

Coexpressed EphA Receptors and Ephrin-A Ligands Mediate Opposing Actions on Growth Cone Navigation from Distinct Membrane Domains

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Summary

Contact-dependent signaling between membrane-linked ligands and receptors such as the ephrins and Eph receptor tyrosine kinases controls a wide range of developmental and pathological processes. Paradoxically, many cell types coexpress both ligands and receptors, raising the question of how specific signaling readouts are achieved under these conditions. Here, we studied the signaling activities exerted by coexpressed EphA receptors and GPI-linked ephrin-A ligands in spinal motor neuron growth cones. We demonstrate that coexpressed Eph and ephrin proteins segregate laterally into distinct membrane domains from which they signal opposing effects on the growth cone: EphAs direct growth cone collapse/repulsion and ephrin-As signal motor axon growth/attraction. This subcellular arrangement of Eph-ephrin proteins enables axons to discriminate between *cis*- versus *trans*-configurations of ligand/receptor proteins, thereby allowing the utilization of both Ephs and ephrins as functional guidance receptors within the same neuronal growth cone.

Introduction

Cells coordinately respond to multiple extrinsic cues to regulate their proper growth, fate specification, and organization. These cell-to-cell interactions are reliant upon signaling pathways that produce unambiguous responses (Hunter, 2000). For instance, during CNS development, neuronal growth cones are guided toward their targets by specific attractive or repulsive cues within and around their migratory pathway, which are recognized by corresponding receptors on the surface of the neuron (Tessier-Lavigne and Goodman, 1996). Intuitively, these guidance signals are based on the segregation of receptors to neurons and ligands to different cells along their axonal pathways, thus ensuring a linear and unambiguous transfer of guidance information. Nevertheless, individual cells frequently coexpress receptors and their cognate ligands, prompting us to ask how meaningful guidance responses are achieved when both components are simultaneously present within the same membrane.

An important class of molecules mediating guidance

responses via contact-dependent signaling is the Eph family of receptor tyrosine kinases (RTKs) and their membrane-linked ligands, the ephrins. Eph RTKs are divided into two classes, EphAs and EphBs, generally reflecting their binding preference for GPI-linked ephrin-A, or transmembrane ephrin-B ligands, respectively (Lemke, 1997). Ephs control cellular adhesion and deadhesion events by linking their activation to local modulations of the assembly and disassembly rates of cytoskeletal components and adhesion structures (Kullander and Klein, 2002). Intriguingly, Eph-ephrin interactions can also elicit reverse signaling events via A and B class ephrin “ligands,” leading to bidirectional responses within both the Eph receptor- as well as the ephrin ligand-bearing cell (Bruckner et al., 1997; Davy et al., 1999; Henkemeyer et al., 1996; Wang et al., 1999). Together, these interactions play a critical role in a wide range of processes including axon navigation, interstitial branch formation, synapse formation, cell migration, vascularization, and hindbrain segmentation (Drescher et al., 1997; Flanagan and Vanderhaeghen, 1998; Palmer and Klein, 2003).

The study of contact-mediated receptor-ligand interactions, such as between the Ephs and ephrins, has focused primarily on models with complementary expression of receptor and ligand. However, studies in many cell types including the CNS have revealed that in contrast to the classical complementarity of Eph and ephrin expression domains, often a more complex situation is encountered when analyzing several receptor/ligand family members, revealing coexpression in various cell types of the developing CNS (Hornberger et al., 1999; Iwamasa et al., 1999). Recently, the known repertoire of Eph-ephrin interactions has been expanded by the finding that ephrin-A5 can bind and activate the EphB2 receptor (Himanen et al., 2004), indicating that the actual degree of overlap between cognate Ephs and ephrins may be much larger than previously appreciated. These observations therefore raise the question of how appropriate instructive signaling responses are achieved in cells expressing both receptors and ligands.

The presence of ephrin-A ligands on retinal ganglion cells (RGCs) has been suggested to mediate the desensitization toward axon withdrawal from exogenous sources of ephrin-As via hypothetical *cis*-interactions with coexpressed Eph receptors (Hornberger et al., 1999). However, the bidirectional nature of Eph-ephrin interactions potentially complicates this interpretation, and therefore it remains unclear whether ephrin-A reverse signaling, similar to other systems (Cutforth et al., 2003; Knoll et al., 2001), also contributes to the navigational behavior of RGC axons. Genetic and in vitro studies demonstrated cell-autonomous requirements for both EphB forward and ephrin-B reverse signaling within the same tissues, indicating that the bulk of the coexpressed proteins are not sequestered by mutual *cis*-interactions (Adams et al., 2001; Gerety and Anderson, 2002; Stein et al., 1998).

EphA forward signaling plays a well-established role

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in directing the navigation of limb-innervating motor axons (Eberhart et al., 2002; Feng et al., 2000; Helmbacher et al., 2000; Kania and Jessell, 2003). Nevertheless, these cells also express ephrin-A ligands (Iwamasa et al., 1999), prompting us to examine how guidance responses are integrated under conditions of Eph/ephrin coexpression. We show that coexpressed EphAs and ephrin-As could be independently *trans*-activated by ligand and receptor, respectively, each of them exerting opposing effects on neuronal growth cone behavior: EphA proteins triggered growth cone collapse, and ephrin-As promoted axon growth and markedly increased growth cone spreading. In both neuronal and nonneuronal cells, EphA and ephrin-A proteins were found to display planar segregation within the plasma membrane. Selective mistargeting of ephrin-A receptor or EphA ligand binding domains into EphA or ephrin-A-enriched membrane domains, respectively, lead to *cis*-interactions and consequent attenuation of *trans*-activated signaling. Thus, the lateral segregation of Eph- and ephrin-mediated activities within the plasma membrane is essential to sidestep potential problems caused by coexpressing cognate receptor/ligand pairs, thereby facilitating unambiguous signaling responses. Consequently, this mechanism allows the simultaneous utilization of Eph and ephrin proteins as functional guidance receptors within the same neuronal growth cone, providing a unique means of enhancing the guidance information encoded by a limited set of components.

Results

Coexpression of EphA Receptors and Ephrin-A Ligands on Developing Motor Axons

EphA3, EphA4, ephrin-A2, and ephrin-A5 have all been reported to be expressed by developing spinal motor neurons (Iwamasa et al., 1999). To determine if these A class Ephs and ephrins are coexpressed or segregate into separate motor neuron populations, we examined the cellular distribution of their mRNAs and proteins. In the embryonic chick spinal cord, high levels of *ephrin-A5* RNA can be detected in most embryonic brachial and lumbar lateral motor column (LMC) motor neurons during the period in which these cells innervate fore- and hindlimb muscles (Figures 1A–1C). Double labeling showed that a large portion of LMC neurons positive for EphA4 protein coexpress *ephrin-A5* (Figures 1B and 1C). High levels of ephrin-A5 and EphA4 proteins were, moreover, codetected by double-immunofluorescence on motor axons, including their distal segments (Figures 1D–1G).

Affinity probe detection utilizing ECD (extracellular domain)-EphA3-Fc, ECD-EphA7-Fc, and ECD-ephrin-A1-Fc chimeric proteins, moreover, detected high levels of EphAs and ephrin-As on the surface of limb-innervating motor and sensory axons between E4 and E6 (Figures 1H and 1I and see Figure S1 in the Supplemental Data available with this article online). Because of the wide binding spectrum of A class Ephs and ephrins (Lemke, 1997), these affinity probes detect the composite of available ephrin-As and EphAs. Both ECD-EphA3-Fc and ECD-EphA7-Fc probes, moreover,

bound to the entire length of identified motor axons that had been labeled with the motor neuron-specific reporter, *Hb9-gfp* (Figures 1J and 1K and S1; Thaler et al., 1999). Finally, triple immunofluorescent staining on chick motor column explants derived from the brachial spinal cord detected high levels of both EphA4 and ephrin-A2 on SC1⁺ motor growth cones and axon shafts (Figures 1L–1N). Taken together, these results demonstrate that individual motor axons coexpress EphAs and ephrin-As.

EphAs and Ephrin-As Signal Independent and Opposite Effects on Growth Cones

To investigate the signaling activities of endogenously coexpressed EphA and ephrin-A proteins, we tested how individual spinal motor neuron growth cones responded to stimulation with exogenous ephrin-A or EphA proteins. Motor column explants were dissected from open book preparations of the brachial spinal cord of chick embryos and cultured for 36 hr. During this culture period, motor axons extended 50–400 μm from the explant and were tipped with well-defined F-actin-rich growth cones (see Figure 2A). Application of preclustered ECD-EphA7-Fc to explants for 3 hr triggered a marked (1.49-fold \pm 0.05) enlargement of growth cones (Figures 2D and 2F) compared to controls treated with human IgG-Fc (Figures 2A and 2C), reflected by a shift toward a distribution of growth cones with larger sizes (Figures 2C and 2F). As expected, similar results were obtained upon stimulation with preclustered ECD-EphA3-Fc protein, which, like EphA7, binds with high affinity to A class ephrins (data not shown).

Double-label immunofluorescence staining revealed that the majority of growth cones in culture expressed both ephrin-A2 and EphA4 (Figures 2B and 2E). This raised the possibility that endogenous ephrin-As might interfere with the ability of EphA4 to be *trans*-activated by ECD-ephrin-As and trigger growth cone collapse. To test this, we applied clustered ECD-ephrin-A1-Fc to motor explants and found that within 1 hr growth cone collapse was triggered in 63% (\pm 0.05%) of the cells, leading to a shift toward a smaller growth cone size distribution (Figures 2F and 2G). Taken together, these results indicate that motor axon growth cones are simultaneously capable of initiating forward signaling via EphA receptors and reverse signaling via ephrin-A proteins, although the signaling pathways activated by EphAs and ephrin-As have opposite effects on growth cones.

Reverse Signaling via Ephrin-A Proteins Stimulates Motor Axon Growth

The induction of growth cone spreading within 3 hr suggested that EphA \rightarrow ephrin-A reverse signaling mediates contact-dependent attraction. To test this possibility, we assayed the effect of chronic stimulation of the reverse signaling pathway on neurite growth. Brachial explants of motor neurons were cultured 36 hr on a substratum containing ECD-EphA7-Fc and compared to explants grown on IgG-Fc. Culture on ECD-EphA7-Fc substrata lead to a strong increase in neurite outgrowth: the average neurite length was increased \sim 2.4-fold compared to control cultures (Figures 2J–

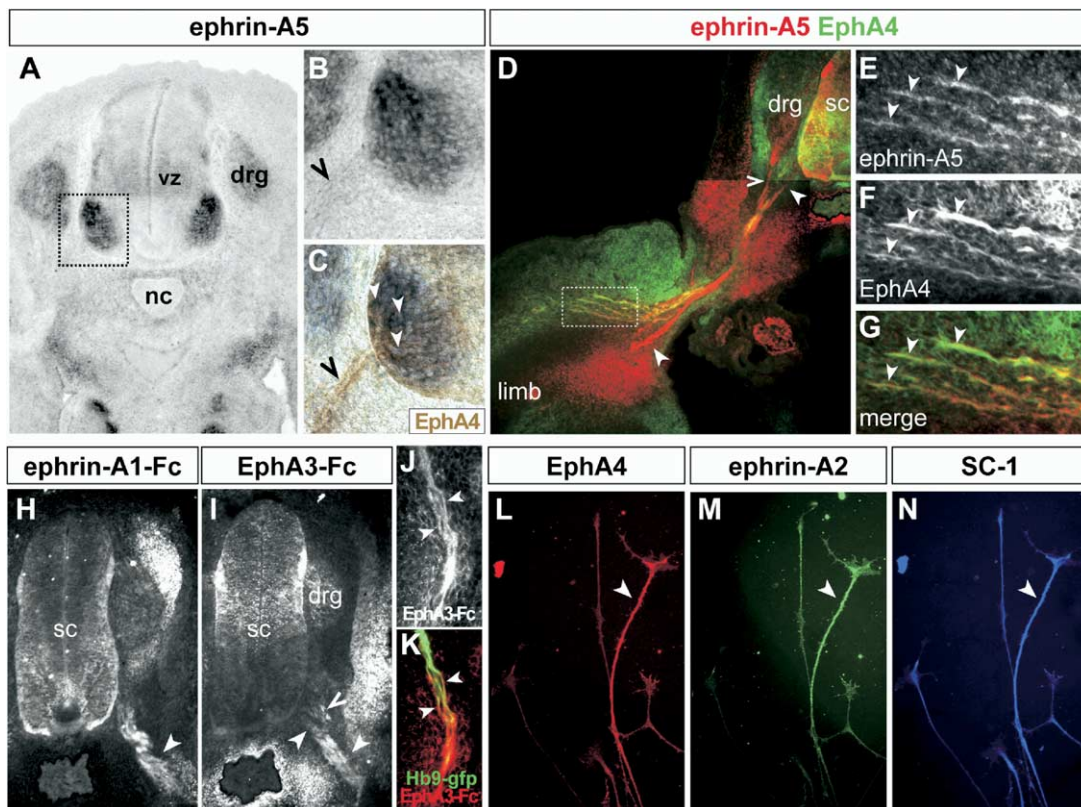


Figure 1. Coexpression of Ephrin-A Ligands and EphA Receptors in Embryonic Motor Neurons and Their Axons

(A–C) Expression of *ephrin-A5* mRNA in the lumbar E5 chick spinal cord.

(A and B) Motor neurons (boxed, [B]) of the LMC express *ephrin-A5* RNA (motor axons, arrowhead). Additional expression in dorsal root ganglion (drg), and medial ventricular zone (vz). nc: notochord.

(C) EphA4 protein (brown) is detected in most *ephrin-A5* (dark blue)-expressing motor neurons (arrowheads).

(D–G) Coimmunodetection of *ephrin-A5* and EphA4 proteins on dorsal limb-innervating LMCI axons (lumbar, E5 chick embryo).

(E–G) Enlargement of boxed area in (D) reveals colocalization of *ephrin-A5* and EphA4 on distal axon segments (arrowheads).

(H and I) Detection of EphA and *ephrin-A* proteins on motor axons exiting the ventral root (closed arrowheads) with *ephrin-A1-Fc* (H) and EphA7-Fc (I) affinity probes, respectively.

(I) *ephrin-As* are also detected on sensory axons (open arrowhead).

(J and K) Detection of *ephrin-As* on distal Hb9-gfp-labeled motor axons (E4 chick embryo, electroporated with Hb9-gfp).

(L–N) Coimmunodetection of EphA4 and *ephrin-A2* on individual cultured motor axons (arrowheads) and growth cones (identified by SC-1, N).

2L). Taken together, these results provide strong evidence that EphA → *ephrin-A*-induced reverse signaling mediates contact-dependent attraction of motor neuron axons.

Ectopic EphAs and Ephrin-As Exert Contrasting Effects on Motor Axon Navigation

To examine the activity of axonal *ephrin-A* and EphA proteins in an *in vivo* assay, we first assayed how motor axon navigation is affected when these proteins are artificially elevated in chick embryos. Forced expression of full-length EphA4, but not of EphA4^{ΔICD} (lacking the intracellular domain; Figure S2P), in spinal motor axons by using *in ovo* electroporation caused a severe reduction of the anterior intercostal ramus (*ica*), while the exterior intercostal ramus (*ice*) appeared unaffected (compare Figures S2A–S2C). This aberrant projection of EphA4-overexpressing axons was likely due to their avoidance of the endogenously *ephrin-A*⁺ inner body

wall mesenchyme (Figure S2Q). In contrast, forced expression of *ephrin-A5* resulted in supernumerary *ic* branches that caused ectopic bridging of adjacent nerves within EphA4-expressing regions of the body wall mesenchyme (Figures S2D and S2R). Unlike native *ephrin-A5*, however, a transmembrane version of *ephrin-A5* (*ephrin-A5*^{ΔGPI-TM}; Figure S2O) failed to induce *ic* bridging (Figure S2E), indicating that GPI anchorage was required and that EphA binding activity per se was not sufficient to produce this phenotype. The results obtained with this *in vivo* gain-of-function assay therefore suggested that EphAs and *ephrin-As* can both influence motor axon navigation in a cell-autonomous and instructive manner.

Removal of Axonal Ephrin-A Protein Abolishes EphA-Induced Growth Cone Spreading

Next, we examined how axonal *ephrin-As* mediate growth cone spreading and neurite outgrowth in re-

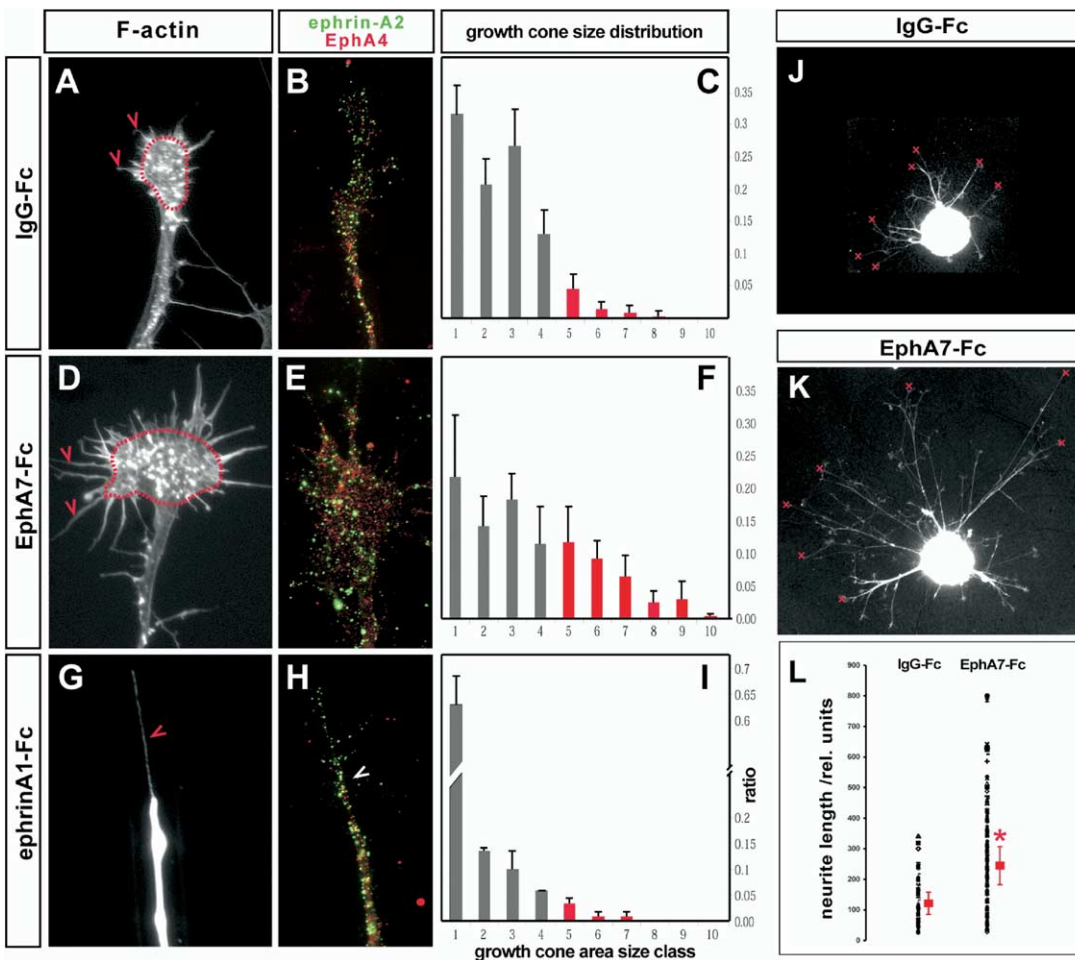


Figure 2. Contrasting Growth Cone Responses Elicited by Coexpressed EphA and Ephrin-A Proteins

(A) Growth cone morphology visualized by rhodamine-phalloidin following 3 hr treatment with IgG-Fc. (B) EphA4 and ephrin-A2 coexpression on control growth cones. (C) Control growth cone size distribution arranged in size classes from 1 to 10 ($n = 6$ explants, for each an average [σ] of 87 growth cones were measured using Openlab software). (D) EphA7-Fc application (3 hr) induces spreading of growth cones. (E) EphA4 and ephrin-A2 coexpressed on growth cones exposed to EphA7-Fc. (F) Growth cone size distribution is shifted toward larger classes (5–10, red; $n = 6$; σ 123 growth cones each). (G) ephrin-A1-Fc induces growth cone collapse within 1 hr. (H) EphA4 and ephrin-A2 coexpressed on collapsed growth cones. (I) Growth cone size distribution accordingly displays shift to the smallest size classes ($n = 3$; σ 119 growth cones each). (J–L) Long-term stimulation with EphA ectodomains promotes motor neurite outgrowth. Motor column explants were cultured for 24 hr on control IgG-Fc (J) or EphA7-Fc (K); neurite length was measured (from base of explants to tip of growth cone). (L) Scatter plot of absolute neurite length. Individual measurements are in black, average length in red ($n = 3$ explants, σ 85 neurites each [IgG-Fc]; $n = 3$, σ 160 neurites [EphA7-Fc]). * $p < 0.001$, Student's t test.

sponse to exogenous ECD-EphAs. We considered two models: (1) ephrin-As could play a direct role as receptors, or (2) ephrin-As might act indirectly by activating EphAs in *cis*, thereby tonically restricting growth cone size. In this scenario, ECD-EphAs may compete for Eph-ephrin *cis*-interactions, thereby relieving a tonic inhibitory signal. In the first case, elimination of ephrin-As would be predicted to lead to a loss of responsiveness of growth cones to exogenous EphAs and consequently their failure to enlarge when challenged with ECD-EphAs, and in the second case, growth cones should shift to a larger default size in the absence ECD-EphAs.

To strip ephrin-As from motor growth cones, explants were incubated with phosphatidylinositol-specific phospholipase C (PI-PLC) to remove GPI-anchored proteins from axonal membranes. This procedure efficiently removed endogenous as well as transfected V5 epitope-tagged ephrin-A5 from the surface of motor neurons without affecting EphA4 (Figures 3A and 3B, see also Figures 4B–4E). We found that the PI-PLC-mediated removal of GPI-anchored proteins had no significant effect on normal growth cone size (Figures 3C and 3E). In contrast, the stripping of ephrin-As from motor axons abolished the ECD-EphA7-Fc-induced spreading response (Figures 3D and 3E). These data therefore sug-

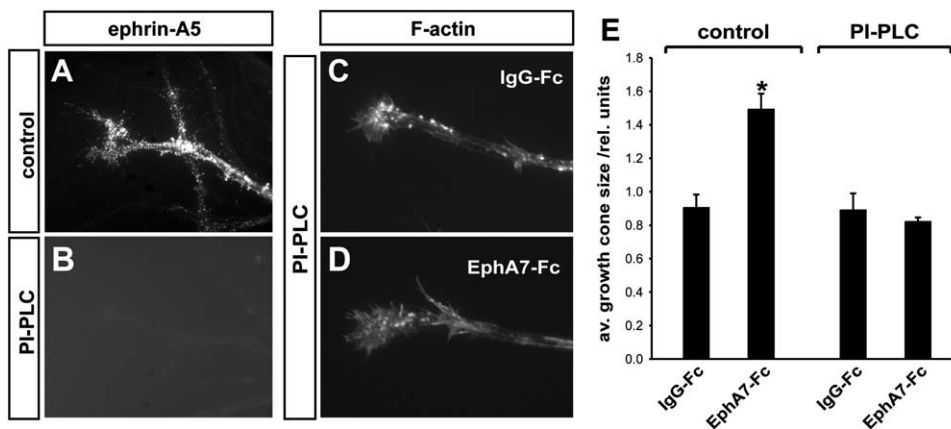


Figure 3. Removal of GPI-Anchored Proteins Abolishes EphA Ectodomain-Triggered Growth Cone Spreading

(A and B) Removal of ephrin-A5 by PI-PLC monitored by immunodetection for ephrin-A5, in control (A) and after PI-PLC (B).

(C and D) Rhodamine-phalloidin-stained IgG-Fc and EphA7-Fc-treated growth cones after PI-PLC treatment.

(E) Average growth cone sizes upon treatment with indicated proteins of chick motor column explants treated with PI-PLC (slightly reduced value for IgG-Fc/PI-PLC compared to IgG-Fc/control is not significant, $p > 0.2$; PI-PLC: $n = 6$ explants, ≈ 208 growth cones each [IgG-Fc]; $n = 6$, ≈ 274 growth cones [EphA7-Fc]; Control: $n = 3$ explants, ≈ 142 growth cones [IgG-Fc]; $n = 4$, ≈ 110 growth cones [EphA7-Fc]). Control: $*p < 0.001$; PI-PLC: difference between IgG-Fc and EphA7-Fc values is not significant, $p > 0.4$.

gest that EphA-induced growth cone spreading is mediated by direct signaling via axonal ephrin-A proteins.

Ephrin-A Levels Influence the Amplitude of Reverse, but Not Forward, Signaling

Graded EphA signaling plays a well-established role in controlling the projections of retinal axons across the optic tectum/superior colliculus (Drescher et al., 1997; Flanagan and Vanderhaeghen, 1998). Therefore, we asked whether the level of ephrin-A, likewise, quantitatively influenced growth cone responsiveness to ECD-EphA-induced spreading. The neural tube of chick embryos was electroporated with a bicistronic expression construct encoding ephrin-A5 and gfp (Figures 4A–4C). Fifty hours postelectroporation (HH stage 23), gfp⁺ explants were isolated and cultured for 36 hr, followed by a 3 hr bath-application of control IgG-Fc or EphA7-Fc proteins. Nontransfected explants treated with EphA7-Fc exhibited a 1.48 (± 0.09)-fold increase in growth cone size compared to controls (Figure 4F). The overexpression of ephrin-A5 enhanced this response, triggering a 1.86 (± 0.10)-fold increase in growth cone size (Figures 4F and 4I). At the same time, comparison of control and ephrin-A5 overexpressing growth cones revealed no significant change in area sizes upon control IgG-Fc protein application (Figure 4F). These findings suggest that the extent of growth cone spreading triggered by exogenous ECD-EphAs reflects the level of ephrin-A present on the cell membrane.

Next, we examined whether elevated levels of ephrin-A5 might antagonize EphA-mediated repulsive signaling via *cis*-interaction. Following the electroporation of chick embryos with ephrin-A5-expression constructs, motor column explants were cultured and treated for 1 hr with preclustered control IgG-Fc or ECD-ephrin-A1-Fc. These explants were treated with nonsaturating ($\sim 1/2$ -max, 100 ng/ml) concentrations of ECD-ephrin-A1-Fc (saturation of the growth cone collapse response

was achieved at levels >200 ng/ml). We found that 100 ng/ml of the EphA ligand ECD-ephrin-A1-Fc induced collapse of 64.0% of the growth cones from control explants, versus inducing collapse of 65.8% of the growth cones extending from ephrin-A5-overexpressing explants (Figures 4G and 4K). Thus, the sensitivity of growth cones to collapse-inducing ephrins was not significantly altered by overexpression of ephrin-A5 (Figure 4G).

The previous findings indicated that the coexpression with ephrin-As does not significantly alter EphA-mediated growth cone collapse. We therefore directly analyzed the activation properties of the EphA receptor under conditions of ephrin-A coexpression. COS-7 monkey kidney epithelial cells stably transfected with either *EphA3* alone or *EphA3* plus *ephrin-A5* were stimulated with the EphA ligand ECD-ephrin-A1-Fc, and receptor activation was subsequently monitored by immunoblotting with phosphotyrosine-specific antibodies following immunoprecipitation. In both *EphA3*, as well as *EphA3* + *ephrin-A5* cells, significant EphA3 phosphorylation was only observed upon (*trans*-) application of ECD-ephrin-A1-Fc (Figure 4H), and the level of EphA3 activation was comparable under both conditions. Hence, coexpression of ephrin-A5 was not sufficient to induce EphA3 receptor activation, nor was the *trans*-activation of EphA3 measurably altered by the simultaneous presence of ephrin-A5. Similarly, coexpression with ephrin-A1 did not lead to altered activation of EphA2 by ECD-ephrin-A1-Fc in cultured human fibroblasts (N.C. and T.H., unpublished data). Together, these data suggest that EphAs can be functionally uncoupled from coexpressed ephrin-As.

EphA and Ephrin-A Proteins Are Segregated within the Growth Cone Membrane

Double-label immunocytochemistry to detect EphA4 and ephrin-A5 on fixed, detergent-treated motor axons

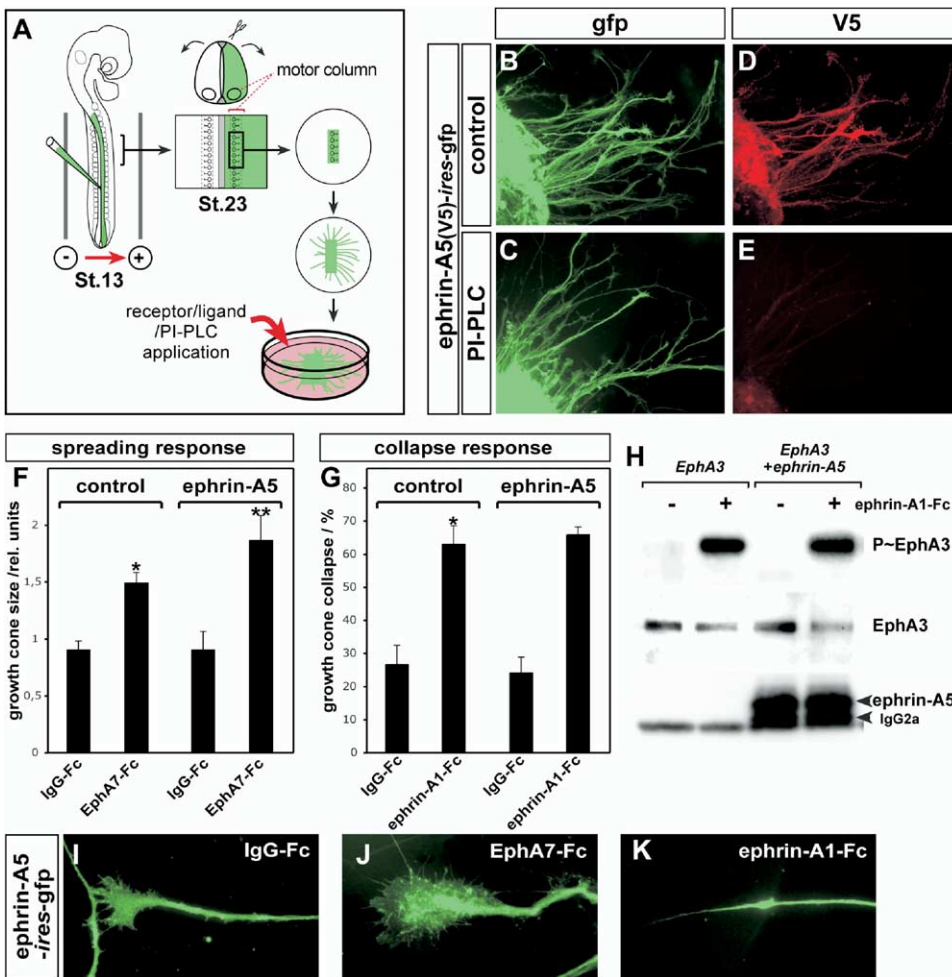


Figure 4. Elevation of Axonal Ephrin-A Levels Enhances EphA-Induced Spreading but Does Not Alter Ephrin-A-Induced Collapse
 (A) Schematic: Strategy used to introduce V5-epitope tagged ephrin-A5 into motor growth cones by in ovo electroporation, with subsequent (gfp⁺) motor column explant dissection and culture.
 (B and C) Transfected explants display gfp expression on cell bodies and axons.
 (B and D) gfp⁺ axons and growth cones concomitantly express ephrin-A5 (detected by V5-antibody).
 (C and E) PI-PLC treatment eliminates the V5-signal.
 (F and G) Sizes of gfp⁺ control and ephrin-A5-overexpressing growth cones after treatment with indicated Fc-fusion proteins.
 (F) Overexpression of ephrin-A5 leads to enhanced EphA7-Fc-triggered spreading (see [J]; **p < 0.001) but does not significantly alter control growth cone size (p > 0.5; ephrin-A5: n = 4 explants, ø92 growth cones each [IgG-Fc]; n = 4, ø113 [EphA7-Fc]). *p < 0.001.
 (G) Overexpression of ephrin-A5 does not significantly alter ephrin-A1-Fc-triggered growth cone collapse (p > 0.5; ephrin-A5: n = 3 explants, ø87 each [IgG-Fc]; n = 4, ø103 [ephrin-A1-Fc]). *p < 0.0001. Slight difference between IgG-Fc values is not significant: p > 0.4.
 (H) Coexpression of ephrin-A5 does not alter trans-activation of EphA3 receptor. Stably transfected COS-7 cells (constructs are indicated) were stimulated (+) with ephrin-A1-Fc or mock treated (-). EphA3 activation (P~EphA3) was monitored by immunoblotting with anti-Phosphotyrosine antibody following immunoprecipitation. IgG_{2a}: band corresponding to mouse anti-V5 antibody used to IP V5-tagged ephrin-A5 and EphA3, detected by HRP-conjugated anti-mouse antibody.
 (I-K) Examples of morphologies of ephrin-A5-overexpressing gfp⁺ motor neuron growth cones treated with indicated proteins.

revealed a nonoverlapping subcellular pattern of the coexpressed proteins (Figures 5A–5D and also see Figure S3). To test whether this observation reflected the relative distribution of coexpressed EphAs and ephrin-As within the native growth cone membrane, we performed fluorescent antibody copatching (Harder et al., 1998) with extracellularly epitope-tagged EphA3(-HA) and ephrin-A5(-Flag), followed by confocal imaging on live growth cones. In this assay, constituents of membrane structures with similar properties and in close proximity are expected to be copatched by antibody crosslinking

while the restricted diffusion between different membrane fractions produces segregated clusters of their respective components. By using this approach, the vast majority of fluorescent patches obtained corresponded to either EphA3 or ephrin-A5, but not both (Figures 5E–5H; 4.3% ± 2.9% EphA3⁺ephrin-A5⁺ patches), thus indicating a mutually exclusive distribution of EphA and ephrin-A protein within the native growth cone membrane. In transfected COS-7 cells, a similar nonoverlapping distribution of EphA3 and ephrin-A5 was observed (Figure 6B; 7.4% ± 1.9%). At the same

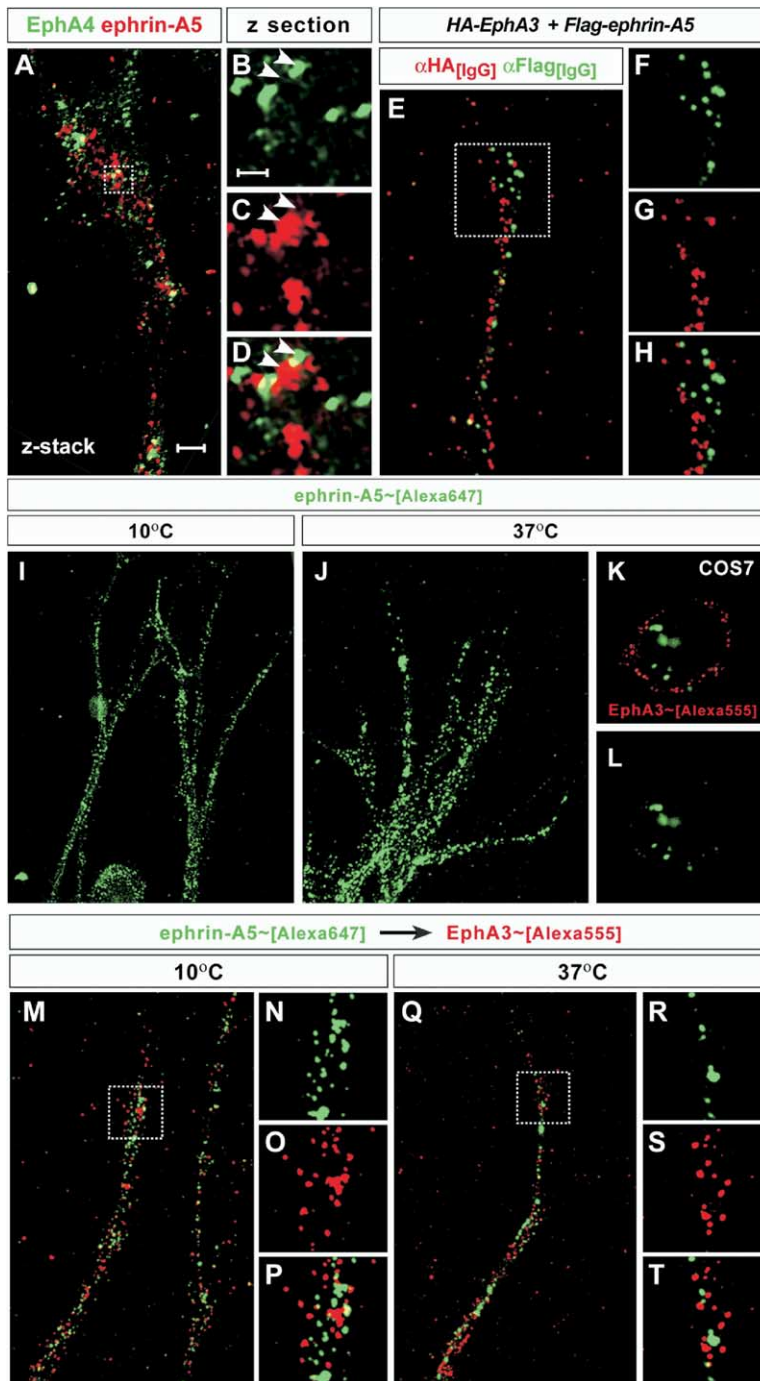


Figure 5. Coexpressed EphA and Ephrin-A Proteins Are Laterally Segregated within the Membrane and Are Simultaneously Accessible to *trans* Binding

(A) Double-immunofluorescence detection of EphA4 and transfected V5 epitope-tagged ephrin-A5 of a fixed detergent-treated motor neuron growth cone. EphA4 and ephrin-A5 staining is mutually exclusive. Stack of 30 optical z plane sections obtained by laser scanning microscopy (LSM). Scale bar: 5 μ m. Boxed area: blow up (B–D). In single optical z plane section, yellow signals in the z stack (A) resolve into individual foci (two arrowheads). Scale bar: 1 μ m.

(E) Fluorescence antibody copatching of double-transfected HA-EphA3 + Flag-ephrin-A5 on live growth cones using epitope tag antibodies. Copatching was induced by secondary antibodies. Boxed area: blow up ([F]–[H]; single z plane section); EphA3 and ephrin-A5 become patched into nonoverlapping foci.

(I) Alexa647-conjugated ephrin-A5-Fc binding on motor axons and growth cones at 10°C.

(J) After incubation at 37°C, larger patches appear on axons, presumably corresponding to endocytosed Alexa647 ~ ephrin-A5-Fc. One microgram/milliliter Alexa647 ~ ephrin-A5-Fc was applied to culture medium for 1 hr at indicated temperatures, followed by PBS wash and confocal microscopy.

(K and L) Sequential application of 1 μ g/ml Alexa647 ~ ephrin-A5-Fc (30 min) followed by Alexa555 ~ EphA3-Fc (30 min) to EphA3 + ephrin-A5 cotransfected COS-7 cells. Most ephrin-A5 appears internalized (green patches), while EphA3-Fc appears bound to plasma membrane (red).

(M–T) Sequential application of Alexa647-conjugated ephrin-A5 followed by Alexa555 ~ EphA3 on live growth cones.

(M–P) Procedure carried out at 10°C reveals concomitant binding of ephrin-A5 (green) and EphA3 (red) to separate domains on surface of the same growth cone. Box in (M) corresponds to (N)–(P).

(Q–T) At 37°C, large patches of presumably endocytosed ephrin-A5 (green) appear (e.g., see Figures S4A–S4D), that (as in [I]–[L]) are mostly negative for EphA3 (red signal). Box in (Q) corresponds to (R)–(T).

time, extensive copatching ($72.8\% \pm 3.3\%$) of coexpressed EphA3 and EphA4 (Figure 6E), as well as ephrin-A2 and ephrin-A5 ($80.1\% \pm 5.2\%$, Figure 6C) was observed. These results indicate that in both motor neurons and nonneuronal cells, coexpressed EphA and ephrin-A proteins display a segregated localization within the plasma membrane.

Coexpressed EphA and Ephrin-A Proteins Are Accessible to *trans* Binding

Our previous results indicated that coexpressed EphAs and ephrin-As can be independently activated in *trans*.

To directly visualize whether the coexpressed EphA and ephrin-A proteins can simultaneously be bound in *trans* by ECD-ephrin-As and ECD-EphAs, live motor neuron growth cones were incubated with directly fluorophore-conjugated ephrin-A5 (Alexa647 ~ ECD-ephrin-A5-Fc), followed by washout to prevent receptor-ligand interaction in the medium, and subsequent incubation with Alexa555 ~ ECD-EphA3-Fc. Under conditions that prevented endocytosis (at 10°C; Bruckner et al., 1999), both cell surface bound Alexa555 ~ ECD-EphA3-Fc probe for ephrin-As and Alexa647 ~ ECD-ephrin-A5-Fc probe for EphAs were observed within separate clus-

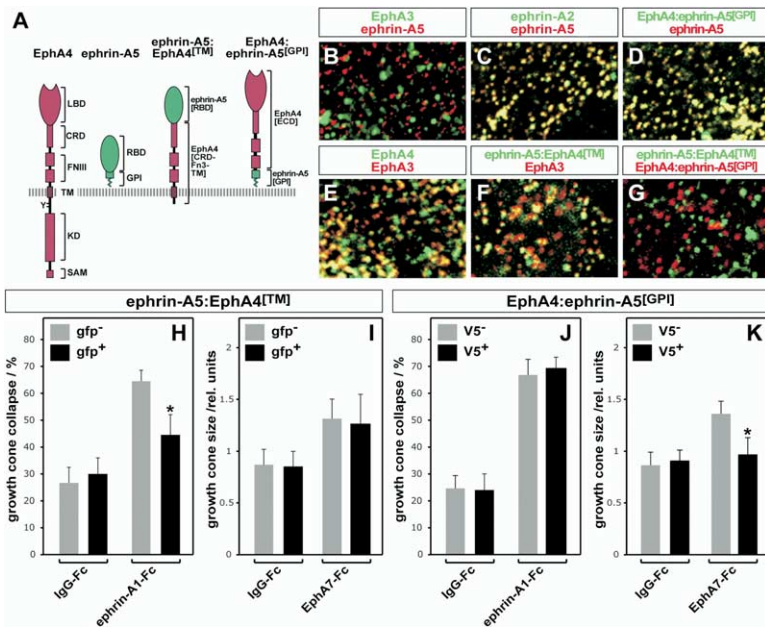


Figure 6. Forced Colocalization with EphA-Ligand or Ephrin-A Receptor Binding Domains Leads to *cis*-Masking and Disruption of Ephrin-A- or EphA-Mediated Signaling

(A) Structure of wild-type EphA4, ephrin-A5, and domain-swapping mutants (see [Experimental Procedures](#)).

(B–G) Fluorescent antibody copatching in COS-7 cells transfected with indicated constructs. The following antibodies were used: anti-HA (rabbit) + anti-Flag (mouse) (B and F), anti-V5 (rabbit) + anti-Flag (mouse) (C, D, and G), anti-V5 (rabbit) + anti-HA (mouse) (E). Constructs: Flag-ephrin-A5 (B–D), V5-ephrin-A2 (C), V5-EphA4:ephrin-A5 (D and G), HA-EphA3 (E and F), V5-EphA4 (E), Flag-ephrin-A5:EphA4 (F and G). Copatching was induced by anti-mouse and anti-rabbit secondary antibodies (see [Experimental Procedures](#)).

(H) ephrin-A5:EphA4 chimera attenuates ECD-ephrin-A1-Fc-triggered growth cone collapse (**p* < 0.01).

(I) ECD-EphA7-Fc-triggered spreading responses are not significantly altered (*p* > 0.8). ephrin-A5:EphA4-ires-gfp-transfected (gfp⁺) growth cones were scored compared to untransfected (gfp⁻) axons (e.g., see [Figures 7A](#)

and 7B) ([H]: *n* = 6 explants, 25 growth cones each [IgG-Fc]; *n* = 3, 37 [ephrin-A1-Fc]; [I]: *n* = 3, 21 [EphA7-Fc]). (K) EphA4:ephrin-A5 chimera attenuates ECD-EphA7-Fc-triggered growth cone spreading (**p* < 0.001). While neither ECD-ephrin-A1-Fc-triggered collapse nor control growth cone size/collapse are significantly altered (*p* > 0.6; *p* > 0.8; [J]) ([J]: *n* = 4 explants, 56 growth cones each [IgG-Fc]; *n* = 3, 78 [ephrin-A1-Fc]; [K]: *n* = 4, 62 [EphA7-Fc]).

ters ([Figures 5I](#) and 5M–5P). Incubation at 37°C resulted in ephrin-A-triggered growth cone collapse ([Figure 5J](#)) and to the appearance of larger-sized Alexa647~ECD-ephrin-A5-Fc⁺ clusters ([Figures 5J](#) and 5M–5P), presumably corresponding to endocytosed complexes ([Zimmer et al., 2003](#)). Similarly, sequential incubation with Alexa647~ECD-ephrin-A5-Fc and Alexa555~ECD-EphA3-Fc on EphA3 + ephrin-A5 COS-7 cells at 37°C revealed large internalized clusters that labeled for ECD-ephrin-A5-Fc, but not for ECD-EphA3-Fc ([Figures 5K](#) and 5L). Interestingly, on both growth cones and COS-7 cells, these Alexa647~ECD-ephrin-A5⁺ clusters remained segregated from Alexa647~ECD-EphA3-labeled foci, indicating that EphA receptor activation did not recruit coexpressed ephrin-A protein into the same membrane domains. Together, these data suggest that on motor neuron growth cones coexpressed EphA and ephrin-A proteins are capable of simultaneously binding ephrin-As and EphAs presented in *trans*, respectively. Moreover, upon activation of EphA receptor, the planar segregation of EphAs and ephrin-As appeared to be preserved.

Differential Membrane Targeting Is Required for Signaling by Coexpressed EphAs and Ephrin-As

We next asked whether the planar separation of coexpressed EphA and ephrin-A proteins is an obligatory requirement for their independent signaling activities. To address this question, we designed chimeric proteins in which (1) the receptor binding domain (RBD) of ephrin-A5 replaced the globular ligand binding domain (LBD) of EphA4 (ephrin-A5:EphA4TM); and (2) the ECD of EphA4 was coupled to the C-terminal portion of ephrin-A5 including the GPI anchor signal sequence

(EphA4:ephrin-A5^{GPI}) ([Figure 6A](#)). These chimeras were tested for their localization within the membrane relative to wild-type EphA4 and ephrin-A5 by fluorescent antibody copatching. Upon cotransfection, wild-type ephrin-A5 copatched with ephrin-A2, but not with EphA3 ([Figures 6B](#) and 6C). EphA3, however, colocalized with EphA4 ([Figure 6E](#)). In contrast to wild-type ephrin-A5, ephrin-A5:EphA4TM extensively copatched with coexpressed wild-type EphA3 ([Figure 6F](#)). Conversely, EphA4:ephrin-A5^{GPI} colocalized with wild-type ephrin-A5 ([Figure 6D](#)). Hence, in the ephrin-A5:EphA4TM and EphA4:ephrin-A5^{GPI} chimeras, the C-terminal portions of EphA4 and ephrin-A5, respectively, were sufficient to retain the typical membrane-targeting properties of the wild-type proteins, despite switching respective ligand/receptor binding domains.

We reasoned that the expression in motor neuron growth cones of the ephrin-A5:EphA4TM chimera, as opposed to wild-type ephrin-A5, would selectively target EphA binding activity to EphA-containing membrane domains, thus potentially interfering with their activation in *trans* by ECD-ephrin-As. Conversely, the expression of EphA4:ephrin-A5^{GPI} was predicted to introduce ephrin-A binding activity into ephrin-A-positive membrane domains, possibly affecting responses toward *trans*-ECD-EphAs. To test these ideas, the ephrin-A5:EphA4TM chimera was introduced in motor neuron growth cones, followed by ECD-ephrin-A or ECD-EphA protein application. In contrast to wild-type ephrin-A5, ephrin-A5:EphA4TM led to a marked (20.1%) reduction in ECD-ephrin-A1-induced growth cone collapse ([Figure 6H](#)), while having no significant effect on ECD-EphA7-induced growth cone spreading nor on control growth cone size ([Figures 6H](#) and 6I). The basis for this signaling defect was predicted to reside in the

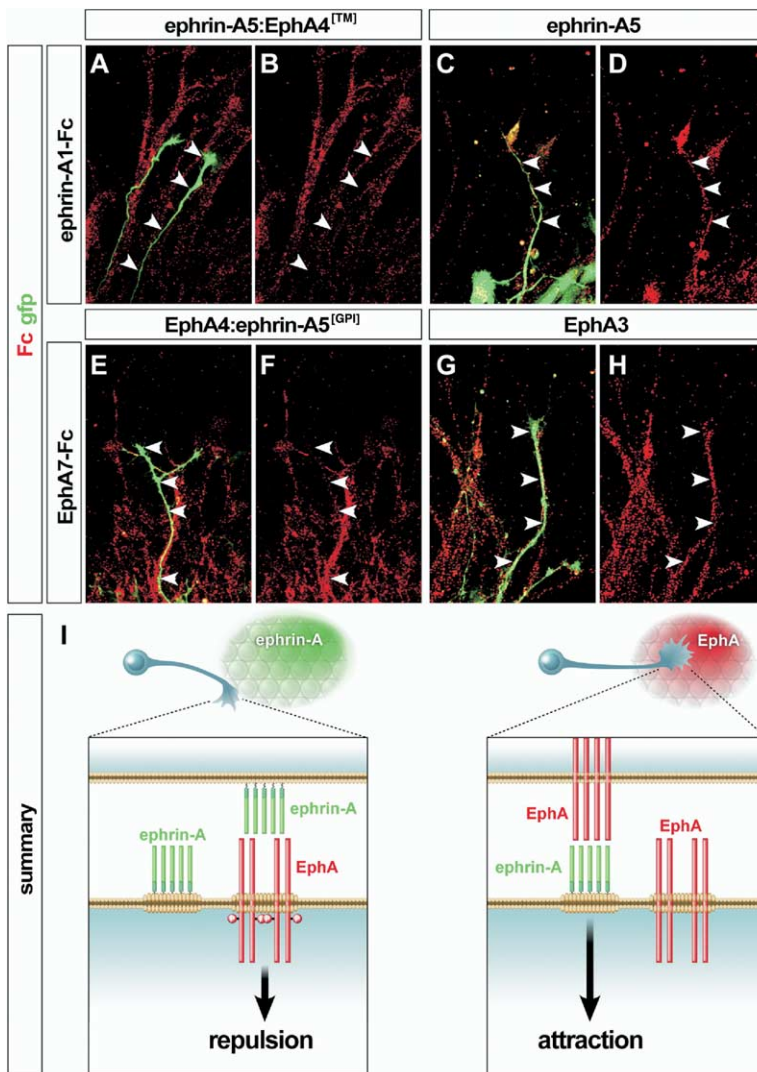


Figure 7. Lateral Segregation of Coexpressed EphAs and Ephrin-As Allows Their Utilization as Independent Axon Guidance Receptors

(A and B) ephrin-A5:EphA4TM(ires-gfp)-transfected (gfp⁺) axons display extremely diminished binding of *trans*-ephrin-As (ephrin-A1-Fc; arrowheads in [B]), indicating masking of endogenous EphAs. Note noncollapsed morphology of gfp⁺ growth cones (A).

(C and D) Axons transfected with native ephrin-A5(ires-gfp) (gfp⁺) retain binding of ephrin-A1-Fc (arrowheads in [D]), indicating absence of masking.

(E and F) EphA4:ephrin-A5^{GPI} leads to diminished binding of EphA7-Fc (arrowheads in [E]). (G and H) Axons transfected with native EphA3(ires-gfp) (gfp⁺) display high levels of EphA7-Fc binding (arrowheads).

(I) The lateral segregation of coexpressed EphAs and ephrin-As within the growth cone membrane frees both proteins for *trans*-interactions, thereby uncoupling their respective signaling activities. Thus, encounter of an EphA/ephrin-A growth cone with an ephrin-A-expressing cell can trigger collapse (left panel), while contact with EphA-expressing cells can trigger attractive responses (right panel).

masking of endogenous EphAs by colocalized ephrin-A5:EphA4TM. Consistent with this idea, ephrin-A5:EphA4TM+ growth cones displayed a dramatic reduction in surface bound ephrin-A1-Fc protein, compared to axons lacking ephrin-A5:EphA4TM extending from the same explant (Figures 7A and 7B)—thus indicating masking of endogenous EphA-LBDs by colocalized ephrin-A5:EphA4TM chimeras.

In contrast to ephrin-A5:EphA4TM, the expression of the EphA4:ephrin-A5^{GPI} chimera led to a dramatic reduction in ECD-EphA7-Fc-mediated growth cone spreading, without significantly affecting ECD-ephrin-A1-Fc triggered collapse responses (Figures 6J and 6K). EphA4:ephrin-A5⁺ axons displayed a severe reduction in the level of surface-bound ECD-EphA7-Fc in contrast to EphA4:ephrin-A5^{GPI}-negative axons extending from the same explant (Figures 7E and 7F), indicating *cis*-masking of endogenous ephrin-As by the chimeric protein. Hence, forced colocalization with EphA-LBDs or ephrin-A-RBDs leads to the *cis*-masking and disruption of ephrin-A- or EphA-mediated signaling. Thus, their spatial arrangement within the plasma

membrane prevents receptor-ligand *cis*-interactions by a mutual exclusion mechanism, thereby uncoupling EphA- and ephrin-A-mediated signaling activities within the same neuronal growth cone.

Discussion

In this paper, we present a mechanism that allows coexpressed Eph receptors and ephrin ligands to function as independent axon guidance receptors within the same navigating growth cone. We provide several lines of evidence indicating that in motor neurons EphAs and ephrin-As partition into separate membrane domains from which they exert opposing effects on neuronal growth cone behavior. This lateral segregation of Ephs and ephrins appears to restrict their ability to interact in *cis*, thereby freeing coexpressed EphAs and ephrin-As to productively signal when cognate receptor-ligand pairs encountered one another in *trans*. Thus, EphA⁺/ephrin-A⁺ growth cones are capable of being attracted to sources of EphA protein while simultaneously being repelled from tissues expressing ephrin-As (Figure 7I).

Membrane Compartmentalization of Ephs and Ephrins

The differential sorting of signaling proteins to specific portions of the cell membrane, such as to the apical versus the basolateral membrane in epithelial cells or the axonal versus somatodendritic membrane in neuronal cells, is essential for many aspects of cellular maturation and function. In the developing CNS, several classes of guidance receptors display restricted localization along the length of the axon (Brittis et al., 2002; Dickson, 2002; Dodd et al., 1988). This proximodistal compartmentalization can serve as a mechanism for determining the timing of guidance receptor function. In the case of the EphAs and ephrin-As, we observed a finer level of compartmentalization into non-overlapping EphA- and ephrin-A-enriched domains within the same segments of the axonal membrane. The partitioning of cell surface receptors and signaling components into specialized submembrane domains with distinct lipid composition is commonly implicated in directing T cell signaling and chemotaxis (Manes et al., 2003; Simons and Toomre, 2000). Recent studies, moreover, suggested that the normal responsiveness of neuronal growth cones toward guidance cues similarly requires the integrity of lipid raft membrane domains (Guirland et al., 2004). In addition to creating platforms for the efficient assembly of signaling complexes, the planar compartmentalization within the plasma membrane is thought to prevent undesired or premature signaling by restricting mutual access of potentially interacting components (Dykstra et al., 2003; Simons and Toomre, 2000). Thus, the lateral segregation of EphAs and ephrin-As appears to represent a unique case of this general mechanism: effectively uncoupling their respective signaling activities by preventing *cis*-interactions of coexpressed receptors and ligands via mutual physical exclusion.

What evidence suggests that the compartmentalization of EphAs and ephrin-As into distinct membrane domains is necessary for these proteins to function as independent receptors? In the developing visual system of the chick, overexpression of ephrin-As within EphA⁺ retinal ganglion cells was found to disrupt EphA-mediated repulsion in response to ephrin-A ligands encountered in *trans* (Hornberger et al., 1999). The desensitization of EphA signaling in these cells is thought to be due to the *cis*-interaction of ephrin-As with endogenously expressed EphAs. Evidence for this type of *cis*-interaction also comes from transfection experiments in HEK293 cells in which EphA and ephrin-A proteins were no longer compartmentalized due to overexpression and/or changing the membrane distribution of ephrin-As by expressing them as transmembrane proteins (Yin et al., 2004). In the present study, we demonstrate that selectively swapping the specific membrane targeting properties of EphA and ephrin-A binding activities results in *cis*-interactions that directly interfere with the signaling activities of endogenous ephrin-As and EphAs. Together, these results demonstrate that under conditions where EphA and ephrin-A proteins are not segregated in the membrane, mutual *cis* binding appears to be kinetically favored over *trans*-interactions. Thus, the sorting into separate EphA- and ephrin-A-enriched membrane domains helps to prevent

cis-masking effects that can occur when cognate receptor/ligand pairs colocalize in cells that coexpress both proteins.

It is, moreover, conceivable that inherent systemic differences in membrane lipid composition, as they are known to occur between various cell types (Simons and Toomre, 2000), could contribute to changes in relative membrane targeting and thereby to altered signaling behaviors of coexpressed EphAs and ephrin-As between different cell types—such as motor neurons and retinal ganglion cells or between mature neurons and those actively extending axons. Furthermore, it seems possible that the contextual modulation in the assembly/disassembly of Eph- and ephrin-enriched membrane domains could serve as a mechanism for simultaneously switching the Eph-ephrin receptor systems on and off. This intriguing possibility is consistent with the observation that in EphB/ephrin-B coexpressing vascular endothelial cells, treatment with polysialic acid facilitated EphB activation in the absence of exogenously applied ephrin-B ligand (Stein et al., 1998). Thus, not only intrinsic cellular properties and/or relative expression levels, but also extrinsic signals that regulate their differential sorting within the plasma membrane, could affect the activities exerted by coexpressed Ephs and ephrins.

Roles of Coexpressed Ephs and Ephrins in Guidance Signaling

The mechanism allowing independent signaling via coexpressed Ephs and ephrins, as elucidated here, should aid in our understanding of Eph/ephrin function in vivo: ephrin-A → EphA forward signaling leading to repulsion and EphA → ephrin-A reverse signaling promoting growth cone attraction (Figure 7I). However, the concomitant presence of independent Eph and ephrin binding activities on the same cell membrane poses in itself an intriguing problem when “double-positive” cells encounter one another. Although this is predicted to lead to the simultaneous signaling of attraction and repulsion, how these divergent signals are integrated is unknown. For instance, will one signal override the other, or will coactivation lead to qualitatively different responses? Recently, the latter scenario has been suggested to occur during urogenital morphogenesis where cosignaling of ephrin-B and EphB, as opposed to EphB alone, may mediate a switch from repulsive to adhesive signaling (Dravis et al., 2004), although the relative subcellular localization of B class Eph and ephrins remains to be elucidated.

The development of limb-innervating motor axons may offer an entry point in the study of some of the in vivo consequences of Eph/ephrin coexpression. Both gain- and loss-of-function experiments linked EphA4 to repelling LMCI axons from the ephrin-A-positive ventral limb mesenchyme, thereby contributing to the specificity in dorso-ventral choice of LMC axons (Eberhart et al., 2002; Helmbacher et al., 2000). Intriguingly, in certain genetic backgrounds, a significant fraction of EphA4 null mutant mice displayed a complete failure of LMCI axons to innervate the dorsal limb, leading to their exclusive selection of a ventral trajectory (Helmbacher et al., 2000). This observation contrasts with the

randomization of dorso-ventral axon choice that would be predicted from the loss of responsiveness of these axons toward a ventral limb repellent. Interestingly, EphA4 is also expressed in the dorsal limb mesenchyme (Helmbacher et al., 2000), while LMCI axons coexpress ephrin-As. Thus, these latter results raise the intriguing possibility that Eph forward and ephrin reverse signaling function coordinately to determine the dorsal choice of LMCI axons.

Distinguishing *cis*- and *trans*-Signaling

The present study provides one of the first examples of the selective segregation of cognate receptor and ligand within the same growth cone membrane and demonstrates their independent roles as axon guidance receptors. Although we focused on the Ephs and ephrins, we speculate that this arrangement may serve as a more generalizable paradigm for molecules mediating cell-cell signaling. The cellular responses exerted by some cell adhesion molecules (CAMs), for instance, can be significantly altered by homo- and/or heterophilic *cis*-interactions (Kamiguchi and Lemmon, 2000). These *cis*-interactions appear to be context dependent, since under many paradigms *trans* binding has to predominate to result in unambiguous adhesion responses by the same molecules. How this selectivity is achieved remains unknown, but it is conceivable that, similar to coexpressed Ephs and ephrins, differential submembrane targeting could principally free coexpressed heterophilic CAMs for the required *trans*-interactions.

Similar to EphAs and ephrin-As, neuropilin and plexin guidance receptors are coexpressed with their cognate semaphorin ligands in spinal motor neurons, while developing motor axons are nevertheless highly sensitive to exogenous semaphorins (Chen et al., 1997; Varela-Echavarría et al., 1997). Interestingly, genetic evidence in *Drosophila* and mice indicated cell-autonomous roles of neuronal transmembrane semaphorin "ligands" (Godenschwege et al., 2002; Leighton et al., 2001), suggesting that similar to the Ephs and ephrins, forward and reverse semaphorin signaling may both operate in the guidance of motor axons. Thus, the possibility of functionally uncoupling receptor- and ligand-mediated signaling activities within the same cell membrane has significant implications for understanding the *in vivo* contribution of these signaling systems for axon guidance decisions, as well as other cell-cell signaling processes.

Experimental Procedures

Immunohistochemistry, Affinity Probe Detection, and In Situ Hybridization

Immunohistochemistry/in situ hybridization on tissue sections was carried out as described (Thaler et al., 2002). Motor column explants were fixed by stepwise replacement of culture medium with 10% sucrose/4% PFA/PBS for 30 min, washed in PBS, incubated 1–2 hr with primary/secondary antibodies in 1% BSA/PBS ($\pm 0.2\%$ Tween 20). The antibodies were as follows: rabbit anti-EphA4 (1:1000, Santa Cruz S-20), goat anti-ephrin-A2/-A5 (1:500, R&D), mouse anti-V5 (1:500, Invitrogen), rhodamine-phalloidin (1 μ g/ml, Mol. Probes). For affinity probe detection, sections were incubated 1 hr at RT with 5–10 μ g/ml ligand/receptor (R&D)/1% BSA/PBS, followed by 1 hr 2 μ g/ml Cy3-anti-human IgG-Fc. Secondary antibodies were from Jackson IR. Covalent fluorophore conjugation of

Fc-chimeras was carried out using Alexa monoclonal antibody labeling kits (Mol. Probes).

In Vitro Motor Axon Guidance Assay

HH stage 23 chick embryos were dissected in ice-cold DMEM/F12 (Gibco)/25% fetal bovine serum after treatment with 0.5% trypsin in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's for 45 min on ice. Following removal of floor plate, explants from the ventral third of the brachial spinal cord-comprising motor column were excised. Culture conditions/medium were as described (Shirasaki et al., 1998). For growth cone collapse/spreading assays, explants were cultured 48 hr on poly-D-lysine and laminin (5 μ g/ml) double-coated coverslips prior to application of Fc-chimeras, preclustered by anti-Fc for 1–3 hr. PI-PLC (1 U/ml, Sigma) was applied for 1 hr prior to assay. Neurite outgrowth assay was as follows: explants were cultured on multicoated coverslip; nitrocellulose was used to obtain uniform carpet of preclustered EphA7-Fc (10 μ g/ml) or IgG-Fc, plus laminin as described (Knoll et al., 2001).

Fluorescence Antibody Copatching

Antibody copatching was essentially carried out as described (Harder et al., 1998). Briefly, medium of live transfected explants or COS-7 cells was stepwise replaced by 1% BSA/PBS, then sequentially incubated for 1 hr at 10°C (Zimmer et al., 2003) with combinations of mouse anti-HA (Sigma), rabbit anti-HA (BD Clontech) or mouse anti-Flag (M2, Sigma) in 1% BSA/PBS followed by washing (PBS). Copatching was induced by Cy3-goat anti-mouse plus Cy5-goat anti-rabbit antibodies (Jackson IR) for 1 hr at 10°C followed by washing and confocal imaging.

Transfection Procedures and Expression Vectors

Transfection/maintenance of chick embryos and cells was carried out as described (Carter et al., 2002; Thaler et al., 2002). Full-length mouse and chick *ephrin-A2/5*, *EphA3/4* cDNAs or derivatives were cloned into *pCAGGS-ires-gfp* or *cDNA3.1* (Invitrogen). For V5-ephrin-A5, V5-tag was inserted just N-terminal of GPI anchor sequence. For V5-ephrin-A2 and Flag-ephrin-A5, epitope tags were inserted between signal sequence (SS) and first residue of RBD. For V5-, Flag-, HA-EphA3, and V5-EphA4, tags were inserted between SS and first residue of the LBD. To generate EphA4 Δ ICD, the intracellular domain (ICD) starting at aa 598 was removed. For ephrin-A5^{TM(Δ GPI)}, GPI anchor sequence was replaced by the TM of EphA3. For ephrin-A5:EphA4^{GPI}, the RBD of chick ephrin-A5 replaced the LBD of EphA4 Δ ICD; for EphA4:ephrin-A5TM, the ECD of EphA4 fused N-terminal to the GPI anchor of mouse ephrin-A5. All constructs were sequenced, expression and membrane-localization confirmed by surface antibody/affinity probe detection.

Receptor Activity Assays

COS-7 cells stably transfected with *EphA3* and *EphA3 + ephrin-A5* were serum starved for 48 hr prior to stimulation (20 min, 5 μ g/ml ephrin-A1-Fc). After lysis at 4°C, EphA3 and ephrin-A5 were immunoprecipitated using anti-V5 and subjected to SDS-PAGE/Western blotting as described (Carter et al., 2002). Activated EphA3 was detected using monoclonal anti-P-Tyr (4G10; UBI).

Supplemental Data

Supplemental Data include four figures and can be found with this article online at <http://www.cell.com/cgi/content/full/121/1/127/DC1/>.

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