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Progesterone receptor membrane component 1 (PGRMC1) expression in murine retina

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Abstract

Purpose—Sigma receptor 1 (σ R1) and 2 (σ R2) are thought to be two distinct proteins which share the ability to bind multiple ligands, several of which are common to both receptors. Whether σ R1 and σ R2 share overlapping biological functions is unknown. Recently, progesterone receptor membrane component 1 (PGRMC1) was shown to contain the putative σ R2 binding site. PGRMC1 has not been studied in retina. We hypothesize that biological interactions between σ R1 and PGRMC1 will be evidenced by compensatory upregulation of PGRMC1 in σ RI^{-/-} mice.

Methods—Immunofluorescence, RT-PCR, and immunoblotting methods were used to analyze expression of PGRMC1 in wild type mouse retina. Tissues from σ RI^{-/-} mice were used to investigate whether a biological interaction exists between σ R1 and PGRMC1.

Results—In the eye, PGRMC1 is expressed in corneal epithelium, lens, ciliary body epithelium, and retina. In retina, PGRMC1 is present in Müller cells and retinal pigment epithelium. This expression pattern is similar, but not identical to σ R1. PGRMC1 protein levels in neural retina and eye cup from σ RI^{-/-} mice did not differ from wild type mice. Nonocular tissues, lung, heart, and kidney showed similar *Pgrmc1* gene expression in wild type and σ RI^{-/-} mice. In contrast, liver, brain and intestine showed increased *Pgrmc1* gene expression in σ RI^{-/-} mice.

Conclusion—Despite potential biological overlap, deletion of σ R1 did not result in a compensatory change in PGRMC1 protein levels in σ RI^{-/-} mouse retina. Increased *Pgrmc1* gene

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expression in organs with high lipid content such as liver, brain, and intestine indicate a possible tissue specific interaction between σ R1 and PGRMC1. The current studies establish the presence of PGRMC1 in retina and lay the foundation for analysis of its biological function.

Keywords

sigma receptor 1; sigma receptor 2; retina; mouse; sigma receptor; neuroprotection; Müller cells; progesterone receptor membrane complex 1

Introduction

Sigma receptors were originally described as opiate receptors based on psychomimetic effects induced by morphine (μ), ketocyclazocine (κ) and SKF-10,047 (σ) (1). Later studies revealed that SKF-10,047 opiate receptor properties resided in binding of (–)-stereoisomers to μ and κ receptors (2). Nevertheless, the ‘ σ ’ designation remained. Subsequent radioligand binding experiments revealed two binding sites: Sigma 1 (σ R1) and 2 (σ R2). The σ R1 and σ R2 binding activity has been detected in a wide variety of tissues including the eye. Studies performed over a decade ago used receptor binding assays to demonstrate the presence of σ R1 and σ R2 in lacrimal gland, iris-ciliary body, and retinal membranes (3-6).

The cloning of σ R1 (7-10) combined with the availability of σ R1 knockout (σ R1^{–/–}) mice have provided essential tools to elucidate its role in retina (11-13). The σ R1 protein is not known to share an homology with any other mammalian protein. It is a 223 amino acid, 25 kDa stress-activated putative molecular chaperone anchored to the ER-mitochondrial membrane with additional localizations at nuclear and plasma membranes (7, 13, 14). Although σ R1 binds to a variety of synthetic drugs, including the specific agonist (+)-pentazocine, the identities of its endogenous ligands are uncertain (7). Neurosteroids such as progesterone and the hallucinogen N,N-dimethyltryptamine (DMT) have been proposed as potential candidates (7, 15-17). In ocular tissues, σ R1 has been identified by molecular and immunohistochemical methods in retinal cells (ganglion, Müller, RPE), optic nerve head, ciliary body/iris, and epithelial cells of cornea and equatorial region of lens (18-20). In the eye, σ R1 is a potential therapeutic target. Lacrimal secretion is stimulated by σ R1 ligands (21), which might prove useful in treatment of dry eye syndrome (4). Topical administration of σ R1-site agonists lowers intraocular pressure and might provide control of ocular hypertension (22, 23). Profound retinal neuroprotection has been demonstrated using σ R1 ligands *in vitro* (24-27) and *in vivo* (28, 29).

Much less is known about σ R2. Until recently, this dearth of studies was due to a lack of molecular tools. Pharmacological studies show that σ R2 has different enantiomer selectivity, with a lower affinity for (+) benzomorphans and a higher affinity for (–) benzomorphans than σ R1 (30). The σ R2 binding site is a ~21.5 kDa protein initially isolated from PC12 tumor cells (30). Overexpression of σ R2 occurs in a variety of cancers including solid tumors and several σ R2 ligands induce tumor cell death (31-35). Aside from receptor binding assays demonstrating its presence in rabbit lacrimal gland (3) and bovine retina (6), no other reports of σ R2 have been published in ocular tissue. Although σ R2 has not yet been cloned, a recent report suggests that the σ R2 binding site is part of the progesterone receptor

membrane component 1 (PGRMC1) protein complex (36). The identity of σ R2 binding site as part of PGRMC1 has met with some controversy, however, with results of alternative studies indicating that PGRMC1 and σ R2 are two different proteins (37, 38).

In light of this controversy, investigations of PGRMC1 and its possible biological interactions with σ R1 are intriguing. PGRMC1 is a heme-binding protein related to cytoplasmic cytochrome b5 domain (M_r 22-28 kDa) (39). Studies have shown that the σ R2 selective probe (WC-21) cross-linked a protein with a similar sequence as PGRMC1 and that binding of a selective σ R2 ligand was blocked by a PGRMC1 protein inhibitor (36). Cells with enhanced PGRMC1 protein expression exhibited higher σ R2 binding, while knock down of PGRMC1 decreased σ R2 binding (36). Additionally, σ R2 and PGRMC1 share similar subcellular locations in the ER, microsomal membranes, mitochondria, lysosome, and cytoplasmic membrane (40-42). Both σ R2 and PGRMC1 have been shown to play a role in the binding of progesterone (17, 43). Both proteins are also cancer biomarkers and potential therapeutic targets (32, 33, 44). Of particular interest are an elegant series of studies from the Cenedella laboratory that have characterized a high-affinity, membrane steroid binding protein with molecular features that appear to be identical to PGRMC1 in lens epithelium (45,46).

Given the recent evidence that the σ R2 binding region is part of the PGRMC1 protein complex, we hypothesize that PGRMC1 is expressed in retina and that biological interactions between σ R1 and PGRMC1 will be reflected in compensatory upregulation of PGRMC1 in σ RI^{-/-} mice. In the present study, we investigated PGRMC1 localization, gene, and protein expression in the eye and in a variety of other tissues from wild type and σ RI^{-/-} mice. The present work provides insight on potential biological overlap between σ R1 and PGRMC1 and lays the foundation for future investigations of the role of PGRMC1 in retina.

Methods

Animals

Wild type (WT) C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) and σ RI knockout (σ RI^{-/-}) mice (47) were used. Details about genotyping and establishment of the mouse colony have been described (11). Maintenance of the animals adhered to institutional guidelines for humane treatment of animals as outlined in the DHEW Publication, NIH 80-23, The Guiding Principles in the Care and Use of Animals and to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Analysis of PGRMC1 by immunofluorescence

Retinal cryosections were fixed in 4% paraformaldehyde for 10 min, washed three times with 0.1% Triton X-100 for 5 min. Slides were blocked (PowerBlock; BioGenex, San Ramon, CA) for 1 h at room temperature, incubated with primary antibodies overnight at 4 °C followed by secondary antibodies for 1 h at 25°C. Sections were examined for immunofluorescence using an Axioplan-2 fluorescent microscope (Carl Zeiss, Göttingen, Germany) equipped with a high-resolution microscope camera. Images were captured and processed using Zeiss Axiovision software (version 4.7; Carl Zeiss). Antibodies included

anti-PGRMC1 (1:200, Cell Signaling Technology, Denver, MA, USA), anti-vimentin (1:200, Millipore, Billerica, MA, USA) to label retinal Müller cells, and anti-Brn3a, a marker for neurons (1:100; Santa Cruz Corp., Santa Cruz, CA). Secondary antibodies (Alexafluor 488 and Alexafluor 555) were obtained from Invitrogen-Life Sciences (Grand Island, NY).

Analysis of PGRMC1 in retinal Müller cells

Müller cells were isolated from 5~7-day-old pups and cultured per our method (19). Briefly, eyeballs were removed, placed in serum-free Dulbecco's modified Eagle medium (DMEM) with penicillin/streptomycin and soaked overnight at 25°C in the dark. They were rinsed in phosphate-buffered saline (PBS) and incubated in buffer containing trypsin, EDTA and collagenase. Retinas were removed from eyeballs, placed in DMEM supplemented with glucose, fetal bovine serum and penicillin/streptomycin and gently pipetted into small aggregates. Cultures were washed vigorously with medium until only a strongly adherent, flat, cell population remained. Cells were used at passage 4-5. The purity of cultures has been verified using antibodies that are known markers of Müller cells (48, 49). Cells were subjected to immunoblotting to detect PGRMC1 and to immuno-cytochemistry following our published methods (13, 49).

Gene expression analysis

Neural retina, RPE/eyecup, brain, heart, lung, liver, and kidney were harvested from $\sigma R1^{+/+}$ and $\sigma R1^{-/-}$ mice. Tissues were homogenized, RNA extracted using TRIzol (Invitrogen, Carlsbad, CA). RNA was transcribed to cDNA using iScript cDNA synthesis kit (Bio-rad, CA, USA). qRT-PCR was performed using SYBR Green Supermix (Bio-Rad, CA, USA). Primers were from Integrated DNA Technologies (CA, USA) (*Pgrmc1*, NM_016783.3, Forward: 5'-TTCCTGCTCTACAAGATCGTTCGC-3', Reverse: 5'-CTTGCCGTTGATGGCCATGAGAAT-3' 167bp; $\sigma R1$, NM_030996, Forward: 5'-CATTCGGGACGATACTGG GC-3', Reverse: 5'-CCTGGGTAGAAGACCTCACTTTT-3' 101bp; *Gapdh*, NM_008084.2, Forward: 5'-CATGGCCTCCAAGGAGTAAGA-3', Reverse: 5'-GAGGGAGATGCTCAGTGTTGG-3', 104 bp). Resulting C_T values were normalized to GAPDH and expression levels were calculated using 2^{-C_T} as reported (13).

Analysis of PGRMC1 by immunoblotting

To detect PGRMC1 levels in retina and eyecup, tissue lysates were subjected to immunoblotting as described (13). Antibodies included anti-PGRMC1 (1:200, Cell Signaling Technology, Denver, MA, USA), anti- $\sigma R1$ (1:500) (50), and anti-GAPDH (1:2000, Millipore, Billerica, MA, USA) used as a loading control. Tissue lysates were prepared by sonication in cell lysis buffer with protease inhibitors. Protein samples were fractionated on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk and exposed to primary antibody at 4°C overnight followed by appropriate secondary conjugated antibody to horseradish peroxidase (1:5000, Santa Cruz Corp., Santa Cruz, CA) at room temperature for 1 h, and developed by Chemiluminescence SuperSignal Western system. Protein levels were quantified by densitometry and expressed as a ratio to GAPDH.

Statistical Analysis

Data were analyzed by three-way ANOVA using GraphPad Prism (La Jolla, CA). The measured variable was gene expression level (or protein level) and the factors (fixed effects) were the two different genes (proteins) being studied ($\sigma R1$ or PGRMC1), the different mouse strains (WT or $\sigma R1$ KO) and the tissue types (e.g. neural retina, RPE/eyecup, liver, kidney etc). Sidak multiple comparison test was the post-hoc test. A p value <0.05 was considered significant.

Results

PGRMC1 immunohistochemical analysis in ocular tissues

Immunofluorescent analyses were performed in ocular tissues to investigate whether PGRMC1 was present and if so in which cell types. Hematoxylin/eosin-stained cryosections (Fig. 1A, C, E) are shown for comparison to cryosections subjected to immunofluorescent labeling using anti-PGRMC1 antibody. The cornea, showed faint PGRMC1 labeling in the epithelium, but not the stroma (Fig. 1A, B). PGRMC1 was present in lens epithelial cells (Fig. 1C, D), but not lens fibers. PGRMC1 was detected in ciliary body epithelial cells and trabecular meshwork cells (Fig. 1E, F). PGRMC1 was abundant in liver (positive tissue control, Fig. 1G). The pattern of expression of PGRMC1 in these tissues was similar to $\sigma R1$ labeling (18).

PGRMC1 localization in retinal sections

We used immunohistochemistry to determine the localization of PGRMC1 in the retina. A hematoxylin/eosin-stained retinal cryosection (Fig. 2A) is shown for comparison to cryosections subjected to immunofluorescent labeling using anti-PGRMC1 antibody (Fig. 2B). There was labeling clearly evident in the RPE, a single layer of cuboidal cells distal to photoreceptor outer segments. There was PGRMC1 labeling of the internal limiting membrane, discrete inner nuclear layer cell soma, and outer limiting membrane, which is consistent with a pattern of labeling for Müller glial cells. To examine whether PGRMC1 labeled Müller cells, we co-labeled retinal sections with vimentin, a Müller cell marker (Fig. 2C). The merged image shows overlap of PGRMC1 with vimentin (Fig. 2D). Immunolocalization of PGRMC1 and vimentin in retinas of WT compared with $\sigma R1^{-/-}$ (KO) showed similar immunolabeling patterns (Fig. 2E,F,G).

Pgrmc1 gene and protein expression in retina

Pgrmc1 and $\sigma R1$ gene expression was analyzed by qRT-PCR in neural retina (NR) and RPE/sclera/eyecup (EC) of WT and $\sigma R1^{-/-}$ mice. Gene expression is shown as the ratio of *Pgrmc1:Gapdh* and $\sigma R1:Gapdh$ (Fig. 3A). In neural retina of WT mice, *Pgrmc1* expression was five-fold greater than expression of $\sigma R1$. In the eyecup of WT mice, *Pgrmc1* expression was four-fold less than $\sigma R1$ (Fig. 3A). *Pgrmc1* and $\sigma R1$ expression were examined in retina and eyecup from $\sigma R1^{-/-}$ mice and as anticipated, $\sigma R1$ was not expressed in $\sigma R1^{-/-}$ neural retina or eyecup (Fig. 3B). A comparison of *Pgrmc1* gene expression in WT versus $\sigma R1^{-/-}$ mice showed a significant increase in *Pgrmc1* gene expression in neural retina from $\sigma R1^{-/-}$ mice compared to WT, but no difference in expression in RPE/eyecup of WT versus $\sigma R1^{-/-}$

(Fig. 3C). We investigated $\sigma R1$ versus PGRMC1 protein levels in neural retina and eyecup of WT and $\sigma R1^{-/-}$ mice by immunoblotting (Fig. 3D). There was abundant $\sigma R1$ in WT mouse retinal tissues and no $\sigma R1$ protein detected in $\sigma R1^{-/-}$ mice tissues. PGRMC1 was abundant in neural retina and eyecup of both WT and $\sigma R1^{-/-}$ mice (Fig. 3D,E). Thus, while there was a significant increase in *Pgrmc1* mRNA in $\sigma R1^{-/-}$ retina, this was not accompanied by increased PGRMC1 protein.

PGRMC1 analysis in retinal Müller cells

The co-immunolocalization of PGRMC1 with the Müller cell marker vimentin, led us to culture Müller cells isolated from mouse retina and to investigate expression of PGRMC1 in these cells. Immunocytochemical analysis detected PGRMC1 and vimentin, but not the ganglion cell marker Brn3 in isolated retinal Müller cells (Fig. 4A). Immunoblotting studies showed that both PGRMC1 and $\sigma R1$ have robust expression in WT cells (Fig. 4B). The PGRMC1 protein level in Müller cells cultured from $\sigma R1^{-/-}$ mice was similar to that found in Müller cells from WT mice (Fig. 4C).

Pgrmc1 gene expression analysis in non-ocular tissues

Because *Pgrmc1* and $\sigma R1$ are present in many tissues, we investigated *Pgrmc1* and $\sigma R1$ gene expression patterns in other tissues compared to that of retina. We analyzed *Pgrmc1* and $\sigma R1$ gene expression in lung, liver, brain, heart, intestine, and kidney of WT and $\sigma R1^{-/-}$ mice. Gene expression is shown as the ratio of *Pgrmc1:Gapdh* and $\sigma R1:Gapdh$. *Pgrmc1* mRNA levels were significantly greater than $\sigma R1$ mRNA levels in lung and liver of WT mice (Fig. 5A). *Pgrmc1* mRNA was expressed in lung and liver of $\sigma R1^{-/-}$ mice (Fig. 5B). Although no difference in *Pgrmc1* expression was observed in lung of WT compared to $\sigma R1^{-/-}$ mice (Fig. 5C), a significant increase in *Pgrmc1* expression in liver occurred in $\sigma R1^{-/-}$ mice relative to WT counterparts. *Pgrmc1* expression was minimal in heart, but robust in brain, intestine and kidney of WT mice (Fig. 5D). In WT mice, *Pgrmc1* mRNA levels were significantly greater than $\sigma R1$ mRNA levels in brain, intestine and kidney (Fig. 5D). In $\sigma R1^{-/-}$ mice, *Pgrmc1* was also expressed in the tissues examined (Fig. 5E). *Pgrmc1* mRNA levels were significantly increased in $\sigma R1^{-/-}$ mouse brain and small intestine compared to WT mice, however, no change in expression levels of *Pgrmc1* in heart and kidney was observed as a consequence of $\sigma R1$ deletion (Fig. 5F).

Discussion

The effect of $\sigma R1$ ligands on retinal degeneration is an important area of study, however, the lack of information on the molecular identity of $\sigma R2$ made it impossible to determine whether $\sigma R2$ might have a compensatory role in the absence of $\sigma R1$. Understanding whether a functional biological overlap exists between $\sigma R1$ and $\sigma R2$ is important as $\sigma R1$ has already been shown to be crucial to retinal health. For example, administration of the $\sigma R1$ agonist (+)-pentazocine provides profound preservation of retinal structure in an *in vivo* diabetic *Ins2^{Akita}* mouse (29). More recent studies from our lab and others have characterized the retinal consequences when $\sigma R1$ is absent (11, 12, 51). The $\sigma R1^{-/-}$ mice exhibit a retinal phenotype characterized by late-onset loss of retinal ganglion cells accompanied by functional changes in the negative scotopic threshold, a measure of ganglion cell function

(11). Previously, many labs spent considerable effort unsuccessfully attempting to clone the elusive $\sigma R2$, thus the recent discovery that the $\sigma R2$ binding site might be contained within PGRMC1 (36) was of great interest and prompted the present work. The importance of characterizing potential biological interactions between PGRMC1 and $\sigma R1$ is underscored by the ongoing controversy concerning the relationship between $\sigma R2$ and the PGRMC1 protein complex (37).

In the current study, we demonstrated the novel expression and localization of PGRMC1 in ocular tissues including retina. Analysis of PGRMC1 protein levels, in the absence of $\sigma R1$, indicated that a biological interaction between $\sigma R1$ and PGRMC1 is unlikely in retina. We cannot, however, rule out the possibility that $\sigma R1$ and PGRMC1 may share overlapping functions in other tissues. Although *Pgrmc1* gene expression in lung, heart, and kidney are insensitive to $\sigma R1$ deletion, lipid rich tissues such as liver, intestine, and brain all showed increased *Pgrmc1* gene expression in response to $\sigma R1$ deletion.

In the present study, we established the retinal expression pattern of PGRMC1 in ocular tissues including retina. Previous ligand-binding studies provided evidence for $\sigma R2$ expression in ocular tissues using lacrimocytes (3) and retinal membranes (6). The work was performed nearly two decades ago when nothing was known about this receptor at the molecular level. Prior retinal studies were performed with whole retinal membranes, thus the distribution of cell specific binding sites was unknown. In non-retinal ocular tissues, PGRMC1 was present in ciliary epithelium and lens. This pattern of expression is similar to that of $\sigma R1$, which is also found in ciliary and lens epithelium (18). The finding that PGRMC1 is present in the lens epithelium supports work from the Cenedella laboratory on a high-affinity, membrane steroid binding protein present in lens. Based on molecular similarities and binding properties, the protein that we are investigating in retina is most likely the one reported over a decade ago in lens epithelium (45,46).

The finding that PGRMC1 is abundantly expressed in retina suggests that an important but as-of-yet undiscovered role exists for this protein. Given that PGRMC1 has been shown to contain the putative $\sigma R2$ binding site, our present findings corroborate previous studies with bovine retinal membranes showing that $\sigma R2$ is an abundant protein in ocular tissue (6). PGRMC1 was localized to Müller and RPE cells, compared to $\sigma R1$ which in addition to Müller and RPE is also strongly expressed in the ganglion cells (18, 19). The strong presence of PGRMC1 in Müller and RPE, two cell types that play critical roles in maintaining proper neuronal function suggests that PGRMC1 has important biological functions in retina.

In the absence of $\sigma R1$, changes in *Pgrmc1* gene and protein expression were tissue specific. In neural retina, increased *Pgrmc1* gene expression in $\sigma R1^{-/-}$ mice suggested a possible interaction between $\sigma R1$ and *Pgrmc1*, however, this was not borne out at the protein level. Deletion of $\sigma R1$ did not alter the expression pattern or levels of the PGRMC1 protein in the retinas of $\sigma R1^{-/-}$ mice relative to WT. In addition, *Pgrmc1* gene expression in lung, heart, and kidney were also insensitive to $\sigma R1$ deletion. In contrast, *Pgrmc1* gene expression in lipid rich tissues including neural retina, liver, intestine, and brain all showed increased *Pgrmc1* gene expression in response to $\sigma R1$ deletion.

The finding that deletion of $\sigma R1$ resulted in tissue specific alterations in *Pgrmc1* gene expression is noteworthy. The insensitivity of PGRMC1 protein to $\sigma R1$ deletion in retina, however, makes the interpretation of this result difficult. The biological relationship between the $\sigma R1$ and the $\sigma R2$ binding site, which has been shown to be part of the PGRMC1 protein complex, is uncertain. The overlap in ligand selectivity between $\sigma R1$ and $\sigma R2$ has led to speculation that the receptors share overlapping/intersecting signaling pathways (2). If PGRMC1 is $\sigma R2$ and if a biological relationship did exist between $\sigma R1$ and $\sigma R2$, then deletion of $\sigma R1$ in mouse might lead to compensatory changes in expression of PGRMC1. In the present study, the increase in *Pgrmc1* gene expression in response to $\sigma R1$ deletion was only observed in lipid rich tissues. The existence of a lipid based biological interaction between $\sigma R1$ and PGRMC1 is a reasonable speculation. For example, $\sigma R1$ has a cholesterol binding region, which may explain its affinity for a variety of drugs and ability to bind progesterone (15). In addition, $\sigma R1$ can localize to lipid rafts and has a known role in determining raft composition (52). On the other hand, PGRMC1 plays a role in regulating cholesterol synthesis and is a component of a multi-protein complex that binds progesterone and other steroids (37). PGRMC1 has also been found in lipid rafts (53).

In summary, this study provides the first molecular evidence that PGRMC1 is present in retina. The strong expression of PGRMC1 in both Müller and RPE cells suggest that PGRMC1 has an important, yet-to-be discovered role in retina. In retina, PGRMC1 function seems likely to be independent of $\sigma R1$ because PGRMC1 protein levels were insensitive to $\sigma R1$ deletion. Whether the retinal phenotype observed in $\sigma IR^{-/-}$ mice (e.g. ganglion cell loss) (11) would be altered in the absence of PGRMC1 is not known, but would be an informative avenue of investigation. There was insensitivity of *Pgrmc1* gene expression to $\sigma R1$ deletion observed also in lung, heart, and kidney. In contrast, a biological interaction between $\sigma R1$ and PGRMC1 may exist in lipid rich tissues including liver, intestine, and brain which all showed a potential compensatory increase in *Pgrmc1* gene expression in response to $\sigma R1$ deletion. The current studies establish the presence of PGRMC1 in retina and lay the foundation for analysis of its biological function.

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Fig. 1. Immunofluorescent detection of PGRMC1 in wildtype (WT) mouse cornea, lens and ciliary body

Eyes harvested from 8 wk old WT mice were processed for cryosections and were incubated with anti-PGRMC1, followed by incubation with Alexafluor 488-labeled secondary antibody (green) as described in the text. Black and white images of hematoxylin/eosin stained sections for (A) cornea showing the epithelium (ep) and stroma (st), (C) lens showing the fibers (f) and epithelium (e), and (E) ciliary body showing trabecular meshwork (TM) and epithelium (ep) are provided for comparison to sections incubated with anti-PGRMC1: (B) cornea, (D) lens, (F) ciliary body. PGRMC1 labeling in liver (G) is shown as a positive control. The blue immunofluorescence labels nuclei using DAPI. Calibration scale bar = 50μm.

Fig. 2. Immunolocalization of PGRMC1 in wildtype and $\sigma R1^{-/-}$ mouse retina

Mouse retinal cryosections were incubated with anti-PGRMC1 or anti-vimentin followed by incubation with secondary antibody as described in the text. (A) Hematoxylin-eosin-stained section for comparison to adjacent retinal immunocytochemistry sections. Several layers of the retina are indicated (gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; onl, outer nuclear layer; is, inner segments; rpe, retinal pigment epithelium). (B) Immunolabeling for PGRMC1 using either Alexafluor 555 (red) or Alexafluor 488 (green, inset) detected PGRMC1 in the innermost region of retina (above the ganglion cell layer) consistent with the inner limiting membrane and around soma of cells in the inner nuclear

layer, and the outer limiting membrane, and labeling of the RPE cells. (C) Immunodetection of vimentin labeled the radially oriented fibers of Müller cells; the merged image (D) for PGRMC1 and vimentin shows some co-localization. Immunolabeling of PGRMC1 in retinas of $\sigma RI^{+/+}$ (WT) mice was compared with that of $\sigma RI^{-/-}$ (KO) mice (E), and the labeling was similar between the two groups. Vimentin co-immunolabeling was performed on retinas of WT and KO mice (F) and the merged image (G) shows the similar pattern of labeling of PGRMC1 and vimentin. The blue immunofluorescence labels nuclei using DAPI. Calibration scale bar = 50 μ M.

Fig. 3. Analysis of *Pgrmc1* expression in retina and RPE/eyecup of $\sigma R1^{+/+}$ (WT) and $\sigma R1^{-/-}$ (KO) mice

mRNA from neural retina (NR) and RPE/eye cup (EC) was isolated from $\sigma R1^{+/+}$ WT (A) and $\sigma R1^{-/-}$ KO (B) mice (age 8 wks) to compare expression levels of the $\sigma R1$ gene to the *Pgrmc1* gene. The expression levels of *Pgrmc1* in NR and EC of $\sigma R1^{+/+}$ mice versus $\sigma R1^{-/-}$ mice (C) were calculated based on the data in A and B. (D) Protein was isolated from NR and EC of $\sigma R1^{+/+}$ and $\sigma R1^{-/-}$ mice and subjected to immunoblotting to detect $\sigma R1$ and PGRMC1 as well as GAPDH (loading control). Quantification of densitometric scans of protein bands is shown (E) data are the ratio of the band densities of $\sigma R1$:Gapdh and *Pgrmc1*:Gapdh determined for four experiments (*** $p < 0.001$).

Fig. 4. Immunofluorescent and immunoblotting analyses of PGRMC1 in wildtype mouse Müller cells

Primary Müller cells were isolated from 5-7 day WT mice. Immunocytochemical analysis (A) showed that the cells were positive for $\sigma R1$, PGRMC1, and vimentin (Müller cell marker), but not Brn3 (retinal ganglion cell marker). Protein was isolated from Müller cells isolated from $\sigma R1^{+/+}$ (WT) and $\sigma R1^{-/-}$ (KO) pups and subjected to SDS-PAGE, followed by immunoblot analysis with an affinity-purified antibody against $\sigma R1$, PGRMC1, and GAPDH (internal loading control) (B). Quantification of densitometric scans of protein bands is shown (C). Each experiment was performed in triplicate (***) $p < 0.001$.

Fig. 5. Analysis of *Pgrmc1* expression in non-ocular tissues of $\sigma R1^{+/+}$ (WT) and $\sigma R1^{-/-}$ (KO) mice

mRNA from lung and liver was isolated from $\sigma R1^{+/+}$ WT (A) and $\sigma R1^{-/-}$ KO (B) mice (age 8 wks) to compare expression levels of the $\sigma R1$ gene to that of *Pgrmc1*. The expression levels of *Pgrmc1* in the $\sigma R1^{+/+}$ mouse lung and liver versus $\sigma R1^{-/-}$ mouse lung and liver (C) were calculated based on the data in A and B. mRNA from brain, heart, small intestine (Int), and kidney was isolated from $\sigma R1^{+/+}$ WT (D) and $\sigma R1^{-/-}$ KO (E) mice to compare expression levels of genes encoding $\sigma R1$ to PGRMC1. The expression levels of *Pgrmc1* in the $\sigma R1^{+/+}$ mouse brain, heart, small intestine and kidney versus $\sigma R1^{-/-}$ mouse brain, heart, small intestine and kidney (F) were calculated based on the data in D and E. qRT-PCR was performed to analyze the expression of *Pgrmc1* or $\sigma R1$ normalized to *Gapdh*. The data are expressed as a ratio of the $\sigma R1$ (or *Pgrmc1*):*Gapdh*. Each experiment was performed in triplicate (** $p < 0.01$, *** $p < 0.001$).