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Axotomized mouse retinal ganglion cells containing melanopsin show enhanced survival, but not enhanced axon regrowth into a peripheral nerve graft

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Abstract

Melanopsin is found in only ~2% of mouse retinal ganglion cells (RGCs), making these RGCs uniquely and directly photosensitive. Given that the majority of RGCs die after axotomy and that grafting of a peripheral nerve to the eye provides a permissive environment for axon regrowth, the present study examined the survival and axonal regrowth of melanopsin-containing RGCs in mice. One month after optic nerve transection and grafting, RGCs with regrown axons were labeled from the grafts and retinae were processed to visualize melanopsin and TUJ1. Melanopsin-positive and negative RGCs were counted and compared to axotomized RGCs from ungrafted eyes and uninjured RGCs. Melanopsin-positive RGCs showed a 3-fold increase in survival rate compared to non-melanopsin RGCs. Despite this enhanced survival, melanopsin-containing RGCs did not show increased axon regrowth into nerve grafts.

Keywords: Retinal ganglion cells; Melanopsin; Regeneration; Mouse; Nerve graft

1. Introduction

If the optic nerve of mature rodents is transected close to the eye, half of the retinal ganglion cells (RGCs) die by the end of the first week (Berkelaar, Clarke, Wang, Bray, & Aguayo, 1994; Robinson, 1994), and this axotomy-induced death continues to the extent that ~90% of RGCs die by one month (Berkelaar et al., 1994; Cenni et al., 1996; Robinson, 1994; Villegaz-Pérez, Vidal-Sanz, Bray, & Aguayo, 1988; Villegaz-Pérez, Vidal-Sanz, Rasminsky, Bray, & Aguayo, 1993). Even with this profound loss, axotomy-induced RGC death is not total. A small percentage of RGCs show long-term survival (Cenni et al., 1996; Villegaz-Pérez et al., 1993), yet the vital characteristics of these few cells have not been fully elucidated.

Attachment of a peripheral nerve graft to the eye at the cut optic nerve stump provides a permissive environment for RGC axons to regrow (Cui & Harvey, 2000; Inoue, Hosokawa, Morigiwa, Ohashi, & Fukuda, 2002; Inoue, Sasaki, Hosokawa, & Fukuda, 2000; Robinson & Madison, 2000; Thanos, Mey, & Wild, 1993; Vidal-Sanz, Bray, Villegaz-Pérez, Thanos, & Aguayo, 1987; Villegaz-Pérez et al., 1988; Watanabe, Sawai, & Fukuda, 1993), and has also been reported to reduce axotomy-induced RGC death in rats, suggesting a trophic effect of the graft (Villegaz-Pérez et al., 1988). Even with this more permissive regrowth environment however, few RGCs that survive for one month regrow axons into a graft.

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Recently, a subset of ganglion cells has been identified that are directly light-sensitive and contain the putative photopigment melanopsin (Gooley, Lu, Chou, Scammell, & Saper, 2001; Hattar, Liao, Takao, Berson, & Yau, 2002; Provencio et al., 2000). These uniquely photosensitive ganglion cells that appear to help regulate the circadian clock (Berson, Dunn, & Takao, 2002; Panda et al., 2002) and other irradiance-based tasks (Hattar et al., 2003) account for only ~2% of all RGCs (Hattar et al., 2002). Given their unique characteristics, we first examined whether melanopsin-containing RGCs showed the same pattern of cell death after optic nerve transection as non-melanopsin RGCs and second, whether the capacity of melanopsin-containing RGCs for axonal regrowth into a peripheral nerve graft was the same as non-melanopsin-containing RGCs. Preliminary results of these finding have been reported in abstract form (Robinson & Madison, 2003).

2. Materials and methods

2.1. Animals

All procedures were approved by the Veterans Affairs Medical Center animal use and care committee. C57BL/6 mice (male and female, 20–22 g) were deeply anesthetized for all the following procedures with a mixture of ketamine, xylazine, and acepromazine in normal (0.9%) saline (respectively, 100, 6, and 1 mg/kg, i.p.). In the present work, each processed retina often contributed to more than one data set (e.g., retrogradely labeled RGCs from a graft combined with melanopsin immunocytochemistry). Accordingly, the number of retinae contributing to each data set is indicated in Results rather than indicating the number of animals prepared.

2.2. Peripheral nerve grafting

A portion (approximately 15 mm) of the femoral nerve from the left hindlimb was harvested using microscissors to serve as an autologous graft. Using an intraorbital approach, the left optic nerve was exposed by longitudinally opening the dural sheath (to prevent damage to the blood supply) and transected approximately 0.5 mm from the eye using microscissors. The trunk of the femoral nerve graft was trimmed and apposed to the ocular stump of the optic nerve and then attached by application of Tisseel VH™ fibrin sealant prepared according to the manufacturer's instructions (Baxter Healthcare Products, Glendale, CA). Preliminary experiments determined that the manufacturer's syringe delivery system was too large for the present application, so the two components (sealer and activator) were each loaded into multiple sterile 1 cc syringes with short 30G needles and stored at −80 °C. Prior to surgery, both components were placed in a 37 °C water bath until needed. After the sealant stiffened, the remaining graft was ligated at it most distal point and placed in a channel in the skull made between the orbit and the left occipital bone. The retinal blood supply was examined by fundoscopy at the end of the procedure after pupil dilation using topical 1% cyclopentolate hydrochloride (Alcon, Ft. Worth, TX). Eyes with a damaged blood supply were excluded from the study. All surgical procedures were performed by the same investigator.

2.3. Retinal ganglion cell axotomy alone

Using the same approach described above, the optic nerve was transected and sealed with fibrin sealant without a peripheral nerve graft.

2.4. Retrograde labeling of regenerating retinal ganglion cells

One month after graft attachment to the eye, mice were reanesthetized and the ligated, distal graft was reexposed. The graft was transected ~10 mm from the eye and a 4% Fluoro-Gold solution (FG, Fluorochrome, Denver, CO) in normal saline was applied to the cut graft to label regenerated RGC axons. The surgical area was then closed and the animal allowed to recover.

2.5. Retrograde labeling of control retinal ganglion cells

For preliminary experiments to determine the number of retinal ganglion cells in control retinae and the distribution of TUJ1 immunoreactivity, 4% FG was applied to the cut optic nerve and the mice allowed to recover.

2.6. Retinal processing

All animals were anesthetized with an overdose of anesthetic three days after application of FG and then perfused through the heart with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Retinae were dissected and prepared as flattened wholemounts, with four radially oriented cuts in each retina and the superior pole of the retina was identified. Wholemounts were postfixed for <1 h and washed for at least 1 h in 0.1 M PBS.

2.7. Immunocytochemistry

Retinae were processed for melanopsin immunocytochemistry using rabbit antiserum UF006 generated against the N-terminus peptide of mouse melanopsin (Panda et al., 2002) in combination with tyramide signal amplification and Alexa Fluor dyes (TSA kits #12 and

15, Molecular Probes, Eugene, OR). For anti-Fluoro-Gold, anti-melanopsin and TUJ1 immunocytochemistries, the choice of Alexafluor dye was arbitrary as both were used successfully. Briefly, retinal wholemounts were incubated in 3% hydrogen peroxide in PBS for 5 min to block non-specific peroxidase activity and then washed extensively in PBS. Retinae were then incubated in blocking buffer (TSA blocking reagent containing 0.1% Triton-X in PBS) for 1 h, followed by incubation in antiserum UF006 (1:5000 in blocking reagent) for 72 h at 4 °C with agitation. After extensive washing in PBS, retinae were incubated in a horse radish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:100 in blocking reagent) for 2 h. Wholemounts were washed extensively and then incubated in amplification buffer containing 0.0015% hydrogen peroxide and tyramide/Alexa Fluor 594 (1:100) for 15 min at room temperature. No labeling was observed when primary antibodies were omitted.

For TUJ1 immunocytochemistry, rabbit polyclonal antibody PRB-435P (1:4000) recognizing neuronal type III/beta tubulin (Covance Research Products, Berkeley, CA), was used in the identical TSA protocol with labeling by tyramide/Alexa Fluor 594 (1:300) for 30 min.

For RGC double-labeling, retinae were again incubated in 3% hydrogen peroxide in PBS for 5 min to block peroxidase activity, washed extensively in PBS, and then incubated in the next primary antibody. For combinations involving melanopsin staining, anti-melanopsin immunoprocessing was always first. For combinations without melanopsin, anti-FG immunoprocessing was first. Preliminary experiments determined that double-labeling color separation could be optimized when RGCs were processed for immunocytochemistry using an antibody against FG (AB153, rabbit polyclonal, Chemicon International, Temecula, CA) in the identical TSA protocol described above with the substitution of labeling by tyramide/Alexa Fluor 594 (1:500) for 15 min. At the end of all processing, the tissue was extensively washed in PBS, mounted onto glass slides, dehydrated through graded alcohols, cleared in xylene and coverslipped with Cytoseal 60 (Richard-Allen Scientific, Kalamazoo, MI).

2.8. Retinal ganglion cell counts

RGCs were counted at a magnification of 400× using a Zeiss Axiophot microscope equipped for fluorescence. Cells were identified using individual and composite filter sets appropriate for Alexa Fluor 594, 488 and FG (Chroma Technology, Brattleboro, VT). Two methods were used to count RGCs. The first method sampled all RGCs within a fixed area (e.g., 230 μm×230 μm at 400×, or the same box at 250×) at three eccentricities in each quadrant (0.5, 1.5, and 2.5 mm from the optic disk). The number of RGCs from these 12 regions/retina was used to calculate the average density for each retina. Total retinal area was determined by outlining the retina using Stereo Investigator 2000 software (Microbrightfield, Inc., Colchester, VT). Total RGCs per retina were then determined by multiplying the density by the area. This sampling method could not be used in grafted retinae to count melanopsin-positive and regenerating RGCs due to their small numbers, so a direct method was used instead. These retinae were completely surveyed using non-overlapping increments to determine the total number of RGCs.

To examine if RGC death was influenced by location within the retina, normal and axotomized RGC density data from the most central location (0.5 mm from the optic disk) was divided by data from the most peripheral location from the disk (2.5 mm) for each quadrant and expressed as a quotient.

2.9. Statistical analysis

Student *t*-tests for paired data were used to compare distribution ratios within groups. Differences were considered statistically significant when $p<0.05$. Analysis of variance (using Student–Newman–Keuls post hoc comparisons) was used to determine differences among groups. Differences were considered statistically significant when $F<0.05$ and $p<0.05$.

2.10. Minipump implantation

All mice with peripheral nerve grafts received dorsal subcutaneous minipumps (Alzet model 1007D, Durect Corporation, Cupertino, CA) containing normal saline at the time of initial surgery. All pumps were removed 7 days later. The pumps were implanted to provide baseline data for future pharmacological manipulation of RGC survival and axon regrowth using this model.

3. Results

In agreement with previous reports of ganglion cell counts in C57BL/6 mice (Inoue et al., 2000; Williams, Strom, Rice, & Goldowitz, 1996), the distribution of FG-labeled retinal ganglion cells in normal mouse retina was highest near the optic disk (~4000 cells/mm^2) and least in the peripheral retina (Fig. 1A, ~2600 cells/mm^2) for an overall density of 3285 ± 75 ($N=5$, mean ± SEM) representing 50,920 ± 1161 RGCs per retina. In contrast, the distribution of melanopsin-positive ganglion cells was reversed, with the highest density (~90 cells/mm^2) at the peripheral edge of the retina and the lowest (~25 cells/mm^2) in the most central retina (Fig. 2, upper panels) for an overall density of 66 ± 7 ($N=6$) representing 1023 ± 104 RGCs per retina.

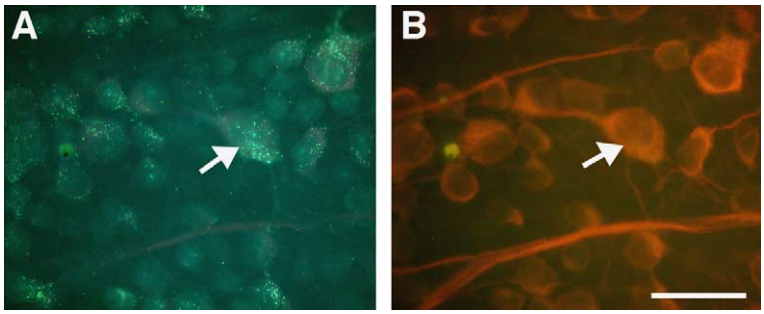

Fig. 1. TUJ1 immunoreactivity co-localizes with retinal ganglion cells. (A) Ganglion cells containing yellow punctate FG (arrow) in a retinal wholemount three days after application of the retrograde tracer to the cut optic nerve. (B) Red TUJ1 immunoreactivity using Alexa Fluor 594 co-localized (arrow) to the same retrogradely labeled cells. TUJ1 staining was used to indicate all surviving ganglion cells without retrograde labeling. Scale bar = 25 μM.

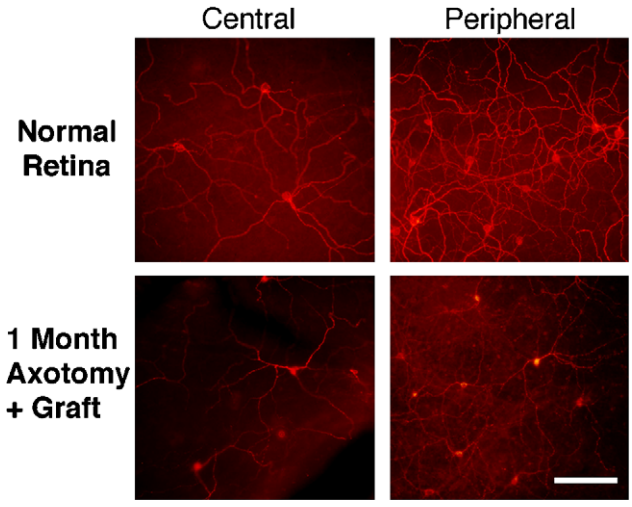

Fig. 2. Melanopsin-positive retinal ganglion cells are reduced one month after axotomy, but their retinal distribution is maintained. In the normal eye melanopsin-positive ganglion cells are least dense in the central retina and most dense in the peripheral retina. One month after axotomy and placement of a nerve graft, melanopsin-positive cells are reduced, but their differential distribution between the central and peripheral retina is maintained. Scale bar = 100 μM.

A comparison of FG-labeled ganglion cells and TUJ1-immunocytochemistry (Fig. 1) revealed complete overlap in RGCs between the two methods. This overlap allowed TUJ1 immunoreactivity to be used to indicate the total number of surviving retinal ganglion cells one month after axotomy in both grafted and ungrafted retinae. In axotomized RGC preparations, TUJ1-positive cytoplasmic processes of Müller glia were also noted at the vitreal surface of wholemounts, but these processes did not interfere with counting RGCs. Ganglion cell density data revealed a significant reduction to 396 ± 27 ($N = 5$) TUJ1-positive ganglion cells/mm^2 (for a total of 6132 ± 455 RGCs per retina) one month after axotomy alone and a similar reduction to 422 ± 52 ($N = 4$) TUJ1-positive ganglion cells/mm^2 (for a total of 6543 ± 802 RGCs per retina) one month after axotomy and grafting (Fig. 3A). However, these reductions were not statistically different from each other. The extent of long-term axotomy-induced cell death described here for all retinal ganglion cells is also in agreement with other reports in rats and mice (Berkelaar et al., 1994; Inoue et al., 2002; Robinson, 1994; Villegaz-Pérez et al., 1988; Villegaz-Pérez et al., 1993).

Unexpectedly, melanopsin-positive ganglion cells survived to a significantly greater degree than non-melanopsin ganglion cells. Approximately 42% of the original population of melanopsin-positive ganglion cells survived after axotomy alone compared to only ~12% of the original non-melanopsin ganglion cell population. In addition, the presence of a peripheral nerve graft did not alter the relative percent survival between the melanopsin-positive and non-melanopsin populations of axotomized ganglion cells (Fig. 4A). Proportionally (Fig. 4B), melanopsin-positive ganglion cells represented only ~2% of all normal RGCs (1023 ± 104 of the 50,920 ± 1161 RGCs). After axotomy alone, their proportional representation increased significantly to ~7% (431 ± 50, $N = 5$) of the ganglion cells that survived (6132 ± 455, $N = 5$). A similar proportional increase in representation was also seen in the nerve graft group, with ~6% (377 ± 30, $N = 5$) of the surviving ganglion cells (6543 ± 802, $N = 4$) being melanopsin-positive. While axotomy reduced the population of melanopsin-positive ganglion cells by approximately half, their increased density in the peripheral retina relative to the central retina was maintained (Fig. 2). Based on immunocytochemistry, melanopsin-positive ganglion cell bodies appeared smaller after axotomy with less dendritic branching (Fig. 2) both in the presence or absence of a peripheral nerve graft.

The ratio of central to peripheral RGC densities was examined to determine if RGC death was related to retinal location. In normal retinae the ratio was 1.56 ± 0.8 ($N = 5$), indicating 156 RGCs per given area centrally for every 100 RGCs in the peripheral retina. One month after axotomy alone, the ratio (2.15 ± 0.33, $N = 5$) was

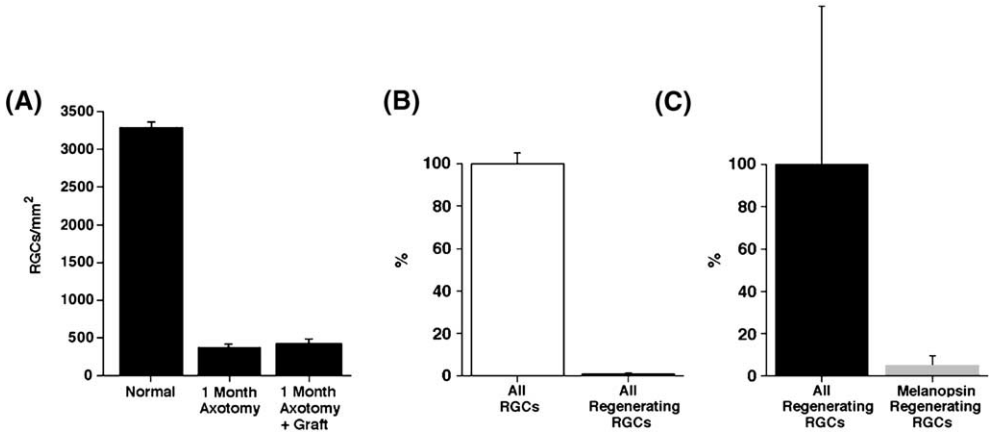

Fig. 3. Axotomy-induced retinal ganglion cell loss and axonal regrowth after nerve graft attachment. (A) Attachment of a femoral nerve graft to the eye did not significantly alter the profound ganglion cell death on month after axotomy. The mean density of ganglion cells was similarly reduced in grafted and non-grafted retinae. (B) Compared to all ganglion cells (100%), the proportion with axonal regrowth into a peripheral nerve graft was only 0.5%. (C) Among the subpopulation of surviving ganglion cells demonstrating axonal regrowth (100%) only 5.1% were melanopsin-positive. Bars in A = SEM, in B, C = SD.

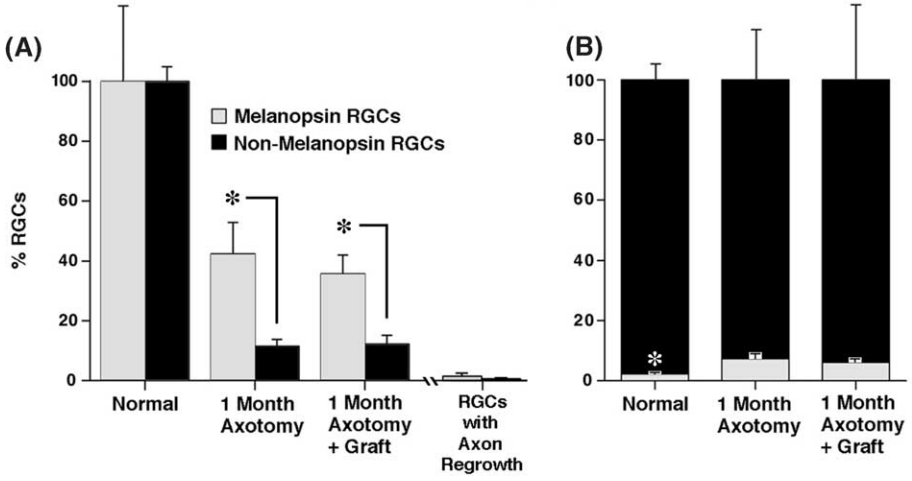

Fig. 4. The proportional survival of melanopsin-positive retinal ganglion cells compared to non-melanopsin ganglion cells after axotomy. (A) Axotomy induced the loss of both types of ganglion cells at one month, but significantly more melanopsin-positive ganglion cells survived at one month (~37% with or ~42% without a nerve graft) compared to non-melanopsin ganglion cells (~12% with or ~11% without a nerve graft). Asterisks denote differences at the $p < 0.05$ level between melanopsin and non-melanopsin ganglion cells with and without nerve grafting using ANOVA and post hoc *t*-tests. The proportion of RGCs with axon regrowth (a subset of the 1 month axotomy+graft data) was not significantly different between melanopsin and non-melanopsin cell types (*t*-test). (B) The increased survival of melanopsin-positive ganglion cells led to a significant increase in their proportional representation among ganglion cells after axotomy: from ~2% of normal RGCs to approximately ~6% and ~7% (grafted and ungrafted) of axotomized RGCs. Asterisk denotes difference at the $p < 0.05$ level between the proportions of melanopsin-positive RGCs in normal and experimental retinae using ANOVA and post hoc *t*-tests. Bars = SD.

not statistically different (*t*-test), indicating that RGC death was proportional across the retina. Similarly, the ratio for melanopsin RGCs in the normal retina was 0.54 ± 0.08 ($N = 5$), and was not significantly different one month after axotomy (0.61 ± 0.08, $N = 4$).

After axotomy, few of the surviving population of retinal ganglion cells regrew axons into a peripheral nerve graft. Only 232 ± 78 ($N = 5$) ganglion cells were retrogradely-labeled (e.g., Fig. 5A) from the nerve grafts one month after attachment. Proportionally (Fig. 3B), this represents only 0.5% of the normal retinal ganglion cell population. When regenerating melanopsin-positive ganglion cells (e.g., Fig. 5B) are normalized to all regenerating RGCs (Fig. 3C), the 12 ± 5 ganglion cells per retina represent only ~5% of the regenerating population.

4. Discussion

We have examined the ability of mouse retinal ganglion cells containing the putative photopigment melanopsin to survive axotomy and regrow an axon into a

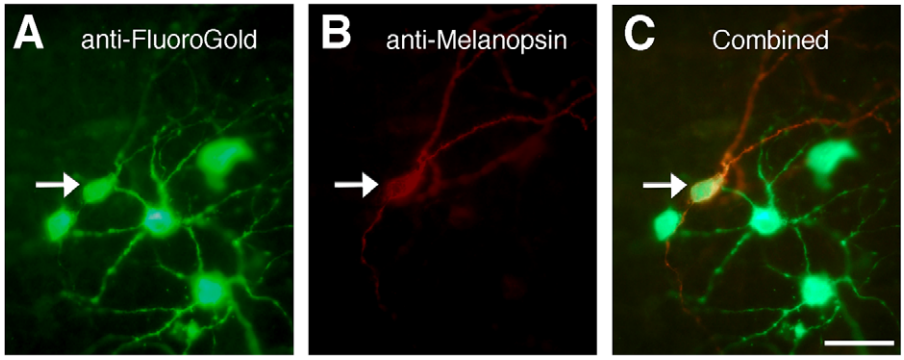

Fig. 5. Melanopsin-positive and non-melanopsin retinal ganglion cells regrow axons into a peripheral nerve graft. (A) One month after graft attachment to the eye, regenerating axons were labeled from the graft with FG and the retinal wholemount was processed for green anti-FG immunoreactivity using Alexa Fluor 488 (arrow). Following processing for red anti-melanopsin immunoreactivity using Alexa Fluor 594 (B), the proportion of regenerating ganglion cells that were also melanopsin-positive (arrow) was assessed (C). Scale bar = 25 μM.

peripheral nerve graft. The main findings are: (1) that this small subset of ganglion cells is less sensitive to axotomy-induced cell death than the rest of the ganglion cell population, and (2) that this increase in survival is not matched by an increased ability to regrow axons into the permissive environment of a peripheral nerve graft.

Axotomy-induced death of mature rodent retinal ganglion cells has been well documented. Even though most mouse and rat ganglion cells die after optic nerve transection (Berkelaar et al., 1994; Cenni et al., 1996; Robinson, 1994; Villegaz-Pérez et al., 1988; Villegaz-Pérez et al., 1993), ganglion cells do not all die at the same time. Half of these cells die rapidly within one week of axotomy (Berkelaar et al., 1994; Robinson, 1994), with a small percentage of viable ganglion cells persisting even months after the injury (Cenni et al., 1996; Villegaz-Pérez et al., 1993).

Given that retinal ganglion cells are a diverse group according to anatomical and physiological classification schemes (e.g., Boycott & Wässle, 1974; Fukuda, 1977), the critical features of those that are injury-resistant are unknown. Previous characterizations of injury-resistant RGCs have associated them with increased cytochrome oxidase activity (von Bussmann, Gary, & Jen, 1993) as well as maintained expression of heat shock protein 27 (Krueger-Naug, Emsley, Myers, Currie, & Clarke, 2002) and transcription factor c-Jun (Robinson, 1994). Whether the surviving mouse melanopsin-containing RGCs also share these other features of injury-resistant RGCs is unknown. Recent work in the mouse has shown non-uniform immediate early gene expression in melanopsin-positive RGCs (Semo, Lupi, Peirson, Butler, & Foster, 2003), suggesting heterogeneity even among this small population of RGCs.

The unique anatomy/physiology of melanopsin-containing RGCs may contribute to their increased survival: the putative photopigment confers intrinsic photosensitivity that is provided through unusually large, yet sparsely branching dendritic fields (Berson et al., 2002). Conversely, the axotomy-induced death of half of the melanopsin-positive population suggests that melanopsin is unlikely to be the sole survival factor. Differential survival among melanopsin-positive RGCs could be reflected in the two anatomically-defined types of these RGCs based on dendritic stratification (Belenky, Smeraski, Provencio, Sollars, & Pickard, 2003), differences in immediate early gene expression (Semo et al., 2003), or on an altered relationship between melanopsin and pituitary adenyl cyclase-activating polypeptide, a neurotransmitter of the retinohypothalamic tract that can be co-expressed in these RGCs (Hannibal, Hindersson, Knudsen, Georg, & Fahrenkrug, 2002).

Interestingly, we observed that the vitreal cytoplasmic processes of Müller glia were labeled by the *neuronal* beta III/tubulin marker TUJ1 only after RGC axotomy (data not shown). Our data analysis using retinal wholemounts allowed us to negate this problem by focusing through these processes onto TUJ1-positive cells within the ganglion cell layer for counting non-melanopsin RGCs, but the possibility remains that other cells in that layer (i.e., normally TUJ1-negative displaced amacrine cells) may also have become TUJ1-positive as a result of retinal remodeling. Although we have no evidence to support this change, the calculation of ~12% surviving non-melanopsin RGCs after axotomy could be an overestimate. The observed TUJ1-positive endfeet mirror recent reports of Müller cell outgrowth associated with retinal reattachment (Fisher & Lewis, 2003 for review). Müller glia are known to provide neuroprotection in the form of growth factors and antioxidants after retinal injury (Garcia & Vecino, 2003). A possible explanation for the preferential survival of some melanopsin-containing RGCs is that they may be more sensitive to the trophic factors provided by activated Müller glia than non-melanopsin RGCs. From an evolutionary perspective of opsins, mouse melanopsin is most closely related to invertebrate opsins (Provencio, Jiang, De Grip, Hayes, & Rollag, 1998). Melanopsin within this class of

RGC may represent a marker for an evolutionarily distinct class of RGC that is more injury-resistant (or in the case of Müller glia-derived support, more responsive) than RGCs that are not intrinsically photosensitive.

If RGC death were to occur in the central retina more than in the peripheral retina, then the increased survival of melanopsin-containing RGCs reported here might be explained by differences in the retinal distribution of non-melanopsin (highest density in the central retina) and melanopsin RGCs (highest density in the peripheral retina). Villegaz-Pérez et al. (1993) examined distribution-related RGC death in the rat and found no difference in proportional RGC death one month after axotomy. Similarly, we found no such difference in the mouse when all RGCs were considered together or for just the melanopsin-positive subpopulation.

In neonatal rodents, supernumerary RGCs undergo naturally-occurring cell death and they are more susceptible than mature RGCs to axotomy-induced cell death (Bonfanti et al., 1996). Whether neonatal melanopsin-containing RGCs are as susceptible to injury as the rest of the RGC population and to what extent, if any, they undergo naturally-occurring cell death remains to be determined. Even without injury, ~2% of the entire RGC population dies monthly in the mouse resulting in a ~40% loss of ganglion cells over a lifetime (Neufeld & Gachie, 2003). A similar loss (~36%) of the melanopsin RGC population has been reported in old mice (Semo et al., 2003), suggesting that they are equally susceptible as the rest of the ganglion cell population to this type of long-term attrition.

The capacity for axotomized RGCs to regrow axons into a conducive environment has also been well documented. While only a small percentage of RGCs survive axotomy, an even smaller percentage of RGCs that survive the injury regrow axons into the permissive environment of a peripheral nerve graft (Cui & Harvey, 2000; Inoue et al., 2002; Inoue et al., 2000; Robinson & Madison, 2000; Thanos et al., 1993; Vidal-Sanz et al., 1987; Villegaz-Pérez et al., 1988; Watanabe et al., 1993). Again, characterizations of these regenerating ganglion cells have not identified a specific cell type (Robinson, 1995; Watanabe et al., 1993). In the rat, quantitative studies using a variety of identification methods and strains have shown up to 9.5% of RGCs can regrow axons into a graft (Robinson & Madison, 2000; Thanos et al., 1993; Vidal-Sanz et al., 1987; Villegaz-Pérez et al., 1988). Compared to the rat, less robust axonal regeneration is demonstrated here in the mouse, with an average of 232 RGCs (0.5% of the total population) per retina. This number of regenerating mouse RGCs is slightly higher than the 0.1–0.3% reported by other groups using the same model system (Cui & Harvey, 2000; Inoue et al., 2002; Inoue et al., 2000). Our previous work with fibrin glue in the rat suggested a positive influence of fibrin glue on regenerating RGCs compared to direct suture attachment of the peripheral nerve graft (Robinson & Madison, 2000). This difference in grafting technique may also explain our slightly increased number of regenerating mouse RGCs.

The lack of correlation shown here between the capacity of RGC survival and subsequent ability to regrow axons into a nerve graft has also been previously demonstrated in the mouse by overexpressing the anti-apoptotic gene Bcl-2 (Inoue et al., 2002). Overexpression of this gene rescued ~65% of the RGC population from axotomy-induced cell death, but the surviving RGCs showed even less robust axon regrowth into a nerve graft than wild-type controls (Inoue et al., 2002).

In the present study, no difference was seen in the number of viable RGCs remaining one month after axotomy with or without the placement of a nerve graft (Fig. 3A). At the same time point in the rat, Villegaz-Pérez et al. (1988) suggested that graft placement increased the number of presumed ganglion cells by ~62%. The lack of a graft effect in the mouse may be attributable to the more precise RGC identification methods used here and/or species differences between the two studies. Variation in the number and distribution of melanopsin-positive ganglion cells does appear to be species-specific. For the C57BL/6 mouse, we concur with Belenky et al. (2003) that these cells are distributed evenly between dorsal and ventral retina and add the observation of a peripheral bias in cell density compared with the central retina; a distribution that is maintained after axotomy (Fig. 2). In the Wistar rat however, these cells were preferentially found in the dorsal retina compared to the ventral retina (Hannibal et al., 2002), while the reverse distribution was recently described in the Mongolian gerbil (Fite, Blanchard, & Bengston, 2003).

Further work will be necessary to determine if the increased survival of melanopsin-containing RGCs is related to their unique photosensitive ability among RGCs. The enhanced survival of RGCs after Bcl-2 overexpression is attributed to the inhibition of downstream caspases in the apoptotic cascade (Inoue et al., 2002). It would be interesting to know if the same mechanisms apply to melanopsin-containing RGCs. Whatever the exact mechanism of enhanced survival is, it is clear that additional interventions will be necessary to enhance axonal regrowth in surviving RGCs.

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