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ORIGINAL PAPER

Enhanced Survival of Melanopsin-expressing Retinal Ganglion Cells After Injury is Associated with the PI3 K/Akt Pathway

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Abstract In the present study, we studied the factors that contribute to the injury-resistant property of melanopsin-expressing retinal ganglion cells (mRGCs). Since phosphatidylinositol-3 kinase (PI3 K)/Akt signaling pathway is one of the well-known pathways for neuronal cell survival, we investigated the survival of mRGCs by applying the PI3 K/Akt specific inhibitors after injury. Two injury models, unilateral optic nerve transection and ocular hypertension, were adopted using Sprague-Dawley rats. Inhibitors of PI3 K/Akt were injected intravitreally following injuries to inhibit the PI3 K/Akt signaling pathway. Retinas were dissected after designated survival time, immunohistochemistry was carried out to visualize the mRGCs using melanopsin antibody and the number of mRGCs was counted. Co-expression of melanopsin and phospho-Akt (pAkt) was also examined. Compared to the survival of non-melanopsin-expressing RGCs, mRGCs showed a marked resistance to injury and co-expressed pAkt. Application of PI3 K/Akt inhibitors decreased the survival of mRGCs after injury. Our previous study has shown that mRGC are less susceptible to injury following the induction of ocular hypertension. In this study, we report that mRGCs were injury-resistant to a more severe type of injury, the optic nerve transection. More importantly, the PI3 K/Akt pathway was found to play a role in maintaining the survival of mRGCs after injury.

Keywords Melanopsin · Retinal ganglion cell · Optic nerve injury · Glaucoma

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Introduction

Differential cell death of retinal ganglion cells (RGCs) suggests that a population of neurons is more resistant to injury. In an animal model of optic nerve transection (ONT), approximately half of the total population of RGCs die in the first week and more than 90% of cells were lost 2 weeks post-operation (Berkelaar et al. 1994; Cheung et al. 2004). Elucidating the underlying factors that attribute to differential cell death can provide insights for neuroprotective strategies.

Melanopsin is a novel photopigment that exclusively expressed in a small population of RGCs (Provencio et al. 2000). This group of neurons is found to be intrinsically photosensitive and involved in non-visual photoperception, including photoentrainment, as they project their axons to the retinohypothalamic tract and suprachiasmatic nucleus (SCN). The distinct pathway allows these melanopsin-expressing RGCs (mRGCs) response to light independent of the input from classical photoreceptors, rods and cones (Berson 2003; Hannibal et al. 2004; Hattar et al. 2002; Provencio et al. 2000).

Recently, mRGCs are shown to be less susceptible to death after complete ONT in mice (Robinson and Madison 2004). Following that, our group found that mRGCs survive relatively longer in the ocular hypertension (OH) model, whereas a significant number of superior colliculus-projecting RGCs (scRGCs) die in the process (Li et al. 2006a). The resistance to injury may be probably due to the extensively branched dendrites and the unique intrinsic properties of melanopsin. By far, there is no definitive answer except postulations. In the present study, we showed that mRGCs demonstrate injury-resistance after ONT and the induction of OH in our rat models. Phosphatidylinositol-3 kinase (PI3 K)/Akt cascades are well-known neuronal survival pathway mediated by upstream growth factor receptors (Kaplan and Miller 1997) or calcium influx (Yano et al. 1998). In the present study, we found that a subgroup of mRGCs that survived after insults expressed phospho-Akt (pAkt). The application of PI3 K/Akt inhibitors attenuated the number of mRGCs in both types of injuries, indicating that PI3 K/Akt is most likely one of the pathways for protecting this group of neurons from death.

Methods

Animals

Sprague-Dawley female rats (250–280 g) were reared in a temperature-controlled room on a 12-h light/12-h dark cycle in the Laboratory Animal Unit of The University of Hong Kong. The animals were basically divided into three groups for the investigation of mRGC survival and the effect of the PI3 K/Akt pathway inhibitors after ONT (Group 1) and the induction of OH (Group 2). The normal group (Group 3) was served as the control (details shown in Table 1).

The animals were anesthetized with intraperitoneal injection of a mixture of ketamine (70 mg/kg) and xylazine (7 mg/kg) during the experiments and were killed with an overdose of sodium pentobarbital (150 mg/kg). All the experimental and animal handling procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were reviewed and approved by the Faulty Committee on the Use of Live Animals in Teaching and Research in The University of Hong Kong.

Table 1 Grouping of the experimental animals

Group		No of animals	ONT	OH	LY[b]	WM[b]	DMSO[b]	Time-point
1	A	9[a]	√					7 days
	B	10[a]	√					14 days
	C	13	√		√			14 days
	D	7	√			√		14 days
	E	6	√				√	14 days
2	A	6		√	√			14 days
	B	4		√		√		14 days
	C	7		√			√	14 days
3		9[a]						

Total $n = 71$, excluding those developed cataract and inflammation

[a] Three of the animals were assigned for immunohistochemistry and TUNEL assay. The remaining was for cell counting

[b] Intravitreal injection was given at Day 0, 4, 7 and 10 after either ONT or OH induction

ONT, optic nerve transection; OH, ocular hypertension; LY, LY294002; WM, Wortmannin; DMSO, Dimethyl Sulfoxide

Retrograde Labeling of scRGCs and the Experimental Procedures of ONT

To compare the differential survival of mRGCs and scRGCs, retrograde labeling of scRGCs was performed and the detailed procedures were described previously (Li et al. 2006a, b). In brief, a piece of gelfoam (Upjohn, Kalamozoo, MI, USA) soaked with 6% Fluoro-Gold (FG; Fluorochrome, Denver, CO, USA) was placed over both exposed superior colliculus after removing the overlying cortex. Unilateral ONT was carried out in Group 1 animals 7 days after the labeling. The eyelids were elevated and an incision was made on the superior conjunctiva. The posterior pole and the optic nerve of the right eye were then exposed. The dural sheath was split longitudinally and the optic nerve was then separated from the dorsal aspect of the sheath. A complete cut of the optic nerve, 1.0 mm behind the globe, was made using a pair of fine iris scissors. Care was taken to avoid the damage of the underlying retinal artery. The animals were then allowed to recover from anesthesia and killed according to the specified time-points listed in Table 1.

Induction of OH

The animals were anesthetized using a mixture of ketamine and xylazine. One drop of proparacaine hydrochloride 0.5% (Alcaine, Alcon, Fort Worth, Texas, USA) was applied to the eyes to desensitize the cornea. OH was induced unilaterally using argon laser (Ultima 2000SE Argon Laser, Coherent, USA) to photocoagulate the limbal and episcleral veins of the Group 2 animals and the procedures were adopted from our previous studies (Li et al. 2006a, b). Approximately 20 laser spots (power 1 W; duration 0.1 s; spot size 50–100 μm) on two episcleral veins and 70 spots on limbal veins (270 degrees around limbus, except nasal side) were applied to disrupt the aqueous outflow of the experimental eyes. A second laser photocoagulation with the same procedures was applied 7 days later to ensure the elevation of intraocular pressure (IOP) was maintained. At the same time, retrograde labeling of scRGCs was performed using the same procedures described before. The animals were killed and the eyes were enucleated according to the time-points specified in Table 1.

Measurement of IOP

IOP was measured with a calibrated tonometer (Tonopen-XL, Mentor Massachusetts, The Netherlands) before the laser treatment, 1 week and 2 weeks after the treatment. The animals were anesthetized using a mixture of ketamine and xylazine. One drop of proparacaine hydrochloride 0.5% (Alcaine, Alcon) was applied to the eyes to desensitize the cornea. To avoid diurnal variations, all IOP measurements were taken consistently at about 10:00 am and 5 to 10 readings were averaged for each measurement. The animals were then put back into cages and kept warm under light for recovery.

Intravitreal Injection of PI3 K/Akt Inhibitors

LY294002 (LY, Sigma-Aldrich Co, St. Louis, MO, USA) and Wortmannin (WM, Sigma) were dissolved in 100% Dimethyl Sulfoxide (DMSO, Sigma) and diluted in sterile phosphate-buffer solution (PBS, 0.01 M, pH7.4) to a final concentration of 2 mM and 0.1 mM, respectively. DMSO was served as the control vehicle. The concentration we chose was referred to similar experiments of previous studies (Cheng et al. 2002; Kilic et al. 2005; Park et al. 2004). A pilot study was carried out to examine whether the inhibitors induce any toxicity effect on mRGCs.

Intravitreal injection was performed adopting the schedule similar to previous studies (Kermer et al. 2000; Kilic et al. 2005), i.e. four times at 0, 4, 7, and 10 days (Group 1C–E and Group 2). For each injection, 3 μl of the solution was slowly injected into the posterior chamber of the eyeballs using a micropipette. To avoid the damage of the lens, the micropipette was inserted at approximately 1.5 mm behind the ora serrata and at an angle pointing to the optic nerve pole. Animals that developed cataract or inflammation were excluded in this study.

Tissue Processing and Immunohistochemistry of mRGCs

For the study of cell survival, the animals were killed and the eyes were enucleated. The retinas were immersed into 4% paraformaldehyde in PBS.

The mRGCs were visualized by using the immunohistochemical procedures published previously (Li

et al. 2006a). Retinas were washed and blocked with 10% normal goat serum in 0.3% Triton X-100 and PBS mixture (PBT) for an hour at room temperature. The retinas were then incubated with polyclonal rabbit melanopsin antibody (1:1,000; Affinity Bioreagents, CO, USA) for 3 days at 4°C. After thorough washes, the samples were incubated with goat anti-rabbit IgG secondary antibody (1:500; Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) for an hour at room temperature. The retinas were flat-mounted on glass slides and cover-slipped for examination.

To study the pAkt expression, paraffin-sectioning was carried out in three animals of Groups 1A and B. After the killing of the animals and removal of anterior parts of the eyes, the whole eyecups were immersed into the fixative solution, and then embedded in paraffin. Approximately 6-μm-thick sections were cut and mounted on gelatin-coated slides. Six retinal sections of each animal were chosen for investigation. Following deparaffinization, the sections were immunostained with the monoclonal anti-pAkt antibody (Cell Signaling Technology, MA, USA) at dilution of 1:150 overnight at 4°C, and followed by the incubation of goat anti-mouse IgG secondary antibody (1:500, Molecular Probes) for 1 h at room temperature. Then, the sections were incubated with melanopsin antibody following the procedures described above. Negative controls were done by omitting the incubation of primary antibody.

Terminal Deoxynucleotidyl Transferase Biotin-dUTP nick end Labeling (TUNEL)

TUNEL assay was used to investigate whether the RGCs expressing melanopsin were apoptotic. Six retinal sections of each animal were chosen for investigation. Immunohistochemistry of melanopsin was performed using the protocol described above. Following that, TUNEL assay (ApopTag, Chemicon International Inc., Temecula, USA) was carried out according to the procedures listed by the manufacturer. Negative control of TUNEL was done by omitting the terminal deoxynucleotidyl transferase (TdT) reaction. Also, the sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) and then cover-slipped for examination.

Sampling and Counting of RGCs

The survival of mRGCs and scRGCs was examined by counting the number of cells that were melanopsin-positive and FG-positive RGCs under a fluorescent microscope (Nikon, Kawasaki, Japan) with an eyepiece equipped with a grid (800×800 μm^2/microscopic field for mRGC counting; 200×200 μm^2/microscopic field for scRGC counting). The sampling area for mRGC counting was larger compared to that of scRGCs because this is to increase the accuracy of counting the small population of mRGCs in the retina. Samples were taken along the median line of each quadrant from optic disc to the peripheral border of the retina at 800 and 400 μm intervals for mRGC and scRGC sampling correspondingly. Each retina was divided into four quadrants and five to ten microscopic fields were counted per quadrant, corresponding to approximately 22% of total mRGCs and 3% of total scRGCs. Images of retina showing mRGCs and FG-positive RGCs (i.e. scRGCs) were captured using a Rhodamine and UV-385 filters of the fluorescent microscope, respectively. To evaluate the distribution of mRGCs, the central and the peripheral retinal regions were defined as the sampling area of 400–2,000 μm and 2,000–4,000 μm from the optic nerve, correspondingly. Single-blinded approach was used in all cell counting and measurements to eliminate any subjective bias during the procedures.

Statistical Analysis

One-way analysis of variance (ANOVA) (Prism v4.0, GraphPad Software, Inc., San Diego, CA, USA), followed by a post-hoc Tukey's Multiple Comparison Test were used for the analysis of the results. Statistically significant difference was set at $P < 0.05$.

Results

Death of mRGCs Following ONT

In this study, the survival of the mRGCs and scRGCs was compared at 7 days (Group 1A) and 14 days (Group 1B) after ONT (7d ONT and 14d ONT). For ease of comparison, the survival of the cells is expressed in percentage, i.e. (the density of RGCs of

the experimental group/the density of RGCs of the control group) ×100%. Approximately 60% of scRGCs survived at 7d ONT, which is comparable to the result in previous studies (Berkelaar et al. 1994; Cheung et al. 2004). The number of mRGCs was also reduced to 40% at this time point. At 14d ONT, scRGC continued to die and only 10% of the total population was left ($P < 0.05$), while interestingly, the number of mRGC was more or less the same as that at 7d ONT ($P < 0.05$) (Figs. 1 and 2a). Data indicate that mRGCs did have injury-resistant ability after the traumatic optic nerve injury.

In the normal retina, there was approximately 2% of RGCs expressing melanopsin. The percentage of proportion [(density of mRGC/density of total RGC) ×100%] remained more or less the same at 7d ONT. However, the percentage of mRGCs increased in proportion to more than 9% of the total RGC population at 14d ONT (Group 1B) compared to those of the control (Group 3) and 7d ONT groups (Group 1A) ($P < 0.001$) (Fig. 2c). This is probably due to the dramatic loss of scRGCs, but not mRGCs, at this time-point.

We then analyzed the number of surviving mRGCs against the retinal topography. Our results demonstrated that the density of the cells at the peripheral retina was relatively higher than that at the central retina, at a ratio of approximately 2:3 for central: peripheral retina, which is consistent with the result of a previous study in mice (Robinson and Madison 2004). The proportion of distribution still remained even though there was a significant loss of mRGCs after the ONT ($P < 0.05$) (Fig. 3a).

The Axotomized mRGCs Expressed pAkt

As mentioned above, PI3 K/Akt pathway is a survival mechanism to protect RGCs from injury, and the activation of the PI3 K/Akt downstream effector, pAkt, is also found to attribute for the survival of RGCs (Cheung et al. 2004; Kretz et al. 2005; Nakazawa et al. 2002; Nakazawa et al. 2003). To verify whether the mRGCs also expressed pAkt in protecting the cells from injury, doubled-staining of immunohistochemistry was carried out using melanopsin and pAkt antibodies. In normal retina, the percentage of mRGCs expressing pAkt was low, approximately 20%. However, the percentage increased significantly at 7d and 14d ONT, about 50–60% of mRGCs expressed pAkt (Fig. 4). Results herein showed that a population, but not all, of mRGCs co-expressed pAkt (Fig. 5). This indicates that melanopsin-expressing cells survived through, at least in part, upregulation of activated Akt.

In this part of study, we sought to know whether the cells expressing melanopsin had signs undergoing apoptosis following injury (7d and 14d ONT). We found that all the TUNEL-positive cells were not melanopsin-positive (Fig. 6a–c). Also, all mRGCs, with and without injury, did not labeled with TUNEL (Fig. 6d–i). The results indicate that the cells expressing melanopsin were not apoptotic.

Effect of PI3 K/Akt Inhibitors on mRGC Survival After Injury

The result in immunostaining of pAkt leads to a speculation that PI3 K/Akt signaling pathway may play a role in mRGC survival. We then used two PI3 K/Akt inhibitors, the LY and WM, to test the hypothesis. A preliminary study was carried out to test the toxicity effect of the inhibitors on the survival of mRGCs. We found that after the intravitreal injection of LY and WM at the dose used, no significant difference was found in the cell density compared to the animals with vehicle injection (DMSO) and the control group (Fig. 7a).

Application of PI3 K/Akt inhibitors attenuated the survival of the mRGCs by 6% and 14% for LY and WM, respectively (Fig. 7b). The effect of WM was more effective in inhibiting the survival of mRGCs compared to the LY ($P < 0.05$ for LY; $P < 0.001$ for WM). Data indicate that inhibiting PI3 K/Akt resulted in reducing injury-resistance in axotomized mRGCs. The pattern of the mRGC distribution in central and peripheral retina did not reveal any significant difference with and without the injection of inhibitors which indicates that the effect of the inhibitors leads to a uniform cell loss across the retina ($P < 0.05$) (Fig. 3a).

Loss of scRGCs is a profound characteristic of OH model as published previously (Chauhan et al. 2002; Grozdanic et al. 2003; Ji et al. 2004, 2005; Mittag et al. 2000). Our recently published study demonstrated that cells expressing melanopsin are less susceptible to damage under the condition of elevated IOP (Li et al. 2006a). In the present study, our result again confirmed that there was no significant loss of mRGCs after the

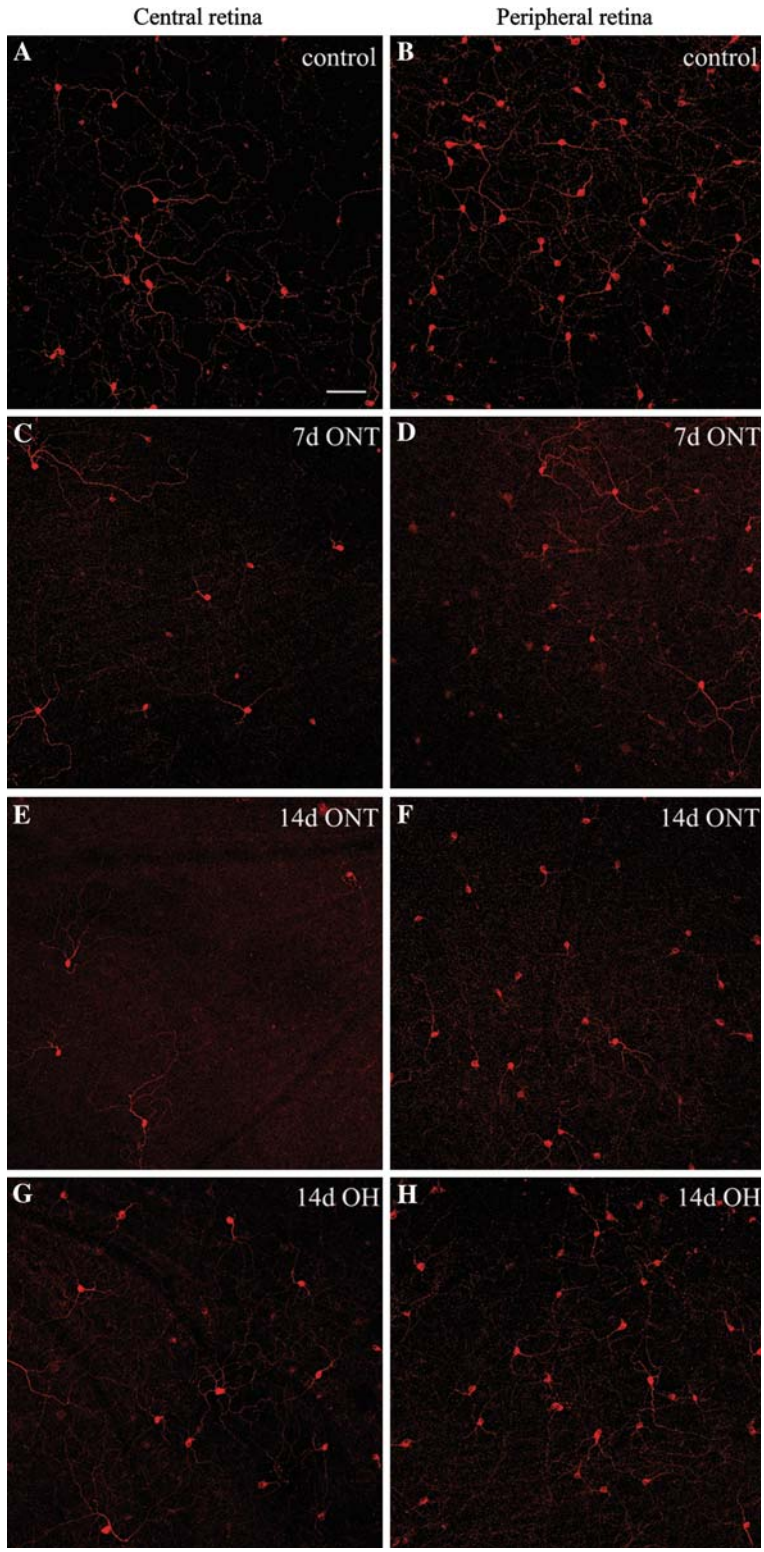


Fig. 1 Representative retinas showing immunostained melanopsin-expressing retinal ganglion cells (mRGCs). (**a**) Control; central retina. (**b**) Control; peripheral retina. (**c**) Retina at 7 days after optic nerve transection (d ONT); central retina. (**d**) Retina at 7d ONT; peripheral retina. (**e**) Retina at 14d ONT; central retina. (**f**) Retina at 14d ONT; peripheral retina. (**g**) Retina at 14 days after the induction of ocular hypertension (d OH); central retina. (**h**) Retina at 14d OH; peripheral retina. Scale bar, 75 μm

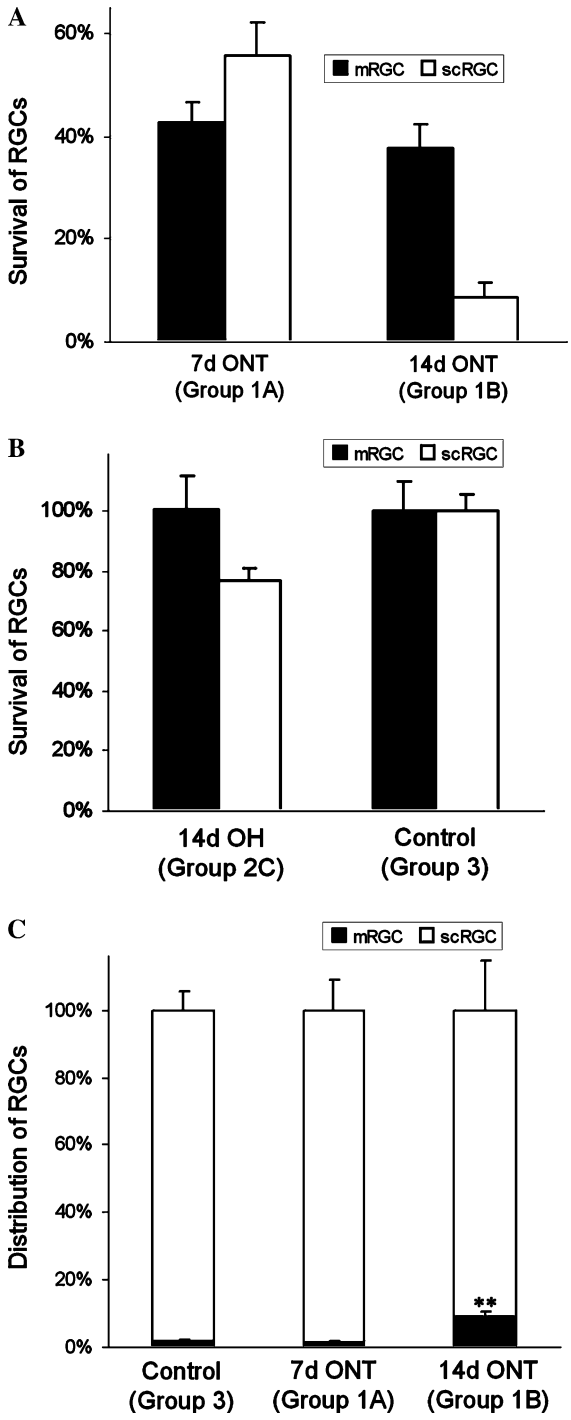


Fig. 2 (**a**) Survival of superior colliculus-projecting retinal ganglion cells (scRGCs) and melanopsin-expressing retinal ganglion cells (mRGCs) following optic nerve transection (ONT). The loss of scRGCs is further increased to approximately 90% at 14 days after ONT (d ONT) and reveals a significant difference compared to 7d ONT ($P < 0.001$). However, the loss of mRGCs at 7d ONT does not show a significant difference compared to the loss at 14d ONT and reveals a delayed cell death ($P < 0.05$). (**b**) Survival of scRGCs and mRGCs following the induction of ocular hypertension (OH). Significant cell loss of scRGCs is observed at 14d OH ($P < 0.001$) but no observable loss of mRGCs is noticed compared to the control ($P < 0.05$). (**c**) Percentage of mRGCs and scRGCs in the total number of RGCs in retinas after ONT. About 2% of cells are mRGCs in control retina and the percentage remains more or less the same at 7d ONT ($P < 0.05$). However, the percentage of mRGCs is increased significantly to approximately 9% at 14d ONT (**$P < 0.001$)

induction of OH (Figs. 1 and 2b). We then sought to find out the possible underlying factor that contributed to the injury-resistant property of these mRGCs. Using the same approach as the ONT study described above, application of PI3 K/Akt inhibitors (both LY and WM) significantly attenuated the survival of mRGCs by 20% compared to that of the control ($P < 0.05$) (Fig. 7c). Again, the application of the inhibitors did not change the pattern of distribution in the central and peripheral retina among the surviving mRGCs ($P < 0.05$) (Fig. 3b).

Discussion

Being part of the central nervous system, the optic nerve, if injured, could lead to an irreversible visual impairment, and the regeneration of axons is hindered by intrinsic and extrinsic factors (Caroni and Schwab 1993; Chen et al. 1995; Schwab et al. 1993). Rescue and protection of RGCs are the primary aims for optic nerve damage. Experimental ONT is one of the useful models to mimic the situation of optic nerve injury because it gives an extensive loss of RGCs within a short period of time after the transection (Berkelaar et al. 1994; Villegas-Perez et al. 1993). However, the axotomized RGCs do not die at the same time and a proportion of them still survives even after a prolonged period (Berkelaar et al. 1994; Villegas-Perez et al. 1993). This indicates that a subpopulation of cells is more tolerable to injury. Investigation of the factors attributing to the injury-resistant property of cells is particularly informative for the protection of RGCs after injury.

Cells that express melanopsin are found to be more injury-resistant to ONT (Robinson and Madison 2004). This group of mRGCs shows approximately a 3-fold increase in survival rate compared to those

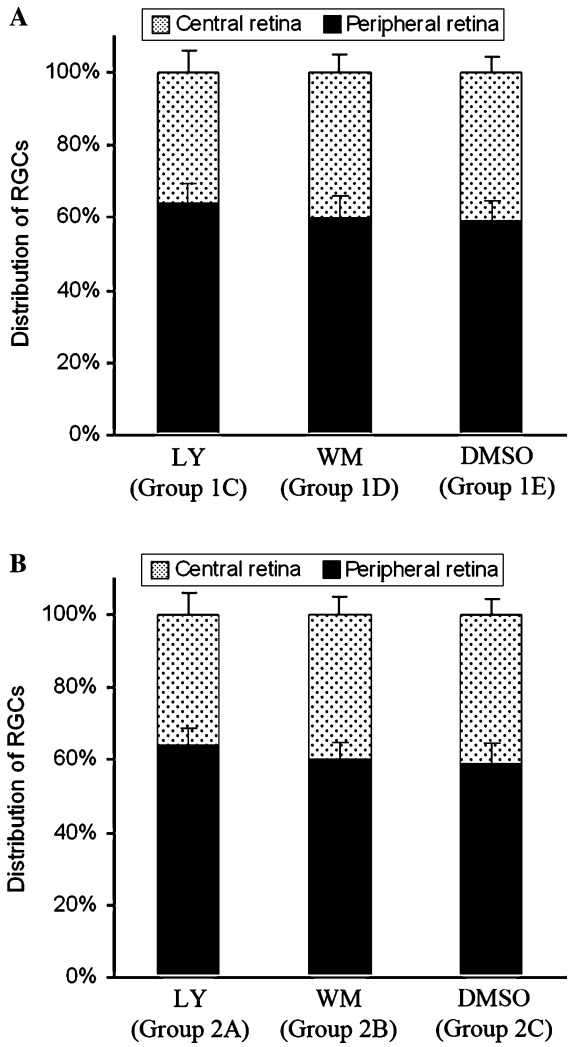


Fig. 3 (**a**) Distribution of the surviving melanopsin-expressing retinal ganglion cells (mRGCs) at 7 days after optic nerve transection (7d ONT) in different conditions: intravitreal injection of LY294002 (LY), Wortmannin (WM), and vehicle (DMSO). No obvious alteration of the distribution pattern is noticed between the eyes with inhibitor and vehicle injected ($P < 0.05$). (**b**) Distribution of mRGCs 14 days after ocular hypertension (14d OH) with intravitreal injection of LY, WM, and vehicle (DMSO). No obvious alteration of the distribution pattern is noticed among the groups ($P < 0.05$). LY, experimental group with LY-injected; WM, experimental group with WM-injected; DMSO, experimental group with DMSO-injected

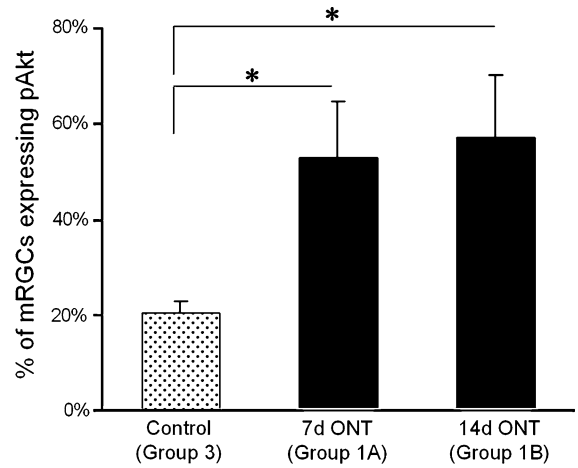


Fig. 4 Percentage of retinal ganglion cells (RGCs) that co-expressed melanopsin and phospho-Akt (pAkt) following optic nerve transection (ONT). The percentage of co-expression is significantly increased at 7 days after ONT (d ONT) and 14d ONT compared to the control (*$P < 0.01$)

without melanopsin expression although no enhancement of axon regeneration into the peripheral nerve graft after ONT was observed (Robinson and Madison 2004). Following that, although the difference is insignificant, the loss of mRGCs were found to be proportionally less than the total number of RGCs in DBA/2 J mice with severe glaucoma damage (Jakobs et al. 2005). Recently, mRGCs are also shown to be less vulnerable to the injury of OH in rats (Li et al. 2006a). In the present study, our data confirm that mRGCs were relatively less susceptible to optic nerve injury and OH. The longitudinal investigation in the present study demonstrated the disparity of cell survival between mRGCs and scRGCs at 14d ONT but not at 7d ONT.

Only a small number of mRGCs are found in the normal retina, approximately 1–2% in rodents (Hattar et al. 2002). Following 2 weeks of ONT, mRGCs showed an increase, to about 9%, in the proportion among the total RGCs in the retina, which is comparable to the result shown in mice (Robinson and Madison 2004). The injury-resistant property of mRGCs was also observed in the OH model. Significant cell loss of scRGC was found but no observable loss of mRGC is noticed. The results are in line with our previous study (Li et al. 2006a).

Resistant to injury-induced damage of neurons is extensively studied, but the factors and underlying mechanism still remain controversial. Signaling pathways such as PI3 K/Akt and Ras/MEK are recognized as prominent survival mechanisms following neuronal damage. PI3 K acts by exerting the inhibitory effect on Bad (Datta et al. 1999) caspase 3 and caspase 9 (Cardone et al. 1998; Kim and Park 2005; Zhou et al. 2000). Various growth factors or

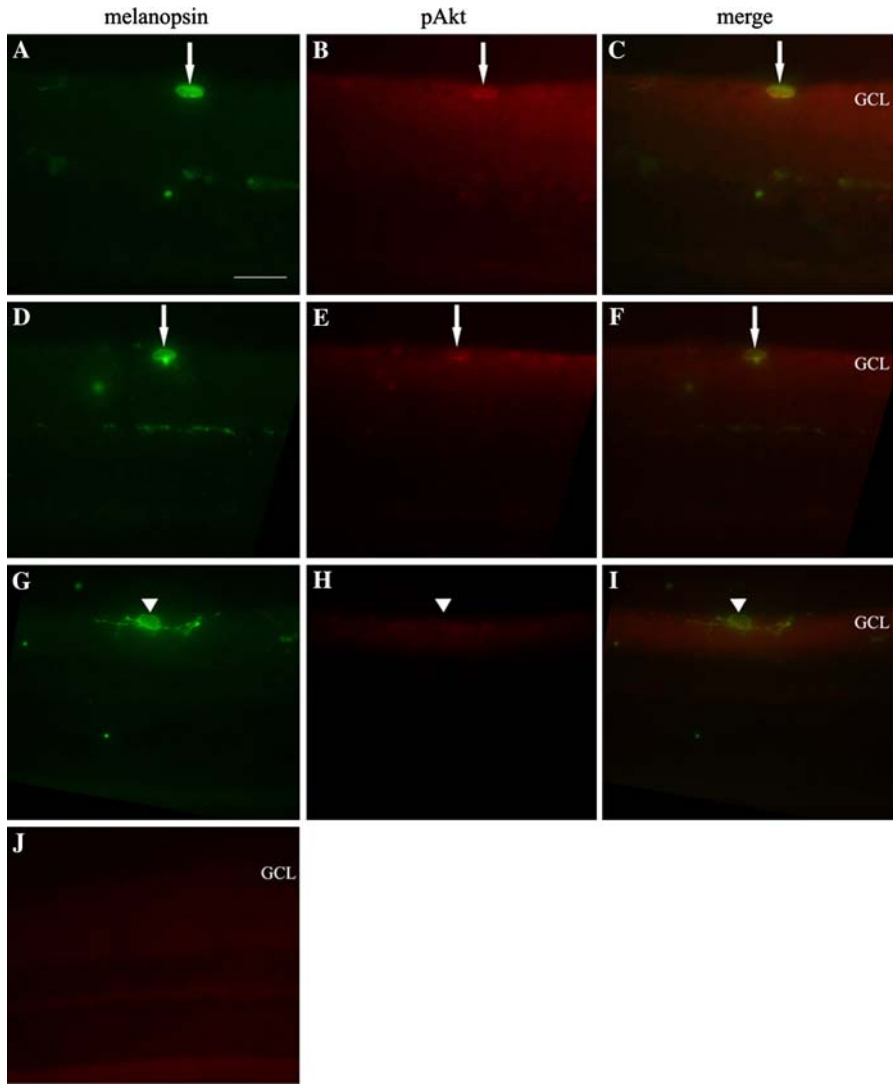


Fig. 5 Doubled-labeling of melanopsin and phospho-Akt (pAkt) in retina after optic nerve transection (ONT). (**a**, **d**, **g**) melanopsin-positive retinal ganglion cells (mRGCs) (green). (**b**, **e**, **h**) pAkt-positive RGCs (red). (**c**, **f** ,**i**) merge images. A population of mRGCs co-expresses with pAkt (**a**–**f**, arrows) whilst some of them are not (**g**–**i**, arrow heads). (**j**) Negative control with primary antibody incubation omitted. GCL, ganglion cell layer; IPL, inner plexiform layer. Scale bar, 30 μm

hormone, such as brain-derived neurotrophic factor (Bonnet et al. 2004; Nakazawa et al. 2002) and erythropoietin (Weishaupt et al. 2004), rescued the injured RGCs via this pathway. Previous studies demonstrated that the injury-mediated activation of PI3 K/Akt pathway probably contributes to the delayed cell death of RGCs after ONT and acute OH (Cheung et al. 2004; Huang et al. 2008; Nakazawa et al. 2002). As anticipated, the surviving mRGCs expressed activated Akt after the injury of ONT. Surprisingly, not all, but a group of the mRGCs did co-express melanopsin and pAkt. Results herein support our hypothesis of the involvement of PI3 K/Akt survival pathway in rescuing the mRGCs from damage. To further verify this phenomenon, the potent and selective P13 K inhibitors, LY and WM, were injected intravitreally into the axotomized eyes. We found that the survival of mRGCs with inhibitors injected was significantly lowered compared to the group with the injection of vehicles. Data indicate that the mRGCs exerted the injury resistance, at least partly, via the PI3 K/Akt signaling mechanisms following transection. The attenuation of cell survival was more in the group of animals receiving WM compared to that of LY. This may probably be due to the specificity of the inhibitor for PI3 K/Akt for LY. Compared to LY, WM does not only exert its inhibitory effect on the PI3 K/Akt pathway, but also on other enzymes such as mitogen-activated protein kinase at certain circumstances (Ferby et al. 1994,

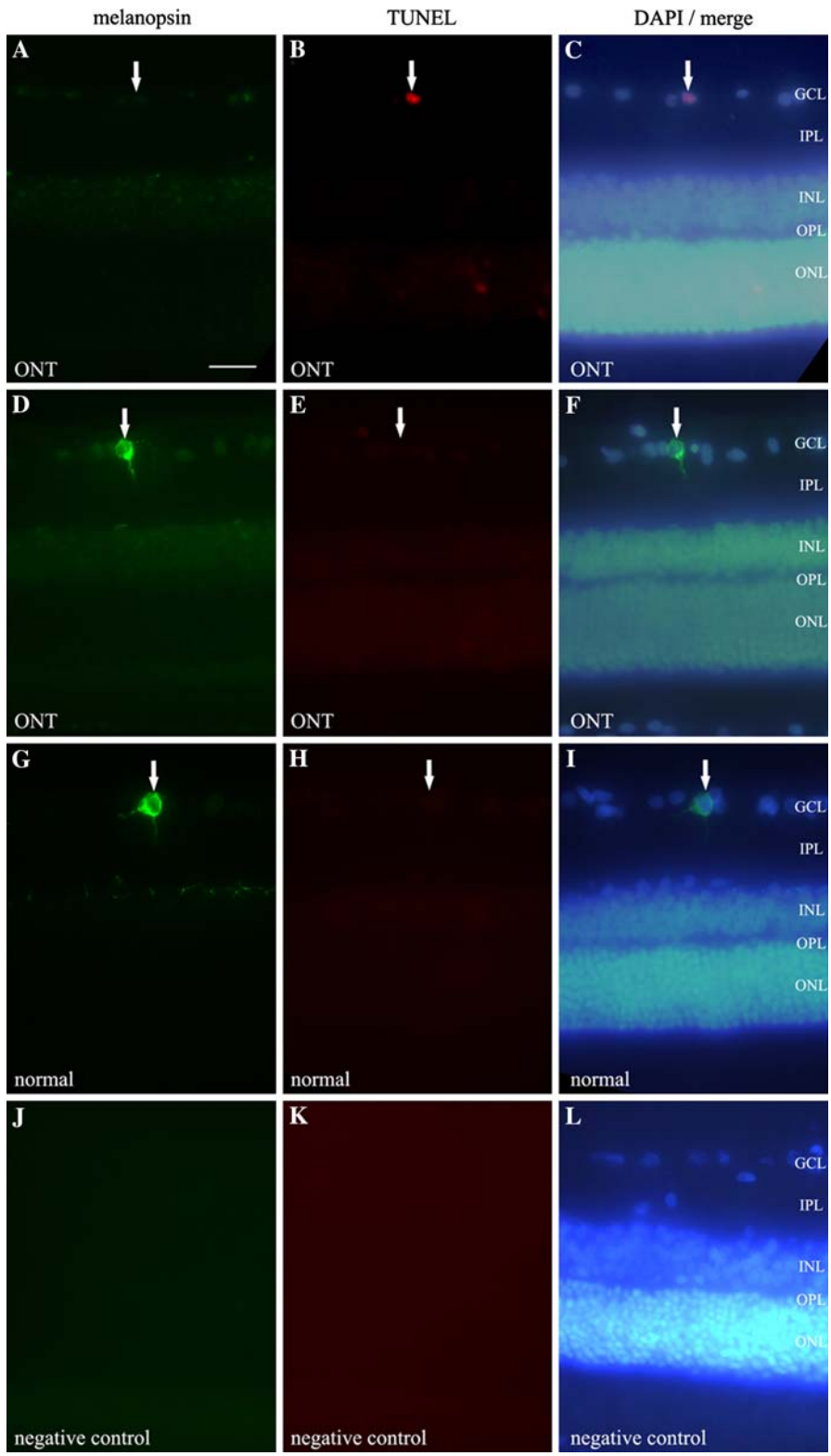
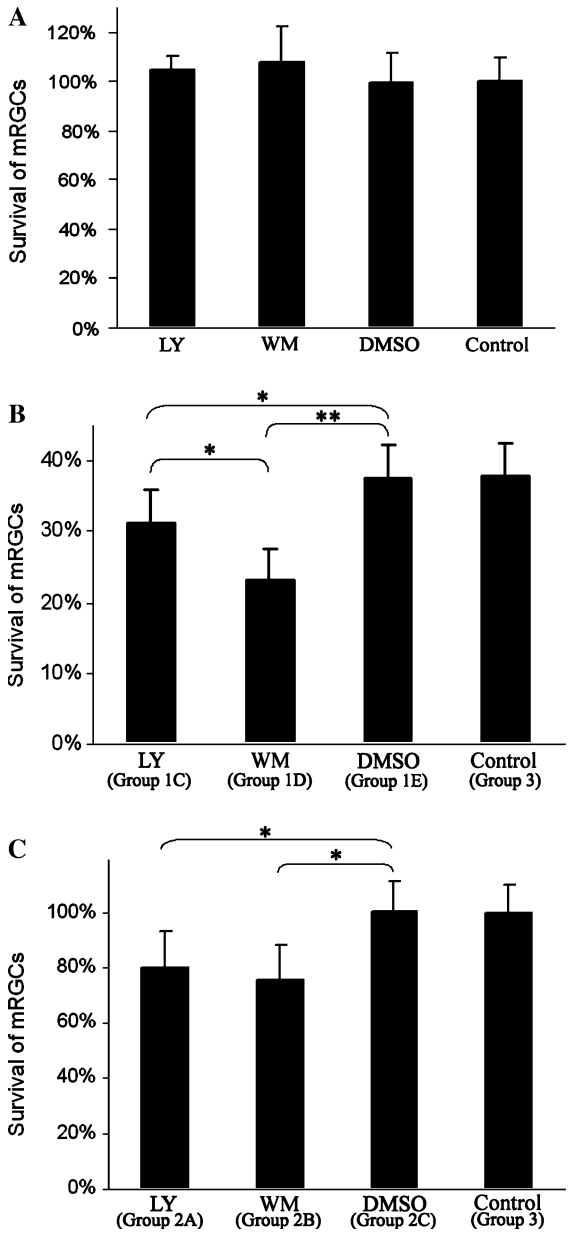


Fig. 6 Triple-labeling of retinal sections with melanopsin (**a**, **d**, **g** and **j**), TUNEL (**b**, **e**, **h** and **k**) and DAPI (**c**, **f**, **i** and **l**). (**a**–**c**) Apoptotic retinal ganglion cells (RGCs) are labeled with TUNEL but not melanopsin-positive after optic nerve transection (ONT). (**d**–**f**) Melanopsin-expressing RGCs (mRGCs) are not labeled with TUNEL after ONT. (**g**–**i**) mRGCs are not labeled with TUNEL in normal retina. (**j**–**l**) Negative control of melanopsin and TUNEL labeling. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nucleus layer; OPL, outer plexiform layer; ONL, outer nucleus layer. Scale bar, 30 μm



◀ **Fig. 7** (**a**) Effect of intravitreal injection of the PI3 K/Akt inhibitors on the survival of melanopsin-expressing retinal ganglion cells (mRGCs) in normal retina. No obvious cell loss is observed among groups ($P < 0.05$). (**b**) Effect of intravitreal injection of the PI3 K/Akt inhibitors on the survival of mRGCs after optic nerve transection (ONT). Significant attenuation of cell survival following the application of both inhibitors (LY, LY294002, $*P < 0.05$; WM, Wortmannin, $**P < 0.001$) is observed. No difference is found between the group with vehicle injection (Dimethyl Sulfoxide; DMSO) and the group with ONT but without any injection ($P < 0.05$). (**c**) Effect of intravitreal injection of the PI3 K/Akt inhibitors on the survival of mRGCs after the induction of ocular hypertension (OH). Significant attenuation of cell survival is observed following the application of both inhibitors ($*P < 0.05$). No difference is found between the group with vehicle injection (DMSO) and the control group ($P < 0.05$). LY, experimental group with LY injected; WM, experimental group with WM injected; DMSO, experimental group with DMSO injected

1996). This may imply that the injury-resistant property of mRGCs is not solely due to the activation of PI3 K/Akt pathway following ONT.

The RGCs that express melanopsin also revealed delayed cell death after the elevation of IOP. In the OH model, inhibiting the PI3 K/Akt pathway significantly reduced the survival of mRGCs by 20%, which was comparable to the cell loss of scRGCs. This indicates that about one-fifth of the mRGCs relied on the activated PI3 K/Akt pathway following the induction of OH. Contrary to ONT, LY and WM did not reveal any differential effect on mRGC survival. The discrepancy might probably due to the extent of injury in these two models. We postulate that optic nerve injury may elicit a more complex response and upregulation of more survival pathways.

In rodent retina, the density of mRGCs appears to be higher in the peripheral region than those in the central (Robinson and Madison 2004). This distribution pattern was not altered even after injury or the intravitreal injection of inhibitors. Our result points out that the injury-induced cell loss of mRGCs was evenly distributed. As mRGC plays an important role in the modulation of photoentrainment, the uniform cell death across the retina may help to maintain the function of receiving light signals and prevent the disturbance of the physiological light/dark cycle following insult.

As discussed in the previous studies (Li et al. 2006a; Robinson and Madison 2004), anatomically long and sparsely branching dendrites, and the intrinsic photosensitive characteristics may be the factors contributing to the resistance in cell injury. Further study is required to examine these possibilities and also explore the upstream regulators and downstream effectors of the PI3 K/Akt pathway.

Conclusion

Delayed cell death was shown to exist in a small population of RGCs that expresses melanopsin after

injury. Our finding confirms that these cells do possess the injury-resistant property in the two injury models. The survival of these neurons is at least in part via the PI3 K/Akt pathway. Further investigation is essential to understand other signaling pathways that facilitate the prolonged survival of mRGCs.

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