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Research Article

Muscle A-Kinase Anchoring Protein-α is an Injury-Specific Signaling Scaffold Required for Neurotrophic- and Cyclic Adenosine Monophosphate-Mediated Survival

Yan Wang [a,b,1], Evan G. Cameron [b,c,1], Jinliang Li [d,e], Travis L. Stiles [b], Michael D. Kritzer [d,e], Rahul Lodhavia [b], Jonathan Hertz [a], Tu Nguyen [b], Michael S. Kapiloff [d,e,*,1], Jeffrey L. Goldberg [a,b,c,**,1]

[a] Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, FL 33136, United States
[b] Department of Ophthalmology, University of California, San Diego, CA 92093, United States
[c] Byers Eye Institute, Stanford University, Palo Alto, CA 94303, United States
[d] Department of Pediatrics, Interdisciplinary Stem Cell Institute, Leonard M. Miller School of Medicine, University of Miami, Miami, FL, 33136, United States
[e] Department of Medicine, Interdisciplinary Stem Cell Institute, Leonard M. Miller School of Medicine, University of Miami, Miami, FL, 33136, United States

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ABSTRACT

Neurotrophic factor and cAMP-dependent signaling promote the survival and neurite outgrowth of retinal ganglion cells (RGCs) after injury. However, the mechanisms conferring neuroprotection and neuroregeneration downstream to these signals are unclear. We now reveal that the scaffold protein muscle A-kinase anchoring protein-α (mAKAPα) is required for the survival and axon growth of cultured primary RGCs. Although genetic deletion of mAKAPα early in prenatal RGC development did not affect RGC survival into adulthood, nor promoted the death of RGCs in the uninjured adult retina, loss of mAKAPα in the adult increased RGC death after optic nerve crush. Importantly, mAKAPα was required for the neuroprotective effects of brain-derived neurotrophic factor and cyclic adenosine-monophosphate (cAMP) after injury. These results identify mAKAPα as a scaffold for signaling in the stressed neuron that is required for RGC neuroprotection after optic nerve injury.

1. Introduction

Neuronal death following injury, including due to trauma or ischemia, remains an important source of long term disability with few adequate therapies. In the eye, blindness can result from the death of retinal ganglion cells (RGCs) that transmit visual information from the retina via the optic nerve to the lateral geniculate, pretectal, and superchiasmatic nuclei of the brain. Failure of central nervous system (CNS) neurons, including RGCs, to survive and regenerate their axons after injury results in part from a lack of adequate trophic signaling. RGC death can be delayed by application of exogenous neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin-4 (NT-4), fibroblast growth factor (FGF), and glial-derived neurotrophic factor (GDNF) (Mo et al., 2002, Unoki and LaVail, 1994, Wang et al., 2013). This trophic signaling can be enhanced by electrical stimulation or elevation of intracellular cyclic adenosine-monophosphate (cAMP) levels (Goldberg and Barres, 2000, Goldberg et al., 2002, Corredor et al., 2012). How cAMP potentiates the neuronal response to trophic factors is not well understood, although inhibiting cAMP-dependent protein kinase (PKA), an enzyme subject to tight spatial and temporal regulation in cells through its localization by the diverse family of "AKAP" scaffold proteins (Scott et al., 2013), blocks the positive effects of electrical activity and cAMP on neuronal survival and axon growth (Goldberg et al., 2002).

Muscle A-kinase anchoring protein (mAKAP; also known as AKAP6) is a 255 kDa scaffold protein localized to the nuclear envelope of neurons and striated myocytes and that binds PKA as well as a large number of signaling enzymes implicated in stress responses (Passariello et al., 2015). mAKAP also binds the cAMP target Epac1, adenylyl cyclases (types II and V), and the cAMP-specific phosphodiesterase 4D3. Because these enzymes participate in negative and positive feedback, cAMP levels are predicted to be tightly regulated around mAKAP "signalosomes," providing local control over the phosphorylation of relevant PKA substrates. Differential PKA localization by scaffolds like mAKAP is likely to be especially important in cell types like neurons where subcellular domains, including cell bodies, axons and dendrites, are often separated across great distances. In neurons, other AKAPs such as AKAP79 function in synaptic signaling (Weisenhaus et al., 2010), but it is unknown whether any

* Correspondence to: M.S. Kapiloff, Departments of Pediatrics and Medicine, Interdisciplinary Stem Cell Institute, Leonard M. Miller School of Medicine, University of Miami, Miami, FL 33136, United States.
** Correspondence to: J.L. Goldberg, Byers Eye Institute at Stanford University, 2452 Watson Ct, Palo Alto, CA 94303, United States.
E-mail addresses: mkapiloff@med.miami.edu (M.S. Kapiloff), jlgoldbe@stanford.edu (J.L. Goldberg).
[1] These authors are designated as co-first or co-senior authors.

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PKA scaffolds have a role in neuronal survival or axon growth. Neurons express the longer α isoform of mAKAP, mAKAPα, which contains an amino-terminal sequence with additional binding sites, e.g. for 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Michel et al., 2005).

The function of neuronal mAKAPα has not been characterized, albeit deletion of the exon encoding the mAKAPα-specific N-terminus resulted in a failure-to-thrive phenotype (Michel et al., 2005). Besides binding enzymes related to cAMP signaling, mAKAPα also binds in neurons the mitogen-activated protein kinases MEK5 and ERK5 that are known to be important for neuronal survival in response to neurotrophins (Michel et al., 2005, Dodge-Kafka et al., 2005, Wang et al., 2006, Watson et al., 2001). Given that mAKAPα can coordinate cAMP and ERK5 signaling, we hypothesized that mAKAPα might be involved in survival or axon growth signaling in neurons, which require both of these signaling pathways to optimally promote these functions in RGCs(Goldberg et al., 2002). We now present evidence that mAKAPα is expressed in RGCs but discovered in fact that mAKAPα does not regulate RGC survival during normal development or in the absence of injury, but rather mediates stress-specific signaling of survival and axon growth in vitro and survival in vivo after optic nerve injury.

2. Materials & Methods

2.1. Animals

All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of California San Diego and University of Miami Institutional Animal Care and Use Committees. All animals were randomly assigned to experimental groups and littermates were used as controls.

For in vitro experiments, litters of Sprague Dawley rats (Harlan Laboratories, Inc., Allen Park, MI) were used at postnatal day (P) 2–4 for isolation of RGCs. For in vivo experiments, 20 female and 20 male (2 months old) wildtype and $mAKAP^{fl/fl}$ C57BL/6 mice were used (Kritzer et al., 2014b). Cre recombination of the conditional mAKAP allele containing LoxP sites surrounding exon 9 results in exon 9 deletion, frame-shift and premature termination of translation within exon 10, 5′ of the exons encoding the docking sites for many relevant mAKAPα-binding partners, including nesprin-1α (conferring nuclear envelope localization), calcineurin, PDE4D3 and PKA (Li et al., 2010, Pare et al., 2005, Kapiloff et al., 1999). Note that no residual mAKAP N-terminal protein fragment was detected following knock-out (Fig. 1C and data not shown). A Math5-directed cre recombinase transgenic mouse was used to direct developmental mAKAPα knock-out in the retina (Yang et al., 2003, Tsien et al., 1996), while injection of AAV2 expressing both cre and GFP marker protein (AAV2-Cre) was used to direct selective, inducible knock-out in adult RGCs (Park et al., 2008). Heart extracts were obtained from mAKAPβ knock-out and control mice of the following genotypes following tamoxifen administration: Tg(Myh6-cre/Esr1*) / $mAKAP^{fl/fl}$ for knock-out and Tg(Myh6-cre/Esr1*) for control. All surgical procedures were performed under general anesthesia via intraperitoneal injection of ketamine (75 mg/kg) and xylazine (15 mg/kg). Mice also received subcutaneous injection of buprenorphine (0.03 mg/kg; Bedford Laboratories) as postoperative analgesic. Eye ointment containing erythromycin was applied to protect the cornea.

2.2. Immunopanning of RGCs

RGCs were purified (>99.5%) from postnatal (P2 to P4) Sprague–Dawley rats through sequential immunopanning, as previously described (Goldberg et al., 2002). RGCs were cultured on poly-D-lysine (PDL; 70 kDa, 10 μg/mL; Sigma, St. Louis, MO) and laminin (1 μg/mL; Invitrogen, Carlsbad, CA) in neurobasal (NB) serum-free defined medium containing insulin (5 μg/mL), sodium pyruvate (1 mM), L-glutamine (1 mM), triiodothyronine (T3; 40 ng/mL; Sigma), N-acetyl cysteine (NAC; 5 μg/mL; Sigma), B27 (1:50), BDNF (50 ng/ml), CNTF (10 ng/ml) and forskolin (5 mM) as described (Meyer-Franke et al., 1995).

2.3. Immunohistochemical Staining of mAKAP in Adult Mice Retina

2-month old mice were euthanized by 100% CO_2 inhalation. Eyes were dissected and embedded in OCT for cryosection (10 μm) immediately. Sections were post-fixed in 4% PFA for 15 min and then washed 3 times in PBS. Retinal sections were blocked in 5% normal goat serum and 0.2% BSA in PBS for 30 min, then incubated for 1 h in the same buffer with FL100 rabbit antibody to mAKAP 245–340 (Li et al., 2013). After washing, retinal sections were incubated with Alexa 594-conjugated goat anti–rabbit secondary antibody (1:500; Invitrogen) for 1 h, before washing and mounting.

2.4. Western Blot Analysis

Protein extracts from acutely purified postnatal rat RGCs, adult mouse brain and heart lysed in 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, and protease inhibitors were quantified using the DC Protein Assay Kit II (Bio-Rad, California cat. 500–0112). Lysates were fractionated by SDS-PAGE and transferred to nitrocellulose membranes as previously described (Kapiloff et al., 1999, Yu et al., 2014). Western blots were developed using horseradish peroxidase-conjugated, donkey secondary antibodies, Supersignal West Chemiluminescent Substrates (Thermo Scientific) and a Fujifilm LAS-3000 imaging system.

2.5. RT-PCR

Total RNA was extracted from RGCs transfected with mAKAP siRNA or whole mouse retinas using a Qiagen RNeasy extraction kit (cat. 74104) and cDNA synthesis carried out using iScript reverse transcriptase (Bio-Rad). Quantitative PCR (qPCR) was performed on the CFX Connect Real-Time PCR System (Bio-Rad) using TaqMan Gene Expression Master Mix (Life Tech; Cat#4369016) with the following conditions: initial denaturation 95°C for 6 min followed by 35 cycles of denaturation (95°C for 45 s), annealing (58°C for 1 min) and extension (72°C for 1 min). HPRT expression was measured as a normalizer for each sample. Results were analyzed by the relative quantity (ΔΔCt) method, as previously described (Thellin et al., 1999, Jiang et al., 2012). The primers were as follows: HPRT (Life Tech; ID# Mm00446968_m1) and AKAP6 (ID#Mm01292745_m1).

2.6. RGC Survival and Neurite Growth Assays

Postnatal RGCs were electroporated immediately after isolation with control On-TARGETplus Non-targeting siRNA #1 (Thermofisher) or mAKAP ON-TARGETplus siRNA oligonucleotides (GAC GAA CCU UCC UUC CGA A UU) as previously described (Corredor et al., 2012). RGCs were then plated at low density (3000–5000 cells/well) on 48-well plates in growth media containing forskolin, BDNF and CNTF. To investigate the role of mAKAP in various neurotrophic signaling pathways we also cultured siRNA transfected RGCs in growth media containing either forskolin (5 μM) alone or forskolin with BDNF (50 ng/mL), CNTF (10 ng/mL), EGF (10 ng/mL), or FGF (20 ng/mL). For survival assays, RGCs were cultured for up to 3 days in growth media and then stained with live cell marker calcein (C3100MP, Life Technologies), dead cell marker sytox (S11368, Life Technologies), and Hoechst nuclear dye (H1399, Life Technologies). RGC survival was expressed as the ratio of total calcein-positive cells to calcein- and sytox-positive cells. For neurite outgrowth assays, RGCs were cultured in growth media for 3 days. After fixation in 4% PFA for 30 min and washing with PBS 3 times, RGCs were stained for βIII-tubulin and analyzed using ImageJ Neurite Tracer or by Cellomics automated microscopy to quantify neurite length. Over 300 cells per condition for each of three biological