Molecular and Cellular Neuroscience 71 (2016) 1–12

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Novel axon projection after stress and degeneration in the *Dscam* mutant retina

K.A. Fernandes [a], S.J. Bloomsburg [b], C.J. Miller [b], S.A. Billingslea [b], M.M. Merrill [b], R.W. Burgess [c], R.T. Libby [a], P.G. Fuerst [b,d,*]

[a] Flaum Eye Institute, University of Rochester Medical Center, Rochester, NY 14642, USA
[b] University of Idaho, Department of Biological Sciences, Moscow, ID 83844, USA
[c] The Jackson Laboratory, Bar Harbor, ME 04609, USA
[d] WWAMI Medical Education Program, Moscow, ID 83844, USA

ARTICLE INFO

Article history:
Received 31 July 2015
Revised 30 November 2015
Accepted 7 December 2015
Available online 10 December 2015

Keywords:
Cell death
Regeneration
Optic nerve
Axon crush
Plasticity

ABSTRACT

The Down syndrome cell adhesion molecule gene (*Dscam*) is required for normal dendrite patterning and promotes developmental cell death in the mouse retina. Loss-of-function studies indicate that *Dscam* is required for refinement of retinal ganglion cell (RGC) axons in the lateral geniculate nucleus, and in this study we report and describe a requirement for *Dscam* in the maintenance of RGC axon projections within the retina. Mouse *Dscam* loss of function phenotypes related to retinal ganglion cell axon outgrowth and targeting have not been previously reported, despite the abundance of axon phenotypes reported in *Drosophila* Dscam1 loss and gain of function models. Analysis of the *Dscam* deficient retina was performed by immunohistochemistry and Western blot analysis during postnatal development of the retina. Conditional targeting of *Dscam* and *Jun* was performed to identify factors underlying axon-remodeling phenotypes. A subset of RGC axons were observed to project and branch extensively within the *Dscam* mutant retina after eye opening. Axon remodeling was preceded by histological signs of RGC stress. These included neurofilament accumulation, axon swelling, axon blebbing and activation of JUN, JNK and AKT. Novel and extensive projection of RGC axons within the retina was observed after upregulation of these markers, and novel axon projections were maintained to at least one year of age. Further analysis of retinas in which *Dscam* was conditionally targeted with *Brn3b* or *Pax6α* Cre indicated that axon stress and remodeling could occur in the absence of hydrocephalus, which frequently occurs in *Dscam* mutant mice. Analysis of mice mutant for the cell death gene *Bax*, which executes much of *Dscam* dependent cell death, identified a similar axon misprojection phenotype. Deleting *Jun* and *Dscam* resulted in increased axon remodeling compared to *Dscam* or *Bax* mutants. Retinal ganglion cells have a very limited capacity to regenerate after damage in the adult retina, compared to the extensive projections made in the embryo. In this study we find that DSCAM and JUN limit ectopic growth of RGC axons, thereby identifying these proteins as targets for promoting axon regeneration and reconnection.

1. Introduction

Damage to retinal ganglion cells (RGCs) and their axons results in visual impairment and blindness, but only limited progress has been made in cell therapy approaches to replace lost RGCs or in stimulating regrowth of RGC axons. Analysis of signaling events in glaucoma mouse models and optic nerve crush, a commonly used acute model for glaucoma (Allcutt et al., 1984a,b), has identified changes in the activation status of JUN, JNK, DLK and AKT as mediators of subsequent cell death and as potential mediators of axon regrowth (Duan et al., 2015; Koistinaho et al., 1993; Watkins et al., 2013). An outstanding question is the nature of the pathways that are activated by these stresses. For example JUN and JNK signaling is involved in cell stress, remodeling and regeneration, and cell death (Fernandes et al., 2012; Vander and Levkovitch-Verbin, 2012; Yoshida et al., 2002), suggesting that upregulation of these pathways may serve to activate an axon remodeling and regenerative response, followed by cell death if this process fails.

In this study we examine RGC stress pathways and maintenance of the RGC axon in the *Dscam* mutant retina. The Down syndrome cell adhesion molecule (DSCAM) protein is a homophilic cell adhesion molecule (Agarwala et al., 2000; Yamakawa et al., 1998) that also serves as a receptor for the axon guidance molecule netrin (Liu et al., 2009; Ly et al., 2008). The *Dscam* gene is required for several features of normal retinal development including: developmental cell death (Fuerst et al., 2008), lamination (Yamagata and Sanes, 2008), dendrite-refinement

* Corresponding author at: 145 Life Science South, Department of Biological Sciences, University of Idaho, Moscow, ID 83844, USA.
E-mail address: fuerst@uidaho.edu (P.G. Fuerst).

http://dx.doi.org/10.1016/j.mcn.2015.12.003
1044-7431/

(Li et al., 2015), and to prevent clustering of cell bodies and dendrites (Fuerst et al., 2009). Axons of *Dscam* mutant RGCs project normally to the optic nerve head (Fuerst et al., 2009), suggesting that alternative netrin receptors guide RGC axons out of the retina (Deiner et al., 1997). Defects in refinement and segregation of RGC axon terminals in the *Dscam* mutant brain have been described, indicating that *Dscam* plays a role in both axon and dendrite organization in retinal neurons (Blank et al., 2011).

Here we report that activation of the stress pathway proteins JUN, JNK and AKT occurs in the postnatal *Dscam* mutant retina, but with a different outcome following stress and axon degeneration compared to other RGC stress and damage models: remodeling and projection of new RGC axons. Axons target the optic disc but fail to exit, and project extensively through the *Dscam* mutant retina.

2. Methods

2.1. Animal care and handling

Mice were housed on a 12-hour light dark cycle and fed ad libitum. Mice taken for study were anesthetized with tribromoethanol and perfused with phosphate buffered saline, pH 7.4. Retinas were hemisected and fixed in 4% buffered PFA at room temperature for 2–4 h. All procedures were performed in accordance with the respective University of Idaho, Jackson Laboratory or Rochester Animal Care and Use Committees.

2.2. Mutant and transgenic mouse lines and genotyping

$Dscam^{del17}$, $Dscam^{2J}$, $Dscam^{F}$ and $Dscam^{FD}$ mice (truncation, protein null, conditional and germ line-targeted conditional, respectively) were genotyped as previously described (Fuerst et al., 2008, 2010, 2012). $Dscam^{del17}$, $Dscam^{2J}$ and $Dscam^{FD}$ mice are collectively referred to as $Dscam^{LOF}$ (loss of function) unless otherwise noted. YFPH mice were acquired from The Jackson Laboratory and genotyped according to JAX protocols. *Pax6α*-Cre mice (generous gift of Dr. Gruss) and *Brn3b*-Cre mice (generous gift of Dr. Van Bennet) were genotyped by PCR for the presence of the Cre gene. The floxed allele of *Jun* was a generous gift of Dr. Behrens (Behrens et al., 2002). Ai9 and tdtomato/GFP reporter mice were acquired from The Jackson Laboratory and genotyped according to JAX protocols.

2.3. Retina sectioning

Fixed retinas were sucrose sunk in 30% buffered sucrose for 1 h, followed by an additional 30 min in 50% buffered sucrose and 50% optimal cutting temperature (OCT) reagent (Tekura Inc.). Retinas were frozen in 100% OCT reagent and cut at 10 μm on a cryostat.

2.3.1. Controlled optic nerve crush (CONC)

Optic nerve injury was performed as previously described (Harder and Libby, 2011; Libby et al., 2005). In brief, optic nerves were crushed for approximately 5 s just behind the eye using self-closing forceps (Roboz RS-5027). Eyes were harvested at 1 day following CONC to assess JUN upregulation.

2.4. Immunohistochemistry

Tissues were incubated in a blocking solution consisting of 7.5% normal donkey serum, 0.1% triton x-100 (sections) or 0.4% triton x-100 (whole retinas) and 0.02% sodium azide, diluted in phosphate buffered pH 7.4 saline (PBS). Antibodies were diluted in blocking solution. Sections were incubated with primary antibody for 2 h (at room temperature) or overnight (at 4 °C). Sections were washed 2× for 15 min in PBS. Secondary antibodies, which were diluted in blocking solution, were applied for 2 h at room temperature, followed by three ten minute washes in PBS. The second wash was supplemented with DAPI reagent to stain nuclei at a dilution of 1:50,000 of a 1 mg/ml stock. Whole retinas were stained in a similar fashion except they were blocked for 1 h and antibodies were incubated for four days (primary) and three days (secondary), and washes were carried out for 2 h at 4 °C. Tissues were mounted on slides with 80% glycerol, in 1× PBS, containing 0.02% sodium azide, and imaged on an Olympus IX81 inverted microscope.

2.5. Western blot analysis

Retinas were homogenized in t-per buffer (Thermo Scientific) supplemented with protease inhibitors and EDTA (Thermo Scientific). Protein concentrations were determined using the Bradford analysis. Polyacrylamide gel electrophoresis and Western blotting were performed as described previously (Schramm et al., 2012). Band densities were compared using image J software.

2.6. Dextran and cholera toxin injection

Cy3-conjugated dextran (10,000 kDA; Life Technologies) was injected into the superior colliculus of mice at postnatal day 8 (P8). Retinas were collected for study at P16. 2 μl of cholera toxin conjugated to alexa-568 (Life Technologies) was injected into a single eye of mice between the ages of P28 and six months. Mice were taken for study two days later.

2.7. Nearest neighbor analysis

Nearest neighbor analysis was performed as previously described using the program winDRP (de Andrade et al., 2014; Keeley and Reese, 2014; Rockhill et al., 2000; Wassle and Riemann, 1978). Soma size was set at 10 μm, with 25 10 μm bins selected for analysis.

2.8. Antibodies used

The following antibodies were used in this study for immunohistochemistry: JUN (Abcam, catalog number ab40766: 1:250), β-III tubulin (the antigen recognized by the TUJ1 antibody) (Sigma Aldrich, catalog number: SDL3D10: 1:1000), neurofilament (Developmental Studies Hybridoma Bank, catalog number 2H3: 1:50), and AP2α (Developmental Studies Hybridoma Bank, catalog number 3B5 concentrate: 1:50). Fluorescent secondary antibodies directed to the appropriate species were used (Jackson Immuno Research, 1:500).

The following antibodies were used for Western blotting: pJUN (Cell Signaling Technology, catalog number D47G9: 1:1000), JUN (Cell Signaling Technology, catalog number 9165: 1:1000), JNKP (Cell Signaling Technology, catalog number 9251: 1:500), AKT473 (Cell Signaling Technology, catalog number 4060: 1:1000), AKT (Cell Signaling Technology, catalog number 4691: 1:1000), GADPH (Synaptic Systems, catalog number 247 002: 1:1000) and goat anti-rabbit:HRP (Cell Signaling Technology, catalog number 7074: 1:25,000).

3. Results

3.1. Postnatal axon remodeling phenotypes in Dscam mutant mice

The axons of RGCs project across the surface of the retina to the optic disc, where they exit the eye and target loci in the rest of the brain. RGC axons projected out of the $Dscam^{LOF}$ retina normally during development (Fuerst et al., 2009); however, RGC axons projecting aberrantly within the adult $Dscam^{LOF}$ retina were subsequently observed (Fig. 1 A and B). These axons branch multiple times within the retina (Fig. 1 C) and course through the synaptic layers of the retina (Fig. 1 D). The developmental timing of this phenotype was assayed.