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Profound defects in pupillary responses to light in TRPM-channel null mice: a role for TRPM channels in non-image forming photoreception

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Abstract

TRPM1 is a spontaneously active non-selective cation channel that has recently been shown to play an important role in the depolarising light responses of ON bipolar cells. Consistent with this role, mutations in the TRPM1 gene have been identified as a principle cause of congenital stationary night blindness. However, previous microarray studies have shown that *Trpm1* and *Trpm3* are acutely regulated by light in the eye of mice lacking rods and cones (*rd/rd cl*), a finding consistent with a role in non-image forming photoreception. In this study we show that pupillary light responses are significantly attenuated in both $Trpm1^{-/-}$ and $Trpm3^{-/-}$ animals. $Trpm1^{-/-}$ mice exhibit a profound deficit in the pupillary response that is far in excess of that observed in mice lacking rods and cones (*rd/rd cl*) or melanopsin, and cannot be explained by defects in bipolar cell function alone. Immunolocalisation studies suggest that TRPM1 is expressed in ON-bipolar cells and also a subset of cells in the ganglion cell layer, including melanopsin expressing photosensitive retinal ganglion cells (pRGCs). We conclude that in addition to its role in bipolar cell signalling, TRPM1 is involved in non-image forming responses to light and may perform a functional role within pRGCs. By contrast, $TRPM3^{-/-}$ mice display a more subtle pupillary phenotype with attenuated responses under bright light and dim light conditions. Expression of TRPM3 is detected in Muller cells and the ciliary body but is absent from pRGCs, and thus our data supports an indirect role for TRPM3 in pupillary light responses.

Keywords

melanopsin; pRGCs; phototransduction; circadian; melastatin

Introduction

Transient receptor potential cation channel, subfamily M, member 1 (TRPM1) forms a spontaneously active non-selective cation channel (Duncan *et al.*, 1998; Kraft & Harteneck, 2005) whose expression is restricted to the retina and skin (Koike *et al.*, 2010). A remarkable body of recent research has confirmed that TRPM1 is expressed in ON bipolar

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cells and is responsible for carrying the inward current that drives the depolarising light responses in these cells (Morgans *et al.*, 2009; Shen *et al.*, 2009; Koike *et al.*, 2010; Morgans *et al.*, 2010). Loss of TRPM1 has been shown to abolish light responses from rod ON bipolar cells and dramatically reduce responses from cone ON bipolar cells in the mouse retina (Morgans *et al.*, 2009; Koike *et al.*, 2010). Furthermore, mutations of the *Trpm1* gene have been identified as a primary cause of stationary night blindness in humans and horses (Audo *et al.*, 2009; Li *et al.*, 2009; van Genderen *et al.*, 2009), a condition characterised by disruption of signalling via ON-bipolar cells. Thus it would seem that TRPM1 performs an essential role in ON bipolar cells and is necessary for the transmission of rod and cone information to the inner retina and transduction of the ON visual pathway (Morgans *et al.*, 2010).

In addition to the rod and cone photoreceptors of the outer retina, research over the last decade has identified an additional class of inner retinal photoreceptor (Freedman *et al.*, 1999; Lucas *et al.*, 1999), consisting of a small subset of photosensitive retinal ganglion cells (pRGCs) expressing the photopigment melanopsin (Hattar *et al.*, 2002; Hankins *et al.*, 2008). These cells signal environmental irradiance, mediating a range of non-image forming responses to light including circadian entrainment, pupil constriction and the regulation of sleep (Freedman *et al.*, 1999; Lucas *et al.*, 2001; Lupi *et al.*, 2008). Unlike the rods and cones, the molecular details of the phototransduction cascade employed by melanopsin pRGCs remain uncertain. Whilst studies have suggested the involvement of a $G\alpha_{q/11}$–type G-protein, phospholipase C and activation of a Trp-type ion channel (Hankins *et al.*, 2008; Do & Yau, 2010), the identification of the genes and proteins involved is unclear. We have previously used microarrays to investigate responses to light in the eye of mice lacking rods and cones (*rd/rd cl*), a system where all remaining responses to light are driven by melanopsin-dependent signalling. This study identified changes in expression of both *Trpm1* and *Trpm3* mRNA and thus identified these genes as potential components of the melanopsin signalling pathway (Peirson *et al.*, 2007). It is therefore possible that TRPM1 channels perform multiple roles in the retina, including the classical image-forming visual pathway as well as non-image forming responses to light associated with melanopsin expressing pRGCs.

To test our hypothesis that TRPM1 and TRPM3 contribute to non-image forming responses to light we assessed pupillary responses in *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice, comparing these responses to those of wildtype mice with normal retina, mice lacking melanopsin (*Opn4*$^{-/-}$) and mice lacking functional rod and cone photoreceptors (*rd/rd cl*). Our results demonstrate that *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice have attenuated pupillary responses consistent with a role for these channels in non image forming photoreception.

Methods

Animals

Trpm1$^{-/-}$ (NIH-1696: LexKO 428) and *Trpm3*$^{-/-}$ (NIH-1697: LexKO 380) mice (n=6, n=5, respectively) were obtained via a Wellcome Trust Knockout Mouse Resource application awarded to MWH & SNP. Mice were supplied via EMMA (www.emmanet.org). *Opn4*$^{-/-}$ (n=3) and *rd/rd cl* mice (n=4) were bred at the University of Oxford (UK) as described

previously (Freedman *et al.*, 1999; Hattar *et al.*, 2002). Wildtype mice (n=5) used in these studies were on a C57BL/6x129 mixed background, the same as *Trpm1*$^{-/-}$, *Trpm3*$^{-/-}$ and *Opn4*$^{-/-}$ animals. All mice were aged over 3 months and were accustomed to handling. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and the University of Oxford Policy on the Use of Animals in Scientific Research.

Microarray analysis

Light pulse experiments and microarray analysis were performed as reported previously (Peirson *et al.*, 2007). Briefly, *rd/rd cl* animals (age 130 ± 16 days) were sacrificed at 0, 30, 60, and 120 min (n=4 per time point) after onset of a 15 min light pulse (fluorescent white light, 1.4 mW/cm^2/s) or sham light pulse. Eyes were then collected under infrared light and immediately snap frozen on dry ice. Total RNA was isolated from whole eyes, *in vitro* transcribed and then hybridized to Mouse Genome 430 v2.0 Genechips (Affymetrix), and the resulting data analysed as previously described (Peirson *et al.*, 2007).

Laser capture microscopy and PCR

Laser capture microscopy and PCR analysis were performed as described previously (Peirson *et al.*, 2007). Briefly, wildtype eyes (ZT 6–12) were snap frozen and sectioned at 20 μm. Slides were briefly fixed in 70% ethanol at −20°C, stained with 20% cresyl violet, dehydrated, and dried at 40°C for 1–2 minutes. Sections of the retinal ganglion cell (RGC) layer were laser dissected with a PALM MicroBeam system (PALM-microlaser, Bernried, Germany), with each preparation containing approximately 30–40 cells. Total RNA was subsequently extracted with a PicoPure RNA extraction kit (Arcturus, Sunnyvale, CA), treated with 1 unit DNase (Sigma), reverse transcribed with random decamers with a RETROscript kit (Ambion), and tested for candidate gene expression with Sybr green I mastermix (Applied Biosystems) with 50 cycles of amplification. Primer sequences used were as follows (5′ to 3′): Trpm1 F GGGTTTGCTGATCTGGGTAA, Trpm1 R TGATGAAAGGTTCGGTGGTT, Trpm3 F TCCTGTCACTGGAGCATCTG, Trpm3 R CACAGCGGTAGCAGCAATAA, β-actin F ACCAACTGGGACGATATGGAGAAGA, β-actin R CGCACGATTTCCCTCTCAGC.

Pupillometry

Animals were housed on a 12:12 light:dark cycle and were tested between ZT 4-8. All animals were dark adapted for 1–2 hours prior to testing. A xenon arc lamp (150W solar simulator, Lot Oriel, UK) with a 480 nm monochromatic filter (Andover, 10 nm half-bandwidth) was used to produce a light intensity of 14.6 log quanta/cm^2/s (173 μW/cm^2/s) (bright light) or 11.6 log quanta/cm^2/s (0.17 μW/cm^2/s) (dim light). Where stated a bright white light stimulus was used (16.6 log quanta/cm^2/s, 13.6 mW/cm^2/s). In all cases, light was transmitted to the eye via a liquid light pipe as an irradiant light stimulus using a 2″ integrating sphere (Pro-lite Technology, UK). Irradiance measurements were made using a radiometrically calibrated spectrophotometer (Ocean Optics, UK). The delivery of the light stimulus was controlled via software which regulated the opening and closing of a shutter in the light pathway (LSZ160 shutter, Lot Oriel UK; custom software supplied by BRSL, Newbury, UK). In order to determine the level of the pupillary light response, images were

collected with a Prosilica NIR sensitive CCD video camera (BRSL, Newbury, UK) at a rate of 10 frames per second. The camera was positioned perpendicular to the contralateral eye which was illuminated by infra-red LEDs (850nm, 10nm half-bandwidth). In this way consensual pupil responses could be measured in response to an irradiant light stimulus. 5 minutes prior to recording, a 1% tropicamide was applied to the stimulated eye. During pupil measurements unanaesthetised animals were temporarily restrained using normal husbandry techniques for the duration of the recording (29 seconds). After brief baseline measurements of the dark adapted pupil (2 seconds), the left eye was exposed to light stimulus for 10 seconds. Recovery data was collected for a post-stimulus period of 17 seconds. Each animal was tested on multiple occasions to minimise any artefacts due to handling. Results were comparable across all tests. To assess the ability of the pupil to constrict fully, a topical solution of 1M carbachol (Sigma) in sterile PBS (pH 7.4) was applied to the cornea. Pupil size was measured after 1 hour of dark adapting before carbachol was administered, and again 15 minutes after application of the miotic. All images were analysed using ImageJ (http://rsbweb.nih.gov/ij).

Immuocytochemistry of retinal sections

Eyes were removed (ZT 6-10) and the lens punctured with a fine needle prior to fixation in 4% paraformaldehyde in PBS at 4°C for 16 hrs. Eyes were then cryoprotected in 30% (w/v) sucrose in PBS at 4°C for 48 hours before embedding in OCT medium (Sakura Finetek) and stored at −80°C prior to use. 18μm tissue sections were prepared at −23^{oC} using a Leica CM1850 cryostat (Leica Microsystems) and collected on Poly-L-Lysine coated slides (Thermo Scientific). Fluorescent immunolabelling was performed using standard techniques. Briefly, retinal sections were permeabilised in PBS with 0.2% Triton X at RT for 20 min and blocked in PBS with 10% normal goat serum (Sigma) with 0.2% Triton X for 1 hour at RT. Primary antibodies were incubated for 16 hrs at 4°C diluted in 2.5% goat serum in PBS with 0.2% Triton X; rabbit polyclonal anti-melanopsin antibody recognising the N-terminus of murine Opn4 (UF006, Advanced Targeting Systems, San Diego, CA, US), 1:2500 (Provencio *et al.*, 2002; Berson *et al.*, 2010; Ecker *et al.*, 2010); chicken polyclonal anti-β-gal antibody (ab9361, Abcam) 1:1000, (Pires *et al.*, 2009). Goat anti-rabbit and Goat anti-chicken Alexa-488 and Alexa-555 labelled secondary antibodies (Life Technologies) were incubated for 2 hrs at RT diluted 1:200 in 2.5% goat serum in PBS with 0.2% Triton X. For double-labelling primary antibodies were incubated simultaneously and secondary antibodies were incubated sequentially. All wash steps were performed using PBS with 0.05% Tween-20. Sections were mounted in Prolong Gold anti-fade reagent containing DAPI (Life Technologies). Immunolabelling of retinal flatmounts with the UF006 melanopsin antibody was performed using similar protocols, with the exception that primary antibody was incubated for 72 hrs, secondary antibody incubated for 16 hrs and Triton-X concentrations increased to 1% for all solutions. Fluorescent images were acquired using an inverted LSM 710 laser scanning confocal microscope (Zeiss) with Zen 2010 image acquisition software (Zeiss). Excitation was 405nm, 488nm and 561nm with emissions collected between 440-480, 505-550 and 600-700nm for DAPI, green and red fluorescence respectively. Collected image stacks typically comprised 6-8 focal planes. Unless stated images show a single focal plane. Pixel size was typically 0.2, 0.2, 1.0μm (x,

y, z). For all images, enhancements of brightness and contrast were performed using ImageJ software.

Statistical analysis

All data are shown ± standard error of the mean. Microarray data were analysed by one-way ANOVA and corrected for multiple tests (Benjamini Hochberg FDR correction) as described previously (Peirson *et al.*, 2007). Statistical comparisons for pupillometry data were performed using a 2-tailed Student's t-test using MS Excel.

Results

Trpm1 and *Trpm3* as candidate genes in melanopsin signalling

Microarray studies have identified *Trpm1* and *Trpm3* as potential candidate genes in melanopsin pRGC signalling (Peirson *et al.*, 2007). *Trpm1* mRNA expression is up-regulated 1.4 fold in response to acute light stimulation in mice lacking rods and cones (*rd/rd cl*) ($F_{3,12}$ =13.4, P=0.00039, P=0.0054 with FDR correction), a model where the only remaining responses to light are driven by melanopsin pRGC-dependent signalling. By contrast, *Trpm3* mRNA is negatively regulated by light in the *rd/rd cl* eye ($F_{3,12}$ =7.38, P= 0.0046, P=0.0079 with FDR correction) (Figure 1A). Laser capture microscopy was used to isolate small groups of retinal ganglion cells (typically 30–40 cells) for PCR analysis (Figure 1B). *Trpm1* mRNA expression was detected in all ganglion cell samples in which housekeeping gene expression (β-actin) could also be detected (11 of 12 samples). *Trpm3* expression was detected in 5 of 12 samples, whereas melanopsin was detected in only 1 sample (Figure 1C). Combined, these results demonstrate that both *Trpm1* and *Trpm3* mRNA are expressed in the ganglion cell layer, and suggest that both TRPM1 and TRPM3 are involved in non-image forming responses to light, potentially participating in the melanopsin signalling pathway. To further investigate the role of TRPM1 and TRPM3 in non-image forming responses to light we compared pupillary light responses (PLR) in $Trpm1^{-/-}$ and $Trpm3^{-/-}$ mice against $Opn4^{-/-}$ mice lacking melanopsin and *rd/rd cl* mice lacking rods and cones.

$Trpm1^{-/-}$ and $Trpm3^{-/-}$ mice show attenuated pupillary responses to light

In order to demonstrate the overlapping contributions of the classical rod cone photoreceptors and the melanopsin system we assessed pupillary responses in *rd/rd cl* mice lacking functional rods and cones and $Opn4^{-/-}$ mice lacking melanopsin. Consistent with previous reports (Lucas *et al.*, 2001; Lucas *et al.*, 2003) our data show that loss of only rod and cones or melanopsin based photoreception in isolation has only a subtle effect on pupillary responses when compared with wildtype mice in which all classes of photoreceptors are functional (Figure 2). In wildtype mice a rapid constriction of the pupil was observed following the onset of bright light stimulation (480nm, 14.6 log quanta/cm^2/s), with pupil area reduced to ~5-10% of dark adapted values. Pupil constriction was maintained throughout illumination and following termination of the light stimuli, with a sustained constriction after the cessation of stimulus presentation (post-illumination pupil response) (Kankipati *et al.*, 2010). $Opn4^{-/-}$ mice have a relatively modest defect in the PLR. The initial phase of constriction was similar in $Opn4^{-/-}$and wildtype mice yet $Opn4^{-/-}$mice

failed to achieve full pupil constriction with the pupil area constricted to ~20% of dark adapted values compared to ~5% for wildtype mice (P=0.0053, t=7.33, df=3). In addition *Opn4*$^{-/-}$mice showed a less pronounced post-illumination pupil response with a more rapid recovery of pupil size compared to wildtype mice. The maintenance of significant pupil constriction in *Opn4*$^{-/-}$ mice shows that rods and cones are capable of driving near complete pupil constriction in the absence of melanopsin function. Consistent with the known role of melanopsin expressing pRGCs, significant pupil constriction was also observed in *rd/rd cl* mice (3-6 months) lacking functional rods and cones. Again the initial phase of rapid constriction was similar between *rd/rd cl* and wildtype mice. Whilst *rd/rd cl* mice failed to achieve full constriction with the 480nm stimulus used, full constriction was achieved using a bright white light stimulus (16 log quanta/cm^2/s, full data not shown). With the 480nm stimulus a reduction in pupil area to ~15% of dark adapted values was achieved (P=0.040, t=3.49, df=3), consistent with the reduction in sensitivity previously described (Lucas *et al.*, 2001). The post-stimulus response was also apparent in *rd/rd cl* mice, with pupil constriction maintained at ~25% of dark adapted values following termination of the light stimulus, consistent with a strong melanopsin contribution to this response (Dacey *et al.*, 2005; Kankipati *et al.*, 2010). In all respects, pupillary responses observed in wildtype, *Opn4*$^{-/-}$ and *rd/rd cl* mice are entirely consistent with previously published data (Lucas *et al.*, 2001; Lucas *et al.*, 2003).

By contrast to both *rd/rd cl* and *Opn4*$^{-/-}$ mice, *Trpm1*$^{-/-}$ mice showed a profound attenuation in pupillary responses to light (Figure 2). The initial phase of rapid pupil constriction was entirely absent with only a small and slow response observed resulting in a maximal pupil constriction of only ~75-80% of dark adapted values after 10 seconds of illumination (480nm, 14.6 log quanta/cm^2/s) (P=0.00015, t=14.04, df=4). Rapid or significant pupil constriction was not observed for any individual *Trpm1*$^{-/-}$ mouse investigated during any trial, with similar results observed on multiple days with multiple handlers. Furthermore, a significant pupil constriction was also absent following stimulation with bright white light (16.6 log quanta/cm^2/s, full data not shown). Overall, the defect observed for *Trpm1*$^{-/-}$ mice is far greater than that observed in mice lacking functional rods and cones or mice lacking melanopsin, but is not completely abolished as has been described in triple knockout mice lacking all retinal photoreceptors (Hattar *et al.*, 2003). Given that either rods and cones or melanopsin pRGCs alone are capable of driving near complete pupil constriction, the large deficit observed in *Trpm1*$^{-/-}$ mice suggests a disruption of both rod/cone signalling (mediated by ON bipolar cells) and melanopsin driven signalling in these mice. *Trpm3*$^{-/-}$ mice also displayed a defect in the PLR when compared to wildtype controls, yet this defect was not as severe as that observed for *Trpm1*$^{-/-}$ mice. *Trpm3*$^{-/-}$ mice exhibited a rapid pupil constriction in response to light stimulation that was maintained throughout illumination and similar to that observed for wildtype, *rd/rd cl* and *Opn4*$^{-/-}$ mice. However, *Trpm3*$^{-/-}$ mice also failed to reach full pupil constriction with a maximum reduction in pupil size of ~80% observed (P=0.041, t=2.98, df=4). This value is similar to that observed for *Opn4*$^{-/-}$ mice and interestingly *Trpm3*$^{-/-}$ mice also displayed a profoundly attenuated post-stimulus response following the termination of light stimulation. This defect was more pronounced in *Trpm3*$^{-/-}$ mice than observed in *Opn4*$^{-/-}$ mice. Application of carbachol (1M) resulted in a complete constriction of the pupil in *Trpm1*$^{-/-}$

and *Trpm3*$^{-/-}$ mice suggesting that the functioning of the ciliary muscle in these mice is grossly unaffected and is physically capable of driving full pupil constriction (Figure 2).

In addition to stimulation with bright light we also examined the pupillary light responses of *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice in response to dim light illumination (480nm, 11.6 log quanta/cm^2/s) (Figure 3). At this intensity of light, the majority of pupil constriction is driven by rod/cone photoreceptors with only a minimal, if any, contribution from melanopsin pRGCs (Lucas *et al.*, 2003; Lall *et al.*, 2010). In keeping with the known role of TRPM1 in ON bipolar cell function and transmission of rod/cone driven signals, pupil constriction was completely absent in *Trpm1*$^{-/-}$ mice following dim light stimulation (P=7.4E-9, df=4, t=168.91). By contrast, *Trpm3*$^{-/-}$ mice showed a notable pupil constriction in response to dim light with pupil area reduced to ~40% of dark adapted values. However, this value was significantly attenuated compared to wildtype mice (P=0.0054, df=4, t=5.47).

In summary, the data presented shows an attenuation of the pupillary light response in both *TRPM1*$^{-/-}$ and *TRPM3*$^{-/-}$ mice. The phenotype observed in *Trpm1*$^{-/-}$ mice is consistent with defects in both ON bipolar cell function and also melanopsin driven responses. *Trpm3*$^{-/-}$ mice show attenuated constriction under bright light and dim light conditions when compared to wildtype controls.

Expression of TRPM1 and TRPM3 in the retina and pRGCs

The structure and morphology of *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ retinae appear anatomically normal, with no obvious indications of retinal degeneration (data not shown). Immunolabelling with an anti-melanopsin antibody revealed that the melanopsin system also appears normal in the retina of both *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice. The levels of melanopsin staining and the morphology of pRGCs were indistinguishable from wildtype controls (Figure 4). No melanopsin labelling was observed in retina of *Opn4*$^{-/-}$ mice (data not shown).

The *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ transgenic mouse models used in this study both incorporate a β-gal reporter encoded by the insertion of a Lac-Z gene into the reading frame of the target genes. We performed immunolabelling with an anti-β-gal antibody on retina from *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice in order to visualise the transgene product and determine the pattern of TRPM1 and TRPM3 expression in the retina. Detection of the β-gal reporter in the *Trpm1*$^{-/-}$ retina revealed expression in cells located within the inner nuclear layer (INL) resembling bipolar cells. However, in addition expression of the β-gal reporter was also detected in a subset of cells in the ganglion cell layer (GCL) (Figure 5). No expression of the β-gal reporter was detected in the ciliary body in *Trpm1*$^{-/-}$ mice. Double labelling with the anti-β-gal antibody and an anti-melanopsin antibody revealed the consistent expression of the β-gal reporter (TRPM1) within melanopsin expressing pRGCs (>90% of all pRGCs observed), with β-gal expression consistently detected in M1 and M2 type pRGCs and also displaced M1 type pRGCs located in the INL (Figure 5). No appreciable staining was observed following incubation of the β-gal antibody with normal wildtype retina not incorporating a β-gal transgene (data not shown). Immunolabelling of the β-gal reporter in *Trpm3*$^{-/-}$ mice revealed expression primarily in structures resembling the end-feet of Muller cells in the GCL, with weaker levels of staining observed in a subset of cells in the

INL consistent with the cell bodies of Muller cells (Figure 6). In addition to expression in the retina, strong expression of the β-gal reporter was also observed in the ciliary body of *TRPM3*$^{-/-}$ mice. Double labelling with an anti-melanopsin antibody shows a lack of β-gal expression within pRGCs of *TRPM3*$^{-/-}$ mice (Figure 6).

Overall the results of our immunolocalisation studies, suggest that TRPM1 is expressed in ON-bipolar cells and also a subset of cells in the GCL, including pRGCs, whereas TRPM3 is expressed in Muller cells and also cells of the ciliary body but is absent from pRGCs.

Discussion

A remarkable body of recent research has confirmed a role for TRPM1 in the depolarising light responses of ON bipolar cells, and shown that mutations in TRPM1 appear to account for about half of all cases of complete congenital stationary night blindness (CSNB1) (Morgans *et al.*, 2010). In addition to this role in the ON visual pathway, our microarray studies suggest that TRPM1 and TRPM3 may play a potential role in responses to light in the absence of rods and cones, presumably mediated by melanopsin expressing pRGCs (Peirson *et al.*, 2007). The visual phenotype of the *Trpm1*$^{-/-}$ mice used in this study has been investigated previously (Morgans *et al.*, 2009). ERG recordings from *Trpm1*$^{-/-}$ mice show a normal a-wave but the b-wave (a measure of ON-bipolar cell depolarisation) is absent (Morgans *et al.*, 2009; Shen *et al.*, 2009). *Trpm1*$^{-/-}$ mice also have visual defects, including a relatively modest reduction in spatial frequency threshold (10%) and reduced contrast threshold (3-fold) compared to normal wildtype controls (Morgans *et al.*, 2009). These authors conclude that *Trpm1*$^{-/-}$ mice have significant but not profound visual impairment, similar to those observed for CSNB. However, to date no study has investigated non-image forming responses to light in *Trpm1*$^{-/-}$ animals. In this study we have used the pupillary light response to assess the function of both rod cone and melanopsin based signalling pathways in *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice.

A role for Trpm1 in non-image forming responses to light

Consistent with previous reports, our data demonstrates that both the rod/cone photoreceptors and melanopsin pRGCs contribute significantly to pupillary responses to light. Only subtle defects were observed in mice lacking melanopsin, or mice lacking rods and cones, demonstrating that whilst melanopsin is required to attain full pupil constriction in response to bright light (Lucas *et al.*, 2003), either pathway is capable of driving significant pupillary constriction. Previous studies have shown that removal of all three classes of photoreceptors is necessary to completely abolish the pupillary light response in mice (Hattar *et al.*, 2003). Remarkably *Trpm1*$^{-/-}$ mice show a profound attenuation of pupillary responses to light, with only very limited levels of pupil constriction observed in response to bright light illumination, and a complete lack of constriction observed under dim light conditions. Given that existing data indicates that expression of *Trpm1* mRNA is restricted to skin and retina, and is absent from the brain (Morgans *et al.*, 2009; Koike *et al.*, 2010), it would seem unlikely that the pupil defect observed in *Trpm1*$^{-/-}$ mice is due to downstream changes in the neural circuits that regulate pupil constriction, but is instead mediated by changes in retinal function. The pupillary defect observed in *Trpm1*$^{-/-}$ mice

was significantly greater than that observed in *rd/rd cl* animals and cannot therefore be explained by the loss of ON-bipolar cell function and disruption of rod and cone driven signals alone. In fact, the defect observed in *Trpm1*$^{-/-}$ mice is most consistent with a defect in both ON bipolar function and also melanopsin driven responses in the retina of these mice. In support of this conclusion, it is worth noting that mGluR6-deficient mice, which are defective in the same ON bipolar signalling pathway as *Trpm1*$^{-/-}$ mice, are still able to attain pupil constriction down to ~25% area (compared with preceding pupil area in darkness) (Iwakabe *et al.*, 1997). Taken together, the profound defects in pupillary responses over and above those observed in the absence of rods/cones or in other transgenic mouse lines with defective ON bipolar cell signalling (Iwakabe *et al.*, 1997; Thompson *et al.*, 2011) strongly suggests a substantial contribution of TRPM1 to pupillary light responses in a manner that is independent of bipolar cell function.

PCR analysis of isolated ganglion cell preparations indicates that *Trpm1* mRNA is expressed in cells of the GCL, although this expression is seemingly not restricted to melanopsin pRGCs (as expression is detected in samples lacking *Opn4* expression). In support of a direct role for TRPM1 in the melanopsin signalling pathway we consistently detected the expression of the TRPM1 β-gal reporter in melanopsin-expressing pRGCs, including M1 and M2 type pRGCs and also displaced pRGCs with cell bodies located in the INL (Berson *et al.*, 2010; Schmidt *et al.*, 2011). However, previous reports detailing the expression of TRPM1 in the retina (*in situ* hybridisation and immunocytochemistry) have reported that expression of TRPM1 is confined to ON-bipolar cells (Morgans *et al.*, 2009; Koike *et al.*, 2010), although one study has reported low but detectable transcript levels in the GCL (Hackler *et al.*, 2010). The reason for the discrepancy between these previous studies and our results are unclear. It is plausible that the detection of the β-gal transgene reporter offers a more sensitive method for determining expression of TRPM1 than those used in previous studies. However, it is worth noting that on close examination of the images shown by Morgans et al, (2009) a case can be argued for a weak level of labelling of the TRPM1-L antibody in the GCL, albeit at lower levels than observed in bipolar cells. Alternatively, it is possible that the β-gal transgene in *Trpm1*$^{-/-}$ mice may report the expression of additional TRPM1 splice variants that are not recognised by the specific probes used previously (Oancea *et al.*, 2009). A further possibility is that our β-gal results are an artefact of the transgenic mouse line used in this study, and that expression of the β-gal reporter is aberrantly expressed in other cell types that do not express TRPM1. It is also possible that changes in the pattern of TRPM1 expression are induced by functional changes in *Trpm1* deficient mice, potentially due to developmental abnormalities or disruption of the transcription control elements that regulate *Trpm1* expression. However, such possibilities fail to explain the light induction of *Trpm1* expression in the *rd/rd cl* retina, the presence of *Trpm1* mRNA in isolated GCL preparations from wildtype mice, or the results of our pupillometry studies where a clear and striking defect in non-image forming responses is evident in *Trpm1*$^{-/-}$ mice. We suggest that low levels of TRPM1 are expressed in a subset of cells in the GCL, which includes melanopsin-expressing pRGCs.

Combined our data suggest that TRPM1 plays a previously uncharacterised role in non-image forming responses to light that is distinct from its role in ON bipolar cells, and is

consistent with a role in melanopsin pRGCs as previously predicted (Peirson *et al.*, 2007). However, the potential cellular functions of TRPM1 in pRGCs (and other ganglion cells) are currently unclear. Stimulation of the melanopsin signalling pathway itself is known to result in activation of a $G\alpha_{q/11}$ type G-protein signalling pathway leading to the activation of PLC-β isoforms and ultimately the influx of Ca^{2+} through an as yet unidentified TRP like channel in the cell membrane (Hankins *et al.*, 2008; Do & Yau, 2010). Previous studies have indicated that the most likely candidates for this TRP-like channel are members of the TRPC channel subfamily, potentially TRPC3, TRPC6 or TRPC7 (Sekaran *et al.*, 2003; Warren *et al.*, 2006; Graham *et al.*, 2008), although recent evidence has shown that intrinsic photoresponses persist in pRGCs lacking each of these channels (in isolation) and suggests only a non-essential role for TRPC6 in melanopsin signalling (Perez-Leighton *et al.*, 2011). Despite the identification of a profound attenuation of pupillary responses to light in *Trpm1*$^{-/-}$ mice, and the consistent detection of the β-gal reporter within melanopsin pRGCs, based on the biophysical and pharmacological properties of the light induced current in pRGCs TRPM1 would appear unlikely to be the as-yet unidentified channel mediating the primary depolarising responses to light in these cells. The depolarising Trp channel in pRGCs is known to be activated by $G\alpha_{q/11}$ type G-proteins and is largely insensitive to agents that influence the $G\alpha_o$ pathway, whereas by contrast TRPM1 is spontaneously active and negatively regulated by the $G\alpha_o$ signalling pathway (Lambert *et al.*; Graham *et al.*, 2008; Do *et al.*, 2009; Morgans *et al.*, 2009; Koike *et al.*, 2010). However, it is possible that multiple TRP-like channels contribute to the cellular functions of pRGCs (Perez-Leighton *et al.*, 2011), including TRPM1, and this channel may regulate resting membrane potential, depolarisation and/or intrinsic photoresponses, potentially in a $G\alpha_o$ dependant manner similar to that seen in ON-bipolar cells (Morgans *et al.*, 2009; Koike *et al.*, 2010; Morgans *et al.*, 2010). It is also possible that TRPM1 contributes to intracellular calcium homeostasis within pRGCs, as has been suggested in melanocytes (Devi *et al.*, 2009). Further electrophysiological studies are required to determine the specific role played by TRPM1 in pRGCs. Moreover, expression of TRPM1 does not appear to be restricted to pRGCs, but was also detected in a significant proportion of other cells in the GCL. We cannot therefore rule out a more generalised role of TRPM1 in ganglion cell function, which is in some way necessary for pRGC signalling. However, the relatively modest reported effects on spatial vision in the *Trpm1*$^{-/-}$ mice suggest that any defect in the visual retinal ganglion cells is minor. Selective deletion of TRPM1 expression within pRGCs, potentially via conditional knockouts, will be necessary to confirm the specific role of TRPM1 in pRGC function and melanopsin phototransduction.

Irrespective of whether or not TRPM1 is indeed a direct component of the melanopsin signalling pathway or influences the pupillary light response via an indirect mechanism, the data described here does demonstrate a significant role for TRPM1 in non-image forming responses to light. The implications of our findings are that human CSNB1 patients with mutations in the TRPM1 gene may also show defects in such responses, potentially impacting on not only pupil constriction but also circadian entrainment and regulation of sleep. Indeed, this may provide a potential diagnostic tool for CSNB1 resulting from TRPM1 mutations. Assessment of pupillary light responses in these patients in combination with sleep monitoring studies will be required to confirm this hypothesis. However it should

be noted that loss of TRPM1 exerts a greater deficit on rod ON-bipolar cell function than cone ON-bipolar function (Morgans *et al.*, 2009), and as humans have a significantly higher proportion of cones compared to the rod-dominated mouse retina, the defects observed in pupil responses from human patients may not be as severe as those observed in *Trpm1*$^{-/-}$ mice.

A role for Trpm3 in non-image forming responses to light

By contrast to TRPM1, the role of TRPM3 in the mammalian retina has not previously been investigated, although *in situ* hybridisation experiments have identified expression of *Trpm3* in the retinal pigment epithelium, inner nuclear layer and ganglion cell layer, in addition to the ciliary body and lens epithelial cells (Karali *et al.*, 2007). We show that *Trpm3*$^{-/-}$ mice exhibit attenuated pupillary responses to bright light, yet in contrast to *Trpm1*$^{-/-}$ animals this defect was relatively subtle and more closely resembles the phenotype observed in *Opn4*$^{-/-}$ mice with a failure to reach full pupil constriction and an exaggerated post-stimulus response (Lucas *et al.*, 2003). However, *Trpm3*$^{-/-}$ mice show an attenuated pupil constriction under both bright light and dim light conditions. This property is not shared by *Opn4*$^{-/-}$ mice that show diminished PLR only at higher light intensities (Lucas *et al.*, 2003). This data indicates that *Trpm3*$^{-/-}$ mice are not a phenocopy of *Opn4*$^{-/-}$ mice and that the mechanisms underlying the PLR defect in these mice are most likely different. This conclusion is supported by the observation that convincing expression of the β-gal reporter was not detected within melanopsin expressing pRGCs in *Trpm3*$^{-/-}$ mice. Furthermore, in agreement with previous *in situ* studies (Karali *et al.*, 2007), high levels of TRPM3 expression were detected in the ciliary body and therefore a defect in muscle function cannot be eliminated as the basis of the pupillary phenotype observed in these mice. Studies in bovine ciliary muscle cells suggest that muscarinic stimulation by carbachol involves two types of non-selective cation channel, and Trp channels have been suggested as potential candidates (Takai *et al.*, 2004). We suggest that TRPM3 may be one of the unidentified channels involved in regulating pupil constriction, but may also perform other roles in the retina. Future studies are required to clarify the level at which TRPM3 is involved in mediating pupillary responses to light.

Conclusions

In summary, here we demonstrate that both *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice show attenuated pupillary light responses. The profound nature of the defect observed for *Trpm1*$^{-/-}$ mice suggests a defect in both rod/cone and melanopsin pRGC-mediated signalling. The expression of TRPM1 in pRGCs indicates that TRPM1 may play a functional role in melanopsin signalling and therefore participate in both the classical ON visual pathway and also non-imaging forming response to light. The pupillary defect observed in *Trpm3*$^{-/-}$ mice has similarities to that observed in *Opn4*$^{-/-}$ mice, although the lack of TRPM3 expression in pRGCs and the strong expression in Muller cells and cells of the ciliary body suggest that this channel may influence pupillary responses and or pRGC function via an indirect mechanism. This conclusion is seemingly supported by responses to dim light illumination, where attenuated pupil constriction is observed for *Trpm3*$^{-/-}$ mice compared to wildtype controls, a property not consistent with the phenotype of *Opn4*$^{-/-}$ mice. Further

experiments will be needed to clarify the roles of both channels in pRGC function and their contributions to non-image forming responses to light.

Acknowledgements

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Abbreviations

β-gal	β-galactosidase
CCD	Charge couple device
DAPI	4′,6-diamidino-2-phenylindole
GCL	Ganglion cell layer
INL	Inner nuclear layer
OCT	Optimum Cutting Temperature
PBS	Phosphate buffered saline
pRGC	Photosensitive retinal ganglion cell
RGC	Retinal ganglion cell
TRPM	Transient receptor potential cation channel, subfamily M

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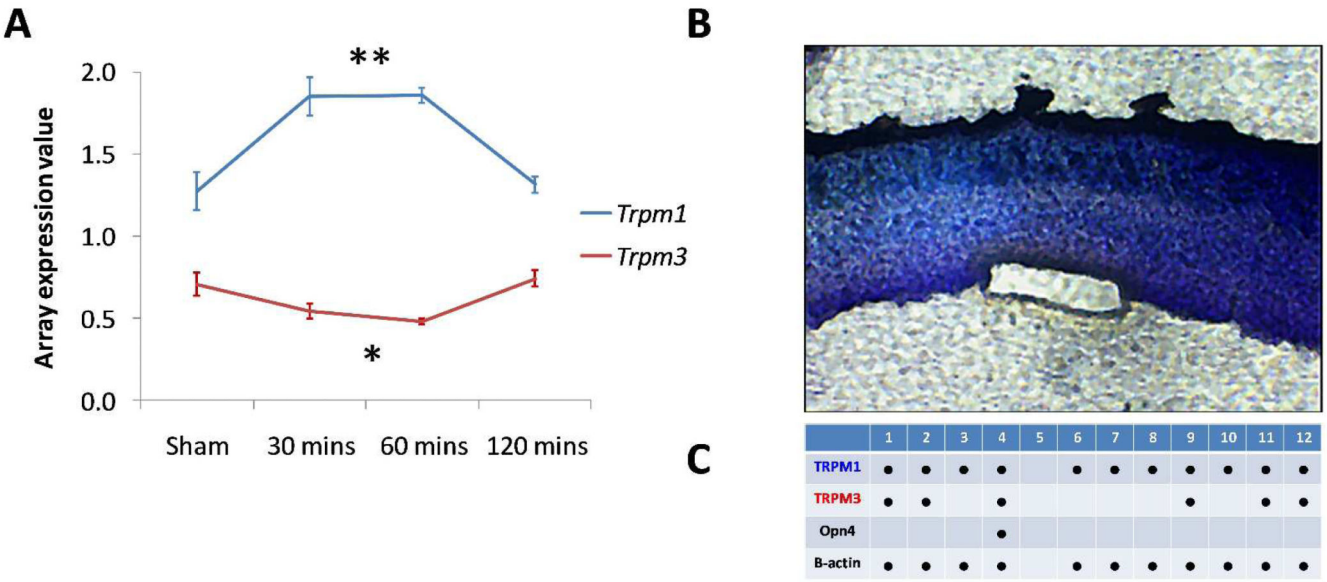

Figure 1.

Expression of *Trpm1* and *Trpm3* mRNA. **(A)** Microarray data show that *Trpm1* and *Trpm3* are acutely regulated by light in the eye of mice lacking rods and cones (*rd/rd cl*). *Trpm1* was significantly upregulated in response to light (**=$F_{3,12}$ =13.4, P=0.00039, P=0.0054 with FDR correction), whereas *Trpm3* was significantly downregulated (*=$F_{3,12}$ =7.38, P= 0.0046, P=0.0079 with FDR correction). **(B, C)** Both *Trpm1* and *Trpm3* mRNA are detected in the retinal ganglion cell layer of wildtype mice, based upon PCR analysis of cDNA from isolated ganglion cell layer preparations. **(B)** Retinal section stained with 20% cresyl violet, following the isolation of a retinal ganglion cell layer preparation using laser capture microdissection (typically 30-40 cells/preparation). **(C)** Summary table showing the expression of *Trpm1*, *Trpm3*, *Opn4* and β-*actin* in 12 ganglion cell layer preparations.

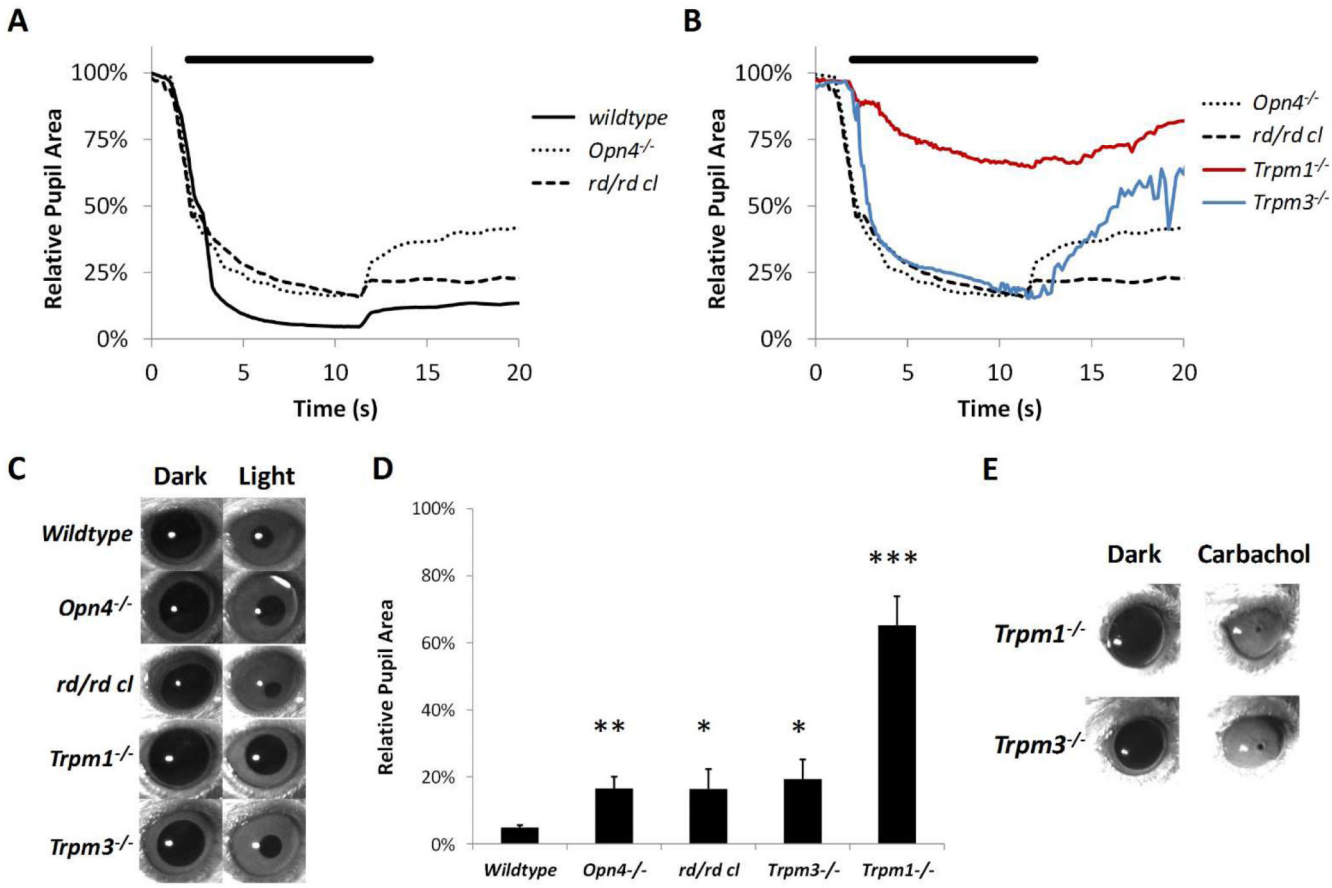

Figure 2.

Analysis of the pupillary light response (PLR) in *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice. **(A)** Pupil constriction profiles recorded from normal wildtype, *Opn4* $^{-/-}$ and *rd/rd cl* mice in response to bright light illumination (480nm, 14.6 log quanta/cm^2/s). **(B)** Pupil constriction profiles recorded from *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice in response to bright light illumination (14.6 log quanta/cm^2/s). Responses from *Opn4*$^{-/-}$ and *rd/rd cl* mice are plotted for comparison. Light stimuli from 2-12 seconds (black bar above traces). Profiles shown are mean of responses from n=3-6 mice. **(C)** Representative images of pupil size recorded before and after bright light stimulation. **(D)** Graph shows the maximal pupil constriction observed following bright light exposure. * = p<0.05, **=P<0.01, ***=P<0.001. **(E)** Images of pupil size observed for *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice before and after application of 1M carbachol.

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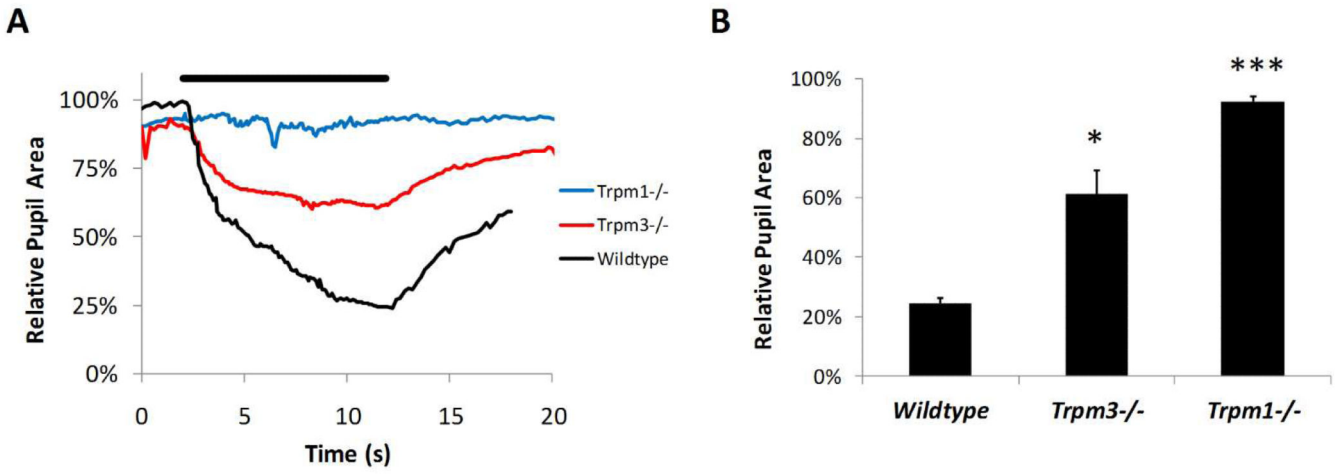

Figure 3.

Analysis of the pupillary light response (PLR) in *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice. **(A)** Pupil constriction profiles recorded from *Trpm1*$^{-/-}$, *Trpm3*$^{-/-}$ and normal wildtype mice in response to dim light illumination (11.6 log quanta/cm^2/s). Profiles shown are mean of responses from n=3-6 mice. **(B)** Graph shows the mean maximal pupil constriction observed following dim light exposure. * = p<0.01, ***=P< 0.5E-8.

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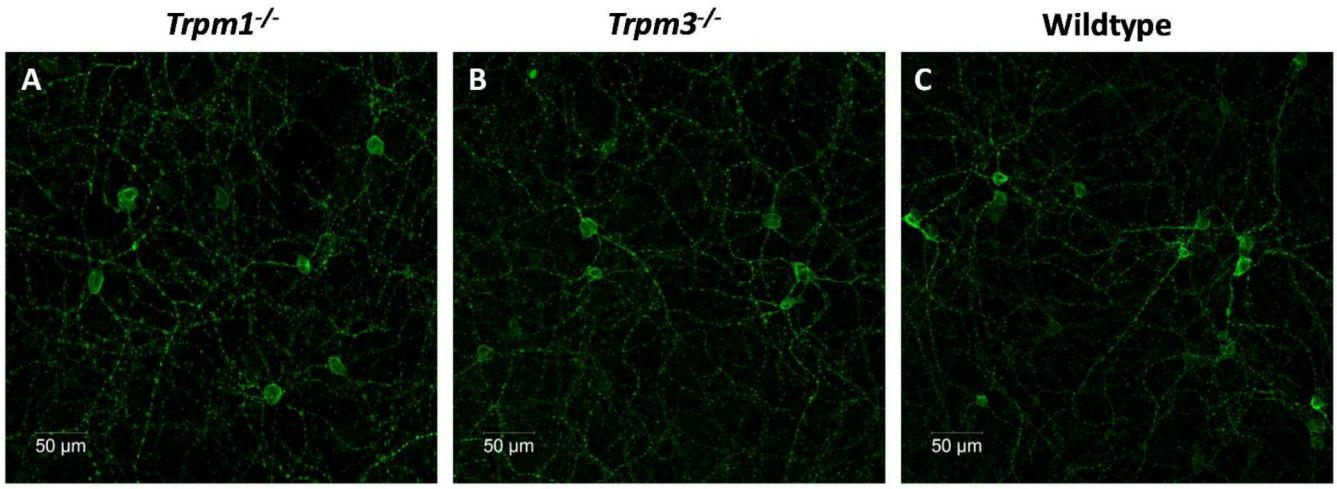

Figure 4.

Melanopsin expression in *Trpm1*$^{-/-}$ and *Trpm3*$^{-/-}$ mice. Confocal images, showing the levels of melanopsin immunoreactivity observed in *Trpm1*$^{-/-}$ (**A**), *Trpm3*$^{-/-}$ (**B**) and normal wildtype whole mounted retina (**C**). Note that no differences were observed, with levels of expression, number of cells and distribution of melanopsin expressing cells similar for all strains of mice.

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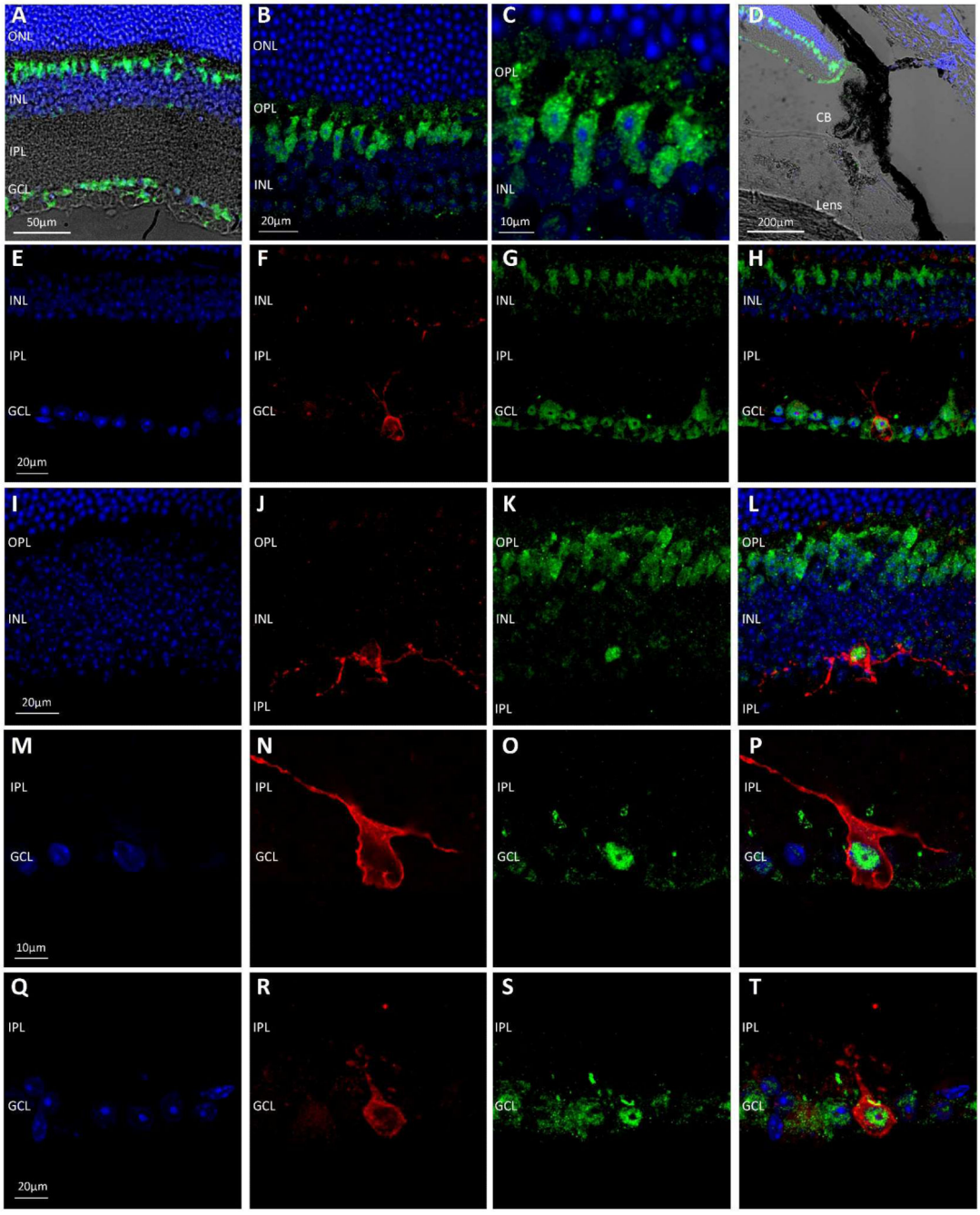

Figure 5.

Localisation of TRPM1 in the mouse retina. **(A-C)** Localisation of TRPM1 expression via detection of the β-gal reporter (green) in the *Trpm1*$^{-/-}$ retina demonstrates expression in cells of the inner nuclear layer (INL) resembling bipolar cells, and in a subpopulation of cells in the ganglion cell layer (GCL). **(D)** TRPM1 (β-gal) expression was not detected in the cells of the ciliary body. **(E-T)** Double staining for β-gal (green) and melanopsin (red) in the *Trpm1*$^{-/-}$ retina demonstrates the consistent co-expression of TRPM1 (β-gal) within melanopsin expressing pRGCs, including M1 type pRGCs, M2 type pRGCs and displaced

pRGCs. DAPI nuclear counter stain is shown in blue. Outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), ciliary body (CB).

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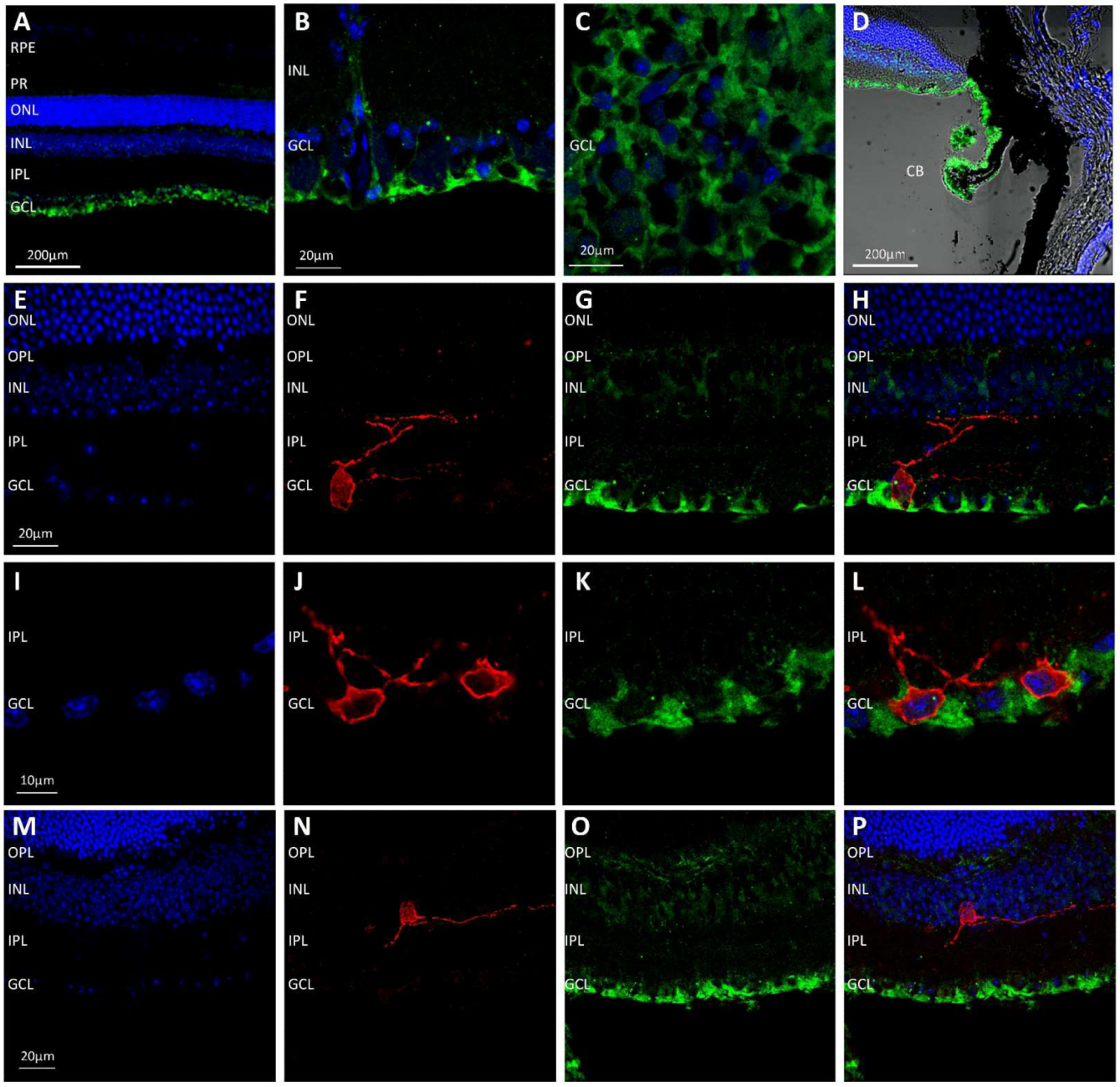

Figure 6.

Localisation of TRPM3 in the retina. **(A-C)** Localisation of TRPM3 expression via detection of the β-gal reporter (green) in the *Trpm3*$^{-/-}$ retina demonstrates strong expression in structures located in the ganglion cell layer (GCL) resembling Muller cell end feet **(D)** Strong expression of the β-gal reporter is detected in the cillary body. **(E-L)** Double staining for β-gal (green) and melanopsin (red) demonstrates the lack of TRPM3 (β-gal) expression within melanopsin expressing pRGCs located in the GCL. **(M-P)** Expression of TRPM3 (β-gal) is absent in displaced pRGCs in the *Trpm3*$^{-/-}$ retina. DAPI nuclear counter stain is shown in blue. Photoreceptors (PR), outer nuclear layer (ONL), outer plexiform layer

(OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), ciliary body (CB).

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