Mpp4 recruits Psd95 and Veli3 towards the photoreceptor synapse

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Membrane-associated guanylate kinase (MAGUK) proteins function as scaffold proteins contributing to cell polarity and organizing signal transducers at the neuronal synapse membrane. The MAGUK protein Mpp4 is located in the retinal outer plexiform layer (OPL) at the presynaptic plasma membrane and presynaptic vesicles of photoreceptors. Additionally, it is located at the outer limiting membrane (OLM) where it might be involved in OLM integrity. In Mpp4 knockout mice, loss of Mpp4 function only sporadically causes photoreceptor displacement, without changing the Crumbs (Crb) protein complex at the OLM, adherens junctions or synapse structure. Scanning laser ophthalmology revealed no retinal degeneration. The minor morphological effects suggest that Mpp4 is a candidate gene for mild retinopathies only. At the OPL, Mpp4 is essential for correct localization of Psd95 and Veli3 at the presynaptic photoreceptor membrane. Psd95 labeling is absent of presynaptic membranes in both rods and cones but still present in cone basal contacts and dendritic contacts. Total retinal Psd95 protein levels are significantly reduced which suggests Mpp4 to be involved in Psd95 turnover, whereas Veli3 proteins levels are not changed. These protein changes in the photoreceptor synapse did not result in an altered electroretinograph. These findings suggest that Mpp4 coordinates Psd95/Veli3 assembly and maintenance at synaptic membranes. Mpp4 is a critical recruitment factor to organize scaffolds at the photoreceptor synapse and is likely to be associated with synaptic plasticity and protein complex transport.

INTRODUCTION

Six classes of neurons build up the complex neuronal network of the retina. Light of various wavelengths is absorbed by rod and cone photoreceptors, followed by the integration of the signal through bipolar, amacrine and horizontal cells and further transmitted to the brain by ganglion cells (1). Polarity of neuronal cells is essential for the retinal integrity. To maintain photoreceptor polarity, protein complexes are recruited towards particular subcellular locations, including the outer limiting membrane (OLM), which consists of the adherens junction and a region with similarity to tight junctions, the so-called subapical region (SAR) (2). The OLM contributes to retinal integrity by connecting photoreceptors to Müller glia cells. As in other polarized cells, members of the membrane-associated guanylate kinase (MAGUK) family are found in complexes at the plasma membrane of several subcellular compartments. Here, these scaffold proteins play an important role in targeting, clustering and anchoring of other proteins. They can assemble combinations of cell adhesion molecules, cytoskeletal proteins (3), receptors, ion channels and their associated signaling components at specific membrane sites (4,5). MAGUK proteins contain multiple protein–protein interaction domains [at least one PSD95, Dlg and ZO-1 (PDZ), an Src homology 3 (SH3) and a guanylate kinase (GUK) domain (5,6)] required for their scaffolding function. MAGUK proteins can be divided in multiple protein–protein interaction domains [at least one PSD95, Dlg and ZO-1 (PDZ), an Src homology 3 (SH3) and a guanylate kinase homolog (GUK) domain (5,6)] required for their scaffolding function. MAGUK proteins can be divided in multiple protein–
domains, additional domains and sequence similarity (5,7). Within the p55 subfamily, seven membrane palmitoylated proteins (MPPs) have been identified (Mpp1–7) of which Mpp4 is present in photoreceptors (8). Mpp4 is a protein of 637 amino acids containing two N-terminal L27 domains, a PDZ domain, an SH3 domain and a C-terminal GUK domain (8). Although its mRNA is also present in heart, spleen, liver and cerebellum, Mpp4 is preferentially expressed in photoreceptors of the mammalian retina and of main interest for this study (8,9).

In photoreceptors, Mpp4 is localized at presynaptic vesicles and the plasma membrane of the photoreceptor synapse at the outer plexiform layer (OPL). Additionally, some antibodies directed against Mpp4 detect protein at the connecting cilia (the region between the inner and outer segments) and the OLM (9,10). At the OLM, Mpp4 is localized at the SAR and so is the Crb complex (11). Crb1 is associated with (the region between the inner and outer segments) and the plasma membrane of the photoreceptor synapse at the connecting cilia (24), but it has not been identified which of these Dlg subfamily, including Dlg1, Psd95, Psd93 and SAP102, are localized at the neuronal synapse (25). Both signals were not detectable in Mpp4 knockout, 47% as heterozygote and 23% as wild-type. The homozygous as well as the wild-type mouse stocks were maintained as a cross of C57BL/6 and 129/Ola (50%/50%). In Mpp4+/− animals, Mpp4 staining using immunohistochemical analysis was observed in the OLM and OPL. Both signals were not detectable in Mpp4−/− retinas (Fig. 3A).

**RESULTS**

**Mpp4 knockout mice**

Homozygous Mpp4−/− mice were obtained by generating a mutant Mpp4 allele that introduced a frame shift in the beginning of exon 7 encoding the PDZ domain of Mpp4 (Fig. 1A). The recombined allele was visualized on Southern blot (Fig. 1B and C). Mpp4−/− mice are indistinguishable from their wild-type littermates; they grew and bred normally. Twenty-two heterozygote intercrosses resulted in 167 pups, with an average of seven to eight pups per litter, of which 29% was genotyped as knockout, 47% as heterozygote and 23% as wild-type. The homozygous as well as the wild-type mouse stocks were maintained as a cross of C57BL/6 and 129/Ola (50%/50%). In Mpp4+/− animals, Mpp4 staining using immunohistochemical analysis was observed in the OLM and OPL. Both signals were not detectable in Mpp4−/− retinas (Fig. 3A).

**Histological morphology of the Mpp4−/− retina**

Under normal light/dark cycle, the Mpp4−/− retina develops and remains histologically normal up to 12 months of age (Fig. 2A–F), except for sporadically displaced photoreceptors. Only in one Mpp4−/− animal out of the five studied, a small area of displaced photoreceptors was observed (Fig. 2C). Continuous exposure to white light of 3000 lux for 3 days did not significantly increase the number of displaced photoreceptors in retinas at 3 months of age. Small areas of photoreceptor displacement were observed in four out of the seven animals, showing one example in Figure 2D. However, this phenomenon was not observed at an earlier time point of 1 month or at the later time points of 6 and 12 months of age (Fig. 2E and F). No photoreceptor displacement was observed in all wild-type animals studied. No morphological defects were found in Mpp4−/− retinas developed in complete darkness when compared with Mpp4+/− retinas at the age of 1 and 3 months (data not shown). Moreover, in vitro culture of retinas isolated at day 0–1 and cultured up to 1 month showed normal development of the retinal layers in Mpp4−/− and Mpp4+/− retinas (data not shown).

**Scanning-laser ophthalmoscopy and ERG**

At the age of 9 and 18 months, fundus visualization with scanning-laser ophthalmoscopy (SLO) did not reveal any
major changes or signs of retinal degeneration in Mpp4−/− compared with Mpp4+/+ retinas (Supplementary Material, Fig. S1). Additionally, a group of six wild-type and six Mpp4−/− animals were exposed to light at 3 months of age and SLO was used to visualize the fundus. Photoreceptor displacement as observed on histological sections could not be detected by SLO (data not shown). The analysis of the retinal vasculature using angiography revealed no changes in the inner retina (Supplementary Material, Fig. S1, FLA) or in the choroid (Supplementary Material, Fig. S1, ICGA).

To investigate the effect of the Mpp4 deletion on retinal function, ERGs were recorded from Mpp4−/− and Mpp4+/+ mice (9 and 18 months of age) under scotopic and photopic conditions. Under both conditions, no significant differences were observed between the retinal electric signals obtained from Mpp4−/− and Mpp4+/+ retinas at 9 and 18 months (Supplementary Material, Fig. S2A–C).

**Mpp4 is not required for correct localization of the Crb complex**

Immunostainings for several proteins located at the OLM were performed. Important members of the Crb-complex such as Crb1 (Fig. 3F), Crb2 and Crb3, the multiple PDZ proteins Mupp1 and Patj (PALS1-associated tight junction protein), the MAGUK proteins Mpp5 (Pals1) and Mpp3, and the polarity protein Par3 were all detectable at the OLM, as well as proteins connected to the adherens junction

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**Figure 1.** Targeted disruption of Mpp4. (A) Mpp4 disrupted by insertion of the targeting vector. Correct homologous recombination deleted the PDZ domain of the Mpp4 gene. (B) XbaI/SpeI Southern blot analysis using a 767-bp PCR fragment probe in the 5′-flanking region (a 14.2-kb fragment for Mpp4+/+ DNA and a 10.5-kb fragment for Mpp4−/− DNA). (C) EcoRI Southern blot analysis using a 256-bp 3′-probe in the 3′-flanking region (a 5.6-kb fragment for Mpp4+/+ DNA and a 5.1-kb fragment for Mpp4−/− DNA). Scale bar: 1 kb.
(ZO-1, β-catenin) or inner segments (F-actin). Staining patterns of all these proteins were identical in Mpp4<sup>−/−</sup> and Mpp4<sup>+/+</sup> retinas (Supplementary Material, Fig. S3 and data not shown).

**Down-regulation of Psd95 and mislocalization of Veli3**

To unravel the function of Mpp4 in the OPL, we investigated proteins that are expressed in the OPL and putatively co-localize with Mpp4. Several of them remained unchanged in their expression and localization. These include the MAGUK proteins: Dlg1 (Fig. 3C) and Mpp3, synaptic vesicle-connected proteins such as synaptogyrin, synaptotagmin and clathrin, snare protein 25 (Snap-25) and the vesicle transporter proteins VGluT1 and VGAT1 (data not shown). However, intensity of Veli3 and Psd95 staining at the OPL were substantially reduced (Fig. 3B and E). In the Mpp4<sup>+/+</sup> retina, Psd95 protein was observed in the photoreceptor synapses of the OPL and weakly in the inner plexiform layer (IPL), as shown in Figure 3E (and data not shown). In Mpp4<sup>−/−</sup> retinas, only the IPL staining remained detectable. Co-localization of Mpp4 and Psd95 is depicted in Figure 3G. Whether the cerebral
Psd95 expression was also influenced by the loss of Mpp4 from the cerebellum was checked by comparing Mpp4<sup>−/−</sup> and Mpp4<sup>+/+</sup> cerebral sections. In the Mpp4<sup>−/−</sup> cerebellum, Mpp4 is observed in the Purkinje cell layer (PCL) and does not co-localize with Psd95. In the Mpp4<sup>+/+</sup> cerebellum, the staining for Mpp4 disappeared from the PCL without influencing the Psd95 staining (Fig. 5C).

The Veli3 antibody produced an intense staining of the photoreceptor synapses in the OPL, whereas less intense Veli3 staining was found at the OLM, cone photoreceptors and a subset of bipolar cells, in accordance with Stoehr et al. (30). In the Mpp4<sup>−/−</sup> retinas, despite the reduced signal for Veli3 in the OPL, the signal remained present at the OLM, cones and bipolar cells (Fig. 3B). Merged signals for Veli3 and Psd95 show co-localization in Figure 3H. The staining patterns for the MAGUKs Dlg1 and Cask were unaltered in Mpp4<sup>−/−</sup> and Mpp4<sup>+/+</sup> retinas (Fig. 3C and D). In contrast to co-localization shown for Veli3 and Psd95, Cask and Psd95 did not co-localize (Fig. 3I). In summary, Mpp4 is essential to recruit Psd95 and Veli3 to the presynaptic plasma membrane of photoreceptors.

**Altered localization of Psd95 visualized by electron microscopy**

Using electron microscopy, no structural differences in rod and cone terminals were observed between Mpp4<sup>−/−</sup> and Mpp4<sup>+/+</sup> retinas. Psd95 was detected at the plasma membrane of Mpp4<sup>+/+</sup> rod and cone spherules. In the rod spherules, the Psd95 signal was concentrated at the lateral plasma membrane and synaptic vesicles (Fig. 4A). In the cone pedicles, strong association of the signal with the basal contacts between photoreceptor and horizontal and bipolar cells was observed, in addition to its association with the lateral
plasma membrane (Fig. 4B). In the IPL, Psd95 was also detected at the plasma membrane of ganglion cells concentrated at dendritic profiles which contain neurotubuli and localize postsynaptically to the ribbon synapse structures of bipolar cells (Fig. 4C). Psd95 was not present at presynaptic vesicles or at the plasma membrane of rod and cone synapses of \textit{Mpp4}^-/- retinas, whereas Psd95 labeling at the cone basal contacts and IPL were similar for \textit{Mpp4}^-/- and \textit{Mpp4}^+/+ animals. Previous results on immuno-EM staining of \textit{Mpp4} demonstrated \textit{Mpp4}-positive signals in the rod spherules and cone pedicles located especially at the lateral membrane and membranes of presynaptic vesicles (11). Thus, Psd95-positive signals became highly reduced at parts of the rod and cone presynaptic membranes corresponding to the \textit{Mpp4} localization.

**Figure 4.** Immuno-electron microscopic detection of Psd95. Vertical cryosections of the retina immunolabeled with Psd95 show: (A) Rod spherules receiving invaginating processes (stars) of unlabeled bipolar or horizontal cell dendrites. \textit{Mpp4}^+/+ rod spherules immunolabeled for Psd95 (left) at the plasma membrane and presynaptic vesicles. Loss of Psd95 from the \textit{Mpp4}^-/- rod spherules (right). (B) Cone pedicles receiving invaginating processes (stars) of unlabeled bipolar or horizontal cell dendrites. \textit{Mpp4}^+/+ labeled cone pedicle base (left, top half) showing Psd95 labeling at the plasma membrane, basal contacts and presynaptic vesicles. Loss of Psd95 signal from the plasma membrane and presynaptic vesicles of the \textit{Mpp4}^-/- cone pedicle leaving only the cone basal contacts positive (arrowheads right). (C) Positive Psd95 labeling in both \textit{Mpp4}^+/+ and \textit{Mpp4}^-/- postsynaptic processes of ganglion cells in the IPL. M, mitochondrion; R, Rod; C, Cone and G, Ganglion cell. Scale bars: 500 nm.

**Loss of Mpp4 causes increased turnover of Psd95**

The association between Mpp4, Psd95, Dlg1, Veli3 and Cask was investigated by immunoprecipitation (IP). Psd95 was coimmunoprecipitated with Mpp4 from mouse retina lysates (Fig. 5A). Input samples for IP showed significantly reduced Psd95 protein levels in \textit{Mpp4}^-/- retina lysates compared with protein levels from \textit{Mpp4}^+/+ retina lysates, which is confirmed by western blot of total retina lysates (Fig. 5B). The reduction in Psd95 was not universal as confirmed by unchanged Psd95 protein levels found in cerebral cortex (data not shown) and cerebellum (Fig. 5A). The interaction between the MAGUK proteins Dlg1 and Mpp4 was demonstrated by coimmunoprecipitation of the two proteins from
Mpp4<sup>−/−</sup> retina lysates (Fig. 5A). In addition, coimmunoprecipitation of Mpp4 and Veli3 confirmed the interaction of Veli3 and Mpp4 as described recently by Stöhr et al. (30). However, in contrast to the reduction in Psd95 protein levels found in the input samples, Veli3 retinal protein levels remained comparable in Mpp4<sup>−/−</sup> and wild-type total retinal lysates. Psd95 protein levels (95 kDa) in Mpp4<sup>−/−</sup> and wild-type total retinal lysates (Fig. 5A). As Dlg1 was not detectable in the input samples, separate western blotting was performed to reveal that Dlg1 protein levels were also comparable in Mpp4<sup>−/−</sup> and Mpp4<sup>+/+</sup> total retina lysates (Fig. 5B). Interaction between Mpp4 and Cask was not detectable by IP and no changes were observed in protein levels of Cask (Fig. 5A). In summary, Mpp4 is connected to Psd95, Dlg1 and Veli3 at the photoreceptor synapse, but has a unique influence on the Psd95 protein turnover.

**DISCUSSION**

The function of MAGUK protein Mpp4 was investigated because this protein is preferentially expressed in the mammalian retina (10) and might be connected to the Crumbs (Crb) complex via Mpp5 (11). These two characteristics provide a reason to hypothesize that Mpp4 is a candidate gene causal to retinal disease. However, aside from sporadic focal morphogenetic alterations around 3 months of age, Mpp4<sup>−/−</sup> knockout mice did not show retinal degeneration and remained structural and functional normal up till the age of 18 months. Additional light exposure or complete darkness did not consistently induce morphogenetic alterations or severe retinal degeneration. The minor morphological effects suggest that Mpp4 might be a candidate gene for mild human retinopathies only. MPP4 mutational studies have been performed on some cohorts of retinal disease patients but no disease-causing mutations were identified (31). By studying the function of Mpp4 in the photoreceptor synapse, we discovered that Mpp4 is involved in the recruitment of Psd95 and Veli3 at presynaptic membranes. Moreover, Mpp4 has a crucial role in Psd95 turnover.

Localization of Crb complex members such as Crb1, Crb2, Crb3, Mpp5, Patj and Muppl at the OLM is independent of...
Mpp4, because localization and immunofluorescent reactivity of Crb complex proteins were not affected by the loss of Mpp4. In a previous study (11), we identified Mpp4 immunoreactivity at electron microscopic level just above the adherens junction at the OLM. At light microscopic level, the Mpp4 immunoreactive signal was observed at the OLM and in the OPL. Stöhr et al. (10) did not detect Mpp4 at the OLM, but detected Mpp4 at the cilium, which were not able to confirm. This discrepancy can be because of the different antibodies and antibody epitope retrieval techniques used. Rosettes, retinal folds and severe retinal disturbances as observed in the Crb1−/− (17) and Crb1rd8 (16) mutant mice were only sporadically found in the Mpp4−/− mice but not in Mpp4+/+ mice. Also, SLO at 9 and 18 months of age revealed no major differences between the retinas of the Mpp4−/− and Mpp4+/+ mice. These results suggest that the supposed docking capacity of Mpp4 for the Crb complex is not an essential function at the site of the OLM or that this function might easily be fulfilled by other (MAGUK) protein(s). Although the precise role of Mpp4 at the OLM is not resolved by studying the Mpp4−/− mouse, it is clear from the same mouse model that Mpp4 has a major role in hemming at least two proteins, PSD95 and Veli3, at the photoreceptor synapse.

At the OPL, Mpp4 co-localizes with several other MAGUK proteins and synaptic vesicle-binding proteins. By comparing the staining patterns of these proteins in Mpp4−/− mice with their patterns in Mpp4+/+ mice, two proteins showed a remarkable reduction in intensity. Staining of PSD95 (SAP90), a MAGUK protein homologue to Drosophila DLG, is normally observed in the OPL and IPL (24). A significant reduction in PSD95 in the OPL without changes in the IPL was observed in the Mpp4−/− retina. Electron microscopic immunostaining of PSD95 confirmed the disappearance of labeling from the rod and cone presynaptic vesicles and plasma membrane, whereas the labeling was still detectable at the cone basal contacts and dendritic contacts in the IPL. Previous results on immuno-EM staining of Mpp4 demonstrated Mpp4-positive signals in the rod spherules and cone pedicles located especially at the lateral membrane and membranes of presynaptic vesicles (11). To confirm that Mpp4 and PSD95 are associated, we immunoprecipitated Mpp4 from retina lysates and found PSD95 to coimmunoprecipitate with Mpp4. Input signals demonstrated that PSD95 protein levels in Mpp4+/− retinas are significantly reduced, whereas its protein levels in the cerebellum, where co-localization with Mpp4 is absent, remained unchanged. The decrease in the amount of retinal PSD95 protein was not due to suppression of transcription by Mpp4, since mRNA levels as detected by quantitative PCR were unaltered (data not shown). The results from this Mpp4−/− mouse model suggest a major role for Mpp4 in the localization, stabilization and regulation of PSD95 protein turnover in the rod synaptic terminal. In the cone pedicles, these mechanisms are partly independent of Mpp4, whereas in ganglion dendrites these mechanisms are fully independent of Mpp4. The function of PSD95 has mainly been investigated in other tissues than the retina. At the postsynaptic density, PSD95 is thought to be involved in assembling signal transduction complexes and in regulating synaptic plasticity. In vitro, PSD95 is connected to subunits of N-methyl-d-aspartate (NMDA)-type glutamate receptors (32,33), Shaker K+/channels (34) and neuronal nitric oxide synthase (nNOS) (35). Lack of PSD95 results in altered synaptic transmission in the hippocampus without obvious changes in morphology and NMDA-receptor currents but causing a substantially lower learning index (36). In Mpp4−/− retinas, photoreceptor synapses with highly reduced PSD95 protein levels were morphologically indistinguishable from those in Mpp4+/+ retinas (36). Although changes in ERG could not be detected, insufficient amounts of Mpp4 and PSD95 might alter the synaptic composition of signal transduction complexes and synaptic plasticity, influencing the synaptic signal transmission on a more subtle scale not detectable by ERG. Moreover, studying the interaction between Mpp4 and PSD95 in rod synapses may unravel a general mechanism involved in the stabilization of PSD95.

The correct localization of Veli3 seems to be dependent on Mpp4 as well. This homologue of Lin-7 (25), previously demonstrated to be a binding partner of Mpp4 (30), was no longer detectable at the presynaptic area of the OPL, while its pattern in the cone bodies, OLM and subset of bipolar cells remained unchanged. Although Veli3 was no longer localized at the synapses of rod and cones in the absence of Mpp4, its retinal protein level was still comparable between Mpp4−/− and Mpp4+/+ mice. Whereas, Mpp4 functions in regulating the turnover of PSD95 and membrane targeting at the photoreceptor synapse, Mpp4 only functions in membrane targeting of Veli3. At the OLM, Veli3 is still correctly localized in Mpp4−/− retina, where it is able to bind Mpp5 (30), a member of the retinal Crumbs complex (17). Our data strengthens the hypothesis that Veli proteins are part of several protein complexes involved in polarization of different cell types and an aid in the assembly of signal transduction complexes (28).

It has been shown that Veli is accompanied by Cask and Mint1, to create the Cask/Mint/Veli (Lin-2/Lin-10/Lin-7) complex present in neurons (26). They bind together in a complex leaving the PDZ domains free to recruit cell adhesion molecules, receptors or other MAGUK proteins. Only two members of this complex are expressed in the photoreceptors, Cask and Veli3. The third member, Mint1, was not detectable at the OPL, but was detectable at the synaptic contacts of the IPL (data not shown). Veli3 localization at the OPL is Mpp4-dependent, whereas Cask localization is Mpp4-independent. The latter can be explained by the lack of direct binding between Cask and Mpp4 in retina lysates and different cellular locations observed for Cask and Mpp4. These results suggest that the Cask/Mint/Veli complex observed in other types of neurons does not exist in photoreceptors.

The localization of Dlg1, a protein closely related to PSD95, and the localization of Mpp3 were not affected by the absence of Mpp4. IP demonstrated that Dlg1 is able to bind to Mpp4 and earlier results showed that Mpp3 (Dlg3) interacts with Dlg1 (SAP97) in the brain (37). We are currently investigating the existence of two complexes: one containing Dlg1 and Mpp4, which exist separately from the second containing Dlg1 and Mpp3 (data not shown). In the neuromuscular junction, Cask is also known to bind Dlg1 (22,38), suggesting that Cask might be involved in the correct localization of Dlg1, however, their staining patterns in the photoreceptor terminals are very different.
So, in Mpp4−/− retina either Mpp3 is able to take over the homing ofDlg1 at the synaptic terminal orDlg1 is functioning as an anchor to Mpp4 and Mpp3 containing complexes ready to get connected to the membrane. The last suggestion leads to the hypothesis thatDlg1, bound to the membrane, serves as an anchor forMpp4 (orMpp3) to recruit different complexes. So, at the photoreceptor synapse, a unique Mpp4/Psd95/Veli3 protein complex probably homed byDlg1 is involved in the retention of proteins to the membrane. Our hypothesis is in agreement with previous studies indicating that Veli proteins andPsd95 are clustered together to recruit receptors towards the membrane (39). Moreover, our data suggest thatMpp4 is essential forPsd95 stabilization and maintenance ofPsd95 at the rod and cone synaptic membrane. So far, the function of the protein complex containingMpp4, Psd95 and Veli3 is unclear. Putative complex members and binding partners ofMpp4, Veli3 andPsd95 at the presynaptic photoreceptor terminal, such as PMCA, a critical regulator of the calcium homeostasis (40), will be studied. Research on the complex interactions, together with electrophysiological experiments onMpp4−/− retina compared with theMpp4+/+ retina, might unravel the putative role of this complex in visual perception or synaptic plasticity.

MATERIALS AND METHODS

Generation of Mpp4−/− mice

Gene targeting was performed as described previously (41). Primers JW54 (5′-GCCTTGCTGAGTCCCATG-3′) and JW55 (5′-GATCATTGCTCAGGGTGCC-3′) were used to amplify a 291-bp fragment from a full-length mouseMpp4 cDNA (11). The cDNA fragment encoding the PDZ domain ofMpp4 was used to screen a λEMBL3 genomic 129/Ola DNA phage library. Forward primer JW95 (5′-GCCTGGCCGCGGATCCCGG-3′) in the λ multiple cloning site and primer JW96 (5′-GACGCTTGATGGTTGCTCC-3′) in exon 7 ofMpp4 were used to amplify a 5.2-kb 5′-targeting arm using a long-distance polymerase chain reaction (PCR) kit (Advantage 2; Clontech). Primer JW97 (5′-ACACTGAAGCTGGTTAATGTGC-3′) was kept in complete darkness up till the age of 1, 3, 6, 9 and 12 months. At each time-point, 4–8 age-matched female animals were used per genotype. The light experiment starts with a dark period of 14–16 h. Thereafter, the animals were placed in a white box and continuously exposed to diffuse white light of 3000 lux for 72 h (TLD-18 W/33 tubes, Philips; 350–700 nm) without pupillary dilation. Immediately after these 72 h of light exposure, the animals were sacrificed by inhalation of CO2 and cervical dislocation. The eyes were enucleated and a blue spot (Alcian blue) was placed on the superior side of each eye for orientation, followed by paraformaldehyde [4% in phosphate-buffered saline (PBS)] immersion-fixation for 30 min. The left eye was cryoprotected with sucrose (5 and 30%) or dehydrated in an ethanol series and embedded in paraffin for immunohistochemical analysis. The right eye was dehydrated in an ethanol series and stored in Technovit (Kultzer, Wehrheim, Germany). The whole right eye was cut in 3-μm sections, stained with 1% toluidine blue and analyzed for the presence of retinal changes. The same procedure of enucleation and fixation was followed for animals raised in complete darkness. Pregnant females of both genotypes (Mpp4+/+ andMpp4−/−) were kept in complete darkness and their offspring was raised in complete darkness up till the age of 1, 3 or 6 months, followed by the histological comparison of the retinas.

Immunohistochemical analysis

Cryosections (7 μm) and paraffin sections (4 μm) were used for the immunohistochemical analysis of several proteins.
located in the OLM and OPL. Epitopes used to raise antibodies against Crb1, Crb2, Mpp3 and Mpp4 are described by van de Pavert et al. (17) and Kantardzhieva et al. (42).

The anti-Mpp4 (AK4 and AK8) antibodies used in western blotting, immunohistochemistry and IP were raised against aa 345–359 and aa 252–268, respectively. For correct antibody epitope retrieval for anti-Mpp4 staining on paraffin sections, the slides were incubated with glycine (0.75 M) at 35°C for 15 min prior to blocking. Primary antibodies against Psd95 and Cask were purchased from Affinity Bioreagents,Dlg1, Muppl, p120, synaptoyrin, synaptotagmin, Snap-25, and β-catenin from BD Transduction Laboratories, VGlut form Synaptic Systems, VGat from Chemicon and Veli-3 from Zymed. Secondary antibodies were IgGs conjugated to Cy3, Alexa488, FITC or TRIC (Jackson ImmunoResearch and Invitrogen). In short, cryosections were rehydrated in PBS and blocked for 1 h in 10% serum, 0.4% Triton X-100 and 1% bovine serum albumin (BSA) in PBS, followed by the incubation overnight at 4°C with primary antibody in 0.3% serum, 0.4% Triton X-100 and 1% BSA in PBS. Sections were washed three times with PBS and incubated with secondary antibody diluted in 0.1% serum and 1% BSA in PBS at room temperature for 1 h. Paraffin sections were deparaffinized. Sections were boiled for 7 min in EDTA buffer (4 mM Tris, 1 mM EDTA, pH 8.0) using the microwave and slowly cooled to room temperature (2 h). After 1 h of blocking with 10% serum and 1% BSA in PBS, the primary antibody (in 1% BSA in PBS) was applied, followed by overnight incubation at 4°C. The sections were incubated with secondary antibody (in 0.1% BSA in PBS) and incubated at room temperature for 1 h. The sections were visualized by confocal laser scanning microscopy (Zeiss 501) and pictures were made with Zeiss LSM image browser v3.2. All stainings were repeated at least three times using four different animals per genotype at two different time-points (3 and 6 months of age).

Electron microscopy and immunohistochemistry

Two Mpp4+/+ and two Mpp4−/− female animals at the age of 6 and 12 months were sacrificed with an overdose of pentobarbital, the chest was opened and a needle was placed in the left cardiac ventricle for retrograde perfusion-fixation (1 min: 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer; pH 7.4; 3–5 min: 1% paraformaldehyde, 1.25% glutaraldehyde in saline; pH 7.4). Eyes were enucleated and fixed in 4% paraformaldehyde buffer (0.125 M Tris–HCl, 21% glycerol, 4% SDS; pH 6.8) through short ultra-sonification (twice) and prolonged shaking at 14°C until tissues were fully homogenized. Samples were stored at −20°C and used as material for immunoblotting.

For IP, eyes of 12 animals per genotype at the age of 3 months were enucleated and after the removal of the anterior segment, lens and vitreous, the retina was isolated as described previously (11). Up to 12 retinas were pooled and homogenized in extraction buffer [10 mM HEPES, 10 mM NaCl, 3 mM MgCl2, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 mM Na3VO4, 1 × Complete protease inhibitors (Roche); pH 7.9]. Lysates were centrifuged at 1000 g until tissues were fully homogenized. Supernatants were centrifuged again at 20 000g to separate the cytosolic and membrane fractions. The membrane fraction was dissolved in lysis buffer [50 mM HEPES, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 1 mM PMSF, 1 × Complete protease inhibitors (EDTA, Roche), 10 μg/ml aproatin (Sigma); pH 7.4]. All lysates were clarified by centrifugation at 20 000g at 4°C for 30 min. Before precipitation, supernatants were incubated with Dynabeads® protein G (Dyna Biotech ASA) coupled to the antibody of interest (coupled to the beads according to the manufacturer) for 2 h at 4°C. Thereafter, the Dynabeads were washed three times with lysis buffer, followed by boiling in sample buffer supplemented with β-mercaptoethanol or alternatively by elution in glycine (pH 1.5) at 37°C for 5 min. The obtained proteins were loaded onto a 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and followed by cryoprotection with sucrose (5 and 30%) and stored at −80°C. Frozen sections of 30–40-μm thick were cut on the cryostat and collected in phosphate buffer (PB). Sections were incubated for 96 h with primary antibody (Psd95, Affinity BioReagents). After rinsing the sections with PB, they were incubated with the secondary antibody ImmunoVision Poly-HRP-Goat Anti-mouse IgG (ImmunoVision Technologies Co., Daly City, CA, USA). A Tris–HCl diaminobenzidine solution containing 0.03% H2O2 was used to visualize the peroxidase present on the secondary antibody and intensified by the gold-substituted silver peroxidase method (43). Sections were rinsed in sodium cacodylate buffer (0.1 mM; pH 7.4) and post-fixed for 20 min in 1% osmium supplemented with 1% potassium ferriyanide in sodium cacodylate buffer (0.1 mM; pH 7.4). After a second wash with sodium cacodylate buffer, the material was dehydrated and embedded in epoxy resin as described earlier. Ultrathin sections were cut from both immunostained and non-stained sections, and photographed using the FEI TECHNAI electron microscope. Pictures were collected with the ImageView soft imaging system and processed using Adobe Photoshop (44).

IP and immunoblotting

For protein detection, brains of Mpp4−/− (n = 6; 6 months of age) and Mpp4+/+ (n = 6; 6 months of age) mice were isolated. The cerebellum was separated from the rest of the brain, washed in PBS and immediately snap-frozen in liquid nitrogen. Tissues were homogenized in SDS sample buffer (0.125 M Tris–HCl, 21% glycerol, 4% SDS; pH 6.8) through short ultra-sonification (twice) and prolonged shaking at 14°C until tissues were fully homogenized. Samples were stored at −20°C and used as material for immunoblotting.

Electron microscopy and immunohistochemistry

Two Mpp4+/+ and two Mpp4−/− female animals at the age of 6 and 12 months were sacrificed with an overdose of pentobarbital, the chest was opened and a needle was placed in the left cardiac ventricle for retrograde perfusion-fixation (1 min: 0.1 M sodium cacodylate in saline; pH 7.4; 3–5 min: 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer; pH 7.4). Eyes were enucleated and fixed in 4% paraformaldehyde, 0.1% sodium cacodylate in saline for 30 min followed by overnight incubation in 0.1 M sodium cacodylate buffer. The next day, the eyes were washed once in 0.1 M sodium cacodylate buffer and osmiumixed for at least 1 h. Subsequently, the eyes were dehydrated for 5–10 min in 30% ethanol (twice), 50, 70, 96 and 100% ethanol (twice for 15 min). The eyes were washed with acetone three times for 15 min and impregnated with epoxy resin/acetone (30/70, 15 min; 50/50, 30 min; 80/20, 30 min). Finally, the eyes were impregnated with epoxy resin 100% for 30 min followed by embedding and polymerization at 35°C for 16 h, 45°C for 8 h and 65°C for at least 24 h.

For immunohistochemistry, animals were retrograde perfusion-fixed as described earlier. Eyes were enucleated and fixed in 4% paraformaldehyde in saline for 30 min followed by cryoprotection with sucrose (5 and 30%) and stored at −80°C. Frozen sections of 30–40-μm thick were cut on the cryostat and collected in phosphate buffer (PB). Sections were incubated for 96 h with primary antibody (Psd95, Affinity BioReagents). After rinsing the sections with PB, they were incubated with the secondary antibody ImmunoVision Poly-HRP-Goat Anti-mouse IgG (ImmunoVision Technologies Co., Daly City, CA, USA). A Tris–HCl diaminobenzidine solution containing 0.03% H2O2 was used to visualize the peroxidase present on the secondary antibody and intensified by the gold-substituted silver peroxidase method (43). Sections were rinsed in sodium cacodylate buffer (0.1 mM; pH 7.4) and post-fixed for 20 min in 1% osmium supplemented with 1% potassium ferriyanide in sodium cacodylate buffer (0.1 mM; pH 7.4). After a second wash with sodium cacodylate buffer, the material was dehydrated and embedded in epoxy resin as described earlier. Ultrathin sections were cut from both immunostained and non-stained sections, and photographed using the FEI TECHNAI electron microscope. Pictures were collected with the ImageView soft imaging system and processed using Adobe Photoshop (44).
after electrophoresis transferred onto nitrocellulose membranes. Membranes were blocked (1% milk, 1% BSA in Tris-buffered saline) and protein detection was performed using primary and secondary antibodies (conjugated to horseradish peroxidase) followed by visualization of the bands by using ECL reagent (Amersham Biosciences).

**ERG and SLO**
ERG and SLO were performed according to previously described procedures (45,46) (Supplementary Material, Figs S1 and S2). Both Mpp4−/− (n = 6; at the age of 9 and 18 months) and Mpp4+/+ (n = 4; at the age of 9 months, n = 5; at the age of 18 months) female mice were dark-adapted overnight and anesthetized with ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg). The pupils were dilated and single-flash ERG recordings were obtained under dark-adapted (scotopic) and light-adapted (photopic) conditions. Brief description of ERG and SLO materials and methods is available in Supplementary Material.

**SUPPLEMENTARY MATERIAL**
Supplementary Material is available at HMG Online.

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**Conflict of Interest statement.** None declared.

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