Chapter 5
Interdependence Among Members of the mGluR6 G-protein Mediated Signalplex of Retinal Depolarizing Bipolar Cells

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Abstract Normal vision depends on signaling from photoreceptors to central visual areas via parallel pathways that are optimized for detecting increments (ON) or decrements (OFF) in light intensity. The divergence of these two pathways occurs at the first synapse. The OFF pathway is mediated via Off-bipolar cells that hyperpolarize in response to light increments because they utilize ionotropic glutamate receptors. On-bipolar cells that initiate the ON pathway utilize metabotropic glutamate receptors to signal via a G-protein cascade to the transient receptor potential melastatin 1 (TRPM1) channel, and depolarize in response to light increments. Several proteins (mGluR6, TRPM1, GPR179, RGS7, RGS11, nyctalopin, LRIT3, Gα0, Gβ3, Gβ5, and R9AP) have been shown to be required for normal functioning of the depolarizing bipolar cell cascade. Here, we use immunohistochemistry in mouse models that lack one or more of these proteins to understand their interdependency. The picture

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that evolves is that of a large complex, in which the removal of any one element results in either delocalization of or decreased expression of other elements.

Light stimulation results in a graded change in sustained release of the neurotransmitter glutamate from photoreceptors. This chemical signal is converted into an electrical signal by two classes of postsynaptic bipolar cells (BCs) that either hyperpolarize (HBCs) or depolarize (DBCs) in response to a light increment. HBCs utilize ionotropic glutamate receptors and maintain the polarity of the photoreceptor signal. DBCs, in comparison, utilize the G-protein-coupled receptor (GPCR) metabotropic glutamate receptor type 6 (mGluR6) to modulate the transient receptor potential melastatin 1 (TRPM1) nonspecific cation channel, inverting the photoreceptor response. The members of this GPCR signal transduction cascade have been identified based on contributions from many laboratories. The focus of this review is to provide an overview of the interdependence of expression among these components of the DBC dendritic mGluR6 to TRPM1 signaling complex, or "signalplex," based on analyses of multiple mutant mouse lines and cascade components by immunohistochemistry.

Defects in most components of the mGluR6–GPCR cascade have been identified in humans and animal models using the noninvasive assay of retinal function, the electroretinogram (ERG). All of these mutants share the same ERG phenotype, a normal a-wave reflecting the function of the photoreceptors themselves, but the absence of a b-wave reflecting the loss of DBC function. An example of an ERG series for the Nyx^{nob}, no b-wave mouse mutant is shown in Fig. 5.1. The ERG defect indicates that nyctalopin (NYX) expression is required for normal DBC function not only in the mouse but also in human patients with complete congenital stationary night blindness (cCSNB). Other members of the DBC signalplex subsequently have been identified based on a comparable loss of the ERG b-wave. These include in mouse models the genes: *Grm6*, *Trpm1, Nyx, Gpr179, Lrit3,* $G\alpha_o$, *Gβ3, RGS7/RGS11* double knockouts, [10, 12, 14, 16, 19, 22, 23, 25, 26, 32, 34, 40, 41]; in cCSNB human patients: *NYX, GRM6, TRPM1, GPR179, LRIT3,* [1–3, 6, 11, 15, 18, 20, 21, 27, 35, 38, 39, 42, 44, 46–50] and in night-blind horse models, *Trpm1* [5].

We know that the GPCR cascade begins when a change in photoreceptor glutamate release is detected by the mGluR6 receptor and ends with the gating of the TRPM1 channel and a depolarizing response in the DBCs. How the other parts of the DBC signalplex function remains incompletely understood, in part because the detailed interaction and stoichiometry of the proteins remain to be defined. In this chapter, we describe the known interdependencies that have been inferred from immunohistochemical analyses of key components in mouse mutants where expression of one or more members of the cascade is absent. We focus in particular on the expression of proteins that form puncta at the tips of DBC dendrites, presumed to be the locus of the DBC cascade. In the case of rod DBCs, discrete puncta are visible, and we focus our analysis on these structures, which can be visualized using immunohistochemistry with confidence (Fig. 5.2). We do not include a description of

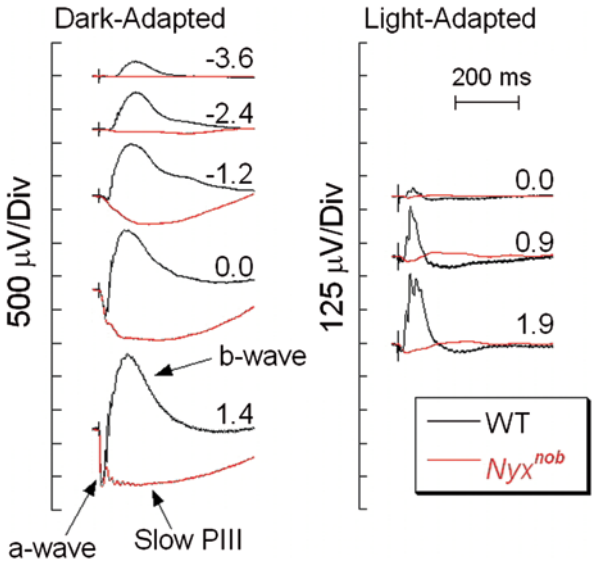

Fig. 5.1 Comparison of WT (*black traces*) and *Nyx*^{nob} (*red traces*) ERGs recorded to strobe flash stimuli presented under dark-adapted conditions (*left*) or superimposed upon a steady adapting field (*right*). WT mice generate a positive polarity b-wave that dominates the response to low luminance stimuli and follows the a-wave in response to high luminance flashes. The b-wave is absent in *Nyx*^{nob} animals. Under dark-adapted condition, the removal of the b-wave reveals slow PIII, an ERG component of Müller cell origin. Values next to each pair of waveforms indicate strobe flash luminance (log cd s/m^2)

proteins such as $G\alpha_o$ or Gβ3, which do not form distinct puncta in the OPL, rather they are expressed throughout the cell [14, 43]. This does not mean they are unimportant for DBC function, as the knockout models produced for each lack an ERG b-wave and DBC function [12–14]. Rather, this feature may indicate they interact with other signalplex components in a transient manner, or are simply not visible by immunostaining when localized to DBC puncta.

Table 5.1 summarizes the expression on the tips of the DBCs of the mGluR6–GPCR components localized to the DBC puncta that have been identified in mutant mouse models to date. Below, we review results obtained in mouse mutants for *Grm6, Trpm1, Gpr179, Nyx,* or *Lrit3* in terms of the impact of a mutant allele on the expression of other signalplex components.

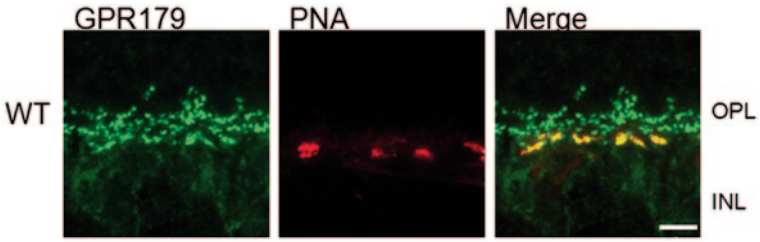

Fig. 5.2 Punctate labeling of rod DBCs in OPL of the mouse retina. Transverse section of WT mouse retina stained for GPR179 (*green*), which localizes to all DBC dendrites, and PNA (*red*) a marker for cone terminals. Note that GPR179 co-localizes with PNA. The *green puncta* in the merged image represent staining at the tips of rod DBCs

Table 5.1 Interdependence of protein expression as puncta on the dendritic tips of DBCs deduced from mutant mouse lines

Protein	Discrete puncta in OPL	*Grm 6*[1]	*Gna o*[2]	*Gnb 3*[3]	*Gng 13*[4]	*R9A P*[5]	*Gb 5*[6]	*RGS 11*[7]	*RGS 7*[8]	*RGS 7/11*[9]	*GPR 179*[nob](10)	*Nyx*[nob] (11)	*TRPM1*[tvrm27](12)	*Trpm1*[13]
mGluR6	Y	–		↓		+	+	+		+	+	+	+	+
$G\alpha_o$	N		–	↓								+		
Gβ3	N			–										
Gγ13	N			↓	–									
R9AP	Y	–		↓		–		↓			–			
Gβ5	Y	–		↓		+	–	+	↓	↓	+			
RGS11	Y	↓		↓		–		–		–	–	+		
RGS7	Y	↓				+		+	–	–	–	+		
GPR179	Y	↓								+	–	+	+	
Nyctalopin	Y	+									+	–	+	+
TRPM1	Y	↓		+						+	+	–	+	–
b-wave		–	–	↓		d	–	d	d	–	–	–	–	–
Synapse (EM)		A		A			A			N/A	N	N		N

References: 1, 2—[12]; 3—[14]; 4, 5, 6, 7, 8, 9, 10—[32, 37], Fig. 5.3; 11—[33], Fig. 5.4; 12—[31]; 13—[19, 25, 33, 40], Fig. 5.3

N normal, *A* abnormal

[a] Indicates the result from the knockout mice. Protein present (+); absent (-), or (↓) on puncta at dendritic tips of DBCs.

[d] b-wave delayed. No symbol indicates data not published.

mGluR6 Expression is Required for the Localization of Multiple Signalplex Components

mGluR6 is encoded by *Grm6*, which was established as the DBC glutamate receptor using *Grm6* knockout mice, created by gene targeting [23]. These mice lack the ERG b-wave and responses to light onset in the superior colliculus. Subsequently, two additional mutants for *Grm6* were identified: $Grm6^{nob3}$ [22] and $Grm6^{nob4}$ [34]. Immunohistochemical results are very similar in all three lines, namely mGluR6 expression is absent from the DBC terminals. Using immunoprecipitation approaches, mGluR6 has been shown to interact with TRPM1 and GPR179 [29]. Figure 5.3 shows the consequences of the absence of mGluR6 on expression of TRPM1, GPR179, RGS11, RGS7, nyctalopin, and R9AP at the tips of DBCs. The impact on the protein level using western blots is similar (Fig. 5.4), although this does vary depending on the specific laboratory. These data and those summarized in Table 5.1 indicate that mGluR6 expression is required for the correct localization of TRPM1, RGS11, Gβ5, and R9AP to the dendritic tips of DBCs [8, 9]. The latter three components are proposed to form a trimeric GAP complex because the loss of any one results in the absence or significant reduction in expression of the other two. The western blot data indicate that the loss of mGluR6 has a relatively moderate impact on GPR179 and TRPM1. In the case of TRPM1, this is because there is a large pool of TRPM1 in the other compartments of the cell, so loss of TRPM1 from the dendrites does not appear to have a major impact on total TRPM1 expression. RGS7 puncta remain in $Grm6^{nob4}$ mice [8], suggesting it is part of another complex, possibly GPR179/Gβ5/RGS7.

Localization of Other Signalplex Components is Independent of TRPM1 Expression

TRPM1 is the nonspecific cation channel modulated by the mGluR6 cascade. The first indication that TRPM1 was the channel required for DBC function came from studies in night-blind Appaloosa horses [4, 5]. In these horses, it was noted that the leopard spotting coat color and night blindness phenotypes were localized to a chromosomal region containing TRPM1. In addition, levels of *Trpm1*–mRNA expression were significantly reduced (several hundred fold) in night-blind horses, as compared to animals with normal vision. The identity of the DBC cation channel as TRPM1 was confirmed by three groups independently using knockout mice [19, 25, 40]. Their data showed that: $Trpm1^{-/-}$ mice lack the ERG b-wave and $Trpm1^{-/-}$ DBCs lack mGlur6-mediated light evoked responses. TRPM1 colocalizes with mGluR6 in DBC puncta, together strongly suggesting that it is a critical component of the DBC signalplex. However, unlike some other components of the complex, TRPM1 is expressed both in the puncta and throughout the entire DBC, where it is located in intracellular compartments [33]. In $Trpm1^{-/-}$ mice, mGluR6, GPR179, RGS11, RGS7, and nyctalopin are all expressed and normally localized (Fig. 5.3).

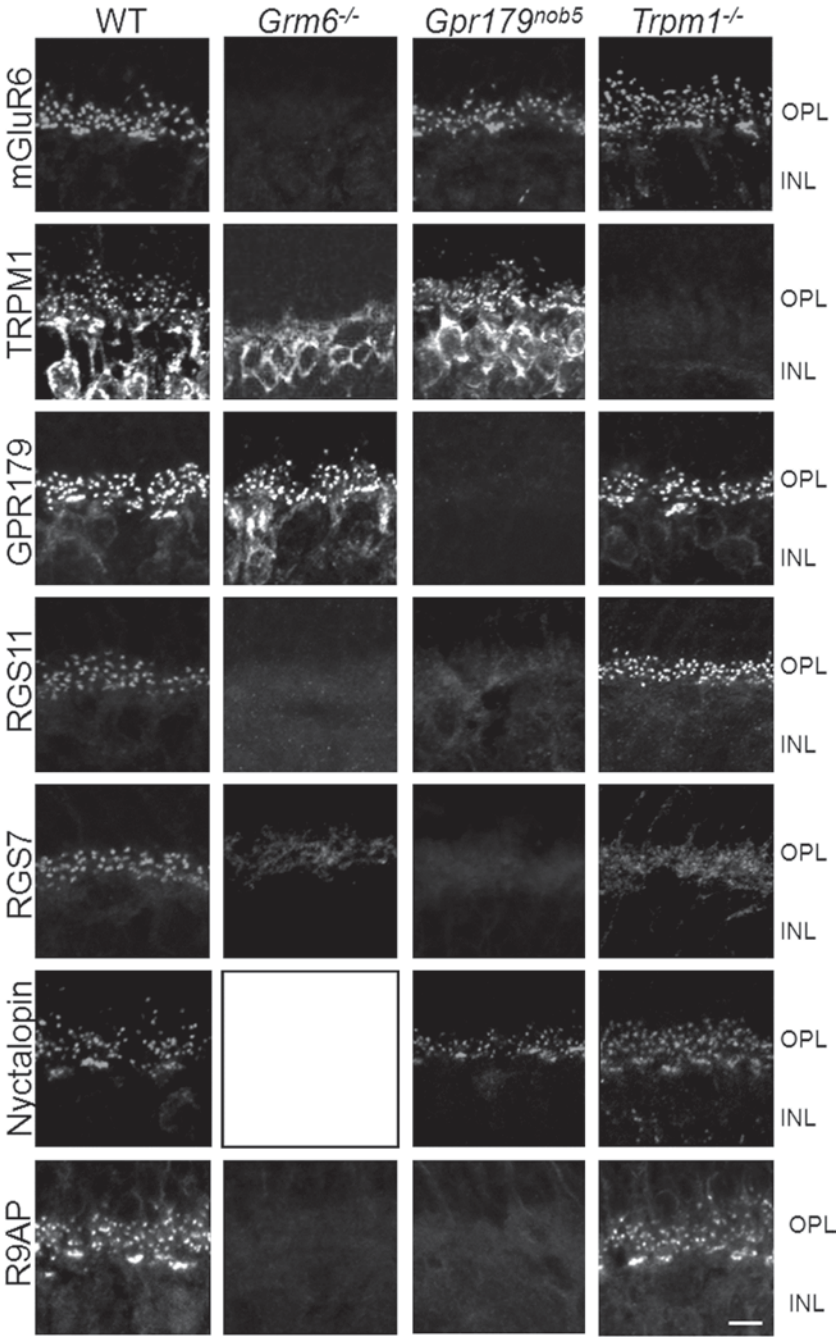

Fig. 5.3 Interdependency of DBC signalplex components revealed by immunohistochemistry in WT, *Grm6*$^{-/-}$, *GPR179*^{nob5}, and *Trpm1*$^{-/-}$mice. Staining in most panels was generated in the authors' laboratory using published methods and antibodies [28, 32, 33]. The image of TRPM1 staining in the *Grm6*$^{-/-}$mice is adapted from Xu et al. [45]. *Open box* indicates that this particular experiment has not been done

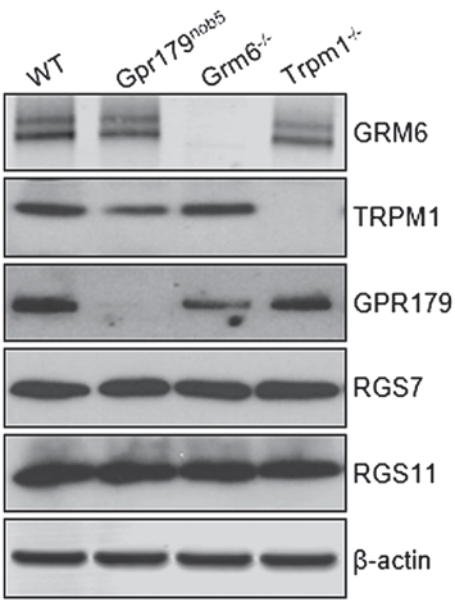

Fig. 5.4 Western blot of key DBC cascade proteins in mutant mouse lines. Western blots show the impact of eliminating three key elements of the DBC signal transduction cascade, GPR179, mGluR6, and TRPM1, on these proteins and the RGS7 and RG11 proteins critical to DBC function. In each mutant, the only protein lost is that produced by the targeted gene. Of particular note, the RGS proteins are present at near normal levels despite their abnormal localization to the DBC dendritic tips of *GPR179*^{nob5} and *Grm6*$^{-/-}$ mice (Fig. 5.2)

While the mechanism by which TRPM1 is gated remains to be firmly established (see Nawy chapter for review), the number of functional TRPM1 channels present in the DBC signalplex may be the limiting factor with respect to the maximal amplitude of the light-evoked DBC response. This conclusion was reached after using a TRPM1 mutant, *Trpm1*^{tvrm27}, resulting from an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen. The *Trpm1*^{tvrm27} mutation is caused by a missense mutation, p.A1068T, in the predicted pore region of the channel, which is presumed to cause the lack of function. As predicted, mice homozygous for the *Trpm1* ^{tvrm27} share the same ERG phenotype with other *Trpm1* knockout lines, namely the lack of a b-wave. In contrast to *Trpm1*$^{-/-}$ mice, the mutant TRPM1^{tvrm27} protein is expressed and localized correctly into puncta on the DBC dendritic tips [31].

The most significant observations from this study arose from comparisons of mice heterozygous for either the *Trpm1*^{tvrm27} or the *Trpm1* knockout allele. In *Trpm1*$^{+/-}$ mice heterozygotes, the b-wave was the same as WT controls, whereas the b-wave of *Trpm1*$^{+/tvrm27}$ heterozygous animals was about 32% smaller. Patch-clamp recordings of *Trpm1*$^{+/tvrm27}$ heterozygous rod DBCs also showed mGluR6-mediated responses that were similarly reduced, a reduction that was not seen in heterozygous *Trpm1*$^{+/-}$ DBCs. These results suggest that the p.A1068T mutation acts as a dominant negative in the tetrameric TRPM1 channel and that the channel in the *Trpm1*$^{+/tvrm27}$ heterozygous DBCs is comprised of WT and mutant subunits. The quantitative reduction of DBC function is consistent with the hypothesis that channels with 0–2 mutant subunits retain function whereas those with 3–4 mutant subunits do not, although individual combinations of mutant and WT subunits also may have different kinetics. How the number of TRPM1 channels present in DBC

puncta might be set is unclear but likely involves yet to be discovered scaffolding components of the signalplex.

Expression of GPR179 is Required for Localization of GAP Complexes to DBC Dendrites

GPR179 is a 7-transmembrane protein. Based on primary sequence data, it has been classified as a member of the GPCR superfamily. It was discovered as an important component of the mGluR6 transduction cascade independently and simultaneously from whole-exome sequencing of patients with cCSNB [2] and from mapping the gene involved in *Gpr179*^{nob5}, a naturally occurring mouse b-wave mutant [32] and it colocalizes with DBC signalplex components (Fig. 5.3). GPR179 expression localizes both RGS7 and RGS11 to the DBC terminals (Fig. 5.3 and Orlandi et al. [28]). RGS7 is likely to interact with Gβ5, as DBCs in mice lacking Gβ5 expression also lack RGS7 expression [7, 8, 24, 36]. Because RGS7 does not interact with R9AP, it is possible that its interaction with GRP179 is critical to both its localization and perhaps function in the DBC cascade. While the specific functions of GPR179 remain to be determined, it is clear that GRP179 plays a critical role in assembling elements of the mGluR6–GPCR signalplex, although both mGluR6 and TRPM1 are localized to the tips of *Gpr179*^{nob5} DBCs (Fig. 5.3). Our recently published data indicate that GPR179 sets the sensitivity of the TRPM1 channel, whereas RGS7/RGS11 sets the sensitivity of the mGluR6 cascade [37].

Nyctalopin is Required for TRPM1 Expression

In 1998, Pardue and colleagues identified a naturally occurring no b-wave mouse mutant. In this mouse, the phenotype was inherited as an X-linked trait [30] and subsequently we showed that it was caused by a deletion mutation in *Nyx* [17], the same gene that causes the X-linked form of human cCSNB [3, 35]. Nyctalopin is a member of the small leucine-rich repeat proteoglycan family of proteins. It is anchored to the cell membrane by either a single transmembrane domain or a GPI anchor in a species-dependent manner. Nyctalopin is comprised of a series of leucine-rich repeats, which are localized to the extracellular space. Efforts by several groups to make selective antibodies to nyctalopin have been unsuccessful, likely resulting from its extensive post-translational modifications. In view of this, we made a transgenic mouse line expressing an EYFP-nyctalopin fusion gene [17], which showed restricted and punctate expression of GFP to DBC terminals and colocalization with mGluR6 puncta. When these transgenic mice were crossed onto the *Nyx*^{nob} background, the expression of the EYFP-nyctalopin fusion protein restored the ERG b-wave. In addition to the absence of the ERG b-wave, results show nyctalopin also interacts with TRPM1 [9, 33]. Together, these results suggest

that *Nyx*^{nob} DBC dysfunction is due to the loss of the TRPM1 channel from the signalplex (Fig. 5.5). How nyctalopin controls TRPM1 expression and localization to the DBC dendritic tips is unclear, but could reflect a role in trafficking TRPM1 or stabilization in the DBC membrane.

LRIT3 is Required for DBC Function

LRIT3 (leucine-rich-repeat, immunoglobulin-like and transmembrane-domain 3 (LRIT3) is the most recently identified member of the mGluR6 signalplex [47]. This discovery was made by whole-exome sequence analysis of cCSNB patients who did not harbor a mutation in any of the known members of the signalplex. *Lrit3* knockout mice lack the ERG b-wave [26] supporting the results from cCSNB patients. While the function of LRIT3 is currently unknown, it is predicted to be an extracellular protein tethered to the membrane by a single transmembrane domain. The extracellular domain contains a LRR domain similar to nyctalopin. Whether LRIT3 interacts with nyctalopin via this domain will be of interest. This similarity also suggests it may be involved in trafficking and/or localization of some critical component of the DBC signalplex, similar to nyctalopin.

Interdependent Expression of RGS11, Gβ5, and R9AP

The DBC light evoked response requires the inactivation of the mGluR6 mediated G-protein cascade. Critical to this process are regulators of G-protein signaling (RGS), protein complexes that act as GTPase-activating proteins (GAPs). Two RGS proteins, RGS7 and RGS11 appear to have redundant function, as expression of both must be eliminated to produce a no b-wave ERG phenotype [10, 41]. In photoreceptors, RGS proteins form complexes with R9AP and Gβ5. In DBCs, this is true at least for RGS11, since *R9AP*$^{-/-}$ mice lack normal localization of RGS11 and reduced expression of Gβ5. Knockout of RGS7 and RGS11 or R9AP leave the primary members of the signalplex, mGluR6, TRPM1, and GPR179 localized correctly (Fig. 5.5).

General Conclusions

This review presents the analyses of a large number of knockout mouse lines using immunohistochemical approaches to examine the interdependent/independence of expression patterns of many components of the mGluR6–GPCR transduction cascade. We believe that the results suggest that the mGluR6–GPCR signalplex is comprised of several subdomains with clear hierarchies. One domain includes

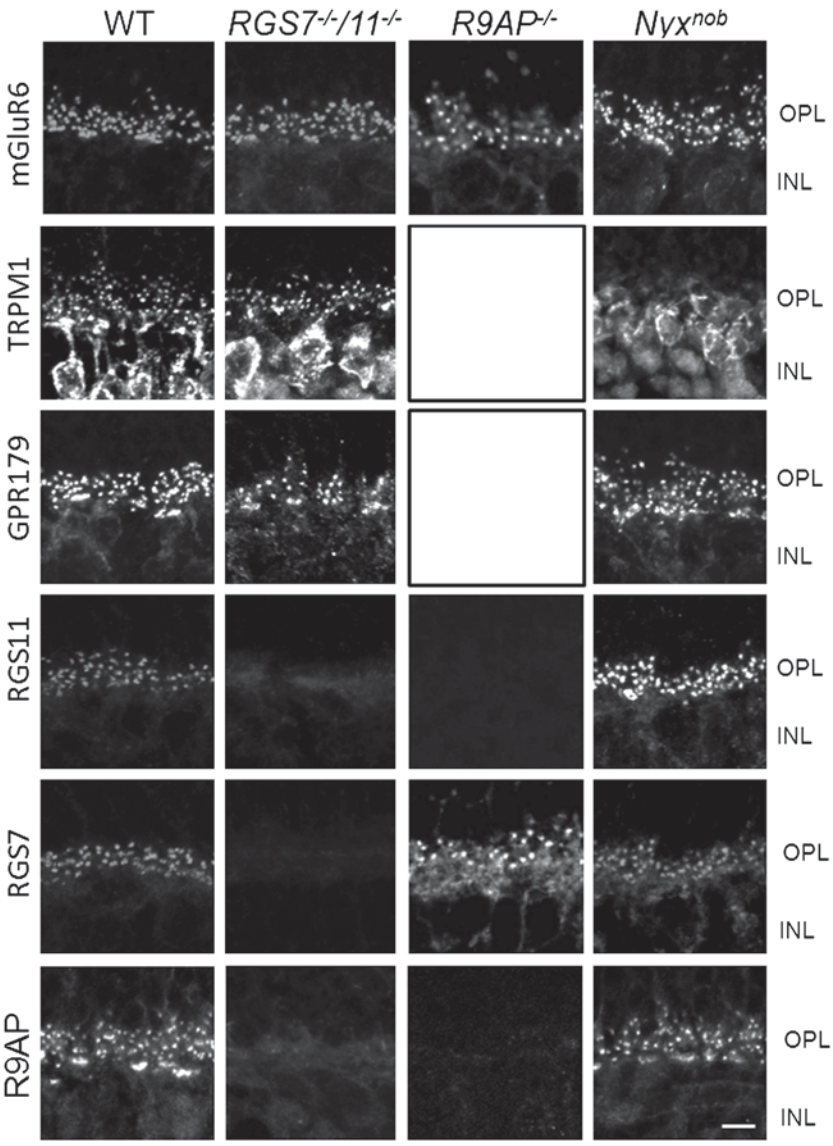

Fig. 5.5 Interdependency of DBC signalplex components revealed by immunohistochemistry in WT, *RGS7*$^{-/-}$/$^{-}$*RGS11*$^{-/-}$, *R9AP*$^{-/-}$, and *Nyx*^{nob} mice. Data for *R9AP*$^{-/-}$ mice adapted from Cao et al. [8]. Open boxes indicate that these particular experiments have not been done

mGluR6, TRPM1, R9AP, RGS11, and Gβ5, which appear to form a complex with several interdependencies, but in which expression of all members is dependent on the presence of mGluR6. A second domain includes R9AP, RGS7, and Gβ5, as the elimination of any of these has no impact on mGluR6 expression. We think that RGS7 and Gβ5 form a third complex with GPR179 and that GPR179 appears to be

a master regulator of the GAP complexes, as its absence causes mislocalization of RGS7/RGS11, R9AP, and Gβ5. Nyctalopin and TRPM1 form a separate subcomplex, and nyctalopin is essential for correct localization of the TRPM1 channel to the DBC membrane. In comparison, elimination of TRPM1 has the least impact on the expression of signalplex components, and its absence leaves the localized expression of every known component intact. This is consistent with the idea that TRPM1 gating is the final step in the DBC signal transduction process.

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