

## On the turning of *Xenopus* retinal axons induced by ephrin-A5

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### SUMMARY

The Eph family of receptor tyrosine kinases and their ligands, the ephrins, play important roles during development of the nervous system. Frequently they exert their functions through a repellent mechanism, so that, for example, an axon expressing an Eph receptor does not invade a territory in which an ephrin is expressed. Eph receptor activation requires membrane-associated ligands. This feature discriminates ephrins from other molecules sculpturing the nervous system such as netrins, slits and class 3 semaphorins, which are secreted molecules. While the ability of secreted molecules to guide axons, i.e. to change their growth direction, is well established in vitro, little is known about this for the membrane-bound ephrins. Here we set out to investigate – using *Xenopus laevis* retinal axons – the properties of substratum-bound and (artificially) soluble forms of ephrin-A5 (ephrin-A5-Fc) to guide axons.

We find – as expected on the basis of chick experiments – that, when immobilised in the stripe assay, ephrin-A5 has a repellent effect such that retinal axons avoid ephrin-A5-Fc-containing lanes. Also, retinal axons react with repulsive

turning or growth cone collapse when confronted with ephrin-A5-Fc bound to beads. However, when added in soluble form to the medium, ephrin-A5 induces growth cone collapse, comparable to data from chick.

The analysis of growth cone behaviour in a gradient of soluble ephrin-A5 in the 'turning assay' revealed a substratum-dependent reaction of *Xenopus* retinal axons. On fibronectin, we observed a repulsive response, with the turning of growth cones away from higher concentrations of ephrin-A5. On laminin, retinal axons turned towards higher concentrations, indicating an attractive effect. In both cases the turning response occurred at a high background level of growth cone collapse. In sum, our data indicate that ephrin-As are able to guide axons in immobilised bound form as well as in the form of soluble molecules. To what degree this type of guidance is relevant for the in vivo situation remains to be shown.

Key words: Axon guidance, Retinotectal projection, Ephrin-A5, EphA receptor, *Xenopus*, Growth turning assay, Growth cone collapse

### INTRODUCTION

In recent years several gene families involved in patterning the nervous system have been identified. Among them is the Eph family of receptor tyrosine kinases and their ligands, the ephrins. This large family is subdivided into the EphA family with EphA receptors interacting with glycosylphosphatidylinositol- (GPI) anchored ephrin-As and the EphB family with EphB receptors binding transmembrane-tethered ephrin-Bs. The Eph family shows a widespread expression during development and has been associated with a variety of different processes ranging from cell migration and angiogenesis to axon guidance (for reviews, see Adams, 2002; Drescher, 2002; Kullander and Klein, 2002; Wilkinson, 2001).

The role of the Eph family in the establishment of neuronal connections has been studied in a number of different model systems, in particular the formation of the retinotectal projection. It is now believed that this topographically organised projection is set up to a large extent on the basis of complementary gradients of Eph receptors and ephrins in both

the retina and the tectum, involving repulsive but also attractive interactions between Eph receptors and ephrins (reviewed by Knöll and Drescher, 2002; Wilkinson, 2000). In ephrin-A mutant mice, mapping across the anteroposterior axis is affected (Feldheim et al., 1998; Feldheim et al., 2000; Frisen et al., 1998), while recent data from in vivo analyses showed an important role of EphB family members in mapping across the mediolateral axis (Hindges et al., 2002; Mann et al., 2002). Functional investigations of topographically expressed transcription factors and signalling molecules in the retina also support the idea of a prominent role of the Eph family in retinotectal mapping (Koshiba-Takeuchi et al., 2000; Mui et al., 2002; Sakuta et al., 2001; Schulte and Cepko, 2000; Schulte et al., 1999).

The way in which the Eph family exerts its function has just started to be uncovered. A characteristic of the Eph family is their capacity for bi-directional signalling such that both Eph receptors and ephrins function as both receptors and ligands (Klein, 2001; Knöll and Drescher, 2002). In vivo, ephrins have to be membrane bound to fulfil either the receptor or ligand role.

As ligands, the membrane attachment is believed to lead to a clustering of the ephrins, which is a necessary requirement for Eph receptor activation, since soluble clustered ephrins (for example as Fc chimeric molecules, as described in the studies here), but not soluble monomeric ephrins, activate Eph receptors (Davis et al., 1994). Also, the function of ephrins as receptors requires membrane anchorage for signalling into the cytosol, which for GPI-anchored ephrin-As most likely requires, in addition, a transmembrane co-receptor (Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001; Knöll et al., 2001).

We were interested in the question of whether the substratum-bound ephrins possess the ability to guide axons, i.e. they have the capability to change the growth direction of an axon without inducing a full growth cone collapse. Most other molecules so far associated with axon guidance are secreted molecules such as netrins, semaphorins and slits (Müller, 1999). They are thought to function *in vivo* by forming gradients through a localised secretion, and to guide axons by a chemoattractive or chemorepulsive guidance mechanism (Tessier-Lavigne and Goodman, 1996). So far only these secreted molecules were active in the 'growth cone turning assay', which represents an assay mimicking some aspects of the *in vivo* situation (Campbell et al., 2001; de la Torre et al., 1997; Hopker et al., 1999; Ming et al., 1997; Ming et al., 1999; Song and Poo, 2001; Song et al., 1998; Song and Poo, 1999). In this turning assay, a gradient of a particular molecule is generated through a pulsed release from the tip of a micropipette. The growth of axons within such a gradient is then analysed using time lapse microscopy.

The response of growth cones to gradients of soluble molecules might be fundamentally different from their response to membrane-bound ephrins, which involves a local cell-cell contact between the responsive growth cone and the ephrin-expressing cell, and which is, as such, classified as a guidance mechanism via contact repulsion or attraction (Tessier-Lavigne and Goodman, 1996). Such an interaction might for example involve reciprocal signalling between these cells. Also the clustering of ligands and receptors and/or their lateral mobility within the membrane might play an important role. These features might be exclusive for membrane-bound molecules.

So far ephrin-As have been investigated in the 'stripe assay' with retinal ganglion cell (RGC) axons, where they force a striped outgrowth of retinal axons, and in the 'collapse assay', where they induce a full growth cone collapse (Davenport et al., 1999; Drescher et al., 1995; Journey et al., 2002; Menzel et al., 2001; Monschau et al., 1997; Shamah et al., 2001; Wahl et al., 2000). However, these results do not address the question of whether ephrins have the ability to induce turning of growth cones, which is believed to be the consequence of a *local* or *partial* growth cone collapse (e.g. Fan and Raper, 1995; Zhou et al., 2002). It is conceivable that ephrin-As for some, as yet unknown, reasons might only be able to induce a general growth cone collapse (thus acting *in vivo* as a stop signal), which would suffice to explain the striped outgrowth in the stripe assay.

Here we show for *Xenopus* retinal ganglion cell growth cones that ephrin-A5 has the ability to induce a specific collapse reaction in the collapse assay and a striped outgrowth in the stripe assay. These results are comparable with those using chick retinal axons (Drescher et al., 1995; Monschau et

al., 1997). Also, ephrin-A5-Fc is able to induce the turning of *Xenopus* retinal growth cones when applied as a soluble clustered molecule and as an immobilised molecule linked to beads. The turning response is substratum-dependent, a feature which has also been shown recently for netrins (Höpker et al., 1999). This turning response occurs under special conditions, that is at higher concentrations and/or steeper slopes of the gradient. Under these conditions a subpopulation of about 20% reacted with turning, while the rest of the growth cones showed a collapse reaction. Thus it has been shown for the first time that ephrin-A5 is in principle able to guide retinal axons *in vitro*.

## MATERIALS AND METHODS

### Culture preparation

*Xenopus laevis* embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Embryonic retinae were prepared from stage 28 embryos (Harris et al., 1985). The eyes were removed, washed in Hank's solution supplemented with antibiotics and the retinae were explanted on laminin- (20 µg/ml) or matrigel-coated culture dishes. The culture medium consisted of 60% Leibovitz L15 (Sigma, St. Louis, MO) supplemented with 1% foetal calf serum and antibiotics (gentamycin, streptomycin) (Gibco, Paisley, Scotland). In the case of the stripe assay, the medium additionally contained 0.4% methylcellulose. Explants were grown at room temperature for 24 hours (collapse assay, modified stripe assay, bead assay and pulsation gradient) or for 48 hours (labelling the axons with AP constructs).

### Staining of retinal growth cones in culture with alkaline phosphatase fusion proteins

Retinae of stage 28 *Xenopus* embryos were explanted on matrigel and incubated at room temperature for 48 hours in culture medium containing 0.4% methylcellulose. Following roughly the method described by Cheng and Flanagan (Cheng and Flanagan, 1994) for chick retinae, cultures were washed in PBS and incubated for 2 hours either with ephrin-A5-alkaline phosphatase fusion protein (ephrin-A5-AP, 5 nM) or, as control, 5 nM AP alone. Then the cultures were washed six times with HBHA (0.5 mg/ml BSA; 0.1% NaN<sub>3</sub>, 20 mM Hepes pH 7.0 in Hank's A solution), fixed in 60% acetone, 3% formaldehyde and washed twice with HBS (20 mM Hepes pH 7.0, 150 mM NaCl). Endogenous phosphatases were inactivated at 65°C for 15 minutes. After washing with AP buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM MgCl<sub>2</sub>), the colour reaction was performed in 4.5 µl nitro-blue-tetrazolium (50 mg/ml; NBT) (Promega, Madison, WI, USA), 3.5 µl 5-bromo-4-chloro-3-indoyle phosphate (BCIP) (50 mg/ml; Promega, Madison, WI, USA) per ml AP buffer. One hour later the staining reaction was stopped by washing with PBS.

### Collapse assay

The collapse assay was performed essentially as described previously (Raper and Kapfhammer, 1990; Cox et al., 1990). Retinal explants of *Xenopus* were grown overnight on laminin (20 µg/ml) coated cover slips. The growth cones were investigated using an inverted phase contrast microscope. By using a computer-controlled scanning stage, up to 15 growth cones could be observed simultaneously in time lapse. Pictures were taken under automatic control every 3-5 minutes, starting 30 minutes before and finishing 60 minutes after the addition of proteins. A growth cone was considered as collapsed when the motile structures, i.e. lamellipodia and filopodia, disappeared and the growth cone stopped growing or even retracted its whole axon shaft.

*In vivo*, ephrin-A5 is membrane anchored. Here we have used a soluble version of ephrin-A5, in which the GPI-anchor was replaced

by the Fc part of a human IgG antibody [named ephrin-A5-Fc (Ciossek et al., 1998)]. As a control protein in these collapse assays, Fc alone was added, which is known to have no or very little collapse-inducing activity (Ciossek et al., 1998).

### Modified stripe assay

The technique for the modified stripe assay has been described previously (Vielmetter et al., 1990; Hornberger et al., 1999). 660  $\mu$ l of a solution of 25 cm<sup>2</sup> nitrocellulose dissolved in 12 ml methanol was dropped in a 6 cm Petri dish and evaporated overnight. The silicon matrix containing channels of 90  $\mu$ m width was pressed on the nitrocellulose-coated Petri dish and the protein solution for the first set of lanes was injected with a Hamilton syringe using 100  $\mu$ l of a solution containing 8  $\mu$ g/ml ephrin-A5-Fc (or Fc for control experiments) clustered with 80  $\mu$ g/ml of an anti-human Fc antibody conjugated with FITC (Sigma, St. Louis, MO). After 30 minutes incubation in 5% CO<sub>2</sub> at 37°C, the silicon matrix was carefully removed, the stripes were washed once with Hank's A solution and then the solution for the second set of lanes was applied (80  $\mu$ l of a solution containing 3  $\mu$ g/ml Fc clustered with 30  $\mu$ g/ml non-labelled anti-human Fc-antibody). After 30 minutes incubation, the stripes were washed twice with Hank's A solution and covered with laminin (20  $\mu$ g/ml) for 1-2 hours. Finally the stripes were washed again and covered with medium until explantation of the *Xenopus* retinae. Owing to the clustering of ephrin-A5-Fc with an FITC-conjugated Fc-antibody, alternating lanes can be distinguished using fluorescence microscopy. After 2 days in culture, explants were fixed in 4% paraformaldehyde and analysed.

### Growth cone turning assay using Ephrin-A5 coated beads

Following the protocol of Kuhn et al. (Kuhn et al., 1995), carboxylated latex beads (4.5  $\mu$ m in diameter) were coated with Protein-A. Remaining active groups were subsequently blocked with BSA. These beads were incubated with ephrin-A5-Fc (clustered with an FITC-conjugated antibody) overnight at 4°C, centrifuged and washed with PBS. To verify the binding of ephrin-A5-Fc, the beads were examined under blue light with 450-490 nm excitation wavelength. Successfully labelled beads showed green fluorescence.

Using a laser tweezers device (600 mW diode laser with a wavelength of 1200 nm, P.A.L.M.), beads were placed in front of *Xenopus* retinal ganglion growth cones. Beads without any or only protein A coating were used in control experiments. Pictures were taken every 30 seconds. The growth cones were observed for about 1.5 hours in each experiment. Subsequently, the turning angle relative to the direction of the last 10  $\mu$ m segment of the axon shaft at the beginning of the experiment and the growth cone collapse rate were determined.

### Growth cone turning in an ephrin-A5 gradient

Gradients of diffusible substances were established as described

previously (Lohof et al., 1992; Zheng et al., 1994). In brief, a stable gradient was produced by ejecting the substance of interest out of a capillary having a tip opening of 1  $\mu$ m. Pulses were created by applying a pressure of 3 psi for 10 mseconds at 2 Hz. The growth cones were positioned at a distance of 100  $\mu$ m – or in the case of ephrin-A5-Fc gradients at a distance of 60  $\mu$ m – from the micropipette tip with an angle of 45° relative to the initial direction of the axon shaft. After establishment of the gradient, pictures were taken every 5 minutes up to the end of the experiment for about 1 hour. Concentration of ephrin-A5-Fc in the capillary was 20  $\mu$ g/ml. In controls, pure medium without guidance molecules was loaded into the capillary.

## RESULTS

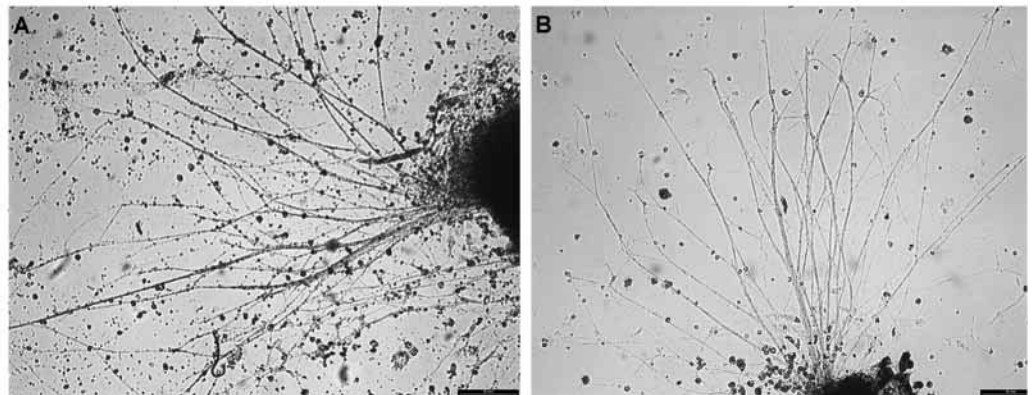
### *Xenopus* RGC and growth cones express EphA receptors

First the expression of EphA receptors on axons and growth cones of *Xenopus* retinal explants was analysed using ephrin-A5-AP, a fusion protein consisting of the extracellular region of chick ephrin-A5 fused to alkaline phosphatase (Ciossek et al., 1998). Because of promiscuous interactions between EphA receptors and ephrin-As as well as conserved Eph/ephrin interactions across species, chick ephrin-A5-AP is well suited to uncover expression of EphA receptors on *Xenopus* retinal axons. Incubation of retinal axons with ephrin-A5-AP and subsequent colour reaction resulted in a strong staining of retinal axons (Fig. 1A). In contrast, cultures treated in the same way with AP only did not stain (Fig. 1B). This shows that EphA receptors are expressed on *Xenopus* retinal ganglion cell axons and growth cones.

### *Xenopus* RGC growth cones collapse after exposure to ephrin-A5-Fc

Given this expression, we then examined whether retinal growth cones from *Xenopus* are sensitive to a growth cone collapse-inducing activity of ephrin-A5, like those from chick (Drescher et al., 1995; Monschau et al., 1997), mouse (Davenport et al., 1999) or zebrafish (Brennan et al., 1997). For this, we used ephrin-A5-Fc, a fusion protein of ephrin-A5 and the Fc part of human IgG, which dimerises in solution. The collapse assay was performed as described previously (Raper and Kapfhammer, 1990; Cox et al., 1990), using time-lapse video microscopy. 'Collapse' is here defined as the disappearance of lamellipodia and filopodia

**Fig. 1.** *Xenopus* retinal axons express EphA receptors. Explants from 2 day *Xenopus* retinae were grown on matrigel for 48 hours. (A) Incubation with ephrin-A5-AP, which binds all EphA receptors, results in a dark labelling of retinal axons. (B) Incubation with AP alone leads to no staining. Scale bar: 100  $\mu$ m.



**Table 1. Growth cone collapse rate of *Xenopus* retinal axons after contact with soluble ephrin-A5-Fc**

ephrin-A5-Fc	% Growth cone collapse
1 µg/ml without clustering	44% (70/158; n=18)
20 µg/ml without clustering	88% (23/26; n=3)
5 µg/ml with clustering	80% (23/29; n=3)
Fc	9% (6/67; n=6)

The growth cone collapse rate using ephrin-A5-Fc is significant as shown by  $\chi^2$ -test ( $P < 0.001$ ). The number of independently performed experiments ( $n$ ), i.e. the number of explants analysed, is indicated.

from the growth cone and a cessation of growth cone advance.

In control experiments using 1 µg/ml Fc only, 6 of 67 (9%, Table 1) of the growth cones showed a growth cone collapse. Similar levels of this 'non-specific' background collapse, which is possibly caused by changes in pH or temperature, have also been seen in other experiments (Drescher et al., 1995; Monschau et al., 1997). In contrast, application of 1 µg/ml unclustered chick ephrin-A5-Fc increased the percentage of collapsed growth cones from 9% to 44% (70 of 158, Table 1). A further rise in collapse rate was achieved when the concentration was raised to 20 µg/ml (also unclustered). Here 88% of the growth cones (23 of 26) showed collapse. 5 µg/ml of clustered ephrin-A5-Fc resulted in the collapse of 80% of the analysed growth cones (23 of 29). These data show that *Xenopus* retinal growth cones are similarly sensitive to ephrin-A5-Fc as, for example, are chick retinal axons (Drescher et al., 1995; Monschau et al., 1997). It also shows that ephrin-A5-Fc can exert its activity as a dimer, while other ephrins, in particular members of the ephrin-B family, require tetrameric or higher oligomerised forms for appropriate receptor activation (e.g. Stein et al., 1998).

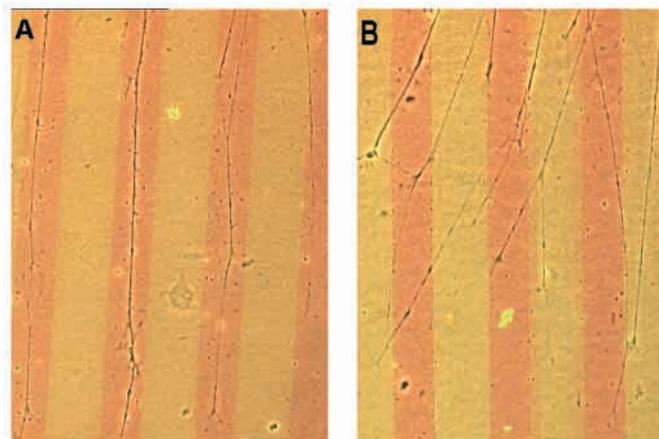
### ***Xenopus* retinal axons avoid ephrin-A5 containing lanes**

To further characterise the effects of ephrin-A5 on *Xenopus* retinal axons, the stripe assay was employed (Hornberger et al., 1999; Vielmetter et al., 1990; Walter et al., 1987). In this assay, neurons are given the choice to extend their axons on either of two substrata that are arranged in alternating narrow lanes. From the behaviour of the outgrowing axons a judgment on relative attraction and/or repulsion between these substrata can be made. The lanes in the stripe assay used here did not contain membranes as in the original stripe assay (Walter et al., 1987), but purified proteins (Vielmetter et al., 1990). To promote the outgrowth of retinal axons, the lanes were coated uniformly with laminin.

Using a substratum consisting of immobilised ephrin-A5-Fc versus Fc, retinal axons strongly preferred to grow on lanes containing Fc, but not ephrin-A5-Fc stripes (Fig. 2A). In control experiments, in which both lanes contained Fc, retinal axons did not show any preference (Fig. 2B). These data indicate that substratum-bound ephrin-A5 has a repellent guidance activity on *Xenopus* retinal axons.

### **Ephrin-A5 coupled to beads induces turning of retinal growth cones**

We subsequently analysed in more detail the behaviour of retinal axons in response to immobilised ephrin-A5-Fc by



**Fig. 2.** In vitro guidance of *Xenopus* retinal axons by ephrin-A5-Fc in the stripe assay. (A) Striped outgrowth of retinal axons is observed when they are given the choice of growing on either ephrin-A5-Fc containing (yellow) or Fc containing lanes (orange). Axons avoid the ephrin-A5 containing lanes. (B) Both stripes have been coated with Fc protein alone. Axons show no preference for either type of lanes. Laminin was used as an outgrowth promoting substratum.

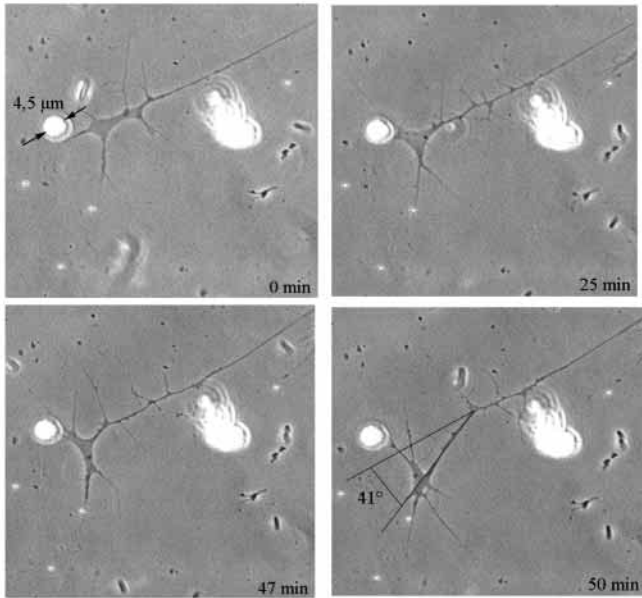
means of video time-lapse microscopy of individual growth cones. Using laser tweezers (Kuhn et al., 1995), it was possible to position ephrin-A5-Fc-coated beads directly in the pathway of growing retinal axons (Fig. 3). We found that ephrin-A5-Fc-coated beads caused, in most cases, either turning or collapse of retinal growth cones (14 out of 15). Control experiments using uncoated beads or beads coated with protein A alone confirmed the specific response to ephrin-A5-Fc, as only 2 out of 12 growth cones showed a growth cone collapse, while the growth direction of the other 10 was unaffected (Fig. 4).

In almost half of the interactions with ephrin-A5-Fc beads, axons reacted with growth cone collapse (7/15), while the other half (7/15) showed a turning response. A typical example of such a turning response is shown in Fig. 3. Whenever there was no collapse reaction, it was possible to determine the turning angle by which growth cones were deflected from ephrin-A5 beads. This was done by measuring the angle between the axon shaft before and after contact and passing the bead (Fig. 3) (Gallo et al., 1997). The analysis revealed that growth cones in contact with ephrin-A5-Fc-coated beads showed on average a turning angle of  $-41^\circ \pm 2.6^\circ$  ( $n=8$ ), whereas the turning angle of growth cones after contact with control beads was merely  $-6^\circ \pm 0.4^\circ$  ( $n=10$ ).

### **Analysis of ephrin-A5 in the growth cone turning assay**

The growth cone turning assay developed by M. Poo and co-workers is regarded as a sophisticated technique to study the ability of a molecule to guide axons in vitro (Lohof et al., 1992; Zheng et al., 1994). We have applied this assay to investigate the ability of ephrin-A5 to induce changes in growth direction of *Xenopus* retinal axons not only when presented in substratum-bound form (see above), but also as a soluble molecule in a gradient.

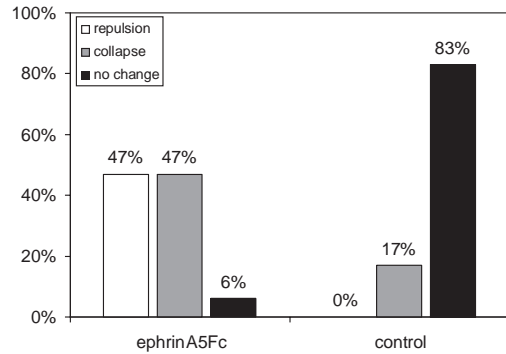
Exposure to gradients of soluble ephrin-A5-Fc did indeed result in a turning of retinal growth cones. This occurred, however, only if the micropipette was moved closer to the



**Fig. 3.** Growth cone turning of retinal axons after contact with an ephrin-A5-coated bead. (Top, left) *Xenopus* retinal growth cone filopodia contact an ephrin-A5-Fc coated bead. The bead is located slightly to the right of the pathway of the growth cone. (Top, right) The growth cone has moved slowly forward and has made extensive filopodial and lamellipodial contact to the bead. (Bottom, left) The growth cone has started to detach from the bead, the central domain of the growth cone has moved away from the bead. (Bottom, right) The growth cone has almost detached from the bead. Part of the growth cone previously in contact with the bead appears to be collapsed. There are only very few filopodial contacts left with the bead. Its growth direction has changed by an angle of  $-41^\circ$  to the left (away from the bead). New filopodia have been formed on the side opposite to the bead. In total, a mean turning angle of  $-41^\circ \pm 2.6^\circ$  was determined for experiments with ephrin-A5-coated beads ( $n=8$ ). In control experiments with non-coated or protein A-coated beads ( $n=10$ ), growth cones did not show a change in growth direction ( $-6^\circ \pm 0.4^\circ$ ;  $n=10$ ). As schematised in the picture (bottom, right), turning angles were measured by determining the angle between the axon shaft before bead contact and after passing the bead. Experiments were performed using laminin as an outgrowth promoting substratum. The pictures were taken using a Zeiss inverted phase contrast microscope with a magnification of 63. The beads were  $4.5 \mu\text{m}$  diameter.

growth cone, such that the distance between pipette tip and growth cone was reduced from  $100 \mu\text{m}$  to  $60 \mu\text{m}$  (Lohof et al., 1992; Zheng et al., 1994). The experiments were performed with unclustered ephrin-A5-Fc at a concentration of  $20 \mu\text{g/ml}$  at the pipette tip (Fig. 5). Using a lower concentration of ephrin-A5-Fc ( $5 \mu\text{g/ml}$ ) and positioning the pipette tip at a distance of  $100 \mu\text{m}$  from the growth cone did not lead to a turning response (data not shown). By decreasing the distance between growth cone and pipette tip, the slope of the gradient gets steeper and the concentration of ephrin-A5-Fc at the growth cones gets higher. We could not use higher concentrations of ephrin-A5-Fc, as these higher concentrations cause aggregation and thus blocked the tip opening such that no stable gradient could be formed (data not shown).

Surprisingly, the responses were substratum dependent. When the assay was performed on dishes coated with



**Fig. 4.** Summary of data from the growth cone turning assay using ephrin-A5-coated beads and laser tweezers. Ephrin-A5-coated or control beads were placed (using laser tweezers) in front of the retinal growth cones. After contact with ephrin-A5-coated beads, roughly half of the observed growth cones showed growth cone collapse (7 of 15; 47%), while the other half reacted with repulsion (7 of 15; 47%). One growth cone did not show any reaction. Control beads (uncoated or coated with protein A) did not elicit any change in growth cone behaviour in most cases (10 of 12; 83%). However, two growth cones showed a growth cone collapse. In all experiments laminin was used as an outgrowth promoting substratum.

fibronectin, retinal growth cones turned away from the pipette, i.e. showed a repulsive response, with a mean turning angle of  $-25^\circ (\pm 2.2^\circ; n=13)$ . When grown on a laminin substratum, this repulsive response was converted into attraction with a mean turning angle of  $+20^\circ (\pm 1.1^\circ; n=12)$  (Figs 5 and 6).

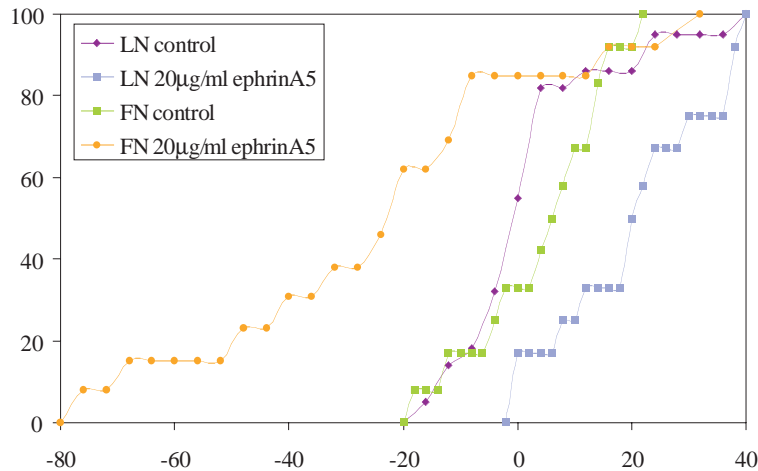
If the pipette was placed  $100 \mu\text{m}$  from the growth cone, as has been done in the experiments with BDNF and netrin 1 (see below), ephrin-A5-Fc applied at  $20 \mu\text{g/ml}$  did not elicit any measurable turning effect on retinal growth cones.

In another set of controls, we analysed *Xenopus* retinal growth cones growing on laminin in BDNF and netrin 1 gradients. In a gradient of BDNF ( $50 \mu\text{g/ml}$  at the pipette tip), growth cones were repulsed with a mean turning angle of  $-37^\circ (\pm 3.6^\circ; n=13)$ , which is in good correlation with data obtained by Höpker et al. (Höpker et al., 1999). In the second set of experiments, myc-tagged netrin 1 (a gift from M. Tessier-Lavigne) at a concentration of  $5 \mu\text{g/ml}$  was applied (Höpker et al., 1999). Here *Xenopus* growth cones were repelled with a mean turning angle of  $-21^\circ (\pm 1.3^\circ; n=16)$ . Netrin 1 typically elicits an attractive response (de la Torre et al., 1997; Ming et al., 1997); however, as shown recently (Höpker et al., 1999), netrin 1 activity is also substratum dependent, such that the growth cone response is converted from attraction to repulsion if axons are grown on laminin instead of e.g. fibronectin.

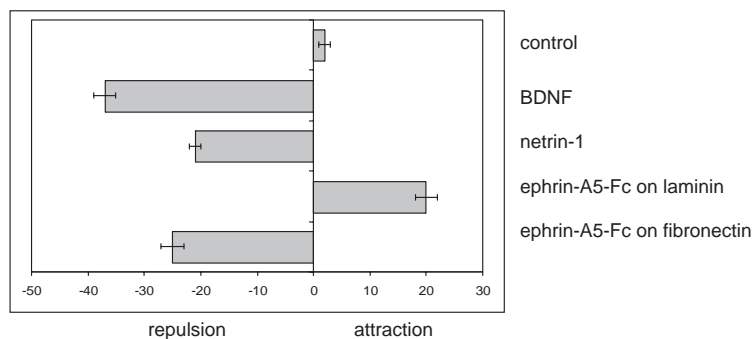
In control experiments with only medium in the pipette, no turning of axons was observed (Fig. 5). The mean turning angles of all experiments are summarized in Fig. 6.

## DISCUSSION

We were interested in understanding in more detail the mechanisms by which ephrin-A5 exerts its function during the set-up of neuronal connections. As ephrins are membrane-bound molecules in vivo, they might act in a different way to secreted molecules such as netrins, semaphorins and slits. Here



**Fig. 5.** Cumulative distribution plot of turning angles of retinal growth cones in response to gradients of various axon guidance molecules. On the x axis, turning angles with negative values indicate repulsion and positive values indicate attraction. The y axis gives the percentage of growth cones with turning angles greater than the corresponding angle on the abscissa. Both BDNF (50 µg/ml) and netrin 1 (5 µg/ml) induce repulsive reactions with growth cones turning away from the pipette (avoiding higher concentrations) (not shown here, see Fig. 6) (Mann-Whitney test:  $P \leq 0.012$ ). In an ephrin-A5-Fc gradient, growth cones show substratum-dependent turning responses. On laminin, growth cones turn towards the pipette (attraction) with a mean turning angle of +20°, on fibronectin the turning behaviour is converted to repulsion with a mean turning angle of -25°. Controls consisted of axons growing on laminin or fibronectin without guidance molecules in the pipette. Here growth cones were neither repelled nor attracted. The turning responses are statistically significant ( $P < 0.003$ , Mann-Whitney test).



**Fig. 6.** Summary of the turning responses of retinal growth cones caused by different axon guidance molecules. Growth cones are repelled by netrin 1 (5 µg/ml) and BDNF (50 µg/ml). Ephrin-A5-Fc at 20 µg/ml induces a substratum-dependent turning on laminin leading to attraction, whereas on fibronectin it leads to repulsion. Shown are mean turning angles (see also Fig. 5). Error bars represent s.e.m.

we show that *Xenopus* retinal axons are sensitive to ephrin-A5 in the 'collapse assay' and the stripe assay. Retinal axons react with turning and collapse after contact with ephrin-A5-Fc-coated beads and, when exposed to a focal source of soluble ephrin-A5-Fc, they showed a substratum-dependent turning response, also at a high background level of collapse reaction.

### Growth cone collapse versus turning

In agreement with data from other species, retinal axons from

*Xenopus laevis* express EphA receptors. In the stripe assay they (consequently) avoid ephrin-A5-Fc-containing lanes and their growth cones collapse when exposed uniformly to soluble ephrin-A5-Fc.

As the stripe assay evaluation is normally done when the striped pattern is already established, it is not possible to determine whether the patterned outgrowth is the result of a mechanism involving growth cone collapse or guidance. In one scenario, it is conceivable that each time an axon hits an ephrin-A5-containing stripe its growth cone collapses, followed by a subsequent regrowth in a slightly different direction, followed again by growth cone collapse, regrowth etc., leading thus to a striped outgrowth. In the other scenario there might be a 'smooth' turning of retinal axons away from ephrin-A5-containing stripes involving no or only a partial growth cone collapse (Fan and Raper, 1995) (B. K. Müller, Diplomarbeit, University of Tübingen, 1988). The former case would indicate that ephrin-A5 might simply act as a stop signal *in vivo*, whereas in the latter case it would function as a true guidance molecule steering growth cones towards their target *in vivo*. On a more general level, an investigation of this question would help to understand more about the way in which growth cones 'work', meaning here how they integrate Eph receptor signalling.

The relationship between growth cone turning and growth cone collapse has been considered for some time now (Fan and Raper, 1995; Walter et al., 1990). On the one hand, there is the more extreme view that there are two classes of molecules, with one class *only* able to induce a full growth cone collapse, while the other class is able to induce growth cone turning. On the other hand there is the view that the same molecule can induce either turning or collapse, depending on the way in which it is presented to the growth cone. Growth cone turning is understood here as a consequence of a local or partial growth cone collapse (Fan and Raper, 1995; Zhou et al., 2002). Mechanistically, in this view a local application of a repulsive guidance molecule would lead to a reorganisation and retraction of actin filaments and microtubules at the site of ligand-receptor activation, while non-activated domains of the growth cone continue to advance further, which would lead finally to a turning away from the repulsive molecules causing the local collapse. The same guidance molecule might also induce a general growth cone collapse if presented uniformly to the growth cone, i.e. from all sides. There might be various parameters that determine stop versus guidance activity, such as the length/strength of receptor activation or the diffusion rates of activated signalling molecules inside the growth cone. Thus, some signalling molecules might readily spread throughout the growth cone, whereas others would be confined to the immediate vicinity of the activated guidance receptor (e.g. Kasai and Petersen, 1994). Therefore, the growth cone collapse-inducing activity of ephrin-A5, as shown here in the collapse assay and in the bead assay, is not necessarily in disagreement with a possible function as a guidance molecule. Interestingly, in ephrin-A5 mutant mice a transient

overshooting of retinal axons beyond their target area, the superior colliculi, into the inferior colliculi has been observed, in addition to topographic targeting errors within the superior colliculi, consistent with a function of ephrin-A5 as both guidance and stop signal (Frisen et al., 1998).

Indeed, as we show here in the case of a focal presentation to *Xenopus* retinal axons, ephrin-A5-Fc in either substratum-bound (i.e. bound to beads) or soluble form induces growth cone turning, albeit on the background of a high percentage of growth cone collapse (see below). It is very possible that, by changing the concentration of ephrin-A5-Fc on the beads, or the shape of the gradient or the angle between the gradient and growth cone, the reaction of growth cones could be shifted more towards growth cone collapse or turning.

In this context, it came as a surprise to us that lower concentrations of ephrin-A5 result in collapse only, whereas higher ephrin-A5 concentrations induce turning (still on a high background level of collapse reaction). We expected rather the opposite, with growth cone collapse at higher concentrations, and turning at lower concentrations. At present we have no clear idea of the meaning of this finding. One interpretation – in contrast to the currently prevailing hypothesis – might be that growth cone collapse and turning are two different processes, which nevertheless can be induced concentration-dependently by the same molecule. It is also possible that the differential steepness of the ephrin-A5-Fc gradient, which is dependent on the distance from the micropipette tip, might be important with respect to growth cone guidance versus collapse (Goodhill and Baier, 1998; Rosentreter et al., 1998).

Nonetheless, our data from the turning assay allow the conclusion that substratum-bound and soluble ephrin-A5 have the ability to function as guidance molecules *in vitro*.

### Substratum dependency of the turning response

The direction of growth cone turning in an ephrin-A5-Fc gradient was dependent on the substratum on which the axons were grown. On laminin we observed an attraction with a turning towards higher concentrations of ephrin-A5. To confirm this turning response, we changed the substratum to fibronectin resulting in a conversion of the turning response from attraction on laminin to repulsion on fibronectin. This substratum dependency supports the view that the turning of retinal axons in response to an ephrin-A5-Fc gradient does not represent an artefact, but is a reflection of the well-known fact that the substratum on which axons are growing plays an important role in the interpretation of guidance molecules. A similar phenomenon has been described, for example, for netrin 1, which results in an attraction on fibronectin (and other substrata) and a repulsion on laminin (Höpker et al., 1999; Song and Poo, 2001) (for reviews, see Song and Poo, 1999).

Generally, our finding strengthens the idea that extracellular matrix molecules, which make up the scaffold within which growth cones migrate *in vivo*, have an important influence on the way in which growth cones interpret guidance cues.

Laminin was also used as an outgrowth-promoting substratum in the stripe assay experiments. Here, however, ephrin-A5 leads to striped outgrowth, which is normally interpreted as a repulsive effect. While at first view these stripe assay results are in contradiction to the data from the turning assay, there are some aspects which might lead to a more coherent picture (see also Holmberg and Frisen, 2002). In the

turning assay only a minor fraction of axons (20%) responded with a turning response (either attractive or repulsive), while the majority of growth cones (80%) collapsed upon interaction with ephrin-A5-Fc on both laminin and fibronectin. A similar situation holds true for the stripe assay. Here also the majority of axons are prevented from invading ephrin-A5 containing stripes. Thus, in sum, in both assays the majority of retinal axons behaves in the same way, i.e. with avoidance of ephrin-A5.

There is a smaller subpopulation of axons showing no striped outgrowth, meaning they are not repelled by ephrin-A5-Fc. Investigating the behaviour of retinal axons in the stripe assay using time-lapse microscopy showed that indeed a fraction of growth cones were not immediately repulsed on hitting the border of ephrin-A5-containing stripes, but grew for some time within the ephrin-A5 stripes and were displaced from these stripes only after some time, if at all (data not shown). This suggests that the repellent response in a fraction of retinal axons is biphasic with an initial attraction or insensitivity towards ephrin-A5 and a second phase involving a repulsive reaction. Given that the turning assay was performed for only one hour, it is possible that only the first phase in the response to ephrin-A5 was observed. It is known that turning responses can be switched from repulsion to attraction, and vice versa, from attraction to repulsion using a large variety of different stimuli (Song and Poo, 2001). Future experiments will be directed towards investigating the functional meaning of this biphasic response of retinal axons.

In sum, we have shown here for the first time that ephrin-As are able to guide axons both in immobilised form and as soluble molecules. Thus, for guiding axons, the membrane attachment of ephrin-As is not necessary. The relevance of these findings for the *in vivo* situation remains to be shown.

A difference between *in vivo* and *in vitro* conditions is potentially the dynamics of ligand clustering. In the *in vitro* experiments the level of oligomerisation of ephrin-A5 is fixed, such that ephrin-A5 is presented, for example, in the growth cone turning assay as a dimer. *In vivo*, in the context of a cell-to-cell interaction, the oligomerisation of ephrin-As is possibly a much more dynamic process. Here ephrin-A and EphA molecules are localised in subdomains of the membrane called lipid rafts, which are very small entities (<70 nm) in the non-activated state and might contain only a few proteins (Simons and Toomre, 2001). However, during activation, which means here the interaction of ephrin-As with EphA receptors, rafts fuse to large (and then microscopically visible) structures. Although the mechanism/s of lipid raft fusion are yet poorly understood, this clustering could finally lead to large assemblies of EphA/ephrin-A complexes, and in turn to a considerably stronger interaction than that occurring in the *in vitro* experiments. In consequence the off-rate, i.e. the separation of ligands and receptors, could be different and could affect the guidance behaviour, for example owing to an increased duration of the response (see also Hattori et al., 2000). It might also be possible that *in vivo* signals from the (activated) ephrin-A-expressing cell are sent to the EphA receptor-expressing axon and that these signals modulate the biological response. Thus it appears that in particular the role of lipid rafts in axon guidance has to be investigated in more

detail to gain a deeper understanding of EphA/ephrin-A-mediated signalling processes.

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