

## Local nonpermissive and oriented permissive cues guide vestibular axons to the cerebellum

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### SUMMARY

Information that originates from peripheral sensory organs is conveyed by axons of cephalic sensory cranial ganglia connecting the sensory organs to appropriate central targets in the brain. Thus, the establishment of correct axonal projections by sensory afferents is one of the most important issues in neural development. Previously, we examined the development of the vestibular nerve that originates from the VIIIth ganglion using a flat whole-mount preparation of the rat hindbrain and developed an *in vitro*, culture preparation that can recapitulate vestibular nerve development (Tashiro, Y., Endo, T., Shirasaki, R., Miyahara, M., Heizmann, C. W. and Murakami, F. (2000) *J. Comp. Neurol.* 417, 491-500). Both

*in vivo* and *in vitro*, the ascending branch of the VIIIth ganglion projecting to the cerebellum reaches the base of the cerebellar primordium and starts to splay out towards the rhombic lip, apparently avoiding the ventral metencephalon. We now examine the nature of cues that guide vestibulocerebellar axons by applying various manipulations to the flat whole-mount *in vitro* preparation. Our observations suggest that local nonpermissive cues and oriented cues play a pivotal role in the guidance of vestibular axons to their central target.

Key words: Axon guidance, Hindbrain, Rat, Vestibular nerve, Organotypic culture, Sensory cranial ganglion

### INTRODUCTION

Sensory information from various peripheral organs is conveyed by sensory ganglia neurons, which connect the sensory organs to appropriate central targets in the brain and spinal cord. The information thus conveyed is required for brain functions, including higher cognitive functions. The establishment of correct axonal projections by sensory afferents is therefore one of the most crucial issues for the establishment of a functional brain.

The dorsal root ganglia (DRG) as well as those from the sensory cranial ganglia flanking the spinal cord and the hindbrain first extend axons longitudinally on entering the neural tube and then grow ventrally towards their second order neurons. Accumulating evidence suggests that long-range diffusible cues from the ventral spinal cord regulate ventrally oriented ingrowth of DRG axons (Fitzgerald et al., 1993). Chemorepulsion by Sema3A secreted from ventral spinal cord, for example, has been indicated to be involved in this process (Messersmith et al., 1995; Püchel et al., 1995). The ventrally oriented growth is achieved by sprouting of collaterals from

their axons, which initially extend longitudinally (Ozaki and Snider, 1997; Zhang et al., 1994). In this process, a secreted molecule, Slit, appears to be involved in axon elongation and branching of sensory afferents (reviewed by Brose and Tessier-Lavigne, 2000; Zinn and Sun, 1999). To reach their final targets, afferents from each sensory ganglion must travel longitudinally and terminate at appropriate segments of the spinal cord of the brainstem. This is particularly important for cranial sensory ganglia, each of which conveys sensory information of a different range of modality. However, the mechanisms guiding sensory axons to their target in the hindbrain are only poorly understood.

Afferent axons from the VIIIth ganglion provide a useful model for studying this issue, because they are well characterized anatomically (Angulo and Merchan, 1990; Larsell, 1923; Voogd, 1995) and developmentally (Ashwell and Zhang, 1992; Ashwell and Zhang, 1998; Morris et al., 1988; Tashiro et al., 2000), and an *in vitro* assay system that can mimic the development of vestibular axons has been established (Tashiro et al., 2000). Both *in vivo* and *in vitro*, axons from the VIIIth ganglion bifurcate to extend

longitudinally on entering the brainstem, like DRG afferents, to innervate the cerebellum and vestibular nuclei (Tashiro et al., 2000).

To understand the guidance mechanisms of primary vestibular afferents to their central target, we have used flat whole-mount preparations of the rat embryo and performed various manipulations, including ablation, transplantation and rotation of hindbrains as well as collagen gel co-culture, and have found that all aspects of the guidance of vestibulocerebellar afferents can be explained by the coordinated action of local permissive and nonpermissive cues.

## MATERIALS AND METHODS

### Animals and hindbrain preparation

Wild-type Wistar rat embryos and green fluorescent protein (GFP) transgenic rat embryos were used. GFP transgenic rat line TgN(acro/act-EGFP)4OsB was one of the seven lines produced by micro injecting a mixture of EGFP cDNA driven by  $\beta$ -actin promoter (Okabe et al., 1997) and acrosin-EGFP fusion cDNA driven by acrosin promoter (Nakanishi et al., 1999) into the pronucleus of SD strain. All tissues from the transgenic line used for the present study emitted green fluorescence under blue excitation light.

The procedures for flat whole-mount preparation followed those of Shirasaki et al. (Shirasaki et al., 1995), with some modifications. In brief, the hindbrain of embryonic day (E) 13 rat embryos (E0 set as day of vaginal plug) was removed with the Vth and VIIIth ganglia attached, and then cut along the dorsal midline. The hindbrain was then opened, whole mounted with the ventricular side downwards, and the Vth ganglion removed. Although the VIIIth ganglion is composed of vestibular and cochlear components, our preparation should not contain the cochlear component for reasons described previously (Tashiro et al., 2000).

In the descriptions hereinafter, the terms 'lateral', 'medial', 'dorsal', 'ventral', 'rostral', 'caudal' and 'longitudinal' refer to positions and directions in the opened and flattened hindbrain.

### Culture

Procedures for organotypic culture of hindbrain followed those of Tashiro et al. (Tashiro et al., 2000). In brief, E13 rat hindbrain was flat whole mounted on collagen-coated membrane (Transwell, Corning Costar, No. 3492; Yamamoto et al., 1989) and cultured in DMEM/F12 medium supplemented with 3.85 mg/ml glucose, 10  $\mu$ g/ml insulin, 100  $\mu$ g/ml transferrin, 20  $\mu$ g/ml streptomycin, 10% FBS, 25 ng/ml brain-derived neurotrophic factor (BDNF) and 12.5 ng/ml neurotrophin 3 (NT3) (Avila et al., 1993). The cultures were maintained for 2-4 days at 37°C in an environment of humidified 95% air and 5% CO<sub>2</sub>. Procedures for explant culture in collagen gels followed those of Shirasaki et al. (Shirasaki et al., 1995). In brief, explants of cerebellar primordium or ventral metencephalon from E13 preparations were embedded in collagen gels with stage matched VIIIth ganglion (VIIIg) and were cultured for three days in the same medium described above.

### Immunohistochemistry

Cultured preparations were fixed with 4% paraformaldehyde overnight at room temperature and processed for immunostaining (see Shirasaki et al., 1996; Tashiro et al., 2000). In brief, the preparations were incubated in rabbit anti-recombinant human parvalbumin (PV) antibody (No. 3865; 1:4000 dilution; Rhyner et al., 1996; Troxler et al., 1999) as a primary antibody for 12 hours, rinsed in normal goat serum (NGS) (diluted 1:100 in phosphate-buffered saline with Triton X-100), incubated with biotinylated secondary anti-rabbit IgG (Vector Labs, 1:200), rinsed and incubated in avidin-biotin peroxidase complex (ABC) (Vector Labs, Vectastain ABC Elite kit). To develop

the staining, tissue was incubated in diaminobenzidine tetrahydrochloride (DAB) (0.05% in Tris-buffered saline) with 0.01% H<sub>2</sub>O<sub>2</sub>. Some preparations were incubated in biotinylated secondary antibody followed by an incubation in Cy3-conjugated Streptavidin (Jackson ImmunoResearch, 1:500) for 3 hours. At least six washes of 1 hour each were performed after each incubation step. Tissues were gently coverslipped and observed under an epifluorescence microscope (Olympus BH2).

### Analysis of neurite growth

In transplantation experiments, one side of the hindbrain was always left intact and used as a control. Preparations in which vestibular afferents on the control side showed ordered and vigorous growth were selected for analysis.

For chemotropic assay using explant cultures in collagen gels, images from a light microscope were incorporated into a computer with a CCD camera (Fujix HC-2000, Fuji Photo Film, Co., Tokyo) and montages of the images were constructed. Images of explants were divided into four quadrants (see Fig. 3I). For each explant, the areas covered by parvalbumin-positive neurites, from the boundary of explants to the outer perimeter of the neurites, were measured in the proximal (p) and distal (d) quadrant with the aid of NIH image software. The average of the ratio p/d was then calculated, which gives a ratio of 1 for radial outgrowth, and the difference from the value 1 was examined using Student's *t*-test. In controls, neurites in arbitrarily divided quadrants from VIIIth ganglion cultured alone were analyzed.

To quantitate direction of axonal growth in the cerebellar primordium, a piece of cerebellar primordium was taken from a GFP transgenic rat and transplanted to the corresponding position of the cerebellar primordium of a wild-type rat with or without 90° rotation. Preparations in which transplants were square-shaped were selected and used for the analysis. Then, the numbers of parvalbumin-positive vestibular axons crossing the caudal and the rostral boundary of a transplant, and those crossing the medial boundary and lateral edge were counted. Axons that ended or faded away in the transplant, those extending from the caudal to lateral boundary and those from the medial to the rostral boundary were excluded from the analysis.

## RESULTS

As reported previously, the VIIIth nerve bifurcates into descending and ascending branches on entering the brainstem (Fig. 1; Tashiro et al., 2000). We focused on the ascending branch in this study, because these can be unambiguously identified as vestibulocerebellar axons (Tashiro et al., 2000).

### Vestibular axons ascend in the absence of target

The cerebellum is the final target of vestibulocerebellar axons. To examine possible involvement of target-derived long-range diffusible cues, we first ablated the rostral hindbrain, including the cerebellar primordium. We found that vestibular axons can ascend in the absence of the target: they reached the cut edge of the hindbrain tissue irrespective of the presence of cerebellar primordium (Fig. 2A; *n*=4). It seems unlikely that substrate-associated directional cues (Nakamura et al., 2000) contribute to their guidance towards the cerebellum, because when a strip of hindbrain tissue residing on the pathway of vestibular axons was cut and rotated by 180° along the rostrocaudal axis, the axons extended rostrally ignoring the rotation (Fig. 2B; *n*=8). Although the axons on rotated tissues appeared to extend somewhat less vigorously compared with unmanipulated preparations (Fig. 2A), the rotation per se does not seem to

affect rostrally oriented growth of the axons, because tissue manipulation without rotation provided similar results (Fig. 2C;  $n=8$ ). Taken together, these results suggest that neither target-derived long-range diffusible cues nor substrate-associated directional cues are required for vestibular afferent guidance towards their final target, the cerebellum. These results lead us to speculate that there is a preferred substrate for vestibular axons extending from the entry point to the cerebellar primordium.

### Ventral metencephalon is a nonpermissive substrate for vestibular axons

After reaching the base of the cerebellar primordium, the vestibular axons splay out towards the rhombic lip without extending into the ventral metencephalon (Tashiro et al., 2000). It is possible that ventral metencephalon prevents ingrowth of the axons. To test this possibility, we transplanted a small piece of an explant of ventral metencephalon into the cerebellar primordium (Fig. 3A). As shown in Fig. 3B,C, vestibular axons in all preparations tested failed to enter the transplanted tissue ( $n=6$ ). In control experiments in which tissue of the cerebellar primordium was excised and returned to its original position, vestibular axons extended across the boundary and showed a pattern of innervation that was indistinguishable from that of normal preparations (data not shown), indicating that transplantation per se does not affect the growth of axons. These results raise the possibility that ventral metencephalon provides a nonpermissive substrate for vestibular axons.

Owing to the ambiguity of host/donor boundary, the transplantation experiment described above does not preclude the possibility that a diffusible factor released from the ventral metencephalon repels vestibular axons at a distance. To ensure that local cues regulate the growth of vestibular axons, we examined whether the axons stop growing at the tissue boundary between transplant and host tissues. For this, we used a GFP transgenic rat as a donor of ventral metencephalon explant. GFP transgenic rat used in the present study expresses GFP under the promoter of  $\beta$ -actin (Nakanishi et al., 1999; Okabe et al., 1997). In this mutant, all cells in the neural tube appeared to express GFP. We found that vestibular axons stopped growing around the donor/host boundary, as judged by fluorescence of GFP (Fig. 3D,E;  $n=6$ ), with some axons slightly invading the transplanted tissue. It is unlikely that tissues of GFP transgenic rat are generally non-permissive for axonal growth, because vestibular axons invaded into the cerebellar primordium of GFP transgenic rat (Fig. 3F;  $n=4$ ). Together, these results suggest that local cues in the ventral metencephalon prevent invasion of vestibular axons into the rostral hindbrain.

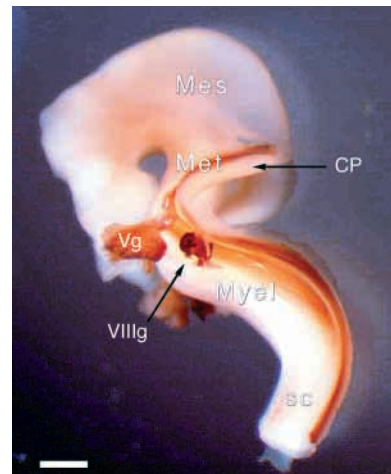
In support of this idea, turning of VIIIth ganglion axons was not observed when they were co-cultured with an explant of ventral metencephalon in collagen gel matrices. Although the length of PV-stained axons emanating from a quadrant of the ganglion opposing the ventral metencephalic explant was somewhat shorter compared with those emanating from the other three quadrants (Fig. 3H,J), turning of axons away from the explant was not observed. Similar results were obtained in preparations that were observed with a phase-contrast microscope without PV staining ( $n=18$ , data not shown). We also found that floor plate explant did not repel vestibular axons

in collagen gels ( $n=13$ , data not shown). In addition, deletion of the floor plate ( $n=8$ ) or ventral metencephalon ( $n=4$ ) in flat whole-mount preparations did not affect the extension of these axons (data not shown). It thus seems unlikely that chemorepulsion from ventral metencephalon contributes to vestibular axon guidance.

### Vestibular axons extend laterally in the absence of rhombic lip

After arriving at the base of the cerebellar primordium, vestibular axons grew into the cerebellar primordium, heading toward the rhombic lip. To test that directional cues of the substratum in the CP guide vestibular axons laterally, the direction of the axonal pathway in the cerebellar primordium was reversed along the mediolateral axis, by transplanting tissue of the cerebellar primordium taken from the contralateral side (Fig. 4A). Vestibular axons invading into the transplanted tissue continued growing toward the rhombic lip ( $n=4$ ), ignoring the rotation (Fig. 4A,B). We next tested whether this directed growth of the axons is caused by diffusible chemoattractant released from their target (the rhombic lip). For this, the rhombic lip was surgically ablated. Vestibular axons still extended toward the cut edges of the cerebellar primordium (Fig. 4C;  $n=7$ ), suggesting that targeted-derived chemoattractant is not required for the laterally directed growth of vestibular axons. In agreement with this view, the VIIIth ganglion, when co-cultured with an explant of the cerebellar primordium that includes the rhombic lip in collagen gels, extended axons radially and showed no biased growth towards the explant of the cerebellar primordium (Figs 3J, 4D).

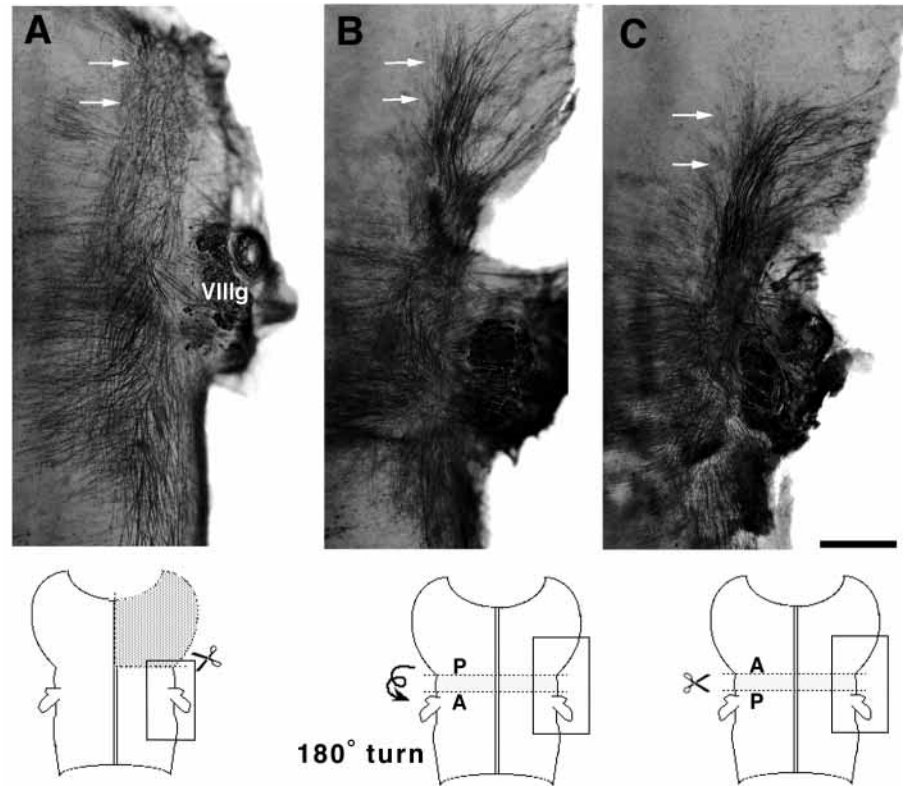
Taken together, these results favor the idea that nonpolarized local cues in the cerebellar primordium play key roles in directing the vestibular axons to the rhombic lip.



**Fig. 1.** Development of vestibular afferents in the rat embryo. Side view of the rat neural tube at embryonic day (E) 14.5. Immunostaining with anti-parvalbumin (PV) antibody. Vestibular afferents originating from the VIIIth ganglion bifurcate into ascending and descending branches that gradually turn dorsally and splay out towards the lateral edge of CP at E14-E15 (Tashiro et al., 2000). CP, cerebellar primordium; Mes, mesencephalon; Met, metencephalon; Myel, myelencephalon; sc, spinal cord; Vg, Vth ganglion; VIIIg, VIIIth ganglion. Scale bar: 800  $\mu$ m.



**Fig. 2.** Neither long-range chemoattractant nor substrate-associated polarized cues are required for rostral growth of vestibular axons. (A) Flat whole-mount preparation of the rat hindbrain whose rostral part including the cerebellar primordium (CP) had been removed and cultured for 2 days. The axons from the VIIIg reached the rostral end, which has been sectioned (arrows). (B) A thin strip was cut along the circumferential axis and was put back to the original position after 180° rotation along the rostrocaudal axis. Vestibular axons extend rostrally as if they ignore the rotation and reach the CP (arrows). (C) Control experiment in which the thin strip was put back to the original position without rotation. The preparations were cultured for 4 days in B and C. Schematic diagrams in the lower panels show corresponding procedures of tissue manipulation. Shaded areas show the regions of manipulation and rectangles represent the areas shown in photomicrographs. Immunostained with anti-PV antibody. Rostral is upwards. Scale bar: 500 µm.



### Vestibular axons respect substratum orientation in the cerebellar primordium

Vestibular axons did not grow randomly within the cerebellar primordium, but appeared to extend with a certain orientation. This observation raises the possibility that these axons are guided by cues with an orientation. To test this idea, a region of the cerebellar primordium was rotated by an angle of ~90°, with the lateral edge reoriented rostrally (see Fig. 5A, inset). We found that a predominant number of axons in the transplant reached the rostral edge in rotated tissue (Fig. 5A), while the majority of the axons ended in the lateral aspect of the transplant in control, unrotated tissue (Fig. 5B). Quantification of the results reinforced this finding (Fig. 5C). These results suggest that growth directionality of vestibular axons in the cerebellar primordium is regulated by some substrate-associated local cues distributed with an orientation. Thus, the guidance of vestibular fibers toward the rhombic lip can be explained by non-polarized local cues distributed with an orientation.

### Mesencephalon provides nonpermissive substrate for vestibular axons

Vestibular axons extended rostrally but never beyond the midbrain/hindbrain boundary. This raises the possibility that the mesencephalon is an unfavorable substrate for these axons. To test this idea, a piece of tissue taken from the cerebellar primordium was transplanted to the rostral part of the mesencephalon (Fig. 6A). Vestibular axons stopped growing at the donor/host boundary and formed a sharp boundary, and failed to grow into the tissue of the mesencephalon (Fig. 6B;  $n=4$ ); Transplantation from the mesencephalon gave rise to the same results irrespective of

the site of explant origin. This observation suggests that nonpermissiveness of mesencephalic tissue prevents invasion of vestibular axons into the midbrain.

There is an alternative possibility that explains the failure of vestibular axons to grow into the mesencephalic tissue, i.e. formation of a barrier for axons at the donor/host boundary. To test this, we transplanted VIIIth ganglion on a mesencephalic transplant. Surprisingly, axons from the VIIIth ganglion extended on the mesencephalic transplant, although they were unable to penetrate the donor/host region (Fig. 6C;  $n=2$ ). These results are consistent with the idea that the failure of the vestibular axons to invade the mesencephalon is due to the presence of a barrier at the mesencephalon/metencephalon boundary.

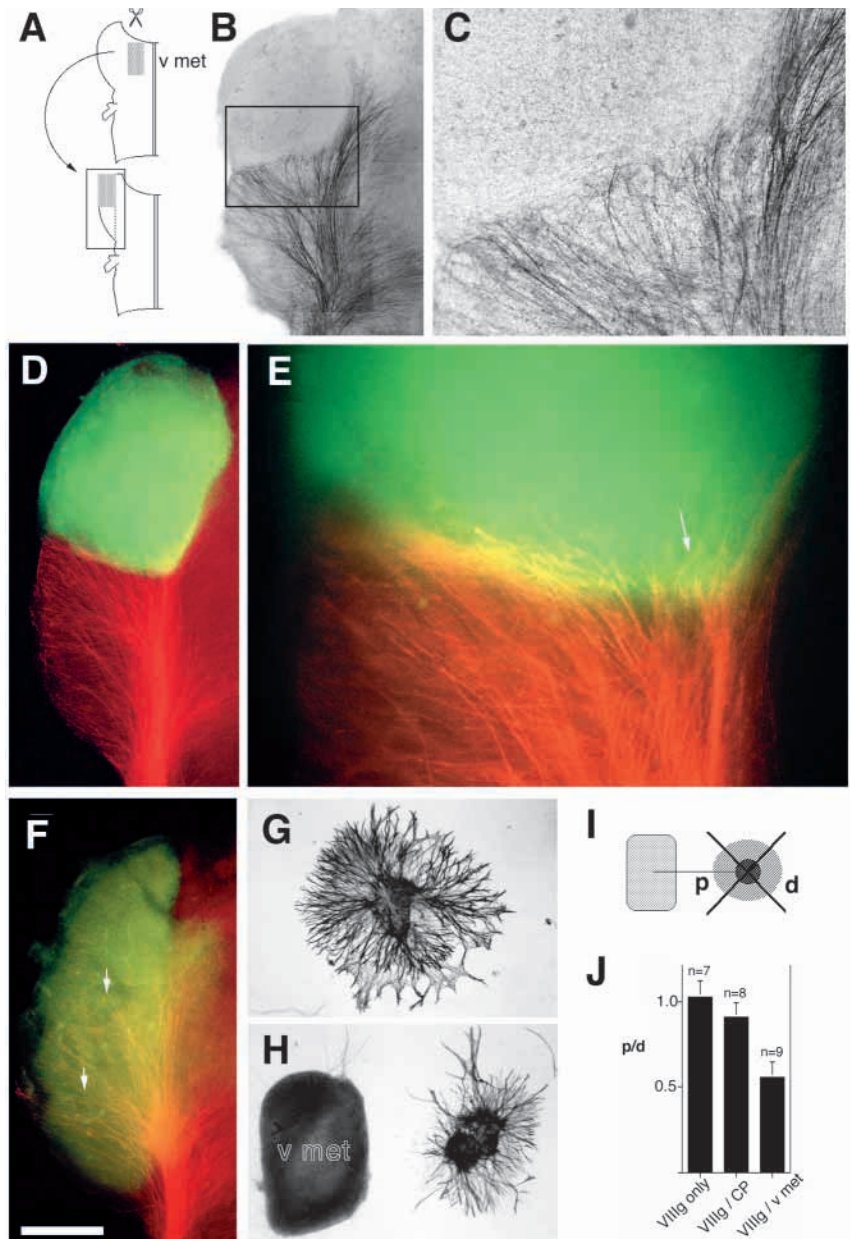
## DISCUSSION

Using a hindbrain flat whole-mount preparation that mimics the *in vivo* environment for axonal growth, the present results demonstrate that local cues play a key role in the guidance of the vestibular axons to their final target, the cerebellum (Fig. 7).

### Longitudinal growth

After entering the brainstem, most vestibular axons, if not all, bifurcate into descending and ascending branches (Tashiro et al., 2000, see also Larsell, 1923, Morris et al., 1988, Naito et al., 1995), suggesting that the axons do not choose either to descend or to ascend. Together with our present finding that rotation of hindbrain substratum along the rostrocaudal axis did not hinder growth of vestibular axons (Fig. 2), this leads us to

**Fig. 3.** Local inhibitory cues in ventral metencephalon suppress the growth of vestibular axons. (A) Transplantation of ventral metencephalic tissue. Tissue from the ventral metencephalon (shaded area) replaced the rostral CP. (B) Low-magnification view of ventral metencephalon transplanted preparation that corresponds to the area outlined by a rectangle in A. (C) High-magnification view of the areas outlined by a rectangle in B. The axons did not extend into transplanted tissue. (D) Tissue of ventral metencephalon from a GFP transgenic rat (green) transplanted to the rostral CP. (E) High-magnification view of D showing donor/host boundary. The donor/host boundary coincided with the boundary of axonal growth. White arrow indicates a labeled axon in the boundary. (F) Control experiment in which entire CP was replaced with CP tissue of a GFP transgenic rat. Axons continued growing across the explant boundary unlike D (arrows in F). Rostral is upwards. Each preparation was cultured for 3 days, immunostained with anti-PV antibody and visualized with DAB (B,C) or Cy3 (D-F). (G-J) Collagen gel culture of the VIIIg with tissue of the ventral metencephalon. (G) VIIIth ganglion cultured alone extended neurites radially. (H) VIIIth ganglion co-cultured with an explant of ventral metencephalon (v met). Neurite outgrowth from the VIIIg appears to be reduced on the side opposite the metencephalic explant. (I) The method of neurite outgrowth quantification. The area of neurite outgrowth from the VIIIg was divided into four quadrants, and the area of neurite on the proximal (p) and distal (d) quadrants were compared. (J) p/d ratio was indicated by filled columns. No significant difference was observed between VIIIg/CP and VIIIg only. Significant difference was observed between VIIIg/v met and VIIIg only ( $P < 0.01$ ). The VIIIg was cultured for 3 days and immunostained with anti-PV antibody. Vertical bars indicate the s.e.m.. Rostral is upwards. Dorsal is leftwards. Scale bar: 610  $\mu\text{m}$  in B; 120  $\mu\text{m}$  in C; 500  $\mu\text{m}$  in D,F; 125  $\mu\text{m}$  in E; 415  $\mu\text{m}$  in G,H.



speculate that there may be a preferred substratum for vestibular axons extending along the longitudinal axis (Katz et al., 1980; Kuwada, 1986).

While a major bundle of axons extended along the longitudinal axis in the present study, ventrally oriented growth of vestibular axons was also observed (Fig. 2). Such axons were observed from early stages in culture (one day in vitro, data not shown), while collaterals of vestibular axons extend ventrally only after extending longitudinally in vivo. Although the exact explanation requires further study, this may suggest that some mechanism that suppresses sprouting of vestibular axons is modulated in the present in vitro preparations.

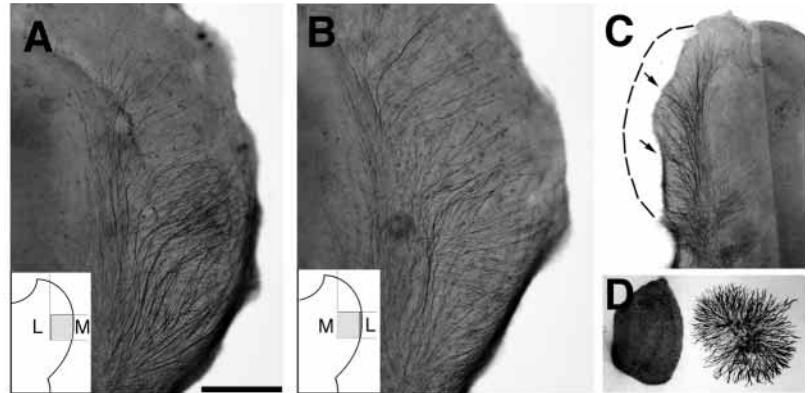
#### Growth towards the cerebellar primordium

In the metencephalon, at the level of the cerebellar primordium, vestibular axons almost never grow into the ventral region (Tashiro et al., 2000). Similar behavior of the axons has been

observed in vitro: very few axons enter the brainstem at this level (Tashiro et al., 2000), suggesting that this region is inhibitory for vestibular axons. In the spinal cord, sensory axons were shown to be repelled by *Sema3A*, which is expressed in the ventral spinal cord (Messersmith et al., 1995; Püchel et al., 1995). Although *Sema3A* appears to be expressed in the ventral hindbrain (Shepherd et al., 1996), it seems unlikely that it is responsible for this nonpermissiveness, because the *Sema3A* receptor, as detected by alkaline phosphatase-conjugated *Sema3A* probe, is not expressed on the VIIIth nerve (Kobayashi et al., 1997). Other repulsive molecules such as *Slit2* and other members of semaphorin family are also expressed in the ventral spinal cord (e.g. Zou et al., 2000). However, their expression in the hindbrain and responsiveness of vestibular axons to these molecules remains unestablished. Moreover, explants of ventral metencephalon did not show repulsion of vestibular axons at a distance in



**Fig. 4.** Laterally oriented growth of vestibular axons is independent of mediolateral polarity of the CP substratum. (A) Mediolateral polarity-reversed preparation. Vestibular axons extend laterally despite the reversed polarity of the substratum. Inset shows mediolateral reversal of polarity. Tissue of CP from the contralateral side of a donor was transplanted to the corresponding site of the CP. This resulted in reversal of mediolateral polarity without changing rostrocaudal polarity of the tissue. (B) Control experiment in which a region of CP was sectioned and put back to its original position. Target-derived chemoattractant may be not involved in vestibular axon guidance towards the rhombic lip. (C) Rhombic lip-ablated preparation. Vestibular axons reached the lateral cut edge of the CP (arrows). Broken line indicates the outline of original rhombic lip. (D) VIIIg co-cultured with CP. Neurites extended radially despite the presence of a CP tissue, without showing biased growth (Fig. 3J). Each preparation was cultured for 3 days and immunostained with anti-PV antibody. Rostral is upwards. M, medial; L, lateral. Scale bar: 500  $\mu\text{m}$  in A,B; 950  $\mu\text{m}$  in C; 850  $\mu\text{m}$  in D.

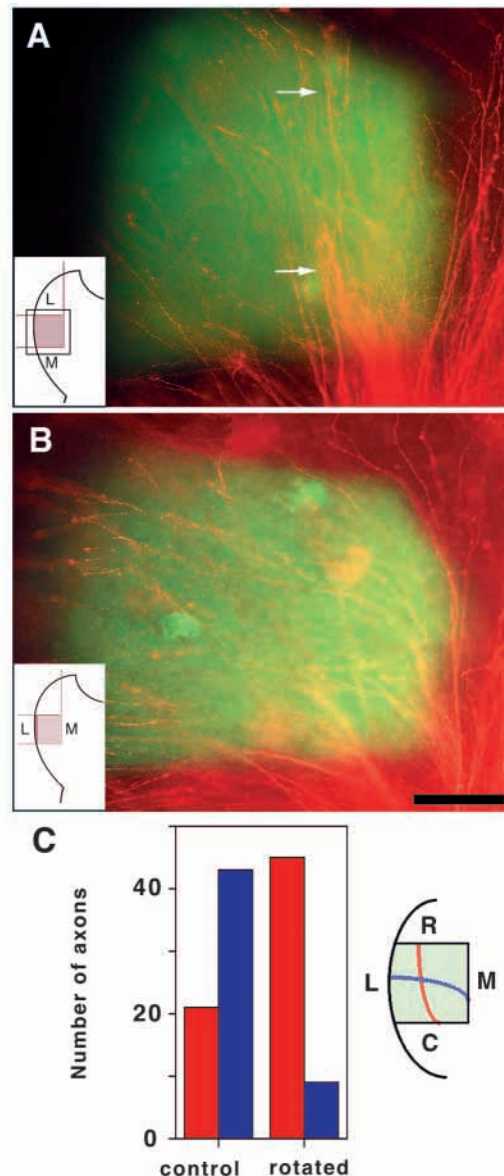


collagen gels (Fig. 3G-J). This observation also precludes the likelihood that ventral metencephalon-derived diffusible cues other than Sema3A repel vestibular axons. Co-culture of vestibular ganglion with floor plate explants in collagen gels also failed to demonstrate chemorepulsion of these axons (data not shown), suggesting that floor plate-derived secreted molecules such as netrin-1 (Colamarino and Tessier-Lavigne, 1995) and Slit (Hu, 1999; Nguyen Ba-Charvet et al., 1999) are not responsible for their guidance. It thus seems unlikely that long-range diffusible cues prevent entry of vestibular axons into the ventral region of the rostral hindbrain, although the possibility that subtle turning of axons was not detected in our assay cannot be precluded. The present findings indicate instead that the ventral metencephalon provides local cues that are nonpermissive or inhibitory for vestibular axons. This idea is supported by transplantation of the ventral hindbrain tissue into the cerebellar primordium: vestibular axons stopped growing around the boundary between host and transplant, with some slightly invading into the transplant (Fig. 3E), providing evidence to suggest that this inhibitory effect of axonal outgrowth does not function at a distance.

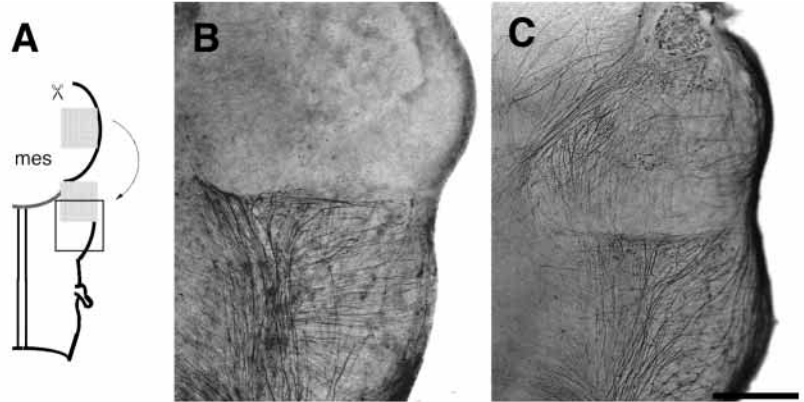
In collagen gel culture, however, extension of axons emanating from the vestibular ganglion was somewhat suppressed on the side opposing the ventral metencephalon,

**Fig. 5.** Cues in CP may be oriented mediolaterally.

(A) Vestibulocerebellar axons change their growth direction in rotated tissue. The axons extend rostrally (arrows) as if they respect the tissue orientation. Tissue of CP from a GFP transgenic rat (represented by shaded area in the inset) was transplanted to a corresponding site of the host CP after approx. 90° rotation. (B) Transplanted tissue of a GFP transgenic rat in a control experiment where tissue of the cerebellar primordium from a GFP transgenic rat was transplanted without rotation. The axons extend laterally as in normal preparations. Each preparation was cultured for 3 days, immunostained with anti-PV antibody and visualized by Cy3. (A,B) Double exposure photos under illumination of the rhodamine filter and GFP filter. Rostral is upwards. (C) Comparison of the number of axons directing rostrally and laterally. Red column represents the number of axons extending from the caudal thorough to the rostral edge (red line in the inset) and blue column represents those from the medial to the lateral edge (blue line in the inset). A total of 64 axons were scored from 9 control cultures and 54 axons from 9 rotated preparations. M, medial; L, lateral; R, rostral; C, caudal. Scale bar: 120  $\mu\text{m}$ .



**Fig. 6.** Vestibular axons fail to extend into mesencephalic tissue. (A) Transplantation of mesencephalic tissue. A piece of alar plate tissue from the dorsal mesencephalon (shaded) replaced the rostral CP. (B) Mesencephalon transplanted preparation. Microphotograph correspond to the areas outlined by rectangles in A. The axons failed to extend into transplanted tissue. Similar results were obtained with transplantation of ventral mesencephalon. (C) Transplantation of VIIIth ganglion on a mesencephalon transplanted preparation. Axons from the VIIIth ganglion extended on the mesencephalic transplant, although they were unable to penetrate the donor/host boundary region. Each preparation was cultured for 3 days, immunostained with anti-PV antibody and visualized with DAB. Rostral is upwards. mes, mesencephalon. Scale bar: 300  $\mu$ m in B; 340  $\mu$ m in C.



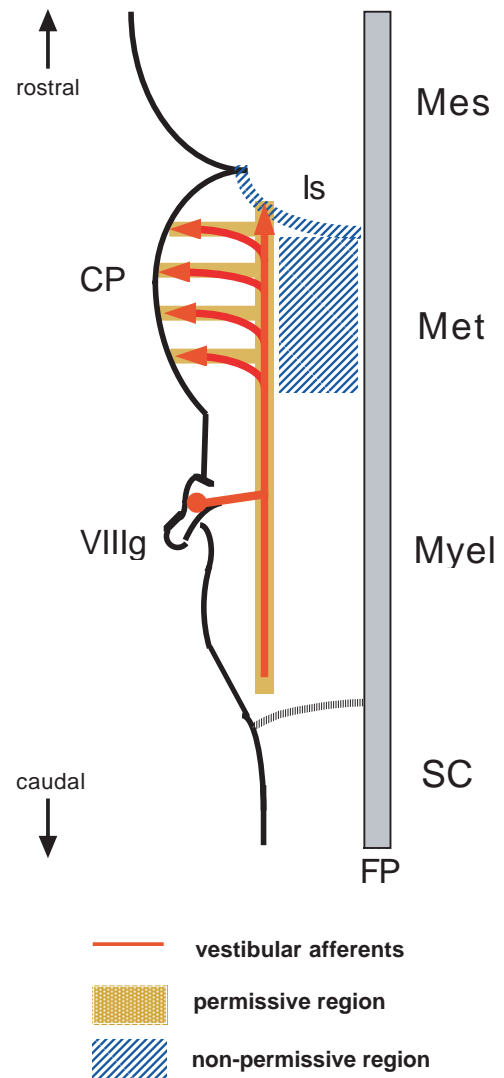
suggesting that some inhibitory diffusible factor(s) is or are released into collagen gels. A possible explanation for this apparent discrepancy is that the putative diffusible factor spreads out efficiently in the gels but not in brain tissues. In any case, the failure of vestibular axons extending into the ventral metencephalon can be accounted for by local inhibitory cues in this region.

#### Growth of vestibular axons in the cerebellar primordium

In the cerebellar primordium, vestibular axons showed ordered growth, lined towards the rhombic lip and oriented almost perpendicularly to the rhombic lip. Again, long-range diffusible factors do not appear to be required for this directed growth, because deletion of the rhombic lip did not affect the directed growth of vestibular axons (Fig. 4C) and tissue explants of the rhombic lip region did not attract vestibular axons in collagen gels (Fig. 4D). Moreover, the substrate does not appear to be polarized along the mediolateral axis, as 180° rotation of the substratum tissue did not affect the directed growth of the vestibular axons towards the rhombic lip (Fig. 4A,B). It is, thus, likely that vestibular axons grow into the cerebellar primordium because it provides a favorable substrate for these axons (but see Chédotal et al., 1997).

An interesting finding is that orientation of vestibular axon growth in the cerebellar primordium was sensitive to rotation of the substratum (Fig. 5). Similar results were obtained in a study of optic tract development (Harris, 1989): when small pieces of the presumptive optic tract were rotated by ~90° either clockwise or counterclockwise, retinal axons that encountered the rotated neuroepithelium turned in correspondence with the direction of rotation. The present results imply that cues that guide vestibular axons are lined with a particular orientation. The fact that vestibular axons are the first axons entering the cerebellar primordium (Ashwell

and Zhang, 1992) precludes the possibility that they follow other axons growing earlier. Although the Bergman glia is a notable oriented structure in the cerebellum, it should be lined perpendicular to the vestibular axons. Thus, some cues yet unidentified are likely to contribute to the orientation of vestibular axons.



**Fig. 7.** Guidance mechanism of vestibulocerebellar afferents. The vestibular fibers entering the brainstem take two major longitudinal pathways, one ascending and the other descending along non-polarized local permissive cues (dark yellow). Non-permissive cues in ventral metencephalon (blue) inhibit ventral growth. Ascending fibers reach cerebellar primordium and splay out towards rhombic lip on oriented permissive cues. CP, cerebellar primordium; FP, floor plate; Is, isthmus; Mes, mesencephalon; Met, metencephalon; Myel, myelencephalon; SC, spinal cord; VIIIg, ganglion VIII.

### Failure of growth into the mesencephalon

Vestibular axons failed to grow into a transplant of tissue from the mesencephalon. This corresponds to their growth pattern *in vivo*, where vestibular axons stay within the hindbrain. Failure of vestibular axons to grow into the midbrain region may be because the entire midbrain region is nonpermissive for vestibular axons. It is also possible, however, that midbrain/hindbrain boundary functions as a barrier for vestibular axons. Our finding that an explant of the VIIIth ganglion placed on transplanted mesencephalic tissue, extended axons on mesencephalic substratum but failed to grow into the donor/host boundary supports the latter possibility. Recent studies have demonstrated that an interaction between Gbx2 expressed in the hindbrain and Otx2 expressed in the midbrain leads to formation of midbrain/hindbrain boundary (Broccoli et al., 1999; Irving and Mason, 1999; Millet et al., 1999). Thus, transplantation of midbrain tissue into the hindbrain might cause formation of a new boundary that hinders the growth of vestibular axons. It would be interesting to examine whether the donor/host boundary expresses isthmus-associated molecules such as fibroblast growth factor 8.

### Role of local cues in the guidance of CNS axons

During the past decade, great progress has been made in understanding the roles of long-range diffusible cues in the guidance of axons. Netrins, originally isolated as floor plate-derived diffusible molecules (Kennedy et al., 1994; Serafini et al., 1994), for example, have been shown to be involved in the guidance of a variety of axons by their chemotropic activity; these include commissural axons in the spinal cord, hindbrain and midbrain (reviewed in Murakami and Shirasaki, 1997), corticofugal axons (Métin et al., 1997), axons of retinal ganglion cells (de la Torre et al., 1997), and trochlear motor axons (Colamarino and Tessier-Lavigne, 1995). Netrins attract or repel these axons *in vitro* at a distance, and trajectories of most of these axons are disturbed in netrin-deficient mice (Bloch-Gallego et al., 1999; Deiner et al., 1997; Deiner and Sretavan, 1999; Serafini et al., 1996). Some members of the semaphorin family proteins have also been shown to repel sensory, sympathetic, hippocampal and olfactory axons in collagen gel at a distance (de Castro et al., 1999; Chédotal et al., 1998; Chen et al., 1998; Messersmith et al., 1995). More recently, Slit also attracted attention as a diffusible chemorepellent of axons (Li et al., 1999; Niclou et al., 2000; Nguyen Ba-Charvet et al., 1999; Ringstedt et al., 2000). These studies strongly suggest that long-range diffusible cues play important roles in axon guidance, although the extent of diffusion of these molecules *in vivo* remains to be determined.

However, numerous studies have demonstrated that extracellular matrix molecules and cell-surface adhesion molecules modulate neurite outgrowth *in vitro*, implicating involvement of local cues in axon guidance. Most of these studies tested the effect of candidate molecules in preparations that are very different from those *in vivo*, obscuring the importance of substrate-associated cues in axon guidance. However, recent evidence has also been accumulating that substrate-bound cues contribute to the guidance of axons in preparations that mimic *in vivo* development (Chédotal, et al., 1997; Godement and Bonhoeffer, 1989; Harris, 1989; Nakamura et al., 2000; Sharma and Frank, 1998; Simon and

O'Leary, 1992; Sugisaki et al., 1996; Tuttle et al., 1998; Walz et al., 1997; Wang et al., 1995), suggesting the importance of substrate-bound (local) cues. The present study has further underlined the importance of local cues in axon guidance by showing that local cues play major roles in the guidance of vestibular axons to their final target. It also provides evidence that oriented cues determine the pattern of axonal growth.

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