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The expression and regulation of chick *EphA7* suggests roles in limb patterning and innervation

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SUMMARY

Eph receptors and their ligands, the ephrins, have been implicated in early patterning and axon guidance in vertebrate embryos. Members of these families play pivotal roles in the formation of topographic maps in the central nervous system, the formation of brain commissures, and in the guidance of neural crest cells and motor axons through the anterior half of the somites. Here, we report a highly dynamic expression pattern of the chick *EphA7* gene in the developing limb. Expression is detected in discrete domains of the dorsal mesenchyme from 3 days of incubation. The expressing cells are adjacent to the routes where axons grow to innervate the limb at several key points: the region of plexus formation, the bifurcation between dorsal and ventral fascicles, and the pathway followed by axons innervating the dorsal muscle mass. These results suggested a role for EphA7 in cell-cell contact-mediated signalling in dorsal limb patterning and/or axon guidance. We carried out experimental manipulations in the chick embryo wing bud to alter the dorsoventral patterning of the limb. The analyses of *EphA7* expression and innervation in the operated wings indicate that a signal emanating from the dorsal ectoderm regulates *EphA7* in such a way that, in its absence, the wing bud lacks *EphA7* expression and shows innervation defects at the regions where the gene was downregulated. *EphA7* downregulation in the dorsal mesenchyme after dorsal ectoderm removal is more rapid than that of *Lmx-1*, the gene known to mediate dorsalisation in response to the ectodermal signal. These results add a new gene to the dorsalisation signalling pathway in the limb. Moreover, they implicate the Eph receptor family in the patterning and innervation of the developing limb, extending its role in axon pathfinding to the distal periphery.

Key words: Chick, Eph, EphA7, Cek11, Ephrins, Neural tube, Axon guidance, Sclerotome, Limb patterning, Mesenchyme, Apical ectodermal ridge, Wnt7a, Lmx-1, Dorsal ectoderm

INTRODUCTION

Eph receptors constitute the largest subfamily of receptor tyrosine kinases, with 14 members already described. These receptors and their ligands, the ephrins, have been recently attributed key roles in regionalisation, cell migration and axon guidance in vertebrate embryos (see Nieto, 1996; Gale and Yancopoulos, 1997; Drescher, 1997, for reviews and Eph Nomenclature Committee, 1997, for nomenclature). They mediate cell-cell contact-dependent signalling, since both receptors and ligands are membrane attached and this is a requisite for ligand/receptor signalling (Davis et al., 1994). The ephrins are subdivided in two subfamilies depending on the type of linkage to the cell surface and their binding specificity. A-class ephrins are attached to the cell membrane by a GPI linkage and bind to EphA receptors, and B-class ephrins contain a transmembrane domain and bind to EphB receptors. There is a high degree of promiscuity in the binding between subfamily members (Bambrilla et al., 1995; Gale et al., 1996) and there are indications of bidirectional signalling of receptors and ligands (Holland et al., 1996; Brückner et al., 1997). In all cases described in the nervous system, receptor-ligand interaction results in axon repulsion (Orioli and Klein, 1997).

Studies of expression patterns in the developing embryo with specific cDNA probes (Nieto et al., 1992; Becker et al., 1994; Ruiz and Robertson, 1994; Ganju et al., 1994, Henkemeyer et al., 1994) or whole embryo binding assays with the subfamily-specific receptor/ligand bodies (Cheng and Flanagan, 1994; Gale et al., 1996) have demonstrated complex and dynamic expression patterns for both ephrins and receptors during development. Although there is a prominent expression in the developing nervous system, ligands and receptors are expressed throughout the embryo (Gale et al., 1996; Flenniken et al., 1996). This is also the case for *EphA7*, which, in addition to its expression within the central nervous system (Araujo and Nieto, 1997), is also expressed during limb development. At early stages, the expression is dorsally restricted and, later on, correlates with the pathway followed by axons innervating the limb. This is not surprising since the same molecules used for the patterning of different regions in the embryo are also

present in the limb (Cohn and Tickle, 1996). Limb development is a well-established model for studying patterning mechanisms and, in particular, chick limb development offers the possibility of easy experimental manipulation, which permits correlations between resulting phenotypes and gene function.

Three signalling systems have been described that confer patterning information along the three axes of the limb. (1) The apical ectodermal ridge (AER), responsible for the outgrowth and patterning in the proximodistal axis (Saunders, 1948; Rowe and Fallon, 1982), whose position is regulated by *Radical fringe* (Laufer et al., 1997; Rodriguez-Esteban et al., 1997) and its action mediated by one or several members of the fibroblast growth factor (FGF) family (Niswander et al., 1993; Fallon et al., 1994; Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). (2) The zone of polarizing activity (ZPA) controls patterning in the anteroposterior axis via the production of Sonic Hedgehog (SHH; Riddle et al., 1993; Chang et al., 1994; López-Martínez et al., 1995). (3) The control in the dorsoventral axis is regulated by the ectoderm. *Wnt-7a* is expressed in the dorsal ectoderm and controls dorsalisation through the induction of the LIM homeobox gene *Lmx-1* in the dorsal mesenchyme (Parr and McMahon, 1995; Riddle et al., 1995; Vogel et al., 1995). Finally, *Engrailed-1*, which is expressed in ventral AER and ventral ectoderm is essential for proper AER position and maturation as well as ventral limb patterning (Logan et al., 1997; Loomis et al., 1996, 1998).

Considering the dual role attributed to members of the Eph family in both regionalisation at early stages and axon guidance at later stages (see Sefton and Nieto, 1997, for a review), we have addressed these two questions for EphA7 during limb development. We have analysed its pattern of expression in detail and experimentally altered its domains of expression. The analyses of the resulting phenotypes indicate that *EphA7* is regulated by a signal emanating from the dorsal ectoderm and that there is a correlation between the normal expression of *EphA7* and normal limb innervation. Together with the guidance of motor axons throughout the somites (Wang and Anderson, 1997), this work provides evidence that members of the Eph family are involved in axon pathfinding outside of the central nervous system and is the first suggestion of their involvement in limb innervation.

MATERIALS AND METHODS

Preparation of embryos

Fertilised chicken eggs were purchased from Ibertec Farm, Valladolid, Spain and from Granja Rodriguez-Serrano, Salamanca, Spain. Eggs were routinely incubated, opened and staged according to Hamburger and Hamilton (1951). All the experimental manipulations were performed at stages 17-24. The specimens were fixed in 4% paraformaldehyde overnight and processed either for whole-mount in situ hybridisation, embedded in agarose and sectioned in a vibratome (100 μm) for slice free-floating hybridisation or embedded in Paraplast and serially sectioned (6 μm) for tissue section hybridisation.

Removal of the dorsal ectoderm

For the removal of the dorsal ectoderm of the wing bud, a cut was made in the dorsal ectoderm following its junction with the AER. After the cut was made, 2 μl of a solution of Nile Blue sulphate (0.5%) in water were applied to the dorsal surface of the limb. After a few seconds, the ectoderm blisters and can be easily removed with fine forceps. The cut made along the length of the AER prevents involuntary AER removal while peeling the dorsal ectoderm. We performed two kinds of dorsal ectodermal removal: (i) exclusively over the dorsal limb surface (DER), and (ii) extending from the distal tip of the wing bud to the dorsal neural tube (DERt). After the operation, the eggs were sealed and returned to the incubator until the embryos were killed. We used the unoperated left wing as a control.

In situ hybridisation in whole embryos and tissue sections

Digoxigenin-labelled antisense riboprobes were prepared and used for in situ hybridisation in whole mount or in slices as described in Nieto et al. (1996). Following hybridisation, whole embryos were fixed in 4% paraformaldehyde, paraffin-embedded and serially sectioned at 15 μm. For the preparation of ^{35}S-labelled riboprobes and hybridisation in tissue sections, we followed the protocol described in Wilkinson and Nieto (1993). Hybridised embryos were cleared in 50% glycerol in PBS and photographed with a Leica M10 stereomicroscope under dark-field illumination, and the sections were photographed with a Zeiss Axiophot microscope. The *EphA7* probe corresponded to nucleotides 2757-3473 of the complete cDNA sequence (Araujo and Nieto, 1997). The chick *Wnt-7a*, *Lmx-1*, *Pax-3* and *Ephrin-A5* probes were kindly provided by C. Tabin, J.C. Izpisua-Belmonte, P. Gruss and U. Drescher, respectively.

Double immunostaining and in situ hybridisation

In double-labelling experiments, whole-mount in situ hybridisation was carried out prior to immunostaining, essentially as described in Nieto et al. (1996) except for the omission of the proteinase K step. The anti-acetylated tubulin antibody (1:500) used corresponds to the TuJ1 antibody described by Moody et al. (1989). Peroxidase activity was detected by incubation in a solution of DAB (0.1%) in PBS containing 0.025% H_2O_2. The embryos were processed after double labelling as described above.

RESULTS

EphA7 expression during early limb development

In our analysis of *EphA7* expression in the chick embryo, apart from a prominent expression in the central nervous system (Araujo and Nieto, 1997), we detected transcripts in the developing limb that, at early stages, were restricted to the dorsal mesenchyme. The analysis of this expression reveals a highly dynamic pattern (Fig. 1). Transcripts were observed in the AER at stage 18 (Fig. 1A,B, arrowheads), the levels decreased from stage 20 and became undetectable by stage 22. In the mesenchyme, weak expression was first observed adjacent to the trunk at stage 19 (not shown). From stage 22, the level of expression increased considerably and the pattern of expression could be divided into different domains. One domain was located adjacent to the trunk spanning the anteroposterior basis of the limb bud (Fig. 1C); we termed it the dorsoproximal domain. The second was located mid-dorsally and was called the dorsomedial domain. It developed from proximal to distal during subsequent stages up to stage 27 (Fig. 1C,E,F). From stage 23, two additional domains of *EphA7* expression were detected running along the proximoanterior and proximoposterior margins of the wing, respectively (Fig. 1E,F). Sectioned wings showed that these domains of expression are restricted to the dorsal mesenchyme; the ectoderm, including the AER (Fig. 1D, arrowheads), is

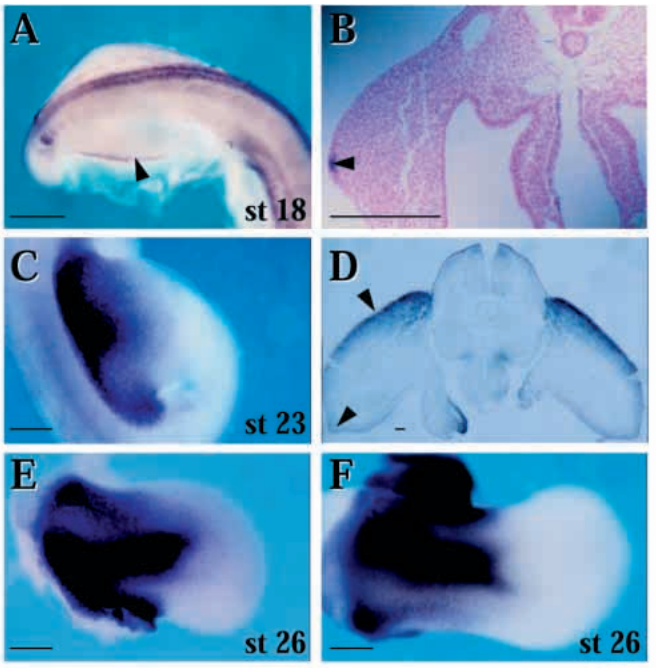

Fig. 1. Expression of *EphA7* during early stages of limb development. (A) Lateral view of a stage 18 embryo tail showing *EphA7* expression in the AER (arrowhead) and in the caudal somites. (B) Transverse section through the limb shown in A, where the expression of *EphA7* can be clearly observed in the AER. (C) Dorsal view of a stage 23 limb bud. (D) Transverse section of the same embryo at the level of the limb, showing *EphA7* expression in the dorsal mesenchyme. Dorsal views of stage 26 wing (E) and limb buds (F), showing the dorsomedial domain of *EphA7* expression advancing distally. The bar indicates 250 μm.

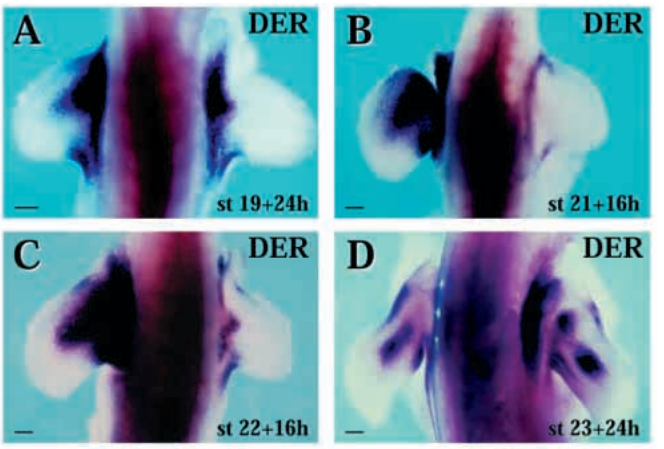

Fig. 2. Regulation of *EphA7* expression by the dorsal ectoderm. (A) 24 hours after removal of the dorsal ectoderm at stage 19, *EphA7* expression has not appeared within the manipulated wing. (B,C) Downregulation of *EphA7* expression is detected on embryos operated from stage 21 to 22 and analysed 16 hours later. (D) From stage 23 onwards, the operation did not affect *EphA7* expression in the manipulated wing. All specimens are viewed at the dorsal aspect and oriented with the anterior to the top. In all the photographs, the operated wing is to the right. The bar indicates 250 μm.

completely devoid of transcripts. This dorsal restriction of expression suggested to us that *EphA7* might have a role in cell-cell contact-mediated signalling during dorsal limb patterning, and prompted us to analyse whether expression was modified after altering the dorsoventral patterning of the limb.

A signal derived from the dorsal ectoderm regulates *EphA7* expression in the wing bud

Dorsalisation in the limb bud has been shown to be controlled by WNT7a, a factor secreted by the dorsal ectoderm (Parr and McMahon, 1995). In order to eliminate WNT7a signalling, we removed the dorsal ectoderm of the right wing (DER) at different stages of development and analysed *EphA7* expression 16-48 hours after the operation. As shown in Fig.2, dorsal ectoderm removal drastically modified *EphA7* expression. When the operation was carried out before the onset of *EphA7* expression in the dorsal mesenchyme (stages 17-19, *n*=15), transcripts were not detected in this region at any time after the operation (15/15, Fig. 2A). In embryos operated at stages 20-22, *EphA7* expression was downregulated in the dorsomedial domain (24/24, Fig. 2B,C), whereas the removal of the dorsal ectoderm at stages 23-24 did not alter *EphA7* expression (14/14, Fig. 2D). These results indicate that a signal derived from the dorsal ectoderm is responsible for the induction and maintenance of *EphA7* expression in the dorsal mesenchyme of the wing bud. Furthermore, they also show that from stage 23 onwards, the dorsal mesenchyme becomes independent of the dorsal ectoderm in its capacity to express the gene.

We analysed the time course of the downregulation of *EphA7* expression. Dorsal ectoderm removal was performed on stage 22 wing buds, when *EphA7* expression is already established in the dorsal mesenchyme. The pattern of expression of *EphA7* remained unaffected 6 hours after the operation (9/9, Fig. 3A), but clear downregulation in the dorsomedial domain of expression was observed 9 hours after dorsal ectoderm removal (6/9, Fig. 3B). No expression in this domain was detected in any of the operated embryos 12 hours after ectoderm removal (*n*=7, not shown).

EphA7 expression is downregulated before *Lmx-1* after dorsal ectoderm removal

WNT-7a mediates dorsalisation through the induction of the LIM homeobox gene *Lmx-1* in the dorsal mesenchyme (Riddle et al., 1995; Vogel et al., 1995). Indeed, *Lmx-1* transcripts are absent in the distal mesoderm 24-48 hours after dorsal ectoderm removal (Riddle et al., 1995; Vogel et al., 1995). Consequently, we were interested to compare the time course of *Lmx-1* downregulation after dorsal ectoderm removal with that of *EphA7*. We hybridised adjacent paraffin sections with probes for *EphA7*, *Lmx-1*, *Pax-3* and *Wnt-7a* at different times after dorsal ectoderm removal. *Wnt-7a* expression was used as a control of the extent of the ectoderm removal (Fig. 3F,I), and *Pax-3* expression as a marker of the myoblasts populating the wing at these stages (Fig. 3E,H). Wings analysed 6 hours after the operation did not show any change in *EphA7*, *Lmx-1* or *Pax-3* expression (Fig. 3A,D,E). As already described, the *EphA7* dorsomedial domain of expression was undetectable 12 hours after ectoderm removal, whereas *Lmx-1* expression was diminished but still detectable 24 hours after the operation (Fig. 3G). Indeed, *Lmx-1* expression was completely downregulated in the distal mesenchyme 32 hours after the operation (not shown). Its expression in the proximal

mesenchyme was not affected, as previously described (Riddle et al., 1995; Vogel et al., 1995). The dorsoproximal domain of *EphA7* expression was not affected by the operation but note that the ectoderm was not removed from this region, as shown by *Wnt-7a* expression (Fig. 3I).

Analysis of cell death in the mesenchyme that might have been induced by the operation (not shown) indicated that some cell death occurred in the dorsal mesenchyme, starting in scattered cells at about 6 hours and peaking at 24 hours after the operation. Because clear downregulation of *EphA7* was observed 9 hours after the operation, preceding ample cell death, we believe that the absence of the dorsomedial domain of expression is not caused by cell death. This is consistent with the persistence of *Pax-3* expression for at least 24 hours (Fig. 3E,H).

Correlation between *EphA7* expression and limb innervation

As already mentioned, several members of the Eph receptor family have been implicated in the formation of topographic maps in the central nervous system and in the formation of forebrain commissures. The pattern of *EphA7* expression in the developing central nervous system correlates with the formation of several axonal tracts (Araujo and Nieto, 1997). Since *EphA7* expression in the developing limb coincides with stages of limb innervation (Hollyday, 1995), we asked whether there was any relationship between its expression domains and the formation of the innervating tracts in the wing. We carried out double-labelling experiments to simultaneously detect *EphA7* expression (in situ hybridisation) and axon trajectories, using anti-tubulin immunohistochemistry (Moody et al., 1987). At stages 22-23, the end of the 'waiting period' (Hollyday, 1995), axons coming from neighbouring spinal nerves converge to form the brachial plexus (Fig. 4A, arrows) at a region surrounded by areas of *EphA7* expression (Fig. 4A, arrows). Later on, axons enter the limb, grow adjacent to a domain of high *EphA7* expression (Fig. 4B, arrow) and diverge to form two bands of loose fascicles that will innervate dorsal and ventral muscle masses, *brachialis superior* and *brachialis inferior* respectively (Hollyday, 1995). The bifurcation of these fascicles also coincides with a region of high *EphA7* expression (Fig. 4D, star). The main dorsal nerve trunks (*brachialis superior* in the wing

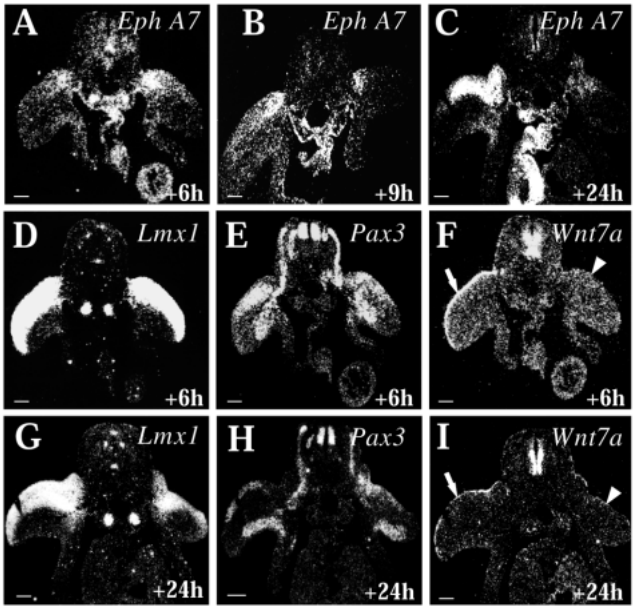

Fig. 3. Effect of dorsal ectoderm removal on the expression of *EphA7, Lmx-1* and *Pax-3*. (A-C) Time course of *EphA7* downregulation. *EphA7* expression is still observed in the dorsal mesenchyme of the manipulated wing 6 hours after the operation (A), whereas 9 hours after ectoderm removal expression is downregulated (B). The absence of transcripts is also observed 24 hours after the operation (C). Dark-field micrographs of adjacent transverse sections (longitudinal section of the wings) through embryos operated at stage 21 and hybridised with *EphA7* (A,C), *Lmx1* (D,G), *Pax3* (E,H) and *Wnt-7a* (F,I) probes. (D) *Lmx1* expression is not altered at 6 hours after the operation and is slightly downregulated after 24 hours (G). *Pax-3* expression remains unchanged in the operated wing (E,H). The hybridisation with *Wnt-7a* (F,I) allows the evaluation of the extent of ectoderm removal. Arrows in F and I indicate *Wnt-7a* expression at the dorsal ectoderm in the control wing and arrowheads demarcate the region deprived of dorsal ectoderm. In all the panels, the operated wing is to the right.

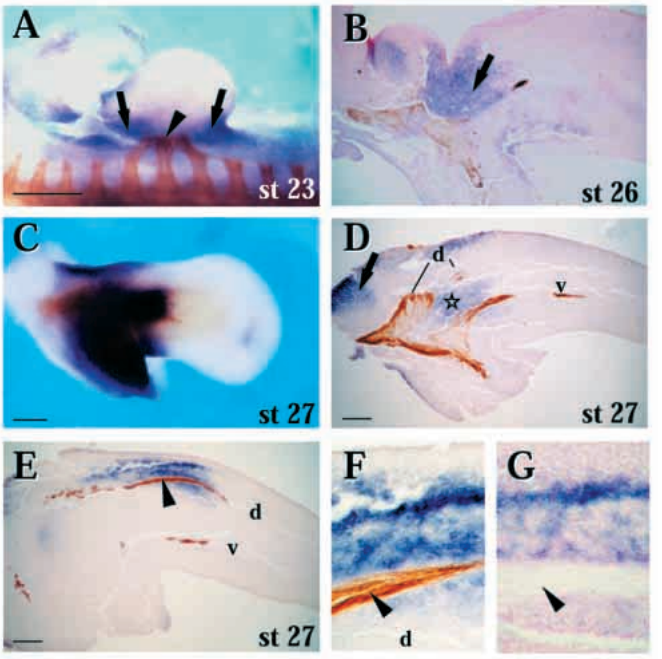

Fig. 4. Correlation between *EphA7* expression and the formation of the axonal tracts during limb innervation. Double-labelled embryos showing *EphA7* expression (blue) and axonal tracts (TuJ1 immunoreactivity, brown). (A) Dorsal view of a stage 23 wing bud showing the brachial plexus (arrowhead) and *EphA7* expression in the dorsoproximal mesenchyme, adjacent to the plexus (arrows). (B) Longitudinal section of a stage 26 wing, illustrating the axon fascicles that enter the wing and the dorsoproximal domain of *EphA7* expression (arrow). (C-E) Dorsal view of a stage 27 limb bud and its longitudinal sections taken at different levels. (D) The axonal tracts and *EphA7* labelling both in the mesenchyme adjacent to the trunk (arrow) and in the mesenchymal cells located at the divergence point between dorsal and ventral nerve trunks (star). (E) The dorsal axonal tract (arrowhead) running along its pathway. This can be better observed in the high-power photograph shown in F. (G) Longitudinal section of a stage 27 limb, illustrating the lack of gene expression along the pathway of the axons. d, dorsal axon fascicle; v, ventral axon fascicle. The bar indicates 250 μm.

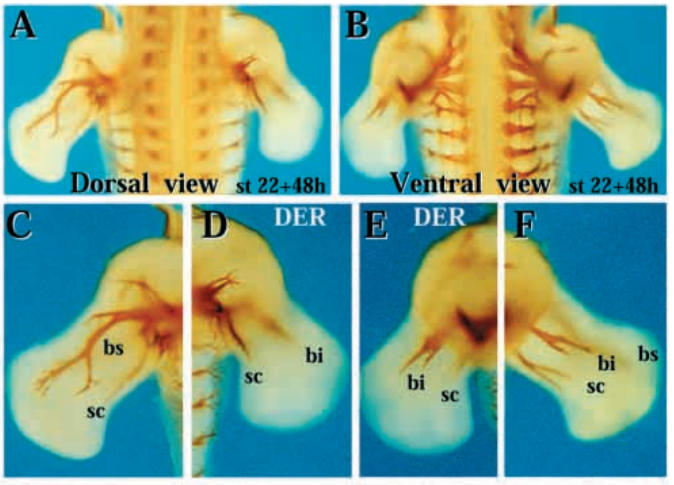

Fig. 5. Dorsal ectoderm removal and dorsal innervation. (A,B) Dorsal and ventral views of an eviscerated embryo that was subjected to dorsal ectoderm removal in the right wing bud at stage 22, and analysed for innervation patterns (β-tubulin staining) 48 hours after the operation. Note that dorsal innervation is absent in the operated wing (A) and that the ventral tracts are not altered (bi and sc, B). (C-F) High power photographs of the wings shown in A and B showing the corresponding nerve tracts. Note that, in the dorsal view of the operated wing (A,D), the *brachialis inferior* can be observed out of plane, and, in the ventral view of the control wing (B,F), the *brachialis superior* is out of focus. DER, dorsal ectoderm removal of the wing bud; bs, *brachialis superior*; bi, *brachialis inferior*; sc, *supracaracoideus*.

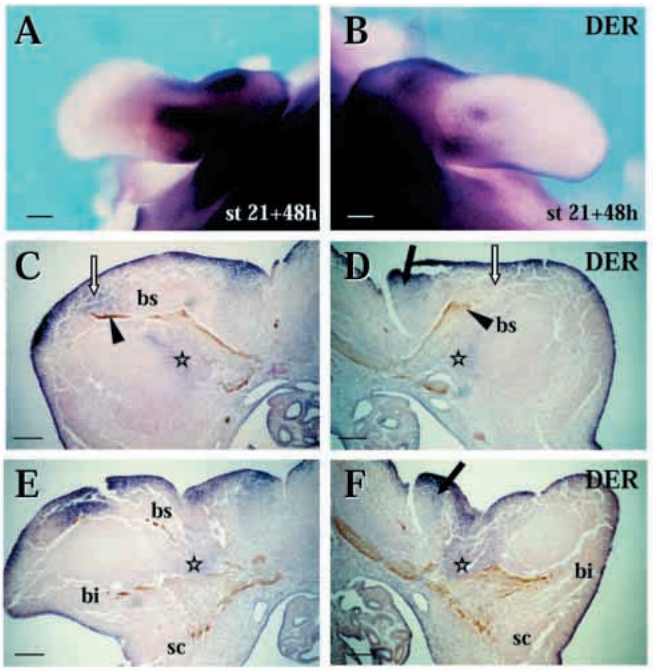

Fig. 6. *EphA7* expression and the formation of the *brachialis superior*. (A,B) Dorsal views of the control and operated wings of an embryo subjected to partial dorsal ectoderm removal in the right wing bud at stage 21, and analysed for *EphA7* expression 48 hours after the operation. (C,E) Sections of the control wing at different levels. *EphA7* expression is detected in the dorsoproximal mesenchyme (white arrow in C) and at the divergence of the dorsoventral fascicles (star). The β-tubulin labelling highlights the dorsal axonal tract (*brachialis superior*, bs; arrowhead in C) and ventral tracts (*brachialis inferior*, bi; *supracaracoideus*, sc in E). The corresponding sections of the operated wing are shown in D and F, respectively. Dorsal innervation is abnormal, showing defects that correlate with the lack of *EphA7* expression in dorsomedial mesenchyme adjacent to the normal pathway followed by these axons (white arrow in C,D). Note that *EphA7* expression is maintained in the dorsoproximal and the dorsoventral divergence regions (black arrows and stars, respectively) and that dorsoventral bifurcation together with ventral innervation in the operated wing are indistinguishable from those in the control wing (compare E and F). DER, dorsal ectoderm removal of the wing bud. The bar indicates 250 μm.

and dorsal crural trunk nerve in the leg) advance distally following a pathway that is surrounded by *EphA7*-expressing cells of the dorsomedial domain (Fig. 4E-G). In conjunction, these data show that axons growing along the above-mentioned pathways never enter regions of *EphA7* expression. The main ventral nerve trunk (*brachialis inferior* in the wing) bifurcates to give rise to an additional ventral nerve trunk, the *supracaracoideus* (see Fig. 6E,F).

EphA7 expression and the formation of the *brachialis superior*

The negative correlation between *EphA7* expression and the pathways followed by axons innervating the limb bud suggest that this gene may be involved in patterning and innervation of the limb. Since we were able to abolish *EphA7* expression adjacent to the pathway followed by the *brachialis superior* after dorsal ectoderm removal, we decided to analyse the dorsal innervation patterns in the operated wings. Right wing buds were denuded of dorsal ectoderm in stage 21 (n=8) or 22 embryos (n=3) and innervation was examined 48 hours after the operation (stage 26-28 embryos). As shown in Fig. 5A,C,D, in wings completely denuded of ectoderm, we could not see the *brachialis superior* emerging from the dorsoventral bifurcation. Ventral innervation was not altered in these operated wings (Fig. 5B,E,F). We then partially removed the dorsal ectoderm of the wing bud (see limit of removal in Fig. 6B) and analysed these embryos for *EphA7* expression and innervation. The dorsomedial domain of *EphA7* expression was downregulated up to the region of ectoderm removal (compare Fig. 6A with 6B), whereas the dorsoproximal expression (adjacent to the trunk, Fig. 6B,D,F, black arrows) was maintained, as was expression in the region located at the divergence between dorsal and ventral axon fascicles (Fig. 6D and F, stars). The *brachialis superior*, which is the main dorsal nerve trunk at these stages (Fig. 6, arrowheads), was present but it was altered in the operated wings. The pathway followed by this fascicle is surrounded by *EphA7*-expressing cells, that downregulate their expression of *EphA7* after dorsal ectoderm removal and, thus, are devoid of transcripts in the operated wing (Fig. 6C,D, white arrows). We could not find axons of the *brachialis superior* growing distally in regions devoid of *EphA7* expression, as seen in control unoperated wings (arrowhead in Fig. 6C). Ventral innervation (both the *brachialis inferior* and *supracoracoideus* nerves) was indistinguishable from that in the control wing as was the bifurcation between dorsal and ventral nerve trunks (compare Fig. 6E with F).

These axons bifurcate adjacent to a domain of *EphA7* expression that was maintained in the operated wings. These results show a correlation between the lack of *EphA7* expression and innervation defects in the developing wing.

EphA7 expression and axon convergence at the plexus region

By removing the dorsal ectoderm of the developing wing bud, we have been able to downregulate *EphA7* expression in the dorsomedial mesenchyme and have shown defects in dorsal innervation pathways. The dorsoproximal domain of *EphA7* expression is adjacent to the area where the plexus forms. Expression in this domain persisted when the ectoderm was only removed from the dorsal limb surface (Figs 2, 3 and 6) and, consistently, the formation of the brachial plexus was normal (not shown). We decided to remove the dorsal ectoderm covering the proximal base of the limb together with that of the wing bud and analyse whether *EphA7* transcription was also downregulated in this region.

Dorsal ectoderm was removed in embryos at different stages from the region adjacent to the AER to the dorsal neural tube (DERt). As previously, when the ectoderm was removed before the onset of *EphA7* expression in the limb (stage 17, n=3), transcripts could not be detected at any time after the operation (not shown). When embryos were operated from stage 23 onwards, *EphA7* expression pattern was not affected (3/3, not shown). Embryos operated between stages 19 and 22 (n=22) were analysed from 12 to 24 hours after the operation for *EphA7* expression and plexus formation. In all the embryos analysed, the dorsomedial domain of expression was absent from 12 hours after the operation and expression at the dorsoproximal domain surrounding the plexus formation area was also affected. In a high proportion of these embryos (17/22), expression was very much reduced when compared to control wings (compare Fig. 7F with G) and, in some of them (5/22), expression was completely abolished (Fig. 7D,E). Analysis of plexus formation indicated that spinal nerve axons failed to converge correctly, occupying a much wider area than in the control wings (Fig. 7A-C), and also failed to enter the wing. In embryos analysed for *EphA7* expression and innervation (Fig. 7D-I), we observed that the degree of the phenotype could be correlated with the residual level of *EphA7* expression. The axons were always excluded from regions expressing the gene and convergence was observed at regions of residual expression (Fig. 7F, arrow).

Ephrin-A5, a cognate ligand of *EphA7*, is expressed in the lateral motor column at brachial and lumbar levels

The biological function of a receptor must be mediated by the interaction with its ligand. Ephrin-A5, together with other A-class ephrins, has been shown to bind in vitro to murine homologues of EphA7 (Gale et al., 1996). The pattern of *ephrin-A5* expression throughout the embryo is compatible with it being the physiological ligand for EphA7 in several systems (Flenniken et al., 1996; our unpublished observations). We have looked at the expression of *ephrin-A5* in spinal motor neurons and found that, the lateral motor column at brachial and lumbar levels express high levels of this ligand (Fig. 8). This indicates that the growth cones of the spinal motor neurons that innervate the wing and the leg (Tsuchida et al., 1994; Ensini et al., 1998) express *ephrin-A5*, suggesting that receptor-ligand interaction may take place during axon pathfinding. Such an interaction would be compatible with the innervation defects that we observed after abolition of *EphA7* expression.

DISCUSSION

Members of the Eph family of receptors and their ligands have been implicated in early patterning (Xu et al., 1995, 1996) and in axonal pathfinding in the central nervous system (Cheng et

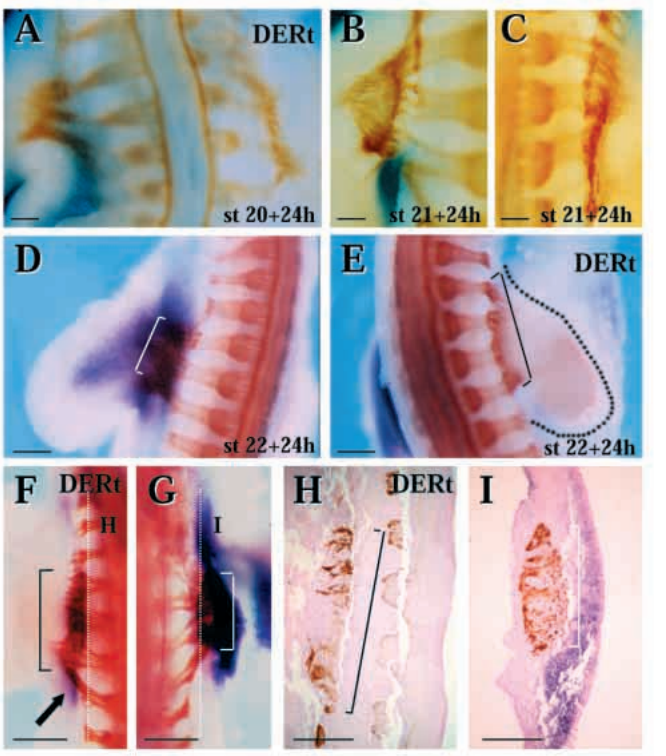

Fig. 7. *EphA7* expression and the formation of the brachial plexus. Dorsal ectoderm was removed from the distal end of the right wing (the AER was left intact) to the trunk region close to the neural tube at stages 20-22 and analysed 24 hours after the operation. (A-C) Dorsal views of the wings after anti-tubulin immunohistochemistry. (A,B) Unoperated left wings; (A,C) the corresponding operated right wings (B,C, correspond to the control and operated wing of the same embryo). (D,E) Dorsal views of the control and manipulated wings of an embryo operated at stage 22 and analysed for *EphA7* expression and β-tubulin staining 24 hours after the operation. (F,G) Ventral views of an embryo similar to that shown in D, E after evisceration. (H,I) Longitudinal sections of the wings shown in F and G, respectively. The dotted lines in F and G indicate the approximate location of the sections shown in H and I, respectively. Spinal axons converge at the brachial plexus in control wings (left wing in A,B,D,G,I). In double-labelled embryos, the region occupied by the plexus along the anteroposterior axis of the wing is indicated by a white bracket (D,G,I). In the operated wings, *EphA7* expression is absent from the dorsal mesenchyme and is very much reduced (F) or completely lost (E) in the most proximal domain running along the anteroposterior axis. In these operated wings, the spinal axons fail to converge at the plexus region, the area occupied by them being indicated by a black bracket (E,F,H). DERt, dorsal ectoderm removal both from the wing and the lateral trunk. The contour of the operated limb is delineated in E. The bar indicates 250 μm.

al., 1995; Drescher et al., 1995; Nakamoto et al., 1996; Gao et al., 1996; Henkemeyer et al., 1996; Orioli et al., 1996; Park et al., 1997; Frisen et al., 1998). Outside of the central nervous system, they have been implicated in the guidance of motor axons through the somites and in the migration of neural crest cells (Wang and Anderson, 1997; Krull et al., 1997; Smith et al., 1997). In this work, we show that the expression and regulation of a member of the EphA subfamily, *EphA7*, suggests roles in early patterning and innervation of the limb.

EphA7 and early limb bud patterning

Experiments in the chick embryo indicate that cells from different parts of the limb bud sort out in vitro, suggesting the existence of surface molecules involved in this segregation process (Ide et al., 1994). Eph family members are good candidates to be involved in this process, as they have been implicated in the restriction of cell movement between hindbrain segments that leads to the sorting out of cells and results in the sharpening of domains with distinct regional identity (Xu et al., 1995; Irving et al., 1996). The expression pattern of *EphA7* in the nervous system is suggestive of its involvement in the regionalisation of the diencephalon and the hindbrain (Araujo and Nieto, 1997). Moreover, we proposed that, in the retina, EphA7 may be involved in a mechanism of cell sorting along the dorsoventral axis between cells expressing and non-expressing the receptor (Sefton and Nieto, 1997). When we found that *EphA7* transcripts were localised in restricted regions of the dorsal mesenchyme of the developing limb bud, we were interested in analysing whether this gene might be involved in cell-cell contact-mediated signalling during dorsal limb patterning. If EphA7 is involved in specifying dorsal territories, removal of the dorsalisation signal should affect its expression. Since WNT7a is the dorsalising signal in the limb that emanates from the dorsal ectoderm (Parr and McMahon, 1995), we abolished the dorsalisation signalling cascade by removing the dorsal ectoderm. This resulted in the downregulation of *EphA7* demonstrating that its expression in the dorsal mesenchyme depends on a signal derived from the dorsal ectoderm. This signal is needed both for the onset and maintenance of its expression, suggesting that mesenchymal cells need to be exposed to the ectodermal signals for an extended period of time. From stage 23 onwards, cells that express the gene become independent from the dorsal ectoderm.

EphA7 is downregulated more rapidly after dorsal ectoderm removal than *Lmx-1*, the gene known to mediate dorsalisation in response to the ectodermal signal. This suggests that *EphA7* expression is unlikely to be regulated by the homeoprotein. It could be that EphA7 is situated upstream of Lmx-1 in the WNT7a dorsalisation cascade, but this seems unlikely because *Lmx-1* is expressed in the limb bud well before *EphA7* and in a broader region (Vogel et al., 1995; Riddle et al., 1995). The possibility exists that WNT7a induces *EphA7* expression through a signalling cascade different from that involving *Lmx-1*. This is also suggested by the fact that, unlike *Lmx-1* (Riddle et al., 1995; Vogel et al., 1995), *EphA7* expression is downregulated after dorsal ectoderm removal in the dorsoproximal mesenchyme. Finally, the signal derived from the dorsal ectoderm that activates *EphA7* expression may be different from WNT7a. In any case, we show that *EphA7* responds to the lack of dorsalisation signals in a very rapid manner, faster than *Lmx-1*, the gene believed to mediate WNT7a signalling.

After removal of the dorsal ectoderm, cell death occurs in the subjacent mesoderm. We wanted to discard the possibility that the absence of *EphA7* transcripts in the dorsal mesenchyme after dorsal ectoderm removal reflected the death of the expressing cells. This is unlikely because our analysis of cell death indicates that there is a reduced number of cells dying that could not justify the complete absence of expression. Furthermore, we detect the dorsal mesenchymal cells by the presence of *Lmx-1* expression after *EphA7* downregulation, which also occurs before cell death is readily appreciated.

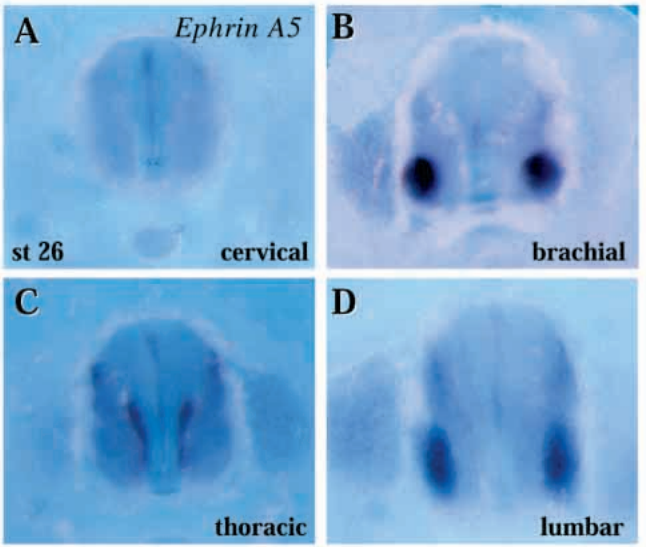

Fig. 8. Expression of *ephrin-A5* in the spinal cord. Transverse vibratome sections of a stage 26 embryo at different anteroposterior levels. (A) At cervical region, no *ephrin-A5* expression is detected. (B) In the brachial region, expression is clearly detected in the ventral horn of the spinal cord, restricted to the lateral motor neuron column. (C) At the thoracic region, expression is observed in the visceral motor neurons. (D) At the lumbar region, as in the brachial region, transcripts are detected at the lateral motor neuron column.

EphA7 and limb innervation

In the hindbrain, after segmental units have been established, *EphA7* expression can be spatiotemporally correlated with the formation of several longitudinal axonal tracts (Araujo and Nieto, 1997). Similarly, in the limb bud, there is a good correlation between *EphA7* expression and the formation of the main nerve trunks during limb innervation. The domains of *EphA7* expression are, at these stages, adjacent to the routes where axons grow to innervate the limb at several key points: the region of plexus formation, the bifurcation between the main dorsal and ventral fascicles, and the pathway followed by axons innervating the dorsal muscle mass. The axons do not enter regions of *EphA7* expression and, considering that axon repulsion is the response observed after Eph receptors-ephrins interactions in the central nervous system (Orioli and Klein, 1997), this suggests that, in the limb, the growth cones may be repelled from regions expressing this receptor. This is compatible with the phenotype observed after the downregulation of *EphA7* expression following ectoderm removal above the region of plexus formation. The normal

convergence of spinal nerves may be in part mediated by the restrictions in navigation imposed by the normal expression pattern of the receptor. The failure in convergence is compatible with the relaxation of the restriction from entering the adjacent regions, now devoid of *EphA7* expression.

Removal of the dorsal ectoderm covering the limb bud downregulates *EphA7* expression in the dorsomedial domain of regions denuded of ectoderm. This expression domain is adjacent to the pathway followed by the *brachialis superior* which, in turn, does not form normally. When *EphA7* expression at the bifurcation between dorsal and ventral nerve trunks was not affected, the routes followed by the axons that were adjacent to these domains were not altered.Ventral innervation, was indistinguishable from that in the control wing, as expected after an operation which only affects the dorsal patterning. All these data show a correlation between *EphA7* expression and innervation of the developing wing, in keeping with the idea that growth cones are excluded from regions of receptor expression. Furthermore, the presence of the receptor and, thus, receptor-ligand interaction are necessary to promote axonal growth, since the absence of *EphA7* expression precludes the growth of the dorsal nerve trunk and the entering of the axons in the limb. The response may be mediated by the interaction of these growth cones expressing high levels of e*phrin-A5*, a ligand that binds EphA7, with the mesenchymal cells expressing the receptor. It is worth noting here that Ohta et al. (1997) have shown inhibition of neurite growth in motor neurons expressing *EphA4* exposed to active forms of ephrin-A2 or ephrin-A5.

In the retinotectal system, e*phrin-A5* and *ephrin-A2* expressed in the tectum have been shown to repel retinal axons (Drescher et al., 1995; Nakamoto et al., 1996). Furthermore, alteration of *ephrin-A2* pattern by retroviral-induced overexpression in the tectum gives rise to modifications of retinal axon mapping in vivo, with temporal axons avoiding the ectopic patches of *ephrin-A2*-expressing cells and projecting to abnormal anterior positions (Nakamoto et al., 1996). The growth cones of the retinal ganglion cells express the receptors (EphA3, EphA5) and the target cells in the tectum express the ligands (ephrin-A2, ephrin-A5). Repulsion and collapse of the growth cones being initiated by the signal transduced by the tyrosine kinase receptor after binding to its ligand (Holland et al., 1998). In our system, the opposite occurs, the motor neuron axons express the ligand and the mesenchymal cells express the receptor. A similar situation has been described during the formation of brain commissures (Henkemeyer et al., 1996), where the receptor (EphB2) is expressed in the cells underneath the path of the axonal fibers and the axons express a ligand for it. *EphB2* mutant mice show defects in the formation of the posterior component of the anterior commissure and evidence has accumulated to suggest that the informative signal is transduced in the axon expressing the ligand (Henkemeyer et al., 1996). This transduction of signals by class B ligands upon binding to their receptors, gives rise to a bidirectional signalling system into both the receptor- and ligand-expressing cells (Holland et al., 1996; Brückner et al., 1997). We propose that a similar mechanism might take place within the A subfamily. Apart from the signal transduced to the cell bearing the receptor (see Holland et al., 1998, for a review), a signal can be transduced to the ligand-expressing cell, in this case, the motor neuron. However, in this system, the ligand is bound to the axonal membrane by a GPI linkage and there is no experimental evidence of A-class ephrins being able to transduce signals. Nevertheless, axonal molecules attached to the membrane by a GPI anchor have been shown to associate with fyn kinase and to transduce signals involved in controlling neurite outgrowth (see Faivre-Sarrailh and Rougon, 1997; Holland et al., 1998 for reviews). The connection between GPI-anchored molecules and cytoplasmic signalling molecules implies an interaction with a transmembrane linker protein, the best candidate to date being Caspr (Peles et al., 1997).

It is possible to explain innervation defects by the lack of target muscles; Martin and Lewis (1986) have described a lack of dorsal soft tissues 6 days after dorsal ectoderm removal, suggesting a lack of dorsal muscles. However, the lack of muscles would affect the final stages of innervation, when the motor neurons need trophic support from the target. Indeed, limbs experimentally devoid of muscles are able to form normal primary nerve trunks (Lewis et al., 1981), including the *brachialis superior*, as expected considering that axon pathfinding and axon final target-recognition are independent events. In our analysis up to 48 hours after operation, we have observed the result of defects in axon guidance rather than defects in specific neuromuscular interaction. In relation to this, at the stages when we find defects in axonal pathways, *Pax-3*-expressing cells are clearly detected in the dorsal compartment of the limb bud.

We cannot discard the possibility that other guidance molecules involved in limb innervation are affected after dorsal ectoderm removal, such as the ligand-receptor pairs HGF-Met or neuropilin-SemD (Ebens et al., 1996; Kitsukawa et al., 1997). However, the phenotype of mutant mice for these molecules are different to those described in this work. Whereas HGF mutant mice show defects in motor axon branching and have normal plexus formation (Ebens et al., 1996), neuropilin mutants show an extreme defasciculation of motor axons (Kitsukawa et al., 1997). Nor can we disregard the fact that the operation affected other members of the Eph/ephrins families. However, known patterns of expression for Eph receptors and ephrins in the limb (Ganju et al., 1994; Ohta et al., 1996, 1997; Patel et al., 1996; Gale et al., 1996; Flenniken et al., 1996, and our unpublished observations for other receptors and ephrins) do not resemble that of *EphA7*, which correlates with the pathways altered in the operated embryos. The expression of *EphA4* in the limb is well documented (Ohta et al., 1996; Patel et al., 1996) and also shows a highly dynamic pattern. It is prominently expressed in the progress zone at early stages and at posterodistal levels and, in relation to tendons formation at later stages, but it also shows a domain of expression at the limb base at E5 (Ohta et al., 1996) which, although is not dorsally restricted, could also be affected by ectoderm removal and participate in the processes that we have described. Taking into account the fact that the motor neurons that innervate both the dorsal and ventral limb express *ephrin-A5*, it is likely that other members of the EphA family might be involved in ventral innervation. Furthermore, motor neurons also express Eph receptors, including *EphA4* (Nieto et al., 1992; Ohta et al., 1996), suggesting that interactions between receptor-expressing growth cones and cells in their pathways expressing the ligand can also participate in limb innervation processes.

The use of the so-called receptor or ligand bodies (soluble

forms of receptors or ephrins) that recognise ligands or receptors in situ in whole embryos, has aided acquisition of an overview of the general expression of ligands and receptors (Gale et al., 1996). Although the pattern observed for EphA receptors using such reagents correlates well with the pattern described for several individual members, it is worth noting here that it does not represent the expression of *EphA7* in the limb or in other developmental systems. Indeed, *EphA7* expression is peculiar when compared to that of other EphA receptors. Whereas these are expressed in a nasotemporal gradient in the developing retina (Marcus et al., 1996), *EphA7* is expressed in two overlapping dorsoventral and centroperipheral gradients (Sefton et al., 1997). Similarly, in the hindbrain, EphA receptors are expressed in specific rhombomeres (Nieto et al., 1992; Ganju et al., 1994; Becker et al., 1994), whereas *EphA7* is expressed in all rhombomeres in different species (Ellis et al., 1995; Taneja et al., 1996; Araujo and Nieto, 1997). We think that it is not likely that the lack of other EphA receptors different from EphA7 might be responsible for the defects that we have observed after dorsal ectoderm removal and thus, we propose that at the stages analysed *EphA7* is involved in the guidance of several axon tracts in the developing limb bud.

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