Multiple influences on the migration of precerebellar neurons in the caudal medulla

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SUMMARY

Neurons destined to form several precerebellar nuclei are generated in the dorsal neuroepithelium (rhombic lip) of caudal hindbrain. They form two ventrally directed migratory streams, which behave differently. While neurons in the superficial migration migrate in a subpial position and cross the midline to settle into the contralateral hindbrain, neurons in the olivary migration travel deeper in the parenchyma and stop ipsilaterally against the floor plate. In the present study, we compared the behavior of the two neuronal populations in an organotypic culture system that preserves several aspects of their in vivo environment. Both migrations occurred in mouse hindbrain explants dissected at E11.5 even when the floor plate was ablated at the onset of the culture period, indicating that they could rely on dorsoventral cues already distributed in the neural tube. Nevertheless, the local constraints necessary for the superficial migration were more specific than for the olivary migration. Distinct chemoattractive and chemorespulsive signal were found to operate on the migrations. The floor plate exhibited a strong chemoattractive influence on both migrations, which deviated from their normal path in the direction of ectopic floor plate fragments. It was also found to produce a short-range stop signal and to induce inferior olive aggregation. The ventral neural tube was also found to inhibit or slow down the migration of olivary neurons. Interestingly, while ectopic sources of netrin were found to influence both migrations, this effect was locally modulated and affected differentially the successive phases of migration. Consistent with this observation, while neurons in the superficial migration expressed the Dcc-netrin receptor, the migrating olivary neurons did not express Dcc before they reached the midline. Our observations provide a clearer picture of the hierarchy of environmental cues that influence the morphogenesis of these precerebellar nuclei.

Key words: Precerebellar neuron, Tangential migration, Inferior olive, Floor plate, Netrin, Mouse

INTRODUCTION

Several neuronal populations are generated in the dorsal rhombencephalon, at the level of the rhombic lip. They migrate following distinct dorsoventral paths identified as the pontine, olivary and superficial migratory streams. They settle in the ventral neural tube and contribute neurons to several precerebellar nuclei. Therefore, the precebellar system permits the study of the cellular and molecular mechanisms involved in the control of cell migration. We have focused on two populations of precerebellar neurons generated in the rhombic lip of rhombomeres 7/8 (or 8) (Cambronero and Puelles, 2000) and destined to form the inferior olive (ION), the lateral reticular (LRN) and the external cuneatus (ECN) nuclei. These neurons circumnavigate at different depths around the medulla following two routes, both orthogonal to the anteroposterior axis (Harkmark, 1954; Altman and Bayer, 1987a; Altman and Bayer, 1987b; Bourrat and Sotelo, 1988; Bourrat and Sotelo, 1990; Bourrat et al., 1989; Tan and Le Douarin, 1991; Ambrosiani et al., 1996). The olivary migration follows a deep parenchymal pathway; the ION neurons stop migrating ipsilaterally and aggregate near the floor plate, whereas their axons cross the floor plate and travel towards the contralateral hemicerebellum where they project. The LRN and ECN neurons, which are generated slightly later, gather under the pial surface forming a superficial migration, which crosses the ventral midline to continue dorsalward on the contralateral side.

The floor plate is a complex ventral midline structure involved in neural tube patterning and in the guidance of both growth cone and cell body migration. The floor plate is the source of several short- and long-range chemoattractants and chemorepellents that are likely to govern both the dorsoventral orientation of the olivary and superficial migrations and their different behaviors at the ventral midline. In addition, it could provide olivary neurons with a stop signal preventing them from crossing the midline. Several diffusible and extracellular matrix molecules have been implicated as mediators of the different activities of the floor plate (Kennedy et al., 1994; Serafini et al., 1994; Klar et al., 1992; Echelard et al., 1993; Brose et al., 1999). Netrin-1, which is expressed in the floor plate at all axial levels (Kennedy et al., 1994) and contributes to the attractive action of the floor plate on growing axons (Tamada et al., 1995; Shirasaki et al., 1995; Shirasaki et al., 1998), has been implicated as a major chemoattractant for ION (Bloch-Gallego et al., 1999) and LRN neurons (Alcantara et al., 2000). In netrin-1 mutant mice the ION neurons are misplaced, their number is reduced and their cerebellar projection is abnormal (Bloch-Gallego et al., 1999). Retrograde tracing experiments suggest that the LRN neurons are less affected in netrin1 mutants (Bloch-Gallego et al., 1999). The developing ION and LRN neurons express several netrin receptors of the Dcc and

Unc-5 families (Ackerman et al., 1997; Bloch-Gallego et al., 1999), which is consistent with a direct role of netrin-1 on their dorsoventral migration.

In the present study we examined directly the influence of the local environment on the behavior of neurons of the olivary and superficial migrations, identified by Brn3.2 and TAG-1 expression, respectively. We compared their responses in bulbar explants when grafted in an ectopic location, to those ectopic fragments of midline structures or to sources of diffusible netrin-1 protein.

MATERIALS AND METHODS

Animals

OFI mice obtained from Iffa Credo (Lyon, France), were mated and the day of vaginal plug detection was considered as E0.5. Pregnant females were killed with an overdose of ether. Embryos, recovered in chilled PBS, were dissected for explantation or fixed overnight at 4°C in 4% paraformaldehyde in 0.12 M sodium phosphate buffer, pH 7.4 (PFA4%). Lightly ether-anaesthetized pregnant mice were injected in the tail vein with a solution of BrdU (Sigma; 2 mg/ml; 20 μg/g) in 0.9% NaCl on gestational days 10.5, 11.5 and 12.5 to identify neuronal birthdates. Embryos were fixed at E13.5, E14.5 or E18.5. DNA-incorporated BrdU was detected by immunocytochemistry.

Immunocytochemistry

Dissected hindbrains or explants were fixed overnight at 4°C in F4% for the detection of e-NCAM, L1 (gifts from Dr Schachner), vimentin (Amersham) and BrdU (Becton Dickinson). Immunocytochemistry was performed on vibratome (100 μm thick) or on cryostat (14 μm thick) sections of hindbrain or on whole explants. Sections or explants were incubated with the first antibody for 48 hours at 4°C, revealed with a biotinylated secondary antibody (1/300, Jackson) for 1 hour at room temperature followed by streptavidin-biotin peroxidase (1/400, Amersham) and visualized with diaminobenzidine and H2O2. For the detection of incorporated BrdU, vibratome sections or explants were treated with 2 N HCl for 45 minutes, and digested for 10 minutes with 20 μg/ml proteinase K. The sections were postfixed in PFA4% containing 0.2% glutaraldehyde and treated as above.

In situ hybridization

E11.5 to E15.5 embryos or explants at the end of the culture period were fixed overnight at 4°C in F4%. Transverse vibratome sections (200 μm thick) of albumin-gelatin embedded dissected hindbrains or fixed explants were dehydrated in methanol and stored at –20°C. In situ hybridization and double-colour in situ hybridization were performed on rehydrated tissue as described (Bally-Cuif and Wassef, 1994). The diluted anti-digoxigenin antibody was adsorbed overnight on a confluent

layer of EBNA-293 cells before being used in experiments involving COS or EBNA cells, as it was found to crossreact with an epitope present in variable amounts on these cells.

Probes

The mouse Brn3.2 template was generated by PCR from mouse genomic DNA based on the published sequence (Turner et al., 1994). A 524 bp fragment between nucleotides 548 and 1071 was amplified and subcloned in pBS. The entire coding region of rat TAG-1 cDNA (Furley et al., 1990) was provided by Dr T. Jessell. pBS plasmid containing sequences from the 3´ UTR region of mouse netrin was provided by Dr T. Serafini. Plasmids containing cDNA fragments of rat F-spondin (gift from Dr Klar), mouse PACAP (gift from Dr Waschek) EphA4, ephrin A5, ephrin B1 and B3 (gift from Dr Gilardi) and SemaF (gift from Dr Chedotal) were also used.

Culture procedures

Preparation of the explants

Explants containing the whole rhombencephalon including the cerebellum were dissected from E11.5 and E12.5 brains in PBS-0.6% glucose, and opened on the dorsal midline. They were cultured for 1-4 days ventricular side down on Biopore membranes (Millipore) floating on DMEM/F12 medium (Gibco) supplemented with antibiotics and with 10% fetal bovine serum and 5-10% horse serum. In some experiments the medium was supplemented with 500 ng/ml of purified chick netrin-1 (gift from Dr Tessier Lavigne) or with BrdU (10 mM, for 30 minutes).

Ablations and transplantations

Fragments of floor plate, rhombic lip or ganglionic eminence were ablated or dissected using tungsten needles and scalpel blades. Transplants were grafted at different levels onto hindbrain explants. Alternatively, hindbrain explants were cut transversally in the region of migration and a floor plate explant or a COS or EBNA-293 cell aggregate was placed against the cut edge. Fragments of E11.5 cerebral cortex or ganglionic eminence were inserted into the region of migration. The transplantation procedure is outlined on the side of each panel. Unless otherwise specified, the explants were cultured for 3-4 days in vitro, fixed overnight in PFA4%, and processed for whole-mount in situ hybridization or immunocytochemistry.

Sources of netrin protein

COS7 cells were transfected with pGNET1myc or pGNET2myc (using Lipofectamine, Gibco BRL) as described by Serafini et al. (Serafini et al., 1994), or using Fugene 6 (Boehringer), according to the manufacturer’s instructions. pTLmEn2m (Joliot et al., 1998) was used as an unrelated control

plasmid. An EBNA-293 cell line stably transfected with a netrin-1 expression plasmid and the control line transfected with the empty vector were also used. Aggregates of EBNA or transfected COS cells were prepared by the hanging drop method (Kennedy et al., 1994) and used as transplants in the same way as tissue fragments. Restricted sources of purified netrin were established in different ways. Affigel beads were rinsed in PBS and incubated overnight with a solution of netrin-1 (5 μg/ml in DMEM/F12) and used as transplants. Low-melting-point agarose in DMEM/F12 was mixed with a solution of purified chick netrin-1 (10 μg/ml final) and some was aspirated into gel-saver tips. After cooling, the blocks or tips were cut into smaller pieces, which were used as sources of netrin-1 protein. BSA was used instead of netrin in control experiments. As a biological test of netrin-like activity, the putative sources of netrin-1 were cocultured in collagen gels at a small distance from rhombic lip fragments and axonal outgrowth was examined at 24 and 48 hours.

RESULTS

The mouse superficial and olivary migrations

The relationship between the superficial and olivary migrations is best illustrated at E13.5, when both migrations have reached the ventral midline (Fig. 1A,B). TAG-1, which encodes a member of the immunoglobulin superfamily involved in axonal guidance (Dodd et al., 1988; Furley et al., 1990; Wolfer et al., 1994), is expressed in neurons of the superficial migration (Fig. 1B,E-G) whereas Brn3.2, which encodes a POU domain transcription factor (Turner et al., 1994), is expressed in the olivary migration (Fig. 1B,H-J). The superficial and olivary migrations are separated by an unlabeled gap on Brn3.2-TAG1 double-labeled sections (Fig. 1B). It should be noted that both markers also label other neuronal populations located more dorsally. Immature axons that travel in the superficial region of the caudal hindbrain could serve as substrate for both migrations (Fig. 2E). In vivo, they separate the olivary migration from the pial surface and from the superficial path described below, which is followed by the superficial migration. In the hindbrain, the floor plate consists of a sheet of specialized radial glia with tightly packed processes that abut the pial surface. In the region of migration, the floor plate seems to be built on piles: the glia fibers loosen and diverge from the midline near the pial surface, delimiting superficial galleries that cross beneath the floor plate (Fig. 1D) and are followed by the neurons of the superficial migration. In the E11.5 caudal hindbrain, TAG-1 mRNA was detected in the subventricular zone and in the hypoglossal nucleus (data not shown). At E12.5, a row of TAG-1 expressing (TAG-1+) cells emerges from the rhombic lip (Fig. 1E). At E13.5 this band, which corresponds to the superficial migration, is continuous across the midline in the caudal hindbrain (Fig. 1F). In addition, a discontinuous layer of superficial TAG-1+ cells was observed in the dorsal neural tube on flat mounts (not shown). At E15.5, while the hypoglossal nucleus was still

labeled, TAG-1 transcripts were no longer detected in the subventricular zone, the superficial migration or the ECN and LRN nuclei (Fig. 1F,G). Thus, in vivo, TAG-1 is expressed transiently in the neurons of the superficial migration and becomes downregulated as they settle into their final territory. At E12.5, Brn3.2 transcripts were expressed in a circumferential band of cells abutting the rhombic lip and located deeper in the parenchyma than the superficial migration (Fig. 1H). The Brn3.3 and TAG-1 positive cells occupied complementary domains in the subventricular layer. At E13.5, the Brn3.2 positive cells gathered in the ventral half of the neural tube (Fig. 1I), in a domain corresponding to the olivary migration (Altman and Bayer, 1987a; Turner et al., 1994), which avoided the floor plate. At E14.5 the Brn3.2 signal was concentrated in the ION (Fig. 1J). Some dorsally located neurons also expressed Brn3.2 at this stage. Later on, Brn 3.2 expression persisted only in the ION. TAG-1 and Brn3.2 were used as markers of the two migrations in subsequent experiments. BrdU injections were used to determine the birthdates of the neurons of the superficial and olivary migrations more precisely than was possible in earlier experiments (Taber Pierce, 1973) (data not shown). The bulk of olivary cells was generated at E10.5, with a smaller population labeled by BrdU injections at E11.5. The BrdU-labeled olivary neurons reached the ventral midline at E13.5. The peak of LRN and ECN neurons production occured 1 day later, at E11.5. BrdU injections at E12.5 also resulted in the labeling of some neurons in these nuclei. The BrdU-labeled neurons of the superficial migration reached the ventral hindbrain by E13.5.

The superficial and olivary migrations in bulbar explants

To examine whether the bulbar migrations could resume in vitro, explants containing the hindbrain including the cerebellum were dissected from E11.5 or E12.5 mouse embryos and cultured for 1-8 days, ventricular side down, in an open book configuration. A 30-minute pulse of BrdU at the beginning of the culture period was used to label the superficial migration. The explants were fixed after 1-3 days in vitro and treated in toto for BrdU immunocytochemistry. In this way, only the most superficial cells of the ventricular and pial surfaces were accessible to the antibodies. The BrdU-labeled cells, which were confined to the vicinity of the rhombic lip after 1 day in vitro (Fig. 2A), progressed towards the midline during the two following days (Fig. 2B-E). 3 days after explantation they formed a sharply delimited band continuous across the midline (Fig. 2C,E). Detection of BrdU or TAG-1 (Fig. 2D) transcripts revealed similar migration patterns on explants dissected at E11.5 (Fig. 2C,D) or E12.5 (Fig. 2F,G). Thus, the migration of cells of the superficial stream proceeded normally in vitro even if slightly slower than in vivo. Further maturation did not proceed in vitro: the band of TAG-1+ or BrdU+ cells did not disappear even after 8 days in culture (data not shown) and the explants maintained the same appearance as at 3 days in culture. In E11.5 explants, Brn3.2 was detected in two longitudinal dorsal stripes, unrelated to the ION, and extending along the whole

hindbrain. After 2 days in vitro, the ION was detected as an isolated medial patch of Brn3.2 expressing (Brn3.2+) cells in the caudal hindbrain. 1 day later, these cells reached the ventral midline, where they accumulated (Fig. 2H). In most explants, a faint labeling for Brn3.2 was also detected in the floor plate region (Fig. 2H). In transverse sections of the explants, the Brn 3.2+ ION was located more ventrally than in vivo and was fused on the midline under the floor plate (Fig. 2H,I). In explants dissected at E12.5 instead of E11.5 and maintained for 3 days in culture, Brn3.2 transcripts were detected in the two ION on both sides of the floor plate but never across (Fig. 2K,L). More commissural axons were detected on transverse sections of E12.5 explants immunostained for L1/Ng-CAM (Fig. 2M) compared to similar explants dissected at E11.5 (Fig. 2J). In addition, no gap was detected between the floor plate and the pial surface of the explant (compare Fig. 2J,M).

Origin of the TAG-1 and Brn3.2 neurons in the rhombic lip and their behavior at the floor plate

Unilateral ablation of the rhombic lip at the beginning of the culture, at E11.5 (n=8) or E12.5 (n=12), resulted in a sharp decrease in the number of dorsal TAG-1+ or BrdU neurons on the operated side (Fig. 3A,B). After 4 days in culture, TAG-1+ neurons that have crossed the midline were detected ventrally on the operated side (Fig. 3A,B). Because most olivary neurons were postmitotic at E11.5 they could have been already located ventrally at the time of explantation and have matured and developed Brn3.2 expression in situ. We sought to eliminate this possibility by extirpating the presumptive source of olivary neurons at the time of explantation. The rhombic lip was ablated unilaterally at E11.5. After 3 days in vitro, the size of the Brn3.2+ area was always decreased on the lesioned site (n=22). When the width of the ablations reached one third of the hindbrain (n=5, Fig. 2C) Brn3.2 expressing cells were no more detected ipsilaterally. Most Brn3.2+ neurons remained ipsilateral to the intact rhombic lip. Some Brn3.2+ cells migrated superficially across the floor plate and gathered beneath it. However, they did not migrate beyond its contralateral limit (Fig. 2C,D). When the rhombic lip was extirpated unilaterally at E12.5 (n=6), Brn3.2+ cells were always observed near the floor plate on both sides although the number of cells was smaller on the operated side (data not shown). This suggested that at E12.5 some olivary cells had already migrated close to the ventral midline and therefore were not eliminated by rhombic lip ablation. Taken together, these observations indicated that the superficial and olivary neurons migrated in vitro, although some alterations could be detected after 3 days in culture. Unless otherwise specified, in the experiments described below, ablations and transplantations were performed at E11.5 and the bulbar explants were cultured for 3 or 4 days before being fixed. The total number of transplanted or ablated explants was over 950. Slightly more than one third

were discarded because of contamination, inefficient transfection, high background staining or poor histochemical reaction, which were the main causes of variability in these experiments. In two thirds of the remaining explants, the experimental design was a variant of that described here, i.e. comparison of the effects of COS- netrin and floor plate on the same explant instead of COS-netrin and COS-control, graft of rosa 26 rhombic lip instead of wild type, etc. The numbers of explants that were treated in the experiments (n) are indicated.

Influence of the floor plate on the olivary and superficial migration

The floor plate was ablated in the region of migration at the beginning of the culture period (Fig. 4A-E). In some cases both sides of the explants fused together on the midline during the culture period. The completion of the ablation was checked using a rat F-spondin probe as a floor plate marker (n=9, Fig. 4A). In most cases (8/9), the Brn3.2+ cells reached the ventral midline but failed to form a central compact structure (Fig. 4A; compare with Fig. 2H). The TAG-1+ neurons also migrated to the ventral edge of the explant in the absence of a floor plate structure (3/3, arrow in Fig. 4E). These observations indicated that, at the onset of their migration, the superficial or olivary neurons do not any longer require the presence of a floor plate to reach the ventral neural tube.

Nevertheless, floor plate-derived signals could be essential in vivo to modulate the rate or direction of migration in different neuronal subpopulations. We therefore examined whether the superficial and olivary migrations could be still responsive to floor plate-derived signals. The chemotropic activity of floor plate fragments was tested on the migrating ION and superficial migration after ablation of the endogenous floor plate from the region of migration in E11.5 hindbrain explants. Floor plate fragments dissected either from the ablated floor plate (n=8, Fig. 4B) or from different anteroposterior levels (n=7+5, Fig. 4C, spinal cord) were inserted perpendicularly to the ventral midline. Both sides of the ectopic floor plates (Fig. 4B,C) were lined with Brn3.2+ cells deviating from their normal trajectory.

At some distance from the ectopic floor plate as well as on the contralateral side, a population of Brn3.2+ cells reached the ventral midline (arrow, Fig. 4B). Brn3.2 (n=7, 7/7, Fig. 4D) and TAG-1 (n=3, 3/3, Fig. 4E) cells accumulated on one side of a floor plate fragment placed against the edge of hindbrain explants cut in the region of migration. This demonstrated that, in previous experiments, the floor plate did not act as an obstacle to the migration but that the migrating cells were indeed deflected from their normal trajectory. Brn3.2+ cells did not accumulate around unrelated neural fragments grafted in the region of migration (cerebral cortex or ganglionic eminence, data not shown). These observations indicated that ectopic floor plate grafts, dissected from hindbrain or spinal cord, attracted both the olivary and superficial neurons.

The signals necessary for olivary migration are widely distributed in the caudal brain

To examine the role of intervening structures in the migration of olivary neurons, rhombic lip fragments were dissected from the caudal hindbrain and transplanted in various ectopic sites: in the most dorsal part of the spinal cord (n=4, 4/4, Fig. 4F) or in the pontine rhombic lip (n=4, 3/4, Fig. 4G). The endogenous floor plate was left in place. After 3 days in culture, a group of Brn3.2+ cells was detected close to the endogenous floor plate ipsilaterally to the grafted rhombic lip. No Brn3.2 staining was detected near the floor plate on the contralateral side. Thus, although the migration domain of olivary cells is delimited in vivo as well as in control explants, these cells were capable of migrating in ectopic environments either in the rhombencephalon or in the spinal cord. In addition, the Brn3.2+ cells always remained ipsilateral to the rhombic lip grafts (Fig. 5C). In contrast, no ectopic TAG-1 positive cells were produced by similar grafts.

Signals from the ventral neural tube and floor plate impair the progression of the olivary neurons

To test the possibility that the floor plate provides a stop signal to the migrating olivary neurons, we grafted fragments of caudal rhombencephalon comprising the floor plate at various distances from the rhombic lip in bulbar explants from which the endogenous floor plate had been eliminated (Fig. 4H,I). After 3 days in culture, the Brn3.2+ cells stopped at the level of an ectopic floor plate placed at short distance from the rhombic lip (n=5, 5/5, arrowhead in Fig. 4H) without crossing it, and therefore without reaching the ventral midline. When the distance between the floor plate and the rhombic lip was increased by placing the floor plate with additional ventral tissue further away from the ventral midline (n=4, 4/4, Fig. 4I), the Brn3.2+ cells crossed the endogenous ventral midline into the adjacent tissue and reached the floor plate (arrowhead in Fig. 4I), where they stopped. In this latter type of graft, the Brn3.2+ cells did not aggregate or cluster against the floor plate as they do in all other types of floor plate grafting, indicating the presence of ventral signals that modify their behavior. Together with the results described above, these observations indicate that the floor plate not only attracts olivary cells during migration but also provides them with a short-range stop signal. Signals from the ventral neural tube could also contribute to slowing down migration as the olivary neurons reach their final destination.

Chemoattractive and repulsive candidates that could mediate the ventral influences on the migrations

Netrin-1 and its Dcc and Unc-5 family receptors are expressed in the caudal hindbrain during the period of migration (Bloch Gallego et al., 1999; Alcantara et al., 2000). We examined whether the pattern of expression of netrin-1 and Dcc was maintained in hindbrain explants. In E11.5 hindbrain

explants maintained for 3 days in vitro, netrin-1 was expressed in the medial region of the explants with a peak of expression at the floor plate, and another more laterally (Fig. 5B), which was consistent with their in vivo pattern (Fig. 5A). In E12.5 explants maintained for 2 days in vitro, Dcc was expressed in a thin medial stripe at the level of the ION and, more laterally, in neurons of the superficial migration (Fig. 5F). This pattern is consistent with the in vivo expression of Dcc in the medial olive and the superficial migration at E13.5 (Fig. 5E). EphA4 was expressed in the olivary migration at E13.5 (Fig. 5C). At the same stage, EphA4 ligands were expressed both in the floor plate (ephrin B3, Fig. 5D; ephrin B1, similar pattern, not shown) and surrounding the olivary migration (ephrin A5, Fig. 5G,H). An inhibitory ensheathing could also be provided by cells expressing Sema4C (Fig. 5I). The superficial migration neurons expressed high levels of the pituitary adenylyl cyclase activating peptide (PACAP), which could modulate various aspects of their intracellular signalling.

Influence of netrin on the olivary and superficial migrations

The influence of ubiquitous or local overexpression of netrin- 1 on the migration of olivary and superficial neurons was examined. E11.5 hindbrain explants were incubated for 30 minutes in culture medium containing (or not) purified chick netrin-1 protein (500 ng/ml), a concentration that elicited a robust axonal outgrowth from rhombic lip explants (compare Fig. 6A and B). After 3 days in culture, the shape of the ION was more regular and compact near the floor plate in netrin- treated explants (n=7,5/7, Fig. 6D) compared to control explants (n=6, Fig. 6C).

Aggregates of COS cells transfected with chick netrin-1 expression plasmids (COS-netrin) or with an unrelated plasmid (ctrl) were used to establish local sources of netrin protein or as controls. COS-netrin aggregates were tested for the production of netrin-1-like activity by evaluating their attractive effect on rhombic lip axons in collagen gels (Fig. 6E). Placed against the edge of bulbar explants, COS-netrin aggregates attracted a population of Brn3.2+ neurons (n=8, 6/8 COS-netrin, 2/8 COS-ctrl). These were restricted to the dorsal neural tube and accumulated at the interface between the COS cell aggregate and neural tube (Fig. 6G). Brn3.2+ cells did not accumulate around small COS-netrin aggregates inserted more ventrally into the migration pathway (not shown). Similarly, local sources of purified netrin protein either adsorbed on affigel beads or mixed with low-melting-point agarose did not deflect the Brn3.2 migration (not shown). TAG-1+ cells accumulated against COS-netrin aggregates and migrated diffusely over their surface (n=3, 3/3, Fig. 6H,I). It was more difficult to interprete the consequence of netrin overexpression more ventrally as insertion of COS aggregates perturbed the migration of TAG-1+ cells. Similar results were obtained with aggregates of a netrin-secreting permanent cell line (EBNA- net). Compared to that of ectopic floor plates, the attractive effect of COS-netrin cells was more restricted to the dorsal neural tube.

DISCUSSION

Previous studies of the olivary and superficial migrations (Bourrat and Sotelo, 1988; Bourrat and Sotelo, 1990; Ono and Kawamura, 1989; Bloch-Gallego et al., 1999) dealt with a later stage of development and started when these neurons had reached the ventral medulla oblongata and begun axonogenesis. The aim of the present study was to understand the factors that influence an earlier step, during which these neurons migrate from their dorsal site of generation to reach the ventral aspect of the neural tube. We found that both the superficial and olivary migrations occurred in an in vitro explant system that maintained several features of their normal environment and allowed for their manipulation.

Influence of the local environment on the olivary migration

Floor-plate ablations did not prevent the migration of olivary neurons towards the midline, showing that local cues that could guide dorsoventral migration were already distributed in the neural tube at E11.5. The presence of a repulsive activity in the rhombic lip was unlikely because many olivary neurons succeeded in reaching the ventral midline in experiments where dorsal neural tube ablations were too narrow. Olivary neurons migrated out of rhombic lip fragments transplanted at more anterior levels of the hindbrain or more posteriorly in the spinal cord, indicating that their migration is not dependent upon caudal hindbrain specific cues. At this stage of development, signals capable of maintaining the motility of olivary neurons or providing them with a permissive substrate are widely distributed along the caudal neural tube. However, these cues are not ubiquitous, as Brn3.2 neurons failed to migrate from rhombic lip explants cocultured with cerebral cortex tissue or in collagen gel or matrigel (I. dD. and M. W., unpublished observation).

Inhibitory signals to olivary migration in the ventral neural tube

Olivary neurons migrating in the spinal cord or the pons always ended as a tight cluster against the floor plate. This contrasts with the diffuse and wider distribution of olivary neurons in the caudal hindbrain. This behavior is even enhanced when supernumerary ventral tissue is inserted in the olivary migration pathway, as illustrated in Fig. 4I. This suggests that cues present in the ventral domain either inhibit olivary migration or interfere with cell-cell adhesion modifications that are necessary for their condensation into distinct nuclei. Several candidate molecules that could mediate the inhibitory behavior of the ventral neural tube on olivary migration have been described. We have observed that ephrin A4 is expressed in the inferior olive at E13.5, whereas several putative ligands are expressed around the olivary migration (ephrin A5), in the floor plate (ephrin B1 and B3) or slightly later between the olivary lamellae (ephrin A5 at E15). The ventral part of mouse rh7/8 contains a large patch of Sema3A expressing cells (Sem D) (Varela-Echavarria et al.,

1997) and several small patches of neurons expressing Sema4C line the olivary migration. The olivary neurons, on the other hand, have been reported to express low levels of the neuropilin 2 semaphorin receptor (Chen et al., 1997) but not neuropilin 1, a selective receptor of Sema3 family members (Kawakami et al., 1996). This complex, though still lacunary, network of inhibitory influences is probably deployed in order to delimit a wide mediolateral outline of the mature ION where the olivary migration settles and begin the complex rearrangements that accompany the morphogenesis of the characteristic ION subdivisions (Bourrat and Sotelo, 1991; Wassef et al., 1992).

The floor plate provides a stop signal to olivary neurons

Two known properties of the floor plate could account for the behavior of olivary neurons whose cell bodies stop at the floor plate, in contrast to their axons, which cross it. On the one hand, the tightly packed radial glia of the midline could behave more like a mechanical obstacle to cell bodies than to axons. Nevertheless, in E11.5 explants, the olivary cells reach the pathway of the superficial migration and find their way beneath and across the floor plate, possibly as a result of the elimination of longitudinal axon tracts in the explants. These neurons are not capable, however, of pursuing their migration on the other side. The floor plate has been shown to act as a switch that changes the localization of adhesion molecules and guidance receptors from internal pools to the cell surface, and vice versa, as growth cones cross the midline (Dodd et al., 1988; Stein and Tessier-Lavigne, 2001). As the olivary neurons cross beneath the floor plate they could similarly receive a short range signal from the floor plate preventing them from migrating contralaterally.

Influence of netrin on the olivary migration

The influence of netrin-1 on the olivary migration was tested in various ways. Purified netrin was diluted in the culture medium or diffused from focal sources; aggregates of netrin- transfected COS cells or a netrin-secreting EBNA-293 permanent cell line served as netrin sources. Netrin had two clear influences: it attracted a restricted subpopulation of Brn3.2-expressing neurons from the dorsal neural tube and, when provided in the culture medium, facilitated migration, resulting in a smoother and more regular outline of the ION nucleus near the floor plate. On the other hand, netrin sources did not mimick floor plate fragments that attracted and stopped Brn3.2 neurons when inserted into their pathway or placed against the edge of hindbrain explants cut in the region of migration. Compared with the phenotype of netrin mutants, the modest influence of exogenous netrin on the in vitro olivary migration is puzzling. We have observed that dorsoventral migration proceeds normally in vitro when the floor plate is ablated at the onset of migration, suggesting that, in the absence of a high ventral source of netrin, the olivary migration relies on the dorsoventral distribution of hindbrain

cues. In netrin mutants, besides the lack of a ventral source of netrin, the dorsoventral organization of the neural tube is probably disturbed before the onset of olivary migration, which could explain the ectopic distribution of clusters of olivary neurons around their normal path. In addition, the direct influence of netrin on migrating olivary neurons could be restricted in time. Dcc transcripts are detected at both ends of the migration but not in migrating olivary neurons. At one end, premigratory olivary neurons in transit in the subventricular zone probably transiently express Dcc transcripts. At the other end, at E13.5, the medial subset of olivary neurons that abuts against the floor plate contains Dcc transcripts. The persistence of the DCC protein in the migrating ION neurons is unknown. COS-netrin cells were only active when placed near the dorsal neural tube, suggesting that the DCC protein is short-lived. On the other hand, we find that addition of soluble netrin to the medium globally improves the migration on ION neurons in vitro, especially in the ventral domain. In these experiments, netrin could influence both the early and late phases of olivary migration, allowing ION neurons to reach the ventral domain before the complete downregulation of DCC protein expression. At subsequent stages of development, Dcc becomes widely expressed in the ION (Bloch-Gallego et al., 1999) and could affect axonal pathfinding.

Influence of the local environment on the superficial migrations

As observed for olivary migration, floor plate ablation did not prevent the superficial migration from reaching the ventral hindbrain. Similarly, rat commissural axons still project ventrally in the absence of floor plate (Placzeck et al., 1990). In contrast to olivary migration, however, establishment of the superficial migration was constrained by the continuity of structural elements, as it did not cross the limit between grafted and host tissue in homo- or heterotopic rhombic lip transplantation experiments. We have recently observed (Kyriakopoulou et al., 2002) that TAG-1 homophilic binding is essential for the migration of superficial neurons in explants. It is likely, however, that other environmental cues are important for the superficial migration. Whereas we find that TAG-1+ cells from the explants are attracted by COS-netrin aggregates and we could confirm that netrin-1 elicited a robust outgrowth of TAG-1+ axons from E11.5 or E12.5 caudal rhombic lip explants cultured in matrigel or collagen gels (Alcantara et al., 2000), very few cells migrated on these axons during the first 3 days in culture. In several instances, glial cells were found to provide a blueprint prefiguring neuron migration pathways: astrocytic sheathing wraps the migrating olfatory neuron precursors (Lois et al., 1996) and loose glial processes prefigure the pontine migration pathway (Ono and Kawamura, 1990). Glial end-feet tunnels, similar to those observed beneath the floor plate, could be a prerequisite for the superficial migration. Meninges, which are removed at the onset of migration, could also be a source of signals, as they are for external granule cells (Ma et al., 1998). We find that TAG-1+ cells prematurely stop migration after 2 days in vitro in our explant system.

Influence of netrin on the superficial migration

We observed that netrin attracts the TAG-1 expressing cells of the superficial migration and that these neurons express Dcc. It was therefore unexpected that the LRN apparently forms and projects normally to the cerebellum in netrin-1 mutants (Bloch- Gallego et al., 1999). We observed that PACAP is expressed at high levels in the superficial migration, whereas the PAC1 receptor has been reported to be expressed ubiquitously in the hindbrain (Washek et al., 1998). A high cytoplasmic concentration of cAMP could increase the sensitivity of the neurons of the superficial migration to a residual netrin signal present in the mutants, or to signalling by another Dcc ligand or cofactor (Ming et al., 1997). Similar to the neurons of the superficial migration, the pontine neurons respond to netrin signals from the onset of their migration but are nevertheless able to reach the ventral pons in netrin mutants (Yee et al., 1999). The present study, through the use of an organotypic system that enabled direct manipulation of the olivary and superficial migrations of the caudal hinbrain, has assessed more precisely the relative importance of structures and molecular signals that influence the migration of these population of precerebellar neurons.

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Figures

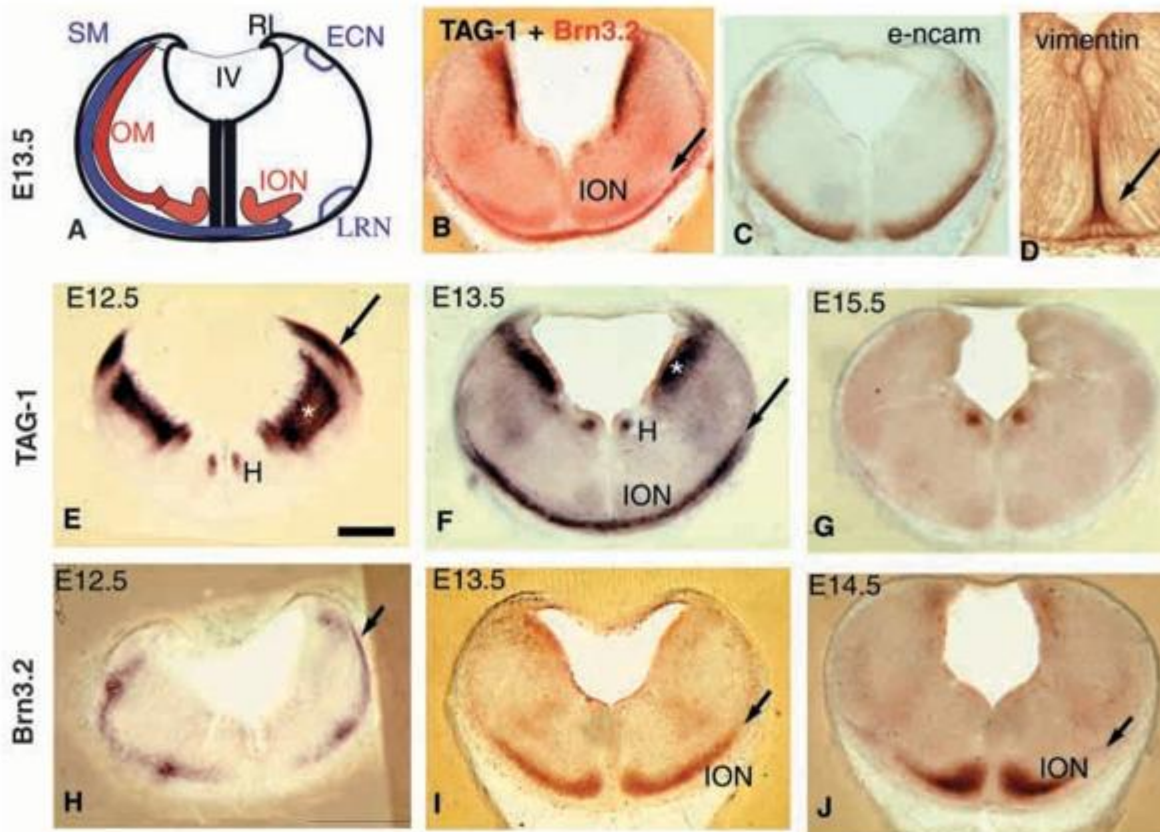
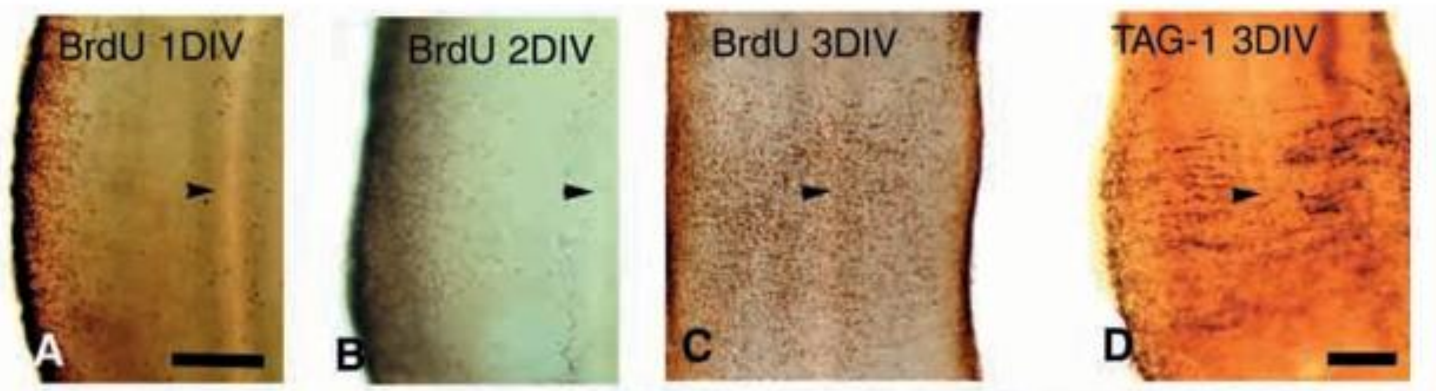
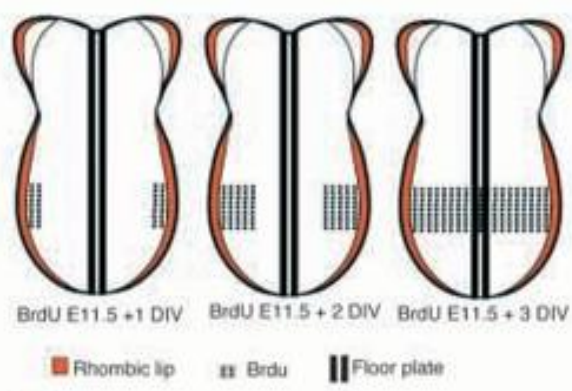
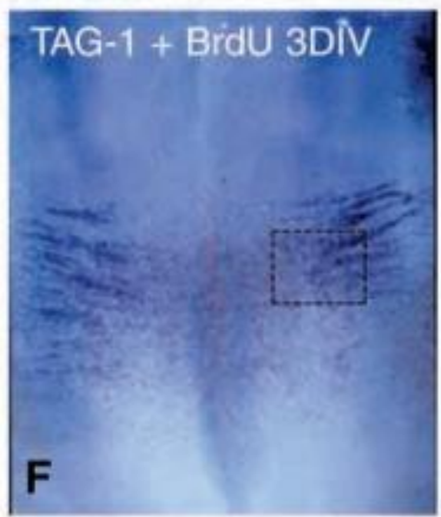
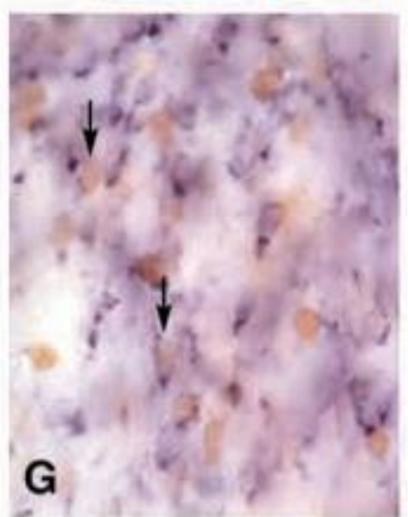


Figure 1. **The superficial and olivary migrations**. (A) Schematic representation of a transverse section through the caudal hindbrain. The olivary migrations (OM) gives rise to the ipsilateral inferior olive (ION), whereas the neurons of the superficial migration (SM) form the contralateral lateral reticular (LRN) and external cuneatus (ECN) nuclei. (B-D) Transverse sections of the caudal hindbrain of E13.5 embryos. (B) A gap separates the superficial (arrow) and olivary (ION) migrations on sections double labeled for TAG-1 (brown) and Brn3.2 (red) transcripts. (C) Underlying the olivary migration is a superficial sheet of axons that express and embryonic form of NCAM (e-ncam). (D) Vimentin antibody labels the specialized radial glia that constitutes the floor plate. This tight bundle loosens when it reaches the pial surface, delimiting a superficial path (arrow). (E-J) Transverse sections of the caudal hindbrain treated for the detection of TAG-1 (E-G) or Brn3.2 (H-J). (E-G) Between E11.5 and E14.5, TAG-1 marks the superficial migration (arrows), the hypoglossal nucleus (H) and the subventricular zone (asterisks in E,F). The neurons of the superficial migration reach the midline at E13.5 (F). By E15.5 (G), TAG-1 expression is downregulated. (H-J) Brn3.2 is expressed in the neurons of the olivary migration (arrows) beginning from E12.5 (H), and more faintly in other neuronal populations of the dorsal hindbrain. Notice that the stream of Brn3.2 neurons seems to originate in a gap in the subventricular expression of TAG-1. The olivary neurons reach the midline at E13.5 (I). Brn3.2 expression in the ION persists at later stages (J). Bar, 300 μm.


BrdU 1DIV
BrdU 2DIV
BrdU 3DIV
TAG-1 3DIV
A
B
C
D


BrdU E11.5 +1 DIV
BrdU E11.5 + 2 DIV
BrdU E11.5 + 3 DIV
Rhombic lip
Brdu
Floor plate
E


TAG-1 + BrdU 3DIV
F


G

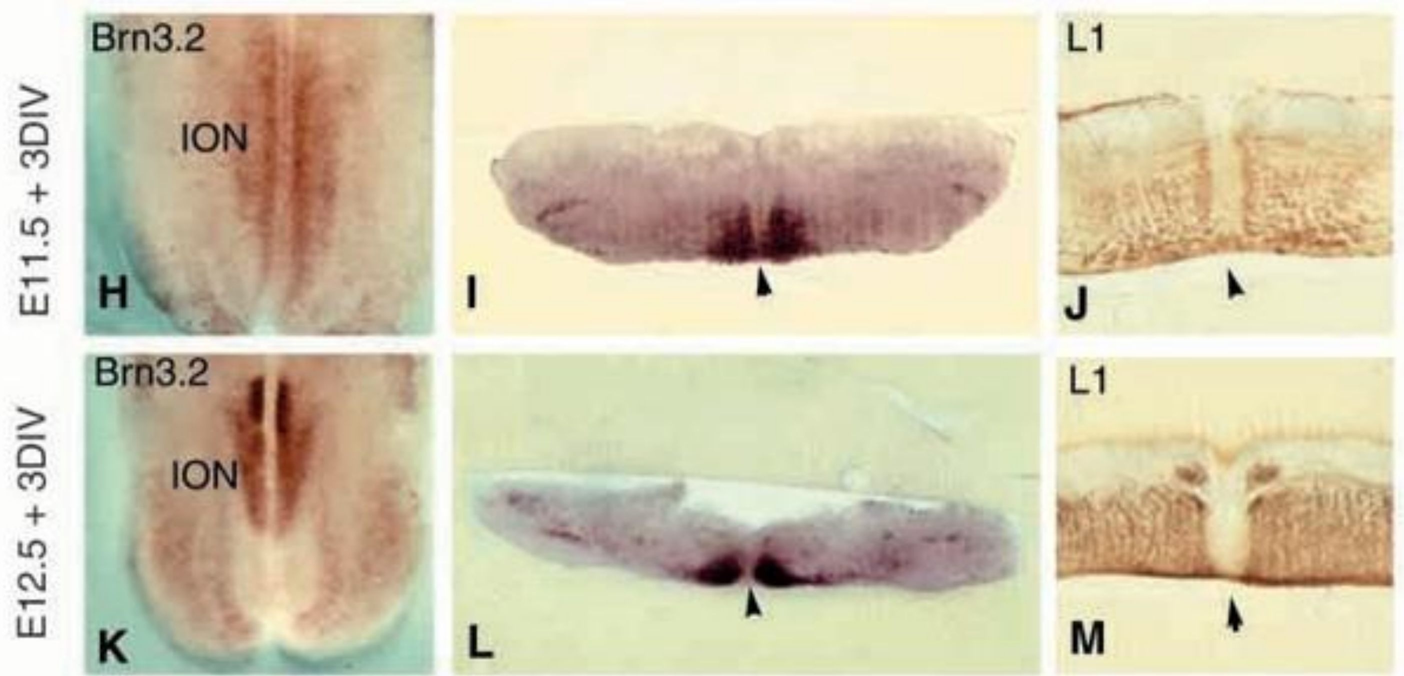

E11.5 + 3DIV
E12.5 + 3DIV
Brn3.2
ION
L1
H
I
J
K
L
M

Figure 2. **The superficial and olivary migrations in vitro**. (A-C) BrdU immunostained bulbar explants viewed from the pial surface. The explants incubated for 30 minutes with BrdU at E11.5 were analyzed after increasing time intervals in culture. The BrdU-labeled cells progress from their origin in the rhombic lip towards the floor plate (arrowheads) as schematized in (E). (D) The neurons of the superficial migration express TAG-1 in vitro. (F-G) E12.5 explants incubated for 30 minutes with BrdU maintained in culture for 3 days. TAG-1 transcripts are detected in blue and BrdU immunoreactivity is shown in brown. (F) The TAG-1-labeled cells form thin rows dorsally and arrange in a more diffuse pattern around the ventral midline. (G) Higher magnification of the area framed in F. The arrows point to TAG-1-labeled cells, which have incorporated BrdU at the beginning of the culture period. (H-M) Whole mounts (H,K) and transverse vibratome sections (I,J,L,M) of bulbar explants dissected at E11.5 (H-J) or E12.5 (K-M) and cultured for 3 days in vitro. Except in (J,M), where axons are labeled by immunostaining for L1, the brown label marks the olivary neurons detected by in situ hybridization of Brn3.2 transcripts. In E12.5 explants (K,L), as in vivo, the inferior olives on both sides are separated by the floor plate whereas they fuse ventrally in E11.5 explants (H,I). This behavior fits in with the presence of a ventral gap at the pial end of the floor plate in E11.5 (J) but not in E12.5 (M) explants. Arrowheads, floor plate.

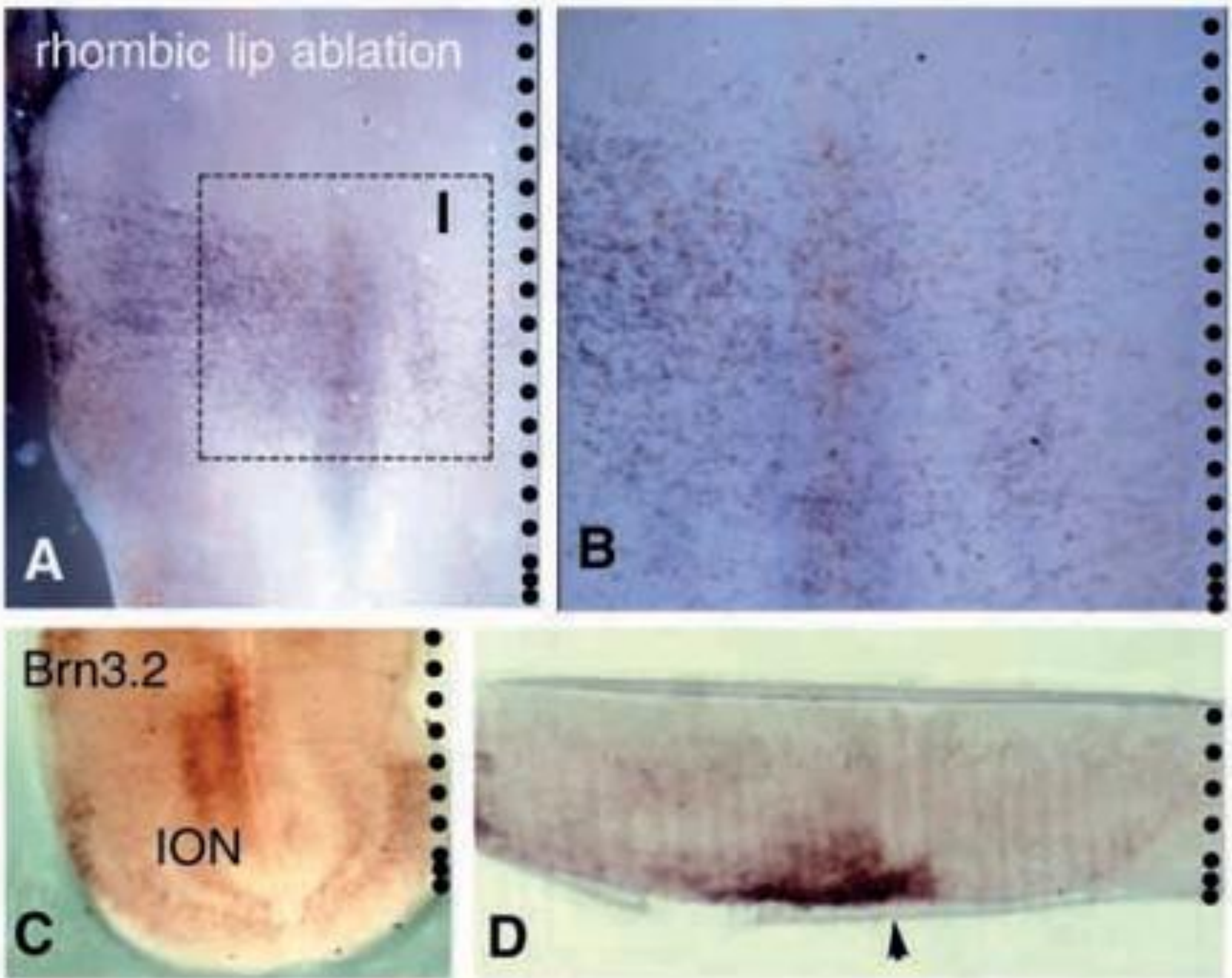


Figure 3. **Consequences of unilateral rhombic lip ablations**. (A,B) Explant in which the left rhombic lip was extirpated at the time of explantation (indicated by dots on the right side of the panels). (B) Higher magnification of the area outlined in (A). Some TAG-1- and BrdU-labeled cells have crossed the floor plate. (C,D) Ablation of the left rhombic lip (dots on the right side of the panels) in E11.5 bulbar explants prevents the formation of the ipsilateral inferior olive, indicating that these neurons were located in the dorsal neural plate at the onset of the culture. (D) Vibratome section through the explant illustrated in (C). The olivary neurons cross beneath the floor plate but stop at its contralateral limit. ION, inferior olive; arrowheads, floor plate.

Rhombic lip TAG-1

Rhombic lip Brn 3.2

Floor plate

migrating neurons

A B C D E

F G H I

RL

Figure 4. **Influence of the floor plate on the olivary and superficial migrations in vitro**. All panels except (E) show bulbar explants dissected at E11.5, cultured for 3 or 4 days, treated for the detection of Brn3.2 transcripts, and viewed from their pial surface. (E) Similar explant dissected at E12.5, cultured for 3 days, and treated for the detection of TAG-1 transcripts. The drawings schematize additional manipulations. The caudal part of the endogenous floor plate was ablated in most cases and both sides of the explants have fused together. (A) Ablation of the floor plate marked in red (F-spondin transcripts) did not prevent the migration of olivary neurons (arrow) towards the midline. (B-D) The olivary neurons accumulate along floor plate transplants dissected from the medulla oblongata (B,D) or the spinal cord (C) of E11.5 embryos. The migration of olivary (D) and superficial (E) neurons is deflected towards a floor plate apposed to the sectioned caudal hindbrain. The inset in E illustrates a higher magnification of the ectopic TAG-1 migration. (F,G) Caudal rhombic lip fragments were transplanted posteriorly (F) or anteriorly (G). They produced Brn3.2+ neurons, which migrated in the ectopic environment (F, spinal cord and G, pons) and accumulated ipsilaterally against the endogeous floor plate. (H,I) Grafts containing floor plates (delineated with dashed lines) were placed at different distances from the rhombic lip (RL). The endogenous midline (asterisk) fused with the additional bulbar tissue. The midline was crossed by olivary neurons but they did not migrate beyond an ectopic floor plate placed closer (H) or farther (I) than the endogenous one. Arrowheads point to the grafted floor plates and arrows to the ectopic ION. Bars, 500 μm (A-E);300 μm (F-I).

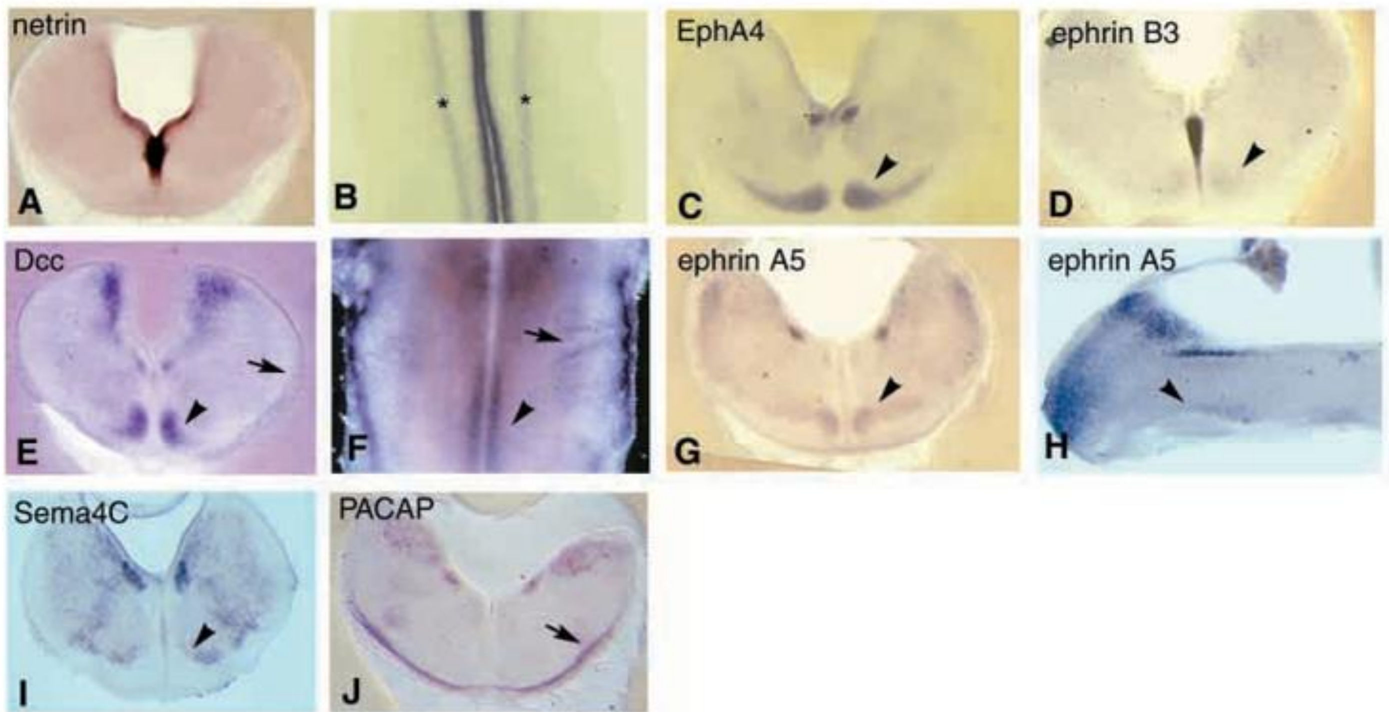


Figure 5. **Chemoattractive and repulsive signalling around the olivary and superficial migrations**. Coronal (A,C,D,E,G,I,J) or sagittal (H) sections through the E13.5 caudal hindbrain. (B,F) E11.5 (B) and E12.5 (F) hindbrain explants cultured for 3 and 2 days, respectively, in vitro. (A,B) Netrin-1 midline expression is maintained in E11.5 explants (B) and is similar to its in vivo pattern (A). (E,F) The Dcc netrin receptor is expressed both in vivo (E) and in vitro (F) in the medialmost olivary neurons (arrowheads) and in migrating neurons of the superficial migration (arrows). (C,D,G,H) EphA4 is expressed in the olivary neurons at the end of migration (C) whereas several EphA4 ligands are expressed in the floor plate (D, ephrin B3, ephrin B1 not illustrated) or surround the forming olivary nucleus (G,H, ephrin A5). The inhibitory influence of Sema4C expressing cells (I) could also funnel the olivary migration into its proper pathway. (J) The superficial migration expresses high levels of PACAP transcripts. The arrowheads point to the olivary migration, the arrows to the superficial migration. Asterisks in B, lines of higher netrin expression.

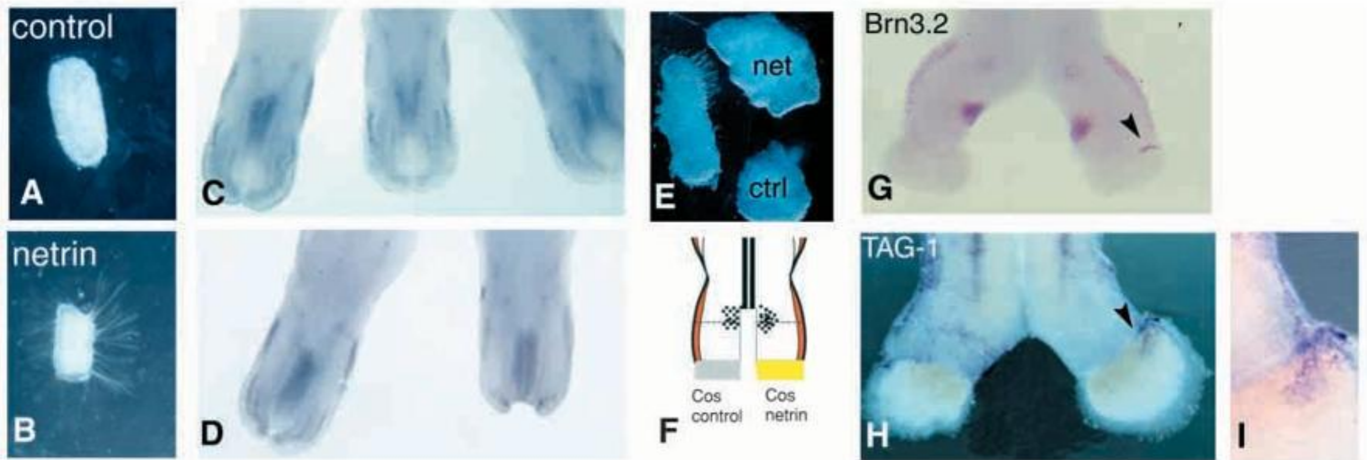


Figure 6. Influence of netrin on the olivary and superficial migrations. (A-D) Influence of 500 ng/ml purified netrin-1 on hindbrain explants. Netrin promoted profuse axonal outgrowth from rhombic lip fragments grown in collagen (B) compared to control explants (A). The shape of the ION was more regular and more neurons reached the ventral neural tube in hindbrain explants treated with purified netrin (D) compared to untreated control explants (C). (E) E11.5 rhombic lip explant cocultured in collagen gel with netrin (net)-transfected COS cells aggregates (COS-netrin) and control (ctrl) aggregates (En-2-transfected COS cell, COS C). Axons extend from the rhombic lip in the direction of Cos- netrin. Cell migration from the rhombic lip explants is negligible and is not observed before 3 days in culture (data not shown). (F-I) Transplantation experiments similar to those illustrated in Fig. 4D,E. Netrin-transfected (arrowheads) and control COS cells aggregates were used instead of floor plates. Netrin attracts a population of Brn3.2+ (G) and TAG-1+ (H) cells from the dorsal neural tube. I is a detail of H, illustrating the TAG-1+ cells that migrate on the surface of the COS-netrin aggregate.