Brief Communications

Repulsive Guidance Molecule Plays Multiple Roles in Neuronal Differentiation and Axon Guidance

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Repulsive guidance molecule (RGM) is a membrane-bound protein originally isolated as a guidance molecule for retinal axons. Three RGM isoforms (RGMa–RGMc) exist in vertebrates. We showed previously that RGMa is a cell-survival factor in the neuroepithelium of chick embryos that suppresses the proapoptotic activity of its receptor neogenin. In the present study, we performed gain- and loss-of-function analysis of RGMa in chick embryos to further investigate RGMa function. We found that RGMa overexpression promotes neuronal differentiation, whereas *RGMa* small interference RNA represses it. Similar experiments conducted at later developmental stages using retroviral vectors reveal that perturbation of *RGMa* expression disturbs the retinotectal projection. Our work provides the first evidence for a role for RGMs in axon guidance *in vivo*. In addition, these results suggest that RGMa exerts multiple functions during neural development.

Key words: **axon guidance; retinotectal; brain development; mapping; neuronal apoptosis; RNA interference**

Introduction

In vertebrates, retinal axons make connections in a topographic manner (Sperry, 1963; McLaughlin et al., 2003). Temporal retinal axons project to the anterior tectum and nasal axons to its posterior part. Sperry (1963) proposed that the development of this topographic projection is governed by ligands and receptors expressed in complementary gradients in the retina and tectum. Accordingly, biochemical analysis revealed that retinal axons are guided by repulsive glycosylphosphatidylinositol-anchored proteins highly expressed in the posterior tectum (Stahl et al., 1990). ephrin-A2 and ephrin-A5 were the first molecules identified as topographic guidance molecules in this system (Cheng et al., 1995; Drescher et al., 1995), recently followed by repulsive guidance molecule isoform a (RGMa) (Monnier et al., 2002). However, there is so far no direct evidence that RGMa guides retinal axons *in vivo*, and the analysis of *RGMa*-deficient mice suggested that RGMa does not guide retinal axons (Niederkofler et al., 2004).

RGMa is expressed in the neural tube before the appearance of retinal axons (Matsunaga et al., 2004), suggesting that it exerts additional roles in CNS development (Matsunaga and Chédotal, 2004). Accordingly, *RGMa* knock-out mice show defects in neural tube closure (Niederkofler et al., 2004). Recently, a transmembrane protein, neogenin, has been identified as a receptor for RGMa mediating its repulsive activity (Rajagopalan et al., 2004). Neogenin was also shown to be a dependence receptor (Mehlen and Thibert, 2004) inducing apoptosis when unoccupied by RGMa (Matsunaga et al., 2004).

We performed here gain- and loss-of-function analysis of RGMa in chick embryos to show that RGMa controls neuronal proliferation, differentiation, and axon guidance in addition to neuronal survival.

Materials and Methods

Expression constructs and in ovo *electroporation.* Most expression constructs, including RGMa small interfering RNA (siRNA), were described previously (Matsunaga et al., 2004). A second RGMa siRNA (RGMa siRNA-2; 5′-AATGCGTGGAGCAGAAAGTGT-3′) was also designed. For long-term expression, RGM siRNAs and a control siRNA (a scramble form of *RGMa* siRNA; 5′-AATCTCGTAGTGTGCTCTATC-3′) were excised from *pSilencer* together with the U6 promoter and subcloned into *RCASBP(B)* vectors (Hughes et al., 1987; Fekete and Cepko, 1993). Hamburger and Hamilton stage 10 (HH10) to HH11 (Hamburger and Hamilton, 1951) chick embryos were electroporated as described previously (Funahashi et al., 1999) or using tungsten needles, with the following constructs: *pMiw*-RGMa (3.0 mg/ml), *pMiw*-RGMa-green fluorescent protein (GFP) (3.0 mg/ml), *pcDNA*-human neogenin (3.0 mg/ml), *pSilencer*-RGMa siRNA (1.5 mg/ml), *pSilencer*-neogenin siRNA (1.5 mg/ml), *pSilencer*-control siRNA (1.5 mg/ml), *pBabe*-p35 (1.0 mg/ml), *RCASBP(B)*-RGMa (2.0 mg/ml), *RCASBP(B)*-alkaline phosphatase (AP) (2.0 mg/ml), *RCASBP(B)*-RGMa siRNA (1.0 mg/ml), *RCASBP(B)*-RGMa siRNA-2 (1.0 mg/ml), and *RCASBP(B)*-control siRNA (1.0 mg/ml). GFP expression vector (*pMiwIII*-GFP, 0.5 mg/ml) was coelectroporated to check the electroporation efficiency.

Bromodeoxyuridine incorporation experiments. Bromodeoxyuridine

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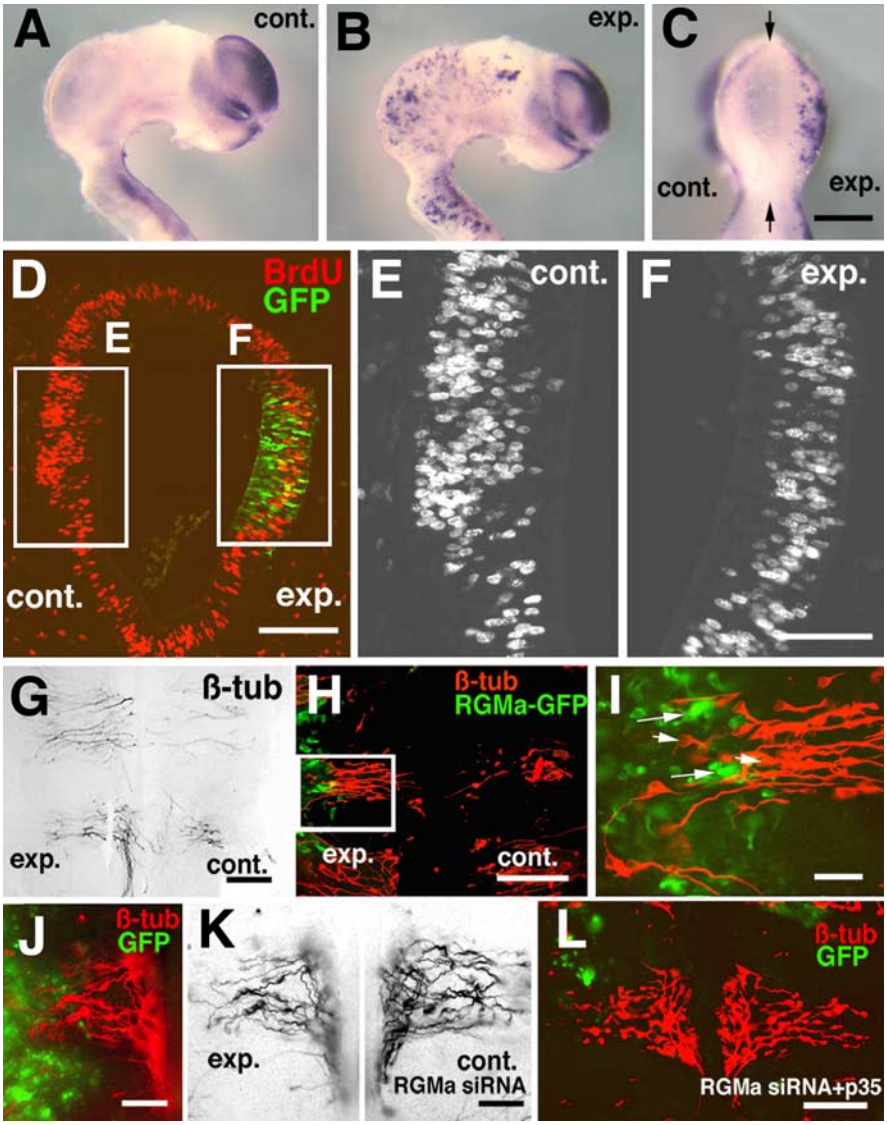


Figure 1. *RGMa* overexpression represses cell proliferation and induces neuronal differentiation. ***A–C***, *In situ* hybridization for *RGMa*, 2 d after *RCASBP(B)*-RGMa electroporation. The staining is weak to help revealing exogenous *RGMa* expression. The control side (***A***), experimental side (***B***), and posterior view (***C***) are shown. Arrows indicate the dorsal midline in ***C***. ***D–F***, BrdU labeling experiment 24 h after *RCASBP(B)*-RGMa and *pMiwIII*-GFP electroporation. ***D*** is an overlay image of BrdU immunostaining and GFP fluorescence. ***E*** and ***F*** are higher magnifications of corresponding areas in ***D***. ***G–I***, Immunostaining for β-tubulin in the rhombencephalon (HH14), 15 h after electroporation of *pMiwIII*-RGMa (***G***) or RGMa-GFP (***H***, ***I***). ***G*** and ***H*** are views from the ventral side of flat-mounted embryos. ***I*** is a higher magnification of the corresponding area in ***H***. ***H*** and ***I*** are overlay panels of GFP and β-tubulin immunostaining. The number of β-tubulin-positive neurons (arrowhead in ***I***) is increased on the experimental side, and they are surrounded by RGMa-GFP-expressing cells (arrows in ***I***). ***J–L***, The number of β-tubulin-immunoreactive neurons in the rhombencephalon is decreased on the experimental side compared with the control side, 15 h after electroporation of *RGMa* siRNA (***J***, ***K***) or coelectroporation of *RGMa* siRNA and p35 (***L***). cont., Control side; exp., experimental side. Scale bars: ***C***, 500 μm; ***G***, ***H***, 200 μm; ***D***, ***J***, ***K***, ***L***, 100 μm; ***F***, ***I***, 50 μm.

(BrdU) (10 mM) was injected in chick embryos as described previously (Matsunaga et al., 2002).

In situ *hybridization.* Probes and procedures for *in situ* hybridization on whole-mount embryos and sections were described previously (Matsunaga et al., 2000; Marillat et al., 2002).

Immunohistochemistry. Whole-mount immunostaining was performed as described previously (Chédotal et al., 1995). Embryos were incubated with mouse anti-βIII-tubulin antibody (1:2000, TuJ1; Babco, Richmond, VA) or rabbit anti-GFP antibody (Invitrogen, Carlsbad, CA). Embryos were cut partially or totally along the dorsal midline and ventral midline and then flat mounted. Immunostaining on sections was performed as described previously (Matsunaga et al., 2001). gag was visualized using rabbit anti-gag polyclonal antibody (1:200; kindly provided by Dr. T. Jaffredo, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7622, Paris, France) or mouse monoclonal antibody (1:100; clone AMV-3C2; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Neogenin expression was detected using a rabbit polyclonal neogenin antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA).

DiI labeling of retinal axons. Retinal fibers were labeled with DiI (Invitrogen) and analyzed as described previously (Itasaki and Nakamura, 1996). A small DiI crystal was inserted into the dorsotemporal retina at embryonic day 15 (E15) to E16 (Nakamoto et al., 1996). After examination of DiI-labeled axons trajectory, 150-μm-thick sections of the tectum were made with a vibratome (Leica, Nussloch, Germany) and counterstained with Hoechst (Sigma, St. Louis, MO).

Results

RGMa overexpression promotes neuronal differentiation

Neuronal differentiation in the hindbrain and midbrain starts at approximately HH11+ and HH14, respectively (Chédotal et al., 1995; Eickholt et al., 2001). In the midbrain, neurons differentiate in an anterior-to-posterior manner (LaVail and Cowan, 1971). *RGMa* expression starts before the onset of neuronal differentiation and is well correlated with the timing of neuronal differentiation particularly in the midbrain (Matsunaga et al., 2004 and data not shown), suggesting that *RGMa* might control neuronal differentiation. To test this possibility, we overexpressed *RGMa* in the diencephalon and myelencephalon of HH10–11 embryos. Similar results were obtained with *pMiw*-RGMa or *RCASBP(B)*-RGMa expression vectors.

We first found that, 48 h after electroporation, the size of the neural tube was reduced on the experimental side (Fig. 1*A–C*). Because RGMa overexpression does not influence cell death (Matsunaga et al., 2004), we tried to determine whether neuronal proliferation was affected. At 24 h after electroporation, the number of BrdU-labeled cells was significantly reduced in the midbrain ($-27.26 \pm 1.48\%$; $n = 4$ of 4) and hindbrain ($-44.64 \pm 13.77\%$; $n = 4$ of 4) (Figs. 1*D–F*, 2*H*), indicating that RGMa reduced cell proliferation. This reduction was transient and was not detected when BrdU was injected 48 h after electroporation (data not shown). Next, we overexpressed RGMa or an RGMa-GFP fusion protein and examined whether RGMa also influenced neuronal differentiation using immunostaining for β-tubulin. We mainly examined the rhombencephalon of HH15 chick embryos because, at this stage, it contains a limited number of neurons. At 15 h after *RGMa* electroporation, the number of

β-tubulin-positive cells was increased on the experimental side in the midbrain (+88.40 ± 14.94%; $n = 4$ of 5) and hindbrain (+40.11 ± 7.98%; $n = 6$ of 8) (Figs. 1*G*, 2*I*), indicating that RGMa promoted neuronal differentiation. β-Tubulin-positive neurons were always observed around GFP-positive cells (Fig. 1*H*,*I*), suggesting that RGMa functions as a ligand and stimulates neuronal differentiation around RGMa-expressing cells. Moreover, we performed loss-of-function experiments by expressing siRNA (Katahira and Nakamura, 2003) for *RGMa* (Matsunaga et al., 2004). BrdU incorporation experiments revealed that suppression of *RGMa* expression had little effects on cell proliferation (+7.14 ± 4.20%; $n =$ 7 of 8) (Fig. 2*H*). In contrast, the number of β-tubulin-positive cells was significantly reduced on the experimental side (−23.01 ± 11.36%; $n = 7$ of 10; $p < 0.001$) (Figs. 1*J*,*K*, 2*I*) and not with control siRNA construct (+1.32 ± 5.65%; $n = 10$ of 10). This suggested that suppressing *RGMa* function reduced neuronal differentiation. To determine whether the decrease of BrdU-labeled cells or β-tubulin-positive cells induced by *RGMa* siRNA could be attributable to increase apoptosis (Matsunaga et al., 2004), we coelectroporated *RGMa* siRNA with the baculovirus p35, a general caspase inhibitor (Thibert et al., 2003). In this case, a comparable decrease in the number of β-tubulin-positive cells (−20.30 ± 2.03% reduction; $n = 5$ of 7; $p < 0.0001$) (Figs. 1*L*, 2*I*) was observed, whereas proliferation was unchanged (+01.74 ± 3.84%; $n = 7$ of 8) (Fig. 2*H*). In the midbrain and hindbrain, many transcription factors or secreted molecules define the territories and polarity along the anteroposterior and dorsoventral axis, such as *Pax6* (paired box gene 6), *En-2* (engrailed 2), *Wnt1* (wingless), *ephrin-A2*, and *Shh* (sonic hedgehog) (Nakamura, 2001). *RGMa* or *RGMa* siRNA overexpression did not affect the expression pattern of these genes, suggesting that RGMa does not affect cell proliferation and differentiation by altering the pattern of cell fate specification in the neural tube (data not shown).

RGMa regulates neuronal differentiation through its receptor neogenin

Because the RGMa receptor neogenin is expressed in the neuroepithelium and postmitotic cells of early chick embryos (Fig. 2*D*,*F*) (Matsunaga et al., 2004), we suspected that neogenin could control neuronal differentiation.

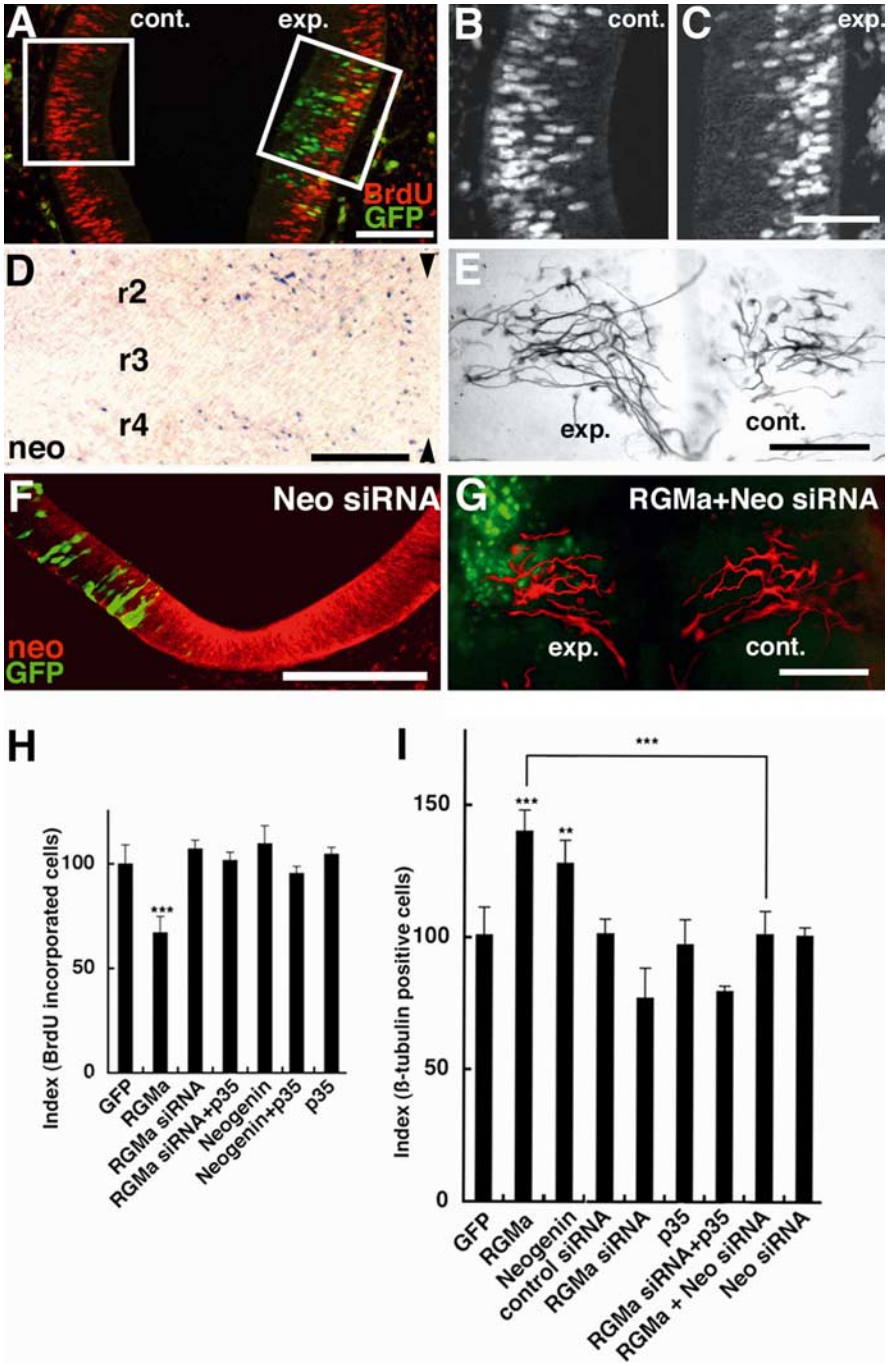


Figure 2. RGMa promotes neuronal differentiation through neogenin signaling. ***A–C***, Neogenin overexpression has no effect on cell proliferation. ***A*** is a combined image of BrdU immunostaining and GFP fluorescence. ***B*** and ***C*** are higher magnifications of the framed areas in ***A***. ***D***, *In situ* hybridization for neogenin in the rhombencephalon of HH15 embryo. *Neogenin* is highly expressed in rhombomeres r2 and r4 in which postmitotic neurons first appear (arrowheads indicate the midline). ***E***, At 15 h after the overexpression of neogenin in the rhombencephalon, the number of β-tubulin neurons is increased on the electroporated side (exp.) compared with the control side (cont.). ***F***, Immunostaining for neogenin and GFP fluorescence in the mesencephalon 24 h after coelectroporation of neogenin siRNA and GFP. Neogenin siRNA represses endogenous neogenin expression on the experimental side (visualized with GFP). ***G***, Coelectroporation of RGMa and *neogenin* siRNA suppresses RGMa induction of neuronal differentiation. ***H***, ***I***, Quantification of BrdU labeling in the midbrain and hindbrain 24 h after electroporation (***H***) or β-tubulin-positive cells in the ventral hindbrain 15 h after electroporation (***I***). The index with SDs ($n = 4–6$) is the ratio of the number of BrdU-labeled cells or β-tubulin-positive cells on the experimental side versus the control side in each experimental condition. $^{**}p < 0.001$; $^{***}p < 0.0001$; others are nonsignificant. The statistical significance was determined by *t* test, using Prism (GraphPad Software, San Diego, CA). Scale bars: ***D***, 200 μm; ***A***, ***E***, ***F***, ***G***, 100 μm; ***C***, 50 μm.

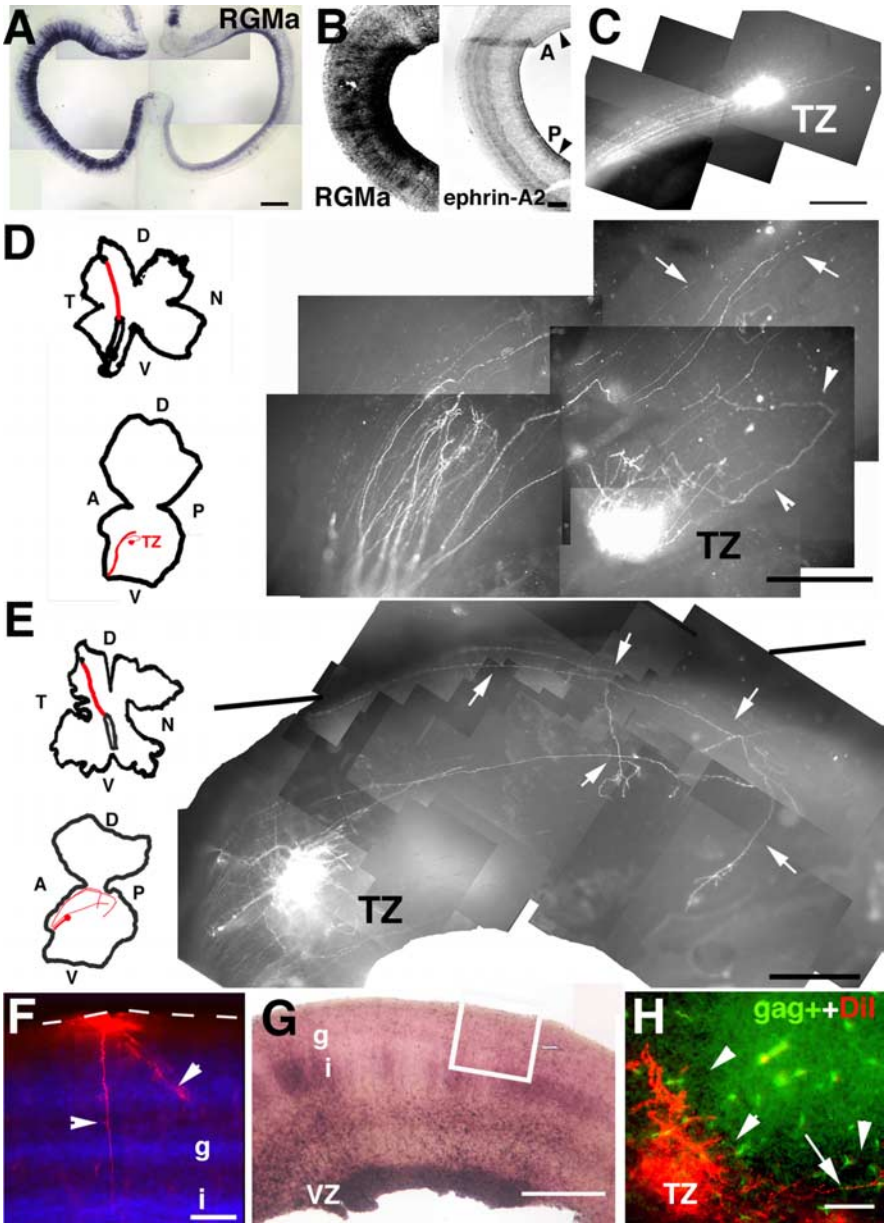


Figure 3. RGMa overexpression perturbs the targeting of retinotectal axons. ***A***, *In situ* hybridization for *RGMa* 6 d after *RCASBP(B)*-RGMa electroporation. Strong *RGMa* expression is seen in the tectum on the experimental side (left). ***B***, *In situ* hybridization of serial sections of the experimental side of an electroporated embryo (7 d after electroporation) for *RGMa* (left) and *ephrin-A2* (right). *Ephrin-A2* (arrowheads) is still expressed in a high-posterior (P) to low-anterior (A) gradient. ***C***, Dorsotemporal retinal axon trajectory in a control, *RCASBP(B)*-AP electroporated embryo. ***D***, ***E***, Two examples of RGMa electroporated embryos. In both cases, many labeled axons project beyond their TZ (arrows in ***D***, ***E***). In one case, some axons make a U-turn before reaching their TZ (arrowheads in ***D***). The line in ***D*** marks the orientation of the vibratome section (***F***). ***F***, Overlay image of DiI and 4′,6′-diamidino-2-phenylindole staining (ventro-middle region of the tectum). DiI-labeled retinal axons (arrowheads) have aberrant projection under layer g. ***G***, *In situ* hybridization for RGMa in the section adjacent to the section in ***F***. The framed area corresponds to the tectum level in ***F***. ***H***, Overlay image of DiI and anti-gag immunostaining [*RCASBP(B)*-RGMa electroporated embryo]. DiI-labeled axons (arrow) avoid the region of high gag expression (green, arrowheads) before ending at their TZ. A, Anterior; D, dorsal; g, layer g in the tectum; i, layer i; N, nasal; P, posterior; T, temporal; V, ventral. Scale bars: ***A–F***, 500 μm; ***G***, ***H***, 100 μm.

To distinguish between the proapoptotic activity of neogenin and its other possible functions, full-length neogenin was coelectroporated with p35. At 24 h after neogenin electroporation, the number of BrdU-labeled cells on the experimental and control sides were almost the same ($-4.25 \pm 3.36\%$; $n = 4$ of 4) (Fig. 2*A–C*), showing that neogenin did not influence cell proliferation. However, the number of β-tubulin-positive cells was increased on the experimental side ($+27.89 \pm 8.80\%$; $n = 5$ of 6), indicating that neogenin induced neuronal differentiation (Fig. 2*E*,*I*). To determine whether neogenin mediates the neuronal differentiation-promoting activity of RGMa, we performed double electroporation of *RGMa* with *neogenin* siRNA. This siRNA construct repressed endogenous neogenin expression ($n = 3$ of 5) (Fig. 2*F*) (Matsunaga et al., 2004). Whereas the number of β-tubulin-positive cells was increased by RGMa, coelectroporation of *neogenin* siRNA suppressed this effect ($+1.28 \pm 8.73\%$; $n = 5$ of 5) (Fig. 2*G*,*I*). *Neogenin* siRNA alone did not have any effect ($+0.71 \pm 3.09\%$; $n = 7$ of 7) (Fig. 2*I*). These results suggest that RGMa promotes neuronal differentiation through neogenin signaling.

RGMa regulates topographic retinotectal projection in chick embryos

By E9.5 (HH36), after retinal axons started to enter the tectum, *RGMa* is expressed in the tectum according to a low-anterior and high-posterior gradient as reported previously (Monnier et al., 2002). *RGMa* expression was particularly strong in the ventricular zone and in layers vi and viii (LaVail and Cowan, 1971) but was not expressed in the stratum opticum and superficial layers of the tectum, in which retinal axons project at later stages (data not shown).

To study the possible role of RGMa in guiding retinotectal axons (Monnier et al., 2002), *RCASBP(B)*-RGMa expression vectors were electroporated in the mesencephalon at HH10 (E1.5) (Fig. 3*A*), and the retinotectal projection was analyzed at E17–E18. RGMa overexpression only transiently affects cell proliferation (this study) and apoptosis (Matsunaga et al., 2004). Accordingly, the size of the tectum was similar on the experimental and control side. In addition, RGMa overexpression did not alter the gross histology and anteroposterior polarity of the tectum, in particular the expression of *En-2* (data not shown) or *ephrin-A2* ($n = 3$ of 3) (Fig. 3*B*). At E17, retinal axons should have formed terminal arbors [terminal zone (TZ)], and overshooting axons and ectopic branches that form during normal development should have been eliminated (Nakamura and O'Leary, 1989; Yates et al., 2001). In control embryos [*RCASBP(B)*-AP], almost all labeled axons from the dorsotemporal quadrant of the retina have their TZ in the anteroventral tectum ($n = 5$ of 5) (Figs. 3*C*, 4*F*). In contrast, in RGMa electroporated embryos, we could detect overshooting axons passing beside their TZ ($n = 15$ of 27) (Figs. 3*D*,*E*, 4*F*). In one case, many retinal axons projected behind their TZ, but some made a U-turn

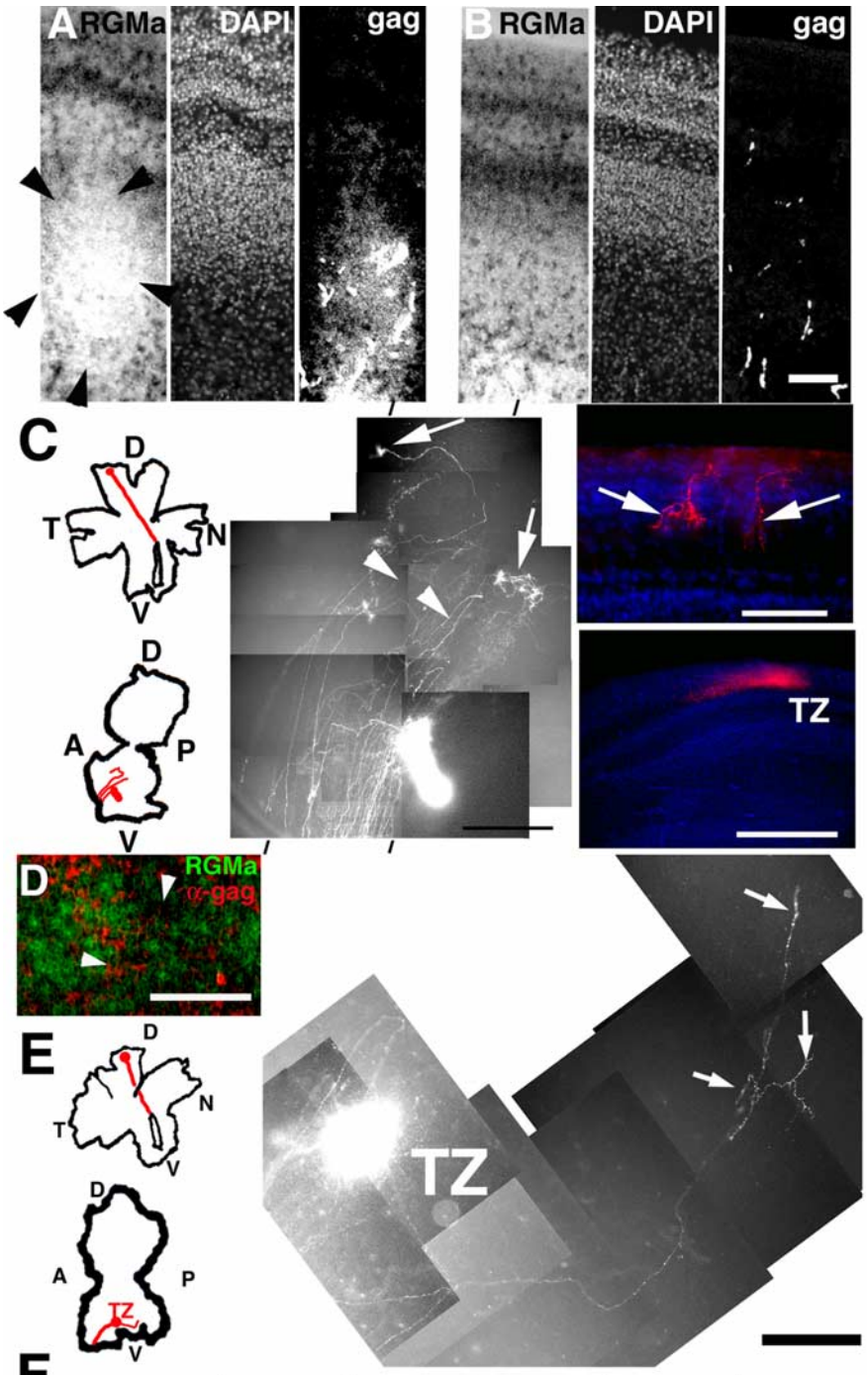
and reached their TZ (Fig. 3*D*). In normal tectum, retinal axons arborize in the superficial layer [above layer g of stratum griseum et fibrosum superficiale (SGFS) or layer viii] (LaVail and Cowan, 1971). In *RGMa* electroporated tectum, layers developed normally, but ectopic DiI-labeled retinal fibers penetrated into deeper layers (Figs. 3*F*, 4*F*). *In situ* hybridization for *RGMa* performed on tectum slices of RGMa electroporated embryos revealed that DiI-labeled axons were surrounding regions with high level of RGMa expression (Fig. 3*F*,*G*). Similar observations were made on tectum sections labeled with anti-gag antibodies: DiI-labeled axons avoid regions of high gag expression level (Fig. 3*H*). This suggests that retinal axons avoid tectum domains expressing high levels of RGMa, as expected from its repulsive action *in vitro*.

We next performed loss-of-function experiments of RGMa using retroviral vectors encoding two distinct *RGMa* siRNAs (RGMa siRNA and siRNA-2). These RGMa siRNA expression constructs strongly repressed endogenous *RGMa* expression ($n = 3$ of 3 for each siRNA) (Fig. 4*A*,*B*,*D*). In embryos electroporated with *RCASBP(B)*-control siRNA, the retinotectal projection developed normally ($n = 8$ of 8) (Fig. 4*F*). In *RCASBP(B)*-RGMa siRNA electroporated embryos, temporal retinal axons focused into TZ as in control embryos, but many axons projected to ectopic positions posterior to their TZ proper ($n = 4$ of 18) (Fig. 4*C*,*F*). However, these ectopic retinal axons still innervated and arborized into normal recipient layers (SGSF) (Fig. 4*C*). Similar results were obtained with *RCASBP(B)*-RGMa siRNA-2 construct (Fig. 4*E*,*F*).

Discussion

RGMa regulates neuronal differentiation

In this study, gain- and loss-of-function experiments revealed that RGMa promotes neuronal differentiation, whereas RGMa siRNA represses it. In the developing midbrain and hindbrain, *RGMa* expression precedes the birth of the first neurons, suggesting that RGMa may control the timing of neuronal differentiation. Neogenin is a receptor for RGMa in axon guidance and cell survival (Matsunaga et al., 2004). We showed here that neogenin overexpression promotes neural differentiation and that *neogenin* siRNA blocked the neuronal differentiation-promoting activity of RGMa. Overall, this suggests that RGMa controls neuronal differentiation through neogenin signaling. Because



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	total experiment numbers	overshooting axons remain	overshooting axons and ectopic	pathway disturbed	no effect
RCAS-AP	5	0	0	0	5
RGMa	27	10	4	1	12
RCAS-siRNA control	8	0	0	0	8
RCAS-RGMa siRNA	18	1	3	0	14
RCAS-RGMa siRNA-2	20	6	3	0	11

Figure 4. RGMa siRNA perturbs the formation of the retinotectal projection. ***A***, ***B***, *RCASBP(B)*-RGMa siRNA represses *RGMa* expression in the tectum 8 d after electroporation (E9.5, HH35). *RGMa* expression is repressed in the electroporated region (arrowheads) that is also immunolabeled with anti-gag antibodies. ***B*** is the control region on the opposite side of the same embryos in which gag expression was not detected. ***C–E***, *RCASBP(B)*-RGMa siRNA overexpression disturbed retinal axon trajectory. Ectopic overshooting axons (arrowheads) and arbors (arrows) are observed (***C***). The right panels are overlay images of DiI and DAPI staining of vibratome sections shown by lines in ***C***. DiI-labeled retinal axons make arbors in ectopic position (arrows). Terminal zone (TZ). ***D***, *In situ* hybridization of an *RCASBP(B)*-RGMa siRNA-2 electroporated embryo for RGMa (green) and immunostaining for gag (red). RGMa is repressed in gag-positive tectum cells. ***E***, *RCASBP(B)*-RGMa siRNA-2 overexpression also disturbed retinal axon projection. Overshooting axon and ectopic branches are seen (arrows). ***F***, Summary of the retinotectal phenotypes after RGMa gain- and loss-of-function experiments. Scale bars: ***B***, 100 μm; ***C***, ***E***, 500 μm; ***D***, 200 μm.

recent data suggest that RGMa is also a bone morphogenetic protein (BMP) coreceptor (Babitt et al., 2005), a perturbation of BMP signaling may also contribute to the observed phenotypes. The function of RGMa in cell differentiation/proliferation and survival (Matsunaga et al., 2004) could explain the neural tube closure defects observed in RGMa knock-out mice (Niederkofler et al., 2004).

A second RGM molecule, RGMb, is expressed in the nervous system in a complementary pattern to *RGMa* in mice (Niederkofler et al., 2004; Oldekamp et al., 2004; Schmidtmer and Engelkamp, 2004) and chick embryos (E. Matsunaga and A. Chédotal, unpublished data). We found that, although RGMb, like RGMa, blocks the neogenin proapoptotic activity (Matsunaga and Chédotal, unpublished data), it does not affect cell proliferation or neuronal differentiation. This result suggests that RGMa and RGMb have distinct function in the neural tube and that the observed defects are specific for RGMa.

RGMa is involves in topographic retinotectal projection in chick embryos

The targeting of temporal retinal axons is perturbed in embryos overexpressing RGMa in the optic tectum. Although many retinal fibers still project to their appropriate TZ, overshooting axons remained and the trajectory of retinal axons is severely perturbed, suggesting an aberrant homing. Interestingly, the perturbation of the layer-specific expression of *RGMa* appears to induce an ectopic retinal axon extension or branching into deeper layers. In addition, loss-of-function experiments with two distinct RGMa siRNA constructs also revealed that many axons projected and arborized at wrong positions in the tectum. These results indicate that RGMa, in addition to control the development of the retinotectal projection along the tectum rostrocaudal axis, may be involved in axonal pruning and the layer-specific arborization of retinal axons possibly by steering them away from tectal layers expressing RGMa. It will be important to study possible direct or indirect interactions between ephrins–Ephs and RGM–neogenin signaling to fully understand the molecular control of retinal axons pathfinding (Frisen et al., 1998; Feldheim et al., 2000; Yamada et al., 2001; Sakurai et al., 2002).

The phenotypic analysis of *RGMa* knock-out mice did not reveal any abnormal projections of visual axons (Niederkofler et al., 2004), and this could be attributable to species differences (McLaughlin et al., 2003; Niederkofler et al., 2004). First, *RGMa* is not expressed in a gradient in the superior colliculus of mouse embryos (Niederkofler et al., 2004). Second, *RGMb* is expressed in the mouse superior colliculus and may also influence the development of the retinocollicular projection. Additional studies, such as the analysis of visual projections in *RGMa*/*RGMb* double-mutant mice, will be required to answer this point. It will also be important to analyze the visual projection in neogenin knock-out mice.

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