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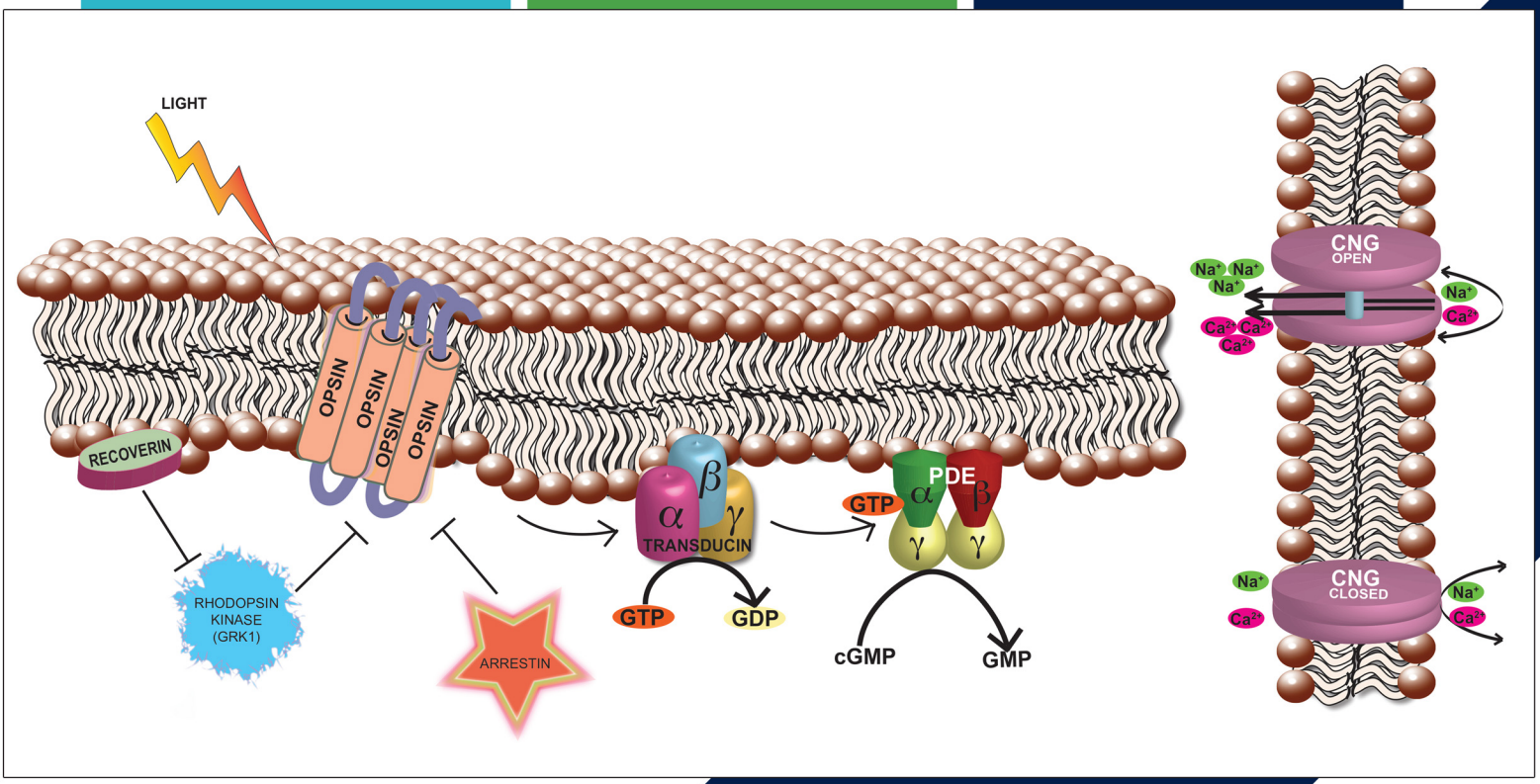
MOLECULAR BASES OF NEURODEGENERATIVE DISORDERS OF THE RETINA



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Molecular Bases of Neurodegenerative Disorders of the Retina

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PREFACE

The interpretation of light signals has fascinated as well as perplexed the minds of geniuses, including Isaac Newton, Albert Einstein and Charles Darwin. The eye is the medium through which our brain interprets the various environmental cues. It also acts as a window to the brain. Ever since humanity has developed an understanding of the ways eyes interpret light signals, many mysteries about its structure, development and function have been unearthed.

Photoreceptors (rods and cones) - which are situated in the back of the eye in a transparent tissue called Retina - form the majority of the neurons that are involved in transmitting light signals to the brain. The unique polarized morphology and physiology of the photoreceptors make them one of the most fascinating cell types. So much so, that they have been used to examine the way our eyes have evolved to interpret visual cues. In fact, photoreceptors are the first responders to light, and thus hold the bulk of our visual power. However, 'with great power comes great responsibility'; photoreceptors have evolved stringent mechanisms to maintain their function over the lifespan of an organism. The light-sensing cellular antenna of the photoreceptors is a modified cilium. Even slight perturbations in the development, structure, or function of the cilium can result in severe blindness disorders (called ciliopathies). Innovative technological advances are rapidly leading to a better understanding of the photoreceptors, particularly with respect to the involvement of the cilia in interpreting the light signal. Moreover, defects in the retinal vasculature and neuronal degeneration are the primary cause of debilitating disorders, such as Diabetic Retinopathy and Age-related Macular Degeneration. Numerous investigations have yielded new insights on the involvement of defective protein trafficking and angiogenesis in the manifestation of various eye diseases. I have compiled information from excellent investigators who have worked diligently and made seminal contributions to the field of photoreceptor development and pathogenesis of neuronal degeneration in the retina.

My sincere thanks go to the authors for contributing chapters and highlighting their comprehensive and cutting-edge research.

Cover art by Manisha Anand, Department of Ophthalmology, UMASS Medical School, Worcester, MA.

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CHAPTER 1

Ciliary Trafficking in Vertebrate Photoreceptors

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Abstract: Cilia are microtubule-based extensions of the plasma membrane of cells. These extensions detect extrinsic cues that are crucial for carrying out a myriad of developmental and homeostatic signaling cascades. Cilia have evolved diverse means to mediate signaling cascades in a cell-type specific manner. In this article, I have summarized the conserved mechanisms of formation of cilia and their structural and functional specialization for light detection.

Keywords: Basal body, Cilia, Photoreceptor, Retina.

INTRODUCTION

The cilia, Latin for 'eyelash', were first described by Anton van Leeuwenhoek in 1675 as *'incredibly thin feet, or little legs, which were moved very nimbly'* [1]. Historically, although cilia were considered vestigial, notable hypotheses were put forth to understand the nature and function of cilia. For example, cilia were thought to be active organelles and that sperm flagella (or cilia) contained fibrils that extended across the entire length of the flagellum. However, these hypotheses could not be tested due to the technical limitations at the time. Cilia and flagella have now been recognized as key players in organism development and homeostasis [2].

Based on the structure and function of the microtubule skeleton, there are two types of cilia: motile cilia and primary (or immotile) cilia. As the name implies, motile cilia are involved in cell movement, such as sperm motility and mucosal clearance by the trachea. They originate from the basal body, the mother centriole in the form of microtubule extensions. The motile cilia possess 9 outer doublet microtubules and a central pair of singlet microtubules, forming a 9+2 array. Unlike motile cilia, the primary cilia display a 9+0 arrangement of microtubules due to the absence of the central microtubule pair. Both types of cilia are involved

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in sensory perception, hence also termed sensory cilia. Although the motile and primary cilia are clearly distinguished based upon their motility and function, there are instances of motile cilia being sensory, such as tracheal cilia, and primary cilia show properties of motility, such as the cilia in the embryonic node [3 - 7].

Cilia act as a hub for G-protein coupled receptors (GPCRs) such as rhodopsin [8 - 10]. A conserved process called Intraflagellar Transport (IFT) regulates cilia growth and function. IFT in cilia participates in signaling pathways by concentrating cognate receptor proteins in the ciliary membrane [6] and involves movement of cargo (membrane receptors) towards cilia tip by anterograde motor Kinesin-II and back to the base driven by cytoplasmic dynein-2 motor. Previous studies have shown that IFT proteins are organized into two distinct complexes: complex A (IFT144, 140, 139, and 122) and complex B (comprising of several IFT proteins, including IFT88, 81, 80, 27, and 20) [11 - 14] (Fig. **1**). Disruption of IFT proteins results in defective cilia formation and associated function.

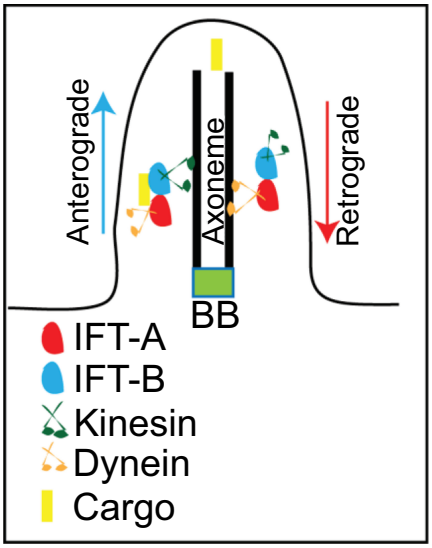

Fig. (1). Cilia: This figure depicts a simplified representation of IFT in cilia. The IFT-B particle carries cargo in anterograde direction to distal tip of cilia using the anterograde motor Kinesin-II whereas dynein and the IFT-A complex carry out retrograde movement.

Given the involvement of cilia in various developmental pathways, their dysfunction is associated with severe human disorders, collectively termed ciliopathies. In fact, involvement of cilia in human diseases was first reported in a disorder of ciliary motility in patients with primary cilia dyskinesia (PCD). Although named as a dysfunction of primary cilia, which are immotile cilia, this disease is caused due to defects in motile cilia [15]. Bjorn Afzelius first identified

this disorder in 1976 in patients that exhibited immotility of bronchial cilia, sperm dysfunction and ear infections [16]. PCD in association with *situs inversus* (reversal of the left-right asymmetry of the body) is also observed in patients with Kartagener syndrome [16 - 18]. In the last decade, ciliary dysfunction has been linked to numerous ciliopathies, including Meckel-Gruber syndrome, Bardet-Biedl Syndrome, Joubert Syndrome, polycystic kidney diseases, Nephronophthisis, Senior-Loken Syndrome, Usher Syndrome, and some forms of Retinitis Pigmentosa (RP) [3].

RP is a clinically and genetically heterogeneous group of disorders characterized by retinal degeneration due to the loss of rod and cone photoreceptors. Photoreceptor dysfunction and degeneration due to ciliary dysfunction is commonly observed in ciliopathies. In this chapter, I will briefly describe the anatomy of the eye and the retina, and focus on the involvement of cilia in the maintenance of photoreceptor structure and function. I will also briefly discuss the involvement of ciliary function in other cell types of the retina that are critical for normal vision.

Eye

The eyeball is located at a strategic position to ensure maximum light detection while securely positioned in the cavity of the orbit. It is largely divided into anterior and posterior chambers. Light enters the anterior part of the eye, which includes the cornea and the pupil, and passes through the lens to the posterior part of the eye, which is the retina; here, it activates a complex set of events. The retina then projects the signal *via* the optic nerve to the lateral geniculate nucleus (LGN) in the brain (Fig. **2**).

There are three major fluid-filled chambers in the eye: anterior chamber, which is between the cornea and the iris; posterior chamber, between the iris and the lens and vitreous chamber, which is located between the lens and the retina and is filled with vitreous humor. The anterior and the posterior chambers are filled with a fluid called aqueous humor, which supplies nutrients to the cornea and the lens and maintains optimum pressure in the eye, called intraocular pressure (IOP). The IOP is regulated by a balanced inflow and outflow of aqueous humor from the anterior chamber into the blood stream. A major pathway for outflow of the fluid is *via* the trabecular meshwork tissue into Schlemm's canal. This outflow is measured to calculate the IOP of the eye, which serves as an important risk factor for developing an incurable blindness disorder called glaucoma [19, 20].

CHAPTER 2

Zebrafish Models of Photoreceptor Ciliopathies

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Abstract: Ciliopathies refer to a genetically and clinically heterogeneous class of disorders that result from defects in the formation or function of the primary cilium. Cilia are the microtubule-based organelles that protrude from the surface of almost all vertebrate cells, including the rod and cone photoreceptors. The photoreceptor sensory cilium consists of the connecting cilium and outer segment with the outer segment forming a unique structure containing thousands of tightly packed disc membranes. Mutations in over 50 genes result in syndromic ciliopathies that can manifest with retinal degeneration, including Bardet-Biedl syndrome (BBS), Joubert Syndrome, Jeune Syndrome, and nephronophthisis (NPHP) or in non-syndromic retinal dystrophies like Leber Congenital Amaurosis (LCA) and Retinitis Pigmentosa (RP). Zebrafish have been widely used as a model system to study ciliopathies, particularly BBS and Joubert Syndrome, and for studying the mechanisms leading to photoreceptor degeneration associated with these disorders. Investigators were drawn to zebrafish due to the rapid growth and transparency of the zebrafish embryo, the differentiation of photoreceptors by 3 days post-fertilization, and the ability to suppress gene function through morpholino knockdown. The genetic heterogeneity of ciliopathies and desire for more accurate genotype-phenotype correlations make zebrafish an appealing model for studying gene- and allele-specific differences in a rapid manner. This review will discuss the current zebrafish models of retinal ciliopathies, evaluate the widespread use of morpholinos as tools to knock down gene function in zebrafish, and make predictions on how zebrafish will contribute in future studies of ciliopathies.

Keywords: *ahi1*, *arl13b*, Bardet-Biedl Syndrome, BBS, *cep290*, Ciliopathies, Cilium, Joubert Syndrome, Photoreceptor, Retina, Retinal Degeneration, Sensory Cilium, Zebrafish.

INTRODUCTION

Cilia are microtubule-based organelles that typically project from the apical surface of almost all vertebrate cells and remain surrounded by the plasma membrane. At the base of each cilium is a protein and microtubule-based structure

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called the basal body. The basal body is a modified centriole that nucleates cilium formation and serves to link the cilium with the rest of the cytoskeleton. Ciliary microtubules organize in pairs to form an axoneme, the structural backbone of the cilium. The axoneme is organized with either a "9+2" or "9+0" geometry, depending on the presence of a central pair of microtubule doublets, although exceptions to these rules do exist [1]. Motile cilia typically have a "9+2" axoneme, while non-motile and primary cilia possess a "9+0" axoneme. For many years, research in cilia largely focused on the role of cilia in cellular locomotion in single-cell eukaryotes and in fluid movement in certain tissues. In the early 2000s, however, interest in ciliary biology exploded when genes encoding cilia or basal body proteins were directly linked to the multi-syndromic diseases termed "ciliopathies". The ciliopathies exhibit a variety of clinical symptoms that typically involve kidney, eye, brain, and skeletal abnormalities, along with other variable phenotypes [2, 3]. To better understand the role of cilia in development and the etiology of disease, scientists often turned to zebrafish as an *in vivo* model to study cilia pathology. Forward genetic screens in zebrafish identified mutations in cilia-related genes, but morpholino oligonucleotides were widely used to knock-down gene function. Since 2004, cilia have been the subject of almost 800 review articles discussing the role of cilia in signaling [4 - 8], development [9, 10], and disease [2, 3, 11], as well perspectives on trafficking mechanisms and motors in cilia [12 - 14], and cilia evolution [15, 16]. With so many authoritative and exhaustive reviews available, it would seem redundant to address these topics again. Missing from these commentaries, however, is a closer inspection of the tools, techniques, and experimental approaches taken when using zebrafish as a model, as well as the resulting phenotypes. In light of new advances with genome editing tools, such as TALE nucleases (TALENs) and CRISPRs, it would be useful to catalog the phenotypes reported for zebrafish cilia mutants and phenotypes obtained from morpholino injections and consider commonalities and differences in these phenotypes. This review will describe the photoreceptor sensory cilium, provide a brief overview of ciliopathy diseases, and finally, examine how researchers have used the zebrafish as a model to understand the genetics and cell biology of ciliopathies, with a particular emphasis on photoreceptors.

The Photoreceptor Sensory Cilium

Vertebrate photoreceptors consist of a light-sensitive outer segment, an inner segment containing the biosynthetic machinery, and a synaptic terminal. The photoreceptor outer segments contain hundreds of tightly stacked disc membranes, with each disc membrane containing thousands of molecules of the visual pigment and the machinery for phototransduction. The visual pigment of rods is the G-protein coupled receptor rhodopsin, while cones express specific

cone opsins with sensitivities to different wavelengths of light. It is estimated that an individual photoreceptor contains approximately one billion opsin molecules, in addition to components of the G-protein cascade necessary for light detection. Protein synthesis, however, occurs in the inner segment and a highly efficient trafficking mechanism transports proteins to the outer segment. The inner and outer segments are connected by a thin, nonmotile cilium. First identified as a bona fide ciliary structure by electron microscopy in 1956 by Eduardo De Robertis [17], the photoreceptor connecting cilium was considered distinct from the outer segment and largely regarded as little more than a passageway for phototransduction proteins destined for the outer segment. Today, the connecting cilium and outer segment are together considered a single "sensory cilium" with a ciliary proteome consisting of almost 2000 proteins [18].

Photoreceptor sensory cilia exhibit many characteristics typical of all cilia, as well as a number of features unique to photoreceptors. The structure of photoreceptor cilia has been studied for years by electron microscopy [17, 19, 20], as well as by freeze-fracture analysis [21], and recently by cryo-electron tomography [22]. Like all cilia, photoreceptor sensory cilia contain an axoneme, a transition zone (*i.e*. the connecting cilium), and a basal body anchoring the cilium to the cytoskeleton by a long, striated rootlet [22]. Photoreceptors contain two basal bodies. The distal basal body, or mother centriole, sits at the top of the rootlet and serves as a template for the microtubule doublets of the axoneme. The proximal basal body, or daughter centriole, aligns perpendicularly with the distal basal body but does not generate an axoneme. Microtubule triplets project apically from the distal basal body and transition into doublets to form the 9+0 nonmotile axoneme, which extends through the transition zone (*i.e*. connecting cilium) and into the outer segment. The microtubule doublets split apart within the distal outer segment and extend to the outer segment tips as microtubule singlets in both mammals and zebrafish [23, 24]. The most distinctive, and unique, feature of the photoreceptor cilium is the massive volume occupied by the disc membranes. Like other primary cilia, the process of Intraflagellar Transport (IFT) is required for the development and maintenance of the photoreceptor connecting cilium and outer segment [24 - 30]. The details of IFT [12, 31], the IFT motors [13, 14, 32], its requirements for photoreceptor sensory cilia [33, 34] have been extensively reviewed elsewhere and interested readers are encouraged to seek out these sources.

The anatomical structure of the photoreceptor cilium and molecular mechanisms governing cilia development and maintenance are highly conserved among vertebrates. As this review focuses on zebrafish, however, a few key distinctions between zebrafish, rodent, and human photoreceptor sensory cilia should be briefly mentioned. First, calyceal processes are present in the photoreceptors of

CHAPTER 3

Inositol Phosphatases in Retinal Ciliary Disorder

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Abstract: Phosphoinositides are phospholipids that regulate signal transduction, endocytosis, and protein trafficking. Each phosphoinositide has a unique pattern of distribution within cellular compartments and is tightly regulated by inositol kinases and phosphatases localized within a specific membrane compartment. Inositol polyphosphate 5-phosphatases regulate phosphoinositide levels and are implicated in human diseases, such as Lowe syndrome and Joubert syndrome. Here we review the pertinent findings of the roles of 5-phosphatases in cilia function and signaling.

Keywords: Cilia, INPP5E, Joubert syndrome, Lowe syndrome, OCRL.

INTRODUCTION

Cilia are evolutionarily conserved hair-like organelles protruding from the plasma membrane (PM) of nearly all post-mitotic vertebrate cells [1 - 3]. A cilium is comprised of a nucleating basal body and a phospholipid membrane-bound axoneme that grows from the basal body during nutrition deprivation. The axoneme can be subdivided into a transition zone, axonemal stalk and a distal ciliary tip. The cilium is conventionally classified into two main types: motile and immotile/primary cilium. In the motile cilia, the outer nine sets of microtubules surround a central inner microtubule pair ("9+2" arrangement), whereas primary cilia lack the central microtubule doublet ("9+0" arrangement) [4]. This review will focus on the primary cilia.

Cilium formation (ciliogenesis) occurs in several stages. Typically it is induced when nutrition is deprived and the cell stops cell division during G1 phase of cell cycle. The mother centriole differentiates into the basal body, which associates with membrane vesicles and migrates to the cell surface where it forms the base of the primary cilium [5]. Next, elongation of the axoneme from the basal body is mediated by the transport of ciliary building proteins, a process called intraflag-

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ellar transport (IFT). Extension of the primary cilium occurs exclusively at the distal tip of the axoneme. Typically, there is only one primary cilium per quiescent cell; however, some cell types can develop a group of primary cilia (*e.g.* choroid plexus of the brain) [6].

Being a sensory organelle, the primary cilium plays a crucial role in embryonic development and adult tissue homeostasis [7]. Upon chemical and mechanical stimulation, the primary cilia can sense changes in the extracellular environment and initiate multiple intracellular signal transduction pathways. Hedgehog (Hhg), Wnt and platelet-derived growth factor (PDGF) signaling pathways are three typical signal transduction pathways with components localized to the primary cilia [8 - 10]. Hhg signaling pathway and its proteins specify tissue patterning in embryonic development and play an important role in post-natal homeostasis [11]. Upon binding to its receptor, the twelve-transmembrane protein Patched (Ptch), Hhg facilitates the activation of Smoothened (Smo), which in turn causes the accumulation of the active form of the transcription factor Gli in the primary cilium [12 - 14]. The disruptions of the Hhg pathway by mutations in genes that regulate ciliogenesis result in developmental disorders.

In the eyes, photoreceptors are a unique type of ciliated cells that specialize in visual phototransduction. The connecting cilium within each photoreceptor is required for the formation of the outer segment in the retina, where photosensory G protein-coupled receptor rhodopsin molecules are densely packed to allow detection of single photons [15]. The cilium is now recognized as a critical structure within the photoreceptors that mediate visual transduction. Defects in the ciliary machinery often result in improper formation of the outer segment of the retina and presents as retinal degeneration in patients [16 - 20].

Phosphoinositides and Inositol Phosphatases

Phosphoinositides, also referred to as phosphatidylinositol lipids, are ubiquitous phospholipid signaling molecules present in all mammalian cells [21]. Phosphoinositides are composed of a hydrophobic fatty acid and glycerol backbone, enabling the insertion into lipid membranes, linked to a soluble six sided inositol head group that may be phosphorylated at the D3, D4 and D5 positions, giving rise to seven different signaling molecules [22]. These signaling molecules regulate vesicular trafficking, cell proliferation and differentiation, protein synthesis and cytoskeletal rearrangements by acting as precursors to second messengers or by recruiting and activating phosphoinositide-binding effector proteins [7]. The intracellular levels of phosphoinositides are tightly regulated, both spatially and temporally, by a complicated and well choreographed interplay between phosphoinositide kinases and phosphatases (Fig. **1**).

Phosphoinositide kinases (*i.e.* phosphoinositide 3-kinase; PI3K) generate phosphoinositide second messengers such as phosphatidylinositol(3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) by phosphorylation of phosphatidylinositol(4,5)-bisphosphate (PtdIns(4,5)P_2). PtdIns(4,5)P_2 is the most abundant phosphoinositide that serves as substrate for various phospholipases C, which generate diacylglycerol (DAG) and the soluble inositol Ins(1,4,5)P_3 by cleavage of PtdIns(4,5)P_2 [23]. Highly conserved inositol polyphosphate phosphatases hydrolyze their specific phosphorylated phosphoinositide substrates and are classified according to the position they dephosphorylate on the inositol ring.

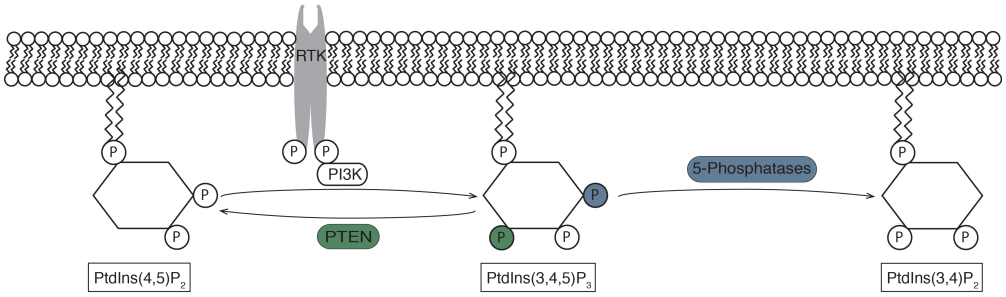

Fig. (1). Phosphoinositide kinases and phosphatases regulate intracellular levels of phosphoinositides. The binding of substrate to growth factor receptor tyrosine kinase (RTK) results in autophosphorylation on tyrosine residues of the receptor. Phosphoinositide 3-kinase (PI3K) activity is initiated by direct binding of PI3K to these phosphotyrosine residues, resulting in transient phosphorylation of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Inositol polyphosphate phosphatases such as the 3-phosphatase PTEN and 5-phosphatases (including OCRL, INPP5B and INPP5E) control phosphoinositide levels by removing a phosphate group from PtdIns(3,4,5)P_3. PTEN removes a phosphate group from the 3-position of the inositol ring generating PtdIns(4,5)P_2, whereas 5-phosphatases form PtdIns(3,4)P_2 by dephosphorylating the 5-position of the inositol ring.

The inositol polyphosphate 5-phosphatases (5-phosphatases) remove the phosphate group from the 5-position of the inositol ring in an Mg^{2+}-dependent mechanism [24]. Five known substrates for 5-phosphatases have been identified: the water soluble inositol phosphate substrates inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4), and the lipids PtdIns(3,5)P_2, PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2. The 5-phosphatase family comprises ten mammalian members, which are classified into four groups based on substrate specificity [25]. Group I hydrolyzes only Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, group II hydrolyzes all known substrates, group III only hydrolyzes substrates with a phosphate group in the D3-position (*i.e.* PtdIns(3,4,5)P_3) and group IV is only active against PtdIns(3,4,5)P_3 and remains poorly understood. All members of the 5-phosphatases family share a conserved 300 amino acid catalytic 5-phosphatase domain. Additional protein-interaction domains confer unique specificity of 5-phosphatases depending on the cell types [26].

CHAPTER 4

Understanding the Pathogenesis of Neurodegeneration in Diabetic Retinopathy (DR)

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Abstract: Diabetic Retinopathy (DR) is the leading cause of irreversible global vision loss. It affects the entire neurovascular unit of the retina, along with gradual neuro-degeneration and neuroinflammation. DR has primarily been considered a microvasculature complication of diabetes, a well-known metabolic disorder. However, recent studies have reported the presence of neurodegenerative changes in the retina of DR patients prior to clinical manifestations. In this review, we have compiled clinical, histopathological, biochemical and genetic evidences that suggest a role of neurodegeneration in DR progression and pathogenesis. These studies indicated neural changes in the retina that have lead to microvascular alterations. Furthermore, the mechanisms underlying the neural changes can help identify drug targets for effective management of the disease, which in turn will help reduce the burden of visual impairments caused by DR.

Keywords: Degenerative disease, Diabetes, Inflammation, Neurons, Retina.

NEURODEGENERATION

Neurodegeneration can be defined as the degenerative changes (both structural and functional) in neurons that lead to progressive loss of neuronal function whilst promoting their death through apoptosis or other mechanisms like autophagy and necrosis [1]. Neuronal damages are irreversible and show detrimental effects on the human body. Neurodegenerative changes include an increased rate of cell death and proliferation of macroglial population (known as reactive gliosis and recognized by the increased expression of glial fibrillary acidic protein (GFAP) and microglial activation [2, 3]. Neurodegeneration has been implicated in the pathogenesis of central nervous system diseases like Parkinson's, Alzheimer's

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and Huntington's disease [4, 5]. The eye is also vulnerable to neurodegenerative changes, as found in the pathogenesis of vision threatening diseases such as glaucoma, retinitis pigmentosa and age related macular degeneration [6 - 8]. However, neuronal damage has not been documented in diabetic retinopathy (DR) [9].

DIABETIC RETINOPATHY

Diabetes is one of the major causes of socio-economic burdens in the developing world. It is comprised of a group of metabolic diseases that affect multiple organs. The retina can be severely affected by the diabetic changes, leading to catastrophic loss of vision (termed Diabetic Retinopathy). DR manifests as damaged vascular as well as neuronal networks causing vitreous hemorrhage, microaneurysms, lipid exudates, cotton wool spots, macular edema and abnormal neovascularization [10].

A recent population-based study (2012) estimated the overall global prevalence of DR to be 34.6%. This number is increasing exponentially [11]. The duration of diabetes and glucose levels are major risk factors that determine the prevalence of DR [12]. The Wisconsin Epidemiologic Study of DR observed that 80% of patients with diabetes developed retinopathy within 15 years of its onset. The study also noted that the prevalence of advanced stage of DR was 67% in people with a longer duration of diabetes (>35 years), while it occurred in only 1.2% of people with shorter disease duration (<10 years) [13]. According to a WHO report, the occurrence of diabetes in India reached 31.1 million people. The WHO report also claims that this number will double each year [14]. The 2007 Andhra Pradesh Eye Disease Study (APEDS) in Southern India suggested an estimate of around 2.77 million people with DR and nearly 0.07 million people with severe DR [15].

Complications of Diabetic Retinopathy

DR is classified in two categories: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). These categories are based on the detectable changes in the retinal microvasculature. NPDR represents the earliest stage of DR. In patients with poor diabetic control, NPDR slowly progresses to the severe proliferative PDR stage. In mild NPDR, patients show one microaneurysm or dot blot hemorrhage in fundus quadrants. In severe NPDR, hemorrhage, venous bleeding and abnormalities in intra-retinal microvasculature are commonly observed [16].

In PDR, the ischemic retina releases growth factors like the vascular endothelial growth factor (VEGF) that induce the proliferation of abnormal vessels in the

retina [17]. The newly formed vessels known as neovessels are fragile and tend to bleed at any time, causing vitreous hemorrhage. These neovessels create tractions in the retina as well as the detachment of the retina from the choroid. Neovascular glaucoma is also a vision-threatening complication of PDR, which is caused by the formation of new vessels, which can block the normal aqueous humor flow in the anterior chamber of the eye [18]. Another major factor of vision loss in DR is diabetic macular edema (DME), which is characterized by the accumulation of leaked fluids from the retinal capillaries in the macula, area of central vision [19].

Therapeutic approaches for DR depend upon the severity of the complications in patients. The most widely used approach during early disease stage is retinal laser photocoagulation [20]. This treatment seals leaked vessels, which redirects the blood supply and reduces overall oxidative damage.

Lately, anti-VEGF therapy has become a preferred strategy in the management of DME. VEGF is essential for many physiological functions in the retina. It plays a major role in vasculogenesis and neurogenesis. It is also an important neuroprotective agent in the retina. However, a side effect of anti-VEGF therapy is neurodegeneration [21].

NEURODEGENERATION IN DIABETIC RETINOPATHY

The retina is a highly metabolically active tissue in the eye. It is a well-organized laminated structure of multiple cell types (Fig. **1**). The vertebrate retina contains two synaptic layers intercalated between three nuclear layers: outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL). The ONL contains rod and cone photoreceptor nuclei, whereas the INL is composed of horizontal, bipolar and amacrine cell nuclei. The GCL contains the nuclei of retinal ganglion cells (RGC) and displaced amacrine cells [22]. The retinal nerve fiber layer (RNFL) forms the innermost layer and is composed of axons of the ganglion cells. The nourishment to neuronal cells in the retina is provided through the blood vessels which are organized in a specific pattern in the retina. The communication between the blood vessels and neuronal cells in the retina is maintained by two types of glial cells: microglia and macroglia [23]. This entire cellular network in the retina gets compromised in DR. A majority of the studies on DR is primarily focused on the changes in the microvasculature of the retina. However, the neuronal damage in DR pathogenesis has largely been overlooked. Various preclinical and clinical studies have also provided plenty of evidence for neuronal damage in diabetic eyes, which is discussed in the next section.

CHAPTER 5

Rhodopsin Traffics to the Rod Outer Segment in the Absence of Homodimeric and Heterotrimeric Kinesin-2

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Abstract: Homodimeric (KIF17) and heterotrimeric kinesin-2 (KIF3A, KIF3B and KAP) molecular motors are essential for anterograde intraflagellar transport (IFT) among invertebrates. Here we show that deletion of KIF3A in embryonic mouse retina interferes with IFT by preventing transition zone and axoneme formation. Absence of outer segments leads to severe mistrafficking of rhodopsin and rapid degeneration. By contrast, deletion of KIF3A in the adult mouse by tamoxifen-induction reveals normal rhodopsin transport to outer segments with failure of outer segment (OS) maintenance. Germline deletion of KIF17, a motor that cooperates with heterotrimeric kinesin-2 among invertebrates, affected neither OS structure nor photoreceptor morphology/ function thereby excluding an essential role of KIF17 in photoreceptor IFT. A KIF3A/KIF17 double knockout phenocopies a rod-specific KIF3A knockout. We conclude IFT is not required for rhodopsin transport to the OS but rather, anterograde IFT mediated by KIF3 participates in photoreceptor transition zone (PTZ) and axoneme formation.

Keywords: Anterograde intraflagellar transport (IFT), Conditional knockouts, Heterotrimeric kinesin-2, Homodimeric kinesin-2, Mouse photoreceptors, Rhodopsin trafficking.

INTRODUCTION

The light-sensitive photoreceptor sensory cilium, regarded as a modified primary cilium, is comprised of OS disc membranes, a basal body (microtubule-organizing center) and an axoneme. Each OS communicates with its inner segment (IS) through a connecting cilium which is a structure equivalent to the transition zone of primary cilia [1]. OS proteins, synthesized in the IS, must traffic through the photoreceptor transition zone (PTZ) to be incorporated into nascent discs. Intra-

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flagellar transport (IFT), a bidirectional ciliary trafficking pathway conserved among invertebrates and vertebrates [2], has been suggested to traffic rhodopsin and other OS proteins together with IFT particles (IFT-A and IFT-B) along the ciliary axoneme [3, 4].

Heterotrimeric kinesin-2 (KIF3, consisting of KIF3A, KIF3B and KAP subunits) [5] and homodimeric kinesin-2 (KIF17) [6] are canonical anterograde IFT motors present in a broad range of species [7 - 9]. KIF17 and KIF3 are thought to cooperate during ciliogenesis in which KIF3 builds the axoneme core and KIF17 the axoneme distal segments (Fig. **1**) [10, 11]. However, interaction of KIF17 with KIF3, and KIF17 contribution to vertebrate ciliogenesis or membrane protein trafficking are unclear. Disruption of KIF3A, the obligatory subunit of KIF3, caused rapid photoreceptor degeneration and OS protein mistrafficking [12, 13]. However, we showed that rod-specific knockout of KIF3A did not prevent trafficking of rhodopsin to the OS even as the OS degenerated [14]. A second anterograde motor candidate in photoreceptors is KIF17, which mediates IFT in *C. elegans* (Osm-3) [11] and was suggested to participate in zebrafish photoreceptor development [15].

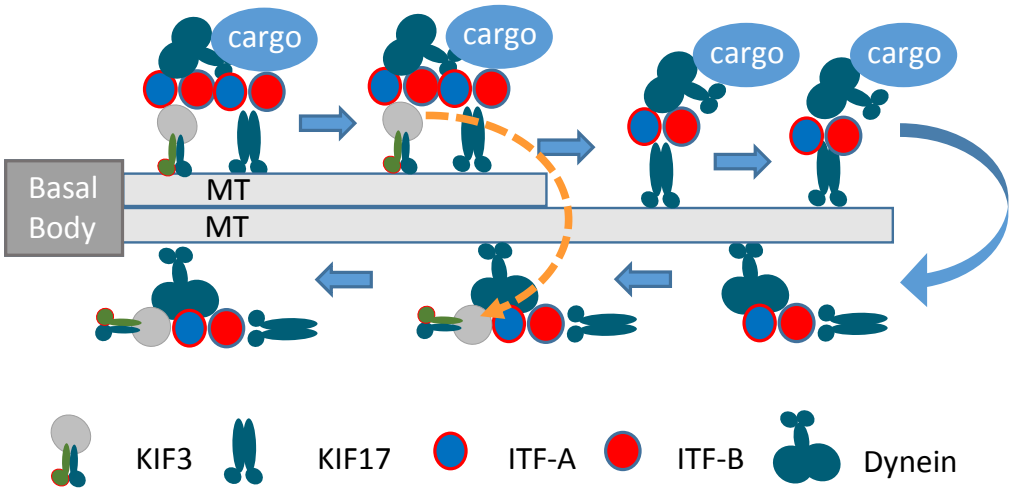

Fig. (1). Intraflagellar transport. KIF3 and KIF17 cooperate in anterograde transport of cargo at the proximal axoneme (MT doublet). Cargo consists of IFT particles, dynein motors, axoneme building blocks and axoneme stabilizing factors. Whereas KIF3 turns around, KIF17 continues to move cargo distally along the MT singlet. Retrograde transport is powered by dynein motors.

This study explores the roles of KIF3 and KIF17 in mouse photoreceptor ciliogenesis and rhodopsin trafficking. We deleted KIF3A in retina during embryonic photoreceptor development and in the adult mouse with tamoxifen-

induction. Retina-specific deletion of KIF3A during early development resulted in failure to form PTZs and OSs. Depletion of KIF3A in adult photoreceptors by tamoxifen-induction resulted in progressive shortening of the OS axoneme despite continued rhodopsin trafficking. Germline deletion of KIF17 did not produce a recognizable retina phenotype up to the age of one year. Our data indicate that KIF3-driven IFT functions primarily in photoreceptor ciliogenesis and axoneme stabilization rather than rhodopsin transport.

Ubiquitous Expression of KIF3A and KIF17

KIF3A and KIF17 are expressed in most mouse tissues, prominently in testes, ovary, lung, brain and thymus, and both are present in the eye (Fig. **2A**). In mouse retina, KIF3A (Fig. **2B**) and KIF17 (Fig. **2C**) are observed abundantly in photoreceptor inner segments (IS) and the outer nuclear layer (ONL).

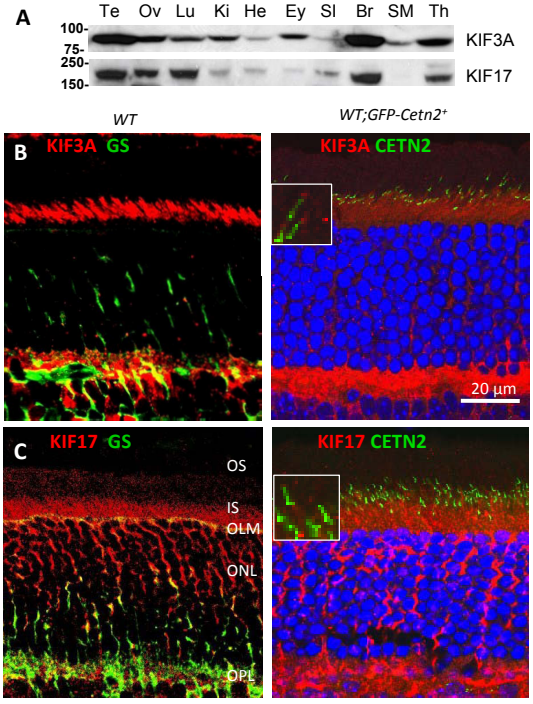

Fig. (2). KIF3A and KIF17 expressions in retina. (A) Multiple tissue immunoblot probed with anti-KIF3A (upper panel) and anti-KIF17 (bottom panel) antibodies, respectively. **(B)** KIF3A in WT (left) and *GFP-Cetn2*$^{+}$ (right) mouse retinas at one month of age. Centrin 2 (CETN2) is a calmodulin-like Ca^{2+}-binding protein associated with centrioles and transition zones. Left, colabeling of anti-KIF3A (red) with anti-glutamine synthase (GS, a Muller cell marker, green). Right inset, enlargement of GFP-CETN2-labeled basal bodies and connecting cilia (PTZ). **(C)** KIF17 (red) expression in WT (left) and *GFP-Cetn2*$^{+}$ (right) mouse retinas. KIF17 label overlaps with GS at ONL in Muller glia, but not with basal bodies (inset). Right inset, basal bodies and transition zones identified by GFP-CETN2. OS, outer segment; IS, inner segment; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer.

CHAPTER 6

The Molecular Links between Mutations in RDS and Diseases of the Retina

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Abstract: The photoreceptor specific tetraspanin protein peripherin-2, also known as retinal degeneration slow (RDS) plays a critical role in the biogenesis and maintenance of both rod and cone photoreceptor outer segments. Over 80 pathological mutations in RDS have been linked with multiple degenerative blinding diseases including retinitis pigmentosa and various forms of macular degeneration. RDS-associated disease is characterized by a diverse set of phenotypes with variability in penetrance, severity, and timing of disease onset. Much insight into the complex pathological mechanisms associated with RDS mutations has been gleaned from work in animal models with disease-causing mutations. In the current review we summarize our current understanding of RDS function in the normal retina and how defects in this function contribute to the associated disease pathologies in human patients.

Keywords: Animal models, Blindness, Choriocapillaris atrophy, Cones, Disease mechanisms, Electroretinography, Macular dystrophy, Microdomain, Morphogenesis, Outer segments, Photoreceptors, Protein complexes, Protein trafficking, RDS, Retinal degeneration, Retinitis pigmentosa, Rim region, Rods, ROM-1, Tetraspanin.

INTRODUCTION

Peripherin-2, also known as retinal degeneration slow (RDS), is a photoreceptor-specific tetraspanin and is necessary for the formation of rod and cone outer segments (OSs) [1 - 7]. More than 80 pathogenic mutations in the human RDS gene (PRPH2) have been identified and lead to a diverse set of mostly dominantly inherited retinal degenerative diseases [8]. RDS-associated pathologies can be broadly characterized as either retinitis pigmentosa (RP), which primarily affects rod photoreceptor cells, or macular dystrophies (MD), which primarily affect central vision, either by direct effects on cone photoreceptors or by causing signi-

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ficant defects in neighboring tissues such as the retinal pigment epithelium (RPE)/choroid which result in macular vision loss [8 - 10]. An excellent resource for RDS-associated pathologies can be found on the Retina International website (http://www.retina-international.org/ files/sci-news/ /rdsmut.htm). Autosomal dominant RP (adRP) represents 20-40% of total cases of RP [11, 12]. Though rhodopsin mutations account for more adRP than any other gene (~25%) [11], RDS is one of the most common associated genes other than rhodopsin (~2.5-9% of adRP cases) [11, 12]. The pathology of MD is complex and represents a broad spectrum of disorders including pattern dystrophies, adult-onset foveomacular vitelliform dystrophy, central areolar choroidal dystrophy, AMD-like late onset MD and multifocal pattern dystrophy simulating STGD1/fundus flaviomaculatus [8], many of which have significant overlap in their clinical presentation. The percentage of MDs linked to RDS mutations ranges from 2-18% [8] depending on the population. A thorough understanding of the RDS protein and its molecular function in OS biogenesis is critical for developing treatments for these important blinding diseases. Here we focus on what is known of the molecular role of RDS in OS biogenesis and how interruption of these processes leads to disease pathology.

RDS PROTEIN, TERTIARY STRUCTURE AND COMPLEX FORMATION

Photoreceptor OSs are highly modified 9+0 primary cilia. The OSs contain hundreds of flattened membrane structures, termed discs in rods and lamellae in cones, which are organized and stacked mostly perpendicular to the incoming light [13]. The discs are encased in a sheath of the plasma membrane in rods but are at least partially contiguous with the plasma membranes in cones [14, 15]. Each flattened disc/lamellae is circumscribed by a rim region with a distinct hairpin structure [13, 14, 16]. RDS localizes to this rim region and plays a critical role in maintaining the curvature and general organization of this highly-ordered lipid structure [17 - 20].

The RDS protein is a 39 kD four pass transmembrane glycoprotein with cytosolic N- and C- termini and two asymmetrical loops (D1 and D2) which project into the intradiscal space in rods and the analogous lamellar lumen/extracellular space in cones [7, 21, 22]. The N-terminus, transmembrane domains and D1 loop of RDS are thought to function as the structural backbone allowing the proper folding and packing of RDS within the disc membrane [23] while the D2 loop and C-terminal domains have established functional roles. The D2 loop mediates covalent and non-covalent intermolecular interactions, facilitating oligomerization, and is the site of the sole RDS N-linked glycosylation [23]. The C-terminal of RDS is a multi-functional domain that helps target RDS to the OS, forms a binding domain

for the GARP subunit of the rod (but not cone) cyclic nucleotide gated channel and melanoregulin, and forms an amphipathic helix involved in the regulation or generation of lipid curvature and membrane fusion in the OS [19, 20, 24 - 32].

Following synthesis, the RDS monomer associates with itself and with its homologue rod outer segment membrane protein 1 (ROM-1) to form both homo- and hetero-tetramers which are the core of the functional oligomers of RDS [33 - 35] (Fig. **1A**). ROM-1 shares a similar tertiary structure with RDS although it lacks any known functional domains within its C-terminus and is not glycosylated [36]. RDS is more abundant in OSs than ROM-1, and ROM-1 is thought to act primarily as an ancillary protein perhaps modulating RDS' function [23, 25, 37 - 39]. ROM-1 is capable of forming tetramers that do not include RDS, and pools of ROM-1 alone have been isolated in detergent resistant rafts from OSs, although no ROM-1 function independent of RDS has been identified [40, 41]. While RDS/RDS RDS/ROM-1 and ROM-1/ROM-1 tetramers are held together through non-covalent interactions, both RDS and ROM-1 also form intermolecular disulfide bonds mediated by a cysteine at position 150 and 153, respectively, within their D2 loops [42]. These intermolecular disulfide bonds allow RDS/RDS and RDS/ROM-1 tetramers to link up into larger oligomers, which are thought to form after exit from the ER, though oligomerization may occur differently in rods and cones [43, 44]. Both RDS and ROM-1 are found in non-covalent tetramers and in disulfide linked intermediate-sized complexes but the largest type of complexes contains only RDS [35, 42] (Fig. **1A**).

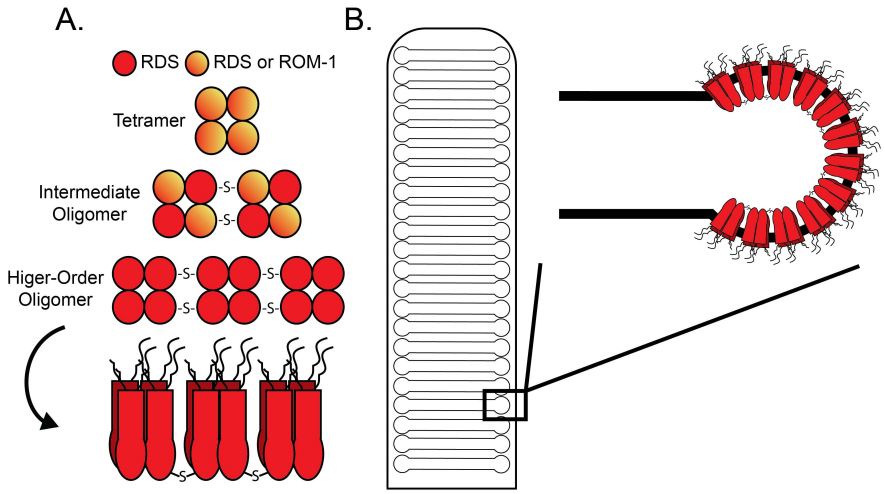

Fig. (1). Diagram describing RDS complex formation. A. RDS and ROM-1 assemble into covalent and non-covalent complexes. Red balls represent RDS and orange balls are either RDS or ROM-1. **B.** Model for assembly of RDS complexes in the membrane. RDS ability to mediate membrane curvature coupled with space measurements suggests that the complexes are oriented around the hairpin. **B** is modified from [20].

CHAPTER 7

Rhodopsin-Regulated Grb14 Trafficking to Rod Outer Segments: Functional Role of Grb14 in Photoreceptors

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Abstract: Growth factor receptor-bound protein 14 (Grb14) belongs to the Grb7 family. It is an adapter molecule, lacking any intrinsic enzyme activity, but mediates protein-protein and protein-lipid interactions. In photoreceptors, Grb14 undergoes a rhodopsin-dependent translocation from the inner segments to the outer segments. In photoreceptors, Grb14 undergoes a light-dependent tyrosine phosphorylation and protects the insulin receptor (IR) phosphorylation, which is neuroprotective. Outer-segment-localized Grb14 also modulates the activity of the cyclic nucleotide gated (CNG) chancel. Thus, Grb14 plays a key role in receiving signals from rhodopsin, and translocating to outer segments, where it regulates IR and CNG channel activities. The present study supports the idea that rhodopsin regulates non-canonical signaling pathways in photoreceptor cells.

Keywords: CNG channel, Grb14, Insulin receptor, PTP1B, Rhodopsin, Rod outer segments, Tyrosine phosphorylation.

INTRODUCTION

Signaling from tyrosine kinases is commonly facilitated by scaffold and adapter proteins, with domains, such as Src-homology-2 (SH2) and phosphotyrosine-binding (PTB), that bind precise phosphotyrosine sites and elements that selectively link to downstream targets that activate cytoplasmic pathways. Growth factor receptor-bound protein (Grb14) is an adapter protein that belongs to the Grb7 family, which includes Grb7 and Grb10 [1, 2]. All Grb7-family members have well-defined regions/domains of a proline-rich motif (PS/AIPNPFPEL), a Ras-associating (RA) domain, a Pleckstrin-homology (PH) domain, a "Between the PH and SH2 domain" (BPS) region, and a Src-homology (SH2) domain (Fig. **1**) [2].

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Decades ago, researchers observed a close resemblance between the RA and PH domains of Grb7 and a cell migration protein, Mig10, from *Caenorhabditis elegans* [3]. The Mig10 protein belongs to the MRL protein family [4, 5]. The most remarkable difference between the Grb7 and MRL proteins is the presence of the BPS domain and a C-terminal SH2 domain in the Grb7 family of proteins [4]. The Grb7 family of proteins has a unique BPS domain [1]. It has been shown that the insulin receptor (IR) associates with the BPS domain of Grb10 and Grb14 [6 - 8]. The PH domains of Grb7 [2] and Grb14 [6] interact with phosphoinositides; however, the protein-lipid interaction in cellular signaling is not fully understood. Through SH2 domains, several receptor tyrosine kinases and many signaling molecules can interact with Grb7, Grb10, and Grb14 [1]. These Grb7 family proteins have been shown to be localized mainly in the cytoplasm, and in certain cases have been observed in the plasma membrane, focal contacts, and mitochondria [2]. The phosphorylation on serine/threonine and tyrosine residues in Grb7, Grb10, and Grb14 has been observed. However, their functional significance has not been elucidated [2]. In this review, we describe Grb14's unique roles in rod photoreceptor cells.

Fig. (1). Domain organization of Grb14. Pro, proline rich region, RA, ras-associating domain, PH, pleckstrin homology domain, BPS, between PH and SH2 domain, SH2, Src-homology region.

Grb14 Role in Insulin Receptor Signaling

Grb14 is a pseudo-substrate inhibitor of the IR [9], and negatively regulates IR signaling through inhibition of its kinase activity [8, 10]. The BPS domain of Grb14 interacts with the IR [8]. These observations were further supported by a genome-wide association study showing that reduced insulin sensitivity in patients with diabetes is strongly associated with single nucleotide polymorphisms in Grb14 [11]. These findings were further supported by research with Grb14 knockout mice, which showed improved glucose homeostasis and enhanced insulin signaling [12], confirming the negative role of Grb14 in insulin signaling. In myocardial tissues, Grb14 is important in the activation of the PI3K/Akt signaling pathway; Grb14 ablation has been shown to result in myocardial infarction [13]. IR signaling is essential for photoreceptor neuroprotection, and ablation of IR in rods leads to stress-induced photoreceptor degeneration, whereas ablation in cones results in cone degeneration without added stress [14, 15]. Interestingly, Grb14 is expressed in the retina, and it inhibits IR kinase activity *in vitro* [10]. However, Grb14 knockout mice exhibited reduced IR activation *in vivo* due to increased activity of protein tyrosine phosphatase 1B (PTP1B) [16]. Similarly, reduced activation of IR due to increased phosphatase activity has been

observed in the liver tissues of Grb14 knockout mice [12]. In the retina, IR activation is light-dependent and is due to light-dependent inhibition of PTP1B activity [17]. The mechanism behind the light-induced activation of IR is light-dependent phosphorylation of Grb14 by a non-receptor tyrosine kinase, Src [16]. The phosphorylated Grb14 binds to PTP1B and inhibits its activity [16]. The non-phosphorylated form of Grb14 has a higher affinity for IR, whereas the phosphorylated form of Grb14 has a higher affinity for PTP1B [16]. The light-induced activation of IR, inhibition of PTP1B activity, activation of Src, and phosphorylation of Grb14 are mediated through a rhodopsin-dependent, G-protein coupled receptor [16]. Rhodopsin activation determines the state of Grb14 phosphorylation and IR-mediated neuroprotection *in vivo* [15, 16]). The mechanism of IR activation by Grb14 is described in Fig. (**2**). Mutations in the rhodopsin gene and mice that are unable to activate rhodopsin exhibit retinal degeneration due to the absence of Src activation, Grb14 phosphorylation, increased PTP1B activity, and subsequent inactivation of IR signaling [15, 16]. These findings suggest that activators of Src or inhibitors of PTP1B may be able to rescue the retinal degeneration phenotype.

Negative Regulatory Role of Grb14 on Insulin Receptor Signaling

Grb14 is an insulin receptor (IR)-interacting protein in the retina [3]. Interestingly, Grb14 is a pseudo-substrate inhibitor of IR [2] and inhibits IR tyrosine kinase activity. Consistent with these findings, a genome-wide association study demonstrated that single nucleotide polymorphisms at Grb14 are strongly associated with reduced insulin sensitivity in diabetic patients [4]. IR signaling is essential for photoreceptors, as deletion of IR [5] or proteins involved in the IR signaling pathway leads to both rod and cone degeneration [6, 7]. In addition to Grb14, IR signaling is also negatively regulated by protein tyrosine phosphatase, PTP1B [8]. Thus, the major question remains: how does the IR overcome inactivation by PTP1B and Grb14 in retinal neurons? This review primarily focuses on the intracellular localization and spatial and temporal regulation of Grb14 on IR signaling.

Light-Dependent Translocation of Grb14 in Rod Photoreceptor Cells

In dark-adapted retina, Grb14 is predominantly localized to photoreceptor inner segments [18]. Upon light-illumination, a certain portion of Grb14 translocates to the rod outer segments [18] (Fig. **3**). The binding of Grb14 to rod outer segment membranes is also light-dependent [18, 19]. This binding does not require transducin signaling, but requires the photobleaching of rhodopsin [18]. Grb14 is predominantly associated with the outer segment plasma membrane, but not the disc membranes [20]. In mice lacking rhodopsin photobleaching, Grb14 is

CHAPTER 8

Cellular Mechanisms of Cone Defects in Cyclic Nucleotide-Gated Channel Deficiency

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Abstract: The cone photoreceptor-specific cyclic nucleotide-gated (CNG) channel is indispensable for cone function. Cones are essential for daylight vision and visual acuity. Mutations in the *CNGA3* and *CNGB3* genes are associated with achromatopsia, cone degeneration, and early-onset macular degeneration, and account for 80-85% of Achromatopsia cases. Patients with CNG channel defects exhibit cone dysfunction and progressive degeneration of cones, as revealed by electrophysiological recordings, psychophysical testing, and morphological examinations. The cellular events and underlying mechanisms of CNG channel deficiency have been explored using mouse models. In this review, we have summarized our current understanding of the modes of cone defects due to CNG channel deficiency.

Keywords: Apoptosis, cGMP, CNG channel, Cone, Endoplasmic reticulum, Mitochondrion, Photoreceptor, PKG, Retina.

INTRODUCTION

The photoreceptor cyclic nucleotide-gated (CNG) channels are located at the plasma membrane of the outer segments and are essential for phototransduction [1 - 4]. In darkness/dim light, binding of the channel ligand cyclic guanosine monophosphate (cGMP) activates CNG channels, allowing a steady depolarizing cation current, mostly mediated by Ca^{2+} and Na^{+}, to flow into the outer segment. Light induces hydrolysis of cGMP by the phosphodiesterase PDE6, resulting in channel closure and membrane hyperpolarization [1, 5, 6]. Interestingly, the light sensitivity, cGMP sensitivity, Ca^{2+} permeability, structural features, and functional modulation are profoundly different between rod and cone CNG channels [1, 4].

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The CNG channel is the only source of Ca^{2+} influx into the outer segments. Therefore, this channel is important for intracellular Ca^{2+} homeostasis, which controls light response, cellular calcium signaling, and cGMP production. Structurally, the CNG channels are members of the pore-loop cation channel super family. They share a structural domain with hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and K^+ channels [6 - 8]. The channel is composed of two subunits, the A and B subunits. The rod channel is composed of CNGA1/CNGB1 and the cone channel has CNGA3/CNGB3. Heterologous expression studies showed that the A subunits form the ion-conducting moiety, while the B subunits act as modulators [9 - 11]. Mutations in photoreceptor CNG channels are found in inherited retinal degenerative diseases [12 - 19].

Mutations in Cone CNG Channel Subunits and Human Cone Diseases

The human genes for CNGA3 and CNGB3 are located in 2q11.2 and 8q21-q22, respectively. Mutations in *CNGA3* and *CNGB3* have been identified in human patients with achromatopsia or rod monochromatism, cone-rod dystrophies, and early-onset macular degeneration [15 - 18]. *CNGA3* mutations were also identified in patients with Leber's congenital amaurosis [20]. To date, about 141 mutations in *CNGA3* and 63 mutations in *CNGB3* have been reported (www.hgmd.cf.ac.uk); these mutations are found in 80-85% of all achromatopsia [16, 17, 21 - 25]. As achromatopsia is primarily caused due to defects in the channel subunits, it is also called a "channelopathy" [26]. Achromatopsia is a severe retinal disorder with a prevalence of approximately 1 in 33,000 individuals. The patients report inability to distinguish colors, reduced visual acuity, photophobia/hemeralopia, and pendular nystagmus. Some patients also exhibit paradoxical pupillary constriction when transitioned from light to dark (Flynn phenomenon) [27]. Electroretinography (ERG) analysis revealed unmeasurable cone function and normal or abnormal rod function [18, 24, 28, 29]. Optical coherence tomography (OCT) [30 - 37] and adaptive optics scanning light ophthalmoscopy (AOSLO) [38, 39] studies have established cone loss in patients with achromatopsia associated with CNG channel mutations. Due to a founder effect, the *CNGB3* mutations are present in increased frequency in the Pingelapese population of the Pacific Islands [40].

Interestingly, patient population studies revealed some epidemiological features. Most achromatopsia cases in European populations arise from mutations in the CNGB3 subunit [18, 19, 21, 41], while mutations in CNGA3 are the leading cause of the disease among Asian populations [22 - 24, 42 - 46]. CNGA3 is the primary subunit, forming the ion-conducting unit. Heterologous expression studies showed that majority of *CNGA3* mutations are loss of function alleles [47 - 57]. Most disease-causing CNGB3 mutations are amino acid substitutions. However, the

frame shift mutation, Thr383fsx, occurs most frequently and accounts for >80% of all *CNGB3* alleles [21, 41, 58, 59]. As the mutation truncates the pore and the C-terminal cytoplasmic domain, it is considered a null mutation. In addition, mutations in CNGA3 and CNGB3 have been found in sheep [60] and dog breeding [61 - 63] models of day blindness, respectively.

No treatment currently exists for CNG channelopathy. Gene therapy has shown promise for phenotype corrections [26]. Successful functional and structural rescue has been demonstrated following gene therapy in *Cnga3*$^{-/-}$ and *Cngb3*$^{-/-}$ mice and in canine models of CNGB3 mutations [61, 64 - 67]. Recently, the ciliary neurotrophic factor (CNTF) was shown to induce transient photoreceptor deconstruction and enhance cone functional rescue in canine models of CNGB3 deficiency [68], but the effect was not supported in human clinical trials [69].

Cone Defects in Mouse Models of CNG Channel Deficiency

The retinal phenotype of CNG channel deficiency has been examined in mouse models. *Cnga3*$^{-/-}$ and *Cngb3*$^{-/-}$ mice show reduced cone function and progressive cone degeneration [70 - 74]. As models of achromatopsia, these mouse lines have been used in studies of cone structure and function [71, 72], cone opsin plasma membrane targeting [70], retinal synaptic plasticity [74 - 76], visual acuity [72, 77], regulation of the channel subunit expression [72], mechanisms of cone degeneration [78], and gene replacement therapy [64 - 66]. Because cones comprise only 2-3% of the total photoreceptor population in the wild-type mouse retina, identification of the cellular alterations and biochemical events in CNG channel-deficient mice is challenging. To overcome this limitation and to better model the pathology in the cone-rich fovea-macular region of the human retina, *Cnga3*$^{-/-}$ and *Cngb3*$^{-/-}$ mice have been crossed with the *Nrl*$^{-/-}$ mouse line [79, 80]. NRL, a rod-specific transcription factor, is essential for rod differentiation [81]. The *Nrl*$^{-/-}$ mice develop a rodless and S-cone-enriched retina. These mice show no rod function but enhanced cone function [81, 82]. Using the *Nrl*$^{-/-}$ retinas, we have shown abundant expression of the cone CNG channel, and have demonstrated that CNGA3 and CNGB3 interact *in vivo* and that the cone CNG channel is a heterotetrameric complex [83]. Similar to their respective single knockout mice, *Cnga3*$^{-/-}$*/Nrl*$^{-/-}$ and *Cngb3*$^{-/-}$*/Nrl*$^{-/-}$ mice show cone dysfunction and cone loss due to apoptosis [79, 80]. In addition to mouse models, canine models of CNGB3 deficiency/mutations have been used to examine CNG channel-associated cone defects and in therapeutic interventions [51, 61, 67, 68].

Mechanisms of Cone Degeneration Due to CNG Channel Deficiency

As cones are the minority photoreceptor population in mammalian retina, examining cone dysfunction represents a major challenge. The double knockout

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Dr. Hemant Khanna obtained his Ph.D. from the University of Delhi, India. After obtaining postdoctoral training at the University of Michigan, Dr. Khanna was appointed as the Assistant Professor in the Department of Ophthalmology, University of Michigan in 2008. His research focused on understanding the molecular mechanism of severe inherited eye diseases. In 2010, Dr. Khanna moved to UMMS where he continues to work on delineating disease mechanisms and development of tools to design treatment paradigms. Dr. Khanna has published more than 50 papers in the international scientific journals and serves on the editorial board of several journals, as well as on the NIH study sections. He is also involved in teaching and mentoring undergraduate and graduate students. Dr. Khanna has received three patents and a Celebrate Invention Award.