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Aberrant axonal projections in mice lacking EphA8 (Eek) tyrosine protein kinase receptors

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We have generated mice homozygous for a mutation that disrupts the gene encoding EphA8, a member of the Eph family of tyrosine protein kinase receptors, previously known as Eek. These mice develop to term, are fertile and do not display obvious anatomical or physiological defects. The mouse *ephA8/eek* gene is expressed primarily in a rostral to caudal gradient in the developing tectum. Axonal tracing experiments have revealed that in these mutant mice, axons from a subpopulation of tectal neurons located in the superficial layers of the superior colliculus do not reach targets located in the contralateral inferior colliculus. Moreover, *ephA8/eek* null animals display an aberrant ipsilateral axonal tract that projects to the ventral region of the cervical spinal cord. Retrograde labeling revealed that these abnormal projections originate from a small subpopulation of superior colliculus neurons that normally express the *ephA8/eek* gene. These results suggest that EphA8/Eek receptors play a role in axonal pathfinding during development of the mammalian nervous system.

Keywords: axon guidance/gene targeting/neuronal development/tyrosine kinase receptors

Introduction

The Eph family of receptors constitutes the largest subgroup within the tyrosine protein kinase receptor family. Fourteen distinct members of this receptor family have been isolated to date (Tuzi and Gullick, 1994; Brambilla and Klein, 1995; Gurniak and Berg, 1996). They recognize a family of membrane-bound ligands, suggesting that they are involved in mediating cell–cell interactions (Pandey *et al.*, 1995; Tessier-Lavigne, 1995). In this manuscript we refer to Eph family receptors and their ligands using a new nomenclature that has been agreed to by the community and is being submitted for publication to *Cell* (M.Tessier-Lavigne, J.Flanagh, N.Gale, T.Hunter and E.Pasquale, personal communication). The ligands have two distinct structures. Whereas some are anchored to the membrane by a glycosyl phosphatidylinositol (GPI) linkage (ephrin-A subfamily; i.e. B61, ELF-1, Ehk1-L, AL1/RAGS, Lerk-4), others possess a single transmembrane domain followed by a short cytoplasmic tail (ephrin-B subfamily; i.e. Elk-L, Htk-L, Elk-L3) (Pandey *et al.*, 1995). Emerging evidence indicates that the Eph receptors bind their cognate ligands with similar affinities in the nanomolar range and rather limited specificity (Gale *et al.*, 1996). Two subfamilies of Eph receptors, EphA and EphB, have been identified based on the relative homologies of their ectodomains. The EphA subfamily (previously known as the Eck subgroup) encompasses eight different receptors including EphA8/Eek. To date, only six members of the EphB subfamily (previously known as the Elk subgroup) have been identified. In general, EphA receptors recognize the GPI-linked ephrin-A proteins, whereas the EphB receptors interact preferentially with transmembrane ephrin-B ligands (Gale *et al.*, 1996). Additional levels of specificity within each of these subgroups are likely to be provided by their respective spatial and temporal patterns of expression (Sajjadi and Pasquale, 1993; Becker *et al.*, 1994; Henkemeyer *et al.*, 1994; Bergemann *et al.*, 1995; Bouillet *et al.*, 1995; Ciossek *et al.*, 1995). It is also possible that, *in vivo*, other factors may influence the recognition specificity between certain Eph receptors and their ligands.

A significant number of Eph receptors are expressed in the nervous system (Brambilla and Klein, 1995). Recent observations, primarily derived from studies of the chicken retino-tectal system, have indicated that Eph receptors and their cognate ligands play important roles in axon fasciculation and may serve as positional labels for axonal guidance. For instance, ephrin-A2/ELF-1, a GPI-anchored ephrin expressed in the tectum, displays a complementary gradient of expression with its cognate EphA3/Mek-4 receptor expressed in the retina (Chen *et al.*, 1995). AL-1/RAGS, another member of the ephrin-A subgroup, has been shown to stimulate axon fasciculation in cultured cortical neurons (Winslow *et al.*, 1995) and to have axon repellent activity on retinal ganglion cells (Drescher *et al.*, 1995). Likewise, ephrin-A2/ELF-1 has also been shown to act as an axonal repellent *in vitro* and to alter the distribution of retinal axons when ectopically expressed *in vivo* (Nakamoto *et al.*, 1996).

Analysis of mice defective for EphB2/Nuk, a receptor that recognizes transmembrane ligands (Henkemeyer, 1994; Gale *et al.*, 1996), revealed a defect in axonal pathfinding of anterior commissure axons (Henkemeyer *et al.*, 1996). Interestingly, analysis of similar mutant mice which retained the extracellular and transmembrane domains of EphB2/Nuk but had its tyrosine kinase region replaced by bacterial LacZ sequences did not exhibit this defect, thus raising the possibility that certain Eph receptors and their transmembrane ligands mediate bidirec-

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tional cell signaling. Consistent with this hypothesis, members of the ephrin-B subgroup are phosphorylated by an as yet unidentified tyrosine protein kinase upon binding to EphB2/Nuk (Holland *et al.*, 1996).

We have isolated recently the mouse homolog of *eek*, a partially cloned rat gene that encodes a member of the Eph family of tyrosine kinase receptors (Chan and Watt, 1991). Its predicted amino acid sequence is more closely related to the EphA than to the EphB subgroup of receptors. Therefore, this receptor has been designated as EphA8. As expected, EphA8/Eek binds to three GPI-linked ligands, ephrin-A2/ELF-1, ephrin-A3/Ehk1L and ephrin-A5/AL-1/RAGS, with similar nanomolar affinities (Park and Sanchez, 1996). Previous studies have indicated that the rat *eek* gene is expressed in the developing nervous system (Chan and Watt, 1991). In the present studies, we have utilized gene targeting techniques to generate mice lacking EphA8/Eek receptors in order to analyze their role in the development of the mammalian nervous system. Our results indicate that EphA8/Eek receptors play an important role in axonal pathfinding for a subset of tectal commissural axons.

Results

Inactivation of the ephA8/eek gene

The mouse *ephA8/eek* locus was disrupted by homologous recombination in embryonic stem (ES) cells by using a strategy that involved the in-frame insertion of sequences encoding the transmembrane region of the human TrkA receptor (Martin-Zanca *et al.*, 1989) followed by the bacterial β-galactosidase gene (*lacZ*) within an exon corresponding to the extracellular region of the EphA8/Eek receptor (Figure 1A). This strategy yielded a mutant allele that encoded a chimeric transmembrane protein of 1205 amino acid residues with a short extracellular domain corresponding to the 116 amino-terminal residues of EphA8/Eek, the TrkA transmembrane domain and a cytoplasmic region consisting of a functional β-galactosidase protein (Figure 1B).

Developmental expression of the ephA8/eek gene

The developmental expression of the *ephA8/eek* gene was determined by X-gal histochemistry in mice heterozygous (+/–) and homozygous (–/–) for the targeted allele. LacZ expression was first detected at embryonic day (E) 10.5. At this time, the highest levels of expression were observed near the midline region of the tectum (Figure 2). Less intense X-gal staining was found in discrete regions of the hindbrain, the dorsal horn of the spinal cord and in the naso-lacrimal groove (Figure 2A). LacZ expression in these regions decreased after E12.5 and was barely detectable in late gestation embryos (E17.5) and in postnatal animals (see below). No new areas of LacZ expression appeared during postnatal development.

Sagittal sectioning of E10.5 embryos revealed that LacZ expression localizes to a highly defined region in the rostral area of the tectum (Figure 3). The intensity of X-gal staining suggests that *ephA8/eek* is expressed in a decreasing rostral to caudal gradient (Figure 3), a property previously observed for other members of the Eph receptor family expressed in the chicken retino-tectal and the mouse hippocampal/septal systems (Chen *et al.*, 1995; Gao *et al.*, 1996). The graded expression of *ephA8/eek* transcripts in the superior colliculus was confirmed by *in situ* hybridization (data not shown). X-gal staining in this region continued to be high in mid and late gestation embryos (E12.5–E17.5) (data not shown). At birth, LacZ expression localized to a subpopulation of superior colliculus cells (Figure 4A and B). X-gal staining of this region decreased dramatically after birth, appearing very weak by postnatal day (P) 5 and no longer detectable after P10 (Figure 4C). To date, none of the antisera elicited against EphA8/Eek receptors has been useful for immunohistochemical studies, thus preventing us from comparing their pattern of expression with that of X-gal staining. However, *in situ* hybridization studies using wild-type mice demonstrated the presence of *ephA8/eek* transcripts in the same structures (data not shown), indicating that insertion of the bacterial *lacZ* sequences within the *ephA8/eek* locus did not alter its expression pattern.

Analysis of LacZ expression in the superior colliculus of postnatal (–/–) mice suggests that the temporal regulation of *ephA8/eek* expression might be slightly altered in these animals. As illustrated in Figure 4A and B, X-gal staining in the superior colliculus of P0 (–/–) mice was considerably more robust than the 2-fold increase expected for these animals when compared with their (+/–) littermates. Moreover, LacZ expression was still detectable in sections from adult (–/–), but not (+/–), animals (Figure 4C and D). These results raise the possibility that EphA8/Eek signaling may down-regulate its own expression. Alternatively, the maturation program of these cells may be altered in the absence of functional EphA8/Eek receptors, perhaps by preventing certain differentiation events that result in the silencing of this gene in normal, mature neurons.

The presence of LacZ expression in (–/–) mice strongly suggests that EphA8/Eek receptors are not necessary for the survival of EphA8/Eek-expressing cells. Morphological examination of these cells in the superior colliculus of either (+/–) or (–/–) mice suggested that they correspond to neurons. They were medium sized, could be stained with Nissl staining and were scattered throughout the superficial layers of the superior colliculus. Moreover, all LacZ-expressing superior colliculus cells in adult (–/–) mice reacted with a specific monoclonal antibody elicited against NeuN, a neuron-specific marker (Figure 5), but not with antibodies specific for astrocyte- or oligodendrocyte-specific markers (data not shown).

A subset of tectal commissural axons are absent in the ephA8/eek (–/–) mice

Mice homozygous for the disrupted *ephA8/eek* allele do not show any overt anatomical or behavioral phenotype. They reach adulthood and are fertile. Based on recent results obtained with other Eph receptors and their cognate ligands (Chen *et al.*, 1995; Drescher *et al.*, 1995; Winslow *et al.*, 1995), we examined whether these (–/–) mutant mice had a defect in axonal pathfinding. In rodents, the superior colliculus is the main target for retinal ganglion cell axons, and serves as an integrator of visual input as well as of other sensory modalities. Superior colliculus neurons send efferent projections to multiple regions, mostly located in the diencephalon, medula and midbrain. *ephA8/eek* expression is restricted to a previously undefined subpopulation of superior colliculus neurons

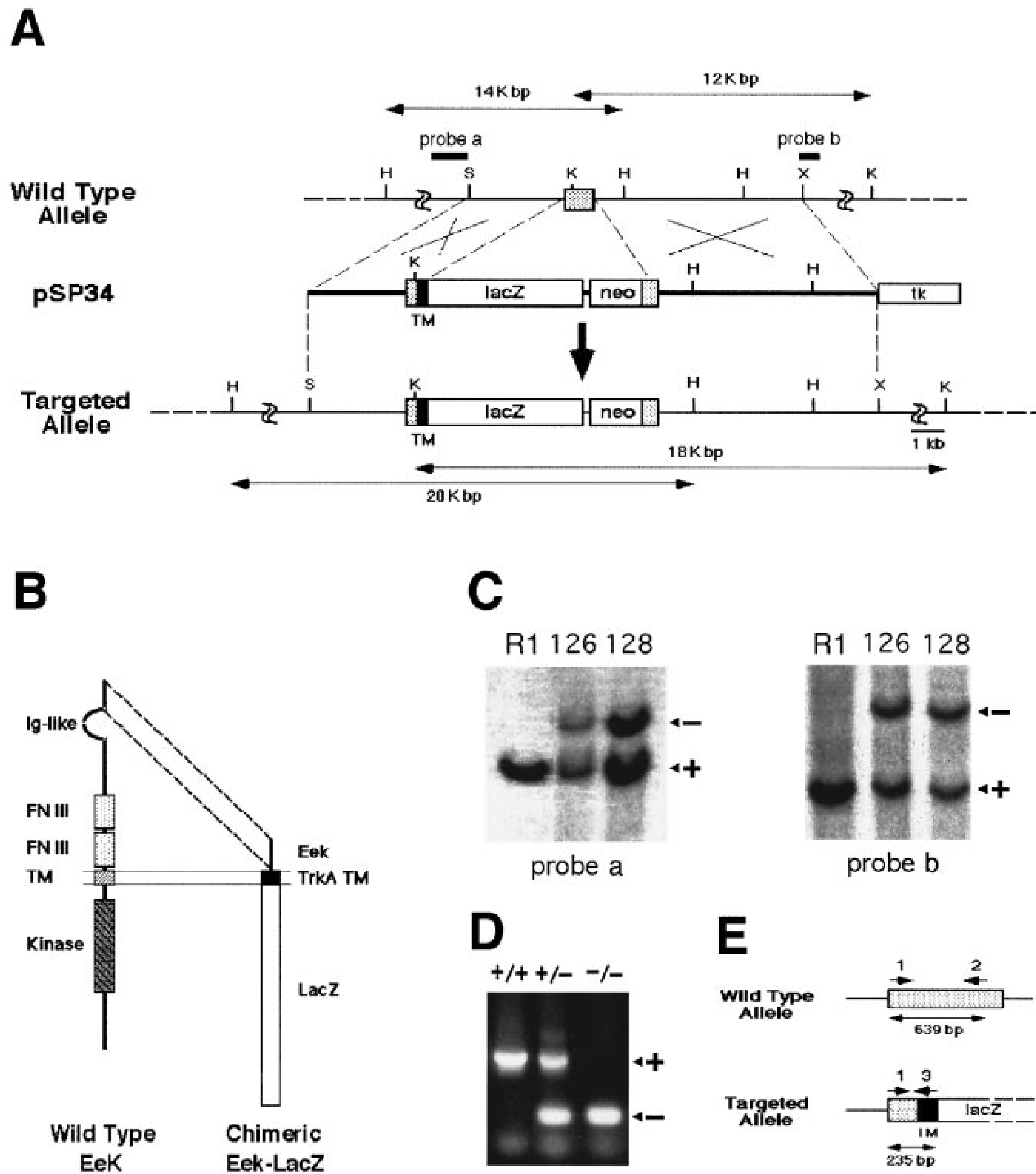

Fig. 1. Generation of *ephA8/eek* (–/–) mutant mice. (**A**) Schematic diagram of the strategy used to target the *ephA8/eek* gene. Top: partial restriction map of wild-type genomic DNA encompassing the targeted exon (dotted box) (H, *Hin*dIII; K, *Kpn*I; S, *Sac*I; X, *Xba*I). This DNA region contains other exons that remain to be mapped. Middle: the targeting vector, pSP34, contains the above indicated exon in which we have inserted in-frame sequences corresponding to the transmembrane region of the human TrkA receptor (residues 401–445, black box) and the bacterial *lacZ* gene (open box, LacZ). pSP34 also contains the *neo* (open box, neo) and thymidine kinase (open box, tk) genes driven by the phosphoglycerate kinase (PGK) promoter. Bottom: schematic diagram of the predicted targeted allele resulting from a homologous recombination event between the wild-type allele and the targeting vector. Sequences used for Southern blot analysis of recombinant ES cell clones (probes a and b) are indicated by black bars. Probe a recognizes *Hin*dIII DNA fragments of 14 (wild-type allele) and 20 kbp (targeted allele). Probe b detects *Kpn*I DNA fragments of 12 (wild-type allele) and 18 kbp (targeted allele). (**B**) Schematic representation of the wild-type EphA8/Eek receptor (left) and the predicted chimeric EphA8/Eek–LacZ fusion protein (right) encoded by the targeted allele depicted in (A). Structural domains include: Ig-like, immunoglobulin-like domain; FN III, fibronectin type III-like domains; TM, transmembrane domain; kinase, catalytic domain; EphA8/Eek, sequences derived from the EphA8/Eek receptor; TrkA TM, transmembrane domain derived from the human TrkA receptor; and LacZ, bacterial β-galactosidase. Dotted lines indicate the region of the EphA8/Eek receptor present in the chimeric EphA8/Eek–LacZ protein. (**C**) Southern blot analysis of the parental ES cells (clone R1) and of two representative recombinant clones (B249-126 and B249-128) used to generate chimeric mice. Probes (a and b) are those described in (A). Migration of *Hin*dIII (probe a) and *Kpn*I (probe b) DNA fragments derived from wild-type (+) and targeted (–) alleles is indicated by arrowheads. The size of these DNA fragments is indicated in (A). (**D**) PCR-aided amplification of DNAs isolated from tails of littermates derived from crosses between (+/–) mice. Migration of DNA fragments derived from wild-type (+) (639 bp) and targeted (–) (235 bp) alleles is indicated by arrowheads. (**E**) Schematic representation of the strategy used to amplify *ephA8/eek* sequences from wild-type and targeted alleles. Primers are indicated by arrows. Boxes are those described in (A).

which are scattered in the superficial layers of this structure. The lack of anti-EphA8/Eek-specific antibodies capable of detecting these receptors by immunohistochemical techniques has prevented us from identifying the axonal projections as well as the targets of these superior colliculus neurons.

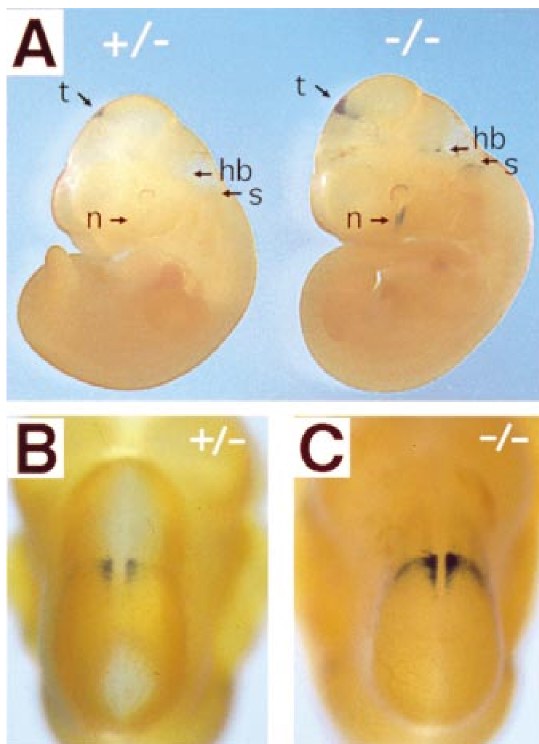

Fig. 2. Expression of the targeted *ephA8/eek* gene in E10.5 embryos. (**A**) Whole mount X-gal staining of (+/–) and (–/–) E10.5 embryos. LacZ expression is seen in the midbrain tectum (t), hindbrain (hb), rostral region of the spinal cord (s) and naso-lacrimal groove (n). Staining is more evident in the (–/–) embryo. No staining could be observed in wild-type littermates (not shown). (**B** and **C**) Vertical view (from above) of the E10.5 (+/–) and (–/–) embryos depicted in (A).

To search for a possible defect in tectal projections, we placed a tiny crystal of the fluorescent dye 1,1′-dioctodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) in the superficial part of the left superior colliculus of (+/+) and (–/–) adult mice. Analysis of serial sections throughout the brain and brainstem of these mice revealed that most projections are present in the (–/–) animals. A possible exception was the caudal region of the contralateral inferior colliculus (Gonzalez-Hernandez *et al.*, 1987), where the number of nerve fibers was reduced significantly (Figure 6A and B). Similar results were obtained with younger animals (P0 and P5) (data not shown). In order to provide additional support for this observation, we performed retrograde labeling experiments in which the DiI crystal was placed in the superficial part of the caudal region of the inferior colliculus of both wild-type and (–/–) mice. As expected, the wild-type animals displayed abundant labeling in the contralateral superior and inferior colliculus (Figure 6C). Similar results were observed in the contralateral inferior colliculus of the (–/–) mice (Figure 6D). However, only sparse labeling could be observed in the contralateral superior colliculus of these mice, even in animals in which there was robust labeling of the adjacent inferior colliculus (Figure 6D). These results indicate that in the absence of EphA8/Eek receptors, a subpopulation of commissural superior colliculus neurons does not innervate their targets in the contralateral inferior colliculus.

Abnormal axonal projections in the spinal cord of ephA8/eek (–/–) mice

The observed decrease of superior colliculus afferents in the caudal region of the inferior colliculus of (–/–) mice

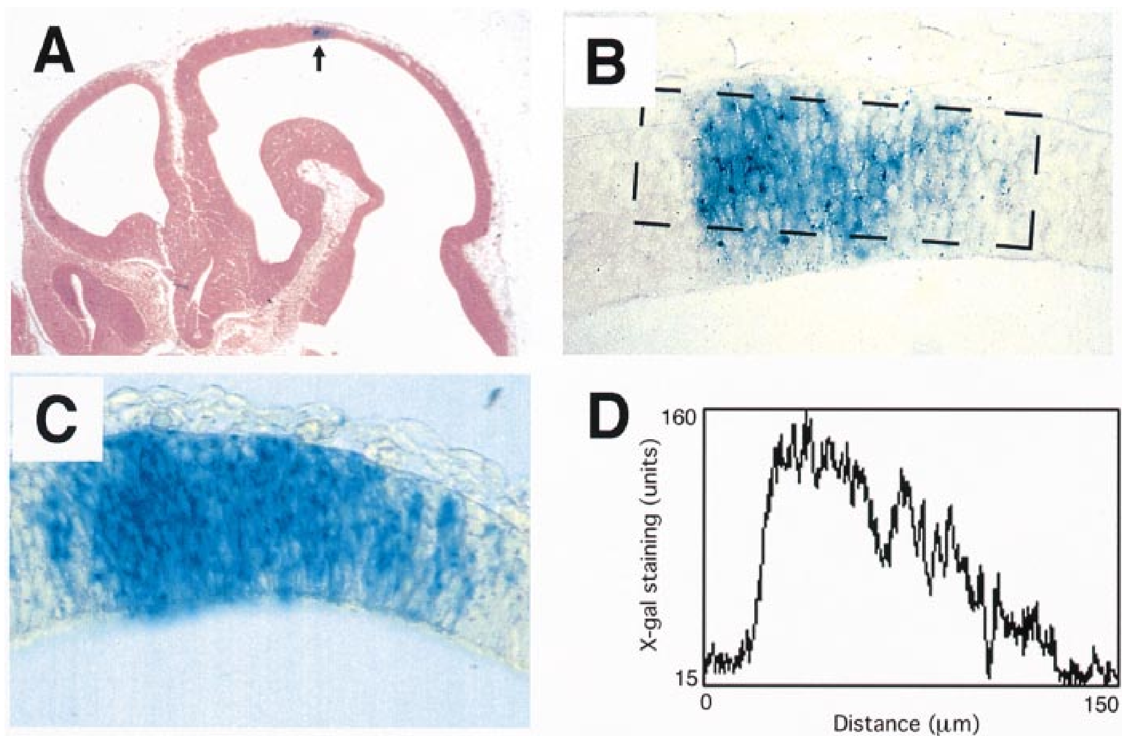

Fig. 3. The *ephA8/eek* gene is expressed along a rostral–caudal gradient. LacZ expression in sagittal sections of (**A** and **B**) (+/–) and (**C**) (–/–) E10.5 embryos. Rostral is to the left. (**D**) Densitometric analysis (NIH Image 1.52 software) of X-gal staining (dotted box) depicted in (B).

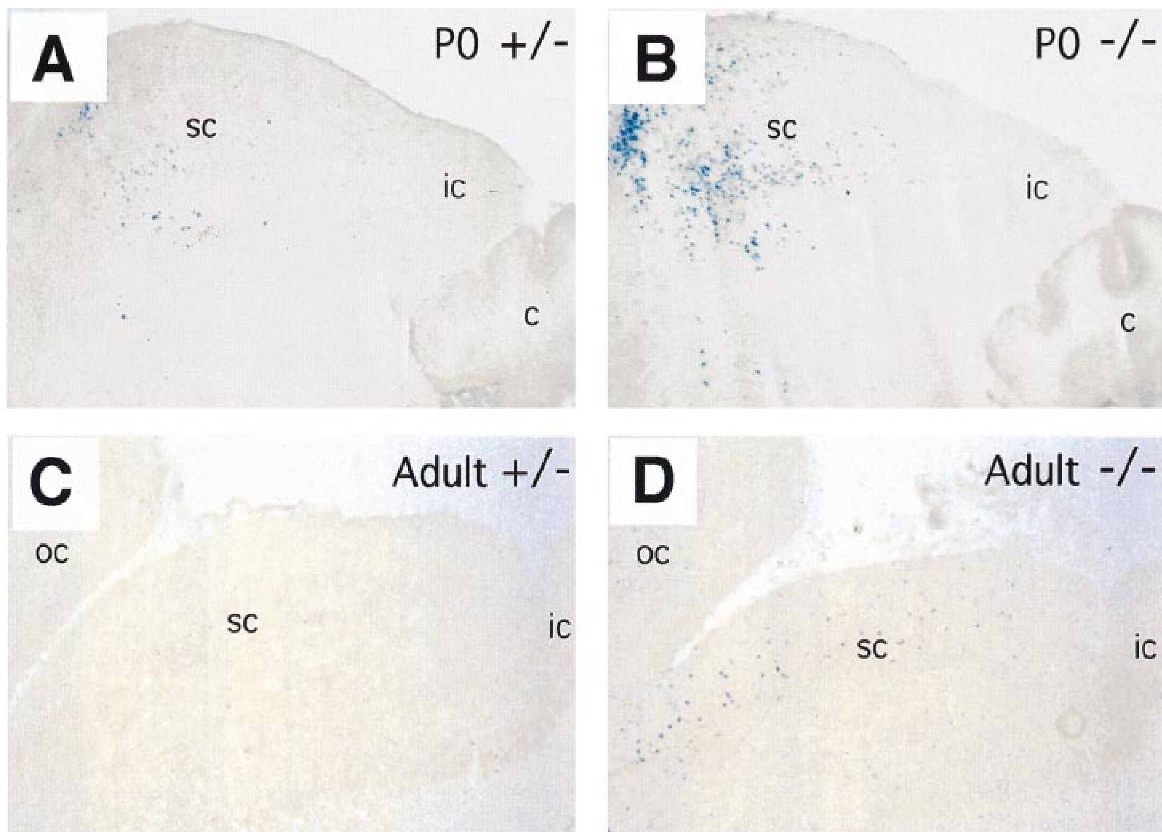

Fig. 4. Expression of the *ephA8/eek* gene in a subpopulation of superior colliculus cells. LacZ expression in (**A**) (+/–) and (**B**) (–/–) P0 mice. LacZ expression in (**C**) (+/–) and (**D**) (–/–) adult (15 weeks) mice. Occipital cortex (oc), superior colliculus (sc), inferior colliculus (ic) and cerebellum (c) are indicated.

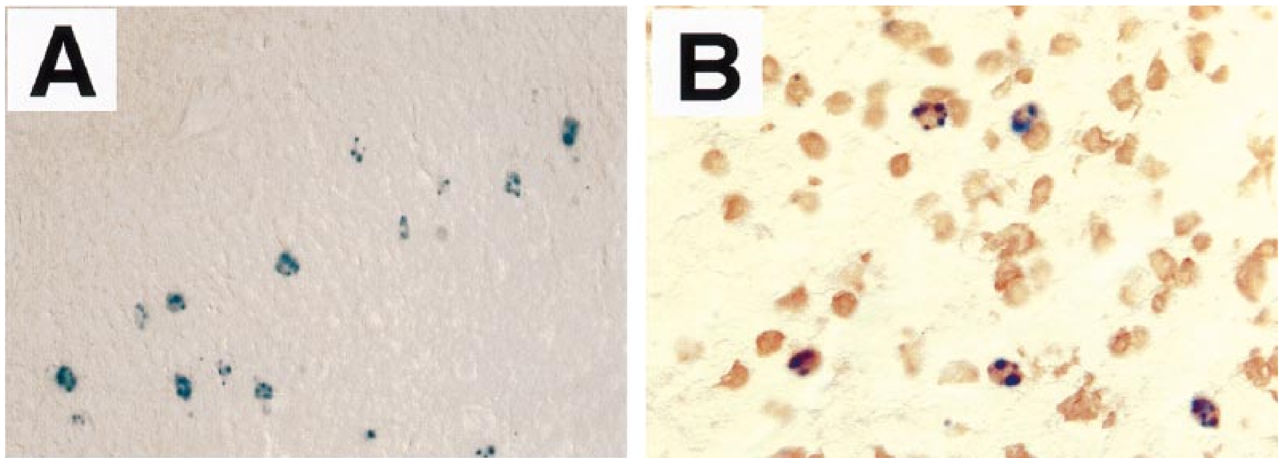

Fig. 5. The *ephA8/eek* gene is expressed in neurons. X-gal staining of a sagittal section of the superior colliculus of an adult (–/–) mouse either (**A**) alone or (**B**) followed by incubation with a neuron-specific NeuN antibody.

may reflect a lack of axonal outgrowth in those neurons deprived of EphA8/Eek receptors. Alternatively, the absence of these receptors may result in misguided axonal growth. To examine this possibility, we analyzed serial sections of the major structures of the central nervous system of (+/+), (+/–) and (–/–) adult mice which had received a tiny DiI crystal in the superficial superior colliculus. No major differences were observed in any of the brain, brainstem or cerebellar structures examined. However, the midcervical spinal cord of (–/–) mice, but not of their (+/+) littermates, showed the presence of a significant tract of DiI-labeled fibers in the ipsilateral ventral funiculus (Figure 7A and B). These fibers project branches to the ventral horn, suggesting that they form connections with a subpopulation of spinal cord neurons (Figure 7B). Similar results were obtained with younger animals (P0 and P5) (data not shown). All (–/–) mice analyzed in this study (n = 15) exhibited reduced innervation of the inferior colliculus from the contralateral superior colliculus and an aberrant fiber tract in the ipsilateral spinal cord, indicating complete penetrance of this phenotype.

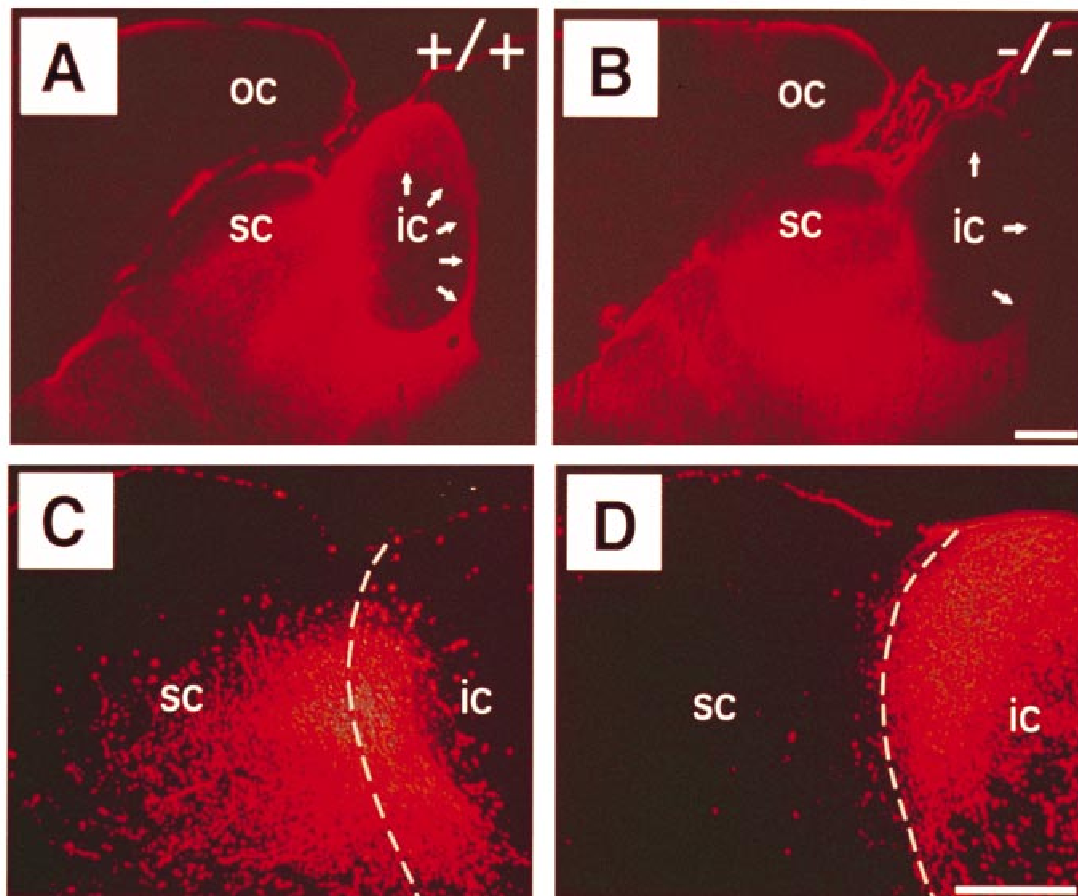

Fig. 6. Axonal projections from the superior and inferior colliculus in (A and C) (+/+) and (B and D) (–/–) mice. (**A** and **B**) Sagittal cryostat sections through the right hemisphere of adult mice which received a small DiI crystal in the superficial region of the left superior colliculus (rostral is to the left). (**C** and **D**) Retrograde labeling of the right superior colliculus in P5 mice which received a small DiI crystal in the superficial region of the left inferior colliculus (rostral is to the left). The occipital cortex (oc), superior colliculus (sc) and inferior colliculus (ic) are indicated. The border between the superior and inferior colliculus is indicated by a dashed line. Scale bar = 500 μm.

To determine the origin of these aberrant axonal tracts, we inserted a DiI crystal in the left ventral funiculus of the midcervical spinal cord of (+/+) and (–/–) animals. In the wild-type animals, the expected structures in the brain, including the cortex, the deep layers of the tectum and the brainstem nuclei, become labeled. Similar results were observed in the (–/–) animals (data not shown). However, when we sectioned the ipsilateral superior colliculus of these animals, only those derived from the (–/–) mutant mice displayed significant retrograde labeling in the superficial layers (Figure 7C and D). These results suggest that the aberrant projections observed in mice lacking EphA8/Eek receptors are likely to originate in the superior colliculus.

Retrograde labeling of ephA8/eek-expressing neurons

To determine whether the aberrant axonal tract found in (–/–) mice originates from *ephA8/eek*-expressing cells, we performed additional retrograde tracing experiments using fluorogold followed by X-gal staining of the same sections. As illustrated in Figure 8, cell bodies of superficial superior colliculus neurons of (–/–) but not of (+/–) mice were retrogradely labeled when mice were injected with fluorogold tracer in the ipsilateral cervical region of the spinal cord. A significant number of these fluorogold-labeled neurons expressed the chimeric EphA8/Eek–LacZ protein as determined by X-gal staining (Figure 8C and D). These observations indicate that at least some of the aberrant axonal projections found in the spinal cord of (–/–) mutant mice originate in those neurons of the ipsilateral superior colliculus that normally express EphA8/Eek receptors. In agreement with the DiI tracing experiments, fluorogold injected in the caudal region of the inferior colliculus retrogradely labeled contralateral X-gal-stained superior colliculus neurons in (+/–), but not in (–/–) mice (Figure 8E–H). These observations indicate that in the absence of EphA8/Eek receptors, at least certain *ephA8/eek*-expressing superior colliculus neurons do not project comissural axons and display ipsilateral projections to the spinal cord not found in wild-type animals.

Discussion

During development, neurons project axons over large distances with high precision. Correct axonal pathfinding is likely to be mediated by complementary positional labels located on the developing axons and cells lining the growth path, as well as on target cells (Sperry, 1963). The molecular nature of these labels is beginning to be unveiled (Tessier-Lavigne and Goodman, 1996). Recent evidence suggests that certain members of the Eph receptor

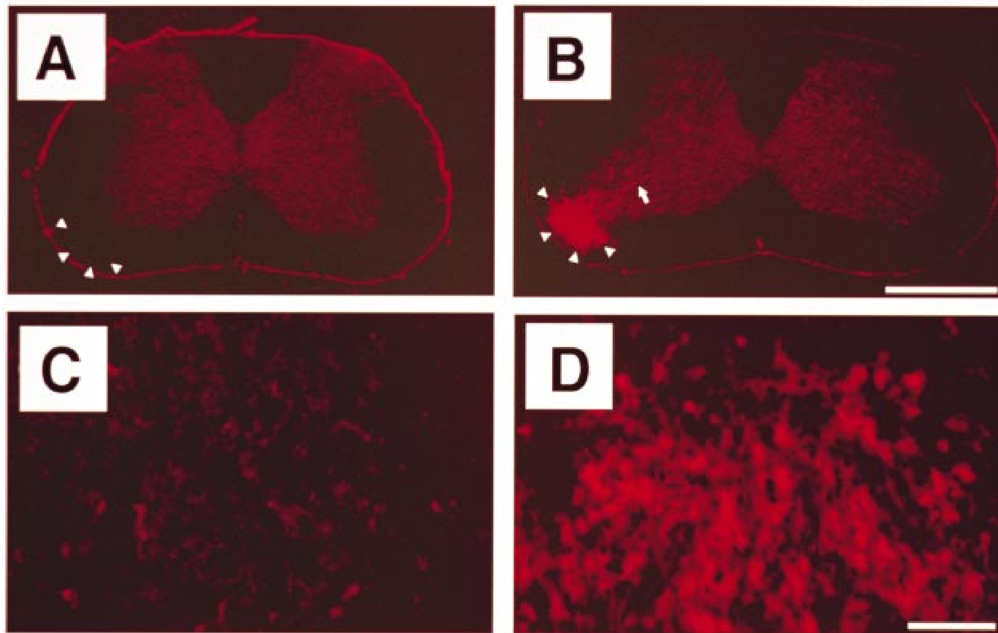

Fig. 7. Axonal projections from the superior colliculus and cervical spinal cord in (A and C) (+/+) and (B and D) (–/–) mice. (**A** and **B**) Cross-sections of the cervical spinal cord of adult mice that received a small DiI crystal in the superficial region of the left superior colliculus. A dense DiI-labeled axon tract is seen in the left (ipsilateral to the DiI application) ventral funiculus of (–/–) adult mice (arrowheads). No such DiI-labeled axons are seen in the (+/+) animals. The white arrow in (B) indicates axonal branches projecting to the ventral horn. (**C** and **D**) Retrograde labeling of the left superior colliculus of P5 mice which received a DiI crystal in the left ventral funiculus of the cervical spinal cord. Serial sectioning through the superior colliculus of these animals only revealed DiI labeling in the (–/–) mice. Scale bars are 500 μm in (A) and (B), 100 μm in (C) and (D).

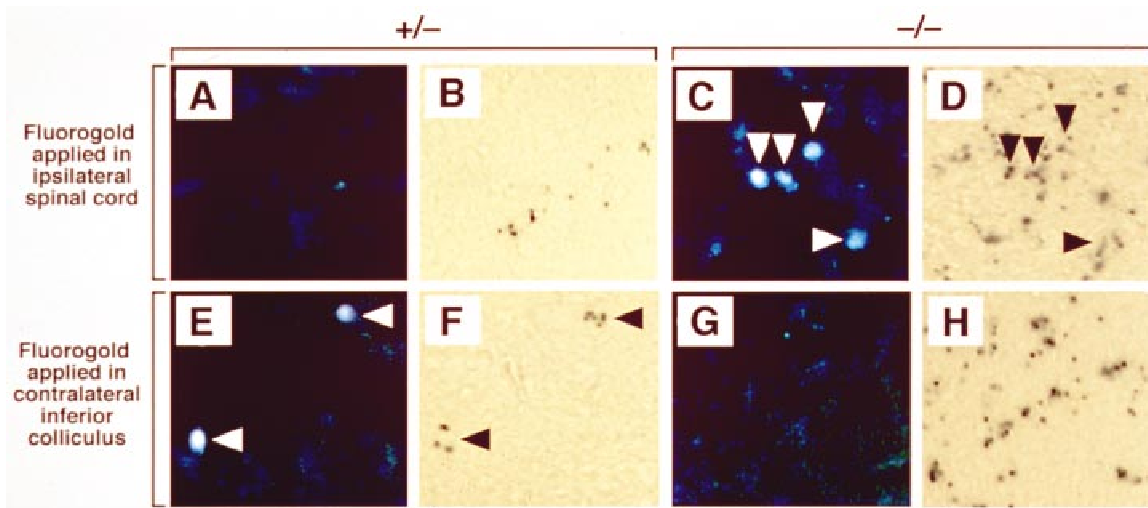

Fig. 8. Retrograde labeling of LacZ-expressing neurons in the superior colliculus of (A, B, E and F) (+/–) and (C, D, G and H) (–/–) mice. (**A** and **C**) Retrograde labeling of superior colliculus neurons after fluorogold application in the ipsilateral cervical spinal cord of P5 mice. (**B** and **D**) X-gal staining of the same sections. (**E** and **G**) Retrograde labeling of superior colliculus neurons after fluorogold application in the contralateral inferior colliculus of P5 mice. (**F** and **H**) X-gal staining of the same sections. Animals were allowed to survive for 16 h after fluorogold application. White arrowheads indicate fluorogold-labeled cells. Black arrowheads indicate X-gal staining in the same cells.

and ephrin ligand families play a role in axonal pathfinding (Chen *et al.*, 1995; Drescher *et al.*, 1995; Nakamoto *et al.*, 1996). The results described in this study, utilizing gene-targeted mice lacking EphA8/Eek tyrosine protein kinase receptors, provide genetic evidence in support of this concept. Specifically, our observations indicate that EphA8/Eek is required for the generation of proper axonal projections during the development of the mammalian nervous system. In the absence of these receptors, certain *ephA8/eek*-expressing superior colliculus neurons do not project commissural axons to putative targets in the contralateral inferior colliculus. In addition, these neurons display ipsilateral projections to the spinal cord not found in wild-type animals.

Our observations do not establish the ontogeny of these aberrant axonal tracts. It is known that during development, CNS neurons often send projections that overshoot their targets to retract later in development (O'Leary and Koester, 1993). As a consequence, some of these projections may reach the cervical region of the spinal cord during embryogenesis. Therefore, it is possible that in the absence of EphA8/Eek receptors, these overextended

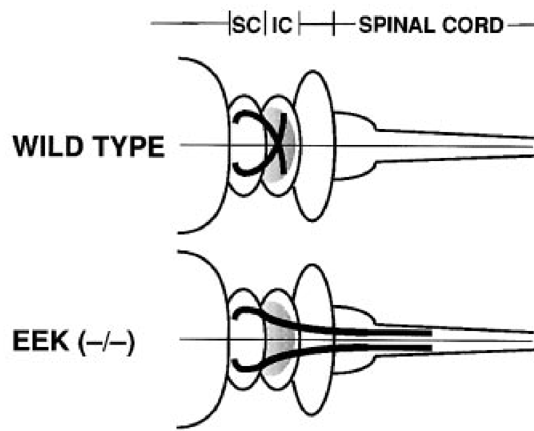

Fig. 9. Schematic diagram of a model depicting the (top) normal and (bottom) aberrant projections (thick solid line) of EphA8/Eek-expressing neurons in (+/+) and (–/–) mice. The shaded area represents a rostral–caudal gradient of expression of the putative ligand(s) of the EphA8/Eek receptors.

projections are not fully eliminated and may account for the abnormal tracts observed in *ephA8/eek* (–/–) newborn or adult mice. This mechanism, however, would not explain the absence of commissural axons innervating the inferior colliculus. It is possible that the absence of EphA8/Eek receptors has different consequences in different subpopulations of neurons and that they are required for both generation of commissural axons and regression of overshooting immature projections. Alternatively, EphA8/Eek receptors may play a role in axonal guidance (Figure 9). These receptors may recognize positional cues and direct the axonal projections of *ephA8/eek*-expressing neurons through the midline and towards targets in the contralateral inferior colliculus. In the absence of EphA8/Eek receptors, the growing axonal projections would not be able to recognize these cues and may follow an aberrant track (specified by other positional cues) away from the midline and towards the ipsilateral spinal cord.

The mechanism by which EphA8/Eek receptors may mediate axonal pathfinding remains to be determined. We recently have established that EphA8/Eek can serve as a receptor for at least three ephrins of the GPI-linked subgroup, ephrin-A2/ELF-1, ephrin-A3/Ehk1L and ephrin-A5/AL-1/RAGS (Park and Sanchez, 1996). Each of these molecules bind EphA8/Eek receptors with similar nanomolar affinity and activate their tyrosine kinase activity with comparable efficiency. Interestingly, ephrin-A2/ELF-1 and ephrin-A5/AL-1/RAGS are expressed in antero-posterior gradients in the developing tectum, with highest levels in the posterior part of the tectum (Chen *et al.*, 1995; Drescher *et al.*, 1995; Nakamoto *et al.*, 1996). Moreover, both these ephrins have repellent activity towards axons that presumably express Eph receptors (Drescher *et al.*, 1995; Nakamoto *et al.*, 1996). Unfortunately, the limited number of neurons expressing EphA8/Eek receptors in mice makes it very difficult to examine the nature of their response to its various ligands in *in vitro* assays. However, if Eph ligands, including ephrin-A2/ELF-1 and ephrin-A5/AL-1/RAGS, would act as repellents for EphA8/Eek-containing axons, they may prevent them from reaching the most caudal region of the inferior colliculus and possibly guide them through the midline towards the contralateral inferior colliculus. If so, neurons lacking EphA8/Eek receptors may be able to continue through the ipsilateral inferior colliculus towards the spinal cord, presumably following other topographic cues intended for different neurons.

Recently, Henkemeyer *et al.* (1996) have reported axonal pathfinding defects in mice lacking EphB2/Nuk, a receptor that preferentially recognizes transmembrane-containing ephrins. In these mice, axons of the anterior commissure fail to reach their targets and project to the floor of the brain. In this system, the axons of the anterior commissure express the transmembrane ligands whereas the EphB2/Nuk receptors are present in the ventral cells over which the anterior commissure axons grow. Therefore, these receptors are likely to mediate a repulsive activity that prevents these axons from reaching the floor of the brain. Interestingly, animals carrying mutant EphB2/Nuk receptors in which their tyrosine kinase domain has been replaced by LacZ sequences do not display this defect, indicating that the catalytic activity of EphB2/Nuk is not critical for anterior commissure guidance. Whereas there are several possible mechanisms to explain these observations, the most intriguing is the possibility that ephrin-B ligands have their own signaling function.

The aberrant projections found in both *ephA8/eek* (–/–) and *ephB2/nuk* (–/–) mutant mice follow defined axonal tracts. These observations suggest that these projections recognize topographic cues even in the absence of specific Eph receptors. How neurons respond to the appropriate set of positional cues remains to be determined. It is possible that the nature of the signals provided by the interaction between certain Eph receptors and their cognate ephrins determines a hierarchical order in which only the strongest signals result in a biological response. Alternatively, such a hierarchy might be determined by the relative levels of expression of a given positional receptor–ligand system. Generation of additional strains of mice defective for other Eph receptors as well as their cognate ephrins should provide relevant information regarding the mechanism by which these signaling molecules contribute to the establishment of precise axonal projections in the developing mammalian nervous system.

Materials and methods

Targeting of the ephA8/eek gene in ES cells

Genomic *ephA8/eek* DNA clones were isolated from a library of 129/Sv mouse DNA. To generate the left arm of the targeting vector, a 3.5 kbp *Sac*I–*Kpn*I DNA fragment encompassing sequences encoding the EphA8/Eek receptor was subcloned into pBluescript and inserted in-frame with sequences corresponding to the transmembrane domain of the human TrkA receptor (amino acid residues 401–445) (Martin-Zanca *et al.*, 1989). These sequences were next subcloned in-frame with a 4.2 kbp DNA fragment encompassing the bacterial *lacZ* gene followed by the polyadenylation signal of SV40. The resulting 7.8 kbp DNA fragment was blunt-ended and subcloned into the *Xho*I site of the pPNT vector (Tybulewicz *et al.*, 1991) to generate the left arm plasmid. The right arm plasmid was obtained by subcloning a 8.2 kbp *Kpn*I–*Not*I DNA fragment of *ephA8/eek* genomic sequences into the pPNT vector. Finally, DNA inserts encompassing the left and right arms were combined to generate the targeting vector (pSP34) depicted in Figure 1A. ES cells (R1 clone) were transfected with pSP34 DNA by electroporation as previously described (Joyner, 1993). Clones carrying the expected mutation were used to generate chimeric mice by blastocyst injection. Chimeras derived from two clones (B249-126 and B249-128) transmitted the targeted allele when bred to wild-type C57Bl/6 mice. The resulting

heterozygous mice subsequently were bred to wild-type C57Bl/6 mice to propagate the mutant allele or among themselves to generate homozygous mice. Therefore, the mice utilized in this study have a mixed genetic background of 129/Sv and C57Bl/6 strains. PCR analysis for routine genotyping used the following oligonucleotides as primers: 5′-TGGG-ACTCCATCAACGAGGTAGACG-3′ (primer 1), 5′-TAGCCAGCACT-GCACACGCACTTGC-3′ (primer 2) and 5′-CACAGCCACCGAGA-CCCCAAAAGGT-3′ (primer 3). Genomic DNA for PCR analysis was extracted from either mouse tails or embryonic tissues using a Qiagen kit.

β-Galactosidase staining and immunohistochemistry

For whole mount staining, embryos were collected in phosphate-buffered saline (PBS) and incubated at room temperature for 30 min in solution B (0.2% glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$ in 0.1 M potassium phosphate, pH 7.4). Embryos were washed three times in solution C (0.01% Na deoxycholate, 0.02% NP-40 in solution B), and incubated at 37°C overnight in solution D (0.5 mg/ml X-gal, 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide in solution C). Embryos were then rinsed and stored in solution C at 4°C. For histochemical analysis, embryos were dehydrated in ethanol, embedded in paraffin and sectioned at 8 μm. The NIH Image 1.52 software was used for image analysis in scanned slides.

For immunohistochemical detection of NeuN, anesthesized mice were perfused with PBS followed by 4% formaldehyde in PBS. Cryostat sections were first processed for X-gal staining using the procedure described above for whole mount embryo staining, and then incubated for 1 h at 37°C with a mouse monoclonal antibody against NeuN (Mullen *et al*., 1992; diluted 1:100) followed by biotin-conjugated horse anti-mouse serum. The Vector ABC Elite kit was used for visualization of bound antibodies.

Axonal tracing

For DiI labeling, mice were perfused with PBS followed by 4% formaldehyde in PBS and the brains with spinal cord were dissected out. A tiny DiI crystal (Molecular Probes) was placed in the superficial left superior colliculus in young animals (P0 and P5) or adult mice. Their brains were incubated in formalin solution at 37°C for 3 weeks for P0 and P5 brains, and 6 weeks for adult brains. The brains were then incubated in 10% sucrose in PBS, and 14 μm serial sagittal sections were cut through the desired region. DiI labeling in the inferior colliculus and rostral spinal cord was performed in a similar way. For retrograde axonal tracing with fluorogold (Molecular Probes), P5 animals were anesthesized by hypothermia and a fluorogold crystal was placed in the left inferior colliculus. The animals were allowed to survive for 16 h and were then perfused with PBS followed by 4% formaldehyde in PBS. Serial sections were cut and photographed in a fluorescence microscope prior to subjecting the same sections to β-galactosidase staining for co-localization studies. Labeling of the rostral spinal cord with fluorogold was performed in a similar way.

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