RATE-LIMITING STEP OF CONE PHOTOTRANSDUCTION RECOVERY AND OGUCHI DISEASE MECHANISMS

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LIST OF ABBREVIATIONS

AST  Active site tether
Arr  Arrestin
BOP  Blue opsin promoter
cAMP  Cyclic AMP
cGMP  Cyclic GMP
CNG  cGMP-gated cation channel
COS  Cone outer segment
CSNB  Congenital stationary night blindness
ERG  Electroretinogram
GAP  GTPase-accelerating protein
GC  Guanylyl cyclase
GCAP  Guanylyl cyclase activating protein
GOF  Gain of function
GOP  Green opsin promoter
GPCR  G-protein-coupled Receptor
GRK  G-protein-coupled Receptor Kinase
hGRK7  Human GRK7
iCre  Codon improved Cre-recombinase
IHC  Immunohistochemistry
INL  Inner nuclear layer
IPL  Inner plexiform layer
LOF  Loss of function
MCV10  GOPR9AP
MCV7  BOPGRK7
MCV8  GOPGRK7
MCV9  BOPR9AP
NCKX  Na\(^+\)/Ca\(^{2+}\), K\(^+\) exchanger
OLM  Outer limiting membrane
ONL  Outer nuclear layer
OPL  Outer plexiform layer
OS  Outer segment
PDE  Phosphodiesterase
PKA  Protein Kinase A
PNA  Peanut agglutinin
PTM  Post-translational modification
R*  Meta-rhodopsin II
R9AP  RGS9-anchoring protein
RGS  Regulator of G-protein Signaling
RK  Rhodopsin Kinase
RLS  Rate-limiting step
ROS  Rod outer segment
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>RPE</td>
<td>Retinal Pigmented Epithelium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SPR</td>
<td>Single photon response</td>
</tr>
<tr>
<td>SWS</td>
<td>Short-wavelength-sensitive</td>
</tr>
<tr>
<td>T</td>
<td>GNAT1/-, transducin knock-out</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>$\tau_D$</td>
<td>Dominant recovery time constant</td>
</tr>
<tr>
<td>$\tau_{rec}$</td>
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ABSTRACT

RATE-LIMITING STEP OF CONE PHOTOTRANSDUCTION RECOVERY AND OGUCHI DISEASE MECHANISMS

By

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Advisor: Ching-Kang Jason Chen, Ph.D.

Retinal photoreceptors provide the first gateway in which light information from the environment is transformed into neuronal signals. The cone and rod photoreceptors are responsible for day and night vision, respectively. Understanding rod and cone phototransduction is to figure out how these cells differ in their temporal and spatial sensitivities to allow perception of a broad dynamic range of stimuli. Phototransduction is mediated through a G-protein signaling cascade. Light absorption by visual pigment triggers the isomerization of 11-cis-retinal covalently attached to these pigments, which are heptahelical transmembrane G-protein-coupled receptors. Isomerization of 11-cis-retinal to all-trans-retinal activates the receptor, which catalyzes the exchange of GDP for GTP on the α subunit of heterotrimeric G-protein called transducin. Activated transducin relieves inhibitory constraint on cGMP-PDE, leading to rapid hydrolysis of cGMP, closure of cGMP gated cation channels, and membrane hyperpolarization. In order for photoreceptor to be responsive to light again, this robust phototransduction pathway must be deactivated in a timely fashion and this involves several reactions simultaneously. First, the activated opsin must be phosphorylated by G-protein-coupled receptor kinases (GRKs) and capped by arrestin binding. Second, activated transducin must hydrolyze bound GTP through intrinsic GTPase activity, which is accelerated by a GTPase accelerating protein (GAP) complex comprised of RGS9-1/Gβ5-L/R9AP. Mutations in human genes involved in these reactions cause various visual defects. Cone, by and large, uses the same set of genes for pigment and transducin deactivations but it has lower sensitivity and faster kinetics than rod and is responsible for high visual acuity. During phototransduction recovery in which multiple reactions take place, the slowest reaction will determine the overall rate of recovery. In rod, this so-called, rate-limiting step has been determined to be transducin deactivation. It is unknown whether cone transducin deactivation also controls the timing of cone
recovery, although we and others have shown that cone possesses a higher level of GAP concentration. In this thesis, the rate-limiting step in cone phototransduction recovery has been unequivocally determined by overexpressing RGS9-1 by 2.7 fold in mouse cones, which results in accelerated cone recovery. Complementarily, we find that ectopically expressing a human cone opsin kinase GRK7 in mouse cones does not affect cone recovery. These results altogether demonstrate that the rate-limiting step of cone recovery is the GTP-hydrolysis of cone transducin, not the opsin phosphorylation by GRKs. By elucidating the rate-limiting step of photoreceptor recovery, we have revealed the importance of G-protein cycling in timing of both rod and cone photoreceptors. This may further be generalized to other physiological processes controlled by heterotrimeric G-proteins.

The proper shutoff of phototransduction is essential for normal vision as recovery defects lead to visual impairment. Even though the reaction catalyzed by GRK1 is not rate-limiting, mutations of this important gene render rhodopsin phosphorylation and deactivation the slowest step in rod recovery and create a pathological condition. GRK1 mutations have been found in Oguchi disease patients, who suffer from congenital stationary night blindness. One of the mutations, V380D, is investigated in detail in this study. Transgenic expression of GRK1 V380D mutant in rods reveals a kinase with reduced expression and catalytic activity. While V380D GRK1 is found capable of inactivating rhodopsin, the reduction in kinase activity leads to a delayed dark adaptation, and is congruent with the night blindness phenotype observed in Oguchi disease patients. Finally, we have also investigated the role of post-translational isoprenylation on GRK1 function. We found that isoprenylation is required for GRK1 membrane association and outer segment targeting. Altogether our data add significantly to understanding the structure and function of GRK1, which is one of the least understood molecules involved in vertebrate phototransduction.
CHAPTER 1

Introduction

Heterotrimeric G-protein signaling mediates the transduction of extracellular stimuli into intracellular responses in many physiological processes. Extracellular stimuli, such as hormones, odors, light, neurotransmitters, are detected by G-protein-coupled Receptors (GPCR) with heptahelical transmembrane motifs (Chen 2005; DeMaria and Ngai 2010). Activated GPCR catalyzes the exchange of GTP for GDP on the α-subunits of trimeric G-proteins, which are composed of α and βγ subunits. Upon activation, Gα-GTP dissociates from Gβγ subunits and both moieties can modulate activity of respective downstream effectors. The termination of a G-protein signaling pathway requires the shutoff of GPCR and deactivation of activated G-proteins, the latter of which is mediated by intrinsic GTPase activity of Gα subunits that turns bound GTP into GDP. The shutoff of GPCR requires phosphorylation of its C-terminal Ser/Thr residues by G-protein-coupled receptor Kinases (GRKs) (Doan, Mendez et al. 2006). GPCR phosphorylation dampens its catalytic activity toward G-protein but complete uncoupling between the two is not achieved until the binding of arrestin to phosphorylated GPCR. (Premont and Gainetdinov 2007; Ribas, Penela et al. 2007). The intrinsic GTPase activity, which is necessary for turning off activated G-protein, is regulated by GTPase accelerating proteins (GAP) (Siderovski and Willard 2005). In rod phototransduction, a prototypical G-protein signaling pathway, the duration of rhodopsin signaling is determined not by GPCR lifetime but by how fast Gα subunit hydrolyzes bound GTP (Krispel, Chen et al. 2006). Genetic defects in G-protein deactivation are relatively rare, but in a few established cases they are accompanied by severe pathological consequences (Spiegel 2000; Lamb and Pugh 2004; Nishiguchi, Sandberg et al. 2004; Lania, Mantovani et al. 2006; Conn, Ulloa-Aguirre et al. 2007).

Phototransduction

Vision, the ability to perceive light, is one of the sensory abilities for living organisms (Figure 1.1). The conversion of photon into electrical signal takes place at photoreceptor outer segment, which lies between the outer nuclear layer (ONL) of the retina and retinal pigmented epithelium (RPE) (Burns and Arshavsky 2005; Chen 2005; Fu and Yau 2007; Larhammar, Nordstrom et al. 2009). Two types of image forming photoreceptors, rod and cone, are found in
Vision is one of the essential sensory systems to allow organisms to interact with their surroundings and is mediated through the eye. Visual perception begins when light enters the eye, passing through the cornea and the lens, and reaches the retina. The retina is a thin neuronal tissue in the back of the eye consisting of multiple cell layers, including photoreceptors. Light energy is captured and converted to electrical signal in the outer segment of photoreceptor, transmitted to the bipolar cells and ganglion cells, and sent down the optic nerve for further processing in the brain.
the outer retina. A third type of light sensitive cells, intrinsically light-sensitive retinal ganglion cells (ipRGC) are found in the inner retina. Rod is responsible for dim light vision and cone is responsible for daylight vision, whereas ipRGC is essential for transmitting non-image forming visual functions (Provencio, Jiang et al. 1998; Fu, Zhong et al. 2005; Sedwick 2010). Image forming photoreceptors have four distinct morphological structures: outer segment (OS), inner segment (IS), cell body, and synaptic terminal. For rod, light absorption occurs at the disc membranes of the OS, which contains millions of photon capturing molecules called rhodopsin. Rhodopsin consists of an apo-protein called opsin and a chromophore, 11-cis-retinal, which is covalently attached to Lys296 residue through an Schiff base linkage. The chromophore acts as an inverse agonist that keeps rhodopsin inactive. In the dark, cyclic GMP (cGMP) concentration inside the OS is high enough to maintain a fraction of cGMP-gated cation channel (CNG) in the open conformation. The influx of cations, Na⁺ and Ca²⁺, through the CNG channel keeps rod in a depolarized state (Figure 1.2). Upon photon capture by rhodopsin, 11-cis-retinal is photoisomerized to all-trans-retinal, resulting in a series of conformational changes in rhodopsin molecule that ultimately leads to an activated form of rhodopsin called Metarhodopsin II (R*), which facilitates the exchange of GDP for GTP on the α subunit of transducin. Tα-GTP activates cGMP phosphodiesterase (cGMP-PDE) by binding and relieving the inhibitory constraints imposed by PDEγ subunits on the catalytic PDEα and PDEβ subunits. Activated PDEαβ is a highly efficient enzyme working at diffusion limit and catalyzes the hydrolysis of cGMP to 5‘GMP (Burns and Arshavsky 2005). The drop in cGMP concentration leads to the closure of the CNG channels and membrane hyperpolarization. This drop in the rod membrane potential decreases the release of glutamate at photoreceptor synapse, which is sensed by retinal bipolar cells and parallel processed in retinal circuits before it is sent to the brain (Figure 1.3). Without phototransduction, a willing brain finds no input signal to decipher.

In order for rod to capture the next photon, all active molecules produced during the phototransduction cascade must be turned off (Figure 1.4). Several reactions take place simultaneously during the so-called recovery phase of phototransduction in order to reset the system. First, R* has to be deactivate to prevent its coupling to transducin. R* is phosphorylated by G-protein-coupled receptor 1 (GRK1) (Chen, Burns et al. 1999), followed by the binding of arrestin to the phosphorylated-receptor (Xu, Dodd et al. 1997; Burns, Mendez et al. 2006) (Figure 1.4A). This two-step reaction uncouples R* from transducin and allows for Schiff base
Figure 1.2. The Outer Segment and the “Dark Current”. The rod outer segment (ROS) consists of a stacked layer of disk membranes surrounded by the plasma membrane. In the dark, the cyclic-nucleotide gated (CNG) cation channel on the plasma membrane is opened by the high level of cGMP and allows Na\(^+\) and Ca\(^{2+}\) influx. At the same time, the Na\(^+\)/Ca\(^{2+}\)/K\(^+\) exchanger (NCKX) pumps 4 Na\(^+\) in, 1 Ca\(^{2+}\) out and 1 K\(^+\) out. The circulation of the cations generates the “dark current” and maintains the photoreceptor in a depolarized state.
Figure 1.2
Figure 1.3. The Disk Membrane and Light Activation. On the photoreceptor disk membrane, the heptahelical receptor, opsin, is covalently bound to the chromophore, 11-cis-retinal, to form rhodopsin. Rhodopsin is coupled to transducin, consisting of Ga-GDP and Gβγ. The phosphodiesterase, PDE6, is also located on the disk membrane and is comprised of the catalytic subunits, PDEαβ, and two inhibitory subunits, PDEγ. During light activation, 11-cis-retinal is photoisomerized to all-trans-retinal, which induces to several conformation changes in rhodopsin to become metarhodopsin II (R*). R* then activates transducin and catalyzes the exchange of GDP for GTP on Ga. Ga-GTP then dissociates from Gβγ and binds to PDEγ and releases the inhibitory action on PDEαβ. Activated PDEαβ hydrolyzes cGMP to 5’GMP and reduces the cGMP concentration in the outer segment, which ultimately closes the CNG channels. Without Ca²⁺ influx and as the NCKX continues to pump out intracellular Ca²⁺, the photoreceptor is hyperpolarized and decreases its glutamate release at the plexiform layer.
hydrolysis to dissociate the isomerized chromophore all-trans-retinal from rhodopsin. Regeneration of 11-cis-retinal is through an elaborated process named “the visual cycle” and involves the adjacent retinal pigment epithelium (Arshavsky 2002; Kusakabe, Takimoto et al. 2009). Second, the G-protein transducin has to be turned off and this is achieved by the intrinsic GTPase activity that removes the gamma phosphate of bound GTP. The intrinsic rate of GTP hydrolysis is rather slow when assayed in vitro and to speed up the rate of transducin deactivation, a GAP complex accelerates the GTPase activity. This GAP complex is comprised of RGS9-anchoring protein (R9AP) (Hu and Wensel 2002; Hu, Zhang et al. 2003; Hu and Wensel 2004; Keresztes, Martemyanov et al. 2004), Regulator of G-protein Signaling 9 isoform 1 (RGS9-1) (Cowan, Fariss et al. 1998; He, Cowan et al. 1998; Chen, Burns et al. 2000), and the long form of the fifth member of the Gβ family (Gβ5-L) (Watson, Aragay et al. 1996; Chen, Eversole-Cire et al. 2003; Krispel, Chen et al. 2003; Rao, Dallman et al. 2007) (Figure 1.4B). Upon GTP-hydrolysis, Tα-GDP loses its affinity for PDEγ. The free PDEγ re-associates with and inactivates PDEαβ. Third, the calcium and cGMP concentration are restored to the dark level through a Ca²⁺-feedback mechanism. To achieve this, the Ca²⁺ sensors, guanylyl-cyclase activating proteins (GCAP) (Mendez, Burns et al. 2001; Burns, Mendez et al. 2002; Howes, Pennesi et al. 2002; Mendez and Chen 2002; Pennesi, Howes et al. 2003), detect the drop in intracellular Ca²⁺ concentration and activate photoreceptor membrane bound guanylyl cyclase (GC) (Lowe, Dizhoor et al. 1995; Karan, Frederick et al. 2010). Guanylyl cyclase catalyzes the de novo synthesis of cGMP from GTP and restores cGMP level. As cGMP concentration increases, the CNG channel reopens to allow the influx of Ca²⁺ and Na⁺ ions (Figure 1.4C). The cation influx restores the so-called “dark current” to the photoreceptor and tonically increases glutamate release at synaptic terminal.

The differences between cone and rod

Both rod and cone phototransduction use cGMP as the second messenger to transduce light, and a similar but distinct set of genes are used. There are many intrinsic differences between the two photoreceptor types and are responsible for their distinct contributions to vision. While rod phototransduction has been well characterized in the past 40 years (Fu and Yau 2007), it is a consensus now that understanding cone activation and deactivation constitutes the next frontier in phototransduction research (Kawamura and Tachibanaki 2008). Rod phototransduction is highly sensitive to light but lags behind in spatial resolution. Cone on the
Figure 1.4. Photoreceptor Recovery. Photoreceptor must recover in order to capture the next photon. Three reactions, receptor termination, G-protein deactivation, and cGMP regeneration, are required for photoreceptor recovery. First, (A) the phosphorylation of the activated receptor (R*) by G-protein-coupled receptor kinase 1 (GRK1) and the subsequent binding of arrestin to p-R* terminate receptor signaling and transducin activation. In mouse, the loss of GRK1 (GRK1−/−) leads to defects in photoreceptor recovery (upper right). Second, (B) the intrinsic GTPase activity of transducin hydrolyzes GTP to GDP to deactivate the G-protein. The GTPase activity of transducin is accelerated by the GAP complex, R9AP/Gβ5-L/RGS9-1. In mouse without RGS9-1 (RGS9−/−), photoreceptor recovery is also delayed (middle right). Lastly, (C) the drop in Ca^{2+} leads to the binding of guanylyl cyclase activating protein (GCAP) to guanylyl cyclase (GC). This binding leads to the activation of GC, which catalyzes the conversion of GTP to cGMP. The restoration of cGMP level opens the CNG channels on the plasma membrane and allow photoreceptor depolarization to regenerate the “dark current”. The lost of GCAPs in mouse photoreceptors also leads to recovery delay.
A. Rhodopsin deactivation

\[ R^* \xrightarrow{GRK1} p-R^* \xrightarrow{Arrestin} Arr-p-R^* \]

B. GTP-hydrolysis of transducin

\[ \text{GAP complex: RGS9-anchoring protein (R9AP)} \]
\[ \text{Regulator of G-protein signal 9 isoform 1 (RGS9-1)} \]
\[ \text{Long form of the fifth member of Gβ subunit (Gβ5-L)} \]

C. cGMP regeneration

\[ \text{Guanylyl cyclase (GC)} \]
\[ \text{Guanylyl cyclase activating protein (GCAP)} \]

\[ \text{CNG-channel reopening and photoreceptor depolarization} \]

Figure 1.4

Howes et al., (EMBO) 2002
other hand has high temporal resolution but it is much less sensitive to light. Rod is capable of responding to single photon (Rieke and Baylor 1998; Doan, Mendez et al. 2006), whereas cone requires approximately 100 times greater intensity of light to generate a photoresponse. Kinetically, rod photoresponses are slower and saturate faster, while in cones they are faster and do not saturate even at high intensities. Structurally, rod and cone photoreceptors have distinct outer segment morphology as their names suggest. The rod-shaped ROS has a diameter and length of 1.4 and 24 µm, respectively, whereas the cone-shaped COS has on average 1.2 and 13 µm (Carter-Dawson and LaVail 1979). The disc membranes in rod are stacked lipid layers that are discontinuous and separated from the plasma membrane. In contrast, cone disk membranes are shorter, formed by membrane invagination and are continuous with the plasma membrane (Shichida and Matsuyama 2009). The smaller OS in cones may contribute to their faster response kinetics as the effective concentration of the phototransduction proteins is increased. At the same time, the open disc formation gives cone a larger surface area for chromophore dissociation and exchange during pigment regeneration. Another key difference between the two photoreceptor types is how they are coupled to their downstream circuitry at the outer plexiform layer (OPL) (Mustafi, Engel et al. 2009). While rod bipolar cells receive inputs from many rods, cone bipolar cell received information from much less cones. In extreme cases in primate retina, one cone bipolar cell is found coupled to only one cone photoreceptor. The rod circuitry is design to maximize sensitivity by having a high conversion factor. Conversely, the low convergence of the cone circuitry results in reduction in sensitivity but enhancement in acuity.

In addition to structural and network differences between rod and cone, almost all the players in the phototransduction cascade, such as visual pigments, transducin, GRKs, arrestin, CNG channel, and Na/K, Ca\(^{2+}\) exchanger are different for cone (Larhammar, Nordstrom et al. 2009; Shichida and Matsuyama 2009). While rhodopsin is the light receptor for rod, different cone pigments with different action spectra are found in cones. In human three types of cones exist: short wavelength (S), medium wavelength (M), and long wavelength (L). The cone composition in other species varies and in mouse only two types of cones are present: S and M cones (Applebury, Antoch et al. 2000). The mouse S-cone opsin and M-cone opsin are maximally sensitive at wavelengths 360 and 508 nm, respectively. Another difference between rod and cone is their OS structure. The open disc structure of COS gives cone a larger surface area to allow for faster chromophore dissociation and exchange in regenerating the visual
pigments, which, in turn, may contribute to faster cone physiology (Mustafi, Engel et al. 2009). In addition to different opsin composition, rod and cone have different transducin subunits. Rod transducin is made up of Gαt1/Гβ1/Гγ1, whereas cone transducin is comprised of Gαt2/Гβ3/cone Гγ (Lerea, Somers et al. 1986; Fung, Lieberman et al. 1992; Lee, Lieberman et al. 1992; Peng, Robishaw et al. 1992). Rod transducin (Gαt1/Гβ1/Гγ1) has been observed to undergo light-dependent translocation from OS to IS (Kerov, Chen et al. 2005; Chen, Wu et al. 2007; Lobanova, Finkelstein et al. 2007). The translocation event is a light-adaptation mechanism for rod to protect itself under bright light condition. However, cone transducin does not normally undergo translocation except when illumination is so bright that bleaches all cone pigments (Lobanova, Herrmann et al. 2010). A recent study reveals that Gαt2 (GNAT2) also moves in a light-dependent manner when placed in rods (Chen, Woodruff et al. 2010). While the molecular mechanism in light-dependent protein translocation in rod remains unclear, a chaperone protein may be responsible in rod trafficking and absent in cone. Furthermore, replacing Gαt2 in Gαt1−/− (GNAT1−/−) rods causes reduced sensitivity, slower activation, but faster recovery kinetics. This study demonstrates that the rod and cone differences can be partially attributed to different transducin α subunits used by cone and rod and that the translocation phenomenon is not dependent on the Gα subunits but on the cells where they reside.

In addition to opsin and transducin, the cGMP-gated channels in cones are also different from the ones in rods. The cGMP-gated channels belong to the cyclic-nucleotide gated (CNG) channel family and are tetrameric channels. Rod CNG channel is made up of 3 CNGA1 and 1 CNGB1 subunits, whereas cone CNG channel is made up of 2 CNGA3 and 2 CNGB3 subunits (Weitz, Ficek et al. 2002; Zheng, Trudeau et al. 2002; Zhong, Molday et al. 2002; Peng, Rich et al. 2004). In heterologous system, CNGA1 and CNGA3 subunits can form functional homomeric channels, while CNGB1 and CNGB3 cannot. CNGB1 and CNGB3 require the CNGA subunits to form functional channel and contribute to variation in channel properties, such as conductance and drug sensitivity. It is obvious that the variation in the components of phototransduction machinery must contribute to the differences between rod and cone. However, the kinetic consequence on what makes a rod a rod or a cone a cone requires detailed characterizations. One way to further elucidate rod-cone differences is to swap phototransduction components between the two cell types, which is an active area of work currently ongoing in the lab.
Unlike rod, isolating mouse cone photoreceptors for biochemical and electrophysiological studies has been difficult due to its rarity (3% of the photoreceptors) and fragility. Meanwhile, wild-type cones in different species are being characterized in recent years to compare its kinetic properties to rods (Tachibanaki, Tsushima et al. 2001; Nikonov, Kholodenko et al. 2006). The cone phototransduction proteins provide for its faster kinetics, but with lower sensitivity. Each reaction in the cone phototransduction cascade has been carefully quantified and overall is less efficient than its corresponding reaction in rods. In carp (freshwater fish), four types of cones are present, red, green, blue and UV-sensitive, which makes up for 2% of its photoreceptor (Kawamura and Tachibanaki 2008). Of these cones, 60% are red cones containing red-sensitive pigments. Comparing the flash intensities required to illicit half-maximal response, it was determined that the overall amplification of carp cone, from pigment activation to cGMP hydrolysis, is ~250 times less efficient than rod. The decrease in efficiency is the combined effect of decreased activation in cone transducin and cone PDE6. In rod, one activated pigment (R*) activates 54 transducin per second. On the other hand, cone transducin is estimated to be activated at a rate of ~2 molecules/s per activated cone pigment. This is a ~30 fold difference in the activation between rod and cone transducin. To account for the overall 250-fold difference in amplification, cone PDE is calculated to have 10-fold decrease in activation efficiency by cone transducin. All these factors contribute to the kinetic differences between rod and cone phototransduction.

While cone amplification is lower resulting in its decreased sensitivity, the fast recovery of cone response provides for its high acuity. The fast response in cone must imply that the reactions responsible for terminating the active species and regenerating the dark current are faster than rod. Carp cones express GRK7 instead of GRK1, the GRK in rods, in the deactivation of cone opsin (Tachibanaki, Tsushima et al. 2001; Shimauchi-Matsukawa, Aman et al. 2005; Tachibanaki, Arinobu et al. 2005; Wada, Sugiyama et al. 2006). After light stimulation, cone opsin is phosphorylated at 5 phosphates per R*/s, which is 50 times higher than the reaction in rods at 0.09 phosphates per R*/s. Interestingly, the opsin phosphorylation occurs in the recovery time course in cone, which suggests pigment deactivation may determine the speed of cone recovery.
In addition to faster opsin deactivation, transducin turn-off and cGMP regeneration must also be faster in cone to re-establish cGMP level and reopen the CNG channel. Studies in the eastern chipmunk, a cone dominant retina, estimated that cone expresses 10-fold higher level of RGS9-1 than rod, which will accelerate cone transducin deactivation (Cowan, Fariss et al. 1998). Also, rod and cone in different species express different sets of GCAP and GC proteins, which may contribute to differences in cGMP synthesis between rods and cones. The role of GCAPs and GCs in the recovery of phototransduction will be further discussed in the next section. Structurally and biochemically, cones have distinct characteristics that give them the unique ability to provide for fast and high temporally resolved vision.

**Mouse Cone Photoreceptors and Nrl<sup>−/−</sup> retina**

To detect light across different wavelength for color vision, cone opsin expression dictates the sensitivity in different cone photoreceptors. S opsin (*Opn1sw*) is expressed in the short-wavelength sensitive (SWS) cones and is maximally stimulated by light between 415-430 nm. M opsin (*Opn1mw*) is expressed in the middle-wavelength sensitive (MWS) cones and is maximally stimulated by light between 530-537 nm (Marc and Sperling 1977; Mustafi, Engel et al. 2009). Mouse cone system does not contain the L cone, unlike primates that contain all three types of cones to mediate trichromatic vision. As a nocturnal species, mouse possesses a rod-dominated retina and L cone may not be needed and hence eliminated during evolution (Jacobs 2009; Mustafi, Engel et al. 2009). The cone distribution across different species also varies, dependent on the species’ nocturnal and diurnal cycle. In human cone system, cone density is concentrated at the fovea to provide for high visual acuity. In contrast, mouse cones are distributed evenly across the retina, with a density of 1.2 x 10<sup>4</sup> cells mm<sup>-2</sup>, mimicking the peripheral region of primate retinas. Furthermore, mouse cone photoreceptors have a unique spatial patterning between S and M cones (Applebury, Antoch et al. 2000). S cones are highly localized in the inferior (ventral) region, while M cones are concentrated in the superior (dorsal) region of the retina. Although S and M are the two cone types in mouse, their cone opsin expressions are not mutually exclusive (Lyubarsky, Falsini et al. 1999; Akimoto, Filippova et al. 2004). It has been shown that β-galactosidase (*lacZ* reporter) driven by the human M cone promoter is expressed in both cone types (Wang, Macke et al. 1992). Message quantification of rod and cone opsin indicates a ratio of 1000:30:10 for rhodopsin:S-opsin:M-opsin mRNAs. The higher S opsin mRNA level over M opsin reflects that there are more S opsin than M opsin per
cone and/or more S cones than M cones. Moreover, S and M cone opsins are co-expressed in the cones in the inferior hemisphere, but less in the superior hemisphere. In the superior hemisphere, M cone opsin is expressed without any S opsin. These results demonstrate that cone distributions are graded and opsin expression is dependent on the promoter strength in each cone photoreceptor. In fact, recent study shows that M-opsin expression increases when S-cone is down-regulated in mouse, confirming that the translational machinery in cones regulates the expression of S and M cone opsin (Daniele, Insinna et al. 2011). Competition on the same translational regulatory elements can determine the level of S and M opsin expression in cone photoreceptor. Based on primary structure, mouse S opsin is ~85% identical to other mammalian SWS pigments (Yokoyama and Radlwimmer 1998; Yokoyama, Radlwimmer et al. 1998). It forms an Schiff base linkage with 11-cis-retinal at Lys291 and has a $\lambda_{\text{max}}$ of 360 nm. The mouse M cone opsin has a $\lambda_{\text{max}}$ of 508 nm and is similar to the red and green cone (L/M) opsin family. Mouse cone opsins have different $\lambda_{\text{max}}$ from primate opsins, which may be attributed to structural variations. The variations in cone distribution and its opsin expression and characteristics can contribute to the sensitivities, spatial resolution and to how different species perceive and interpret their environment.

It has been suggested mouse cone is the ancestral photoreceptor in which rod derives from (Jacobs 2009; Mustafi, Engel et al. 2009). Cone makes up 3% of the photoreceptors in the mouse retina, where their nuclei reside in the outer half of the ONL and sit close to the outer limiting membrane (OLM) (Carter-Dawson and LaVail 1979). Under light microscopy, COS diameter is thicker and more transparent compared to ROS. COS may have evolved as the disc membrane become separated from the plasma membrane to become the OS found in rod photoreceptors. This step in photoreceptor evolution is required as animals adapt to darker environments. As mentioned in previous sections, rod has similar but a distinct set of phototransduction proteins that vary from cone. Therefore, there must be a transcription switch that determines the differentiation between rod and cone during development. One such molecules is neural retina leucine zipper (Nrl), a transcription factor that has been found to upregulate protein expression of rod-specific genes and indirectly suppresses cone-specific genes through its interaction with Nr2e3 (Mears, Kondo et al. 2001). NRL contains a basic leucine zipper motif, which recognizes and binds Nrl response element (NRE). Along with other transcription factors such as Crx, Nrl associates with the NRE on rhodopsin promoter to
upregulate rhodopsin expression. The role of Nrl in photoreceptor development has been verified in the knockout mouse, which has a ‘rodless’ or cone-like retina (Daniele, Lillo et al. 2005; Nikonov, Daniele et al. 2005). The photoreceptors in Nrl\(^{−/−}\) have similar sensitivity and nuclear structures to wild-type cones. In particular, the nuclear structures show a pattern of heterochromatin, similar to the ones in wild-type cones. mRNA and protein analysis demonstrate that Nrl\(^{−/−}\) photoreceptors have cone-specific protein expression, such as cone transducin, S opsin, and cone arrestin, whereas rod-specific proteins (rhodopsin and rod transducin) are not detectable. While Nrl\(^{−/−}\) photoreceptors share many hallmarks of a typical cone, Nrl\(^{−/−}\) ‘cones’ appear to have disorganized disk morphology with shorter outer segments. Also, these ‘cones’ undergo a slow degeneration, evident by an age-dependent decrease in their ERG responses. Nrl\(^{−/−}\) photoreceptors seem to adopt a S-cone phenotype as its ERG amplitude is greater than WT response when stimulated with a 400 nm light, which is absorbed by S opsin. The increase in ERG responses corresponds with a higher S opsin expression in Nrl\(^{−/−}\) retina. In contrast, M cone opsin expression does not change comparing WT and Nrl\(^{−/−}\) photoreceptors, which is reflected in similar ERG responses when stimulated with a 530 nm stimulus. The enhanced S cone phenotype in Nrl\(^{−/−}\) retina suggests that the default fate of photoreceptor progenitors may be S cone photoreceptors. The “cone-like” photoreceptors in Nrl\(^{−/−}\) retina have been useful in the study of the biochemical and physiological properties of cone photoreceptor.

Physiologically, cone properties in WT and genetically modified mouse have been investigated using Electroretinogram (ERG), which does not give single-cell resolution (Ekesten, Gouras et al. 1998; Lyubarsky, Chen et al. 2000). On a single cell level, rod photoreceptors have been well characterized through suction pipette recording on the outer segments (Baylor, Lamb et al. 1979). However, the characterization in cone kinetic has been difficult due to its rarity and frailty of COS in the mouse retina. Instead of using the outer segments, the inner segments have been exploited to record cone photoresponses in recent studies (Nikonov, Daniele et al. 2005; Nikonov, Kholodenko et al. 2006). The inner segment (IS) contains K\(^{+}\) channels to carry the outward membrane current, which is transduced from the OS. After light stimulation, the closure of CNG channels at the OS reduces the inward membrane current and hyperpolarizes the cell. The hyperpolarization then continues to the IS, which eliminates the outward current through the K\(^{+}\) channel. This change in outward current is captured through suction pipette recording. In addition, the recordings are carried out in the presence of a 500 nm background light to suppress
the rod response. To discern the type of cone under recording condition, cone responses from 361 and 501 nm flashes are compared. As most mouse cones express both S and M cone opsins, the one with the higher expression would be maximally stimulated by the corresponding wavelength of light. The responses are derived from S cone if the cone is maximally stimulated at 361 nm, or M cone if maximally stimulated at 501 nm. The location of the S and M cones also corresponds with their light sensitivity, with M cone in the superior region and S cone in the inferior region. IS recordings on sliced wild-type retina have demonstrated that S and M cones have similar flash kinetics and response properties, despite their differences in cone opsin expression (Nikonov, Kholodenko et al. 2006). Wild-type cones respond with a 0.2% decrease in membrane current per photoisomerization. The sensitivity (the amount of light needed to obtain half-maximal response) of mouse cones is $2 \times 10^5$ photon $\mu m^{-2} s^{-1}$, which is more than 40 fold lower than a mouse rod at 350 photon $\mu m^{-2} s^{-1}$. S and M cones have time-to-peak ($t_{peak}$) values of 73 and 63 ms and a dominant recovery time constants ($\tau_D$) of 73 and 68 ms, which are considerably faster than rod responses with $t_{peak}$ of 205 ms and $\tau_D$ of 235 ms. Similar parameters are also obtained in GNAT1<sup>−/−</sup> animals, except with a slower $\tau_D$ of ~110 ms. The cone recording in wild-type mouse is performed in the presence of background light, while GNAT1<sup>−/−</sup> cone is recorded without background light since deleting transducin removes rod photoresponses (Calvert, Krasnoperova et al. 2000). Light adaptation has been demonstrated to accelerate the recovery of rod photoreceptors. Similarly, WT cone recovery becomes accelerated under light adaptation, hence the lower $\tau_D$ in wild-type cones compared to GNAT1<sup>−/−</sup> cones. Comparing rod and cone, the cone recovery of 110 ms in GNAT1<sup>−/−</sup> animal is two times faster than WT rods around 235 ms. Using the cone IS, it is now possible to study cone physiologically in WT and genetically manipulated mice and to understand it unique role in the visual system.
CHAPTER 2
Deactivation of cone transducin rate-limits the recovery of cone photoreceptors

Introduction

The recovery of photoreceptors after light stimulation requires (1) receptor deactivation by GRK1 phosphorylation and arrestin binding (Chen, Burns et al. 1999; Lyubarsky, Chen et al. 2000), (2) GTP hydrolysis by transducin, which is accelerated by a GAP complex containing RGS9-1/Gβ5-L/R9AP (Chen, Burns et al. 2000; Krispel, Chen et al. 2003; Nishiguchi, Sandberg et al. 2004; Krispel, Chen et al. 2006), and (3) restoration of cGMP level to re-open CNG channel to resume the circulating “dark-current” (Burns, Mendez et al. 2002). Disruption in any of the reactions during recovery results in prolonged activation. Mutations in genes involved in some of these recovery steps have been identified in human and in animals, which lead to various forms of visual defects including bradyopsia, congenital stationary night blindness, Leber’s congenital amarousis, cone/rod dystrophy (Cideciyan, Zhao et al. 1998; Nishiguchi, Sandberg et al. 2004; Cideciyan 2010; Karan, Frederic et al. 2010; Michaelides, Li et al. 2010). Studying genetic modified mice have provided insights on the reactions important for photoreceptor recovery and mechanisms involved in the pathology of some of these diseases. Mouse without GRK1 (GRK1−/−) or arrestin (Arr1−/−, or Arr4−/−) has a severe delayed rod recovery, confirming that GRK1 and arrestin are the main players responsible for deactivating R* in rod (Xu, Dodd et al. 1997; Chen, Burns et al. 1999; Lyubarsky, Chen et al. 2000). Without GRK1 or arrestin, R* remains active for a very long period of time and because it takes just a few hundred R* to saturate rod’s photoresponse, rod sensitivity in affected individuals does not return until all R* is deactivated by spontaneous decay. This takes minutes instead of milliseconds. Recessive mutations in GRK1 and arrestin have been identified in patients of Oguchi Disease, a type of congenital stationary night blindness (Fuchs, Nakazawa et al. 1995; Cideciyan, Zhao et al. 1998; Hayashi, Gekka et al. 2007; Oishi, Akimoto et al. 2007). These patients have difficulties with night vision, which correlates with defective R* deactivation. We will set aside Oguchi Disease and its relation with GRK1 for now, and will discuss it further in Chapter 3.

Similar to defective rhodopsin phosphorylation or arrestin binding that prolongs rhodopsin lifetime that delays rod recovery, additional loss of function manipulations in different
recovery steps may also prolong phototransduction. As the RGS9-1/Gβ5-L/R9AP GAP complex facilitates transducin turn-off, defects in any components of this GAP complex may delay rod recovery. In photoreceptors of RGS9\(^{-/-}\), Gβ5\(^{-/-}\) or R9AP\(^{-/-}\) mouse, they possess very similar delay in rod recovery as transducin deactivation is not assisted and depends on a slower endogenous rate of GTP hydrolysis by transducin α subunit (Chen, Burns et al. 2000; Krispel, Chen et al. 2003; Keresztes, Martemyanov et al. 2004). It is now known that the level of R9AP determines the overall levels of Gβ5-L and RGS9-1 and that some components like RGS9-1 and Gβ5-L are obligate partners to each other because in the absence of one, the other becomes unstable and is subject to proteolysis (Chen, Eversole-Cire et al. 2003; Keresztes, Martemyanov et al. 2004). In RGS9\(^{-/-}\), Gβ5\(^{-/-}\) and R9AP\(^{-/-}\) photoreceptors, the GAP complex is similarly abolished to result in the reduction of transducin GTPase activity. In fact, the recovery time constants, \(\tau_{\text{rec}}\) and \(\tau_D\), are slowed from 180 and 270 ms in control rods to 2600 and 9000 ms in rods lacking the GAP complex. Such an increase in recovery time constant in the absence of the GAP complex may reflect the basal GTPase activity of transducin in rod differs under dim and bright flash conditions, where \(\tau_{\text{rec}}\) and \(\tau_D\) are determined, respectively. Alternatively, this may suggest that under dim light condition where much less activated transducin is present in rod, some other protein may facilitate transducin GTP hydrolysis (Chen, Burns et al. 2000).

Another indispensable reaction for rod recovery is the restoration of cGMP level in order to reestablish the “dark current” (Fu and Yau 2007). This is accomplished by light-dependent regulation of guanylyl cyclase activating proteins (GCAPs), members of the Ca\(^{2+}\)-binding protein family, on guanylyl cyclases (GCs). GCAPs are molecular switches on GC activity during dark to light transition. Under high Ca\(^{2+}\) level in the dark, Ca\(^{2+}\)-bound GCAPs inhibit GCs and the rate of cGMP synthesis is low. After light exposure, the drop in cGMP level after PDE6 activation leads to the closure of the CNG channels and the decrease in Ca\(^{2+}\) influx. In the duration that the channels are closed, the Na\(^{+}\)/Ca\(^{2+}\)/K\(^{+}\)-exchanger (NCKX) continues to extrude intracellular Ca\(^{2+}\) out of the OS at the ratio of 4Na\(^{+}\)/1Ca\(^{2+}\)/1K\(^{+}\). The combine effect of channel closure and Ca\(^{2+}\) efflux after light illumination results in the drop in Ca\(^{2+}\) concentration and membrane hyperpolarization (Yau and Nakatani 1984; Luo, Xue et al. 2008). As photoactivation drives Ca\(^{2+}\) down to the nanomolar (~30-50 nM) range (Fain, Matthews et al. 2001), unbound GCAP then stimulates GCs to increase the rate of cGMP synthesis, restoring cGMP level back to the
dark state to reopen the CNG channel and allows cation influx and photoreceptor depolarization. There are two GCAPs (Cuenca, Lopez et al. 1998; Howes, Bronson et al. 1998) and two GCs (Liu, Seno et al. 1994; Cooper, Liu et al. 1995; Lowe, Dizhoor et al. 1995) in mouse photoreceptors. GC1 and GCAP1 are present in rod and cone, whereas GC2 and GCAP2 are only present in rod. GC1 is activated by both GCAP1 and GCAP2, whereas GC2 is activated by GCAP2. The GCAP1 and GCAP2 proteins are ablated in mouse simultaneous as their genes are in a tail-to-tail arrangement. In animals lacking both GCAPs (GCAP−/−), rod recovery is delayed, since cGMP synthesis cannot be restored in a timely manner after Ca2+ level decreases (Mendez, Burns et al. 2001; Howes, Pennesi et al. 2002; Pennesi, Howes et al. 2003). Consistent with lack of Ca2+-feedback to restore cGMP concentration, the GCAP−/− rods have larger amplitudes and its SPR amplitude is five times higher than WT. Furthermore, the loss of GCAP abolishes the rod’s sensitivity to the large range of light stimulus. Recovery delay is rescued by restoring GCAP1 expression in rod and cone (Howes, Pennesi et al. 2002). On the other hand, restoring GCAP2 expression GCAP−/− restored the recovery kinetics at saturating light flashes, but not at sub-saturating flashes (Mendez, Burns et al. 2001). These evidences suggest that GCAP1 has a more dominant role than GCAP2 in rod photoreceptors. GCAP2 may be less sensitive to Ca2+ changes in rods, hence its inability to properly recover rod at lower flash intensities. Overall, GCAPs regulation on GCs is important in setting the rod’s light sensitivity and for the Ca2+-feedback on regenerating the cGMP level and the “dark current”.

It is evident the three reactions described above are required for photoreceptor recovery and any deficiency in these steps results in the loss of the photoreceptor’s sensitivity and compromises spatiotemporal resolution of phototransduction. However, there was a big debate in the timing and interplay of these recovery reactions before 2006, in which under normal condition which of these reactions rate-limits the overall rate of recovery. It is conceivable that the slowest reaction will determine how fast photoreceptor can recover. Genetic ablation of genes responsible for any of these reactions may artificially slow down the recovery; therefore it does not reveal whether a particular reaction is rate-limiting under normal condition. Because the Loss of function (LOF) approach is not suitable to find the rate-limiting step, a gain of function (GOF) approach is devised to tackle the question by transgenically increasing the concentration of the enzyme responsible for the slowest step to alter the overall recovery of phototransduction. It has been found by overexpressing R9AP in rod that the concentration of RGS9-1 and Gβ5-L
can be elevated (Krispel, Chen et al. 2006). By increasing the level of GAP complex, the GTP-hydrolysis of transducin is accelerated and rod recovery is sped up. The $\tau_{\text{rec}}$ and $\tau_D$ are sped up from 190 and 246 ms in control rods, to 74 and 80 ms, respectively. Furthermore, the degree of recovery acceleration is a function of GAP overexpression. In a more recent study a recovery time constant of 54 ms was recorded with a 6-fold overexpression of GAP (Chen, Woodruff et al. 2010). A decrease in recovery time constant (faster recovery) demonstrates that GTP-hydrolysis of transducin is the slowest step in the recovery phase of rod phototransduction. At the time I joined the lab, it became clear to me that one of the obvious standing question was whether cone photoreceptor shares the same rate-limiting step for its recovery. This is an important question to answer because it has been shown that cone possesses a higher RGS9-1 level than rods in many species examined including human, bovine, mouse and chipmunk. In the chipmunk case, the level of overexpression in cone reaches ten-fold (Cowan, Fariss et al. 1998). Can such a high amount of GAP complex in cone change the identity of the rate-limiting step of cone recovery? If the turn-off of cone transducin does not rate-limit cone recovery, what is the real rate-limiting step?
Methods

Transgenic constructs and mouse husbandry

In this study, a similar GOF approach in Krispel et al is used to overexpress the GTPase-accelerating protein (GAP) complex in mouse cone photoreceptors. Two transgenic constructs, MCV9 and MCV10, are generated to drive R9AP overexpression in S and M cones, respectively. MCV9 construct consists of the 520 bp human blue opsin promoter (Akimoto, Filippova et al. 2004), flanked by R9AP and terminated with a protamine polyadenylation (pA) signal. The blue opsin promoter is subcloned into pBluescript (Stratagene, Santa Clara, CA) using KpnI and XhoI, along with the pA signal using BamHI and NotI, to generate the pBOP1.1 plasmid. R9AP is placed downstream to the promoter in pBOP1.1 using XhoI and BamHI. The transgenic construct was linearized with KpnI and NotI and submitted to the VCU Mouse Core Facility for nuclear injection. MCV10 was generated in a similar manner deriving from pGOP1.1 plasmid, which contained the 5 kbp green opsin promoter (Akimoto, Filippova et al. 2004). The presence of MCV9 and MCV10 transgenes are confirmed with PCR on digested mouse tail DNA using the primer sets described in Table 2.1. The amplification conditions for PCR are: 94°C for 5 minutes, then 35 cycles of 94°C for 1 minute, 64°C for 1 minute, and 72°C for 1 minutes and followed with a final extension of 72°C for 5 minutes. For MCV10, six founders are generated with line 1, 5, and 6 overexpressing RGS9-1. Line 6 is carried forth for characterization. R9AP+/−, MCV9 and MCV10 are mated into GNAT1−/− (designated as ‘T’) (Calvert, Krasnoperova et al. 2000) to remove the rod interference and facilitate single-cell recordings. At the same time, MCV9 and MCV10 were crossed into R9AP−/− background to allow western quantification of the RGS9-1 overexpression in cones. Genotyping conditions for the animals used in this study are listed in Table 2.1.

The opsin promoters are utilized to drive cone-specific expression of human GRK7 (hGRK7). The hGRK7 cDNA is inserted into pBOP1.1 and pGOP1.1 between the promoter and the pA signal with XhoI and BamHI to generate the pBOPGRK7 (MCV7) and pGOPGRK7 (MCV8) constructs. The constructs were linearized with KpnI and NotI to remove the pBluescript backbone and submitted to the VCU Mouse Core facility for nuclear injection. The presence of the MCV7 and MCV8 transgenes was confirmed with PCR on digested tail DNA using the following primer sets described in Table 2.1. The amplification conditions for detecting
Table 2.1. Genotyping information. The table lists the genotyping information for the different strains used in this thesis. MCV7, 8, 9, and 10 are transgenic mouse line, whereas, GNAT1, R9AP, Nrl and GRK1 are strains for endogenous mouse proteins. The temperature ($T_m$) listed next to the strain name is the annealing temperature used during PCR.
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<th>Strains (Tm, °C)</th>
<th>Transgene</th>
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<td>hGRK7-2, 5’-ACC ACC TTT CCC CAG CAC TCT GAA CT-3’</td>
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<td>MCV8 (64)</td>
<td>GOP1.1, 5’-GAG ACA GTT TTC TAC AGC CT-3’</td>
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<td>hGRK7-2</td>
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<td>MCV10 (64)</td>
<td>GOP1.1, R9AP230</td>
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<th>Primers (KO)</th>
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<td>GNAT-R, 5’-GCC GGC GGA GTC ATT GAG CTG GTA-3’</td>
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<td>R9AP-F1, 5’-TGG GGA GAA GCT AAC CAG ACT A-3’</td>
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<td>R9AP-KOR1, 5’-AGT CCC ATC TTT GCC TTT ACT G-3’</td>
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<td>NRL (64)</td>
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<td>Neo4, 5’-CCT GCG TGC AAT CCA TCT T-3’</td>
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the transgenes were: 94°C for 5 minutes, then 35 cycles of 94°C for 1 minute, 64°C for 1 minute, and 72°C for 1 minutes and followed with a final extension of 72°C for 5 minutes. For MCV8, 8 founder lines were identified and line 2 and 8 had GRK7 expression with IHC. Based on the fluorescent intensity, line 2 expressed higher level of GRK7 than line 8 and hence was carried forth for characterization. For characterization, MCV7 and MCV8 animals were mated in GRK1^{-/-} (Chen, Burns et al. 1999) to remove the endogenous GRK1.

**Immunohistochemistry**

A small burn mark was placed on the superior side of the mouse cornea before the eyes were dissected in 1X PBS (Phosphate-buffer saline, pH 7.4). A small incision was made on the cornea near the ora serrata to allow fluid permeation. The eyeball was fixed overnight in 4% paraformaldehyde (PFA) in 1X PBS at 4°C. After fixation, the cornea and the lens were carefully removed and the eyecup was cryo-protected in 30% sucrose in 1X PBS at 4°C. After the eyecup settled in the sucrose solution, it was embedded and frozen in TBS (Triangle Biomedical Sciences, Durham, NC, USA) at -80°C. Frozen eyecup blocks were sectioned at 24 µm and placed on microscope slides. For immunostaining, sections were washed three times with 1X PBS at five minutes each. Washed sections were blocked with 10% goat serum in PBT (0.3% Triton X-100 in 1X PBS) for 1 hour at room temperature (RT). Blocking solution was removed before adding the primary antibody. Primary antibodies, CT318 (1:200) and FITC-conjugated PNA (1:500), were diluted with 10% goat serum in PBT, applied over the section, and incubated overnight at RT. After incubation, sections were washed three times with PBT at five minutes each. After washing, Alexa-568 conjugated goat-anti-rabbit secondary antibody was diluted at 1:1000 with 10% goat serum in PBT, added to the sections and incubated for two hours at RT. After incubation, sections were washed three times with PBT at ten minutes each. After washing, sections were protected with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA), mounted with cover slips, and sealed with nail polish. Confocal images were taken with Zeiss META LSM 510 confocal microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) in the VCU Microscopy Core Facility (Department of Anatomy and Neurobiology, Virginia Commonwealth University). Images were viewed on LSM Image Browser program (Carl Zeiss Imaging, Thornwood, NY, USA) without any post-hoc adjustments.

**Immunostaining of flat-mount retina (Perform by Mr. Yen-Lin Chen)**
Mouse eyeball was dissected in the similar fashion as for IHC. After the removal of the cornea and the lens, sharp, fine scalpel was used to cut the retina into four quadrants. The superior and inferior quadrants were retained and the other two was discarded. The retina from the quadrants was carefully peeled away from RPE using a fine brush and flattened onto nitrocellulose filter paper with the photoreceptor side facing up. The flattened retinas were fixed in 4% PFA in 1X PBS overnight at 4°C. After fixation, staining was performed in 0.6 mL microfuge tubes. Retina was washed three times with 1X PBS at five minutes each. Similar procedure was carried out as for IHC with a few modifications. The primary antibodies incubation was performed with gentle rocking at 4°C for two days to allow antibody penetration. After mounting and sealing, images were taken immediately using the Zeiss META LSM 510 confocal microscope at 63X magnification.

Immunoquantification

ImageJ software (NIH, USA) was used to trace the RGS9-1 signals in rods and cones to determine their average fluorescent intensities in the retinal cross section and flat-mount retinas on single optical slices. The cone RGS9-1 intensities were selected based on the surrounding PNA signals. To quantify the relative RGS9-1 expression in T (GNAT1−/−), 9T (MCV9 in GNAT1−/−), 10T (MCV10 in GNAT1−/−), and 910T (MCV9 and MCV10 in GNAT1−/−), the cone signal was normalized to rod level in both the superior and inferior hemisphere of the retina. Then to determine the level of RGS9-1 overexpression in the transgenic lines, the relative levels were normalized to the cone level in T. Statistics were performed in SigmaPlot and One-way Anova was used to compare the level of overexpression across different genotypes. Significance is established with p ≤ 0.05.

Western Blot

Mice were euthanized with CO₂, followed with cervical dislocation. Eyeballs were removed with forceps and placed in 1X PBS for dissection. The cornea and lens were removed before separating the retina from the retinal pigmented epithelium (RPE). For one retina, it is homogenized in 75 µL 1X PBS and solubilized in 75 µL LSB Buffer, followed with incubation at 95°C for 10 minutes. Retinal proteins were resolved on a 12% Tris-HCl polyacrylamide gel and electrophoresed in 1X SDS-PAGE buffer at 150V for 100 minutes. Following SDS-PAGE, the proteins were transferred onto nitrocellulose membrane in 1X transfer buffer at 250 mA for
120 minutes. The membrane was stained with 0.1% Ponseu S in 5% glacial acetic acid to ensure the completion of protein transfer. Ponseu S was destained with distilled water and afterwards, the membrane was blocked in blocking buffer (10% milk in 1X TBST) for 1 hour at room temperature (RT). Primary antibodies (CT318, 1:6k, GAPDH, 1:50k) were diluted in blocking buffer and incubated with the membrane for 2 hours at RT. After primary antibody incubation, the membrane was washed four times in 1X TBST at 5 minutes each. HRP-conjugated goat-anti-rabbit secondary antibody was diluted (1:30k) in blocking buffer and incubated with the membrane for 1 hour at RT. Following secondary antibody, the membrane was washed six times in 1X TBST at 10 minutes each before developing it using the SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Blot image was captured on film and on the Kodak Image Station 440 CF.

Light stimuli (Provided by Dr. Lihui Cao)

The light source was a 75-W Xenon arc lamp, modulated by a heat filter and arrays of calibrated neutral-density and 10-nm-band-pass interference filters. The beam was controlled by an electronic shutter and delivered to the microscope via a fiber-optic light guide. It was passed through an iris to control the spot size, with a circle area of 400 µm in diameter, and focused with a 40X objective. Flashes were delivered 10 ms in duration. The light intensity was periodically calibrated with a radiometer.

Retinal slice recording (Provided by Dr. Lihui Cao)

Mice at 30 to 60 days of age were reared in cyclic light, and dark-adapted overnight prior to an experiment. Mice were anesthetized by Avertin, and eyes enucleated, hemisected under infrared illumination. The retina was then attached to a nitrocellulose filter paper (Millipore, pore size 0.22 um), the pigment epithelium side down. The pigment epithelium was then peeled off with another filter paper quickly, now with the photoreceptor side up. The isolated retina on the filter paper was transferred to a chamber and fixed to its bottom with silicon grease. The chamber was filled with the standard solution. Most of the experiment performed under the Locke’s solution: 112.5 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, 10 mM HEPES, 0.02 mM EGTA, 20 mM NaHCO₃, 3 mM Na₂-succinate, 0.5 mM Na-glutamate, 10 mM glucose). The chamber was placed on the stage of a handmade mechanical slicer, and cut vertically in 150-200 µm thickness together with the filter paper. Several slices were then
transferred to the neighboring recording chamber and rollover with both ends fixed with silicon grease. A slice hold-down (a platinum-iridium frame strung) was sometimes used to prevent the retinal slice from floating.

Experiments were carried out under precisely controlled temperature, which is 35.5-36.5°C, monitored by a thermistor placed closely to the recording cell in the chamber. The bath solution was temperature-controlled before perfusion and heated up again when running close to the chamber, running at 100ml/hr. Using silanized suction pipettes with openings around 3.5-4.5 µm, several cell bodies and the conjoined inner segment were sucked in. This protocol was first developed by Dr. Nikonov and used with some modifications (Nikonov, Daniele et al. 2005). Membrane current was measured with a current-to-voltage amplifier (Axon 200B; Axon instruments). All signals were low-pass filtered at 20 Hz (8-pole Bessel) and sampled at 10 kHz. The inner segment of the cone circulating current is an outward current, carried primary by K⁺ channels. Here all the data are presented in the conventional manner as positive-going.

Brief flashes (10ms) of 380 nm light were delivered at 2.6s intervals. The effective collecting area, Ac, of an outer segment for incident light approximately perpendicular to the longitudinal axis of the outer segment is given by 

\[ Ac = 2.303 Q \alpha f d^2 l / 4 \]

where Q is the quantum efficiency of the isomerization, \( \alpha \) is the transverse specific optical density of the outer segment, and \( f \) is a factor that depends on the polarization of the incident light, \( d \) and \( l \) are the diameter and length of the outer segment, respectively. Here we adopted the previous measurements published by Nikonov (Nikonov et al, 2006), in which Ac of transducin knockout (GNAT1⁻/⁻) mouse is 0.2 µm², and Nrl⁻/⁻ mouse is 0.11 µm².

**Determination of Pepperberg time constant (Provided by Dr. Lihui Cao)**

Assuming that light-activated PDE activity decays exponentially and the guanylyl cyclase activity is maximal and equal for the duration of the saturated response, the slope of the saturation time as a function of the natural log of the flash strength provides a estimate of the time constant for the decay of PDE activity, the slower component of the rhodopsin or transducin shutoff (Pepperberg, Cornwall et al. 1992). Saturation time was defined as the period over which the flash suppressed 40% of the dark current.
Results

Relative level of RGS9-1 in mouse rod and cone photoreceptors

It was reported in cone-dominant chipmunk retinas that RGS9-1 level is ~10 times higher in cone than in rod (Cowan, Fariss et al. 1998). The overexpression was calculated based on the ratio of RGS9-1 protein expression normalized to opsin concentration in the cone-dominant chipmunk retina versus rod-dominant mouse and bovine retina. However, the overexpression may have been overestimated since 25% of the photoreceptors in chipmunk are rods. The RGS9-1 level observed in chipmunk retina is, then, partially derived from rods and can lead to an overestimation of the relative RGS9-1 expression. To date, while numerous immunohistochemical examination of RGS9 expression in retinas of multiple species were reported, it has not been systematically determined how much more RGS9-1 mouse cone may have over rod. The quantification of cone RGS9-1 level has been challenging because in rod-dominated retina such as mouse and bovine, cone cells make up a small fraction of total retinal photoreceptors. In the case of mouse, the number is ~3%. We sought to determine relative level of RGS9 in mouse cone and rod by performing quantitative IHC on mouse retinal sections using an anti-RGS9-1 antibody and a cone marker, peanut agglutinin (PNA). Qualitatively, it is very easy to see that RGS9-1 expression is indeed higher in cone (Figure 2.2A and 2.3A, ‘T’). To quantify the RGS9-1 level in cone, we compared and quantified the immunofluorescent intensities between rod and cones on both flat-mount retina and retinal sections. On a single optical slice of 0.39 µm, the rod and cone intensities (~4-8 cells) were outlined with the ‘draw’ option in ImageJ and the average intensities were calculated. Using this approach, we determined that RGS9-1 level in cones is approximately 1.6-fold over rods (Figure 2.2B, 2.3B, ‘T’). Our quantification of cone RGS9-1 level is considerably lower than the reported value in chipmunk (Cowan, Fariss et al. 1998), in which it was estimated to be 10-fold higher in cone than rod. This difference in RGS9-1 level in cones can conceivably be attributed to species difference between chipmunk and mouse. However, it is also possible that our immunofluorescence quantification may underestimate the level of cone RGS9-1 expression due to signal saturation in cone. The linear range of the IHC staining with CT318 (anti-RGS9-1 antibody) was not established in the immunoquantification of RGS9-1. If the staining is above the linear range, the 1.6-fold overexpression of cone RGS9-1 over rod is then an underestimation of the relative RGS9-1 level.
Figure 2.1. Transgenic Constructs for Cone RGS9-1 Overexpression and GRK7 expression. (A) A cartoon representation of the distribution of the two types of mouse cone photoreceptor, middle (M) wavelength and short (S) wavelength sensitive cones. M-cones are distributed in higher density in the superior hemisphere of the retina, while S-cones are distributed in the inferior hemisphere. In the central region, there is a gradient population of M and S cones as the two cell types crosses. (I, inferior; S, superior) (B) green opsin and blue opsin promoters, are used to drive the expression of human GRK7 in mouse cones. MCV7 transgenic construct consists of the blue opsin promoter flanked by human GRK7 coding region, followed with pA signal to promote S-cone specific expression. MCV8 transgenic construct is design in a similar manner with the green opsin promoter for M-cone specific expression. (C) To overexpress RGS9-1 in mouse cone photoreceptor, R9AP was placed downstream to the blue (MCV9) and green (MCV10) opsin promoters, followed by the pA signal to drive S and M-cone specific expression, respectively. The number below the constructs define the number of founder lines, then the number of transmitters and the number of expressers
Figure 2.1
Figure 2.2. Immunohistological characterization of RGS9-1 level in T, 9T, 10T, and 910T animals. (A) Confocal projected images of retinal sections stained with anti-RGS9-1 antibody (CT318, red) and a cone marker (PNA, green). Images were taken with 63X magnification at both the superior and inferior region in the T, 9T, 10T, and 910T retina. Qualitatively, RGS9-1 can be overexpressed in the S and M cones using the blue and green opsin promoters. Scale bar is 10 µm. (B) The fluorescence quantification of the rod and cone RGS9-1 signals on retinal cross sections in (A). Cone RGS9-1 signal is selected based on the surrounding PNA on individual optical slice and captured in ImageJ software. Cone RGS9-1 signals are normalized to the rod signals within a single optical slice, with rod expression being 100%. When normalized to rod RGS9-1, wild-type cone has 1.8 times higher amount of RGS9-1 in the superior and inferior hemispheres. The RGS9-1 level in the superior and inferior regions is 1.9 and 2.7 times higher in the 9T retina, 4.8 and 4.2 times higher in the 10T retina, and 5.4 and 4.2 times higher in the 910T retina. Error bar equals standard error of mean (SEM) (C) The cone RGS9-1 levels in 9T, 10T and 910T are normalized to the control cone signals to determine the overexpression level compared to cones. Relative to wild-type cones, the RGS9-1 level in the superior and inferior region of the retina is 1.1 and 1.6 times higher in the 9T cones, 2.7 and 2.4 times higher in the 10T cones, and 3.0 and 2.4 times higher in the 910T cones. Quantitatively, cones with various levels of RGS9-1 overexpression have been established with transgenesis in the 9T, 10T and 910T animals.
Figure 2.2

A.

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Figure 2.2
Figure 2.2

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RGS9-1 overexpression in MCV9 and MCV10 transgenic lines

In mouse rod, the concentration of the GAP complex is determined by R9AP level, it is not known whether this is also true in cone. Assuming that R9AP expression level determines the overall GAP level in cones, we placed R9AP cDNA downstream of the blue and green opsin promoters (Figure 2.1C) and sought to overexpress GAP complexes specifically in cones. This resulted in the production of MCV9 and MCV10 transgenic lines overexpressing RGS9-1 in the S and M cones, respectively. Six MCV10 founders were generated that contained the transgene and among which lines 1, 5, and 6 were found to have a higher intensity of RGS9-1 staining in their cones. Line 6 was carried forth for further characterization because the homogeneity of RGS9-1 overexpression across retina. To enable electrophysiological recordings, MCV9 and MCV10 animals were both crossed into GNAT1−/− (T) background to generate T, 9T, 10T and 910T. Putting them in the T background removes rod signals and facilitates cone recordings by removing the need to use background light to suppress rods, which may inadvertently cause light adaptation and/or rod/cone cross talk through electrical synapse involving GAP junctions. Eliminating the caveat concerning light adaptation is also important because one of the well-established effects of light adaptation is accelerated recovery (Ekesten, Gouras et al. 1998). In the mouse retina, S-cone is distributed in higher density in the inferior hemisphere, while M-cone is distributed more in the superior hemisphere. As the blue opsin promoter drives S-cone specific expression, the cone RGS9-1 overexpression in the MCV9 was found predominantly in the inferior hemisphere. However, the cone RGS9-1 overexpression driven by the green opsin promoter in MCV10 was found in both superior and inferior hemispheres (Figure 2.2A). The homogenous cone overexpression of RGS9-1 in MCV10 may be due to the integration site of the MCV10 transgene, or the use of a larger promoter (5 kb vs. 550 bp), or both. It is also possible that the cones in the inferior region do contain green opsin promoter activity, which permits RGS9-1 overexpression in the inferior cones of MCV10. This speculation is consistent with the finding that all cones contain certain amount of M cone promoter activity, while S cone promoter activity is the strongest in the inferior hemisphere (Applebury, Antoch et al. 2000; Akimoto, Filippova et al. 2004).

Qualitatively, we have now established two transgenic lines overexpressing RGS9-1 in mouse cones. However, how much overexpression have we achieved in MCV9 and MCV10? Using the approach mentioned above, RGS9-1 staining was performed on the cross sections of T,
9T, 10T and 910T retinas to compare their intensities. We normalized the cone signal to that of neighboring rods on single optical slice of 0.39 µm. In the control retina (T), RGS9-1 is 1.8 fold higher in cones over rods in both the superior and inferior hemisphere (Figure 2.2A, B, ‘T’). In the 9T animal (MCV9 in T), cone RGS9-1 is 1.9 and 2.7 fold higher than rod in the inferior and superior hemispheres, respectively (Figure 2.2A, B, ‘9T’). In the 10T animal (MCV10 in T), cone RGS9-1 is 4.8 and 4.2 fold higher than rod in the inferior and superior hemispheres, respectively (Figure 2.2A, B, ‘10T’). By crossing MCV9 and MCV10 together (910T), cone RGS9-1 is 5.4 and 4.2 fold higher than rod in the inferior and superior hemispheres, respectively (Figure 2.2A, B, ‘910T’). By normalizing the overexpression level of RGS9-1 in 9T, 10T and 910T to the control cone (T), inferior and superior cones have 1.1 and 1.6 fold higher in 9T, 2.7 and 2.4 fold higher in 10T, and 3.0 and 2.4 fold higher in 910T, respectively (Figure 2.2C).

Using transgenesis, we have established that the cones of 910T have 3-fold higher level of GAP complex than wild-type cones.

**RGS9-1 immunoquantification on flat-mount retina**

Using IHC, we quantified the level of RGS9-1 overexpression in our transgenic animals. It is understandable that immunofluorescence may generate high variation in quantification due to sample preparation, quenching efficiency, oxidation, and staining variation. To minimize any artifact, all samples were processed at the same time and confocal images were taken immediately after staining. Furthermore, we decided to quantify RGS9-1 level on flat-mount retina in addition to retinal cross sections and normalized the cone RGS9-1 fluorescent intensity to rod signal. In control (T) flat-mount retina, cone RGS9-1 is 1.4 fold higher than rod (Figure 2.3, ‘T’). In the transgenic animals, cone RGS9-1 in the inferior and superior hemispheres is express at 1.7 and 2.5 fold higher in 9T, 2.7 and 2.4 fold higher in 10T, and 3.9 and 3.1 fold higher in 910T than rod, respectively (Figure 2.3B). In addition, the level of overexpression was obtained by normalizing to control cone signal (T). Relative to wild-type cone, inferior and superior hemispheres of 9T have 1.2 and 1.8 fold higher expression of RGS9-1, 10T have 1.9 and 1.7 fold higher expression, and 910T have 2.7 and 2.2 fold higher expression, respectively (Figure 2.3C). Overall, immunoquantification on flat-mount retina has slightly lower expression than the results from retinal cross sections. The quantification determined on flat-mount retina may be more reliable since it is possible to encircle and capture the RGS9-1 immunofluorescence.
Figure 2.3. Immunoquantification of RGS9-1 level on flat-mount retina in T, 9T, 10T, and 910T animals. (A) To further demonstrate RGS9-1 overexpression in the 9T, 10T and 910T animals, flat-mount retinas were stained with the anti-RGS9-1 antibody (CT318, red) and costained with the cone marker, PNA. Confocal images were taken at 63X magnification immediately after staining. Images were taken at the superior and inferior hemispheres of the retinas. Scale bar equals 10 µm. (B) Higher magnification images are taken at 8X zoom on the flat-mount retinal pictures in (A). Scale bar equals 2 µm. (C) Quantification on the RGS9-1 fluorescence on the flat-mount retinal images in (B) were analyzed with ImageJ software. On single optical slice, Cone RGS9-1 fluorescence intensities was selected based on the surrounding PNA staining and normalized to rod signals. Based on RGS9-1 immunofluorescence, wild-type cones express 1.4 times more RGS9-1 than rods. Compared to the rod signals, the cone RGS9-1 level in the superior and inferior regions are 1.7 and 2.5 fold higher in the 9T animal, 2.7 and 2.4 fold higher in the 10T animal, and 3.9 and 3.1 fold higher in the 910T animal. Error bar equals SEM. (D) Quantification of cone RGS9-1 level determined in (C) normalized to wild-type cone signal. Compared to wild-type cone, RGS9-1 are overexpressed in the superior and inferior regions by 1.2 and 1.8 fold in the 9T retina, 1.9 and 1.7 fold in the 10T retina, and 2.7 and 2.2 fold in the 910T retina. Based on the quantification, cone RGS9-1 can be overexpressed as high as 2.7 fold over wild-type cones. Error bar represents SEM.
Figure 2.3
Figure 2.3
Figure 2.3
**C.**

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<td>37/95</td>
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*All rod signal normalized to 100%*

---

**Figure 2.3**
RGS9-1 Ratio (Normalized to Cone)

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<td>Ratio</td>
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* Statistics performed with One-way ANOVA-Tukey test

**Figure 2.3**
of cone and rod photoreceptors on single optic slice. The overexpression level determined on flat-mount retina will be used in this study to determine whether increasing RGS9-1 level in cone photoreceptor will speed up its recovery.

**Transgenic overexpression of RGS9-1 in a ‘cone-like’ retina of Nrl<sup>−/−</sup> mouse**

While immunoquantification of RGS9-1 on retinal sections and flat-mount retina revealed GAP overexpression in MCV9 and MCV10 transgenic animals, it is possible, to corroborate this finding by checking RGS9-1 overexpression in the so-called ‘cone-like’ retina of Nrl<sup>−/−</sup> animals. The photoreceptors in the Nrl<sup>−/−</sup> retina resemble closer to cones than rod, as demonstrated by an upregulation of cone-transducin (GNAT2) and the absence of rod-transducin (GNAT1) (Figure 2.4A). By crossing the MCV9 and MCV10 transgenes into Nrl<sup>−/−</sup> animals, retinal RGS9-1 levels in the resulting animals with expression driven by the blue and green opsin promoter were found to increase (Figure 2.4). After quantification, RGS9-1 levels in MCV9 and MCV10 are overexpressed by 3-fold and 4.2-fold, respectively, compared to control Nrl<sup>−/−</sup> retina. We found a similar and expected increase in Gβ5 levels correspondingly by 2.7-fold and 3.5-fold in MCV9 and MCV10 animals, respectively (Figure 2.4B). As a negative control, the Gβ5-S levels are the same across all animals analyzed. Gβ5-S is not found in mouse photoreceptor and hence does not increase when R9AP is overexpressed in them. The fold of increase in RGS9-1 level is higher in Nrl<sup>−/−</sup> background (by Western) than in WT background (by immunofluorescence). We have no explanation why, but would like to point out that while both determination methods have their own shortcomings (see below for Nrl<sup>−/−</sup> background), one consistent finding is that we do indeed generate transgenic mouse lines with elevated RGS9-1 levels in cones of both MCV9 and MCV19 transgenic animals.

While cone-specific RGS9-1 overexpression in MCV9 and MCV10 is confirmed in Nrl<sup>−/−</sup> background by immunoblot analysis, the conclusion has a few caveats. First, Nrl<sup>−/−</sup> photoreceptors have been reported and confirmed in our hand that they undergo a slow age-dependent degeneration. Analysis of a six-month old MCV9 animal in Nrl<sup>−/−</sup> background reveals a decrease in cone-transducin, most likely due to photoreceptor degeneration (Figure 2.4A, lane 4). The degeneration in Nrl<sup>−/−</sup> photoreceptor complicates the data interpretation while studying the biochemical and physiological properties derived from Nrl<sup>−/−</sup> animals. Second, the RGS9-1 level in Nrl<sup>−/−</sup> photoreceptor is 50% less than that of WT level (Figure 2.4A, lane 1, 2 and 7),
Figure 2.4. Western analysis on RGS9-1 overexpression in MCV9 and MCV10 transgenic animals in Nrl<sup>+/−</sup> background. (A) Western blot demonstrating the increase in RGS9-1 overexpression in MCV9 and MCV10 in Nrl<sup>+/−</sup>. In the ‘cone-like’ retina of Nrl<sup>+/−</sup>, cone transducin expression is upregulated, whereas rod transducin expression is absent. In Nrl<sup>+/−</sup>, RGS9-1 overexpression from MCV9 and MCV10 transgenes can be detected with Western blot. However, due to the slow degeneration of Nrl<sup>+/−</sup> photoreceptors (lane 4 with the drop in cone transducin at 6 months old), quantification of RGS9-1 overexpression has to be considered carefully. Also, the level of RGS9-1 expression in Nrl<sup>+/−</sup> photoreceptor is actually lower than the WT control, suggesting that the Nrl<sup>+/−</sup> photoreceptor has some abnormality. (B) Quantification of RGS9-1 and Gβ5-L overexpression from the MCV9 and MCV10 transgenes in Nrl<sup>+/−</sup> background. RGS9-1 expression in MCV9 and MCV10 is 3.12±0.56 and 4.23±0.11 fold higher than Nrl<sup>+/−</sup> control. Gβ5-L expression in MCV9 and MCV10 is 2.63±0.04 and 3.45±0.09 fold higher than Nrl<sup>+/−</sup> control. The level of RGS9-1 and Gβ5-L are increased in the presence of the transgenes, MCV9 and MCV10. Error bar represents SEM.
Figure 2.4
which is unexpected and puzzling. As previously published results and further analyzed in this study, WT cone RGS9-1 level is higher than rod. This is not the case in the ‘cone-like’ photoreceptor in the Nrl<sup>−/−</sup> animals, suggesting that these cells are different from WT cones. Due to these concerns encountered, we decided to abandon any further analysis involving Nrl<sup>−/−</sup> background.

**Western analysis of mouse cone RGS9-1 level**

Can we overcome the difficulty of quantifying protein levels in mouse cone due to its scarcity in the retina? Furthermore, can we reliably quantify the level of protein that is expressed in both rod and cone, such as the GAP complex? One approach that immediately comes to mind is to label mouse cone genetically by a fluorescent protein such as EGFP. If an animal like that can be made, one can dissociate the retina, isolate enough fluorescently labeled cones and perform Western blotting for protein quantification. To do so, we have recently generated several transgenic mouse lines called GGFP using the construct shown in Figure 2.5A. One of them, GGFP-C, displays homogeneous cone labeling across retina (Figure 2.5B and C). This line may be used in future experiments attempting to assess the level of any protein in mouse cones. For proteins that are expressed in both rod and cones, it is possible to examine the level of expression in cone if expression in rod can be selectively removed. To achieve this, we take advantage of the fact that RGS9-1 and Gβ5-L are obligate partners in vivo and that knocking out Gβ5-L eliminates RGS9-1 level. Therefore by removing one of the two obligate partners in rod photoreceptor, the level of RGS9-1 in cones can be examined by quantitative Western blotting.

In the lab, Dr. Jason Chen has successfully flanked the Gβ5 gene in the so-called Gβ5<sup>F/F</sup> mice. By crossing Gβ5<sup>F/F</sup> mice into the iCre75 background, in which a codon-improved Cre-recombinase (iCre) is specifically expressed in rod photoreceptors, Gβ5-L can be conditionally removed from rods (Figure 2.6B). To enable Western blotting determination of RGS9-1 level in cones of MCV9 and MCV10 transgenic animals, we mated MCV9 and MCV10 into the R9AP<sup>−/−</sup> background, which remove the endogenous RGS9-1 in both rod and cone and reveal only the transgenic cone RGS9-1 level (Figure 2.6B). We have obtained data to show that this strategy works. In the 10R9AP<sup>−/−</sup> retina (MCV10 in R9AP<sup>−/−</sup>), the transgenic RGS9-1 is specifically expressed in mouse cones, as confirmed in IHC by PNA co-staining (Figure 2.6C). This data shows that the RGS9-1 detected by Western blotting from 10R9AP<sup>−/−</sup> retina is derived
Figure 2.5. Characterization of GGFP-C animals. (A) The GGFP transgenic construct used to drive GFP expression in M-cones using the green opsin promoter. (B) Confocal image of GGFP-C retinal sections. Cone-specific expression of GFP is confirmed with two cone markers, cone-arrestin (VCU011) and PNA. GFP expression is found throughout the cone and concentrated in the cell body and synaptic layer. (C) Quantification of GFP-expressing cones in GGFP-C. The number of GFP+ cells are counted in the superior and inferior region of the retina and normalized to VCU011 and PNA signals. Based on the GFP+/VCU011 and GFP+/PNA signal, the cone-specific GFP expression in GGFP-C is homogenous.
Figure 2.5
Figure 2.6. Determination of RGS9-1 expression in various genetic background. (A) GAP concentration in WT and R9AP\textsuperscript{+/-}. As R9AP level regulates the concentration of the GAP complex in rods, animals with one copy of R9AP have only 50% of RGS9-1 and G\(\beta\)5-L proteins, while G\(\beta\)5-S remains unchanged. The 50% drop in R9AP\textsuperscript{+/-} is used to estimate the GAP concentration in cone. (B) In R9AP\textsuperscript{-/-}, RGS9-1 expression is abolished. By crossing MCV10 into R9AP\textsuperscript{-/-} to generate 10R9AP\textsuperscript{-/-}, any RGS9-1 expression is the result of transgene expression. (C) The RGS9-1 expression in WT and 10R9AP\textsuperscript{-/-} on IHC. RGS9-1 expression is found in rod and cone, whereas RGS9-1 is expressed only in the cones of 10R9AP\textsuperscript{-/-}. S, Superior; I, Inferior. (D) Revealing cone RGS9-1 level by removing RGS9-1 only in rods. In iCre75F/F animal, the rod-specific Cre-recombinase removes G\(\beta\)5 and consequently, the RGS9-1 in rod photoreceptor. The RGS9-1 signals remaining on immunoblot derive from cone photoreceptors. Compared to iCre75/G\(\beta\)5\textsuperscript{F/F}, RGS9-1 and G\(\beta\)5-L level in 10R9AP\textsuperscript{-/-} are expressed at 4.5 and 10-fold higher. Adjusting for the endogenous RGS9-1, MCV10 in WT background is estimated to have 5.5 fold higher RGS9-1 than non-transgenic cones. (E) A summary on the RGS9-1 quantification in different transgenic animals with various techniques. It seems that the Western blot quantification is two-fold higher than the quantification with immunofluorescence.
**Figure 2.6**

A. Western blot analysis showing the differential expression of RGS9-1, Gβ5, and GAPDH in WT and R9AP+/− mice.

B. Bar graph depicting the intensity of RGS9-1, Gβ5-L, and Gβ5-S normalized to GAPDH. Asterisks indicate *P < 0.05, significant difference, t-test.

C. Immunohistochemical images comparing WT and 10R9AP+/− mice for S and L channel staining.
**Figure 2.6**

### D.

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### E.

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**Figure 2.6**
exclusively from cones. For quantification purpose, RGS9-1 level in iCre75/Gβ5^{F/F} animal is considered as WT cone level. Comparing 10R9AP	^{−/−} and iCre75/Gβ5^{F/F} animals, the RGS9-1 and Gβ5-L expression is found to have increased by 4.5-fold and 10-fold in 10R9AP	^{−/−} retina. In R9AP	^{−/−}, the endogenous RGS9-1 and Gβ5-L protein expression are abolished. Therefore, the actual RGS9-1 and Gβ5-L overexpression of the MCV10 transgene is adjusted to 5.5-fold and 11-fold over WT cones. The characterization of MCV9 and MCV910 in R9AP	^{−/−} background is currently underway, so is the determination of GAP level in R9AP	^{+/−} line in the iCre75/Gβ5^{F/F} background. We envision that this strategy will most likely produce the most reliable quantitative data for us to establish a titration of RGS9-1 level in cones and the degree of recovery acceleration (see below).

**Analysis of Cone ERG recovery using cone-derived B wave responses**

Before single cone recording is established using suction-pipettes, cone recovery is analyzed by using a double-flash ERG protocol (Figure 2.6A). A test flash of 1.63 log cd S/m^2 is applied on a light-adapted mouse and after a specific interstimulus interval (ISI) from 100 to 2000 ms, the same flash is applied and the remaining cone response is recorded. The response from the second flash is indicative of the cone recovery from the first flash. Normalizing to the maximal response and plotting the response recovery to the ISI, a recovery curve is obtained (Figure 2.7A). By fitting the recovery curve with the exponential equation, \( f(x) = 1 - e^{-\frac{x-t_0}{\tau}} \), two parameters, \( t_0 \) and \( \tau \), are obtained. \( \tau \) is the time constant of the recovery curve, whereas \( t_0 \) is the critical time in which there is no recovery (the x-intercept). By performing the double flash ERG on T, 9T, 10T, and 910T animals, a smaller \( t_0 \) is observed in all the RGS9-1 overexpressing animals. The critical time in T is 91.2 ms and is decreased to 76 and 73 ms in 9T and 10T, respectively. The \( t_0 \) is further decreased to 55 ms in 910T, suggesting the additive effect of the RGS9-1 overexpression from both transgenes. The smaller the critical time, the faster the cone has recovered and stays in saturation for a shorter period of time. In addition, the presence of MCV7 transgene in 9T and 10T animals does not further decrease \( t_0 \).

**RGS9-1 overexpression causes faster cone recovery (performed by Dr. Lihui Cao)**

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Figure 2.7. Photopic double flash ERG to determine cone recovery. (A) To assess cone recovery with ERG, light-adapted animals are recorded with a double flash protocol. A test flash is applied and after a certain interstimulus interval (ISI), a probe flash is applied and the cone response is recorded. Subtracting the oscillating potential (OP) and normalizing the B wave response from each ISI to the maximum response, a recovery curve is constructed with the cone-derived ERG B wave. Fitting the curve with the single exponential equation, the critical time ($t_0$) is obtained. The $t_0$ is the time in which no recovery is observed. The shorter the $t_0$, the less time the cone stays in saturation, which indicates a faster recovery. Here a representative recovery fit is displayed for MCV9 and MCV10, which demonstrate faster recovery in both transgenic lines. (B) Representative response traces of different genotypes at different ISI. (C) Analysis on the double flash recording of different genotypes. In T cones, the $t_0$ is 91 ms. In 9T and 10T, the $t_0$ is 76 and 73 ms, respectively, which suggest a faster recovery in cones with RGS9-1 overexpression. Furthermore, 910T has a $t_0$ of 55 ms, suggesting a synergistic effect of MCV9 and MCV10 transgene. While the data suggest cone deactivation is the slowest step in cone recovery, photopic double flash is not a direct assessment of cone recovery.
Fit equation: \( f(x) = 1 - \exp((-x-t_0)/\tau) \)
Figure 2.7
Figure 2.7

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<th>MCV10T (n=2)</th>
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Cone-derived ERG b-wave response is not a direct measurement of cone photoresponse. Measuring cone ERG a-wave, which is a direct measure, is difficult and subject to great variability due to the rarity of cones in mouse retina. To accurately measure cone photoresponses, we have worked with Dr. King-Wai Yau’s lab at the Johns Hopkins University. Dr. Lihui Cao, who is a postdoctoral fellow in Dr. Yau’s lab, works closely with us and uses a cutting-edge retinal slice recording technique to record photoresponses from cones. In retinal slice recordings, a loose seal is first established by pulling in several nuclei, including the IS, into the recording pipette, as shown in Figure 2.8A. A background light strong enough to saturate rods but dim enough to leave a fraction of cones unstimulated is then turn on, followed by a flash of varying intensity to stimulate cone responses. Dr. Cao reported to us that response amplitude and recovery kinetics recorded this way varied greatly, presumably because of the health of the retinal slices and the effect of background illumination needed to saturate rods. We then decided to mate all of our animals into the GNAT1−/− animals, in which rod transducin gene is deactivated and hence rod response is genetically removed, to simplify this rather difficult and time-consuming recording procedure (Figure 2.8B). This improvement allows us to obtain reliable cone responses with light stimulus at 380 nm with intensity ranging from 1504, 3500, 6660, 22500, 70500, 140000, 340000, 850000 photons µm⁻². Plotting the response amplitude vs. flash intensity, the sensitivity, which is the intensity that produces a half-maximum response, is obtained (Figure 2.8C). Furthermore, two recovery time constants (τrec and τD) can be extracted by analyzing the recovery phase of the response waveforms in dim and bright flash. The dominant recovery time constant (τD) is determined by Pepperberg plot by fitting the time to 40% recovery vs. flash intensity with a linear function to obtain its slope (Figure 2.8D). For τrec, the falling phase of dim flash response is fitted with an exponential equation to obtain the time constant (Figure 2.8E).

When recording is performed on 910T cones to assess the effect of RGS9-1 overexpression on cone recovery kinetics, an accelerated recovery is easily seen as compared to T (Figure 2.9). The τD in 910T is 30.6 ms, compared to 64.3 ms in T cones (Figure 2.9C and D; Table 2.2). The smaller τD (faster recovery) in 910T confirms that the GTP-hydrolysis of cone transducin rate-limits the recovery of cone phototransduction. Similarly, τrec is decreased to 22.2 ms in 910T, compared to 37.0 ms in T cones (Figure 2.9E and F; Table 2.2). In addition to the recovery kinetics in T and 910T, the τD from R9AP+/− cones is determined to be 93 ms in the
Figure 2.8. Provided by Dr. Lihui Cao. Light response families and kinetic analysis of S-dominant mouse cones. (A) DIC picture of a retinal slice, suction pipette recordings were done from mouse cell bodies of the inner segment. (B) Light-response family of a representative transducin knockout (GNAT1⁻⁻) mouse cone photoreceptor. The responses are normalized as the gray line above which is not light sensitive and presumed to be a voltage-activated current. The responses are averaged from 5-40 traces. All the S-cone recordings are done under 380nm light, since the 360nm light is not strong enough to saturate it. (C) The amplitude vs. intensity has been fitted with an exponential saturation function to extract half-saturating flash intensity $\sigma$. In this case, it is 10500 photons $\mu$m⁻². (D) The “Pepperberg” or dominant recovery time constant (Td) was estimated by fitting with a straight light on the semi log plot to different recovery time (20%, 30% and 40%) of the saturating flashes. The traces for analysis should be normalized first with the maximum of the light response (gray line in left panels). (E) Recovery time constant of dim light was fitted with a first exponential function. (F) The recovery time constant of the saturated light can be fitted with second exponential function, which shows the first decay of 67.9 ms, and the second decay of 361ms.
Figure 2.8

A. 

B. 

C. 

D. 

E. 

F.
Figure 2.9. Faster recovery in cones with higher RGS9-1 overexpression. (A-F provided by Dr. Lihui Cao) Normalized response traces from a GNAT1−/− cone (A) and a 910T cone (B). Note the shorter recovery time in the 910T cone. The dominant recovery time constant (τD) is estimated by calculating the slope on the dependence of the time to reach 40% recovery across different light intensities between 910T and T cones in (C) and (D). In 910T (RGS+, GNAT1−/−), the smaller τD indicates faster recovery in cones with higher RGS9-1 overexpression. The dim flash recovery time constant (τrec) is obtained by fitting the dim flash responses with a single exponential function in (E) and compared between 910T and T (F). Similar to τD, the τrec is smaller in cones with RGS9-1 overexpression. Faster recovery in 910T confirms that cone deactivation rate-limits the recovery phase of cone phototransduction. (G) Plotting the τD from cones with titrating level of RGS9-1 level and fitting with a single exponential equation to obtain the upper limit of cone recovery. (H) In the attempt to generate cones with higher RGS9-1 concentration >2.7-fold, cone photoreceptor is observed to undergo early degeneration as RGS9-1 and PNA intensities disappear. Due to the degeneration, cones with higher RGS9-1 concentration cannot be established.
Figure 2.9

**A.** Gnat1\(^{-/-}\) vs Gnat1\(^{-/-}\) RGS9\(^{+}\) response to light intensity.

**B.** RGS9\(^{+}\) response to light intensity.

**C.** Time to 40% recovery as a function of light intensity.

**D.** 

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**E.** 

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<td>(\tau_{rec}) (ms)</td>
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Figure 2.9

G.

H.
presence of background light. The $\tau_D$ of 93 ms in R9AP$^{+/+}$ is probably influenced by light adaptation, which has been shown to speed up recovery in photoreceptors. To obtain a more accurate measure, we also mated R9AP$^{+/+}$ into the GNAT1$^{-/-}$ background and cone recordings in R9AP$^{+/+}$T (R9AP$^{+/+}$ in GNAT1$^{-/-}$ background) animal reveals a $\tau_D$ of 131 ms (data not shown). Using the quantification on flat-mount retina and values of $\tau_D$ in R9AP$^{+/+}$, T, and 910T, an exponential function can be fitted to our data set (Figure 2.9G) that reveals an asymptote $\tau_D$ value of ~25 ms when RGS9-1 is overexpressed in cones. To further prove that the acceleration of cone recovery can be pushed to reach the asymptote, we produce homozygous transgenic animals with two copies of MCV9 and/or MCV10 transgene. Recordings from the cones of these animals show highly variable response amplitudes or in some cases, no responses at all. IHC analysis on retinal sections of these animals then revealed that cones in the so-called 99T and 1010T mouse retinas degenerate as the number of PNA-positive cells are significantly decreased (Figure 2.9H). This data suggest that overexpression of RGS9-1 may cause cone degeneration, or that both transgenes were inserted into loci that contains genes essential for cone survival. Despite the obvious drawback, the acceleration in cone recovery observed in the 910T animals confirms our ERG analysis that GTP-hydrolysis of cone transducin rate-limits the recovery phase of cone phototransduction.

The generation and distribution of human GRK7-expressing cones in MCV7 and MCV8 transgenic mouse lines

To test the effect of increasing GRK level on the recovery of cone photoreceptor, human GRK7 expression is specifically expressed in mouse cones using the blue opsin and green opsin promoter (Figure 2.1B). Human GRK7 is used to increase the GRK activity in mouse cones instead of bovine GRK1 for the following reasons: (1) GRK7 is a superior kinase than GRK1 and (2) since GRK7 is not expressed in mouse, its transgenic expression can be easily identified without any endogenous protein influence. To confirm GRK7 expression in mouse cone photoreceptor, IHC staining is performed on the retinal section of MCV7 and MCV8 transgenic animals with the Y722NP antibody (the characterization of Y722NP antibody is described in Chapter 3). In both transgenic lines, GRK7 expression is specific to mouse cone photoreceptor and found in COS, as demonstrated by the presence of the kinase surrounded by the cone marker, PNA (Figure 2.10A). The GRK7 expression in MCV7 and MCV8 has distinct distribution
Figure 2.10. Human GRK7 (hGRK7) expression in mouse cone photoreceptors. (A) Confocal images of human GRK7 expression in MCV7−/− (MCV7 in GRK1−/− background) and MCV8−/− (MCV8 in GRK1−/− background) retina. In MCV7−/− and MCV8−/−, GRK7 expression (red) is restricted to cone photoreceptors as indicated with the overlap with PNA, a cone marker (green). Images were taken in both the superior and inferior regions of the retina. The inferior region of MCV7−/− retina contains more GRK7-expressing cones than the superior region, consistent with gradient expression of the S-cone previously reported in the mouse retina. The ratio of hGRK7-positive cells to PNA is nearly 1:1 in the inferior region of MCV7−/− animals. In the cones of MCV8−/−, hGRK7 expression is distributed near uniformity across the retina as confirmed by PNA. The expression pattern in MCV8 may reflect the strength and distribution of the green opsin promoter in the mouse retina or the high copy number of the transgene. (B) The transgenic hGRK7 expression in the cones of MCV7 and MCV8 are quantified and normalized against PNA. In the superior region, hGRK7 is expressed at 35±7% and 88±4% in MCV7 and MCV8, respectively, while in the inferior region, it is expressed at 96±2% and 88±2% in MCV7 and MCV8, respectively. Student t-test was used to determine the significant in the expression differences.
Figure 2.10

A. Merge

GRK7, Inferior

GRK7

PNA

MCV7, Superior

MCV8, Inferior

MCV8, Superior

MCV7, Inferior

GRK7

PNA

Merge
**Table 2.10**

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<td>0.35 ± 0.07 (3)</td>
<td>0.96 ± 0.02 (3)</td>
</tr>
<tr>
<td>Inferior</td>
<td>0.88 ± 0.04 (3)</td>
<td>0.88 ± 0.02 (3)</td>
</tr>
</tbody>
</table>

*Figure 2.10*
profile. Based on the ratio of GRK7 to PNA, GRK7 expression in MCV7 is present in 96% of the cones in the inferior hemisphere and in 35% of the cones in the superior hemisphere (Figure 2.10B). In contrast, GRK7 expression in MCV8 is distributed homogeneously in both the superior and inferior hemispheres at 88%. With MCV7 and MCV8 transgenic mice, the GRK activity in mouse cone photoreceptor can be increased.

**Exploring the potential of cone pigment deactivation mediated by GRKs as the second rate-limiting step in cone recovery**

With the 910T animals, we have shown that RGS9-1 overexpression can accelerate the recovery of cones. This soundly establishes that cone-transducin turn-off rate-limits cone recovery. As a complementary approach and a control to this finding, we also sought to test the effect of overexpressing GRKs in mouse cone recovery by expressing human GRK7 in cones of MCV7 and MCV8 transgenic animals (Figure 2.10). Initial characterization of MCV7 and MCV8 transgenic animals is described in Chapter 3. For cone recovery, MCV7 is first mated into GNAT1−/− to generate 7T and to assess its cone recovery. The $\tau_D$ and $\tau_{rec}$ are 62.1 and 45.8 ms in the 7T cones (Figure 2.11 and Table 2.2), which is similar to the 64.3 and 37.0 ms obtained for cones of the transducin knockout animals (Figure 2.9). The lack of recovery acceleration between the two types of cone suggests that cone opsin phosphorylation may not be the rate-limiting step of mouse cone recovery.

We then sought to determine whether opsin deactivation might be the second slowest step in cone recovery using the 7T and 8T animals. In a condition when RLS is accelerated enough that it is no longer the slowest step, the second slowest step may determine the overall rate of cone recovery. Currently, we have not discerned whether the level of RGS9-1 overexpression in 910T cones has been elevated enough to allow us to study the second slowest step. As mentioned earlier, further attempts in increasing RGS9-1 level beyond 910T result in cone degeneration (Figure 2.9H). Also, based on the fit in Figure 2.9G, the recovery in 910T has not reached the asymptotic value of 25.1 ms. To test whether opsin deactivation becomes rate-limiting in 910T cones, the recovery time constants between 7910T and 910T cone responses are compared. The $\tau_D$ and $\tau_{rec}$ are 33.0 and 25.4 ms in 7910T cones, respectively, and they are not significantly different from the 30.6 and 22.2 ms of 910T cones (Table 2.2). We conclude that opsin phosphorylation by GRKs may not accelerate cone recovery in 910T. Since it cannot be determined whether the recovery in 910T has reached the rate of the
second RLS, the termination of cone opsin as the second slowest step in cone recovery remains a possibility.
Figure 2.11. Mouse cone recovery is unaffected with increasing GRKs concentration. (Provided by Dr. Lihui Cao). Representative response traces of single cone recording from T (A) and 7T (B). Recovery time constants, $\tau_D$ (C) and $\tau_{\text{rec}}$ (D), are compared between T and 7T. No significant changes are detected between WT cones and GRK7-expressing cones, suggesting that cone opsin phosphorylation does not rate-limit cone recovery.
Figure 2.11

A. hGRK7+ Gnat1−

B. Gnat1−

C. Mode: 1st On

D. Mode: 1st On

\[ \tau_{m} = 53.0 \text{ ms} \]

\[ \tau_{r} = 50.1 \text{ ms} \]
Table 2.2. Provided by Dr. Lihui Cao. Physiological properties of cones of GNAT1<sup>−/−</sup>, and MCV-9T mice. Column 1 gives the genotypes of the mice and numbers of cells from which recordings were made. Column 2-7 present parameters of these two genotyping cones: \( R_{\text{max}} \), the saturating amplitude of light response; \( t_{\text{peak}} \), the time to peak of the dim-flash response; \( t_{\text{int}} \), integration time; \( \tau_D \), the dominant recovery time constant; \( A \), the amplification constant; \( Q_{1/2} \), the half saturating flash intensity; \( S_f \), the sensitivity of the normalized dim-flash response, specified as percent of the saturating response per (photon \( \mu m \))<sup>2</sup>). The sensitivity was measured with 360 nm flashes. An outer segment volume of 14 \( \mu m^3 \) and collecting area of 0.2 \( \mu m^2 \) at 360 nm were assumed for these S-dominant cones to estimate the amplification constant. Student t-test were performed to test the difference between genotypes in each parameter: significant difference exists between \( \tau_D \), but there is no difference among genotypes in other parameters.
Table 1.2
Physiological properties of cones of different mouse lines in GNAT1<sup>-/-</sup> background

<table>
<thead>
<tr>
<th>Mice #</th>
<th>Genotype</th>
<th>$R_{max}$</th>
<th>$t_{peak}$</th>
<th>$t_{int}$</th>
<th>$S_F$</th>
<th>$\tau_D$</th>
<th>$\tau_{rec}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (n=24)</td>
<td>Gnat&lt;sup&gt;/-&lt;/sup&gt;</td>
<td>8.3 ± 0.6</td>
<td>83.9 ± 2.5</td>
<td>78.9 ± 3.1</td>
<td>3.1E-4 ± 3.2E-5</td>
<td>64.3 ± 3.0</td>
<td>37.0 ± 2.6</td>
</tr>
<tr>
<td>7T(n=18)</td>
<td>GRK7&lt;sup&gt;+&lt;/sup&gt;/Gnat&lt;sup&gt;/-&lt;/sup&gt;</td>
<td>8.7 ± 0.8</td>
<td>87.8 ± 5.5</td>
<td>76.2 ± 4.5</td>
<td>3.4E-4 ± 4.0E-5</td>
<td>62.1 ± 4.7</td>
<td>45.8 ± 6.7</td>
</tr>
<tr>
<td>910T (n=13)</td>
<td>RGS9&lt;sup&gt;+&lt;/sup&gt;/Gnat&lt;sup&gt;/-&lt;/sup&gt;</td>
<td>6.5 ± 0.4</td>
<td>78.4 ± 2.8</td>
<td>61.9 ± 3.0 (*)</td>
<td>9.7E-5 ± 9.7E-6 (*)</td>
<td>30.6 ± 2.0 (*)</td>
<td>22.2 ± 3.2 (*)</td>
</tr>
<tr>
<td>7910T (n=11)</td>
<td>RGS9&lt;sup&gt;+&lt;/sup&gt;/GRK7&lt;sup&gt;+&lt;/sup&gt;/Gnat&lt;sup&gt;/-&lt;/sup&gt;</td>
<td>6.7 ± 0.6</td>
<td>87.8 ± 5.3</td>
<td>67.1 ± 2.3 (*)</td>
<td>1.1E-4 ± 1.9E-5 (*)</td>
<td>33.0 ± 2.3 (*)</td>
<td>25.4 ± 1.1 (*)</td>
</tr>
</tbody>
</table>
Discussion

The blue and green opsin promoters are used in this study to overexpress RGS9-1 and GRK7 in the two types of mouse cones. RGS9-1 overexpression can be established by overexpressing R9AP, indicating that similar to rod, R9AP concentration controls the level of the GAP complex in cone. On the other hand, GRK7 is used to increase the overall GRK activity in cones because it is a superior kinase in-vitro. The fact that mouse as a species lacking a GRK7 gene facilitates the detection of the transgenic GRK7 expression in our mouse lines. Based on the design, MCV7 and MCV9 should drive GRK7 and RGS9-1 expression in mouse S cones with the blue opsin promoter, whereas MCV8 and MCV10 should drive GRK7 and RGS9-1 expression in mouse M cones with the green opsin promoter. The S and M cones make up the 3% of photoreceptor in mouse retina. Due to the rarity of cones, IHC on retinal cross section is used to determine the overexpression of RGS9-1 and the expression of GRK7 in the transgenic cones. The expression pattern of transgenic RGS9-1 and GRK7 is dependent on the type of the cone opsin promoter because S and M cones are found to have distinct localization in mouse retina. Using the blue opsin promoter, the overexpression of RGS91 and GRK7 expression in S cone are concentrated in the inferior hemisphere and decreased towards the superior hemisphere. In contrast, the protein expression in M cones is distributed across the superior and inferior hemisphere with slightly higher level in the superior cones. The S cones in the inferior region contain both blue and green opsin promoter activity, whereas the ones in the superior region contain mostly green opsin promoter with fewer blue opsin promoter activity. Using the blue and green cone opsin promoters, the overexpression of the GAP complex and GRKs is established in mouse cone photoreceptors.

As mentioned, the low number of cones makes it difficult to quantify the level of RGS9-1 overexpression in MCV9 and MCV10 compared to WT cones. However, since the overexpression of RGS9-1 is detectable on retinal sections, the immunofluorescence signal of the cone RGS9-1 is normalized to rod level and quantified accordingly. Furthermore, the same RGS9-1 quantification is carried out on flat mount retina. In WT, the RGS9-1 expression in cone is 1.8 and 1.4 fold higher than rod on retinal section and flat-mount retina, respectively. The quantification is slightly variable, which can be attributed to the penetration capacity of the CT318 antibody between cross section and flat-mount retina. Averaging the two quantifications, the WT cone RGS9-1 is 1.6-fold higher than rod, which is significantly lower than the estimated
10-fold increase in RGS9-1 in chipmunk with a cone-dominant retina. This difference in the quantification of RGS9-1 level in cone and rod can be partially attributed to species variation. Revisiting the study by Cowan et al., the RGS9-1 intensity in chipmunk and mouse retina is not the same between rod and cone, suggesting that the fold of RGS9-1 overexpression in chipmunk may not reflect the same overexpression in mouse retina (Cowan, Fariss et al. 1998). In addition, the determination in chipmunk may be an overestimation. Only 75% of the photoreceptors in chipmunk are cones, suggesting that the RGS9-1 quantified on Western blot has significant contribution from chipmunk rods. On the other hand, there are many known caveats on using immunofluorescence to quantify protein expression. Among them, it is possible that the staining may not be within linear range, which may cause the cone signal to saturate and underestimate the cone RGS9-1 quantification. While the linear range of CT318 (anti-RGS9-1 antibody) on IHC is not established in this study, immunostaining and image acquisition are performed simultaneously to minimize variations between experimental conditions. The higher level of RGS9-1 in MCV9 and MCV10 suggests that the condition used in IHC is not saturated, but it does not indicate that the quantification is performed in the linear range. Therefore, we settle for a RGS9-1 level in wild-type mouse cones as 1.6-fold higher than rod, which is much lower than the conventional wisdom.

While quantification by IHC has its own bias and we cannot really conclude with certainty beyond having made mouse lines with overexpressed RGS9-1 in cones, alternative means are deemed necessary if we are to obtain the actual level of overexpression in these mouse cones. We have finally found two alternative ways to achieve this quantification. First, using the ‘cone-like’ retina of the Nrl+/− mice, the RGS9-1 overexpression in the MCV9 and MCV10 transgenes is confirmed and quantified to be 3 and 4.2 fold. The RGS9-1 signals are derived purely from the ‘cone-like’ photoreceptor and there is no rod signal contamination in Nrl+/− background. The fold of transgene overexpression in Nrl+/− background is two times higher than the immunofluorescence quantification. However, we have less confidence in this method because in Nrl+/− background, these ‘cone-like’ photoreceptors adopt an enhanced S cone identity and hence the quantification may not correlate with the overexpression in WT background. Furthermore, the slow degeneration in Nrl+/− may reduce the level of RGS9-1 depending on mouse age when the determination is made. Second, removing the endogenous RGS9-1 with R9AP+/− or the RGS9-1 in rods using the rod-specific Cre-recombinase to deleted the rod GAP
complex (iCre75, Gβ5Flox/Flox), the RGS9-1 expression in cone photoreceptor can be determined and quantified with immunoblot. Surprisingly, the RGS9-1 expression from MCV10 transgene is 5.5 fold higher than WT cone, about two times higher than the immunofluorescence quantification. Further experimentation is required to ensure that no leaky transgene expression is presented in the rods of MCV10 animals. The quantification in R9AP+/− and iCre75/Gβ5F/F animals seems to be a superior method than using Nrl+/− animals because morphology of R9AP+/− and iCre75/Gβ5F/F cones resemble more closely to that of WT cones. One consistent finding using Western blot to quantify the RGS9-1 level in MCV9 and MCV10 is that the fold of overexpression is two times higher than using immunofluorescence. It is possible that the immunofluorescence quantification is off by a factor of 2. Whether protein quantification using immunofluorescence is lowered by a factor of two may be assessed with further controls by comparing rod with known RGS9-1 overexpression. Since the quantification in R9AP+/− and iCre75/Gβ5F/F is still ongoing, in this thesis, the immunofluorescence data is used to correlate the RGS9-1 overexpression and its effects on cone recovery.

Our experimental design allows us to combine MCV9 and MCV10 transgenic lines together in 910T to achieve a higher level of RGS9-1 overexpression in cones, which is determined to be 3- and 2.7-folds base on quantification on retinal section and flat mount retina, respectively. Functionally, cones with higher RGS9-1 concentration have faster recovery compared to WT cones. The MCV9 and MCV10 animals are crossed into GNAT1−/− background to eliminate rod phototransduction. The identification of cone responses is facilitated in the GNAT1−/− background. More importantly, no background light is needed to pre-saturate rod responses before recording cone, thus preventing complications associated with light-induced recovery acceleration. The recovery of cone photoreceptor is assessed with ERG and single cell recording, the former is non-invasive but indirect and the latter is direct but invasive. With double flash ERG, the cone-derived B wave response is used to assess cone recovery. Fitting the normalized recovery curve with a single exponential equation, the critical time (t₀), the time in which cone responses remain unrecovered, can be determined. Smaller t₀ suggests faster recovery. Compared to WT, cone recovery in 9T and 10T are equally accelerated and further speed up in 910T cones, suggesting the additive effect of RGS9-1 overexpression in 910T to further increase cone transducin deactivation. The acceleration observed in cones with higher RGS9-1 concentration lends strong support to the notion that GTP-hydrolysis of cone transducin
may be the rate-limiting step in cone recovery. While double flash ERG recording confirms the faster recovery in cones with RGS9-1 overexpression, the smaller $t_0$ derived from double flash ERG is not a direct measurement of cone recovery since the recovery curve is generated from the cone-derived bipolar cell response. To directly measure the recovery of cone photoreceptor, suction recordings are performed on isolated retinas from T, 9T, 10T and 910T animals. Cone recordings demonstrated WT cones recovery is accelerated in 910T with 2.7-fold RGS9-1 overexpression. The recovery time constants in dim flash and bright flash responses are both sped up to 31 and 22 ms, respectively, in cones with higher GAP concentration, confirming that cone transducin deactivation dictates the recovery phase in cone phototransduction in all ranges of light intensities. While 2.7-fold increase in RGS9-1 concentration can speed up cone recovery, it is unknown whether the $\tau_D$ of 31 ms in 910T cones reflects the boundary in which the cone transducin deactivation is sped up enough that the second cone RLS becomes the slowest step. Fitting the dependence of cone recovery time constant to the fold of RGS9-1 concentration, we obtain a theoretical upper limit in cone recovery as 25.1 ms. If the upper limit of cone recovery has been reached, then increasing RGS9-1 concentration beyond 910T shall have little or no influence on the $\tau_D$ of cone recovery. However, the attempts to further increase RGS9-1 level by generating multiple copies of transgene (99T, 1010T, 9910T, 91010T, and 991010T) result in an unexpected and disappointing cone degeneration phenotype, which in most cases can be seen as early as one month of age. This degeneration suggests that by increasing the deactivation of cone transducin, an unknown mechanism is triggered that leads to cone apoptosis. It is possible that the degeneration is mediated through the increase of R9AP, Gβ5-L, RGS9-1, or any combination of these proteins, which pinpoints to an unknown role of the GAP complex in maintaining photoreceptor health. It is difficult to discern which GAP component is the trigger of cell degeneration. Also, it is unknown whether this cone degeneration is light dependent and further studies are required to determine if dark adaptation or removing cone phototransduction can prevent cone death. This is not the first report in which photoreceptor degeneration is observed in genetically modified photoreceptors. In mice without GRK1 and/or arrestin, photoreceptors degenerate due to prolonged rhodopsin activation. Also, rods with overexpressed GRK1 degenerates in the presence of bright light, suggesting that early recovery may lead to photoreceptor degeneration (However, a BAC construct was used to overexpress GRK1 and may cause side effects depending on the integration site and the content of the construct). While it is
evident that higher RGS9-1 (>3-fold) expression causes cone degeneration, the mechanism by which apoptosis is triggered in cone photoreceptor requires further investigation.

As cone recovery is rate-limited by the deactivation of cone transducin, cone opsin phosphorylation by GRKs must occur faster than the GTP-hydrolysis of cone transducin. Therefore, increasing GRK activity should not affect the recovery time constants in cone phototransduction. In this study, GRK7 is used to increase the opsin phosphorylation. However, since GRK7 is not present in mouse genome, it is essential to confirm that GRK7 is indeed functional in mouse cones, which will be described in detail in the following chapter. Suffice it to say that GRK7 is confirmed to be functional in mouse cone photoreceptors; the effect of increasing GRKs on cone recovery can be assessed. Single cell recording on 7T cones shows those cones with GRK7 expression has similar $\tau_D$ and $\tau_{rec}$ compared to T cones. The generation of the 8T animals is underway and will be characterized in the similar manner. The recovery in 8T cones is expected to be similar to 7T and T cones. The similar recovery observed between GRK7-expressing cones and WT cones demonstrates that cone opsin deactivation is not the RLS in cone recovery. Possibilities remain that the cones recorded in 7T animals may not contain GRK7 expression since the kinase expression is concentrated in the inferior hemisphere in MCV7. To minimize recording from non-GRK7 expressing 7T cones, cones are selected from the inferior region. Cone recording from 8T or 78T can further solidify the point that cone opsin phosphorylation by GRKs does not rate-limit the recovery of cone phototransduction.

As cone opsin phosphorylation by GRKs is not the slowest step in cone recovery, however, it may be the second slowest step. To test this, the recovery time constants of 910T and 7910T cones are compared. If cone transducin deactivation is sped up enough that the second RLS becomes the slowest step, then increasing the enzyme level for the second RLS can further accelerate cone recovery. Therefore, if 910T cone with 2.7-fold of RGS9-1 overexpression and a recovery time of 31 ms is the fastest that the first RLS can be sped up, then the second RLS can be studied. By overexpressing GRKs in 910T cones (7910T), cone opsin deactivation is increased under the condition in which cone transducin turnoff is accelerated. In 7910T cones, the $\tau_D$ and $\tau_{rec}$ is similar to 910T, suggesting that cone opsin phosphorylation is not the second slowest step in cone recovery. However, this interpretation has a few caveats. It is not established that the deactivation of cone transducin in 910T cones has sped up to become faster than the
second RLS. Therefore, the lack of recovery acceleration in 7910T may be because the GTP-hydrolysis of cone transducin is still the slowest step in recovery in 910T cones. Due to the cone degeneration induced by early recovery, it may be difficult to assess the second RLS in cone recovery until further manipulations can prevent the apoptosis in cone photoreceptors.
Conclusion

It has been determined that transducin deactivation rate-limits the recovery of mouse rod phototransduction because by increasing the concentration of the GAP complex, rod recovery can be accelerated from ~200 ms to as fast as 54 ms. Rod and cone share some, while possessing distinct cell-specific phototransduction components, it is unknown whether the identity of RLS in rod recovery also applies to cone. Higher level of the GAP complex in cones of many species should conceivably contribute to a faster recovery time course and it has the potential to alter the identity of the RLS in cone recovery. However, an additional kinase, GRK7, is also co-expressed with GRK1 in the cones of certain species, which increases the kinase activity in cone photoreceptors. Such a higher kinase level may also promote a faster opsin phosphorylation and may contribute to accelerate recovery in cone phototransduction. Altogether, it is not an easy task to predict which might be the actual slowest step in cone photoreceptor recovery. The success of using a GOF approach by either overexpressing RGS9-1 or GRKs to reveal the RLS of cone phototransduction recovery has been adopted here and unequivocally identified that cone transducin deactivation is indeed the slowest step during the recovery of cone phototransduction.
CHAPTER 3
The Role of GRKs in Oguchi Disease

Introduction

The activity of G-protein-coupled-Receptor Kinase 1 (GRK1) was first described in the light-dependent phosphorylation of rhodopsin in the outer segment (OS) of rod photoreceptors (Bownds, Dawes et al. 1972; Kuhn and Dreyer 1972; Frank, Cavanagh et al. 1973). As GRK1 was unstable and sensitive to biochemical manipulations, isolating it in sufficient quantity and good quality was difficult despite attempts from many labs over a period of 30 years (Maeda, Imanishi et al. 2003). The GRK1 gene was cloned in the early 90s (Lorenz, Inglese et al. 1991; Zhao, Haeseleer et al. 1997), and since then many important findings have elucidated GRK1’s roles in phototransduction recovery, light and dark adaptation, and in stationary night blindness of human Oguchi disease. GRK1 is post-translationally modified by isoprenylation and phosphorylation. Furthermore, other signaling molecules, such as recoverin and protein kinase A, also regulate GRK1 function. Many data have been collected to show that GRK1 is essential for the deactivation of photoactivated rhodopsin and timely termination of rod and cone photoresponses. However, the intermolecular interactions between GRK1 and visual pigments were not clear until recently when the GRK1 crystal structure was solved and provided further insight. We summarize here recent discoveries on GRK1 and its regulation and function in retinal photoreceptors. Questions that need further attentions will also be discussed.

G-protein-coupled receptors (GPCR) with heptahelical transmembrane domains are a large group of cell surface proteins enabling a cell to sense its environment by coupling to heterotrimeric G-proteins. A wide range of physiological processes such as senses, hormonal actions, and neurotransmission employ GPCRs. The termination of GPCR signaling requires a protein family known as G-protein-coupled-receptor kinases (GRKs), which phosphorylate activated GPCR and are responsible for homologous desensitization. GRKs belong to the family of Serine-Threonine protein kinases and phosphorylate C-terminal serine and threonine residues located at C-terminal tails of activated GPCRs. Following receptor phosphorylation, the interaction with G-protein is weakened, and a subsequent binding of arrestin to phosphorylated receptors completely prevents them from coupling to G-proteins. In most non-photoreceptor cells, arrestin binding initiates receptor endocytosis to prolong and enhance desensitization (Dorn
Controlling GPCR activity by this simple and elegant deactivation mechanism is important for many cellular responses, as loss-of-function mutations in GRKs have lead to a variety of pathologic conditions.

There are seven members in the GRK family that can be further subdivided into three groups: GRK1-like, GRK2-like, and GRK4-like. GRK1-like group consists of GRK1 and GRK7, which are expressed specifically in retinal photoreceptors. GRK2-like group contains GRK2 and GRK3, which are ubiquitously expressed. GRK4-like group is comprised of GRK4, GRK5 and GRK6. GRK4 expression is restricted to kidney, cerebellum, and testis, while GRK5 and GRK6 are expressed ubiquitously. All GRKs share a central kinase domain of approximately 270 amino acids, typical to that of other Ser/Thr kinases. The N-terminus of GRKs contains an RGS Homology (RH) domain, which is found in Regulators of G-protein Signaling (RGS) proteins (Ribas, Penela et al. 2007; Dorn 2009). The RH domain of GRK1 and GRK2 has ~20-25% homology to that of the R7 subgroup of RGS proteins (data not shown). GRK2 has been shown to interact with G\textsubscript{aq} family through its RH domain, but with low GTPase accelerating protein (GAP) activity (Ribas, Penela et al. 2007). The physiological function of GRK1 RH domain remains unclear. The C-terminal regions of GRKs are most dissimilar with varying sizes and different protein motifs that contribute to the diversity of GRKs. GRK2 is critical in cardiac physiology, as its inactivation leads to cardiac abnormality and embryonic lethality in mice (Dorn 2009). GRK1 and GRK2 are the most extensively studied GRK members. GRK1 is required for visual pigment deactivation in rod and cone (Chen, Burns et al. 1999; Lyubarsky, Chen et al. 2000). The specific expression of GRK1 in retinal photoreceptors provides an opportunity to study GRK’s function under a physiological context and has been useful in understanding the general actions of other GRKs in eukaryotic cells.

GRK7, a close relative of GRK1, was cloned from mammalian species (Weiss, Raman et al. 1998) and quite interestingly, the expression patterns of GRK1 and GRK7 vary from species to species. GRK1 is expressed in both rod and cone photoreceptors, while GRK7 is expressed only in cones and not in rods of human (Chen, Zhang et al. 2001; Weiss, Ducchesshi et al. 2001). In species such as pigs and dogs, their cones only express GRK7. In rodents, GRK7 is absent and both rods and cones rely on GRK1 for receptor deactivation. Studying the differences between
GRK1 and GRK7 has provided insights into the structure/function relationship of GRKs and the pathological mechanism of Oguchi Disease.

**Role of GRK1 in phototransduction, Oguchi Disease, and light-dependent degeneration**

Photoreceptors without GRK1 take a very long time to dark adapt. In GRK1−/− photoreceptors, receptor deactivation is so slow that it artificially becomes the slowest step during the recovery phase of rod and cone phototransduction (Chen, Burns et al. 1999; Lyubarsky, Chen et al. 2000). Inactivating one copy of GRK1 also causes a noticeable delay in rod recovery, suggesting that GRK1 level may be critical in setting the duration of rod’s photoresponses. However, while the loss-of-function approach clearly demonstrates the importance of GRK1 in rod recovery, it does not have the power to reveal whether R* phosphorylation dominates the recovery time course in normal rods. To determine whether a reaction is rate-limiting in a cascade of events, a gain-of-function approach such as increasing the concentration of the enzyme that catalyzes the slowest step is more appropriate (Nikonov, Engheta et al. 1998; Kennedy, Sowa et al. 2003). If rhodopsin deactivation rate-limits rod recovery, enhancing rhodopsin phosphorylation by overexpressing GRK1 should accelerate rod recovery. In Krispel et al., several transgenic lines with varying levels (up to four fold) of GRK1 overexpression were made but the rods showed no statistically significant changes in the activation and deactivation phases to WT rods. These negative results suggest that rhodopsin deactivation is not the normal rate-limiting step in rod recovery. In fact, using a similar gain-of-function approach where the ternary GAP complex of R9AP/Gβ5-L/RGS9-1 is overexpressed in rods, the recovery can be sped up. This indicates that transducin deactivation is the overall rate-determining step in rod recovery (Krispel, Chen et al. 2006). In rods with four-fold increase of RGS9-1 concentration, the recovery time constants (τD and τrec) are decreased to 70 ms compared to 250 ms in WT rods. Recently, Chen et al. observed a recovery time constant of 54 ms in rods with a six-fold increase of RGS9-1 overexpression, again significantly different from the 185 ms found for WT controls (Chen, Woodruff et al. 2010). With transducin turn-off as the slowest step, is rhodopsin deactivation the second slowest step in rod recovery? The criterion to reveal the second rate-limiting step is to sufficiently speed up transducin GTP-hydrolysis until the second step becomes the slowest step. Is the six-fold higher RGS9-1 overexpression enough to speed rod recovery to a point that the overall rate is now determined by the second slowest
step? If yes, the 54 ms time constant reflects how fast GRK1 can work on R*. If not, one has to assume that GRK1 works even faster. As a comparison, the recovery time constant of GRK1−/− rods is between 4,000 to 5,000 msec. Using GRK+/− or reconstitution GRK1−/− rods with lower amount of GRK1 (Chen, Woodruff et al. 2010), rod recovery can be modulated to be faster than GRK−/− but slower than WT rods. In a study by Doan et al., the recovery time of single photon response (SPR) in GRK+/− is twice that of WT rods. Similarly, the recovery time constants are increased (slower recovery) in rods with lower amounts of GRK1 in both dim and bright flash conditions. By reducing the level of the kinase, rhodopsin deactivation becomes slower than transducin deactivation during the recovery phase. In the condition where rhodopsin deactivation becomes rate-limiting, any manipulations that regulate rhodopsin lifetime may now be studied with single rod recordings. Recent demonstrations of studying SPR variability in GRK1+/− /Arrestin1+/− rods and recoverin modulating rhodopsin lifetime during light-adaptation are examples of using rods with lower GRK1 level to study rhodopsin deactivation (Doan, Azevedo et al. 2009; Chen, Woodruff et al. 2010).

Proper shutoff of rhodopsin requires phosphorylation by GRK1, followed by arrestin binding. Recent study has proposed and demonstrated that the relative concentration between GRK1 and arrestin controls the kinetics of SPR by modulating rhodopsin lifetime (Doan, Azevedo et al. 2009). It has been shown that arrestin has a low-affinity towards unphosphorylated rhodopsin and may compete with GRK1 in rhodopsin binding during recovery. To demonstrate the competition, SPRs are recorded from GRK1+/− and GRK1+/− /Arrestin−+/− rods, in which the level of GRK1 and arrestin is decreased to below 50% of WT level. In rods with reduced GRK1, the recovery of the SPR is twice that of WT. When arrestin level is decreased in GRK+/− rods (GRK1+/−/Arrestin−/+), the recovery time is restored to WT level. These results suggest that decreasing arrestin favors GRK1 binding and its activity on R*. The competition between GRK1 and arrestin influences the time course of rhodopsin deactivation and controls the variability of SPR. It is interesting to note that in this study (Doan, Azevedo et al. 2009), rhodopsin deactivation time course is longer, whereas in other reports (Krispel, Chen et al. 2006; Chen, Woodruff et al. 2010; Gross and Burns 2010) transducin turn-off is the slower reaction. It was speculated by Doan et al. that the differences between the two results can be attributed to the differences in Ames and Locke’s solutions. However, Gross et al. demonstrated that the acceleration in transducin deactivation through RGS9-1 overexpression
can be recapitulated in both Ames and Locke’s solutions. The reason for this puzzling discrepancy remains unclear.

Photoreceptor is susceptible to damage and degenerates in the presence of light delivered at high intensity or for a prolonged period (Hao, Wenzel et al. 2002). Genetic manipulation in mouse in recent years has identified several proteins that protect photoreceptors from light damage. One of them is GRK1. In the first report of the GRK1−/− mouse, outer segment defects and photoreceptor degeneration occur under regular vivarium lighting conditions and can be prevented by rearing them in total darkness (Chen, Burns et al. 1999). Photoreceptor lacking arrestin also displays light-induced OS defects and degeneration. In the double knockout (GRK1−/−/Arrestin−/−) mouse, photoreceptor becomes extremely sensitive to light damage, which occurs at exposures as short as one minute (Choi, Hao et al. 2001; Hao, Wenzel et al. 2002; Krishnan, Lee et al. 2008). The susceptibility to light damage in GRK1−/−, Arrestin−/− and double knockout photoreceptors presumably arise from the prolonged activation of rhodopsin. The degeneration in these animal models has been shown to activate both transducin-dependent and transducin-independent pathways to cause cell death (Hao, Wenzel et al. 2002; Fan, Sakurai et al. 2010). In the presence of bright light, photoreceptor without transducin still degenerates in a pathway that signals through activated rhodopsin. In contrast, the degeneration seen in GRK1−/− and Arrestin−/− photoreceptors under dim light condition is mediated by constant transducin activation. By mating these animals with GNAT1−/−, the degeneration under dim light can be prevented (Hao, Wenzel et al. 2002). These evidences showcase the protective properties of GRK1 and arrestin in rods against light-damage by timely rhodopsin deactivation.

Since the loss of GRK1 leads to prolonged rhodopsin lifetime and cell death, can increasing GRK1 protect photoreceptor against degeneration? In the recent study by Whitcomb et al., BAC transgenic technique was used to overexpress GRK1 in rod photoreceptor to approximately three fold (GRK1+). Correspondingly, rhodopsin phosphorylation is also increased in GRK1+ rods. Retinal morphology in GRK1+ animals is similar to WT under regular illumination. However, when the animals are exposed to intense light (10,000 lux) for 12 hours, GRK1+ photoreceptors display faster degeneration (Whitcomb, Sakurai et al. 2010). These data are contrary to what one may expect because GRK1 overexpression does not provide protection against light-induced degeneration, instead, hyper-phosphorylation of rhodopsin may accelerate
an unknown signaling event that leads to cell death. GRK1 concentration apparently needs to be maintained optimally within photoreceptors as an increase or loss of GRK1 can accelerate light-induced photoreceptor degeneration. While retinal degeneration has been described in many mouse strains in which they become useful models of human macular degeneration and retinitis pigmentosa, the cellular mechanisms that trigger apoptosis have been less well understood. Future studies are needed to determine the signaling pathway(s) leading from rhodopsin light absorption to photoreceptor cell death to shed light into these dark and debilitating human conditions.

It is evident from animal studies that the loss of GRK1 results in severe defects in photoreceptor function. Similarly, mutations in GRK1 and arrestin genes have been found in human patients suffering from Oguchi Disease, an autosomal recessive congenital stationary night blindness (CSNB). Oguchi Disease was first described in 1907 and is characterized with a unique fundus appearance of golden-grayish discoloration (Carr and Gouras 1965), which disappears after dark-adaptation for more than three hours. This fundus characteristic is termed Mizuo Phenomenon. It is observable under optical coherence tomography (OCT) that the distribution of the fundus discoloration coincides with the accumulation of activated rhodopsin at the shortened outer segment layer (Hashimoto and Kishi 2009). Color vision in Oguchi patients is typically normal, which indicates that cone function is unaffected. ERG analyses of Oguchi patients demonstrate severe delay in scotopic recovery and elevated rod thresholds, while their photopic responses are by and large unaffected (Carr and Gouras 1965; Cideciyan, Zhao et al. 1998; Zhang, Zulfiqar et al. 2005). Various GRK1 mutations have been identified and are listed in Table 3.1. Ectopically expressing GRK1 with Oguchi mutations (V380D, Ser536 (4-bp del), and exon 5 deletion) in COS7 cells has demonstrated a reduced ability to phosphorylate R* (Cideciyan, Zhao et al. 1998; Khani, Nielsen et al. 1998). However, these mutations may also destabilize GRK1 in photoreceptors and this remains to be tested. As losing kinase activity or protein expression lead to defective rhodopsin phosphorylation and a prolonged dark adaptation time in Oguchi disease patients, one interesting discrepancy is worth noting here. Mouse without GRK1 has severe recovery defects in both cone and rod, while cones of most Oguchi disease patients appear spared. This is because of the expression of GRK7 (Maeda, Imanishi et al. 2003) in human cones. It is also interesting to note that cone ERG responses are diminished in patients with P391H GRK1 mutation, suggesting that this particular mutation may have a dominant effect.
**Table 3.1.** GRK1 mutations in Oguchi Disease reported to date. The list of GRK1 mutations that have been identified clinically in patients with Oguchi Disease.
### Table 3.1: GRK1 mutations in Oguchi Disease reported to date

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Protein</th>
<th>Cone/Rod function</th>
<th>Gender/Age</th>
<th>Family History</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family 61029 (#11)</td>
<td>c.827+623_883del</td>
<td>Partial Exon 3 deletion (1.1 kb)</td>
<td>Normal/delay</td>
<td>male, 19</td>
<td>Ashkenazi Jewish</td>
<td>(Cideciyan, Zhao et al. 1998)</td>
</tr>
<tr>
<td>Family 61029 (#13)</td>
<td>c.827+623_883del</td>
<td>Partial Exon 3 deletion (1.1 kb)</td>
<td>Normal/delay</td>
<td>female, 16</td>
<td>Pakistan</td>
<td>(Zhang, Zulfiqar et al. 2005)</td>
</tr>
<tr>
<td>Family 61029 (#15)</td>
<td>c.827+623_883del</td>
<td>Partial Exon 3 deletion (1.1 kb)</td>
<td>Normal/delay</td>
<td>female, 13</td>
<td>Pakistan</td>
<td></td>
</tr>
<tr>
<td>JU#0008 (III-2)</td>
<td>P391H</td>
<td>Mutation in catalytic domain</td>
<td>Reduced/delay</td>
<td>male, 35</td>
<td>Japanese</td>
<td>(Hayashi, Gekka et al. 2007)</td>
</tr>
<tr>
<td>JU#0008 (III-3)</td>
<td>P391H</td>
<td>Mutation in catalytic domain</td>
<td>Reduced/delay</td>
<td>female, 31</td>
<td>Japanese</td>
<td></td>
</tr>
<tr>
<td>Yamamoto, Nobouski</td>
<td>Unknown</td>
<td>Unknown (shorter OS)</td>
<td>Normal/delay</td>
<td>male, 31</td>
<td>Japanese</td>
<td>(Hashimoto and Kishi 2009)</td>
</tr>
</tbody>
</table>
in these cones affecting both GRK1 and GRK7 (Hayashi, Gekka et al. 2007). The possibility that the photoreceptors with P391H mutation suffer some degree of degeneration leading to the cone defect remains, as shown in GRK1−/− mouse photoreceptors. The clinical presentation of Oguchi patients seems indistinguishable across the different mutations, although the molecular consequences of these mutations to GRK1 may be quite different.

**Structure/Function of GRK1**

Crystal structures of modified and inactive GRK1 have been solved to provide some knowledge about the structure-function relationship of the kinase. Furthermore, one of the signaling molecules that modulate GRK1 function in photoreceptor is recoverin (Chen 2002; Komolov, Senin et al. 2009). Recoverin interacts with GRK1 in a Ca2+-dependent manner (Chen, Inglese et al. 1995), which will be discussed in the following section. Recent studies have revealed the molecular interaction between recoverin and GRK1. In Ames et al., the NMR structure of the first 25 amino acid of GRK1 (RK25) complex with Ca2+-bound recoverin is solved. Residues 4-16 of RK25 form an amphipathic α-helix, where the hydrophobic surface interacts with a conserved groove lined with hydrophobic residues in recoverin. This N-terminal groove of recoverin, comprised of nine hydrophobic residues conserved across other neuronal calcium sensors, is implicated in target recognition (Ames, Levay et al. 2006). This N-terminal groove is exposed through the Ca2+-myristoyl switch to allow recoverin binding to GRK1. The residues in GRK1 responsible for binding recoverin are V9, V10, A11, A14, and F15, which form a network of hydrophobic interactions to confer a Kd of 1.4 µM with recoverin. Mutations of these hydrophobic residues abolish GRK1 binding to recoverin (Ames, Levay et al. 2006). In addition, the N-terminal residues of GRK1 are required for rhodopsin binding (Higgins, Oprian et al. 2006). Taken together, the binding of recoverin to GRK1 at the N-terminal domain prevents GRK1 from association to rhodopsin when Ca2+ level is high. As the calcium level drops in photoreceptors upon illumination, myristoyl-group on recoverin is sequestered within the hydrophobic groove, resulting in dissociation of recoverin from GRK1 and allowing GRK1-rhodopsin interaction.

While GRK1 is responsible for the phosphorylation of R*, the molecular interaction between kinase and the GPCR remains unclear. In the GRK family, the structures of GRK2 and GRK6 are solved (Boguth, Singh et al. ; Lodowski, Pitcher et al. 2003; Lodowski, Tesmer et al. 2009).
Recently, six GRK1 crystal structures are reported in various distinct conformations to demonstrate the key elements important for kinase activity and interaction with R* (Singh, Wang et al. 2008). The GRK1 in the study does not have the last 13 residues (GRK1535), which include the isoprenylation site. In these structures, the GRK1 kinase domain is similar to a typical Ser/Thr kinase (AGC Kinase), which contains a large (residues 181-268) and small (residues 269-454) kinase lobe and a C-terminal kinase extension. The active site is formed within a cleft between the large and small kinase lobe and complex with two Mg$^{2+}$ and the nucleotide, ATP or ADP. At the active site, the surface of the large lobe is lined with basic residues to bind with rhodopsin’s C-terminal peptides. The small lobe contains the phosphate-binding loop (P-loop) to interact with the triphosphate of ATP. However, the P-loop (Gly-rich B1-B2 turn) in GRK1 is observed to shift away from the nucleotide-binding site when compared to GRK2 and GRK6. In fact, GRK1’s P-loop resembles more closely to the structure in Protein Kinase A without any bound nucleotide (Apo-PKA). In the apo-GRK1 structure, the kinase domain adopts a more flexible conformation as compared to nucleotide-bound GRK1. It is hypothesized that the kinase domain adopts a rigid conformation upon nucleotide binding and become fully closed and active upon binding to R* at the second and third cytoplasmic loops.

Residues 455-511 forms the C-terminal Kinase Extension domain in GRK1 and contains three regions: the C-terminal (large) lobe tether (residues 455-471), an active site tether (AST, residues 472-480), and an N-terminal (small) lobe tether (residues 498-511). The N-terminal and C-terminal lobe tethers have been previously described, while AST has not, due to its sequence variability among GRKs and AGC kinases. GRK1’s AST can only be observed in the nucleotide-bound structure and becomes disorder in the apo-kinase, similar to the one in PKA. GRK1 AST includes the “tail loop” (Asp472-Tyr477), which packs closely to the active site, and is predicted to participate in substrate stabilization. The AST is connected to the N-terminal lobe tether through residues 481-489, which contains the autophosphorylation sites (Ser488/Thr489). The phosphorylation status of the two residues is unclear in the structure, although they are phosphorylated in mass spectrometric analysis in the presence of ATP (Palczewski, Buczylik et al. 1992; Singh, Wang et al. 2008). It is speculated that the autophosphorylation may participate in electrostatic interaction with Arg222 and Lys221 to stabilize the kinase domain.
The N-terminal structure of GRK1535 is visible in one of the six crystals (form I, PDB 3C4W). The N-terminus (up to residue 32) forms extensive contacts with the RH domain and some with the kinase domain. Two novel phosphorylation sites at Ser5 and Thr8 are identified, but only Ser5 is phosphorylated in native bovine GRK1. Whether phosphorylation of these sites affects GRK1 activity during phototransduction remains to be determined. Interestingly, the N-terminal structure determined by crystallography is different from the RK25 amphipathic helix determined by NMR that complex with Ca\(^{2+}\)-recoverin. Recently, a more ordered GRK6 structure revealed the N-terminal region as a helix that interacted with the C-terminal Kinase Extension (C-tail) to stabilize the large and small kinase lobe and adopt a near active conformation (Boguth, Singh et al.). A set of residues from the small lobe (Arg190), N-terminal helix (Ala8, Asn9, Leu12, and Leu13), and C-tail (Ile472 and Lys475) forms a network of hydrophobic interactions and hydrogen bonds to stabilize the kinase. As these residues are highly conserved in GRK1, it is likely a similar intramolecular network is required for GRK1 to become activated. It is also possible that the amphipathic helix formation of GRK1 requires the presence of recoverin, the GPCR, or other cofactors. Further study is required to resolve the structure and interaction of N-terminus with other regions during GRK1 activation.

The RH domain of GRK1 is made up of nine \(\alpha\)-helices that are typical of the RGS domain, with two additional GRK-specific helices. More importantly, GRK1 are observed in crystals to dimerize at a hydrophobic surface in the RH domain. The dimer interacting residues are conserved in all GRKs except GRK2 and GRK3. However, GRK1 runs as a monomer on size exclusion chromatography and analytical ultracentrifugation. In addition, mutations (D164A, L166K) in the binding interface do not affect the biochemical properties of GRK1. These results suggest that dimer formation in the crystal structure may just be a beautiful artifact of high GRK1 concentration. In light of this, a few caveats have to be taken in account with the structures of the truncated GRK1 (GRK1535). The affinity (\(K_m\)) and activity (\(V_{max}\)) of GRK1535 deviates from the full-length GRK1, which suggests the kinase domain in GRK1535 does not reflect the native conformation. Furthermore, the structure of the N-terminal domain in GRK1535 is different from the RK25 bound to Ca\(^{2+}\)-recoverin (Ames, Levay et al. 2006; Singh, Wang et al. 2008). It is possible that the N-terminal helix formation is induced through the interaction with Ca\(^{2+}\)-recoverin and needs further investigation. To resolve these discrepancies,
crystal structure of the full-length and active GRK1 is needed to fully understand the intermolecular and intramolecular interactions between GRK1, recoverin, and rhodopsin.

**Regulations of GRK1 activity**

Several signaling molecules have been identified to regulate GRK1 function in photoreceptors. As demonstrated through biochemical and structural analysis, recoverin interacts with GRK1 and negatively regulates GRK1 in a light-dependent manner as the calcium concentration changes (Chen 2002; Komolov, Senin et al. 2009). In the dark, high level of cGMP opens the CNG channel on the plasma membrane and maintains a high Ca$^{2+}$ concentration in the OS. The high Ca$^{2+}$ level induces exposure of the myristoyl group of recoverin and allows it to translocate to OS. At the disk membrane, recoverin inhibits GRK1 activity through interactions of the N-terminal domains. As light stimulation leads to a drop in calcium concentration, the myristoyl group of recoverin becomes embedded within the protein and recoverin loses its ability to associate with the membrane and perhaps diffuses out of OS (Strissel, Lishko et al. 2005). Without calcium, the inhibition of GRK1 is relieved to allow R* phosphorylation. While studies have shown that recoverin myristoylation is not required for its inhibition on GRK1, it does enhance the cooperative effect of recoverin on GRK1 (Chen, Inglese et al. 1995). In mouse photoreceptors lacking recoverin, the recovery is faster compared to wild-type rods (Makino, Dodd et al. 2004). In contrast, addition of exogenous recoverin prolongs photoresponses through enhanced inhibition of GRK1 and increased R* lifetime (Gray-Keller, Polans et al. 1993; Erickson, Lagnado et al. 1998). However, there was one study in which titrating the calcium concentration in the OS does not affect R* phosphorylation (Otto-Bruc, Fariss et al. 1998). This argues against a role of recoverin in modulating R* lifetime through GRK1. To further test whether recoverin influences R* lifetime through regulating GRK1, Chen et al. underexpressed GRK1 to make rhodopsin deactivation the slowest step in rod recovery. Under such condition, the presence of background light accelerates rod recovery but this effect disappears when recoverin is removed from photoreceptors. In the absence of recoverin, the inhibitory control on GRK1 is removed and rhodopsin phosphorylation occurs faster (Chen, Woodruff et al. 2010). Thus recoverin is indeed a negative regulator of GRK1 in photoreceptors.

In addition to recoverin regulation, PKA has also been found to phosphorylate and negatively regulate GRK1 (Horner, Osawa et al. 2005). *In vitro*, GRK1 is phosphorylated at...
Figure 3.1. Current understanding of GRK1 function in photoreceptors. In the dark, there is a high Ca$^{2+}$ and cGMP concentration in the OS. High Ca$^{2+}$ is detected by and bound to the Ca$^{2+}$-binding protein, recoverin (Rec). Ca$^{2+}$ binding induces the exposure of the myristoylated group of Rec to allow membrane association on the disk membrane. Membrane-bound Rec then bind to the N-terminus of GRK1 to inhibit its activity towards rhodopsin, the heptahelical GPCR. At the same time, GRK1 is phosphorylated and inhibited by Protein Kinase A (PKA, not shown) on Ser$^{18}$ as PKA is activated by high cAMP level. After light stimulation, 11-cis-retinal is photoisomerized to all-trans-retinal and leads to rhodopsin activation (R*). R* and downstream signaling events lead to channel closure and decrease in Ca$^{2+}$ and cGMP (cAMP) level. The drop in cAMP prevent PKA-dependent inhibition on GRK1. In addition, drop in Ca$^{2+}$ leads to the sequester of the myristoylated group in Rec. Non-myristoylated Rec releases GRK1, allowing the kinase to phosphorylate the C-terminal residues on R*. GRK1 is isoprenylated on Cys$^{558}$ residue for its membrane localization.
Ser21 when incubated with the catalytic subunit of PKA or PKA-holoenzyme stimulated with cAMP. Furthermore, urea-stripped ROS incubated with GRK1 and PKA has a 50% decrease in rhodopsin phosphorylation. The importance of Ser21 phosphorylation is seen with the phospho-mimetic mutation (S21E) of GRK1, which has decreased activity towards rhodopsin. In contrast, S21A GRK1 is resistant to PKA inhibition and constitutively phosphorylates rhodopsin. These evidences suggest a role of PKA in phosphorylating and regulating GRK1 activity in photoreceptor. Under high Ca\textsuperscript{2+} influx in the dark, calmodulin is activated and stimulates cAMP synthesis in photoreceptor. High cAMP level activates PKA to stimulate its phosphorylation on GRK1 at Ser21. Since the N-terminal residues of GRK1 are important in rhodopsin interaction, the phosphorylation at Ser21 introduces a charge residue and obstructs receptor-kinase recognition. Future studies are required to test PKA’s action on GRK1 activity in vivo and its modulation on phototransduction.

One of the post-translational modifications on GRK1 is isoprenylation, which is the covalent attachment of an isoprenoid group to a C-terminal cysteine residue. The modification allows the protein to associate with and function at the membrane. Isoprenylation occurs at the CaaX box motif (C, cysteine; a, any aliphatic residue; X, the residue that determines the type of isoprenoid group attached) (McTaggart 2006). The very C-terminus of GRK1 contains a CaaX box motif (C\textsuperscript{558}VLS) to promote isoprenylation (Inglese, Glickman et al. 1992). The last three residues are proteolytically removed and a farnesyl group (15 carbon unit) is covalently linked to cysteine through a thioester bond. A methyl group is then added to complete the reaction. In COS7 cells, GRK1 isoprenylation is abolished by the C558S mutation, and the protein loses its ability to associate with the plasma membrane. In addition, GRK1 without isoprenylation has reduced (~25%) R* phosphorylation activity, indicating that GRK1 isoprenylation is needed for full activity by anchoring the kinase to the disk membrane. It is unclear whether GRK1 isoprenylation and membrane association affects intrinsic kinase activity, which can be examined by measuring the degree of autophosphorylation of the C558S GRK1 mutant. By changing the terminal serine to leucine at position 561 (Ser561Leu), the farnesylation of GRK1 can be changed to geranylgeranylation (20 carbon unit). When incubating geranylgeranylated GRK1 with urea-stripped ROS extract, the kinase is found to constitutively anchor to the disk membrane independent of light condition, whereas farnesylated GRK1 only localizes to membrane in the presence of light. Activated rhodopsin may serve as a docking site for
farnesylated GRK1 to associate to the disk membrane, whereas geranylgeranylated GRK1 is self-sufficient to anchor to the membrane.

Similar to other kinases, GRK1 is found to be autophosphorylated at two residues, Ser488 and Thr489, at the C-terminus. Over the years, experiments studying GRK1 have used the autophosphorylation status as a method to assess the intrinsic kinase activity and compare it to the kinase’s capability to phosphorylate R* (Horner, Osawa et al. 2005; Higgins, Oprian et al. 2006). These sites were identified on purified GRK1 from bovine retina through peptide digestion and mass-spectrometric analysis (Palczewski, Buczyliko et al. 1992). Alanine or aspartate substitution at either one of the residues does not affect the phosphorylation of the other residue, suggesting that there is no hierarchy between the autophosphorylation reactions (Palczewski, Ohguro et al. 1995). The alanine mutants of GRK1 have less affinity for ATP, while phospho-mimetic mutants have similar affinity for ATP as regular GRK1. These evidences suggest that the phosphorylation at Ser488/Thr489 may affect the conformation of the catalytic pocket during phosphorylation. To test the effect of autophosphorylation on GRK1, a synthetic peptide that corresponds to the autophosphorylation region is incubated with purified GRK1. The peptide did not affect GRK1’s ability to phosphorylate rhodopsin as the kinase has low activity towards the peptide. These evidences suggest that the autophosphorylation domain in GRK1 does not serve as an auto-inhibitory domain, which makes GRK1 unique among the protein kinases. Unphosphorylated GRK1 (Ser488 and Thr489 to alanines) have been shown to preferentially phosphorylated Ser338 on R*, whereas regular GRK1 phosphorylates Ser338 and Ser343. It is speculated that autophosphorylation influences the affinity and enhances the dissociation of GRK1 from phosphorylated rhodopsin to allow arrestin binding (Buczyliko, Gutmann et al. 1991), but its exact physiological role remains to be elucidated.

It is evident that GRK1 is the kinase responsible for light-dependent rhodopsin phosphorylation and the timely R* deactivation in photoreceptor recovery. The regulation of GRK1 activity through post-translational modifications and other signaling molecules (PKA and recoverin) are important. However, the molecular mechanisms of GRK1 activity on photoreceptor function have not been studied in detail. In this part of thesis, I planned on investigating the effects of GRK1 post-translational modifications (isoprenylation and autophosphorylation) on photoreceptor recovery. More importantly, several GRK1 mutations
have been implicated in the pathogenicity of Oguchi Disease. However, their effects on GRK1 function have not been clearly elucidated and are also studied here. Several mutations, P388H, K216R, C558S, V380D, and STAA, are generated (Table 3.2) and studied in cultured cell lines and in the rods of transgenic mice. In animals with transgenic expression of mutant GRK1 in rod photoreceptors, I hope to determine the mechanisms on how P388H and V380D mutations cause the night-blindness phenotype in Oguchi Disease. Also, it has been suggested that the presence of GRK7 in human cones preserves the day vision in Oguchi patients. Therefore, as mouse does not contain a GRK7 gene, human GRK7 is transgenically expressed to confirm its functionality and to generate a mouse model of Oguchi Disease. Through these studies, the role of PTMs and Oguchi mutations on GRK1 function and photoreceptor function can be elucidated and further our understanding on the structure-function relationship of GRKs.
Methods

Antibody

To study the kinase activity of GRK1, previous works have used radioisotope labeling to measure the chemical reactions mediated by GRK1 autophosphorylation and rhodopsin phosphorylation on purified OS. To circumvent from using radioisotope, the polyclonal peptide antibodies, A4102 and A4101NP, were raised in rabbit against the peptide sequence, CQDVGAFS$^{488-489}$VKGVAF, which corresponds to GRK1 autophosphorylation site. A4102 was raised against the phosphorylated peptide at Ser and Thr, whereas A4102 was raised against the non-phospho peptide. The peptide was conjugated to keyhole lamprey hemocyanin prior to immunization. (Yenzym Antibodies, Burlingame, CA). A4102 is purified from rabbit serum first through the nonphospho-peptide conjugated to Sulfo-link agarose beads (Pierce, Rockford, IL), then through the phospho-peptide column to ensure the antibody is only specific to the phosphorylated-GRK1. A4101NP is the antibody eluted from the non-phospho-peptide column. Both antibodies are specific to GRK1 on Western Blot and IHC using in-vitro and in-vivo methods. Furthermore, A4102 recognizes specifically phosphorylated S488 and T489, as no band is detected on Western blot in the single and double mutants (S488A, T489A, and STAA). It has been shown that GRK1 can autophosphorylated the non-mutated residue in the single autophosphorylation mutant. With the development of the A4102 antibody, this is the first time a phospho-antibody is raised toward autophosphorylated GRK1.

To study GRK7, the polyclonal peptide antibody Y722NP was raised in rabbit against the peptide sequence, CAEIDDFSEVRGVE, which corresponds to GRK7 autophosphorylation site. The peptide was conjugated to keyhole lamprey hemocyanin prior to immunization. (Yenzym Antibodies, Burlingame, CA). The antibody was affinity purified against the column conjugated to the corresponding peptide and stored at -20°C. Total of 6 mL of Y722NP antibody was purified at a concentration of 1.08 mg/mL. The specificity of Y722NP to GRK7 was confirmed with Western blot on rods expressing GRK7 (GRK723).

Mutagenesis and cloning

Mutagenesis of bovine GRK1 in pBluescript is performed with Stratagene Quikchange Kit (Stratagene, Santa Clara, CA) according to manufacture’s instructions. The primers used for generating the GRK1 mutations are listed in Table 3.2. Mutations are confirmed by sequencing
with VCU Molecular Biology Core Facility. GRK1 mutants are subcloned into pcDNA(GFP) plasmid and pFASTBac1 with SalI/NotI for expression studies in HEK293, COS7 and High Five Insect cells. To express the GRK1 mutants in mouse rod photoreceptors, GRK1 harboring the mutations are subcloned into pRho4.4 with SalI and BamH1, which contain the 4kb rhodopsin promoter and the protamine polyadenylation (pA) signal. The GRK1 mutants are placed downstream to the rhodopsin promoter and before the pA signal. The transgenic construct is released from the pBluescript backbone with BssHII. The digested DNA is sent to VCU L.T. Christian III Transgenic Mouse Core Facility for nuclear injection.

**Ectopic expression of GRK mutants in cultured eukaryotic cells**

HEK293 and COS7 cells are cultured in a similar condition. Cells are cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 20 mM L-glutamine, 1 mM sodium pyruvate, and 100 units/mg Penicillin/Streptomycin. High Five (Hi5) insect cells are cultured in Sf-900 II SFM media supplemented with 10% FBS. All expression studies are performed in 6 well plates. Transfection of pcDNA(GFP) with GRK1 mutations is performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and harvested 24 hours later for analysis. For proteasome inhibition, transfected cells are treated with 100 µM Mg132 in DMSO and harvested 24 hours later.

**Mouse husbandry**

Animal studies were performed under protocols approved by the Virginia Commonwealth University IACUC. Integration of the GRK1 mutant transgene is genotyped with PCR with the primers, RH1.1, 5’-TCA GTG CCT GGA GTT GCG CTG TGG, and RK-b1, 5’-TCG TCG GCC GTG TCG TAG TCC TCG, to produce a 450 base pair product. The amplification conditions for detecting the transgene are: 94°C for 5 minutes, then 35 cycles of 94°C for 1 minute, 64°C for 1 minute, and 72°C for 1 minutes and followed with a final extension of 72°C for 5 minutes. Animals are reared in 12hr cyclic dark/light cycles.

**RNA extraction and reverse-transcriptase PCR (RT-PCR)**

RNA is extracted in Trizol reagent (Invitrogen, Carlsbad, CA) following manufacture procedures. RT-PCR on purified RNA is performed with Superscript III system (Invitrogen, Carlsbad, CA) following manufacture’s instructions. Detection of transgene cDNA is performed
with RH1.1 and RKB1 primers (bRK) for the 5’-end and PrmlB and bRK-CDGLRTH primers (bRKnw) for 3’-end of the transgene. GNAT1-WT Primers (Table 2.1) is used to control for DNA contamination as the primer set covers a small intron. Expresser is confirmed with the presence of PCR product in the 5’-end and 3’-end transgene.

**Western Blot**

Mice were euthanized with CO₂, followed with cervical dislocation. Eyeballs were removed with forceps and placed in 1X PBS for extraction. The cornea and lens were removed before separating the retina from the retinal-pigmented epithelium (RPE). For one retina, it is homogenized in 75 μL 1X PBS and solubilized in 75 μL LSB Buffer, followed with incubation at 95°C for 10 minutes. Retinal proteins were resolved on a 12% Tris-HCl polyacrylamide gel and electrophoresed in 1X SDS-Page buffer at 150V for 100 minutes. Following SDS-Page, the proteins were transferred onto nitrocellulose membrane in 1X transfer buffer at 250 mA for 120 minutes. The membrane was stained with Ponseau S (0.1% w/v Ponseau S in 5% glacial acetic acid) to ensure the completion of transfer. Ponseau S was washed away with distilled H₂O. For immuno detection, the membrane was blocked in blocking buffer (10% milk in 1X TBST) for 1 hour at room temperature (RT). Primary antibody was diluted in blocking buffer and incubated with the membrane for 2 hours at RT. After primary antibody incubation, the membrane was washed four times in 1X TBST at 5 minutes each. The animal-specific secondary antibody was diluted (1:30k) in blocking buffer and incubated with the membrane for 1 hour at RT. Following secondary antibody, the membrane was washed six times in 1X TBST at 10 minutes each before developing it using the SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Blot image was captured on film and on the Kodak IS440 CF machine.

**Immunohistochemistry**

A small burn mark was placed on the superior side on the mouse cornea before the eyes were dissected in 1X PBS (Phosphate-buffer saline, pH 7.4). A small incision was made on the cornea near the ora serrata to allow fluid permeation. The eyeball was fixed overnight in 4% paraformaldehyde in 1X PBS at 4°C. After fixation, the cornea and the lens were carefully removed and the eye cup was cryo-protected in 30% sucrose in 1X PBS at 4°C. After the eyecup settled in the sucrose solution, it was embedded and frozen in TBS (Triangles Biomedical Sciences, Durham, NC, USA) at -80°C. Frozen eyecup blocks were sectioned at 24μm. For
immunostaining, sections were washed three times with 1X PBS at five minutes each. Washed sections were blocked with 10% goat serum in PBT (0.3% Triton X-100 in 1X PBS) for 1 hour at room temperature (RT). Blocking solution was removed before adding the primary antibody. Primary antibody was diluted with 10% goat serum in PBT, applied over the section, and incubated overnight at RT. After incubation, sections were washed three times with PBT at five minutes each. After washing, secondary antibody was diluted with 10% goat serum in PBT, added to the sections and incubated for two hours at RT. After incubation, sections were washed three times with PBT at ten minutes each. DAPI was used for nuclear staining at a final concentration of 500 ng/mL and applied during the secondary incubation. Finally, sections were protected with ProLong Gold antifade reagent (Invitrogen, California), mounted with cover slips and sealed with nail polish. Confocal images were taken with Zeiss META LSM 510 confocal microscope in the VCU Microscopy Core Facility.

**Electroretinogram (ERG)**

ERG is recorded with the LKC UTAS-3000 System (LKC Technologies, Gaithersburg, MD). All procedures are performed in the dark with night-vision goggle. Dark-adapted mouse is anesthetized with ketamine/xylazine mixture (150/10 mg/kg; intra-peritoneal) and then the eyes dilated with tropicamide drops for 10 minutes. After dilation, the animal was placed in the ganzfeld with a water-heated plastic coil to maintain the body temperature at 35-37°C. Two gold-plated-electrodes are placed in front of the cornea and 1X PBS was applied to allow electrical contact. The ground electrode was placed on the forehead of the mice. Background light is turned on for 10 minutes to suppress the rods and increase the cone response. After light adaptation, a 1.63 log cd s m⁻² stimulus was delivered at 1Hz to assess the photopic response of the retina. 90 responses were averaged and stored by the EMWin software. Data was exported into and analyzed in Microsoft Excel software. Due to the small ERG a-wave amplitude from mouse cones, the b wave amplitude derived from the cone responses was quantified across different genotypes to compare the functionality of replacing GRK1 with GRK7.

**Statistical analysis**

All statistics were performed using Sigma Pro 7.0. One-way Anova test or student t-test were performed across different groups/genotypes to obtain statistical significance of p value < 0.05.
Retina fractionation

Two fresh retinas were extracted as described above (“Western Blot”) from one animal and placed together in a 1.5 mL microfuge tube. All procedures were performed on ice until solubilization with 2X LSB and heat treatment. The retinal sample was resuspended in 150 µL 1X PBS and sonicated with (machine) for 1 cycle of 5 pulses. 50 µL of the retinal extract was removed, mixed with 50 µL 2X LSB and labeled as total extract (T). The remaining 100 µL retinal extract was ultracentrifuged at 100,000 x g for 1 hour at 4°C to separate the soluble and membrane contents. The supernatant (S) was carefully removed (~100 µL), placed in a new tube, and mixed with 1:1 2X LSB. The pellet (P) fraction was resuspended with 100 µL 1X PBS and also mixed with 100 µL 2X LSB. All LSB treated samples were incubated at 95°C for 10 minutes. 8 µg of each extracted was loaded on 12% SDS-PAGE gel for Western Blot analysis. Complete separation of soluble and membrane fractions were determined by RGS9-1 and GAPDH localization. RGS9-1 is completely membrane localized, while GAPDH is a cytosolic marker.

Outer Segment (OS) Isolation

To determine proteins in outer segment localization, outer segments were isolated from 5 mice retinas. 6 retinas were isolated from 3 mice, one was used as a total protein control and the other 5 retinas were used to purify OS. The 5 retinas were pooled in 500 µL 47% sucrose in ROS buffer (20 mM MOPS pH 7.4, 60 mM KCl, 30 mM NaCl and 2 mM MgCl2) and vortex at low intensities for 2 minutes. The agitation force detached outer segments from the photoreceptor, which then partitioned into the 47% sucrose. After vortex, the solution was centrifuged at 10,000 x g for 30 minutes at 4°C. The supernatant was removed to a new tube and mixed 1:1 with ROS buffer, which diluted the sucrose to 23.5%. The pellet was resuspended in another 500 µL 47% sucrose in ROS buffer and OS extraction was repeated. The supernatants were pooled and centrifuged at 20,000 x g for 30 minutes at 4°C. At 23.5% sucrose, the OS is denser and can be pelleted. The pellet containing OS was resuspended in 75 µL ROS buffer and mixed with 75 µL 2X LSB. 8 µL of ROS extract was resolved on 12% SDS-Page gel to determine the protein content.

Rhodopsin bleaching time course
To determine the amount of rhodopsin bleached when exposed to the background light (30 cd•m\(^2\)) LKC UTAS-3000 System, spectrum analysis was performed on dark-adapted retinas exposed to background light at various time points. All procedures were performed in the dark with night goggle until exposure to background light in the ganzfeld. Retinas are extracted and pooled from 10 dark-adapted C57BL/6 (Jackson Laboratory, Bar Harbor, Maine) mice. During extraction, retinas were kept on dry ice until completion. 20 retinas were homogenized in 500 µL Buffer A (10% Triton X-100 in 1X PBS). Retinal Extract was quickly centrifuged to pellet insoluble content. 25 µL of the extract represented the rhodopsin content from one retina and was diluted with Buffer A to the final volume of 500 µL for spectrophotometer reading. To test rhodopsin bleaching rate, one retina (500 µL) was exposed to background light for 0, 1, 2, 5, and 10 minutes. Rhodopsin absorbance is recorded with spectrophotometer at 498 nm (A\(_{498}\)) before (A\(_{pre}\)) and after background exposure (A\(_{post}\)). After completion of the last time point, all samples are exposed to a bright-light box for 5 minutes to bleach all the rhodopsin. A\(_{498}\) reading is taken again on all samples (A\(_{bleached}\)). The percent of rhodopsin bleached at each time point is calculated with equation 1, 

\[
\% \text{Rhodopsin bleached} = \frac{A_{pre} - A_{bleached}}{A_{pre} - A_{bleached}}.
\]

All time points are performed in triplicates.

**Time course of dark adaptation**

To test the recovery of mouse photoreceptor after 50% bleach, animals are dark-adapted overnight before performing ERG. Dark-adapted animals are anesthetized with ketamine/xylazine (150/10 mg/kg; intra-peritoneal) and their eyes dilated with tropamide drops for 10 minutes. Mouse is placed into the ganzfeld in the same procedure in the method ‘ERG’. A 1.4 log cd S/m\(^2\) flash is triggered to obtain the fully dark-adapted response. Afterwards, the backlight (30 cd m\(^2\)) is turn on for two minutes to reach 50% rhodopsin bleach. After turning the light off, log 1.4 log cd S/m\(^2\) flash is triggered at time 1, 2, 5, 10, 15, 30, 45 and 60 minutes to assess the recovery from bleach. The ERG A wave amplitude is used to measure the photoreceptor recovery and is normalized to the pre-bleach response.
Results

Characterization of Y722NP, a GRK7-specific peptide antibody

During the generation of the MCV7 and MCV8 transgenic lines, the polyclonal Y722NP antibody was generated against the synthetic peptide, CAEIDDFSEVRGVE, which corresponds to GRK7 autophosphorylation site. Comparing GRK723 (rod-specific GRK7 expression) to control retinal extracts, Y722NP is specific to GRK7 on Western Blot (Figure 3.2A). Furthermore, the antibody is specific to GRK7 on MCV7 retinal sections with IHC (Figure 3.2B) and does not have any non-specific signals, as no immunofluorescence is detected in GRK1−/− retina.

Functional replacement of GRK7 for GRK1 in mouse cones

To test whether human GRK7 is functional in mouse cones, MCV7 and MCV8 are crossed into GRK1−/− background to generate the MCV7−/−, MCV8−/− and MCV78−/− animals. By removing the endogenous GRK1 in cone photoreceptor, the function of GRK7 in MCV7 and MCV8 cones can be assessed with photopic ERG. Under light-adapted condition, the cone responses from 1Hz flicker flash of 1.63 log cd S/m² are recorded from WT, MCV7−/−, MCV8−/−, MCV78−/− and GRK1−/− animals (Figure 3.3). After quantification of the cone-derived ERG B wave amplitude, flicker responses are 196 µV in WT cones, 105 µV in MCV7−/− cones, and 145 µV in MCV8−/− and MCV78−/− cones. The flicker response is abolished in GRK1−/− cones down to 18 µV. The expression of GRK7 in cone photoreceptors restored the flicker responses in cones lacking GRK1. The flicker response amplitude in MCV7−/− cones is less than the one in MCV8−/− cones (Figure 3.3B), which corresponds with the different GRK7 expression pattern between MCV7 and MCV8 (Figure 2.10). Interestingly, the responses amplitude in MCV8−/− and MCV78−/− cones are very similar, confirming that the GRK7 expression in MCV8 is almost in all the cones and therefore, additional GRK7 from the MCV7 transgene does not further increase the response amplitude. However, the restoration of the cone responses in MCV8−/− and MCV78−/− does not reach the WT responses, suggesting that some defects are presented in these transgenic cones.

Generation of GRK1-specific antibodies, A4101NP and A4102.
Figure 3.2. Characterization of Y722NP, a GRK7 specific polyclonal antibody. (A) Western blot analysis on retina with rods expressing human GRK7 using the rhodopsin promoter (GRK723). Retinal extract was resolved on 12% SDS-Page and transferred onto nitrocellulose membrane. Y722NP detects a band in GRK723 retina (+ lane), which is absent in the control retina (- lane). This demonstrate the specific of Y722NP antibody to GRK7. (B) On IHC, Y722NP is specific for GRK7 on retinal sections. Two dilution was tested (1:50, 1:100) on IHC to test the staining conditions. Y722NP can detect GRK7 expression (red) in the cones of MCV7−/− as the sections were co-stained with PNA (green). The expression of GRK7 is cone-specific as all positive cells are surrounded with the cone-specific marker, PNA. Images were taken at 40X with the fluorescence microscope. The peptide antibody, Y722NP, is specific to GRK7 in Western Blot and IHC.
Figure 3.2

GRK7-specific antibody

**Antibody Info (From Yenzym)**
- Rabbit #Y722 (Affinity Purified Rabbit Ab)
- Against pS490-GRK7
- Purified through S490 (non-phospho)
- 0.18 mg/ml x 6.00 mL (1.08 mg total)
- Lot: 80508
Figure 3.3. The ERG flicker response of WT, MCV7−/−, MCV8−/−, MCV78−/−, and GRK1−/− animals. (A) Animals were dark-adapted overnight and then light-adapted for 10 minutes before recording. A 1.63 cd/m² stimulus was delivered at 1Hz and the 90 responses were averaged. Representatives waveforms are shown here. Wild-type cones are able to respond to 1Hz stimulus, while animals lacking GRK1 cannot respond due to the delay in cone recovery. Cone responses are restored when hGRK7 is expressed in GRK1−/− cones. The rescue in ERG flicker response in hGRK7-expressing cones demonstrates GRK7 can functionally replace GRK1 in mouse cones and deactivate cone opsins in a timely manner. (B) The flicker responses across difference genotypes are quantified. Due to the small a wave amplitude of cone ERG response, the derived B wave is used in the quantification. The response for WT is 195.89±8.24 µV, MCV78−/− is 144.64±5.47 µV, MCV7−/− is 104.69±7.55 µV, MCV8−/− is 144.88±4.36 µV, and GRK1−/− is 17.60±2.47 µV. While the expression of hGRK7 in cones can substitute for the loss of GRK1, the responses of MCV78−/− is lower than WT. The lack of complete rescue in MCV78−/− response can be attributed to the degeneration effects of GRK1−/− as its outer segment is shortened and may exert influences on the functionality of the cone photoreceptors. In addition, MCV8−/− cones has higher amplitude than MCV7−/− because hGRK7 expression is more uniform in MCV8 than MCV7, which facilitates more cone responses. Similarly, the lack of further rescue in MCV78−/− is also due to the homogenous expression of hGRK7 in MCV8−/−, so additional hGRK7 expression from MCV7 in the same cones have no influence on the flicker response. One-way Anova statistical test was applied to determine the significance in the flicker response amplitude (p < 0.05).
Figure 3.3
Figure 3.4. Characterization of GRK1 antibodies, A4101NP and A4102 antibody \textit{in-vivo} and \textit{in-vitro}. Western blot on \textit{in-vitro} kinase assay of purified GRK1 to determine the phospho-specificity of A4102. (A) The phospho-peptide used to generate A4102 antibody. (B) \textit{In-vitro} phosphorylation assay performed on recombinant GRK1 treated with either Mg$^{2+}$-ATP or Protein-Phosphatase-2A (PP2A). The phosphorylation level and protein level are assessed on Western Blot. G8 controls for the total GRK1 protein. Phosphorylated GRK1 runs as higher MW species, which is recognized by A4102. (C) A4102 \textit{in-vivo} specificity on WT and GRK$^{-/-}$ retinas on a peptide binding assay. A4102 antibody is only specific to phospho-peptide and is depleted from supernatant, preventing binding on Western blot. A4102 is also specific for GRK1, as no protein signal is detected in GRK1$^{-/-}$ retina. A4102 antibody is specific to phosphorylated-GRK1 (pGRK1) on Western Blot. Next, IHC staining is performed on WT and GRK1$^{-/-}$ sections using G8 (D, 1:100), A4101NP (E, 1:100), and A4102 (F, 1:300) antibodies. Sections are co-stained with PNA-Fitc (1:500) to mark cone photoreceptors. Confocal images are taken at 63X magnification. The A4101NP and A4102 antibodies stains for GRK1 in the similar manner as G8, and no signal is detected in GRK1$^{-/-}$.
Figure 3.4

A. 
H₃N-CQDVGAFSpTpVKGVAF-COOH

B. 

<table>
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<tr>
<th></th>
<th>A4102</th>
<th>G8</th>
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<tr>
<td>PP2A</td>
<td>+</td>
<td>−</td>
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<tr>
<td>ATP</td>
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C. 

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<td>Nonphospho Peptide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho Peptide</td>
<td></td>
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D. 

Figure 3.4
Figure 3.4
To study GRK1 autophosphorylation at S\textsuperscript{488} and T\textsuperscript{489}, the phospho-antibody, A4102 and A4101NP, were generated. A4102 and A4101NP were raised against a phospho-peptide and the non-phospho-peptide corresponding to GRK1 autophosphorylation site (Figure 3.4A). The specificity of A4102 antibody is tested with a kinase assay by treating recombinant GRK1 with either ATP or Protein-Phosphatase-2A (PP2A). On Western blot, A4102 only detect GRK1 under the treatment of ATP, which promotes GRK1 autophosphorylation. No phosphorylated protein can be detected under treatment of Protein Phosphatase 2A (Figure 3.4B). To further test A4102 specificity, retinas from WT and GRK1\textsuperscript{−/−} mice are compared. A4102 does not detect any signal in GRK1\textsuperscript{−/−} retina (Figure 3.4C, top panel). Also, an antibody depletion assay is performed by incubating the A4102 antibody with agarose beads coupled to the phosphorylated or unphosphorylated peptide. After centrifugation, the supernatant above the beads is used as primary antibody for Western blot as shown in figure 3.4C. GRK1 detection is lost when A4102 binds to beads with phosphorylated peptide and is depleted from the supernatant (Figure 3.4C, bottom panel). More importantly, A4102 does not recognize the unphosphorylated GRK1, since it does not bind to the unphosphorylated peptide and remained in the supernatant to recognize GRK1 on immunoblot (Figure 3.4, middle panel). On retinal sections, A4101NP (Figure 3.4E) and A4102 (Figure 3.4F) also recognize GRK1 in the OS of photoreceptors, similar to the commercial GRK1 antibody, G8 (Figure 3.4). None of the three antibodies (G8, A4102, A4101NP) detect any signals in GRK1\textsuperscript{−/−} sections, confirming their specificity to GRK1 on IHC. A4101NP antibody recognizes the non-phosphorylated and phosphorylated GRK1, whereas A4102 is specific to phosphorylated-GRK1 (pGRK1). A4102 is a useful reagent to monitor changes in GRK1 autophosphorylation, which reflects the intrinsic kinase activity.

The light/dark transition on GRK1 autophosphorylation

To test whether GRK1 autophosphorylation is modulated by dark and light condition, a dark adaptation time course is performed. WT mice are dark-adapted and their retinas extracted at times 0, 0.5, 1, 12, 24 and 240 hours. After 240 hours in the dark, one animal was placed into ambient light for 1 hour before the retinas were harvested. Comparing autophosphorylated GRK1 (A4102) to total GRK1 (G8) (Figure 3.5A), the autophosphorylation decreases by ~50% within the first 30 minutes of dark-adaptation (Figure 3.5B, time point 2), which then returns to basal level after one hour. The implication of the drop in autophosphorylation requires further investigation. It is possible during dark adaption, GRK1 activity increases in order to


**Figure 3.5. GRK1 autophosphorylation time course.** WT animals are dark-adapted overnight and their retinas extracted at various time points. Retinas are extracted under infrared light. (A) Western blot of retinal extract at various time points to assess the level of autophosphorylation during light to dark transition. (B) Quantification of GRK1 autophosphorylation during dark-adaptation. Level of phosphorylation is determined by the ratio of p-GRK1(A4102)/total GRK1 (G8). There is little or no difference in GRK1 autophosphorylation during light and dark adaptation.
Figure 3.5
phosphorylate and deactivate R*. If autophosphorylation has an inhibitory effect on GRK1 activity, then reducing autophosphorylation (through a protein phosphatase) can increase GRK1 activity. As GRK1 kinase activity increases, autophosphorylation then returns to normal level and provides a negative feedback on GRK1 activity.

**Ectopic expression of GRK1 mutants.**

Six residues, K\textsuperscript{216}, V\textsuperscript{380}, P\textsuperscript{388}, S\textsuperscript{488}, T\textsuperscript{489}, and C\textsuperscript{558}, in GRK1 are selected to study their roles in GRK1 function. K\textsuperscript{216} is the catalytic residue important in the kinase activity. P\textsuperscript{388} (P\textsuperscript{391} in human) and V\textsuperscript{380} are residues mutated in the human Oguchi Disease (Table 3.1). S\textsuperscript{488}, T\textsuperscript{489}, and C\textsuperscript{558} are residues involved in the PTMs for GRK1. S\textsuperscript{488} and T\textsuperscript{489} are found to be autophosphorylated, whereas C\textsuperscript{558} is the isoprenylation site for GRK1. Sequence alignments are performed on human GRK1 to GRK7 (Figure 3.6) using the ClustalW2 software. The alignment shows that K\textsuperscript{216} and P\textsuperscript{388} are conserved across all seven GRK members. The V\textsuperscript{380} is unique to GRK1, whereas C\textsuperscript{558} is conserved in GRK1 and GRK7. The autophosphorylated residues are presented across GRK members with some variations. GRK7 has one of the autophosphorylated residue as E, which is phosphomimetic to S and T residues. In the GRK2-group, the autophosphorylated residues are expanded to DEED, which are all phosphomimetic. These evidences suggest autophosphorylation may play a role in GRK regulation and function.

To study GRK1 autophosphorylation, the S\textsuperscript{488} and T\textsuperscript{489} residues are mutated to alanines to generate the STAA mutant. Also, to test the effects of other mutations, GRK1 harboring K216R, C558S, P388H, and V380D are generated (Table 3.2). K216R mutation abolishes GRK1 kinase activity (Horner, Osawa et al. 2005) and C558S eliminates GRK1 isoprenylation (Inglese, Glickman et al. 1992; Inglese, Koch et al. 1992). P391H and V380D are missense mutations found in patients with Oguchi Disease (Yamamoto, Sippel et al. 1997; Khani, Nielsen et al. 1998; Hayashi, Gekka et al. 2007). As mentioned, human GRK1 P391 correspond to P388 in bovine GRK1. These five mutants are first studied with ectopic expression in cultured cells. In High Five insect cells, GRK1 mutants have robust expression (Figure 3.7A, top panel). However, all of the GRK1 mutants are not autophosphorylated, except for C558S mutant (Figure 3.7A, middle panel), suggesting that these mutations abolish GRK1 activity. The absence of autophosphorylation in the K216R mutant is expected, as the lysine residue is responsible for kinase activity. On the other hand, the C558S mutant is autophosphorylated, which indicates that
Figure 3.6. GRK family alignment with ClustalW2 software. The seven members of the G-protein coupled receptor kinase family are aligned to assess the important residues in the GRKs. Five specific residues (highlighted in red) are selected for further characterization in cultured cells and in transgenic mouse lines. The residues proline (P, Oguchi mutation) and lysine (K, Kinase-dead mutation) are conserved across all GRK members, whereas valine (V, Oguchi mutation) is specific to GRK1. Furthermore, GRK1 and GRK7 are the only GRKs with the cysteine residues for isoprenylation modification. It seems all GRKs have the autophosphorylation residues in serine and threonine residues, or some phospho-mimetic residues (glutamic or aspartic acid), suggesting autophosphorylation is important for GRK signaling.
Figure 3.6
Table 3.2. GRK1 mutant summary. Five GRK1 mutants are generated for expression studies in cultured cells and transgenic mice. P391H and V380D mutations are found in patients suffering from Oguchi Disease. P391 for human GRK1 corresponds to P388 in bovine GRK1. C558S mutation abolishes the ability for GRK1 to undergo isoprenylation at the cysteine residue. K216R mutation renders GRK1 catalytically inactive (Kinase-Dead). STAA mutation replaces the autophosphorylated residues S\textsuperscript{488} and T\textsuperscript{489} in GRK1 to alanine residues. These mutations are generated with Stratagene Quickchange kit with the specific primers in column 3. GRK1 mutants are subcloned into pcDNA(GFP) plasmid for cell transfection and into pRho4.4 plasmid for transgenic expression in mouse rod photoreceptor. The expression pattern of GRK1 mutants in HEK293 cells and in transgenic animals are listed in column 4 and column 5 and 6, respectively. The percentage of GRK1 mutant expression in HEK293 cells is normalized to the WT level. The transmission rate of GRK1 transgenic strains is determined by the number of expresser/number of founders.
<table>
<thead>
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<th>Mutation</th>
<th>Designation (reference)</th>
<th>Primers</th>
<th>HEK293</th>
<th>Transgenic</th>
<th>Transmission Rate</th>
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<td>P388H</td>
<td>Oguchi</td>
<td>5’-GAGATGATCGCGCCAGAGGTCTACTTTCGAGGCC-3’ 5’-CCTCTGGCCCGATCATCTCGTACAGCGT-3’</td>
<td>&lt; 10%</td>
<td>-</td>
<td>4/5</td>
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<td>C558S</td>
<td>Isoprenylation</td>
<td>5’-TGTGCTTTCTAGATCGATAAGCTTGATAT-3’ 5’-TTATCGATCTAGGAAAGCACAGACATGCCCGAC-3’</td>
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<td>&lt; 10%</td>
<td>-</td>
<td>1/4</td>
</tr>
<tr>
<td>V380D</td>
<td>Oguchi</td>
<td>5’-TTACTTCGCGCTGGGTAGACACGCTTGATAGTG-3’ 5’-ACCCAGCGCGAAATCTACGAAAAGTCTG-3’</td>
<td>&lt; 10%</td>
<td>+</td>
<td>6/10</td>
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<tr>
<td>STAA</td>
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<td>5’-TCCAGGATGTTGGTGCCCTTGCCCGGCGGTCAAGGGCCGTGGCC-3’ 5’-GAAGGCCACCCACATCTGGATATTCTTTCGATA-3’</td>
<td>50%</td>
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Figure 3.7. GRK1 mutant expression in cultured cells. (A) Recombinant WT and mutant GRK1 are expressed in High Five (Hi5) insect cells using the Bac-to-Bac Baclovirus expression system. Cell extracts are resolved on 12% SDS-Page, transferred onto nitrocellulose membrane, and stained with antibodies (G8, A4102 and actin). Note that C558S mutant has a higher molecular weight than WT GRK1. The shift is due to the lack of isoprenylation preventing the cleavage of CAAX motif. The STAA mutant confirms the phospho specificity of A4102 antibody. (B) GRK1 constructs in pcDNA(GFP) are transfected in COS7 cells and harvested 24 hours later. First lane is WT retinal extract for control. The mutants, P388H, K216R and V380D, have a complete reduction in GRK1 expression, whereas STAA and single phosphorylation mutant have partially reduced expression. The loss of expression in these GRK1 mutants are different from the pattern in Hi5 cells. This can be attributed to the differences in cell type, where Hi5 cells may not have the proper degradation machinery as mammalian cell lines. Also, the single phosphorylation mutant also confirm that A4102 antibody is specific to the double phosphorylated residues at Ser488 and Thr489. To test whether the loss of protein can be rescued by inhibiting the proteasome complex, (C) the same GRK1 constructs are transfected to HEK293 cells and harvested 24 hours later. Transfected cells treated with the proteasome inhibitor Mg132 at 100 µM are denoted with ‘+’. Cell extracts are resolved on 12% SDS-Page and blotted for GRK1 expression and autophosphorylation. GFP is used as a transfection control. (D) Quantitative analysis of protein expression and autophosphorylation. All signals are normalized to untreated WT. All mutants except C558S have reduced protein expression. Mg132 treatment restores some levels of protein expression, but not autophosphorylation.
Figure 3.7

A. Hi5 Insect cells

*GRK1 mutants are stable

B. COS7

*GRK1 mutants are not stable
Figure 3.7
isoprenylation is not required for GRK1 activation. The C558S mutant has a larger molecular weight and runs higher than WT GRK1 due to the lack of proteolytic cleavage of the AAX residues.

To further examine GRK1 mutants in mammalian system, the mutations are cloned into pcDNA(GFP) plasmid and expressed in COS7 (Figure 3.7B) and HEK293 (Figure 3.7C). GFP is used as transfection control. In contrast to the expression profile in Hi5 cells, GRK1 mutants K216R, P388H and V380D have ~90% reduction in protein expression compared to WT GRK1 (Figure 3.7B and C, G8). The reduction in protein expression and kinase activity in GRK1 with kinase-dead mutation (K216R) and the Oguchi mutation (V380D) is consistent with previous reports (Figure 3.7B, lane 5 and 6; Figure 3.7C, lane 7 and 9) (Khani, Nielsen et al. 1998; Horner, Osawa et al. 2005). On the other hand, this is the first observation on the loss-of-protein phenotype for the P388H mutation (Figure 3.7B lane 3; Figure 3.7C lane 3). The reduction in GRK1 expression caused by P388H and V380D mutations may provide the mechanism for the loss of night vision in Oguchi patients. Loss of GRK1 prolongs the lifetime of R* and prevent the timely recovery of photoreceptors. Furthermore, abolishing GRK1 kinase activity also resulted in loss of protein, suggesting that kinase activity may be required for GRK1 stability and autophosphorylation may confer GRK1 stability. Indeed, loss of autophosphorylation results in ~50% reduction in GRK1 (Figure 3.7C, lane 11; Figure 3.7D). However, autophosphorylation do no account for the complete stability of GRK1 and points to a separate mechanism to stabilize the kinase. In addition, single autophosphorylation mutants, S488A and T489A, have reduced GRK1 expression (Figure 3.7B, lane 8 and 9). The multiple molecular species of WT GRK1 fall into a single lower form in STAA, S488A and T489A, suggesting that the larger GRK1 species are phosphorylated. The different expression profiles in P388H, V380D, K216R and STAA mutants in insect and mammalian cells can be attributed to the differences in the protein degradation machinery between the cell types.

Consistent in all cell types tested, GRK1 without isoprenylation does not have the CAAX motif cleaved, resulting in a larger GRK1 protein (Figure 3.7A, B, and C). Also, GRK1 lacking isoprenylation is autophosphorylated; however, after intensity analysis, there is a ~50% drop in the phosphorylation level (Figure 3.7D). It is conceivable that GRK1 may have lower kinase activity before isoprenylation and assume full activity once it is isoprenylated and localized to
the membrane. This observation is consistent with the literature that GRK1 activity on purified, light-activated ROS requires farnesylation (Inglese, Glickman et al. 1992; Inglese, Koch et al. 1992).

**The loss of GRK1 protein expression, but not autophosphorylation, can be restored by the treatment of the proteasome inhibitor, Mg132, in HEK293 cells.**

The loss of protein seen in P388H, V380D and K216R mutants may be the consequence of protein misfolding. Misfolded proteins are tagged with ubiquitin and subsequently degraded by proteasome (Ardley 2009). To test whether proteasome machinery is responsible in degrading the GRK1 mutants, HEK293 cells expressing WT and mutant GRK1 are treated with the proteasome inhibitor, Mg132, at 100 µM. Transfected cells are dosed at every 6th hour for 24 hours (Figure 3.7C and D). Mg132 treatment does not affect the level of WT GRK1 level (lanes 1 vs. 2), while the treatment partially restored ~50% of three mutants, P388H (lanes 3 vs. 4), V380D (lanes 7 vs. 8), and K216R (lanes 9 vs. 10). However, no change is observed with treatment of STAA mutant (lane 11 vs. 12). The lack of rescue in the STAA mutant suggests that the proteasome machinery does not mediate the reduction in GRK1 level through the loss of autophosphorylation. This observation may also explain why only ~50% of GRK1 can be restored with Mg132 treatment in GRK1 containing K216R, V380D, or P388H mutations. Although Mg132 inhibited the degradation of GRK1 mutants, it did not restore the autophosphorylation. The lack of autophosphorylation indicates that the restored GRK1 protein does not have activity.

**Generation and characterization of mutant GRK1 transgenic mouse lines.**

To study the GRK1 mutants *in-vivo*, the P388H, C558S, K216R, V380D, and STAA mutants (Table 3.2) are expressed in mouse rods with the rhodopsin promoter. Since all GRK1 mutants, except C558S, are expected to have reduction in protein expression, it is difficult to determine the protein expression from the transgene due to the presence of endogenous GRK1. On the other hand, the lack of transgenic protein expression is usually caused by the integration of the transgene into a genetically silenced region. To circumvent the complication of using protein expression as confirmation of transgene expression, PCR is performed on the transgene cDNA with bRK and bRKnew primer sets, which bind to the 5’end and 3’end of the transgene, respectively, to determine transgene expression (Figure 3.8-3.13). GNAT1-WT primers are used.
to control for DNA contamination as the removal of intron results in smaller PCR fragment for GNAT1 cDNA. The transmission rate of the five transgenic lines is summarized in Table 3.2.

For P388H transgenic line, five founders are generated with four expression lines. Line 2 has the highest RNA expression (Figure 3.8B), while no GRK1 protein is detected by immunoblotting (Figure 3.8A). TGbRK and TGbRK700/− are used as positive controls. TGbRK has WT bovine GRK1 expression in mouse rod, whereas TGbRK700/− is TGbRK in GRK1/− background. On Western blot, Bovine GRK1 has a larger MW than mouse GRK1, which is seen in Figure 3.8A. The presence of RNA expression without protein expression suggests that P388H mutation destabilizes GRK1.

Next, K216R transgenic line generated four founders with only one expresser. Line 4 has the highest RNA expression (Figure 3.9). Line 4 is crossed into GRK1/− for further analysis. For V380D transgenic line, ten founders are generated with six transmitters (Figure 3.10). Out of the six transmitters, line 1 and line 5 have the highest RNA expression (Figure 3.10B and C). Western analysis of V380D line 1 and 5 in WT background do not reveal any higher running molecular species (Figure 3.10A). However, the protein expression in V380D line 1 may have a slight increase in the genotype positive mice (Figure 3.10A), which suggests some GRK1 expression from the mutant transgene. Any mutant GRK1 expression can be clearly determined by removing the endogenous mouse GRK1. V380D line 1 is crossed into GRK1/− background for analysis and discussed further in the later sections. For STAA transgenic line, ten founders are generated with five transmitters. Out of the five, lines 2 and 7 have high RNA expression (Figure 3.11C). Similar to the P388H and V380D transgenic lines, no larger molecular species is found in STAA lines on Western blot (Figure 3.11A and B). STAA lines 2 and 7 are crossed into GRK1/− background to study the transgene expression.

Lastly, C558S transgenic line generated four founders and only one expresser. C558S expression reveals the presence of the higher molecular species, which confirms the expression of the transgene (Figure 3.12A, G8). In addition, C558S mutant is also autophosphorylated (Figure 3.12A, A4102), consistent with the observation that GRK1 without isoprenylation is catalytically active. Quantitative analysis shows that C558S has two-fold increase in GRK1 expression over WT (Figure 3.12B, G8/GAPDH), confirming transgenic protein expression.
Figure 3.8. P388H transgenic analysis with Western Blot and RT-PCR. (+) indicates the confirmation of transgene transmission by PCR. Retinas were extracted and homogenized in 1X PBS and solubilized with 2X LSB. (A) Immunoblot analysis to screen for bovine GRK1 runs higher than endogenous mouse GRK1 as seen in TGbRK and TGbRK700−/−. No higher molecular weight species were detected in any of the P388H transgenic positive animal. (B) mRNA Analysis of P388H transgenic mice. RNA from mouse retinas are extracted by Trizol following manufacturer’s protocol and reverse-transcriptase PCR is performed with Superscript III. mRNA levels are detected from P388H transgenic positive mice. Transducin (GNAT1) is used to control for DNA contamination. Mice harboring the P388H transgene also express the mRNA for bovine GRK1. The lack of transgene protein expression, but a presence of its mRNA, suggests that P388H mutation destabilizes GRK1. TGbRK is rod-specific bovine GRK1 expression in wild-type mouse, whereas TGbRK700−/− is TGbRK expression in GRK1−− background to remove the endogenous GRK1 protein.
Figure 3.8

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<table>
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</table>
Figure 3.9. K216R transgenic analysis with RT-PCR. (+) indicates the confirmation of transgene transmission by PCR. RNA are purified from mouse retina using the Trizol reagent. cDNA synthesis is done with Superscript III. PCR is performed on the cDNA with bRK (RH1.1 and RKb1) and bRKnew (Prm1B and bRK-CDGLRTH) to detect the bovine GRK1 transgene. bRK and bRKnew detect presence of the 5’ and 3’ ends of the transgene, respectively. Out of the four K216R lines, line 2 did not transmit (not shown), whereas line 3 stop transmitting after few litters. Line 1 showed transgene expression on the 5’end, but not the 3’end, which is then considered a non-expresser. Line 4 showed robust transgene expression was mated in GRK⁻⁻ background to study its protein expression.
Figure 3.9
Figure 3.10. V380D transgenic analysis with Western Blot and RT-PCR. Analysis performed in the same protocol as previous figure. Out of the ten V380D transgenic lines, 6 transmitted their transgenes. (A) Western analysis of V380D line 1 and 5 in WT background. Immunoblots are probed with G8 and A4102 for total GRK1 and p-GRK1, whereas CT215 and Actin controls for protein loading control. Line 1 shows an increase in its GRK1 expression in the genotyping positive animal (although no upper band was observed). Line 5 does not show any increase in GRK1 protein in transgenic positive animals. 95SIL is a control retina for transgenic GRK1 expression of the S561L mutant, which has ~10 fold increase in GRK1 expression. RNA analysis are performed on line 1 and 2 (B), 3 and 5 (C), and 6 and 7 (D). Line 1 and 5 show a robust RNA detection on the 5’ and 3’ end of the transgene. Line 3, 6 and 7 are terminated. GNAT1 WT primer set is used as a control for DNA contamination. Line 1 and 5 are mated into GRK1−/− background to assess their protein expression in rod photoreceptors.
<table>
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<tr>
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<td>P388H+ RNA</td>
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**Figure 3.10**
Figure 3.11. STAA transgenic analysis with Western Blot and RT-PCR. Analysis performed in the same protocol as previous figures. Out of the ten STAA transgenic lines, 5 transmitted the transgene. Western analysis are performed on STAA line 1 and 5 (A), and 3 and 6 (B) to assess GRK1 overexpression. No significant increase in GRK1 expression is observed in any of the lines, when compared to 95SIL, TGbRK and TGbRK700--. (C) RNA analysis are then performed on line 1, 2, 3, 6 and 7 to assess the RNA expression of the transgene. Base on RNA analysis, line 2 and 7 show consistent expression in both the 5’ and 3’ end of the transgene. Line 1, 3 and 6 breeding are terminated. Line 2 and 7 are mated into GRK1-- background to assess their mutant expression in rod photoreceptors. GNAT1 is used to control for DNA contamination.
Figure 3.11
Figure 3.12. Western analysis on the retina of C558S transgenic animals.
Genotypes were confirmed by PCR. Retinas were extracted in 1X PBS and solubilized with 2X LSB (A) Western analysis of C558S positive mice in WT background. GAPDH and CT215 are loading controls for G8 and A4102, respectively. C558S+ animals have a upper running band compared to WT, confirming the expression of bovine GRK1 transgene. (B) Quantification of panel A. C558S expression is two fold over its transgenic WT littermates and ~1.5 fold less than TGbRK. However, the autophosphorylation levels are reduced in C558S mutant when normalized to total GRK1 content.
Figure 3.12
However, the autophosphorylation of C558S mutant is reduced compared to WT (A4102/G8), which implies isoprenylation may affect GRK1 kinase activity.

By removing the endogenous mouse GRK1, the expression of mutant GRK1 expression are isolated and further characterized (Figure 3.13). In GRK1\(^{+/−}\) background, V380D (Figure 3.13A), STAA (Figure 3.13B and C), and C558S (Figure 3.13E) have protein expression. V380D and C558S are discussed further in later sections. STAA line 5 does not have any expression and its mating is discontinued (Figure 3.13A). STAA lines 2 and 7 have protein expression, as expected from the RNA expression (Figure 3.13B and C; Figure 3.11). However, STAA line 2 displayed a mixed expression profile, as few genotype positive STAA animals have no mutant expression (Figure 3.13B). Integration of STAA transgene in line 2 must have occurred at two separate regions, where one of them is silenced. This line is terminated because it is difficult to isolate the pure expresser from the non-expresser. Also, STAA line 7 has mutant expression at 15% of GRK1\(^{+/−}\) rods, which implies ~7.5% of the WT animals (Figure 3.13D). The low expression of STAA can be attributed to the low copy number of the transgene or the instability of GRK1 due to the loss of autophosphorylation. By comparing the protein to the RNA level in the transgene, the relative stability of GRK1 mutant can be compared to WT level. Unfortunately, the TGbRK700\(^{+/−}\) animal was lost during this study, which prevents the comparison of the mutant bovine GRK1 to the WT bovine GRK1. Furthermore, the endogenous WT GRK1 RNA level cannot be compared to the transgene RNA level due to their different promoters. Mouse GRK1 is under the endogenous GRK1 promoter, whereas the 4kb rhodopsin promoter drives transgene GRK1 expression. Under the different promoter, the WT and transgene GRK1 DNA transcription are not under the same regulatory elements. Therefore, the characterization of P388H and K216R transgenic lines has been discontinued. The higher GRK1 expression in V380D and C558S animals (Figure 3.13E) facilitates their characterization in GRK1\(^{+/−}\) background to study their effects on GRK1 functions and rod phototransduction.

**Characterization of V380D line 1 in GRK1\(^{+/−}\) background (V380D)**

The GRK1 mutation, V380D, is first identified in patients suffering from Oguchi Disease (Table 3.1). Rod-specific expression of V380D GRK1 is established with the transgenic construct in Figure 3.14A. Western analysis is performed in WT, V380D, and GRK1\(^{+/−}\) retina to ensure no changes in other phototransduction components (GC, PDE\(α\), RGS9-1, G\(β5\)-L, and
Figure 3.13. Western analysis on transgenic GRK1 mutants in GRK1\textsuperscript{-/-} background. (A) Immunoblot was performed on retinal extracts in V380D\textsuperscript{-/-} line 1 and STAA\textsuperscript{-/-} line 5 with G8 (GRK1), CT318 (RGS9-1), and GAPDH. GRK1 mutant expression is detected in V380D\textsuperscript{-/-} line 1, but not in STAA\textsuperscript{-/-} line 5. The doublet formation of V380D protein is different from the WT GRK1 in GRK1\textsuperscript{+/-}, suggesting the Oguchi mutation may affect the PTM of GRK1. Reduction in RGS9-1 is seen in STAA\textsuperscript{-/-} retina, suggests that line 5 has no expression and acts like GRK1\textsuperscript{-/-} to result in degeneration. (B) Immunoblot analysis on the retinas of STAA\textsuperscript{-/-} line 2 animals demonstrates STAA has low GRK1 expression (*). However, the expression is mixed as some of the genotype positive animals do not express the protein. (C) Protein expression of GRK1 STAA in STAA\textsuperscript{-/-} line 2 and 7. Since line 7 has GRK1 expression, line 2 mating is terminated as its expression is mixed. Immunoblot is performed with A4101NP antibody, but no protein expression is detected in the STAA retina. This is due to the loss of antibody recognition as STAA mutation abolishes the epitope for the A4101NP antibody. There is non-specific band from A4101NP (**). (D) The quantification of the immunoblot in C. STAA expression in line 7 (~15%) is comparable to line 2 (~23%), normalized to GRK1 level in GRK1\textsuperscript{+/-} retina (~50% to WT, data not shown).
Figure 3.13
Figure 3.14. Characterization of V380D mutation in GRK1<sup>−/−</sup> background. (A) the V380D transgenic construct includes the rhodopsin promoter, followed with bovine GRK1 cDNA harboring the mutation, terminating with a protamine polyadenylation signal. Transgenic V380D animals are mated into GRK1<sup>−/−</sup> background to remove the endogenous GRK1 protein. (B) The immunoblot on WT, V380D, and GRK1<sup>−/−</sup> retinal extracts probed with the following antibodies, K285 (anti-GC, 1:5k), PA1-720 (anti-PDE6α, 1:1k), G8 (anti-GRK, 1:1.5k), CT318 (anti-RGS9, 1:5k), CT215 (anti-Gβ5, 1:5k), GAPDH (1:40k), and DSC-Rv (anti-recoverin, 1:10k) to detect the phototransduction proteins. Note the lower expression of mutant GRK1 in V380D in the middle lane. (C) Semi-thin sections of WT, V380D and GRK1<sup>−/−</sup> mouse retina to assess their morphology. Note the normal photoreceptor morphology in V380D compared to WT, whereas GRK1<sup>−/−</sup> has distorted and reduced ONL. V380D expression can prevent the degeneration seen in GRK1<sup>−/−</sup> retina.
A. 

B. 

C. 

Figure 3.14
recoverin) (Figure 3.14B). Note the decrease in GRK1 expression in V380D compared to WT. Semi-thin plastic section of WT, V380D, and GRK1<sup>−/−</sup> demonstrates that V380D expression can prevent the photoreceptor degeneration seen in GRK1<sup>−/−</sup> (Figure 3.14C). The degeneration in GRK1<sup>−/−</sup> retina has reduced nuclear layer, and distorted IS and OS. The restoration of photoreceptor morphology in V380D suggests that the mutant GRK1 is functional.

Quantitatively, GRK1 expression in V380D is 40% compared to WT level, whereas autophosphorylated GRK1 in V380D is 20% when normalized to total GRK1 protein (Figure 3.15A). Altogether, V380D is calculated to have 8% catalytic active GRK1 compared to WT. The reduced autophosphorylation in V380D implies the mutant has defective activity. With IHC on retinal cross sections, V380D mutant is mislocalized throughout the photoreceptor (Figure 3.15B, left panel), whereas the autophosphorylated-GRK1 is only found in the ROS (Figure 3.15B, right panel). While V380D mutation renders GRK1 partially defective, the residual functional kinase can still be properly targeted to the ROS as demonstrated by IHC. To test whether V380D can properly deactivate R*, rhodopsin phosphorylation assay is performed with the phospho-rhodopsin antibody, α-p334, on mouse section (Figure 3.15C). Like WT, V380D mutant can phosphorylate R*, as detected by α-p334 antibody, in a light-dependent manner. Furthermore, light-dependent R* phosphorylation is abolished in GRK1<sup>−/−</sup> animal as expected. These evidences confirm that the residual functional V380D GRK1 is properly localized to ROS and can function to terminate R* signaling.

To further assess the functionality of V380D rods, ERG is performed on dark-adapted mouse (Figure 3.16). Overall, no gross differences in the ERG A and B waves are observed between WT and V380D animals (Figure 3.16A and B). By analyzing the A wave parameters, effective delay (t<sub>eff</sub>), amplification (A) factor, and the A wave amplitude are determined (Figure 3.16C). The t<sub>eff</sub> is a fitted parameter which indicates the time delay between the flash and the negative deflection of the A wave response. The A factor is the measurement of the amplification events during the activation phase of phototransduction. WT and V380D have t<sub>eff</sub> of 2.79 and 1.72 ms, A factor of 424 and 361 s<sup>−2</sup>, and A wave amplitude of 424 and 361 µV, respectively. Only t<sub>eff</sub> shows a statistical difference between WT and V380D. It is not clear how the V380D mutation in GRK1 can lead to shorter delay in A wave response and further study is required to understand this change in photoreceptor property. B wave analysis determines the implicit time
Figure 3.15. V380D expression and function in mouse rods. (A) V380D GRK1 is expressed at 40% of WT GRK1 and its autophosphorylation is 20% compared to the total GRK1. Consequently, the amount of catalytically active GRK1 in V380D is only 8% of the WT GRK1. V380D has doublet in GRK1 expression, with the upper species being the autophosphorylated form. The lower amount of autophosphorylated species may suggest a deficient in catalytic activity in the mutant GRK1. (B) IHC staining of V380D retinal sections with A4101NP (1:100, GAR-568/red) AND A4102 (1:300, GAR-568/red) antibodies. V380D has homogenous expression of the mutant GRK1 in rod photoreceptor. GRK1 expression is mislocalized and distributed throughout the photoreceptor in different compartments, whereas autophosphorylated GRK1 is only found in the OS, suggesting that the catalytically active GRK1 is properly targeted to the OS for its function. (C) To assess the function of V380D in rods, light-dependent phosphorylation of R* by GRK1 is tested with the phospho-rhodopsin antibody, α-p334 (1:100, GAR-568/red), on IHC. Light-dependent phosphorylation of R* is observed in V380D after 15 minutes light exposure, similar to WT GRK1. This phosphorylation event is specific to GRK1 as no phosphorylation is detected in GRK1-/- animal. GRK1 harboring V380D has residual functional kinase that is able to localize to OS and function to deactivate R*. V380D animal also has a transgene for cone-specific GFP expression. DAPI staining is applied at 500 ng/mL for nuclear staining. Confocal images are taken at 63X magnification with a scale bar of 10µm.
Figure 3.15

A.

<table>
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<tr>
<th>WT</th>
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</tr>
<tr>
<td>p-GRK1</td>
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<tr>
<td>total-GRK1</td>
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</tr>
</tbody>
</table>

B.

- WT (n=3)
- V380D (n=3)

C.

- Dark
- Light

* Student test; p < 0.05
Figure 3.16. Scotopic ERG on dark-adapted WT and V380D mice. (A) Representative waveforms of the ERG responses from WT and V380D mice at four different intensities: -2.78, -1.23, -0.03, and 1.4 cd S/m². Overall, the ERG responses are similar between WT and V380D. (B) The representative traces of the A wave analysis between WT and V380D. The initial falling phase of the A wave responses from -1.23, -0.81, -0.40, and -0.03 cd S/m² are normalized to the 1.4 cd S/m² response and fitted with the equation $f(x) = \exp(-0.5*\phi*A*(x-t_{eff}))^2$, the Lamb and Pugh model. Fits are performed with IgorPro 5.0 software. The A wave parameters, amplitude, effective delay ($t_{eff}$), and amplification (A) factors are summarized in (C). The A wave amplitude at 1.4 cd S/m² is 424 and 361 µV for WT and V380D, respectively. The $t_{eff}$ is 2.8 and 1.7 ms for WT and V380D, respectively, which is significantly different. The reason for this difference requires further characterization. The A factor is 16 and 19 s⁻² for WT and V380D, respectively. The statistics are performed in SigmaPlot 7.0 and significance is established with $p<0.05$. (D) B wave analysis on the ERG responses from WT and V380D mice. There are slightly changes in the B wave amplitude and implicit time at the dim flashes, which may suggest some photoreceptor differences and require further characterization. However, overall, the ERG responses are grossly normal and V380D can respond in timely manner.
Figure 3.16
Figure 3.16
C. **A Wave Parameters**

- **Effective Delay (ms)**
  - **WT (n=6)**: 2.79±0.07
  - **V380D (n=6)**: 1.72±0.24*

- **A Wave Amplitude (µV)**
  - **WT**: 424±31.3
  - **V380D**: 361±20.9

- **A Factor (s⁻²)**
  - **WT**: 16.0±1.43
  - **V380D**: 19.2±2.56

* *-test: P<0.05

---

**Figure 3.16**
D. B wave

![Graph showing implicit time and amplitude for WT and V380D](image)

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* P < 0.05

Figure 3.16
and amplitude, of the responses from bipolar cells (Figure 3.16D). The implicit time, the time required for maximum B wave response, is faster in V380D than WT animals at the low and high intensities. On the other hand, the B wave amplitude of V380D is reduced at the lower intensities. It is unknown how these minor changes in B wave properties arise. It is possible there are slight changes in photoreceptor and bipolar cell properties due to the V380D mutation. Overall, the ERG responses between V380D and WT animals are similar.

**Characterization of C558S line 2 in GRK1−/− background (C558S)**

The requirement of isoprenylation for GRK1 membrane association has been established in cell culture. However, the regulation of isoprenylation on GRK1 has not been established in-vivo. Using the rhodopsin promoter, the C558S GRK1 mutant is expressed in mouse rod photoreceptor (Figure 3.17A), without affecting the expression of other phototransduction proteins. Quantification analysis reveals that C558S GRK1 is expressed at 82% compared to WT level; however, the autophosphorylation status of C558S is reduced to 21% when normalized to total GRK1 protein (Figure 3.17B). Furthermore, IHC on C558S retinal sections with A4101NP and A4102 antibodies shows that the expression of C558S mutant is not homogenous. Qualitatively, the expression is in ~60% of the photoreceptors (Figure 3.17C). The expression is rod-specific, as the GRK1 signal does not overlap with PNA, a cone-specific marker. Furthermore, the GRK1 staining is distributed throughout the photoreceptor in C558S sections (Figure 3.17C), suggesting that GRK1 without isoprenylation is mislocalized and cannot be targeted to the ROS.

**GRK1 localization in WT, V380D, and C558S retinas**

To test the effect of the mutations on GRK1 localization, the membrane and cytosolic contents are isolated from light-adapted WT, V380D, C558S, and GRK1−/− retinas (Figure 3.18). The indication of proper separation between membrane and cytosolic contents is confirmed by RGS9-1 and GAPDH. RGS9-1 is a membrane protein, whereas GAPDH is completely soluble. In WT retina, GRK1 is mostly membrane-bound (Figure 3.18A). V380D mutant localization is evenly distributed between the membrane and cytosolic fractions, indicating a defect in membrane association compared to WT GRK1 (Figure 3.18A). In contrast, C558S mutant is completely soluble and cannot localize to the membrane, confirming the role of isoprenylation in GRK1 membrane association (Figure 3.18B). In addition to membrane fractionation, rod outer
Figure 3.17. Characterization of C558S mutant in GRK1<sup>−/−</sup> background. (A) The immunoblot on WT, C558S, and GRK1<sup>−/−</sup> retinal extracts probed with the following antibodies: K285 (anti-GC, 1:5k), PA1-720 (anti-PDE6α, 1:1k), G8 (anti-GRK, 1:2k), A4101NP (anti-GRK1, 1:2k), A4102 (anti-pGRK1, 1:5k), CT318 (anti-RGS9, 1:5k), CT215 (anti-Gβ5, 1:5k), GAPDH (1:40k), and DSC-Rv (anti-recoverin, 1:10k) to detect the phototransduction proteins. Note the difference in the affinity of G8 and A4101NP in detecting the lower band of GRK1. (B) The quantification in GRK1 expression between WT and C558S. C558S is expressed at 82%, but autophosphorylation is reduced to 21% when normalized to total GRK1. This may imply that isoprenylation is required to fully activate GRK1. (C) IHC staining of C558S and GRK1<sup>−/−</sup> sections with A4101NP and A4102 antibodies. Without isoprenylation, GRK1 is mislocalized and distributed throughout the IS, cell body, and the synaptic terminal of rod photoreceptors. However, the expression of C558S transgene is not homogenous, but ~50% of the retina by estimation. GRK1 isoprenylation is required for GRK1 membrane association in ROS.
Figure 3.17
Figure 3.17
Figure 3.18. The localization of GRK1 mutants in V380D and C558S. (A, B) Retinas are homogenized and their membrane and cytosol contents separated with ultracentrifugation. In (A), the partition between WT and V380D is compared. Majority of the WT GRK1 is localized to the membrane, whereas V380D GRK1 has a equal distribution between membrane and soluble fraction. The reduced membrane association by V380D mutation suggests a defect in protein distribution. The complete partition of membrane and soluble fractions is confirmed with RGS9-1 and GAPDH localization. RGS9-1 is found to be all membrane-bound, whereas GAPDH is soluble. In (B), the partition between WT, C558S and GRK⁻/⁻ retina is compared. C558S GRK1 is found completely in the soluble fractions, confirming the role of isoprenylation for GRK1 membrane association. T: total extract, P: pellet fraction, S: soluble fraction. (C, D) To determine the protein localization in ROS, ROS is isolated from mouse retinas using the sucrose gradient, although not at complete purification. Comparing WT and V380D in C, OS contains both the WT and V380D GRK1. The V380D protein in OS further confirm the ability of the mutant GRK1 to localize to the OS and deactivate R*. On the other hand, comparing WT and C558S in D, no GRK1 is detected in the OS of the C558S photoreceptor. This further solidifies the role in isoprenylation in localizing GRK1 to the OS membrane. RGS9-1 presence and the lack of GAPDH confirm the purification of OS. T: total extract, P: pellet fraction, OS: purified outer segment fraction.
Figure 3.18
segments (ROS) are isolated to determine the proper targeting of GRK1. While the ROS purification is not 100%, the isolated ROS from WT retina has a robust amount of GRK1 expression (Figure 3.18C), along with RGS9-1 expression. On the other hand, a low amount of V380D mutant is detected in isolated ROS, corresponding to the autophosphorylated GRK1 found in the ROS on IHC that can deactivate R*. (Figure 3.15A and B). For C558S mutant, no GRK1 is detected from the isolated ROS (Figure 3.18D), further confirming the role of isoprenylation in targeting GRK1 to ROS.

**Single rod recording in WT, V380D, C558S, and GRK1−/− animals**

To assess the function of the transgenic rods in the GRK1 mutant lines, we collaborated with Drs. King-Wai Yau and Lihui Cao to record the rod photoresponses using suction pipette recordings. Analysis from isolated rod recordings demonstrated similar $t_{\text{peak}}$ and sensitivity ($\sigma$) in the photoresponses between WT and V380D animals (Figure 3.19). The $\tau_{\text{rec}}$ and $\tau_D$ are 283 and 221 ms for WT rods, 1060 and 329 ms for V380D rods, and 5800 and 10700 ms for GRK1−/− rods (Figure 3.19). The recovery times in dim ($\tau_{\text{rec}}$) and bright flashes ($\tau_D$) are larger in V380D photoreceptors, due to either a reduction in GRK1 expression and/or a decrease in catalytic efficiency. However, the delay in V380D rod is not as severe as GRK1−/−, confirming that V380D mutant can phosphorylate R* to recover rod. Interestingly, the recovery of V380D rods is slower in dim flashes compared to bright flashes, suggesting that V380D GRK1 activity is dependent on the flash intensities.

On the other hand, the recovery time constants, $\tau_{\text{rec}}$ and $\tau_D$, of C558S rods are severely prolonged to 5700 and 7900 ms (Figure 3.20A and B), respectively. While C558S mutant expression is not homogenous (Figure 3.17C), it is possible that some of the rods recorded do not have any mutant GRK1 expression. By recording enough rods in C558S retina, responses from rods with and without the mutant protein are obtained. Comparing all the rods recorded, no variations in photoreceptor recovery is observed; therefore we conclude that rods with C558S mutant expression respond similarly to GRK1−/− rods. The severe recovery delay in C558S rods corresponds with the absence of GRK1 in ROS as the lack of isoprenylation prevents GRK1 membrane association.

**Recovery analysis of V380D and C558S animals**
Figure 3.19. Single-rod recordings for WT, V380D, and GRK1<sup>−/−</sup>. (A)
Representative waveform of flash responses from WT, V380D and GRK1<sup>−/−</sup> rods. Qualitatively, V380D rod recovery is delayed compared to WT, but not as severe as GRK1<sup>−/−</sup>. The $\tau_{\text{rec}}$ (dim flash recovery time constant) and $\tau_D$ (dominant recovery time constant) are 283 and 221 ms for WT rods, 1060 and 329 ms for V380D rods, and 5800 and 10700 ms for GRK1<sup>−/−</sup> rods. The recovery of V380D is severely delayed in dim flashes compared to bright flashes, which can be attributed to recoverin occupying most of the mutant GRK1 since its level is low (~8% active V380D) compared to WT GRK1. The sensitivity between WT and V380D is unaffected.
Summarized data of all the RK mutants

<table>
<thead>
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<th></th>
<th>$t_{\text{peak}}$ (ms)</th>
<th>$t_{\text{int}}$ (ms)</th>
<th>$\tau_{\text{rec}}$ (ms)</th>
<th>$\tau_D$ (ms)</th>
<th>$\sigma$ (photons $\mu$m$^{-2}$ s$^{-1}$)</th>
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<tr>
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<td>213 ± 22</td>
<td>468 ± 60</td>
<td>283 ± 35</td>
<td>221 ± 10</td>
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<td>V380D$^{-/-}$ (n=16)</td>
<td>188 ± 5.3</td>
<td>1250 ± 42 *</td>
<td>1060 ± 61 *</td>
<td>329 ± 5.5 *</td>
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<td>7300 ± 1100</td>
<td>5800 ± 800</td>
<td>10700 ± 2700</td>
<td>33 ± 8.0</td>
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- SEM
- * indicated significant difference bet group 1 and 2; All the characters in group 3/4 are significantly different with wt except $\sigma$. Group 3 and 4 are not significantly different.

Figure 3.19
Figure 3.20. Recovery defect of C558S mutant. (A) Single rod recordings on WT, C558S, and GRK1\(^{-/-}\) animals. Representative traces reveal that C558S has severe delay in recovery, similar to GRK1\(^{-/-}\), suggesting that C558S is non-functional since GRK1 without isoprenylation cannot be transported to ROS to deactivate R*. (B) Rod kinetics across WT, C558S, and GRK1\(^{-/-}\). The \(\tau_{\text{rec}}\) and \(\tau_{\text{D}}\) in C558S is severely prolonged to the level of GRK1\(^{-/-}\), while the sensitivity of their rods are similar to WT. (C) Recovery assay with ERG is performed with C558S and GRK1\(^{-/-}\) after an initial flash of 1.4 cd S/m\(^2\). Recovery is measured with another 1.4 cd S/m\(^2\) flash after 15, 30, 45 minutes of dark-adaptation. (D) The B wave recovery is plotted against dark-adaptation time after first flash. C558S recovery time course is similar to GRK1\(^{-/-}\), suggesting C558S, while still retain some kinase activity, cannot deactivate R* in the ROS.
A.

WT

C588S<sup>-/-</sup>

GRK1<sup>-/-</sup>

B.

Summarized data of all the RK mutants

<table>
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<tr>
<th></th>
<th>$t_{\text{peak}}$ (ms)</th>
<th>$t_{\text{int}}$ (ms)</th>
<th>$\tau_{\text{rec}}$ (ms)</th>
<th>$\tau_D$ (ms)</th>
<th>$\sigma$ (photons $\mu$m&lt;sup&gt;2&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;)</th>
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<td>7300 ± 1100</td>
<td>5800 ± 800</td>
<td>10700 ± 2700</td>
<td>33 ± 8.0</td>
</tr>
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- SEM
- * indicated significant difference bet group 1 and 2; All the characters in group 3/4 are significantly different with wt except $\sigma$. Group 3 and 4 are not significantly different.

Figure 3.20
Figure 3.20
While it is clear that V380D animals can respond to light under scotopic ERG conditions and its rods have recovery delays on the single cell resolution, the question remains on how V380D mutation in GRK1 causes the night-blinding phenotype in Oguchi Disease. Under scotopic condition for ERG, each stimulus only bleaches a small percentage of rhodopsin. However, in human Oguchi patients, the delay in dark-adaptation time is observed when the subjects transition from light environment into dark condition where a large percentage of R* needs to be deactivated and regenerated. To test the ability for the mouse to dark-adapt, the time needed for photoreceptors to recovery after exposure to bleaching light is measured (Figure 3.21). WT animals are dark-adapted overnight and their retinas extracted and exposed to the background light in the LKC UTAS-3000 ERG machine for 0, 1, 2, 5, 10 minutes. The free rhodopsin contents are measured at 498 nm, the $\lambda_{\text{max}}$ of unbleached rhodopsin, and plotted against exposure times (Figure 3.21A). It is determined that 50% of the rhodopsin are bleached by two minutes of background light exposure, which is the time used in the subsequent experiment for rhodopsin bleaching before dark-adapting the animals (Figure 3.21E). To obtain a baseline (pre-bleach), ERG response is recorded from dark-adapted animals with a 1.4 log cd S/m² (10db) flash. Then the background light is turn on for two minutes. After turning off the background light, recovery from the 50% bleach is measured with the same 1.4 log cd S/m² flash at various time points. For WT animals, recovery is reached by 10 minutes of dark-adaptation, whereas V380D animals recover slower, restoring only 74% of the pre-bleach amplitude at one hour of dark-adaptation (Figure 3.21B). This delay in recovery recapitulates the dark-adaptation defect in Oguchi patients. In comparison, photoreceptors without GRK1 cannot recovery beyond 15% of the pre-bleach amplitude, which most likely derives from cones since their recovery is faster than rods. For V380D animals, the delay in dark-adaptation is due to the low level of the active GRK1. R* phosphorylation becomes limiting as the V380D mutant has only 8% active GRK1. To further confirm this hypothesis, recovery time course was recorded from Bark7 and 95Bark7 animals, which have only 15% GRK1 expression (Chen, Woodruff et al. 2010). The recovery in Bark7 and 95Bark7 animals are similarly delayed as observed in V380D animals (Figure 3.21C), further suggesting that R* cannot be deactivated in a timely fashion when a high concentration of R* and a low amount of active GRK1 are present. As a control, the pre-bleach A wave amplitudes are similar in all the animals tested (Figure 3.17D), except for GRK1$^{-/-}$. The reduced A wave response in GRK1$^{-/-}$ animals has been reported and is due to the photoreceptor
Figure 3.21. Dark adaptation experiment. (A) Dark-adapted WT retinas are extracted and exposed to the background light on the LKC UTAS-3000 ERG machine to assess the rhodopsin bleaching time course. Unstimulated rhodopsin is measured by the absorbance at 498 nm. Completed bleached rhodopsin measurement is established with 15 minutes light exposure and subtracted from each measurement. $A_{498}$ is measured at time 0, 1, 2, 5, 10 minutes. Result shows that at 2 minutes, 50% of rhodopsin is bleached. This time exposure is used in the subsequent experiment as the rhodopsin bleaching time before recovery. (B) The time course to assess the recovery of rod photoreceptor after 50% bleach by background light. Animals are dark-adapted overnight and a pre-bleach flash at 1.4 log cd S/m$^2$ is obtained for unbleached A wave response. Background light is applied for 2 minutes to reach 50% bleach. After the background light is removed, the recovery is probed by a test flash of 1.4 log cd S/m$^2$ at 1, 2, 5, 10, 15, 30, 45, and 60 minutes. The ERG A wave is used to measure the recovery of rods and normalized to pre-bleach flash. Comparing WT, V380D, and GRK1$^{-/-}$, WT recovers from bleach around 10 minutes after dark-adaptation, whereas V380D only recovery ~75% after 1 hour. GRK1$^{-/-}$ has severe recovery defect after bleach. (C) Additional bleaching experiments perform on Bark7, which has 15% GRK1 expression, and 95Bark7, which has six-fold GAP overexpression and 15% GRK1 expression. Bark7 and 95Bark7 has similar recovery profile as V380D, suggesting the low amount of GRK1 causes dark-adaptation delay. (D) The dark-adaptation A wave amplitude across different genotypes. All strains have similar A wave amplitude ~420 µV, except for GRK1$^{-/-}$ due to rod degeneration. (E) Representative recovery profile in all genotypes.
Figure 3.21
Figure 3.21
degeneration caused by the absence of GRK1. The delay in dark-adaptation caused by V380D mutation provides a mechanism for the night-blindness in Oguchi patients.

Next, the photoreceptor recovery in C558S animals is measured with ERG. Since single cell recordings demonstrate that photoreceptors with C558S mutant recover similar to GRK1<sup>−/−</sup> photoreceptors, we tested the ability of C558S animals to recovery from a single bright flash. (Figure 3.20C and D). Dark-adapted animals are stimulated with a 1.4 log cd S/m² flash and recovery is assessed at different time point after dark-adaptation. As seen in Figure 3.20D, C558S photoreceptor recovery is similar to that of GRK1<sup>−/−</sup> animals, suggesting that GRK1 without isoprenylation is unable to terminate R* signaling due to its inability to target to the ROS.
Discussion

Oguchi Disease is a type of congenital stationary night blindness (CSNB), characterized by a delay in dark-adaptation lasting more than three hours. Mutations in GRK1 and arrestin genes have been identified in Oguchi patients. Since GRK1 and arrestin are involved in deactivating R*, it is conceivable that defects in R* phosphorylation and arrestin binding result in prolonged R* signaling and delayed recovery. The recovery defect is observed in mouse photoreceptors lacking GRK1 or arrestin, confirming the pathogenicity of GRK1 and arrestin mutations in Oguchi Disease. However, the recovery delay in GRK1−/− rods and cones suggests that GRK1−/− animal is completely blind, which is not consistent with the night-blindness phenotype in Oguchi Disease. It has been found that GRK7, an ortholog of GRK1, is expressed in the cones all mammalian species except murine animals. Therefore, GRK7 may have redundant function to GRK1 in phosphorylating cone opsin and preserving the cone vision in Oguchi Disease. The functionality of human GRK7 in mouse cones is verified in MCV7, MCV8 and MCV78 animals in GRK1−/− background. The replacement of GRK7 over GRK1 in mouse cones restores the cone-derived B wave response, which supports that human GRK7 can phosphorylate and deactivate cone opsin. This is expected as many species such as fish and other vertebrates also express GRK7 in their cones; hence sometimes GRK7 is referred to as “opsin kinase”. Furthermore, the functionality of GRK7 in mouse cones confirms the mechanism in which Oguchi patients retain their cone function to prevent complete blindness. The cone response amplitude from MCV7−/− is 72% \( \frac{105 \mu V}{145 \mu V} \) to the response from MCV8−/−. This is due to the difference in the number of cones that have GRK7 expression between MCV7 and MCV8. With decreased S cones in the superior hemisphere of mouse retina, the GRK7 expression is correspondingly less in the superior region in MCV7. On the other hand, M cones are homogenously distributed in mouse retina to drive GRK7 expression in most of the cones. In fact, the ratio of GRK7-expressing cones between MCV7 and MCV8 is 74% \( \frac{0.35 + 0.96}{0.88 + 0.88} \), which corresponds closely to the ratio of the B wave response amplitude between MCV7−/− and MCV8−/− animals. This calculation suggests that cone response amplitude is proportional to the number of GRK7-expressing cones in mouse retina. Furthermore, the response amplitude does not increase in the MCV78−/− cones because all the cones already have GRK7 expression from
the MCV8 transgene. The expression and response profile of MCV7 and MCV8 confirm that all cones have M opsin promoter activity, whereas S opsin promoter activity is higher in the inferior than superior hemisphere. However, the response amplitude in the MCV8−/− or MCV78−/− is not restored to the WT level. It has been shown that GRK1−/− photoreceptors have shortened rods and leads to reduced A wave response. The shortened photoreceptor OS may cause the reduction in B wave response by changing the glutamate release at the cone synaptic terminals. It is also possible that in GRK1−/− background, the constant R* activation in rod photoreceptor dampens the cone responses through electrical communication by the gap junction. By putting back GRK1 in the rods of MCV78−/− animals to prevent ROS shortening, cone recordings can then be obtained to determine whether GRK7 can fully restore the cone-derived B wave responses.

The function consequences of the five GRK1 mutants are studied in ectopic expression and in transgenic mouse models. In cells culture, the mutant expression profiles are different between insect and mammalian cell lines, where some of the mutants (K216R, P388H and V380D) are stably expressed in insect cells but become destabilized in mammalian cells. The change in protein stability can be attributed to the different degradation machinery between the cell types. As mammalian cultures are closely related to the mammalian in-vivo environment, the reduced expression suggests that these mutations destabilize GRK1. Interestingly, the decreased protein expression in the kinase-dead mutant, K216R, is opposite from the reported literature, where other groups are able to observe the mutant expression. Closer inspection on the technical details points to a difference in the expression time. Other groups have expressed the mutant upward of 48-72 hours, where in this study the transfected cells are harvested at 24 hours. At longer expression time, the mammalian cells may have reached stationary phase of growth and the degradation machinery may have shut down, masking the instability of the mutant protein. At earlier time points (<24 hours), it is evident that the stability of GRK1 requires the kinase activity. Nonetheless, ectopic expression of mutant proteins has to be carefully interpreted, as the cultured cell environment may not reflect the native conditions.

To study the role of autophosphorylation on GRK1 function, we have generated the A4102 antibody to monitor the autophosphorylation level and the intrinsic kinase activity. GRK1 autophosphorylation changes significantly in the first 30 minutes of dark-adaptation, suggesting its role in modulating GRK1 activity during light transitions. To further examine the
physiological role of GRK1 autophosphorylation, the STAA mutations are introduced into
GRK1 to abolish the autophosphorylation sites. In insect cells, the STAA mutant is stably
expressed but not autophosphorylated, further confirming the specificity of A4102 antibody. As
expected, autophosphorylation requires the intrinsic kinase residue as no phosphorylation is
detected in insect cells with K216R mutant expression. Although the expression profile of these
two mutants changes when expressed in mammalian cell lines, several points can still be
concluded from the mammalian culture study. First, GRK1 may require kinase activity for
protein stability as GRK1 expression is abolished with K216R mutation. Second,
autophosphorylation by the intrinsic catalytic activity provides for partial GRK1 stability as
STAA mutation reduces GRK1 expression by ~50%. Other phosphorylation sites in GRK1 may
be present to stabilize the protein, or the catalytic residue may have non-kinase role in the
structural stability of GRK1. To further examine the role of the K216R and STAA mutations on
GRK1 function, transgenic mouse lines are generated to express these mutants in mouse rods
with the rhodopsin promoter. However, the characterization of K216R and STAA transgenic
lines are met with several obstacles. The expression of GRK1 harboring K216R and STAA
mutations is low despite the presence of transgene RNA, suggesting that kinase activity plays a
role in GRK1 stability. To quantify the instability effects of GRK1 mutants, the ratio protein
expression and RNA expression is compared between WT and GRK1 mutants. However, after
several experimentations (data not shown), the RNA level in WT GRK1 and transgenic GRK1
mutant are found to be very different. The differences in RNA level can be attributed to the
promoters that drive GRK1 expression. WT mouse GRK1 is under the endogenous GRK1
promoter, whereas rhodopsin promoter drives the expression of bovine GRK1 mutants. In fact,
the mRNA level under the rhodopsin promoter is extremely low compared to mouse GRK1
promoter. Due to this difficulty, the quantification of protein to RNA ratio is terminated in
K216R and STAA transgenic lines. Furthermore, the transgenic expression of STAA mutant in
mouse rods is low (15-23% compared to GRK+/- level) and displays a ‘mix-expression’ pattern in
GRK1+/- background. The ‘mix-expression’, where some STAA+ animals have GRK1 expression
while some do not, is one downfall in transgenic mouse model when random integration
produces two transgene integration sites where one region is genetically silenced and the other
one is active. Due to the low/no expression and ‘mix-expression’ of GRK1, the characterization
of K216R and STAA transgenic mouse lines is currently discontinued.
Next, the effects of Oguchi mutations, P388H and V380D, on GRK1 function are investigated. When expressed in insect cells, both mutants affect the catalytic efficiency as demonstrated by the loss of autophosphorylation. In COS7 and HEK293 cells, mutant kinase expression is highly reduced, similar to the kinase-dead mutant. Sequence alignment shows that P^{388} is a conserved residue cross all GRK members in the catalytic domain, implying that it is important in GRK1 kinase activity. V^{380} is unique to GRK1, whereas other GRK members have a cysteine residue in the same location. While this residue is not conserved for GRK1, the introduction of a negatively charged residue into the catalytic domain by the V380D mutation may affect its kinase domain. The downregulation of GRK1 expression in the V380D mutant is consistent with published results. On the other hand, this is the first evidence demonstrating that P388H mutation causes GRK1 instability. The in-vivo effects of P388H and V380D mutations are assessed in transgenic mouse models. When crossed into GRK1^{−/−} background, no GRK1 protein expression is detected from the P388H transgene, despite high RNA level. In P388H animals, similar to K216R and STAA strains, the protein to RNA ratio is hard to determine and its characterization is terminated. It remains a possibility that the P388H mutation results in GRK1 instability and degradation, causing the rod recovery defect and the night-blindness phenotype in Oguchi Disease.

Previous study by (Khani, Nielsen et al. 1998)) has demonstrated that V380D expression in COS7 cell is highly reduced and the mutant protein is unable to phosphorylation R* in purified ROS preparation. To our surprise, V380D expression in transgenic mouse rods is expressed and is catalytically active. The V380D transgenic animals express the mutant GRK1 protein homogenously at 40% of the WT level. However, the autophosphorylation of V380D GRK1 is reduced to 20% when normalized to total GRK1 protein. Taken together, only 8% of the V380D mutant is catalytically active when compared to the WT level. These evidences confirm that GRK1 harboring V380D mutation remains catalytic active, although at reduced capacity. In addition, the low mutant expression can prevent and rescue the degeneration caused by the loss of GRK1. This observation provides an explanation on how human photoreceptors in Oguchi Disease remain viable and does not degenerate in the manner seen in mouse GRK1^{−/−} photoreceptor. Interestingly, the catalytically active kinases (autophosphorylated GRK1) in V380D photoreceptor are detected only in the ROS, whereas the defective GRK1 (unphosphorylated GRK1) is distributed throughout the photoreceptor. The residual mutant
GRK1 in ROS also functions in the light-dependent phosphorylation of R*, contrary to the published data described above. This difference is most likely due to the different protein regulation between COS7 cell and mammalian photoreceptor. Further physiological comparison demonstrates that WT and V380D animals have similar ERG responses, confirming that V380D photoreceptors can respond properly in fully dark-adapted state. As expected from the reduced activity and expression in V380D animals, the recover of rod photoreceptor is delayed compared to WT. In dim flash, the recover of V380D photoreceptor is delayed by ~3.75 fold, whereas in bright flash, the delay is only ~1.5 fold. The delay is more pronounced in dim flash condition because the regulation of GRK1 by recoverin. Under dim flash condition, the Ca^{2+} change is minimal and GRK1 remains associated to recoverin. As there are only 8% active GRK1 in V380D rods, the ratio of recoverin to the mutant GRK1 is greater than the WT level. Therefore, recoverin sequesters most of the V380D GRK1 and the free GRK1 concentration is likely very low, resulting in a longer recovery delay than bright light, in which recoverin releases all of the GRK1 protein when Ca^{2+} is low. The reduced expression and activity in V380D mutant cause rod recovery to be delayed up to one second in fully dark-adapted condition, however, the dark-adaptation time (light to dark transition) in Oguchi patients is prolonged to greater than three hours. It is possible that single cell recording may not properly demonstrate the full recovery defect in V380D rods. To test the dark-adaptation ability in mouse, the time required for photoreceptors to recover from 50% bleached rhodopsin is measured. After light exposure for two minutes, WT animals can fully dark-adapt by 10 minutes, whereas V380D animals need more than one hour to recovery. Further extrapolation from this data suggests that if rhodopsin is bleached close to 100%, V380D can take more than two hours to fully dark-adapted, which is similar to the recovery defects documented in human Oguchi patients. In addition, a similar dark-adaptation delay is recorded from Bark7 animals, which have ~15% GRK1 expression (Chen, Woodruff et al. 2010). This confirms that reduced GRK1 expression lead to delay in rhodopsin deactivation when the R* to kinase ratio is increased. When GRK1 level is reduced and becomes limited, the kinase cannot keep up with the demand of R* phosphorylation when a large amount of R* are generated, which results in dark-adaptation defect. Biochemically, V380D has reduced activity but can still function in rhodopsin phosphorylation and main a healthy photoreceptor. Physiologically, reduce V380D mutant expression causes rod recovery delay and dark-adaptation delay.
defects. Together, these evidences provide a new mechanism in the pathogenicity of human Oguchi Disease.

Lastly, the GRK1 harboring C558S mutation is the only mutant that has consistent expression in all the cell types tested. *In-vitro* data has demonstrated that isoprenylation is required for GRK1 to localize to the membrane and deactivate R* (Inglese, Glickman et al. 1992). Isoprenylation is a PTM unique to the GRK1-family including GRK1 and GRK7, whereas other GRK members use other mechanisms for membrane association. For example, GRK2 associates with Gβγ to localize to the membrane (Ribas, Penela et al. 2007). To confirm the role of GRK1 isoprenylation in photoreceptor function, GRK1 with C558S mutation is expressed in mouse rod photoreceptors and studied in GRK1−/− background. While the protein distribution of C558S mutant is not homogenous in mouse retina, the transgenic mouse line is still useful for studying GRK1 isoprenylation. The C558S GRK1 is expressed at 82% compared to WT level and its autophosphorylation is reduced to 21% compared to total GRK1 level, which suggests that the lipid modification is not required for GRK1 activity. However, the reduced autophosphorylation in C558S mutant implies that full activation of the kinase requires isoprenylation and/or membrane association. In addition, GRK1 without isoprenylation is detected in the cytosol and is redistributed throughout the rod photoreceptor. Furthermore, C558S mutant is unable to associate with the lipid membrane and cannot be transported to ROS. Functionally, since GRK1 without isoprenylation cannot be targeted to ROS, the mutant kinase cannot deactivate R* during recovery and the C558S rods behaves similarly to the ones in GRK1−/− animals. These data confirm that isoprenylation is required for GRK1 to associate with the membrane and be targeted to the OS. Since GRK1 without isoprenylation is absent from the OS, the kinase cannot phosphorylate and terminate R* even though the mutant remains catalytically active.
Conclusion

It is evident that any conclusions drawn from ectopic expression studies must be analyzed with careful consideration. Variations in cell regulation and signaling mechanisms may influence the phenotype observed across different cell types. The conclusions from cell culture experiments must be verified in native tissues. As GRK1 is responsible for light-dependent rhodopsin phosphorylation and the timely R* deactivation in phototransduction recovery, understanding the molecular details of GRK1 function is essential as changes in GRK1 activity has significant consequences on photoreceptor recovery. Five transgenic mouse lines are generated to study the consequences of the mutations, P388H, K216R, C558S, V380D, and STAA, on GRK1 function in photoreceptors. Due to technical issues derived from transgenesis, such as mixed expression, low/no protein and RNA expression, the characterization of P388H, K216R and STAA transgenic lines are discontinued. However, the preliminary work in this thesis suggests that P388H, K216R and STAA mutations play a role in GRK1 stability.

In addition, through transgenic, biochemical, and physiological techniques, the mechanisms leading to the night-blinding Oguchi Disease are clearly described. Human GRK7 is functionally redundant to GRK1 and can preserve cone vision when GRK1 is defective in human Oguchi patients. Contrary to previous findings, GRK1 harboring V380D mutation has reduced expression and activity, and can still deactivate R* in a light-dependent manner and prevent the photoreceptor degeneration observed in GRK1−/− animals. In V380D animals, the reduced kinase activity causes a dark-adaptation delay, recapitulating the symptom observed in patients with Oguchi Disease.

Last, the role of GRK1 isoprenylation in photoreceptor function is elucidated. The C558S mutation abolishes GRK1 isoprenylation. The mutant has normal, but patchy, GRK1 expression with reduced activity. Without isoprenylation, GRK1 cannot associate with the membrane nor target to the ROS. These data suggest that isoprenylation is required for full activation of the kinase, either by the lipid modification or the docking to the membrane near rhodopsin. Due to the lack of ROS targeting, photoreceptor with non-isoprenylated GRK1 behaves like GRK1−/− photoreceptor and has severe delay in recovery. Isoprenylation is an essential modification that allows GRK1 to localize to the outer segment and exert its function in rhodopsin deactivation.
CHAPTER 4

Future Directions

It is determined that rod and cone share the similar RLS in photoreceptor recovery, as the GTP-hydrolysis of transducin/cone transducin. As demonstrated in this thesis, studying cone phototransduction is more difficult due to the rarity and fragility of cones in mouse retina. To further understand how each phototransduction player contributes to the different response kinetics between rod and cone, the cone components can be swapped into rod photoreceptor using genetic manipulations with knock-in, knockout and/or transgenic constructs. Recent study (Chen, Woodruff et al. 2010) expressing cone transducin in rod photoreceptor without rod transducin has demonstrated that the Gα subunit contribute partially to the differences in the sensitivity, amplification, and recovery kinetics between rod and cone. Therefore, it is essential to swap the opsins, Gβγ subunits, and the CNG subunits between rod and cones to further understand the underlying factors on what makes rod a rod and cone a cone.

As demonstrated by the degeneration observed in cones with high level of RGS9-1, photoreceptors are high susceptible to molecular or metabolic changes which can trigger cell programmed death. Understanding photoreceptor degeneration is an essential frontier in vision biology as 1 in 2000 people is estimated to suffer some forms of retinal degenerative diseases (Sohocki, Daiger et al. 2001), especially when there is a lack of therapy. While many degeneration models have been observed and documented in mouse and human, the molecular mechanisms leading to cell death remain elusive. Future investigation is required to monitor the pro-apoptotic pathways during photoreceptor degeneration and determine therapeutic methods in preventing or delaying cell apoptosis.

Lastly, the findings from V380D and MCV78 animals provide a new mechanism in the pathogenesis of Oguchi Disease and confirm the role of GRK7 in preserving cone functions. Also, the role of isoprenylation in GRK1 membrane translocation is confirmed in mouse rods. These results increase our understanding on the structure-function relationship of GRK1. As mentioned, the P388H, K216R and STAA mutations require further investigation. To facilitate the characterization of GRK1 mutations, knock-in constructs can be used to introduce the mutations in mouse and to study the mutants under the same regulatory elements as WT GRK1.
Future characterization of P388H can reveal the mechanism on how the mutation causes Oguchi Disease. Similarly, studying the STAA mutant can demonstrate how autophosphorylation influences the function and stability of GRK1. Also, as we have established GRK7-expressing cones in MCV7 and MCV8, any functional variations between GRK1 and GRK7 can be studied through biochemical and physiological techniques.

Through the use of genetic, biochemical, and physiological techniques, we have confirmed that cone transducin deactivation is the RLS in cone recovery. Also, a modified disease mechanism in human Oguchi patients is described in detail and further demonstrates the importance of timely opsin termination for normal vision. As we continue to study the rod/cone differences, photoreceptor degeneration, and the molecular details of phototransduction, one day we will have a complete understanding in photoreceptor biology and be able to prevent retinal diseases.
List of References


Vita

Frank Sungping Chen was born on May 17, 1982 in Oakland, California. He was brought to Taiwan by his parents when was three years old. In Taipei, He attended the Affiliated Experimental Elementary School of National Chengchi University until fifth grade. At age 11, he returned to Virginia and attended Crossfield Elementary School, Lanier Middle School and Fairfax High School. After high school, he entered college at the University of Virginia at Charlottesville, VA, where he received his Bachelor of Science in Chemistry with a specialization in Biological Sciences in 2005. In 2006, he began his graduate studies at Virginia Commonwealth University in Richmond, VA. In May 2007, he was awarded the Pre-health Post-baccalaureate Certificate Degree in Biochemistry. Afterwards, he continued to work on his Ph.D. degree in Biochemistry at Virginia Commonwealth University. In August 2011, he will continue forth to study medicine at Eastern Virginia Medical School.

Frank enjoys weight lifting, running, working on computers and traveling.