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Adult Stem Cells, Tools for Repairing the Retina

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Abstract

Purpose: Retinal degenerative diseases lead to the death of retinal neurons causing visual impairment and blindness. In lower order vertebrates, the retina and its surrounding tissue contain stem cell niches capable of regenerating damaged tissue. Here we examine these niches and review their capacity to be used as retinal stem/progenitor cells (RSC/RPCs) for retinal repair.

Recent Findings: Exogenous factors can control the *in vitro* activation of RSCs/PCs found in several niches within the adult eye including cells in the ciliary margin, the retinal pigment epithelium, iris pigment epithelium as well as the inducement of Müller and amacrine cells within the neural retina itself. Recently, factors have been identified for the activation of adult mammalian Müller cells to a RPC state *in vivo*.

Summary: Whereas cell transplantation still holds potential for retinal repair, activation of the dormant native regeneration process may lead to a more successful process including greater integration efficiency and proper synaptic targeting.

Keywords

retinal stem cells niche; retinal regeneration; retinal adult stem cells

Introduction

Retinal degenerative diseases, such as age-related macular degeneration (AMD), retinitis pigmentosa and glaucoma, all of which result in the loss of retinal cells are among the leading causes of irreversible blindness. (1–4). Similar to treatments of other parts of the central nervous system (CNS), treatments to repair the human retina following degenerative diseases remain a challenge because unlike species of lower vertebrates, the human retina lacks a regenerative pathway. One possible approach for treatment of these blinding diseases is to replace cells that are lost via transplantation. However, the success of transplantation approaches significantly depend on the identification of suitable sources of donor cell populations and finding ways to manipulate them *in vitro* to the appropriate development stage prior to transplantation. RSCs/PCs with the potential to produce either neural retinal

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Conflict of Interest

Aladdad and Karl E. Kador declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

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progenitors or RPE progenitors exist in the developing eye of all vertebrates and remain active in lower vertebrates throughout life (5, 6). The stem cells proliferate embryonically and thus may help to build the retina initially, but in postnatal mammals they clearly do not proliferate to regenerate the retina in response to injury. However, a number of different cellular sources of RSCs/PCs have been identified in the adult mammalian eye, raising the possibility of autologous transplantation if these cells could be expanded and induced to differentiate into desirable retinal cell types. These include pigmented cells in ciliary body, iris pigment epithelium (IPE), and retinal pigment epithelium (RPE), and Müller cells within the retina (7–12). Therefore, this review provides an update on the nature and origin of these different niches for RSCs/PCs found within the eye.

RSCs/PCs in the ciliary marginal zone (CMZ)

Lower vertebrates, such as fish and amphibians, continue to generate new retinal neurons throughout life from highly proliferative multipotent RSCs found in the CMZ, along the peripheral of the neural retina (13). These cells respond to retinal injury and can generate all retinal cell types, including RPE, photoreceptors, and inner retinal neurons (14). Although, retinogenesis in birds is completed and all neurons are generated by hatching (15), hatched chicks have a zone of proliferating cells at the peripheral margin of the retina, similar to that of fish and amphibians (16). However, the regeneration ability of CMZ-derived RPCs in chick is limited to amacrine and bipolar cells without evidence for the generation of photoreceptor, horizontal or ganglion cells and only in small quantity (16). This restriction can be overcome by exogenous growth factor stimulation such as insulin-like growth factor-I, and epidermal growth factor (EGF) combined with fibroblast growth factor-2 (FGF2), suggesting that extrinsic and not intrinsic factors limit the neurogenic potential of the CMZ-derived RPCs in the chicks (16). Furthermore, in contrast to fish and amphibian, the progenitors of the chick retina do not increase their rate of proliferation in response to retinal damages and contribute to retinal regeneration (16). Until recently, it was thought that adult mammalian eye lacked the regenerative capacity due to the absence of the CMZ (8, 17). However, several studies showed that the pigmented epithelium layer of the ciliary body (CB), a structure analogous to the lower vertebrate CMZ, in adult mammalian eye contains a small population of quiescent cell that display stem cell properties, are able to proliferate *in vitro* (18–21), and are capable of responding to a genetic injury *in vivo* (22). The CB is derived from neuroepithelium and located between the retina and the RPE. It is composed of two layers: the inner transparent layer is continuous with the neural tissue of the retina and the outer pigmented layer is continuous with the RPE (8, 23). Recent studies have shown that activation and proliferation of retinal progenitor-like cells within the adult mouse CB occur in response to retinal ganglion cell (RGCs) injury (24). Furthermore, a subpopulation of those cells were positively stained for homeodomain protein Chx10 and recoverin, which is normally expressed in photoreceptors and bipolar cells of the retina (24). Brenda *et al.* showed that RSCs within the pigmented CB, have undergone more self-renewing divisions and generated larger RSCs population in adult mice with inherited defects that impair the normal development of RPE progenitor ($Mitf^{mi/mi}$) or neural retina progenitor ($Chx10^{orJ/orJ}$) populations compared to the wild-type controls (25). Moreover, RSCs extracted from $Mitf^{mi/mi}$ mice were expanded *in vitro* to form pigmented ciliary spheres that have the ability

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to differentiate to neural cell types (including photoreceptor) and glial lineages. Also, *Chx10*$^{or J/or J}$ pigmented ciliary spheres gave rise to Müller glia and neural cell types including RGCs, amacrine and photoreceptors (25). Pigmented ciliary spheres (pigmented neurospheres) express RSCs markers such as *Pax6*, *Six3*, and *Rx* (26), and can be induced to differentiate into different retinal cell types, including rod photoreceptor cells (PRCs), bipolar cells, RGCs and Müller glia (8, 25), indicating that multipotent behaviour of these cells in the CB. In adult pig, primary cells from CB were able to proliferate and form neurospheres *in vitro*. Furthermore, these cells differentiated into cells that expressed the stem/progenitor marker *Sox2*, neuronal progenitor marker Pax6 and neuronal marker β-III-tubulin, but they fail to express specific retinal markers (27). It has been suggested that specific differentiation of CB-derived progenitor cells to generate functional retinal precursor cells is highly dependent on the conditions of differentiation protocols and cellular environment *in vivo* (28). Therefore, pre-differentiated or re-programmed pig CB-derived cells may provide a valuable source for retinal repair (29). RSCs were also derived from human retinal ciliary margin and were able to generate all of the different retinal cell types *in* vitro. Moreover, these cells were able to survive, migrate, integrate, and differentiate into PRCs after transplantation into adult mice (30). Pigmented CE-derived stem cells from fetal human tissue were proliferated in presence with VEGF and expressed of neural stem cells markers Notch, Jagged, N-Cadherin and β-Catenin (31). Although these studies suggest that the pigmented CE in the adult mammalian eye contains a population of cells that can be expanded in culture and exhibit characteristics of stem cells, such as self-renewal and the ability to express markers characteristic of different types of retinal neurons (32), it is unclear that these cells represent the naive, undifferentiated and unpigmented stem cells found in the CMZ of lower vertebrates or in the neurogenic regions of adult mammalian brain (33). Furthermore, the capacity of these cells to expand and self-renew gradually decrease with higher passage number (27), and eventually fail to differentiate into photoreceptors (34). Therefore, these findings suggest that the pigmented CE in adult mammalian eye does not contain rare population of RSCs, but that pigmented CE cells can acquire RPC-like characteristics, such as the capability to proliferate, self-renew, and express RSCs markers while retaining differentiated cell characteristics (34–36). However, a recent study refuted the claim by showing that retinal neurospheres (RNS) expressed Nanog at higher levels compared to embryonic stem (ES) cells and CE. Moreover, these RNS expressed retinal progenitor genes such as *Pax6*, *Chx10*, *Nestin* and *Rax*, suggesting that RNS cells have retinal progenitor characteristics (37). Debbio et. al. have also proven the potential of the stem cells in adult CE, as they possess the capacity to generate functional neurons, that express sodium and potassium channels, and are able to differentiate into RGCs and rod-PRCs (28). A recent report showed that pigmented CE-derived RSCs expressed P-Cadherin, which is important to the formation and proliferation of RSCs *in vitro*, and not is required for maintenance of RSCs *in vivo*. Thus, P-Cadherin expression is functionally important for RSCs and is not necessarily indicative of the CE tissue of origin (20).

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RSCs/PCs in the retinal pigment epithelium (RPE)

RPE consists of a monolayer of hexagonal-shaped pigmented epithelial cells that lie between Bruch's membrane and the outer neural retina, and plays multiple critical roles in the vertebrate eye, mainly supporting the function and survival of the light-sensitive PRCs (38–40). Several studies in different vertebrate species, including mammals, have indicated that embryonic RPE is capable of transdifferentiation and generating new neurons (41, 42). During transdifferentiation, RPE-derived cells begin to lose their pigmentation, expand in culture, and express stem cell markers. These RPE-derived stem cells (RPESCs) are able to generate new retinal neurons and glial cells (43–45). Interestingly, RPE in amphibians but not in fish possess the ability to transdifferentiate into neurons and glial cells (46–48). Although several *ex-vivo* studies have demonstrated the neuronal transdifferentiation capability of RPE in adult rats and humans (49–53), these adult stem cells in RPE have been used only as an allograft of unsorted adult RPE and their function as RPCs has not been clearly documented *in vivo* (reviewed by Chichagova *et al.* (54)). Adult rat RPE demonstrated the capacity to proliferate in situ but not to transdifferentiate into the diverse cell types found in the retina (55). However, adult RPE has been de-differentiated *in vitro* to a fetal RPE or proliferative stem cells with a greater replicative and regenerative capacity (50). Human fetal neuronal retinal progenitor sheets with its RPE were transplanted subretinally into severely retinal degenerated rats have shown good integration with the host, developed into mature PRCs and other retinal cells, and induced improvement in vision (56). These sheets of human fetal retinal progenitors with its RPE transplants also improved vision in humans with diabetic retinopathy (57). In comparison with human fetal RPE, adult human hRPESC-derived RPE survived and retained cell polarity when transplanted subretinally in rabbits (58). Recent reports showed that subretinal transplantation of human RPE cells derived from RPESCs into royal college of surgeons (RCS) rats prevents the loss of PRCs that occurs in these animals (59). Additional studies demonstrated that the developmental stage of human RPESC-derived RPE significantly influences the efficacy of RPE cell replacement. Specifically, transplantation of cells of an intermediate 4-week stage of RPE differentiation most consistently preserved vision in RCS rats compared to 8- or 2-week stage RPE cells (60).

RSCs/PCs in the iris pigment epithelium (IPE)

IPE are derived from the same embryonic origin as the neural retina and iris tissue has been known for its significant ability to regenerate lens in many vertebrate species; including human (61–63). Several studies demonstrated the evidence that the iris epithelial cells from amphibians (61), birds (64), pigs (27), and rodents (65, 66) exhibit RPCs properties and have the potential to generate retinal-specific cells *in vitro*. Only the inner IPE layer contain nestin-positive RPCs, which can proliferate and differentiate into diverse retinal cell types in the presence of growth factors, whereas nestin-negative cells do not proliferate and have limited neuronal potency (65). Comparative studies in adult pigs showed that neurospheres generated from dissociated cells of the iris and the pars plana were 2–3 folds greater than those isolated from the CB, suggesting that these regions may be particularly rich in stem cells. Furthermore, adult pig IPE cells in both spheres and monolayer cultures were able to differentiate into neurons and glia (27). Iris tissue derived from adult rat eye showed the

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ability to generate cells expressing differentiated neuronal markers (67). Furthermore, the *Crx* gene transfer into these cells induced the expression of rhodopsin, a specific marker for rod-PRCs (68). Recent studies showed that the combination of *Crx*, *Rx* and *NeuroD*, could convert human IPE cells into photosensitive PRCs (69). Recently, new tissue culture protocols, which consist of dispase treatment and matrigel embedding, have been tested with adult porcine IPE-derived RSCs. These cultures showed intense differentiation of IPE-RSCs to neuronal and rod photoreceptor-like cells without the need for serum or growth factors such as FGF2 and IGF2, in the culture (70). Thus, these studies indicate that IPE consists of at least two different RPCs populations, with one having the ability to differentiate into diverse retinal cell types without gene transfer or additives in culture. However, many of these cells maintain properties of differentiated epithelial cells and lack the capability to differentiate into retinal neurons (71). Therefore, further studies are needed to confirm the differentiation potential of IPE cells to fully functional PRCs before they can be used clinically.

RSCs/PCs -like Müller glia (MG) cells in the retina

MG cells are fully differentiated radial-like glial cells which span the entire thickness of the retina. They play a critical role in supporting retinal neurons and their functions, including: maintaining neuroretinal tissue; contributing to the formation of the blood-retinal barrier; and regulating neuronal signalling processes (72, 73). In response to injury, MG cells de-differentiate, proliferate, express RPCs markers, and form a protective barrier between damaged and healthy tissues. These activated MG cells help in wound healing process, modulation of immune and inflammatory responses, producing new neurons and glia and promoting neuronal survival (72, 74). In adult fish, it is well-established that retinal neurons, including cone and rod PRCs, can be regenerated from MG cells (75–78). Zebrafish is the best-studied and has been shown to contain multipotent MG cells which can self-renew, express RPCs markers such as: *Pax6*, and regenerate all retinal neuronal and glial cell types after injuries (75, 79, 80). MG cells in Japanese ricefish medaka (Oryzias latipes), exhibit a restricted capacity to regenerate the retina. However, recent studies showed that restoration of Sox2 expression— a protein which is necessary and sufficient for regeneration in MG in zebrafish as well as in the induction of pluripotent stem cells— in ricefish MGs can drive a regenerative response to injury (81–83). In postnatal chicken, upon extensive amacrine death by neurotoxin, N-methyl-d-aspartate (NMDA), the MG cells become activated and transdifferentiated into amacrine and bipolar cells (84). Furthermore, selective injury to RGCs led MG cells to generate $Brn3^+$ RGCs, in the presence of bFGF and insulin (85). Damage to adult rat retina led to activation of the dormant MG cells, proliferation and expression of RSCs markers such as Sox2 or other retinal-specific cell markers (75). Similarly, MG cells in the damaged mouse retina can be induced to proliferate when exposed to an agonist for α7 nicotinic acetylcholine receptor and to express RPC markers and can be stimulated to generate retinal neurons upon treatment with FGF and insulin or EGF (86, 87). These findings suggest that both extrinsic and intrinsic cues can guide the differentiation of MG cells to retinal-specific cells (87, 88). Recent studies showed that MG-specific overexpression of the proneural transcription factor (Ascl1), together with the use of a histone deacetylase inhibitor, which altered the epigenetic profile of the MG cells genome,

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enables adult mice-derived MG cells to generate retinal neurons *in situ*, that integrate with host retinal neurons and respond to light after retinal injury (89). Several *In vitro* studies identified a population of human retina-derived MG cells that are able to differentiate into RPE cells and retinal neurons, including those with PRCs markers (90–93). In comparison with non-pigmented CE cells, human-derived MG cells survived in culture, independent from the presence of EGF, and acquired markers of retinal neurons, including both RGCs and PRCs. However, both cell populations also expressed genes of retinal progenitors including, *Sox2*, *Pax6*, *Chx10* and *Notch* (94). Upon, transplantation of human MG-derived PRCs into rat model of photoreceptor degeneration, these cells were able to migrate and integrate into the host outer nuclear layer (ONL). Furthermore, electroretinography data showed an improvement in PRCs function (95). In light of these data, further investigations could lead to consideration of MG cells as a potential source of neural regeneration for retinal degenerative diseases in adult mammals, including humans.

Lgr5 positive Amacrine Cells as RPCs

A single study has described leucine-rich repeat containing G-protein receptor 5 (Lgr5) amacrine cells as a potential source of retinal progenitor cells in the adult mouse retina (96). Amacrine cells are a post mitotic interneuron found in the inner nuclear layer and the retinal ganglion cell layer of the retina primary responsible for modulating signals from the bipolar cells to RGCs. Lgr5, which is involved in regulation of the Wnt pathway and has been used as a marker for adult stem cells in other tissues has been localized in glycinergic amacrine cells in the adult rodent retina (97–99). Using Lgr5 promoted eGFP labelled mice coupled with the thymidine analog EDU it was observed that Lgr5 positive cells will spontaneously proliferate within the adult retina including in the absence of injury. Further it was observed that these cells would then transdifferentiate into other retinal neurons or MGs. The Lgr5 phenotype in these proliferating cells is of particular interest as Wnt signalling has previously been shown to induce MGs to proliferate and transdifferentiate into neurons (100). While these results and the existence of a neuronal cell capable of spontaneously proliferating is exciting, there is a need for this data to replicated that ensure that these cells are a source of RPCs and not newly differentiated cells from an MG source (100).

Conclusions

The regeneration of the retina through the replacement of cells leading to the restoration of lost function is an exciting possibility in the treatment of degenerative eye diseases. While this challenge may appear daunting, an evolutionary precedent for this regeneration exists in fish and amphibians where the retinogenesis process is maintained after embryonic development and the retina continues to grow throughout their life through the addition of new neurons into the retina mainly from RSCs in CMZ, rod progenitors in the INL and ONL, and MG cells. In birds, a higher order vertebrate, retinogenesis at the peripheral margin of retina is limited, but it can be enhanced by extrinsic growth factors. In adult mammals, including human, while retinal cells have not been observed to proliferate or regenerate for cell loss or injury *in vivo*, several studies illustrated the ability of the pigmented cells in CB to transdifferentiate into RPCs *in vitro*. Furthermore, MG cells have demonstrated the capacity to generate different retinal neurons *in vitro* as well as in

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genetically modified animals *in vivo*. Therefore, pigmented cells within the CB and MG cells should be identified as a potential source for retinal regeneration and vision restoration. Despite the progress that has been made in identification of extrinsic cues and intrinsic determinants involved in RPC differentiation, further understanding of the signalling pathways that regulate specific differentiation of these cells into desired phenotypes (e.g., photoreceptors, RPE) specifically for cells activated *in situ* is needed. Many *in vitro* studies have demonstrated the ability of RPCs to differentiate into diverse retinal neurons and glial cells, but this is limited after transplantation, where the integration and differentiation are dependent on host environment including the maturity of the retina, the immune response and the inhibitory molecules secreted by the degenerated retina. Whereas, alteration in delivery system and gene reprogramming could improve RPC transplantation, the activation of this dormant *in situ* regenerative process through the addition of extrinsic factors (such as FGF2, EGF, insulin or Wnt signal modulation) or the alteration of intrinsic signals (such as the overexpression of Ascl1) may provide a more successful strategy for vision restoration.

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Table 1:

Factors involved in retinal stem cell proliferation, differentiation and identification.

Gene Name	Gene Symbol	Function	References
Achaete-scute family bHLH transcription factor 1	Ascl1	Transcription factor which plays a role in the neuronal commitment and differentiation. Used for the reprogramming of Müller glia to retinal progenitors that underlies retinal regeneration.	(89)
β-Catenin (Catenin beta-1)	CTNNB1	A subunit of the cadherin protein complex and acts as intracellular signal transducer in the Wnt signalling pathway. Has been shown to be a cell-cell or cell-matrix contact-regulated inducer of neurogenesis and is required for neural differentiation.	(31, 101)
N-Cadherin (neural cadherin)	NCAD	Transmembrane protein that involves in upregulation of adhesion and extracellular matrix proteins accompanies the transformation of neuro epithelial cells (NE) into radial glia.	(31)
P-Cadherin (Cadherin-3)	CDH3	Calcium-dependent glycoprotein involved in cell-cell adhesion and is important to the formation and proliferation of RSCs *in vitro*.	(20)
Class III β-Tubulin	TubB3	Neuronal specific class of the tubulin proteins that form the microtubules of the cytoskeleton. It has been used to identify and separate neurons in brain tissues from glial cells. Considered a RGCs marker due to their neuronal origin.	(27, 102)
Cone-rod homeobox protein	Crx	Photoreceptor-specific transcription factor which plays a role in the differentiation.	(68, 69)
Epidermal growth factor	EGF	Stimulates cell growth and differentiation by binding to its receptor, EGFR.	(16)
Fibroblast growth factors	FGF2 and bFGF	Involved in mitogenic and cell survival activates. Also, involve in embryonic development, cell and tissue growth or repair.	(16, 85)
Homeobox protein Chx10	Chx10	Critical for progenitor cell proliferation and bipolar cell determination in the developing retina.	(94, 103)
Insulin-like growth factor 2	IGF2	Hormone involved in the regulation of cell proliferation, migration, differentiation, and survival.	(70)
Jagged1	Jag	Acts through Notch1 to promote self-renewal and neurogenic potential of multipotent NSCs.	(31, 104)
Leucine-rich repeat-containing G-protein coupled receptor 5	Lgr5	Involved in regulation of the Wnt pathway and has been used as a marker for adult stem cells in other tissues. Has been localized in glycinergic amacrine cells in the adult rodent retina.	(97–100)
Microphthalmia-associated transcription factor	Mitf	Member of the basic helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor family. *Mitf* has been shown to regulate a broad range of genes, ranging from genes important for pigment production, to genes involved in cell cycle regulation, migration and survival. In the mouse, *Mitf* is expressed early and uniformly in optic vesicle cells as they evaginate from the developing neural tube.	(25, 105)
Nanog		Transcription factor critically involved with self-renewal of undifferentiated stem cells. Also, play an important role in maintenance of pluripotency.	(37, 106)
Neuroectodermal Stem Cell Marker	Nestin	Type VI intermediate filament protein used as a marker for neural stem cells.	(37, 65)
Neuronal Differentiation 1	NeuroD1	Basic helix loop helix transcription factor that promotes neuronal differentiation and development.	(69)
Notch		Transmembrane receptor protein that regulates the proliferation, migration, and differentiation of neural crest cells during the embryonic development.	(31, 94)
Paired box protein 6	Pax6	A key regulatory gene of the eye and brain that is present during embryonic development and promotes neurogenesis. Mutation of the *Pax6* gene is known to cause various disorders of the eyes.	(26, 27, 37, 75, 79, 80, 94)
POU Class 4 Homeobox 2	Pou4F2 or Brn3	Plays an important roles in differentiation, survival, and axonal elongation during the development of RGCs and their specific subtypes.	(85, 107)

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Gene Name	Gene Symbol	Function	References
Recoverin	Rcvrn	Neuronal calcium-binding protein that is normally expressed in PRCs functioning as a calcium sensor that regulates rhodopsin phosphorylation through inhibition of rhodopsin kinase.	(24)
Retinal Homeobox	Rax or Rx	Essential for vertebrate eye development. *Rx* function is required for the specification and maintenance of retinal progenitor cells (RPCs). Also necessary during retinal regeneration and loss of function leads to a lack of eye development.	(26, 69, 108)
SIX homeobox 3	Six3	Plays a crucial role in retinal development during embryonic stage by activation of *pax6* and *sox2*.May also play a role in lens development.	(26)
SRY-box 2	Sox2	Transcription factor and the earliest marker of the neural plate. Expressed in proliferating cells and those that acquire glial fates. Regulates NSC multipotency and proliferation capacity. Plays an important roles in both human and mouse retinal development. Assists in the induction of amacrine and müller glial cells in mouse *RPCs*.	(75, 81–83, 94)
Vascular endothelial growth factor	VEGF	Sub-family of the platelet-derived growth factor family of cysteine-knot growth factors. Involved in both vasculogenesis and angiogenesis.	(31)
WNT signaling gene family or Wingless-type MMTV integration site family or Wingless/ Integrated	Wnt	Wnt signals are present in early and developed stages of various tissues. Wnt proteins regulate cell proliferation, mediate signalling between neighboring cells and control stem cell proliferation and self-renewal. It also proved to regulate proliferation and neurogenic potential of Müller Glial cells via a Lin28/*let-7* miRNA-dependent pathway in adult mammalian retinas.	(100)