Current Biology 17, 1363–1372, August 21, 2007 ©2007 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2007.07.045

Article

Microarray Analysis and Functional Genomics Identify Novel Components of Melanopsin Signaling

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Summary

Background: Within the mammalian retina, there exists a third photoreceptive system based upon a population of melanopsin (*Opn4*) expressing photosensitive retinal ganglion cells (pRGCs; also termed ipRGCs or intrinsically photosensitive RGCs). Here, we use a microarray-based approach, which we term transcriptional recalibration, coupled with functional genomics to identify downstream targets of melanopsin signaling.

Results: In a mouse with genetically ablated rods and cones (*rd/rd cl*), approximately 30% of the ocular transcriptome is transiently regulated in response to nocturnal light exposure (3112 genes). A total of 163 of these genes were associated with the "intracellular signaling" gene ontology term. On the basis of their similarity to invertebrate phototransduction genes, 14 were selected for further study. Laser capture microdissection demonstrated that eight of these genes (*Gnas*, *Gnb2l1*, *Gnaq*, *Prkcz*, *Pik3r1*, *Inadl*, *Slc9a3r1*, and *Drd1a*) colocalized with melanopsin. The impact of genetic ablation of one of these genes, protein kinase C zeta (*Prkcz*), was assessed. $Prkcz^{-/-}$ animals show attenuated phase-shifting responses to light, reduced period lengthening under constant light, and attenuated pupillary responses at high irradiances, as well as impaired light-induced gene expression in the suprachiasmatic nuclei (SCN). These attenuated responses are indistinguishable from the deficits observed in melanopsin knockout mice.

Conclusions: Here, we show that (1) *Prkcz* plays an as yet unidentified role in melanopsin signaling, (2) the proteins of seven further light-regulated genes emerge as strong candidates in melanopsin signaling, and (3) transcriptional recalibration may provide a powerful new approach for dissecting unmapped signaling pathways.

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Introduction

Mice lacking both rod and cone photoreceptors (*rd/rd cl*) retain multiple nonvisual responses to light, including phase shifting of circadian behavior, acute suppression of pineal melatonin, and pupil constriction [1–3]. The photoreceptors mediating these responses are a subset of photosensitive retinal ganglion cells (pRGCs) that express melanopsin (OPN4) [4–7]. Unlike hyperpolarization responses of the rods and cones of the outer retina, light triggers depolarization of pRGCs associated with marked changes in intracellular Ca^{2+} [6, 7]. Recent in vitro studies using cells transfected with melanopsin provide compelling evidence that this protein is the photopigment of the pRGCs [8–10]. The pRGC phototransduction cascade is pertussis toxin insensitive, leading to the suggestion that melanopsin may utilize an invertebrate-like signaling cascade [10, 11]. Furthermore, pharmacological experiments suggest that melanopsin signals via a G_q-type G protein coupled to phospholipase C, resulting in TRP channel activation [9, 10, 12]. These studies, although highly informative, explored melanopsin phototransduction in different cell lines and so may not faithfully reflect the native transduction cascade of melanopsin pRGCs [8, 13].

To investigate the phototransduction mechanisms of pRGCs in vivo, we followed changes in the ocular transcriptome of mice lacking rods and cones (*rd/rd cl*) in response to a 15 min light pulse by using microarray hybridization. Transcriptional regulation is unlikely to be involved in the primary events of phototransduction, which are based upon rapid posttranslational phenomena such as protein interaction and modification [14]. However, we predicted that the perturbation of the genetic network encoding the phototransduction cascade by an acute stimulus would produce a transcriptional adjustment or "recalibration" of those genes whose protein products contribute to this primary signaling event [15, 16]. We successfully applied this method to identify novel candidate genes involved in melanopsin signaling. A detailed analysis of one of these genes, the atypical C-type protein kinase *Prkcz*, in *Prkcz*-deficient mice showed that this kinase plays a critical role in melanopsin signaling.

Results

Light-Induced Transcriptional Responses

Within 60 min after nocturnal light exposure, 3245 probe sets in the *rd/rd cl* eye (out of ~45,100 probe sets represented on the MG430v2 array) demonstrated significant changes in expression levels. These probe sets corresponded to 3,112 out of a total of 10,274 genes reliably identified as being expressed, corresponding to ~30% of the *rd/rd cl* ocular transcriptome. Hierarchical clustering demonstrated similar numbers of upregulated and downregulated genes (1567 versus 1678, respectively). Moreover, the effects of light on gene expression were

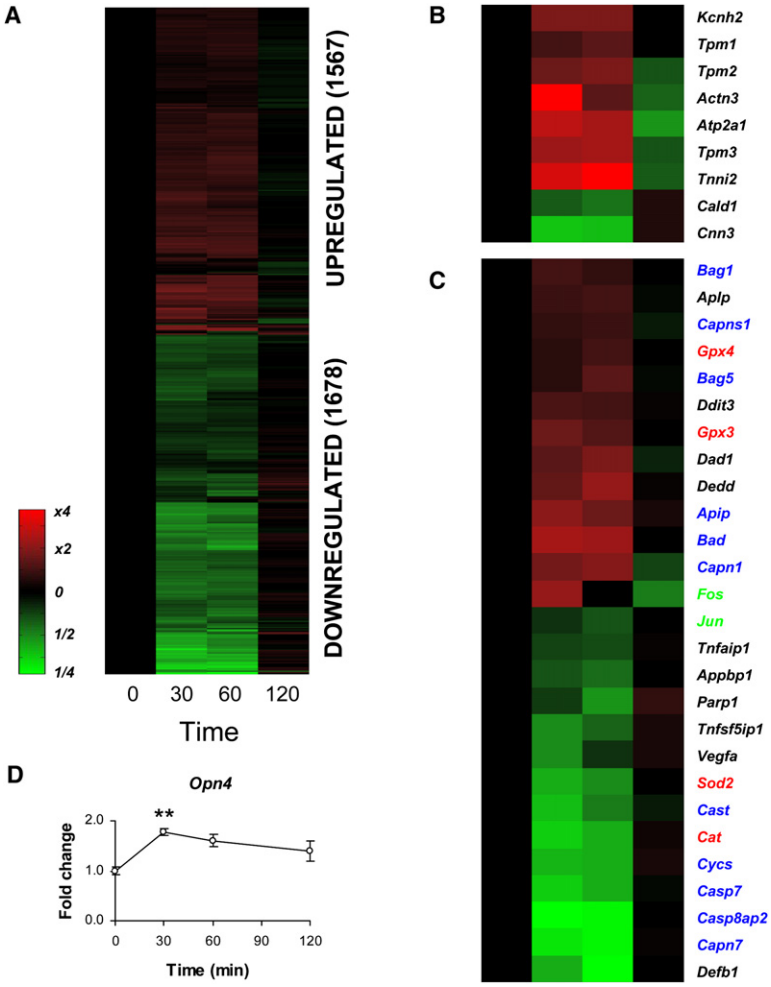

Figure 1. Approximately 30% of the Ocular Transcriptome of the *rd/rd cl* Eye Is Significantly Modulated in Response to Light

(A) Clustergram of genes regulated by light in the *rd/rd cl* eye. Note that changes in ocular transcription were transient, and by 120 min, most transcripts exhibited similar expression levels as the dark-housed sham controls ($n = 4$, $p < 0.05$ after multiple test correction). Red indicates increased expression, and green indicates decreased expression when compared to the sham control group (black).

(B and C) Transcriptional recalibration of genes associated with iris muscle contraction (B) and neuroprotection (C) after light exposure (all data normalized to the corresponding sham group; $n = 4$). Highlighted apoptotic genes are associated with AP-1 complex (green text), oxidative stress (red text), and *Bcl2*/caspase/calpain apoptotic pathways (blue text).

(D) *Opn4* expression was transiently induced in the *rd/rd cl* eye in response to nocturnal light exposure, when assayed by qPCR with primer sets designed to exons 2–3 of the predicted mouse transcript (mean ± SEM, $n = 4$, $p < 0.01$).

transient and by 120 min mRNA levels were again broadly comparable to those of the control group (Figure 1A). To validate the microarray data, we determined the profile of expression of several of the regulated genes by qPCR on the same nonamplified RNA samples. Overall, both the dynamics and the degree of transcriptional change were similarly described by both methods (Figure S1A in the Supplemental Data available online). Among these genes was the immediate early gene *Fos* that, as described previously [17], exhibited a rapid upregulation peaking at ~30 min and returning to baseline levels by 120 min.

Multiple pathways should be modulated by light within the eye. For example, pRGC-mediated pupil constriction [3] should lead to a recalibration of transcripts associated with the contraction of muscle proteins within the iris. One would also predict that even a transient bright-light stimulus may trigger neuroprotective pathways within the eye [18]. Therefore, the data were mined for genes associated with the known pathways mediating muscle contraction and neuroprotection. Nine transcripts linked to muscle contraction were identified (Figure 1B), including the muscle thin filament proteins tropomyosin 1-3 (*Tpm1-3*) and the actin-binding proteins caldesmon 1 (*Cald1*) and calponin 3 (*Cnn3*). Additionally, 81 transcripts associated with neuroprotective processes were transiently light regulated (Figure 1C), including a number of proteins that are involved in reactive oxygen species metabolism (*Sod2*, *Gpx3-4*, and *Cat*) and that have been implicated in lipid peroxidation and phototoxicity in the retina [19]. Furthermore, transcripts associated with both the AP-1 (*Fos* and *Jun*) and apoptosome complexes (*Apif* and *Cycs*) were identified. Both caspases (*Casp7*, *Casp8ap*) and calpains (*Capn7*, *Capns1*) were found to be light modulated, as were genes associated with *Bcl-2*-mediated cell death (*Bag1*, *Bag5*, and *Bad*) [18, 20]. We have yet to determine whether the light modulation of these neuroprotective pathways is triggered by the direct energetic effects of light or whether they are mediated via the pRGC system. Functional genomic approaches, of the sort we describe below, will be required for determining whether these genes are strong candidates for further study. These results are consistent with our prediction that specific signaling pathways are regulated by light.

Light modulation of the melanopsin signaling pathway in the *rd/rd cl* eye was initially addressed by analysis of the melanopsin gene itself. Interestingly, melanopsin was qualified as "not present" from the microarray data. However, the Affymetrix MG430v2 chips used in this study contain only a single probe set for melanopsin, and this set maps to the 3′ terminal sequence

Table 1. Classification of Molecular Functions of Intracellular-Signaling-Cascade Genes

Classification	Number	Transcripts
Small GTPases	34	Arfrp1, Arf4, Arf6, Arl3, Arl4a, Arl6, Arl10c, Cdc42, Diras1, Hras1, Mras, Rab1, Rab2, Rab4b, Rab5a, Rab6, Rab8b, Rab10, Rab11b, Rab14, Rab18, Rab24, Rab27a, Rab28, Rab33b, Ralb, Ran, Rap1a, Rap1b, Rheb, Rhoc, Rit2, Rnd3, and 5430435G22Rik
Heteromeric G protein subunits	7	Gna13, Gnaq, Gnas, Gnb1, Gnb2l1, Gng3, and Gng13
Guanyl-nucleotide exchange factors	5	Rabif, Rapgef2, Rasgrf1, Sos2, and Vav3
Other proteins[a]	13	Ctnnal1, Ddit3, Dnaja1, Gtpbp4, Hspa5, Kif16b, Mt1, Mt2, Nenf, Nup62, Pbp, Pkd2, and Smad1
Protein kinases	20	Araf, Braf, Crk, Lats2, Mapk8, Mapk10, Mast1, Mast2, Mast4, Nlk, Pik3r1, Pik3r3, Pink1, Prkcz, Raf1, Rock1, Srpk2, Stk3, Tlk1, and Ulk1
Other enzymes[b]	21	Akr1b3, Car8, Ddah2, Edd1, Egln2, Gucy1a3, Gucy1b3, Magi1, Ndufb2, Nudt4, Plce4, Ppib, Ppp2ca, Ptpn11, Thop1, Timp2, Usp8, Vcp, Wsb1, Ywhae, and Ywhag
PX adaptor proteins	10	Snx1, Snx2, Snx5, Snx6, Snx9, Snx13, Snx14, Snx16, Snx17, and Snx19
PDZ adaptor proteins	9	Arhgef12, Dvl1, Erbb2ip, Inadl, Pclo, Pdlim4, Rhpn2, Sipa1l1, and Slc9a3r1
Other adaptor proteins	30	Arhgap1, Arhgap5, Arl1, Cdgap, Cblb, Cnih4, Dab1, E430034L04Rik, G431001E03Rik, Gab1, Gadd45b, Gdi2, Grb7, Gulp1, Mapk8ip2, Mapkbp1, Nck1, Ncoa2, Ptplad1, Rabl4, Rasa1, Rasa2, Socs5, Sh2bpsm1, Snag1, Socs7, Spag9, Statip1, Trim23, and Ywhaq
Receptor proteins	6	Cd81, Dcbld2, Drd1a, Fgfr3, Nisch, and Nr3c1
Unknown classification	8	Asb13, Derl1, Pea15, Rasl11b, Rsu1, Spsb3, Wsb2, and 5730407K14Rik
Total	163	

Molecular function classifications of light-regulated genes connected to intracellular signal transduction (163 genes; overrepresentation significance p = 0.0047).

[a] Including channels, cytoskeleton proteins, globins, growth factors, heat shock proteins, lipid-binding proteins, metallothioneins, and transcription factors.

[b] Including carbonate dehydratases, dehydrogenases, guanylate cyclises, guanylate kinases, hydrolases, isomerases, monooxygenases, oxidoreductases phopholipases, phosphatases, prolyl hydroxylases, proteases, protein ligases, and ubiquitin ligases.

of the mouse transcript (nucleotides 1553–2116 of NM_013887). RT-PCR with primers targeting the 5′ region of the melanopsin transcript on the same RNA samples used for the microarrays demonstrated that melanopsin was indeed expressed in the *rd/rd cl* eye. These results, and additional unpublished findings (J. Bellingham and S. Halford, personal communication), provide evidence for the existence of a short melanopsin isoform in the mouse, an isoform that lacks a significant portion of the 3′ coding region. To determine whether melanopsin exhibits transcriptional recalibration, we undertook qPCR analysis on cDNA from the *rd/rd cl* eye by using primers designed to exons 2–3 of mouse melanopsin. This gene was shown to be present and was significantly and transiently upregulated after light exposure (Figure 1D). Collectively, the data presented in Figures 1B–1D are all consistent with the hypothesis that microarray measures of transcriptional recalibration provide a means to identify candidate components of unmapped signaling pathways.

Light Modulation of Intracellular Signaling Cascades

Among the light-regulated genes, the gene ontology (GO) class "intracellular signaling cascade" was significantly overrepresented (p = 0.0047, based on hypergeometric distribution) and provided the basis for subsequent analyses (Tables S1 and S2). The 163 genes of this biological process category were further classified for molecular function (Table 1). A total of 14 genes were chosen for further investigation on the basis of existing knowledge of G protein-coupled and invertebrate phototransduction signaling pathways. These included G protein subunits and related genes (*Gnas*, *Gna13*, *Gnb2l1*, *Gng13*, and *Gnaq*), genes related to phosphoinositide signaling (*Plce2*, *Prkcz*, *Pik3r1*, and *Pik3r3*), genes coding for PDZ-domain-containing proteins (*Inadl*, *Slc9a3r1*, and *Magi1*) as well as a receptor of the GPCR superfamily and a Trp-like ion channel (*Drd1a* and *Pkd2*). For all candidates, expression in the *rd/rd cl* retina was confirmed by qPCR (Figure S1B). We performed RT-PCR on RNA extracted from laser-capture-microdissected ganglion cells to determine which of the candidates were associated with melanopsin (Figure 2A). Six out of 14 of the selected genes were excluded from further analysis because they failed to colocalize in any preparations (Figure 2A) and thus left eight remaining candidates (*Gnas*, *Gnb2l1*, *Gnaq*, *Prkcz*, *Pik3r1*, *Inadl*, *Slc9a3r1*, and *Drd1a*).

Protein Kinase C Zeta Is Expressed in pRGCs

Prkcz was chosen as our first candidate for functional analysis on the basis that it has an unknown function in the mammalian eye and showed a light-induced expression profile very similar to melanopsin (Figure 1D and Figure S1A). Furthermore, in *Drosophila*, PKCs have been implicated in the phototransduction cascade [11, 21], and in the *rd/rd cl* eye, *Prkcz* was the only PKC regulated by light. PKCζ was expressed in a subset of retinal ganglion cells (Figure 2B). A further association of PKCζ and melanopsin was demonstrated with immunocytochemistry. Colabeling of PKCζ and *Opn4*-Gal in *Opn4-Gal* heterozygous retinal sections showed expression of PKCζ at the extracellular membrane of all Gal-positive cells (Figure 2C). Note that although PKCζ

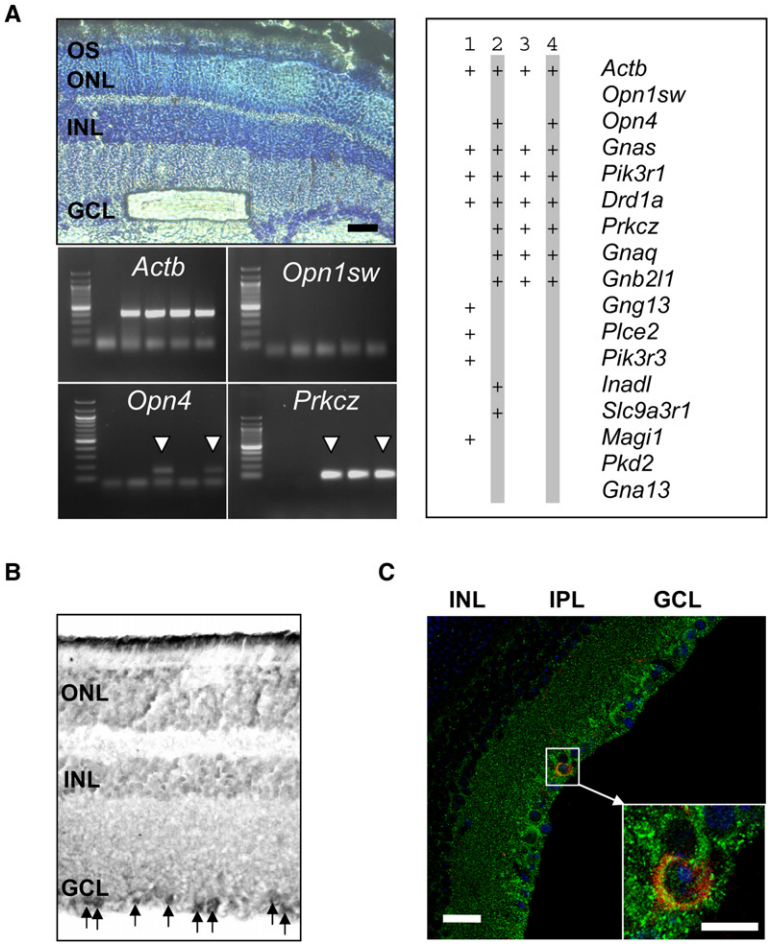

Figure 2. Expression Analysis Implicates PKCζ as a Candidate for Melanopsin Signaling

(A) PCR analysis of retinal ganglion cell layer sections (upper left) and then agarose gel electrophoresis of PCR products from four LMPC cDNA preparations of wild-type retinal ganglion cells (lower left). *Actb* (β-Actin) served as a positive control for viable cDNA, and *Opn1sw* (UV-S cone opsin) served as a negative control for photoreceptor layer contamination. White arrows indicate those preparations containing *Opn4*. The right panel illustrates those genes expressed in each of the four (1–4) LMPC preparations. Presence indicated by plus signs. Shaded columns indicate those LMPC preparations in which *Opn4* was detected.

(B) PKCζ protein is expressed in a subset of retinal ganglion cells (arrows).

(C) Coexpression of melanopsin (red) and PKCζ (green) proteins in photosensitive retinal ganglion cells. Confocal imaging of double-labeled wild-type retina sections shows that PKCζ is expressed in the retinal ganglion cell layer including all melanopsin-positive cells (red; yellow indicates colocalization). Blue coloration corresponds to DAPI staining of nuclei. The inset shows a single OPN4-positive cell at higher magnification (abbreviations are used as follows: GCL, ganglion cell layer; IPL, inner plexiform layer; and INL inner nuclear layer; scale bars indicate 20 μm).

was always coexpressed with melanopsin, it is also expressed in a population of nonmelanopsin retinal ganglion cells (Figures 2B and 2C).

Prkcz Is Expressed in the Retinal Pigment Epithelium

Melanopsin expression has been reported previously in the retinal pigment epithelium (RPE) [22]. To determine whether *Prkcz* shows synonomous expression within this tissue, we undertook a series of RT-PCRs. Our results show that both melanopsin and *Prkcz* are both expressed within the RPE (Figure S2). These data are consistent with the hypothesis that PKCζ is involved in melanopsin signaling and that the RPE may also show melanopsin-based photodetection.

Nonvisual Photoresponses Are Attenuated in *Prkcz*$^{-/-}$ Mice

As described previously, *Prkcz*$^{-/-}$ mice are viable and show a normal anatomical and behavioral phenotype [23]. These animals were used for assessing the function of this kinase in pRGC-regulated light responses. Melanopsin localization and transcript levels were normal in *Prkcz*$^{-/-}$ eyes (Figure 3A), precluding the possibility that *Prkcz* deficiency affects nonvisual photoresponses by regulating melanopsin expression directly. Phase shifting of circadian-wheel-running activity in response to nocturnal light exposure, period lengthening in constant light (LL), and pupil constriction in response to high irradiances are known to be attenuated in the absence of melanopsin [24–26]. In *Prkcz*$^{-/-}$ animals, exposure to a single 15 min light pulse (white light; 400 lux/250 μW/cm^2/s) at CT 14 produced a 43% decrease in the magnitude of phase delays of locomotor activity when compared to wild-type littermate controls (Figures 4A and 4B). This finding is mirrored by a ~40% reduction in light-induced *Per1* gene expression within the SCN of *Prkcz*$^{-/-}$ animals (Figure S3). Free-running periods (τ) in constant darkness (DD) were not affected in *Prkcz*$^{-/-}$ mice (23.35 ± 0.128 hr versus 23.31 ± 0.096 hr in wild-type controls; Figure 4C). However, when animals were kept in constant light conditions (LL), the lengthening of τ that accompanies bright-light exposure was strongly attenuated in *Prkcz*$^{-/-}$ mice (Figure 4C). In the normal murine brain, there are few sites that express *Prkcz* [27]. In this study, we confirmed that *Prkcz* is absent from the suprachiasmatic nuclei (SCN) (Figure S4). This strongly suggests that the observed defects in circadian photosensitivity are not due to loss of function of PKCζ at the level of the SCN.

The effects of light on pupil constriction were also examined in *Prkcz*$^{-/-}$ mice. Both sustained and transient pupil responses were significantly attenuated in 8- to 10-month-old *Prkcz*-deficient animals (Figure 5), again duplicating the phenotype of melanopsin knockout mice [24]. *Prkcz* transcripts have not been detected in any of the midbrain and hindbrain nuclei that regulate

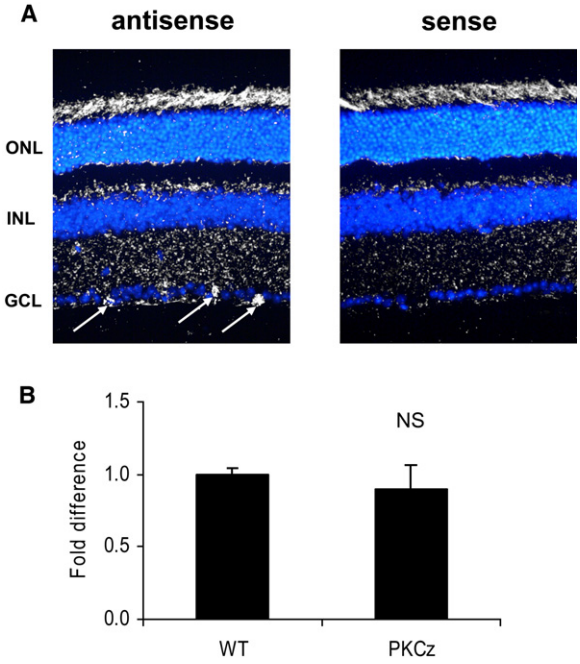

Figure 3. Melanopsin Is Expressed Normally in the *Prkcz*$^{-/-}$ Retina

(A) In situ hybridization. Melanopsin-positive retinal ganglion cells are indicated by arrows.

(B) qPCR on melanopsin levels in the *Prkcz*$^{-/-}$ eye revealed no significant difference from those found in wild-type animals (mean ± SEM, n = 4, Student's unpaired t test, p > 0.05).

light-induced pupil constriction, such as the olivary pretectal nuclei (OPN) [28–30] (H.O., unpublished data). This strongly suggests that the observed defects in pupil constriction are due to retinal deficits rather than any loss of PKCζ in brain areas that control the pupil.

Finally, the application of 1 M carbachol produced maximal pupil constriction in both genotypes, indicating that the decreased sensitivity to high irradiances was not the result of impairing pathways associated with iris muscle contraction (Figure 5B). The observed defects in pupil responses were also present in younger animals (Figure S5). Thus, the circadian phenotype of *Prkcz*$^{-/-}$ mice is indistinguishable from that of melanopsin knockout animals (Table 2).

Discussion

The elucidation of novel signaling pathways by classical approaches can be a demanding and often extremely time-consuming task. By contrast, modern genomic technologies allow for the rapid analysis of whole transcriptomes under various physiological conditions. The increased sensitivity of contemporary microarray technology has enabled a greater resolution of changes in gene expression, as well as allowed the use of biological replicates rather than data filtering based upon arbitrary fold-change criteria [31–33]. This enables the detection of subtle changes such as those expected in response to the depletion of messenger proteins after transient activation of a defined signaling pathway. Such an approach—which we term transcriptional recalibration—was used in this study to identify novel components of the melanopsin signaling pathway. By placing the microarray data within the broad context of invertebrate GPCR phototransduction, followed by colocalization experiments, we identified eight intracellular signaling candidates that might be involved in

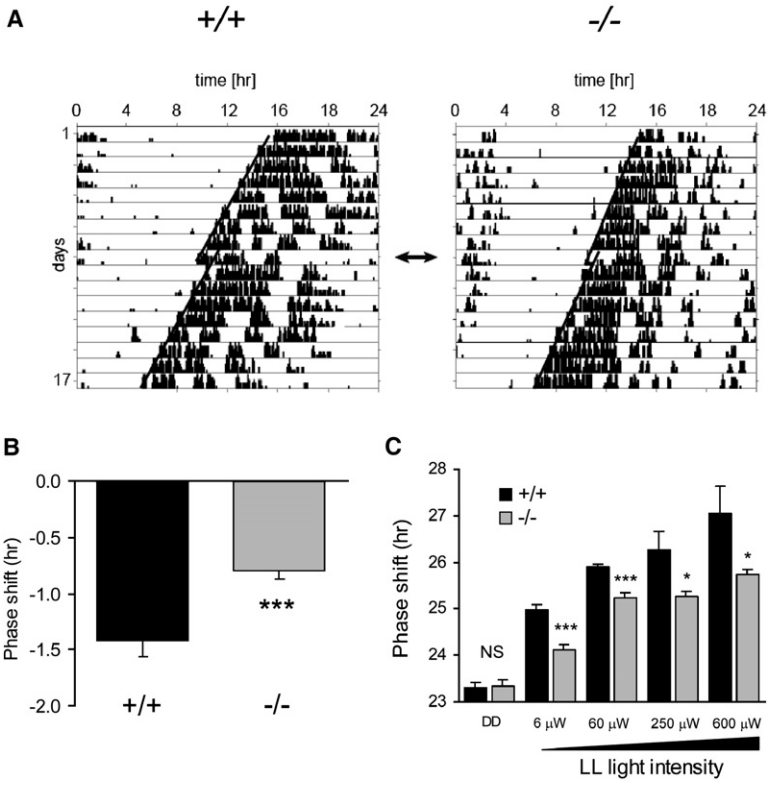

Figure 4. *Prkcz*$^{-/-}$ Mice Show Attenuated Behavioral Responses to Light

(A) Representative actograms of wild-type (+/+) and *Prkcz*$^{-/-}$ mice (−/−) in DD. A single white light pulse (15 min, 400 lux/250 μW/cm^2/s) was administered at day 9 of the experiment (marked by the black arrow) and thereby caused a phase delay of locomotor activity in both genotypes.

(B) Phase-shifting responses to a 15 min light pulse as shown in (A). Phase delaying was impaired in *Prkcz*$^{-/-}$ mice (0.79 ± 0.08 hr versus 1.42 ± 0.15 hr in wild-type animals; mean ± SEM; p < 0.001; unpaired Student's t test; n = 13).

(C) Whereas the free-running period length in DD was unaffected in *Prkcz*$^{-/-}$ mice, period lengthening in LL was attenuated at all light intensities tested (mean ± SEM; *p < 0.05; ***p < 0.001; unpaired Student's t test; n = 4–7).

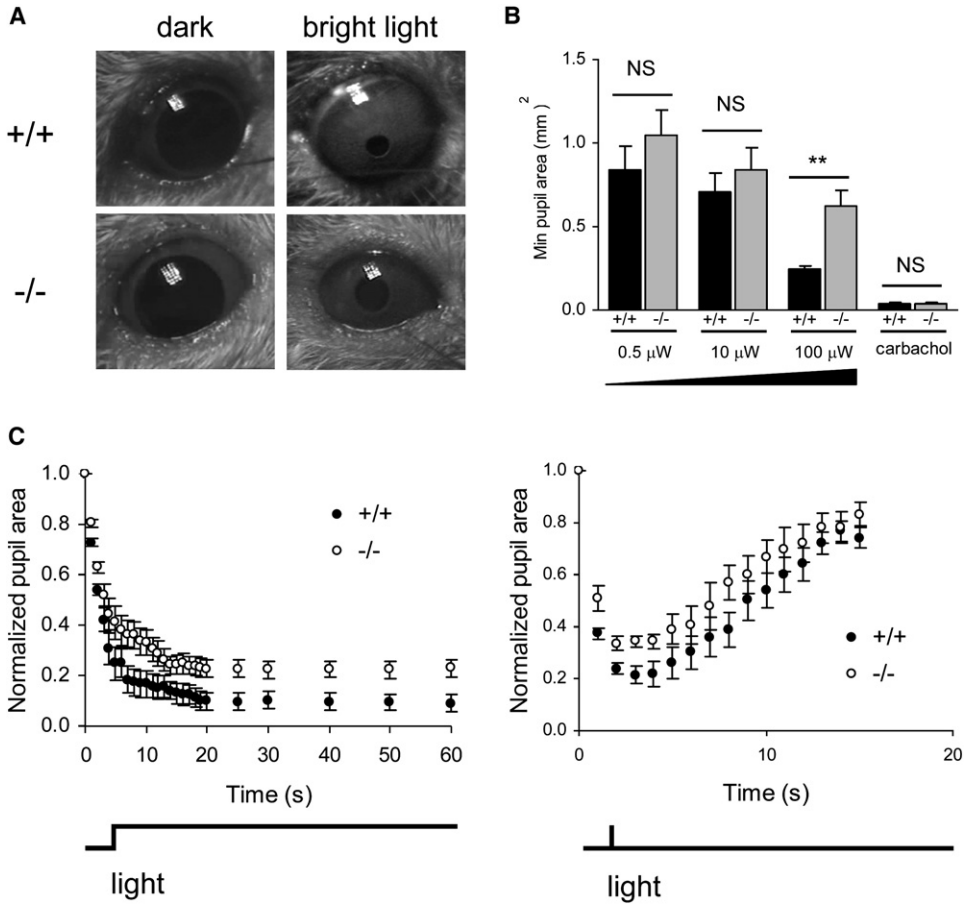

Figure 5. Impaired Pupil Responses to Bright Light Exposure in *Prkcz*$^{-/-}$ Mice

(A) Wild-type (+/+) and *Prkcz*$^{-/-}$ mice (−/−) showed pupil constriction in response to 1 min of bright (100 μW/cm^2) monochromatic (~460 nm) light, but the constriction was less in *Prkcz*$^{-/-}$ than in wild-type animals (left panel). There was no difference in dark-adapted pupil size between the two genotypes.

(B) Summary of results showing the minimum pupil areas (mean ± SEM, n = 8) attained by the two genotypes during dim (0.5 μW/cm^2), medium (10 μW/cm^2), and bright (100 μW/cm^2) light exposure and after application of 1 M carbachol. The minimum pupil size attained by *Prkcz*$^{-/-}$ mice was significantly larger than that of wild-type animals only in bright light illumination ($p < 0.01$; Student's unpaired t test; n = 8).

(C) Pupillary responses of *Prkcz*$^{-/-}$ mice were attenuated under both continuous and transient (25 ms) light exposure. The average pupil area normalized with respect to the initial dark value is plotted (mean ± SEM; n = 8).

melanopsin-pRGC signaling (Figure 6). Functional genomic studies on one of these candidates, the novel C-type protein kinase PKCζ (*Prkcz*), were conducted by analysis of a range of nonvisual responses to light in *Prkcz*-deficient mice. Our experiments demonstrate that the responses in *Prkcz*- and melanopsin-deficient animals are remarkably similar. Collectively, these data provide strong evidence that PKCζ plays an essential role in melanopsin signaling.

These deficits cannot be attributed to the loss of rod and cone inputs to the SCN because *rd/rd cl* mice do not show attenuated phase-shifting responses to light. Although *Prkcz* is also expressed in a subset of nonmelanopsin retinal ganglion cells (Figures 2B and 2C), the pupillary defects cannot be attributed to this additional cell population. If PKCζ were a critical factor in the light responses of all ganglion cells, then pupil responses to dim (scotopic) and medium (mesopic) intensity stimuli would be equally affected. This is not the case. It is only at high irradiances that the deficit is observed (Figure 5B). Thus *Prkcz*$^{-/-}$ mice mirror the irradiance-dependent deficit found in melanopsin knockout animals [3]. A role for PKCζ in regulating melanopsin expression can also be excluded because both the number and distribution of melanopsin-expressing ganglion cells are unchanged in *Prkcz*$^{-/-}$ animals (Figure 3). Finally, *Prkcz* is not expressed within the SCN or midbrain and hindbrain nuclei that regulate light-induced phase-shifts and pupil constriction. This argues that the attenuated responses observed are not due to the absence of *Prkcz* in these brain areas. Thus, the most parsimonious explanation of our data is that PKCζ plays a critical, yet undefined, role in melanopsin signaling.

As discussed above, several previous studies using in vivo as well as cell-based expression systems suggest that pRGCs functionally resemble invertebrate rhabdomeric photoreceptors [9, 34–36]. By analogy to the *Drosophila* phototransduction cascade [21], we suggest that PKCζ may influence ion-channel activity via participation in an INAD-like signaling complex (including PLC-, PKC-, and PDZ-domain-containing scaffolding proteins; Figure 6). Alternatively, it could act by

Table 2. Comparison of Circadian Phenotype of *Opn4*$^{-/-}$ and *Prkcz*$^{-/-}$ Mice

Response	Gene	+/+	−/−	Δ	Reference
Tau in DD					
	Opn4	23.7 hr	23.8 hr	-	[26]
	Opn4	23.7 hr	23.7hr	-	[25]
	Prkcz	23.3 hr	23.3 hr	-	This study
Phase Shift[a]					
	Opn4	−2.7 hr	−1.7 hr	−37%	[26]
	Opn4	−1.4 hr	−0.8 hr	−43%	[25]
	Prkcz	−1.4 hr	−0.8 hr	−43%	This study
Tau in LL[b]					
	Opn4	+1.5 hr	+0.9 hr	−40%	[26]
	Opn4	+1.55 hr	+1.0 hr	−35%	[25]
	Prkcz	+2.59 hr	+1.87 hr	−28%	This study
Pupillary response					
	Opn4	0.05	0.20	−16%	[24]
	Prkcz	0.10	0.23	−14%	This study

Comparison of circadian phenotype of *Opn4*- and *Prkcz*-deficient mice.
[a] Based on 30 min light pulse of 70–280 lux white light (50–170 μW/cm^2/s); [26]), 15 min of 480nm light [25], or 15 min 400 lux white light (250 μW/cm^2/s; this study).
[b] Based on LL white light levels at approximately 100 lux (60 μW/cm^2/s).

regulating the activity of some other critical component of the melanopsin signaling cascade. Intriguingly, unlike eye-PKC (inaC) in *Drosophila* [37], mammalian PKCζ is an atypical PKC, lacking both Ca^{2+}- and DAG-binding domains, thereby precluding a direct activation of PKCζ via phospholipase C [38]. Interestingly, two PI3 kinase subunit-encoding genes (*Pik3r1/3*) have also emerged from our screen, and PI3 kinase has been shown to directly and indirectly activate atypical PKCs in different tissues [39]. Thus, extrapolating between *Drosophila* phototransduction and mammalian pRGC signaling is not entirely straightforward, and the elucidation of PKCζ function could provide critical insight into how the invertebrate and pRGC light-signaling pathways differ.

In summary, we have applied a novel microarray-based approach to identify new elements of melanopsin signaling. A number of candidate genes were identified, including an atypical C-type protein kinase *Prkcz*. The phenotypic characterization of *Prkcz*$^{-/-}$ mice demonstrated a critical role for this gene in melanopsin-regulated light responses. We suggest, therefore, that transcriptional recalibration may provide a new method of investigating unknown signaling pathways and that the functional analysis of *Prkcz* and the other light-regulated genes identified in this study might provide an improved understanding of the molecular processes underlying nonvisual photoreception in the mammalian retina.

Experimental Procedures

Gene-Expression Microarrays and Statistics

C3H/He male mice (age 130 ± 16 days) lacking both rods and cones (*rd/rd cl*; [1, 40]) were housed under a reversed 12:12 LD cycle for 2 weeks with food and water ad libitum. A light pulse of 1.4 mW/cm/s

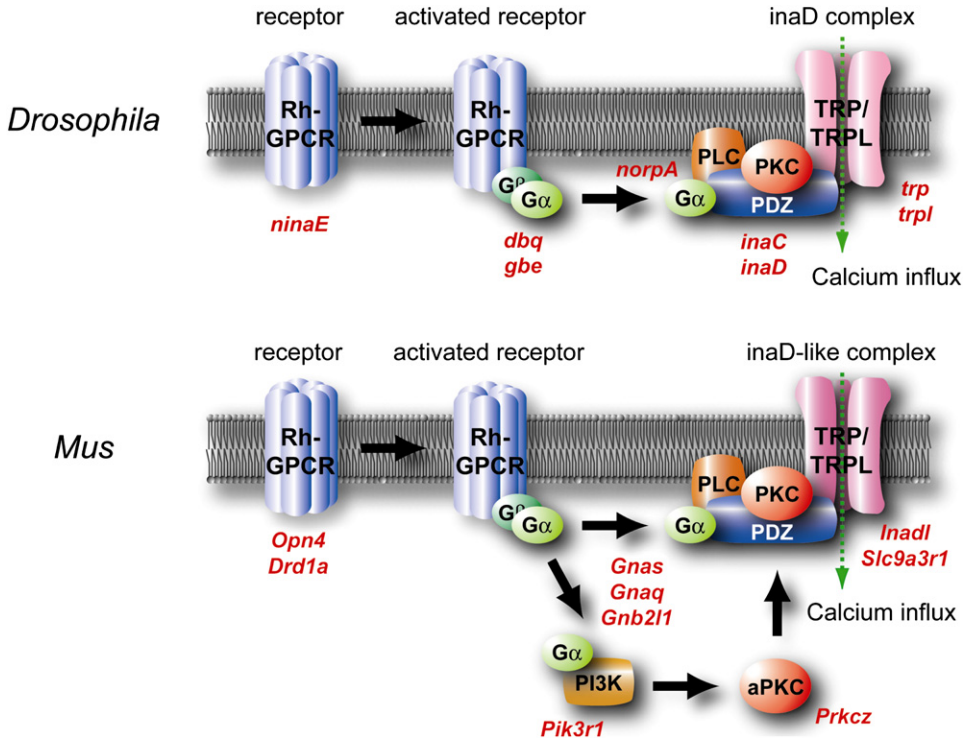

Figure 6. Comparison of *Drosophila* Phototransduction and Putative Mouse Melanopsin Signaling

PKCζ may be involved in the modulation of ion-channel activity (possibly via an INAD-like signaling complex) or via modulation of some other critical component of the melanopsin phototransduction cascade (see text for details). Potential roles of further candidates identified in this study are indicated in red.

(3000 lux) was administered at zeitgeber time (ZT) 16 for 15 min, with fluorescent white light. Animals were sacrificed by cervical dislocation at 30, 60, and 120 min after the onset of the light pulse. At each time point, four light-pulsed and one to two sham controls were used (two at 30 min, one at 60 min, and one at 120 min), with sham animals undergoing similar treatment but without light exposure. Paired eyes were collected in darkness with an infrared viewer and snap frozen on dry ice. Total RNA was extracted from whole eyes with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and cleaned with an RNAeasy kit (QIAGEN, Hilden, Germany). Total RNA quality was assessed with an Agilent Bioanalyser, and RNA was amplified and in vitro transcribed with an RNA amplifaction kit and IVT reagents (Affymetrix, Santa Clara, CA). Labeled RNA quality was again assessed with an Agilent Bioanalyser. A total of 16 cRNA samples were hybridized to Mouse Genome 430 v2.0 Genechips (Affymetrix, Santa Clara, CA), with four biological replicates per time point for the light-treated and one to two replicates for the control group. Hybridization and scanning was carried out at the MRC microarray center (Hammersmith Hospital, London, UK). Only probe sets called as present across all 16 arrays were analyzed, giving a raw dataset of 16,321 probe sets. Raw data were normalized with a log2 transform with median stabilization. Variance was highly comparable across all 16 arrays, and as such variance stabilization transforms were not applied. Differences across the experimental time course were analyzed by one-way ANOVA with the false discovery rate (FDR) multiple test correction [41]. Subsequent analysis was conducted with MatLab 7.1 software with bioinformatics toolkit (Mathworks, Natick, MA), NetAffx (Affymetrix), and Entrez Gene (NCBI) databases. GO terms for light-regulated probe sets were compared against GO representation on 430 v2.0 genechips. Overrepresentation significance was determined on the basis of a hypergeometric distribution (calculations performed with MatLab 7.1 software, Mathworks).

Quantitative PCR

RNA samples were prepared as described for microarray hybridization. cDNA was synthesized with a RetroScript kit (Ambion, Austin, TX), and quantitative PCR (qPCR) was conducted with both Sybr green I and FAM-labeled TaqMan probes and an SDS7700 thermal cycler (Applied Biosystems, Foster City, CA). Relative quantification of transcript levels was done as described previously [42]. The geometric mean of six housekeeping genes was used for normalization (*Gapdh*, *Actb*, *Rplp1*, *Hprt1*, *B2m*, and *Tbp*). Primer sequences are provided in the Supplemental Data.

Laser Microdissection and Pressure Catapulting

Wild-type 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 min. Sections of the retinal ganglion cell (RGC) layer were laser dissected with a PALM MicroBeam system (PALM-microlaser, Bernried, Germany), and each preparation contained approximately 40–50 cells. Total RNA was subsequently extracted with a PicoPure RNA extraction kit (Arcturus, Sunnyvale, CA), treated with 1 unit DNase (Sigma-Aldrich, St. Louis, MO), reverse transcribed with random decamers with a RETROscript kit (Ambion), and tested for candidate gene expression with Sybr green I mastermix (Applied Biosystems, Foster City, CA) with 50 cycles of amplification. PCRs were run in real time for ensuring that different primer sets did not exhibit differences in amplification efficiency.

Immunocytochemistry

Immunostaining of PKCζ protein on wild-type retinal sections was performed as described [43]. In brief, 4-month-old animals were sacrificed by cervical dislocation at ZT 4–6. Eyes were dissected and fixed in 4% PFA at 4°C ON. After dehydration and paraffin embedding, 8-μm-thick microtome sections were mounted on slides, dewaxed, blocked with 1% H_2O_2 and 10% goat serum, and incubated with polyclonal rabbit PRKCZ antibody (1:1000 in Tris/0.05% Tween) [44] at 4°C overnight. Secondary antibody incubation, avidin/biotin amplification, and DAB/Nickel visualization were carried out with the Vectastain Elite ABC kit according to the manufacturer's protocol. *Opn4*$^{-/+}$ (*tau-LacZ*$^{-/+}$) mice [5] were enucleated (ZT 6–12), and the eyes were fixed in 4% paraformaldehyde overnight at 4°C; this was followed by cryoprotection with 30% sucrose in PBS. Tissues were rinsed in PBS before being embedded in OCT, frozen in isopentane cooled in liquid nitrogen, sectioned at 20 μm with a cryostat (Leica CM 3050 S), and stored at −80°C. After thawing and blocking in 10% normal donkey serum/1% BSA in PBS/0.1% Na-azide/0.3% Triton X-100 for 1 hr at 4°C, slides were incubated with PKCζ antiserum [44] diluted 1:500 in blocking solution for 2 days at 4°C. After washing four times in PBS/0.3% Triton X-100 at room temperature, sections were incubated with Alexa Fluor 488 nm conjugated to donkey anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:200 for 2 hr. Slides were, washed twice in PBS, blocked again for 1 hr at 4°C, and incubated with primary β-GAL antibody (Biotrend, Koln, Germany, 1:1,000 in blocking solution) overnight at RT. Slides were then washed four times, incubated with Alexa fluor 568 nm conjugated to donkey anti-goat IgG (Vector Laboratories, 1:200) for 2 hr, mounted with Vectashield fluorescent mounting medium with DAPI (Vector Laboratories), and visualized under a confocal microscope (Leica TCS SP, Leica Microsystems, Bensheim, Germany).

In Situ Hybridization

Adult male wild-type and *Prkcz* deficient animals [23] were entrained to a 12 hr light:12 hr dark cycle (LD 12:12) for at least 2 weeks and sacrificed 2 hr after "lights off " (ZT 14) under a 15 W safety red light by cervical dislocation. Eyes were quickly removed, and brains dissected out and fixed overnight in 4% paraformaldehyde. After dehydration, glass bodies were removed from the eyes, and tissues were cleared in xylene and embedded in paraffin. Eight-micrometer-thick sections were prepared on a microtome (Leica Microsystems) and stored at 4°C until use. In situ hybridization with ^{35}S-UTP-labeled antisense RNA probes was performed as described [45]. The probes spanned nucleotides 308–1151 of the melanopsin transcript (GenBank accession NM_013887) and nucleotides 2831–3891 of the *Per1* (GenBank accession AF022992) transcript. After hybridization and washing, slides were dipped in Kodak NTB-2 emulsion (Kodak, Stuttgart, Germany) and counterstained with Hoechst dye (Sigma-Aldrich, Seelze, Germany). Dark-field and blue fluorescence micrographs of the same area were captured on a Leica DMR microscope (Leica Microsystems) with an Olympus DP50 CCD camera (Olympus, Hamburg, Germany) and overlaid with Photoshop software (Adobe, San Jose, CA).

Activity Monitoring

Prkcz knockout mice were generated as described previously [23]. General mouse handling and activity monitoring were conducted in accordance with standard protocols [46, 47]. Adult wild-type and *Prkcz*-deficient animals were entrained to LD 12:12 for at least 14 days in custom-made single-cage isolation boxes and subsequently released into constant darkness (DD) for another 2 weeks. Activity onsets and individual period lengths in DD were determined with the ClockLab software package (Actimetrics, Evanston, IL). For light pulses, animals received 15 min of bright white light (~400 lux/250 μW/cm^2/s) at CT 14 and were subsequently kept in constant darkness for at least 10 more days so that phase shifts could be assessed by comparison of regression lines through the onsets before and after light exposure. For constant light (LL) experiments, animals were initially entrained to LD 12:12 and released into LL of increasing intensity (14 days per condition). Free-running period lengths were determined by χ^2 periodogram analysis on day 4–13 of each condition.

Pupillometry

Pupillometry on adult wild-type and *Prkcz*-deficient animals was performed as described [24]. In brief, animals were entrained to LD 12:12 and tested at ~ZT 6–8 after 1 hr of dark adaptation. Pretreatment measurements were obtained with an IR-sensitive CCD video camera equipped with macro optics with infrared (>850 nm) LED illumination (Sony, Tokyo, Japan). Pupil reflexes were triggered by 1 min exposure to a 460 nm LED light source or by 25 ms exposure to a bright white halogen lamp covered by a diffusing sphere constructed from a ping pong ball. For carbachol responses, a drop of 1 M carbachol in PBS (Sigma-Aldrich, Seelze, Germany) was applied to the eyes of dark adapted animals with a Pasteur pipette. Constriction was measured 5 min after drug application.

Video stills were captured with Windows movie maker software (Microsoft, Redmond, WA), and pupil areas were estimated with Photoshop (Adobe Software).

Supplemental Data

Additional Experimental Procedures, five figures, and two tables are available at http://www.current-biology.com/cgi/content/full/17/16/1363/DC1/.

Acknowledgments

The authors would like to thank the staff at the MRC Microarray center at the Hammersmith hospital, Manuel Graeber, Linda Moran, and Federico Turkheimer for access to LMPC as well as many useful discussions and finally Jane Howarth at Affymetrix UK. H.O. would additionally like to thank Uwe Herzig and Nadine Naujokat for their help with the pupillometry setup. Foundling *Opn4*$^{+/-}$ animals used for colocalization studies were kindly provided by Samer Hattar and King-Wai Yau at Johns Hopkins, Baltimore. The authors would also like to thank Professor Roger Hardie (University of Cambridge) for his constructive comments on a draft of this manuscript. This work was funded by a Wellcome Trust program grant to R.G.F. H.O. was supported by an Otto Hahn Medal fellowship from the Max Planck Society.

Received: May 17, 2007
Revised: July 11, 2007
Accepted: July 12, 2007
Published online: August 16, 2007

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