

# Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons

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## SUMMARY

The Eph family of tyrosine kinase receptors has recently been implicated in various processes involving the detection of environmental cues such as axonal guidance, targeted cell migration and boundary formation. We have inactivated the mouse *EphA4* gene to investigate its functions during development. Homozygous *EphA4* mutant animals show peroneal muscular atrophy correlating with the absence of the peroneal nerve, the main dorsal nerve of the hindlimb. This phenotype is also observed, although with a lower penetrance, in heterozygotes. During normal hindlimb innervation, motor axons converge towards the sciatic plexus region at the base of the limb bud, where they must choose between dorsal and ventral trajectories within

the limb. Among the axons emerging from the sciatic plexus, dorsal projections show higher levels of EphA4 protein than ventral axons. In *EphA4* mutant mice, presumptive dorsal motor axons fail to enter the dorsal compartment of the limb and join the ventral nerve. Our data therefore suggest that the level of EphA4 protein in growing limb motor axons is involved in the selection of dorsal versus ventral trajectories, thus contributing to the topographic organisation of motor projections.

Key words: Eph, Ephrin, Motor neuron, Axonal navigation, Limb, Mouse, Tyrosine kinase

## INTRODUCTION

Control of axon growth and guidance plays an essential role in the development of the nervous system. The topographic organisation of motor projections to the periphery provides a good example of the complexity of the guidance system, since neurons of a single type, sharing common developmental origin, neurotransmitter usage and physiological functions, display unique navigational properties enabling them to connect distinct targets. The anteroposterior and mediolateral positions of motor neurons in the spinal cord correlate with the pathway followed by their axons and with the location of their target muscle in the limb (reviewed in Pfaff and Kintner, 1998). Motor neurons are organised in longitudinal columns in the spinal cord. The medial motor column (MMC) extends along the AP axis, and innervates body wall and back muscles (Tsuchida et al., 1994). The lateral motor columns (LMC) are induced at brachial and lumbar levels, and innervate limb muscles. The LMCs are further divided into subcolumns, called motor pools, which innervate individual muscles. Motor pools belonging to the medial or lateral parts of the LMC (mLMC and lLMC) respectively innervate muscles derived from the ventral and dorsal primary muscle masses of the limbs (Landmesser, 1978). The future identities of motor neurons (i.e., the knowledge of which target muscles they are going to innervate) are established by early inductive events, occurring

before motor neurons exit the cell cycle (Lance-Jones and Landmesser, 1980), so that at the time of axon initiation, motor neurons are already programmed to innervate a specific target muscle (Matise and Lance-Jones, 1996). Hence, when the target field is experimentally mislocated, motor neurons actively search for the new location, reorient their trajectory and finally find their appropriate target (Lance-Jones and Landmesser, 1981; Laing, 1984; Ferns and Hollyday, 1993). These experiments also show that the limb bud contains cues that are interpreted by the axons and enable them to find and select the territory in which they are programmed to migrate. Motor axons are therefore expected to express molecules on their growth cone that can read these cues along their pathway.

Several families of proteins involved in various aspects of axonal guidance have been identified. Among them, the Eph family of tyrosine kinase receptors and their membrane-bound ligands, the ephrins, are believed to mediate guidance decisions by contact repulsion (reviewed by O'Leary and Wilkinson, 1999). Both Eph receptors and ephrins can be divided into two subfamilies, according to their structural similarities and binding affinities (Gale et al., 1996). Ephrin-A ligands are anchored to the membrane with a glycosyl phosphatidylinositol (GPI) group, and preferentially bind EphA receptors, while ephrin-B ligands contain transmembrane and cytoplasmic domains, and bind EphB receptors. An exception to this rule is EphA4, which binds ephrin-A as well as ephrin-B2 and ephrin-

B3 ligands (Bergemann et al., 1998). Our knowledge of the role of Eph receptors and ephrins is based on various experimental approaches including growth cone collapse assays (Drescher, 1997), stripe assays (Drescher, 1997), ectopic expression during development (Xu et al., 1999) and gene knockout (reviewed by O'Leary and Wilkinson, 1999). In particular, these molecules have been implicated in the establishment of topographically ordered projections in the retinotectal system (Drescher, 1997). Furthermore, targeted inactivations of several receptors and ligands have revealed pathfinding defects in the central nervous system (Orioli et al., 1996; Frisen and Barbacid, 1997; Park et al., 1997; Dottori et al., 1998; Frisen et al., 1998; O'Leary and Wilkinson, 1999). However, these phenotypes were surprisingly limited considering the extensive and complex expression patterns of these genes in the nervous system. This restriction presumably reflects a high degree of redundancy within the two families, consistent with the existence of promiscuous interactions between receptors and ligands (Gale et al., 1996). Besides its role in axonal guidance, the Eph receptor-ephrin signalling system has been implicated in angiogenesis notably in arterial/venous domain demarcation (Wang et al., 1998; Adams et al., 1999) and in the segmental restriction of cell intermingling in the developing hindbrain (Xu et al., 1995, 1999).

In this paper, we report the investigation of the function of the *EphA4* gene by targeted inactivation. *EphA4* was initially identified as a tyrosine kinase receptor expressed in a rhombomere-specific manner in the mouse embryonic hindbrain (Gilardi-Hebenstreit et al., 1992; Nieto et al., 1992). *EphA4* has been reported to have a complex and dynamic expression pattern in the nervous system including the developing forebrain, the hindbrain and the spinal cord, but also in non-neural tissues throughout development and adulthood (Mori et al., 1995; Martone et al., 1997). In the chick embryo, spinal cord *EphA4* was shown to be expressed in motor neurons innervating the forelimb and the hindlimb, but not in thoracic motor neurons (Fukushima et al., 1996; Ohta et al., 1996). A targeted mutation of *EphA4* has been described recently and shown to affect the development of the corticospinal tract and the formation of the anterior commissure (Dottori et al., 1998). Here, we demonstrate that, in addition to these phenotypes, *EphA4* mutant mice present a defect in hindlimb innervation resulting in abnormal gait and posture. We show that the latter phenotype is due to misrouting of motor axons normally fated to invade the dorsal limb territory. This defect demonstrates that EphA4 is involved in the choice between dorsal and ventral axonal trajectories of hindlimb motor neurons. In addition, our observation that different levels of EphA4 protein are expressed in dorsal and ventral hindlimb motor axons in wild-type animals suggests that this role in pathway selection is achieved by differential reading of the environment owing to the level of EphA4 protein on growth cones.

## MATERIALS AND METHODS

### Generation of *EphA4* mutant mice

A 11 kb fragment was initially cloned from a 129 Ola mouse strain genomic library using a probe derived from an *EphA4* cDNA (Gilardi-Hebenstreit et al., 1992; A. Chestier and S. S.-M., unpublished data).

It covered the 5' part of *EphA4*, including the first three exons and 1 kb of 5' flanking sequences. A 1 kb piece corresponding to the 5' end of this fragment was subsequently used to rescreen the same library, allowing the isolation of a 20 kb fragment, extending 9.5 kb upstream and 10 kb downstream of the first exon and which was used for the targeting construct. ES cells (CK35 line, a generous gift from C. Kress and C. Babinet (Camus et al., 1996)) were maintained, transfected and selected as described previously (Schneider-Maunoury et al., 1993). 400 G418-resistant colonies were screened to detect the homologous recombination event by PCR (using primers 1 and 2) and Southern blotting (see Fig. 1). The PCR primers were as follows: 3' external primer (1) mapping downstream to the *XhoI* site, which delimitates the targeting vector, 5'-GGGGACTTCTGATGCCTGGAGTCC-3', and internal primer (2) covering the frameshift mutation in exon 3, 5'-AAGAAGTGTCCACTCACCGTTCGCG-3'. Among the 30 independent targeted ES clones identified, four were injected into C57BL/6J blastocysts and 10 to 15 chimeras were obtained in each case. Heterozygous animals were obtained upon crossing the chimeras with C57BL6×DBA2 or 129 Sv mice and identified by analysis with primers (1) and (2). Germline transmission was obtained with all four ES lines. In subsequent studies, genotyping of wild-type, heterozygous and homozygous animals was performed by PCR analysis of tail or yolk sac DNA using the following set of primers: 5' primer (a), 5'-TTCTGCCACTGCTATTGGTCACGAG-3'; 3' primer (b) located in the *EphA4* first intron, 5'-AACTGGTCTGAGCTCCAGAAGACC-3'; and 3' primer (c) located in the *lacZ*-coding sequence, 5'-GATGGGCGCATCGTAACCGTGCATC-3'.

### Western blotting analysis

The heads of freshly collected E13.5 to 15.5 embryos were homogenised in lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, containing protease inhibitors), and clarified by centrifugation. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electroblotted to a polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL). The membrane was incubated overnight with a rabbit polyclonal antibody directed against the C-terminal part of EphA4, kindly provided by D. Wilkinson (Irving et al., 1996), at 1:10,000 dilution and subsequently with a peroxidase-conjugated anti-rabbit secondary antibody (SIGMA, 1:10,000 dilution). Binding of the latter antibody was revealed with the enhanced chemiluminescence detection system (Amersham).

### Immunocytochemistry and in situ hybridisation

Embryos were collected and fixed overnight in 4% paraformaldehyde (PFA), embedded in 4% agarose (for EphA4 immunostaining), or in albumin/gelatine, and vibratome sectioned (40 µm thick sections for immunohistochemistry, 70 µm for in situ hybridisation). The sections were dehydrated in methanol, rehydrated and processed for immunocytochemistry (Schneider-Maunoury et al., 1993) or in situ hybridisation (Prince and Lumsden, 1994). Immunostaining was performed using a rabbit polyclonal antibody directed against the 11 C-terminal amino acids of EphA4 (1:20,000; Becker et al., 1995) and a mouse monoclonal antibody directed against Islet-1/2 (1:200, 39-4D5 from DSHB). The RNA probes were derived from the following DNA fragments: a 1.2 kb fragment (position 652-1834) from the *EphA4* cDNA (Gilardi-Hebenstreit et al., 1992), a 1.5 kb fragment from the mouse *Lim-1* gene (clone 31; Fujii et al., 1994), and a 355 bp fragment (position 477 to 832) from the rat *islet-1* gene (kind gift from F. Sedel).

### Histology and motor neuron counts

For motor neuron counting, P4 animals were perfused in 4% PFA, 0.25% glutaraldehyde. The spinal cord was dissected out and the preserved dorsal and ventral roots were used as landmark to define the L1-L5 lumbar region, which was paraffin embedded. Serial sections (10 µm thick) were obtained and stained with toluidine blue.

In each section, motor neurons were counted only when the nucleoli were visible in the nucleus, to avoid counting the same motor neuron twice. The L3-L4 and L5 regions were defined according to internal landmarks in the spinal cord. The L2/L3 transition is marked by the appearance of a dorsal part in the LMC (see figure 6B). The appearance of sacral motor neurons in a column slightly dorsal to the LMC marks the transition to the L5 segment. According to this method, the AP length of the L3-L4 region was estimated at 960  $\mu\text{m}$ , approximately, and the L5 segment at 400  $\mu\text{m}$ .

### Whole-mount neurofilament staining

Whole-mount nerve staining was performed as described (Maina et al., 1997). Briefly, E12.5 and E13.5 embryos were fixed overnight at 4°C in Dent's fixative (1:4, DMSO/methanol), bleached in H<sub>2</sub>O<sub>2</sub> (1:2, 30% H<sub>2</sub>O<sub>2</sub>/Dent's fixative) at room temperature and incubated overnight with a mouse monoclonal antibody directed against neurofilaments (2H3, DSHB) at a 1:5000 dilution. After washing with TBS (10 mM Tris-HCl pH 8.0, 100 mM NaCl), the embryos were incubated overnight with the secondary antibody (goat anti-mouse IgG-conjugated with horseradish peroxidase, A-4416, Sigma, 1:100 dilution). After several washes in TBS, the embryos were stained with diaminobenzidine and cleared by dehydration in methanol followed by incubation in 50% BABB (1:2, benzyl alcohol/benzyl benzoate) and finally in 100% BABB.

### Motor neuron tracing

For DiI tracing, a solution of the carbocyanine dye DiI (Molecular probes, 0.5% in ethanol) was diluted 1:10 in 0.3 M sucrose. This solution was injected with a capillary into the PEL muscle of E15.5 embryos fixed in 4% PFA and DiI was allowed to diffuse overnight at 37°C in 4% PFA. DiI staining was detected under epifluorescence using a Leica MZ10 dissecting microscope.

## RESULTS

### EphA4-deficient mice display a hindlimb phenotype

To investigate EphA4 function during mouse development, we inactivated the gene by homologous recombination in ES cells. Exon 1, encoding the first 30 amino acids, and a part of the first intron were replaced by a cassette containing the *lacZ*-coding sequence, followed by the neomycin gene under the control of the phosphoglycerate kinase I promoter (*lacZ/pgk-neo* cassette), so that the *lacZ*-coding sequence was placed immediately downstream of and in frame with the first codon of *EphA4*. In addition, a frameshift mutation was introduced into EphA4 exon 3, to ensure that unpredictable transcription of the mutated gene would not lead to the production of a functional protein (Fig. 1A). Homologous recombinant ES cell clones were identified by Southern blotting analysis using a 3' external probe (Fig. 1A,B). The presence of the frameshift mutation within exon 3 was verified by PCR analysis (Fig. 1A and data not shown). Further characterization of the mutated alleles was performed by Southern analysis using 5' external, *neo* and *lacZ* probes (Fig. 1A and data not shown). Four independent ES clones carrying the expected mutated allele were injected into blastocysts and led to germline transmission and establishment of mutant lines. All the analyses described in this paper were performed on at least two of these lines and gave identical results. Search for EphA4 transcripts (data not shown) and protein, by western blotting (Fig. 1C) or immunohistochemistry (Fig. 1D,E), established that the mutated allele was null.

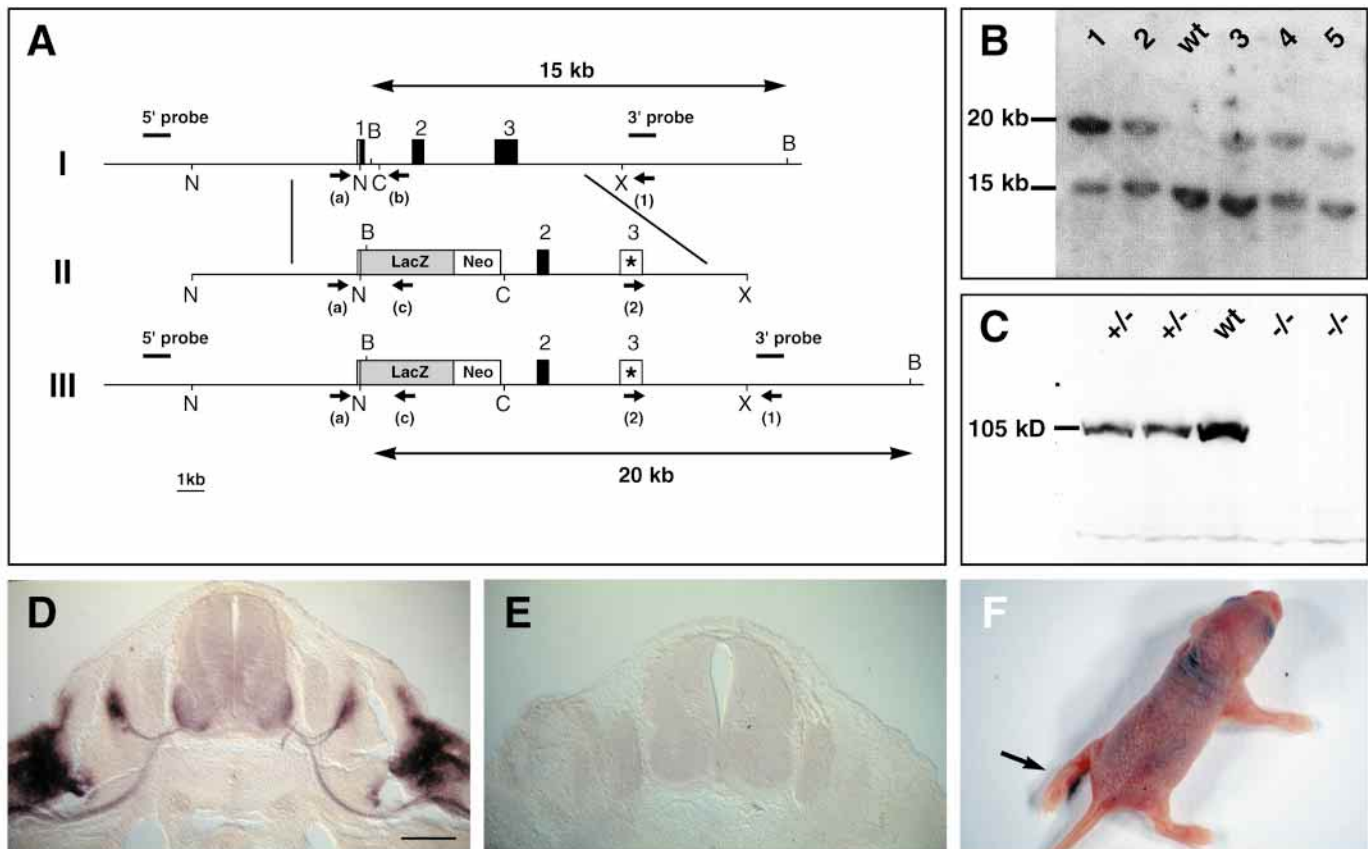
Heterozygous animals were viable and fertile, but some of

them showed a striking abnormal hindlimb position (club-foot) phenotype (Fig. 1F), which led to an unusual gait in older animals. Club-foot deformity is thought to be caused by muscle weakness or imbalance and can result from muscular or neuronal defects (Handelsman and Badalamente, 1981; Nonaka et al., 1986; Yoshimura et al., 1988; Feldbrin et al., 1995). The club-foot phenotype observed in *EphA4* mutant mice thus suggested a failure of foot flexion and digit extension. Heterozygotes can be affected unilaterally or bilaterally, with about 30% of the hindlimbs showing the phenotype. Homozygotes were obtained in Mendelian ratio upon crosses between heterozygotes. They displayed the same club-foot phenotype at birth, and unusual gait later on. The penetrance of this phenotype was enhanced as compared to the heterozygotes, with 88% of the hindlimbs affected. These data suggest that EphA4 is required, in a dose-dependent manner, for the control of normal hindlimb posture and that it may regulate either motor innervation or hindlimb muscular development.

### Loss of dorsal hindlimb innervation in *EphA4* mutants

The muscles responsible for foot flexion and digit extension, collectively called peroneal muscles, are located in the anterior and lateral compartments of the distal hindlimb. Gross anatomical observation of new-born homozygous mutant animals (data not shown) and histological analysis of hindlimb sections at E14.5 (Fig. 2A,B) revealed that all muscles of the distal hindlimb had developed normally and were present in their expected location. However, examination of hindlimb muscular anatomy in adult heterozygous and homozygous mutant animals revealed a complete atrophy of peroneal muscles in affected hindlimbs, but not in non-affected hindlimbs (data not shown). In *EphA4*<sup>-/-</sup> animals, the volume of the other limb muscles, including their antagonists in the posterior compartment of the distal limb, appeared similar in affected and non-affected limbs. However, they appeared reduced in size when compared to those of wild-type animals, although this difference was less dramatic than that seen for the peroneal muscles (data not shown).

The peroneal group of muscles corresponds to the entire target field of the peroneal nerve. We therefore investigated whether this nerve might be affected by the mutation. We first performed a detailed analysis of hindlimb innervation during development by whole-mount immunocytochemistry using an antibody directed against neurofilaments (2H3). Limb-innervating axons are provided by motor neurons located in the lumbar segments L1 to L5. Spinal nerves from L1, L2 and L3 converge to form the femoral (lumbar) plexus in the anterior part of the hindlimb bud and mainly innervate the thigh. Spinal nerves from L3, L4 and L5 converge to form the sciatic (sacral) plexus, in the posterior part of the limb bud, which will mainly innervate the distal part of the limb and the foot. Each plexus constitutes a strategic orientation point where motor axons choose between a dorsal and a ventral trajectory into the limb. Axons emerging from the sciatic plexus and entering the dorsal compartment of the limb bud (dorsally projecting axons) will form the peroneal nerve and innervate muscles derived from the dorsal part of the distal limb bud. These muscles will later give rise to the peroneal group of muscles. Axons following the ventral pathway will constitute the tibial nerve and



**Fig. 1.** Targeted disruption of the *EphA4* locus. (A) Schematic representation of the wild-type allele (I), the targeting vector (II) and the targeted allele (III), indicating the expected restriction fragments revealed with the 3' external probe. The first three *EphA4* exons are shown as black boxes. The targeting strategy involved the replacement of a 1.2 kb *NcoI*-*Clal* fragment containing exon 1, which encodes the first 30 amino acids of the protein, with a *lacZ*-*pgk*-*neo* cassette. The *lacZ* open reading frame is placed in frame with the initiating codon of *EphA4*. In addition, a frameshift mutation was introduced in exon 3 (white box with a star). The positions of the different primers for PCR are indicated. B, *Bam*HI; C, *Clal*; N, *Nco*I; X, *Xho*I. (B) Southern blotting analysis of wild-type (wt) and recombinant ES clones. *Bam*HI-digested genomic DNA was analysed with the 3' external probe, revealing the presence of the 20 kb fragment corresponding to the targeted allele in the five independent recombinant ES cell lines. (C) Western blotting analysis of lysates of E14.5 embryo heads with an antibody directed against EphA4. The genotypes of the embryos are indicated above the lanes. No EphA4 protein is detected in *EphA4*<sup>-/-</sup> embryos, while its level is reduced in heterozygous embryos as compared to wild type. (D,E) Immunohistochemical detection of EphA4 protein in vibratome sections in the brachial region of wild-type (D) and *EphA4* homozygous mutant (E) E11.5 embryos, using an antibody directed against EphA4. (F) *EphA4*<sup>+/-</sup> P5 mouse showing an abnormal positioning of the left hindlimb (arrow). Scale bar, 400  $\mu$ m in D,E.

innervate the ventral compartment and its derived muscles, which have functions antagonistic to the peroneal muscles. Peroneal axons first emerge from the sciatic plexus at E11.5 (Fig. 3A). In E11.5 *EphA4*<sup>-/-</sup> embryos, the developing ventral tibial nerve appears normal, whereas few or none of the axons take the direction normally followed by the peroneal nerve (Fig. 3A,B). At E12.5, the tibial nerve is divided into several ventral branches, which appear normal in all *EphA4*<sup>-/-</sup> hindlimbs (Fig. 3C,D, arrowheads). In contrast, the peroneal nerve, which at this stage divides into two branches in the dorsal part of the wild-type limb (Fig. 3C, arrows), is completely absent from most *EphA4*<sup>-/-</sup> hindlimbs (Fig. 3D). Analyses at later stages (E14.5-E15.5) confirmed the absence of the peroneal nerve in most hindlimbs (Figs 2C-F, 3E,F; Table 1), but surprisingly revealed that one muscle of the lateral compartment of the distal hindlimb, the peroneus longus (PEL) muscle, normally innervated by the peroneal nerve, was contacted in mutants by an aberrant branch coming from a ventral nerve (Fig. 3E,F; Table 1). This branch defasciculated

from the sural nerve near the ankle, and turned sharply in the opposite direction (Fig. 3F). The innervation of the PEL muscle by this ectopic branch was demonstrated by retrograde labelling: DiI injection into the PEL muscle of E15.5 embryos led to labelling of the peroneal nerve in wild-type embryos

**Table 1. Statistical distribution of the phenotypes of *EphA4* mutants revealed by neurofilament staining of dorsal muscle innervation at E12.5 to E15.5**

Genotype	n	Severity of the phenotype (%)			
		1	2	3	4
Wild type	20	100	0	0	0
<i>EphA4</i> <sup>+/-</sup>	26	46	19	19	16
<i>EphA4</i> <sup>-/-</sup>	16	6.25	6.25	13	75

The phenotypes were classified in four types: (1) normal, (2) normal or near-normal peroneal nerve, presence of an ectopic branch, (3) peroneal nerve extremely reduced, presence of an ectopic branch, and (4) peroneal nerve absent, presence of an ectopic branch. *n* is the number of hindlimbs analysed.

(Fig. 3G), and of the ectopic branch in affected limbs of *EphA4*<sup>-/-</sup> or *EphA4*<sup>+/-</sup> embryos (Fig. 3H). In contrast to the PEL muscle, the muscles of the anterior compartment of affected distal hindlimbs were never found to be innervated (data not shown).

In conclusion, the selective atrophy of peroneal muscles that appears progressively after birth in *EphA4*<sup>-/-</sup> animals correlates with the absence of the peroneal nerve. Lack of electrical stimulation associated with this aneural character is likely to be the direct cause of the atrophy. Analysis of limb nerve pattern during development suggests that the absence of

the peroneal nerve is due to the fact that axons do not enter the dorsal compartment of the limb bud after emerging from the sciatic plexus.

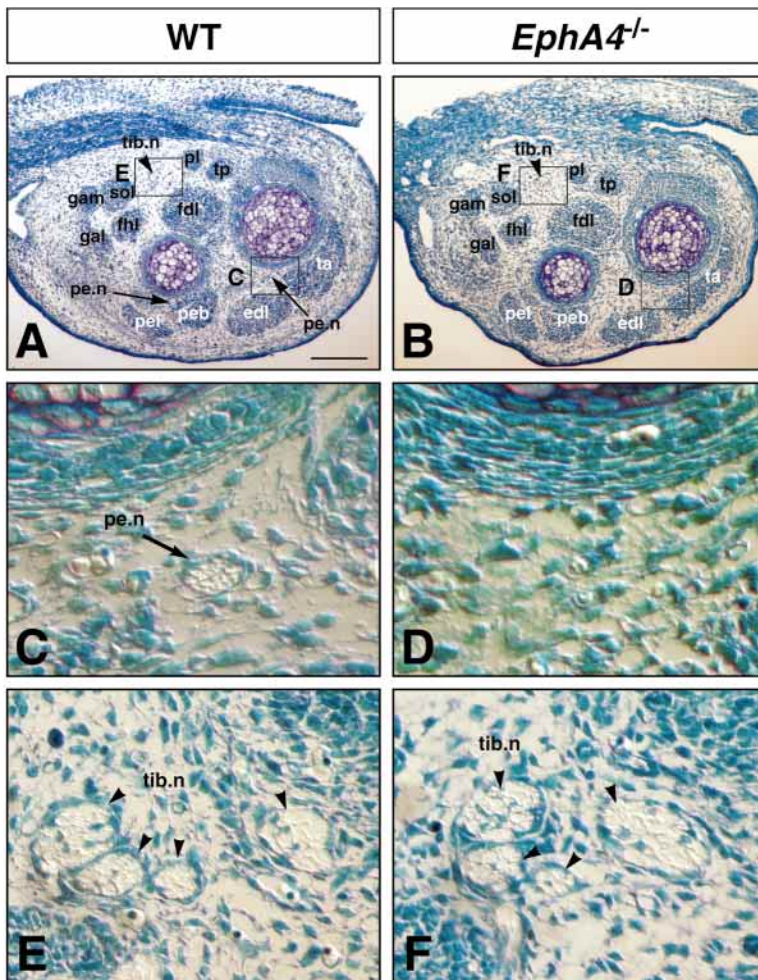
### ILMC neurons are present in *EphA4*<sup>-/-</sup> mice but do not send axons into the dorsal mesenchyme of the hindlimb bud

The absence of the peroneal nerve in *EphA4*<sup>-/-</sup> mice could have resulted from abnormal pathfinding decisions of the axons at the exit of the sciatic plexus or from the elimination of the corresponding motor neuron population. To determine whether this population is preserved in the mutants, we have taken advantage of the fact that motor neurons belonging to different subtypes progressively segregate from each other and form subcolumns that become spatially recognisable at stage 29 in the chick embryo in the brachial region (Socanathan and Jessell, 1998), and at E14.5 in the mouse lumbar region (Fig. 4, data not shown). Furthermore, at this stage in the chick, motor neurons from the lateral and medial parts of the LMC are distinguishable owing to the different combinations of LIM genes that they express (Tsuchida et al., 1994). ILMC neurons, which innervate dorsal muscles, express *Lim-1* and *islet-2*, whereas mLMC neurons, which innervate ventral muscles, express *islet-1* and *islet-2* (Tsuchida et al., 1994; Ensini et al., 1998).

We therefore performed a series of immunolabellings experiments at E14.5-15.5 in the L3-L5 segments of the mutant spinal cord using an antibody directed against *islet-1* on serial transverse sections of the L3-L5 region. This antibody weakly recognises *islet-2* as well and makes it possible to define mLMC (Fig. 4, arrowheads) and ILMC (Fig. 4, arrows) as strongly and weakly positive domains, respectively. In the homozygous mutant spinal cord, both neuronal populations were observed at the expected positions, although their sizes appeared slightly reduced (Fig. 4). This suggests that the ILMC motor neurons are present and correctly specified and that they occupy their expected location in the spinal cord. These data therefore support the idea that the peroneal axons have been misrouted due to the absence of EphA4.

### Different levels of EphA4 in dorsally versus ventrally projecting axons

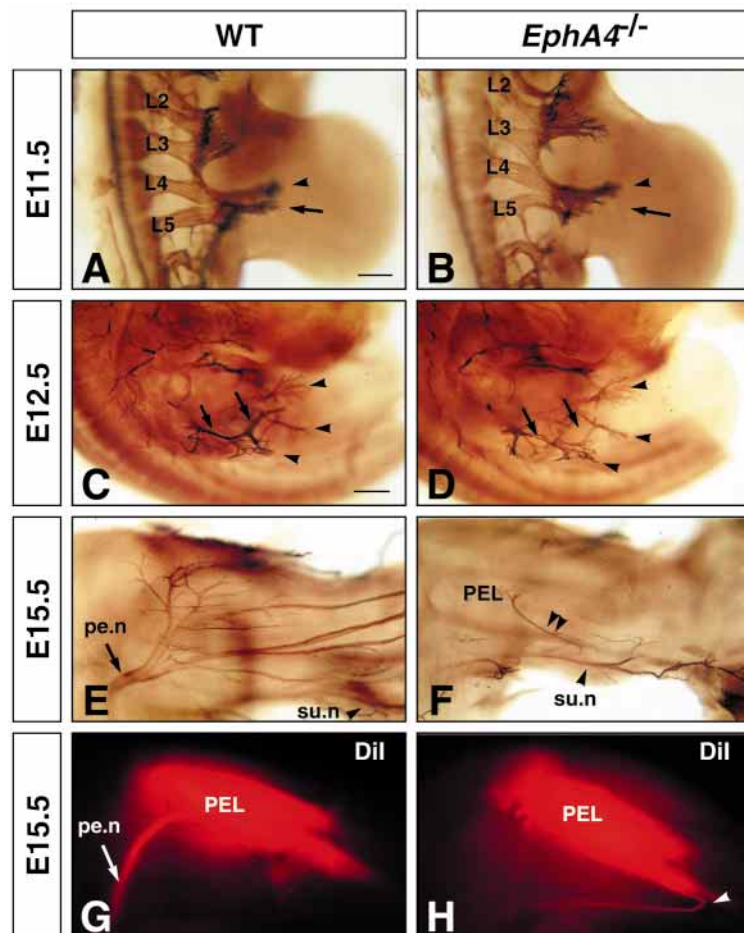
To investigate the involvement of EphA4 in the navigation of peroneal axons, we examined its expression in the spinal cord of mouse embryos at E11.5, the stage at which selection of dorsal versus ventral trajectories occurs (Fig. 3A). In contrast to later stages of development (see below), *EphA4* mRNA was detected along the entire length of the spinal cord (data not shown). The domain of highest expression corresponded to the motor columns (Fig. 5A). Lower levels of expression were observed in the mantle layer, the ventricular zone and in a stream of cells joining the ventricular zone to the motor column. Immunohistochemistry performed with an antibody directed against EphA4 (Becker et al.,



**Fig. 2.** Normal hindlimb muscle formation and absence of the peroneal nerve in *EphA4*<sup>-/-</sup> E14.5 embryos. Transverse sections of hindlimbs, at the level of the shank, from wild-type (A,C,E) and *EphA4*<sup>-/-</sup> (B,D,F) embryos were counterstained with toluidine blue. (A,B) All muscles identified in the wild-type embryo (A) can be recognized and have a normal appearance in the *EphA4*<sup>-/-</sup> embryo. The muscles (or their tendons) of the anterolateral (peroneal) and of the posteromedial (tibial) compartments are indicated by their initials in white and in black, respectively. (C-F) Enlargements of the boxed areas indicated in A and B. Examination of the hindlimb nerves indicates that while the tibial nerve (E,F) is present, the peroneal nerve (C,D) is not found in the expected region of *EphA4*<sup>-/-</sup> hindlimbs. ta, tibialis anterior; edl, extensor digitorum longus; peb, peroneus brevis; pel, peroneus longus; pl, plantaris; tp, tibialis posterior; fdl, flexor digitorum longus; fhl, flexor hallucis longus; sol, soleus; gam, gastrocnemius medialis; gal, gastrocnemius lateralis; tib.n, tibial nerve; pe.n, peroneal nerve. Right is anterior and top is medial. Scale bar: 200 μm in A,B; 25 μm in C,D; 32 μm in E,F.

1995) on transverse sections was consistent with the *in situ* hybridisation data in the spinal cord (Figs 1D, 5B; data not shown). In addition, EphA4 was also detected in axons innervating the dorsal axial musculature and the limbs (Figs 1D, 5B).

Examination of the posterior part of the hindlimb bud



**Fig. 3.** Absence of dorsal hindlimb innervation in *EphA4*<sup>-/-</sup> embryos. (A-F) Neurofilament staining of hindlimbs from wild-type (A,C,E) and *EphA4*<sup>-/-</sup> (B,D,F) embryos. (A,B) Dorsal view, right side, of E11.5 embryos showing limited distal extension of dorsal axons (arrows) as compared to ventral axons (arrowheads) in the mutant embryo. (C,D) Lateral view of E12.5 embryos. In the distal part of the limb, the sciatic nerve divides into a ventral portion, the tibial nerve (out of focus, arrowheads), and a dorsal portion, the peroneal nerve (two arrows). The peroneal nerve is completely missing in the mutant limb. (E,F) Hindlimbs from E15.5 embryos whose skin and cutaneous innervation have been removed to visualise the lateral compartment of the distal limb (shank). In the wild-type limb (E), the peroneal nerve comes from the thigh, and branches to innervate the group of muscles in the anterolateral part of the shank. In the mutant limb (F), the peroneal nerve is absent, and muscles of the anterolateral shank are not innervated, except by a small abnormal branch (double arrowhead) that defasciculates from the sural nerve and turns back to innervate the PEL muscle. The sural nerve, because of its more ventral position, is barely in the focal plane of the picture of the wild-type hindlimb (E). (G,H) Retrograde tracing of axons innervating the PEL muscle by Dil injection of E15.5 embryos. In the mutant (H), the arrowhead indicates the labelled ectopic branch. L2-L5, spinal nerves from lumbar segments L2 to L5; pe.n, peroneal nerve; su.n, sural nerve; PEL, peroneus longus. Scale bar, 300  $\mu$ m in A,B, 400  $\mu$ m in C-H.

indicated that the spinal nerves were also strongly EphA4-positive until the sciatic plexus (Fig. 5B). Beyond the plexus, axons entering the dorsal compartment of the limb bud were still strongly labelled, whereas axons projecting ventrally were only weakly positive (Fig. 5B,D,F,G). In contrast, immunostaining with an anti-neurofilament antibody led to equivalent labelling of both dorsal and ventral bundles of axons (Fig. 5C,E,H,I). In addition, in the proximal part of the dorsal limb bud, the mesenchyme towards which dorsal axons are projecting was also strongly positive for EphA4 (Fig. 5B,D).

In conclusion, during the initial period of limb innervation, *EphA4* expression in spinal cord motor neurons is not restricted to limb-innervating segments. Nevertheless, the existence of a differential level of EphA4 protein in dorsal and ventral axons emerging from the sciatic plexus suggests that EphA4 may participate in the selection of dorsal versus ventral trajectories.

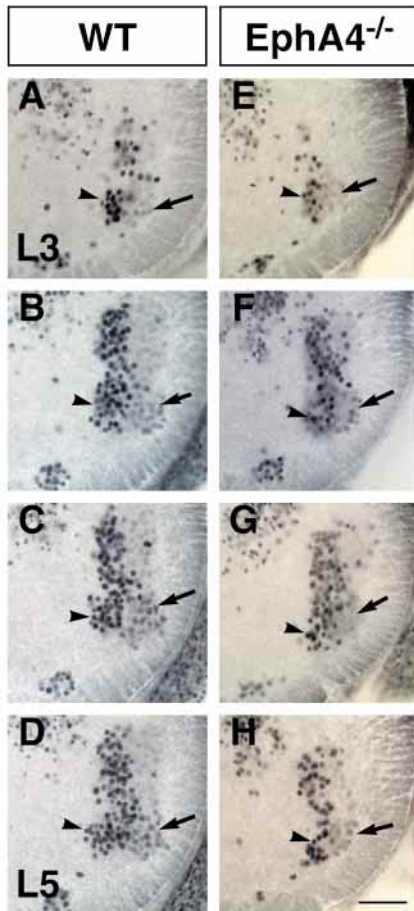
#### Dorsally fated axons join the ventral nerve in *EphA4* mutant embryos

To investigate the trajectory of misrouted dorsal axons in *EphA4* mutants, we took advantage of the fact that *lacZ* expression reflects normal *EphA4* expression and that  $\beta$ -galactosidase activity is not restricted to cell bodies and but also observed along the axons. We first compared the pattern of  $\beta$ -galactosidase activity in sections at the level of the sciatic plexus of E11.5 *EphA4*<sup>+/-</sup> embryos between affected and non-affected cases, i.e., when the dorsal nerve was absent or present. When the dorsal nerve had formed normally, it was composed of X-gal-positive axons, while the ventral nerve demonstrated a much weaker  $\beta$ -galactosidase staining (Fig. 6A). Anti-neurofilament staining confirmed the presence of both nerves in the same section (Fig. 6B). In contrast, in *EphA4*<sup>+/-</sup> embryos in which no dorsal nerve was detected (Fig. 6D), the ventral nerve was clearly X-gal positive (Fig. 6C). Similarly, in 12 *EphA4*<sup>-/-</sup> embryos, which were all lacking the dorsal nerve, the ventral nerve always appeared X-gal positive (Fig. 6E).

These data indicate that, in *EphA4* mutants, there is a correlation between the absence of the dorsal nerve and the presence of Xgal-positive axons, presumably corresponding to dorsally fated neurons, in the ventral nerve. This suggests that, in these cases, the dorsal axons have been misrouted at the level of the sciatic plexus and have joined the ventral nerve.

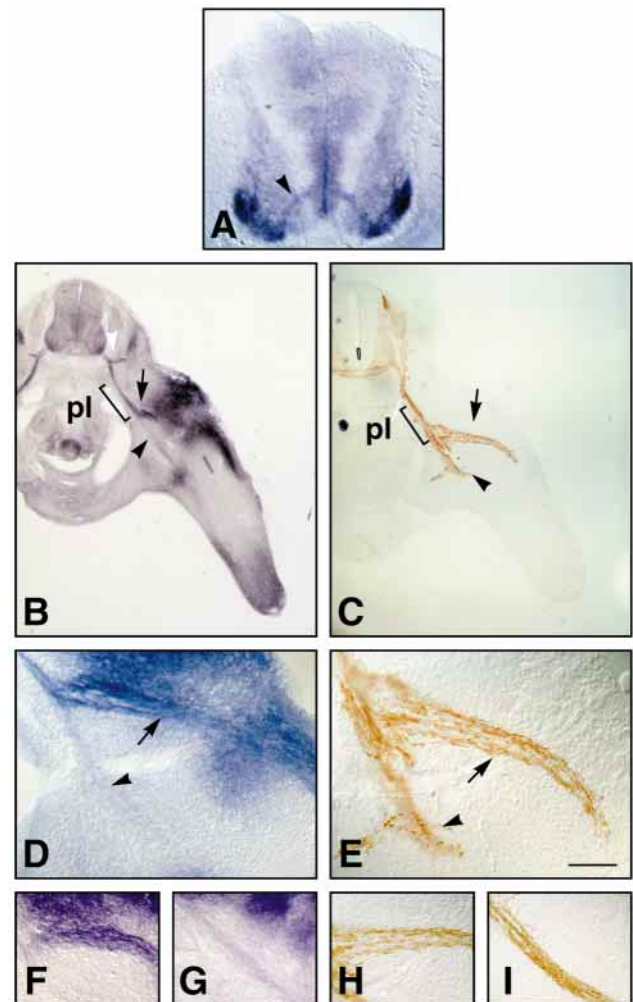
#### Loss of motor neurons independent of the navigation defect in *EphA4* mutant mice

We noticed a slight reduction in the number of LMC neurons in the L3-L5 region of E14.5 *EphA4*<sup>-/-</sup> embryos (Fig. 4). Such a phenotype was not observed at E13.5 (data not shown). Evaluation of the number of neurons weakly or strongly positive for the antibody directed against islet-1 at later stages of development (E15.5, E16.5) indicated a general reduction in the number of LMC neurons in segments L2 to L5 of mutant animals (data not shown). These data were



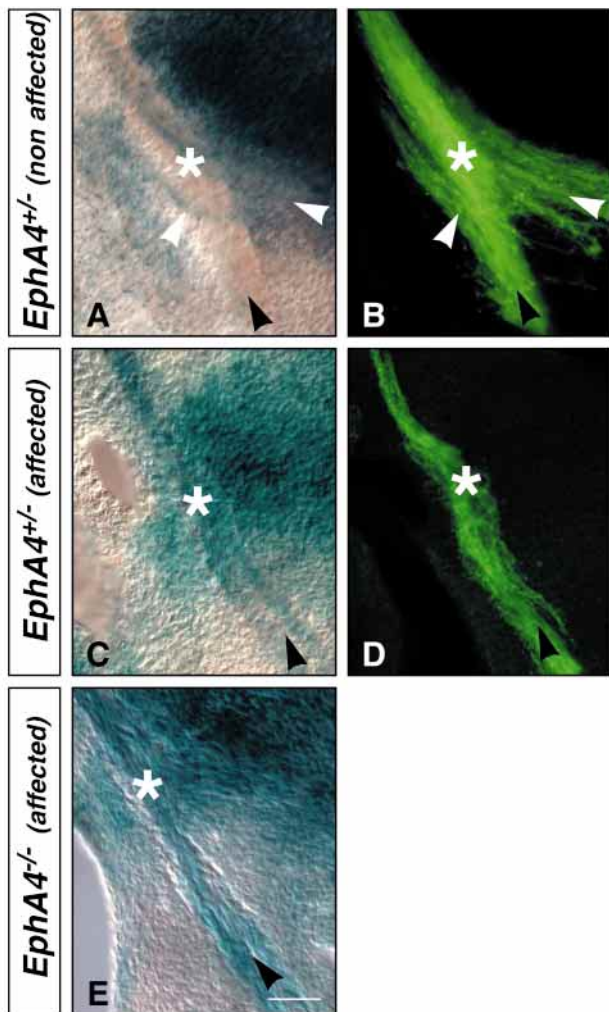
**Fig. 4.** Analysis of motor pool organisation in the E14.5 lumbar spinal cord. Transverse sections regularly spaced from the L3 (A,E) to L5 (D,H) segmental levels were analysed by immunohistochemistry with an antibody directed against islet-1/2 on wild-type (A-D) and *EphA4*<sup>-/-</sup> (E-H) embryos. Dorsal is to the top, medial to the left. The medial pools of strongly labelled motor neurons (arrowheads) correspond to the mLMC (islet-1+, islet-2+), and the lateral, weakly labelled pools (arrows) to the lLMC (islet-1-, islet-2+). In *EphA4*<sup>-/-</sup> embryos, although slightly reduced, the two pools of motor neurons are present at the expected positions. Scale bar, 100  $\mu$ m.

confirmed by quantification of the number of LMC neurons on serial spinal cord sections at postnatal day 4 (P4) (Fig. 7). In the L3 and L4 segments, we found a reduction of 23% and 35%, respectively, in the number of motor neurons in heterozygous and homozygous mutants, compared to wild type ( $P=0.0004$  and  $P<0.0001$ , respectively, *t*-test; Fig. 7A). However, since the L3 and L4 segments contain the peroneal motor pool, such a reduction could be a secondary consequence of the pathfinding error of peroneal axons. To investigate this possibility, we quantified separately motor neuron numbers in spinal cord sides corresponding to limbs that did not present a club-foot phenotype (non-affected sides), since the peroneal nerve is normal in these cases (data not shown). We also observed significant reductions in non-affected sides, although less dramatic: 17% in heterozygotes and 28% in homozygotes, compared to wild type ( $P=0.002$  and  $P<0.0001$ , respectively; *t*-test, Fig. 7A). Furthermore, in the L5



**Fig. 5.** Different levels of EphA4 protein on dorsal and ventral limb motor axons. (A) Expression of *EphA4* in the E11.5 spinal cord, detected by in situ hybridisation on transverse vibratome section. *EphA4* is expressed at high levels in motor columns. Weaker expression is observed in the mantle layer, the ventricular zone and a stream of cells connecting the ventral ventricular zone and the motor column (arrowhead). (B-I) EphA4 (B,D,F,G) and neurofilament (C,E,H,I) immunohistochemistry on transverse sections of E11.5 embryos, at the level of the sciatic plexus. (B-E) A high level of EphA4 is detected in the dorsal part of the proximal limb bud and along spinal nerves until the sciatic plexus (bracket in B,C). Motor axons innervating the epaxial musculature (white arrowhead in B) are also strongly immunoreactive. Beyond the sciatic plexus, axons innervating the dorsal region of the limb show high levels of EphA4 protein (arrow), whereas ventral axons are only weakly labelled (arrowhead). (F,H and G,I) Adjacent sections of the dorsal and ventral branches, respectively. Neurofilament staining indicates that differences in the numbers of axons are not likely to explain the difference in EphA4 labelling between the dorsal and ventral branches. Scale bar, 100  $\mu$ m in A,D-I; 400  $\mu$ m in B,C.

segment, which does not contribute significantly to the peroneal nerve, 19% and 41% reductions were observed in heterozygous and homozygous animals, respectively ( $P=0.0008$  and  $P<0.0001$ , respectively, *t*-test; Fig. 7B). These data suggest that the deficit in motor neurons in the LMC of *EphA4* mutant mice is largely independent of the peroneal



**Fig. 6.** Presumptive dorsal axons follow the ventral trajectory of the tibial nerve when the dorsal peroneal nerve is absent. Transverse sections of E11.5 *EphA4*<sup>+/+</sup> (A–D), and *EphA4*<sup>-/-</sup> (E) embryos, at the level of the sciatic plexus, revealed for  $\beta$ -galactosidase activity (A,C,E) and neurofilament immunofluorescence (B,D). (A,B) *EphA4*<sup>+/+</sup> embryo, presenting a non-affected limb, in which both dorsal (white arrowheads) and ventral (black arrowhead) nerves can be seen emerging from the sciatic plexus (\*). The dorsal axons contain  $\beta$ -galactosidase activity, whereas the ventral nerve is X-gal negative. (C,D) Limb from a *EphA4*<sup>+/-</sup> embryo in which the dorsal nerve is absent. Anti-neurofilament staining (D) reveals the ventral nerve, which is X-gal positive (arrowhead in C), whereas no dorsal branch is observed in this section or in the neighbouring ones. (E) *EphA4*<sup>-/-</sup> embryos are also devoid of the dorsal nerve and display  $\beta$ -galactosidase activity in the ventral nerve. Scale bar, 100  $\mu$ m.

nerve pathfinding defect and reflects another function of *EphA4*.

To investigate whether this phenotype reflected the *EphA4* expression pattern, we analysed the expression of the gene in the spinal cord between E12.5 and E15.5. From E12.5, *EphA4* expression is downregulated in MMC neurons and becomes restricted to subpopulations of neurons in the brachial and lumbar LMC (Fig. 8; data not shown), consistent with observations in the chick embryo (Fukushima et al., 1996; Ohta et al., 1996). At E14.5 and E15.5, at the L3–L5 levels, *EphA4*

is expressed in most LMC neurons, as demonstrated by the comparison with the *Lim-1* and *islet-1/2* expression patterns (Fig. 8D–F and data not shown) and by double labelling for *islet-1/2* and  $\beta$ -galactosidase (Fig. 8G). These data indicate that *EphA4* is expressed at similar levels in both ILMC and mLMC neurons after the period of establishment of ventral and dorsal axonal pathways and suggest that this expression might be required to prevent the loss of motor neurons observed in the mutant.

## DISCUSSION

In this study, we have investigated two phenotypes that result from the targeted inactivation of the *EphA4* gene: an abnormal navigation of the axons of the ILMC of the hindlimb at early stages of limb innervation and a general reduction in the number of hindlimb motor neurons at later stages.

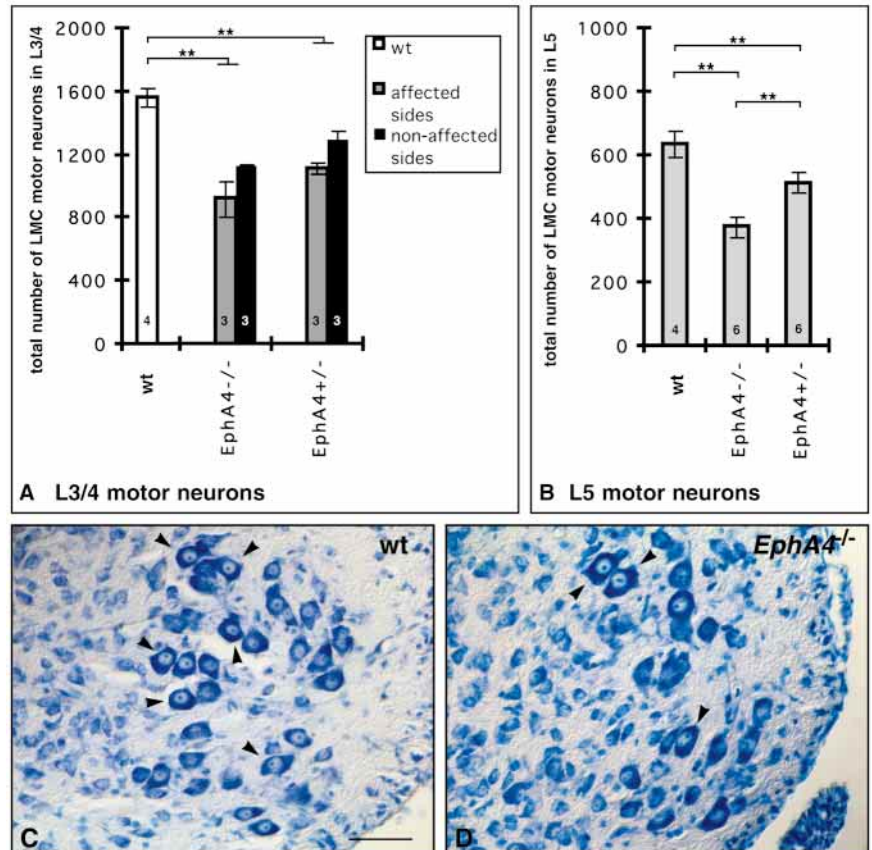
### *EphA4* level in LMC neurons participates in the selection of dorsal/ventral axonal pathfinding

Our observation of the absence of dorsal hindlimb innervation in *EphA4* mutants, combined with the presence of ILMC neurons expressing the expected identity markers and the abnormal  $\beta$ -galactosidase labelling of the ventral nerve, indicate that the mutation affects axonal pathfinding rather than cell survival or identity. An important question at this stage is whether the effect of the mutation is cell autonomous. While *EphA4* is expressed in limb motor neurons at the time of the pathfinding decision (E11), the protein is also present in the limb mesenchyme, particularly in its dorsal part (Fig. 4B,C). However, we have made the striking observation that the *EphA4* protein is present at different levels on dorsal and ventral axons (Fig. 4B,C; see also (Ohta et al., 1996)). In addition, two *EphA4* ligands, *ephrin-A2* and *ephrin-A5*, have been shown to be expressed in the chick limb bud at the time of dorsal/ventral pathway selection (Ohta et al., 1997), and examination of their protein distribution in the chick hindlimb has shown that *ephrin-A5* is present exclusively in the ventral compartment, and higher levels of *ephrin-A2* are present ventrally than dorsally (C. Krull, personal communication). These ligands have been shown *in vitro* to inhibit the outgrowth of motor axons from lumbar and brachial, but not thoracic, levels (Ohta et al., 1997). This correlates with *EphA4* expression at the stage used for motor neuron cultures and therefore suggests that *ephrin-A2* and *ephrin-A5* can be repulsive for *EphA4*-expressing motor axons.

Taken together, these data suggest the following model for the control of hindlimb dorsal/ventral axonal pathfinding, assuming that the axons can only choose one of these two pathways at the bifurcation point. Dorsally fated axons, expressing high levels of *EphA4*, are repelled by the ventral compartment expressing *EphA4* ligands, and therefore grow preferentially in the less repulsive dorsal compartment. It is interesting to note that both *EphA4* (Fig. 4) and *EphA7* (Araujo et al., 1998) are expressed in the dorsal limb compartment. Differential masking of the low level of ligand present in the dorsal compartment, due to co-expression with these two Eph receptors, might be an additional mechanism by which the dorsal compartment is permissive for the growth of axons expressing high levels of *EphA4* (an example of an Eph



**Fig. 7.** Loss of motor neurons independent of the pathfinding defect. (A,B) Quantification of motor neuron numbers in the lumbar LMCs. The bars represent, for each genotype (wild-type and mutant P4 animals), the average total number of LMC motor neurons over a region spanning either L3 and L4 (A), or L5 (B). Motor neurons were counted for every section, and numbers from each sections were cumulated. The analysis was performed on the cumulated L3 and L4 segments (A), where the majority of peroneal motor neurons are located and on the L5 segment (B), which contains only very few peroneal motor neurons. (A) Affected sides (showing abnormal position of the hindlimb) were treated separately from non-affected ones. (B) The differences between affected and non-affected sides were non-significant and they were grouped. Error bar=standard error,  $**P \leq 0.0002$  (*t*-tests). The figures in the bars indicate the number of animal sides analysed in each case. (C,D) Examples of toluidine-blue-stained wild-type (C) or *EphA4*<sup>-/-</sup> (D) spinal cord sections showing some motor neurons with one or several nucleoli (arrowheads). Scale bar, 50  $\mu$ m in C,D.

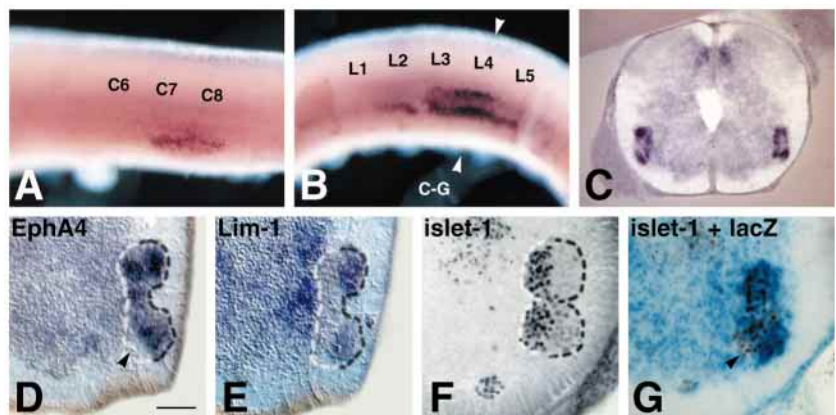


receptor masked by a ligand has been provided by Hornberger et al., 1999). Due to their lower level of EphA4, ventrally fated axons are less sensitive to ligand-mediated repulsion. Since their trajectory is, nevertheless, restricted to the ventral pathway, they must recognise other cues, resulting either in growth cone attraction towards the ventral pathway or repulsion from the dorsal one.

According to this model, in *EphA4*<sup>-/-</sup> embryos, the ventrally fated axons are not affected and follow the ventral pathway. The dorsally fated axons are not prevented from following the ventral pathway and they would be expected to choose randomly between ventral and dorsal pathway. However, according to our data, they almost systematically take the ventral route. This suggests that dorsally fated axons are also subjected to a second signalling system promoting the choice of the ventral route. This second guidance system may be identical to the one postulated in the previous paragraph for ventrally fated axons. In wild-type dorsally fated axons, repulsion by the dorsal compartment through EphA4 signalling must overcome this additional system and allow selection of the dorsal pathway.

*EphA4* is also expressed in forelimb motor neurons, suggesting that it might play a role there in motor axon pathfinding. Neurofilament staining of forelimbs from E14.5-15.5 *EphA4*<sup>-/-</sup>

embryos revealed a strong reduction of the dorsal nerve equivalent of the peroneal nerve in approximately 70% of the cases, although there was never a complete elimination (data not shown). These data are therefore consistent with EphA4



**Fig. 8.** Expression of *EphA4* during the period of motor neuron death. *EphA4* expression was analysed by in situ hybridisation on whole-mount E13.5 spinal cords (A,B, views of the brachial and lumbar regions, respectively) or on vibratome transverse sections (C,D) of E14.5 spinal cord at the level indicated by the arrowheads in B. Additional sections at the same level were analysed by in situ hybridisation with a *Lim-1* probe (E), by immunohistochemistry with the antibody directed against islet-1/2 (F), and by immunohistochemistry with the same antibody, combined with revelation of  $\beta$ -galactosidase activity (G). Note the co-expression of *EphA4* with *Lim-1* in the lLMC and with *islet-1* in the mLMC, with the exception of a small negative medioventral region (arrowheads in D,G). Scale bar, 200  $\mu$ m in A-C, 100  $\mu$ m in D-G.

playing similar roles in the forelimb and the hindlimb, but the more moderate phenotype observed in the forelimb suggests a less critical function, possibly due to functional redundancy with other genes.

### **EphA4 may constitute an effector of motor neuron identity genes**

Distinct categories of motor neurons have been defined on the basis of the type and location of their target muscles in the periphery (Landmesser, 1978; Tsuchida et al., 1994). In each category, motor neurons are programmed very early to contact their appropriate target (Lance-Jones and Landmesser, 1980; Matisse and Lance-Jones, 1996). This specificity is believed to be controlled at the transcriptional level by the expression of distinct combinations of transcription factors of the Lim family (reviewed in Pfaff and Kintner, 1998), and to be mediated by differential expression of receptors for axon guidance molecules. Our observation of different levels of EphA4 expression in dorsally and ventrally projecting axons suggests that *EphA4* is under the control of cell identity genes (e.g. *Lim-1*, *islet-1*). In particular, the high level of expression in axons of motor neurons fated to project along a dorsal trajectory correlates with expression of the Lim-1 transcription factor, while the low level in axons fated to choose a ventral pathway correlates with expression of *islet-1*. The inactivation of *islet-1* and *Lim-1* in the mouse led to a complete absence of motor neurons (Pfaff et al., 1996) and embryonic lethality (Shawlot and Behringer, 1995), respectively, preventing the analysis of their role on motor axon pathfinding. However, the role of *Lim-1* has recently been studied in chimeric embryos obtained by injection of ES cells homozygote for a new *Lim-1/tau-lacZ* null allele into wild-type embryos (A. Kania and T. Jessel, personal communication). The navigation of axons from *Lim1*<sup>-/-</sup> motor neurons is affected, but the phenotype is distinct from that of *EphA4*<sup>-/-</sup> embryos; after reaching the bifurcation point, dorsally fated neurons randomly choose dorsal or ventral pathways (A. Kania and T. Jessel, personal communication). Such behaviour is consistent with our model as long as, in dorsally fated neurons, Lim-1 is required both for *EphA4* expression, which prevents the choice of the ventral route, and for sensing of the postulated second signalling system, which in contrast promotes this pathway.

### **Reduced motor neuron survival in *EphA4* mutants**

During development, most populations of neurons are produced in excess, with a subsequent elimination of supernumerary neurons by apoptosis. This process of naturally occurring neuronal death is thought to allow a perfect matching of neuron numbers with the size of the target field. In the case of mouse motor neurons, this process takes place between E13.0 and E18.5 (Lance-Jones, 1982), and leads, in the case of the lumbar LMC, to a reduction to about 60% of the initial number of motor neurons (Lance-Jones, 1982).

Quantification of motor neuron number revealed a reduction in *EphA4* mutants as compared to wild-type animals, which did not correlate with the navigation defect of dorsally fated neurons and affected the entire lumbar LMC (Fig. 5). This difference between mutant and wild-type animals becomes apparent between E13.5 and E14.5, and is likely to be due to an increase in the apoptosis occurring during the normal period of cell death. The general decrease in the number of

L2-L5 motor neurons could explain the reduction in volume of all distal hindlimb muscles observed in *EphA4*<sup>-/-</sup> animals. EphA4 may therefore be involved in promoting motor neuron survival. Is such a function cell-autonomous or is the expression of trophic factors modified in the mutant limb? We favour the first possibility for the following reasons. (i) In the mutant, preliminary analyses did not reveal any obvious defect in limb muscle and Schwann cell morphology, two important sources of neurotrophic factors (data not shown). (ii) We have shown that *EphA4* is expressed in a large part of the LMC during the period of motor neuron death. (iii) One EphA4 ligand, ephrin-A1, has been shown to have neurotrophic activity in cultures of dissociated cells from embryonic rat spinal cord as well as in cultures enriched in motor neurons (Magal et al., 1996).

### **Multiple neuronal phenotypes associated with the *EphA4* mutation**

*EphA4* expression in the embryo is not restricted to hindlimb motor neurons; therefore, additional phenotypes could have been expected. A likely candidate for being affected was the hindbrain, where *EphA4* is expressed in alternate segments (Gilardi-Hebenstreit et al., 1992; Nieto et al., 1992) and where ephrin-Eph receptor signalling has been shown to be involved in the restriction of cell intermingling (Xu et al., 1995, 1999; Mellitzer et al., 1999). However, we have not observed any obvious defects in the hindbrain of homozygous *EphA4* mutants (data not shown).

During the course of our analysis, the generation and study of another *EphA4* targeted allele has been reported (Dottori et al., 1998). These authors demonstrated that animals homozygous for this allele showed an abnormal synchronous (kangaroo-like) movement of the hindlimbs, which they linked to a reduction of the dorsal funiculus, the pathway of the corticospinal tract (CST) in rodents. They also observed an aberrant trajectory of CST axons within the medulla and spinal cord, accounting for the reduction of the dorsal funiculus. Finally, they observed a frequent loss of the anterior commissure in homozygous animals. Consistent with the observations of Dottori and colleagues, we found that mice homozygous for our mutant allele of *EphA4* also show synchronous movements of left and right hindlimbs, correlating with a strong reduction of the dorsal funiculus, and are missing the anterior commissure (data not shown). We did not observe these defects in heterozygous animals.

Therefore, besides the observation of the same phenotypes as those noted by Dottori and collaborators in *EphA4* mutant mice, we have detected additional defects in axonal navigation and survival of hindlimb motor neurons. What could be the cause of this discrepancy? One possibility is that it might be due to differences in genetic background. Our mutation was maintained in a mixed background (C57Bl6×DBA2), while Dottori and colleagues used C57Bl6 mice. A second major difference resides in the alleles generated. In our study, we eliminated the first exon, introduced a frameshift mutation in the third exon, and verified that the protein was absent in limb motor neurons at the precise stage when the axonal pathway defect occurs. Dottori and colleagues generated a mutation that eliminates the third exon and showed that the protein was absent from the hindbrain at E8.5. However, the possibility that unexpected splicing events could generate enough EphA4

protein in motor neurons to rescue the phenotype cannot be ruled out.

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