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Intraretinal RGMa is involved in retino-tectal mapping

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The repulsive guidance molecule (RGMa) is involved in controlling the topography of retinal ganglion cell axons along the anterioposterior axis of the tectum. Here, we generated a new RGMa-monoclonal antibody and show that it is expressed in the developing retina, suggesting that it may regulate retinal axon pathfinding. We tested this hypothesis by using *in ovo* electroporation to either overexpress or downregulate RGMa in the eye. Anterograde labeling of retinal axons entering the optic tecta revealed abnormal phenotypes when RGMa expression is perturbed. These included the absence of terminal zone, the premature stalling of arborization of fibers, overshooting of terminal zone, aberrant axonal turns in the optic tectum and abnormal projections into deeper tectal layers. Moreover, RGMa overexpression frequently leads to intraretinal pathfinding errors. Thus, these data suggest that RGMa expression on retinal axons is a major determinant of topographic targeting in the retino-tectal projection and in the retina.

Keywords: Retino-tectal projection; Repulsive Guidance Molecule RGMa; Axon guidance; Retina; Tectum; Pathfinding; DiI labeling; RNA interference

Introduction

The pathfinding of axons during the development of the nervous system is under the control of a complex set of environmental guidance cues. Embryological, genetic and tissue culture experiments have demonstrated that these guidance factors exert four kinds of action on growth cones, classified as chemorepulsion, chemoattraction, contact dependent repulsion and contact dependant attraction (Tessier-Lavigne and Goodman, 1996).

The Repulsive Guidance Molecule (RGM) was the first protein identified that acts as a contact-dependent repulsive factor (Stahl et al., 1990). Five years ago the successful cloning of chick RGMa opened new fields of investigation (Monnier et al., 2002; Mueller et al., 2006; Isenmann et al., 2003). RGMa is the first member of a novel family of GPI-anchored proteins of which 3 mammalian members, RGMa, RGMb, and RGMc, were identified (Niederkofler et al., 2004). *In situ* hybridization in the mouse embryo reveals that RGMa and RGMb are predominantly expressed in distinct, mostly non-overlapping patterns in the central nervous system, while RGMc is mainly expressed in skeletal and heart muscles (Niederkofler et al., 2004).

In vitro, RGMa possesses activities similar to some members of the Ephrin family (in particular Ephrin A5 and Ephrin A2), causing growth cone collapse and inhibiting outgrowth of temporal retinal ganglion cell (RGC) axons (Drescher et al., 1994; Stahl et al., 1990; Monnier et al., 2002; Mueller et al., 2006). Recently, Rajagopalan and colleagues (2004) identified the transmembrane protein Neogenin as a high-affinity receptor for RGMa. The primary role of Neogenin in the nervous system may be the transduction of RGM signals at the level of the growth cone. Consistent with selective axonal responsiveness to RGMa, Neogenin is expressed in a high temporal, low nasal gradient in the retina (Rajagopalan et al., 2004). This data taken together with the high posterior, low anterior expression pattern of RGMa in the optic tectum led to the hypothesis that RGMa is involved in guiding retinal neurons towards their targets in the optic tectum. RGMa may act as a repulsive guidance cue, confining the terminal zones of temporal axons to the more anterior positions in the optic tectum. More recent *in vivo* studies using gain and loss-of-function analysis brought further evidence that the RGMa gradient in the optic tectum controls retinal axon guidance (Matsunaga et al., 2006).

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In the present study, we are attempting to complete the characterization of the role of RGMa during the establisment of chick-retinotectal projection. Beyond its expression in the tectum, we show that RGMa is expressed on retinal ganglion cell axons projecting into the tectum. We reasoned that retinaly expressed RGMa might play a prominent role in the guidance of RGC axons. To address this issue, we have chosen the approach of overexpressing or downregulating RGMa in the developing chick eye, combined with labeling techniques to assay the effects of misexpression on topographic mapping of retinal axons. Here we show that retinal overexpression and downregulation of RGMa led to topographic targeting errors of temporal retinal axons in the optic tectum. These errors included aberrant arbor and fiber projections, the absence of terminal zones, as well as branching into deeper tectal layers. In addition, RGMa overexpression induced pathfinding errors in the retina.

Results

Localization of the RGMa protein in the developing chick retina

To study the RGMa expression pattern, we established an anti RGMa-producing cell line by immunizing mice with an RGMa peptide spanning the residues 195 to 349. IgG producing cell lines were screened first in an ELISA assay to assess binding to RGMa followed by Western Blotting to ensure specificity. Fig. 1A shows Western Blots using the 8B6 antibody, which specifically recognizes RGMa in membrane preparations from COS-7 cells transfected with RGMa. Western Blot analysis performed on chick embryonic retinae (E6) detected one band with a molecular weight of 33 kDa, which is similar to the band detected in the chick tectum (33 kDa; Stahl et al., 1990; Monnier et al., 2002). To ensure that the 8B6 antibody specifically recognizes RGMa, we performed Western blotting on its closest relative, RGMb, which has more than 50% homology at the amino acid level (Niederkofler et al., 2004). In this analysis, 8B6 did not recognize chick RGMb (Fig. 1B), which is indicative of a high level of specificity for RGMa. To determine the exact site of expression of RGMa in the retina, we performed *in situ* hybridization on sections (Fig. 1C). Interestingly, we found that RGMa is expressed all over the retina with strong expression levels in the retinal ganglion cell (RGC) layer (Fig. 1C). Immunohistochemical analysis using the 8B6 antibody confirmed that RGMa is expressed all over the retina (Fig. 1D), moreover, a strong staining in the retinal fiber layer was observed, which indicates that RGMa is expressed on retinal axons. In these experiments the level of expression of RGMa seems homogenous in the different parts of the retina and no temporo-nasal gradient of expression was observed (data not shown). To confirm that RGMa is expressed on retinal axons, we performed immunostaining on retinal explants using the 8B6 antibody. In these experiments 8B6 displayed a strong staining on retinal fibers as well as on growth cones, where it may influence axonal guidance (Figs. 1F, G).

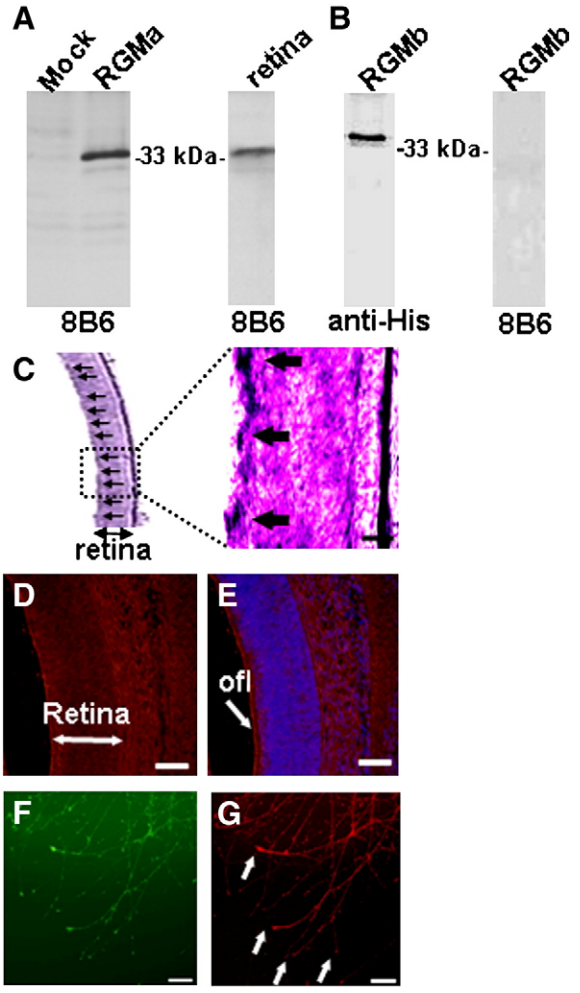

Fig. 1. RGMa is expressed on Retinal fibers during development. (A) Western Blot analysis of RGMa expression. In Mock transfected COS-7 cells, the monoclonal antibody 8B6 does not show any band. In COS-7 cells transfected with RGMa, 8B6 recognizes a 33 KDa band, which corresponds to the molecular weight of RGMa. Western Blots, performed with 8B6, demonstrate RGMa expression in the E8 chick retina. (B) In COS-7 cells transfected with His-RGMb, the anti-His antibody recognizes a 34 kDa band which is not recognized by the 8B6 antibody. (C) In situ hybridization performed on E9 retinal sections display a strong staining in the retinal ganglion cell layer (arrow). The right panel represents a higher magnification of the retina presented in the left. Arrows indicate retinal ganglion cells that show strong staining. (D) E6 chick retinal sections stained with the 8B6 antibody show RGMa staining all over the retina. (E) Chick E6 sections stained with DAPI (blue) and 8B6 (red) show strong RGMa expression in the optic fiber layer (ofl). (F) Retinal explants were stained with Alexa-labeled phalloidin. (G) Fibers and growth cones (arrows) from explants stained with the anti-RGMa 8B6 display strong labeling. Scale bars: 150 μm.

Ectopic-RGMa localization after electroporation

The predominant expression of RGMa on retinal cell axons at early stages of retinal development suggests that RGMa is involved in retinal axon pathfinding. To assess the role of RGMa in the formation of the retino-tectal map, we performed gain- or loss- of function analysis of RGMa in the developing chick retina using electroporation either of an RGMa-expressing plasmid (RCAS-BP-RGMa) or of an RGMa-siRNA construct, respectively. In order to restrict RGMa perturbation to the eye, we narrowed the application of the electric field to the vicinity of the optic vesicle using a tungsten needle as electrode. After injection of plasmid DNA (2 mg/ml) in the optic vesicle, at development stage Hamilton–Hamburger 10–12 (H–H stage10–12; Hamburger and Hamilton, 1951), the microelectrode was placed beneath the optic vesicle and an electric pulse was applied (Momose et al., 1999). Under these conditions, electroporation of GFP plasmid reveals that expression was restricted to the eye (Fig. 2A) and that almost all the cells located in the neural retina and