EphrinA-EphA receptor interactions in mouse spinal neurulation: implications for neural fold fusion

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ABSTRACT The molecular basis of neural fold adhesion and fusion is a poorly understood aspect of neurulation. Cell surface glycosyl phosphatidylinositol (GPI)-anchored proteins have been implicated in neural fold adhesion, with ephrinAs particularly attractive candidates in view of the cranial neural tube defects observed in mice lacking ephrinA5 or the EphA7 receptor. Here, we demonstrate that ephrinsA1, A3 and A4, as well as several EphA receptors, are expressed in the closing mouse spinal neural tube. Most ephrinAs and EphA receptors were found to be expressed in multiple tissues in the caudal region, whereas EphA2 receptor was expressed specifically at the apices of the neural folds just prior to onset of neural tube fusion. Using mouse whole embryo culture, we found that cleavage of GPI-anchored molecules from the embryonic cell surface resulted in delay of spinal neural tube closure. Injection of EphA1 and EphA3 fusion proteins intra-amniotically into cultured embryos was used to specifically disrupt ephrinA-EphA receptor interactions, and led to inhibition of spinal neural tube closure, without adverse effects on growth or developmental progression. These treatments did not disturb neural plate bending or neural fold elevation, both of which are critical for spinal neural tube closure. Our findings demonstrate that ephrinA-EphA receptor interactions are required for closure of the mouse spinal neural tube, and support the hypothesis that ephrinA-EphA receptor interactions may participate in the molecular recognition events that culminate in adhesion and fusion of the tips of the neural folds during spinal neurulation.

KEY WORDS: *morphogenesis, neural tube, embryo culture, adhesion, GPI anchor*

Introduction

Neurulation, the formation of the neural tube, is a fundamental early component in the establishment of the vertebrate central nervous system. Many studies have examined the cellular and molecular processes that underlie the initial shaping of the neural plate, and its bending during elevation of the neural folds (Colas and Schoenwolf, 2001; Schoenwolf and Smith, 1990; Copp *et al.*, 2003). Much less attention has been paid to the subsequent event of midline neural fold fusion, which occurs progressively along the entire axis of the neurulating embryo, and which marks the completion of neural tube closure.

In amphibians, birds and mammals, a zone of altered cell morphology with numerous rounded cellular blebs has been observed along the tips of the neural folds, immediately prior to fusion. These surface alterations are suggested to reflect the cellular events that enable adhesion between the folds (Waterman, 1975; Waterman, 1976; Rice and Moran, 1977; Lawson and England, 1998). Moreover, lamellipodial and filopodial cellular protrusions have been observed emerging from the tips of the neural folds and linking with each other across the midline (Geelen and Langman, 1979). Similar observations have been made in other morphogenetic systems, for example the closing palatal shelves and fusing eyelids, in which membrane specialisations are observed at the sites of incipient adhesion and

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fusion (Gato *et al.*, 2002; Tao *et al.*, 2005).

Molecular clues to the events of neural fold adhesion and fusion have come from studies in which the apposing tips of the neural folds were found to be covered with extracellular carbohydrate-rich material which stains with ruthenium red (Sadler, 1978) and contains alpha-D-mannose, alpha-D-glucose and N-acetyl-D-glucosamine sugar residues (Smits van Prooije *et al.*, 1986). Removal of this extracellular material using phospholipase C in whole embryo culture resulted in neural tube closure defects (O'Shea and Kaufman, 1980). Since cell surface glycosyl phosphatidyl-inositol (GPI) linkages are amongst the targets of phospholipase C (Andrews *et al.*, 1988), this finding raises the possibility that GPI-anchored molecules may participate in neural fold fusion.

Independent evidence implicating GPI-anchored molecules in neural fold fusion has come from the finding of cranial neural fold fusion defects in mice lacking ephrinA5 or EphA7 (Holmberg *et al.*, 2000). EphrinA5, like other members of the ephrinA family, is a GPI-anchored cell surface ligand that interacts with members of the transmembrane EphA receptor family. Although best known for their repulsive effects, for example in retinal axon guidance (Drescher, 1997), ephrinA-EphA interactions can also be attractive in some cellular contexts, as was suggested for the putative role in cranial neural tube closure (Holmberg *et al.*, 2000).

Despite the evidence implicating ephrinA-EphA interactions in cranial neural fold adhesion and fusion, it is notable that defects of spinal neurulation were not observed in the ephrinA5 and EphA7 knockouts (Holmberg *et al.*, 2000). Indeed, to date there has been no systematic study of either the expression or function of the ephrinA-EphA system during spinal neural tube closure. In the present study, we show that several ephrinAs and EphA receptors are expressed during mouse spinal neurulation. By treating cultured embryos with phosphatidylinositol-specific phospholipase C, we confirm that GPI-anchored molecules are required for spinal neural tube closure. Moreover, by applying exogenous EphA fusion proteins to neurulation stage embryos in culture, we demonstrate a requirement for binding between ephrinAs and endogenous EphA receptors in neurulation. This study provides the first evidence for a role of ephrinA-EphA interactions in closure of the mouse spinal neural tube.

Results

EphrinA1 *and* A3 *are expressed during mouse spinal neurulation*

To gain insight into the ephrinAs that might participate in mouse spinal neurulation, we studied the distribution of their mRNAs by whole mount *in situ* hybridisation. At E8.5, both *ephrinA1* and *A3* are strongly expressed in the caudal region (Figure 1A,G), including the region of open neural folds, termed the posterior neuropore (PNP). In sections, *ephrinA1* transcripts are detected in the open neural plate of the PNP but with reducing intensity rostrally, towards the site of neural fold fusion (Figure 2A-D). In contrast, *ephrinA3* is expressed more uniformly throughout the E8.5 PNP (Figure 2E-H), although with most intense expression in paraxial mesoderm. By E9.5, *ephrinA1* transcripts become confined to the ventral tissues of the PNP and tail bud (Figure 1B,C; Figure 2I-L), whereas *ephrinA3* expression extends more rostrally into the trunk region (Figure 1H,I). In the PNP region,
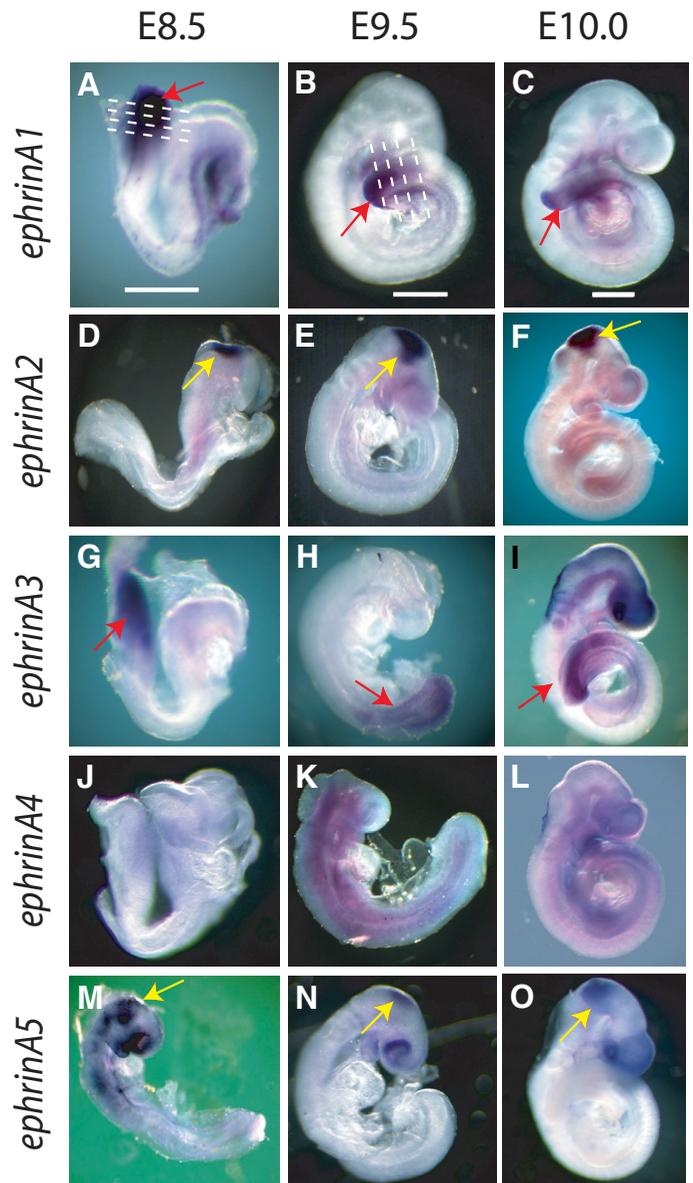
Fig. 1. Expression of *ephrinAs* during mouse neurulation. *Whole mount* in situ *hybridisation of embryos at E8.5 (6-8 somites)* **(A,D,G,J,M),** *E9.5 (12-17 somites)* **(B,E,H,K,N)** *and E10 (25-26 somites)* **(C,F,I,L,O).** EphrinA1 *and* ephrinA3 *transcripts are localised to the posterior neuropore (PNP) region (red arrows in A-C and G-I), with* ephrinA1 *additionally expressed in the branchial arches.* EphrinA2 *and* ephrinA5 *mRNAs are localized to the midbrain (yellow arrows in D-F and M-O), with* ephrinA5 *additionally expressed in the optic vesicle and telencephalon.* EphrinA4 *is expressed ubiquitously, with an increase in intensity at E9.5 (J-L). Dashed lines (A,B) indicate plane of sections in Fig. 2. Scale bars, 0.5 mm.*

ephrinA3 is expressed most strongly in the lateral paraxial mesoderm, with low intensity expression also in the neural plate (Figure 2M-P). Expression of *ephrinA1* was also detected in the branchial arches at both E8.5 and E9.5 (Figure 1A,B), whereas *ephrinA3* becomes expressed in the brain by E10 (Figure 1I).

EphrinA2 and *A5* are both strongly expressed in a broad cranial domain at E8.5 (Figure 1D,M), with expression gradually becoming localized to the midbrain as neurulation progresses through

E9.5 to E10 (Figure 1E,F,N,O). In addition, *ephrinA5* is expressed in the developing telencephalon (Figure 1O). There is no expression of either of these *ephrins* in the PNP region. Expression of *ephrinA4* is ubiquitous throughout neurulation. E8.5 embryos exhibit low intensity expression (Figure 1J) but by E9.5 *ephrinA4* has been up-regulated and this more intense expression continues at E10 (Figure 1K,L).

Hence, several *ephrinAs* are expressed in the PNP during neurulation, consistent with a role for these GPI-anchored molecules in spinal neural tube closure.

Multiple ephrin receptors are expressed in the spinal neural tube during neurulation

To survey the expression of ephrin receptors (Ephs) during mouse neurulation, RT-PCR was initially performed on cranial and caudal portions of E8.5 and E9.5 embryos (Figure 3). This showed that, of the *EphA* receptors studied, all are expressed in the cranial region during this developmental period. In the caudal region, *EphA1, A2, A4* and *A5* are expressed strongly, while *EphA3* and *A8* are also detectable. Expression of *EphA7* is not observed.

Whole mount *in situ* hybridisation, together with analysis of transverse sections, was used to document the distribution of *EphA1, A2, A4* and *A5* transcripts in the E9.5 caudal region. *EphA1* has a rather general expression pattern, being expressed throughout the neuroepithelium, paraxial mesoderm, notochord and dorsal hindgut (Figure 4A-D), although not in ventral tissues. *EphA5* is expressed particularly in the PNP region (Figure 4M)

and sections reveal that transcripts are present strongly throughout the neuroepithelium and at low level in other tissues (Figure 4N-P).
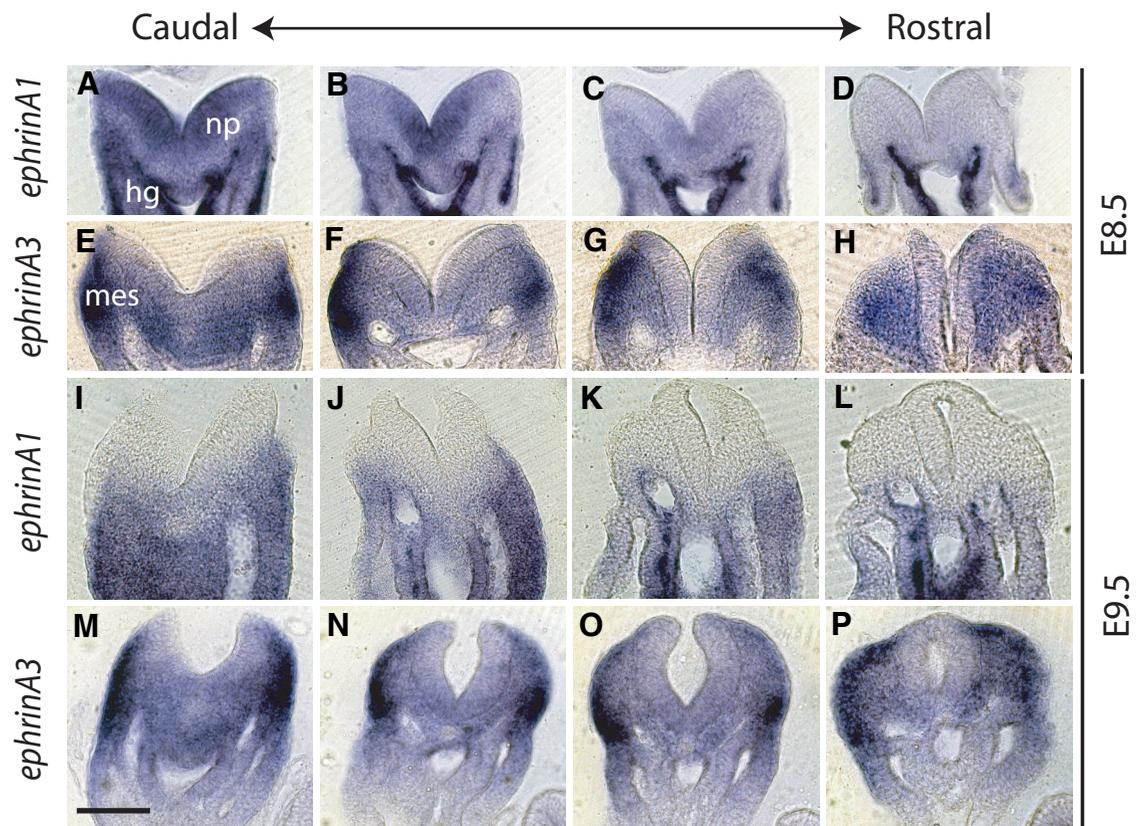
In contrast, *EphA2* and *EphA4* exhibit tissue-specific expression. *EphA2* is expressed strongly in the neuroepithelium and notochord, but not in the paraxial mesoderm and hindgut, with a striking variation in expression pattern along the spinal axis (Figure 4E-H). In the open PNP, *EphA2* transcripts are present throughout the neuroepithelium but more rostrally, towards the site of neural fold adhesion and fusion, *EphA2* expression becomes highly restricted with transcripts detected only in the apposing tips of the neural folds (Figure 4H,Q). This very specific pattern of *EphA2* expression was reproducible in multiple embryos at this stage (Figure 4Q, insets). The expression pattern of *EphA4* is almost complementary to *EphA2*, with strong expression in the paraxial mesoderm and only low intensity, patchy expression in the neural plate (Figure 4I-L). Nevertheless, there appears to be enhanced expression of *EphA4* at the neural fold tips when adhesion and fusion are underway (Figure 4L).
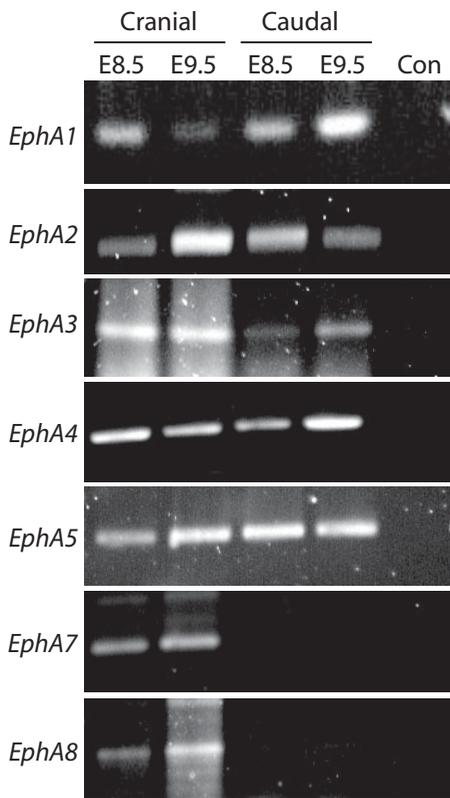
Hence, while several EphA receptors are expressed in the closing spinal neural folds, the specific localisation of *EphA2* and *EphA4* transcripts is particularly suggestive of a role in neural fold adhesion and fusion. To examine the localization of EphA2 expression in greater detail, we performed immuno-electron microscopy on E9.5 embryos. This analysis confirmed that EphA2 is present at the tips of the neural folds and suggests, moreover, that it is expressed mainly in the surface ectoderm, which forms the leading edge of the neural fold at the

Caudal ⟵⟶ Rostral

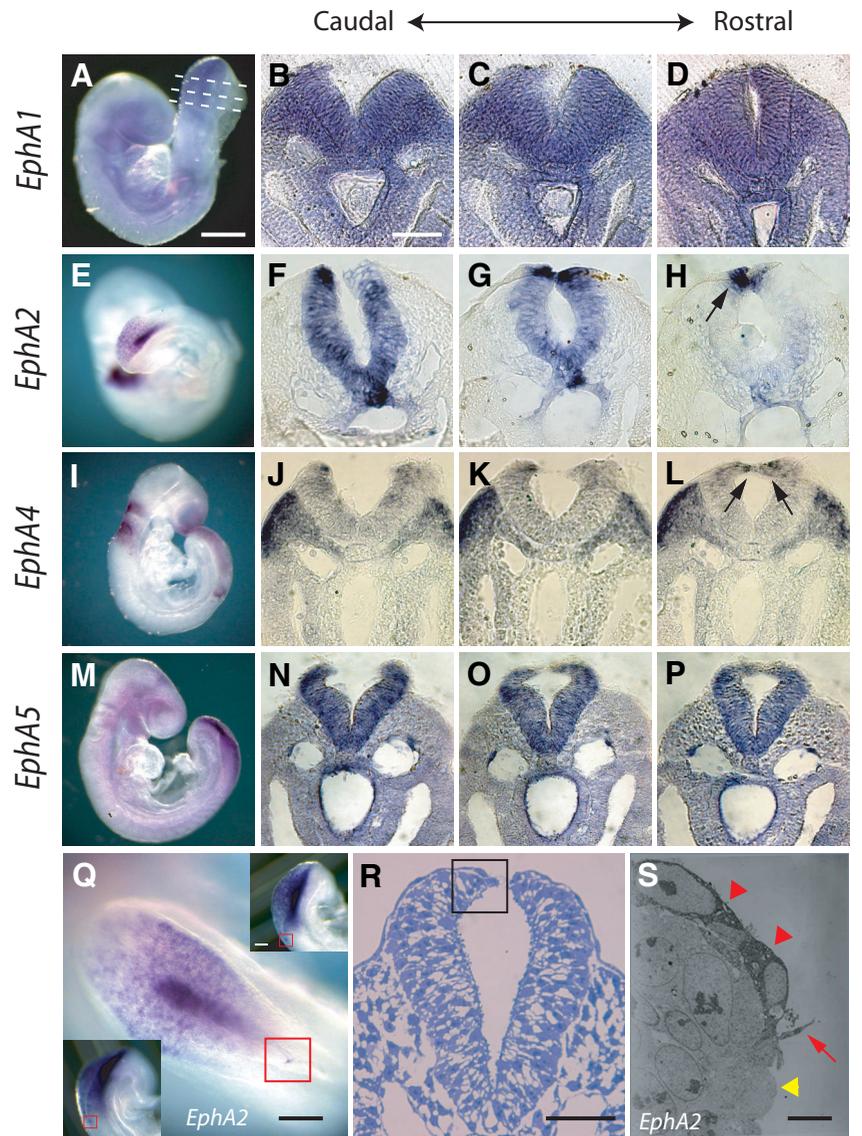**Fig. 2. Expression of *ephrinAs* in the mouse posterior neuropore (PNP).** *Transverse sections through the PNP region of E8.5* **(A-H)** *and E9.5* **(I-P)** *embryos hybridized as whole mounts (indicated by dashed lines in Fig. 1 A,B) for expression of* ephrinA1 **(A-D, I-L)** *and* ephrinA3 **(E-H, M-P)**. *Successive sections are in caudal to rostral sequence, left to right. EphrinA1 is expressed in the caudal-most neural plate at E8.5, whereas expression becomes down-regulated more rostrally, near the point of neural fold adhesion and fusion* **(A-D)**. *Ventrally, expression is intense in the mid/hindgut. At E9.5* **(I-L)**, *ephrinA1 is expressed ventrally in lateral plate mesoderm and hindgut, whereas transcripts are absent from the neural plate. EphrinA3 transcripts are present at E8.5* **(E-H)** *and E9.5* **(M-P)** *particularly in the lateral paraxial mesoderm and, less intensely, in the neural plate. Intensity of expression does not vary along the PNP axis. Abbreviations: hg, hindgut; mes, paraxial mesoderm; np, neural plate. Scale bar: 0.2 mm.*

Fig. 4 (Right). Expression of *EphA* receptors during mouse neurulation. (A-P) *Whole mount* in situ *hybridisation expression analysis of* EphA1, A2, A4 *and* A5 *in whole E9.5 embryos* **(A,E,I,M)** *and vibratome sections through the posterior neuropore (PNP, plane of section as in A) shown in caudal to rostral sequence, left to right* **(B-D, F-H, J-L, N-P)**. EphA1 *is expressed throughout the neural plate, presomitic mesoderm, notochord and hindgut endoderm* **(A-D)**; EphA2 *is expressed throughout the neural plate and notochord* **(E-H)**, *except at the site of neural fold fusion, where expression is solely at the neural fold tips (arrow in H).* EphA4 *is expressed strongly in the presomitic mesoderm and weakly in the neural plate, with strongest expression in the dorsal neural folds* **(I-L)** *particularly at the site of neural fold fusion (arrows in L). Note expression in rhombomeres 3 and 5.* EphA5 *is expressed uniformly throughout the neural plate with lower intensity expression in other tissues* **(M-P)**. **(Q)** *Dorsal view of PNP region of whole mount hybridisation for* EphA2 *mRNA, as in (E). Note expression throughout neural plate, with focal site of hybridisation at the point of neural fold fusion (boxed), corresponding to the localized signal in (H). Insets in (Q) show two further embryos, each with a focal site of* EphA2 *expression at the point of neural fold fusion (boxed).* **(R,S)** *Immuno-electron microscopy of* EphA2 *expression at the neural fold tip (S). Toluidine blue-stained plastic section* **(R)** *shows location of thin section in (S) (boxed). Note* EphA2 *signal in the surface ectoderm cells (red arrowheads), and in a lamellipodium-like protrusion which extends towards the opposite neural fold (red arrow). Neuroepithelial cells immediately adjacent to the surface ectoderm do not express* EphA2 *(yellow arrowhead). Scale bars, 0.5 mm in A (also E, I, M); 0.2 mm in B (also C, D, F-H, J-L, N-P); 0.1 mm in Q,R; 9 μm in S.*

site of closure, rather than in adjacent neuroepithelial cells (Figure 4R,S).

GPI-anchored cell surface molecules are required for neural tube closure

To determine whether GPI-anchored ephrinAs and their EphA receptors play a functional role in neural tube closure, we re-investigated earlier findings that cleavage of GPI-anchored molecules from neurulation-stage mouse embryos cause neural tube closure defects in culture (O'Shea and Kaufman, 1980). The previous study used non-specific phospholipase C, but we increased the specificity of the treatment by using

phosphatidylinositol-specific phospholipase C (PIPLC), which cleaves the majority of phosphatidylinositol anchored molecules from the cell surface (Low *et al.,* 1988; Hornberger *et al.,* 1999). As a control, we probed for surface ephrinAs using an EphA3-AP affinity probe and confirmed that PIPLC does indeed cleave GPI-anchored proteins, including ephrinAs, from the neuroepithelial surface of cultured embryos (Figure 5A,B).

E8.5 embryos (6-8 somite stage) were injected intra-amniotically (Figure 6A,B) with varying concentrations of PIPLC, followed by culture for 8 h. PNP length was then determined as a measure of the normality of spinal neurulation. An enlarged PNP at E9.5-E10.5 strongly correlates with subsequent development of open spina bifida (Copp, 1985). Embryos that were injected with 5 mU and 10 mU PIPLC exhibited markedly enlarged PNPs, compared with PBS-injected controls (Figure 6C-F and data not shown). Both PNP length (Figure 7A) and width (data not shown) were significantly enlarged in embryos exposed to 5 or 10 mU PIPLC compared with controls. On the other hand, PIPLC had no effect on PNP length or width at lower concentrations.

A question arises about the specificity of this action of PIPLC, since retardation of PNP closure was observed at concentrations where the enzyme first became limiting for developmental progression and growth. Hence, crown-rump length (but not somite number) was significantly reduced in embryos exposed to 5 mU PIPLC, while both crown-rump length and somite number were adversely affected at 10 mU PIPLC (Figure 7B,C). Moreover, blisters on cranial neural folds and a wavy closed neural tube were observed in some embryos, indicating non-specific toxicity of PIPLC (Figure 6E). As an alternative approach, therefore, we next employed a more specific intervention in ephrinA-EphA function during mouse spinal neurulation.

Perturbation of ephrinA-EphA interaction inhibits spinal neural tube closure, without altering the pattern of neural plate bending

In order to investigate a possible role for GPI-anchored ephrins in PNP closure, exogenous EphA1 or EphA3, fused to the immunoglobulin Fc domain or alkaline phosphatase (AP) respectively, were injected into the amniotic cavity of E8.5 embryos. Exogenous EphA fusion proteins have been shown to block the binding of ephrinAs to their endogenous EphA receptors (Ciossek *et al.,* 1998; Coulthard *et al.,* 2001). After 8 h culture, embryos treated with either EphA3-AP or EphA1-Fc exhibited a significantly greater PNP length and width compared with control embryos that received the same concentration of Fc or AP alone (Figures 6G-J; 7D,E). Importantly, with this intervention there was no effect on crown-rump length or somite number of the cultured embryos (Figure 7F,G), arguing for a specific effect of the EphA fusion proteins on PNP closure, rather than a general retarding effect on development. These findings demonstrate a requirement for ephrinA-EphA interactions in closure of the spinal neural tube in mouse embryos.

We found that EphA2 is expressed specifically at the apices of the neural folds just prior to and during neural fold fusion in the spinal region. Taken together with the results of the EphA fusion protein perturbation experiments, this might indicate a functional role for ephrinA-EphA interactions in the process of neural fold adhesion and fusion. Alternatively, ephrinA-EphA interactions could be required for another aspect of neurulation. For example,
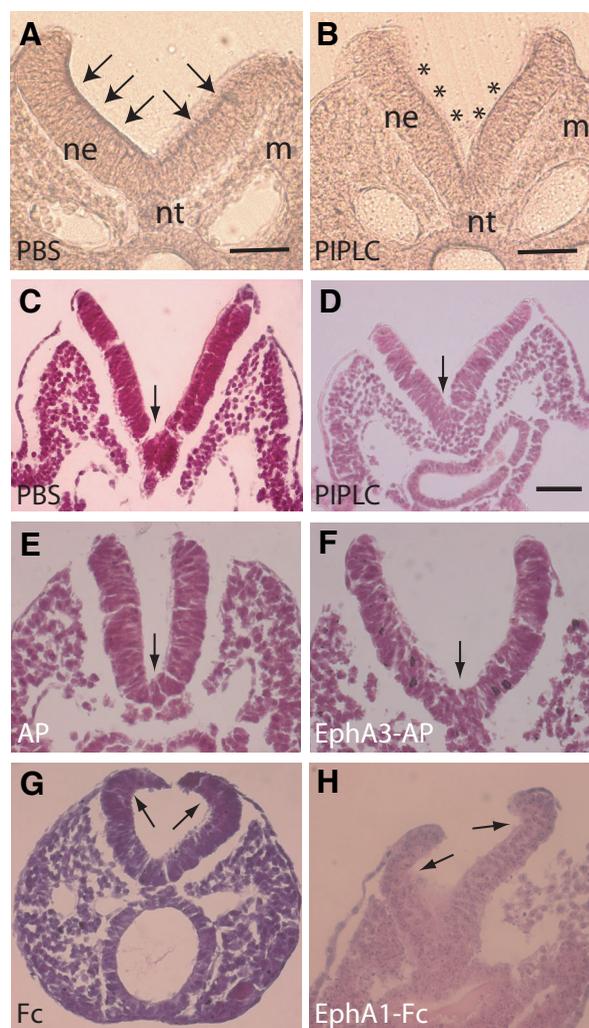
Fig. 5. Phosphatidylinositol-specific phospholipase C (PIPLC) treatment removes cell surface ephrinAs, whereas pattern of neural plate bending is not disturbed by PIPLC or EphA fusion proteins. *(A,B) Receptor affinity probe assay using EphA3-AP to determine the effect of PIPLC treatment on surface ephrinAs in the open PNP region. Enhanced signal is observed on the apical surface of neuroepithelial cells in the PBS-treated control embryo (arrows in* **(A)***) whereas this surface signal is reduced after treatment with 5 mU PIPLC (asterisks in* **(B)***). Non-surface staining (comparable in A,B) represents background in this assay since the EphA3-AP probe does not penetrate. Hence, PIPLC cleaves off ephrinAs, and presumably other GPI-anchored proteins, from cultured embryos. Abbreviations: m, paraxial mesoderm; ne, neuroepithelium; nt, notochord. (C-F) E8.5 embryos exposed in vitro to PBS* **(C)***, 5 mU PIPLC* **(D)***, AP* **(E)** *or EphA3-AP* **(F)** *all exhibit normal bending of the neural plate at the median hinge point (arrows). Note the widely open neural folds in the EphA3-AP treated embryo (F).* **(G,H)** *Slightly more advanced embryos, at E9.0, exhibit normal dorsolateral hinge point formation (arrows) after exposure to either Fc* **(G)** *or EphA1-Fc* **(H)***. Scale bars, 0.05 mm in A,B; 0.1 mm in D (also C, E-H).*

neural fold bending, which is required for elevation and apposition of the neural folds, might be inhibited in the absence of ephrinA-EphA interactions. To test this idea, we prepared transverse sections through the PNP region of embryos treated *in vitro* with PIPLC, EphA3-AP or EphA1-Fc, and their respective controls.
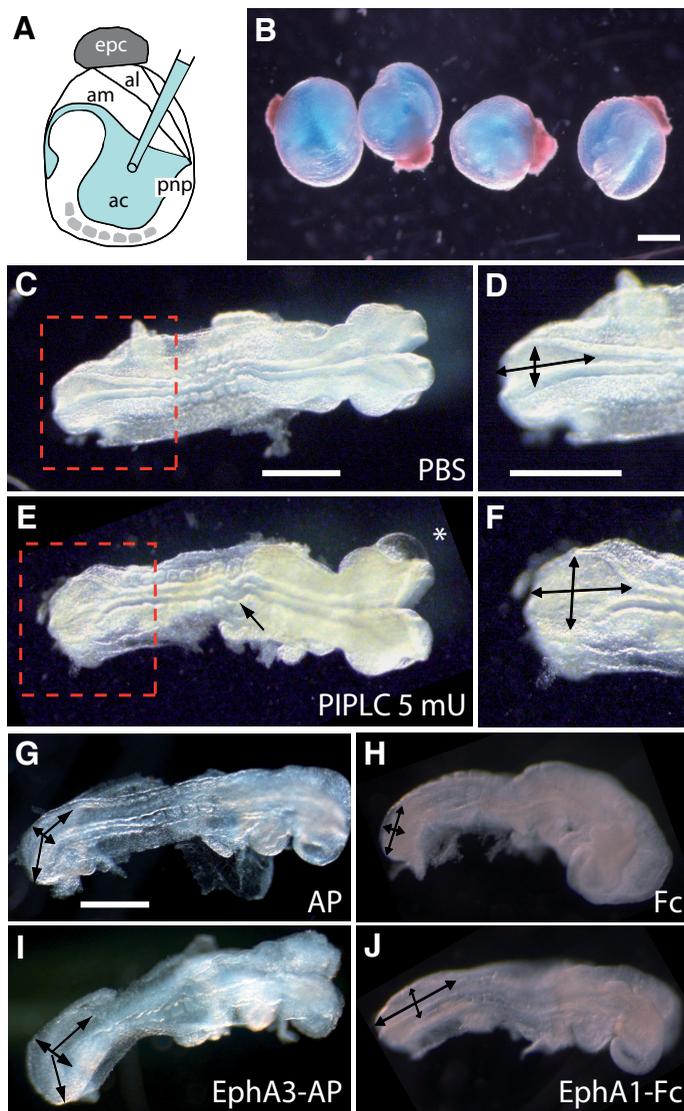
Fig. 6. Removal of glycosyl phosphatidylinositol (GPI)-anchored molecules, or blocking of cell surface ephrinA function, disturbs spinal neurulation. *(A,B) Method of delivery of PIPLC and EphA fusion proteins to cultured embryos. Diagram demonstrating intra-amniotic injection* **(A)** *and whole E8.5 embryos with intact yolk sac and amnion, pre-culture, immediately following intra-amniotic injections of PIPLC plus Fast Green* **(B)***. (C-J) Embryos, with extraembryonic membranes removed, at end of 8 h culture period. Magnified images of PNP region (red boxes) in* **(C,E)** *are shown in* **(D,F)** *respectively. PNP length and width are indicated by arrows. Embryo injected with PBS/Fast Green demonstrates normal sized PNP* **(C,D)** *whereas embryo injected with 5 mU PIPLC/Fast Green exhibits enlarged PNP in both length and width dimensions* **(E,F)***. The wavy neural tube (arrow in E) and surface blister on left cranial neural fold (asterisk in E), both indicate mild toxicity of PIPLC treatment. In contrast, embryos injected with EphA3-AP* **(I)** *and EphA1-Fc* **(J)** *fusion proteins do not differ in morphology from their AP* **(G)** *and Fc* **(H)** *controls, apart from a longer and wider PNP. Abbreviations: ac, amniotic cavity; al, allantois; am, amnion; epc, ectoplacental cone; pnp, posterior neuropore. Scale bars, 1 mm in B; 0.5 mm in C (also E),D (also F), G (also H-J).*

Caenorhabditis elegans where ventral closure depends on function of an Eph tyrosine kinase (George *et al.,* 1998). The finding of midbrain neural tube closure defects in mice lacking ephrinA5 or EphA7 function (Holmberg *et al.,* 2000) points to a further role of ephrin-Eph interactions during morphogenesis, and the results of the present study provide support for this suggestion. Not only are ephrinAs and EphA receptors expressed during the critical stages of spinal neurulation in the mouse embryo, but also perturbation of interactions in the ephrinA-EphA system leads to specific disturbance of neural tube closure. The localization of EphA2 transcripts at the tips of the neural folds is particularly suggestive that the ephrinA-EphA system may function in adhesion and fusion of the neural folds, during the final stage of neurulation. Since EphA2 can bind any of the ephrinA ligands (Pasquale, 2005), it remains unclear which precise ephrinA-EphA2 interactions might be functional in mouse neurulation.

How may ephrinA-EphA interactions mediate neural fold adhesion and fusion?

Ephs are integral membrane proteins whereas ephrinAs are linked to the plasma membrane via a GPI anchor. Ephs and ephrins expressed on different cells can 'trans-interact' (Davis *et al.,* 1994; Pasquale, 2005) and it seems possible that neural tube closure requires two-way interactions of this type. Ephs and ephrins on one neural fold might interact with ephrins and Ephs on the opposing fold. If this is confirmed, neural fold adhesion would differ from tissues such as the optic tectum, in which Ephs and ephrins are reciprocally compartmentalized (Drescher, 1997). If trans-interaction is indeed the mode of functioning of the ephrinA-EphA system in neural tube fusion, the question then arises whether ephrinA-EphA interactions serve a purely adhesive function, drawing the neural fold tips together and enabling physical union across the midline, or whether there is also a role for down-stream signaling within the cells of the neural fold tips. EphrinAs interact with EphA receptors in a highly promiscuous manner (Gale *et al.,* 1996; Flenniken *et al.,* 1996), giving rise to bidirectional signalling. Events downstream of the EphA receptor tyrosine kinase are designated 'forward' signalling, while events downstream of the GPI-anchored ephrinA ligand are termed 'reverse' signalling. Since ephrinAs do

E8.5 embryos exposed to 5 mU PIPLC (Figure 5D) or EphA3-AP (Figure 5F) show apparently normal bending of the neural plate at the median hinge point, compared with PBS- or AP-treated controls (Figure 5C,E). More advanced embryos, at E9.0, treated with EphA1-Fc exhibit normal dorsolateral hinge points (Figure 5H), which are characteristic of the PNP at this later stage, compared with Fc-treated controls (Figure 5G). Hence, neural fold morphogenesis is not overtly disturbed by treatments that cause delay in PNP closure, further implicating the process of neural fold adhesion as a step in neurulation that possibly requires EphA-ephrinA interactions.
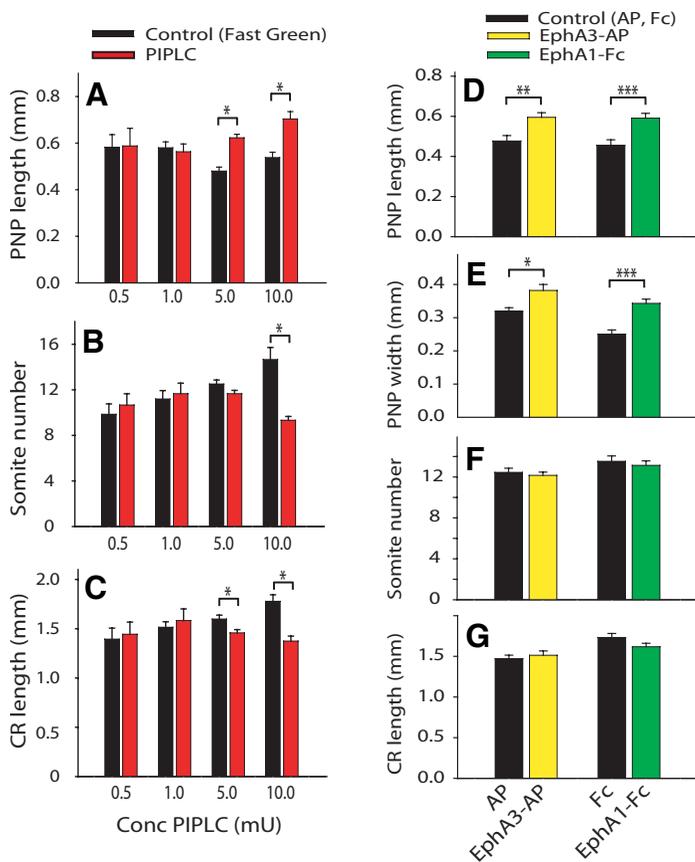
Discussion

Ephs and ephrins have been implicated in epithelial adhesion and fusion events in several developmental systems. In mice, cloacal fusion (Dravis *et al.,* 2004), skull suture fusion (Merrill *et al.,* 2006) and atrioventricular valve and septal fusion (Stephen *et al.,* 2007) have all been shown to involve ephrin-Eph interactions. Similar functions are apparent in other species such as

Fig. 7. Effect of phosphatidylinositol-specific phospholipase C (PIPLC) and EphA fusion proteins on posterior neuropore (PNP) closure, embryonic development and growth. (A-C) *Dose-response analysis of PIPLC treatment effects on PNP length* **(A)**, *somite number* **(B)** *and crown-rump length* **(C)**. *Compared with Fast Green (FG)-treated control embryos, PNP length is significantly increased in embryos exposed to PIPLC (ANOVA; p = 0.008). Pairwise differences are statistically significant only at 5 and 10 mU (* in A; p < 0.05). In addition, somite number is significantly reduced in embryos exposed to 10 mU PIPLC (* in B; p < 0.05), and crown-rump (CR) length is significantly reduced in embryos exposed to 5 and 10 mU PIPLC (* in C; p < 0.05). **(D-G)** Effect of treatment with EphA3-AP and EphA1-Fc fusion proteins on PNP length* **(D)**, *PNP width* **(E)**, *somite number* **(F)** *and crown-rump length* **(G)**. *Compared with AP and Fc controls, both EphA3-AP and EphA1-Fc significantly increase PNP length (D: **, p < 0.005; ***, p < 0.001) and width (E: *, p < 0.05; ***, p < 0.001), whereas there is no effect on somite number or crown-rump length (F,G). Values are mean ± SEM. Sample sizes: (i) PIPLC experiments: 0.5 mU, 6 PIPLC/7 FG; 1.0 mU, 6 PIPLC/5 FG; 5.0 mU, 45 PIPLC/31 FG; 10.0 mU, 6 PIPLC/6 FG; (ii) EphA fusion protein experiments: 19 EphA3-AP/20 AP; 22 EphA1-Fc/15 Fc. Fusion protein concentrations: EphA3-AP, 15 nM; EphA1-Fc, 13 nM. In preliminary studies, these concentrations were the maximum compatible with normal development in culture.*

not have a direct linkage to the cytoplasm, signaling is thought to be mediated by tyrosine kinases that are associated with lipid rafts containing ephrinAs (Murai and Pasquale, 2003).

In our intra-amniotic perturbation experiments, we used EphA1-Fc and EphA3-AP fusion proteins which bind endogenous ephrinA ligands, blocking their physical interaction with endogenous EphAs and thereby inhibiting forward signalling via the EphA tyrosine kinases (Ciossek *et al.,* 1998). This treatment was able to significantly delay the progression of PNP closure, suggesting a role for either EphA-ephrinA physical interaction or forward signaling, or both, in neural tube closure. Further studies will be needed to distinguish between these two possible functions of EphA-ephrinA interactions in spinal neurulation. Since soluble EphA-Fc and EphA-AP fusion proteins adopt a dimeric conformation (Ciossek *et al.,* 1998), they could have generated reverse signalling in these studies, via their binding to endogenous GPI-anchored ephrinAs. Since PNP closure was delayed, we conclude that reverse ephrinA signalling is unlikely to be necessary for spinal neural tube closure. A further possibility, that excessive reverse ephrinA signalling is induced and that this contributes to the PNP closure delay, cannot be discounted at present.

EphA-ephrinA interactions and development of cellular processes at the fusion site

Neural fold fusion involves the formation of lamellipodial processes at the neural fold tips immediately prior to the initial contact (Waterman, 1975; Waterman, 1976; Rice and Moran, 1977; Geelen and Langman, 1979; Lawson and England, 1998). Our electron microscopic observations of mouse spinal neurulation confirm this finding and raise the question as to whether EphA-ephrinA interactions may be involved in regulating this cellular behaviour. The small GTPase Rac is known to induce membrane ruffling and lamellipodia formation both in cultured fibroblasts (Nobes and Hall, 1999) and in morphogenetic events such as dorsal closure in *Drosophila* (Woolner *et al.,* 2005). Inhibition of Rac function suppresses lamellipodia formation, while Rac overexpression causes excessive numbers of protrusions. In tumour cells, EphA2 is known to promote cell motility (Fang *et al.,* 2005) whereas EphA2-deficient cells fail to activate Rac upon stimulation with ephrinA1 (Brantley-Sieders *et al.,* 2004). Hence, there may be a direct correlation between expression of EphA2 on the neural folds tips and occurrence of lamellipodial protrusions.

The situation is likely to be more complex than this, however. In kidney epithelial cells, hepatocyte growth factor-induced branching morphogenesis is associated with cellular protrusion, and yet stimulation of EphA receptors by ephrinA ligands causes the withdrawal of cellular processes from morphogenetically active epithelia, with suppression of Rac1 (Miao *et al.,* 2003). It appears that stimulation of EphA2 by ephrinA1, at least in tumour cells, leads to enhanced turnover of EphA2 from the cell surface and reduced migratory activity. Shp2, a cytoplasmic tyrosine phosphatase and tumour suppressor protein, is recruited to sites of EphA2-ephrinA1 interaction (Miao *et al.,* 2000), leading to disassembly of EphA2 from an active complex with focal adhesion kinase. Interestingly, spina bifida has been reported in mice null for Shp2 (Saxton and Pawson, 1999), raising the possibility that ligand-dependent suppression of forward signaling via EphA2 might be of functional importance in neural tube closure. Clearly, further studies are required to fully determine the role of EphA-ephrinA interactions in the regulation of lamellipodial protrusions during adhesion and fusion of the neural tube.

Functional redundancy in EphA-ephrinA interactions during neurulation

EphA2 shows a particularly strong temporal and spatial correlation with neural fold fusion in the mouse PNP. A gene targeted

knockout of EphA2 (Brantley-Sieders *et al.,* 2004) and three EphA2 gene-trap mutant lines (Mitchell *et al.,* 2001; Naruse-Nakajima *et al.,* 2001; Chen *et al.,* 1996) have been described, but in no case were neurulation defects observed. The EphA2 knockout displays impaired ephrinA1-induced angiogenesis *in vivo*, while homozygotes for one of the EphA2 gene trap mutants are described as having short, kinked tails (Naruse-Nakajima *et al.,* 2001). Hence, there is currently no evidence that loss of EphA2 function alone leads to failure of spinal neurulation. Similarly, of the several EphA4 mutants described, hind limb and gait defects are observed, but no overt neural tube defects (Coulthard *et al.,* 2002). EphA1, and A5 were also found to be expressed in the closing PNP, but no mutants for these genes have yet been described. It seems possible, therefore, that EphA receptors exhibit functional redundancy during spinal neurulation.

The situation is less conclusive for the ephrinAs. While the ephrinA3 knockout mouse displays only defects of the olfactory system (Cutforth *et al.,* 2003), there are currently no described knockouts or gene-trap mutants for ephrinA1 or A4, both of which were expressed in the closing PNP. It remains to be determined whether a single ephrinA might prove to be absolutely required for spinal neural tube closure, or whether functional redundancy will be the case here, as well as for the EphAs.

In conclusion, this paper has demonstrated a requirement for ephrinA-EphA interactions in closure of the mouse spinal neural tube. We also present circumstantial evidence to support the idea that ephrinA-EphA interactions may participate in the molecular recognition events that culminate in adhesion and fusion of the tips of the neural folds.

Materials and Methods

In situ hybridisation and RT-PCR

Whole-mount *in situ* hybridisation, followed by preparation of either 10 μm paraffin sections or 50 μm vibratome sections, was performed using digoxygenin-labelled cRNA probes (Copp *et al.,* 1999). Previously published probes were used for ephrinA1, A2, A3 and A5, and for EphA2 and A4 (Gale *et al.,* 1996; Flenniken *et al.,* 1996). New cDNA probes were prepared by RT-PCR on total RNA extracted using TRIzol reagent (Invitrogen, UK) from E8.5 and E9.5 CD1 embryos. Amplified fragments were cloned into the pGEM-T vector (Promega, UK) and sequenced to confirm identity. Primer sequences were: EphA1: forward 5'CAAGATTGCAAGACTGTGGC 3' and reverse 5'CCTCCCACATTACAATCCCA 3', amplifying a 514 bp fragment (nucleotides 2004 to 2517; GenBank NM_023580); EphA5: forward 5' GGAGAACGGCTCCTTAGACA 3' and reverse 5' GAGCCACAGCGTCCATTGAA 3', amplifying a 728 bp fragment (nucleotides 2662 to 3389; GenBank NM_007937). Additional primer sequences for RT-PCR are available upon request.

Immuno-electron microscopy

Deciduas containing embryos were quickly dissected from the uterus in DMEM containing 10% FBS, then transferred into cold fixative (0.01% glutaraldehyde, 2% PFA, 0.1 M sucrose in 0.05 M sodium cacodylate, 5 mM calcium chloride buffer, pH 7.4) and dissected further until all extra-embryonic tissues had been removed. Embryos were rinsed in fixative and kept overnight in the same solution. While on ice, embryos were washed for 3 h in 0.01% Triton-100 buffered PBS, and then incubated overnight at 4ºC in primary rabbit anti-EphA2 antibody (Zymed Invitrogen, UK) diluted 1:500 in 10% normal goat serum in PBS. After washing, embryos were incubated overnight at 4ºC in horseradish peroxidase-conjugated goat anti-rabbit IgG, diluted 1:100 in 0.01% Triton-100 buffered PBS, before detection with diaminobenzidine solution (Vectastain, Vector Labs, UK). Embryos were then rinsed twice for 5 minutes in 0.05 M sodium cacodylate, transferred to 1% osmium tetroxide buffered in 0.1 M sodium cacodylate for 2 h, washed in 0.5% aqueous uranyl acetate for 30 minutes at 4ºC, and then washed twice for 5 minutes each with distilled water. Embryos were dehydrated through an ethanol series, followed by 3 changes of propylene oxide, then resin/propylene oxide at 3 different ratios (1:2; 1:1; 2:1) for 30 minutes each, before embedding in epoxy resin (Agar 100). Ultra-thin sections were cut on a RMC MT6000 ultramicrotome, using a Diatome diamond knife. Sections were collected on 300 mesh copper grids, then stained briefly in 0.01 M lead citrate and examined using a JEOL 1010 electron microscope. Semi-thin sections cut for light microscopy were stained with 1% toluidine blue/ 1% borax.

Receptor Affinity Probe (RAP) assay

To detect endogenous ephrinA ligands, EphA3-AP fusion protein was used in a RAP assay (Haj *et al.,* 1999). Embryos were dissected free of extra-embryonic membranes in ice-cold Hank's Balanced Salt Solution (HBSS) and dehydrated to 100% methanol. After re-hydration, embryos were washed in buffer 3 (HBSS containing 20 mM HEPES pH 7.0, 0.5 mg/ml BSA), then incubated overnight in 30 nM EphA3-AP fusion protein at 4ºC. After extensive washing in buffer 3, embryos were fixed in 4% PFA for 3 minutes, followed by washing in buffer 4 (HBSS containing 20 mM HEPES pH 7.0, 150 mM NaCl). After incubation at 65ºC to inactivate endogenous alkaline phosphatase, embryos were stained for alkaline phosphatase activity using NBT/BCIP. Embryos were then incubated in 20% gelatine at 65ºC for 30 min, embedded, and soaked in 4% PFA for 3-5 days at 4ºC. Vibratome sections (40-50 μm) were mounted in 80% glycerol/PBS.

Embryo microinjection and culture

Non-mutant random-bred CD1 mice (Charles River, UK) were paired overnight and females were checked for copulation plugs the following morning, designated embryonic day (E) 0.5. Embryos were explanted at E8.5 and those with 6-8 somites were selected for culture in rat serum (Cockroft, 1990). Embryos were allocated randomly to treatment groups to minimise the effect of litter-litter variations. Phosphatidylinositol-specific PIPLC (P5542, Sigma-Aldrich, Poole UK)(Low *et al.,* 1988) and fusion proteins EphA3-AP (gift of Dr Uwe Drescher) and EphA1-Fc (R&D Systems, Abingdon UK) were injected directly into the amniotic cavity in order to bypass the yolk sac. Fast Green (0.5% solution) served as an easily visualised injection marker, with phosphate buffered saline (PBS) as the diluent for the enzyme or fusion protein. Microinjection was performed using a hand-held glass micropipette, pulled using a model P-87 Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, USA). Needle tips were positioned in the amniotic cavity by traversing the yolk sac and amnion (Hartig and Hunter, III, 1998), with a total injection volume of 1-2 μl. Embryos were discarded if most of the injected substance was not retained in the amniotic sac, or if there was significant leakage from the yolk sac. Following 8 h culture, embryos were examined for presence of yolk sac circulation, as an indicator of viability, and somites were counted as a measure of developmental progression. Crown-rump length was determined as a measure of embryonic growth, and was defined as the maximum linear distance from top of the embryonic brain to base of the spinal region, without altering embryonic curvature. Length and width of the posterior neuropore were measured as described previously (Copp, 1985). Statistical analysis was by Student's t-test, computed using SigmaStat v.2 (SPSS Inc.).

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