Ephrin A/EphA controls the rostral turning polarity of a lateral commissural tract in chick hindbrain

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Ephrin A/EphA controls the rostral turning polarity of a lateral commissural tract in chick hindbrain

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Most post-crossing commissural axons turn into longitudinal paths to make synaptic connections with their targets. Mechanisms that control their rostrocaudal turning polarity are still poorly understood. We used the hindbrain as a model system to investigate the rostral turning of a laterally located commissural tract, identified as the caudal group of contralateral cerebellar-projecting second-order vestibular neurons (cC-VC). We found that the caudal hindbrain possessed a graded non-permissive/repulsive activity for growing cC-VC axons. This non-permissiveness/repulsion was in part mediated by glycosyl-phosphatidylinositol (GPI)-anchored ephrin A. We further demonstrated that ephrin A2 was distributed in a caudal-high/rostral-low gradient in the caudolateral hindbrain and cC-VC axons expressed EphA receptors. Finally, perturbing ephrin A/EphA signalling both in vitro and in vivo led to rostrocaudal pathfinding errors of post-crossing cC-VC axons. These results suggest that ephrin A/EphA interactions play a key role in regulating the polarity of post-crossing cC-VC axons as they turn into the longitudinal axis.

KEY WORDS: Commissural axons, Ephrin A, Longitudinal polarity, Hindbrain, Chick

INTRODUCTION

In bilaterally symmetric organisms, neuronal information from one half of the nervous system is communicated to the other half by commissural axons that grow circumferentially across the midline and turn into longitudinal paths towards their targets. The mechanisms that guide the commissural axons towards and across the ventral midline have been studied extensively; they rely on the concerted actions of chemoattractants, chemorepellents and cell-adhesion molecules, distributed along the dorsoventral axis of the neural tube (Tessier-Lavigne and Goodman, 1996; Murakami and Shirasaki, 1997; Stoeckli and Landmesser, 1998; Salinas, 2003; Guthrie, 2004). By contrast, little is known about the mechanisms that control the pathfinding of commissural axons after midline crossing, in particular, those that regulate their rostrocaudal polarity along the longitudinal axis.

Two recent studies have shown that Wnt4 and sonic hedgehog (Shh) control the rostrocaudal polarity of post-crossing spinal commissural axons in rodent and chick, respectively, by forming rostrocaudally orientated gradients in and around the ventral midline (Lyuksyutova et al., 2003; Bourikas et al., 2005). Wnt4 and Shh gradients might account for the longitudinal polarity of most spinal commissural axons, which turn rostrally in close contact to the contralateral surface of the floor plate. However, the trajectories of post-crossing commissural axons are less stereotypical than previously thought. In chick and rodent spinal cord, some post-crossing commissural axons have also been shown to grow caudally or bifurcate rostrocaudally, and some turn longitudinally at a distance away from the floor plate within the ventral spinal cord (Oppenheim et al., 1988; Yaginuma and Oppenheim, 1991; Erskine et al., 1998; Imondi and Kaprielian, 2001; Kadison and Kaprielian, 2004). This diversity is even more pronounced in the developing hindbrain, a wider structure along the dorsoventral axis than the spinal cord. Hindbrain commissural axons not only turn within the ventral aspect of the neural tube, but also at intermediate and dorsal positions, into either rostral or caudal directions (Glover and Petursdottir, 1991; Clarke and Lumsden, 1993; Glover, 1993; Shirasaki et al., 1995; Shirasaki and Murakami, 2001). We envisage that multiple molecular mechanisms, possibly involving components with distinct spatial distributions, account for the diversity of post-crossing commissural projections in the hindbrain. Thus, the hindbrain represents a complex model that is well suited for uncovering novel molecular mechanisms that regulate the guidance of post-crossing commissural axons.

In the present study, we have focused on commissural axons that turn longitudinally at a distance from the ventral midline. The axons, which we identified as coming from the caudal group of the contralateral second order vestibular neurons (cC-VC) (Diaz and Puelles, 2003), turn rostrally at a dorsal position in the hindbrain. Here, we show that caudal hindbrain possesses a graded non-permissive/repulsive activity for growing cC-VC axons. This activity is mediated at least in part by ephrin A. We present multiple lines of evidence that demonstrate ephrin A/EphA signalling plays a crucial role in controlling the rostrally directed turning of post-crossing cC-VC axons.

MATERIALS AND METHODS

Chick embryos and staging

Fertilised eggs (Takeuchi egg farm) were incubated in a humidified incubator at 38.5°C to desired stages. Embryonic staging was based on Hamburger and Hamilton (Hamburger and Hamilton, 1951). Animal studies were carried out in accordance with the guidelines of the Animal Studies Committee of Osaka University.

Organotypic culture and grafting

Organotypic culture of chick hindbrains was carried out as previously described (Chédotal et al., 1997; Zhu et al., 2003).

In transplantation experiments, grafts from donor and host hindbrains were prepared in ice-cold Gey's Balanced Salt Solutions (GBSS, Sigma) with flame sharpened tungsten needles. The grafts were soaked in GBSS containing 30 μg/ml 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO,

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Molecular Probe, Invitrogen) for 5 minutes. Both the graft and the host were transferred onto millicell culture inserts (CM, Millipore) and the graft was positioned using a fire-polished pulled glass pipette. The borders of the grafts were discerned by DiO signals and bright-field illumination.

Anterograde- and retrograde-labelling

Small crystals of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiI) or 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) (Molecular Probes, Invitrogen) were inserted by a glass micropipette with a broken tip, on cultured hindbrains or open-book hindbrains fixed in 4% paraformaldehyde (PFA)/0.1 M phosphate-buffered saline (PBS, pH 7.4). The position of the root of vestibuloacoustic ganglion (gVIII) was marked by Carmine (Wako), and DiI or DiD crystals were inserted at a specific distance from the Carmine mark, guided by a scaled eyepiece graticule. For fixed hindbrains, samples were stored in 4% PFA for 4 weeks at room temperature. Hindbrains were organotypically cultured for 1 day in vitro (div) to 2 div after dye insertion, before fixation. All samples were mounted in Mowiol (Calbiochem).

PI-PLC, hFc, EphA3-Fc and ephrin A2 functional blocking antibody treatment in vitro

Phosphatidylinositol-specific phospholipase C (PI-PLC, Molecular Probes, Invitrogen) at a final concentration of 200 mU/ml was added to the culture medium at the onset of culture, with a fresh supplement of PI-PLC after 1 div. Human Fc fragment of IgG (Jackson ImmunoResearch) or recombinant mouse EphA3-Fc Chimera (R&D Systems) were added at a final concentration of 1500 ng/ml and 2000 ng/ml, respectively, to the culture medium at the onset of culture. A function-blocking antibody against chick ephrin A2 (B3) (a kind gift from Dr H. Tanaka) (Yamada et al., 2001) was used at a final concentration of 20-30 μg/ml.

Immunohistochemistry and antibodies

Immunohistochemistry on flat whole-mount hindbrains was performed as described (Tashiro et al., 2000), except that 0.04% $NiSO_4$ was added to diaminobenzidine tetrahydrochloride (DAB; 0.1% in Tris-buffered saline) solution. Samples were cleared in 90% glycerol for imaging.

Fluorescence immunohistochemistry was performed on 20 μm frozen sections, as described above, with a few modifications: (1) treatment of 0.3% H_2O_2 was omitted; (2) Alexa594-conjugated streptavidin (Molecular Probe, 1:1000) was used; (3) Triton-X100 was used at 0.2%; and (4) slides were mounted in Mowiol (Calbiochem) with 2.5% 1,4-diazabicyclo-[2,2,2]octane (DABCO, Sigma).

Primary antibodies used were a monoclonal antibody against a chick neurofilament associated protein (3A10) (from the Developmental Studies Hybridoma Bank, The University of Iowa) and a monoclonal antibody against chick ephrin A2 (kind gift from Dr H. Tanaka) (Yamada et al., 2001). Secondary antibody used was biotinylated horse anti-mouse IgG (H+L) (1:200, Vector Laboratories).

Affinity probe in situ binding

Chick ephrin A2-alkaline phosphatase (AP) and chick EphA3-AP were kind gifts from Dr H. Tanaka. Affinity probe in situ binding on whole-mount chick hindbrains or frozen sections was carried out largely as previously described (Cheng et al., 1995) with some modifications: the blocking buffer was composed of Hanks' Balanced Salt Solution (HBSS):PBS at 1:1 ratio, with 10% sheep serum. For AP in situ on frozen sections, 30 μm sections were air dried for 2 hours and fixed in 99.5% ethanol for 30 seconds, followed by 5 washes in PBS. The rest of the procedure follows the protocol for the whole-mount AP in situ binding.

RNA in situ hybridisation and probes

In situ hybridisation on whole-mount chick hindbrains was performed as previously described (Henrique et al., 1995). The following plasmid templates were used to generate digoxigenin-labelled anti-sense or sense riboprobes. Plasmids containing chick *ephrin A2*, *ephrin A5* and *ephrin A6* were kind gifts from Dr H. Tanaka (Iwamasa et al., 1999), Dr U. Drescher (Drescher et al., 1995) and Dr N. Wada (University of California, Irvine), respectively. Partial cDNA fragments of chick *EphA3*, *EphA4*, *EphA5* and *EphA7* were generated by RT-PCR from total RNA prepared from E6 chick hindbrains. Primers for RT-PCR correspond to nucleotide positions on cDNA: 831-1620 of EphA3; 410-1219 of *EphA4*; 826-1641 of *EphA5*; and 865-1507 of *EphA7*. The cDNA fragments were cloned into pGEM-T Easy Vector (Promega) for subsequent riboprobe preparation.

In ovo electroporation

In ovo electroporation was carried out essentially as described previously (Nakamura and Funahashi, 2001). Briefly, stage 20-21 eggs were windowed and extra-embryonic membranes were removed from the hindbrain area. Plasmid solution (1 μl) coloured with Fast Green (w/v 0.05%) was injected into the IVth ventricle. Two silver wire (0.3 mm diameter) electrodes as anode and cathode were placed in parallel on either side of the hindbrain and five square pulses of 15 V, 50 ms duration were applied with an Electro Square Porator (ECM830, BTX). The embryos were cooled immediately with a few drops of cold PBS. Eggs were sealed for further incubation until E6.

Two avian retroviral constructs used for electroporation are RCASB(P)-EphA3ΔC (a kind gift from Dr J. G. Flanagan and Dr M. Nakamoto) (Nishida et al., 2002; Feldheim et al., 2004) and RCASB(P) mock vector [EphA3ΔC insert was deleted from RCASB(P)-EphA3ΔC by *Cla*I digestion and the vector re-ligated]. Full-length chick ephrin A2-coding sequences were amplified by RT-PCR and cloned into an expression vector pCAGGS (Niwa et al., 1991). Each of the above three constructs (1 mg/ml in PBS) was co-electroporated with an EGFP expression vector pCAGGS-EGFP (Hatanaka and Murakami, 2002) at 2:1 ratio.

Image recording

Fluorescence and bright field images were captured with a charge-coupled digital (CCD) camera (Axiocam, Zeiss) linked to an upright microscope (BX-60, Olympus). In some cases, fluorescence images were obtained by a laser-scanning confocal microscope (MRC 1024ES, BIORAD).

Data quantification and statistics

Quantitative measurement on fluorescence images was performed using MetaMorph (Version 6.1, Universal Imaging Corporation). Mann-Whitney U-test was subsequently performed on the processed data.

In the cases of caudal turning error of cC-VC axons after addition of PI-PLC and EphA3-Fc in vitro, it was unfeasible to perform quantitative measures as described above because of the retrogradely labelled cells right below the turning point. These samples were arbitrarily divided into two categories: samples with all axons turning rostrally and samples with axons turning caudally. The datasets fit binomial distribution; thus, Fisher's test was used for statistical analysis.

RESULTS

To investigate the mechanisms that control the rostrocaudal turning of hindbrain commissural axons, we first systematically surveyed the commissural tracts in developing chick hindbrains. One tract drew our attention because of its two features: it turns into a rostral longitudinal path within the lateral hindbrain (Fig. 1), and it appears to be one of the earliest tracts that grow along this longitudinal pathway (Fig. 1J,K). Thus, we decided to use this axon tract as our model system for investigating the guidance mechanisms controlling the longitudinal polarity of laterally turning commissural axons.

Developmental course of a cerebellar-projecting lateral commissural tract in chick hindbrain

The developmental course of the lateral commissural tract was first revealed by DiI anterograde-labelling on fixed hindbrains in open book configurations at various stages. Small crystals of DiI were inserted into the dorsal hindbrain 700-1200 μm caudal to the root of vestibuloacoustic ganglion (gVIII) (schematics in Fig. 1A). At stage 24, DiI labelled a tract that crossed the midline and grew rostrally within the lateral aspect of the contralateral hindbrain (Fig. 1B). At

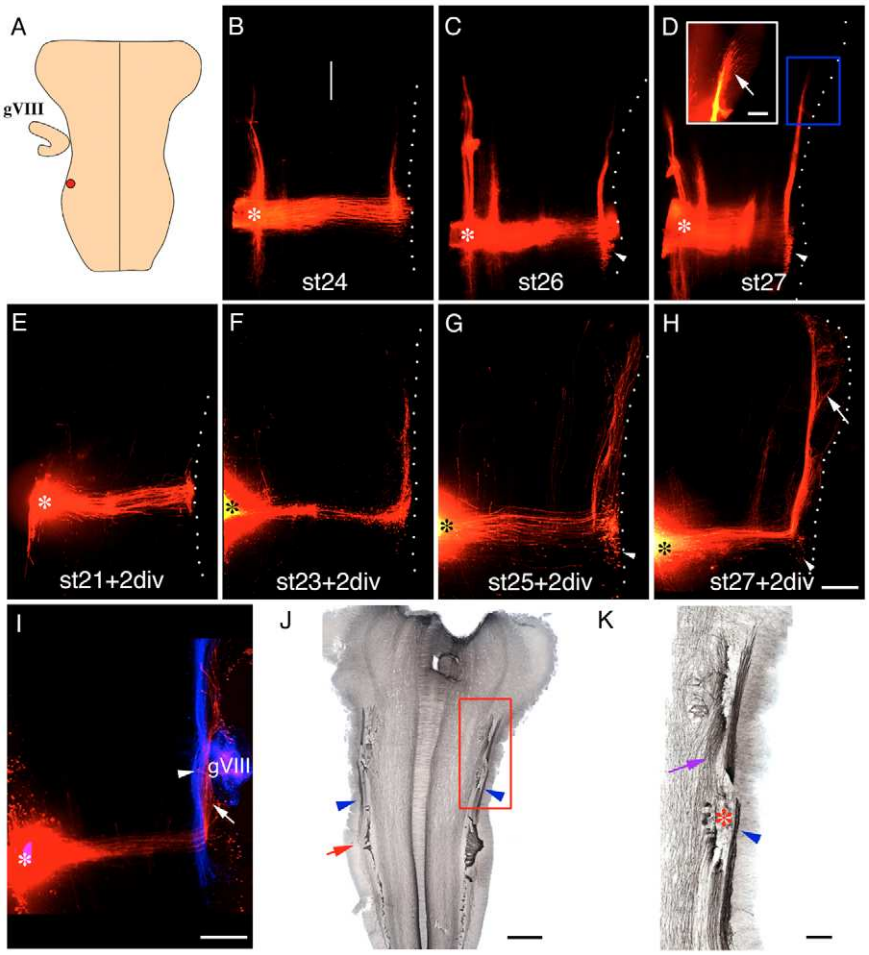

Fig. 1. Development of a lateral commissural tract in chick hindbrains. (**A**) Schematic showing an open-book hindbrain preparation, with DiI anterograde labelling. DiI crystals were inserted into the lateral margin of the caudal hindbrain, 700-1200 μm caudal to the gVIII root. (**B-D**) DiI anterograde labelling on fixed hindbrains at stage 24 (*n*=3), stage 26 (*n*=3) and stage 27 (*n*=4), respectively, showing the progression of a laterally located commissural tract. (D) The labelled commissural tract grew to the base of the cerebellar plate. High magnification of the blue-boxed area in D shows that some axons from the tract turn to invade the cerebellar plate (white arrow in the inset in D). (**E-H**) DiI anterograde labelling of organotypically cultured hindbrains at stage 21 (*n*=3), stage 23 (*n*=8), stage 25 (*n*=10) and stage 27 (*n*=3), cultured for 2 days in vitro (div). The lateral commissural tract labelled by DiI anterograde labelling in the organotypic cultures closely resemble that in the fixed samples both in its appearance and developmental course. At stage 27+2 div (H), axons could be seen to leave the longitudinal tract and invade the developing cerebellar plate. White arrowheads in C,D,G,H indicate retrogradely labelled neurons contralateral to the DiI injection sites. (**I**) An organotypically cultured stage 26 hindbrain, with the lateral commissural tract labelled by DiI (white arrow) and the central projection of the right gVIII labelled by DiD (white arrowhead). The lateral commissural tract was located lateral to the central projections of gVIII (*n*=6). (**J**,**K**) Immunohistochemistry using an antibody against a neurofilament-associated protein (3A10) on a stage 24 flat-mounted hindbrain. (K) A higher magnification view of the boxed area in J. Blue arrowheads in J,K indicate the position of the lateralmost longitudinal tract. This lateralmost tract appears to converge from a turning point ~900-1000 μm caudal to gVIII (indicated by a red arrow in J). Three longitudinal tracts located in the lateral extreme of the hindbrain can be seen in K: the central projection of gV (purple arrow) and of gVIII (root of gVIII indicated by red asterisk), and the lateral commissural tract (blue arrowhead). White vertical line in B indicates the midline. The asterisks in B-I indicate the injection sites of DiI. Scale bar: 400 μm in B-J; 100 μm in the inset in D and in K.

stage 26, the tract extended further rostrally and reached the base of the cerebellar plate (Fig. 1C). By stage 27, some axons defasciculated from the longitudinal tract and turned into the cerebellar primordium (Fig. 1D). The development of this lateral commissural tract could be recapitulated by DiI anterograde-labelling on organotypically cultured hindbrains (Fig. 1E-H), although the labelled tract in cultures appeared to be less fasciculated than that in vivo. The possibility of following the growth of this tract in vitro allowed us to investigate the tissues and molecules that influence the pathfinding of this tract in organotypically cultured hindbrains (see later sections).

We next investigated the relative position of this commissural tract in relation to other longitudinal tracts within the lateral longitudinal fascicle (LLF). First, we compared the position of this tract with that of the central projection of gVIII, labelled with DiI and DiD, respectively, on organotypically cultured stage 26 hindbrains. We found that the lateral commissural tract was positioned further laterally to the gVIII central projection (Fig. 1I). We then immunostained flat-mount chick hindbrains of various stages with 3A10, an antibody against neurofilament associated protein. At stage 24, three neurofilament-positive longitudinal tracts ran side by side within the lateral aspect of the hindbrain (Fig. 1J,K). The medial-most and the middle tracts were the central projections of the trigeminal ganglion (gV) and gVIII, respectively. The lateralmost of the three appeared to converge from commissures at a turning point about 900-1000 μm caudal to the root of gVIII, and the appearance of the tract corresponded well with that of the DiI-labelled lateral commissural tract at a similar stage (compare Fig. 1J with Fig. 1B). Neurofilament staining at later stages showed that the lateralmost tract reached the base of the cerebellar primordium at stage 26, and began to innervate the cerebellum from stage 27 (data not shown), again consistent with that of DiI anterograde labelling. We therefore concluded that the lateralmost 3A10-positive longitudinal tract was the same tract as revealed by DiI anterograde labelling. At stage 24, the 3A10-positive lateralmost tract appeared to grow separately from its nearest neighbour – the gVIII central projections and other longitudinal tracts (Fig. 1J,K), implying that it could be a pioneering tract exploring this lateralmost territory of the hindbrain. Further anatomical characterisation led to the identification of this tract as that formed by a caudal group of the contralateral cerebellar-projecting second order vestibular neurons (cC-VC) (Diaz and Puelles, 2003) (see Fig. S1 in the supplementary material).

Caudal hindbrain possesses a graded nonpermissive/repulsive activity for rostrally growing cC-VC axons

To investigate the mechanisms that control the rostral turning of cC-VC axons, we focused on the guidance potential of hindbrain tissue posterior to the turning point of cC-VC axons. The tissues in this location were grafted to intersect the rostral path of the cC-VC tract, in organotypically cultured stage 25-26 hindbrains. Behaviour of cC-VC axons was then observed following anterograde-labelling of the tract as described in Fig. 1. When the graft was taken from about 1.2 mm caudal to gVIII, a position close to but caudal to the turning point of the cC-VC axons, many axons stalled or turned away before entering the graft, yet a small percentage of axons continued to grow into and through the graft (Fig. 2A,D, *n*=12). A graft from a further caudal position (about 1.8 mm caudal to gVIII) caused a more marked effect; almost all cC-VC axons stalled or turned away without entering the graft (Fig. 2B,D, *n*=12). By contrast, when a piece of tissue was cut from the rostral path of cC-VC and put back to its original position, most labelled cC-VC axons grew through the graft (Fig. 2C,D, *n*=10). These results suggest that caudal hindbrain posterior to the turning point of cC-VC axons is inhibitory (nonpermissive/repulsive) to cC-VC axons. Furthermore, the degree of this inhibition is high caudally and low rostrally.

PI-PLC and EphA3-Fc treatment alleviate the caudal inhibition

Inhibitory axon guidance cues in the CNS have been attributed to a limited number of receptor-ligand families, namely Eph-ephrin, Neuropillin/Plexin-Semaphorin, Robo-Slit and Unc5H-Netrin families (Yu and Bargmann, 2001; Dickson, 2002). Among these, both the ephrin A and Semaphorin VII families are GPI-anchored molecules. The GPI anchor can be enzymatically cleaved by PI-PLC, which in turn result in the removal of these membrane-attached ligands. We first tested whether PI-PLC treatment can alleviate the inhibitory activity in the caudal hindbrain, using the grafting scheme depicted in Fig. 2B. Without adding PI-PLC, almost all axons failed to enter the graft as shown in Fig. 2B (Fig. 3A1, *n*=12). Addition of PI-PLC to the culture medium alleviated the caudal inhibition, as indicated by invasion of many cC-VC axons into the graft (Fig. 3A2, *n*=20). This suggests GPI-anchored molecule(s) is involved in mediating the caudal inhibition.

Among the GPI-anchored inhibitory molecules, ephrin A family members are involved in a diverse range of guidance events in the CNS, often with their graded distribution (Flanagan and Vanderhaeghen, 1998; Wilkinson, 2000; Wilkinson, 2001). To test the potential involvement of ephrin As in the caudal inhibition, we used the soluble recombinant mouse EphA3-Fc to block specifically ephrin A binding to its receptors (Yates et al., 2001; Dufour et al.,

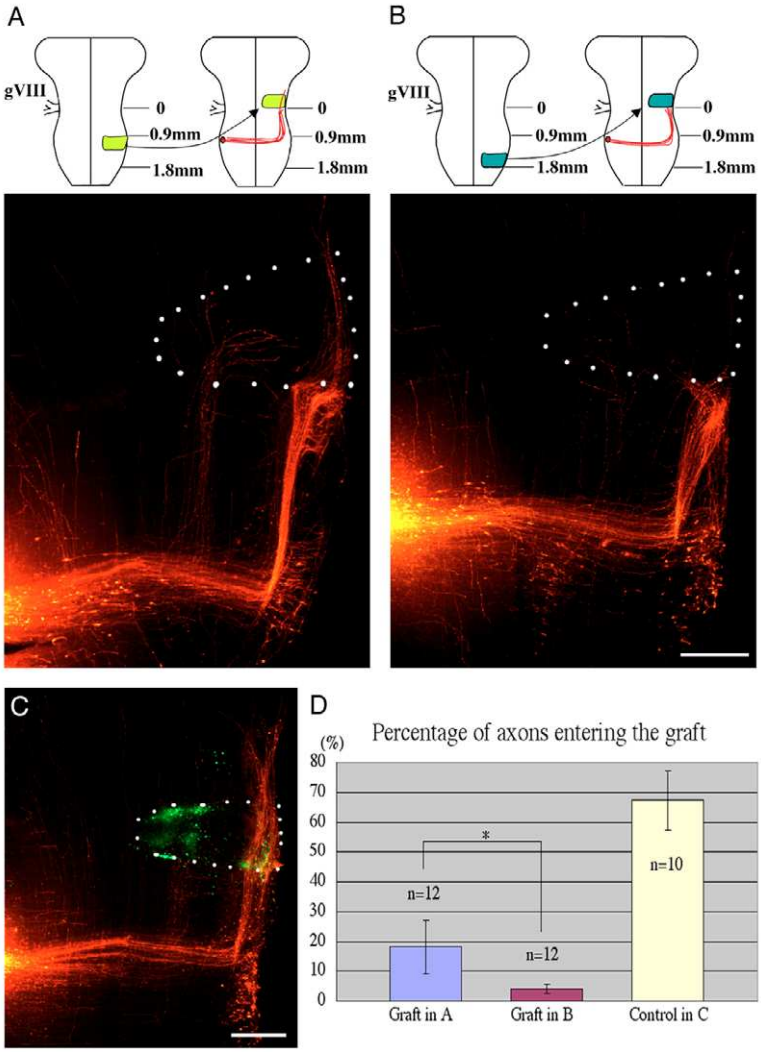

Fig. 2. Caudal hindbrain possesses a graded nonpermissive/repulsive activity for the cC-VC axons. (**A**,**B**) Two types of grafting experiments. The positions of the grafts were approximately 1.2 mm (A) and 1.8 mm (B) caudal to gVIII, respectively. Almost all cC-VC axons stalled or turned away before entering the graft in B (*n*=12), whereas a small proportion of the cC-VC axons still grew through the graft in A. (**C**) A piece of rostral hindbrain along the cC-VC path was cut and put back as control. Majority of cC-VC axons could grow through the graft (*n*=10). All grafts were soaked in DiO before transplanting into the hosts, thus the borders of the grafts were discerned by DiO signal combined with bright-field illumination. DiO signal was displayed only in C, but removed for better illustration of axons in the grafts in other images. White dots outline the border of grafts in A-C. (**D**) Quantification of the percentage of axons entering the graft (**P*<0.0001, Mann-Whitney *U* test) (error bar indicates s.d.). Scale bar: 400 μm.

2003). When purified human Fc (hFc) region of IgG was added to the medium, almost all cC-VC axons failed to enter the graft (Fig. 3B1, *n*=7). By contrast, many cC-VC axons grew through the graft when EphA3-Fc (without clustering) was added to the culture medium (Fig. 3B2, *n*=7), although the effect of EphA3-Fc was slightly weaker than PI-PLC. Inhibition imposed by the more rostral graft as in Fig. 2A could also be alleviated by EphA3-Fc (data not shown). The caudal inhibition could also be reduced when a function-blocking antibody against chick ephrin A2 (B3) (Yamada et al., 2001) was added to culture medium, although the effect is weaker than PI-PLC and EphA3-Fc (see Fig. S2 in the supplementary material). Taken together, these results suggest that ephrin A is involved in mediating the inhibitory effect of the caudal hindbrain.

Distribution of ephrin A ligand and EphA receptor in stage 25 chick hindbrain

The involvement of ephrin A in mediating the inhibitory activity of the caudal hindbrain, presumably through the EphA receptor, predicted the expression of these molecules in the caudal hindbrain. To test this, we first investigated the distribution of ephrin A on stage 25 hindbrains, using EphA3-AP affinity probe in situ binding, and found a strong ephrin A activity in the caudal hindbrain (Fig. 4A, *n*=3). Ephrin A activity was also observed in the rostral cerebellum, consistent with previous reports (Karam et al., 2000; Nishida et al., 2002). In the caudal hindbrain, ephrin A was enriched in the lateral half of the neuroepithelium and its expression appeared to be graded – low in rostral and high in caudal. The rostral extent of the gradient reached ~700 μm caudal to the gVIII root. Thus, both the position of ephrin A active domain and its graded nature in the caudal hindbrain correlate with the characteristics of the inhibition revealed by the grafting experiment (Fig. 2).

We next examined EphA receptor activity by ephrin A2-AP in situ binding. In the lateral half of the hindbrain, EphA distribution appeared to be roughly complementary to that of the ephrin A activity (Fig. 4B, *n*=9). It was distributed in a lateral column extending rostrally into the caudal cerebellum, and caudally into r8 with a tapering end. The caudolateral EphA-positive domain in r6, r7 and possibly rostral r8 was well correlated with the position of retrogradely labelled cC-VC neurons at the equivalent stage (Fig. 4B, compare with Fig. S1A,B in the supplementary material).

To confirm that EphA receptors were indeed expressed in cC-VC neurons, we first retrogradely labelled cC-VC neurons on organotypically cultured stage 25 hindbrains (as in Fig. S1A in the supplementary material). Such hindbrains were cryosectioned and those sections containing retrogradely labelled neurons were imaged immediately (Fig. 4C, top panel), followed by ephrin A2-AP in situ binding, to reveal EphA activity (Fig. 4C, middle panel). These two images were then overlaid to compare the localisation of DiI signal with AP signal on the same section (Fig. 4C, bottom panel). DiI-positive cC-VC neurons were located in the EphA-positive area around the lateral mantle zone (Fig. 4C, arrowhead). Furthermore, cC-VC commissure was positive for EphA signal (Fig. 4C, white and black arrows). Thus, EphA is expressed in the cC-VC axons in a manner that it can interact with the ephrin A in the caudal hindbrain.

Ephrin A2, EphA5 and EphA3 are the main components of the ephrin A/EphA activity in the developing cC-VC system

We next examined which EphA receptors and ephrin A ligands were expressed in the cC-VC neurons and the lateral caudal hindbrain, respectively. In situ hybridisation was performed on stage 25 flat-mount hindbrains, with antisense probes of the following chick cDNAs: *EphA3*, *EphA4*, *EphA5*, *EphA7*, *ephrin A2*, *ephrin A5* and *ephrin A6*. Among these, only *EphA5* and *EphA3* were expressed in the presumptive location of cC-VC neurons (Fig. 5A,B; data not shown) with *EphA5* stronger in the lateral region, while *EphA3* was more medial. Of the three known members of *ephrin A* in chick, only *ephrin A2* was expressed in the lateral region of the hindbrains, while *ephrin A5* and *ephrin A6* were expressed in the ventral half of the hindbrain and around the floor plate, respectively (data not shown). We further studied the expression of ephrin A2 protein with an antibody against chick ephrin A2. On a lateral parasagittal section of

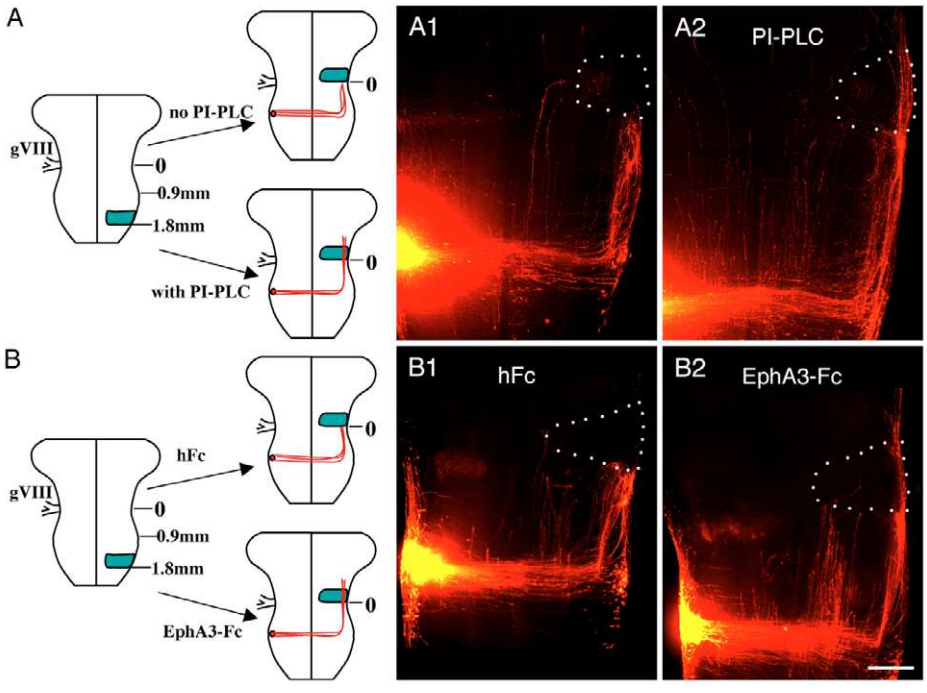

Fig. 3. PI-PLC and EphA3-Fc could alleviate the inhibitory activity of the caudal hindbrain. (**A**,**B**) The experimental procedures followed illustrated using the same grafting scheme as in Fig. 2B. With normal culture medium (A1) or with only hFc added to culture medium (B1), almost all cC-VC axons were prevented from entering the caudal graft. However, adding PI-PLC (A2) or unclustered EphA3-Fc (B2) to the culture medium led to many axons entering the graft, reducing the inhibition in the graft. White dots outline the grafts. Scale bar: 400 μm.

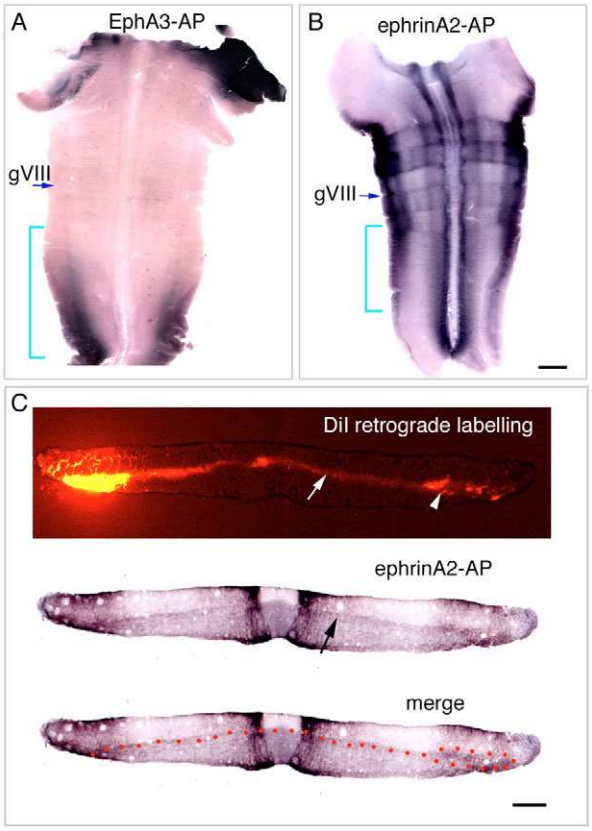

Fig. 4. Distribution of EphA and ephrin A in stage 25 hindbrains. (**A**) EphA3-AP in situ binding on a stage 25 hindbrain revealed ephrin A activity. The blue bracket indicates the rostrocaudal extent of the lateral ephrin A gradient in the caudal hindbrain. (**B**) Ephrin A2-AP in situ binding on a stage 25 hindbrain revealed EphA activity. The blue bracket indicates EphA3-positive domain correlating with the position of presumptive cC-VC neurons. A ventral column of cells adjacent to the floor plate is also EphA positive (see also lower panels in C). (**C**) Top panel shows a transverse section of a hindbrain with its cC-VC neurons retrogradely labelled (retrogradely labelled cC-VC neurons indicated by white arrowhead, and cC-VC commissures indicated by white arrow). The same section was subjected to ephrin A2-AP in situ binding, shown in the middle panel in C; black arrow shows the EphA-positive commissure. The top and middle panels were superimposed and DiI signals were traced onto the AP signals by red dots (bottom panel). cC-VC neurons and their axons appear to be EphA positive. Scale bar: 400 μm in A,B; 100 μm in C.

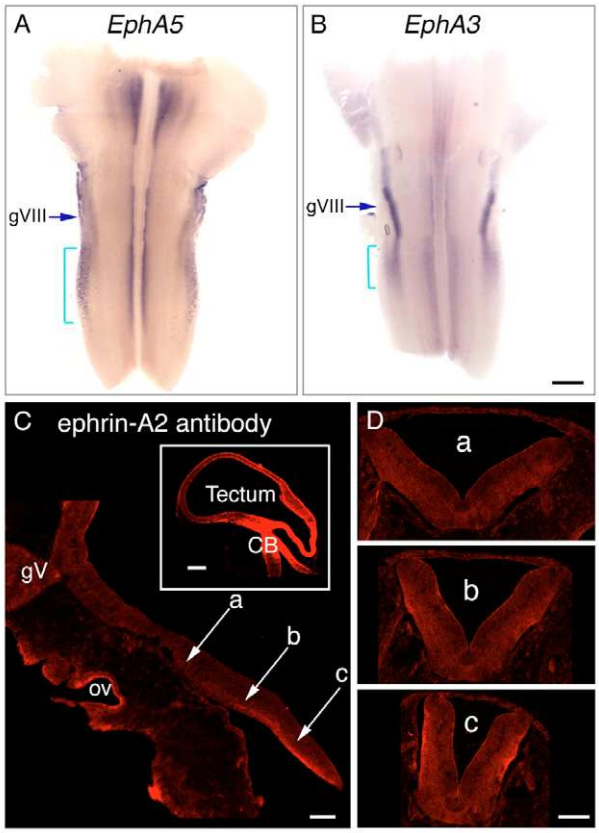

Fig. 5. *EphA5* and *EphA3* are expressed in presumptive cC-VC neurons, and ephrin A2 protein is distributed in a gradient in caudal hindbrain. (**A**,**B**) *EphA5* and *EphA3* in situ hybridisation on stage 25 hindbrains, respectively. The blue brackets in both images indicate expression domains correlating with the location of cC-VC neurons. (**C**) Ephrin A2 immunohistochemistry on a lateral parasagittal section of a stage 26 brainstem. The graded distribution of ephrin A2 in the caudal hindbrain is indicated by arrows a, b and c. Inset shows ephrin A2 protein in tectum and cerebellum from the same brain. (**D**) Ephrin A2 immunostaining on three transverse sections from a stage 26 hindbrain. a-c correspond approximately to positions a-c in C. CB, cerebellum; gV, trigeminal ganglion; ov, otic vesicle. Scale bar: 400 μm in A,B; 200 μm in C, inset in C and D.

a stage 26 brainstem, we detected a caudal-high/rostral-low gradient of ephrin A2 in the tectum, consistent with previous studies (inset of Fig. 5C) (Cheng et al., 1995; Drescher et al., 1995). In the caudal hindbrain, ephrin A2 was distributed posterior to the otic vesicle, in a rostral-low/caudal-high gradient (Fig. 5C). On transverse sections, ephrin A2 immunoreactivity was detected in the lateral half of the hindbrain with its signal stronger in caudal sections (Fig. 5D). From these data, we conclude that ephrin A2, EphA5 and EphA3 are the main components that account for the ephrin A/EphA activities detected by the AP-fusion proteins in the developing cC-VC system.

Ectopic expression of ephrin A2 causes stalling and turning of cC-VC axons

We next explored whether a source of ephrin A2 could directly affect the growth of cC-VC axons. An ephrin A2 expression vector together with EGFP was electroporated in ovo into the rostral hindbrain at stage 18-20. Hindbrains were taken for culture at stage 27-28, and cC-VC axons were anterogradely labelled. Ectopic expression of ephrin A2 as visualised by EphA3-AP in situ binding showed good co-localisation with the co-electroporated EGFP (data not shown). Ectopic expression of EGFP alone had no effect on the growth of the cC-VC axons (Fig. 6A, n=15/16), whereas expression of ephrin A2 caused most labelled axons to stall at or turn away from the border of the ectopic ephrin A2 domain (Fig. 6B,C, n=15/17). These data provide direct evidence that a source of ephrin A2 could elicit stalling and turning of cC-VC axons in ovo.

Perturbing ephrin A/EphA signalling lead to inappropriate caudal turning of some cC-VC axons

Our findings that ephrin A contributes to the laterally located inhibitory activity for cC-VC axons in the caudal hindbrain raise the possibility that ephrin A is involved in the rostral turning polarity of cC-VC axons. We tested out this possibility by perturbing ephrin A/EphA signalling both in vitro and in ovo.

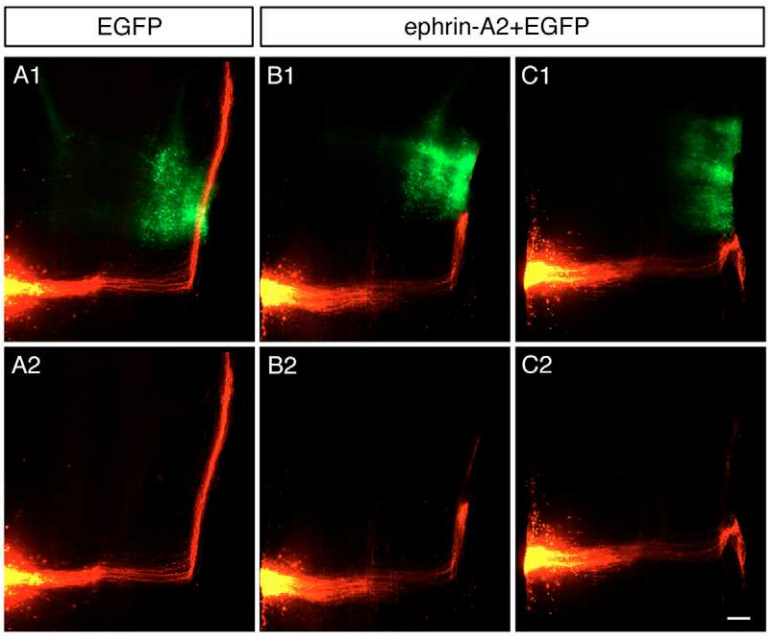

Fig. 6. Ectopically expressed ephrin A2 induces stalling and turning of cC-VC axons. EGFP alone or together with full-length chick ephrin A2 were electroporated in ovo at around stage 20. Electroporated hindbrains were taken for culture at stage 27-28, and cC-VC axons were anterogradely labelled with DiI. (**A1**) EGFP alone has no effect on cC-VC axons (*n*=15/16). (**B1**,**C1**) At the interface of an ectopic ephrin A2 domain, most cC-VC axons either stalled (B1,C1) or turned away to extend caudally (C1) (*n*=15/17). (**A2-C2**) Same images as the A1-C1, respectively, with EGFP signals removed. A small amount of axons grew into the ephrin A2 expression domain in both B2 and C2, suggesting the possibility that cC-VC axons might represent a heterogeneous population with respect to their responsiveness to ephrin A. Scale bar: 200 μm.

In vitro perturbation was achieved by treatment of organotypically cultured stage 25-26 hindbrains with PI-PLC or EphA3-Fc. In control preparations, DiI labelled cC-VC axons turned rostrally within the lateral aspect of the hindbrain in most cases (*n*=20/25) (Fig. 7A), although some of them (*n*=5/25) occasionally turned caudally for a short distance (data not shown). By contrast, PI-PLC treatment resulted in the caudal turning of some cC-VC axons in the majority of samples examined (*n*=25/32) (Fig. 7B), with only 7/32 samples displaying the rostral turning of the entire cC-VC axon population (data not shown). When recombinant EphA3-Fc, a more specific antagonist for ephrin A signalling was applied, inappropriate caudal turning of some cC-VC axons took place in more than half of the samples (*n*=17/27) (Fig. 7D). This effect is not due to the Fc part of the fusion protein, because when hFc alone was added, cC-VC axons turned rostrally in the majority of samples (*n*=21/29) (Fig. 7C).

To perturb ephrin A/EphA signalling in ovo, we employed a retroviral vector encoding a mutant EphA3 with truncated cytoplasmic domain (RCASB-EphA3ΔC) (Nishida et al., 2002; Feldheim et al., 2004). Overexpression of exogenous EphA3ΔC in the developing chick cerebellum causes a dramatic reduction of endogenous ephrin A ligand activity in this tissue, possibly owing to the masking of the endogenous ephrin A by the ectopic EphA3ΔC (Nishida et al., 2002). We therefore introduced RCASB-EphA3ΔC by in ovo electroporation unilaterally into the caudal hindbrains of stage 20-21 chick embryos. EGFP was co-electroporated to discern the electroporated domain. We first confirmed by EphA3-AP in situ binding that a marked reduction of ephrin A activity was caused by ectopic expression of EphA3ΔC (*n*=8/8) (Fig. 8J), but not by electroporation of RCASB mock vector (*n*=4/4) (data not shown). We then analysed cC-VC axon trajectories of the electroporated hindbrains at stage 27-28 by DiI anterograde labelling. Samples with electroporated domains spanning the cC-VC turning point were selected for further analysis. When an RCASB mock vector was electroporated, cC-VC axons turned rostrally in the majority of samples (*n*=41/55) (Fig. 8A-D). However, when RCASB-EphA3ΔC was electroporated, caudal-turning axons were detected in the majority of samples (*n*=33/49) (Fig. 8E-I). DiI-labelling at stage 27-28 appeared to result in fewer or no retrogradely labelled cells, allowing us to perform quantitative analysis by measuring the fluorescence intensity of caudal- and rostral-turning axons using Metamorph. The degree of inappropriate caudal turning was represented by the ratio of caudal versus rostral turning axons and is

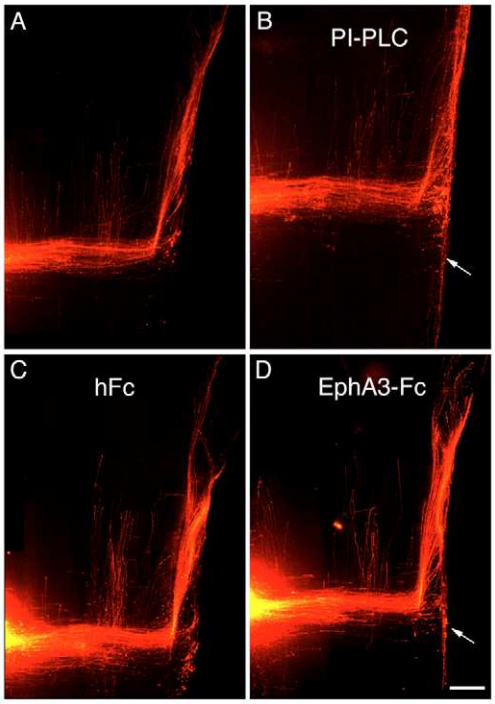

Fig. 7. Blocking ephrin A/EphA signalling in vitro led to some cC-VC axons turning caudally. cC-VC axons were anterogradely labelled on organotypically cultured stage 25-26 hindbrains. (**A**) Without PI-PLC, in 20/25 samples, all axons turned rostrally. (**B**) With PI-PLC, in 25/32 samples, some axons turned caudally (white arrow). ($P<0.0001$, Fisher's test). (**C**) With hFc, in 21/29 samples, all cC-VC axons turned rostrally. (**D**) With EphA3-Fc, in 17/27 samples, some cC-VC axons turned caudally (white arrow) ($P<0.015$, Fisher's test). Scale bar: 400 μm.

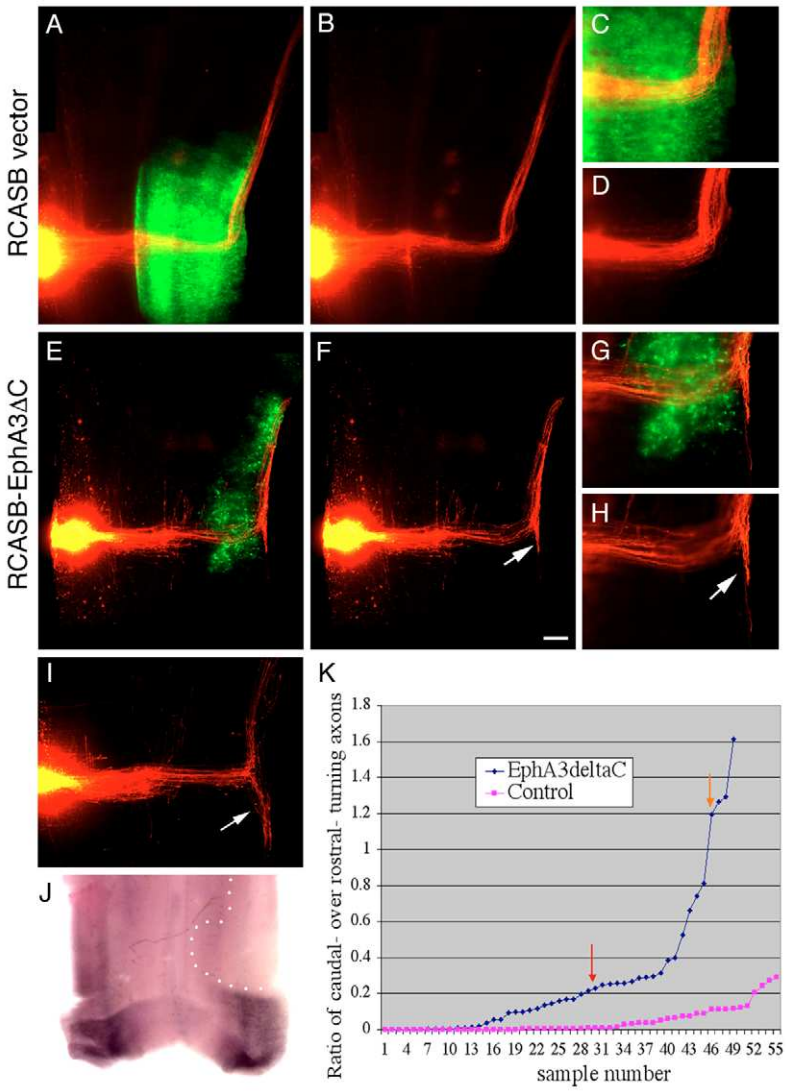

Fig. 8. Interfering with ephrin A/EphA signalling in ovo led to inappropriate caudal turning of some cC-VC axons. RCASB vector or RCASB-EphA3ΔC was electroporated in ovo into caudal hindbrain at stage 20-21. An EGFP expression vector was co-electroporated to reveal the domain of successful electroporation. At stage 27-28, electroporated hindbrains were taken for culture and cC-VC axons were anterogradely labelled. (**A-D**) RCASB vector was electroporated. (**E-H**) RCASB-EphA3ΔC was electroporated. EGFP signals in A,E indicate the domain of electroporation. (B,F) The same images as in A,E, respectively, with EGFP signals removed to better reveal the axons at their turning point. (C,D,G,H) Higher magnifications of axon turning regions of A,B,E,F, respectively. (**I**) A different sample with a higher degree of caudal-turning error when RCASB-EphA3ΔC was electroporated. (**J**) EphA3-AP in situ binding on a hindbrain co-electroporated with RCASB-EphA3ΔC and EGFP. The electroporated domain of this sample indicated by EGFP is outlined with white dots. (**K**) Quantification of caudal turning error in hindbrains electroporated with RCASB-EphA3ΔC versus RCASB mock vector (Control). The degree of caudal turning is represented as the ratio of caudal- over rostral-turning axons. Data are represented in a scatter chart, with the sample values sorted in an ascending order. The red and orange arrows indicate the data points of samples shown in E-H and in I, respectively. Arrows in F,H,I indicate axons turning caudally. Samples with RCASB-EphA3ΔC showed significantly higher incidence and amount of inappropriate caudal turning (P<0.0001, Mann-Whitney U-test). Scale bar: 200 μm in A,B,E,F,I,J; 100 μm in C,D,G,H.

summarised in Fig. 8K. The samples with EphA3ΔC showed both higher incidence and higher percentage of inappropriate turning axons, with the caudal turning axons being on average 27% of those that turned rostrally.

The in ovo perturbation data, together with the in vitro data employing PI-PLC and EphA3-Fc, suggest that ephrin A/EphA signalling in the caudal hindbrain is crucially involved in the control of the rostral turning polarity of the EphA-expressing cC-VC axons.

DISCUSSION

In this study, we have demonstrated that ephrin A/EphA signalling plays a role in the control of the rostral turning polarity of a laterally located hindbrain commissural tract. To our knowledge, this represents the first demonstration that ephrin A/EphA signalling regulates the polarity of post-crossing commissural axons.

The function of ephrin A gradient in the caudal hindbrain

The developing cC-VC axons extend circumferentially at the border of the ventricular and mantle zone. Upon reaching the contralateral lateral hindbrain, cC-VC axons course towards the pial surface and execute rostral turning into longitudinal axis (Fig. 4C; see Fig. S1B in the supplementary material). The rostral extent of an ephrin A gradient, demonstrated by EphA3-AP in situ binding and ephrin A2 immunohistochemistry, reaches the region within which the cC-VC axons turn rostrally. This raises the possibility that ephrin A controls the rostral turning polarity of cC-VC axons by its gradient, rather than forming a caudal inhibitory barrier. Two observations support this possibility: (1) cC-VC axons were only partially inhibited when a piece of caudal hindbrain located posterior and close to their turning point was transplanted to intersect the axonal path (Fig. 2A); and (2) in ovo introduction of RCASB-EphA3ΔC to a region caudal to but not overlapping the cC-VC axon turning point did not disrupt the rostral turning of cC-VC axons (data not shown). Therefore, we envisage that cC-VC axons, upon entering their presumptive turning zone, encounter a rostral-low/caudal-high ephrin A gradient across the rostrocaudal span of their growth cones, which is in turn translated into a preference for turning rostrally. The importance of an ephrin A gradient has been demonstrated in the establishment of topographic projection of several CNS circuits, including the projection of retinal ganglionic axons to various cortical and subcortical targets, the hippocamposeptal system and the thalamocortical projection (Vanderhaeghen et al., 2000; Yue et al., 2002; McLaughlin et al., 2003; Garel and Rubenstein, 2004). The role of ephrin A in topographic projection is generally thought to rely on its ability to cause growth cone collapse and retraction of

EphA-expressing axons in a concentration-dependent manner. Yet whether ephrin A can guide axons by inducing growth cone turning has remained unclear. Here, we showed in vivo that ectopically expressed ephrin A2 could induce turning as well as stalling of cC-VC axons. Our finding gains support from recent in vitro studies that showed that, in addition to growth cone collapse, ephrin A5 bound to beads or in the form of a gradient could induce the turning of *Xenopus* and chick retinal axons (Weinl et al., 2003; Weinl et al., 2005). At present, it remains unknown which condition preferentially induces turning versus collapse in our system. Nevertheless, these data render support to our model that EphA-expressing axons could be induced to change their growth polarity upon growing into a gradient of ephrin A.

It is conceivable that the caudal ephrin A gradient also controls the rostrocaudal polarity of other axon tracts that grow longitudinally through the lateral hindbrain, especially those destined for dorsal structures, such as the cerebellum. One candidate is the inferior olivary axons that extend from the caudal hindbrain towards the cerebellum through the lateral hindbrain (Zhu et al., 2003). The high level of ephrin A in the caudal extreme hindbrain could also function as an inhibitory barrier that obliges the commissural axons emanating from the caudal end of the hindbrain to turn at an intermediate position (Y.Z. and F.M., unpublished). A similar role has been proposed for ephrin-B, in the control of commissural axons in the chick and mouse spinal cord (Imondi and Kaprielian, 2001). Finally, the trajectory of gVIII descending afferent, which grows within the ephrin A gradient and stops at the caudal extreme of the hindbrain, raises the possibility that ephrin A gradient might control the topography of the gVIII afferents to the secondary vestibular neurons, as well as defining the caudal limit of its growth.

Rostrocaudal turning polarity of post-crossing commissural axons

Post-crossing commissural axons make rostrocaudal turning errors when their pre-contact with the floor plate was perturbed genetically, surgically or pharmacologically (Stoeckli and Landmesser, 1995; Matise et al., 1999; Zou et al., 2000; Shirasaki and Murakami, 2001). However, the molecular cues that directly instruct the rostrocaudal turning of post-crossing commissural axons have remained elusive until recently when Wnt4 and Shh gradients adjacent to the floor plate were found to direct the rostral turning of spinal commissural axons in mice and chick, respectively (Lyuksyutova et al., 2003; Bourikas et al., 2005). In both cases, the spinal commissural axons turn rostrally immediately after crossing the floor plate. However, the developing hindbrain as a relay station between the spinal cord and the higher brain structures, hosts a higher diversity of commissural tracts, many of which turn longitudinally at a distance further from the floor plate, within the lateral aspect of the hindbrain. We postulated that molecular cues distributed in the lateral hindbrain exist to control the longitudinal turning polarity of these lateral axon tracts. Our results have demonstrated a rostral-low/caudal-high gradient of ephrin A in the caudolateral hindbrain and that ephrin A/EphA signalling is important to ensure the rostral turning polarity of a lateral hindbrain commissural tract.

Several observations point to the possibility that additional factors might function in concert with ephrin A/EphA in the control of the rostral turning of cC-VC. First, the effect of EphA3-Fc appears to be weaker than PI-PLC treatment in alleviating the caudal inhibition (Fig. 3). Second, cC-VC axons might be a heterogeneous population in their sensitivity to ephrin A, as evidenced by the small percentage of cC-VC axons entering the ectopic ephrin A2 domain (Fig. 6). Yet all cC-VC axons turn rostrally, implying mechanisms other than ephrin A might control the rostral turning of these ephrin A insensitive cC-VC axons. Third, the average percentage of axons turning caudally upon disruption of ephrin A/EphA signalling was only around 27% of the rostral-turning axons. Shh is not a very likely candidate to control the rostrocaudal turning of laterally located commissural axons, as its activity is largely confined to the ventral region of the developing neural tube (Marti et al., 1995; Briscoe and Ericson, 2001). Wnt4 and Wnt3a have been previously shown to express in a caudal-high/rostral-low manner in the lateral hindbrains of developing chick (Hollyday et al., 1995). If they were to control the rostral turning of lateral commissural axons, they would have to function as chemorepellents, rather than having the chemoattractive role demonstrated for Wnt4 in the rostral turning of spinal commissural axons. Indeed, a recent study has shown that the caudally directed growth of corticospinal axons is controlled by the repulsive gradients of Wnt1 and Wnt5a, acting via the Ryk receptors (Liu et al., 2005). Thus, Wnt proteins might act either as repellents or attractants, depending on whether they act through Ryk or Frizzled receptors, respectively. Furthermore, Sema3C also appears to be distributed high in the caudal hindbrain at st27 (Chilton and Guthrie, 2003). The role of these molecules in controlling rostrocaudal turning of commissural axons awaits further investigation.

Conclusion

Taking advantage of the relatively large size of the developing hindbrain, we used transplantation on organotypically cultured hindbrains to dissect out the guidance properties of caudal lateral hindbrain at a gross level, with respect to a developing lateral hindbrain commissural tract, cC-VC. This led us to identify an ephrin A gradient in the caudal lateral hindbrain that plays a role in the control of rostrocaudal turning polarity of cC-VC. The diverse trajectories of hindbrain commissural axons may require a combination of different guidance cues that intricately pave the hindbrain to set the routes for these tracts. In addition, the high diversity could be achieved through each axon tract displaying different responsiveness to the same guidance cue(s).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/19/3837/DC1

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