Published in final edited form as:

Mol Cell Neurosci. 2011 June ; 47(2): 131–136. doi:10.1016/j.mcn.2011.03.008.

EphrinA6 on chick retinal axons is a key component for p75^{NTR}-dependent axon repulsion and TrkB-dependent axon branching

Subathra Poopalasundaram¹, Katharine J.M. Marler, and Uwe Drescher*

MRC Centre for Developmental Neurobiology, King's College London, New Hunt's House, Guy's Campus, London SE1 1UL, UK

Abstract

A characteristic of the ephrin/Eph family is their capacity for bi-directional signalling. This means that an ephrin, for example, can function either as a ligand for an Eph 'receptor', or as a receptor for an Eph 'ligand'. A system in which this phenomenon is well studied is the retinotectal projection in which the guidance of retinal ganglion cell (RGC) axons to their target area in the tectum is controlled by both Ephs and ephrins expressed in gradients in both the retina and tectum.

Here we have analysed the receptor function of ephrinAs on RGC axons in further detail by focussing on ephrinA6, which is the most strongly expressed ephrinA in the chick retina. EphrinAs are GPI-anchored proteins and therefore require the interaction with transmembrane proteins to exert this receptor function. Previous work has shown that ephrinAs interact on RGC axons in cis with the neurotrophin receptors p75^{NTR} and TrkB. P75^{NTR} then was shown to be necessary for the repulsion of ephrinA-expressing RGC axons from an EphA substrate and for the downregulation of axon branching. In turn, an interaction of ephrinAs with TrkB as well as an increase in axonal ephrinA expression augments the axon branch-promoting activity of TrkB. We now show that ephrinA6 is the necessary ephrinA component of the repulsive ephrinA/p75^{NTR} receptor complex on chick RGC axons as axons lacking ephrinA6 no longer avoid an EphA matrix in stripe assay experiments. We also demonstrate that the branch-promoting activity of TrkB is dependent on ephrinA6 as a knockdown of ephrinA6 renders RGC axons insensitive to BDNF, the high affinity ligand for TrkB.

In sum our data further strengthen the hypothesis that a fine-tuned interplay of ephrinAs with p75^{NTR} and TrkB is important for the guidance and branching of RGC axons.

Keywords

Retinotectal projection; Ephrin; p75^{NTR}; TrkB; Axon guidance; Axon branching

© 2011 Elsevier Inc. All rights reserved.

*Corresponding author. Fax +44 207 848 6798, s.poopalasundaram@medsch.ucl.ac.uk (S. Poopalasundaram), kate.marler@kcl.ac.uk (K.J.M. Marler), uwe.drescher@kcl.ac.uk (U. Drescher).

¹Present address: Centre for Neuroendocrinology, Royal Free Campus, University College London, Rowland Hill Street, London NW3 2PF, UK.

Introduction

The retinotectal projection is a well-suited model system to understand molecules and mechanisms controlling topographic mapping. In this system retinal ganglion cell (RGC) axons from the temporal retina project onto the anterior tectum, while nasal RGCs project to the posterior tectum. In the perpendicular axis dorsal and ventral retina connect to lateral and medial tectum respectively. The retinotectal map develops in a number of steps involving activity-independent and activity-dependent processes. The initial ingrowth of retinal axons into the tectum in chick and mammals proceeds via an initial overshooting and subsequent topographically specific axon branching (Huberman et al., 2008; Lemke and Reber, 2005; O'Leary and McLaughlin, 2005).

The key players in retinotectal map development are members of the Eph family of receptor tyrosine kinases and the ephrins. A characteristic of this family—which is made up of the EphA and an EphB subfamilies—is their capacity to signal bi-directionally that is both Ephs and ephrins can function in a context dependent manner as either receptors or ligands (Klein, 2009). This potential is also realised for the retinotectal projection, where ephrinAs, for example, function as receptors when expressed in the retina, and as ligands when expressed in the tectum (Rashid et al., 2005). Overall multiple complementary EphA/ephrinA gradients control the steering of retinal axons to their correct location in the tectum along the antero-posterior axis. Thus there are gradients of EphA “receptors” in the retina (temporal>nasal) and ephrinA “ligands” in the tectum/SC (anterior<posterior), and of ephrinA “receptors” in the retina (nasal>temporal) and EphA “ligands” in the tectum (anterior>posterior). The idea has been put forward that the balance between signalling from these two gradient systems determines the location of topographically-specific branching of retinal axons (Marler et al., 2008; McLaughlin and O'Leary, 2005).

So far a functional characterisation has mostly focussed on ephrinA2 and ephrinA5. Nonetheless, in chick an additional ephrinA, ephrinA6, has been identified (Menzel et al., 2001). EphrinA6 shows clear differences in its amino acid sequence and affinity for EphA receptors when compared to ephrinA2/5, suggesting that ephrinA6 might have role/s divergent from those of ephrinA2/5. EphrinA6 is strongly expressed in the retina but only weakly in the tectum (Menzel et al., 2001) which is opposite to that of ephrinA2 and ephrinA5 which are more strongly expressed in the tectum than the retina (Menzel et al., 2001). Moreover, ephrinA6 is expressed not only in the RGC layer but also in all other layers, suggesting an involvement in establishing and/or stabilising intra-retinal organisation, while ephrinA2 and ephrinA5 are mostly confined to the RGC layer (Hornberger et al., 1999).

The function of ephrinA6 has been investigated so far only with regard to its tectal expression that is in its ligand function. Menzel et al. (2001) showed that ephrinA6 exerts a growth cone collapse inducing activity which is stronger for temporal than nasal axons, which correlates with the higher expression of EphA receptors on temporal than nasal RGC axons.

The aim of this investigation was to study the function of ephrinA6 on RGC axons. Previous stripe assay experiments have shown that RGC axons avoid an EphA substrate indicating that ephrinAs on RGCs can function as receptors and exert a repellent axon guidance function (Rashid et al., 2005). However, ephrinAs are GPI-anchored molecules (Davy et al., 1999; Huai and Drescher, 2001) and require therefore a transmembrane co-receptor/s to fulfil this receptor function. For this recently the neurotrophin receptor p75^{NTR} was identified (Lim et al., 2008). Thus the repulsion of RGC axons from an EphA substrate (such as in stripe assays with alternating EphA7-Fc versus Fc lanes) is abolished using retinæ from p75^{NTR} mutant mice (Lim et al., 2008). The ephrinAs involved here were so far only little investigated (Rashid et al., 2005).

In addition, ephrinAs interact on RGCs also with other molecules, and modulate the function of cis-expressed EphA receptors (Carvalho et al., 2006) and TrkB receptors (Marler et al., 2008). TrkB contributes to retinotectal mapping by controlling retinal axon branching (Cohen-Cory and Fraser, 1995). An increase in the expression of ephrinAs on RGC axons further promotes TrkB signalling leading to an enhanced axon branching and synaptogenesis, suggesting that the level of ephrinA expression is important for TrkB function (Marler et al., 2008).

Our data presented here suggest that ephrinA6 is prominently involved in both guidance and branching functions of retinal axons, that is ephrinA6 is necessary for p75^{NTR}-dependent repellent axon guidance, and for TrkB-mediated branching as knockdown of ephrinA6 leads to an abolishment of axon guidance and a substantial downregulation of axon branching.

Results

Characterisation of RNAis mediating a knockdown of chick ephrinA6

EphrinA6 is the most prominently expressed ephrinA in the chick retina showing a high-nasal-to-low-temporal gradient. It is expressed in the ganglion cell layer as well as in other retinal layers (Menzel et al., 2001).

In order to address the function of ephrinA6, we sought to generate RNAis which efficiently knock-down ephrinA6. Using suitable software, a set of sequences from the ephrinA6 cDNA were selected and cloned into a U6 promoter based RNAi expression vector (Das et al., 2006). This vector also contained a CMV promoter-driven RFP expression cassette enabling the later identification of successfully electroporated retinal ganglion cells.

To analyse the efficiency of these sequences to knock-down their target genes, a chick ephrinA6 expression construct tagged with an FLAG epitope (ephrinA6^{FLAG}) was co-transfected with each of these RNAi vectors into CHO cells. Two days later cell lysates were prepared and the expression level of ephrinA6^{FLAG} protein was analysed in western blots (Fig. 1A). Using this approach we identified one RNAi, RNAi (iA6-2), which almost completely knocked-down ephrinA6^{FLAG}, while another one, RNAi (iA6-1), was less efficient in ephrinA6^{FLAG} downregulation. Expression of an RNAi vector with a scrambled sequence or the RNAi vector alone had no effect on ephrinA6^{FLAG} expression. The specificity of the RNAi for ephrinA6 was shown in co-transfection experiments of RNAi

(iA6-2) with an ephrinA2^{FLAG} expression construct in which the ephrinA2^{FLAG} expression level was not affected (Fig. 1B).

In a similar way we generated a series of RNAis for ephrinA2 (see Experimental Methods). Transfection and western blot analysis led to the identification of RNAi (iA2-4), which out of four different RNAis tested, most strongly knocked down ephrinA2^{FLAG} (Fig. 1B) while it had no effect on ephrinA6^{FLAG} expression (Fig. 1A).

Retinally expressed ephrinA6 function in repellent axon guidance

Next we used these RNAis to study the function of retinally expressed ephrinAs in guidance of RGC axons. Thus we performed stripe assay experiments after electroporation of RNAis in retinal cells which knocked-down ephrinA6 or ephrinA2 (Fig. 1).

For this, single retinal cells were prepared from nasal third of E6 chick retinae, electroporated with different RNAi constructs and plated on a matrix of alternating lanes of EphA7-Fc versus Fc overlaid with laminin to provide an outgrowth-promoting substrate (Rashid et al., 2005). After incubation for 2 days, the growth decision of RGC axons was evaluated. Electroporation of control RNAi plasmid did not affect the outgrowth decision and nasal axons were repelled from growing in EphA7-Fc containing stripes (Fig. 2A, D). In addition, we observed that also cell bodies were repelled from EphA7-Fc containing lanes.

When performing the stripe assay after electroporation of the RNAi which knocks down ephrinA6 (iA6-2; Fig. 1), the striped outgrowth of RGC axons was lost, in that axons almost randomly grew on both types of stripes (Fig. 2B, D). Experiments done in parallel with retinal axons electroporated with RNAi (iA6-2) and plated on a matrix of Fc versus Fc also did not show a guidance of retinal axons (Fig. 2C, D). These experiments showed that ephrinA6 expressed on RGC axons is a necessary component of a receptor complex mediating repulsion from EphA7-Fc containing stripes.

In contrast, electroporation of RNAi (iA2-4) into retinal axons targeting ephrinA2 did not lead to an abolished striped outgrowth (Fig. 2E). The slight reduction in outgrowth preference was statistically not significant (Fig. 2E).

Retinal ephrinA6 function in the axon branching assay

We next characterised the function of retinal ephrinA6 in the axon branching assay (Marler et al., 2008). In this assay, single retinal cells from E8 chick embryos are plated on a uniform laminin/merosin substrate and are analysed for their axon branching behaviour after 4 days in culture in the presence versus absence of BDNF. The increase in branching during this time period covers the critical phase of topographic branching in the tectum which occurs between E8 and E12 (Mey and Thanos, 1992). In accord with this, we did not observe any significant branching if using retinal axons from earlier developmental time points which correspond to the time window at which the majority of RGC axons are still growing towards the tectum ((Mey and Thanos, 1992); data not shown).

Previously we had shown that branching of RGC axons requires activation of the TrkB receptor, as knockdown of TrkB via RNAi prevents a BDNF-induced increase in branching

(Marler et al., 2008). Corresponding *in vivo* data showed that injection of BDNF but not of NGF into the tectum results in an increase in axon branching (Cohen-Cory and Fraser, 1995). Furthermore, an increase in ephrinA5 expression on retinal axons leads to an increase in TrkB signalling and increased axon branching (Marler et al., 2008). Thus it appears that TrkB and ephrinAs together control and promote the level of axon branching. It was expected therefore that a downregulation of ephrinAs reduces the level of branching of RGC axons.

To explore this idea, we have investigated the consequences of a knockdown of ephrinA6 on axon branching. We electroporated RNAi (iA6-2) into retinal cells and analysed the number of branches per axon after 4 days in culture in the presence of BDNF. Here then we did not observe the increase in axon branching (Fig. 3A, C) normally seen after application of BDNF. Electroporation of an RNAi vector containing a scrambled RNAi sequence did not disrupt the BDNF-mediated increase in axon branching (Fig. 3B, C). Electroporation of the RNAi knocking down ephrinA2 using RNAi (iA2-4) did not affect RGC axon branching levels (Fig. 3D). In all these experiments we did not observe an effect of RNAi expression on retinal axon length (data not shown; (Marler et al., 2008)).

In sum, ephrinA knockdown and overexpression data (Marler et al., 2008) show that the branch-promoting function of TrkB on RGC axons is sensitive to axonal ephrinA6 expression.

Discussion

During development of the retinotectal projection, multiple EphAs and ephrinAs with complex expression patterns in the retina and tectum are involved in targeting RGC axons to their correct termination zones in the tectum/SC. Our analysis shows that expression of ephrinA6 on chick retinal ganglion cell axons plays a key role in repellent axon guidance and interstitial branching.

In vivo analysis of ephrinA function

In rodents, there are three ephrinAs, ephrinA2, -A3 and -A5, which are expressed both in the retina and SC. Analysis of knockout mice showed that incremental inactivation of multiple ephrinAs resulted in progressively more severe perturbations of the topographic map. Thus in ephrinA2/5 double mutants topographic targeting of RGC axons is more severely disturbed than in single mutants (Feldheim et al., 2000; Feldheim et al., 1998), and the phenotype in the ephrinA2/A3/A5 knockout is even stronger than in the ephrinA2/A5 mutant (Pfeiffenberger et al., 2006). Since these data are derived from global knockouts of ephrinAs, they do not allow conclusions as to whether these phenotypes are caused by the retinal (“receptor”) or/and tectal (“ligand”) ephrinA functions.

To fulfill a receptor function on RGC axons, ephrinAs as GPI-anchored molecules require transmembrane co-receptor/s to link to the cytosol. Recently, ephrinAs were demonstrated to interact *in cis* with EphAs (Carvalho et al., 2006; Marquardt et al., 2005; Seiradake et al., 2010), and with the neurotrophin receptors p75^{NTR} and TrkB (Lim et al., 2008; Marler et al., 2008). The interaction of ephrinAs with p75^{NTR} and TrkB is modulated by their

(pro)neurotrophin ligands, thus ephrinAs interact with p75^{NTR} in the presence of proBDNF, the high affinity ligand for p75^{NTR}, while they interact with TrkB in the presence of BDNF, the high affinity ligand for TrkB (Marler et al., 2010). We have shown that proBDNF and BDNF exert antagonistic functions on axon branching (Marler et al., 2010) raising the possibility that local processing of proneurotrophins and these selective ephrinA/receptor interactions are important for retinotectal map development (for further details see Marler et al., 2010).

EphrinA6/p75^{NTR} control repellent guidance

Aside from a later role of ephrinA/p75^{NTR} in the control of branching, this receptor complex also plays an earlier role in repellent axon guidance during the initial ingrowth of retinal axons into their target area. RGC axons show a striped outgrowth on an EphA7-Fc versus Fc matrix due to repellent signalling via ephrinA/p75^{NTR} and a loss of p75^{NTR} expression in the retina abrogates this behaviour and results in a disturbance of topographic mapping (Lim et al., 2008; Marler et al., 2010). This showed that p75^{NTR} is a necessary component of an ephrinA-containing repulsive axon guidance receptor complex on RGC axons *in vitro* and *in vivo*.

Until now the identity of the ephrinA component was unknown but we show it to be in chick ephrinA6: knock-down only of this molecule completely abolished the striped outgrowth of RGC axons on an EphA7-Fc versus Fc substrate. In contrast, knockdown of ephrinA2 fails to disturb the patterned outgrowth; thus we conclude that an ephrinA6/p75^{NTR} complex mediates the repulsion of chick RGCs from an EphA substrate.

The lack of effect of an ephrinA2 knockdown in, for example, repellent axon guidance might indicate that ephrinA2 does not interact with p75^{NTR}, and has a divergent function, e.g. interacts with other co-receptors which serve other functions than p75^{NTR}. However, the much stronger retinal expression of ephrinA6 compared to that of ephrinA2 and ephrinA5 (Fig. 2, Hornberger et al., 1999; Menzel et al., 2001) makes it rather likely that ephrinA6 is the key component of this complex because it is the most strongly expressed ephrinA, and thus can compensate for the downregulation of other less strongly expressed ephrinAs.

EphrinA6/TrkB controls axon branching

Besides a function of an ephrinA/p75^{NTR} complex in axon guidance and branching, ephrinA interacts also with TrkB to promote/modulate RGC axon branching though we did not observe an effect on repellent guidance after TrkB knockdown (data not shown; (Marler et al., 2010)).

For example, an interference with the TrkB/ephrinA interaction abolishes branching, and overexpression of ephrinAs augments BDNF-mediated branching (Marler et al., 2008; Marler et al., 2010). We show now that a downregulation of ephrinA6 drastically reduces BDNF-evoked axon branching (Fig. 3C) while ephrinA2 knockdown again has no effect (Fig. 3D). Therefore, TrkB mediated RGC axon branching strongly depends on its co-expression with ephrinA6.

Thus we propose a model for map development in which branching anterior to future TZs is suppressed by p75^{NTR}/ephrinA6 activated by tectal anteriorNposterior EphA gradients while branching posterior to future TZs is suppressed by axonal EphAs activated by tectal ephrinA2 and ephrinA5 expressed in posterior Nanterior gradients (Yates et al., 2001). Branches thus will form preferentially where branch-suppression is at a minimum. We think that during ingrowth of retinal axons, proBDNF is the prevalent neurotrophin, meaning that ephrinAs interact preferentially with p75^{NTR} which leads to a suppression of branching while TrkB is inactive since its signalling depends on a BDNF-induced interaction with ephrinAs. In the region of minimal branch suppression axonal branches are formed and contact (dendrites of) tectal neurons. Presumably via a Hebbian mechanism (Debski and Cline, 2002), proteases are released from tectal neurons leading to a processing of proBDNF to BDNF. This inactivates p75^{NTR} signalling since ephrinAs now form a BDNF-induced complex with TrkB which promotes further branching and the development of elaborate TZs. This model proposes a molecular mechanism of how topographic mapping develops by an interplay between activity-independent and activity-dependent mechanisms (see also Marler et al., 2010).

Outlook

At present it can only be speculated why different ephrinAs are the important players in the retina and the tectum that is ephrinA6 in the retina and ephrinA2/5 in the tectum. A possible explanation might be that topographic map development requires a complex differential spatio-temporal regulation of ephrinA expression in the retina versus the tectum which might be best realised via separate ephrinA-encoding genes allowing a higher diversity and flexibility in expression control (Menzel et al., 2001).

Experimental methods

Reagents and antibodies

BDNF was purchased from Promega. Anti-FLAG antibody was obtained from Sigma, and α -tubulin was from Sigma. The secondary antibodies, goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP were obtained from GE Healthcare. Laminin and all tissue culture reagents were from Invitrogen. Merosin was from Millipore. Recombinant EphA7-Fc chimera was obtained from R&D Systems and Fc control was from Calbiochem.

Cloning of chick ephrinA2 and -A6 for expression studies

Chick ephrinA2 and -A6 cDNAs were obtained by PCR and cloned into a CMV promoter containing expression vector (Sigma) with an NH₂-terminal signal peptide followed by a FLAG tag. The cDNA fragments were sequence-verified and used in expression studies.

Cloning of RNAi for the knockdown of ephrinA6 and ephrinA2

Target sequences selected for ephrinA6 were TAGTGACTGAGCCGCGTAATA for RNAi (A6-1) and CAGGTTTCCTGCAGGACGATTA for RNAi (A6-2). The corresponding DNAs were synthesised and cloned into the chick RNAi vector, pRFPRNAi (Das et al., 2006).

Accordingly, the ephrinA2 target sequence finally chosen was GCCGTCTATTGGAACCGCAGCA (iA2-4). The efficiency of the RNAi constructs in knocking down their corresponding genes was determined by transfection and western blotting as described in the main text (Section 3.1).

Cell culture and transfection

Chinese Hamster Ovary (CHO) cells were purchased from ECACC and cultured in DMEM containing Glutamax and 10% (v/v) foetal bovine serum and 1% (v/v) penicillin/streptomycin. For transfection, cells were split and plated with 500,000 cells per 60 mm plate. Cells were transfected with 1 µg ephrinA DNA and 4 µg RNAi vector (or parental vector DNA) using LipofectAMINE transfection reagent (Invitrogen). Transfections were performed according to manufacturer's protocols. 48 h later media were removed and cells washed in ice cold PBS prior to the addition of lysis buffer (1% (v/v) Triton X-100, 25 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% (v/v) Glycerol, 1 mM of each PMSF, sodium vanadate, sodium pyrophosphate, glycerol pyrophosphate, sodium fluoride, and complete protease inhibitor with EDTA (Roche)).

Primary culture of retinal ganglion cells for branching assays

Single cells were prepared from the nasal third of embryonic day 8 (E8) chick retinae and electroporated with 6 µg of pRFPRNAi, RNAi (iA6-2), RNAi (iA6-1), RNAi (iA2-4), RNAi with a scrambled sequence or the parental vector, as indicated. Cells were plated on cover slips coated with poly-L-lysine, laminin (10 µg/ml) and merosin (2 µg/ml) as indicated and cultured for 4 days in vitro (4 DIV) in neurobasal media supplemented with 2% (v/v) B27, 1 mM glutamine, 5 µM forskolin and 1% (v/v) penicillin/streptomycin. BDNF at a final concentration of 5 ng/ml was added on 1 DIV and 3 DIV, and cultures were fixed with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) after 4 DIV. Cultures were permeabilised with 0.1% (v/v) Triton X-100 and stained with phalloidin-FITC (Sigma) at a concentration of 1 µg/ml to aid in identification of branches. RGCs were routinely identified by staining with antibodies specific for this cell type (Brn3A, Thy-1) and were typically those with the longest axons, as described in Marler et al. (2008).

Primary culture of retinal ganglion cells for stripe assays

Electroporated cells from nasal retinal thirds were prepared as described above and plated on a substrate of alternating lanes of EphA7-Fc and Fc (or Fc and Fc as a control) for stripe assays. Concentrations used were 30 µg/ml for EphA7-Fc and Fc. One type of stripe in each setup was labelled by the addition of Fc-FITC to allow distinction of the lanes. Matrices giving rise to 50 µm wide stripes were used. As specified above, RGCs were routinely identified by staining with antibodies specific for this cell type (Brn3A, Thy-1) and were typically those with the longest axons. For further details see Marler et al. (2008). Scoring of stripe assay results: Axons were scored as 0, if the majority of axons showed no preference for either stripe, 1 if they showed some avoidance of the EphA7-Fc stripe, and 2 for a strong preference for growing on Fc stripes, i.e. almost or fully avoiding the EphA7-Fc stripes. The evaluation of all branching and stripe assays was done blind to the protein matrix used and constructs transfected.

Acknowledgments

This work was supported by grants from the Wellcome Trust and the BBSRC. We thank Sarah Guthrie and Philip Gordon-Weeks for valuable comments on the manuscript.

References

- Carvalho RF, Beutler M, Marler KJ, Knoll B, Becker-Barroso E, Heintzmann R, Ng T, Drescher U. Silencing of EphA3 through a cis interaction with ephrinA5. *Nat. Neurosci.* 2006; 9:322–330. [PubMed: 16491080]
- Cohen-Cory S, Fraser SE. Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. *Nature.* 1995; 378:192–196. [PubMed: 7477323]
- Das RM, Van Hateren NJ, Howell GR, Farrell ER, Bangs FK, Porteous VC, Manning EM, McGrew MJ, Ohyama K, Sacco MA, et al. A robust system for RNA interference in the chicken using a modified microRNA operon. *Dev. Biol.* 2006; 294:554–563. [PubMed: 16574096]
- Davy A, Gale NW, Murray EW, Klinghofer RA, Soriano P, Feuerstein C, Robbins SM. Compartmentalized signaling by GPI-anchored ephrinA5 requires the fyn tyrosine kinase to regulate cellular adhesion. *Gen. Dev.* 1999; 13:3125–3135.
- Debski EA, Cline HT. Activity-dependent mapping in the retinotectal projection. *Curr. Opin. Neurobiol.* 2002; 12:93–99. [PubMed: 11861170]
- Feldheim DA, Kim YI, Bergemann AD, Frisen J, Barbacid M, Flanagan JG. Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. *Neuron.* 2000; 25:563–574. [PubMed: 10774725]
- Feldheim DA, Vanderhaeghen P, Hansen MJ, Frisen J, Lu Q, Barbacid M, Flanagan JG. Topographic guidance labels in a sensory map to the forebrain. *Neuron.* 1998; 21:1303–1313. [PubMed: 9883724]
- Hornberger MR, Dutting D, Ciossek T, Yamada T, Handwerker C, Lang S, Weth F, Huf J, Wessel R, Logan C, et al. Modulation of EphA receptor function by coexpressed ephrinA ligands on retinal ganglion cell axons. *Neuron.* 1999; 22:731–742. [PubMed: 10230793]
- Huai J, Drescher U. An ephrinA-dependent signaling pathway controls integrin function and is linked to the tyrosine phosphorylation of a 120 kDa protein. *J. Biol. Chem.* 2001; 276:6689–6694. [PubMed: 11053419]
- Huberman AD, Feller MB, Chapman B. Mechanisms underlying development of visual maps and receptive fields. *Annu. Rev. Neurosci.* 2008; 31:479–509. [PubMed: 18558864]
- Klein R. Bidirectional modulation of synaptic functions by Eph/ephrin signaling. *Nat. Neurosci.* 2009; 12:15–20. [PubMed: 19029886]
- Lemke G, Reber M. Retinotectal mapping: new insights from molecular genetics. *Annu. Rev. Cell Dev. Biol.* 2005
- Lim YS, McLaughlin T, Sung TC, Santiago A, Lee KF, O’Leary DD. p75(NTR) mediates ephrin-A reverse signaling required for axon repulsion and mapping. *Neuron.* 2008; 59:746–758. [PubMed: 18786358]
- Marler KJ, Becker-Barroso E, Martinez A, Llovera M, Wentzel C, Poopalasundaram S, Hindges R, Soriano E, Comella J, Drescher U. A TrkB/EphrinA interaction controls retinal axon branching and synaptogenesis. *J. Neurosci.* 2008; 28:12700–12712. [PubMed: 19036963]
- Marler KJ, Poopalasundaram S, Broom ER, Wentzel C, Drescher U. Proneurotrophins secreted from retinal ganglion cell axons are necessary for ephrinA-p75NTR-mediated axon guidance. *Neural Dev.* 2010; 5:30. [PubMed: 21044296]
- Marquardt T, Shirasaki R, Ghosh S, Andrews SE, Carter N, Hunter T, Pfaff SL. Coexpressed EphA receptors and Ephrin-A ligands mediate opposing actions on growth cone navigation from distinct membrane domains. *Cell.* 2005; 121:127–139. [PubMed: 15820684]
- McLaughlin T, O’Leary DD. Molecular gradients and development of retinotopic maps. *Annu. Rev. Neurosci.* 2005; 28:327–355. [PubMed: 16022599]
- Menzel P, Valencia F, Godement P, Dodelet VC, Pasquale EB. Ephrin-A6, a new ligand for EphA receptors in the developing visual system. *Dev. Biol.* 2001; 230:74–88. [PubMed: 11161563]

- Mey J, Thanos S. Development of the visual system of the chick—a review. *J. Hirnforsch.* 1992; 33:673–702. [PubMed: 1494045]
- O’Leary DD, McLaughlin T. Mechanisms of retinotopic map development: Ephs, ephrins, and spontaneous correlated retinal activity. *Prog. Brain Res.* 2005; 147:43–65. [PubMed: 15581697]
- Pfeiffenberger C, Yamada J, Feldheim DA. Ephrin-As and patterned retinal activity act together in the development of topographic maps in the primary visual system. *J. Neurosci.* 2006; 26:12873–12884. [PubMed: 17167078]
- Rashid T, Upton AL, Blentic A, Ciossek T, Knoll B, Thompson ID, Drescher U. Opposing gradients of Ephrin-As and EphA7 in the superior colliculus are essential for topographic mapping in the mammalian visual system. *Neuron.* 2005; 47:57–69. [PubMed: 15996548]
- Seiradake E, Harlos K, Sutton G, Aricescu AR, Jones EY. An extracellular steric seeding mechanism for Eph-ephrin signaling platform assembly. *Nat. Struct. Mol. Biol.* 2010; 17:398–402. [PubMed: 20228801]
- Yates PA, Roskies AL, McLaughlin T, O’Leary DD. Topographic-specific axon branching controlled by ephrin-as is the critical event in retinotectal map development. *J. Neurosci.* 2001; 21:8548–8563. [PubMed: 11606643]

Fig. 1.

Characterisation of RNAis to knock-down ephrinA6 and ephrinA2. Selected RNAis were co-transfected with expression vectors encoding FLAG-tagged chick ephrinAs into CHO cells. Two days later, cell lysates were prepared and analysed by western blots using anti-FLAG antibodies. (A) RNAi (iA6-2) leads to a full knock-down of ephrinA6^{FLAG} (upper panel), an RNAi for ephrinA2 (iA2-4) does not affect expression of ephrinA6^{FLAG}. To normalise for the amount of protein loaded, the same filter was reprobbed and analysed with an antibody to α-tubulin (lower panel). (B) Out of four RNAis tested, RNAi (iA2-4) leads to the strongest knockdown of ephrinA2^{FLAG} shown here. The RNAi for ephrinA6 (iA6-2) does not affect the expression level of ephrinA2^{FLAG}. A control RNAi containing a scrambled RNAi sequence and the parental vector did not show an effect on ephrinA2^{FLAG} expression levels.

Fig. 2.

Knockdown of ephrinA6—but not of ephrinA2—in RGC axons abolishes repellent guidance in stripe assay experiments. Retinal cells from E6 chick embryos were electroporated with RNAi constructs containing an RFP cassette and plated on a matrix of alternating lanes of EphA7-Fc vs. Fc, or Fc vs. Fc in control experiments. 2 days later their axon outgrowth behaviour was quantified. (A) Retinal axons electroporated with a control RNAi vector are repelled from EphA7-Fc lanes (green) on an EphA7-Fc vs. Fc matrix. (B) Retinal cells, electroporated with the RNAi (iA6-2) show a random axonal growth on a matrix of EphA7-Fc vs. Fc. (C) Retinal axons, electroporated with RNAi (iA6-2) show a random axonal growth on a matrix of Fc vs. Fc. (D) Quantification of outgrowth preference in ephrinA6 knockdown experiments. (E) Quantification of the experiments on knockdown of ephrinA2.

The experiments are done in the same way as for the analysis of ephrinA6. The graph in (D) represent the data from three, that in (E) from two independently performed experiments. Within each of these experiments, for each condition $n=3$ different stripe assays were evaluated. For each of these stripe assays the behaviour of single axons was scored (for RNAi (iA6-2) >60 neurons per condition; for RNAi (iA2-4) >35 neurons per condition). First generated stripes are labelled by addition of Cy3-Fc. Error bars denote S.E.M and significance is indicated as *** $p<0.001$. Statistical analysis was done in Graph pad using one way ANOVA and Tukey post hoc test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Knockdown of ephrinA6—but not ephrinA2—abolishes BDNF induced branching. Retinal cells from E8 chick embryos were electroporated with vectors containing RNAi (iA6-2), RNAi (iA2-4) or RNAi (c) containing a scrambled RNAi and plated on a laminin/merosin substrate for 4 days in the presence or absence of 5 ng/ml BDNF. After fixation, the number of branches per axon was determined. 3 independent experiments were performed with $n > 30$ for each condition. (A) Branching pattern of an RGC axon electroporated with RNAi (iA6-2) in the presence of BDNF. (B) Branching pattern of an RGC axon electroporated with RNAi (c) in the presence of BDNF. (C) Quantification of the number of branches for

axons expressing RNAi (iA6-2) or RNAi (c), in the presence or absence of BDNF. (D) Quantification of the number of branches for axons expressing RNAi (iA2-4) or RNAi (c), in the presence or absence of BDNF. The average axon length was not affected when using these different RNAis. For further details see Experimental methods, and Marler et al. (2008). Error bars denote S.E.M and significance is indicated as *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$; n.s.: not significant. Statistical analysis was done in Graph pad using one way ANOVA and Tukey post hoc test.