic factor in transplanted embryonic retinae." Brain Res Dev Brain Res **81**(2): 325-7.

Paskowitz, D. M., M. M. LaVail and J. L. Duncan (2006). "Light and inherited retinal degeneration." Br J Ophthalmol **90**(8): 1060-6.

Peichl, L. and J. Gonzalez-Soriano (1994). "Morphological types of horizontal cell in rodent retinae: a comparison of rat, mouse, gerbil, and guinea pig." Vis Neurosci **11**(3): 501-17.

Peterman, M. C., D. M. Bloom, C. Lee, S. F. Bent, M. F. Marmor, M. S. Blumenkranz and H. A. Fishman (2003). "Localized neurotransmitter release for use in a prototype retinal interface." Invest Ophthalmol Vis Sci **44**(7): 3144-9.

Peterman, M. C., J. Noolandi, M. S. Blumenkranz and H. A. Fishman (2004). "Localized chemical release from an artificial synapse chip." Proc Natl Acad Sci U S A **101**(27): 9951-4.

Pittler, S. J., C. E. Keeler, R. L. Sidman and W. Baehr (1993). "PCR analysis of DNA from 70-year-old sections of rodless retina demonstrates identity with the mouse rd defect." Proc Natl Acad Sci U S A **90**(20): 9616-9.

Pizzorusso, T., N. Berardi and L. Maffei (2007). "A richness that cures." Neuron **54**(4): 508-10.

Prusky, G. T., P. W. West and R. M. Douglas (2000). "Behavioral assessment of visual acuity in mice and rats." Vision Res **40**(16): 2201-9.

Punzo, C., K. Kornacker and C. L. Cepko (2009). "Stimulation of the insulin/mTOR pathway delays cone death in a mouse model of retinitis pigmentosa." Nat Neurosci **12**(1): 44-52.

Putignano, E., G. Lonetti, L. Cancedda, G. Ratto, M. Costa, L. Maffei and T. Pizzorusso (2007). "Developmental downregulation of histone posttranslational modifications regulates visual cortical plasticity." Neuron **53**(5): 747-59.

Reme, C. E., C. Grimm, F. Hafezi, A. Marti and A. Wenzel (1998). "Apoptotic cell death in retinal degenerations." Prog Retin Eye Res **17**(4): 443-64.

Rhee, K. D. and X. J. Yang (2010). "Function and mechanism of CNTF/LIF signaling in retinogenesis." Adv Exp Med Biol **664**: 647-54.

Rizzo, J. F., 3rd (2011). "Update on retinal prosthetic research: the Boston Retinal Implant Project." J Neuroophthalmol **31**(2): 160-8.

Rosenzweig, M. R. (1966). "Environmental complexity, cerebral change, and behavior." Am Psychol **21**(4): 321-32.

Rosenzweig, M. R., E. L. Bennett, M. C. Diamond, S. Y. Wu, R. W. Slagle and E. Saffran (1969). "Influences of environmental complexity and visual stimulation on development of occipital cortex in rat." Brain Res **14**(2): 427-45.

Rosenzweig, M. R., E. L. Bennett, M. Hebert and H. Morimoto (1978). "Social grouping cannot account for cerebral effects of enriched environments." Brain Res **153**(3): 563-76.

Rosenzweig, M. R., D. Krech, E. L. Bennett and J. F. Zolman (1962). "Variation in environmental complexity and brain measures." J Comp Physiol Psychol **55**: 1092-5.

Royo, P. E. and W. B. Quay (1959). "Retinal transplantation from fetal to maternal mammalian eye." Growth **23**: 313-36.

Sale, A., M. C. Cenni, F. Ciucci, E. Putignano, S. Chierzi and L. Maffei (2007). "Maternal enrichment during pregnancy accelerates retinal development of the fetus." PLoS One **2**(11): e1160.

Sale, A., J. F. Maya Vetencourt, P. Medini, M. C. Cenni, L. Baroncelli, R. De Pasquale and L. Maffei (2007). "Environmental enrichment in adulthood promotes amblyopia recovery through a reduction of intracortical inhibition." Nat Neurosci **10**(6): 679-81.

Sale, A., E. Putignano, L. Cancedda, S. Landi, F. Cirulli, N. Berardi and L. Maffei (2004). "Enriched environment and acceleration of visual system development." Neuropharmacology **47**(5): 649-60.

Schlichtenbrede, F. C., L. da Cruz, C. Stephens, A. J. Smith, A. Georgiadis, A. J. Thrasher, J. W. Bainbridge, M. W. Seeliger and R. R. Ali (2003). "Long-term evaluation of retinal function in Prph2Rd2/Rd2 mice following AAV-mediated gene replacement therapy." J Gene Med **5**(9): 757-64.

Sharma, R. K., T. E. O'Leary, C. M. Fields and D. A. Johnson (2003). "Development of the outer retina in the mouse." Brain Res Dev Brain Res **145**(1): 93-105.

Sieving, P. A., R. C. Caruso, W. Tao, H. R. Coleman, D. J. Thompson, K. R. Fullmer and R. A. Bush (2006). "Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants." Proc Natl Acad Sci U S A **103**(10): 3896-901.

Soucy, E., Y. Wang, S. Nirenberg, J. Nathans and M. Meister (1998). "A novel signaling pathway from rod photoreceptors to ganglion cells in mammalian retina." Neuron **21**(3): 481-93.

Spolidoro, M., A. Sale, N. Berardi and L. Maffei (2009). "Plasticity in the adult brain: lessons from the visual system." Exp Brain Res **192**(3): 335-41.

Stasheff, S. F. (2008). "Emergence of sustained spontaneous hyperactivity and temporary preservation of OFF responses in ganglion cells of the retinal degeneration (rd1) mouse." J Neurophysiol **99**(3): 1408-21.

Stieger, K. and B. Lorenz (2010). "Gene therapy for vision loss -- recent developments." Discov Med **10**(54): 425-33.

Strettoi, E., C. Gargini, E. Novelli, G. Sala, I. Piano, P. Gasco and R. Ghidoni (2010). "Inhibition of ceramide biosynthesis preserves photoreceptor structure and function in a mouse model of retinitis pigmentosa." Proc Natl Acad Sci U S A **107**(43): 18706-11.

Suzuki, T., M. Mandai, M. Akimoto, N. Yoshimura and M. Takahashi (2006). "The simultaneous treatment of MMP-2 stimulants in retinal transplantation enhances grafted cell migration into the host retina." Stem Cells **24**(11): 2406-11.

Tian, N. and D. R. Copenhagen (2003). "Visual stimulation is required for refinement of ON and OFF pathways in postnatal retina." Neuron **39**(1): 85-96.

Toda, K., R. A. Bush, P. Humphries and P. A. Sieving (1999). "The electroretinogram of the rhodopsin knockout mouse." Vis Neurosci **16**(2): 391-8.

van Praag, H., G. Kempermann and F. H. Gage (2000). "Neural consequences of environmental enrichment." Nat Rev Neurosci **1**(3): 191-8.

Vaquero, C. F., A. Velasco and P. de la Villa (1996). "Protein kinase C localization in the synaptic terminal of rod bipolar cells." Neuroreport **7**(13): 2176-80.

Wang, D. Y., W. M. Chan, P. O. Tam, S. W. Chiang, D. S. Lam, K. K. Chong and C. P. Pang (2005). "Genetic markers for retinitis pigmentosa." Hong Kong Med J **11**(4): 281-8.

Wang, M., T. T. Lam, M. O. Tso and M. I. Naash (1997). "Expression of a mutant opsin gene increases the susceptibility of the retina to light damage." Vis Neurosci **14**(1): 55-62.

Weaver, A., R. Richardson, J. Worlein, F. De Waal and M. Laudenslager (2004). "Response to social challenge in young bonnet (Macaca radiata) and pigtail (Macaca nemestrina) macaques is related to early maternal experiences." Am J Primatol **62**(4): 243-59.

Wen, R., T. Cheng, Y. Song, M. T. Matthes, D. Yasumura, M. M. LaVail and R. H. Steinberg (1998). "Continuous exposure to bright light upregulates bFGF and CNTF expression in the rat retina." Curr Eye Res **17**(5): 494-500.

Weng, S., W. Sun and S. He (2005). "Identification of ON-OFF direction-selective ganglion cells in the mouse retina." J Physiol **562**(Pt 3): 915-23.

Whiteley, S. J., H. Klassen, P. J. Coffey and M. J. Young (2001). "Photoreceptor rescue after low-dose intravitreal IL-1beta injection in the RCS rat." Exp Eye Res **73**(4): 557-68.

Williamson, L. L., A. Chao and S. D. Bilbo (2012). "Environmental enrichment alters glial antigen expression and neuroimmune function in the adult rat hippocampus." Brain Behav Immun **26**(3): 500-10.

Wilson, R. B., K. Kunchithapautham and B. Rohrer (2007). "Paradoxical role of BDNF: BDNF+/- retinas are protected against light damage-mediated stress." Invest Ophthalmol Vis Sci **48**(6): 2877-86.

Woodruff, M. L., A. P. Sampath, H. R. Matthews, N. V. Krasnoperova, J. Lem and G. L. Fain (2002). "Measurement of cytoplasmic calcium concentration in the rods of wild-type and transducin knock-out mice." J Physiol **542**(Pt 3): 843-54.

Wright, A. F., C. F. Chakarova, M. M. Abd El-Aziz and S. S. Bhattacharya (2010). "Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait." Nat Rev Genet **11**(4): 273-84.

Yang, Y., Mohand-Said, S., Danan, A., Simonutti, M., Fontaine,V., Clerin,E., Picaud, S., Leveillard, T., and Sahel, J.A. (2009).

Yao, J., K. L. Feathers, H. Khanna, D. Thompson, C. Tsilfidis, W. W. Hauswirth, J. R. Heckenlively, A. Swaroop and D. N. Zacks (2011). "XIAP therapy increases survival of transplanted rod precursors in a degenerating host retina." Invest Ophthalmol Vis Sci **52**(3): 1567-72.

Zrenner, E. (2002). "The subretinal implant: can microphotodiode arrays replace degenerated retinal photoreceptors to restore vision?" Ophthalmologica **216 Suppl 1**: 8-20; discussion 52-3.

9 PUBBLICATION LIST

1. Pang J.J., Dai X., Boye S.E., Barone I., Boye S.L., Mao S., Everhart D., Dinculescu A., Liu L., Umino Y., Lei B., Chang B., Barlow R., Strettoi E., Hauswirth WW. Long-term retinal function and structure rescue using capsid mutant AAV8 vector in the rd10 mouse, a model of recessive retinitis pigmentosa. Mol Ther. 2011 Feb;19(2):234-42. Epub 2010 Dec 7.

10 ABSTRACT LIST

1. Strettoi E., Barone I., Novelli E. Environmental Enrichment delays photoreceptor degeneration in a mouse model of Retinitis pigmentosa. ARVO abstract 2010 (4052/A445).

2. Liu L., Barone I., Dai X., Lei B., Boye S.L., Chiodo V., Chang B., Hauswirth W.W., Strettoi E., Pang J.-J. Gene therapy preserves inner retinal neurons and their connectivity in rd10 mice, a model of recessive Retinitis pigmentosa with PDEβ mutations. ARVO abstract 2010 (3112/A336).

3. Strettoi E., Barone I., Piano I., Gargini M.C., Novelli E. Environmental Enrichment extends the time window of useful vision in a mouse model of Retinitis pigmentosa. ARVO abstract 2011 (4336/D1080).

11 ACKNOWLEGMENTS

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.