Migration, Integration and Maturation of Photoreceptor Precursors Following Transplantation in the Mouse Retina

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Retinal degeneration leading to loss of photoreceptors is a major cause of untreatable blindness. Recent research has yielded definitive evidence for restoration of vision following the transplantation of rod photoreceptors in murine models of blindness, while advances in stem cell biology have enabled the generation of transplantable photoreceptors from embryonic stem cells. Importantly, the amount of visual function restored is dependent upon the number of photoreceptors that migrate correctly into the recipient retina. The developmental stage of the donor cells is important for their ability to migrate; they must be immature photoreceptor precursors. Little is known about how and when donor cell migration, integration, and maturation occurs. Here, we have performed a comprehensive histological analysis of the 6-week period following rod transplantation in mice. Donor cells migrate predominately as single entities during the first week undergoing a stereotyped sequence of morphological changes in their translocation from the site of transplantation, through the interphotoreceptor matrix and into the recipient retina. This includes initial polarization toward the outer nuclear layer (ONL), followed by formation of an apical attachment and rudimentary segment during migration into the ONL. Strikingly, acquisition of a nuclear architecture typical of mature rods was accelerated compared with normal development and a feature of migrating cells. Once within the ONL, precursors formed synaptic-like structures and outer segments in accordance with normal maturation. The restoration of visual function mediated by transplanted photoreceptors correlated with the later expression of rod α -transducin, achieving maximal function by 5 weeks.

Introduction

RETINAL DEGENERATIONS LEADING to the loss of photoreceptors are a major cause of untreatable blindness in the United Kingdom. Inherited retinal dystrophies affect 1 in 3,000 of the population, and age-related macular degeneration affects 1 in 10 people over 60 years of age. There are few effective clinical treatments and none are able to replace lost photoreceptor cells and restore visual function. By transplanting cells isolated from the developing retina at a stage when rod photoreceptors are immature, we have shown that the non-neurogenic, adult retinal environment is able to incorporate and maintain new photoreceptors for many months [1,2]. These new photoreceptors form synaptic connections with inner retinal neurons, generate outer segments, and are capable of restoring rod-mediated vision in models of retinal degeneration [3–6]. Importantly, we were able to show that these results are only achieved using postmitotic photoreceptor precursor cells but not progenitor cells or photoreceptors at other stages of development [1,3,7,8], a finding supported by others [9,10].

These findings have renewed interest in the potential to generate new photoreceptor precursors from stem cell sources [11–13]. Ground-breaking studies that utilize 3D culture systems have shown that embryonic stem cells (ESCs) self-organize and mimic the morphological development of the retina [14,15]. We have recently used a similar approach to generate transplantable ESC-derived rod precursor cells that integrate within the recipient retina and are virtually indistinguishable from wildtype rods [16].

These findings provide strong justification for continued investigation into photoreceptor-replacement strategies. A number of important features have also arisen from these investigations; the extent of visual function restored is critically dependent upon the number of photoreceptors that migrate correctly into the recipient retina [3,4] and the presence of nonintegrated donor cells in the subretinal space is detrimental [2], so achieving high efficiency of donor cell migration and integration is of paramount importance. Understanding how and when photoreceptor precursors migrate into and integrate within the recipient retina is central to our ability to improve integration efficiency. In the vast majority

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of published reports, recipient eyes have been examined 3 weeks postinjection, by which time migration and integration into recipient retinas is complete. Subsequently, the integrated cells can survive for up to 1 year postinjection [2]. However, the timing and modes of transplanted cell migration, integration, and maturation are unknown. Here, we present the first study that examines the period immediately following transplantation (48 h) until 6 weeks posttransplantation in order to determine the time course of these processes.

Materials and Methods

Animals

C57Bl/6J, *Nrl.GFP*^{+/+} (kind gift of A. Swaroop; [17]), and *Gnat1*^{-/-} (kind gift of J. Lem; [18]) mice were housed in University College London facilities and kept on a standard 12:12 light:dark cycle. The recipient animals were female and aged between 6 and 10 weeks of age at the time of transplantation. Donor mice were of either sex. Animal experiments were conducted according to the Society for Neuroscience policy on the use of animals in neuroscience research.

Retinal dissociation and transplantation

Postnatal day (P) 4–6 *Nrl.GFP* retinas were dissociated using a papain-based dissociation kit (Worthington Biochemical, Lorne Laboratories). Reagents were made up according to manufacturer's instructions and cells were prepared as described previously [1,3]. Briefly, retinas were dissociated in papain solution [20 U/mL; Earl's balanced salt solution (EBSS); antibiotic/antimycotic solution (1:100)] at 37°C/5% CO₂ for 45 min. Cells were gently triturated, passed through a 70-μm strainer, and centrifuged at 200g for 5 min. Pellets were resuspended in 10% ovomucoid inhibitor solution (OMI) containing DNase I (100 U/mL) and incubated for 5–10 min before layering over OMI and centrifuging at 100g for 5 min. The cell pellet was resuspended at 2 × 10⁷ cells/mL in EBSS/1% FCS before undergoing FACS sorting. *Nrl.GFP*⁺ cells were made up to a final concentration of 2 × 10⁵ live cells/μL in a solution containing DNase I (100 U/mL) in EBSS and kept on ice.

Subretinal transplants

Recipient mice were anesthetized with an intraperitoneal injection of 0.2 mL of a mixture of Dormitor (1 mg/mL medetomidine hydrochloride; Pfizer Pharmaceuticals), ketamine (100 mg/mL; Fort Dodge Animal Health), and sterile water in the ratio 5:3:42. Surgery was performed under direct visual control using an operating microscope. Cell suspensions (1 μL injection of 2 × 10⁵ live cells/μL) were administered as a single injection into the subretinal space in the superior ocular quadrant using a sterile 34-gauge hypodermic needle (Hamilton), as described previously [1,3]. Recipients were terminated at 48, 72, and 120 h, and 1, 2, 3, or 6 weeks after injection. Eyes were dissected to remove the cornea, iris, and lens and fixed in 4% (for cell counting) or 1% (for immunostaining) paraformaldehyde in phosphate-buffered saline (PBS) before cryoprotection in 10% sucrose and embedding in OCT

(TissueTek). Eye-cups were sectioned at 15–18 μm. Careful surgical notes were taken and eyes were omitted from further analysis if there was clear evidence of an injection having been administered intravitreally or if there was major reflux of the cell suspension at the time of injection (typically <5% of injections and defined as eyes with <250 GFP⁺ cells remaining in the subretinal space).

Immunohistochemistry

Frozen retinal sections were air-dried, rehydrated with PBS, and incubated in blocking solution (BS; 2% goat serum, 0.1% Triton X-100, and 1% bovine serum albumin in PBS) at room temperature (RT). Sections were incubated overnight at 4°C in primary antibody (monoclonal anti-CtB2 [ribeye] 1:100; BD Biosciences catalogue No. 612044 and polyclonal anti-rod α-transducin, 1:500; Santa Cruz Biotech SC-389) in BS and washed 3 × 10 min in PBS. Labeling was detected using the appropriate fluorescent Alexa 546-tagged secondary antibody (1: 500; Molecular Probes, Invitrogen) for 2 h. Sections were then washed 3 × 10 min and counterstained using Hoechst 33342 and mounted under coverslips.

TUNEL staining for apoptotic cell bodies

TUNEL staining using an apoptosis detection kit was performed according to the manufacturer's instructions (Chemicon Apoptosis Detection Kit, S7165; Millipore) and sections were counter-stained with Hoechst 33342 (2 μM).

Confocal microscopy

Images were taken on a Leica TCS SPE confocal microscope at ~0.5–0.7 μm intervals. Images show either single confocal sections or maximum projection images of xyz confocal stacks through retinal sections, ~15-μm thick. All image stacks included z-sections above and below the integrated cell(s) in which no GFP was seen, ensuring that the entirety of the cell(s) was included in all analyzed images.

Integrated cell counts

Counts of integrated cells were taken using an Observer Z.1 fluorescence microscope (Zeiss) in a blinded manner. The use of the *Nrl.GFP* mouse as a source of donor cells provides a genetic marker for the identification of rod photoreceptors [17,19,20]. Cells were considered “integrated” if the whole cell body was correctly located within the outer nuclear layer (ONL). Integrated cell number was typically determined by counting every third section throughout the whole eye and multiplied to give a total number per retina.

Quantification of proportions of transplanted *Nrl.GFP*⁺ rods adopting teardrop and unipolar morphologies in the interphotoreceptor matrix

Cells within the interphotoreceptor matrix (IPM) region were defined as those that had migrated or been dispersed away from the cell mass and were located in the IPM, which is readily visible as faint autofluorescence exhibited by retinal inner/outer segments (eg, Fig. 3Bi). Cell counts were carried out as described previously.

Quantification of the proportions of transplanted cells possessing rudimentary segments and synaptic bulb-like structures

The proportions of integrated *Nrl.GFP*+ve cells possessing a rudimentary segment or synaptic bulb-like structure were quantified by examination of confocal image z-stacks taken to encompass the region of transplantation in each retina and analyzed using Leica LAS AF software. Cells were classified as integrated if cell bodies were within the recipient ONL. Rudimentary segments were defined as *Nrl.GFP*+ve processes that extend apically from the outer limiting membrane (OLM) into the IPM. Synaptic bulb-like structures were defined as rounded *Nrl-GFP*+ve cellular specializations located within the outer plexiform layer (OPL). The numbers of rudimentary segments and synaptic bulb-like structures were expressed as proportions of the numbers of integrated cells. It should be noted that, due to differing angles of cryosectioning relative to the retina, some segments and synaptic specializations observed in a given confocal stack could not be readily attributed to a given cell body within the same stack. The percentages calculated therefore represent ratios of segment and synaptic specializations to integrated cells rather than exact proportions of cells possessing these structures.

*Quantification of the proportion of integrated *Nrl.GFP*+ve cells expressing rod α -transducin*

The percentages of integrated transplanted *Nrl.GFP*+ve donor cells expressing rod α -transducin were quantified in adult *Gnat1*^{-/-} recipients. Numbers of integrated transplanted cell bodies, *Nrl.GFP*+ve segments, and rod α -transducin-expressing segments were counted through the depth of confocal “z stacks,” taken as earlier. Numbers of rod α -transducin+ve segments were then expressed as a proportion of total *Nrl.GFP*+ve segments.

*Quantification of the proportion of integrated *Nrl.GFP*+ve cells expressing ribeye*

The number of *Nrl.GFP*+ve synaptic bulb-like structures and the proportion of these that colocalized with ribeye expression were counted through the depth of confocal “z-stacks,” taken as earlier, in wildtype recipients.

Macrophage grading

When examined at >3 weeks post-transplantation, the majority of transplanted retinæ display little or no sign of host reaction [2,3]. However, a minority (~15%) showed signs of inflammation and are associated with graft failure. When examined at earlier time-points we found that significantly higher numbers of retinæ exhibited signs of inflammation at 72 (50%) and 120 h (70%) post-transplantation ($N=7$ out of 14 at 72 h; $N=19$ out of 27 at 120 h). This indicates that transplantation is frequently associated with a transient inflammatory reaction, which peaks at 120 h post-transplantation but in the majority of cases resolves by 2–3 weeks post-transplantation. The previous grading system was inadequate for assessing early time-points. We therefore assessed macrophage recruitment using a revised version of the grading scale described previously [2]. Grade 1 demonstrated no to occasional macrophages in the subretinal

space. Grade 2a involved greater numbers of macrophages in the subretinal space, particularly around the cell mass and ramified macrophages in the retina around the site of transplantation, but with no obvious reduction in size of the donor cell mass. Grade 2b was as earlier, but with loss of donor cell mass and occasional amoeboid macrophages in cell mass. Grade 3 demonstrated many amoeboid macrophages in the subretinal space and the retina, combined with severe loss of donor cell mass, loss of underlying recipient ONL, and the presence of autofluorescent cell debris. Only those eyes in which the cell mass was absent/substantially reduced and macrophage presence was very high (Grade 2b and Grade 3) were omitted from the analysis. Those where the cell mass was clearly present but there were also signs of moderate inflammation (Grade 2a) were included. Confirmation of macrophage identity was performed using immunohistochemistry (data not shown). Representative retinal sections were immunostained for biotin-conjugated rat anti-CD68 (1:250; AbD Serotec) or rat anti-F4/80 (1:250; Abcam), and anti-rat and streptavidin Alexa Fluor 546 (1:500; Molecular Probes), respectively.

Nuclear architecture analysis

Nuclei were stained using Hoechst 33342 (2 μ M), enabling chromocenters to be visualized as nuclear regions of intense staining. Numbers of chromocenters were scored following published protocols [21] for cells that had integrated into the ONL and for cells that remained in the subretinal space in confocal xyz stacks of regions displaying moderate transplanted cell integration.

Optomotor response

Gnat1^{-/-} animals received dual transplants, one to the superior retina and one to the inferior retina, in one eye only and underwent optomotor testing, as described previously [3] using the Cerebral Mechanics Optomotry™ system. Contrast sensitivities of treated and control eyes were measured using a double-blind two-alternative forced choice procedure. Briefly, each mouse was placed on a pedestal located in the center of four inward-facing LCD computer monitor screens and was observed by an overhead infrared video camera with infrared light source. After acclimatization, contrast sensitivity was determined under scotopic conditions (<0.003 cds/m²). Contrast sensitivity was measured at a spatial frequency of 0.064 cycles/degree and at a rotation speed of 0.75 Hz, settings previously determined as optimal for maximal stimulation in wildtype mice under scotopic conditions [22]. A staircase paradigm program was used to measure independent contrast sensitivity thresholds in both eyes, defined as the % contrast yielding $\geq 70\%$ correct observer responses. Contrast sensitivity was defined as 100 divided by the lowest percent contrast yielding a threshold response. Animals were dark adapted overnight and at least three recordings were taken on the same day at -3, 3, 5, 7, 14, 21, 28, 35, and 42 days post-transplantation. Control measurements under photopic conditions before and after transplantation were included as positive controls for surgical trauma (data not shown). Animals were light adapted (60 cds/m²) for 30 min prior to testing and the initial stimulus was a 0.128 cycles/degree sinusoidal pattern at 100% contrast.

Statistics

All means are stated $\pm$ the standard deviation (SD), unless otherwise stated. N =number of eyes and n =number of cells examined, where appropriate. Statistical significance was assessed using Graphpad Prism 5 software, and applying paired t -test, 1- and 2-way ANOVA with Tukey's or Bonferroni's correction for multiple comparisons, or Kruskal–Wallis test with Dunn's correction, where appropriate. In figures, * P <0.05, ** P <0.01, and *** P <0.001.

Results

Transplanted postmitotic rod precursors migrate into the recipient retina within the first week post-transplantation

To determine the time course of transplanted donor cell migration into the recipient retina, transplanted wildtype adult mice were terminated at 48, 72, and 120 h, and 1, 2, and 3 weeks post-transplantation and the number of *Nrl.GFP*+ve cells located within the recipient ONL was quantified (Fig.

FIG. 1. Transplanted donor rod precursor cell integration into the recipient outer nuclear layer (ONL) occurs over a period of several days. **(A)** The number of *Nrl.GFP*+ve cells integrated within the recipient ONL increases with time post-transplantation, with the peak of integration occurring between 1 and 2 weeks post-transplantation. $\blacktriangle$ indicates individual data points. **(B)** % of TUNEL+ve, *Nrl.GFP*+ve cells within the ONL does not change between 48 h and 2 weeks post-transplantation. ** P <0.01, Kruskal–Wallis test, Dunn's correction.

1A). Substantial integration takes place during the first 4 days post-transplantation, with an average of 1072 ± 1420 ($N=14$), 3828 ± 5504 ($N=13$), and 4002 ± 6542 ($N=19$) integrated *Nrl.GFP*+ve donor cells per eye at 48, 72, and 120 h, respectively. By 1 week, recipient eyes had an average of 6878 ± 9011 ($N=15$) integrated *Nrl.GFP*+ve cells, a level that was maintained at 2 weeks (6492 ± 4571 ; $N=13$) post-transplantation. Integration at 2 and 3 weeks post-transplantation was significantly higher than at 48 h (P <0.01; Kruskal–Wallis test and Dunn's multiple-comparison correction). A slight decrease in the total number of integrated cells was observed at 3 weeks compared with 2 weeks (5909 ± 3125 ; $N=17$) although this was not statistically significant. Nonetheless, given that the donor cells are harvested during the first week of postnatal development, a period when apoptosis also occurs [23], we examined whether the decrease observed at 3 weeks reflected developmental cell death. To address this, TUNEL staining was performed on transplanted recipient eyes at 1, 2, and 3 weeks post-transplantation (Fig. 1B). No significant difference was observed in the percentage of integrated TUNEL+ve *Nrl.GFP*+ve cells at any of the time-points examined (1.8 ± 2.7 at 1 week, 5.1 ± 4.0 at 2 weeks, and 1.8 ± 1.9 at 3 weeks post-transplantation, $N=4, 4$, and 7 , respectively; P >0.18, ANOVA). Examples of eyes with strikingly high integration (more than 18,000 integrated *Nrl.GFP*+ve cells per eye) were observed at various time-points post-transplantation (Fig. 1A) although such results were observed infrequently (4 out of 74 eyes).

By 2 weeks post-transplantation, the minimum number of integrated cells per eye was markedly higher than at earlier time-points. None of the analyzed eyes had <1,500 integrated cells at 2 or 3 weeks post-transplantation, in contrast to those taken before 1 week, where examples of very low levels of integration (<50 cells per eye) were observed in 15%–30% of eyes (range 23–31,417, median 2715 at 1 week post-transplantation; range 1697–18,615, median 4674 at 2 weeks post-transplantation) (see Fig. 1A). In keeping with this there was a significant difference in variance of integrated cell counts across time (Bartlett's test for equal variances; P <0.001). Together, these results indicate that the peak of integration typically occurs by 1 week post-transplantation and is complete by 2 weeks, but there can be considerable variation in the timing of migration of individual transplanted cells into the recipient ONL.

Migration in the subretinal space and IPM

Studies of migration of immature neurons in the cortex have demonstrated that cells undergoing specific modes of migration adopt characteristic morphologies [24,25]. We therefore sought to characterize the morphologies exhibited by transplanted *Nrl.GFP*+ve rod precursors as they migrated away from the transplanted cell mass and into the recipient ONL over time. Transplanted donor cells undergo a transition from amorphous to rod-like morphology and a spatial translocation from the site of transplantation in the subretinal space, through the IPM to the edge of the ONL (Fig. 2) and into the recipient ONL (Fig. 3). The proposed sequences described later are based on examination by confocal microscopy of >50,000 cells from 74 eyes between 48 h and 2 weeks post-transplantation, with a minimum of 11 eyes per time-point.

FIG. 2. Transplanted donor rod precursor cells migrate away from the donor cell mass through the IPM by process extension and nuclear translocation. **(A)** Schematic of proposed sequence of events and the different morphologies (numbered) adopted by donor cells as they migrate away from the cell mass, through the IPM, toward the recipient neural retina. Note that Morphology 4a likely represents a population of donors that fail to cross the outer limiting membrane (OLM) into the recipient outer nuclear layer (ONL). **(B)** Representative examples of cells displaying the morphologies described in **(A)**. Arrowheads denote basally directed neuritis; open arrowheads denote apically directed processes. Scale bar = 10 μ m. **(C)** Cells progress through Morphologies 1–3 over time post-transplantation. **Ci**: high magnification, representative examples of Morphologies 1–3. **Cii, iii**: proportion of migrating cells displaying Morphologies 2 and 3 at different times post-transplantation. Scale bar = 5 μ m. **Cii, iii**: % of cells analyzed displaying Morphologies 2 and 3. Arrows indicate cell bodies. $**P < 0.01$. Color images available online at www.liebertpub.com/scd

FIG. 3. Transplanted donor cells migrate through the recipient ONL by process extension and nuclear translocation. **(A)** Schematic of proposed sequence of events and the different morphologies (numbered) adopted by donor cells as they migrate into the recipient ONL. **(B)** Examples of cells displaying the numbered morphologies described in **(A)**. Arrowheads denote basally directed neurites. Scale bar = 10 μ m. **(C)** % of integrated *Nrl.GFP*⁺ cells found in the outer, middle, and inner portions of the ONL over time. Integrating donor cells do not undergo bidirectional movement (apical-basal-apical) and Morphology 8a thus represents a group of cells that occupy the most basal aspect of the ONL. Arrows indicate cell bodies. Color images available online at www.liebertpub.com/scd

Nrl.GFP⁺ cells that had migrated away or become detached from the donor cell mass were observed to adopt a sequence of morphologies (Fig. 2) culminating in Morphology 5, where the cell body is located at the apical face of the ONL, with a rudimentary segment extending apically but no basal process (Fig. 2A). Initially, rounded cells (Morphology 1; Fig. 2Bi, ii) orientate relative to the OLM, first becoming teardrop shaped with the tip pointing basally (Morphology 2; Fig. 2Bi, iii). This process begins to extend toward the OLM and the cell becomes unipolar (Morphology 3; Fig. 2Bi, iv). The cell body translocates via the basally directed process toward the OLM. Bipolar intermediates were also observed (Morphology 3a), although only very rarely. Upon approach to the OLM, the cell body becomes the leading portion of the

cell (Morphology 4; Fig. 2Bv) and is the first to translocate across the OLM (Morphology 5; Fig. 2Bvi, vii), rather than cell entry being via a leading process. The apically directed, trailing process may act as a precursor for the inner segment (see Migration through the recipient ONL, below). Approach to the OLM was also correlated with a qualitative lowering in GFP fluorescence (Fig. 2Bvi; Morphologies 4 and 5).

In addition to the morphologies described just now, a small subset of cells located in the subretinal space were observed to adopt a unipolar morphology with a basal process extending all the way to the OLM (Morphology 4a; Fig. 2A, Bii). It is possible that the nucleus could translocate through this OLM-contacting process, to culminate in Morphology 5. However, we propose that this actually represents a population of cells that fail to integrate. First, Morphology 4a was observed as late as 6 weeks post-transplantation, indicating that a proportion of cells may become paused in this state rather than progressing through a sequence. In contrast, Morphology 4 was only ever observed shortly after transplantation (<1 week). Second, cells adopting Morphologies 4 and 5 undergo nuclear condensation, a characteristic of transplanted rod precursor integration (Fig. 2A; see below). In contrast, the cells exhibiting Morphology 4a retained immature uncondensed nuclei. Finally, Morphology 4a was largely restricted to cells integrating toward the anterior margins of the eye, while integration is seen throughout the retina.

Cells adopted teardrop and unipolar morphologies (Morphologies 2 and 3) at the earliest stages of migration away from the donor cell mass. To assess whether a progression through these morphologies takes place, we quantified the proportions of teardrop and unipolar cells at 48 h ($N=6$ eyes, $n=2210$ cells per eye [average]), 72 h ($N=5$ eyes, $n=2229$ cells per eye), 1 week ($N=4$ eyes, $n=3214$ cells per eye), and 2 weeks ($N=6$ eyes, $n=2458$ cells per eye) post-transplantation (Fig. 2Ci–iii). Proportions of both Morphology 2 (teardrop) and Morphology 3 (unipolar) peaked at 72 h post-transplantation, with an average of $7.2\% \pm 4.5\%$ of cells analyzed having Morphology 2 (teardrop) and $1.7\% \pm 1.0\%$ having Morphology 3 (unipolar) ($N=6$ retinas), compared with 48 h, when $4.9\% \pm 2.1\%$ of cells had adopted Morphology 2 and $1.4\% \pm 0.9\%$ had Morphology 3 ($N=6$). This was followed by a decrease to $3.9\% \pm 2.2\%$ and $1.1\% \pm 0.7\%$, respectively, at 1 week ($N=4$) and $1.5\% \pm 1.2\%$ and $1.0\% \pm 0.5\%$, respectively, at 2 weeks post-transplant ($N=5$). A one-way ANOVA with Bonferroni's multiple-comparison post-test revealed a statistically significant drop in the percentage of teardrop-shaped cells from 72 h to 2 weeks ($P < 0.05$), indicating that this morphology is likely to be transiently adopted by migrating cells. No statistically significant difference was observed in percentages of unipolar cells over time, although the trends observed were similar to those for teardrop-shaped cells. The vast majority of remaining cells exhibited Morphology 1 with no obvious orientation with respect to the ONL, and most likely reflect the donor cells that fail to integrate [3].

Migration through the recipient ONL

Having migrated to the edge of the OLM, transplanted rod precursors reach the apical face of the ONL and uni-

versally adopt Morphology 5. The proposed sequence of events that occur as cells migrate through the ONL is shown schematically in Fig. 3A. Typical examples of these are shown in Fig. 3B. Rather than a leading process, as often seen in migrating neuroblasts [24,26,27], the cell body is the first part of the transplanted photoreceptor cell to enter the ONL. On no occasion did we observe cell bodies located in the subretinal space with processes extending past the OLM. Cells entering the ONL (Morphology 5) already possess a trailing process, which resembles a rudimentary segment and is maintained within the IPM as the cell migrates through the ONL. Having entered the ONL, cells extend a basally directed neurite through which the cell body moves (Morphology 6; Fig. 3Bi, arrowheads). Morphology 7 (Fig. 3Bii) represents cells whose somata have translocated to the basal tip of this extended neurite. Further rounds of process extension and cell body movement may then occur (Morphology 8; Fig. 3Biii) before the cell body comes to its final location and the leading process reaches the OPL and terminates in a synaptic bouton-like structure in the OPL (Morphology 9; Fig. 3Biv).

Morphology 8a (Fig. 3Biv) represents cells in which the cell body has traversed the entire ONL and is located at the edge of the OPL. This could indicate a bidirectional movement of cell bodies, as has been reported for developing cones [28], occurring at the turning point in the direction of somal movement or, alternatively, these cells may have failed to initiate appropriate braking mechanisms to halt somal movement. The fact that this morphology was seen as late as 6 weeks post-transplantation would indicate that the latter is the case. However, to further examine whether cells migrate in a bidirectional or unidirectional manner, the proportion of *Nrl*.GFP+ve donor cells located in the outer, middle, and inner thirds of the recipient ONL was quantified over time (Fig. 3C; $N=5$ eyes, $n=341$ cells at 48 h; $N=4$, $n=397$ at 120 h; and $N=4$, $n=393$ at 2 weeks). We found neither a significant shift in cell positions over time nor any difference in the proportions of cells located in the inner, outer, and middle ONL at any time-point (2-way ANOVA with Bonferroni's correction). This suggests that cells move in a unidirectional manner from the OLM to become distributed throughout the depth of the ONL and that Morphology 8a is likely to represent a minority of cells that fail to terminate migration appropriately. Of note, on no occasion were *Nrl*.GFP+ve cell bodies found within the INL or GCL, similar to previous studies [1,3].

Alternative modes of integration

As well as the migration and integration of individual cells described previously, we found evidence for en masse entry of transplanted cells into the recipient retina through a merging of parts of the cell mass with the recipient ONL (Fig. 4A). This may result from a streaming of donor cells and/or engulfment of a portion of the donor cell mass by the recipient ONL as part of the recovery process after accidental trauma incurred by the retina during subretinal injections, similar to that seen in response to damage in the ONL in uveitis [29] (S.E. Barker, personal communication) and laser injury [30]. A qualitative assessment indicates that examples of en masse integration were seen in

FIG. 4. Donor cell integration can occur by mechanisms other than single-cell migration. (A) Example of regions of en masse integration of donor cells (arrows) at 72 h post-transplantation. Such events were more typically associated with ONL trauma. Scale bar = 25 μm. (B) Example of resolved ONL structure and incorporated donor cells at 3 weeks post-transplantation. Scale bar = 25 μm. (C) Examples of columns (i, ii) and clusters (ii, iii) of integrated cells. Columns typically have a single attachment point at OLM (arrowheads), while clusters may have multiple (arrows). Scale bar = 10 μm. SRS, sub-retinal space; INL, inner nuclear layer; GCL, ganglion cell layer. Color images available online at www.liebertpub.com/scd

approximately one-third of the transplants examined ($N=74$) across all time-points. Certain features are common to both mass and individually migrating transplant cells, including condensation of nuclei and a transient decrease in GFP fluorescence and that GFP+ve cells only ever contained a single nucleus. When integration is complete, such areas could be identified as clearly demarcated blocks of several hundred *Nrl.GFP*+ve cells integrated within the recipient ONL (Fig. 4B; see also [3,7]).

In addition to areas of en masse integration, integrated transplanted cells were frequently observed to form small columns of 2–5 cells (Fig. 4Ci, ii) as well as larger clusters of 5–20 cells (Fig. 4Cii, iii). Such events may arise as a result of small gaps or breaks in the recipient OLM since we have previously shown that donor cell integration is improved after transplantation into pharmacological or genetic models of OLM disruption, compared with wildtype [31,32]. Further inspection revealed that the apical attachments of cells in small columns are indeed frequently located at the same point (Fig. 4Ci, ii, arrowheads), while those in the larger clusters were not (Fig. 4Cii, ii, arrows). The presence of multiple apical attachments may indicate that breakages in the OLM are not the primary cause of clustering, which could instead be due to interactions between rods that facilitate their integration.

Transplanted rod precursor cells exhibit morphological features of segment and synapse formation in accordance with normal developmental timing

Normal photoreceptor maturation is influenced both by intrinsic and extrinsic cues. Given that donor cells are

transplanted into the fully mature retina, in which developmental relevant environmental cues would be expected to be absent, we sought to assess the differentiation and functional maturation of transplanted rods post-transplantation, compared with normal development. The *Nrl.GFP*+ve rod precursor cells used as donors were sorted from P4–6 retinas. Although these cells are postmitotic [17], the eyes from which they are derived are neither functionally nor structurally mature. Rod outer segment discs first begin to form around P6 [33] and occasional photoreceptor termini are visible by electron microscopy in the nascent OPL by P4 but synaptic maturation continues until P14 [34]. The time at which donor cells are taken for transplantation is, thus, from a period in which synaptic specializations and segments are beginning to be formed.

We first quantified the percentage of integrated cells possessing a segment and/or synaptic bouton-like structures at 48 h, 120 h, and 2 weeks post-transplantation (Fig. 5A). As early as 48 h post-transplantation, all *Nrl.GFP*+ve donor cells that correctly integrated within the recipient ONL possessed a rudimentary segment ($101\% \pm 8\%$ at 48 h, $N=6$ retinas, $n=552$ cells; $107\% \pm 28\%$ at 120 h, $N=5$, $n=875$; and $95\% \pm 8\%$ at 2 weeks, $N=4$, $n=660$). By contrast, only $36\% \pm 27\%$ of integrated cells had a synaptic bouton-like structure at 48 h post-transplantation, significantly fewer than the number with a rudimentary segment ($P<0.001$). This increased to $79\% \pm 10\%$ at 120 h and $74\% \pm 14\%$ at 2 weeks. This quantification supports two key features of the proposed progression of cell morphologies during migration and integration, namely, the formation of rudimentary segments before synapses and that all donor cells migrating through the recipient ONL possess such a segment.

FIG. 5. Transplanted rod precursors mature at a rate similar to that of developing rods with the exception of greatly accelerated nuclear architecture. **(A)** Almost all integrated *Nrl.GFP*⁺ve cells possess a rudimentary segment. Formation of synaptic bouton-like structures increases over time post-transplantation. **(B)** Ribeye **(i)** and rod α -transducin **(ii)** expression in the developing retina. Presumptive OPL (arrowheads) and OS (arrows) indicated. Scale bar = 50 μ m. **(C)** Expression of ribeye within the synaptic bouton-like structures of *Nrl.GFP*⁺ve integrated cells mirrored normal development and was present from the point of synaptic bouton formation onward. **(D)** Expression of rod α -transducin in the rudimentary segments of *Nrl.GFP*⁺ve integrated cells was delayed relative to normal development but increased over time post-transplantation. **(E)** Examples of ribeye **(i)** and rod α -transducin **(ii)** expression in integrated cells at 48 h and 2 weeks post-transplantation and **(iii)** rod α -transducin expression at 10 days in nonintegrated cells remaining in the subretinal space. Scale bars = 10 μ m **(ei)**; 2 μ m **(ei, inset)**; and 20 μ m **(eii, iii)**. **(F)** *Nrl.GFP*⁺ve cells in the SRS had many more chromocenters than those within the recipient ONL, which displayed mature nuclear architecture from 48 h post-transplantation. **(G)** Examples of multichromocenter nuclear architecture in donor cells in the SRS at 48 h post-injection **(i)** and condensed, single chromocenter nuclear architecture in integrated cells **(ii)**. Donor cells that remain in the SRS display a more mature nuclear architecture by 2 weeks post-transplantation **(iii)**. Many donor cells transplanted into an immature recipient display immature nuclear architecture 3 weeks post-transplantation **(iv)**. Scale bars = 10 μ m, insets $\times 2.5$ zoom. SRS, subretinal space; NBL, neuroblastic layer; INL, inner nuclear layer; GCL, ganglion cell layer; OS, outer segment; IS, inner segment; OPL, outer plexiform layer. *** $P < 0.001$. Color images available online at www.liebertpub.com/scd

Ribeye and rod α -transducin are expressed by transplanted cells at a time equivalent to and later than normal rod photoreceptor development, respectively

As markers of synapse and segment formation, we examined the expression of the ribbon synapse protein ribeye and the late-expressed phototransduction marker rod α -transducin, respectively. In the developing wildtype retinae, expression of ribeye was first detected at P4, presenting as a thin band of punctate staining at the inner edge of the prospective ONL, at the level of the developing OPL. Expression increased, becoming clearer between P6 and P11, and was seen as strong punctate staining throughout the OPL in the adult retina (Fig. 5Bi). Rod α -transducin was not detectable in the developing wildtype retina until P11, being expressed in both the cell bodies and nascent segments (Fig. 5Bii; arrowhead) at this time. In the adult retina, expression was restricted to the outer segments (Fig. 5Bii).

Given the discrete nature of ribeye staining, we were able to determine the proportion of synaptic bouton-like structures made by integrated *Nrl*.GFP+ve cells that expressed ribeye at each time-point post-transplantation. The percentage of *Nrl*.GFP+ve synaptic bulb-like structures that were ribeye +ve was determined in wildtype mice at a range of time-points post-transplantation (Fig. 5C). Ribeye was expressed in the correct anatomical location by 48 h post-transplantation and >95% of *Nrl*.GFP+ve synaptic bulb-like structures were ribeye +ve at all time-points examined (Fig. 5E). Thus, there appears to be little or no time delay between the establishment of a synaptic bulb-like structure and expression of ribeye within that structure.

Rod α -transducin is highly expressed in wildtype rod photoreceptors (see Fig. 5B) and so it was not possible to accurately quantify the proportion of *Nrl*.GFP+ve segments that express rod α -transducin at any given time post-transplantation, although examples of integrated rod α -transducin+ve *Nrl*.GFP+ve segments were occasionally observed as early as 120 h post-transplantation (data not shown). For this reason, and because we also wished to determine when transplanted donor cells first become functional, we used the *Gnat1*^{-/-} mouse model of stationary night blindness as a recipient. In this model, the rods lack the phototransduction protein rod α -transducin, and are nonfunctional and degenerate slowly [18]. We have previously shown that photoreceptor precursor cells transplanted into the *Gnat1*^{-/-} retina show equivalent levels of integration and segment and synaptic bouton-like structure formation as those transplanted into wildtype recipients [3,4]. Further, rod α -transducin expression is particularly pertinent to assessment of the onset of function, as this marker is one of the latest to be expressed during development of a range of rod-specific photo-transduction and outer segment structural proteins examined (rhodopsin, rod arrestin, recoverin, and peripherin-2; data not shown) [35].

Transplanted *Gnat1*^{-/-} mice were terminated at 48 h ($N=2$, $n=301$), 120 h ($N=5$, $n=668$), 1 week ($N=7$, $n=1219$), 10 days ($N=6$, $n=761$), 2 weeks ($N=6$, $n=614$), and 5–6 weeks ($N=4$, $n=743$) and the percentage of integrated rod α -transducin+ve *Nrl*.GFP+ve segments was determined. No rod α -transducin expression was seen before 120 h post transplantation, when just $1\% \pm 1\%$ of segments were rod α -transducin+ve. This increased to $6\% \pm 5\%$ at 1

week, $16\% \pm 9\%$ at 10 days, $51\% \pm 15\%$ at 2 weeks, and $95\% \pm 6\%$ at 5–6 weeks (Fig. 5D, E). Since rod α -transducin expression is robust in the majority of rods by P11 in development, it would thus appear that its expression is delayed following transplantation into the adult environment. In contrast, almost all transplanted donor cells remaining in the subretinal cell mass displayed robust rod α -transducin by 10 days post-transplantation (Fig. 5Eiii).

Maturation of rod photoreceptor nuclear architecture is greatly accelerated

During our analysis of transplanted cell morphologies, we noticed that integrated donor cells possess a very different nuclear architecture to the cells remaining in the subretinal space. This change most likely reflects developmental maturation, where rod photoreceptor nuclei typically have 13 chromocenters at birth and take at least a month to condense these into a single chromocenter [21]. We quantified this difference by determining the number of chromocenters per transplanted cell at 48 h, 72 h, and 2 weeks post-transplantation, both for *Nrl*.GFP+ve donor cells integrated within the ONL and for those that remained in the subretinal space (Fig. 5F, G). By 48 h post-transplantation, $96 \pm 3\%$ of *Nrl*.GFP+ve cells within the ONL had just 1 or 2 chromocenters per nucleus. Conversely, the majority of cells ($78 \pm 12\%$ at 48 h) in the subretinal space had three or more chromocenters per nucleus, a significant difference maintained at all time-points examined, although more cells remaining in the subretinal space displayed a mature nuclear architecture than at earlier time-points (Fig. 5Giv). Intriguingly, many cells that display Morphology 4, which are in the process of migrating through the IPM, also displayed a more mature nuclear architecture, with only one or two chromocenters (Fig. 5Giii). Thus, the acquisition of an adult rod nuclear architecture is highly accelerated in migrating donor cells relative to normal development [21,36,37] and those remaining in the subretinal space. Intriguingly, re-analysis of previous experiments in which P1–3 donors were transplanted into adults or recipients of the same developmental stage [1] revealed that even at 3 weeks post-transplantation, integrated cells transplanted into immature recipient retained a more immature nuclear architecture (Fig. 5Giv), with an average of 3.2 ± 1.1 chromocenters per cell ($N=3$, $n=15$) compared with when transplanted into adult recipients. This indicates that the recipient environment plays an important role in rod nuclear maturation.

Onset of visual function mediated by transplanted rod precursor cells correlates with rod α -transducin expression

To examine when transplanted rod photoreceptor precursors begin to contribute to visual function, we assessed optokinetic head-tracking behavior in *Gnat1*^{-/-} mice at multiple time-points post-transplantation (Fig. 6). The optokinetic response, an instinctive head-tracking behavior exhibited by rodents in response to a moving stimulus, is a sensitive and readily repeatable measure of visual function, which requires no prior training or anesthesia [3,4,38–40].

Optokinetic response was assessed –72 h, 72 h, 120 h, 1, 2, 3, 4, 5, and 6 weeks post-transplantation in 10 *Gnat1*^{-/-}

FIG. 6. Onset of visual function following rod photoreceptor transplantation, as assessed using optokinetic head-tracking. (A) Scatter plot of mean ($\pm$ SD) contrast sensitivity threshold for all animals plotted against days post-transplantation. $\diamond$ Outlier which was excluded from statistical analysis. (B) Mean contrast sensitivity threshold at last time-point assessed, plotted against number of integrated *Nrl.GFP*⁺ rod precursors. (C) Mean ($\pm$ SD) normalized contrast sensitivity for animals 3–10, plotted against days post-transplantation. Maximal function was reached between 28 and 42 days post-transplantation. Animals 1 and 2 were omitted from the analysis since these were only tested to 21 days post-transplantation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Color images available online at www.liebertpub.com/scd

mice, which received dual transplants in one eye only. Under scotopic conditions, head tracking was absent in all *Gnat1*^{-/-} mice prior to treatment ($N = 10$). Conversely, age-matched wildtype controls recorded under the same conditions had a mean contrast sensitivity threshold of 15.03 ± 0.62 ($N = 11$; also see [3]). No discernible response was observed until 1 week post-transplantation, when a small, quantifiable but nonsignificant contrast sensitivity threshold was detected upon presentation of the rotating stimulus to the treated eye only. Mean contrast sensitivity continued to improve in all animals and was significantly better than pretransplantation in the treated eyes at 2 ($P < 0.05$), 3 ($P < 0.01$), 4, 5, and 6 (all $P < 0.001$) weeks post-transplantation (Fig. 6A). The mean contrast sensitivity threshold was 1.01 ± 0.01 at baseline, compared with 1.03 ± 0.02 ($N = 7$) at 72 h post-transplantation, 1.03 ± 0.03 ($N = 2$) at 120 h, 1.10 ± 0.04 ($N = 8$) at 1 week, 1.19 ± 0.14 ($N = 10$) at 2 weeks, 1.22 ± 0.13 ($N = 7$) at 3 weeks, 1.42 ± 0.19 ($N = 8$) at 4 weeks, 1.38 ± 0.16 ($N = 6$) at 5 weeks, and 1.34 ± 0.18 ($N = 5$) at 6 weeks post-transplantation (Fig. 6A).

The amount of function that is restored post-transplantation is closely correlated with the number of transplanted photoreceptor precursors that correctly integrate within the recipient ONL [3,4]. We therefore determined the number of integrated cells present within the recipient ONL immediately after the last time-point tested for each animal. A positive, linear correlation was observed, similar to those described previously [3,4], where those animals with higher contrast sensitivity threshold (ie, better discrimination between similar shades of gray) had higher levels of integrated cells (Fig. 6B). Overall, the thresholds recorded here were a little lower than might have been expected on the basis of the number of cells integrated [3]. This may be due to the requirement to test repeatedly on any given day rather than on separate occasions, as in the previous study, which may lead to the animals becoming disinterested in the stimulus [40].

Given that integration and thus contrast sensitivity thresholds were not equivalent across all the animals tested, the contrast sensitivity threshold measurements acquired at each time-point were normalized against the maximum contrast sensitivity threshold value achieved for that individual animal. This analysis supports the absolute contrast sensitivity measurements, again indicating that function reaches $\sim 50\%$ of maximum at around 3 weeks and maximal function is attained around 5 weeks post-transplantation (Fig. 6C). These data correlate well with the proportion of integrated donor cells expressing rod α -transducin, where 50% of integrated cells were rod α -transducin +ve at 2 weeks post-transplantation but maximal expression was not observed until 5–6 weeks post-transplantation.

Discussion

Retinal repair by transplantation represents a promising therapeutic strategy for the treatment of photoreceptor degeneration. Post-mitotic photoreceptor precursors are capable of migrating into and integrating within the recipient diseased retina [1,9] and restoring vision [3,4]. Given that function is directly dependent upon the number of correctly integrated cells, it is important to understand how and why only precursor cells migrate into the recipient retina, while

other stages do not. Similarly, knowing the timing of maximal donor cell migration is of particular importance when designing strategies that transiently manipulate the recipient retina and/or donor cell behavior in order to promote donor cell migration and integration [31,32,41,42]. Here, we find that donor cell migration occurs within the first week post-transplantation, predominantly as single entities and involving a stereotyped sequence of morphologies, typical of locomoting cells elsewhere in the central nervous system (CNS). Transplanted donor cells mature in a manner consistent with normal rod photoreceptor development, with the exception of accelerated maturation of the nuclear architecture. Onset of visual function is tightly linked with the formation of outer segments and expression of the phototransduction protein rod α -transducin. Together these findings provide the basis from which to begin to determine the molecular mechanisms involved in the migration of immature photoreceptor precursors post-transplantation.

Migration of transplanted donor cells

While there is a wealth of information regarding the migration of immature neurons in the developing CNS, very little is known about how neurons migrate and engraft following transplantation. In the retina itself, we are just beginning to understand how retinal progenitor cells migrate [43–45] and how certain populations of retinal neurons, including ganglion cells, migrate immediately after terminal mitosis [46,47]. Transplanted rod precursors adopted a stereotyped sequence of morphologies that indicate different modes of migration at different times. The migration from the donor cell mass to the outer edge of the ONL is clearly an atypical migration path that does not occur in normal development. Initially, all cells are rounded and amorphous. A proportion of these then polarize and extend a process toward the ONL. This phase of migration takes place within the subretinal space and IPM, regions lacking in cell bodies, and may involve interactions with extracellular matrix (ECM) proteins. Neuroblasts undergoing tangential migration in the rostral migratory stream also migrate as individual cells. The migrating cells colocalize with the ECM protease matrix metalloprotease-9 (MMP-9), indicating that MMPs are involved in their migration through the ECM [48]. Interestingly, application of MMP activators has been shown to enhance the migration and incorporation of transplanted neural progenitor cells into the retina [49].

In the developing retina, immature cones first move basally, after birth at E14–18, before migrating apically between P7 and P9. While the apically directed migration appears to be an active process and requires LINC complex proteins [50,51], it is not yet clear whether the basally directed movements involve migration or whether the cell bodies are simply pushed basally by the birth of new cells at the apical surface. Similarly, nothing is known regarding whether and how immature rods migrate in development. The findings presented here, however, begin to indicate certain key features of rod migration. The rod precursors used in the current study are transplanted into mature, adult recipients, that they become distributed throughout the entire depth of the recipient ONL would suggest that active migration, as opposed to passive displacement, is involved. This would indicate an involvement of force-generating

mechanisms, such as microtubule-based motors or actomyosin contractility and/or other cell types, although further investigations will be required to determine this.

Many postmitotic cells undergo both radial and tangential dispersion [52,53]. However, rod migration in development has been proposed to take place exclusively in the radial direction [54]. Here, the ubiquitous presence of an apical attachment, as well as the integration of photoreceptors in columns, would preclude substantial tangential migration and suggest that transplanted rod migration is also predominantly in the radial direction. During the development of many neuronal cell types, including retinal ganglion cells and cortical neurons, the apical attachment is lost shortly after terminal mitosis and prior to the initiation of migration. However, in contrast to these cell types whose adult locations are remote from the site of terminal mitosis, photoreceptors maintain a connection to the apical surface of the retina and their migration may thus resemble that of neuroepithelial progenitors rather than postmitotic neurons. Indeed, a striking feature of transplanted rod precursor migration is the acquisition and maintenance of an apical attachment and rudimentary segment from the earliest stage of migration into the recipient ONL. The adherens junctions of the OLM, as well as providing adhesive contacts between photoreceptors and Muller cells, are a source of polarity signals that control morphogenesis and lamination [55]. Similar signals may be required for the integration of transplanted precursors into the appropriate retinal layer with correctly localized synapses and segments. Further, the Par3- α PKC-Par6 polarity complex of the adherens junctions is known to interact with small Rho-GTPases whose role in cytoskeletal remodeling and neuronal migration is well documented [56,57].

En masse integration of transplanted photoreceptors is likely to result in substantial displacement of endogenous cells in the recipient ONL in the surrounding area. It is unclear how this affects synaptic connectivity between both donor and endogenous photoreceptors and the cells of the INL. Considerable remodeling of the ONL has also been observed in the mammalian retina during recovery from structural damage [29]. Similar damage-repair mechanisms may permit restructuring of the recipient ONL/INL after en masse integration, but examination of the synaptic maps formed by transplanted donor cells will be of great interest.

Maturation of donor cells and onset of function

We, and others, have previously shown that donor cells must be postmitotic at the time of transplantation in order to migrate [1,3,9,16]. Nonetheless, a significant proportion of transplanted rod precursors fail to integrate, remaining in the cell mass. Such failures can be only partially mitigated by manipulating endogenous barriers such as the OLM [31]. The *Nrl*.GFP+ve population isolated by FACS comprises a heterogeneous population whose birth times range from approximately E12 to 4–6 h before donor animals are terminated [17]. The capacity to migrate may thus be restricted even within the postmitotic precursor population. Defining whether such a window exists is of crucial importance for improving transplantation efficiency, for the identification of accurate cell-surface markers [8,10], and for the isolation of a homogeneous population of integration-competent donor cells from the otherwise heterogeneous populations that arise from stem cell

differentiation protocols [11,13,16,58]. Here we found that cells that migrate into the recipient retina are universally rod α -transducin^{-ve}, despite the presence of rod α -transducin⁺ cells in the subretinal space for at least 6 weeks post-transplantation (indicating that survival of more mature cells is not an issue). Similarly, in a recent study using ESC-derived precursors, we found that those taken at days 26–29 of culture were rod α -transducin^{-ve} and significantly better at integrating than those taken from day 34 of culture, which were typically rod α -transducin⁺ [16]. These data suggest that rod α -transducin or a similarly temporally expressed gene might represent a marker for defining the end of the integration competent window, although further experiments are required to demonstrate this conclusively.

Since donor cells are transplanted into the adult eye, which presumably lacks developmental restricted environmental cues, we sought to determine whether this affected their differentiation. Upon entering the recipient ONL, donor rod precursors universally exhibited rudimentary segments. However, the elaboration of outer segments, as assessed by rod α -transducin expression, was delayed until 10–14 days post-transplantation (equivalent to P16–21 in normal development), compared with normal development, where rod α -transducin expression is robust by P11. Expression of ribeye was tightly correlated with synaptic bouton-like structure formation, with little or no delay between the establishment of a synaptic bulbs and expression of ribeye within that structure. In this respect, transplanted cells appear to recapitulate events observed in photoreceptor development, where synaptic ribbons are observed in the perinuclear region prior to synaptogenesis and in synaptic bulbs from the earliest stages of OPL formation [33,34].

We chose ribeye and rod α -transducin as markers of maturation since synapse formation and expression of components of the phototransduction pathway are key determinants in permitting visual function. Rod α -transducin is one of the last components of the phototransduction pathway to be expressed in development and is later than a number of proteins involved in outer segment formation, such as peripherin-2 [59]. Further studies at the ultrastructural level, similar to those performed by Ader and colleagues at 3 weeks post-transplantation [5], would be required to confirm the time-course of outer segment elaboration. Nonetheless, the onset of visual function, as assessed by optokinetic head tracking, correlates very well with the expression of rod α -transducin in the integrated donor cells. The optokinetic response was used as it is readily repeatable in the same animal without prior training. However, it will also be of interest to establish the onset of other measures of visual function at the retinal and cortical level, as examined previously at 3 weeks post-transplantation [3].

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