Controlling the number of melanopsin-containing retinal ganglion cells by early light exposure

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Abstract

A small percentage of retinal ganglion cells (RGCs) express melanopsin and are intrinsically photosensitive (ipRGCs). Whether light can affect the development of ipRGCs is not clear. In the rat retina, we found constant light exposure during the first postnatal week significantly increased the number of melanopsin immunopositive ipRGCs. This increase was durable and specific for melanopsin immunopositive ipRGCs. BrdU labeling showed no proliferation of the melanopsin immunopositive ipRGCs during constant light exposure. Retrograde labeling from the superior colliculus showed that no other types of RGCs were induced to express melanopsin. Light exposure was effective in increasing melanopsin immunopositive ipRGCs only when it coincided with the apoptotic phase of RGC development. However, daily intravitreous injection of tetrodotoxin, blocking action potentials, abolished the light induced increase of melanopsin immunopositive ipRGCs. These findings indicate that early light exposure can increase the number of melanopsin immunopositive ipRGCs through a process dependent on intrinsic photosensitive spiking activity. Furthermore, the increase of melanopsin immunopositive ipRGCs is potentially induced by apoptosis suppression in ipRGCs or enhanced expression of melanopsin.

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1. Introduction

The discovery of a subtype of retinal ganglion cells (RGCs) utilizing a novel opsin, melanopsin, as a photopigment to generate intrinsic light responses has allowed for rapid progress in the past decade toward understanding the non-image forming visual system (Provenzio et al., 1998; Provenzio et al., 2000; Berson et al., 2002; Hannibal et al., 2002; Hattar et al., 2002; Panda et al., 2002; Provenzio et al., 2002; Hattar et al., 2003; Chen et al., 2011). It has been reported that constant light exposure suppresses melanopsin expression in the adult retina (Hannibal, 2006; Hannibal et al., 2007). Melanopsin-containing RGCs exhibit light responses at birth and therefore are the earliest light responsive neurons in the retina (Sekaran et al., 2005; Tu et al., 2005). Similar to many other types of RGCs, ipRGCs are overproduced and the population decreases in early development. We postulated that light affects the development of ipRGCs and investigated the effects of constant light exposure on developing and mature ipRGCs.

2. Methods

2.1. Animals and retina preparation

Albino Sprague Dawley rats obtained from the animal care facility of the Institute of Biophysics, Chinese Academy of Sciences, were used in this study. Use and handling of animals were strictly in accordance with the institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. During constant light exposure (LL) treatments, rats were exposed to a continual luminance of 400 lux. Control rats were maintained on a 12 h light/dark cycle (LD) with the same (400 lux) luminance during the day.

Animals were deeply anaesthetized with an i.p. injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) and perfused with warm saline transcardially. Eyes were enucleated, marked at the nasal pole, and fixed for 10 min in 4% paraformaldehyde in 0.01 M...
PBS. Then retinas were isolated from the pigment epithelium while maintaining the reference at the nasal pole and were post-fixed for 1 h at room temperature.

2.2. Immunohistochemistry

After rinsing with 0.01 M PBS, retinas were incubated with primary antibodies in medium containing 0.01 M PBS with 1% BSA and 0.5% Triton-X 100 for 2 days at 4 °C. Then retinas were rinsed with 0.01 M PBS and incubated with secondary antibodies in the same medium with 0.05% DAPI overnight at 4 °C. Retinas were rinsed and whole-mounted with Vectashield (Laboratories, H-1000, Vector, Burlingame, CA). Images were acquired on an Olympus FV500 confocal microscope with a 10× objective (UPlanApO, N.A. = 0.40).

For retinal sections, retinas were cryoprotected in 30% sucrose in 0.01 M PBS overnight and frozen in OCT. Tissue was sectioned to a thickness of 18 μm using a cryostat (Leica CM, 1950). The sections were incubated with primary antibodies overnight, rinsed three times and incubated with secondary antibodies and 0.05% DAPI for 2 h at room temperature. After rinsing, the sections were cover-slipped with Vectashield. Images were acquired on an Olympus FV500 confocal microscope with a 60× oil-immersion objective (Plan Apo, N.A. = 1.40).

2.3. Antibodies

Primary antibodies used in this study were: rabbit anti-melanopsin (1:400, PA1-781, Affinity BioReagents, Golden, CO), rabbit anti-PKCα (1:10,000, P4334, Sigma–Aldrich, St Louis, MO), mouse anti-neurofilament (1:200, N1042, Sigma–Aldrich, St Louis, MO) and mouse anti-BrdU (1:200, B8434, Sigma–Aldrich, St Louis, MO).

Secondary antibodies were all obtained from Jackson ImmunoResearch Laboratories (West Grove, PA): donkey anti-rabbit FITC (1:200, 711-095-152), donkey anti-mouse TRITC (1:200, 711-025-151) and donkey anti-rabbit Cy5 (1:200, 711-175-152).

2.4. In situ hybridization

A 539 bp rat PACAP fragment was amplified by RT-PCR (307 bp to 845 bp, primers: 5’-TACGCGACCAAGGGTGGCC-3’ and 5’-ACGTTAGCTGGCAACTCATGCT-3’) from rat retinal total RNA and cloned into pGEM-T (VT202-01, Tiangen, Beijing, China). Digoxigenin-labeled riboprobe was prepared by in vitro transcription reactions (11277073910, Roche, Mannheim, Germany) with linearized vector DNA as the template using SP6 (for antisense probe) or T7 (for sense probe) polymerase (10810274001 and 10881767001, Roche, Mannheim, Germany) following the manufacturer’s instructions.

In situ hybridization of the whole mount retina was performed as described by Z Bao et al. (Bao and Cepko, 1997). Briefly, after fixation overnight in 4% PFA in 0.01 M PBS at 4 °C, rat retinas were rinsed and treated with 10 μg/ml proteinase K (1092766, Roche, Mannheim, Germany) for 15 min at room temperature. After 1 h pre-hybridization at 70 °C, the retinas were hybridized with PACAP riboprobes (0.2 μg/ml) overnight at 70 °C. Then retinas were washed and incubated overnight at 4 °C with sheep anti-digoxigenin-AP (1:10,000, 11093274910, Roche, Mannheim, Germany). After rinsing, the retinas were incubated with NBT/BCIP reagents (1133323001 and 11333221001, Roche, Mannheim, Germany) for color detection following product instructions. Images were acquired by a Nikon E800 microscope using a 4 × objective (Plan Fluor, N.A. = 0.13) or 10 × (Plan Apo, N.A. = 0.45) objective.

2.5. BrdU labeling

Rats reared in LL condition were injected i.p. daily with 50 mg/g body weight BrdU (B5002, Sigma–Aldrich, St Louis, MO) from P0 to P7 under hypothermia. Rats were returned to normal condition from P8 and sacrificed at P20. The retinas were prepared and cryosectioned. Sections were rinsed with 0.01 M PBS, incubated in 4 N HCl for 15 min at room temperature, stained with mouse anti-BrdU antibody (1:200, B8434, Sigma–Aldrich, St Louis, MO) and counterstained with rabbit anti-melanopsin antibody and rabbit anti-PKCα antibody.

2.6. Intraocular injection

From P0 to P7, rats were anaesthetized by hypothermia, the eyelid was opened with a small incision, and 0.5 μl tetrodotoxin (0.4 mM, T8024, Sigma–Aldrich, St Louis, MO) in 3.5 mM citrate buffer (pH = 4.8) was injected into the vitreous body with a glass micropipette daily. Rats in the control group were injected with citrate buffer only.

2.7. TUNEL

Whole-mount retinas were fixed for 20 min in 4% paraformaldehyde in 0.01 M PBS at room temperature. After incubation in 0.01 M PBS containing 2% Triton X-100 at 37 °C for 1 h, the retinas were stained with TUNEL reaction mixture at 37 °C for 1 h following the manufacturer’s instructions (Roche, In Situ Cell Death Detection Kit, 12156792001, Boehringer Mannheim, Mannheim). After TUNEL staining, whole-mount retinas were counterstained with antibodies against melanopsin.

2.8. Retrograde labeling

Animals were anesthetized and placed in a stereotaxic device (Narishige Scientific Instruments, Tokyo). A craniotomy was performed above the injection sites (SC: −6 and −7 mm AP, ±1.4 mm ML, 3.5 and 4.5 mm DV). Then 0.1 μl 4% FluoroGold (80014, Biotium, Hayward, CA) was injected at each point using a Hamilton syringe, which was kept in place for 10 min after injection. The animals recovered for 5 days before being sacrificed for examination of RGCs.

2.9. Quantification of the retinal ganglion cells

The micrographs of whole mount retinas were acquired by a Nikon E800 microscope with a 4 × objective (Plan Fluor, N.A. = 0.13) and individual images were stitched together to form a whole-retina montage. Cell counts were made by at least two individuals blind to the experimental condition and the average of the two observers was used.

To account for area difference, whole-mount retina was divided into four quadrants; micrographs were taken along the midline of each quadrant from the optic disc to the periphery at an interval of 1.4 mm ML, 3.5 and 4.5 mm DV). The micrographs were examined of RGCs.

2.10. Statistical analysis

RGC density (cells/mm²) or number is presented as mean ± standard error of the mean (SE), unless stated otherwise. One-way ANOVA (Origin 7.5) or Independent-samples t test (SPSS 11.5) was used for the analysis of the results. Statistical significance was set at p < 0.025 based on the Bonferroni correction for multiple comparisons when the t-test was applied or p < 0.05 for ANOVA analysis.
3. Results

3.1. Constant light exposure down regulates melanopsin expression in adult retinas

We reared adult rats (LD30) in an environment with constant light exposure for 20 days (LL20). Immunohistochemical analysis showed a clear reduction in melanopsin labeling. The meshwork of melanopsin positive dendrites visible in the control retinas (Fig. 1A) was notably absent in light treated retinas, which only displayed few primary dendrites (Fig. 1B). The number of somas positive for melanopsin was also significantly reduced in light treated retinas compared to controls, especially in the superior and temporal quadrants (Fig. 1E and F). These findings are consistent with earlier studies of melanopsin expression in adult rat retinas (Hannibal et al., 2005).

It is not clear whether the reduction in number of melanopsin immunopositive ipRGCs is due to the down regulation of melanopsin expression to a level below antibody detection as interpreted in the previous study or to the loss of ipRGCs. To address this question, we returned animals exposed to constant light for 20 days to normal light–dark condition for 10 days (LL20 + LD10). The number of melanopsin immunopositive ipRGCs and the intensity of melanopsin immunostaining recovered to normal (Fig. 1C–G).

Fig. 1. Constant light exposure reversibly suppressed the level of melanopsin immunostaining in adult retina. (A–C) Representative micrographs of temporal retinas 2.5 mm from the optic nerve head illustrating melanopsin immunostaining of ipRGCs. (A) In control retinas (LD50), melanopsin positive somas and processes were clearly visible. (B) Fewer melanopsin immunopositive somas were noted in retinas receiving a 20-day constant light exposure (LD30 + LL20). (C) Following ten days of normal light/dark cycle conditions, after a 20-day constant light exposure, the number of melanopsin immunopositive somas and intensity of melanopsin immunostaining recovered (LD20 + LL20 + LD10). Scale bar: 200 μm (D, E, F and G) reduction and recovery in mean densities (cells/mm²) (±SE) of melanopsin immunopositive ipRGCs in different retinal quadrants. Eccentricity is listed relative to the optic disc. Significant differences were observed in the superior, temporal, and inferior quadrants. (***: p < 0.001, n for each group listed in D).
supporting that the apparent reduction in melanopsin immunopositive ipRGCs was due to the down regulation of melanopsin expression rather than the loss of melanopsin immunopositive ipRGCs.

3.2. Constant light exposure in the early postnatal period increases the number of melanopsin immunopositive ipRGCs

When P0 animals were subjected to constant light for 20 days, a similar reduction in the intensity of melanopsin immunostaining in the retina was also observed at the end of the treatment period (Fig. 2B, E and F). When we returned the LL20 animals to the normal environment for 10 days, the melanopsin positive somas and dendrites became clearly visible (Fig. 2C). Surprisingly, the number of melanopsin immunopositive ipRGCs in the LL20 + LD10 retinas did not simply return to the control level, as we observed in adult retinas, but greatly exceeded the number of melanopsin immunopositive ipRGCs in the control retinas, especially in the superior and temporal quadrants (Fig. 2C, E and F). This finding is consistent with data from a recent report studying the effects of different light conditions on melanopsin-expressing ipRGCs in albinos (González-Menéndez et al., 2010a,b).

To check whether constant light exposure induced increases in other type of RGCs, we examined the population of neurofilament-positive RGCs. The number of neurofilament-positive RGCs with large somas was not affected by exposure to constant light in early
postnatal stage (Fig. 3A–D), indicating the increase in number of melanopsin immunopositive ipRGCs was specific.

To determine if the increase of melanopsin immunopositive ipRGCs in LL20 + LD10 retinas was a sustained change, we extended the recovery period to 30 days after a 20-day exposure to constant light. The increase of melanopsin immunopositive ipRGCs persisted in temporal and superior quadrants of retinas after 30 days LD recovery (Fig. 4B and C), indicating this was not a transient change. These findings contrast with the data presented by González-Menéndez et al., (2010a,b) showing the number of ipRGCs was increased by light exposure but then returned to normal after an extended recovery period in mice.

3.3. An increased population of ipRGCs contributes to the increased number of melanopsin immunopositive ipRGCs

Since increased immunostaining with melanopsin could result from both increased melanopsin expression and increase in number of melanopsin immunopositive ipRGCs, we examined the number of melanopsin immunopositive ipRGCs with a melanopsin independent marker. It has been shown that pituitary adenylate cyclase-activating polypeptide (PACAP) is selectively expressed in ipRGCs (Hannibal et al., 2002). Similar to LL20 + LD10 treated retinas, we found LL8 + LD12 treated retinas demonstrated significantly increased numbers of melanopsin immunolabeled cells compared to control retinas (data not shown). We confirmed the number of melanopsin immunopositive ipRGCs by performing in situ hybridization of PACAP. More PACAP positive cells were detected in retinas exposed to constant light in the early postnatal period (LL8 + LD12, Fig. 5), supporting that the increased melanopsin immunopositive ipRGCs number is independent of melanopsin immunostaining.

To summarize, we showed exposure to constant light reversibly suppressed melanopsin expression in adult and neonatal rat retinas. However, exposure to constant light in the early postnatal period led to an increase in the number of melanopsin immunopositive ipRGCs that was not observed in adult retinas and that was specific and sustained for at least 30 days. An increase in the number of melanopsin immunopositive ipRGCs was confirmed by PACAP in situ hybridization. In the following sections, we consider several potential mechanisms that could account for the increase in number of melanopsin immunopositive ipRGCs: proliferation, conversion, overexpression and suppression of apoptosis.

3.4. Light exposure does not induce proliferation

RGCs are among first neurons to differentiate in the retina and the proliferation of RGCs ends around birth (Cepko et al., 1996; McNeill et al., 2011). We injected BrdU daily during the first postnatal week under constant light exposure to determine if proliferation of ipRGCs was induced. BrdU positive cells were observed in the outer nuclear layer (ONL) and the inner nuclear layer (INL) (Fig. 6A). Some cells were double-labeled with PKCs, showing that dividing photoreceptors and rod bipolar cells were reliably labeled. Very few BrdU positive cells were observed in the ganglion cell layer. None of the 127 melanopsin immunopositive ipRGCs we examined were labeled with BrdU, indicating that the light-
induced increase of melanopsin immunopositive ipRGCs was not due to increased proliferation of ipRGCs.

3.5. Light exposure does not induce non-melanopsin containing cells to express melanopsin

Another possible explanation for the increase in the number of melanopsin immunopositive ipRGCs is that non-ipRGCs were induced to express melanopsin by constant light exposure. The majority of RGCs project to the superior colliculus (SC) (Vaney et al., 1981; Linden and Perry, 1983), but only about 10% of ipRGCs project to the SC (Hattar et al., 2006; Baver et al., 2008; Pickard and Sollars, 2010). The projection of ipRGCs appears to be independent of the presence or absence of melanopsin (Lucas et al., 2003). Therefore, if constant light exposure induces melanopsin expression in non-ipRGCs, one would expect an increased percentage of SC projecting ipRGCs. We investigated this possibility by retrograde labeling of RGCs from the SC. Despite the increase in the number of melanopsin immunopositive ipRGCs in the retina, the percentage of SC projecting melanopsin immunopositive ipRGCs remained unchanged after constant light exposure (Fig. 6B), demonstrating that the increase of melanopsin immunopositive ipRGCs was not due to the conversion of non-ipRGCs to ipRGCs.

![Fig. 4.](image-url) The effect of constant light exposure on the number of melanopsin immunopositive ipRGCs was specific and sustained. (A, B, C and D) The increase in the number of melanopsin immunopositive ipRGCs in retinas receiving a 20-day exposure to constant light persisted after 30 days recovery in normal light/dark cycle (LL20+LD30). The mean (±SE) increase remained significant in the superior (B) and temporal (C) quadrants of the retina. (**: p < 0.01, ***: p < 0.001; n listed in the bar for each group).

![Fig. 5.](image-url) Constant light exposure in the early postnatal period increased the number of PACAP positive RGCs. (A1, A2, B1, B2) Representative high magnification (A1 and A2) and low magnification (B1 and B2) micrographs of in situ hybridization of PACAP mRNA on whole-mount rat retina. The density (cells/mm²) of PACAP positive cells in control group (A1 and B1) was lower than the density (cells/mm²) in LL8+LD12 (A2 and B2) group in a similar region of superior retina. Scale bars: 200 μm. (C) Mean (±SE) total number of PACAP positive cells in the control and LL8+LD12 rat retinas. The number of PACAP positive cells increased significantly after early exposure to constant light from P0 to P7 and recovery in normal light/dark cycle for 12 days, consistent with the melanopsin immunostaining results (Fig. 2.). (*: p < 0.001, n listed in the bar for each group).
Retinal cryosections were quadruple labeled with melanopsin (cyan), BrdU (green), DAPI (blue) and PKC to label ipRGCs, indicating this effect was activity dependent. (*: from P0 to P7. TTX blocked the light induced increase of melanopsin immunopositive ipRGCs, the 127 ipRGCs examined, supporting the absence of ipRGCs proliferation after birth. Scale bar: 40 μm). Only a few BrdU positive cells were detected in the ganglion cells layer (GCL), and they never colocalized with any of the 127 ipRGCs examined, supporting the absence of ipRGCs proliferation after birth. Scale bar: 40 μm. BrdU positive cells were sampled in the region 3 mm from optic disc in the temporal and nasal quadrants of the retina. The percentage of melanopsin positive cells and FG− melanopsin positive cells were injected daily with BrdU from P0 to P7 during the period of constant light exposure, and then returned to normal conditions (12 h light/dark cycle) for 12 days (LL8 + LD12). As repeated intraocular injection of TTX may induce changes in eye diameter, we showed the total number of melanopsin immunopositive ipRGCs rather than the density in specific regions.

3.6. TTX blocks the increase of melanopsin immunopositive ipRGCs induced by constant light exposure

To examine the possible role of spiking activity resulting from activation of melanopsin by light exposure, we performed daily injection of a voltage gated Na+ channel blocker, tetrodotoxin (TTX). We previously showed that daily injection of TTX is sufficient to block spiking activities of RGCs in the retina throughout the treatment period (Sun et al., 2011). TTX completely blocked the light-induced increase in melanopsin immunopositive ipRGCs (Fig. 7). As repeated intraocular injection of TTX may induce changes in eye diameter, we showed the total number of melanopsin immunopositive ipRGCs rather than the density in specific regions.

3.7. Apoptosis of melanopsin immunopositive ipRGCs occurs during early postnatal development

One of the remaining possibilities is that constant light exposure increases the number of melanopsin immunopositive ipRGCs through suppression of apoptosis that normally occurs during development (Potts et al., 1982; Perry et al., 1983). It is difficult to demonstrate the reduction of apoptotic melanopsin immunopositive ipRGCs directly because the level of melanopsin expression is greatly down regulated by constant light exposure, as we have shown earlier. Attempts to use antibodies against PACAP, a melanopsin-independent ipRGC marker, never worked in our experiments. This is likely due to low transcription or expression of PACAP during the early postnatal period. In situ hybridization only showed very weak PACAP signal in early postnatal retinas (data not shown). While we were unable to directly test apoptosis in our population of melanopsin immunopositive ipRGCs, we have obtained some evidence addressing the hypothesis of apoptosis suppression. Many TUNEL positive cells were identified in P3 and P5 retinas and some were clearly double labeled with antibodies against melanopsin (Fig. 8A and B). Very few apoptotic cells were seen at P8 and they were found only in the far peripheral retina. Therefore ipRGCs undergo apoptosis and the apoptotic phase is very similar to that of other types of RGCs (Young, 1984; Linden and Pinto, 1985).

Next we maintained animals under one of two paradigms: LL8 + LD22 and LD8 + LL10 + LD12. Eight days light exposure from P0 significantly increased the number of melanopsin immunopositive ipRGCs, whereas 10 days exposure from P7 did not (Fig. 8C–F). As previously discussed, twenty days constant light exposure after P20 down regulated the intensity of melanopsin immunostaining but did not change the number of melanopsin immunopositive ipRGCs either (Fig. 1D–G). Therefore, light exposure was effective in increasing melanopsin immunopositive ipRGCs only when it coincided with the apoptotic phase of RGC development.

Given that there is no proliferation of melanopsin immunopositive ipRGCs or conversion of other RGCs into ipRGCs and that apoptosis of ipRGCs occurs around P3 and P5, one would expect that the peak number of ipRGCs appears at birth. If light exposure suppresses apoptosis of ipRGCs during the first postnatal week, then retinas receiving constant light exposure should have more ipRGCs than the control retinas, with the upper limit approaching the number of ipRGCs at P0. Therefore, we compared the total number of melanopsin immunopositive ipRGCs in neonatal, normal P20, and LL8 + LD12 retinas. Interestingly, while more melanopsin
immunopositive ipRGCs were found in LL8+LD12 retinas than P20 retinas as expected, the number of melanopsin immunopositive ipRGCs was very similar between P0 and P20 retinas reared under normal conditions (Fig. 7). These data seems to contradict our finding that apoptosis occurs in ipRGCs in normal P3 and P5 retina, however the population of melanopsin immunolabeled cells represents only a subset of ipRGCs (Ecker et al., 2010) and it has been shown that P0 retina have twice as many intrinsically photosensitive RGCs than adult retina (Sekaran et al., 2005). Thus, these data are likely a byproduct of the limitations of our antibody’s ability to label ipRGCs and the dynamic process of melanopsin expression in these cells rather than an absence of apoptosis.

4. Discussion

In this study, we showed that constant light exposure reversibly suppressed melanopsin expression in adult retina, consistent with a few earlier studies (Hannibal, 2006; Hannibal et al., 2007). Notably, constant light exposure in the early postnatal period followed by return to normal light conditions increased the number of melanopsin immunopositive ipRGCs, and this effect was sustained for at least 30 days. A transient increase in melanopsin immunopositive ipRGCs following early postnatal constant light exposure has been reported in mice, however this is the first report of a sustained effect (González-Menéndez et al., 2010a,b). Furthermore, the mechanism(s) for this observation has never been explored. We found that the increase of melanopsin immunopositive ipRGCs was not induced by proliferation of existing ipRGCs or conversion of other RGCs to ipRGCs. Further experiments showed that melanopsin immunopositive ipRGCs were increased by constant light only if treatment occurred before P8. This effect was shown to be dependent on intrinsic photosensitive spiking activity. One possible mechanism for this light-sensitive increase in the number of melanopsin immunopositive ipRGCs is that constant light suppresses...
melanopsin immunopositive ipRGCs apoptosis through light sensitive spiking activity.

Many lines of evidence suggest that the light induced increase of melanopsin immunopositive ipRGCs may be due to suppression of apoptosis. First, ipRGCs are overproduced early in development. Calcium imaging and multielectrode array recordings showed four times as many neurons exhibit intrinsic light responses in P0 retinas compared to adult retinas, and twice as many when gap junctions were blocked (Sekaran et al., 2005; Tu et al., 2005). In adult retinas, ipRGCs are only coupled with amacrine cells (Perez de Sevilla Muller et al., 2010). If this is the same early in development, and the gap junction blocker suppresses intrinsic light responses from coupled amacrine cells (Sekaran et al., 2005), then ipRGCs are overproduced by at least two fold early in development, similar to many other types of RGCs (Williams et al., 1990). Second, as we showed in this study, ipRGCs did undergo apoptosis at a similar period with other types of RGCs. Third, constant light exposure was only effective in increasing the number of ipRGCs when it coincided with the period of apoptosis during RGC development.

Suppression of apoptosis could be mediated in part by activation of ipRGCs. As mentioned above, the discrepancy between the number of melanopsin immunopositive cells and intrinsically photosensitive cells, suggests that low levels of melanopsin expression below antibody detection, are sufficient to produce photosensitivity. Thus, ipRGCs with decreased melanopsin labeling during constant light exposure could continue to be light responsive. Spiking activity has been reported to suppress apoptosis, as reviewed by Mennerick and Zorumski (2000). Constant light exposure may result in constant but low level activation of ipRGCs due to their intrinsic light sensitivity. This hypothesis is consistent with our experiments showing that blocking spiking activity with TTX during constant light exposure abolished the light induced increase of ipRGCs. Light-induced activity appears to rescue some ipRGCs that normally undergo apoptosis.

Despite the evidence supporting suppression of apoptosis as the mechanism for the phenomenon we observed in our study, other mechanisms cannot be ruled out. Another possibility is that melanopsin is expressed in more cells following early exposure to constant light. An interesting finding in our study was that number of melanopsin immunopositive ipRGCs was similar between P0 and P20 retinas from rats housed under normal lighting conditions and significantly increased in retinas from rats treated with constant light in the early postnatal period. A recent paper reported similar numbers of ipRGCs in newborn and adult mice (González-Menéndez et al., 2010a,b), which is consistent with our data. These authors concluded that there was no apoptosis occurring in ipRGCs, but our results show that ipRGCs undergo apoptosis around P3 and P5. To reconcile this discrepancy, one needs consider a second process: increasing melanopsin expression. As previously discussed, calcium imaging showed twice as many ipRGCs in P0 retinas compared to adult retinas (Sekaran et al., 2005), while immunostaining showed similar numbers between the two groups in both our data and those of González-Menéndez et al. (2010a,b). Therefore, a portion of ipRGCs that showed intrinsic light responses are not detectable by antibodies. This conclusion is supported by a recent finding that intrinsic light responses can be detected in several subtypes of ipRGCs which are negative for antibody staining (Ecker et al., 2010). Taken together, it is possible that the increase of melanopsin immunopositive ipRGCs following constant light exposure and recovery results from enhanced expression of melanopsin is in a subset of ipRGCs in which the expression of melanopsin was initially undetectable.

Regardless of the underlying mechanism, this novel finding that stimulation by constant light during a formative period leads to a sustained increase in the population of melanopsin immunopositive ipRGCs brings up many important and unanswered questions. An increase in this population of cells has now been demonstrated in both neonatal mice and rats subjected to constant light conditions. Does this phenomenon occur in other species, and if so, what is the durability of the effect? Whether this response is adaptive or mal-adaptive remains to be determined and could be explored by testing functions requiring input from ipRGCs such as pupillary response and circadian rhythm. While constant light conditions do not exist naturally, they may be found in places like hospitals and homes where newborn babies reside and potentially affect ipRGCs in humans. Our data show constant light exposure affects the development of ipRGCs, and understanding this phenomenon may help us better understand the processes regulating early retinal development of intrinsically photosensitive RGCs.

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