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Research Paper

Endogenous Mobilization of Bone-Marrow Cells Into the Murine Retina Induces Fusion-Mediated Reprogramming of Müller Glia Cells

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ABSTRACT

Müller glial cells (MGCs) represent the most plastic cell type found in the retina. Following injury, zebrafish and avian MGCs can efficiently re-enter the cell cycle, proliferate and generate new functional neurons. The regenerative potential of mammalian MGCs, however, is very limited. Here, we showed that *N*-methyl-D-aspartate (NMDA) damage stimulates murine MGCs to re-enter the cell cycle and de-differentiate back to a progenitor-like stage. These events are dependent on the recruitment of endogenous bone marrow cells (BMCs), which, in turn, is regulated by the stromal cell-derived factor 1 (SDF1)-C-X-C motif chemokine receptor type 4 (CXCR4) pathway. BMCs mobilized into the damaged retina can fuse with resident MGCs, and the resulting hybrids undergo reprogramming followed by re-differentiation into cells expressing markers of ganglion and amacrine neurons. Our findings constitute an important proof-of-principle that mammalian MGCs retain their regenerative potential, and that such potential can be activated via cell fusion with recruited BMCs. In this perspective, our study could contribute to the development of therapeutic strategies based on the enhancement of mammalian endogenous repair capabilities.

1. Introduction

Müller glia cells (MGCs) are the main glial cell type of the retina, where they fulfill typical functions of glia in the brain. In particular, they are responsible for the maintenance of retinal structure; they also actively participate in the regulation of ion homeostasis and in neurotransmitter recycling (Bringmann et al., 2009; Reichenbach and Bringmann, 2013; Newman and Reichenbach, 1996). Additionally, MGCs can act as adult stem cells, especially in early vertebrates such as teleost fish (Lenkowski and Raymond, 2014; Nagashima et al., 2013), but also in chicken (Fischer and Reh, 2001, 2003; Gallina et al., 2016). In these animals, MGCs respond to injury by reactivating expression of retinal stem cell genes. Like retinal progenitor cells, they re-enter the cell cycle, proliferate, and eventually re-differentiate into functional retinal neurons (Thummel et al., 2008; Fausett and Goldman, 2006; Fischer and Reh, 2001, 2002). Following pharmacological damage of either ganglion or photoreceptor cells, adult murine MGCs can also re-enter the cell cycle and contribute to neuronal regeneration (Karl et al., 2008; Wan et al., 2008; Ooto et al., 2004; Todd et al., 2015). However, the frequency of such events is extremely low. As a consequence, proliferative MGCs are unable to fully rescue retinal functionality (Karl and Reh, 2010).

The reasons underlying limited regenerative potential of MGCs in mammals have not been clearly elucidated yet. However, a number of studies have identified some of the key events that could contribute to the enhancement of endogenous mammalian regeneration. In particular, proliferation of murine MGCs can be stimulated by overexpression of Ascl1, a transcription factor essential during retinal development (Pollak et al., 2013). Pharmacological perturbation of specific signaling pathways can also stimulate the neural regenerative potential of mammalian MGCs; these include, for instance, Wnt/β-catenin, EGF, FGF, and insulin pathways (Karl et al., 2008; Osakada et al., 2007; Close et al., 2006; Todd et al., 2015; Ooto et al., 2004; Tao et al., 2016; Yao et al., 2016).

Furthermore, we have previously shown that MGCs can undergo de-differentiation and re-entry into the cell cycle following fusion with transplanted hematopoietic stem and progenitor cells (HSPCs) (Sanges et al., 2013, 2016). Importantly, fusion events are largely damage-dependent and result in the formation of tetraploid BM-derived

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hybrids. Interestingly, the reprogramming efficiency of newly generated hybrids is significantly increased when the Wnt/β-catenin pathway is activated in the HSPCs prior to transplantation (Sanges et al., 2013, 2016).

Bone-marrow cells (BMCs, including HSPCs) have been extensively shown to contribute to the repair of damaged tissues and organs (Wang et al., 2003; Orlic et al., 2001; Weimann et al., 2003b; Lagasse et al., 2000; Kale et al., 2003; Jackson et al., 2001). This can be attributed to their plasticity and their ability to change identity via either transdifferentiation or cell fusion-mediated events. In particular, endogenous BMCs have been shown to fuse with several cell types, including glia (Altarche-Xifro et al., 2016), neurons (Johansson et al., 2008; Alvarez-Dolado et al., 2003), hepatocytes (Vassilopoulos et al., 2003; Pedone et al., 2017), cardiomyocytes (Nygren et al., 2004), and gut cells (Rizvi et al., 2006; Davies et al., 2009).

Changes in cellular identity are intimately coupled to the recruitment of BMCs to the site of damage. Several cytokines have been proposed to facilitate BMC recruitment. Among these, stromal-derived factor 1 (SDF1, also known as C-X-C motif chemokine 12 - CXCL12) seems to play a crucially important role (Cheng and Qin, 2012; Nervi et al., 2006; Aiuti et al., 1997).

SDF1 specifically binds to the C-X-C motif chemokine receptor type 4 (CXCR4). The SDF1/CXCR4 signaling axis has already been extensively implicated in the regulation of BMC homing and mobilization (Nervi et al., 2006; Kollet et al., 2003). Robust mobilization of CXCR4-expressing HSPCs in the peripheral blood occurs when plasma levels of SDF1 are increased (Hattori et al., 2001). Additionally, SDF1 is one of the major players in the regulation of HSPC trans-endothelial migration (Aiuti et al., 1997). Interestingly, SDF1 expression is also strongly up-regulated following damage in several tissues, including the liver, the brain, and the retina (Mocco et al., 2014; Lima E Silva et al., 2007; Pedone et al., 2017).

We decided to use a model of *N*-methyl-D-aspartate (NMDA)-induced excitotoxicity, characterized by apoptosis of ganglion and amacrine neurons (Lucas and Newhouse, 1957; Siliprandi et al., 1992; Sucher et al., 1997). We investigated damage-induced recruitment of endogenous BMCs into the retina, showing its dependence on the activity of the SDF1/CXCR4 pathway. We also showed that NMDA-damage could stimulate MGCs to dedifferentiate, and that such dedifferentiation was the result of fusion events involving MGCs and endogenously mobilized BMCs. Resulting hybrids were found to contribute to the replacement of damaged neurons, generating calretinin-positive ganglion and amacrine cells.

Overall, our data suggests that cell fusion is one of the mechanisms underlying MGC plasticity. MGC ability to de-differentiate and proliferate is strongly dependent on the recruitment of their fusion partners, endogenous BMCs. Migration of BMCs is, in turn, regulated by the SDF1/CXCR4 signaling axis. As a result, modulation of the SDF1/CXCR4 pathway can affect MGC plasticity, and the extent to which MGCs can contribute to regeneration of damaged retinal tissue.

2. Materials and Methods

2.1. Animal Care and Treatment

Mice were maintained under a 12-h light/dark cycle with access to food and water *ad libitum*, in accordance with the Ethical Committee for Animal Experimentation (CEEA) of the Government of Catalonia. The CEEA of the Parc de Recerca Biomèdica de Barcelona (PRBB, Spain) reviewed and approved all animal procedures. Additionally, procedures and experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Kilkenny et al., 2011).

Male and female animals between 8 and 12 weeks were used for the study. They were assigned randomly to the various treatment groups. A minimum of three mice per treatment group was used. General anesthesia was induced when needed with intraperitoneal injection of ketamine (70 mg/kg) and medetomidine (10 mg/kg). Anesthesia was reversed with atipamezole (2 mg/kg). At endpoints, mice were euthanized using CO_2.

2.2. Transgenic Mouse Lines

We used the following transgenic mouse lines: Vav-Cre (Stadtfeld and Graf, 2005); CAG-RFP (Long et al., 2005); GFAP-Cre (Zhuo et al., 2001); Calr-Cre (Taniguchi et al., 2011); R26Y (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J) (Srinivas et al., 2001).

Additionally, we generated GFAP-Cre/R26Y and Calr-Cre/R26Y mice. These strains were generated by crossing R26Y mice with either GFAP-Cre or Calr-Cre ones. As a result, mice possessed both the Rosa 26-LoxP-stop-LoxP-YFP [R26Y] transgene and either the GFAP-Cre or the Calr-Cre transgene.

2.3. Sub-lethal Irradiation and Bone Marrow Transplantation

Bone marrow (BM) transplantation was carried out as previously reported (Fazel et al., 2006). Briefly, 8–12 weeks-old mice received total body irradiation with 9 Gγ (double dose of 4,5 Gγ) six weeks prior to retinal damage and/or drug treatment. 3–4 h after irradiation, they received an intravenous injection of 1×10^7 BM cells from young donor mice. Total BM cells were obtained by gently flushing femurs and tibias with PBS.

R26Y/$BM^{CRE\text{-}RFP}$ chimeric animals were generated by replacing the BM of transgenic mice carrying the R26Y allele with the BM of CAG-RFP/Vav-Cre donor mice.

GFAP-Cre/BM^{R26Y} chimeric mice were generated by replacing the BM of GFAP-Cre mice with the BM of donor transgenic mice carrying the R26Y allele.

Calr-Cre/R26Y/BM^{RFP} mice were generated by replacing the BM of Calr-Cre/R26Y mice with that of donor CAG-RFP mice.

2.4. Retinal Damage and Drug Treatment

Mice were anaesthetized and intravitreally injected with 2 μl of either NMDA (20 mmol/μl; Sigma) or PBS, as a control. Briefly, a 30-G needle was used to carefully make a small incision at the upper temporal ora serrata. The 33-gauge needle of a Hamilton's syringe was then inserted into the incision to inject PBS or NMDA into the vitreous. The needle was left in place for 10 s before being retracted to avoid reflux. Eye samples were isolated 24 h (24 hpi), 4 days (4 dpi) or 3 weeks (3 wpi) post-injection.

To investigate the effects of SDF1/CXCR4 signaling modulation, we intravitreally injected 1 μl of SDF1 (50 ng/μl, Sigma) immediately after PBS or NMDA treatment. Control eyes were injected with an equivalent volume of PBS. To block migration of bone marrow cells, mice received intraperitoneal injections of the CXCR4 antagonist AMD3100 (1 mg/kg, Sigma A5602). AMD3100 injections were performed every 24 h and for a maximum of three consecutive days, starting the same day of the NMDA treatment (a single injection for mice sacrificed at 24 hpi, and three injections in total for mice sacrificed either 4 dpi or 3 wpi).

2.5. FACS Sorting of Müller Glia Cells and Hybrids for Gene Expression Analysis

For FACS analysis and sorting, retinae were dissected from the enucleated eyes and disaggregated in trypsin for 20–30 min at 37 °C. Retinal samples were then mechanically triturated, filtered, pelleted, and resuspended in PBS supplemented with 2% fetal bovine serum (FBS). A solution of 6-diamidino-2-phenylindole (DAPI, Sigma 10236276) was also added to exclude dead cells from the analysis (5 mg/ml, used 1:1′000). Flow cytometry analysis was performed in a LSR Fortessa (Becton

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