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Rho kinase is required to prevent retinal axons from entering the contralateral optic nerve

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ABSTRACT

To grow out to contact target neurons an axon uses its distal tip, the growth cone, as a sensor of molecular cues that help the axon make appropriate guidance decisions at a series of choice points along the journey. In the developing visual system, the axons of the output cells of the retina, the retinal ganglion cells (RGCs), cross the brain midline at the optic chiasm. Shortly after, they grow past the brain entry point of the optic nerve arising from the contralateral eye, and extend dorso-caudally through the diencephalon towards their optic tectum target. Using the developing visual system of the experimentally amenable model *Xenopus laevis*, we find that RGC axons are normally prevented from entering the contralateral optic nerve. This mechanism requires the activity of a Rho-associated kinase, Rock, known to function downstream of a number of receptors that recognize cues that guide axons. Pharmacological inhibition of Rock in an in vivo brain preparation causes mis-entry of many RGC axons into the contralateral optic nerve, and this defect is partially phenocopied by selective disruption of Rock signaling in RGC axons. These data implicate Rock downstream of a molecular mechanism that is critical for RGC axons to be able to ignore a domain, the optic nerve, which they previously found attractive.

1. Introduction

A growing axon's distal tip, the growth cone, is directed through a series of guidance choice points to a final target by repellent and attractive guidance cues. The response of a growth cone to any given guidance cue is context dependent, such that a single cue can be attractive to axons in one circumstance and repulsive in another. This is true at the floorplate of the spinal cord, where the axons of dorsal commissural neurons are attracted to the ventral floor plate by the attractive cues Netrin (Ntn) and Sonic Hedgehog (Shh), but are repelled by Shh upon crossing the floor plate (Chedotal, 2011). Similarly, the presence or absence of laminin alters the response of the retinal ganglion cell (RGC) axons to Ntn expressed within the optic nerve head (ONH) (Hopker et al., 1999). RGC axons make a sharp turn into the ONH, repelled by Ntn co-presented with laminin on the vitreal surface of the retina. These axons are subsequently attracted towards the laminin poor, Ntn-rich ONH interior.

RGC axons leave the eye, extend through the optic nerve, enter the ventral brain and then cross to the contralateral side of the brain at a ventral midline structure called the optic chiasm. After crossing the ventral midline, RGC axons encounter the environment of the optic nerve entering the brain from the other (contralateral) eye, yet bypass the nerve and continue dorsally through the diencephalon enroute to their major midbrain target, the optic tectum. The molecular mechanisms that ensure that RGC axons from one eye do not grow into the optic nerve of the other eye, which is permissive to RGC axon growth, are not well understood.

Rho-associated kinase (Rock) is a downstream effector of several axon guidance cues. Rock is activated downstream of the guidance receptors Plxna1 and Epha4, and downregulated downstream of the Ntn receptor, Deleted in Colorectal Cancer (Gallo, 2006; Hall and Lalli, 2010). Rock signaling is controlled by Rho GTPases, and feeds into effector proteins that include Myosin light chain, Lim kinase-1 and Collapsin response mediator protein-2, which act on the cytoskeleton (Gallo, 2006; Loudon et al., 2006; Schofield and Bernard, 2013). We show that *Xenopus laevis* RGCs and their growth cones express Rock protein during development of the optic projection. Pharmacological inhibition of Rock activity reveals that Rock is required for RGC axons of *X. laevis* to extend past the brain entry point (BEP) of the contralateral optic nerve. With Rock inhibition a subset of RGC axons grow aberrantly into the optic nerve and eye on the contralateral side of the embryo. These data are partially phenocopied by overexpression of wildtype (wt)-Rock2, indicating that tightly controlled Rock activity in growth cones functions to prevent a population of RGC axons from exiting the brain and erroneously extending into an area, the optic nerve, which is seen earlier in their trajectory as attractive.

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2. Materials and methods

2.1. Animals

Adult female *X. laevis* were injected with Human Chorionic Gonadotropin (Intervet) to stimulate egg production for in vitro fertilization. Embryos were reared in 0.1× Marc's Modified Ringers solution (MMR; 0.1 M NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.5) and the temperature was varied between 16 and 23 °C to control the rate of development. Stages were assessed according to guidelines described by Nieuwkoop and Faber (Nieuwkoop and Faber, 1994), and optic tract development was staged according to Chien and colleagues (Chien et al., 1993). Frogs and embryo procedures were approved by the Animal Care and Use Committee, University of Calgary.

2.2. Identification of rock1

The human *ROCK1* and *Xenopus tropicalis rock1* nucleotide sequences were used to identify expressed sequence tags (ESTs) from the *X. laevis* genome (Gurdon Institute's Sequence Database, Cambridge). One EST (Clone IMAGE, 6641103) mapped to the 3′ end of *rock1*. A reverse primer AGCTGGCTTTTCCAGATGAAGTTTTTGC was designed based on this EST, and 5′ rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR, Clonetech) was used to amplify the *rock1* coding sequence from cDNA (whole embryos Stages 32 to 40). Gene walking in the 3′ to 5′ direction was used to identify a 4083 base pair (bp) sequence that encodes a full-length Rock1 protein. ClustalW (Goujon et al., 2010; Larkin et al., 2007) and Jalview 2 (Waterhouse et al., 2009) web applications were used to align and annotate rock sequences from multiple species.

2.3. In situ hybridization

Probe synthesis and in situ hybridizations were performed as outlined by Sive et al., 2000. The following plasmids were linearized and used as templates for the synthesis of Digoxygenin-labeled riboprobes: *pBSK-xfgfr1* and *pCS2-xslit2* as per Atkinson-Leadbeater et al., 2010, *pGEMT-xlhx9* from S. Retaux (Institut Alfred Fessard, Gif sur Yvette, France) (Bachy et al., 2001), and *pBSK-ntn1* from C. Holt (Univ. Cambridge, England). For *xrock1*, the plasmid Clone IMAGE, 6641103 was linearized with Sma1 enzyme and transcribed with T7 RNA polymerase. The antisense *xrock2* riboprobe was synthesized from a plasmid (J. Han, Pohang Univ., Korea) encoding full-length *Xenopus rock2* (NCBI Accession NM_001087476.1) by linearizing with Ava1 enzyme and transcribing with T7 RNA polymerase.

Wholemount in situ hybridization was performed as described previously (Sive et al., 2000). Briefly, embryos were fixed in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, 4% formaldehyde) overnight at 4 °C and stored in 100% ethanol at −20 °C. Embryos were rehydrated and permeabilized with Proteinase-K (Sigma, 10 μg/mL), and hybridized at 60 °C to 65 °C with riboprobe in hybridization buffer (10% MEMFA, 50% formamide, 0.1 g/mL dextran sulfate, 1 mg/mL torula RNA (Sigma), 1× Denhardt's solution). Embryos were blocked in 2% Boehringer Mannheim Blocking (BMB) reagent (Roche), and incubated overnight at 4 °C with alkaline phosphatase-coupled anti-DIG antibody (Roche, 1:2000). In situ signal was detected with a colorimetric reaction using BM purple (Roche) or NBT/BCIP (Roche). Staining was stopped by fixation in modified Bouin's fix (10% formaldehyde, 5% glacial acetic acid).

For in situ hybridization on sections, Stage 33/34 and 35/36 embryos were fixed in MEMFA overnight at 4 °C, then sunk in 30% sucrose and embedded in Optimal Cutting Temperature compound (OCT, Tissue-Tek). Twelve micrometer transverse sections through the brain and eyes were cut with a cryostat (Microm) and placed on glass slides. Slides were incubated at 60 °C to 65 °C in hybridization buffer containing DIG-labeled sense or antisense riboprobes. The slides were washed and anti-DIG antibody (Roche, 1:2500) conjugated to alkaline phosphatase was used to detect the riboprobe with NBT/BCIP staining. Slides were washed and mounted with Aquapolymount (Polysciences Inc.). Here, and elsewhere in the paper, photographic images were taken with a Zeiss Stemi SV 11 microscope with AxioCam HRc camera or Zeiss Axioplan2 microscope with MRc camera and Axiovision software. Images were compiled and edited with Adobe Photoshop.

2.4. Eye explant cultures

Stage 24 embryos were collected and anesthetized in Modified Barth's Saline (MBS, 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM HEPES, pH 7.5) containing 0.4 mg/mL tricaine (ethyl 3-aminobenzoate methanesulfonic acid, Sigma). Intact eye buds were placed on coverslips coated with poly-L-ornithine and 10 μg/mL laminin (Sigma), grown for 24 h at 22 °C in culture media (60% L15 (Invitrogen), 0.01% (*w*/*v*) bovine serum albumin (BSA, Sigma), 1% antibiotic/antimycotic (Invitrogen)). Of note, sister embryos had reached Stage 35/36, with axons in the middiencephalon over the same period. Cultures were then fixed in 2% paraformaldehyde (PFA).

2.5. Immunostaining

Immunostaining was performed as described previously (McFarlane et al., 1995). Briefly, for eye bud cultures, fixed explants were washed with phosphate buffered saline (PBS) containing 0.25% Triton-X100 (Sigma) and 0.2% BSA, blocked with 5% goat serum, and incubated with primary antibody. For immunostaining on slides, whole embryos were fixed in 4% PFA at 4 °C, sunk in 30% sucrose and embedded in OCT reagent. Twelve μm thick tissue sections were cut transversely through the brain and eyes using a cryostat (Microm), then washed with PBS containing 0.5% Triton-X100 and 0.2% BSA, blocked with 5% goat serum, and incubated with primary antibody. Samples were mounted with Aquapolymount and imaged. Primary antibodies included rabbit polyclonal anti-human ROCK1/2 (Millipore 07–1458, 1:1000), rabbit polyclonal anti-human Rock1 (Abcam ab97592, 1:200), rabbit polyclonal anti-*Xenopus* Rock2 (1:2000) (a gift from J. Liu (Univ. of Ottawa) (Farah et al., 1998)) (see Supp. Fig. 1 for epitope/immunogen sequences), mouse monoclonal Islet1/2 (Developmental Studies Hybridoma Bank, 39.4D5), and mouse monoclonal anti-myc (9E10, 1:1000). Secondary antibody (Alexa 488 or Alexa 546, Invitrogen) was used at 1:500 for explants and 1:1000 for tissue sections.

2.6. Western blot

Proteins extracted from *Xenopus* embryos Stages 28–40 were separated on a 6% polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) and immunoblotted with rabbit polyclonal anti-human ROCK1/2 antibody (Millipore, 1:1000), anti-human Rock1 antibody (Abcam, 1:1000), and rabbit polyclonal anti-*Xenopus* Rock2 antibody (Farah et al., 1998, 1:1000). Antibodies were pre-incubated for 30 min at room temperature with homogenized Stages 16–24 embryos. Goat anti-rabbit peroxidase-conjugated secondary antibody was used to detect protein expression by enhanced chemiluminescense (Perkin Elmer Corp, UK).

2.7. Exposed brain preparation

The exposed brain preparation was performed as described previously (Chien et al., 1993). Stages 33/34 and 35/36 embryos were anesthetized in 1X MBS containing 0.4 mg/mL tricaine (pH 7.4). The outer skin and dura lining the brain were peeled back on the left side of the embryo, exposing the neuroepithelium to MBS with or without the water-soluble Rock inhibitors Y-27632 (Sigma) and HA-1077 (Sigma), or forskolin (dimethyl sulfoxide) (gift from the Giembycz lab,