The embryonic cerebellum contains topographic cues that guide developing inferior olivary axons

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SUMMARY

The formation of the olivocerebellar projection is supposed to be regulated by positional information shared between pre- and postsynaptic neurons. However, experimental evidence to support this hypothesis is missing. In the chick, caudal neurons in the inferior olive project to the anterior cerebellum and rostral ones to the posterior cerebellum. We here report in vitro experiments that strongly support the existence of anteroposterior polarity cues in the embryonic cerebellum.

We developed an in vitro system that was easily accessible to experimental manipulations. Large hindbrain explants of E7.5-E8 chick embryos, containing the cerebellum and its attached brainstem, were plated and studied using axonal tracing methods. In these cultures, we have shown that the normal anteroposterior topography of the olivocerebellar projection was acquired, even when the cerebellar lamella was detached from the brainstem and placed again in its original position. We also found that, following various experimental rotations of the anteroposterior axis of the cerebellum, the rostromedian inferior olivary neurons still project to the posterior vermis and the caudolateral neurons to the anterior vermis, that now have inverted locations. Thus, the rotation of the target region results in the rotation of the projection. In addition, we have shown that the formation of the projection map could be due to the inability of rostromedian inferior olivary axons to grow in the anterior cerebellum. All these experiments strongly indicate that olivocerebellar fibers recognize within their target region polarity cues that organize their anteroposterior topography, and we suggest that Purkinje cells might carry these cues.

Key words: Purkinje cell, inferior olive, climbing fiber, cerebellum, projection map, chick

INTRODUCTION

In the olivocerebellar system, axons from the inferior olivary nucleus (IO) in the caudal brainstem terminate as climbing fibers in the opposite hemicerebellum, synapsing on the dendritic tree of Purkinje cells (PCs), and also on neurons of the deep nuclei (Brodal and Kawaamura, 1980; Van der Want et al., 1989). The exact function of this projection is still openly debated. While for some investigators the inferior olive organizes movement in time (Welsh et al., 1995), for others it is implicated in motor learning (Ito, 1989). Anatomical and physiological studies have shown that olivary axons innervate the cerebellum in an orderly fashion (for a recent review see Buissere-Delmas and Angaut, 1993), as is the case in many parts of the central nervous system (Udin and Fawcett, 1988; Goodman and Shatz, 1993). Most of the studies have focused on one feature of the projection: climbing fibers originating from different IO subnuclei terminate into distinct mediolateral compartments of the cerebellar cortex, forming adjacent parasagittal bands (the longitudinal organization of the cerebellum). Moreover, nearby clusters of IO neurons do not always project to adjacent cortical bands. Thus, the projection is discontinuous, with sharp boundaries, and ordered with respect to the local origin of the olivary neurons (Azizi and Woodward, 1987; Buissere-Delmas and Angaut, 1993). In addition to this mediolateral topography, it has been demonstrated that the projection is also organized along the anteroposterior axis of the cerebellum. For instance, in the rat, different IO subnuclei can project to a different anteroposterior level of the same parasagittal subdivision of the cerebellar cortex (Furber and Watson, 1983). This anteroposterior topography is particularly striking in the chick, where the rostral IO maps to the posterior cerebellar lobules and the caudal IO to the anterior cerebellar lobules (Furber, 1983).

The cellular and molecular mechanisms underlying the formation of ordered olivocerebellar projections remain unknown (Sotelo and Wassef, 1991). Work carried out in our laboratory indicates that the navigation of olivocerebellar axons within the developing cerebellar parenchyma is regulated by positional information shared between clusters of IO neurons and their corresponding mediolateral compartments of PCs (Wassef and Sotelo, 1984; Wassef et al., 1985; Chédotal and Sotelo, 1992; Wassef et al., 1992a,b,c; Chédotal et al., 1996). Although all these results favor chemoaffinity mechanisms (Sperry, 1963) in the acquisition of the olivocerebellar projection map, the existence of cerebellar axon guidance cues needs further demonstration.
Using surgical manipulations performed in vitro, the aim of the present work is to reverse the anteroposterior axis of the developing cerebellar plate of chick embryos and to examine presumptive modifications in the olivocerebellar projection. In the chick, IO neurons are generated in the so called 'rhombic lip' in the alar plate of the rhombencephalon (Harkmark, 1954), from the third to the fifth day of incubation (E3-E5; Armstrong and Clarke, 1979). During E5-E7, they migrate from the dorsal to the ventral aspect of the medulla through a process of superficial cell migration (Harkmark, 1954, 1956; Tan and Le Douarin, 1991). Their axons cross the floor plate and grow through the lateral aspect of the caudal brainstem, before entering the cerebellar plate at E8.5-E9 and contacting PCs by E10 (Chédotal et al., 1996). We have developed an in vitro system in which we could test, using focal injections of the carboxyamine dyes Dil and DTA, the consequence of an experimental rotation of the anteroposterior axis of the cerebellum on the formation of the olivocerebellar projection. We found that such cerebellar rotations result in an equivalent inversion of the olivocerebellar projection map, demonstrating that the formation of the olivocerebellar topography depends on cerebellar directional informations. We have also shown that axons from the rostromedian IO grow exclusively in tissue derived from the posterior cerebellum, in accordance with their behavior in vivo, whereas axons from the caudolateral IO can invade both anterior and posterior cerebellar halves in vitro, forming compressed olivocerebellar maps.

MATERIALS AND METHODS

Organotypic cultures
Fertilized JA57 hens’ eggs obtained from local farms were incubated at 38°C in a humidified atmosphere. After 7.5-8 days of incubation (E7.5-E8), embryos were collected, staged (Hamburger and Hamilton, 1951), and decapitated. The heads were put into ice-cold Gey’s balanced salt solution with 5 mg/ml glucose (GBSS) and the brains quickly dissected out and left in GBSS. The brain region between the tecto-cerebellar and medullo-spiral junctions (therefore including the cerebellar anlage and the IO) was isolated (see Fig. 1A). The right and left cerebellar plates, which were fused rostrally, were separated with fine tweezers, whereas the caudal portion of the medulla oblongata was cut, and opened along the dorsal midline (Fig. 1B). 3-4 explants were processed successively and then transferred simultaneously onto the membrane of a 30 mm Millipore culture insert plate (pore size 0.4 μm; Millicell CM, Millipore; Stoppani et al., 1991) in 100 mm culture dishes containing 3 ml of medium composed of 50% Eagle’s Basal Medium (BME), 25% Hank’s Balance Salt Solution (HBSS), 5 mg/ml glucose, 10 mM glutamine and 25% (first week) or 15% (following weeks) horse serum, all purchased from Life Technologies. The explants were positioned with their ventricular side down in contact with the membrane of the Millicell, and excess GBSS was carefully removed with a Pasteur pipette (Fig. 1C). The Petri dishes were then placed in an atmosphere of humidified 5% CO₂ with twice weekly replacement of the medium.

Control and cut-unrotated explants
In control cases (n=94) the explant was cultured from 1 to 45 days. In cut-unrotated explants (Fig. 2A, n=15), one cerebellar plate remains untouched, while the other one was cut with fine tweezers along the junction between cerebellum and medulla oblongata, before being replaced in its original position. For all manipulations (see below), the dorsoventral polarity of the cerebellar plate was retained. Moreover, explants (which derive from E7.5-E8 embryos) were operated after half an hour in vitro, and therefore before the arrival of IO axons to the cerebellum, which occurs around E9 in vivo (Chédotal et al., 1996).

In vitro cerebellar rotations
To investigate whether the embryonic cerebellum contains cues that can regulate the anteroposterior positioning of developing olivocerebellar axons, the anteroposterior polarity of one cerebellar plate was reversed. In type 1 rotations (Fig. 2B, n=106) the left cerebellar plate of a first explant was removed, then the right cerebellar plate of a second donor explant was excised, rotated along the anteroposterior axis, and substituted to the ablated cerebellar plate of the first explant (see Fig. 2B). In this type of rotation the anteroposterior polarity of the transplant was inverted while its mediolateral polarity was preserved. In type 2 rotations (n=15), the left cerebellar plate of an explant was equally excised, and its anteroposterior polarity was reversed, before being repositioned in its original location. In this type of rotation, both anteroposterior and mediolateral axes were inverted (Fig. 2C).

Single anterior and single posterior hemi-cerebella
The posterior or anterior halves of each cerebellar plate were excised, giving rise to single posterior (n=8) or single anterior (n=8) explants (Fig. 2D,E).

Immunohistochemical study of the explants
We immunostained some control explants with a mouse monoclonal anti-calbindin D₂₈k (CaBP) antibody (1:10,000, Swant, Switzerland), to determine whether IO neurons and PCs were surviving in this in vitro system, and to check for their localization. CaBP labels PCs and IO neurons in the embryonic (Chédotal et al., 1996), post-hatching and adult chick brains (Rogers, 1989). After 1 or 2 weeks in vitro, the explants were fixed with 4% paraformaldehyde (4% PF) in 0.12 M phosphate buffer (pH 7.2-7.4), for 1-2 hours at room temperature (RT). After fixation, the explants were washed with PBS, transferred in a 5 ml tube, reacted with the CaBP antibody, and revealed using a fluorescein (FITC)-conjugated anti-rabbit antibody (1:100, Silenus) according to Chédotal and Sotelo (1992).

Investigation of the olivocerebellar projection map formation in vitro
The topography of the olivocerebellar projection was examined in control and manipulated explants using anterograde and retrograde axonal tracing methods.

Fig. 1. Method for hindbrain explant culture and attached cerebellum. (A) Dorsal view of the hindbrain of an E7.5-E8 chick embryo. The dashed lines and thin arrows indicate regions of the tissue to be cut with tweezers. The left and right cerebellar plates were separated and turned over (curved arrows). (B) Schematization of the explant once open and ready for culture, viewed from the ventricular side. (C) Explant (shaded) incubating on the membrane of a Millicell insert. The ventricular side of the explant is facing down towards the porous membrane. Three explants were cultured on the same insert. cer, cerebellar plate; fp, floor plate; O₂, oxygen.
Anterograde labelling of inferi or olivary axons

Under a dissecting microscope, a fine crystal of DiI was inserted at the level of the right IO (n=15). The explants were incubated for a further 72-96 hours before fixing with 4% PF. DiA was also assayed for anterograde axonal staining (not shown), but the rate of successfully labelled axons was very low, as previously observed (Chien et al., 1995). In a few cases (n=4), the cerebellar plates of DiI-labelled explants were carefully photographed before being processed for CaBP immunostaining. During this procedure, in which Triton X-100-containing solutions were used, DiI was irretrievably removed from traced axons. Finally, to determine the pathways pursued by olivocerebellar axons in the manipulated cultures, DiI injections in the right IO were performed in type 1 rotation explants (n=12). The injections were done in the caudolateral or in the rostromedial part of the olive.

All explants were whole mounted with Mowiol (Calbiochem), observed and photographed with an Axiopt phot microscope (Zeiss).

RESULTS

Embryonic olivary axons project to Purkinje cells in vitro

The global morphology of hindbrain explants did not change significantly during culture, except for an overall flattening of the tissue, which was still 500-800 μm thick after 3 weeks in vitro (data not shown). Fig. 3A shows a control hindbrain explant, cultured for 2 weeks and immunolabelled with anti-calbindin antibodies. Numerous CaBP-immunoreactive PCs were detected in the cerebellum. Their cell bodies were arranged in a wide peripheral crescent (see Fig. 3A), and the axons ended in a more median region, close to the brainstem junction, devoid of CaBP-positive cell bodies and most probably corresponding to deep nuclear neurons. In the brainstem, many neurons were also CaBP positive, either dispersed or aggregated in distinct nuclei. One of the largest nuclei observed in the caudal portion of the brainstem, immediately adjacent to the floor plate (see Fig. 3A), was identified as the IO nucleus (Rogers, 1989). Its neurons had just finished their migration from the rhombic lips the day (E7.5-E8) explants were put into culture (Rogers, 1989; Harkmark, 1954, 1956; Tan and Le Douarin, 1991; A. Chédotal and C. Sotelo, unpublished observations).

In vivo, IO axons enter the cerebellum around E9 (Chédotal et al., 1996), and therefore they were still growing in the brainstem when explanted. To confirm that this CaBP-positive nucleus was the inferior olive and that its neurons projected to the cerebellum in vitro, we performed axonal tracing experiments. After a unilateral injection of fast-blue in one cerebellar plate, only one nucleus, adjacent to the midline (Fig. 3B), was retrogradely labelled in the contralateral side of the brainstem (bilateral injections lead to bilateral and symmetrical labelling, not shown; see Fig. 6A). Moreover this nucleus, composed of neurons that project into the contralateral cerebellar plate, exhibited a location and morphology corresponding to the nucleus previously identified as the IO by its CaBP immunoreactivity (Fig. 3A,B). Finally, when a fine crystal of DiI was inserted in this region of the brainstem, large axonal plexuses were anterogradely labelled in the contralateral cerebellar territory (Fig. 3C). We cannot exclude that some of these axons may be mossy fibers, some of which enter the cerebellum about this time in vivo (Okado et al., 1987). Retrograde staining of the IO could be obtained after 1 day in vitro (DIV) (see below), suggesting that in culture the growth rate of IO axons is close to the one they have in vivo.

In certain cases, the explants were carefully photographed following DiI tracing and then labelled with anti-calbindin antibodies. Most of the DiI-labelled axons were terminating in the region of the cerebellum containing CaBP-immunoreactive PCs, which were surrounding their cell body and dendrites.
Some olivary axons were also detected in the deep nuclei, their other cerebellar target. Moreover, electrophysiological studies have shown that in such explants, the electrical stimulation of the olivocerebellar tract produces typical climbing fiber responses in PCs (A. Chédotal, C. Sotelo, A. Lohof and J. Mariani, unpublished observations). In conclusion, the results showed that in this in vitro system, olivary axons grow, enter their target territory and synapse on PCs.

**A simple olivocerebellar map is established in vitro**

We tried to determine if, in vitro, the olivocerebellar projection was topographically organized along the anteroposterior axis of the cerebellum. As previously mentioned, large dye injections result in the labelling of the whole IO (Fig. 3B). Therefore, using control explants (n=62), we implanted (after 1 week in vitro) a small crystal of DiI close to the posterior margin of the cerebellar plate and a crystal of DiA close to its anterior margin (Fig. 4A). After 2 days, DiI- (93% of the cases) and DiA- (99% of the cases) labelled neurons were detected in the IO. Interestingly, the two labelled domains were not overlapping, but complementary: DiI-labelled neurons were found in an inverted L-shaped, median region, juxtaposed to the floor plate, almost extending on the entire rostrocaudal length of the IO (see Fig. 4B). In a complementary fashion, DiA-labelled neurons were only detected in an ovoid region, more caudal and lateral than the DiI-labelled domain (Fig. 4B).
Some lateral and median IO neurons were not traced (compare Figs 3B and 4B) as they probably project to the median portion of the cerebellum, where no tracer was injected. The number of labelled IO neurons varied between explants, which could be due to a different neuronal survival, or more probably to a variability in dye crystal sizes and injection sites. In 1% of the cases no IO neurons were labelled, and in 7% of the cases only the caudolateral IO domain was labelled. The organization of this in vitro olivocerebellar map is summarized in Fig. 4C: to make it simpler we can conclude that, in vitro, neurons located in the caudolateral IO project to the anterior cerebellum, whereas rostromedian IO neurons project to the posterior cerebellum. This map resembles the one that has been described in vivo by Furber (1983; see Introduction and Discussion). Interestingly, the map was identical in explants injected with DiI only after an hour in vitro (n=17) and observed after short time survival (Fig. 8A,B). No retrogradely labelled neurons were detected before 22 hours in vitro, the time necessary for the olivary axons to enter the cerebellum and for the retrograde transport of the marker. Thus, from the beginning of the formation of the olivocerebellar projection, the anteroposterior topography was already acquired (compare Figs 8A,B and 4B). The map still formed with explants derived from E6 embryos, and it was also observed after a month in vitro (not shown).

To confirm that the caudolateral and rostromedian IO subdivisions were projecting to distinct anteroposterior cerebellar territories, we injected tiny DiI crystals in the caudolateral or the rostromedian subdivisions of the IO, in order to anterogradely trace their projection into the cerebellum. When the caudolateral olive was injected, dense axonal plexuses were detected in the anterior third of the contralateral cerebellum (Fig. 5A). These axons formed a tight fascicle along the junction between brainstem and cerebellum, and when reaching their most anterior position in this fascicle entered the anterior cerebellum (Fig. 5A), where they desfascicated abruptly (anterior cerebellar entry point). After an injection in the rostromedian IO, labelled axons also followed the same tight fascicle along the border between medulla oblongata and cerebellum. However, when reaching the median region of the fascicle, the labelled axons bent abruptly towards the cerebellum and followed a posterior direction, to terminate within the posterior third of the cerebellar plate (median cerebellar entry point, see Fig. 5B). Only occasionally, a few labelled axons had entered the posterior region of the cerebellar plate directly through a posterior entry point.

The results of retrograde axonal tracing experiments were equivalent when cutting unrotated explants were labelled: in all 15 cases the two IO domains were traced, and the map was identical to the one described for control explants (Fig. 6A,B). Thus, in this in vitro system, IO axons can grow normally through the wound formed at the interface between the cut cerebellum and the medulla oblongata. In addition, when the excised cerebellum was repositioned at ectopic locations along the brainstem (n=6), no IO axon could be retrogradely labelled (not shown), suggesting that olivary axons are probably not attracted by long-range cerebellar signals.

**Topography of the olivocerebellar projection following an inversion of the anteroposterior polarity of the cerebellar plate**

In a first set of experiments, rotation type 1, the cerebellar plate was turned 180° along the anteroposterior axis while its mediolateral polarity was preserved, and the resulting map was analyzed 8 days later (Fig. 7A-D). Following such rotation, we observed (68 cases out of 69) that neurons of the caudolateral IO domains were now DiI-labelled, demonstrating that they sent their axons in the posterior part of the rotated cerebellum. Similarly, rostromedian IO neurons were DiA-labelled (54 cases out of 69), showing that they now project to the anterior part of the rotated cerebellum. In the remaining 16%, no tracing of the rostromedian domain was obtained. In those experiments with short survivals (n=25), the resulting inverted topography appeared between 30 and 60 hours after explantation and cerebellar rotation (Fig. 3C,D). Thus, from the beginning of its formation, the projection map is oriented according to cerebellar intrinsic origin and not its actual position.

In a second set of experiments, rotation type 2, both antero-
In this type of rotation, IO axons were forced to enter the rotated cerebellum through the pial surface and the external granular layer, instead of following their normal route through the brainstem-cerebellum frontier, and therefore were directly invading the region where PC bodies were located. The results were similar to those obtained in type 1 rotation experiments. In 14 cases out of 15, the caudolateral olive was DiI-labelled and in all these cases it projected to the posterior part of the rotated cerebellum (Fig. 7F,G). In 6 cases out of 15 the rostromedian olive was DiA-labelled, and in all cases it was sending axons to the anterior part of the rotated cerebellum. Contrary to rotation type 1, in most cases, only the most rostral part of the rostromedian olivary territory was labelled (compare Fig. 7B-D and FG). In conclusion, we found that when the cerebellar primordium is rotated 180° along its anteroposterior axis, the olivocerebellar projection map is similarly rotated.

We also have studied the pathway followed by olivary axons in some type 1 rotation cases (n=12), by injecting DiI in the caudolateral or rostromedian inferior olive. Anterogradely labelled axons crossed the interolivary commissure and ascended through the lateral aspect of the medulla oblongata to reach the junction between the medulla oblongata and the cerebellum, as in control cases. Once in this region, equivalent to the inferior cerebellar peduncle, labelled axons originating from the caudolateral olive ascended till the median cerebellar entry point (Fig. 9A). The axons bent, entered the cerebellar plate and desfasciculated within the now posterior region, corresponding to the anterior hemicerebellum (Fig. 9A). Conversely, axons originated from the rostromedian olive followed the entire medullocerebellar junction up till the anterior entry point (Fig. 9B). These axons arborized within the now anterior region, which corresponded to the posterior cerebellum. Thus, in type 1 rotation experiments, not only the anteroposterior maps are rotated but the cerebellar entry points are also inverted, suggesting that the axonal guidance cues have a cerebellar location.

**Axons from the rostromedian inferior olive do not grow in anterior cerebellum**

We finally examined how IO axons would behave when confronted with a single anterior or a single posterior cerebella (Fig. 10). Single posterior cerebella received projections from the entire IO (6 cases out of 8), but a ‘compressed’ map still formed, with caudolateral IO neurons projecting to the anteriormost portion of the posterior cerebellum and rostromedian IO neurons projecting to its posteriormost portion (Fig. 10A-C). In one of the remaining two cases no tracing was observed, whereas in the other one only the caudolateral IO domain was DiA-labelled. On the other hand, only neurons from the caudolateral olive project to single anterior cerebella (Fig. 10D-F; 8 cases out of 8): DiI- and DiA-labelled neurons were interspersed, but we did not determine whether some were double-labelled.

**DISCUSSION**

We have cultured and manipulated explants of hindbrain chick embryos, comprising the IO and the cerebellum, to determine...
The developing cerebellum contains topographic cues involved in the pathfinding of olivary axons along its anteroposterior axis. In this in vitro system, olivary axons grow, enter their target territory through distinct entry points, and terminate at the level of their usual target cells, i.e. PCs and neurons of the deep nuclei. In addition, we showed that IO axons do not grow randomly but that a crude map, similar to the one observed in vivo, is established from the initiation of the formation of the projection: neurons from the rostromedian olive project to the posterior cerebellum, whereas neurons of the caudolateral olive project to the anterior cerebellum.

Several attempts had been previously made to reconstruct in vitro the connection between climbing fibers and PCs. Coronal slices of embryonic brainstem have been co-cultured either with dissociated PCs (Hirano, 1990a,b) or cerebellar slices (Gähwiler, 1978; Blank et al., 1983; Knöpfel et al., 1990, 1991; Mariani et al., 1991). In all these conditions, some climbing fibers seem to be able to establish functional synapses on PCs. But none of these systems allow one to study the formation of the olivocerebellar topography, because only random fragments of IO and cerebellum, or dissociated neurons, are co-cultured. Moreover, the axotomized olivary axons are confronted with PCs that are more mature than the one they contact during normal development and on which climbing fibers have already synapsed. Our in vitro system has none of these drawbacks, as the integrity of the entire olivocerebellar pathway is preserved.

This study demonstrates that neurons of anterior and posterior cerebellar territories are not equivalent implying that, during development, some genes are differentially regulated in these two domains. The existence of such an anterior/posterior boundary in the cerebellum has been already suggested, following several approaches. The first arguments came from the phenotypic analysis of cerebellar mutant mice in which anterior or posterior cerebellar domains are differentially affected. In the mutants meander tail (Ross et al., 1990; Napieralski and Eisenman, 1993; Salinas et al., 1994; Eisenman and Arlinghaus, 1994), rostral cerebellar malformation (Lane et al., 1992), swaying (Thomas et al., 1995) and tottering/learner (Hess and Wilson, 1991), the cerebellar abnormalities are mostly restricted to the anterior domain. In addition, two studies conducted in transgenic mice, with the L7/pcp-2 (Oberdick et al., 1993) or the En-2 (Logan et al., 1993) promoters, have also provided direct evidence for a genetic control of this anteroposterior compartmentation. In the case of L7/pcp-2, the mutation of a POU domain in the promoter region results in a restriction of the transgene expression to PCs in posterior lobules, while with a non-truncated promoter all PCs express the transgene. Similarly, the mutation of the engrailed promoter is followed by a restriction of β-galactosidase expression to posterior cerebellar lobules. Other studies have shown anteroposterior variations in the expression pattern of certain developmental genes, coding for transcription factors, by postmitotic cerebellar neurons. In mouse and chick, En-1, En-2, Wnt-7B, Gli and Otx-1 all have high levels of expression in neurons of the anterior cerebellum (Franzt et al., 1994, Millen et al., 1995; Millet and Alvarado-Mallart, 1995), whereas Otx-2 expression is almost restricted to neurons of the posterior domain (Franzt et al., 1994), with lobule VI as a limit. One of those genes could control the expression of the olivocerebellar anteroposterior guiding cues. Nevertheless, only En-2 anteroposterior differential expression is observed in the cerebellar neuroepithelium before the first postmitotic neurons are formed (and before the first olivary axons enter the cerebellum; Millet and Alvarado-Mallart, 1995).

We have shown that the inversion of the anteroposterior polarity of the cerebellar plate before the arrival of IO axons, is followed by an equivalent inversion of the olivocerebellar projection map. The embryonic cerebellum therefore contains directional cues that can be read by IO axons and that distinguish the posterior cerebellar lobules from the anterior ones. Similar observations have been made in the retinotectal projection. In non-mammalian vertebrates, the optic tectum receives axons from the retina stereotypically in such a way that the retinal temporonasal axis corresponds to the tectal rostrocaudal axis (reviewed by Udin and Fawcett, 1988; Holt and
the anterior entry point (arrow). These axons arborize within the anterior hemicerebellum. Thus, the entry points and the map are inverted. Scale bar, 400 μm in A and B.

Fig. 9. Olivocerebellar projection map in rotation type 1 experiments (10 DIV), anterograde axonal tracing. (A) The Dil tracer has been inserted in the caudolateral olive (arrowhead in the inset). Labelled axons ascend, forming a fascicle in the lateral aspect of the contralateral brainstem, and run at the medullo-cerebellar junction. These axons enter the cerebellum through the median entry point (arrow), bend caudally, desfasciculate and innervate the posterior half of the hemicerebellum. (B) In this case, Dil was positioned in the rostromedian olive (arrowhead in the inset). The labelled axons follow a similar pathway to A, but they ascend all along the fascicle at the medullo-cerebellar junction, entering the cerebellum through the anterior entry point (arrow). These axons arborize within the anterior hemicerebellum. Thus, the entry points and the map are inverted. Scale bar, 300 μm in A and B.

Harris, 1993; Goodman and Shatz, 1993). Using this system as a model, Sperry (1963) first proposed that growing axons find their appropriate target neurons because both expressed matching positional markers. This chemoaffinity hypothesis has been tested experimentally by studying the retinotectal projection following changes of the size and/or the orientation of the tectum. In adult goldfish (see references in Yoon, 1977 and Holt and Harris, 1993) and postmetamorphic Xenopus (Levine and Jacobson, 1974), the visual projection to pieces of tectum rotated along their anteroposterior axis shows a corresponding rotation. In addition, we found that olivary axons project to their correct cerebellar territory a few hours after they have entered the cerebellum in intact and in rotated explants. This demonstrates that the olivocerebellar projection does not emerge from an initially more diffuse projection, contrary to what has been reported for other systems such as the retinoculucellar projection of rodents (see Simon et al., 1994). This also extends previous findings, which indicated that in the rat embryo, the growth of olivocerebellar axons in the cerebellar primordium is initially organized along the mediolateral axis (Chédotal and Sotelo, 1992; Wassef et al. 1992b). The present results suggest that the cerebellum contains enough information to entrain the olivocerebellar map to the proper polarity. Therefore it reinforces Sperry’s hypothesis by extending its application to another projection system (see below).

One major question raised by the present observations has not yet been fully answered: which cerebellar cells bear the topographic cues? When IO axons invade the cerebellar plate, it contains glial cells and only three categories of postmitotic neurons, namely PCs, deep nuclear neurons and Golgi cells (Feirabend et al., 1985; Kanemitsu and Kobayashi, 1988). Although glial cells could be involved, and the present results do not invalidate this possibility, all the published data suggest that cerebellar neurons bear the cues for the positioning of olivocerebellar axons (see Sotelo and Wassef, 1991; Millen et al. 1995). Granule cell precursors are present in the external granular layer (EGL) which covers the cerebellar plate (Hanaway, 1967). As in vivo, IO axons do not contact the EGL before reaching PCs (Chédotal et al., 1996), therefore EGL neuroblasts are most probably not involved in the guidance of IO axons. On the other hand, although PCs (Sotelo and Wassef, 1991) and deep nuclear neurons (Hawkes et al., 1992) have both been suggested to be the organizer of the olivocerebellar projection, only PCs fulfill the theoretical prerequisites necessary to play such a key role. First, in adult rats, both the olivocerebellar projection map and the map resulting from PC biochemical heterogeneity are congruent (Gravel et al., 1987; Wassef et al., 1992c), and the latter map forms independently of olivocerebellar axons (Wassef et al., 1990). More importantly, during development, there are early independent and simultaneous processes of biochemical parcelling of the source (Wassef et al., 1992b) and target neurons (Wassef and Sotelo, 1984; Wassef et al., 1985), and this double compartmentation can match through their projection map (Wassef et al., 1992c). Finally, in the rat embryo at least, growing olivocerebellar axons do not enter the cerebellar parenchyma in a chaotic manner, but rather they immediately contact, through distinct entry points, correlated bands of migrating PCs (Chédotal and Sotelo, 1992). The results obtained in the present study with rotation type 2 indicate that even when olivocerebellar axons do not follow their normal pathway within the developing cerebellum, and are immediately confronted with PC dendrites and cell bodies upon their arrival in the cerebellar plate, they

Fig. 10. Illustration of the results of single anterior and single posterior cerebella experiments. Single posterior cerebella (A-C) received projections from both IO domains. A compressed map is established, with the rostromedian olivary neurons projecting to the most posterior region of the posterior cerebellum, and the caudolateral IO projecting to the most anterior region of the posterior cerebellar half. In single anterior cerebella (D-F), interspersed Dil and DiA-labelled neurons were found exclusively in the caudolateral IO, whereas no labelled neurons was observed in the rostromedian territory. Scale bar, 300 μm.
succeed in establishing a normal projection map. These results are also in favor of PCs, rather than deep nuclear neurons, being the cerebellar cells bearing positional information recognized by IO axons.

It is important to determine the mechanisms by which the cerebellum controls the positioning of olivary axons. We found that in a ‘no-choice’ situation, the behavior of IO axons originating from the rostromedial and caudodorsal domains differed. While single posterior cerebella were invaded by olivary axons originating from both domains, only axons from the caudomedial IO were growing in single anterior cerebella. This suggests that either the growth of axons from the rostromedial IO is promoted by molecules restricted to the posterior cerebellum, or that they are repelled by molecules expressed in the anterior cerebellum. The formation of compressed maps in single posterior cerebella could suggest that a molecule, repellent for olivary axons, is expressed in the cerebellum in a decreasing gradient from anterior to posterior. In single posterior cerebella, olivary axons would still be able to detect a higher concentration of the repellent gradient in the most anterior part of the posterior cerebellar half. Again, this situation parallels the one seen in the retinotectal system. Bonhoeffer and colleagues have established that, when given a choice, axons from the temporal retina grow preferentially on membrane carpets derived from anterior tectum rather than posterior, whereas nasal axons do not distinguish between the two types of membrane (Walter et al., 1994). They subsequently demonstrated that this preference was due to the presence on posterior tectal membrane of repellive factors and, more recently, they have fully characterized one candidate repellent (Drescher et al., 1995). Our study suggests that the anterior cerebellum, like the posterior tectum, might contain a repellive factor. It would be interesting to determine if the molecules, in particular the repellive ones, involved in the patterning of the retinotectal projection are also implicated in the formation of the olivocerebellar projection map. Within this line, it is important to recall that posterior tectum and anterior cerebellum are the two domains expressing the highest levels of the En-2 transcription factor, which appears to lie upstream of the molecules responsible for the acquisition of the antero-posterior polarity in the retinotectal system (Itasaki and Kawamura, 1996; Rétaux et al., 1996).

The results reported here reveal a faster and better ability of caudolateral olivary axons to project properly after cerebellar rotation. This axonal difference is not easy to explain; however, as a result of their new anatomical situation, caudolateral olivary axons are exposed to the presumptive repellive influence of the anterior cerebellum from their arrival to the medullo-cerebellar interface. This negative influence, in addition to an increase in the length of the pathway, might explain the obtained results. In any case, the observed modification of cerebellar entry points in type I rotation experiments strongly suggests that olivary axons respond to cues and affinities that are located within the cerebellum.

In conclusion, the present study strongly suggests that the cerebellum contains topographic cues which control the antero-posterior positioning of IO axons. Nevertheless, the olivocerebellar projection map is far more complex and fragmented (see Introduction), because IO axons must not only recognize their anteroposterior position in the cerebellum, but also the sagittal stripe in which they project. In this respect, we have recently shown that the homophilic cell-adhesion molecule BEN/SCI1/DM-GRASP is expressed by corresponding clusters of IO neurons and PC stripes (Chédotal et al., 1996). Therefore, the recognition of the stripe identity could rely on cell-cell adhesion.

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