

A role for S1P signalling in axon guidance in the *Xenopus* visual system

Laure Strohlic, Asha Dwivedy, Francisca P. G. van Horck, Julien Falk and Christine E. Holt*

Sphingosine 1-phosphate (S1P), a lysophospholipid, plays an important chemotactic role in the migration of lymphocytes and germ cells, and is known to regulate aspects of central nervous system development such as neurogenesis and neuronal migration. Its role in axon guidance, however, has not been examined. We show that sphingosine kinase 1, an enzyme that generates S1P, is expressed in areas surrounding the *Xenopus* retinal axon pathway, and that gain or loss of S1P function in vivo causes errors in axon navigation. Chemotropic assays reveal that S1P elicits fast repulsive responses in retinal growth cones. These responses require heparan sulfate, are sensitive to inhibitors of proteasomal degradation, and involve RhoA and LIM kinase activation. Together, the data identify downstream components that mediate S1P-induced growth cone responses and implicate S1P signalling in axon guidance.

KEY WORDS: Sphingosine 1-phosphate, Retinal ganglion cell, Axon pathfinding, Growth cone, Collapse, Guidance cue

INTRODUCTION

Lysophospholipids (LPs), such as sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA), are emerging as a new class of lipid mediators involved in a wide range of cellular processes such as cell proliferation, differentiation, apoptosis and chemotaxis (Spiegel et al., 2002; Anliker and Chun, 2004; Ishii et al., 2004). Initially recognized as components of cell membrane biosynthesis, the recent discovery of their ability to act as extracellular signals has pointed to an active role in cell-cell interactions. LPs bind to a family of G-protein coupled receptors (Spiegel et al., 2002; Hla, 2003; Spiegel and Milstien, 2003; Ishii et al., 2004). LP signalling plays a crucial role in the directed migration of a diverse array of cell types, such as lymphocytes, smooth muscle cells and germ cells, in both vertebrates and invertebrates (Renault and Lehmann, 2006). S1P, for example, acts as a chemoattractant in lymphocyte trafficking via the S1P₁ receptor in mice (Matloubian et al., 2004).

Directed migration is a key process in the establishment of nerve connections in the central nervous system (CNS). Axons from retinal ganglion cells (RGCs) in the eye exhibit an impressive ability to grow in a directed fashion to their targets in vivo and display chemotropic turning responses to extracellular gradients of guidance cues in vitro (de la Torre et al., 1997; Dingwell et al., 2000). Although numerous axon guidance molecules have been identified, the process of axon navigation is not fully understood (Dickson, 2002). LP receptors and their ligands are expressed in the developing CNS and several studies suggest that LPs play important roles in CNS development (Herr and Chun, 2007). The possibility that LPs play a role in guiding axon growth is indicated by experiments in vitro showing that LPA induces a rapid growth cone collapse of chick dorsal root ganglion (DRG) neurons and *Xenopus* RGC neurons, and stimulates neurite retraction in murine embryonic cortical neurons and in rat cerebellar granular neurons (Campbell and Holt, 2001; Ye et al., 2002; Fukushima, 2004). S1P induces opposite effects on neurite outgrowth in PC12 cells and rat DRG

neurons, promoting neurite extension via the S1P₁ receptor and the small GTPase Rac, and neurite retraction via the S1P₂ and S1P₅ receptors and Rho activation (Toman et al., 2004).

In the present study, we have investigated the role of S1P signalling in axon guidance in the visual pathway in *Xenopus*. We show that the growth cones of RGC axons exhibit strong repulsive chemotropic responses to S1P in vitro. S1P appears to work through a heparan sulfate-sensitive signalling pathway that involves the S1P₅ receptor, RhoA and Lim kinase activation, and proteasomal function. In vivo, sphingosine kinase 1 (SphK1), an enzyme that generates S1P, is expressed deep to the optic tract, and disruption of S1P function in vivo causes retinal axons to make pathfinding errors, particularly leading them to dive away from their normally superficial route at the tectal boundary. This work establishes sphingolipids as a new class of putative repulsive axonal guidance molecules in the brain and provides a framework for understanding how they might function.

MATERIALS AND METHODS

Embryos

Xenopus laevis embryos were obtained by in vitro fertilization, raised in 0.1 × Modified Barth's Saline at 14–20°C and staged according to the tables of Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Retinal cultures

Eye primordia were dissected from stage 24, 32 or 40 embryos and cultured at 20°C for 24 hours in culture medium (60% L15 + antibiotics, Gibco) on coverslips coated with poly-L-lysine (10 µg/ml, Sigma) and laminin (10 µg/ml, Sigma). In all the collapse (except for the stage-dependent collapse assay) and turning assays, stage 32 embryos were used.

Collapse assay

Collapse assay was performed as described previously (Luo et al., 1993). Various concentrations of S1P (Sigma) dissolved in methanol and diluted in culture medium or control medium (culture medium + methanol) were added to the cultures for 10 minutes. Cultures were then fixed in 2% paraformaldehyde (PFA) + 7.5% sucrose for 30 minutes and the number of collapsed growth cones counted. Values are presented as percentage of growth cone collapse ±s.e.m. from a minimum of four independent experiments. Statistical analysis was performed using a two-tailed Mann-Whitney *U* test. In all the experiments, unless notified, the S1P concentration used was 0.1 µM. LPA (1 µM) was purchased from Sigma.

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Growth cone turning assay

Stable gradients of S1P were formed as described (Lohof et al., 1992; de la Torre et al., 1997) by pulsatile ejection of S1P (300 nM) using a micropipette with a tip opening of 1 μm . Growth cones from 24-hour cultures of stage 32 retinal explants were positioned 100 μm from the tip opening at an angle of 45° relative to the initial direction of the axon shaft and observed at 20 \times magnification. Pictures were taken every 10 minutes for 1 hour. Turning angles were measured using Openlab software (Improvision). Statistical analysis was performed using a Kolmogorov-Smirnov test.

Pharmacological agents

The following pharmacological reagents were bath applied to cultures immediately prior to the application of S1P in the collapse and turning assays: lactacystin (10 μM ; Calbiochem; a specific inhibitor of the proteasome), N-acetyl-Leu-Leu-NorLeu-Al (LnLL; 50 μM ; Sigma; a proteasome inhibitor), anisomycin (40 μM ; Sigma; inhibits the peptidyltransferase activity on the ribosome), cycloheximide (25 μM ; Sigma; inhibits the translocation reaction on ribosomes), Y-27632 (Rho kinase inhibitor; 10 μM ; Sigma), bovine heparan sulfate (HS; 0.1 mg/ml; Sigma), heparin (0.1 mg/ml; Sigma), heparinase I (2.5 U/ml; Sigma).

Antibodies

Polyclonal antibodies against the first extracellular loop or the second cytoplasmic domain of human S1P₅ were purchased from Abcam (ab 13130) and IMGENEX (IMG-171371), respectively (diluted 1:100 for immunostaining and 1:500 for western blot). The IMGENEX human peptide sequence shares 89% and 83% identity with rat and mouse peptide sequences, respectively. Antibodies raised against the phosphorylated form of eIF4EBP-1 and against ubiquitin-conjugated proteins (FK2) were obtained from Cell Signaling Technology and Affiniti Research Products Limited, respectively (diluted 1:100). Antibodies against phosphorylated and total forms of LIM kinase were from Cell Signalling and BD Transduction Laboratories, respectively (diluted 1:100). Monoclonal antibody against acetylated tubulin was purchased from Sigma (diluted 1:500). The monoclonal antibody against neural cell adhesion molecule (NCAM, 6F11) recognizes the extracellular domain of NCAM (diluted 1:10) (Sakaguchi et al., 1989).

Immunohistochemistry

Twenty-four hour cultures of stage 32 retinal explants were incubated with S1P or control medium for 10 minutes, fixed in 2% PFA/7.5% sucrose, permeabilized with 0.1% Triton X-100, blocked in 10% goat serum, then labelled with primary antibodies and Cy3 or FITC secondary antibodies (1:1000, Chemicon) in 5% goat serum for 1 hour each, and mounted in FluoroSave™ (Calbiochem). Non-collapsed growth cones were visualized at 100 \times on a Nikon Optiphot inverted microscope. Using phase optics to avoid biased selection of fluorescence, a growth cone was randomly selected and an image was captured using a Hamamatsu digital CCD camera. A fluorescent image was then captured, the exposure time being kept constant and below greyscale pixel saturation. The quantification of fluorescence intensity was performed as described (Piper et al., 2006). Data are presented as percentage of control fluorescent intensity \pm s.e.m. Samples from four independent experiments were analyzed. Statistical analyses were performed using a two-tailed Mann-Whitney *U* test. Staining on eye and brain sections or wholemount embryos were performed as described previously (Walz et al., 1997).

Western blot analysis

Stage 32 and 40 *Xenopus* embryos heads or eyes, and E18 mouse brains were lysed in RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.1% SDS) and centrifuged for 10 minutes at 21,000 *g* at 4°C. Supernatants were solubilized in SDS-PAGE sample buffer, boiled for 5 minutes and run on a 12% SDS-PAGE, using a Bio-Rad Laboratories Mini Protean III slab cell. Proteins separated by gel electrophoresis were then transferred to nitrocellulose membranes (Schleicher and Schuell). Western blots using anti-S1P₅ antibodies were performed as described elsewhere (Strochlic et al., 2001), revealed with enhanced chemiluminescent detection (ECL+; Amersham Pharmacia Biotech), and exposed to Fuji X-ray films. For peptide blocking experiments, the S1P₅ antibody was incubated with the corresponding blocking peptide for 3 hours at room temperature.

In situ hybridization

In situ hybridization on stage 40 *Xenopus* brains was performed as described previously (Campbell et al., 2001). The *Xenopus* SphK1 image clone (number 6862150) was purchased from the MRC Geneservice (Cambridge, UK).

Exposed brain preparations and visualization of the optic projection

Exposed brain experiments were performed as described previously (Chien et al., 1993). Surgical procedures were carried out in 0.4 g/l MS222 (3-aminobenzoic acid ethyl ester methanesulphonate salt; Sigma) to anaesthetize embryos. Briefly, stage 35/36 embryos were immobilized by pinning to a Sylgard petri dish and the epidermis, dura and eye were removed from the left side of the head, exposing the underlying intact diencephalon. Embryos were transferred to experimental or control solutions in 1.3 \times MBS/0.1 g/l MS222 and allowed to develop to stage 40/41 at room temperature. For horseradish peroxidase (HRP) labelling, retinal axons from the right eye were labelled with HRP (type VI; Sigma), then fixed in 4% PFA. Brains were dissected out, reacted with diaminobenzidine (DAB; Sigma) and mounted projection side up in PBS with a coverslip supported by two reinforcement rings (Avery). Brains were then divided into classes according to their phenotypes and expressed as a percentage of the total number of brains (N) analyzed. N,N-dimethyl sphingosine (DMS) and FTY720 were purchased from Sigma and Cayman Chemical, respectively.

For trypan blue assay, exposed brains were incubated for 5 minutes in 0.4% trypan blue solution (Invitrogen), washed extensively in PBS, then fixed in 4% PFA. Dead cells were identified in wholemount brains.

Statistics

Statistical analyses were performed using Graphpad InStat3. Each experiment was conducted a minimum of four times.

RESULTS

S1P induces rapid collapse and repulsive turning of *Xenopus* retinal growth cones

Repulsive guidance cues cause the rapid withdrawal of filopodia in growth cones, providing a quantitative 'collapse' assay for measuring chemotropic responses. *Xenopus* retinal growth cones collapse transiently in response to LPA (Campbell and Holt, 2001; Campbell et al., 2001); therefore, the first question we addressed was whether S1P causes growth cone collapse. Indeed, S1P was found to induce dose-dependent collapse in growth cones from stage 32 retinal explants (cultured for 24 hour). Concentrations as low as 0.8 nM induced significant collapse (approximately 40%), and maximum collapse levels (62%; Fig. 1A) were reached at 10 μM . The collapse response was transient and was first detected at 5 minutes (32%), rising to peak levels at 10 minutes (53%), and gradually declining thereafter to control levels by 60 minutes (Fig. 1B-E).

We next asked whether a gradient of S1P could guide axon growth using the growth cone turning assay (Lohof et al., 1992; de la Torre et al., 1997). At a low concentration (0.3 nM in the micropipette corresponding to \sim 300 pM at the growth cone allowing for a dilution factor of 1000), S1P elicited repulsive turning in growth cones away from the micropipette (mean turning angle \sim -16°, Fig. 1F-I). These data show that growth cones are highly responsive to S1P and that a gradient repels their growth.

S1P₅ and heparan sulfate mediate S1P-elicited growth cone collapse

The S1P₅ receptor mRNA is detected in the optic tract in rat and is implicated in S1P-induced neurite retraction in PC12 cells (Im et al., 2000; Toman et al., 2004). Thus, we hypothesized that this receptor might be involved in S1P-elicited responses. First, we investigated whether retinal growth cones express the S1P₅ receptor. Because the

Xenopus S1P₅ sequence has not been reported, we used two antibodies raised against distinct portions of the 43 kDa human S1P₅ receptor. The first, S1P₅-EC, is directed against the first extracellular loop and the second, S1P₅-IC, is directed against the cytoplasmic domain (see Materials and methods). Both antibodies cross-reacted with the *Xenopus* S1P₅, recognizing a single 43 kDa band in *Xenopus* head and eye lysates (Fig. 2A,B) that was blocked by pre-incubation with sequence-specific peptides (Fig. 2C). Immunostaining of transverse eye sections (stage 39) using the S1P₅-IC antibody revealed a signal that was especially intense in the RGC layer (GCL) (Fig. 2D). Immunostaining of stage 32 retinal explants showed strong punctate labelling in growth cones, including the filopodia, that was abolished by pre-incubating the antibody with the corresponding blocking peptide (Fig. 2E, white dashed box).

To test whether the S1P₅ receptor plays a role in S1P-elicited growth cone responses, we performed collapse assays in the presence of the S1P₅-EC antibody. Retinal cultures were pre-

incubated with S1P₅-EC receptor or control IgG antibodies (20 μ g/ml) for 30 minutes before applying S1P for 10 minutes. Treatment with the S1P₅-EC receptor antibody, but not the control IgG, abolished the S1P-induced collapse (Fig. 2F). The S1P₅-EC antibody alone induced a small non-specific increase in growth cone collapse that was not significantly different from control (Fig. 2F). LPA-induced collapse was unaffected by the S1P₅ antibody verifying the specificity of the effect (Fig. 2G). These findings indicate that retinal growth cones express the S1P₅ receptor and that it may mediate, at least partially, S1P-induced collapse.

Heparan sulfates (HSs) can modulate receptor-ligand interactions (Van Vactor et al., 2006). We therefore wondered whether HS might play a role in S1P-elicited responses. To address this, we first performed collapse assays in the presence of HS (bovine) or heparin added immediately prior to S1P application for 10 minutes. This treatment abolished growth cone collapse induced by S1P suggesting that HS is important for S1P signalling (Fig. 2H).

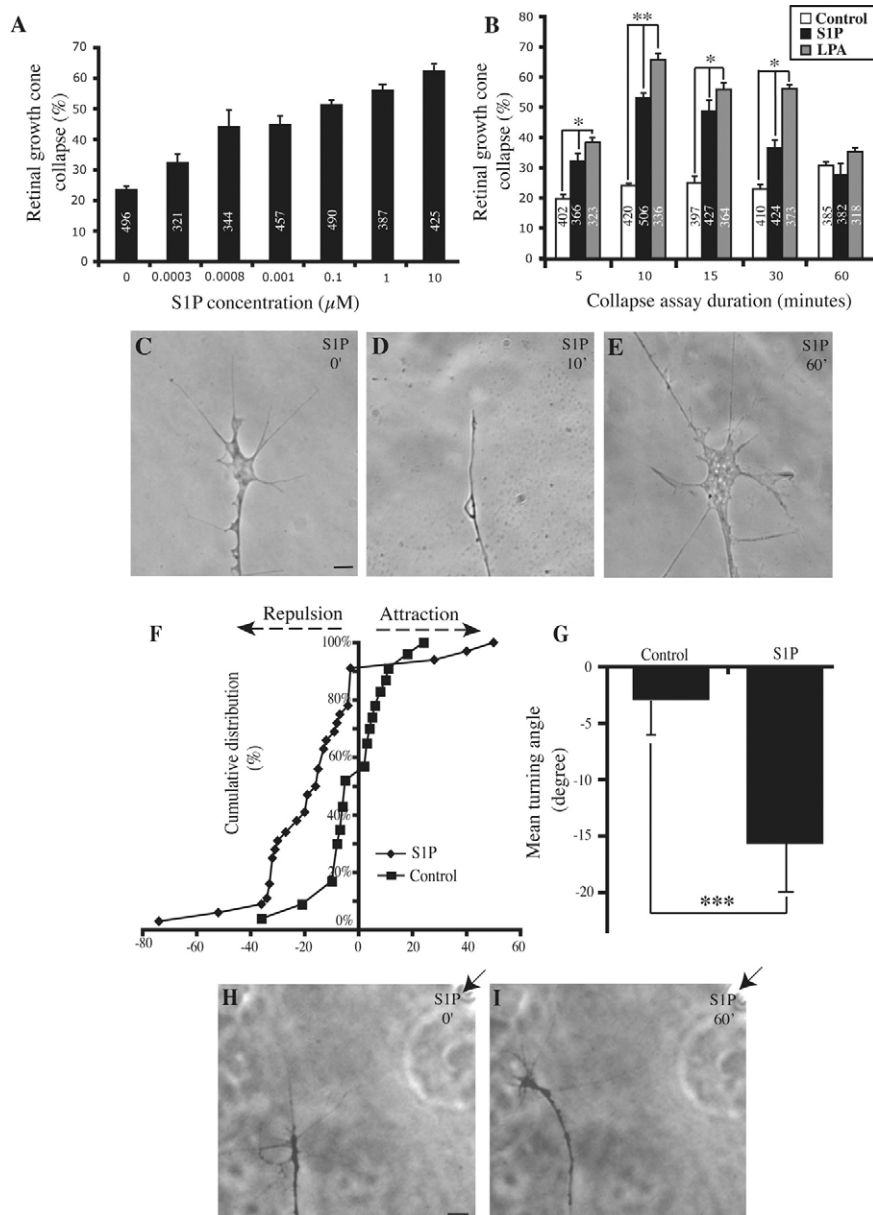
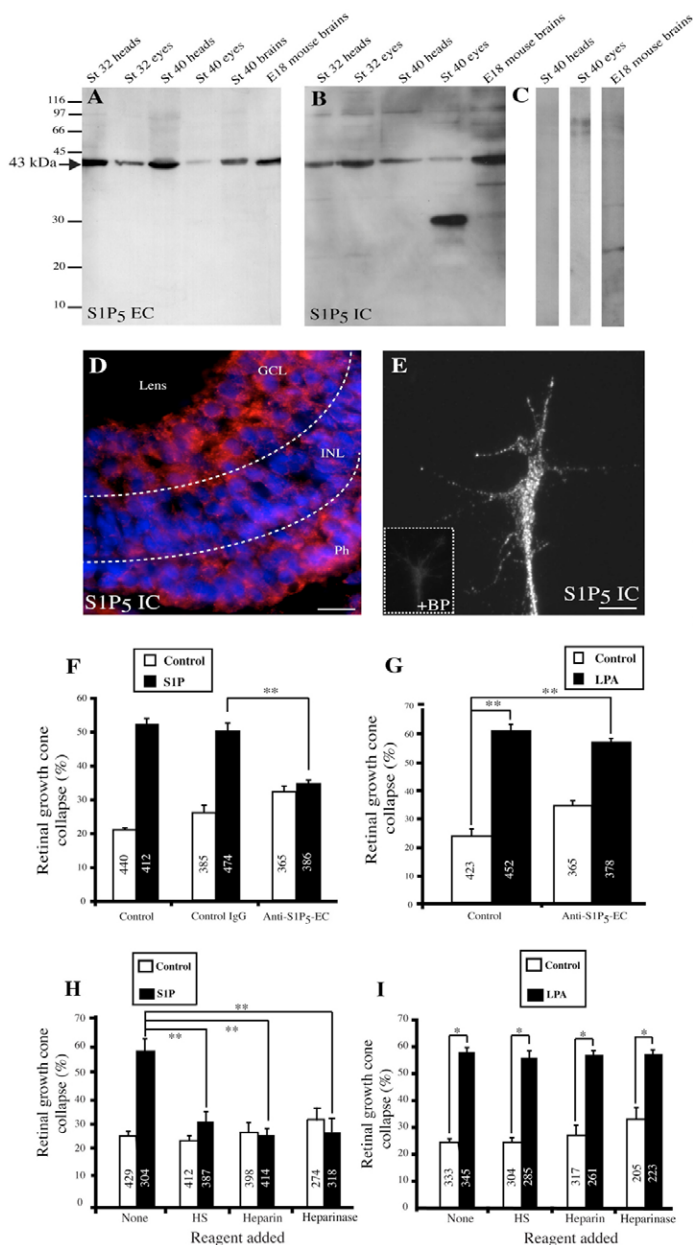


Fig. 1. S1P induces rapid collapse and repulsive turning of *Xenopus* retinal growth cones.

(A) S1P caused collapse in a dose-dependent manner of stage 32 retinal growth cones cultured for 24 hours. (B) S1P (0.1 μ M) and LPA (1 μ M) induced a transient collapse of stage 32 retinal growth cones cultured for 24 hours. (C) The morphology of a retinal growth cone before bath application of S1P. (D) A collapsed retinal growth cone 10 minutes after S1P application. (E) Recovery of a growth cone after 60 minutes of S1P treatment. For D,E, the concentration of S1P used was 0.1 μ M. (F,G) Cumulative frequency graph showing the distribution of growth cone turning angles after 60 minutes in the presence of a S1P or control gradient. Most of the turning angles are negative compared with control, indicating that S1P is repulsive to growth cones. (G) Mean turning graph from data in F. (H) 24-hour cultured stage 32 retinal growth cone before being exposed to a S1P gradient. (I) After 60 minutes the growth cone is repelled by a gradient of S1P. Numbers inside bars indicate growth cones tested. * P <0.05, ** P <0.01, Mann-Whitney U test. Scale bars: in C, 10 μ m for C-E; in H, 20 μ m for H,I.



Retinal explants were then treated with heparinase 1 at a concentration known to remove HS from the surface of retinal axons for 3 hours prior to the addition of S1P for 10 minutes (Walz et al., 1997). Heparinase treatment abolished S1P-induced growth cone collapse, indicating that endogenous HS participates in S1P-induced retinal growth cone collapse (Fig. 2H). Heparinase treatment alone was found to induce a small increase (5%) in growth cone collapse over control levels suggesting that endogenous HS may provide minor protection against collapse. Although statistically significant in this experiment, this increase has not been detected previously (Walz et al., 1997; Piper et al., 2006) and the level of collapse (30%) falls within the normal control range of collapse (20–35%). The increase may, therefore, represent variability in heparinase purity. To determine the specificity of the HS effect, collapse assays were performed using LPA. HS, heparin or heparinase did not affect the growth cone collapse response induced by LPA suggesting that both lysophospholipids signal via different pathways (Fig. 2I).

Fig. 2. S1P₅ and heparan sulfates mediate S1P-elicited growth cone collapse. (A, B) Western blot from E18 embryonic mice brain extracts, and *Xenopus* brain, head or eye lysates of stage 32 and 40 embryos, probed with S1P₅ antibodies (S1P₅-EC and S1P₅-IC). One band of 43 kDa corresponding to the molecular weight of S1P₅ was detected. In stage 40 eye lysate, the S1P₅-IC antibody detected an additional band of 30 kDa that may correspond to a degradation product. (C) Western blot from E18 mice brain extracts and *Xenopus* stage 40 head and eye lysates using the S1P₅-IC antibody incubated with the corresponding blocking peptide. No band of 43 kDa was detected. (D) Transverse cryostat sections (12 μm) of a stage 39 eye immunostained with the S1P₅-IC antibody (red) together with DAPI (nuclei dye, blue). S1P₅ expression was detected in all the layers, including the retinal ganglion cell layer, which was intensively labelled. (E) Expression of S1P₅ in growth cone from stage 32 embryos cultured for 24 hours using the S1P₅-IC antibody. Pre-incubation of the antibody with the corresponding peptide blocked the signal expression (white dashed box). (F) The S1P₅-EC antibody inhibited S1P-induced retinal growth cone collapse. S1P₅-EC antibody was bath-applied in culture for 30 minutes before the addition of S1P. (G) S1P₅-EC antibody did not affect LPA-induced growth cone collapse. (H) S1P-induced retinal growth cone collapse is blocked by the addition of heparan sulfate (HS), heparin or heparinase treatment. HS and heparin were added immediately prior to the S1P application in stage 32 retinal explants cultured for 24 hours. Retinal explant cultures were treated with heparinase for 3 hours before S1P application. (I) LPA-elicited growth cone collapse was not affected by HS, heparin or heparinase. LPA concentration: 1 μM. Numbers inside bars indicate growth cones tested. **P*<0.05, ***P*<0.01, Mann-Whitney *U* test. Scale bars: D, 20 μm; E, 10 μm. GCL, ganglion cell layer; INL, inner nuclear layer; Ph, photoreceptor layer.

S1P-induced responses are sensitive to inhibitors of proteasomal degradation

Chemotropic responses of *Xenopus* retinal growth cones to LPA have been shown to involve local protein degradation (Campbell and Holt, 2001; Campbell and Holt, 2003). Therefore, we investigated whether S1P-mediated collapse also depends on protein degradation by performing collapse and turning assays in the presence of proteasomal inhibitors [N-acetyl-Leu-Leu-NorLeu-Al (LnLL) and lactacystin (Lacta)]. As was previously shown for LPA, S1P-induced collapse was blocked by inhibitors of proteasomal degradation but not by protein synthesis inhibitors (Fig. 3A). Moreover, repulsive turning to a S1P gradient was abolished in the presence of LnLL and Lacta (applied immediately prior to the start of the turning assay; Fig. 3B,C). Proteins targeted for degradation by the proteasome are tagged with ubiquitin (Hershko and Ciechanover, 1998). Quantitative immunofluorescence (Q-IF) analysis using antibodies that specifically recognize ubiquitin-protein conjugates (FK2) revealed that, within 10 minutes, S1P caused an approximately 80% increase in the FK2 immunofluorescence signal whereas levels of eIF4EBP-1-P signal (a marker for translation initiation) remained unchanged (Fig. 3D,E). These data indicate that, like LPA, S1P induces chemotropic responses in growth cones via a pathway that requires proteasomal degradation, not translation.

S1P signalling pathway involves the activation of RhoA and LIM kinase, a degradation target

The Rho family of small GTPases link guidance signals to cytoskeletal rearrangements (Luo, 2000; Dickson, 2001). To investigate whether S1P and LPA signalling depends on RhoA

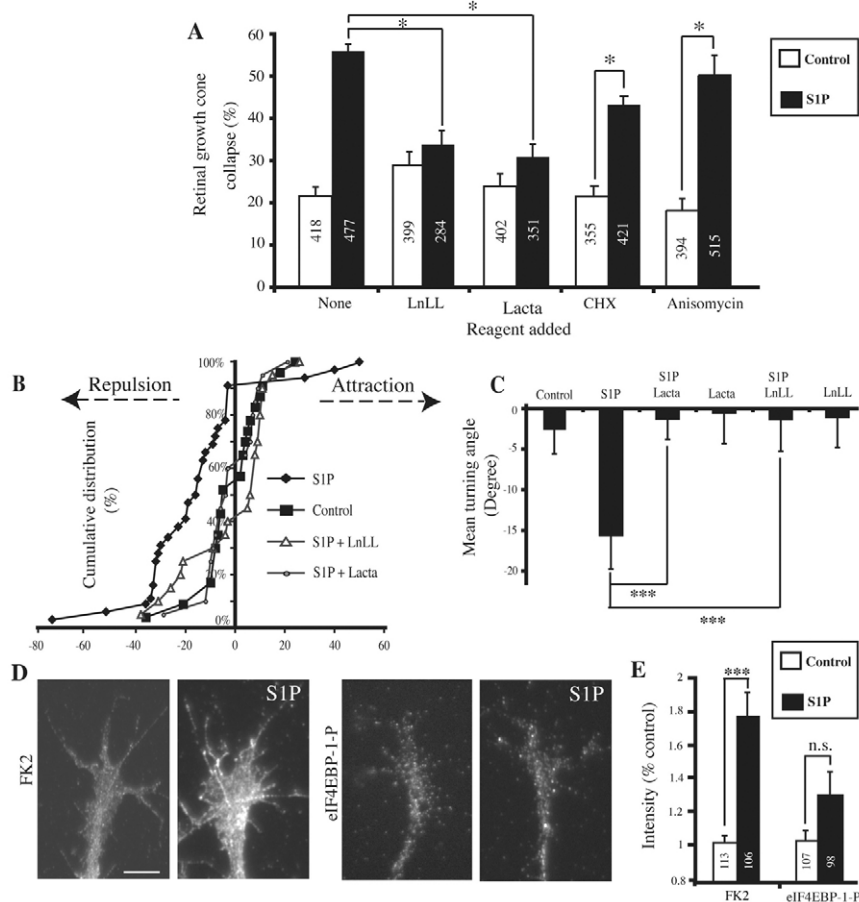


Fig. 3. S1P-induced responses are sensitive to inhibitors of proteasomal degradation.

(A) S1P-induced growth cone collapse (stage 32 embryos cultured for 24 hours) is blocked by proteasome inhibitors (LnLL, Lacta). Conversely, translation inhibitors (CHX, Anisomycin) had no effect on S1P-induced growth cone collapse.

* $P < 0.05$, Mann-Whitney U test. (B,C) Cumulative distribution of turning angles after LnLL and Lacta treatment. S1P-induced repulsive turning of growth cones is blocked by proteasomal inhibitors. (C) Mean turning graph from data in C. *** $P < 0.005$, Kolmogorov-Smirnov test.

(D,E) Quantitative immunofluorescence analysis using antibodies directed against ubiquitin-protein conjugates (FK2) or the phosphorylated form of eIF4EBP-1 (eIF4EBP-1-P).

(D) Representative pictures of FK2 and eIF4EBP-1-P immunoreactivities within growth cones. (E) S1P caused a significant increase in FK2 signal when compared with the control level, whereas the eIF4EBP-1-P signal intensity remained unchanged. Numbers inside bars indicate growth cones tested. *** $P < 0.005$, Mann-Whitney U test. n.s., non significant. Scale bar in D: 10 μm .

activation, collapse and turning assays were performed in the presence of the RhoA kinase inhibitor Y-27632. This treatment abolished both S1P and LPA-elicited growth cone collapse (Fig. 4A), and S1P turning (Fig. 4B,C), suggesting that RhoA activation is an important step in S1P and LPA signalling pathways. If RhoA is required for S1P and LPA signalling in retinal growth cones, then it is also likely that established effectors of RhoA, such as LIM kinase, are involved (Kalil and Dent, 2005). To investigate whether S1P and LPA signalling involves LIM kinase, we performed Q-IF analysis after 2 minutes and/or 5 minutes of S1P or LPA treatment of stage 32 retinal cultures using antibodies directed against either the phosphorylated or the total form of the protein (LIMK-P or LIMK). We found that at 2 minutes (well before most growth cones have started to collapse) S1P induced an ~60% increase in LIMK-P, whereas the total LIMK signal remained unchanged, suggesting that S1P induces rapid activation of LIM kinase (Fig. 4D,E). Interestingly, the LIMK-P signal was not affected by LPA treatment, indicating that LPA signalling does not require LIM kinase activation and that the two guidance cues activate specific pathways in the growth cone (Fig. 4D). However, at 5 minutes, unexpectedly, S1P induced an ~30% decrease in both phosphorylated and total forms of LIM kinase (Fig. 4D,E). Given that S1P-induced growth cone collapse requires proteasomal protein degradation, and that LIM kinase can be polyubiquitinated and targeted to the proteasome in growth cones of primary hippocampal neurons (Tursun et al., 2005), we wished to determine whether this decrease is dependent on protein proteasomal degradation. To test this, lactacystin was bath-applied for 10 minutes before the S1P treatment, then Q-IF was performed on growth cones using LIM kinase antibodies.

Lactacystin treatment abolished the decrease in Q-IF with both antibodies (phosphorylated and total), indicating that the proteasomal degradation pathway is responsible for the S1P-stimulated reduction in LIM kinase (Fig. 4D,E). Given that S1P₅ is expressed in retinal growth cones, we investigated whether S1P₅ is colocalized with the downstream effector phosphorylated LIM kinase. Double immunostaining experiments on retinal growth cones using S1P₅IC and LIMK-P antibodies showed that the phosphorylated (active) form of LIM kinase is significantly colocalized with S1P₅ (quantitative analysis of the colocalization was done using the Intensity Correlation Analysis plugin; Pearson's correlation coefficient: $R_r = 0.745 \pm 0.02$; split Mander's colocalization coefficients: $M_1 = 0.92 \pm 0.012$ and $M_2 = 0.96 \pm 0.007$; $n = 35$ growth cones analysed; see Fig. S1A-C in the supplementary material). This result is consistent with the idea that S1P₅ and the active form of LIM kinase are part of the same signalling pathway. Collectively, these data suggest that S1P and LPA-elicited retinal growth cone collapse require RhoA activation, and that S1P signalling involves LIM kinase activation followed by local proteasomal degradation of LIM kinase.

Gain and loss of S1P signalling cause axon pathfinding errors in vivo

If S1P is involved in RGC axon guidance in vivo, it should be present in the developing *Xenopus* visual pathway. There are, however, few antibodies to sphingolipids and none to S1P that can be used for immunocytochemistry. We therefore examined the distribution of sphingosine kinase (SphK), the enzyme that specifically phosphorylates sphingosine to produce S1P (Saba and

Hla, 2004). In situ hybridization (ISH) performed on stage 40 *Xenopus* wholemount brains showed that SphK1 is expressed widely in the forebrain and midbrain, except in areas close to the dorsal midline (Fig. 5B). When wholemount brains were processed for both ISH and anterograde horseradish peroxidase (HRP)-filling to visualize the RGC axons and the SphK1 signal simultaneously, the axons appeared to be growing in SphK1-positive territory (Fig. 5C). However, transverse sections of the diencephalon revealed that SphK1 is predominantly in the intermediate zone of the neuroepithelium, deep to where retinal axons travel, and is particularly highly expressed in this intermediate zone at the diencephalon/tectal border (Fig. 5D,E). Therefore, the superficially located retinal axons appear to grow in a SphK1-poor area where they are bounded by SphK1-rich territory.

To test whether S1P signalling is involved in retinal axon guidance in vivo, we exposed the developing optic tract to reagents that specifically interfere with S1P signalling during the 12-18 hour period when the RGC axons first pioneer the optic tract (Chien et al., 1993). RGC axons normally follow a stereotyped pathway through the diencephalon and then enter the optic tectum (Fig. 6A,E). With exogenous application of S1P, RGC axons showed

abnormally short projections (Table 1B). When RGC axons in exposed brains did reach the optic tract/tectal border, they often failed to enter the tectum and instead veered sharply in dorsal or ventral directions (Fig. 6B,F). 5 μ M S1P caused 44% of the projections to veer aberrantly at the tectal border, whereas 10 μ M S1P caused this phenotype in the majority of cases (75%, Table 1A). We then wished to determine whether a loss of S1P function also results in axon pathfinding errors. To investigate this, we exposed optic tracts to N, N-dimethyl sphingosine (DMS), a well-known inhibitor of the SphK enzymes (Edsall et al., 1998). Indeed, with exogenous DMS application, axons bypassed the tectum in 50% of the embryos, similar to that observed with the S1P application (Fig. 6C,G; Table 1A). Importantly, when 1 μ M S1P (sub-threshold concentration for inducing axon pathfinding errors) was added along with DMS it rescued, at least partially, the DMS effect, as most axons reached the tectum (addition of S1P decreased by \sim 2-fold the tectum bypass phenotype compared with DMS; see Fig. S2A-C in the supplementary material). These data indicate that either gain or loss of S1P function in vivo results in target recognition errors and raise the possibility that S1P helps to guide axons into the tectum.

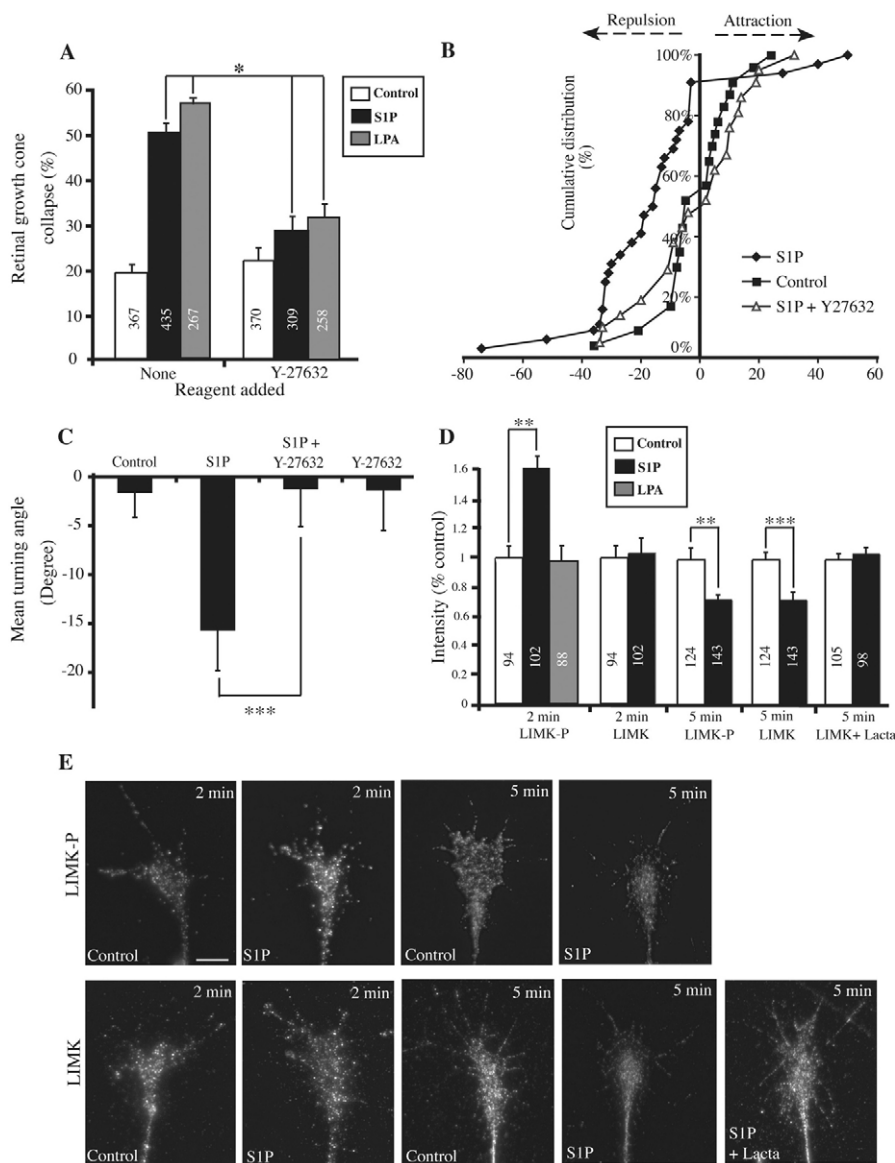


Fig. 4. S1P signalling pathway involves the activation of RhoA and LIM kinase, a degradation target. (A) S1P- and LPA-induced retinal growth cone collapse are blocked by RhoA kinase inhibitor (Y-27632). Y-27632 was added immediately prior to the addition of S1P in stage 32 retinal explants cultured for 24 hours. * $P < 0.05$, Mann-Whitney U test. (B,C) Cumulative distribution of turning angles after Y-27632 treatment. The repulsive response elicited by S1P is inhibited by application of Y-27632 prior to the start of the turning assay. (C) Mean turning graph from data in B. *** $P < 0.005$, Kolmogorov-Smirnov test. (D) Quantitative immunofluorescence analysis of LIMK-P and LIMK immunoreactivities. A 2-minute S1P treatment caused a significant increase in LIMK-P, whereas LIMK signal remained unchanged. LIMK-P signal was not affected by a 2-minute LPA treatment. At 5 minutes, S1P induced a significant decrease of both LIMK-P and LIMK immunoreactivities within growth cones when compared with control. This decrease is abolished by lactacystin treatment. (E) Representative pictures of LIM kinase-P (LIMK-P) and LIM kinase (LIMK) immunoreactivities within growth cones treated with S1P for 2 or 5 minutes, or when lactacystin was applied 10 minutes before S1P application. Numbers inside bars indicate growth cones tested. ** $P < 0.01$, *** $P < 0.005$, Mann-Whitney U test. Scale bar in D: 10 μ m.

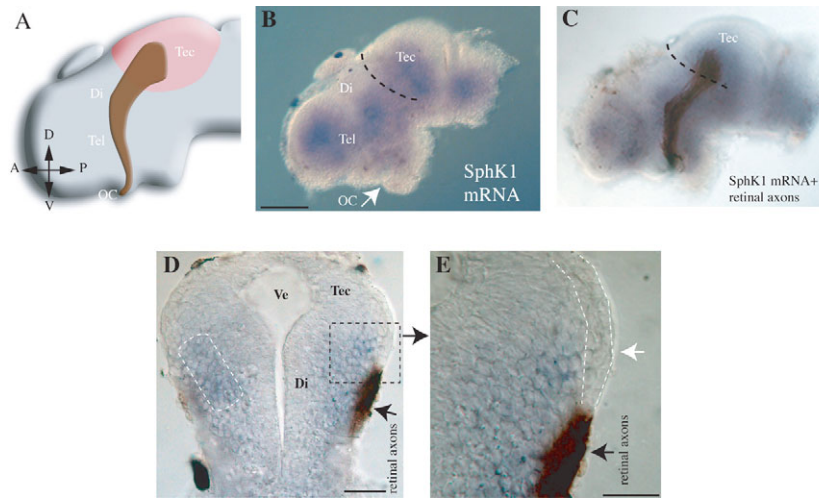


Fig. 5. Sphingosine kinase 1 mRNA expression in the developing optic pathway. (A) Schematic diagram of a stage 40 *Xenopus* brain showing the optic pathway followed by retinal axons leaving the eye. OC, optic chiasm; Tel, telencephalon; Di, diencephalon; Tec, tectum. (B,C) Lateral views of stage 40 *Xenopus* brains, showing *SphK1* mRNA expression alone (B) or together with retinal ganglion cell (RGC) axons visualized by HRP (brown, C). The dashed line qualitatively demarcates the anterior tectal border. (D,E) Transverse sections (60 μm) through the diencephalon and tectum of a stage 40 embryo showing *SphK1* mRNA expression (B, blue) and RGC axons. Cells surrounding the migratory route of RGC axons are intensively labelled (dashed white box, D). (E) High magnification of D. *SphK1* mRNA signal is absent in axon tracts (dashed white line and arrow). Ve, ventricle. Scale bars: in B, 100 μm for B,C; in D, 100 μm ; in E, 50 μm .

Exogenously applied S1P could indirectly impair RGC axon pathfinding by perturbing the neuroepithelial substrate. Thus, S1P-treated brains (5 μM) were also labelled with a neuronal cell marker, neural cell adhesion molecule (NCAM), and an early axon tract marker, acetylated tubulin (Walz et al., 1997), and examined as wholemounts. No obvious differences were detected in the pattern or intensity of marker expression between S1P-treated and control brains (see Fig. S3A-D in the supplementary material). S1P-treated (5 μM) and control brains were also exposed to trypan blue, a vital dye that stains dead cells. No significant difference in the number of labelled cells between S1P-treated and control brains was observed (forebrain, 22 ± 4 cells in the S1P-treated embryos compared with 18 ± 4 cells in controls, $P > 0.2$; tectum, 26 ± 4 cells in S1P-treated embryos compared with 21.5 ± 4 cells in controls, $P > 0.2$; $n = 12$ embryos analyzed per condition). These results indicate that exogenous S1P does not exert its effect by disrupting the general organization of neuroepithelium or scaffold axon tracts, or cause widespread cell death.

Finally, we investigated whether interfering with S1P receptor function also affects axon pathfinding. To perturb receptor signalling, we used FTY720, a sphingosine analogue that is phosphorylated *in vivo* by SphKs and acts as an agonist to S1P receptors including S1P₅ (Gardell et al., 2006; Herr and Chun,

2007), although it is also reported to act as a functional antagonist (Jo et al., 2005; LaMontagne et al., 2006). FTY720 (5 μM) caused a striking axon pathfinding phenotype, similar to that found with S1P treatment, indicating that FTY720 may act as an agonist to S1P receptors in this system (Fig. 6D,H; Table 1A). Examination of the projection in transverse sections shows that the HRP-labelled axons stray away from the pial surface and penetrate deep into the neuroepithelium, particularly at the optic tract/tectal border where axons of exposed brains make major pathfinding errors (Fig. 6I-M). Indeed, measurements of the width (superficial-deep dimension) of the axon projection in transverse sections show that the FTY720 treatment increases the spread of axons by about twofold, indicating that many axons travel abnormally deep in the neuroepithelium (Fig. 6N). Together, these results suggest that S1P may help to confine growing retinal axons to the superficial neuroepithelium by repelling them from the deeper layers.

DISCUSSION

We have investigated the role of S1P signalling in *Xenopus* RGC axon pathfinding. *In vitro*, S1P induces the collapse and repulsive turning of retinal growth cones. These chemotropic responses are HS dependent, and are mediated via protein proteasomal degradation and RhoA and Lim kinase activation. Moreover, *in vivo*

Table 1. Quantitative analysis of the retinal axon pathfinding phenotypes

A. Percentage of total embryos showing either a normal trajectory, a shortened phenotype or target recognition errors

	Phenotypes		
	Normal trajectory	Shortened	Target recognition errors
Control ($n=35$)*	87% (30)	14% (5)	0% (0)
S1P 5 μM ($n=34$)	29.5% (10)	29% (9)	44% (15)
S1P 10 μM ($n=8$) [†]	12.5% (1)	12.5% (1)	75% (6)
DMS 12 μM ($n=27$)	52% (14)	0% (0)	48% (13)
FTY720 5 μM ($n=28$)	29% (8)	21% (6)	50% (14)

B. S1P effects on the outgrowth of RGC axons[‡]

Axon length	400-500 μm (A)	250-400 μm (B)	0-250 μm (C)
Control ($n=15$)	87%	13%	0%
S1P 5 μM ($n=34$)	44%	44%	12%
S1P 10 μM ($n=8$)	37.5%	50%	12.5%

* n , total number of embryos analyzed. Numbers in parentheses indicate number of embryos.

[†]Concentration producing a general toxic effect on embryonic development.

[‡]The extent to which fibers had grown along from the optic chiasm towards the tectum was measured in each embryo. The embryos were divided into three classes (A, B and C) according to the extent of outgrowth measured. The number of brains showing a given class of outgrowth is expressed as a percentage of the total number of brains analyzed (n).

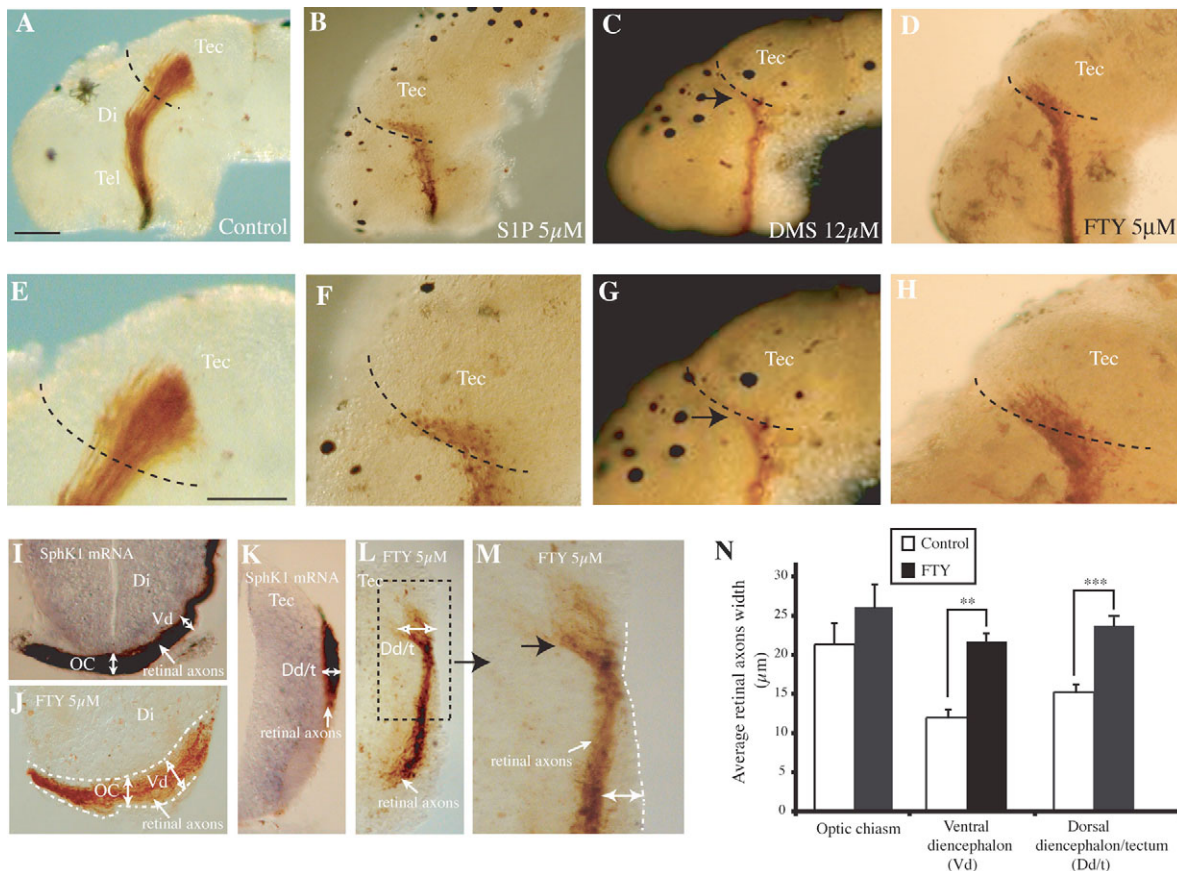


Fig. 6. Gain and loss of S1P signalling cause axon pathfinding errors in vivo. (A-H) HRP-filled optic projection of RGC axons in a lateral view of wholemount brain. Brains were exposed to S1P, DMS or FTY720 for 16 hours from stage 35/36 to 40/41. E-H are higher magnification views of A-D. (A,E) Control projection formed a defined optic tract in the diencephalon and crossed into the tectum. (B,F) An example of retinal axons exposed to S1P (5 μ M) that failed to reach the tectum causing a bypass phenotype. (C,D,G,H) Projections exposed to DMS (12 μ M; C,G) or to FTY720 (5 μ M; D,H) failed to enter the tectum and axons bifurcated dorsally around the target area (black arrow in C and G). (I-M) Transverse sections of brains treated with FTY720 (J,L,M) showing the trajectory of HRP-labelled axons (brown) through the diencephalon/tectum compared with a control brain (SphK1 ISH; I,K). (M) Higher magnification view of L (black dashed box in L). Axons exposed to FTY720 penetrated deeper into the neuroepithelium (white dashed lines and arrows). (N) Quantitative analysis of the axon projection width (superficial-deep dimension). Three measurements were made: at the optic chiasm (OC), ventral diencephalon (Vd) and dorsal diencephalon/tectum (Dd/t) (see white arrows in I-L). FTY720 treatment increased the spread of axons by about twofold in the Vd and Dd/t. ** $P < 0.01$, *** $P < 0.005$; Mann-Whitney U test. $n = 5$ brains analyzed. Tel, Telencephalon; Di, Diencephalon; Tec, Tectum. Scale bars: in A, 100 μ m for A-D,L; in E, 50 μ m for E-H,M.

interference with S1P signalling leads to pathfinding errors. Collectively, the data support the hypothesis that S1P acts as a chemotropic cue for retinal axons that helps to guide them towards their final destination in the optic tectum.

To be classified as an axon guidance molecule for RGC axons, a candidate molecule must fulfil several criteria: (1) it must be able to steer axon growth in vitro and in vivo; (2) interfering with its function should result in axon guidance and/or targeting errors in vivo; and (3) it should be expressed at the right time and place for growing axons to encounter it. Our in vitro and in vivo data showing that S1P induces the collapse and turning of retinal growth cones, and that perturbation of S1P signalling causes target recognition errors satisfy the first two criteria. The finding that FTY720 induced axon pathfinding errors supports the hypothesis that S1P acts as a ligand exerting its effect through binding to receptors in the growth cone and not as a second messenger. Regarding the third criterion, although it was not possible for us to monitor S1P directly, the pattern of expression of *SphK1* mRNA indicates that S1P is likely to be expressed in the right place to influence RGC axon growth. In *Drosophila*, the Wunen genes encode phosphatidic acid

phosphatases that are involved in generating an extracellular gradient of S1P that directs the migration of germ cells (Zhang et al., 1997). This raises the intriguing possibility that a gradient(s) of S1P may exist in the neuroepithelium, providing polarized guidance information to growing axons.

Retinal axons require multiple cues that instruct them to grow, navigate and stop when they reach their target (Dingwell et al., 2000). S1P signalling may be involved in two aspects of axon guidance: (1) limiting growth to the superficial neuroepithelium; and (2) target recognition. The finding that retinal axons travel aberrantly in deep locations in the neuroepithelium after perturbing S1P receptor function suggests that S1P may normally act to repel retinal axons from deeper regions of the neuroepithelium, providing a possible mechanism to limit their growth to a superficial path. S1P repellent activity in vivo is also supported by the 'short tract phenotype' observed in S1P-treated brains. Indeed, we can speculate that this phenotype arises as a result of 'surround repulsion' (Keynes et al., 1997) giving rise to retarded axon growth. DMS treatment, however, would not be expected to slow down axon growth because it reduces the inhibitory influence of S1P.

S1P may also play a role in target recognition, as axons in brains with disrupted S1P signalling consistently show pathfinding errors at the entrance to the tectum. Target recognition is also regulated in part by FGF signalling (McFarlane et al., 1995; McFarlane et al., 1996) and, interestingly, the tectal bypass phenotype that S1P induces is similar to that observed when FGF signalling is disrupted. Indeed, S1P is known to induce *FGF2* mRNA expression in rat astrocytes and in C6 glioma cells (Sato et al., 1999; Sato et al., 2000), and, reciprocally, it has recently been shown that FGF2 causes extracellular release of S1P in cerebellar astrocytes (Bassi et al., 2006), whereas S1P₅ gene expression is downregulated by FGF2 in PC12 cells (Glickman et al., 1999). Thus, cross-talk between the S1P and FGF signalling pathways may be critically involved in target recognition by RGCs.

In addition, our *in vitro* results show that HS is involved in S1P- but not LPA-elicited growth cone collapse. HS is a key cofactor implicated in the binding of various guidance cues to their specific receptors and can modulate ligand-receptor interactions. For example, HS is required for FGF/FGFR and Slit/Robo signalling interactions (Yayon et al., 1991; Hu, 2001; Steigemann et al., 2004). Interestingly, HS addition or removal also causes tectal recognition errors similar to those reported here (McFarlane et al., 1995; Walz et al., 1997; Irie et al., 2002). Although the exact mechanism is not fully understood, it is intriguing to speculate that HSs modulate the interactions between S1P and its receptor. By contrast, HSPGs such as syndecan have been shown to interact with inositol phospholipids and are involved in the transactivation of the SphK1 to produce S1P (Couchman, 2003; Kaneider et al., 2003; Kaneider et al., 2004). This suggests that HS plays a complex role in S1P signalling *in vivo* that may include regulating the production of S1P.

The immunodetection and functional antibody data support the idea that S1P₅ is the receptor involved in S1P-elicited repulsive responses in retinal growth cones. S1P signalling is mediated via at least three families of G proteins, G_i, G_q and G_{12/13} (Ishii et al., 2004; Chun, 2005). S1P₅ is coupled to the three G proteins, like the other S1P receptors (except S1P₁), and is known to activate RhoA via G_{12/13}, which is consistent with our finding that S1P-induced growth cone responses are blocked by RhoA inhibition (Chun, 2005). However, because S1P₅ has not been identified in the *Xenopus* gene database, the results should be interpreted with caution. The only *Xenopus* S1P receptor identified is S1P₁, but this receptor is known to be coupled to G_i and to activate Rac, not RhoA, and is, therefore, not a likely candidate for the RhoA-mediated chemotropic responses reported here (Anliker and Chun, 2004). Other candidate receptors are S1P₂ and S1P₃. Indeed, S1P₂ expression is detected in extending axons from neurons in the developing rat brain (MacLennan et al., 1997), and overexpression of S1P₂ and S1P₃ in PC12 cells promotes neurite retraction via RhoA stimulation (Toman et al., 2004). Further characterization will be needed to identify definitively which specific S1P receptors mediate the growth cone responses in *Xenopus*.

Our data demonstrate that S1P- and LPA-mediated chemotropic responses are sensitive to RhoA inhibition. In line with this, S1P-elicited neurite retraction in PC12 cells and in rat primary oligodendrocyte cultures requires RhoA activation mediated by several S1P receptors, including S1P₅ (Toman et al., 2004; Jaillard et al., 2005). Furthermore, in *Xenopus* spinal neurons, chemorepulsion induced by a gradient of LPA is abolished upon expression of a dominant-negative RhoA (Yuan et al., 2003). Many factors that influence axon growth, including slits, semaphorins, ephrins and netrins, are known to regulate the activity of Rho GTPases (Wahl et al., 2000; Whitford and Ghosh, 2001; Wong et al.,

2001; Li, X. et al., 2002) and perturbation of the activity of Rho GTPases leads to axon pathfinding defects (Dickson, 2001; Li, Z. et al., 2002; Yuan et al., 2003).

Proteasomal degradation is required for LPA- and netrin-induced responses in *Xenopus* retinal growth cones (Campbell and Holt, 2001; Campbell and Holt, 2003), yet the proteins that are degraded remain unknown. Interestingly, our finding that S1P causes a degradation-dependent decrease in LIM kinase identifies LIM kinase, a RhoA effector, as a possible candidate (Kalil and Dent, 2005). In hippocampal neurons, LIM kinase can be polyubiquitinated and targeted to the proteasome in growth cones (Tursun et al., 2005). However, it is puzzling that a two-minute S1P stimulation in growth cones resulted in an increase in LIMK-P immunoreactivity, as it is known that LIM kinase activation results in the phosphorylation and inactivation of cofilin, thus inhibiting actin depolymerization (Arber et al., 1998; Yang et al., 1998). However, recycling of cofilin may be necessary for growth cone collapse (Aizawa et al., 2001), as once cofilin depolymerizes actin it becomes sequestered. Exposure of DRG neurons to Sema3A induces phosphorylation of LIM kinase, which transiently phosphorylates cofilin (Aizawa et al., 2001). Our results show that by 5 minutes of S1P stimulation, LIM kinase immunoreactivity in growth cones is decreased. This proteasomal degradation of active LIM kinase could then shift the balance further in favour of active cofilin, leading to actin depolymerization and collapse. Another possibility is that the degradation of LIM kinase might be necessary to regulate the level of signalling proteins needed for the collapse response, thus allowing a transient chemotropic response by the growth cone. Indeed, our data show that the collapse response to S1P is transient, as only 37% of the growth cones were collapsed by 30 minutes of S1P stimulation compared with 53% at 10 minutes. However, these ideas are speculative and much remains to be done to understand how this pathway is used by S1P.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/2/333/DC1>

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