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Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation

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Sek4 and Nuk are members of the Eph-related family of receptor protein-tyrosine kinases. These receptors interact with a set of cell surface ligands that have recently been implicated in axon guidance and fasciculation. We now demonstrate that the formation of the corpus callosum and anterior commissure, two major commissural axon tracts that connect the two cerebral hemispheres, is critically dependent on Sek4 and Nuk. While mice deficient in Nuk exhibit defects in pathfinding of anterior commissure axons, *sek4* mutants have defects in corpus callosum formation. The phenotype in both axon tracts is markedly more severe in *sek4/nuk[1]* double mutants, indicating that the two receptors act in a partially redundant fashion. *sek4/nuk[1]* double mutants also exhibit specific guidance and fasciculation defects of diencephalic axon tracts. Moreover, while mice singly deficient in either Sek4 or Nuk are viable, most *sek4/nuk[1]* double mutants die immediately after birth primarily due to a cleft palate. These results demonstrate essential and cooperative functions for Sek4 and Nuk in establishing axon pathways in the developing brain, and during the development of facial structures.

Keywords: axon fasciculation/axon guidance/Eph-related receptors/palate formation/tyrosine kinase

Introduction

Receptor protein-tyrosine kinases (RTKs) play essential roles in cell proliferation, differentiation, migration and survival. The largest group of RTKs is the Eph-related receptor family, named after its prototypic member (Hirai *et al.*, 1987). At least 13 different members of this family have been identified in vertebrates (for reviews, see Tuzi and Gullick, 1994; Brambilla and Klein, 1995). Eph-related receptors bind to a set of ligands, named Lerks (Pandey *et al.*, 1995), which in order to be functionally active require membrane attachment either by a glycosylphosphatidylinositol (GPI) anchor or by a single hydrophobic transmembrane domain (Davis *et al.*, 1994). It is therefore thought that Lerks and their Eph-related receptors mediate intimate cell-to-cell signalling, rather than long-range interactions.

Recent evidence suggests that one of the GPI-anchored ligands, AL1/RAGS, can regulate both axon fasciculation and guidance *in vitro*. In a neuron/astrocyte co-culture assay, axon fasciculation was blocked by either soluble AL1/RAGS or a soluble receptor fusion protein (Winslow *et al.*, 1995). Using both growth cone collapse and stripe assays, AL1/RAGS has also been shown to have a repulsive effect on retinal ganglion cell axons, suggesting that this ligand may be an important axon guidance signal that participates in organizing topographic projections of retinal axons to the optic tectum (Drescher *et al.*, 1995; for a review, see also Tessier-Lavigne, 1995). A second GPI-anchored ligand, Elf1, and its receptor, Mek4, may also play a role in the retinotectal system, based on their complementary expression gradients in the optic tectum and retina, respectively (Cheng *et al.*, 1995). Using a dominant-negative approach, it was shown that one of the receptors for Elf1, Sek1/Rtk1, has a role in patterning of the developing forebrain and in regulating cell movement in the segmented hindbrain (Xu *et al.*, 1995, 1996).

Binding and functional assays suggest that Eph-related receptors and their ligands fall into two groups. The two transmembrane-type ligands, Lerk2 and Lerk5/Elf2, specifically interact with a subgroup of four Eph-related receptors (the 'Elk subgroup'), including Sek4, Nuk, Elk and Htk [see Brambilla and Klein (1995) for alternative names in other species]. In contrast, the GPI-anchored ligands (B61, Lerk3, Lerk4, Elf1 and AL1/RAGS) bind a distinct subgroup of receptors (the 'Eck subgroup'), which includes Eck, Ehk1, Mek4 and Sek1. Within each subgroup, there is considerable cross-reaction between receptors and ligands (Bartley *et al.*, 1994; Cheng and Flanagan, 1994; Davis *et al.*, 1994; Bergemann *et al.*, 1995; Brambilla and Klein, 1995; Brambilla *et al.*, 1995; Kozlosky *et al.*, 1995). However, expression patterns of ligand and receptor pairs are only partially overlapping, suggesting that each receptor may bind distinct ligands in different tissues (Sajjadi and Pasquale, 1993; Becker *et al.*, 1994; Cheng and Flanagan, 1994; Henkemeyer *et al.*, 1994; Bergemann *et al.*, 1995; Bouillet *et al.*, 1995; Cheng *et al.*, 1995; Ciossek *et al.*, 1995; Kilpatrick *et al.*, 1996). Since little cross-reactivity has thus far been observed between the two subgroups of ligands and receptors, these may have related but complementary functions *in vivo*.

The clear importance of RTKs in the developing murine nervous system (Klein, 1994; Gassmann *et al.*, 1995) has prompted us to analyse the functions of Eph-related receptors. We have initially focused on the Elk subgroup,

because expression studies have suggested an important role for these receptors in the early phases of neural development and axonogenesis (Lai and Lemke, 1991; Becker *et al.*, 1994; Henkemeyer *et al.*, 1994; Pasquale *et al.*, 1994).

A cDNA clone encoding a fragment of the Sek4 receptor (Tyro6) was first identified in the rat and shown to be specifically expressed in neural tissue. Expression was found to be highest during mid-embryogenesis and to decrease gradually towards birth (Lai and Lemke, 1991). Subsequently, partial and full-length mouse *sek4* cDNAs were isolated, and this gene was shown to be expressed in r3/r5 rhombomeres of the hindbrain, in specific regions of the di- and telencephalon, and in mesodermal and neural crest derivatives (Becker *et al.*, 1994). During late embryogenesis, Sek4 expression was found to be restricted to central nervous sytem (CNS) ventricular linings, and to be upregulated compared with early embryonic stages in many non-neuronal tissues, including lungs, kidney and gut (Ciossek *et al.*, 1995). The mouse Nuk receptor (Henkemeyer *et al.*, 1994), also known as Sek3 (Becker *et al.*, 1994) and Cek5 in chicken (Pasquale, 1991), is expressed in the developing brain, and on peripheral nervous system (PNS) axons during early neuronal pathfinding and fasciculation (Henkemeyer *et al.*, 1994).

Since Sek4 and Nuk are both expressed in r3/r5 rhombomeres, regions of the developing di- and telencephalon, and the mature cerebellum, they may have common functions in these structures (Lai and Lemke, 1991; Becker *et al.*, 1994; Henkemeyer *et al.*, 1994; D.Orioli and R.Klein, unpublished results). This suggestion, together with the fact that Sek4 and Nuk receptors are both activated by the same transmembrane ligands (Bergemann *et al.*, 1995; Brambilla *et al.*, 1995, 1996; Gale *et al.*, 1996), led us to generate mice either singly or doubly deficient in Sek4 and Nuk receptors (see also Henkemeyer *et al.*, 1996).

We now show that Sek4 and Nuk receptors have partially overlapping functions in the guidance of a subset of commissural axons of the CNS, and that these receptors also cooperate in the fasciculation of at least one longitudinal axon tract connecting thalamic nuclei with the ventral midbrain. Whereas *sek4* and *nuk* single mutants are viable, the majority of mice deficient in both Sek4 and Nuk receptors die after birth, primarily due to a cleft palate, demonstrating essential and cooperative functions of Sek4 and Nuk outside the nervous system.

Results

Inactivation of the sek4 gene

The *sek4* gene was inactivated by homologous recombination in mouse embryonic stem (ES) cells, by replacing most of the exons of the kinase domain with a PGK promoter-driven neomycin cassette (Figure 1). Chimeric mice were obtained by injecting recombinant ES cells into C57Bl/6J blastocysts. Crossing of germline targeted heterozygous mice produced *sek4–/–* homozygous animals with a frequency of ~25%, indicating that mice lacking functional Sek4 receptors developed to birth. To examine Sek4 expression in the mutant mice, we performed reverse transcription–polymerase chain reactions (RT–PCRs) using RNA extracted from E16.5 embryonic heads. Combinations of primers corresponding to exon sequences upstream and downstream of the deletion amplified the expected 984 bp DNA fragment from RNAs isolated from wild-type and *sek4+/–*, but not from *sek4–/–* mice, indicating that the exons encoding the kinase domain were not transcribed from the mutant allele (Figure 1C). Using primer pairs upstream of the deletion in the *sek4* gene, in the region encoding the juxtamembrane domain, we were unable to detect the expected 183bp DNA fragment in *sek4–/–* mice, suggesting that the truncated mRNA was unstable. The *sek4* mutation therefore represents a loss-of-function allele.

sek4–/– mice have a defective corpus callosum

sek4–/– mice were viable and fertile, and showed no obvious abnormal behaviour up to the age studied so far (>18 months). However, histological analysis of sections of newborn brains revealed that the corpus callosum, the main axon tract connecting the left and right cerebral hemispheres, was not formed in a significant fraction (3/8) of *sek4–/–* mice. In contrast, this axon tract was normal in all nine wild-type mice analysed. Although spontaneous agenesis of the corpus callosum has been reported in some inbred strains of mice, including 129/ReJ and 129/J (Lipp and Wahlsten, 1992), the *sek4* mutation was analysed in a 129/sv×C57Bl/6J background, which does not exhibit a significant incidence of agenesis of corpus callosum (Wahlsten, 1989). As shown in Figure 2B, callosal axons grow out in the correct orientation from the periphery of the brain towards the midline, and the fibres fasciculate, yet they fail to cross the midline and instead form large bundles, known as Probst's bundles (Probst, 1901). In two animals, callosal axons left the Probst's bundles to migrate along the midline epithelium in an anterior/posterior axis (Figure 2C, see inset), suggesting that Sek4 plays a direct role in guiding callosal axons across the midline.

Sek4 and Nuk receptors cooperate in corpus callosum formation

We have recently shown that mutations in the *nuk* gene result in pathfinding defects of another forebrain axon tract, the anterior commissure (see below) (Henkemeyer *et al.*, 1996). To investigate whether the low penetrance of corpus callosum defects was due to compensation by Nuk, we crossed *sek4–/–* mice with nuk^{1}*–/–* mice to produce double mutants. Whereas nuk^{1}*–/–* mice rarely showed an abnormal corpus callosum (one abnormal and one partially affected, out of eight mice analysed), almost all (8/9) *sek4–/–;*nuk^{1}*–/–* double mutants had an abnormal corpus callosum, indicating that Sek4 and Nuk receptors cooperate in pathfinding of callosal axons. Immunostaining using an antiserum against the neural cell adhesion molecule L1 (Rathjen and Schachner, 1984) revealed that callosal axons fail to cross the midline in *sek4–/–;*nuk^{1}*–/–* double mutants (Figure 3B). Immunostaining against the neural cell adhesion molecule Tag-1/axonin-1 (Wolfer *et al.*, 1994) showed that, in some cases, a few callosal fibres find an alternative path to the contralateral side via the hippocampal commissure (Figure 3D). Those more posterior sections also demonstrated a high degree of specificity, since the hippocampal commissure was generally not affected in *sek4–/–;*nuk^{1}*–/–* brains, suggesting that

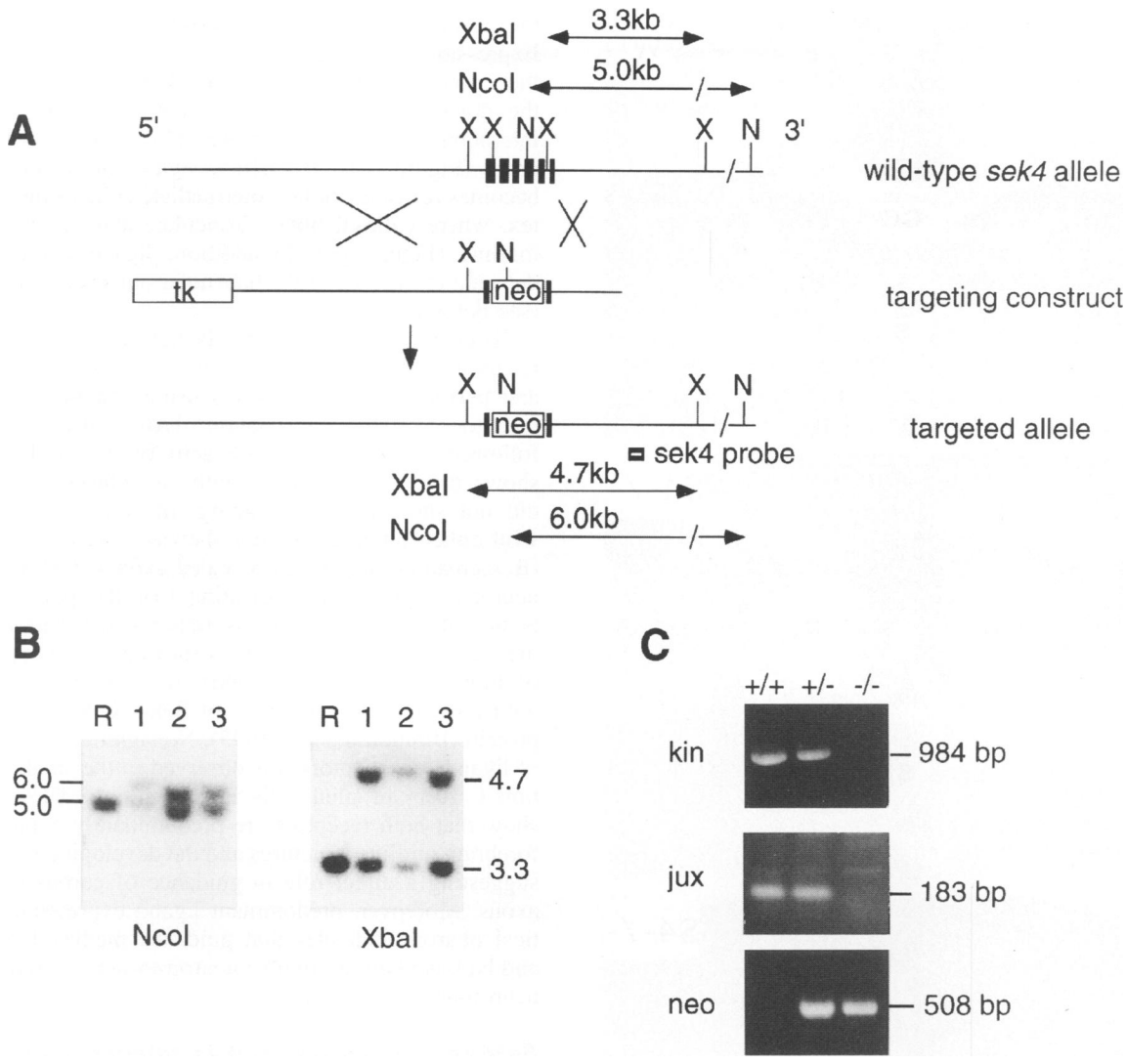

Fig. 1. Targeting of the *sek4* gene. (**A**) Schematic diagram of a region of the wild-type *sek4* locus (top), the targeting construct (middle) and the targeted *sek4* allele (bottom). Vertical closed boxes represent those exons of the *sek4* locus that were mapped, the most 5′ exon starting with nucleotide position 1951 in the human homologue HEK2 (Böhme *et al.*, 1993) and the most 3′ exon containing the stop codon. Transcription of both PGK promoter-driven neomycin resistance (neo) and thymidine kinase (tk) cassettes was in the opposite orientation to the *sek4* gene. Putative crossovers between the endogenous *sek4* locus and the targeting vector are indicated by crossed lines. Cleavage sites for restriction endonucleases *Xba*I (X) and *Nco*I (N) are indicated. The *sek4* probe used in genomic Southern analysis is shown as an open box. The 3.3 and 4.7 kb *Xba*I and 5.0 and 6.0 kb *Nco*I DNA fragments diagnostic for the wild-type and targeted alleles, respectively, are indicated as horizontal arrows. (**B**) Southern blot analysis of genomic DNA from wild-type R1 (R) and three representative recombinant ES cell clones (1–3). (**C**) RT–PCR analysis of mRNA extracted from wild-type (+/+), heterozygous (+/–) and homozygous mutant (–/–) embryos using primers corresponding to exon sequences either flanking the deletion in the kinase domain (kin), 5′ of the deletion, in the region of the juxtamembrane domain (jux), or corresponding to sequences within the neo gene (neo). The sizes of PCR amplified DNA fragments are indicated.

the phenotype is caused by defects in axonal guidance, rather than being due to a general midline defect. Mid-sagittal sections through *sek4–/–;nuk*^{1}*–/–* brains confirmed the absence of callosal fibres at the midline (Figure 3F). Occasionally, a few callosal fibres were seen to have been deflected by 90° and continued to grow anteriorly, similar to the situation in *sek4–/–* single mutants (Figure 3H), but the majority of the fibres appeared to terminate within the Probst's bundles. The markedly more severe phenotype in *sek4–/–;nuk*^{1}*–/–* double mutants, compared with the defects in the single mutants, indicates that the functions of Sek4 and Nuk receptors are largely redundant in corpus callosum formation.

To investigate which structures and cell types of the forebrain express Sek4, Nuk and their physiological ligands, we performed a series of *in situ* hybridization, immunostaining and *in situ* binding experiments. In coronal sections of E13.5 embryos, Sek4 transcripts are present throughout the ventricular and intermediate zones of the forebrain with the highest levels in the region closest to the midline. Figure 4A shows the highest levels of Sek4 transcripts in the cingulate cortex, the hippocampal anlage, and the ventral aspect of the third ventricle (the pre-optic area), through which the anterior commissure axons will travel on their way to the contralateral side. At E16.5, sek4 mRNA transcripts are highest in the ventricular and subventricular zones and cortical layers, but are absent from the intermediate zone where most of the callosal fibres migrate (Figure 4C). The specificity of the *in situ* hybridization signal was shown by using the

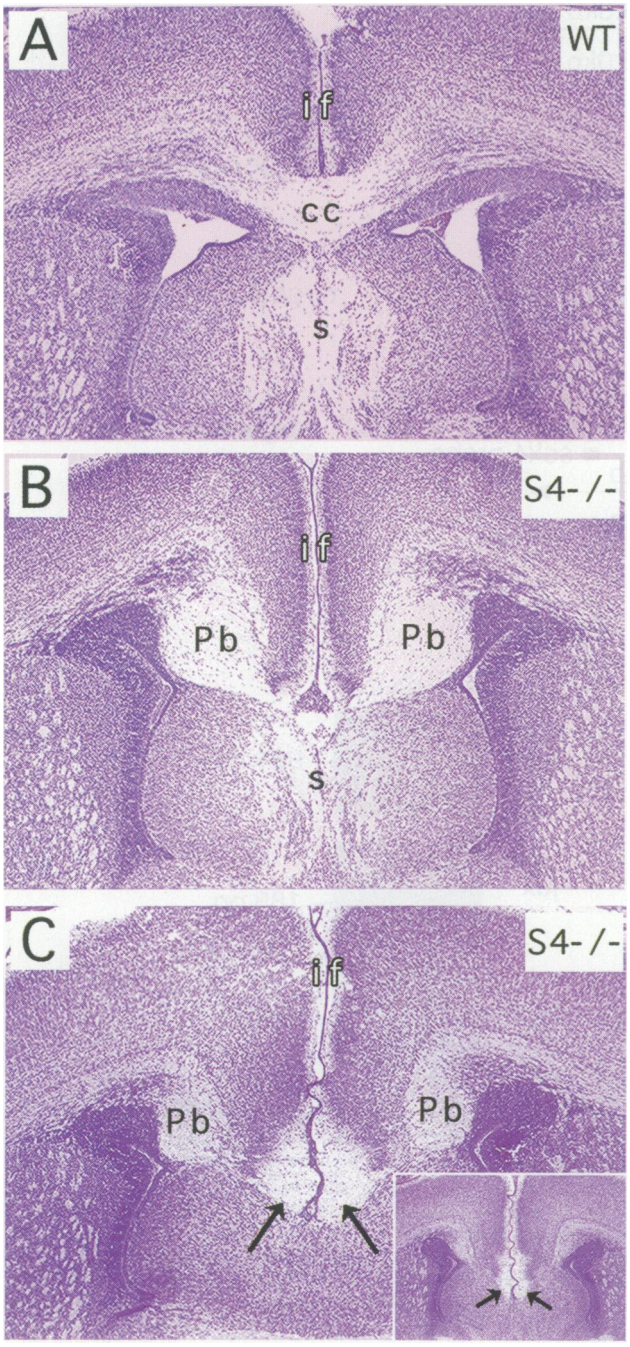

Fig. 2. *sek4–/–* mice have a defect in corpus callosum formation. Cresyl violet-stained coronal sections of the forebrain (at the level of the medial septum, s) from P0 wild-type (WT) (**A**) and two different *sek4–/–* mice (S4–/–) (**B** and **C**). Axon tracts, including Probst's bundles (Pb) in acallosal brains appear unstained. The *sek4* mutant shown in (B) exhibits the typical appearance of Probst's bundles with most fibres terminating within the bundles. (C) Another *sek4* mutant with an acallosal brain. However, many callosal axons are exiting the Probst's bundles, migrate to the midline epithelium, and are deflected in an anterior direction (arrows), as demonstrated by the more anterior section in the inset of (C). Abbreviations: cc, corpus callosum; if, interhemispheric fissure; Pb, Probst's bundle; s, septum. Magnifications: 50×.

same antisense probe on *sek4–/–* embryos (see Figure 8). Nuk expression at E14.5 overlaps with sek4 mRNA expression in many areas of the forebrain (Henkemeyer *et al.*, 1996) and, at E16.5, appears to be associated with the leading edge of the callosal fibres (Figure 4D). Expression of the ligands, using an antibody recognizing the common C-terminal tail of Lerk2/5, is highest in the dorsolateral cortex and absent at the base of the interhemispheric fissure where callosal axons will later cross (Figure 4B). At E16.5, ligand immunoreactivity becomes restricted to the intermediate zone of the neocortex, where callosal fibres fasciculate and migrate to the midline (Figure 4E). In addition, ligand expression is detected in the area of the habenular–interpeduncular tract (see below).

To characterize more directly the cell types in which receptors and ligands act, we cultured E14 cortical neurons and performed binding assays using soluble ligand and receptor–alkaline phosphatase (AP) fusion proteins followed by detection of AP activity *in situ*. Figure 4F shows that cells incubated with an unfused AP protein did not show positive staining. In contrast, incubation with either a Lerk2-AP (not shown) or Lerk5-AP probe (Bergemann *et al.*, 1995) revealed axonal staining in few neurons (Figure 4G), indicating that the population of Sek4/Nuk-expressing cells is rather small. Surprisingly, the majority of cortical neurons showed strong expression of transmembrane-type ligands, revealed by incubation with the chicken homologue of Sek4, Cek10–AP fusion protein (Brambilla *et al.*, 1995). No significant expression of ligands or receptors was observed in the small population (~10%) of glial cells in the culture. These results show that both receptors are predominantly expressed in forebrain midline structures and the developing neocortex, suggesting a direct role in guidance of corpus callosum axons. Moreover, predominant ligand expression in cortical neurons indicates that guidance mediated by Sek4 and Nuk is likely to involve neuron-to-neuron, rather than neuron-to-glia interactions.

Sek4 cooperates with Nuk in anterior commissure axon guidance

The organization of the second main telencephalic commissure, the anterior commissure, is abnormal in nuk^1*–/–* mice (Henkemeyer *et al.*, 1996). A large fraction of axons extended by neurons from the temporal cortex failed to form the pars posterior (acP) of the anterior commissure (Cajal, 1988), but instead were deflected towards the floor of the forebrain. To investigate whether Sek4 receptors also have a role in pathfinding of anterior commissure axons, we analysed coronal sections of newborn brains from *sek4–/–* and *sek4–/–*;nuk^1*–/–* double mutants. All eight *sek4–/–* mice had normal acP axon bundles identical to wild-type mice (see Figure 5A). However, *sek4–/–*; nuk^1*–/–* mice (all nine mice analysed) showed a more severely affected acP axon tract than nuk^1*–/–* mice. In many nuk^1*–/–* mice, the most rostral fibres migrate in a correct horizontal path towards the midline (Figure 5C), whereas more caudal fibres take an abnormal route to the floor of the forebrain (Figure 5E) (see also Henkemeyer *et al.*, 1996). In *sek4–/–*;nuk^1*–/–* double mutants, kept on the same 129×C57Bl/6 genetic background, basically all acP axons are misdirected in an abnormal ventral path (Figure 5D and F). The absence of correctly guided acP axons is also evident in parasagittal sections immunostained for L1 (Figure 5H). Mice homozygous mutant for *sek4* and heterozygous mutant for *nuk* exhibited a normal

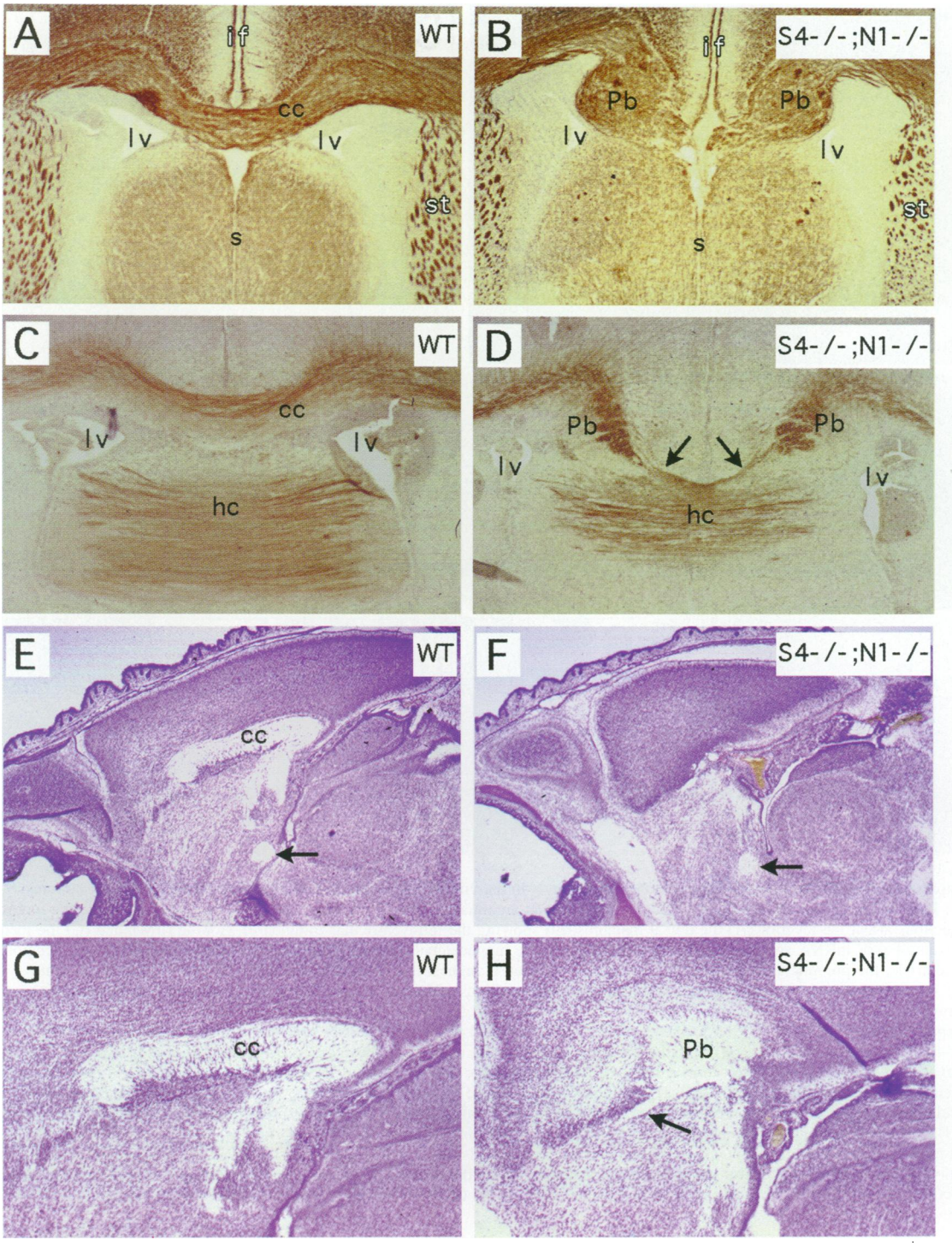

Fig. 3. Agenesis of the corpus callosum in *sek4/nuk* double-mutant mice. Coronal (**A–D**), midsagittal (**E** and **F**) and parasagittal sections (**G** and **H**) of P0 wild-type (A, C, E and G) and *sek4–/–;nuk*^{1}*–/–* mice (B, D, F and H). (A and B) Sections at the level of the medial septum (s) stained for the neural cell adhesion molecule L1. Probst's bundles (Pb) stain positive for L1 and appear brown. No fibres are crossing the midline in *sek4–/–; nuk*^{1}*–/–* mice. (C and D) Sections at the level of the hippocampal commissure (hc) stained for the neural cell adhesion molecule Tag-1. Callosal axons finding an alternative path via the hippocampal commissure (hc) are indicated with arrows. Axons of the hippocampal commissure do not show a defect in guidance across the midline. (E and F) Cresyl violet-stained sections through the midsagittal plane. Note the absence of the corpus callosum (cc) in (F). The normal position of the anterior commissure (pars anterior) is indicated by an arrow in both (E) and (F). (G and H) Cresyl violet-stained parasagittal sections of the same animals as in (E) and (F). Callosal axons growing anteriorly out of the Probst's bundles in *sek4–/–; nuk*^{1}*–/–* mice are indicated by an arrow (H). Abbreviations: cc, corpus callosum; hc, hippocampal commissure; if, interhemispheric fissure; lv, lateral ventricle; Pb, Probst's bundle; s, septum; st, striatum. Magnifications: A and B, 62.5×; C, D, G and H, 80×; E and F, 31.25×.

anterior commissure (n = 3) (data not shown), indicating that anterior commissure axon pathfinding is not dosage dependent for the *nuk* gene. These results indicate that Sek4 by itself is not essential, but cooperates with Nuk in anterior commissure guidance.

Interestingly, the most anterior part of the anterior

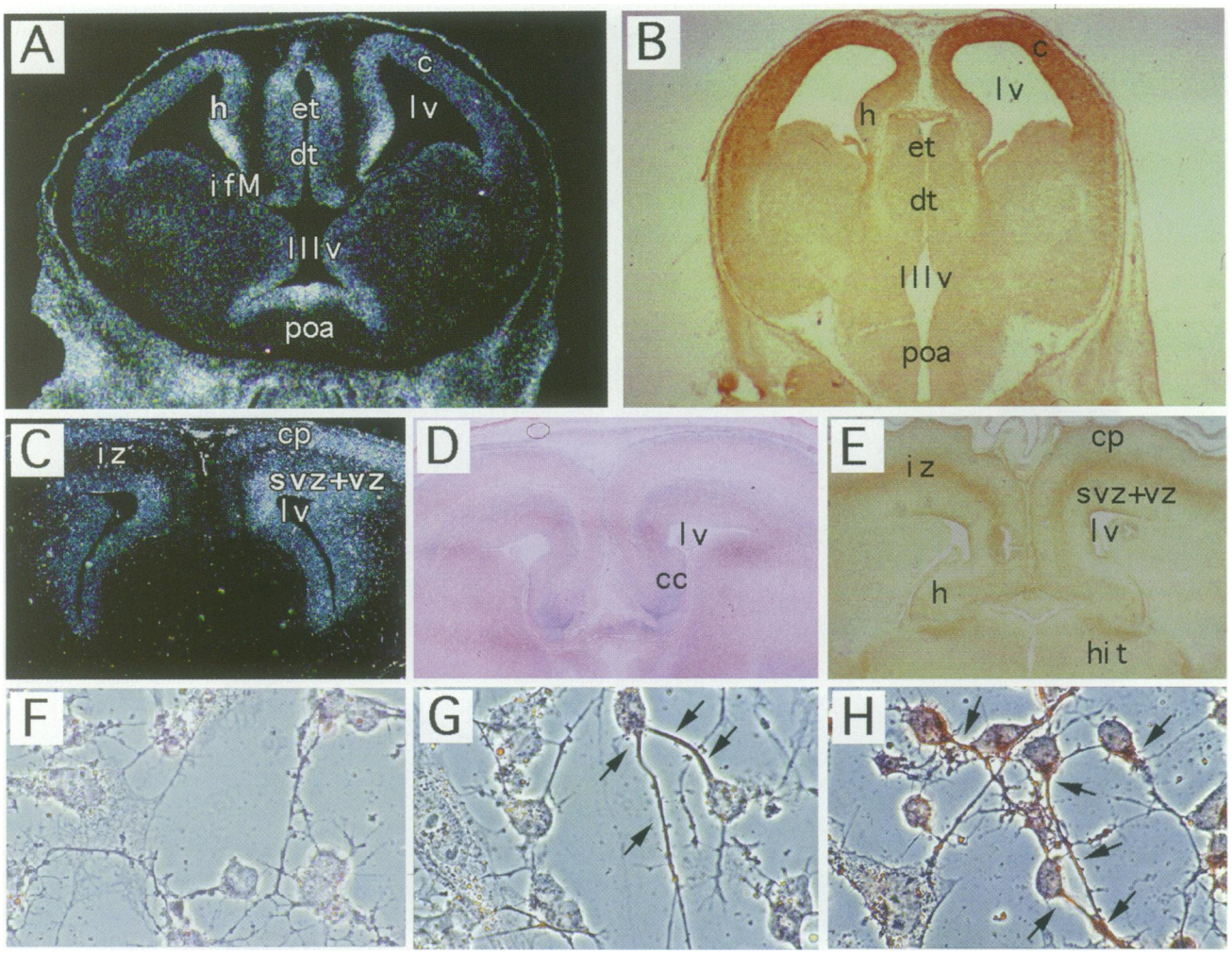

Fig. 4. Expression of Sek4, Nuk and transmembrane-type ligands in the embryonic forebrain and dissociated cortical neuron cultures.
(**A** and **C**) Dark-field views of *in situ* hybridization analyses using an antisense *sek4* probe on E13.5 (A) and E16.5 (E) wild-type mouse embryos. Coronal sections are at the level of (A) the dorsal thalamus (dt) showing the intraventricular foramen of Monroe (ifM), and (C) the medial septum. Highest levels of *sek4* mRNA are found in the cingulate cortex, the hippocampal anlage, dorsal thalamus and epithalamus, and a region ventral to the third ventricle, the preoptic area (poa). At E16.5, *sek4* expression is highest in cortical plate, subventricular and ventricular zones.
(**B** and **E**) Transmembrane-type ligand immunostaining on coronal sections of E14.0 (B) and E16.5 (E) at the level of (B) the thalamus just posterior of the intraventricular foramen of Monroe, (E) the anterior hippocampus. At E14.0, ligand expression is observed in a dorsoventral gradient, with highest expression dorsolaterally. At E16.5, ligand expression is restricted to the intermediate zone (iz) of the cerebral hemispheres and the habenular–interpeduncular tract (hit). (**D**) Nuk-βgal activity at the leading edge of the developing corpus callosum (cc) of a E16.5 $nuk^{lacZ/+}$ mouse (Henkemeyer *et al.*, 1996). (**F–H**) Short-term cultures of E14.0 embryonic cortical neurons were incubated with either unfused alkaline phosphatase (AP) protein (F), Lerk5-AP (G) or Cek10-AP (H), the chicken homologue of Sek4. Cultures were washed, fixed and stained for bound AP activity. Positive cells can be observed by the presence of reddish staining (arrows). Note that few cells express receptor (G), whereas most neurons express ligands (H). Whereas the signal in immature neurons (Dotti *et al.*, 1988) is mainly localized to cell bodies, staining in polarized, mature neurons is highest in axons. Little staining, if any, is found in glial cells. Red crystals in the cytoplasm are also seen in the unfused AP control (F).
Abbreviations: c, neocortex; cp, cortical plate; dt, dorsal thalamus; et, epithalamus; h, hippocampus; hit, habenular–interpeduncular tract; iz, intermediate zone; poa, preoptic area; svz, subventricular zone; vs, ventricular zone; IIIv, third ventricle. Magnifications: A, 31.25×; B, 40×; C–E, 62.5×; F–H, 1250×.

commissure (acA), which connects olfactory nuclei with granule cells of the olfactory bulbs, developed normally in nuk^{1}–/– mice (Henkemeyer *et al.*, 1996). All *sek4*–/–; nuk^{1}–/– double mutants also had an intact acA tract, as evidenced by Tag-1, which specifically stains acA axons (see Figure 5J for sagittal view and Figure 5B for coronal view). This indicates that the developing acA tract does not require functional Sek4 or Nuk receptors. This analysis also showed that acA axons do not mix with the Tag-1-negative acP axons (Figure 5I). In the absence of acP axons in *sek4*–/–;nuk^{1}–/– mice, Tag-1-positive pars anterior axons appear less well bundled (Figure 5J). Hence, Sek4 and Nuk may play a specific role in fasciculation of acA axons of the anterior commissure.

Differential effects of sek4/nuk mutations on epithalamic commissures

Since *in situ* hybridization analysis indicated the presence of *sek4* transcripts in the dorsal diencephalon, we studied two interhemispheric projections in the epithalamus: the habenular and posterior commissures. The former connects the habenular nuclei, which are relay centres between tel- and mesencephalic regions and the limbic and motor systems (Herkenham and Nauta, 1977; Hamill and

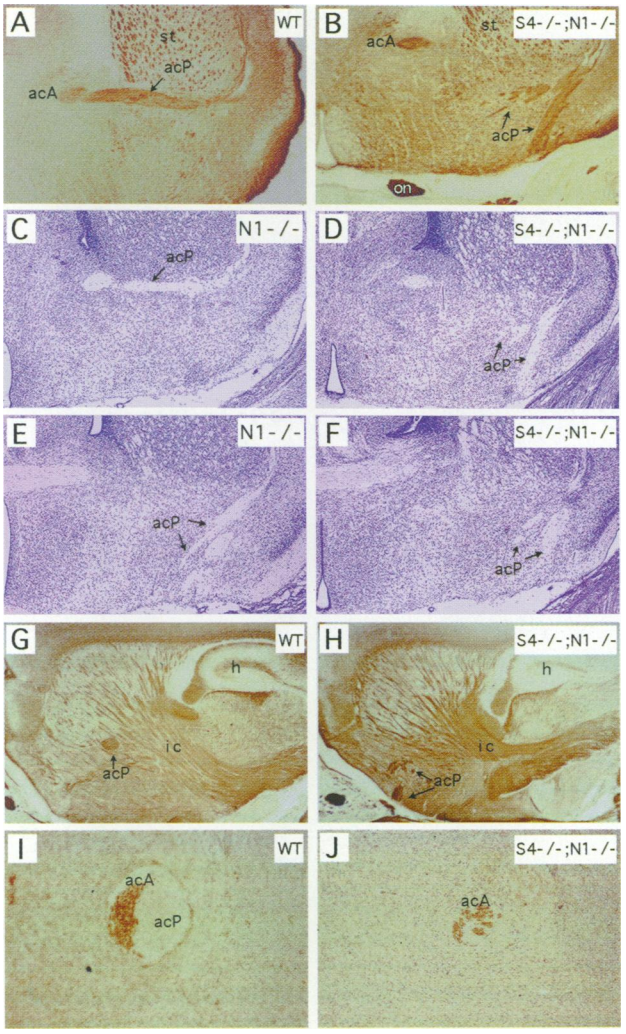

Fig. 5. Sek4 and Nuk co-operate in guidance of anterior commissure axons. Coronal (**A–F**), parasagittal (**G** and **H**) and midsagittal (**I** and **J**) sections of P0 wild-type (A, G and I), *nuk*^{1}*–/–* mice (N1–/–) (C and E) and *sek4–/–;nuk*^{1}*–/–* mice (B, D, F, H and J). (A and B) Coronal sections at the level of the anterior commissure showing the left half of the brain, stained for L1. Anterior commissure (pars posterior) axons (acP) taking an aberrant ventral route are indicated by arrows in (B). Note the normal appearance of the pars anterior of the anterior commissure (acA) in (B). (C–F) Cresyl violet-stained sections comparing the pathfinding defect in acP axons between *nuk*^{1}*–/–* mice and *sek4–/–;nuk*^{1}*–/–* double mutants. Whereas the most rostral axons of the acP tract often migrate in a correct horizontal path in *nuk*^{1}*–/–* mice (C), the more caudal acP axons show the aberrant ventral route (E). In contrast, both rostral (D) and caudal axons (F) of the acP tract of *sek4–/–;nuk*^{1}*–/–* double mutants show abnormal pathfinding behaviour. (G and H) Parasagittal sections at the level of the internal capsule (ic) stained for L1. Ventral position of aberrantly guided anterior commissure axons (acP) of *sek4–/–;nuk*^{1}*–/–* mice are indicated by arrows. (I and J) Cross-section at the midline through the anterior commissure, stained for Tag-1. Note that in (I), only the acA tract expresses Tag-1 and that axons of the acA axons do not mix with acP axons in wild-type brain. In (J), very few remaining Tag-1 negative axons mix with Tag-1-positive acA axons in *sek4–/–;nuk*^{1}*–/–* mice. Abbreviations: acA, anterior commissure, pars anterior; acP, anterior commissure, pars posterior; ic, internal capsule; on, optic nerve; st, striatum. Magnifications: A–F, 62.5×; G and H, 40×; I and J, 160×.

Jacobowitz, 1984). The latter connects a complex of thalamic nuclei that are thought to be involved in eye movements and the pupillary light reflex (Carpenter *et al.*, 1970). Based on cresyl violet and L1 immunostaining, all *sek4–/–;nuk*^{1}*–/–* mice (n = 8) had an abnormal habenular commissure. Whereas more rostral axons appeared to have properly crossed to the contralateral side (Figure 6B), more posterior fibres failed to cross the midline and instead took an aberrant longitudinal path (Figure 6B) or formed bundles on the ipsilateral side reminiscent of Probst's bundles at the callosal midline (Figure 6D). The corresponding single mutants exhibited a normal (7/7 *sek4–/–* and 4/7 *nuk*^{1}*–/–* mice) or mildly affected habenular commissure (3/7 *nuk*^{1}*–/–* mice), suggesting that Sek4 and Nuk cooperate in targeting these commissural axons across the midline. In contrast, the posterior commissure, which is located just ventral and posterior of the habenular commissure (Figure 6A and C), but connects different thalamic nuclei, was not affected in *sek4–/–;nuk*^{1}*–/–* mice (Figure 6B and D).

In E14.5 wild-type mouse embryos, Sek4 mRNA transcripts were abundant in the medial habenula, where the cell bodies of habenular commissural axons are located, and less highly expressed in more lateral thalamic regions (Figure 6E). Nuk is highly expressed throughout the thalamus and hypothalamus (Henkemeyer *et al.*, 1996). At E16.5, Lerk2/5 ligand immunoreactivity was detected only in a specialized midline structure called the subcommissural organ and was absent from commissural axons (Figure 6F). These results indicate that Sek4 and Nuk are required for the appropriate migration of habenular commissural axons, but not of axons in the neighbouring posterior commissure.

Axon fasciculation defects in sek4/nuk double-mutant mice

One pair of axon bundles, originating from the habenular nuclei and running along the anterior/posterior axis, showed defects in fasciculation in *sek4–/–;nuk*^{1}*–/–* mice (Figure 7B). This habenular–interpeduncular tract contains afferent inputs from the habenular nuclei, and projects to the midbrain interpeduncular nucleus, which is a component of the limbic system (Hamill and Jacobowitz, 1984). Tag-1 stains a subset of fibres in the habenular–interpeduncle tract. In sections from wild-type mice, long Tag-1-positive fibres projected from the habenular nuclei and fed into an already tightly bundled axon tract (Figure 7C). In contrast, very short and loosely bundled Tag-1-positive fibres could be seen in all (n = 7) *sek4–/–;nuk*^{1}*–/–* mice (Figure 7D). In contrast, the corresponding single mutants generally had well-fasciculated axon bundles (Figure 7E and F). Seven out of eight *sek4–/–* mice and 5/7 *nuk*^{1}*–/–* mice had a normal habenular–interpeduncular tract, and only one *sek4–/–* and two *nuk*^{1}*–/–* mice were partially affected. These results indicate that both receptors have cooperative functions in fasciculation of the habenular–interpeduncular tract. In contrast to commissural axons, the longitudinal habenular–interpeduncle tract axons did not show a defect in guidance as visualized by either cresyl violet (Figure 7H) or Tag-1 immunostaining (not shown) of parasagittal sections. The phenotype in the habenular–interpeduncle tract correlates with both receptor expression (Figure 6E; M.Henkemeyer, unpublished results) and ligand expression (Figure 4E) in the region of the tract. These data indicate that Eph receptors can control axon fasciculation independently of pathfinding *in vivo*.

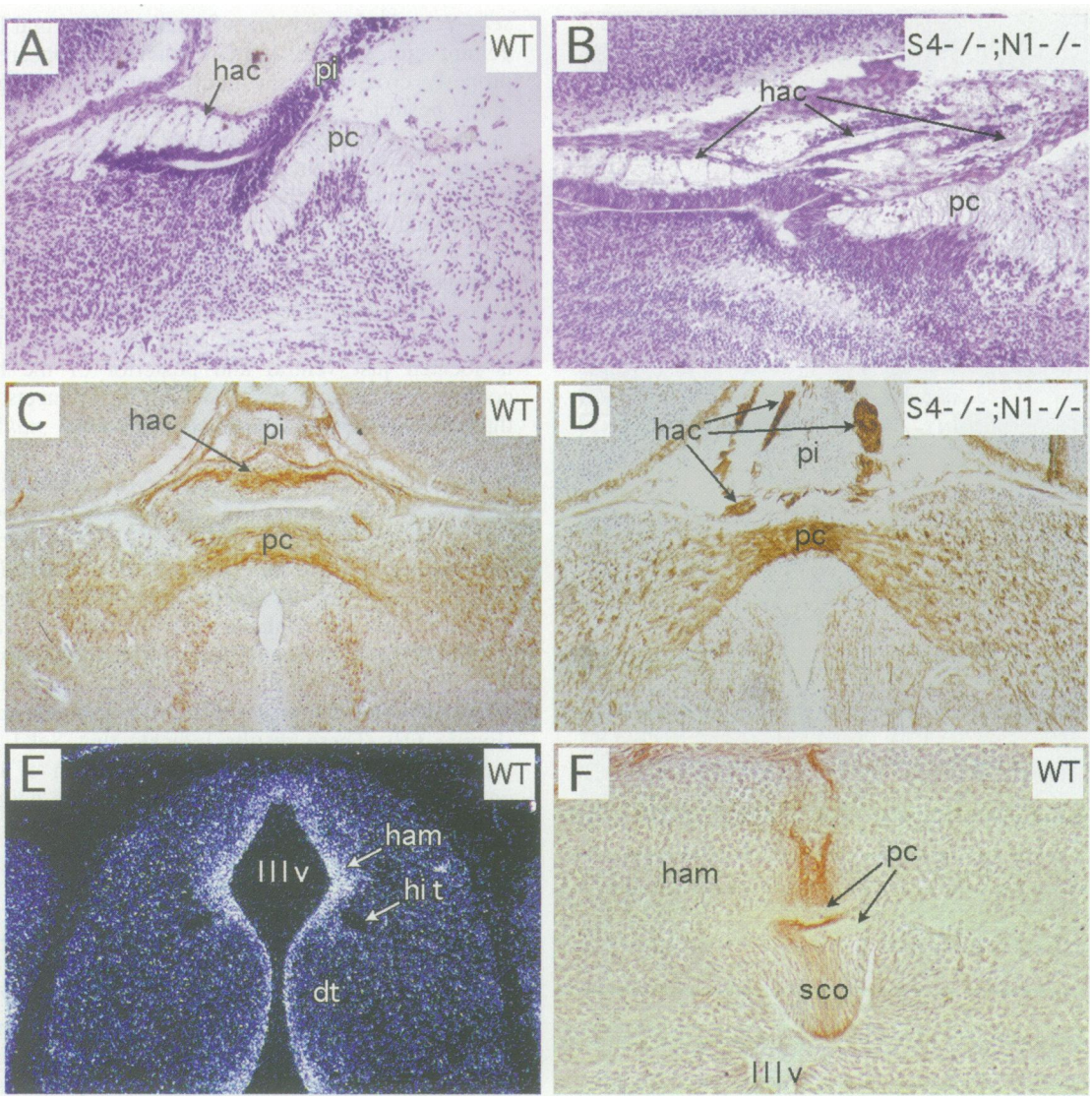

Fig. 6. Differential effects on epithalamic commissures. (**A** and **B**) Midsagittal and (**C** and **D**) coronal sections through the epithalamus of P0 wild-type (A and C) and *sek4–/–;nuk*^{1}*–/–* mice (B and D) stained by cresyl violet (A and B) and for neural cell adhesion molecule L1 (C and D). Habenular commissural axons that have failed to traverse the midline and have been deflected to grow along the anterior/posterior axis are indicated with arrows in (B). The same abnormal longitudinal fibres are visible as large bundles (arrows) in (D). No guidance defect is observed in posterior commissure axons (pc). (**E** and **F**) Coronal sections through the dorsal thalamus of E14.5 (E) and E16.5 (F) wild-type mouse embryos. (E) Dark-field view of *in situ* hybridization analysis using an antisense *sek4* probe. Note the intense signal in the medial habenula. The transversally sectioned habenular–interpeduncular tract (hit) is indicated (arrow). (F) Ligand immunostaining showing intense signal in the subcommissural organ. Note the absence of any signal in posterior commissural axons which traverse through the midline. Abbreviations (see also Figure 3): hac, habenular commissure; ham, medial habenula; pc, posterior commissure; pi, pineal gland (epiphysis); sco, subcommissural organ. Magnifications: A, B and E, 160×; C and D, 125×; F, 320×.

sek4/nuk double-mutant mice show perinatal lethality due to cleft palate

When generating *sek4–/–;nuk*^{1}*–/–* double mutants, it became readily apparent that the majority (~80%) died within 24–48 h after birth. A small fraction of these double-mutant mice (~15%) appeared to die from dehydration due to loss of peritoneal fluid through a herniation at the umbilicus (data not shown). However, a major fraction of double mutants died from an inability to take up milk properly, due in part to a failure in secondary palate closure (see below). We have observed a few survivors, but these were severely growth retarded and rarely reached adulthood (data not shown). Mice heterozygous mutant for one gene and homozygous mutant for the second (e.g. *sek4+/–;nuk*^{1}*–/–*) were partially affected, with two-thirds of the animals being viable and fertile, and one-third dying immediately after birth. These results show that Sek4 and Nuk together are required for postnatal viability. All of the wild-type (n = 9) and single-mutant mice (n = 8) had a normal palate and showed normal feeding behaviour, whereas 10/22 *sek4–/–;nuk*^{1}*–/–* mice had cleft palate. Mice heterozygous mutant for one of the targeted genes and homozygous mutant for the second gene also had cleft palate, but with a low penetrance (3/14 *sek4+/–; nuk*^{1}*–/–* and 1/10 *sek4–/–;nuk*^{1}*+/–* mice) that correlated with the observed perinatal mortality. In many cases, transverse and sagittal sections of double homozygotes revealed only rudimentary palatal shelves throughout the entire length of the oral cavity (Figure 8).

To investigate whether Sek4 and Nuk have a direct role

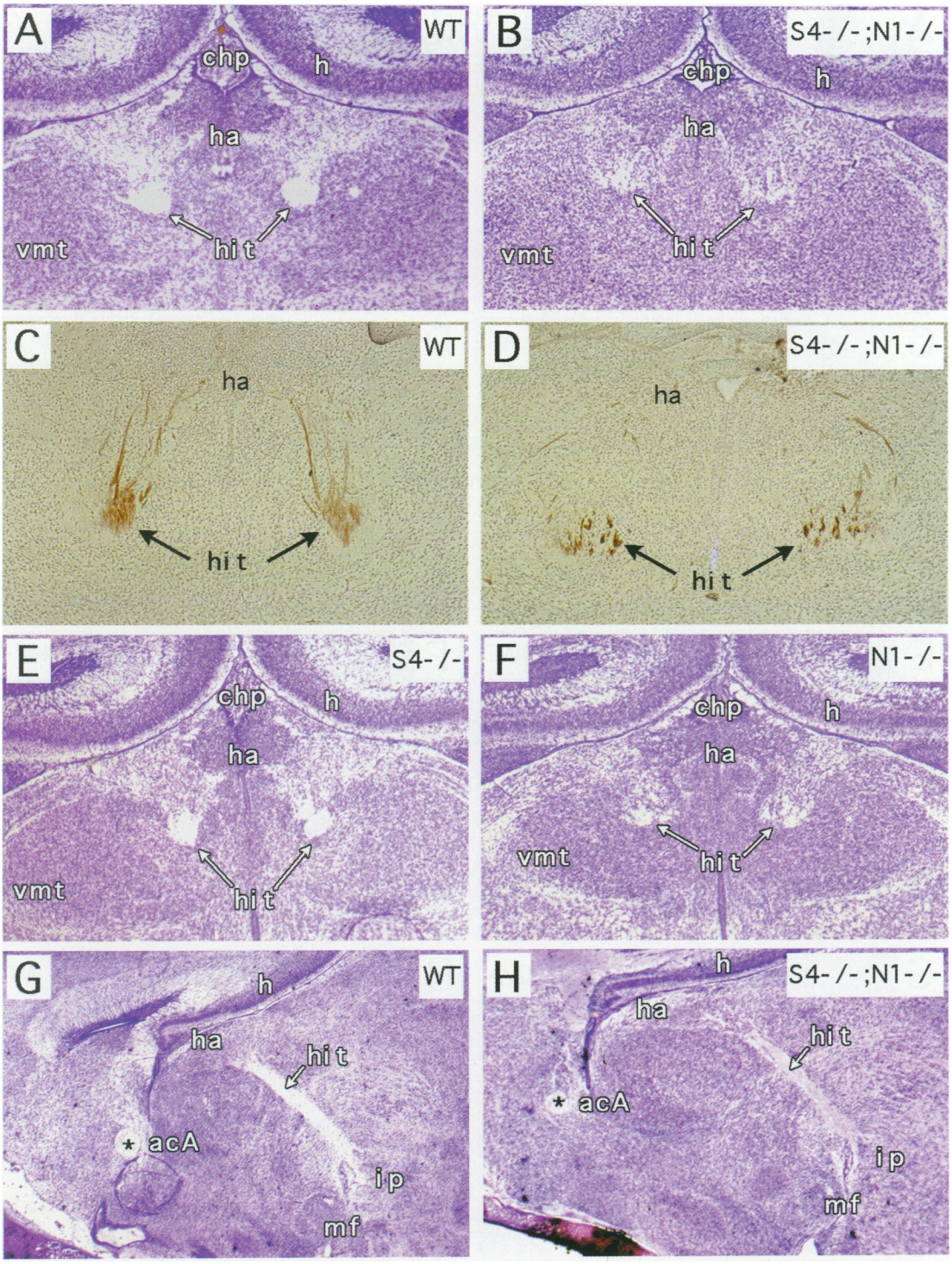

Fig. 7. Defect in axon fasciculation of the habenular–interpeduncular tract. (**A–F**) Coronal sections at the level of the habenula (ha) from P0 wild-type (A and C), *sek4–/–;nuk*^{1}*–/–* mice (B and D), *sek4–/–* (E) and *nuk*^{1}*–/–* mice (F) stained with either cresyl violet (A, B, E and F) or with Tag-1 specific antibodies (C and D). Note the tightly bundled habenular–interpeduncular tract (hit) axons (arrows) in wild-type brains (A and C), compared with loosely packed fibre bundles in *sek4–/–;nuk*^{1}*–/–* mice (B and D). Fasciculation is only partially affected in single mutants (E and F). (**G** and **H**) Cresyl violet-stained midsagittal sections at the level of the habenular–interpeduncular tract from P0 wild-type (G) and *sek4–/–;nuk*^{1}*–/–* mice (H). The correctly guided axons of the habenular–interpeduncular tract are marked with arrows. The normal pars anterior of the anterior commissure is indicated with an asterisk. Abbreviations: acA, pars anterior of anterior commissure; chp, choriod plexus; ha, habenula; h, hippocampus; hit: habenular–interpeduncular tract; mf, mesencephalic flexure; ip, interpeduncular nucleus; vmt: ventromedial thalamic nucleus. Magnifications: A and B, 80×; C and D, 125×; E and F, 62.5×; G and H, 31.25×.

in palate development, we analysed their expression and that of their ligands during mid-embryogenesis. In wild-type E13.5 embryos, we observed high levels of Sek4 mRNA transcripts throughout the facial mesenchyme, with somewhat lower levels in the developing palatal shelves, which at this stage have extended vertically and are located

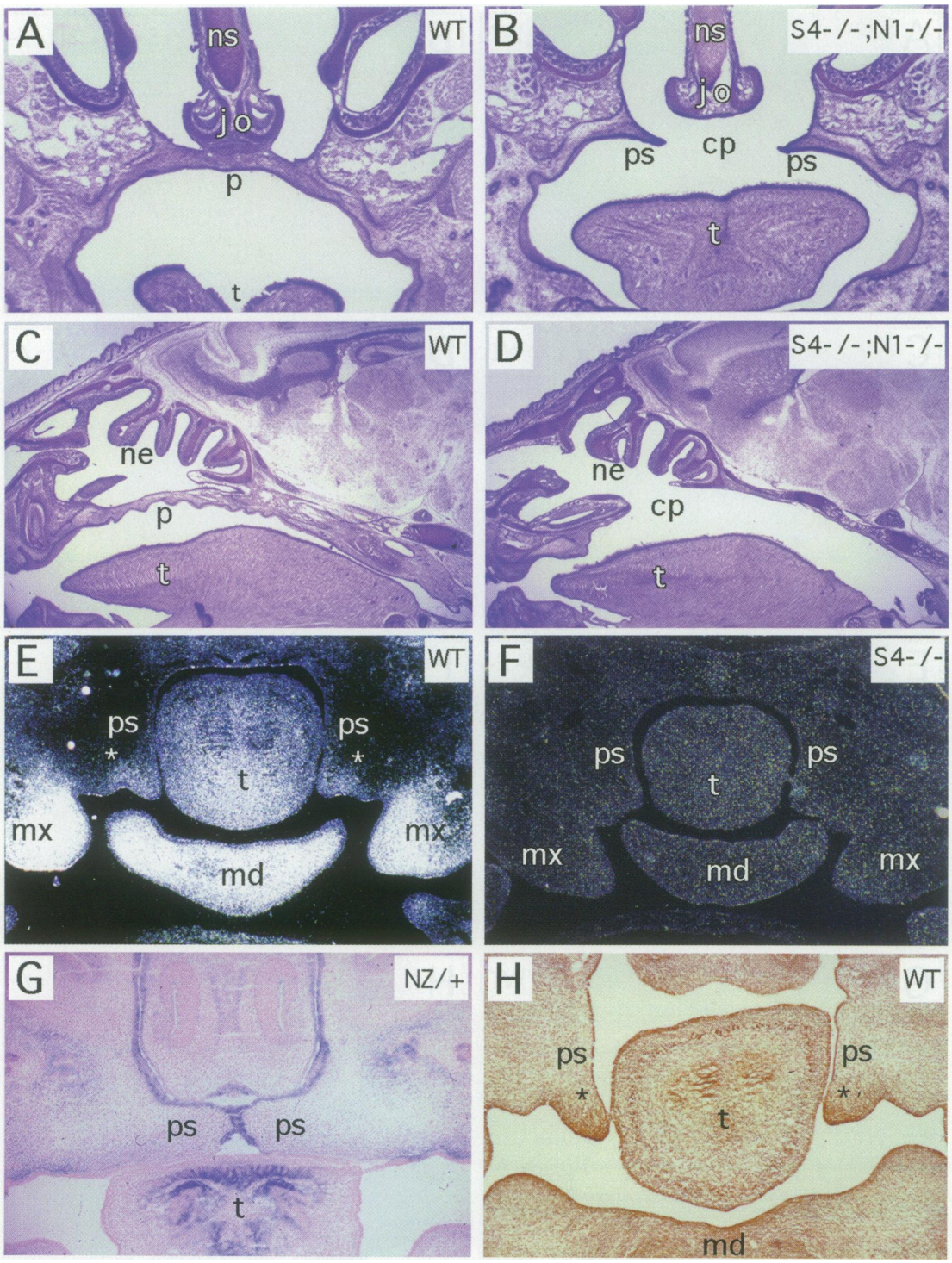

Fig. 8. Redundant functions for Sek4 and Nuk in palate formation. Cresyl violet-stained sections from P0 wild-type (WT) (**A** and **C**) and *sek4–/–; nuk*^{1}*–/–* mice (**B** and **D**). (A and B) Coronal sections at the level of Jacobson's organ (jo). Note the cleft palate (cp) in *sek4–/–;nuk*^{1}*–/–* mice. (C and D) Midsagittal sections demonstrating the complete absence of the secondary palate in *sek4–/–;nuk*^{1}*–/–* mice (D). (**E** and **F**) Dark-field views of *in situ* hybridization analyses on wild-type (E) or *sek4–/–* (S4–/–) (F) E13.5 mouse embryos using an antisense *sek4* cRNA probe. Sek4 expression in vertical palatal shelves (ps) is indicated with an asterisk in (E). (**G**) Nuk expression by staining for βgal activity in heterozygous E14.5 *nuk*$^{lacZ/+}$ mice (NZ/+) (Henkemeyer *et al.*, 1996) at which stage palatal shelves have just started to fuse. (**H**) Immunostaining for the transmembrane-type ligands, Lerk2 and Lerk5, in wild-type E14.0 mouse embryos. High ligand expression in tips of vertical palatal shelves (asterisk). Abbreviations: cp, cleft of palate; jo, Jacobson's organ; md, mandible; mx, maxilla; ne, nasal epithelium; ns, nasal septum; p, secondary palate; ps, palatal shelves; t, tongue. Magnifications: A, B, E and F, 62.5×; C and D, 31.25×; G and H, 80×.

laterally of the tongue (Figure 8E) (Ferguson, 1988). Nuk expression was analysed by βgal activity in E14.5 *nuk*^{lacZ}/+ embryos (Henkemeyer *et al.*, 1996), at which stage the palatal shelves had elevated and started to fuse at the midline (Figure 8G). Nuk-βgal activity was found at the midline epithelial seam and dorsal edge of palatal shelves. In addition, the nasal epithelium and tongue expressed high levels of Nuk-βgal protein, with weaker

staining in the palatal shelf mesenchyme. The expression of Lerk2/5 ligands was detected throughout the oral and nasal cavities, the tongue and at the tips of palatal shelves (Figure 8H). The signal was competed with the immunizing peptide (not shown). Lerk2 expression in the palate has also been detected by *in situ* hybridization analysis (Bouillet *et al.*, 1995). The presence of both receptors and their ligands in the developing palate suggests that the cleft palate phenotype is a direct consequence of a lack of ligand–receptor interaction and signalling. The palate phenotype is very specific, since we have been unable to find abnormalities in other structures derived from either mesenchymal or neurogenic neural crest despite high levels of Sek4 and Nuk expression.

Discussion

Overlapping functions of Sek4 and Nuk

We provide genetic evidence that the cues that control axon guidance and fasciculation in the mammalian brain are in some cases provided by receptor tyrosine kinases of the Eph family. Whereas *nuk*^{1} single mutants have defects in pathfinding of anterior commissure axons (Henkemeyer *et al.*, 1996), *sek4* single mutants exhibit defects in corpus callosum formation, albeit with low penetrance. Both defects are markedly more severe in *sek4/nuk* double mutants, demonstrating that Sek4 and Nuk have overlapping functions in the guidance of axons in commissural projections of the forebrain. This partial redundancy is likely due to the co-expression of Sek4 and Nuk in the same structures, and to the fact that both receptors recognize the same transmembrane-type ligands, Lerk2 and Lerk5. These results suggest that combinations of Eph receptors are used in establishing specific axon pathways in the vertebrate brain. Given the large number of potential Eph receptor–ligand interactions, these cell surface molecules may be among the major determinants of specific pathfinding and fasciculation events throughout the developing nervous system, and indeed may determine the precise formation of topographic connections (Sperry, 1963).

We also show that mice homozygous for both the Sek4 and Nuk mutations generally die perinatally, whereas mice homozygous for either mutation alone are viable and long lived. This lethality is most likely due to a cleft palate defect, observed specifically in the double homozygotes, and to a lesser extent in animals homozygous for one receptor mutation and heterozygous for the other. These findings support the interpretation that Sek4 and Nuk have redundant functions, and provide direct evidence that Eph receptors have critical functions during embryonic development outside of the nervous system.

How do Sek4 and Nuk cooperate in regulating axon guidance in the central nervous system?

The formation of a normal corpus callosum requires the concerted development of several structures at or near the telencephalic midline (Silver *et al.*, 1982, 1993; Wahlsten and Bulman-Fleming, 1994). Since these structures appeared normal in most *sek4/nuk*^{1} double mutants (data not shown), we have no evidence for a general morphological defect in the development of the midline. Rather, we favour the idea that agenesis of the corpus callosum is due to a specific inability of the growing axons to cross over to the contralateral side of the brain.

Consistent with a defect in the migration of callosal fibres across the midline, we show that both Sek4 and Nuk receptors, as well as their ligands, are specifically expressed in the region surrounding the developing commissure. Surprisingly, in dissociated cultures of embryonic cortical neurons the majority of cells expressed transmembrane-type ligands, while a much smaller subpopulation expressed the cognate receptors. This is in contrast to the GPI-anchored ligand, RAGS/AL1, which was recently shown to be expressed in astrocytes (Winslow *et al.*, 1995). This finding indicates that Sek4 and Nuk are primarily involved in neuron-to-neuron signalling in the CNS.

Sek4 and Nuk may act directly within developing commissural axons of the CNS to control growth cone motility. However, the findings that transmembrane-type ligands are expressed in commissural axons (Henkemeyer *et al.*, 1996) and cultured cortical neurons (see Figure 4) raises the possibility that the role of Sek4 and Nuk in guiding axons may not always be cell autonomous. In this latter case, Sek4 and Nuk could act indirectly by regulating the expression of guidance factors, such as cell adhesion molecules. It is also possible that Sek4 and Nuk regulate the viability or trophic status of the neurons; however, our identification of long axon bundles migrating along abnormal longitudinal paths in the brains of double-mutant mice is not consistent with neuronal atrophy. We instead favour a model in which both the ligands and receptors can function as signalling molecules to regulate axon guidance (Henkemeyer *et al.*, 1996).

Not much is known about the physiological downstream effectors of Eph receptors. Interestingly, mice deficient in p190RhoGAP exhibit similar midline guidance defects in the forebrain (J.Settleman, personal communication). p190RhoGAP associates with p120RasGAP bound to activated receptor tyrosine kinases (Settleman *et al.*, 1992) and may therefore be a link between Ras- and Rho-mediated signalling pathways. Activated Eph receptors can bind p120RasGAP (S.Holland, M.H.Henkemeyer and T.Pawson, unpublished results; J.Settleman, personal communication), suggesting that some of the guidance functions of Sek4 and Nuk may be mediated by p190RhoGAP and subsequent rearrangements in the actin cytoskeleton.

Sek4 and Nuk have distinct functions in different commissures

The guidance defect observed in the corpus callosum of *sek4–/–* and *sek4/nuk*^{1} double mutants differs in an informative way from that observed in the anterior commissure of *nuk*^{1} single mutants. In *nuk*^{1} single mutants, the initial outgrowths of most pars posterior axons forming the anterior commissure do not migrate in their normal path from the temporal cortex to the midline, but rather are misguided ventrally towards the floor of the brain (Henkemeyer *et al.*, 1996). In contrast, the initial outgrowth of cortical axons destined to form the corpus callosum of *sek4–/–* and *sek4/nuk*^{1} double mutants appears normal. However, these axons fail to migrate across the midline to the contralateral cortex. Thus, Sek4 and Nuk do not have an essential role in the initial guidance of callosal axons, but both receptors are required for these axons to

traverse the midline. This suggests that other guidance cues are sufficient for initial pathfinding, be they additional Eph receptor family members (Zhou *et al.*, 1994; Kilpatrick *et al.*, 1996) or distinct types of guidance molecules such as netrins or semaphorins (Dodd and Schuchardt, 1995; Kennedy and Tessier-Lavigne, 1995).

The underlying basis for this difference between the anterior commissure and corpus callosum phenotypes is probably related to the distinct patterns of expression of each receptor in the affected structures. Particularly, Nuk is highly expressed in cells of the brain ventral to the anterior commissure axons, and only weakly, if at all, in the commissural axons themselves (Henkemeyer *et al.*, 1996). In contrast, Nuk is most highly expressed in the midline region of the developing corpus callosum, where the defect in callosal axons occurs (see Figure 4D).

We have also uncovered an overlapping function for Sek4 and Nuk in the guidance of a subset of epithalamic axons forming the habenular commissure. In the double mutants, the posterior fibres of this tract were deflected from the midline and appeared to grow inappropriately along the anterior/posterior axis, while the anterior fibres crossed normally to the contralateral side. As with the anterior commissure and corpus callosum, expression of both Sek4 and Nuk was detected in the habenular region of the thalamus. Taken together, these data indicate that Sek4 and Nuk are required for the formation of specific commissural axon pathways in the mouse brain.

Sek4 and Nuk have redundant functions in axon fasciculation

Our results also show that Sek4 and Nuk cooperate in the fasciculation of fibres comprising the habenular–interpeduncular tract. Two main groups of axons are present in this longitudinal pathway. The majority of the axons originate from cells in the medial habenula (Hamill and Jacobowitz, 1984), whereas a second population of axons come from other regions of the brain, including the basal forebrain. These latter axons course through the rostral thalamus before descending caudally into the habenular–interpeduncular tract (Woolf and Butcher, 1985). A potential explanation for this reduced fasciculation in the *sek4/nuk*^{1} double mutants is that these mice have structural alterations within the thalamus or habenular nucleus, which in turn would interfere with the course of the axons as they migrate and funnel into the habenular–interpeduncular tract. However, a more likely explanation is that Nuk and Sek4, with their ligands, may play a direct role in axon bundling, e.g. by inducing their mutual association or by repelling them from surrounding cells. This would be consistent with the finding that AL1 affects axon fasciculation in culture (Winslow *et al.*, 1995). It is also possible that Nuk and Sek4 may act indirectly by regulating the expression of cell adhesion molecules, such as Tag-1.

The critical functions of Eph receptors are not restricted to the nervous system

The absence of both functional Sek4 and Nuk receptors results in a failure of palate fusion along the midline. The consequent cleft palate is likely to be primarily responsible for the observed perinatal lethality of the double homozygotes. Development of the neural crest-derived secondary palate is a complex process that begins with the vertical growth of bilateral palatal shelves from the maxillary process. At E14.5, palatal shelves rapidly elevate to a horizontal position above the tongue, and subsequently fuse at the midline (Ferguson, 1988). Disturbance of any of these events may result in a cleft palate. As rudimentary horizontal palatal shelves were observed in most *sek4/nuk*^{1} double-mutant mice, we conclude that the initial palatal outgrowth and elevation is likely to proceed in the absence of functional Sek4 and Nuk receptors. As both Sek4 and Nuk are specifically expressed at the edges of the palatal shelves, these receptors likely have a direct function in palate formation, either in promoting fusion of the midline epithelial cells, or in the epithelial to mesenchymal transformation that occurs after fusion (Shuler *et al.*, 1992). At least two other members of the Eph-related receptor family, Ehk3/Ebk and Tyro4, are highly expressed in the embryonic palate (Ellis *et al.*, 1995; Valenzuela *et al.*, 1995; Kilpatrick *et al.*, 1996). Overlapping functions of related receptors may be one possible explanation for the observed incomplete penetrance of cleft palate in *sek4/nuk*^{1} double-mutant mice.

In addition to Eph receptors, other factors are known to be important for palate development, notably retinoic acid (RA). Cleft palate phenotypes can be induced both by the administration of large doses of RA and by loss-of-function RA receptor mutations (Lammer *et al.*, 1985; Lohnes *et al.*, 1994). Interestingly, Lerk2, one of the ligands for Sek4 and Nuk, was recently isolated in a screen for RA-responsive genes and its expression was shown to be upregulated in cultured cells after RA treatment (Bouillet *et al.*, 1995). It is tempting to speculate that some of the actions of RA in the development of craniofacial structures are in fact mediated by Lerk2 and its cognate Eph receptors of the Elk subclass.

Physiological roles for Eph receptors

The family of Eph receptors is remarkable for the large number of its members, for their highly restricted patterns of expression, and for their ability to bind cell surface ligands. Here, we have provided direct genetic evidence to show that Nuk and Sek4, two Eph receptor family members, are required for the guidance or fasciculation of specific axon tracts in the mouse CNS. It seems likely that Eph receptors will play similar roles in the human brain, and might therefore be involved in agenesis of the corpus callosum in man (Aicardi *et al.*, 1987; Delezoide *et al.*, 1990; Jeeves, 1990; Kessler *et al.*, 1991; Fischer *et al.*, 1992), especially where this is connected to a median cleft face/palate syndrome (DeMyer, 1975). Furthermore, our results show that Eph receptors have functions outside the nervous system, consistent with the possibility that these molecules and their ligands play a general role in regulating cell–cell interactions during vertebrate embryonic development.

Materials and methods

Targeting in ES cells

The pPNT-based replacement-type targeting vector (Tybulewicz *et al.*, 1991) consisted of 7.5 kb of *sek4* genomic sequence upstream and 1.5 kb downstream of the neo cassette. The deleted region of 1.75 kb included four entire and one partial exon of the tyrosine kinase domain, spanning

amino acids (aa) 652 (immediately downstream of the conserved G-X-G-X-X-G) to 964 in the HEK2 coding region (Böhme *et al.*, 1993). Targeting of mouse R1 ES cells (Nagy *et al.*, 1993) was carried out as previously described (Wurst and Joyner, 1993). Screening of $G418^R$, $GANC^R$-resistant ES cells was by PCR (Joyner *et al.*, 1989) using a 5′ primer (5′-AAG CGC CTC CCC TAC CCG GTA-3′) corresponding to a region in the PGK promoter upstream of the neo gene and a 3′ primer (5′-CCC TGC ACT CTC ACC CTG CC-3′) corresponding to genomic sequences located 36 bp downstream of the 3′ end of the short arm of the targeting vector. PCR-positive ES cell clones were subsequently subjected to Genomic Southern Blot analysis using standard procedures. DNA (10 μg) was digested with *Nco*I or *Xba*I, electrophoresed on a 0.7% agarose gel, blotted, and hybridized with a ^{32}P-labelled 431 bp DNA fragment located immediately downstream of the 3′ end of the targeting vector and a ^{32}P-labelled *neo* probe. ES cells heterozygous for the targeted *sek4* allele were injected into C57Bl/6J blastocysts, which were then transferred into the uteri of pseudopregnant CD1 females. The resulting chimeras from one such ES cell line gave germline transmission after breeding to C57Bl/6J mice.

RT–PCR analysis

E16.5 embryos were collected from a cross of two *sek4*+/– mice and total RNA was extracted from wild-type, *sek4*+/– and *sek4*–/– embryo heads using the guanidine-thiocyanate method according to standard procedures. Forty micrograms of RNA were reverse transcribed into cDNA using oligo(dT) primers and Superscript Reverse Transcriptase (BRL). A 984 bp DNA fragment was amplified using *sek4*-specific primers, one 14 bp upstream (5′-GCTGGTGAGTTTGGGGAAGTG-3′, pos. aa 641–647 in HEK2 cDNA) and the other immediately downstream of the deleted region of the targeted allele (5′-GTGACCCCAATCCTTA-GCAG-3′, pos. aa 965–971 in HEK2 cDNA). Two other *sek4*-specific primers (upstream primer: 5′-CCCGATGCAGAATACACAGAG-3′, pos. aa 589–595; downstream primer: 5′-CGGCACACTTCCCCAAACTCC-3′, pos. 643–649 in HEK2 cDNA) specific for the juxtamembrane region were used to amplify a 183 bp DNA fragment. Two primers specific for the *neo* gene (upstream primer: 5′-GACGTTGTCACTGAAGCGGGAA-GGG-3′; downstream primer: 5′-AATCGGGAGCGGCGATACCGT-AAAGC-3′) were used to amplify a 508 bp DNA fragment. All DNA fragments were analysed in 1.5% agarose gels.

Histology and immunostaining

For histological analysis and immunostaining for neural cell adhesion molecules L1 (gift from M.Schachner) and Tag-1/axonin-1 (gift from P.Sonderegger), newborn mice (P0) were decapitated and fixed in 4% phosphate-buffered paraformaldehyde at 4°C. After 24 h, the solution was replaced with Tris/azide buffer containing 30% sucrose. Tissues remained at 4°C until they sank to the bottom of the tubes. Heads were mounted in tissue freezing medium (Tissue-Tek) and serial frozen sections, either coronally or sagittally, were taken at a thickness of 14 μm, thaw mounted onto gelatine-coated slides, allowed to air dry and further processed. Histological staining of the tissue was carried out with 0.1% cresyl violet solution according to standard protocols. Immunohistochemistry was performed using the ABC Vectastain kit as previously described (Wolfer *et al.*, 1994). Briefly, the tissue was incubated overnight with the diluted primary antibodies, either 1:500 diluted anti-Tag-1 or 1:100 diluted L1 antiserum. After several washes with Tris-buffered saline (TBS), the appropriate biotinylated secondary antibodies, rabbit anti-goat IgG and goat anti-rabbit IgG (Vector Labs), respectively, were applied and incubated again overnight. After incubation with ABC reagent, peroxidase was reacted with 0.02% DAB and 0.05% hydrogen peroxide in Tris buffer. All incubations were carried out at room temperature (RT).

For immunostaining for Lerk2 and Lerk5 (St Cruz, C18), embryos were collected from wild-type matings and fixed in 4% phosphate-buffered paraformaldehyde at 4°C for 3–6 h. Cryoprotection was done as for newborn animals, serial coronal sections were taken at 12 μm. For immunostaining, the antibodies were diluted 1:100. For peptide blocking experiments, primary antibodies were pre-incubated for 2 h at RT with 10-fold (by weight) excess of peptide antigen in TBS. Slides were mounted using Eukitt (Riedel-de-Haën) and observed under the light microscope (Zeiss Axiophot). Photographic slides were scanned (Nikon Coolscan) and the digitalized images assembled using Adobe Photoshop.

In situ hybridization

In situ hybridization analysis was carried out essentially as described (Klein *et al.*, 1989). Briefly, for the generation of a *sek4*-specific cRNA probe, a 735 bp long DNA fragment of Sek4 (pos. amino acid 641–885 of HEK2; Böhme *et al.*, 1993) was amplified by RT–PCR and subcloned into pBluescript. This plasmid was linearized with *Bam*HI and transcribed into a single-stranded antisense probe using T3 RNA polymerase (Promega) in the presence of ^{35}S-labelled αCTP (Amersham). Wild-type and *sek4*–/– embryos were collected at different stages and genotyped using extra-embryonic tissue. Embryos were fixed in 4% phosphate-buffered paraformaldehyde at 4°C for 3–6 h, dehydrated in graded ethanol solutions and embedded in paraffin. Serial coronal sections were taken at 8 μm and mounted onto gelatine-coated slides at 42°C. Proteinase K-treated tissues were hybridized with the *sek4* antisense probe (3×10^4 c.p.m./μl) at 52°C for 16 h and stringent washes were in 0.1× SSC for 15 min at 50°C. After dehydration, slides were dipped in photographic emulsion (Kodak, NTB-2) and exposed for 14 days at 4°C. Developed slides were stained with 0.2% toluidine blue and mounted as described above.

Alkaline phosphatase (AP) in situ analysis

Embryonic mouse (E14.0) cerebral cortices were dissected into Hanks' balanced salt solution (HBSS) containing 6.9 mM HEPES (pH 7.2). HBSS was removed and the tissue incubated for 15 min at 37°C with HEPES containing trypsin (Gibco). The trypsin solution was removed, replaced with HBSS and the tissue was triturated in 2.5 ml of the same solution with a fire-polished glass Pasteur pipette. The cell suspension was diluted to 2×10^6 cells/ml in minimal essential medium (MEM) supplemented with 10% heat-inactivated horse serum, 0.6% glucose, 2 mM glutamine and plated on 60 mm culture dishes (4 ml) containing polylysine-coated (1 mg/ml) coverslips. After 19 h of incubation in 5% CO_2 at 37°C, the cells were washed with HBSS supplemented with 0.5 mg/ml bovine serum albumin (BSA), 0.1% NaN_3 and 200 mM HEPES (pH 7) (HBHA) and then incubated for 2 h at RT in the same buffer containing either 3 nM unfused AP protein, Cek10–AP (Brambilla *et al.*, 1995) or Lerk5–AP (Elf2–AP) (Bergemann *et al.*, 1995). Coverslips were washed three times with HBSS, fixed for 1 min with 60% acetone, 3.7% formaldehyde, 20 mM HEPES and then washed again with HBSS. Endogenous cellular phosphatases were inactivated by heating the cells for 2 h at 65°C. The presence of bound exogenous AP was detected by incubating the cells overnight at 30°C with AP buffer containing 10 mM homo-arginine, 0.33 mg/ml NBT and 0.17 mg/ml BCIP (Cheng and Flanagan, 1994). Coverslips were then washed with phosphate-buffered saline (PBS), mounted with PBS–glycerol (1:1) and analysed under the light microscope.

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