Navigation of trochlear motor axons along the midbrain-hindbrain boundary by neuropilin 2

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Summary

Trochlear motor axons project dorsally along the midbrain-hindbrain boundary (MHB) to decussate at the dorsal midline. We report on the roles of neuropilin 2 and its ligands in the molecular mechanisms controlling this trajectory. In chick embryos, neuropilin 2 was expressed in the neuroepithelium of the dorsal isthmus in addition to the trochlear neurons, and Sema3F transcripts were localized along the caudal margin of the midbrain. Misexpression of Sema3F demonstrated that Sema3F displays repulsive activity in vivo that guides the trochlear motor axons along the MHB. An unexpected result was that misexpression of neuropilin 2 canceled the midbrain-evoked repulsion, allowing trochlear motor axons to cross the MHB and invade the tectum. A binding assay with neuropilin 2 ectodomain revealed the existence of neuropilin 2 ligands in the midbrain, which were masked by ectopic neuropilin 2. We therefore propose that neuropilin 2 neutralizes the repulsive activity in order to steer trochlear motor axons towards the dorsal decussation point. Taken together, our results suggest that the interaction of neuropilin 2 with its ligands has crucial roles for establishing trochlear trajectory along the MHB.

Key words: Chick, Trochlear nerve, Axon guidance, MHB, Neuropilin 2, Sema3F

Introduction

Navigation of growing axons, mediated by local guidance cues, is crucial for the establishment of a functional neuronal circuit. A number of chemoattractant and chemorepellant molecules have been reported to have function in this way (reviewed by Tessier-Lavigne and Goodman, 1996). Class 3 semaphorins are one family that act as diffusible guidance molecules in the peripheral and central nervous system (Luo et al., 1993; Messersmith et al., 1995; Püschel et al., 1995; Taniguchi et al., 1997; Shepherd et al., 1997; Varela-Echavarria et al., 1997; Chédotal et al., 1998). Sema3A acts as an inhibitory guidance cue to steer growth cones of both sensory and sympathetic axons (Luo et al., 1993; Fan and Raper, 1995; Messersmith et al., 1995; Taniguchi et al., 1997; Kitsukawa et al., 1997), whereas Sema3F repels sympathetic axons, but not sensory axons (Chen et al., 1998). The difference in sensitivity to these ligands is explained by the receptor type expressed in the growth cone. Sensory axons express only neuropilin 1, a receptor for Sema3A, while sympathetic axons express both neuropilin 1 and neuropilin 2 (Takagi et al., 1987; Takagi et al., 1991; Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). The Sema3F signal is mediated predominantly by neuropilin 2 (Chédotal et al., 1998; Chen et al., 1998; Giger et al., 1998).

In contrast to the segmental pattern of spinal nerves, each cranial nerve shows a specific fiber organization and function. The trochlear nerve, which is the fourth cranial nerve (IV), consists of a somatic motor component whose nucleus is located in the rostral part of the rhombomere 1 (Lumsden and Keynes, 1989; Mastick and Easter, 1996). The trochlear nerve is unique among cranial nerves in that its axons project dorsally to decussate at the dorsal midline and exit the brain contralaterally. During the dorsal projection the axons elongate along the midbrain-hindbrain boundary (MHB). This trajectory suggests that the interneuromeric boundary may affect the organization of the axonal tract (Chédotal et al., 1995; Mastick and Easter, 1996). However, the molecular mechanism that regulates the establishment of the trochlear axon trajectory along the MHB remains undefined.

It was reported that neuropilin 2 mutant mice exhibit disorganization of several cranial nerves and spinal nerves. In homozygous mutant mice, the trochlear motor axons showed aberrant trajectory, or were missing, although their nucleus was present (Giger et al., 2000; Chen et al., 2000). Trochlear neurons express neuropilin 2, and trochlear motor axons were repelled by Sema3F-transfected cells in vitro (Giger et al., 2000). Moreover, recent paper reported that most of the trochlear nerve were absent in Sema3F-null mutant mice (Sahay et al., 2003). It was thus suggested that neuropilin 2-mediated Sema3F repulsion may guide trochlear axons.

In this study we examined the distribution of neuropilin 2 and Sema3F mRNA in chick embryos. Unexpectedly, neuropilin 2 expression was detected not only in the trochlear nucleus but also in the neuroepithelium of the dorsal isthmus. Sema3F expression was localized to the caudal part of the midbrain along the MHB, and in the rhombic lip. This
expression pattern suggested that Sema3F repulsion may guide trochlear axons along the MHB. However, it was not clear if neuropilin 2 in dorsal isthmus is involved in trochlear axon guidance. To resolve this, we have used a gain-of-function approach in chick embryos, and further explored the roles of neuropilin 2 and Sema3F in trochlear axon guidance. In vivo electroporation revealed that Sema3F indeed repels trochlear axons in vivo, suggesting that Sema3F provides an inhibitory barrier to guide these axons along the MHB. By contrast, ectopic neuropilin 2 inactivated this repulsive activity, i.e. the invasion of trochlear axons into the tectum occurred after neuropilin 2 misexpression. Binding assay using a soluble neuropilin 2 has demonstrated that ectopic neuropilin 2 cancels neuropilin 2 binding activity distributed in the midbrain. We suggest that neuropilin 2 can neutralize the midbrain-evoked repulsive activity in the dorsal isthmus to navigate trochlear axons toward the decussation point. We finally propose a model in which the interaction of neuropilin 2 with its ligands plays decisive roles in trochlear axon guidance.

Materials and methods

Whole-mount immunostaining and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). After removal of the epidermis and mesenchyme, the specimens were immunostained in PGT (PBS-0.2% gelatine-0.25% Triton X-100) containing 5% heat-inactivated goat serum. Primary antibodies against neurofilament (3A10; Developmental Studies Hybridoma Bank or NF-M; Chemicon), ISL1 (40.3A4; DSHB), myosin (MF20; DSHB), GFP (Molecular Probes), and Alexa488 or Alexa594-conjugated secondary antibodies against mouse or rabbit IgG (Molecular Probes) were used. For double staining with MF20 and 3A10, FITC-conjugated anti-mouse IgG2b and Texas Red-conjugated anti-mouse IgG (Molecular Probes) were used as secondary antibodies, respectively. The stained specimens were cleared in 40% glycerol and viewed under a fluorescence dissection microscope (MZ FLIII, Leica).

Flat-mounted embryos were observed under a fluorescence microscope (BX51, Olympus) and photographed with a cooled CCD digital camera (ORCA-ER, Hamamatsu). Fluorescence images were also captured on a confocal laser scanning unit (CSU10, Yokokawa). Three-dimensional images were reconstructed from serial z-axis images using volume rendering software (Aquaconsoms, Hamamatsu). Rotated images were captured and processed by a confocal laser scanning system (FLUVIEW, Olympus). Red fluorescent color of Alexa594 or Texas Red was converted to magenta in figures. For immunohistochemistry, embryos were fixed in 4% paraformaldehyde and embedded in paraffin wax. Sections (5 μm) were reacted with primary (3A10) and secondary (HRP-conjugated anti-mouse IgG) antibodies. The specimen was colored with 3,3′-diaminobenzidine (DAB) substrate and counter-stained with anti-mouse IgG) antibodies. The specimen was colored with 3,3′-diaminobenzidine (DAB) substrate and counter-stained with anti-mouse IgG antibodies. The specimen was colored with 3,3′-diaminobenzidine (DAB) substrate and counter-stained with anti-mouse IgG

Cloning of neuropilin 2 and Sema3F

A cDNA fragment for chick neuropilin 2 was amplified from E3 chick brain mRNA by RT-PCR using the degenerate primers (5′- MRMGAICTGCAAGATATGAYT-3′ and 5′-GTTTCTCCAGTTG- TGCAGCT-3′). A PCR product of 2.1 kb was identified as a chick homologue of neuropilin 2 covering the b1 domain to cytoplasmic domain, and was subsequently used to synthesize a DIG-labeled anti-sense RNA probe for Sema3F. The same fragment was used to isolate the full-length coding region by screening an E2 chick cDNA library. The amino acid sequence and its corresponding coding sequence were registered in the DNA data bank (Accession Number, AB072930). The gene trees were constructed by CLUSTALW alignment (Thompson et al., 1994).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described previously (Watanabe and Nakamura, 2000). Digoxigenin- or FITC-labeled RNA probes were synthesized according to the manufacturer’s protocols (Promega; Ambion). Some specimens were subsequently reacted with 3A10 antibody to detect axonal fibers.

Construction of expression vectors and in ovo electroporation

Chick Sema3F-coding region was inserted into the epitope tagging vector pCMV-Tag5B (Stratagene). The coding region with a C-terminal Myc tag was inserted into a pMiwII expression vector derived from pMiwSV, which carries the chick β-actin promoter and RSV enhancer (Wakamatsu et al., 1997). The coding region of neuropilin 2 was inserted into the pMiwIII expression vector, modified from pMiwII at the multiple cloning sites.

Fertile chick eggs were incubated at 38°C in a humidified atmosphere. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). In vivo electroporation was performed using stick electrodes as described previously (Funahashi et al., 1999; Nakamura et al., 2000). The Sema3F construct (pMiwII-Sema3F-c-myc) or the neuropilin 2 construct (pMiwIII-NP2) was mixed with the GFP expression vector (pEGFP-N1; Clonetech) in a final concentration at 0.5 μg/μl and 1 μg/μl. DNA solution of 0.1-0.2 μl in Tris-EDTA buffer was injected into the central canal with a micropipette. For transfection at stage 14, a pair of electrodes (0.5 mm diameter, 1.0 mm length and 4 mm distance between the electrodes; Unique Medical Imada, Natori, Japan) was placed on the dorsal (anode) and ventral (cathode) sides of the embryos to achieve dorsal-specific transfection. Unilateral transfection at stage 11 was performed as previously described (Watanabe and Nakamura, 2000). A rectangular pulse of 25 V for 50 ms was emitted four times at 1 second intervals by the electroporator (CUY21, Tokiwa Science, Fukuoka, Japan). GFP fluorescence coincides with the expression sites of the transgene (Momose et al., 1999).

Dil-labeling of trochlear axons

Normal or neuropilin 2-transfected embryos were fixed with 4% paraformaldehyde and brain tissues were dissected out. Trochlear nucleus were immunostained using an anti-ISL1 primary antibody (40.3A4; DSHB) and the secondary antibody (HRP-conjugated anti-mouse IgG; Jackson) without detergents throughout the process. Incubation in DAB substrate in the presence of 0.03% cobalt chloride faintly colors the trochlear nucleus, where small crystals of Dil were placed. Then the specimens were incubated at 37°C until trochlear axons were labeled anterogradely.

AP fusion protein-binding assay

A DNA fragment for neuropilin 2 ectodomain without signal sequence, transmembrane domain and cytoplasmic domain was amplified from chick neuropilin 2 coding-region by PCR using the primers (5′-AGCTTTGGCGGCAGACAGCTCAGCCG-3′ and 5′-GGATCTCTGGAAAGCTGATGCGTT-3′). A HindIII-BamHI fragment of amplified DNA was subcloned into HindIII-BglII
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site of pApTag5 (GenHunter) to produce neuropilin 2 ectodomain fused with alkaline phosphatase at its C terminus. This construct was transfected into 293T cells with TransFast (Promega) according to manufacture’s protocol. Cell culture supernatant containing AP fusion protein (NP2dC-AP) was used as a probe for binding reaction as described below.

Brain tissues were dissected out in PBS, blocked with 10% fetal bovine serum in PBS for 5 minutes, and then incubated with 50% dilution of NP2dC-AP for 1 hour at room temperature. After washing three times with PBS, tissues were fixed in 4% paraformaldehyde for 1 hour, and washed three times again in PBS. Then endogenous AP activity was heat-inactivated at 65°C for 3 hours and AP activity was detected by NBT/BCIP staining for several hours. The staining reaction was stopped within 1 hour for detection of abundant Sema3F misexpressed by electroporation. The procedure was modified from Flanagan et al. (Flanagan et al., 2000).

Some embryos were subsequently reacted with 3A10 antibody to detect axonal fibers.

Results

Trajectory of trochlear nerves during development

We first examined the spatial and temporal trajectory patterns of trochlear axons by whole-mount immunostaining with anti-neurofilament antibodies NF-M and 3A10 (Serafini et al., 1996). The trochlear nucleus lies in the rostral part of rhombomere 1, dorsal to the floor plate at the level of medial longitudinal fasciculus (Fig. 1A). From the nucleus, trochlear axons project dorsally to the decussation point at the MHB. Before stage 16, dorsally projecting axons were not detected in the midbrain-hindbrain region (data not shown). A small number of such axons could be detected by stage 17 in the ventral isthmus (Fig. 1B). These were identified as trochlear axons because the cell nuclei were ISL1 positive and the axons perpendicularly crossed the medial longitudinal fasciculus. By stage 18, the number of neurons increased, and the axons...
extended more dorsally (Fig. 1C). By stage 19, the number of neurons had markedly increased and the foregoing axons from the rostral population of the nucleus had already crossed the lateral longitudinal fasciculus to extend in the dorsal isthmus (Fig. 1D). The axons of newly differentiated neurons extended in the rostrodorsal direction and followed the foregoing axons. By stage 20, the axons approach the dorsal midline (Fig. 1E). The entire trajectory was clearly visualized in the rostral view of a thick transverse section (Fig. 1F). During the dorsal projection from stage 17 to stage 20, a number of axons directed towards the dorsal decussation point, but axons that intrude into the midbrain were not detected. Trochlear axons crossed the dorsal midline during stage 20-21. By stage 23, all axons had already sprouted out contralaterally and were extending towards the target (Fig. 1G,H). By stage 26, the axons had reached the precursor of the superior oblique muscle for innervation (Fig. 1I,J).

Trochlear axon bundles were discernible in the sagittal section at stage 24, when the trochlear nerve had decussated at the dorsal midline. In the parasagittal section, two axonal bundles were observed at the dorsal MHB (Fig. 1K,L); one is from the ipsilateral nucleus running in the wall of the neural tube and the other is from the contralateral nucleus, which had already come out of the brain (Fig. 1L). At the midline, only one bundle was observed within the brain, which indicates that two bundles were decussating (Fig. 1M). In summary, trochlear axons initially project dorsally along the MHB and decussate at the midline within the brain before exiting from the contralateral side.

**Expression of neuropilin 2 in trochlear neurons and neuroepithelium of the dorsal isthmus**

It has been suggested that neuropilin 2 is implicated in trochlear axon guidance. In mutant mice homozygous for neuropilin 2, the projection of trochlear motor axons was aberrant or missing (Giger et al., 2000; Chen et al., 2000). Sema3F is the instructive agent for the neurons that express neuropilin 2, a functional receptor for Sema3F (Chédotal et al., 1998; Chen et al., 1998; Giger et al., 1998). Actually, Sema3F can repel trochlear axons in vitro (Giger et al., 2000). We have isolated a chick neuropilin 2 cDNA, whose deduced amino acid sequence is highly homologous to human and mouse neuropilin 2. Molecular phylogenetic analysis showed the amino acid sequence clustering with human and mouse counterparts, indicating that it corresponds to chick neuropilin 2 (Fig. 2A). We have also isolated a chick Sema3F cDNA, whose deduced amino acid sequence is clustered with human and mouse Sema3F (81% identity for both; Fig. 2B).

We examined the expression of neuropilin 2 by whole-mount in situ hybridization in chick embryos in relation to trochlear nerve development. During the dorsal projection of trochlear axons, neuropilin 2 was expressed in the oculomotor nucleus and also in the trochlear nucleus (Fig. 3C,D). Unexpectedly we found neuropilin 2 expression in the dorsal isthmus (Fig. 3A-D). The expression was prominent at stage 14 before its onset in the trochlear nuclei (Fig. 3A), and was sustained during the dorsal projection of trochlear axons. Trochlear axons extended toward the dorsal isthmus, where neuropilin 2 signal was highly positive (Fig. 3D,E). Distinct from its expression in the cranial nerve nucleus in the mantle layer, neuropilin 2 expression in the dorsal isthmus was localized in the neuroepithelium (Fig. 3F). The distal region of extending axons coincided with neuropilin 2 positive neuroepithelium at stage 19-20 (Fig. 3G). It is clear from the flat-mount staining that trochlear axons pass through the neuropilin 2-positive domain to reach the decussation point (Fig. 3J-L).

We also found that, although the entire oculomotor nucleus was highly positive for neuropilin 2, the signal in the trochlear nucleus was limited to the rostral population of the trochlear neurons at stage 19 (Fig. 3J-L). In comparison to ISL1 staining in the entire trochlear nucleus (Fig. 3H,I), neuropilin 2 expression was prominent only in the rostral population of the trochlear neurons (Fig. 3J-L). This observation suggests that neuropilin 2 positive trochlear neurons are located at the rostral nucleus and send forgoing axons dorsally.

**Expression of Sema3F along the midbrain-hindbrain boundary**

Subsequently, we examined the expression of Sema3F in chick embryos focusing on the midbrain-hindbrain region. Faint expression of Sema3F was observed throughout the ventral midbrain from stage 10-11 (Fig. 4A). The expression became intense after stage 12 (Fig. 4B), having extended dorsally to the caudal part of the midbrain by stage 14 (Fig. 4C). At stage 16, which is just before the emergence of the first trochlear axons (Fig. 1B), the expression was confined to the most caudal part of the midbrain (Fig. 4D). During the dorsal projection of the trochlear axons, the caudal limit of Sema3F expression was clearly demarcated, as if it provided a linear boundary along the MHB (Fig. 4D-F).

To reveal the exact position of Sema3F expression in the caudal midbrain, its expression was compared with that of other gene markers. It was reported that Otx2 and Gbx2
expression abuts at the MHB. *Fgf8* expression is induced at the interface of *Otx2* and *Gbx2* expression, overlapping with *Gbx2* expression. Hence, *Otx2* and *Fgf8* expression also abuts at the MHB (Hidalgo-Sánchez et al., 1999; Katahira et al., 2000). Double staining for *Otx2* and *Sema3F* showed that the caudal limit of the *Sema3F* expression corresponded to that of *Otx2* expression, and abutted on the rostral limit of the *Gbx2* expression domain (Fig. 4E,I,J). Consequently, *Sema3F* expression was rostral to the *Fgf8* expression in the isthmus (Fig. 4H). The results indicate that during the dorsal projection of trochlear axons at stage 19. (J-L) Staining of flat-mount specimen for *neuropilin 2* (J, dark blue) and neurofilament (K, magenta) at st19. (L) Negative image of J was merged with K. Trochlear nucleus along the rostrocaudal axis is indicated by double-headed arrows. *neuropilin 2* is expressed in the neuroepithelium of the dorsal isthmus, in addition to the oculomotor and rostral trochlear nucleus. III, oculomotor nucleus; IV, trochlear nucleus; dl, dorsal isthmus; ne, neuroepithelium layer; Mid, midbrain; Hind, hindbrain. Scale bars: 200 μm for A-E,H-L; 50 μm for F,G.

**In vivo repulsive action of Sema3F on trochlear axons**

Assuming that Sema3F protein is an inhibitory guidance cue that provides repulsive activity, it is likely that Sema3F excludes trochlear axons to guide them along the MHB. The question remains, however, as to whether Sema3F protein is repulsive in vivo for trochlear axons. To address this, Sema3F was transfected by in ovo electroporation into dorsal isthmus during projection of trochlear axons in the hindbrain.

First, correct translation of Sema3F protein was ascertained in vivo by translation of Myc tag at the C terminus of the Sema3F construct. Co-electroporation of the Sema3F plasmid (pMiwII-Sema3F-c-myc) and a GFP vector (pEGFP-N1) allowed us to monitor the transfection by GFP fluorescence (Momose et al., 1999). Electroporation was carried out at stage 14 to misexpress Sema3F in the MHB region prior to the
In summary, the results show that Sema3F protein provides functional guidance activity in vivo by repelling trochlear axons during the dorsal projection.

**Neuropilin 2 misexpression causes invasion of trochlear axons into tectum**

As neuropilin 2 is expressed in the neuroepithelium in the dorsal isthmus, but not in the mantle layer, it is unlikely that neuropilin 2 serves as the ligand sensor for axons at this site. Nevertheless, its spatial expression pattern strongly suggests that it may be involved in the guidance of trochlear axons in the dorsal isthmus. To explore its role in the neuroepithelium, we misexpressed neuropilin 2 in the MHB and observed the effects on axon pathfinding.

Full-length neuropilin 2 cDNA inserted in the expression vector, pMiwIII-NP2 was electroporated into the brain at stage 11 together with a GFP expression vector (pEGFP-N1). In the transfected side at stage 22, it was notable that so many axons were deflecting and invading the tectum. In the MHB and observed the effects on axon pathfinding.

Projection of trochlear axons. In the normal brain, trochlear axons had crossed the dorsal midline by stage 23 (Fig. 5A).

In the experimental brain, trochlear axons did not cross the dorsal midline (Fig. 5B). Axons from the trochlear nucleus contralateral to the Sema3F-misexpressing side encountered the ectopic Sema3F-transfected domain, as assessed by GFP fluorescence, just before crossing of the dorsal midline. All the axons were repelled at a certain distance of the Sema3F-transfected domain, which resulted in a change of direction of nearly 180° (Fig. 5C,D). These axons then exited the brain from the ipsilateral side and appeared to follow the proper trochlear nerve route to elongate toward the ipsilateral target muscle (Fig. 5B,C). However, in the Sema3F-misexpressing side, the axonal elongation seemed to be stuck en route of its projection (Fig. 5B). Trochlear axons showed such abnormal trajectories in all the Sema3F-transfected embryos (10/10 GFP-positive embryos; Table 1).

We have further tried to monitor the behavior of axons when they encountered ectopic Sema3F. To visualize spatial axonal trajectory, the brain tissues were flat-mounted and immunofluorescence images were captured on a confocal laser scanning system to process three-dimensional rendering. In normal embryos, trochlear axons which had crossed the lateral longitudinal fasciculus gathered in dorsal isthmus (Fig. 5E). Interestingly, the distal portion of trochlear axons including growth cones turned to the neuroepithelium (Fig. 5F), which is consistent with the observation of the immunostained section (Fig. 3F,G). Distal tip of axons in the neuroepithelium were localized dorsal to the lateral longitudinal fasciculus (Fig. 5E,F). In Sema3F-misexpressing side, trochlear axons ceased dorsal elongation when they encountered ectopic Sema3F and turned to the neuroepithelium (Fig. 5G,H). When transfection was made more ventrally, trochlear axons encountered Sema3F in ventral isthmus, turned once to the neuroepithelium and then reversed to exit the brain (Fig. 5I-K). Trochlear axons behaved as if they avoided repulsive cues, but finally they were expelled out from the brain.

In summary, the results show that Sema3F protein provides functional guidance activity in vivo by repelling trochlear axons during the dorsal projection.

**Neuropilin 2 misexpression causes invasion of trochlear axons into tectum**

As neuropilin 2 is expressed in the neuroepithelium in the dorsal isthmus, but not in the mantle layer, it is unlikely that neuropilin 2 serves as the ligand sensor for axons at this site. Nevertheless, its spatial expression pattern strongly suggests that it may be involved in the guidance of trochlear axons in the dorsal isthmus. To explore its role in the neuroepithelium, we misexpressed neuropilin 2 in the MHB and observed the effects on axon pathfinding.
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We have confirmed that neuropilin 2 misexpression did not affect Sema3F and Fg8 expression (data not shown).

In immunostaining specimens, it appeared that aberrant axons in neuropilin 2-transfected embryos extended not only from rostral but also from caudal trochlear nucleus, i.e., both rostral and caudal trochlear axons have invaded the tectum (Fig. 6E,F,H). We have examined the projection of trochlear axons from the rostral and caudal part of the nucleus. For this purpose, Dil crystals were put in nucleus after visualization of the nucleus by ISL1 staining. In neuropilin 2-transfected embryos, both rostral and caudal trochlear axons extended rostrally to invade the tectum (Fig. 6K). In particular, axons from each part of the nucleus independently entered the tectum rather than making thick fasciculi. It suggests that as the pre-existing rostral axons deflected rostrally, the caudal axons could not join the rostral axons, and consequently crossed the MHB independently. In normal embryos, trochlear axons from Dil labeled nucleus extended dorsally toward the decussation point (Fig. 6J).

**Neuropilin 2 masks its ligand to navigate trochlear axons**

The result of neuropilin 2 misexpression indicates that the inhibitory barrier at the MHB was weakened or dismissed by the transfection of neuropilin 2 so that trochlear axons could invade the tectum. It is plausible that ectopic neuropilin 2 has bound its ligand and canceled its repulsive activity against the trochlear axons. To verify the possibility, we have examined the distribution of neuropilin 2 ligand in normal and neuropilin 2-transfected embryos.

Distribution of the ligand can be visualized by receptor-ligand interaction with a soluble AP fusion protein (Flanagan et al., 2000). For this purpose, neuropilin 2 ectodomain fused to the N terminus of AP (NP2dC-AP) was prepared, and reacted with brain tissues. First, we checked if NP2dC-AP actually detect the ligand distribution of neuropilin 2 in vivo. After co-electroporation of GFP and Sema3F, the GFP-positive region, i.e., the Sema3F-misexpressing region, coincided with NP2dC-AP staining, indicating that this probe can detect the ligand distribution in the brain (Fig. 7G,H). Then, potential

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<tr>
<td>Sema3F + GFP</td>
<td>10</td>
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<td>Repelled (misexpression side)</td>
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*n.d., not determined (malformation of the embryo).
neuropilin 2 ligand distribution was examined on normal embryos with NP2dC-AP. At stage 17, when trochlear axons began to elongate, strong AP staining was found in the whole midbrain and the rhombic lip, but the isthmic region was devoid of staining (Fig. 7A-C). This staining pattern was kept until stage 19 when trochlear axons approach the decussation point (Fig. 7D-F). Thus, trochlear axons could proceed dorsally in the isthmic region which was devoid of the neuropilin 2 ligand. Treatment with intact AP protein gave no apparent staining as observed after NP2dC-AP treatment (data not shown).

Finally, the distribution of neuropilin 2 ligand was examined on neuropilin 2-transfected embryos. In the GFP-positive region where neuropilin 2 was misexpressed, binding activity with NP2dC-AP was significantly reduced or dismissed in compared with the untransfected side (6/7 GFP-positive embryos; Fig. 7I,J). Transfection of GFP did not affect the binding activity of NP2dC-AP (6/6 GFP-positive embryos; data not shown). The results suggest that transfected neuropilin 2 in the midbrain masks its ligand against neuropilin 2-positive trochlear axons so that they could invade the tectum.

Discussion
Trochlear axons originate from the nucleus at the ventral isthmus, project dorsally and then exit the brain from the contralateral dorsal surface after decussating at the dorsal midline. During the dorsal elongation in hindbrain, trochlear axons are under the control of elaborate guidance mechanisms that allow them to extend toward the single decussation point. In this study, we have explored how this unique trajectory is established during development, focusing on the roles of neuropilin 2 and Sema3F. We have obtained the following results in the present study. Sema3F was expressed at the most caudal part of the midbrain, and in the rhombic lip. However, neuropilin 2 was expressed in the trochlear nucleus and also in the neuroepithelium of the dorsal isthmus, where the distal region of trochlear axons converge before the decussation. Misexpression of Sema3F revealed that Sema3F repels trochlear axons in vivo. Misexpression of neuropilin 2 caused invasion of trochlear axons into the tectum. A binding assay with neuropilin 2 ectodomain showed that neuropilin 2 ligands are distributed in the midbrain and that it is masked by ectopic neuropilin 2. Based on these results, possible roles of the neuropilin 2 and its ligand in trochlear axon pathfinding are discussed below.

Neuropilin 2 conveys chemorepulsion to trochlear axons
Neuropilin 1 and neuropilin 2 are the receptors that directly bind to class 3 semaphorins. As only neuropilin 2 is expressed in the trochlear nucleus during the dorsal projection (Y.W., unpublished), neuropilin 2 is the only known receptor for class 3 semaphorins in trochlear axons. However, caudal populations of the trochlear motoneurons do not express neuropilin 2 (see Fig. 3H-L). It is thus inferred that trochlear axons from neuropilin 2-negative caudal nucleus should be
Navigation of trochlear axons along MHB insensitive to the repulsive activity. Accordingly, foregoing axons from the rostral trochlear nucleus may act as pioneer axons and the axons from the caudal nucleus may follow them. This idea is supported by the axonal phenotype of Sema3F-transfected embryos, because both rostral and caudal trochlear axons turned back when they met ectopically transfected Sema3F. Sema3F-insensitive axons may have followed the preceding axons that had been repelled by the ectopic Sema3F.

Existence of neuropilin 2 ligands as the barrier for trochlear axons

Sema3F repelled trochlear axons in vivo at a distance from the Sema3F-transfected domain, indicating that diffusible Sema3F molecules serve as the inhibitory barrier for trochlear axons in the midbrain by preventing them from crossing the MHB. However, expression of Sema3F mRNA in caudal midbrain was not obvious along the dorsal MHB. Therefore, it is not known Sema3F is sufficient to provide the barrier all along the MHB. To resolve this issue, we have examined the distribution of neuropilin 2 ligand, using a soluble neuropilin 2 ectodomain-AP fusion protein as a probe. In the hindbrain, strong AP staining was found in the rhombic lip, which coincided with Sema3F mRNA expression. However, the staining was seen in the whole midbrain, which was different from the line shape expression of Sema3F mRNA in the caudal margin of the midbrain. This indicates that another ligand(s) for neuropilin 2 should be involved in trochlear axon guidance as the repulsive cue. The most plausible candidate is Sema3C (formerly SemE or Collapsin 3), which is the nearest family member of Sema3F (see Fig. 2B) and it can bind to neuropilin 2 (Chen et al., 1997). We have examined expression of Sema3C mRNA in mid-hindbrain region, but we have recognized it only in the oculomotor nucleus, which is not comparable with the broad distribution of neuropilin 2 ligand in the midbrain (Y.W., unpublished). Therefore, in chick embryo, unidentified
ligand(s) for neuropilin 2 may participate in trochlear axon guidance together with Sema3F.

It is noteworthy that Sema3F transfection caused typical effects on trochlear trajectory. When trochlear axons encountered ectopic Sema3F en route, the axons ceased elongation and the distal axons turned to neuroepithelium. It seemed as if axons avoided surrounding repulsive agents. In fact, they were caught between the ectopic Sema3F dorsally, and the floorplate which provides repulsive activity to trochlear axons (see Fig. 5G-K) (Colamarino and Tessier-Lavigne, 1995; Serafini et al., 1996). In some cases, these axons subsequently changed the course to reverse direction and went out of the brain. Interestingly, in normal embryos, distal regions of trochlear axons including growth cones similarly converge in neuroepithelium, where neuropilin 2 is expressed. As neuropilin 2 can neutralize the repulsive activity of its ligands as discussed below, trochlear axons may have converged in the neuroepithelium to avoid the repellants in embryos. However, it remains elusive how trochlear axons subsequently decussate and exit the brain.

**Neuropilin 2 neutralize the repulsive activity of its ligands**

In the neuropilin 2-transfected brain, the trochlear axons acted as if there was no inhibitory barrier and crossed the MHB, while Sema3F mRNA localization at the MHB was unchanged. Therefore, the aberrant axonal projection should be due to functional loss of the inhibitory barrier at the MHB after neuropilin 2 misexpression. It is known that neuropilin 2 can form a homodimer or multimer and bind to Sema3F in vitro in the absence of other components, such as plexins (Chen et al., 1998; Feiner et al., 1997; Nakamura et al., 1998; Takahashi et al., 1999; Tamagnone, 1999). As exogenous neuropilin 2 binds to Sema3F in vivo, it is plausible that the ectopic neuropilin 2 bound to endogenous ligands in the midbrain, and masked them from the neuropilin 2-positive trochlear axons. Consequently, the repulsive activity of the neuropilin 2 ligands might be cancelled. Without the inhibitory barrier, trochlear axons could penetrate the MHB and invade the ipsilateral tectum region. This interpretation is strongly supported by the result that the binding affinity of the neuropilin 2 ligands with neuropilin 2 ectodomain was lost in neuropilin 2-transfected midbrain. These results suggest that neuropilin 2 masked its ligands to neutralize their repulsive activity against trochlear axons.

There is another possibility that ectopic neuropilin 2 may homophilically interact with neuropilin 2 positive trochlear axons to navigate them into the tectum. This idea comes from the fact that neuropilin 2 in cultured cell line can homophilically interact with exogenous neuropilin 2 in trans (Chen et al., 1998). If it is true in vivo, misexpressed neuropilin 2 must interact with exogenous neuropilin 2. However, in our experiment, misexpressed neuropilin 2 did not bind with a soluble neuropilin 2 ectodomain and instead bound with its ligands (see Fig. 7J). Thus, the aberrant troclear trajectory should be caused by the ligand-receptor binding rather than receptor-receptor interaction.

In this context, it is particularly interesting that in normal development neuropilin 2 is expressed in the neuroepithelium of dorsal isthmus. As a membrane-bound receptor, neuropilin 2 must be immobilized on the neuroepithelial cell surface in the dorsal isthmus and be able to bind its ligands. Importantly, the ligands including Sema3F are distributed in the midbrain and the rhombic lip, adjacent to the neuropilin 2 expression domain. Based on the results of neuropilin 2 misexpression, we propose that neuropilin 2 traps repulsive cues in the dorsal isthmus and neutralizes its activity. This may provide the ligand-free space for trochlear axons to pass through the dorsal isthmus despite of being surrounded by repulsive sources.

Our results suggest that neuropilin 2 has a novel biological activity to function in axon guidance, albeit indirectly by masking its ligands. A similar mechanism is suggested in the modulation of ligand distribution by receptor binding for axonal guidance in *Drosophila*. The Netrin receptor, Frazzled can capture and accumulate secreted Netrin protein to navigate photoreceptor axons (Hiramoto et al., 2000). A common feature between the two guidance mechanisms of Frazzled and neuropilin 2 is that the membrane-bound receptor traps diffusible ligand on the regions where axons extend, and this process does not necessarily induce intracellular signaling events. This may provide a general mechanism to modify the distribution of ligand to achieve elaborate axon guidance.

**Other guidance cues for trochlear axon guidance along the MHB**

It was observed that trochlear axons elongate dorsally and simultaneously deflect in a rostral direction throughout the dorsal projection. When the inhibitory barrier of the midbrain was blocked by neuropilin 2 misexpression, both rostral and caudal trochlear axons deflected toward the rostral direction. These results indicate that some attractant or repellant cue exists for the general rostral direction of trochlear axons. In rodent embryos, broad expression of Sema3F mRNA in the hindbrain suggest that Sema3F could be a hindbrain-derived repulsive agent (Giger et al., 2000). In chick embryos, neuropilin 2 ligands seem to be diffused in the hindbrain (see Fig. 7A,J). However, Sema3F may be inadequate to explain the hindbrain-derived repulsion because chick neuropilin 2 expression is absent in caudal trochlear nucleus. In addition, this rostral guidance activity was not cancelled after neuropilin 2 misexpression.

It was reported recently that Fgf8 protein had acted as a chemoattractant for trochlear axons in vitro and in vivo (Irving et al., 2002). Fgf8 could be a candidate for the general rostral guidance cue for trochlear axons, because its mRNA is localized at the rostral margin of the hindbrain along the MHB, just caudal to Sema3F expression domain. In this context, trochlear axons may be attracted rostrally towards the Fgf8-expressing domain, the source of Fgf8 protein. However, after neuropilin 2 misexpression, distribution of Fgf8 mRNA was similar to that in the normal embryo, indicating that aberrant trochlear axons initially extended towards the Fgf8-expressing domain, but subsequently crossed it; i.e. the axons moved away from the Fgf8 source. This is not explained solely by the model of Fgf8-evoked chemoatraction, as trochlear axons finally project away from the source of Fgf8 protein. Therefore, we suspect that other guidance cue(s) distinct from Fgf8 is also involved in the guidance of chick trochlear axons toward the rostral direction.
Roles for neuropilin 2 and its ligands in trochelean axon guidance

Based on the results presented here, we propose a model for the trochelean axon guidance as schematically summarized in Fig. 8A. Trochelean axons project dorsally in response to the repulsive signal from the floor plate (Colamarino and Tessier-Lavigne, 1995; Serafini et al., 1996). During the dorsal projection, trochelean axons incline to the rostral direction in response to unidentified rostral guidance cue(s). As repulsive cues including Sema3F exist in the midbrain, they do not cross the MHB, but instead elongate along the MHB. In the dorsal isthmus, trochelean axons are surrounded by the sources of repellants in the midbrain and the rhombic lip. Nevertheless, they can proceed to reach the decussation point, as neuropilin 2 in the dorsal isthmus may bind to its ligands to neutralize their repulsive activity. The trochelean axons that do not express neuropilin 2 also incline to the rostral direction, and join the foregoing axons in the dorsal isthmus to reach decussation point. Thus, when the neuropilin 2 is misexpressed in the midbrain, the midbrain-evoked repulsive activity is cancelled and trochelean axons deflected to the tectum by crossing the MHB (Fig. 8B). The trochelean axons cannot join the rostral foregoing axons, because deflection seems to have occurred in the ventral isthmus before joining, so that the caudal axons independently invade the tectum. In this regard, neuropilin 2 has dual functions in axon guidance: neuropilin 2 in trochelean neurons plays a direct role in midbrain-evoked repulsive guidance along the MHB; and neuropilin 2 in the neuroepithelium indirectly provides an axonal pathway toward the dorsal decussation point by canceling the repulsion.

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