Development/Plasticity/Repair

Multiple Effects of Ephrin-A5 on Cortical Neurons Are Mediated by Src Family Kinases

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The Eph receptor tyrosine kinases and their membrane-bound ligands, the ephrins, are involved in a variety of developmental processes such as axonal guidance, cell migration, cell adhesion, proliferation, and differentiation. In addition to repulsive effects, ephrins can also induce attractive responses. Up to now, little was known about the underlying signaling mechanisms that regulate attractive versus repulsive effects. In this study, we show that ephrin-A5 enhances the motility of cortical neurons that is dependent on the activity of Src-family kinases (SFKs). Ephrin-A5 further changes the adhesive properties of neurons by inducing the formation of cell aggregates. Using the stripe assay, we found that the motogenic effect of ephrin-A5 is the result of repulsive ephrin-A interactions. Blocking SFK function leads to a conversion of repulsion into adhesion, suggesting that SFKs can act as a biological switch for the response of EphA receptors. Finally, we discovered a ligand-induced release of membrane particles containing EphA receptors, suggesting membrane ripping as a novel mechanism to overcome the "ephrin paradox" of repulsion after high-affinity receptor–ligand binding.

Key words: **ephrin-A5; membrane ripping; adhesion; SFKs; motility; receptor release**

Introduction

The Eph receptor tyrosine kinases and their membrane-bound ligands, the ephrins, represent one of the first identified families of wiring molecules for the formation of precise neuronal connections in the CNS (Cheng et al., 1995; Drescher et al., 1995). There are now many studies showing that Eph/ephrin interactions play important roles for growth cone guidance, axonal and dendritic branching, spine formation, and neural crest cell migration [for recent reviews, see Dickson (2002), Knoll and Drescher (2002), Klein (2004), and Pasquale (2005)]. Moreover, recent work also provided evidence for the involvement of ephrins in diverse processes such as cell proliferation, differentiation, adhesion, and angiogenesis. Initially, the effects exerted by ephrins on growing neurites and migrating neurons were found to be exclusively repulsive, but there is now also compelling evidence for attractive effects of ephrins (Castellani et al., 1998; Holmberg et al., 2000; Huai and Drescher, 2001; Mann et al., 2002; Eberhart et al., 2004).

Ephrins and Eph receptors are divided into two classes, A and B, based on binding specificities and sequence homologies. The two classes of ephrins are further distinguished by their mode of attachment to the cell membrane. A-ephrins are anchored to the membrane via a glycosylphosphatidylinositol linkage, whereas B-ephrins are anchored via a transmembrane domain. Eph/ephrin interactions regulate migration, repulsion, and adhesion by activating Rho family GTPases that are key regulators of actin–membrane interactions and the dynamic reorganization of actin cytoskeleton (Schwartz et al., 1995; Hall and Nobes, 2000; Noren and Pasquale, 2004). Rho family GTPases can be further controlled by Src-family kinases (SFKs) (Kullander and Klein, 2002), which in turn are known to bind to the juxtamembrane region of Eph receptors via well conserved phosphorylated tyrosine residues (Ellis et al., 1996; Zisch et al., 1998). SFKs were reported to increase the tyrosine phosphorylation of EphA receptors and are therefore involved in the downstream signaling of EphA receptor-mediated axonal guidance (Knoll and Drescher, 2004). SFKs are also known to regulate cytoskeletal arrangements by binding to cortactin, an activator of the actin-related protein 2/3 (Arp2/3) complex that is required for the formation of actin networks (Weaver et al., 2001). Moreover, Src was shown to reduce cell adhesion by phosphorylating R-Ras, a positive regulator of the integrins (Zou et al., 2002). R-Ras was further described to be phosphorylated after EphB2 activation in COS cells that lead to deadhesion of the cells from the matrix (Dail et al., 2006).

In addition to cytoskeletal rearrangements that are necessary for the Eph/Ephrin-induced effects, repulsion and migration require termination of receptor–ligand binding between cells. This is the often-discussed "ephrin paradox": how can repulsion or migration occur after high-affinity binding of membrane-bound ephrin ligands with transmembrane Eph receptors? For the A-system, a metalloprotease-mediated cleavage of the ephrin-A ligands after receptor binding was described (Hattori et al., 2000; Janes et al., 2005), whereas for the B-system, bidirectional endo-

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cytosis of entire receptor–ligand complexes was reported (Marston et al., 2003; Zimmer et al., 2003).

In this study, we first describe that a member of the A-ephrins, ephrin-A5, enhances the motility of cortical neurons in an SFK-dependent manner. Thus, in addition to directing the migration of neurons (Santiago and Erickson, 2002; Nomura et al., 2006), ephrins can also enhance the motility of neuronal cells. Second, we show that ephrin-A5 has an effect on the adhesive properties of cortical neurons. Whereas cortical neurons form large aggregates on a homogeneous ephrin-A5 substrate, in the stripe assay they are repelled by ephrin-A5. Inhibition of SFKs converts the antiadhesive properties of ephrin-A5 into adhesive properties, indicating that SFKs act as a biological switch between repulsive and adhesive effects of ephrin-A5. Finally, we found that the repulsive effects of the ephrin-A system are accompanied by the release of activated EphA receptors in membrane particles after binding to the ephrin-A5 ligands. This membrane ripping is an additional mechanism to overcome the ephrin paradox.

Materials and Methods

Preparation of dissected cortical neurons. Cortices from embryonic day 14 (E14) mouse embryos (day of insemination is E1) were dissected and collected in ice-cold HBSS supplemented with 0.65% glucose. After incubation with 0.025% trypsin in HBSS for 17 min at 37°C, the tissue was dissociated into single cells by trituration and filtered through a nylon gauze to remove cell aggregates. Cell density was adjusted to 150 cells/mm^2. Neurons were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.4 mM L-glutamine at 37°C, 5% CO_2 in a humid atmosphere for 48 h. Drug treatment was performed for 48 h applying 5 μM pyrazolopyrimidine (PP2) or PP3 (Merck Biosciences, Nottingham, UK) to the culture medium. All animal procedures were performed in agreement with the institutional regulations of the University of Jena (Jena, Germany).

NIH3T3 cells. NIH3T3 cell lines transfected with a retroviral vector, pLIG, containing the human ephrin-A5 full-length cDNA and a G418 resistance, or with the vector alone (control) were grown in DMEMF/12, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Ephrin-A5-transfected cells grew in the presence of 0.2 mg/ml G418.

Functional assays. For the binding assay with soluble ephrin-A5-Fc (R & D Systems, Wiesbaden-Nordenstadt, Germany), 8 μg/ml recombinant protein preclustered with 80 μg/ml anti-human IgG-Alexa488 (Invitrogen, Karlsruhe, Germany) was applied to cortical neurons growing on 20 μg/ml laminin and 5 μg/ml poly-L-lysine after 2 d *in vitro* for 30 min at 37°C before fixation. To obtain homogeneous substrates of ephrin-A5-Fc, glass coverslips were coated with 100 μl of 8 μg/ml recombinant ephrin-A5-Fc (R & D Systems) preclustered with 80 μg/ml anti-human IgG (Alexis Biochemicals, Lausen, Switzerland) in PBS or 3 μg/ml Fc (Alexis Biochemicals) preclustered with 30 μg/ml anti-human IgG for 1 h at 37°C. After washing with PBS, the coverslips were overlaid with 20 μg/ml laminin and 5 μg/ml poly-L-lysine for 1 h at 37°C. Stripe assays were performed as described previously by Vielmetter et al. (1990) using silicone matrices for stripe formation obtained from the Max-Planck Institute for Developmental Biology (Tübingen, Germany). Glass coverslips were placed on the silicone matrix, and 50 μl of a 8 μg/ml ephrin-A5-Fc solution preclustered with 80 μg/ml anti-human IgG-Alexa488 (Invitrogen) or, as a control, 3 μg/ml Fc clustered with 30 μg/ml anti-human IgG-Alexa488 in PBS, was injected into the matrix channels. After incubation at 37°C for 1 h, the coverslips were washed with PBS, covered with 100 μl of a preclustered Fc solution (3 μg/ml Fc protein and 30 μg/ml unlabeled anti-Fc antibody; Alexis Biochemicals), and incubated at 37°C for 1 h. After washing with PBS, the coverslips were coated with 20 μg/ml laminin and 5 μg/ml poly-L-lysine for 1 h at 37°C. Coculture experiments were performed plating NIH3T3 cells (15 cells/mm^2) on laminin (20 μg/ml)/poly-L-lysine (5 μg/ml)-coated coverslips. After 1 h at 37°C and 5% CO_2, dissociated cortical neurons (300 cells/mm^2) were added.

Time-lapse recordings. Live-cell imaging of cortical cells was performed from 3 to 18 h *in vitro* using a Zeiss (Oberkochen, Germany) Axiovert S100 microscope equipped with a temperature-controlled carbon dioxide chamber (37°C; 5% CO_2). To avoid evaporation, a thin layer of mineral oil covering the medium was used. Phase-contrast images were taken every 6 min over a period of 15 h with a video camera (XC 003P; Sony, Tokyo, Japan). MetaMorph Imaging System 4.0 software allowed automatic recording of eight independent fields.

Antibodies and immunostaining. Cells were fixed with 4% paraformaldehyde in PBS for 30 min, followed by washing with PBS and 0.2% Triton X-100, and blocked with 2% BSA and 0.2% Triton X-100 in PBS. Cultures were then treated with primary antibodies: mouse anti-phosphotyrosine 99 monoclonal antibody, 1:500; rabbit anti-EphA4 polyclonal antibody, 1:800; rabbit anti-c-Src, 1:500 (all from Santa Cruz Biotechnology, Heidelberg, Germany) in blocking solution for 1 h at room temperature and washed with PBS with 0.2% Triton X-100. Secondary antibodies were applied (goat anti-mouse IgG Cy3, 1:800; goat anti-mouse IgG Cy2, 1:100; goat anti-rabbit IgG Cy3, 1:600; Jackson ImmunoResearch Europe, Newmarket, UK) in the blocking solution for 45 min at room temperature. Controls without using primary antibodies resulted only in background signals.

In situ *hybridization of cortical single cells.* The protocol for *in situ* hybridization was modified from Weth et al. (1996). Fixation occurred for 20 min with 4% paraformaldehyde in PBS, pH 7.4, at room temperature. The digoxigenylated riboprobes were used at a concentration of 3 ng/μl. Hybridization was performed overnight at 62°C.

Analysis of in vitro *assays.* For the quantification of cell aggregation, pictures were taken with a Zeiss Axiovert S100 inverted microscope (50 pictures per coverslip) using a 20× phase-contrast objective [numerical aperture (NA), 0.05; PlanNeofluar; Zeiss]. Total number of cells, number of aggregated cells, and number of aggregates were counted (aggregation was defined as contact of the somas of two or more neurons). For the stripe assays, pictures were taken using the 20× phase-contrast objective (NA, 0.05; PlanNeofluar; Zeiss) in combination with fluorescence excitation to visualize the stripes. To determine the distribution of neurons on the stripes, only the location of the cell body was taken into account. To quantify signals obtained with the mouse anti-PY99 and rabbit anti-EphA4 antibodies from neurons growing on ephrin-A5-Fc substrate, phase-contrast pictures (40× phase-contrast objective; Plan-Neofluar, NA, 0.75; 1.6× Optovar; Zeiss) and alternating fluorescence photographs were taken. Using ImageJ software, fluorescence intensities and area of signals respective to the area of cell clusters were measured. Results (mean ± SEM) are presented in percentages. Student's *t* test was used for statistical comparison. Cocultures and colocalization were analyzed with a Zeiss laser-scanning microscope (LSM) 510 and the LSM 510 software using a differential interference contrast 63× oil objective (NA, 1.4; Zeiss).

Biochemistry/immunoprecipitation. Mechanically dissociated cortices were preincubated with 10 μM PP2 or PP3 (Merck Biosciences) for 25 min at 37°C. Cells were shortly centrifuged and minced in lysis buffer containing the following: 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 2.5 mM Na-pyrophosphate, protease inhibitor mixture (1 mM PMSF, 5 μg/ml pepstatin, 25 μg/ml aprotinin, 15.4 μg/ml DE, and 25 μg/ml leupeptin), and 1% Triton X-100 under sonication at 4°C for 10 min. After incubation on ice for 30 min with occasional shaking, the lysates were centrifuged. Supernatant was preincubated with protein A-Sepharose (GE Healthcare, Freiburg, Germany) for 1 h at 4°C. Precipitation with 8 μg/ml ephrin-A5-Fc (R & D Systems), 8 μg/ml Fc (Alexis Biochemicals), or 5 μg/ml mouse anti-SrcGD11 (Millipore, Eschborn, Germany) was performed overnight at 4°C. Precipitates were recovered with protein A-agarose (GE Healthcare), washed three times with lysis buffer without protease inhibitors in MicroSpin columns (GE Healthcare), and resuspended in sample buffer.

Immunoblots. After separation of proteins with SDS-PAGE, they were blotted to Hybond nitrocellulose membranes (GE Healthcare). Western blot was performed using HRP-conjugated secondary antibodies (Jackson ImmunoResearch Europe) and ECL chemiluminescent and chemifluorescent detection (GE Healthcare). The following primary antibodies

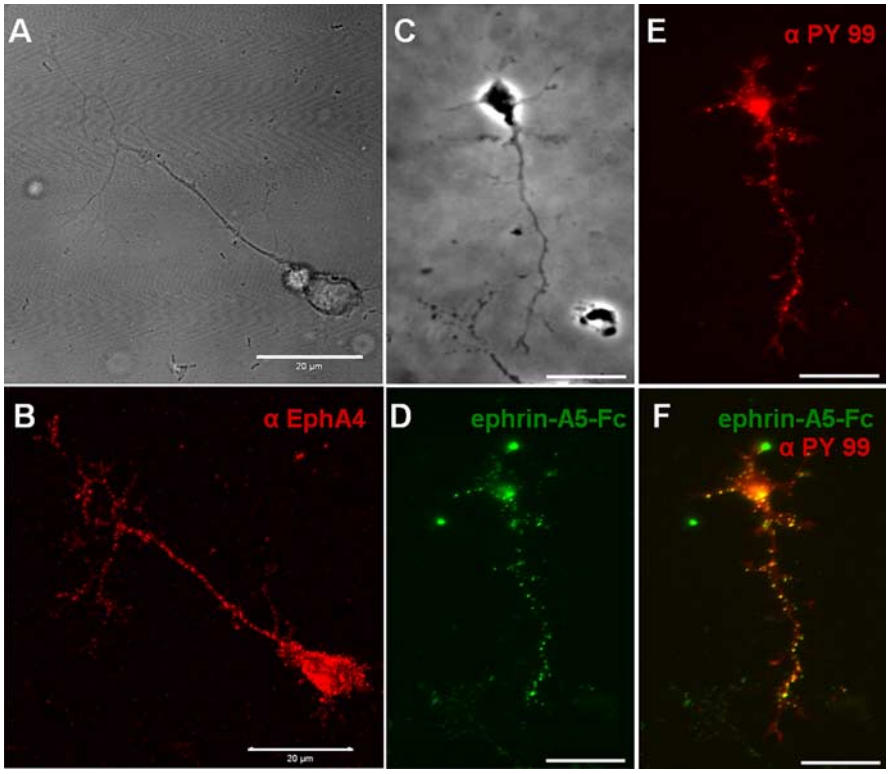

Figure 1. Cortical neurons (E14; 2 div) show activated EphA receptors after stimulation with clustered ephrin-A5-Fc. ***A***, Transmitted-light photograph of a cortical neuron growing on laminin/poly-L-lysine shown in ***B***. ***B***, Anti-EphA4 immunostaining of cortical neurons revealed EphA4 expression along their neurites and soma. ***C***, Phase-contrast microphotograph of the same cell as in ***D–F***. ***D–F***, Stimulation of neurons with preclustered ephrin-A5-Fc using an Alexa488-labeled antibody for 30 min (***D***) followed by PY99 staining (***E***) resulted in colocalized signals (***F***, yellow), indicating activated Eph receptors. Scale bars: ***A***, ***B***, 20 μm; ***C–F***, 25 μm.

were used: rabbit anti-EphA4 polyclonal antibody, 1:500 (Santa Cruz Biotechnology); mouse anti-SrcGD11, 1:1000 (Millipore); and rabbit anti-Src [pY^{418}], 1:500 (Biosource Europe, Nivelles, Belgium). As a positive control for Src we used a nonstimulated A431 cell lysate (Millipore).

Electroporation. Dissociated neurons were incubated in 50 μg of pEYFP-MEM vector for 5 min on ice followed by electroporation in 0.2 cm cuvettes (pulse generator EPI 2500; 1 square-wave pulse, 0.1 ms, 700 V; Dr. Fischer, Heidelberg, Germany). Ice-cold culture medium was added immediately after electroporation. Cells were incubated on ice for 10 min before plating on coverslips.

Results

Ephrin-A5 activates EphA receptors of cortical neurons

Our *in vitro* experiments were designed to examine possible effects of ephrin-A5 on the aggregation and motility of cortical neurons prepared from the somatosensory cortex of mice at E14. In a first set of experiments, we verified that these neurons express functional EphA receptors. Previous data revealed that four EphA receptors (EphA3, EphA4, EphA5, and EphA7) are expressed in the developing cortex at E14 (Niehage et al., 2005). Consistent with these results, *in situ* hybridization of dissociated cortical neurons showed that these EphA receptors are also expressed *in vitro* (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Each of these EphA receptors is expressed by ~70% of the cells, indicating that cortical neurons can express more than one type of EphA receptor (supplemental Fig. 1*E*, available at www.jneurosci.org as supplemental material).

For EphA4, we confirmed its expression also at the protein level. As shown in Figure 1, *A* and *B*, EphA4 receptors are localized along the neurites and somata of cortical neurons. In addition, immunoprecipitation with recombinant ephrin-A5-Fc protein and subsequent immunoblotting with an EphA4 antibody further demonstrated that cortical neurons contain EphA4 receptor protein (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material). To test whether ephrin-A5 can activate EphA receptors of these cells, we stimulated the neurons after 2 d *in vitro* (div) with 8 μg/ml soluble ephrin-A5-Fc preclustered with an Alexa488-labeled anti-Fc antibody for 30 min before fixation. As illustrated in Figure 1, *C* and *D*, the neurons displayed a strong clustered labeling of the binding sites. Phosphotyrosine immunostaining of the ephrin-A5-Fc-stimulated cells with an anti-PY99 antibody resulted in similar clustered signals (Fig. 1*E*). Superimposition of these staining patterns indicated that in most cases, the ephrin-A5-binding sites were colocalized with phosphotyrosine immunoreactivity (Fig. 1*F*). This suggests that recombinant clustered ephrin-A5-Fc activates EphA receptors of embryonic cortical neurons *in vitro*.

Ephrin-A5 induces aggregation of cortical neurons

To investigate the functional roles of ephrin-A5-Fc-induced EphA receptor activation of dissociated cortical neurons, we plated cells for 2 div on homogeneous ephrin-A5-Fc or Fc control substrates that were overlaid with laminin and poly-L-lysine as growth-promoting substrates. We first determined the density of neurons that allowed robust neurite outgrowth of dissociated cortical neurons. At a density of 150 cells/mm^2, almost all cells extended neurites after 2 div, whereas with lower cell numbers, neurite outgrowth was reduced (data not shown). At this cell density, on the control substrate, 48% of the cells grew isolated, and 52% of the cells were aggregated (Fig. 2*A*, left column, *B*). Aggregation was defined as contact of the somata of two or more cells. However, when neurons were plated on homogeneous ephrin-A5-Fc substrate for 2 div, there was a strong increase in cell aggregation (Fig. 2*A*, right column). More than 90% of the neurons formed aggregates. In addition, the number of cells per aggregate on ephrin-A5-Fc (4.4 ± 0.1) was significantly increased compared with the control (2.7 ± 0.08; $p < 0.001$). To examine the time course of cell aggregation on ephrin-A5-Fc substrates, we fixed the cells after different times *in vitro* (Fig. 2*A*). As indicated in Figure 2*B*, an increased number of aggregated neurons on ephrin-A5-Fc could be observed already after 10 h *in vitro*.

To examine in more detail how the aggregation of the cortical neurons occurs, we recorded time-lapse movies of neurons on ephrin-A5 and control substrates (supplemental movies 1 and 2, available at www.jneurosci.org as supplemental material). We started time-lapse imaging 3 h after plating the cells on the different substrates and recorded them for the next 15 h. We observed that on the ephrin-A5-Fc substrate, cells extended and

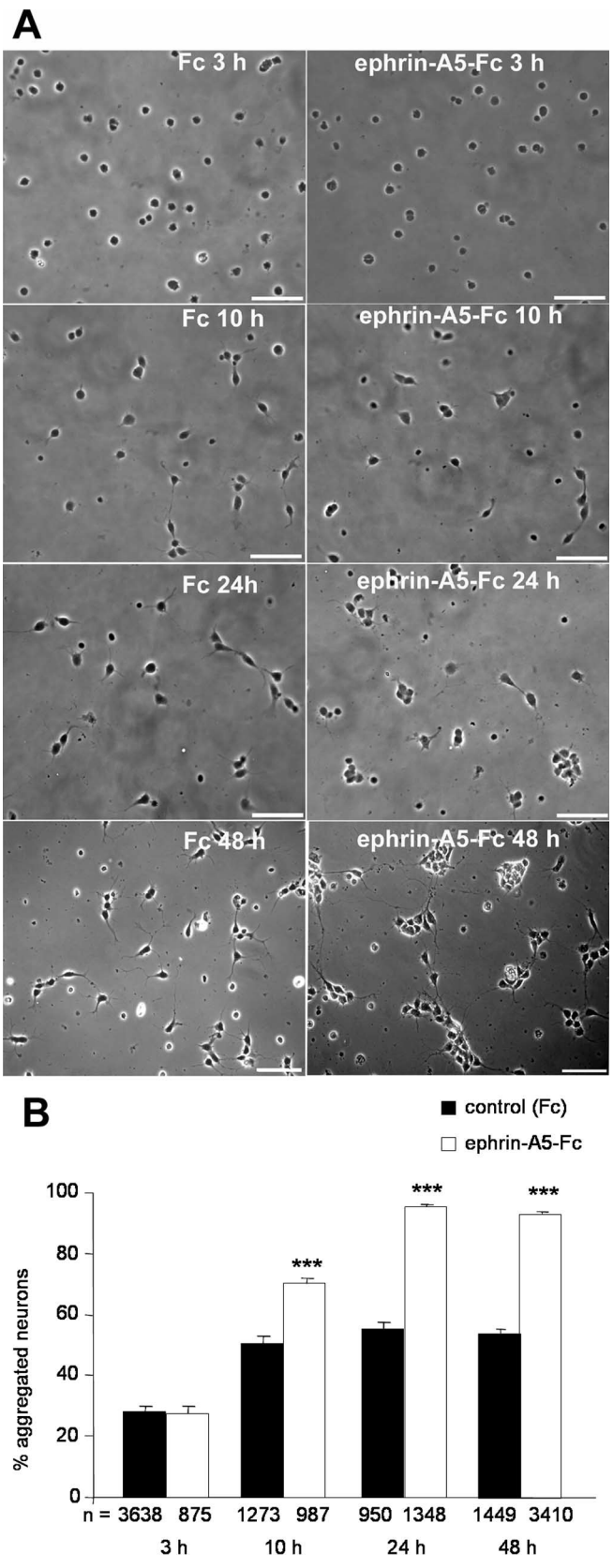

Figure 2. Clustered recombinant ephrin-A5-Fc increases motility and aggregation of cortical neurons (E14) *in vitro*. ***A***, Phase-contrast microphotographs of neurons growing either on homogeneous clustered ephrin-A5-Fc (right column) or control protein (Fc; left column) fixed at different times (3, 10, 24, and 48 h) *in vitro*. ***B***, Quantification of aggregated cortical neurons on the ephrin-A5-Fc and control substrate at different times *in vitro*. Aggregation was defined as contact of somas of two or more neurons. Scale bars, 50 μm. ***$p < 0.0001$.

retracted neurites more often than on the control substrate, which led to more cell contacts, followed by cell migration and aggregation of the contacting neurons. During the 15 h period of time-lapse imaging, on the ephrin-A5-Fc substrate, 16 ± 1.05% of the neurons ($n = 1901$) made contacts with nearby cells, and 87 ± 1.9% ($n = 313$) of these contacts resulted in cell movements leading to cell attachment. In contrast, during the same time period on control substrates, only 12 ± 1% ($n = 1043$) of the cells contacted surrounding neurons, and only 67 ± 5.2% ($n = 116$) of these contacts resulted in cell aggregation. Thus, the increased cell–cell contacts ($p < 0.01$) and the enhanced contact-dependent aggregation ($p < 0.001$) lead to a significant increase in cell–cell attachment of neurons on ephrin-A5-Fc substrates (35% aggregated neurons; $p < 0.001$) compared with the control substrate (13.5% aggregated neurons) in 15 h. Together, these data suggest that ephrin-A5 increases cell motility and aggregation of cortical neurons *in vitro*.

Next, we wanted to know whether ephrin-A5 has similar effects on other cell types. For this, we used a fibroblast cell line known to express EphA receptors endogenously (Miao et al., 2000). This was confirmed with immunostaining against EphA4 (supplemental Fig. 3*B*,*D*, available at www.jneurosci.org as supplemental material). Cells on control substrate showed a spread morphology and adhered effectively to the substrate, whereas fibroblasts on homogenous ephrin-A5-Fc dramatically changed their morphology and became rounded (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). The rounding of these cells indicates that ephrin-A5 induces detachment of the fibroblasts from the substrate, as was already described for B-ephrins (Dail et al., 2006).

SFK downstream signaling of EphA receptors are involved in ephrin-A5-Fc-induced cell aggregation

Next, we wanted to identify possible signaling molecules that are involved in ephrin-A5-Fc-induced aggregation of cortical neurons. Knoll and Drescher (2004) suggested an important role of SFKs in the downstream signaling of EphA receptor-mediated repulsive axon guidance in the retinotectal system. It is known that in rodents, four different SFKs (Src, Lyn, Fyn, and Yes) are widely expressed in the nervous system (Thomas and Brugge, 1997). SFKs bind to the juxtamembrane region of Eph receptors via two well conserved phosphorylated tyrosine residues modulating function and/or binding properties of other signaling molecules (Ellis et al., 1996; Zisch et al., 1998). It was also shown that SFKs only marginally affect the autophosphorylation but profoundly increase further tyrosine phosphorylation of EphA receptors (Knoll and Drescher, 2004).

Because Src is strongly expressed in the cortex (Heidinger et al., 2002; Palmer et al., 2002), we performed immunostaining with a c-Src antibody to confirm that Src is also expressed in cortical neurons *in vitro* (supplemental Fig. 2*E*, available at www.jneurosci.org as supplemental material). To study a possible involvement of SFKs in ephrin-A5-stimulated aggregation, we used PP2 to inhibit SFK function. PP2 is a chemical compound that is known to block SFKs (Hanke et al., 1996; Vindis et al., 2003). An immunoblot against phosphorylated Src showed a reduced phosphorylation level of Src in cortical tissue after incubation with PP2, indicating that SFK function was indeed inhibited (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material). To study the consequences of SFK inhibition, we plated cortical neurons on homogenous ephrin-A5 substrates and added 5 μM PP2 into the medium. On the control substrate,

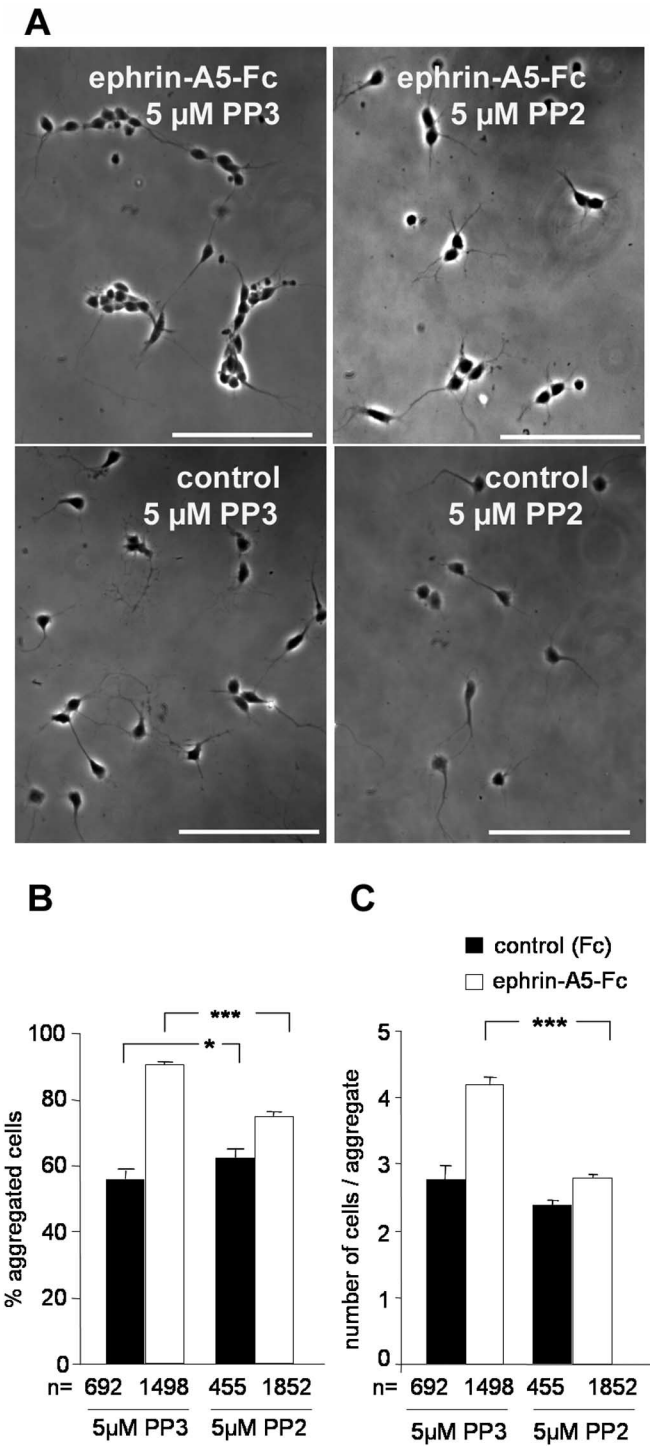

Figure 3. Ephrin-A5-Fc-induced aggregation of dissociated cortical neurons (E14) *in vitro* is dependent on SFK activity. ***A***, Phase-contrast microphotographs of neurons growing on homogeneous ephrin-A5-Fc and control substrate after 2 d *in vitro* in the presence of either 5 μM PP2 (right column) or 5 μM PP3 (control; left column). ***B***, ***C***, Quantification of the number of aggregated cells (***B***) and the size of aggregates (***C***) revealed a significantly reduced ephrin-A5-Fc-induced cell aggregation after blocking of SFKs. ***B***, On the control substrate, aggregation of neurons was slightly increased after blocking of SFKs. Scale bars, 100 μm. *$p < 0.01$; ***$p < 0.0001$.

PP2 slightly increased the number of aggregated cells (Fig. 3*B*; 56 ± 2.5% with 5 μM PP3; 62.2 ± 2.9% with PP2). In contrast, on the ephrin-A5-Fc substrate, SFK inhibition significantly reduced aggregation and the motility of cortical neurons. Both the size of aggregates (2.8 ± 0.08 cells per aggregate) and the number of aggregated cells (74.5 ± 1.3%) were significantly decreased in the presence of PP2 (Fig. 3*B*,*C*). PP3 did not affect neurite growth and cell migration, neither on control nor on the ephrin-A5-Fc substrate (Fig. 3*A–C*). Thus, SFK signaling is involved in ephrin-A5-induced cell aggregation and motility.

SFK inhibition switches the repulsive effect of ephrin-A5 into adhesion

The experiments described so far demonstrated that ephrin-A5 enhances aggregation of cortical neurons in an SFK-dependent way. Time-lapse recordings revealed that increased aggregation after EphA receptor activation is achieved through enhanced neurite outgrowth, more contacts of neurites with surrounding cells, and translocation of the cell somas. The increased cell motility and aggregation could be the result of repulsive interactions that were often described for Ephrin forward signaling (Flanagan and Vanderhaeghen, 1998; Wilkinson, 2001; Knoll and Drescher, 2002). To test whether ephrin-A5 represents a repellent substrate for cortical neurons, we performed the stripe assay according to Vielmetter et al. (1990), using ephrin-A5-Fc clustered with a labeled anti-Fc antibody and unlabeled control protein (Fc). When neurons had a choice between the different substrates, >80% of the cells avoided ephrin-A5-Fc-containing stripes and grew preferentially on the control lanes (Fig. 4*B*,*C*). This effect was concentration dependent; a preferred cell accumulation of the neurons on the control stripes was still observed at a concentration of 0.08 μg/ml ephrin-A5-Fc, and lower concentrations had no effect (Fig. 4*C*). Alternating stripes of labeled and unlabeled control protein also resulted in equal distribution of the cells (Fig. 4*A*,*C*).

A possible explanation of the uneven distribution of the cortical neurons in the stripe assay could be that the survival of the cells on the control stripes is enhanced compared with their survival on the ephrin-A5 stripes. Depaepe et al. (2005) reported that regulated apoptosis of cortical progenitor cells can be mediated through Ephrin-A–EphA receptor interactions. However, using an apoptosis assay [TUNEL (terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling)], we found no increase in the apoptosis of cortical neurons on ephrin-A5 lanes compared with neurons on control stripes (data not shown). Likewise, time-lapse recording from 3 to 18 h *in vitro* revealed no enhanced cell death on the ephrin-A5 stripes. Rather, as on homogenous substrates, we observed an enhanced aggregation of neurons on ephrin-A5-Fc-containing lanes (data not shown). Although on the control lanes only 20 ± 2.8% of the neurons aggregated, the number of aggregated cells increased to 80 ± 2.8% on ephrin-A5-Fc-containing stripes ($p < 0.01$; $n = 138$). Contact-mediated aggregation could also be seen between cells located on adjacent stripes. In these cases, 86 ± 1.9% of the contacts resulted in a translocation of the cell from the ephrin-A5 stripe to the control stripe, whereas only 14% of the translocations occurred from the control lanes to the ephrin-A5-Fc stripes ($p < 0.01$; $n = 28$). Although less frequent (in ~30% of the total translocation events), we also observed cell movements across the stripes without cell attachment. When neurons migrated between the stripes, 77 ± 1.7% of the cells moved from the ephrin-A5 stripes to the control stripes, and only 23 ± 1.7% of the cells moved in the opposite direction (data not shown). Together, these results strongly suggest a repulsive effect of ephrin-A5 on cortical neurons.

We also tested whether ephrin-A5 also acts as a repulsive cue for EphA-expressing fibroblasts. In contrast to cortical neurons when these cells were growing on alternating stripes of ephrin-

A5-Fc and control lanes, they showed no preference (supplemental Fig. 3*E*,*F*, available at www.jneurosci.org as supplemental material). Thus, ephrin-A5 can induce cell detachment but has no repulsive effects on this type of cell. The differential response of fibroblasts and cortical neurons to ephrin-A5 in the stripe assay indicates that detachment of cells from the substrate alone is not sufficient to elicit motility and aggregation.

Next, we examined whether we could block the ephrin-A5-induced effects in the stripe assay by inhibiting SFK activity. For this, we performed the stripe assay in the presence of 5 μM PP2 in the medium; 5 μM PP3 was used in control experiments. We expected that an abolishment of the repulsive effects of ephrin-A5 would result in an equal distribution of neurons in the stripe field. However, inhibition of SFK function not only abolishes the repulsive effect of ephrin-A5, it even leads to a preferential growth of cortical neurons on the ephrin-A5 stripes. As illustrated in Figure 5, *B* and *C*, in the presence of SFK inhibitor, ~80% of the cortical neurons grew on ephrin-A5-containing lanes after 2 div. Together, these data indicate that ephrin-A5 has a repulsive effect on cortical neurons that can be switched into attraction after SFK inhibition.

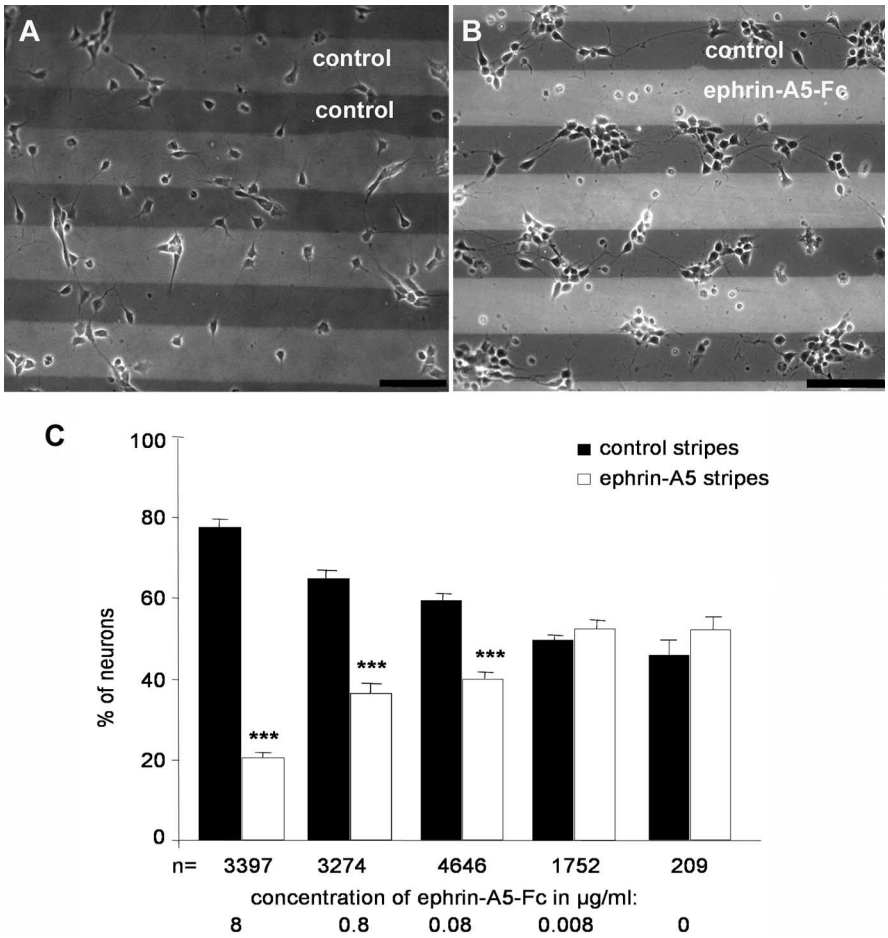

Figure 4. Ephrin-A5-Fc has a repulsive effect on dissociated neurons in the stripe assay after 2 d *in vitro*. ***A***, Neurons on alternating stripes of Alexa488-labeled and unlabeled control protein (3 μg/ml) show no preferential growth. ***B***, On alternating stripes of Alexa488-labeled ephrin-A5-Fc (8 μg/ml) and unlabeled control protein (3 μg/ml), neurons grow preferentially on the control stripes and avoid ephrin-A5-Fc stripes. ***C***, Quantification of the distribution of neurons on alternating stripes using different concentrations of ephrin-A5-Fc. The repulsive effect of ephrin-A5-Fc shows a concentration dependency. Scale bars, 50 μm. ***$p < 0.0001$.

Release of EphA receptors after binding to the ephrin-A5 substrate

The adhesive effects of ephrin-A5 on cortical neurons after inhibition of SFK activity could be a result of the high-affinity binding between the ephrin ligand and its Eph receptors. This then raises the obvious question of how the Eph/Ephrin system can mediate repulsive effects between cells with normal SFK activity, when the binding between ligand and receptor is an adhesive event. For the ephrin-A system, regulated metalloprotease-mediated cleavage of ephrin-A ligands after complex formation with their receptors was described by Hattori et al. (2000). For the ephrin-B system, Zimmer et al. (2003) reported cell contact-induced bidirectional endocytosis of entire receptor–ligand complexes during cell retraction. A first hint for an additional mechanism (the removal of ligand–receptor complexes from the cell surface) came from immunostaining of the EphA4 receptor on cortical neurons plated on the ephrin-A5 substrate. On this substrate, EphA4 immunostaining was not only detected on the cell surfaces, but there was also an intense punctuate staining in a halo surrounding the cells (Fig. 6*B*,*B*′). A very similar staining pattern was observed with the PY99 antibody directed against phosphotyrosines (Fig. 6*D*,*D*′). This is in striking contrast to the staining pattern on the control substrate, where EphA4 and phosphotyrosine immunoreactivity is only observed on cell bodies and neurites, but never on the substrate surrounding the cells (Fig. 6*A*,*A*′,*C*,*C*′). Double immunostaining with anti-EphA4 and PY99 antibodies revealed a colocalization of both signals on the ephrin-A5 substrate around the neurons (supplemental Fig. 4*A*–*D*, available at www.jneurosci.org as supplemental material). The punctuate immunostaining on the substrate could only be detected after membrane permeabilization (data not shown). Thus, when cortical neurons detach from the ephrin-A5 substrate, they apparently release membrane particles containing tyrosine-phosphorylated EphA receptors. This release mechanism might be a prerequisite for the repulsive effects of ephrin-A5 that mediate migration and aggregation of cortical neurons.

Next, we asked whether the release of EphA receptors by membrane ripping is a mere passive event or whether it also requires a signal transduction pathway. Because the repulsive effects described in the present study depend on SFK signaling, we examined the EphA4 receptor release on ephrin-A5 substrates after blocking SFK activity with PP2. As illustrated in Figure 7, compared with the control peptide PP3, the addition of PP2 to the medium reduced the area with immunolabeling around the neurons on the ephrin-A5 substrate by 38% with the EphA4 antibody (Fig. 7*D*) and by 23% with the PY99 antibody (Fig. 7*A*–*C*,*E*). More importantly, there was also a concomitant decrease in the intensity of the immunostaining. After PP2 treatment there was a 1.3-fold reduction in the intensity of the EphA4 signal (Fig. 7*F*) and a 1.4-fold reduction in the intensity of the phosphotyrosine labeling (Fig. 7*G*). This indicates that SFK activity is at least

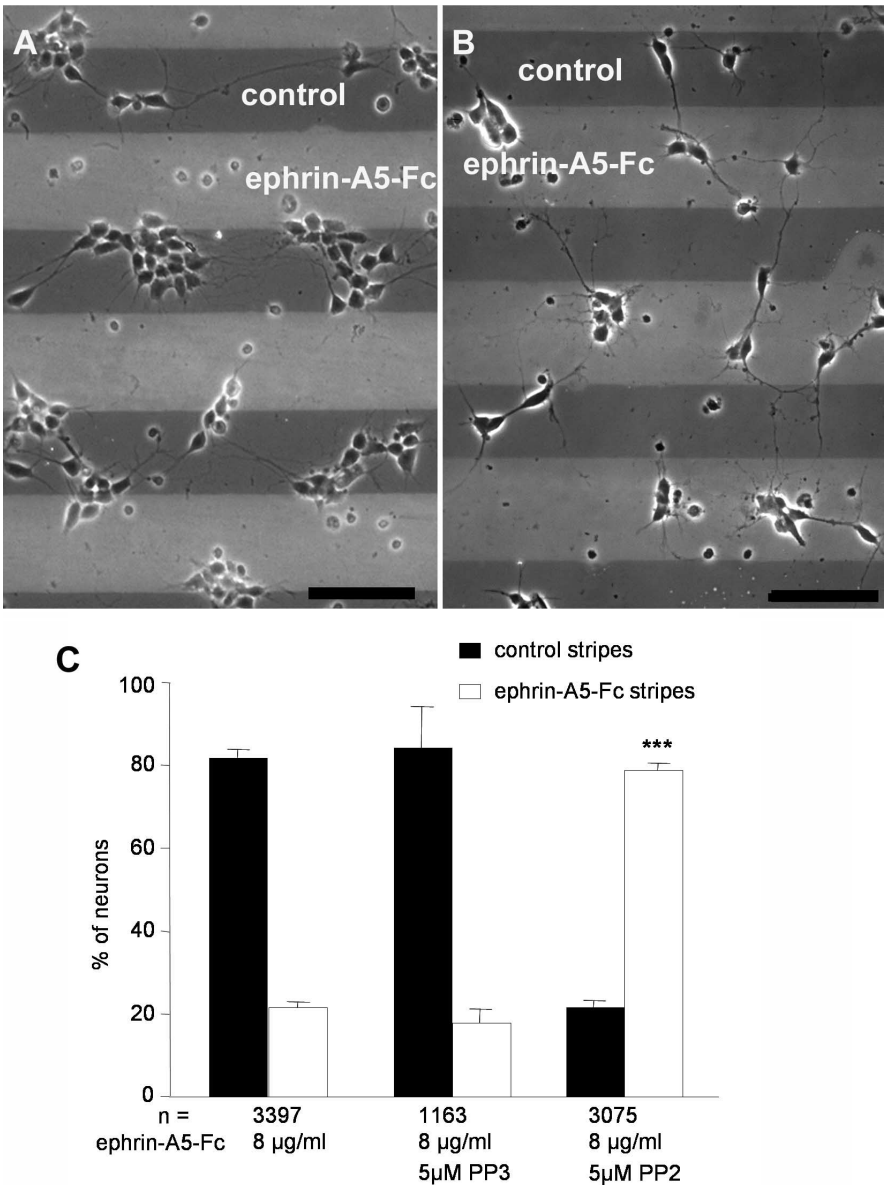

Figure 5. SFKs are involved in repulsive EphA signaling of cortical neurons. Phase-contrast microphotographs of neurons on alternating ***A***, ***B***, Alexa488-labeled ephrin-A5-Fc and unlabeled Fc stripes show the conversion of the repulsive effect of ephrin-A5-Fc (***A***) into attraction after PP2 application (***B***). ***C***, Quantification of the distribution of neurons on alternating stripes under control conditions and in the presence of PP2 and PP3. After PP2 treatment, neurons grew preferentially on ephrin-A5-Fc lanes. Scale bars, 50 μm. ***$p < 0.0001$.

in part involved in the regulation of EphA receptor release of cortical neurons.

Release of activated EphA receptors after cell contact

The experiments described above provided evidence for a release mechanism of EphA receptors on a two-dimensional ephrin-A5 substrate. To examine whether EphA receptors are also released after contact with cells expressing ephrin-A ligands, we cocultured cortical neurons with ephrin-A5-expressing NIH3T3 fibroblasts. The ephrin-A5 mRNA expression of the fibroblasts was controlled using reverse transcription-PCR (data not shown). The functionality of the ephrin-A5 protein was determined in time-lapse recordings of cocultures, in which cortical neurites showed a significant higher number of retractions after contact with ephrin-A5-transfected fibroblasts than in coculture with control fibroblasts (data not shown). We then verified that ephrin-A5 expressed in fibroblasts could activate EphA receptors in cortical neurons. Double immunostaining with EphA4 and PY99 antibodies revealed colocalized clusters of signals at the contact sites between cortical neurons and ephrin-A5-transfected fibroblasts (supplemental Fig. 5*E–H*, available at www.jneurosci.org as supplemental material) that could not be observed at the contact sites of cortical neurons with untransfected fibroblasts (supplemental Fig. 5*A–D*, available at www.jneurosci.org as supplemental material). To test whether cortical neurons also release membrane particles containing EphA receptors after contact with ephrin-A5-expressing cells, we transfected cortical neurons with a membrane-targeted enhanced yellow fluorescent protein (EYFPmem). Near the contact sites between ephrin-A5-expressing fibroblasts and EYFPmem-transfected neurons (Fig. 8), green fluorescent membrane particles could be detected on the side of the transfected fibroblasts (Fig. 8*D*,*J*). These membrane particles were not observed in cocultures of EYFPmem-transfected neurons with control fibroblasts (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). Immunostaining revealed that many of the green fluorescent membrane particles on the side of ephrin-A5-transfected fibroblasts could be labeled with anti-EphA4 antibodies (Fig. 8*B–D*) and anti-PY99 (Fig. 8*G–J*). Thus, EphA receptor release by neurons can also occur after contacts with Ephrin-A ligand-expressing cells.

Discussion

In this study, we examined the effects of ephrin-A5 on the migration and adhesion of embryonic cortical neurons. On a homogeneous ephrin-A5 substrate, the motility of these neurons is enhanced, and they form large aggregates. Blocking SFK function resulted in decreased aggregation of neurons on homogeneous ephrin-A5. These effects appear to be a feature of repulsive interactions, because in the stripe assay, cortical neurons are repelled from the ephrin-A5-containing lanes. The repulsive effect is mediated by SFKs. Blocking SFK activity converted ephrin-A5-induced repulsion into attraction. Finally, we demonstrate that repulsive effects of ephrin-A5 are accompanied by a release of small membrane fragments from the neurons that contain activated EphA receptors. We suggest that this membrane ripping is a mechanism to overcome adhesion after high-affinity binding of ephrin-A ligands and EphA receptors to perform repulsive interactions and migration.

Effects of ephrin/Eph signaling on cell aggregation and migration were previously studied mostly in non-neuronal cells. For instance, Eph/Ephrin-mediated cell aggregation was found in an interleukin-3-dependent cell line (Bohme et al., 1996). Eph-

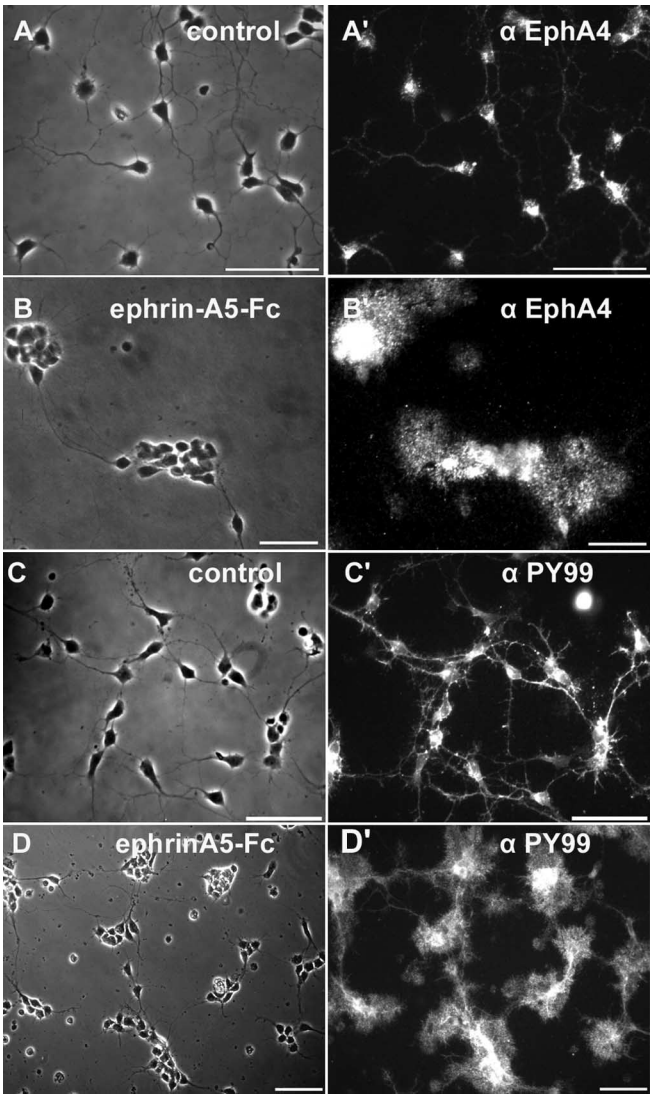

Figure 6. Neurons growing on homogeneous ephrin-A5-Fc release activated EphA receptors to the substrate. ***A′***, On control substrate, EphA4 immunoreactivity is restricted to the neurites and somas of the neurons. ***B′***, On ephrin-A5-Fc, the EphA4 immunostaining of neurons resulted in an extensive staining of the cells and the surrounding substrate. ***C′***, ***D′***, A similar staining pattern of the cells and the surrounding ephrin-A5-Fc substrate could be observed using an anti-PY99 antibody (***D′***), whereas on the control substrate, the PY99 immunoreactivity is restricted to the cells (***C′***). ***A–D***, Corresponding phase-contrast microphotographs. Scale bars, 50 μm.

rin-B1 or EphA4 activation of human platelets promotes stable aggregation, whereby EphA4 forms complexes with Fyn and Lyn, two members of Src-family kinases (Prevost et al., 2002). Moreover, EphB1 and EphA2 activation increases cell migration of endothelial cells (Huynh-Do et al., 2002; Nagashima et al., 2002; Vindis et al., 2003). The EphB1-mediated regulation of migration depends on c-Src activity (Vindis et al., 2003). This is consistent with our findings that ephrin-A5-induced aggregation and motility of cortical neurons requires the activation of SFKs.

Cell–substrate detachment is necessary for ephrin-A5-induced aggregation, motility, and repulsion. A recent study demonstrated that activation of the EphB2 receptor in COS cells inhibited cell matrix adhesion by phosphorylation and inhibition of R-Ras, a positive regulator of integrins, leading to rounding and detachment of cells from the matrix (Dail et al., 2006). We could observe a similar effect after EphA-receptor activation of a fibroblast cell line. However, as was shown in the stripe assay, ephrin-A5-Fc did not elicit a repulsive response of the fibroblasts, although ephrin-A5-Fc induced rounding and detachment of these cells. This then indicates that cell–substrate detachment alone is not sufficient to mediate repulsion.

In the present study, we show that ephrin-A5 can act as both a repulsive and an attractive cue for a single population of cells. Previous work already provided evidence for the formation of adhesive complexes of ephrin-A5 with the EphA7 receptor (Holmberg et al., 2000). In these experiments, the expression of EphA7 splice variants lacking kinase domains shifted the ephrin-A5-induced repulsion to adhesion. It has been proposed that because ephrins and Eph receptors are membrane bound, suppression of signal transduction by kinase dead EphA receptors can turn these proteins into adhesion molecules. Our results suggest that the conversion of ephrin-A5-induced repulsion to adhesion can also depend on different signal transduction mechanisms. When SFK signaling is intact, ephrin-A5 exerts repulsive effects on cortical neurons, whereas in the absence of SFK signaling, cortical neurons preferentially attach to ephrin-A5 substrate. This indicates that the activation state of SFKs can dictate repulsive versus attractive responses of A-ephrins. This is reminiscent of the results of a recent study that suggest a key role for FAK/Src signaling in differential response of Sema 3B-mediated attraction or repulsion (Falk et al., 2005).

The formation of adhesive EphA/Ephrin-A complexes after suppressing or changing EphA-signal transduction pathways raises the question of how migration or repulsion can occur after high-affinity ligand–receptor binding between cells. Previous work for the A-system showed regulated cleavage of ephrin-A ligands after binding of receptors (Hattori et al., 2000; Janes et al., 2005). For the B-system, bidirectional endocytosis of the entire receptor–ligand complex was described previously (Marston et al., 2003; Zimmer et al., 2003). This paradox of performing repulsion or migration after quasi-irreversible receptor–ligand binding is of course not restricted to the Eph/ephrin signaling. The same question can also be asked for migratory processes using high-affinity receptor–ligand interactions in general, in which cells first make contact with the substrate but then extend processes in the forward direction and liberate the contacts at the opposite end. For migrating fibroblasts, it was reported that they release membrane particles containing integrins to the substratum while moving forward. This mechanism was called "membrane ripping" and was described as a major mechanism for release of integrins of fibroblast *in vitro* and *in vivo* (Bard and Hay, 1975; Chen, 1981; Regen and Horwitz, 1992). Membrane ripping occurs only at the rear end of the fibroblasts, a region in which integrin–cytoskeletal linkages tend not to form, whereas at the front end, the membrane is supported by integrin–cytoskeletal connections (Schmidt et al., 1993, 1995). The less-supported membrane at the rear of fibroblasts can be pulled out from the cell and enables membrane ripping (for review, see Lauffenburger and Horwitz, 1996).

Until now, membrane ripping has not been described for other cell types. In our *in vitro* assays, we could observe released membrane particles containing activated EphA receptors not only on a two-dimensional ephrin-A5 substrate, but also after cell–cell contact with ephrin-A5-expressing fibroblasts that mediate repulsion. The release of EphA receptors was reduced after SFK inhibition, suggesting an involvement of Src-family kinases in this process. In fibroblasts, Src was reported to weaken or

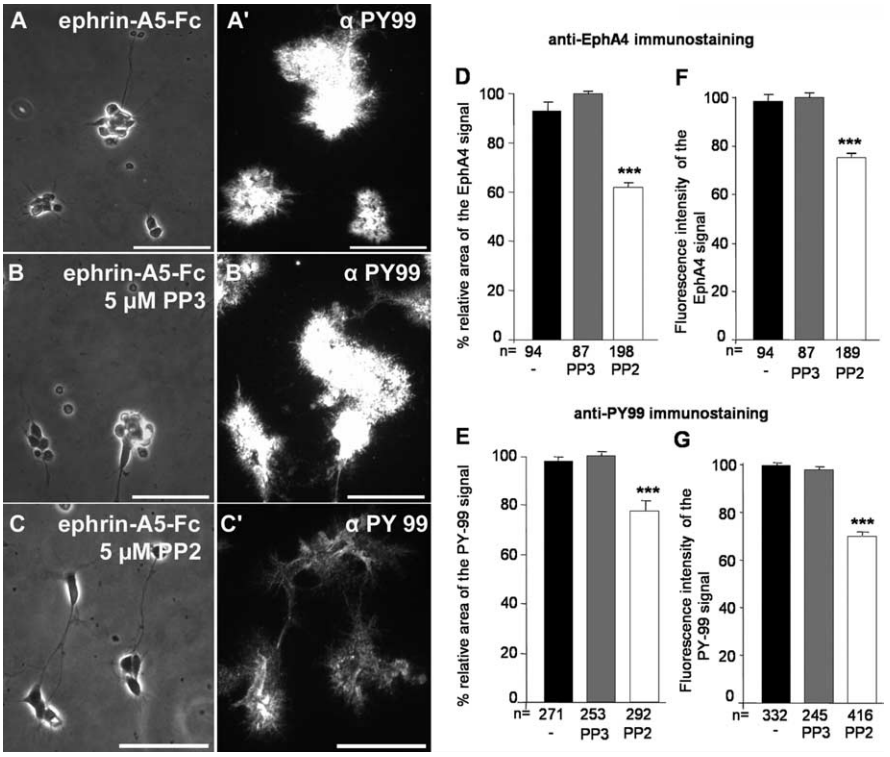

Figure 7. SFK activity is involved in the regulation of EphA4 receptor release of cortical neurons. ***A′–C′***, Anti-phosphotyrosine immunostaining of neurons growing on ephrin-A5-Fc after 2 d *in vitro* in control medium (***A′***) and in the presence of 5 μM PP3 (***B′***) and 5 μM PP2 (***C′***). ***A–C***, Respective phase-contrast microphotographs. ***D***, ***E***, Quantification of the area of the EphA4 (***D***) and PY99 (***E***) signal relative to the size of the aggregates showed a decrease after PP2 treatment. ***F***, ***G***, Furthermore, the fluorescence intensity decreased for the anti-EphA4 (***F***) and anti-PY99 (***G***) immunostaining after blocking of SFKs. Scale bars, 50 μm. ***$p < 0.0001$.

disrupt links between integrins and the force-generating cytoskeleton, which is necessary for cell movement (Felsenfeld et al., 1999). It is known that SFK activity results in an inactivation of cortactin, which in turn leads to a decreased activity of the Arp2/3 complex (Huang et al., 1997). The Arp2/3 complex is an important mediator for the assembly of actin that initiates and cross-links new actin filaments (Huang et al., 1997). The reduced Arp2/3-dependent branching activity observed after cortactin inactivation is thought to trigger a cofilin-mediated breakdown of the actin cytoskeleton (Weed and Parsons, 2001). Cortactin was shown to interact with EphA receptors in muscle cells (Lai et al., 2001) and retinal ganglion cells (Knoll and Drescher, 2004). Relating these findings to the SFK-dependent EphA receptor release from cortical neurons, SFKs could be involved in a local weakening of the cytoskeletal–membrane bonds by inactivating cortactin after ligand-induced EphA receptor activation, thereby allowing the release of membrane particles that contain EphA receptors. Thus, the release of EphA receptors is one mechanism that enables repulsive interactions after receptor–ligand binding.

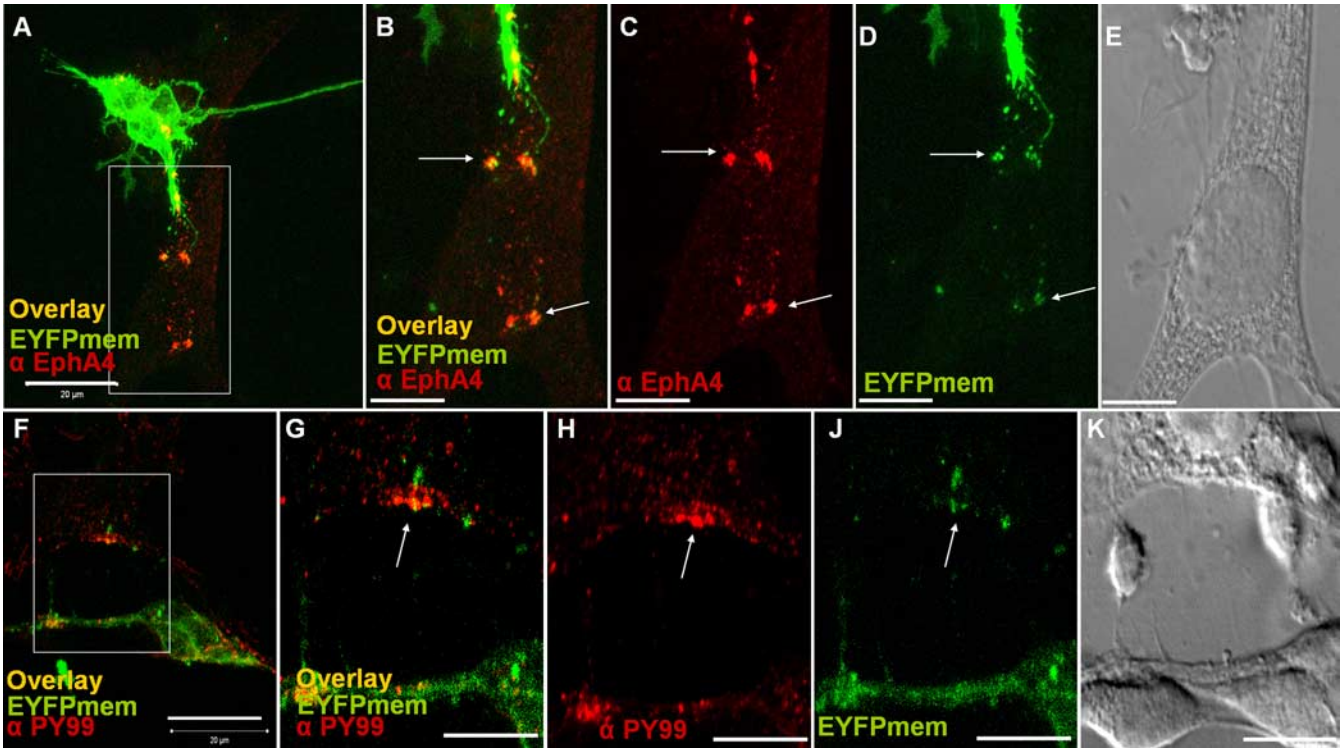

Figure 8. Neuronal membrane particles, including EphA receptors, are released after binding ephrin-A5 of fibroblasts. ***A***, ***F***, Cocultures of EYFPmem-transfected neurons with ephrin-A5-expressing NIH3T3 fibroblasts. ***B–E***, ***G–K***, Magnified sections of the contact sides respective to the marked boxes in ***A*** and ***F***. ***D***, ***J***, Green membrane particles of EYFPmem-transfected neurons could be seen on the site of fibroblasts (arrows). ***A–C***, ***F–H***, EphA4 (***C***) or PY99 immunostaining (***H***) reveal a colocalization (***A***, ***B***, ***F***, ***G***) with many of the neuronal membrane particles (arrows). Scale bars: ***A***, ***F***, 20 μm; ***B–E***, ***G–K***, 10 μm.

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