Research Summary

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Vertebrate eyes mediate both image-forming and non-image-forming visual functions. Image-forming vision enables the animal to detect and track objects in the visual world. Non-image-forming vision provides a measure of the ambient luminance for the purposes of synchronizing the animal's biological clock with the surrounding light-dark cycle (circadian photoentrainment), controlling the pupil size, etc. Retinal rods and cones are photoreceptors responsible for image-forming vision. In mammals, recent evidence indicates that non-image-forming vision is mediated not only by rods and cones, but also by a novel class of photoreceptors – the intrinsically photosensitive retinal ganglion cells (ipRGCs).

In my postdoctoral research，I have used a combination of mouse and *Xenopus* genetics with electrophysiology in addressing issues on visual pigments in rods/cones (Project 1) and ipRGCs (Project 2), as well as the downstream signaling pathway (Project 3). The experimental methods include molecular biology, single-cell recording, electroretinagram, biochemistry, cell biology, and animal behavior. These techniques will also be used in my future research as described later.

Project 1: Role of visual pigment properties in the differences between rods and cones.

Rods and cones detect light with visual pigments, which consist of an apoprotein (opsin) covalently bound to a vitamin A-based chromophore, typically 11-*cis*-retinal. Upon photon absorption, 11-*cis*-retinal isomerizes to all-*trans*-retinal, after which the opsin undergoes conformational changes to become active, ultimately leading to the generation of an electrical response. Rods and cones mediate nighttime and daytime vision, respectively. Although the two types of photoreceptors share a similar phototransduction pathway, cones are typically 100-fold less sensitive than rods and their response kinetics is several-fold faster, with the underlying mechanisms still largely unknown. Certain differences between rod and cone pigments had been described, such as a 10-fold shorter lifetime of the active conformation of cone pigment and its higher rate of spontaneous isomerization, but their contributions to the differences between rod and cone responses remained speculative. To address the role of visual pigment in the rod/cone differences, we used transgenic-frog methods to express human or salamander red cone pigment in *Xenopus* rods, and human rod pigment in *Xenopus* cones [1]. Surprisingly, rod and cone pigments when present in the same cell produced light responses with identical amplification and kinetics, thus ruling out any difference in their signaling properties. However, red cone pigment isomerized spontaneously 10,000 times more frequently than rod pigment. This high spontaneous activity is expected to adapt the native salamander cones even in darkness, making them less sensitive and kinetically faster than rods.

Because *Xenopus* and salamander use 11-*cis*-3, 4-dehydroretinal (A2 retinal) as the chromophore for visual pigments, whereas birds and land-based animals use 11-*cis*-retinal (A1 retinal), we asked whether the same red cone pigment property existed with A1 chromophore. We addressed this question by expressing human red cone opsin in mouse rods [2]. Consistent with our findings from transgenic *Xenopus*, the light responses triggered by transgenic red cone pigment and by native rod pigment were similar in amplitude and kinetics. However, in this case, we did not

detect an increase in dark noise associated with the presence of transgenic red cone pigment in mouse rods, indicating a substantial stabilizing effect of A1 chromophore compared with A2 chromophore. By bleaching a larval salamander red cone, which contains a mixture of A1 and A2, and regenerating it with only A1, we found that the cell's dark noise likewise decreased. Therefore, in land-based mammals using A1, thermal isomerization of cone pigment appears to have an insignificant role in setting cone sensitivity in contrast to the scenario in some lower vertebrates such as amphibians using A2 (see above).

The above results suggest that, depending on the chromophore content, cone pigment plays different roles in the different photoresponse between rods and cones.

Project 2: Role of melanopsin in intrinsically photosensitive retinal ganglion cells (ipRGCs).

IpRGCs are a novel class of photoreceptors that are important for non-image-forming vision such as circadian photoentrainment and pupillary light reflex (PLR). In this newly emerging field, there had been controversy about whether melanopsin, an opsin-like protein, was indeed the pigment underlying the intrinsic light response of ipRGCs, although it was clear that melanopsin was critical for the process because melanopsin-ablated (*Opn4*$^{-/-}$) ipRGCs lost photosensitivity. One suggestion had been that melanopsin was not the signaling pigment, but a photoisomerase that photoconverted all-*trans*-retinal to 11-*cis*-retinal for regenerating a yet unidentified pigment.

To address the role of melanopsin, we studied mice lacking RPE65, the isomerohydrolase that was the key enzyme for the RPE (retinal pigment epithelium) retinoid cycle. Rod and cone pigments relied on the RPE retinoid cycle to synthesize 11-*cis*-retinal to replace photoisomerized all-*trans*-retinal in order for vision to proceed continuously. We found that *rpe65*$^{-/-}$ ipRGCs were ~20- to 40-fold less photosensitive than normal at both single-cell and behavioral (PLR) levels. The low sensitivity of ipRGCs could be rescued by exogenous 9-*cis*-retinal (an 11-*cis*-retinal analog), indicating the requirement of a vitamin A-based chromophore, i.e. 11-*cis*-retinal, for ipRGC photosensitivity. In contrast, 9-*cis*-retinal was unable to compensate for the loss of melanopsin to restore the intrinsic photosensitivity of *Opn4*$^{-/-}$ ipRGCs, arguing against melanopsin functioning merely in photopigment regeneration. Interestingly, exogenous all-*trans*-retinal was also able to rescue the low sensitivity of *rpe65*$^{-/-}$ ipRGCs, suggesting that melanopsin could be a bistable pigment, similar to many invertebrate pigments exhibiting the dual function of photopigment and photoisomerase. These results strongly suggest that melanopsin is the photopigment in the ipRGCs [3].

Project 3: Role of $G_{\beta\gamma}$ in phototransduction cascade.

Heterotrimeric G proteins act as molecular switches that couple various membrane receptors to intracellular effector molecules. A G protein becomes activated upon the receptor-stimulated binding of GTP to its G_α subunit and becomes inactivated when its bound GTP is hydrolyzed to GDP. Although the role of the G_α subunit in this process has been well studied, the cellular functions of the $\beta\gamma$ subunits are less well understood. We used phototransduction as a model system to address the role of $G_{\beta\gamma}$ in G protein signaling. We expressed a cone G_α subunit (*gnat2*) as a transgene in mouse rods and bred the resulting mice into a rod G_α knockout (*gnat1*$^{-/-}$) background [4]. The produced mouse (*gnat2*$^{+/-}$*gnat1*$^{-/-}$) expressed cone G_α instead of rod G_α in

rod photoreceptors. Biochemical assays showed that the interaction between cone G_α and the receptor, rhodopsin, was normal, but the coupling was defective between transgenic G_α and endogenous rod $G_{\beta\gamma}$. *Gnat2*$^{+/-}$*gnat1*$^{-/-}$rods were on average over 100 times less photosensitive than wild-type rods, likely due to inefficient interaction between cone α and rod $G_{\beta\gamma}$. Interestingly, overnight dark-adapted *gnat2*$^{+/-}$*gnat1*$^{-/-}$ mouse rods showed significantly prolonged shutoff, caused by the lack of available $G_{\beta\gamma}$ in the outer segment, where phototransduction took place. In dark-raised *gnat2*$^{+/-}$*gnat1*$^{-/-}$ mouse, however, the time course of its rod response became largely normal, and most of the $G_{\beta\gamma}$ subunits were located in the outer segment. These results suggest that $G_{\beta\gamma}$ dimers are not only important for efficient activation of α subunit, but also for proper termination of phototransduction cascade. This finding could have general implications for heterotrimeric G protein mediated signaling pathways.

Future Plan

My future research plan will focus on two areas:

Proposal 1: Pigment regeneration for melanopsin.

Rod and cone pigments rely on the retinoid cycle primarily located in the RPE for regeneration, though cones could have a different cycle involving the Müller cells [5]. In contrast, melanopsin is suggested to be like many invertebrate pigments by having two photoconvertible stable states, bound to 11-*cis*-retinal and all-*trans*-retinal. Therefore, 11-*cis*-retinal can simply be photoconverted from all-*trans*-retinal. The long term objective is to understand the retinoid cycle for the newly discovered ipRGCs and its interaction with rod and cone retinoid cycles.

1.1. Characterize the *in vivo* properties of the putative two photoconvertible states of melanopsin. I shall expand on my previous success in characterizing the ipRGC function with pupillary light reflex (PLR) measurement on genetically engineered mouse lines. Since both rods and cones contribute to mouse PLR, the measurement will be done on mutant mouse, which has non-functional rods and cones. I shall first verify the existence of two stable states of melanopsin by using different light adapting conditions. Preliminary experiments suggest this is the case. I shall then determine the maximum spectral sensitivity for each of the putative two states. PLR measurement will then be used to determine the lifetime of the two states, and to establish the quantitative dependence of the pigment state populations and transitions on stimulus and adaptation wavelength, intensity, and duration. This knowledge will be helpful in understanding the physiological function of ipRGCs.

1.2. Investigate the interaction between ipRGC retinoid cycle and rod/cone retinoid cycles.

a) Test the "chromophore sink" hypothesis for explaining the reduced sensitivity of *rpe65*$^{-/-}$ ipRGCs. Due to a disruption of RPE retinoid cycle, rods in *rpe65*$^{-/-}$ mice contain abundant free opsin (without chromophore), which can potentially hijack the scarce supply of chromophore causing ~20- to 40-fold reduced sensitivity of ipRGCs (see Project 2). To test this hypothesis, rod opsin is genetically removed by breeding *rpe65*$^{-/-}$ mouse into rod opsin null background (*rho*$^{-/-}$) to generate *rpe65*$^{-/-}$*rho*$^{-/-}$ mouse line. A control mouse line shall be generated by crossing *rpe65*$^{-/-}$

with rod Gα knockout (*gnat1*$^{-/-}$). The resulted mouse line, *rpe65*$^{-/-}$*gnat1*$^{-/-}$, contains free rod opsin but without rod signaling capability. Because cone sensitivity is negligible in *rpe65*$^{-/-}$ mouse, the ipRGC sensitivity in *rpe65*$^{-/-}$*gnat1*$^{-/-}$ and *rpe65*$^{-/-}$*rho*$^{-/-}$ mouse can be compared with PLR measurement to determine the role of free rod opsin in the reduced sensitivity of *rpe65*$^{-/-}$ ipRGCs.

b) Test the hypothesis that 11-*cis*-retinal is transported to the ipRGCs through Müller cells. IpRGCs could obtain chromophore from either RPE (RPE retinoid cycle) or Müller cells (novel cone retinoid cycle). In both cases, because ipRGCs are physically far away from RPE and the cone retinoid cycle is located in Müller cells, the most likely route of chromophore delivery is through Müller cells because they span the full thickness of the retina. Two retinoid carrier proteins, cellular retinaldehyde-binding protein (CRALBP) and cellular retinol-binding protein type I (CRBP I), are expressed in both Müller and RPE. These two carrier proteins are involved in the flow of retinoids in RPE cycle. The function of the two carrier proteins in Müller is unknown. I shall examine the ipRGC function in CRALBP and CRBP I knockout mice, both of which are available, by PLR. If the ipRGC function is affected, selective gene targeting of CRALBP and CRBP in Müller will be done using *Cre*-lox recombination system. The resulting conditional knockout mice will be assayed by PLR on the intrinsic photoresponse of ipRGCs. As a by-product, the cone function of the mutant mice will be measured by "Paired-flash" cone derived ERG analysis to look for specific effects on the cone retinoid cycle, which is important for our daytime vision.

c) A novel retinoid cycle for melanopsin? Mammals are blind at birth for image-forming vision because rods and cones are not photosensitive until around P12 – a time shortly before eye opening in rodents. Recent studies showed that ipRGCs are photosensitive in the newborn retina (P0) [6]. However, RPE retinoid cycle that supplied chromophore to rod and cone pigments is not expected to be functional at P0, and Müller cells are not developed yet. The interesting questions are: how the P0 ipRGCs obtain their chromophore? Is there a separate retinoid cycle for the ipRGCs? I shall first check whether some of the key components of the RPE cycle, for example RPE65, are expressed at P0 by western blot. By measuring *c-fos* induction in SCN (suprachiasmatic nucleus) and calcium imaging on retinal slices, I shall also compare the photosensitivity of P0 ipRGCs between *rpe65*$^{-/-}$ and wild-type controls to determine the role of RPE retinoid cycle for ipRGCs at P0. In addition, I shall study the flow of the ipRGC retinoid cycle by HPLC analysis with radiolabeled retinoids, to help understand its molecular pathway.

Malfunctions in retinoid cycles cause vision impairment, for example, *rpe65*$^{-/-}$ mouse is a well known model for a blinding disease of infancy called Leber congenital amaurosis. Impairment of ipRGCs can affect myriad physiological functions such as adjusting our internal clock to different time zones when traveling, sleep-wake cycle regulation, and a seasonal form of depression. The above proposals will be very helpful to understand the basis of these disease states, therefore useful in designing rational treatments.

Proposal 2: Role of $G_{\beta\gamma}$ in G protein signaling.

Malfunction in G proteins are implicated in numerous human diseases, including cancer. While most studies on heterotrimeric G proteins focus on the function of G_{α}, relatively little is known about the role of $G_{\beta\gamma}$. Nevertheless, $G_{\beta\gamma}$ can have a profound impact on G protein signaling. For

example, my previous research (see above) has implicated $G_{\beta\gamma}$ in the termination of G protein signaling. I propose three strategies to further study the function of $G_{\beta\gamma}$ in phototransduction, a model system for G protein cascades. A better understanding of the role of $G_{\beta\gamma}$ in G protein signaling would facilitate designing therapies for pathologies involving G proteins.

2.1. Establish loss-of-function assay for $G_{\beta\gamma}$. I would like to apply my expertise gained from research in using transgenic *Xenopus* by performing morpholino antisense oligonucleotide (MO) knockdown on $G_{\beta1}$ subunit in *Xenopus* rods. Targeted animals will be firstly assessed by western blot for the efficiency of $G_{\beta1}$ knockdown. ERG and single-cell recordings will be used to analyze the phenotype particularly regarding the termination step of phototransduction. The expression level of $G_{\beta1}$ in rods can be altered by varying the concentration of injected MO. This is especially desirable because a complete loss of $G_{\beta1}$ may render the photoreceptor non-photosensitive. Since $G_{\beta1}$ is widely expressed, a complimentary approach is to knockdown the G_{γ} subunit in *Xenopus* cones, which has a much more restrictive expression, to avoid undesirable effect on other systems. If necessary, conditional knockout of $G_{\beta1}$ specifically in mouse rods shall also be considered.

2.2. Investigate the molecular mechanism of $G_{\beta\gamma}$ in the termination of phototransduction by biochemical approach. In retinal rods, the GAP (GTPase accelerator protein) complex, $G_{\beta5}$-RGS9-1, is required to accelerate the slow intrinsic GTPase activity of rod G_{α}. The interesting question is how $G_{\beta\gamma}$ affects the termination of G_{α}-GTP, which in turn affects the time course of phototransduction. Is that simply by stabilizing G_{α} in the inactive GDP bound state? Does $G_{\beta\gamma}$ contribute to the GAP activity of RGS9-1? What is the relation between RGS9-1 and $G_{\beta\gamma}$? Biochemical assays can help elucidate these questions. I plan to use both multiple and single-turnover GTPase assays [7] to determine the effect of $G_{\beta\gamma}$ on the termination of G protein activity with purified components including G_{α}, $G_{\beta\gamma}$, $G_{\beta5}$-RGS9-1 from either bovine retina or *E. coli* as recombinant proteins.

2.3. Determine the spatial-temporal information of G_{α} interacting with $G_{\beta\gamma}$ and $G_{\beta5}$-RGS9-1 by *in vivo* imaging. Techniques that can unfold the *in vivo* interaction of G_{α} with its partner $G_{\beta\gamma}$ and GAP complex, $G_{\beta5}$-RGS9-1, in G protein signaling could provide crucial clue on the role of $G_{\beta\gamma}$ in this process. To achieve this goal, I plan to use FRET (Fluorescence Resonance Energy Transfer) imaging that is capable of resolving molecular interactions and conformations with a spatial resolution in 1-10 nm range. I shall first use two-chromophore FRET microscopy to analyze the interaction between G_{α} and $G_{\beta\gamma}$ with transgenic *Xenopus* expressing CFP-tagged rod G_{α} and YFP-tagged $G_{\beta1}$ in rods. This technique has been successfully applied by Johns Hopkins researchers to study receptor-mediated activation of heterotrimeric G-proteins [8]. The large size and sturdiness of *Xenopus* rods make them especially suitable for *in vivo* imaging. I shall then image the interaction between G_{α} and $G_{\beta5}$-RGS9-1. Finally, I shall use three-chromophore FRET [9], which is developed to analyze multiprotein interactions in living cells, to analyze the sequential interaction between the three components, G_{α}, $G_{\beta\gamma}$ and $G_{\beta5}$-RGS9-1 in transgenic *Xenopus* rods. I plan to carry out some of the initial experiments while I am still at Hopkins with the help of experts here.

References

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