Deletion of myosin VI causes slow retinal optic neuropathy and age-related macular degeneration (AMD)-relevant retinal phenotype

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Abstract The unconventional myosin VI, a member of the actin-based motor protein family of myosins, is expressed in the retina. Its deletion was previously shown to reduce amplitudes of the a- and b-waves of the electroretinogram. Analyzing wild-type and myosin VI-deficient Snell’s Waltzer mice in more detail, the expression pattern of myosin VI in retinal pigment epithelium, outer limiting membrane, and outer plexiform layer could be linked with differential progressing ocular deficits. These encompassed reduced a-waves and b-waves and disturbed oscillatory potentials in the electroretinogram, photoreceptor cell death, retinal microglia infiltration, and formation of basal laminar deposits. A phenotype comprising features of glaucoma (neurodegeneration) and age-related macular degeneration could thus be uncovered that suggests dysfunction of myosin VI and its variable cargo adaptor proteins for membrane sorting and autophagy, as possible candidate mediators for both disease forms.

Keywords Bipolar cell · Inner retina · Outer retina · Synapse · Stereocilia · Translocator protein TSPO · Choriocapillaris · Mouse model

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Introduction

The unconventional myosin VI, a member of the actin-based myosin motor protein family [1], is a reverse-directed myosin motor that moves towards the minus end of actin filaments [2]. Myosin VI is expressed in many tissues and cell types including hair cells of the cochlea [3] and retinal photoreceptor cells and the retinal pigment epithelium [4, 5]. In hair cells of the cochlea, myosin VI displays dual functions: (1) targeting of protein cargoes to stereocilia, the disturbance of which leads to deafness [6], (2) targeting of cargoes that couple exocytosis and endocytosis, the disturbance of which leads to cell surface shrinkage and disturbed replenishment of vesicles [7, 8]. In the central nervous system, previous studies suggested a role of myosin VI for coupling the membrane cycle towards cellular degrading processes (autophagy) and functional relevant membrane sorting through coupling to different cargo adaptor proteins [9]. In the retina of myosin VI-deficient Snell’s Waltzer mice, a-wave and b-wave amplitudes of the electroretinogram (ERG) were found to be reduced, although the overall retinal morphology and photoreceptor ultra-structure appeared normal [5]. However, the details of the pathology behind the reduced amplitudes of the a-wave and b-wave of the ERG described in this myosin VI mutant [5] and another mutant carrying a missense mutation in the motor domain of myosin VI [10] are elusive.

To study the role of myosin VI in the retina, we here performed scotopic and photopic ERG measurements in combination with high-resolution fluorescence and electron microscopy studies in young and aged myosin VI mutant mice. A subtle but nonetheless clear ocular phenotype with characteristics that resemble late onset optic neuropathy as well as age-related macular degeneration (AMD) has been observed. Deficits in myosin VI mutants included subretinal pigment epithelia basal laminar deposits [11], photoreceptor cell death, and microglial infiltration in the outer and inner retina [12–14]. Thus, this study suggests that disturbance of the myosin VI-mediated membrane cycle process may be a potential risk factor for the generation of AMD, glaucoma, or the described co-morbidity of both [15].

References

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Materials and methods

Animals

Homozygous (sv/sv, myosin VI\(^{sv/sv}\)) and heterozygous (+/sv, myosin VI\(^{+/sv}\)) Snell’s Waltzer myosin VI-deficient mutant mice [3, 16] on a C57BL/6J background and appropriate littermate controls (wild-type) [5, 16] of both genders at ages of 1–1.5, 5–8, and more than 12 months were analyzed functionally and morphologically.

The care and use of animals were approved by the University of Tübingen, Veterinary Care Unit and the Animal Care and Ethics Committee of the regional board of the Federal State Government of Baden-Württemberg, Germany, and followed the guidelines of the EU Directive 2010/63/EU for animal experiments.

Genotyping of the \(rd8\) mutation

Genomic DNA was isolated from mouse external ear biopsies using the QIAamp DNA Mini Kit (Qiagen). The \(rd8\) mutant and wild-type allele were distinguished after PCR amplification (5’- GCCCTGGTTTGAGGAAGAA ACTTGGAGACAGCTACAGTTCATAT-3’, 5’- GCCCC ATTTGCACACTGATGAC-3’) by the presence of a NdeI restriction site, present only in the \(rd8\) mutant allele. Digestion of the 244 bp PCR product with NdeI yields two characteristic fragments of 199 and 45 bp.

Electrophysiology

ERG measurements were performed in 1-, 5–7-, and 12-month-old wild-type, and +/sv and sv/sv myosin VI mutant mice (n = 10 for each group). Animals were dark adapted for at least 24 h. They were anesthetized by an intraperitoneal injection of a mixture of ketamine and xylazine (120 mg/kg ketamine, 10 mg/kg xylazine). The cornea of the eyes of anesthetized mice was desensitized with a drop of Novesine (Novartis Ophthalmics). The upper eyelids were retracted slightly by a surgical silk thread. The animals were placed onto a heated platform (37 °C) during the measurements to keep their body temperature constant. Gold wire ring electrodes placed onto the corneas of both eyes served as working electrodes. A gold wire ring electrode was placed in the mouth to serve as a reference electrode. A stainless steel needle electrode was inserted into the tail of the animals for grounding. The pupils were dilated with a drop of Tropicamide (Novartis Ophthalmics). All these manipulations were performed under dim red light, without bringing the animals into ambient light after dark adaptation. The red light was switched off after finishing all the stages of animal preparation. After an additional 5 min to allow the pupils to dilate, measurement was started using the commercial RetiPort32 device from Roland Consult Systems (Brandenburg, Germany). Standard ERG measurements were performed, with scotopic flash ERG at up to eight different light intensities from 0.0003 to 100 cd/m\(^2\), an additional run for scotopic oscillatory potentials at 100 cd/m\(^2\), photopic 30 Hz Flicker at 3 cd/m\(^2\) after 10 min of light adaptation, a photopic flash ERG, and photopic oscillatory potentials. The light intensity used for the flashes in the photopic ERG measurements was 100 cd/m\(^2\). Data were collected over 160 ms per measurement, with 512 data points per measured waveform. The analog filters of the ERG device were set to the frequency ranges of 0.5–200 Hz for both scotopic and photopic flash ERG, 50–500 Hz for oscillatory potentials and 10–50 Hz for 30 Hz Flicker. In addition, the waveforms of the oscillatory potentials were digitally filtered offline using a DSP filter included in the software of the ERG device (−15 dB for f < 10 Hz). Amplitudes of a-waves were measured from the baseline to the bottom of the a-wave trough, whereas b-wave amplitudes were measured from the bottom of the a-wave trough to the peak of the b-wave. ERG measurements were performed simultaneously on both eyes in each animal, and the eye giving the better parameters was chosen for data evaluation. Values of amplitudes and implicit times of oscillatory potentials are given as so-called oscillatory indices, i.e., values of amplitudes and implicit times of the first four oscillations were summed up. Statistical significance of differences between the values obtained in the three experimental groups was checked by the Mann–Whitney test using the Prism 6 program by GraphPad Software, Inc.

Histology and cell death labeling

For the analysis of cell death markers, wild-type, and +/sv and sv/sv mutant mice were used at 1.5 and 5–6 months of age. Eyes were enucleated, fixed with 4 % paraformaldehyde, cryoprotected in graded sucrose solutions, and embedded with Jung tissue freezing medium (Leica Microsystems, Wetzlar, Germany). The frozen eyes were sectioned (12 μm) in an HM560 cryotome (Microm, Walldorf, Germany). Detection of dying cells was performed as reported previously [17] using the terminal deoxynucleotidyltransferase (TdT) dUTP nick end labeling (TUNEL) assay (Roche Diagnostics, Mannheim, Germany). Microscopy was performed on a Zeiss Imager Z1 Apotome Microscope; images were captured with Zeiss Axiovision 4.7 software (Zeiss, Wetzlar, Germany).

For quantification of cell death, pictures were taken from three entire sagittal sections for at least three different
animals for each genotype using the Mosaic mode of Axiovision 4.7 at 20× magnification. The average area occupied by a photoreceptor cell (i.e., soma size) for each genotype was determined by counting DAPI-stained nuclei in 9 different areas (50 × 50 µm) of the retina. The total number of photoreceptor cells was estimated by dividing the outer nuclear layer area by this average cell size. The number of positively labeled cells in the outer nuclear layer was counted manually.

Data were evaluated using Graph Pad Prism software (GraphPad Software, La Jolla, CA, USA) and ANOVA analysis with a Kruskal–Wallis test and Dunn’s multiple comparison post-test. Data are presented as a mean ± standard error of the mean (SEM).

**Immunohistochemistry**

Retinae from adult (5–8- and 12–24-month-old) wild-type, and +/sv and sv/sv mutant mice for immunolabeling experiments were used. Retinae prepared for cryosections were dissected and fixed for 2 h with 2 % paraformaldehyde, dehydrated in increasing concentrations of sucrose, and then embedded in Tissue-Tek, and cryosectioned at a thickness of 12 µm. Sections were embedded with Vectashield mounting medium with DAPI (Vector Laboratories). Antibodies against myosin VI (rabbit, Santa Cruz, diluted 1:200), calbindin (mouse, Sigma-Aldrich, diluted 1:100), VGLUT1 (guinea pig, Synaptic Systems, diluted 1:100), PKC α (mouse, Novus Biologicals, diluted 1:100), AP-2 (mouse, BD Transduction, diluted 1:100), IBA-1 (rabbit, Wako, diluted 1:100), and translocator protein TSPO, also known as peripheral benzodiazepine receptor PBR (rabbit, abcam, diluted 1:100) were used. Primary antibodies were detected with Cy3-conjugated (Jackson ImmunoResearch Laboratories) or Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes). Immunohistochemistry was repeated at least twice. Sections were viewed using an Olympus BX61. Images were acquired using a CCD camera and analyzed with cellSens Dimension software (OSIS GmbH, Münster, Germany). To increase spatial resolution, retinal sections were imaged over a distance of 8 µm within the outer and inner plexiform layer in an image-stack along the z-axis (z-stack). Typically, z-stacks consisted of 30 layers with a z-increment of 0.276 µm, for each layer one image per fluorochrome was acquired. z-stacks were 3-dimensionally deconvoluted using CellSens RIDE module with the Nearest Neighbour algorithm (OSIS). Figures show composite images, which represent the maximum intensity projection over all layers of the z-stack. Images were processed with Photoshop CS (Adobe Systems). Subcellular localization of immunohistochemically labeled myosin VI was visualized with a Zeiss 510 Meta confocal laser scanning microscope (Zeiss, Göttingen/Jena, Germany).

For quantification of immunopositive-IBA-1 microglia, immunohistochemistry was performed on retinal sections of 6–8-month-old (n = 3 animals) and 12–18-month-old (n = 3 animals) wild-type mice and 6–8-month-old (n = 4 animals) and 15–24-month-old (n = 4 animals) sv/sv myosin VI mutant mice. IBA-1 immunopositive cells were counted in the outer nuclear layer (ONL) and ganglion cell layer (GCL) using an integrated microscopic counting chamber to fix the region of interest (ROI). 15 different ROIs were analyzed for each retinal section. The number of IBA-1 positive cells per 100 µm retinal length was averaged for the ONL and RGC for each retinal section. The counting procedure was done 2–4 times for each animal. Finally, the average of IBA-1 immunopositive microglia cells for the ONL and the RGC per animal was calculated. Statistical analysis was performed using the 2-way ANOVA test with α = 0.05, followed by post hoc tests with Bonferroni adjustment for genotype and age. Data are presented as mean ± standard deviation and number of animals.

**Transmission electron microscopy (TEM)**

Ultrastructural analysis of murine photoreceptor cells were performed as previously described [18]. Ultrathin sections were counterstained with ethanolic uranyl acetate and lead citrate, and analyzed in transmission electron microscopes (Tecnai 12 BioTwin, FEI, The Netherlands; LEO EM912 Omega electron microscope, Zeiss, Göttingen, Germany). Images were obtained with a CCD camera (SIS MegasView3; Surface Imaging Systems, Herzogenrath, Germany) and a slow-scan CCD camera (PROSCAN, Germany; analySIS pro imaging software, version 3.2) and processed with Adobe Photoshop CS (Adobe Systems).

**Results**

**Retinal function is affected in myosin VI mutant mice**

In previous studies, it has been reported that the absence of myosin VI leads to reduced a-wave and b-wave amplitudes of the ERG response in myosin VI-deficient sv/sv mice aged approximately 1.5 and 8.5 months [5]. Pathologic changes in outer retinal function can be detected individually for rod and cones upon differentiating scotopic and photopic Ganzfeld ERG [19]. In the present study, measurements performed in 1-month-old sv/sv mice were inconspicuously displaying normal ERG waves. However, in 5–6-month-old sv/sv mice, we found a significant
reduction of a-wave and b-wave amplitudes for both scotopic and photopic conditions (data not shown) implying an effect on rod and cone photoreceptors observed in sv/sv mice within the first 6-month life span.

Genotyping of the rd8 mutation in the myosin VI mutant mice

Recently, it has been reported that the rd8 mutation in the Crb1 gene may confound ocular-induced mutant phenotypes, being responsible for multiple previously described retinal dystrophy or photoreceptor degeneration mouse models, instead of the predicted gene of interest [20, 21]. To rule out the possibility that the observed ocular phenotype in the myosin VI mutant mouse was due to the rd8 mutation, DNA samples from three wild-type control mice, three +/sv and three sv/sv mutant mice were analyzed for the rd8 mutation by PCR. As a positive control, we used DNA sampled from the Ccl2/Cx3cr1 double knockout (CCDKO) mouse line which is homozygous for the rd8 mutation. The rd8 allele was absent in all tested experimental animals (Fig. S1). Thus, an involvement of the rd8 mutation in the ocular phenotype in myosin VI mutant mice can be ruled out.

Myosin VI immunoreactivity is found in the pigment epithelium, the outer limiting membrane and outer plexiform layer

In 5–6-month-old wild-type mice, myosin VI protein immunoreactivity was localized in the retinal pigment epithelium (RPE), at the level of the outer limiting membrane (OLM) and the photoreceptor inner segments, the outer nuclear layer (ONL), and at the level of photoreceptor axon terminals in the outer plexiform layer (OPL) (Figs. 1b, c, 2, 7). Since RPE cells are a complex polar structure with apical microvilli contacting photoreceptor outer segments and basal infoldings contacting the Bruch’s membrane and the choroid [22], we analyzed the subcellular localization of myosin VI in RPE cells and found that myosin VI is mostly expressed at the level of the basal infoldings (BI) indicating expression predominantly in the basal compartment of RPE cells (Fig. 1d, e). At the level of the outer plexiform layer (OPL), a co-stratification of myosin VI was found with the calcium-binding protein calbindin (Fig. 2a), a marker for mouse horizontal cells and their processes contacting photoreceptor axon terminals [23]. Within the OPL, myosin VI immunoreactivity was also found at the level of the vesicular glutamate transporter 1 (VGLUT1) (Fig. 2b), a specific marker for glutamatergic photoreceptor terminals [24]. Additionally, myosin VI was partly co-localized with protein kinase C (PKCα) immunoreactivity (Fig. 2c), a specific marker for rod bipolar cells and their dendrites contacting rod terminals [23]. These findings provide undescribed hints for an expression of myosin VI in presynaptic and/or postsynaptic neurite elements of cells stratifying in the OPL. A co-localization of myosin VI within this region with the adaptor protein 2 (AP-2) (Fig. 2d), known to be involved in clathrin-mediated endocytosis through assembly with myosin VI [25], supports this observation. In contrast, myosin VI immunoreactivity in sv/sv mutant mice of the same age was absent (Fig. 2a–d). Taken together, myosin VI immunoreactivity is found in the RPE, the OLM, the ONL, and the OPL.

Photoreceptors degenerate in myosin VI mutant mice

Photoreceptor loss is a characteristic feature of blinding diseases, including hereditary forms such as retinitis pigmentosa [26], but also in AMD [27–29]. To test whether the observed changes of retinal function [5] correlated with a degenerative phenotype, TUNEL staining was performed on retinae from 1.5- to 5–6-month-old +/sv and sv/sv mutant mice with wild-type littermates as control. Although there was a trend at the age of 1.5 months (Fig. S2A, C), we observed a small, but significant increase of cell death of photoreceptors in the outer nuclear layer (ONL) in 5–6-month-old sv/sv mutants (Fig. S2B, D). This finding suggests that loss of myosin VI triggered a slow progressive photoreceptor neurodegeneration.

Myosin VI deletion leads to basal laminar deposits in the sub-retinal pigment epithelium

At 5–6-months of age, the outer retina of heterozygous +/sv mice (Fig. 3a) showed no morphological (Fig. 3a, Fig. S2B, D) and functional ERG phenotype (data not shown). In contrast, same-aged sv/sv mutant mice clearly revealed morphological differences: sv/sv mice exhibited a reduced fenestration of the choriocapillaris (CC) [30] and an increasing distance between remaining fenestrae (Fig. 3c), which together with the increased photoreceptor cell death [28] (Fig. S2B, D) may indicate an AMD-relevant phenotype.

To analyze this feature in more detail, serial sections were collected from 4 wild-type mice, and 9 +/sv and 15 sv/sv mutant mice (5–8-month-old). Typically, as shown for wild-type mice, the apical microvilli of the RPE face the photoreceptor outer segments (OS) (Fig. 4a). Also typically, the basal invaginations of the RPE are firmly attached to the inner layer of Bruch’s membrane, which separates the RPE from the fenestrated endothelium of the CC as shown within different regions of serial sectioned wild-type mice (Fig. 4a). In contrast to this ordered
In 10 out of 15 retinae of sv/sv mutant mice (exemplarily shown in Fig. 4b), basal laminar deposits were observed that likely arise from lipoprotein debris [31]. Basal laminar deposits were associated with irregular protrusions of the choriocapillary endothelium (Fig. 4b). Out of 9 +/sv mice between 5 and 8 months, 4 mice developed basal laminar deposits, indicating that heterozygous mice between 5 and 8 months exhibited already an increased risk for altered RPE morphology (data not shown).

**Progression of functional deficits in outer and inner retina function upon myosin VI deletion over age**

In heterozygous +/sv mutants, ERGs are normal at an age of 1 month but showed a slightly, yet not significant reduced a-wave and b-wave amplitudes later at an age of 5–7 months (data not shown). In combination with the first morphological retinal changes observed in 5–7-month-old +/sv mutants, this indicates a slow progression of a functional ocular phenotype in myosin VI mutant mice over age. Therefore, ERG measurements were performed in an experimental series in at least 12-month-old wild-type, and +/sv and sv/sv mutant animals. Different from 5–7-month-old mice, 12-month-old heterozygote (+/sv) mice now have already developed a profound ocular phenotype, indicating a progression of the pathology with age. Scotopic a-wave (Fig. 5a, b) and b-wave amplitudes (Fig. 5a, c) were significantly decreased in homozygous sv/sv mice at light stimulus intensities from 0.3 to 100 cd s/m² and from 0.0003 to 100 cd s/m², respectively. This also occurs, however, with a significant less pronounced phenotype, for the heterozygous gene deletion, and not in +/sv mice for light intensities of 0.03 cd s/m² and lower (Fig. 5c). For photopic ERG measurements, the amplitudes of a-waves and b-waves were also significantly smaller in +/sv mice than in wild-type littermates and comparatively reduced again in sv/sv mutant mice (Fig. 5a, d). No differences were found between implicit times obtained in the three animal groups in scotopic measurements (data not shown).

**Fig. 1** Immunolabeling of myosin VI in wild-type retina. a Scheme of the retina showing retinal layers and cell types. BM Bruch’s membrane, RPE retinal pigment epithelium, OS photoreceptor outer segment, OLM outer limiting membrane, ONL outer nuclear layer, OPL outer plexiform layer, INL inner nuclear layer, IPL inner plexiform layer, GCL ganglion cell layer, C cone, R rod, HC horizontal cell, AC amacrine cell, M Müller cell, BC bipolar cell, GC ganglion cell. b Immunolabeling of myosin VI (red) in wild-type retina shows myosin VI immunoreactivity in the RPE, OLM, ONL, and OPL. Blue DAPI nuclear labeling. c Merged images of myosin VI (red) immunolabeling and the corresponding differential interference contrast (DIC) image in wild-type retina showing the myosin VI labeling in the RPE cells, OLM, ONL, and OPL. d Confocal microscopic image (cLSM) of the subcellular localization of myosin VI in the RPE. e Merged image of the myosin VI immunolabeling in (d) and DIC. The high-magnification cLSM images show myosin VI immunoreactivity in the basal compartment of RPE cells. The myosin VI immunofluorescence signal in the OLM is out of the focal plane. The shown retinal myosin VI expression pattern was found in 7 different wild-type animals. Scale bars 10 μm.
shown), while for photopic b-waves, the implicit time in sv(sv) mice was significantly delayed (Fig. 5d), indicating delayed post-photoreceptor responses of cone- but not rod-mediated signals. Amplitudes of ERG responses to photopic 30 Hz flicker stimuli were also significantly reduced in +/sv and sv(sv) mutant mice (Fig. 5a, d). 30 Hz flicker latencies were almost the same in wild-type mice and +/sv mutants (33.9 ± 2.7 vs. 33.1 ± 4.3 ms), however, significantly longer in sv(sv) mutants (39.3 ± 4.7 ms, \( p = 0.009 \)). Overall, these findings point to both photoreceptor dysfunction and disturbed inner retinal function in myosin VI mutant mice.

To assess how the altered b-wave amplitudes, which originate downstream of the photoreceptors, revealed dependency on photoreceptor function, represented by the a-wave, we analyzed the so-called b/a ratio. This ratio is believed to correlate with the total number of functioning retinal elements [32, 33]. Interestingly, scotopic b/a ratios were increased in both myosin VI mutants, while the photopic b/a ratio was decreased for sv(sv) animals, but not for +/sv animals (Fig. 5e). This indicates that the b-wave amplitude is relatively larger than the a-wave for scotopic stimuli in heterozygous and homozygous myosin VI mutant mice, but smaller than the a-wave for photopic stimuli in homozygous myosin VI mutants. Relatively larger and smaller b-wave amplitudes for scotopic or photopic light stimuli, respectively, point to enhanced rod-mediated signals and deteriorated cone-mediated signals on the bipolar cell/amacrine cell level in myosin VI mutant retina.

To validate altered inner retina function deficits, scotopic and photopic oscillatory potentials (OP) were measured as an useful tool to study pathologies related to inner retinal activity, including damage of the optic nerve, retinal ganglion cell loss or its dysfunction or vascular pressure difference during retinal diseases such as glaucoma [34]. Oscillatory potential amplitudes to scotopic (Fig. 5a, f) and photopic (Fig. 5a, g) stimuli were significantly reduced in both myosin VI mutants, with a larger amplitude decrement in sv(sv) mice. Similar as for photopic b-wave responses, also the implicit time of photopic OP was significantly delayed (Fig. 5g) confirming disturbed signal transduction at the level of bipolar and/or retinal ganglion cells for cone- but not rod-mediated responses. When OP amplitudes to scotopic stimuli were correlated with the amplitudes of a-waves and b-waves (OP/a and OP/
b ratios, respectively), an increased amplitude ratio was obtained for sv/sv mutant mice (Fig. 5h) confirming that scotopic a-waves and b-waves were reduced more than scotopic OP signals in mutant myosin VI mice. In conclusion, our ERG measurements in over 12-month-old myosin VI mutant mice indicate (1) both rod and cone dysfunction in myosin VI mutant mice, (2) reduced inner retinal activity for cone-mediated signals, and (3) enhanced inner retinal activity for rod-mediated signals. Thus, depletion or reduction of myosin VI protein likely has differential effects on cone- and rod-mediated activity in the inner myosin VI mutant retina.

**Myosin VI depletion is correlated with microglia infiltration**

Our results suggest a neurodegenerative phenotype in both the outer and inner retina when expression of myosin VI is precluded or reduced. Retinal degeneration is often accompanied by a migration of microglia into the ONL and the ganglion cell layer (GCL) [12, 13, 35]. Thus, we analyzed the immunoreactivity for the microglial marker IBA-1 in retinal sections of 6–8-month-old (Fig. 6a) and 12–24-month-old (Fig. 6b) wild-type, +/sv (data not shown) and sv/sv mutant mice using high-resolution convoluted fluorescence microscopy. IBA-1 immunoreactivity is typically seen in a ramified pattern in both synaptic layers, the outer plexiform layer (OPL) and inner plexiform layer (IPL), as shown in 6–8-month-old wild-type mice (Fig. 6a). 6–8-month-old +/sv mice, however, did also show an infiltration of IBA-1-positive microglia in the ONL and GCL compared with the wild-type (data not shown). In comparison, at that age, IBA-1 expression was increased in sv/sv mutants (Fig. 6c, 6–8 month). At an age of 12–24 months, sv/sv myosin VI mutant mice exhibited an elevated level of IBA-1 immunoreactivity in the ONL and GCL (Fig. 6c, 12–24 months). In the ONL, no age-dependent differences in IBA-1 immunoreactivity in sv/sv mutant mice were found (Fig. 6c, ONL). In contrast, in the GCL, aged wild-type and sv/sv myosin VI mutant mice showed a significant increase in IBA-1 immunoreactivity (Fig. 6c, GCL).
The translocator protein TSPO has been recently described to be up-regulated in reactive retinal microglia [14, 36]. Therefore, to confirm microglia reactivity, we next analyzed the immunoreactivity for TSPO in retinal sections of 6- and 15-month-old wild-type and sv/sv mutant mice (Fig. 6d, e). In wild-type mice, TSPO immunoreactivity is...
Deletion of myosin VI causes slow retinal...
are possibly the result of a disturbed lysosomal degradation pathway and an incomplete photoreceptor disk membranes clearance.

Additional support for myosin VI being involved in the resorting of membrane during autophagy processes in the RPE comes from the abnormal microglia distribution in the...
outer retina, which may be a further characteristic feature of AMD [35, 45]. Indeed, morphological studies with retinas of older AMD patients that are already devoid of an intact RPE revealed activated microglia in the photoreceptor layer and subretinal space. In this condition (which is different to our myosin VI-deficient mouse where the RPE is still present), subretinal microglia incorporated rhodopsin-positive particles, indicating phagocytosis of dead photoreceptors [35]. Independent from the presence of a RPE, microglia cells translocate to the subretinal space during aging in the murine and human retina [46], where they are suggested to evoke directly low-grade progressive degeneration [12, 13] and secretion of angiogenic factors [47]. Also, the increased rate in cell death in the ONL in homozygous myosin VI mutant mice confirms photoreceptor degradation. However, compared to other hereditary retinal degeneration models [26], the cell death rate in myosin VI-deficient mice is rather low, which may correspond to the very protracted degeneration process in human AMD.

As shown in the Results section, a functional decline is found in the mutant mice, manifesting itself by a reduction of all amplitudes, both scotopic and photopic. In contrast, changes in implicit times were observed only in cone-driven post-receptor responses, i.e., implicit times of photopic OP, photopic b-wave, and photopic 30 Hz flicker. There are several ocular conditions that lead to a delay of cone-related implicit time, such as hereditary photoreceptor degeneration [48], birdshot retinochoroidopathy [49], central areolar choroidal dystrophy [50], disturbance in ocular blood pressure [51], the Laurence-Moon-Bardet-Biedl syndrome [52], diabetic retinopathy [53] and congenital stationary night blindness [54], to name a few. Moreover, the decrease in photopic 30 Hz flicker amplitudes and delays in implicit time has been considered a predictor of progressed neovascularization in patients with central vein occlusion [55]. It remains to be elucidated which pathology leads to the changes of electroretinographic parameters we observed in our study.

Interestingly, Dab2, a myosin VI adaptor protein, has been shown to be expressed in the retina, where it is discussed to be involved in VEGF receptor endocytosis and angiogenesis in the retina [56]. Future studies may thus reinvestigate the AMD-related phenotype described here through disturbed flicker responses, microglia infiltration and basal laminar deposits in the context of possible Dab2 dysfunctions.
Myosin IV mutant mice exhibit characteristics similar to a glaucoma phenotype

Glaucoma is considered a multi-factorial disease [57], and many of the proposed mechanisms, are traditionally linked to elevated intraocular pressure (IOP)-related factors. In addition, the activation of microglia may facilitate disease progression and retinal ganglion cell loss independent of IOP elevation. While AMD and glaucoma typically occur in separate patient groups [58], both diseases may present a co-morbidity [15].

We found that myosin VI is expressed in the OPL that is a dense network of synapses between horizontal cells, bipolar cells and photoreceptors. Localization of myosin VI close to calbindin, a marker for horizontal cells, and in part with PKCα that labels rod bipolar cell dendrites in the outer retina [23] suggests a role of myosin VI adaptor proteins for cargo and protein resorting during the synaptic transmission process in the outer retina. The role of myosin VI may include maintenance of vesicle transport back and forth to the endoplasmatic reticulum within the photoreceptor and/or horizontal cell synapses [9]. The partial co-localization of myosin VI in the OPL with AP-2 that couples myosin VI to clathrin-mediated endocytosis [8, 25] confirms synaptic localization in photoreceptor and/or horizontal cell synapses. Only recently, a role of myosin VI in inner hair cell synapses for coupling of endocytosis and exocytosis as well as for maintenance of surface membrane has been shown [7, 8]. The disturbance of outer retinal synaptic activity due to the myosin VI deficiency may result in altered inner retinal activity: Oscillatory potentials mirror inner retinal function, including inhibitory circuits between amacrine cells and bipolar cells/ganglion cells [33, 59, 60]. Thus, the decreased photopic b/a ratio and the reduced oscillatory potentials for photopic stimuli in aged heterozygous and homozygous myosin VI mutant mice have to be regarded as disturbed inner retinal circuits to cone-evoked signals, subsequent to failure of proper myosin VI-based cargo of vesicles in cone photoreceptors and/or horizontal cells. In line with our observation in the myosin VI mutant retina, outer and inner retinal circuits alter their function after photoreceptor degeneration and show oscillatory activity patterns in the rd1 retina [61, 62].

Oscillatory potential components may also correlate with retinal arteriolar caliber in patients with diabetes [63], suggesting that there is a connection between retinal dysfunction and microvasculature changes. Within this context, the elevated b/a ratio and increased OP/a and OP/b ratios in response to scotopic stimuli need special consideration. In an earlier study, Kerfgoat and Lovasik [64] provided evidence that the rod system is more vulnerable than the cone system to transient alterations of the retinal vascular perfusion pressure (RVPP). When the susceptibility of scotopic OP to altered retinal perfusion was analyzed in volunteers, a defined component of the OP did exhibit pronounced gain in amplitude when retinal vascular pressure was increased [64]. It thus may be interesting in future to investigate scotopic response amplitudes of OP in myosin VI mutant mice in the context of increased vascular pressure. The significant increase of IBA-1-labeled microglial cells at the level of the ganglion cells observed in myosin VI mutants hints at pathological/degenerative processes in the inner retina. In line with that, activated microglia cells have been identified in glaucoma models [65–67], where they may point to stress signals sent from neurons to the glial cells involved in low-grade progressive degeneration and optic neuropathy [12, 13, 18].

Among the various adaptor proteins that interact with myosin VI during the process of autophagosome maturation is optineurin [68]. Sequence variants in the gene for optineurin have been reported to be associated with normal tension glaucoma, a subtype of primary open-angle glaucoma [69, 70]. Expression studies showed that optineurin is not only found in nerve fibers and retinal ganglion cells, as expected for a glaucoma-candidate gene, but also in the RPE. A possible differential spatio-temporal distribution of the myosin VI-bound adaptor proteins in either IPL or OPL may thus couple the entire cycle of sorting of membranes back to the plasma membrane and delivery to degradation processes [9].

Taken together, the present study not only introduces myosin VI as a gene potentially responsible for genetic predispositions of either AMD or glaucoma. The finding, moreover, untangles the endocytotic sorting process per se as a potential cause of these disastrous diseases, as this process is highly sensitive to metabolic demand, mitochondrial damage, age or stress, and could explain the multi-factorial disease of AMD and glaucoma. Not only myosin VI but also cargo adaptor proteins such as Dab2, GIPC, Tom1, optineurin, NDP52, or T6BP [9] may be considered in the future as other candidate genes involved in hereditary AMD forms or genetic predispositions. The human gene MYO6, located on chromosome 6q13, maps with the deafness locus DFNA22 and DFNB37 [71–73]. A single case of a family member with DFNB37 that suffered from retinitis pigmentosa [71] is reported. Whether an ocular phenotype occurs over age in either DFNB37 or DFNA22 families is currently unknown, as the currently clinical observed members are still young [71].

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