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RESEARCH ARTICLE

EphA/ephrin A reverse signaling promotes the migration of cortical interneurons from the medial ganglionic eminence

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ABSTRACT

Inhibitory interneurons control the flow of information and synchronization in the cerebral cortex at the circuit level. During embryonic development, multiple subtypes of cortical interneurons are generated in different regions of the ventral telencephalon, such as the medial and caudal ganglionic eminence (MGE and CGE), as well as the preoptic area (POA). These neurons then migrate over long distances towards their cortical target areas. Diverse families of diffusible and cell-bound signaling molecules, including the Eph/ephrin system, regulate and orchestrate interneuron migration. Ephrin A3 and A5, for instance, are expressed at the borders of the pathway of MGE-derived interneurons and prevent these cells from entering inappropriate regions via EphA4 forward signaling. We found that MGE-derived interneurons, in addition to EphA4, also express ephrin A and B ligands, suggesting Eph/ephrin forward and reverse signaling in the same cell. *In vitro* and *in vivo* approaches showed that EphA4-induced reverse signaling in MGE-derived interneurons promotes their migration and that this effect is mediated by ephrin A2 ligands. In EphA4 mutant mice, as well as after ephrin A2 knockdown using *in utero* electroporation, we found delayed interneuron migration at embryonic stages. Thus, besides functions in guiding MGE-derived interneurons to the cortex through forward signaling, here we describe a novel role of the ephrins in driving these neurons to their target via reverse signaling.

KEY WORDS: Interneuron migration, Cortical interneurons, Ephrin, Reverse signaling, Mouse

INTRODUCTION

The correct functioning of the cerebral cortex depends on the precise balance between excitatory and inhibitory neurotransmitter systems. Excitation is mediated via glutamate by pyramidal cells, the projection neurons of the cortex, and by a special class of local neurons in cortical layer 4, the spiny stellate cells. Inhibition is performed via γ-aminobutyric acid (GABA) by cortical interneurons. These interneurons regulate the degree of glutamatergic excitation, filter the input and synchronize the output of projection neurons. There are about five times more glutamatergic neurons than GABAergic neurons in the neocortex; this ratio is preserved across many mammalian species, suggesting that the numerical balance of excitatory and inhibitory neurons is crucial for normal brain function and behavior. Even though GABAergic interneurons constitute a clear minority of the cells in the neocortex, disturbances in their development, and hence the delicate equilibrium between excitation and inhibition, can lead to devastating neurological or neuropsychiatric diseases (reviewed by Sanacora et al., 2000; Rennie and Boylan, 2003; Levitt et al., 2004; Lewis et al., 2005; Batista-Brito and Fishell, 2009; Marín, 2012).

Cortical projection neurons are generated in the ventricular zone (VZ) of the dorsal telencephalon and then migrate radially to form the laminated neocortex (Rakic, 1995). By contrast, cortical GABAergic interneurons are born in the VZ of the ventral telencephalon in three different regions: the MGE and CGE as well as in the POA. Interneurons originating from these domains then migrate tangentially along a deep (DMS) and a superficial migratory stream (SMS) through the basal telencephalon towards their cortical destinations (Lavdas et al., 1999; Parnavelas et al., 2000; Anderson et al., 2001; Marín and Rubenstein, 2001; Nery et al., 2001; Wichterle et al., 2001; Ascoli et al., 2008; Batista-Brito et al., 2008; Gelman et al., 2009; Corbin and Butt, 2011).

The cellular and molecular mechanisms that regulate and guide interneuron migration out of the basal telencephalon into the neocortex are beginning to be described. Different groups of signaling molecules, including semaphorins and slits, act as repulsive cues for migrating interneurons (Marín et al., 2001; Andrews et al., 2008). By contrast, different isoforms of neuregulin act as short- and long-term attractants that demarcate the migratory route of cortical interneurons (Flames et al., 2004). Another group of signaling molecules that is widely and distinctively expressed in the basal telencephalon during interneuron migration are the ephrins and their receptors, the Eph receptor tyrosine kinases. There is increasing evidence that members of the Eph/ephrin family play important roles in guiding migrating interneurons to the cerebral cortex. For example, ephrin A3 and ephrin A5 are expressed in flanking regions of the pathway of MGE-derived interneurons and prevent these neurons from entering inappropriate regions (Zimmer et al., 2008; Rudolph et al., 2010). These effects are based on typical ligand-receptor interactions, i.e. an ephrin ligand activates a signaling cascade in cells expressing an Eph receptor. In addition to this forward signaling, a distinctive feature of the Eph/ephrin system is that in many cases Eph receptors can also activate ephrin ligands, a mechanism called reverse signaling (Davy and Soriano, 2005; Lim et al., 2008). We previously reported that bi-directional forward and reverse signaling mediated by EphA4 and ephrin B3 segregates migrating POA- and MGE-derived interneurons in the SMS and DMS, respectively (Zimmer et al., 2011).

We found that EphA4 as well as ephrin A and B ligands are co-expressed by MGE-derived interneurons, suggesting Eph/ephrin forward as well as reverse signaling in the same cell. These two mechanisms might have different effects on the migration of cortical interneurons as described previously for growing motor axons, in which forward signaling mediates repulsion, whereas reverse signaling mediates attraction (Marquardt et al., 2005; Suetterlin et al., 2012). Therefore, in the present study, we examined the

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functional role of EphA4 reverse signaling. For this, we applied a battery of sensitive bioassays that showed a migration-promoting effect of EphA4 on MGE-derived interneurons that is mediated via ephrin A2 reverse signaling. Analysis of EphA4 loss-of-function transgenic mice and knockdown (KD) of ephrin A2 in MGE-derived cells revealed a delayed migration of cortical interneurons at embryonic stages *in vivo*.

RESULTS

Eph/ephrins are differentially expressed in the developing basal telencephalon

In our previous work, we focused on the repercussions of complementary expression patterns of EphA4 and ephrin ligands in guiding MGE-derived interneurons through the basal telencephalon (Zimmer et al., 2008; Zimmer et al., 2011). As illustrated in Fig. 1A, EphA4 mRNA and protein is strongly expressed along the entire DMS, encompassing the subventricular zone of the MGE and lateral ganglionic eminence (LGE), during interneuron migration. EphA4 is also expressed at the striatal mantle zone and displays a weaker signal in the ventricular zone of the ganglionic eminences. Our previous studies as well as unpublished data reveal an expression of ephrin ligands along the migratory trajectory of MGE-derived interneurons, indicating co-expressions of receptors and ligands by the same types of cells (Zimmer et al., 2011). Thus, Eph/ephrin interactions do not only delineate the migratory pathway but might also occur between neighboring cells within the deep migratory stream. The functional consequences of this co-expression of ephrin ligands and Eph receptors are not known. In the present study, we show that Eph/ephrin interactions have a motogenic function on interneurons in the DMS.

Eph/ephrin interactions in MGE-derived neurons

To get an overview of potential Eph/ephrin interactions of MGE-derived cells, we performed binding assays with Eph receptors and ephrin ligands. The combination with calbindin immunostaining allowed us to identify the expression of Eph/ephrin proteins in a subpopulation of cortical interneurons. Binding of the recombinant ligands and receptors was detected with fluorescent anti-human IgG antibodies, which resulted in a dotted signal as illustrated in Fig. 1B for dissociated calbindin (CB)$^+$ as well as CB$^-$ interneurons from an embryonic day (E) 14.5 MGE after treatment with recombinant EphA4-Fc protein. This signal was specific to Eph/ephrin binding as it was not detected under control conditions when we used Alexa488-labeled Fc protein alone (data not shown).

The mammalian Eph/ephrin system consists of receptor tyrosine kinases subdivided into nine EphAs and five EphBs. A-type receptors bind to all A-type ephrins (ephrin A1-5), which are tethered to the cell membrane by a GPI anchor. B-type receptors interact with all B-type ephrins (ephrin B1-3), which have a transmembrane domain and a short cytoplasmic region. Exceptions are EphA4, which can bind to both A-type and B-type ligands, and EphB2, which can also interact with ephrin A5 (reviewed by Martinez and Soriano, 2005). For the binding assays, Fc-tagged Eph receptors (EphA3, EphA4, EphB1 and EphB3) and Fc-tagged ephrins (ephrin A3, A5, B1 and B3) were used. Analysis of MGE-derived cells that were cultured with recombinant Eph/ephrin proteins revealed that these cells bind ephrin-A and -B ligands as well as EphA and EphB receptors. The binding affinities differed among the recombinant proteins as well as the cell type. Quantification indicated that most CB$^+$ interneurons express ephrin B ligands and a large proportion ephrin A ligands (Fig. 1C; supplementary material Table S1). The proportion of Eph and ephrin binding for all MGE cells was always lower than for the CB subpopulation. However, these data indicate that to certain degree a co-expression of ephrin ligands and Eph receptors is present in the same cohort of MGE-derived cells.

Previous work showed that EphA4 forward signaling prevents MGE-derived interneurons from entering inappropriate regions (Zimmer et al., 2008; Rudolph et al., 2010). As indicated by the binding studies, these neurons also express ephrin ligands, enabling these cells to perform reverse signaling. To check if EphA4 can trigger reverse signaling in MGE-derived interneurons, we first tested whether EphA4-Fc induces the recruitment of downstream effectors to its binding site. It has been demonstrated that endogenous Src family kinases and RhoGTPases are recruited and activated through Eph/ephrin reverse signaling (reviewed by Xu and

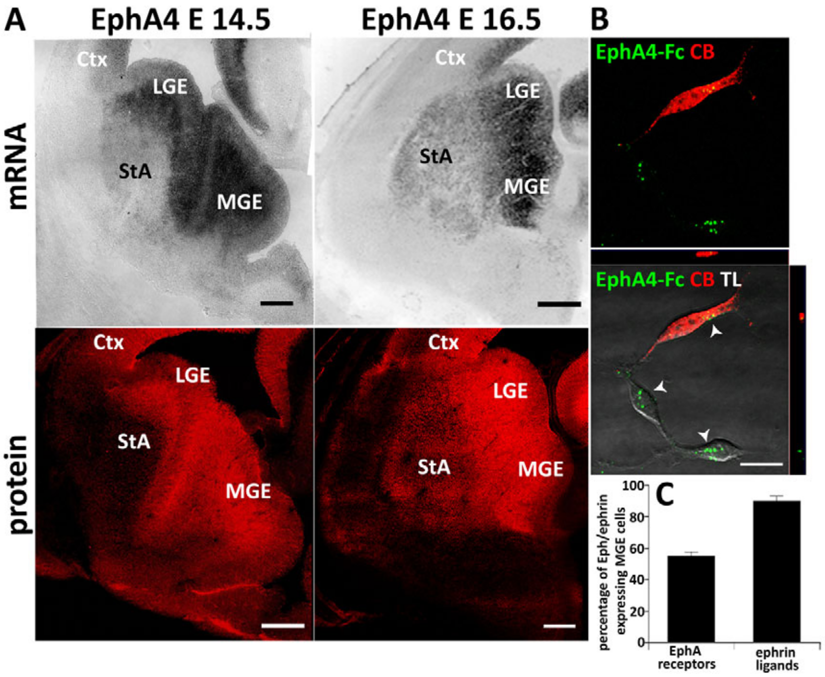

Fig. 1. EphA4 expression and binding in the basal telencephalon. (A) *In situ* hybridization (top) and immunohistochemistry (bottom) on coronal sections revealed an expression of EphA4 mRNA and protein on the DMS of tangentially migrating cortical interneurons at E14.5 and E16.5. Scale bars: 200 µm. (B) Confocal micrographs with *x*- and *y*-line scans in a single optical section of dissociated calbindin positive (CB$^+$) and calbindin negative (CB$^-$) MGE-derived interneurons that bind immunolabeled EphA4-Fc (green dots). Scale bar: 20 µm. (C) Binding studies revealed that at least 50% of MGE-derived CB$^+$ interneurons express EphA receptors and 90% express ephrin ligands (mean+s.e.m.). Ctx, cortex; StA, striatal anlage; TL, transmitted light.

Henkemeyer, 2012). As shown in supplementary material Fig. S1A, pSrc, Fyn and pY350 immunosignals are equally distributed throughout MGE-derived interneurons. Incubating them with Alexa488-labeled EphA4-Fc resulted in robust dotted staining at the initial segment of their leading process (supplementary material Fig. S1B). Multiple phosphorylation events of receptor tyrosine kinases were visible at the EphA4 binding site as indicated by colocalization of EphA4-Fc and pY350 immunosignals (supplementary material Fig. S1B, first lane). Additionally, the Src family kinase Fyn as well as phosphorylated Src showed overlapping signals with the recombinant EphA4, indicating recruitment of Src family kinases through the binding of EphA4-Fc (supplementary material Fig. S1B, second and third lane). This binding was reduced when treated with the inhibitor of Src family kinases PP2 (supplementary material Fig. S1C).

Response of cortical interneurons to EphA4 in the stripe assay

We were then interested in potential functional consequences of EphA4-induced reverse signaling in MGE-derived interneurons. Therefore, these cells were cultured in stripe assays to examine the response of the neurons when they are given a choice between alternating lanes coated with pre-clustered EphA4-Fc and laminin poly-L-lysine stripes alone (control stripes). Although interneurons were equally distributed directly after plating, after 2 days *in vitro* (DIV) about two-thirds of the cells were found on the control stripes and only one-third on the EphA4 stripes (Fig. 2A). The increased number of neurons growing on control stripes was observed with different concentrations of EphA4-Fc, ranging from 5 µg/ml to 48 µg/ml (Fig. 2C). By contrast, the cells exhibited an equal distribution on the two types of stripes in the control situation, where 48 µg/ml Fc solutions rather than EphA4-Fc was injected in the silicone matrix that produces the stripes (Fig. 2B,C). A straightforward interpretation of these experiments is that EphA4 elicits reverse signaling that exerts a repulsive effect on cortical interneurons, as shown previously for EphA4 forward signaling with ephrin ligands, such as ephrin A5, A3 and B3 (Zimmer et al., 2008; Rudolph et al., 2011; Zimmer et al., 2011).

Response of cortical interneurons to EphA4 in the Boyden chamber assay

To examine further the effect of EphA4-Fc treatment on MGE-derived cells, transwell chemotaxis experiments were performed (Fig. 2D). These assays have widely been used to study the response of various neuronal cell types, including cortical interneurons, to guidance cues such as neurotrophins and ephrins (Santiago and Erickson, 2002; Camarero et al., 2006; Perrinjaquet et al., 2011). Thus, dissociated cells from the MGE of E14.5 embryos were placed in the upper compartment of a Boyden chamber and exposed to pre-clustered EphA4-Fc. We also used this assay to examine previously characterized attractants and repellents for cortical interneurons. All signaling molecules were added to the upper, the lower or both chambers. After 6 hours *in vitro*, cells that had migrated from the upper into the lower compartment were counted. To measure the baseline migration of the cells, in all experiments Fc was added as a control and the number of migrating cells on that condition was set to ‘1’ and the cell number of all other treatments was calculated relative to the control.

Brain-derived neurotrophic factor (BDNF) was previously described as a stimulator for interneuron migration, thereby acting as a chemoattractant for cortical interneurons (Behar et al., 1997; Polleux et al., 2002; Berghuis et al., 2005). Consistent with these reports, when BDNF was added to the lower compartment, there were more migrating cells than in control conditions. Conversely, when BDNF was added to the upper compartment, compared with control conditions, fewer neurons migrated through the membrane separating the two compartments (Fig. 2E). We reported previously that ephrin A5 exerts repulsive effects on migrating cortical interneurons (Zimmer et al., 2008). In accordance with these results, we found that in the Boyden chamber assay ephrin-A5-Fc exerts the opposite effect to BDNF on migrating MGE cells. Adding clustered ephrin-A5-Fc to the lower chamber decreased, and adding ephrin-A5-Fc to the upper chamber increased the number of cells migrating through the membrane compared with control conditions (Fig. 2E).

The effects of EphA4 elicited on MGE neurons in the Boyden chamber assay were quite different from the effects observed with BDNF or ephrin-A5-Fc. As illustrated in Fig. 2E, the addition of 10 µg/ml pre-clustered EphA4-Fc either to the lower or the upper compartment increased the number of cells migrating through the membrane compared with the control in each case. Also, more interneurons migrated to the lower compartment when EphA4-Fc was present in both chambers without producing any gradient across the membrane compared with the control conditions with Fc in both compartments. Consequently, EphA4 acts neither as an attractant nor as a repellent for interneurons. What EphA4 does is to increase the general motility of MGE neurons, thereby acting as a motogenic factor. Thus, although at a first glance the results from the stripe assays described in the previous section suggest that EphA4 is a repellent for interneurons, the data from the chemotaxis experiments imply a different interpretation of these experiments. Because EphA4 increases the motility of cortical neurons, they are more mobile on EphA4 stripes than on control stripes; thus, they more often move from EphA4 lanes to the control lanes than in the opposite direction. As a result, more cells are located on the control than on the EphA4 stripes, as observed in our experiments.

EphA4 is a motogenic factor for cortical interneurons

We next used different cell migration assays to directly test whether EphA4 promotes the motility of MGE neurons. We first prepared MGE explants at E14.5 and cultured them in a 3D-matrix. The explants were exposed to pre-clustered recombinant EphA4-Fc protein or, as a control, to Fc protein, each at a concentration of 10 µg/ml. In the control, after 1 DIV the interneurons displayed a robust outgrowth from the explants and the migration index (calculated as described in Materials and methods) was ~1.8 (Fig. 2F,I). However, when EphA4-Fc was added to the medium, there was a threefold increase in the migration index compared with the control situation with Fc protein (Fig. 2G,I). We also tested the possible effects of ephrin-A5-Fc, which has previously been described as a repellent for cortical interneurons, in the same assay. As illustrated in Fig. 2H,I, addition of 10 µg/ml pre-clustered ephrin-A5-Fc had no effect on the migration index. Thus, guiding and driving cortical interneurons are separate effects that are mediated by different molecules.

Recently, we and others identified two different corridors for tangentially migrating cortical interneurons in the basal telencephalon: the DMS originating from the MGE and the SMS originating mainly from the POA (Gelman et al., 2009; Hernández-Miranda et al., 2010; Zimmer et al., 2011). Because our *in situ* hybridization and immunohistochemical data demonstrate that EphA4 is predominantly expressed along the DMS, but not on the SMS, we also tested whether the effect of EphA4-Fc on interneurons is cell type specific. For this, we prepared explants from the two migratory streams (Fig. 2J, scheme) and then performed the

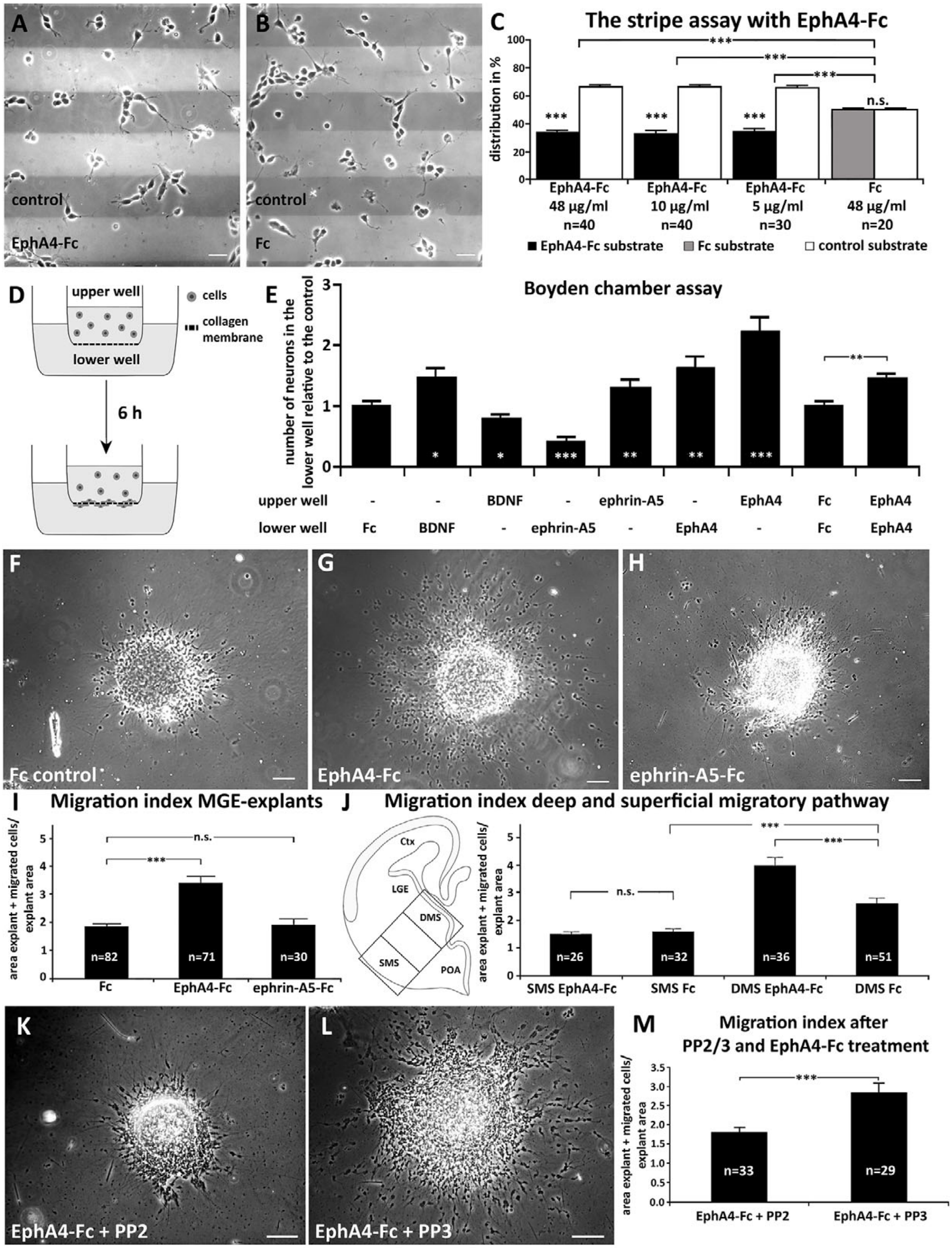

Fig. 2. EphA has a motogenic effect on MGE-derived cells *in vitro.* (A,B) Photomicrographs of interneurons cultured on alternating EphA4 and control (A) as well as Fc and control (B) stripes after 2 DIV. Scale bars: 20 µm. (C) Quantification of the stripe assay after 2 DIV. (D) Schematic of the Boyden chamber assay. (E) The number of cells in the lower well was calculated relative to the control situation, which was set to '1', when recombinant Fc protein was added to the lower well. (F-H) Photomicrographs of EphA4-Fc (G), Fc (F) and ephrin-A5-Fc (H) treated MGE explants in the outgrowth assay after 1 DIV. Scale bars: 100 µm. (I) Quantification of the migration index of MGE-derived explants in the outgrowth assay after 1 DIV. (J) Scheme of the dissection of explants from the DMS and the SMS. Analysis of the migration index of explants from different subdomains indicate that only cells from the DMS but not the SMS respond to recombinant EphA4-Fc. Ctx, cortex. (K,L) Photomicrographs of MGE explants after treatment with EphA4-Fc and the Src inhibitor PP2 (K) and the control treatment with EphA4-Fc and PP3 (L). Scale bars: 100 µm. (M) Quantification of Src inhibition in the outgrowth assay. ****P*<0.001, ***P*<0.01, **P*<0.05, n.s., *P*≥0.05 by Student's *t*-test; error bars represent s.e.m.

outgrowth assay. As depicted in Fig. 2J, these experiments indicate that cells in the SMS are slower than those from the MGE, and that only the cells from the DMS, but not the SMS, respond to recombinant EphA4-Fc. These data support the hypothesis that these two migratory streams are composed of different subsets of interneurons.

As shown above and reported previously, Src family kinases (SFKs) are recruited to EphA4-Fc binding sites in MGE-derived neurons (Zimmer et al., 2011). To test whether the motogenic effect of EphA4-Fc is mediated by SFK signaling, we inhibited SFKs with PP2 in the outgrowth assay. In the presence of PP2, there was no significant effect of EphA4-Fc on the migration index. By adding a combination of PP2 and EphA4-Fc we obtained the same migration index as with Fc treatment alone (Fig. 2K,M; 1.8±0.15). By contrast, the presence of the control peptide PP3 and EphA4-Fc increased the cell outgrowth from MGE explants (migration index with PP3 and EphA4-Fc: 2.84±0.25, *n*=29; Student's *t*-test: $P<0.001$; Fig. 2L,M). Thus, the motogenic effect of EphA4-Fc requires SFK activity.

To examine the motogenic activity of EphA4-Fc on cortical interneurons under conditions that more closely resemble the *in vivo* situation, we next performed homotopic grafting experiments in organotypic brain slices. In these studies, MGE explants from EGFP-expressing mice were transplanted on the MGE of slices from wild-type mice, as illustrated in Fig. 3A, and EphA4-Fc or Fc was

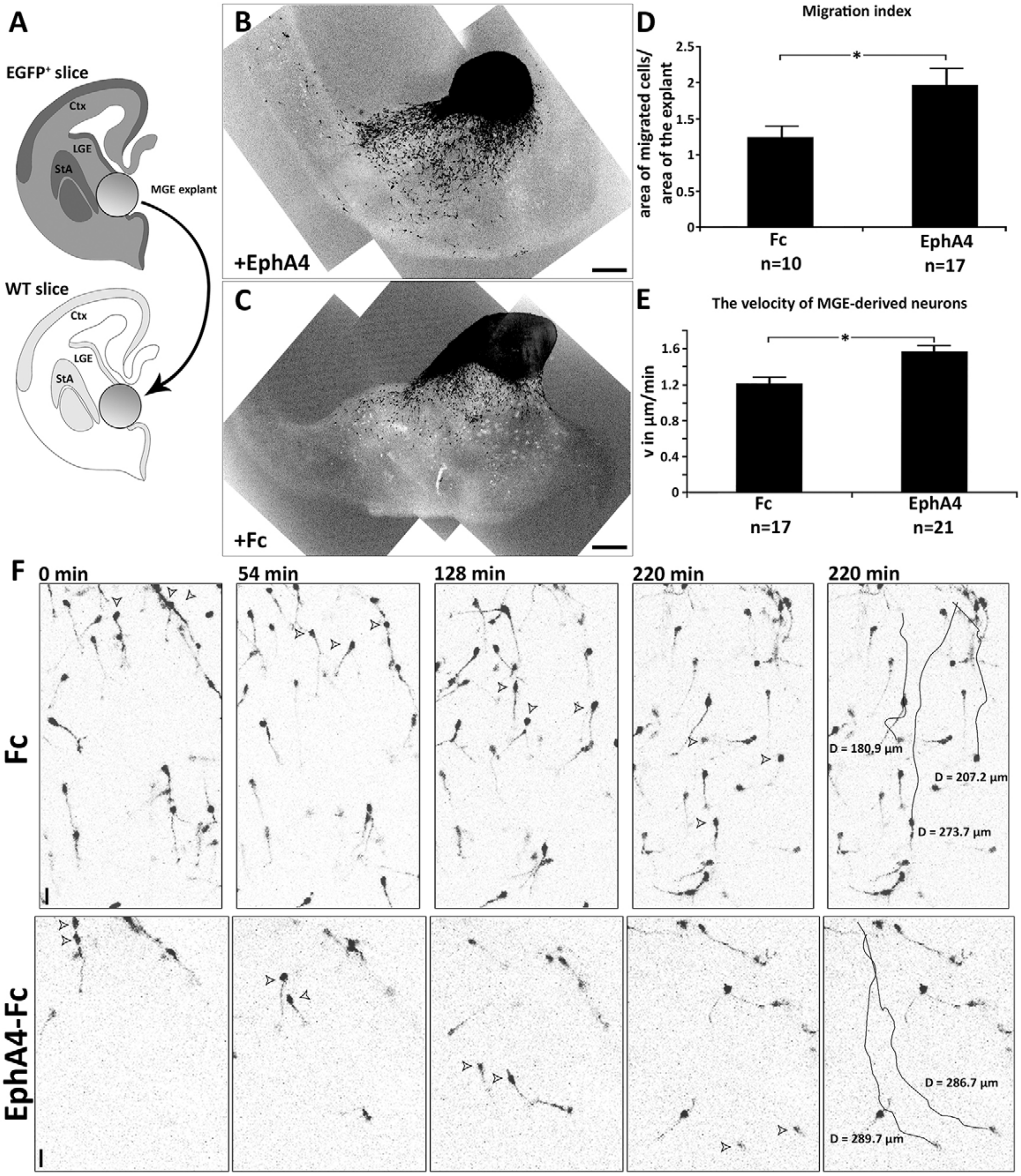

Fig. 3. EphA4 enhances the motility of MGE-derived cells *in vitro*. (A) Schematic of the grafting experiments. Ctx, cortex; StA, striatal anlage. (B,C) Tiled inverted fluorescent photomicrographs of EphA4-Fc- and Fc-treated coronal brain slices with an EGFP$^+$ MGE explant after 2 DIV. Scale bars: 100 µm. (D) Treatment of the slices with EphA4-Fc increased the migration index compared with the control. (E) The velocity of migrating cells was increased when recombinant EphA4 was present in the medium. (F) Inverted fluorescent photomicrographs of time-lapse microscopy on grafting experiments. Arrowheads indicate tracked interneurons. D, migration distance. Scale bar: 20 µm. $^*P<0.05$ by Student's *t*-test. Error bars represent s.e.m.

added to the medium. In order to analyze the effects of EphA4-Fc treatment on the dynamics of interneuron migration, we combined these experiments with time-lapse video microscopy. This allowed us to investigate the general migration pattern and to quantify the distance and velocity of migrating cells in the organotypic environment of the basal telencephalon.

There was no difference in the overall migration pattern between control and EphA4-Fc treated slices after 2 DIV. However, as illustrated in Fig. 3B,C, addition of 10 µg/ml of pre-clustered recombinant EphA4-Fc significantly increased the motility of the cells compared with control conditions. Using the same analysis as described above for MGE explants in a 3D-matrix, we found a significant increase of the migration index in organotypic slices (Fig. 3B-D). To determine the migratory speed directly, we performed quantitative analysis of the velocity of grafted EGFP-MGE cells in organotypic slices using time-lapse recordings. By adding 10 µg/ml of recombinant EphA4-Fc into the media, the speed of the migrating neurons increased significantly from 1.20±0.07 µm/minute on control conditions to 1.54±0.15 µm/minute (Fig. 3E,F; supplementary material Movies 1, 2). Thus, consistent with the data presented above, EphA4 increases the motility of MGE-derived neurons in organotypic slices.

EphA4 loss of function results in a delayed migration of interneurons *in vivo*

The previous *in vitro* assays strongly suggest that EphA4 acts as a motogenic factor for cortical interneurons. To assess directly the role of EphA4 *in vivo*, we used an EphA4 knockout mouse line to examine interneuron migration in homozygous ($EphA4^{-/-}$) and heterozygous ($EphA4^{+/-}$) mutants, as well as in wild-type (WT) animals. For this, we prepared sections from E14.5 litters and immunostained them with antibodies against CB. The CB$^+$ cells showed no obvious defects in the migration pattern among the different genotypes as they migrated in the DMS, as well as the SMS, and avoided the VZ. We then measured the distance of CB$^+$ interneurons within the cortex, starting from the pallial-subpallial boundary (PSPB) to the front of CB$^+$ neurons, relative to the cortical length (measured from the PSPB to the dorsal-most aspect of the cortex). In brains of WT littermates, interneurons in the deep stream migrated 42±2% of the cortical length, whereas in the homozygous mutants CB$^+$ interneurons migrated only 29±1% of the cortical length (Fig. 4A,B). Heterozygous EphA4 mutants exhibited a milder effect; in this case, CB$^+$ interneurons reached 36±1% of the cortex (Fig. 4A,B). Together, these data suggest a delayed migration in EphA4-deficient mice and support the idea that EphA4 serves as a motogenic factor for MGE-derived cortical interneurons.

The motogenic effect of EphA4 is mediated by ephrin A2 reverse signaling

Next, we investigated which ephrin ligand mediates the motogenic effect of EphA4 on MGE-derived interneurons in the DMS. The ephrin ligands that have previously been described as repellents for cortical interneurons, such as ephrin A5, A3 and B3, are unlikely candidates as they are expressed only in the flanking regions of the DMS. One possible mediator for the motogenic function of EphA4 is ephrin A2. *In situ* hybridization and immunohistochemical staining show that ephrin A2 mRNA and protein are present along the DMS, where EphA4 is also expressed (Fig. 5A). Double immunostaining of dissociated MGE-derived cells with EphA4 and ephrin A2 antibodies revealed a co-expression of these molecules on them. This would allow cell autonomous (cis) as well as cell-cell (trans) Eph/ephrin signaling. A deeper analysis of these neurons showed that 97.0±0.3% of the EphA4 and ephrin A2 signals were segregated into distinct membrane domains (n=10 cells from the DMS; Fig. 5B, arrowheads), indicating that cis-interactions are very unlikely in this case.

To test whether ephrin A2 mediates the motogenic effect of EphA4, we used RNA interference to reduce the ephrin A2 level in migrating MGE-derived interneurons using ephrin A2 siRNA *in vitro* and an ephrin A2 shRNA construct for *in utero* electroporation. The knockdown efficiencies were measured by western blot analysis (supplementary material Fig. S2A). Additional binding assays with recombinant EphA4-Fc on ephrin A2 siRNA- as well as ephrin A2 shRNA-transfected MGE-derived cells resulted in a decreased number of EphA4 binding sites compared with control transfected cells (supplementary material Fig. S2B). Double transfection of ephrin A2 shRNA with mut-ephrin-A2 expression vectors, carrying three silent mutations in the shRNA-binding domain to avoid degradation by RNA interference machinery, increased the number of EphA4-binding sites compared with shRNA transfection alone. Thus, the RNA interference constructs sufficiently decrease the interactions between ephrin A2 and EphA4, which can be rescued by a mut-ephrin-A2 expression vector that is not affected by the shRNA (supplementary material Fig. S2B′).

To investigate ephrin A2 as a possible mediator of the motogenic effect of EphA4, ephrin A2 siRNA-transfected MGE-derived cells were first analyzed on the stripe assay containing alternating EphA4-Fc and control stripes as shown before. After 2 DIV, 59.7±0.8% of the untransfected cells were found predominantly on the control stripes whereas only 53.7±1.2% of the ephrin A2 siRNA-transfected cells grew on this stripe (Fig. 5C,C′; Student's t-test: P<0.001, n=89 analyzed photomicrographs). Neurons transfected with control siRNA

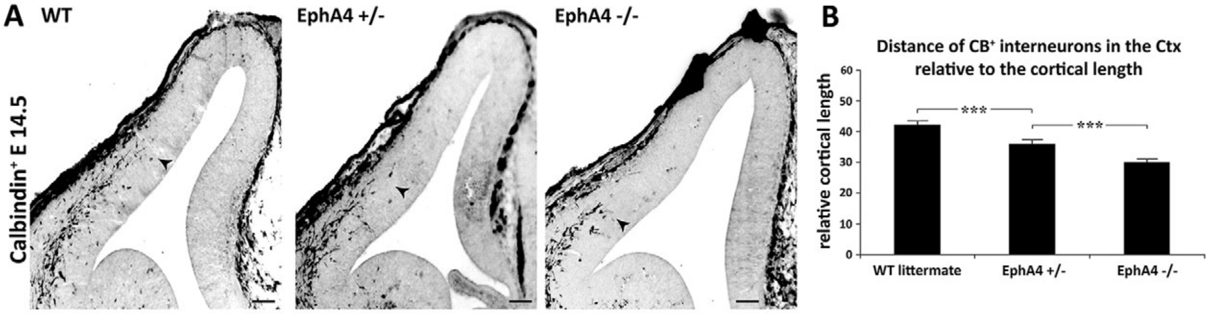

Fig. 4. Delayed migration of CB$^+$ cortical interneurons in EphA4 knockout mice. (A) Photomicrographs of sections from E14.5 wild-type (WT), heterozygous knockout (KO) ($EphA4^{+/-}$) and homozygous EphA4 KO ($EphA4^{-/-}$) cortices that were immunostained against CB. Arrowheads indicate the front of migrating cortical interneurons. Scale bars: 100 µm. (B) Analysis of the relative migration distance revealed a delayed migration of CB$^+$ interneurons in the cortex of homo- and heterozygous EphA4 knockout animals compared with the WT littermates. ***P<0.001 by ANOVA. Error bars represent s.e.m.

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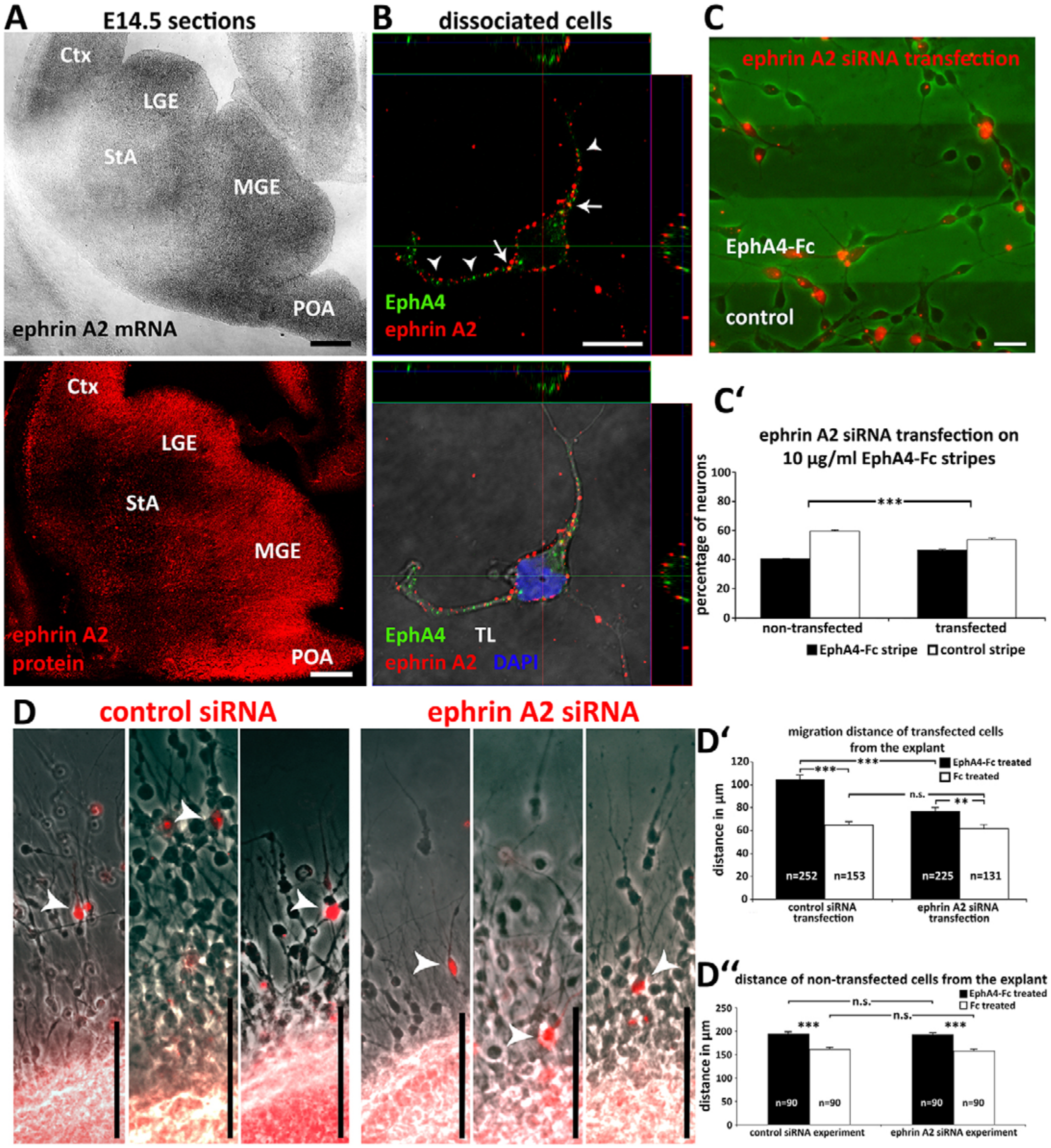

Fig. 5. Ephrin A2 mediates the motogenic effect of EphA4 on MGE-derived interneurons. (A) Photomicrographs of E14.5 brain sections after *in situ* hybridization with ephrin A2 riboprobes (top) and immunohistochemistry with ephrin A2 antibodies (bottom). Ctx, cortex; StA, striatal anlage. Scale bars: 200 µm. (B) Confocal micrograph and *x*- and *y*-scans in a single optical section of a double immunostained dissociated MGE-derived cell with antibodies against ephrin A2 and EphA4. Arrows indicate overlapping and arrowheads non-overlapping immunosignals. Scale bar: 10 µm. TL, transmitted light. (C) Photomicrograph of ephrin A2 siRNA-transfected and non-transfected interneurons on a stripe assay with alternating EphA4-Fc and control stripes. Scale bar: 20 µm. (C′) Ephrin A2 KD in MGE-derived interneurons resulted in a decreased effect of EphA4 in the stripe assay. (D) Photomicrographs of MGE-derived cells treated with EphA4-Fc and transfected with control or ephrin A2 siRNA that migrated out of MGE explants. Arrowheads indicate transfected cells. Scale bars: 100 µm. (D′) KD of ephrin A2 in MGE-derived cells caused a decreased migration distance of EphA4-Fc treated, but not Fc-treated interneurons. (D″) Non-transfected interneurons always migrated longer distances from the explant when treated with EphA4-Fc compared with Fc. ***$P<0.001$, **$P<0.01$, n.s., $P\geq0.05$ by Student's *t*-test. Error bars represent s.e.m.

showed the same distribution as untransfected cells (data not shown). These experiments indicate a decreased impact of EphA4 in ephrinA2 siRNA-transfected cells.

Based on the indication that ephrin A2 mediates the effect of EphA4 on MGE-derived interneurons, MGE explants were transfected with ephrin A2 or control siRNA and an outgrowth assay with 10 µg/ml pre-clustered EphA4-Fc or Fc in the medium was performed as described above. As shown in Fig. 5D,D′ after 1 DIV, control transfected cells cultured in EphA4-Fc-containing medium on average migrated 60% further than cells cultured in medium that contained Fc alone (mean distance from the explant of control transfected, EphA4-Fc treated cells: 104±4 µm; mean distance from the explant of control transfected, Fc treated cells: 65±4 µm; Student's *t*-test: $P<0.001$). This effect was strongly decreased when EphA4-Fc-containing medium was added to cells transfected with ephrin A2 siRNA. Here, the cells migrated only 24% further than did cells treated with Fc protein alone (Fig. 5D,D′; ephrin A2 siRNA-transfected, EphA4-Fc-treated cells: mean distance from the explant: 77±4 µm; ephrin A2 siRNA-transfected cells, Fc treated: mean distance from the explant: 62±4 µm; Student's *t*-test: $P<0.01$). The knockdown of ephrin A2 did not alter the migration of the neurons per se, as in the presence of the Fc protein they migrated as far as control-transfected cells did (Fig. 5D′; 62±4 µm after ephrin A2 siRNA transfection, 64±4 µm after control transfection; Student's *t*-test: $P \geq 0.05$). To check if the migration of non-transfected cells was different among the experimental conditions, the distance of the ten cells that migrated the furthest from the explants was examined. When EphA4 was present in the medium, the migration distance of non-transfected cells was extended compared with Fc treatment. Independently from the experimental conditions, EphA4-Fc-treated cells migrated on average 194 µm away from the explants, whereas Fc-treated cells migrated on average 160 µm away from the explants (Fig. 5D″). Together, these results demonstrate that ephrin A2 knockdown strongly reduces the motogenic effect of EphA4 *in vitro*, supporting the notion that ephrin A2 mediates EphA4-induced reverse signaling in MGE-derived cortical interneurons.

Ephrin A2 knockdown results in delayed interneuron migration *in vivo*

To validate ephrin A2 knockdown in MGE-derived interneurons of living animals, we applied *in utero* electroporation combined with RNA interference in E13.5 embryos. Cell type-specific transfection of MGE-derived interneurons was achieved by oblique holding of the forceps electrodes during surgery and by using an Lhx6-EGFP reporter construct, allowing the expression of EGFP in post mitotic MGE-derived interneurons (Du et al., 2008). Double transfection of the Lhx6-EGFP reporter construct with control shRNA in a 1:4 ratio resulted in a robust number of $EGFP^+$ interneurons in the cortex of E16.5 embryos. $EGFP^+$ interneurons that migrated to the LGE and to the cortex were clearly discernible (Fig. 6A). Their total number was set to 100% and the distribution of $EGFP^+$ interneurons in the LGE and those that proceeded to the cortex was analyzed. Under control conditions, 46±2% of $EGFP^+$ interneurons were found in the LGE and 54±2% migrated to the cortex (Fig. 6A,C). By contrast, double transfection of Lhx6-EGFP with ephrin A2 shRNA in a 1:4 ratio caused a reduced number of $EGFP^+$ interneurons in the cortex. Now 65±3% of $EGFP^+$ interneurons were found in the LGE and only 35±3% proceeded to the cortex (Student's *t*-test: $P<0.001$; Fig. 6B,C). We also attempted to rescue the migration defect caused by ephrin A2 knockdown. Therefore, the ephrin A2-deficient MGE-derived interneurons were additionally transfected with a mut-ephrin-A2 expression vector, carrying silent mutations in the ephrin A2 shRNA binding site. After 3 days *in utero*, the distribution of $EGFP^+$ cells in the LGE and in the cortex was not significantly different from the control conditions (Student's *t*-test: $P \geq 0.05$). In the rescue experiment, 50±2% of the $EGFP^+$ interneurons were found in the LGE and 50±2% in the cortex. Thus, knockdown of ephrin A2 in MGE-derived cells led to a delayed migration of cortical interneurons *in vivo* that could be rescued by expression of mut-ephrin-A2.

DISCUSSION

Numerous studies have traced the long migratory pathways that interneurons follow from their origin in the basal telencephalon to the cortex (reviewed by Park et al., 2002; Ayala et al., 2007; Valiente and Marín, 2010; Antypa et al., 2011). Progress has also been made on the identification of molecular mechanisms that guide and regulate interneuron migration. In the present study, we found a novel role of EphA4 during interneuron migration. Using different *in vitro* assays we found that EphA4 has a motogenic effect on migrating MGE-derived interneurons. This effect was cell type specific and mediated through EphA4/ephrin A2 reverse signaling, where EphA4 acts as a ligand on the migrating interneurons. Consistent with our notion that EphA4 is a motogenic factor, we found delayed migration of cortical interneurons in an EphA4-deficient mouse line.

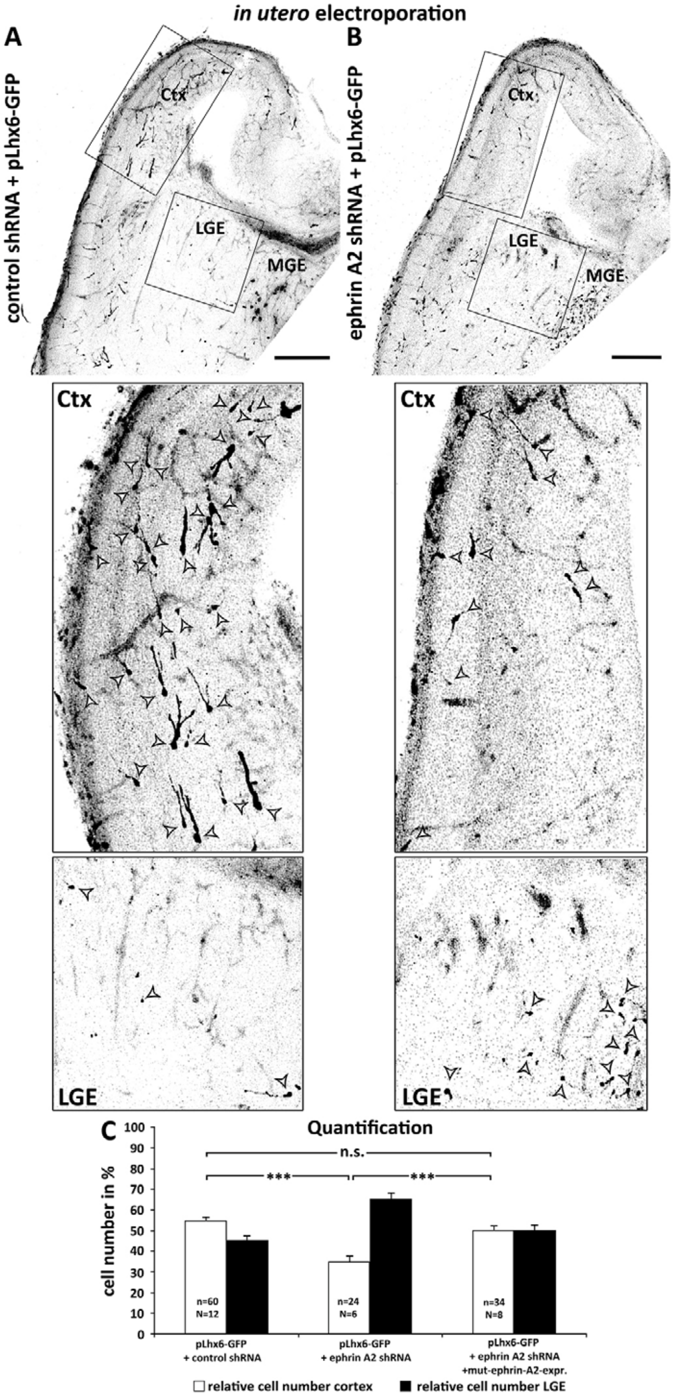

Fig. 6. Ephrin A2 KD leads to a delayed migration of MGE-derived interneurons *in vivo*. (A,B) Inverted confocal micrographs of E16.5 vibratome sections after *in utero* electroporation at E13.5 with control shRNA + Lhx6-EGFP (A) or ephrin A2 shRNA + Lhx6-EGFP (B). Scale bars: 200 µm. Boxed areas of cortex (Ctx) and LGE are shown at higher magnification below. (C) Quantification of $EGFP^+$ interneurons in the LGE and in the cortex. Compared with the control, ephrin A2 KD led to a significant reduction of interneurons that migrated to the cortex, which could be rescued by co-transfection of ephrin A2 shRNA with mut-ephrin-A2 expression vectors. n.s., $P \geq 0.05$, $^{***}P<0.001$ by Student's *t*-test. *n* refers to the number of slices and *N* to the number of brains. Error bars represent s.e.m.

Multiple guidance cues channel migrating interneurons into their appropriate pathways

Once interneurons have initiated their migration, their precise migratory pathways towards and into the cortex are defined by a wide variety of chemorepulsive and chemoattractive cues. Interneuron guidance can be contact mediated, with a permissive pathway flanked by non-permissive boundaries. In addition, gradients of diffusible attractants and repellents emanating from target and non-target regions, respectively, also regulate the directional migration of interneurons. One important class of membrane-bound guidance molecules are members of the Eph/ephrin family. Many previous studies on axonal guidance provided evidence that both A and B ephrins can repel growing axons (reviewed by Pasquale, 2008; Klein, 2009), although there is also increasing evidence that some ephrin ligands can have attractive effects on specific sets of axons (Castellani et al., 1998; Mann et al., 2002; Bolz et al., 2004; Uziel et al., 2008). Recent studies have revealed that the Eph/ephrin signaling system can also direct migrating interneurons. For example, ephrin A3, which is expressed in the striatum, prevents MGE-derived cortical interneurons from invading this inappropriate region (Rudolph et al., 2010). Likewise, ephrin A5, which is expressed in the VZ of the ganglionic eminences, the dorsal border of the DMS, also acts as a repellent for these neurons (Zimmer et al., 2008). The repulsive effects of ephrin A3 and A5 are, at least in part, mediated by forward signaling via the EphA4 receptor. Thus, the corridor of migrating cortical interneurons originating from the MGE is flanked by two repulsive boundaries, with ephrin A3 forming the ventral and ephrin A5 forming the dorsal border of this pathway.

Eph/ephrin signaling also contributes to the separation of different migratory pathways of cortical interneurons. Distinct subtypes of interneurons are generated in specific domains within the basal telencephalon: the MGE, CGE and POA (reviewed by Batista-Brito and Fishell, 2009; Gelman and Marín, 2010). We have recently shown that MGE- and POA-derived cortical interneurons migrate in spatially segregated streams towards the cortex. Ephrin B3, expressed on interneurons generated in the POA that migrate superficially, i.e. ventral of the striatum, acts as a repellent for EphA4-expressing interneurons generated in the MGE that migrate dorsally of the striatum (Zimmer et al., 2011).

The present study revealed a novel role of EphA4 during interneuron migration. In addition to mediating repulsive guidance through forward signaling, EphA4 can also have a motogenic effect on MGE-derived interneurons through reverse signaling. This appears to be unique for EphA4, as other members of the Eph/ephrin family, which also act as repulsive cues for cortical interneurons, such as ephrin B3, ephrin A3 and ephrin A5, do not affect the motility of MGE-derived interneurons in several *in vitro* assays (Fig. 2H,I; data not shown). Thus, our results point to a clear distinction between guiding and driving factors for cortical interneurons. Strikingly, the effects of EphA4 are cell type specific: it can act as a repulsive cue for POA-derived interneurons and as a motogenic factor for MGE-derived interneurons, both via reverse signaling.

Molecular motors and breaks for migrating interneurons

Previous studies have already proposed different soluble molecules as motogenic factors for cortical interneurons (reviewed by Hernández-Miranda et al., 2010). For example, it has been suggested that members of the neurotrophin family, BDNF and neurotrophin 4 (NT4; now known as neurotrophin 5, Ntf5), stimulate interneuron migration *in vitro* (Polleux et al., 2002). Analysis of mice with the targeted deletion of the gene encoding TrkB (Ntrk2), the cognate receptor for BDNF, revealed a reduction in the number of CB$^+$ cortical interneurons. Although these data would be consistent with the notion that BDNF acts as motogenic factor, there are alternative explanations for the reduction of interneurons in TrkB-deficient animals. There is compelling evidence that BDNF signaling affects different aspects of interneuron differentiation and maturation, including downregulation of CB (Jones et al., 1994; Fiumelli et al., 2000). Thus, the reduction of CB$^+$ neurons in TrkB mutants might be merely due to a downregulation of cell markers rather than an actual defect in interneuron migration. Our data obtained with the Boyden chamber assay indicated that BDNF, in contrast to EphA4, exerts an attractive rather than a motogenic effect on cortical interneurons.

In addition to extrinsic factors, Wichterle and colleagues first proposed that MGE-derived neurons have an intrinsic potential for migration (Wichterle et al., 1999). These authors examined the migratory capacity of neurons from embryonic explants of the MGE, LGE and cortex *in vitro* and also after transplantation of these explants in embryonic and adult brains *in vivo*. We found in our outgrowth assays with MGE explants that cell migration arrested after about 4 DIV, when neurons reached an isolated position without cell contact. In addition, we also observed that dissociated MGE cells cultured either in two- or three-dimensional substrates only rarely performed nuclear translocation. By contrast, in the grafting experiments interneurons exhibited normal migration all the way up into the cortex. Based on the results presented here, one possible explanation of these findings is that cell contact of MGE neurons is required to trigger their migration to the cortex.

Within the Eph/ephrin system, the ligands and receptors that are expressed on different cells can interact in trans. Additional binding can occur, when receptors and ligands are expressed on the same cell, which is called cis interaction. For example, in axons of retinal ganglion cells Eph receptors and ephrin ligands are co-expressed and they interact on the cell surface to achieve a topographic mapping in the tectum (Hornberger et al., 1999; Suetterlin et al., 2012). However, for motor axons it has also been shown that Eph receptors and ephrins are co-expressed on the same axon, but in separate membrane domains, which prevents cis interactions. In this case, the Eph receptors and ephrin ligands signal opposing effects on the growth cone: EphAs mediate repulsion through forward signaling and ephrin As attraction through reverse signaling (Marquardt et al., 2005). Given that EphA4 is co-expressed with ephrin ligands on MGE-derived interneurons (Fig. 5B), a cis interaction could mediate the motogenic effect of EphA4 in a cell-autonomous manner. However, as mentioned above, only 3% of the ephrin A2 and EphA4 proteins were colocalized on the cell surface. Moreover, dissociated interneurons do not perform nuclear translocation without extrinsic factors, and a knockdown of ephrin A2 alone does not change the migration ability of the interneurons. Thus, it seems more likely that the interneurons are activated by neighboring cells via trans interactions rather than by cell-autonomous cis interactions. By preventing cis interactions, EphA4 forward signaling can mediate the effects of repulsive guidance cues, such as ephrin A3, A5 and B3, and at the same time EphA4 reverse signaling can trigger motogenic effects in neighboring cells via ephrin A2.

In addition to signaling molecules promoting interneuron migration on the way to their targets, there are other factors that arrest neuronal migration after the cells have reached their final target. We recently found that ephrin B3/EphB1 reverse signaling can also act as a stop signal for migrating POA-derived striatal neurons, after they have reached their target (Rudolph et al., 2011).

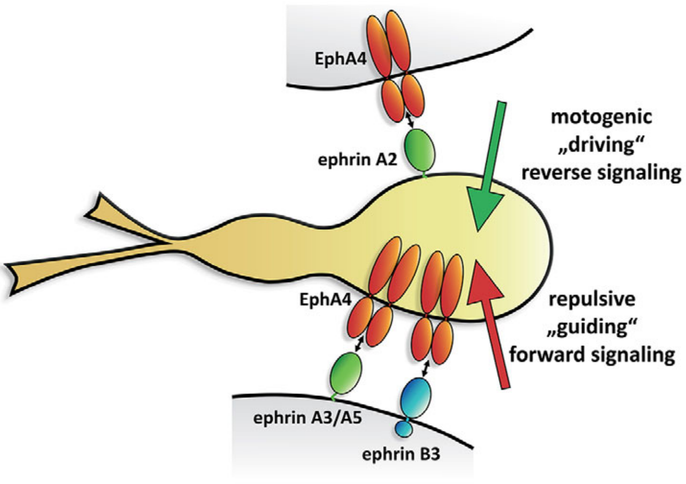

Fig. 7. Schematic of EphA4 signaling in MGE-derived interneurons. Cortical interneurons from the MGE express ephrin A and ephrin B ligands as well as the receptor tyrosine kinase EphA4. Membrane-bound ephrin ligands, such as ephrin A3/A5 and ephrin B3, are expressed in inappropriate regions of migrating cortical interneurons and act as repellents for these cells through EphA4 forward signaling (red arrow). In addition to these guiding effects by contact-mediated ligand-receptor interactions, EphA4 expressed on neighboring cells can also induce reverse signaling in MGE-derived interneurons. EphA4 reverse signaling has a motogenic effect on MGE-derived interneurons and drives these cells to the cortex (green arrow).

Thus Eph/ephrin signaling does not only provide directional cues for cell migration, but it can also provide stop and go signals for migrating neurons. It will be interesting to decipher how members of the Eph/ephrin system convey these different functions by acting on downstream signaling molecules that regulate cytoskeletal elements involved in directing and regulating interneuron migration.

Conclusions

The results presented here, in combination with our previous studies, demonstrate that members of the Eph/ephrin family play very specific and diverse roles in regulating the migration of cortical interneurons generated in different regions of the basal telencephalon towards their final destination in the cerebral cortex. This is illustrated in the summary schematic for EphA4 (Fig. 7). This receptor is expressed on MGE-derived cortical interneurons and prevents, via forward signaling triggered by either ephrin A3, ephrin A5 or ephrin B3, the invasion of these neurons into improper territories (Zimmer et al., 2008; Rudolph et al., 2010; Zimmer et al., 2011). As reported in the present paper, ephrin A2 ligands are also expressed on MGE-derived interneurons and EphA4 can elicit a motogenic activity on these cells via reverse signaling.

MATERIALS AND METHODS

Animals

EGFP-expressing C57BL/6 (Okabe et al., 1997), EphA4-PLAP (Leighton et al., 2001), C57BL/6 and NOR mice were used. The day of insemination was considered as E1. For immunohistochemical studies, mice were additionally staged at a later point (Theiler, 1989). All animal procedures were performed in accordance with the guidelines for the care and use of laboratory animals of Friedrich-Schiller-University, Jena, Germany.

In situ hybridization

Digoxigenin-labeled RNA-probes for murine EphA4 (nucleotides 75-761, NM_007936.1) and ephrin A2 (nucleotides 162-1178, NM_007909.3) were used as described previously (Zimmer et al., 2008).

Primary cell culture

E14.5 MGEs were dissected and dissociated as described previously (Rudolph et al., 2010). Neurons were cultured in single cell medium, plated on two-dimensional coverslips and used for the stripe assay and the Boyden chamber assay. To examine the response to recombinant proteins cells were cultured without fetal bovine serum.

Immunohistochemistry and labeling of MGE-derived neurons

Freshly prepared cryosections (18 µm) or MGE-derived neurons were stained as shown previously (Zimmer et al., 2011) with the following primary antibodies: rabbit anti-EphA4 (Santa Cruz, sc-921; 1:500), goat anti-EphA4 (GT15035, Neuromics; 1:500), rabbit anti-ephrin A2 (sc-912, Santa Cruz; 1:200), rabbit anti-calbindin (CB-38a, Swant; 1:2000), rabbit anti-pY350 (sc-18182, Santa Cruz; 1:500), rabbit anti-pSrc [pY418] (44660G, Invitrogen; 1:250), rabbit anti-Fyn (sc-28791, Santa Cruz; 1:100). Secondary antibodies were: Cy3-conjugated goat anti-rabbit (111-165-003, Dianova; 1:2000), donkey anti-rabbit DyLight488 (711-485-152, Jackson ImmunoResearch; 1:500), donkey anti-goat Cy3 (705-165-147, Jackson ImmunoResearch; 1:1000).

The distance of CB$^+$ interneurons in E14.5 cortices was measured from the PSPB to the front of CB$^+$ interneurons using ImageJ and calculated relatively to the cortical length that was measured from the PSPB to the dorsal-most aspect of the ventricle. In order to ensure that we used reproducible anterior/posterior regions, we only used slices in which MGE and LGE were clearly distinct. Four independent litters were used.

Analysis of Eph/ephrin-labeled MGE-derived cells

For the analysis of the ephrin A2 KD efficiency, cells were cultured in 300 µl OptiMEM (Gibco) containing 6 µl Lipofectamine2000 (Invitrogen), 1.5 µl Alexa555-labeled control siRNA (Invitrogen) and 3 µl ephrin A2 siRNA (Ambion Applied Biosystems; s65339) in a 3:1 ratio or 3 µl Alexa555-labeled control siRNA alone. The medium was changed to neurobasal medium after 2 hours. After 2 DIV, cells were treated with 5 µg/µl recombinant ephrin, Eph (R&D Systems) or Fc proteins (human IgG F(c) fragment; Rockland Immunochemicals) in culture medium for 1 hour. For visualization, proteins were clustered with 10 µg/µl Alexa488 goat-anti-human IgG (Molecular Probes) for 30 minutes. Cells with at least three green dots were considered to be labeled by the recombinant Eph/ephrin protein and set as a percentage of all DAPI-stained MGE-derived cells. Additionally, the Eph/ephrin-labeled CB$^+$ cells were counted and set as a percentage of the CB subpopulation of cortical interneurons.

Stripe assay

Stripe assays were performed according to Vielmetter et al. (Vielmetter et al., 1990) and Rudolph et al. (Rudolph et al., 2010) using silicone matrices, obtained from the Max-Planck Institute for Developmental Biology (Tübingen, Germany).

Boyden chamber assay

Trans filter chemotaxis assays (Chemicon) with 8 µm pore size were performed. MGE neurons were plated in the upper compartment at a density of 900,000 cells/ml serum-free medium (Invitrogen) either supplied with 10 µg/ml Fc protein (Rockland Immunochemicals) or 10 µg/ml recombinant proteins (R&D Systems). The Fc, the ephrin-A5-Fc and the EphA4-Fc proteins were pre-clustered for 30 minutes with 10 µg/ml anti-human IgG (Invitrogen). After 6 hours, cells were fixed and stained with DAPI. Cells that migrated through the membrane were counted. Sixty frames of at least three independent experiments were analyzed. The number of cells that migrated through the membrane in control conditions was set to '1' and the cells that migrated into the lower compartment were calculated relative to 1.

Outgrowth assay

The outgrowth assay was performed as described previously (Steinecke et al., 2012). For the dissection of explants from the DMS and the SMS, brains were cut into 225-µm coronal sections. Slices were kept in GBSS with

0.65% D-glucose and the micro domains were dissected and cut into 200×200 µm pieces. Explants were cultured in medium containing 10 µg/ml Fc protein or 10 µg/ml recombinant EphA4-Fc (all pre-clustered for 30 minutes with 10 µg/ml anti-human IgG) for 1 DIV. Migration index was calculated by the area of outgrown cells relative to the area of the explant.

Preparation of slice cultures

Grafting experiments were performed as described previously (Zimmer et al., 2008). Explants from the VZ of the MGE of corresponding EGFP-slices were transplanted homotopically on WT slices. EphA4-Fc (10 µg/ml) or Fc protein (5 µg/ml) (pre-clustered for 30 minutes with 10 µg/ml anti-human IgG) were added. Slices were fixed after 2 DIV. To measure the velocity of the cells, time-lapse movies were taken using an LSM510 (Zeiss).

Fibroblast cell culture and western blotting

Ephrin A2-expressing NIH3T3 cells were grown under standard cell culture conditions and transfected with Alexa555-labeled control (Invitrogen), ephrin A2 siRNA (Ambion Applied Biosystems; s65339) or ephrin A2 shRNA constructs using Lipofectamine2000 (Invitrogen). The transfection efficiency was calculated and cells were lysed after 32 hours. Western blot analysis was performed using the following antibodies: rabbit anti-ephrin A2 (sc-912, Santa Cruz; 1:200), mouse anti-actin (sc-58673, Santa Cruz; 1:200), rabbit anti-GAPDH (sc-25778, Santa Cruz; 1:200), biotinylated goat anti-rabbit (BA-1000, Vector Laboratories; 1:400), goat anti-mouse (B9904, Sigma; 1:400). Bands were detected using 3,3′-diaminobenzidine as substrate.

Vector cloning

Ephrin A2 shRNA constructs were designed using the siRNA sequence that has been extended by two additional nucleotides (379/380) and cloned into pNeoU6/pNeoU6-GFP. The murine ephrin A2 sequence was cloned into pEGFP-N3 and site-directed mutagenesis was performed to insert three silent mutations within the shRNA recognition site (nucleotide 105 C→A, 108 C→T and 114 C→T).

Knockdown of ephrin A2 in interneurons

MGE explants were cultured for 6 hours in 300 µl OptiMEM (Gibco) containing 6 µl Lipofectamine2000 (Invitrogen), 1.5 µl Alexa555-labeled control (Invitrogen) and 3 µl ephrin A2 siRNA (Ambion Applied Biosystems; s65339) in a 3:1 ratio or 3 µl Alexa555-labeled control siRNA alone. The outgrowth assay was continued as described above. The distance of the transfected cells from the edge of the explants was measured using ImageJ.

In utero electroporation

Timed pregnant mice were treated with 4 mg/kg Carprofen for 20 minutes before being deeply anesthetized with a mixture of fentanyl (0.05 mg/kg), midazolam (5 mg/kg) and metedomidine (0.5 mg/kg). After the uterine horns were exposed, pLhx6-IRES-GFP together with control or ephrin A2 shRNA constructs in a 1:4 ratio (2 µg/µl DNA final concentration) were injected into the lateral ventricles of the embryos. For rescue experiments, injection solution consisted of pLhx6-IRES-GFP, ephrin A2 shRNA constructs, and plasmids encoding for a mutated ephrin A2 protein in the ratio 1:4:7 (2 µg/µl DNA final concentration). Electroporation (5 pulses of 40 V, 100 mseconds duration) was performed using a forceps electrode. After 3 days, *in utero* embryonic brains were fixed and vibratome sections (150 µm) were prepared followed by immunostaining using rabbit anti-GFP (A6455, Invitrogen; 1:1000) antibodies. The number of EGFP$^+$ cells in the LGE and the cortex was counted and the sum was set to 100%. At least three independent operations were performed for each condition.

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Competing interests

The authors declare no competing financial interests.

Author contributions

A.S., C.G., G.Z. and J.R. performed the research; A.S. and J.B. designed the research and wrote the paper.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.101691/-/DC1

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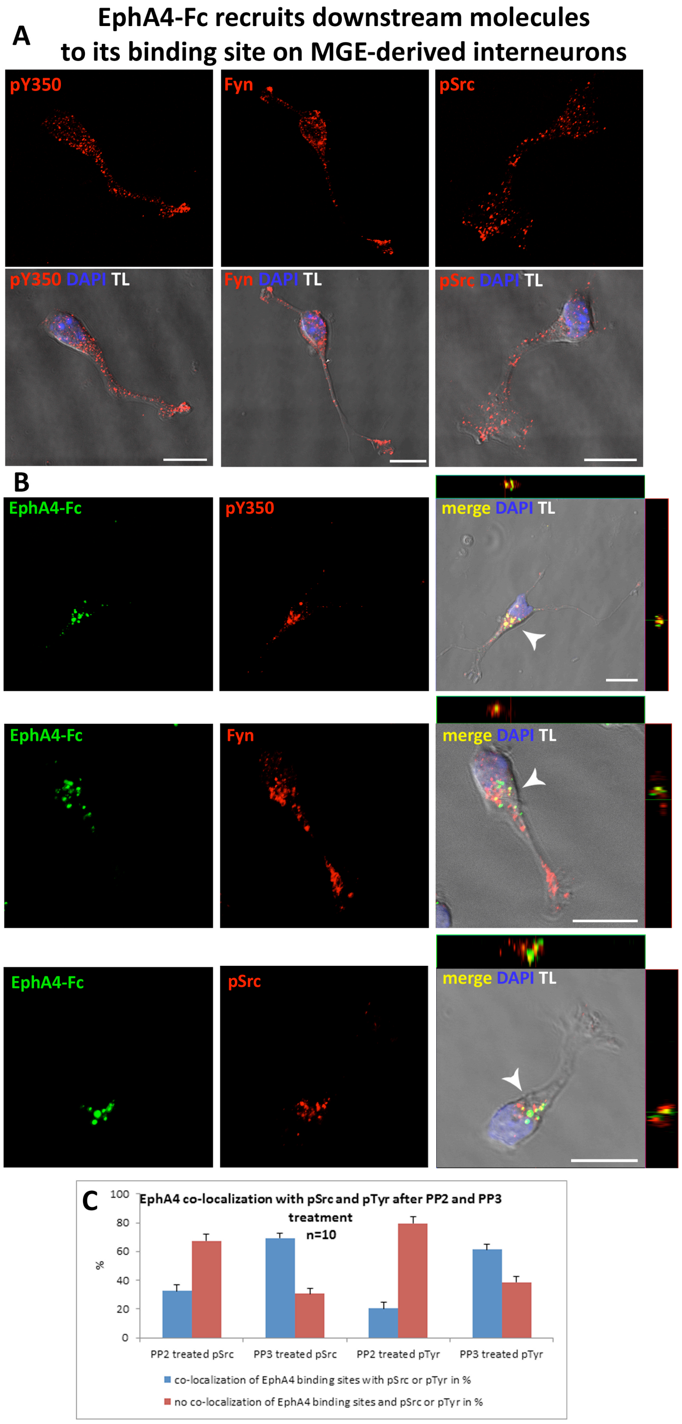

Fig. S1: Recruitment of downstream effectors to the EphA4 binding site

Earlier studies demonstrated that multiple mediators are involved in the transmission of Eph/ephrin reverse signaling, such as the transmembrane receptors TrkB or p75NTR, which are co-expressed with ephrin ligands in lipid rafts. In (A) confocal micrographs of MGE-derived interneurons immunostained with antibodies against pY350, Fyn, and pSrc are shown. The pSrc, Fyn, and pY350 immuno signals are equally distributed throughout the cell. In (B) confocal micrographs with x-y line scans in a single optical section of MGE-derived interneurons treated with recombinant EphA4-Fc (green) and immuno stained with pY150, Fyn, and pSrc antibodies (red) are presented. Arrowheads indicate the initial segments of the leading processes, where fluorescence labeled EphA4-Fc overlapped with pY350, Fyn, and pSrc immuno signals, while the rest of the cell showed only weak immuno signals. TL: transmitted light. Scale bars: 10 µm. This co-labeling was reduced when the cells were treated with the inhibitor of Src family kinases PP2, a chemical compound that is known to block activation of SFKs, including Src and Fyn (Hanke et al., 1996). In comparison to the treatment with the control substance PP3 the co-localization of EphA4 with pSrc or pY350 signals decreased from 69 ± 4% to 33 ± 4% for pSrc and from 61 ± 4% to 20 ± 4% for pY350 signals (C).

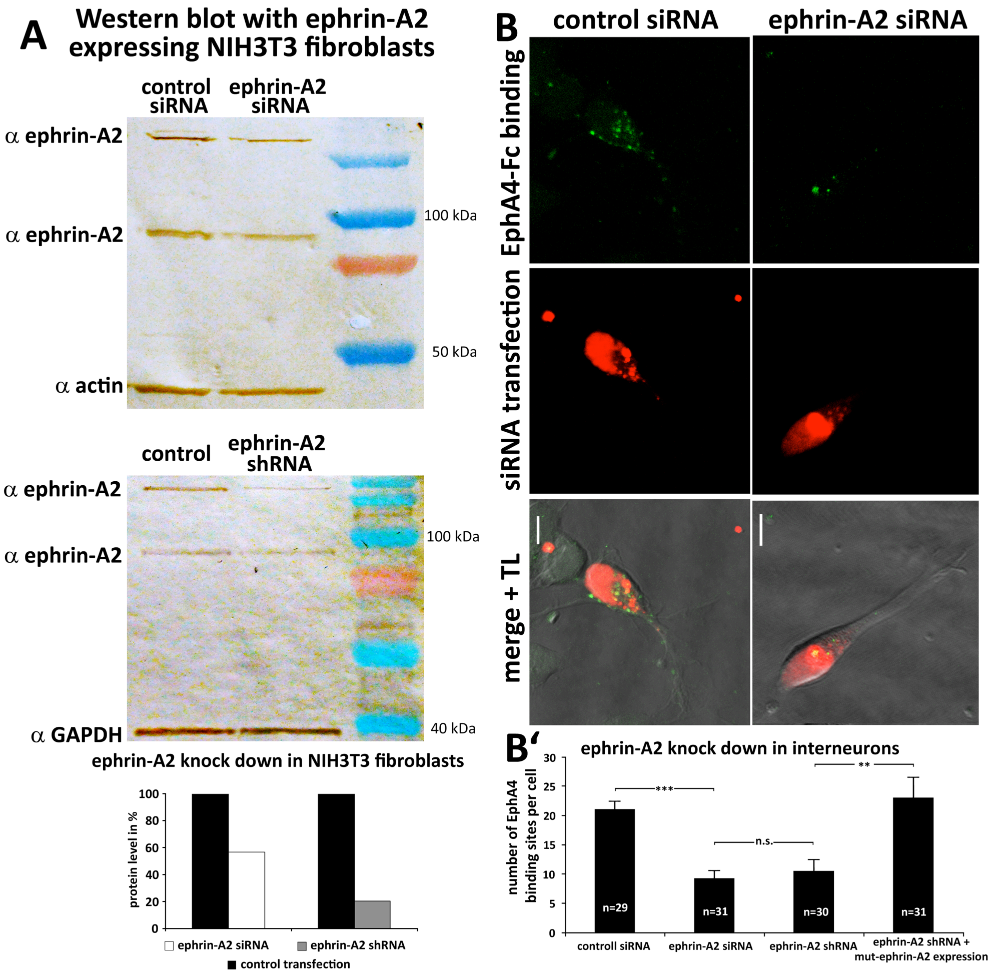

Fig. S2: KD efficiency of ephrin-A2-siRNA and -shRNA

(A), Ephrin-A2-siRNA and -shRNA decrease the ephrin-A2-level in ephrin-A2 expressing NIH3T3 fibroblasts as shown by western blot analysis. To quantify the KD efficiency we calculated the grey value of ephrin-A2-bands relative to the control bands (Actin or GAPDH). Additionally this ratio was calculated relative to the transfection efficiency of NIH3T3 fibroblasts that was about 35%. Ephrin-A2-siRNA decreased the level of ephrin-A2-protein by 43% compared to a control transfection with scrambled siRNA and the ephrin-A2-shRNA showed an 80% decrease of ephrin-A2. (B), Confocal micrograph of MGE-derived cells that were transfected with control or ephrin-A2-siRNA and treated with pre clustered EphA4-Fc. (B'), Binding assays on ephrin-A2-siRNA or -shRNA but not double transfected shRNA + mut-ephrin-A2-expression-vectors resulted in a decreased number of binding sites compared to control transfection. Student's t-test: *** $p<0.001$, ** $p<0.01$, n.s. $p\geq0.05$. Error bars: SEM.

Movie 1. Time-lapse video microscopy of EGFP$^+$ MGE-derived cells on WT-slices treated with Fc-protein as a control. Asterisks indicate tracked cells. Time is presented in minutes. (see main text for details)

Movie 2. Time-lapse video microscopy of EGFP$^+$ MGE-derived cells on WT-slices treated with EphA4-Fc-protein. Asterisks indicate tracked cells. Time is presented in minutes. (see main text for details)

recombinant protein	proportion of total MGE cells (%)	proportion of calbindin positive MGE cells (%)
ephrinA5-Fc	**55 ± 2**	**85 ± 2**
ephrinA3-Fc	**54 ± 3**	**75 ± 2**
EphA3-Fc	**10 ± 1**	**38 ± 3**
EphA4-Fc	**10 ± 1**	**36 ± 3**
EphA6-Fc	**10 ± 1**	**43 ± 4**
ephrinB1-Fc	**34 ± 2**	**40 ± 3**
ephrinB3-Fc	**54 ± 2**	**77 ± 4**
EphB1-Fc	**81 ± 2**	**98 ± 1**
EphB3-Fc	**85 ± 2**	**96 ± 2**

Table S1: Binding study with recombinant Eph/ephrin proteins on MGE-derived cells

Analysis of the binding assay indicated that almost all CB$^+$ interneurons bind EphBs and about 55% of the CB$^+$ neurons bind EphAs. Thus most CB$^+$ interneurons from the MGE express ephrin-B ligands and a large proportion ephrin-A ligands. As the MGE generates various cell types, including also striatal and globus pallidus neurons, the present data indicate that different populations of neurons express different sets of Eph receptors and ephrin ligands and to certain degree a co-expression of ephrin ligands and Eph receptors in the same cohort of cells. Results are presented as a percentage ± SEM.