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The migration of cerebellar rhombic lip derivatives

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SUMMARY

We have used cell labelling, co-culture and time-lapse confocal microscopy to investigate tangential neuronal migration from the rhombic lip. Cerebellar rhombic lip derivatives demonstrate a temporal organisation with respect to their morphology and response to migration cues. Early born cells, which migrate into ventral rhombomere 1, have a single long leading process that turns at the midline and becomes an axon. Later born granule cell precursors also migrate ventrally but halt at the lateral edge of the cerebellum, correlating with a loss of sensitivity to netrin 1 and expression of *Robo2*. The rhombic lip and ventral midline express *Slit2* and both early and late migrants are repelled by sources of Slit2 in co-culture. These studies reveal an intimate relationship between birthdate, response to migration cues and neuronal fate in an identified population of migratory cells. The use of axons in navigating cell movement suggests that tangential migration is an elaboration of the normal process of axon extension.

Movies available on-line

Key words: Chick, netrin, slit, Robo, GFP, Time-lapse confocal microscopy

INTRODUCTION

The orderly dispersal of cells from their origin at the ventricular surface of the neural tube is crucial in determining the organisation of the overlying mantle of the brain. Where radial neuronal migration predominates, the diversity of neurones in the mantle directly reflects the temporal and regional organisation of the underlying precursor pool, exemplified in various laminar structures such as the mammalian cortex. Tangential migration – the active dispersal of neurones perpendicular to the radial axis – results in the mixing of cells from different ventricular origins. Because of the technical difficulties in tracing cell movement from a defined ventricular source to a specific target, the scale and significance of tangential migration are largely unknown. Nevertheless, examples of tangential migration point to its importance in both the formation of specific brain structures such as the olfactory bulb (Wichterle et al., 1997) and the recruitment of specific neuronal subtypes to a given brain region, for example GABAergic interneurones in the dorsal forebrain (Anderson et al., 1997; Anderson et al., 2001; Zhu et al., 1999). To understand better the mechanism of tangential migration and its role in generating neuronal diversity we have examined in detail the origin of migratory cells from a defined axial segment of the embryonic rhombic lip.

The rhombic lip, which comprises the interface between dorsal neuroepithelium and the roofplate of the fourth ventricle, is the source of several migratory populations of neurones and migratory cerebellar granule cell precursors (reviewed by Wingate, 2001). The former contribute to the various precerebellar nuclei of the hindbrain (Harkmark, 1954; Rodriguez and Dymecki, 2000), while the latter condense as the external germinal layer (EGL) of the cerebellum (Alder et al., 1996; Wingate and Hatten, 1999). Migrants emerge as the early overt segmentation of the hindbrain disappears. Different neuronal populations arise from different axial levels of the rhombencephalon. In chick, rhombomeres (r)2-8 give rise to the medial pontine nucleus (Marín and Puelles, 1995; Rodriguez and Dymecki, 2000; Yee et al., 1999) which projects to granule cells. The inferior olive, which projects to Purkinje cells, is derived from r8 and anterior spinal cord (Cambronero and Puelles, 2000). Migratory cerebellar granule cell precursors are generated exclusively from the most anterior hindbrain segment, r1, alongside a small group of ventrally migrating neurones destined for nuclei within the lateral pontine region (Köster and Fraser, 2001; Wingate and Hatten, 1999). Within r1, the cues that organise the different migration paths of these rhombic lip derivatives are unknown.

The molecular cues that might regulate migration from the rhombic lip have received attention in several recent studies that suggest a close relationship between axon guidance and cell migration. For caudal rhombic lip derivatives (r2-8), the ventral midline secreted protein netrin 1 (Serafini et al., 1994) is both a demonstrable chemoattractant (Alcántara et al., 2000; Bloch-Gallego et al., 1999; Yee et al., 1999) and is necessary in vivo for the correct formation of precerebellar nuclei (Bloch-Gallego et al., 1999). By comparison, the role of netrin 1 in regulating granule cell migration from the cerebellar rhombic

lip is unclear: co-culture studies suggest that granule cell precursors are insensitive to netrin 1 (Alcántara et al., 2000), despite expressing the netrin receptors DCC and *Unc5H3* (Engelkamp et al., 1999). However, mutation (Ackerman et al., 1997; Leonardo et al., 1997; Przyborski et al., 1998) or downregulation (Engelkamp et al., 1999) of *Unc5H3* leads to an ectopic, rostral migration of granule cells into the midbrain, suggesting a possible chemorepulsive role for netrin 1. Chemorepellents within the rhombic lip or the roofplate of the fourth ventricle may also be important cues. From its expression in mouse, a strong candidate is Slit2 (Yuan et al., 1999), a ligand of the Robo receptor (Brose et al., 1999; Kidd et al., 1999), which directs cell migration and axon extension in a variety of contexts (reviewed by Brose and Tessier-Lavigne, 2000).

In this study, we characterise the generation and morphology of cerebellar rhombic lip derivatives using an acute dye-labelling strategy. We also examined the mode of migration by constructing green fluorescent protein (GFP)-labelled chimaeric embryos. We used co-culture strategies to define the molecular cues that regulate cell movement. A strict temporal organisation underlies the production of different rhombic lip derivatives in r1. This is reflected in a loss of sensitivity to netrin 1 as migrating cells switch from a ventral, extra-cerebellar fate to a dorsal granule cell precursor fate. All migratory cells are repelled by Neuro2a cells (a source of Slit2). Moreover, *Slit2* is expressed at the rhombic lip and ventral midline throughout this period. While granule cell precursors retract processes and divide again within the EGL, the leading processes of ventrally migrating cells turn and extend longitudinally at the ventral midline as axons. This suggests that the leading processes of these migrating cells are themselves axons.

MATERIALS AND METHODS

In situ hybridisation and probes

Chick embryos at embryonic day (E) 4-7 were harvested in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in 0.01 M phosphate buffer (PFA) at 4°C. The cerebellum and hindbrain region was partially dissected and subjected to in situ hybridisation (Myat et al., 1996). Plasmid templates used to generate digoxigenin-labelled antisense riboprobes were obtained from a number of sources: netrin 1/netrin 2 (gifts from M. Tessier-Lavigne, UC Stanford), *Robo1/Robo2* (gifts of A. Klar, The Hebrew University-Hadassah Medical Scholl, Jerusalem), *erbB4* (Dixon and Lumsden, 1999) and *Pax6* (Goulding et al., 1993).

Partial cDNA clones for chicken *Slit1/Slit2* were generated by RT-PCR using degenerate primers corresponding to the peptide sequences NPFNCNC(Q/H)LAW (Slit5′) and NGTSFHGCIRN (Slit3′) conserved between mouse and human Slit1 and Slit2 proteins. The sequence of the primers was:

Slit5′, 5′-AA(C/T)CC(A/T/G/C)TT(C/T)AA(C/T)TG(C/T)AA-(C/T)TG(C/T)CA(A/T/G/C)CT(A/TG/C)GC(A/TG/C)TGG-3′; and Slit3′, 5′- (A/G)TT(A/T/G/C)CG(A/T/G)AT(A/G)CA(A/G)TG(A/G)-AA(A/G)CT(A/T/G/C)GT(A/T/G/C)CC(A/G)TT-3′.

Total RNA (1 μg), isolated from the brain and spinal cord of E6 chick embryos using Trizol reagent (Life Technologies), was subjected to reverse transcription using the first-strand cDNA synthesis kit (Pharmacia) following the manufacturer's instructions. PCR amplification (30 cycles consisting of 30 seconds at 92°C, 45 seconds at 58°C and 4 minutes at 68°C) was conducted with each primer at a concentration of 2 nM and 100 ng of cDNA, using 2 U of r*Tth* polymerase (Perkin Elmer) in buffer XL II (Perkin Elmer) containing 2 mM Mg-acetate. PCR products of the expected size (1.9 kb) were cloned into pcDNA3.1/V5-his-TOPO (Invitrogen) and sequenced (Genome Express, Paris). Two distinct clones corresponding to *Slit1* and *Slit2* were identified and subcloned into pBluescript KS+ (Stratagene) as *Bam*HI/*Xba*I and *Kpn*I/*Xho*I fragments, respectively. Riboprobes were synthesised with T7 RNA polymerase after linearisation of the plasmid template with *Bam*HI (*Slit1*) or *Kpn*I (*Slit2*).

DiI labelling of rhombic lip in cerebellar explants

Explants were dissected from embryos at E4-7 in Tyrode's saline (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 3.5 mM NaH_2PO_4, 0.1 mM $MgCl_2$, 5.5 mM D-glucose). Briefly, the pia and roofplate were removed and the neural tube opened along the dorsal midline. The rhombic lip of the cerebellum was labelled with 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DiIC_{12}$) as described previously (Wingate and Hatten, 1999). Flattened whole-mounted explant preparations were embedded in 2 mg/ml rat tail collagen (Roche) and cultured in Neurobasal medium containing 2 mM GlutaMAX-I and Antibiotic/Antimycotic Solution (Life Technologies, complete medium) for 24 hours at 37°C with 5% CO_2 in a humidified incubator. Cultured explants were fixed in PFA and mounted in 95% glycerol in PBS prior examination by confocal microscopy.

Co-culture assays on cerebellar explants

Hindbrain explants were prepared and cultured as described above. Rhombic lip was identified as previously described [see Fig. 3 by Wingate and Hatten (Wingate and Hatten, 1999)] and empirically, as the only region of dorsal cerebellum to generate migratory cells in culture (data not shown). Rhombic lip and floor plate fragments were dissected away using flame-sharpened tungsten wire (0.1 mm diameter; Goodfellow) and labelled with 5μM CellTracker™ (Molecular Probes) Green CMFDA (5-chloromethylfluorescein diacetate) or Orange CMTMR (5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine) in complete medium for 10 minutes at 37°C/5% CO_2. After washing in complete medium, tissue fragments (rhombic lip and floor plate) and cell aggregates (see below) were arranged on the pial surface of explanted hindbrains before the collagen had polymerised. Heparin at a concentration of 50 ng/ml was added to Neuro2a co-cultures to potentiate the activity of Slit2 (Brose et al., 1999; Nguyen Ba-Charvet et al., 1999).

Cell lines and generation of cell aggregates

Cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM high glucose, Life Technologies) supplemented with 10% foetal calf serum. The stable 293-EBNA cell line expressing chicken netrin1 (Shirasaki et al., 1996) was selected during every fourth to fifth passage for the maintenance of episomal plasmids by the inclusion of antibiotics (250 μg/ml geneticin/200 μg/ml hygromycin B). The mouse neuroblastoma cell line Neuro2a is known to express Slit2 (Hu, 1999).

Cell aggregates were generated by overnight culture in 'hanging-drops'. Briefly, cells at confluence were trypsinised and pelleted by centrifugation (5 minutes at 500 ***g***) prior to re-suspension at a density of 3×10^7 cells/ml in culture medium. Hanging drops (15 μl each) were suspended by surface tension from the lid of a 35 mm dish containing 2 ml of PBS to prevent drying. Cells were labelled by the inclusion of CellTracker™ (20 μM) during this period. After washing in complete medium, cell aggregates were dissected in to small pieces and used in co-culture assays as described above.

Microsurgical construction of GFP-labelled chimaeras and analysis by confocal microscopy

A plasmid containing the proviral sequences of the subgroup-B

retroviral vector RCASBP(B)-*egfp* was constructed by replacing a 1 kb *Asp*718-*Sal*I fragment, which contains the region encoding the hypervariable domain of the env glycoprotein, of RCASBP(A)-*egfp* with that of RCASBP(B). RCASBP(A)-*egfp* was a gift of C. Cepko (Harvard Medical School, Boston) and contains the *egfp* gene from pEGFP-1 (Clontech) cloned as an *Nco*I-*Xba*I (blunted) fragment in the *Nco*I-*Sma*I sites of the pSlax shuttle vector (Morgan and Fekete, 1996).

Using sharp surgical scissors, the shells of chicken eggs (hi-sex white) incubated to E2 were windowed and the RCASBP(B)-*egfp* plasmid (1 mg/ml in water containing 0.0015% Fast Green) was injected into the rostral hindbrain. Cells were electroporated by applying one to three 20 milliseconds/10 V square waveform pulses between electrodes placed ventral and dorsal to the donor neural tube. Embryos were dissected out and dorsal r1 isolated using flame-sharpened tungsten wire. This tissue was then isotopically transplanted into E2 host eggs (hi-sex brown) in ovo (Wingate and Lumsden, 1996) and the resultant GFP-chimaeras were re-incubated until E6.

Embryos were rapidly dissected and the hindbrain was flattened pial surface downwards, within a glass ring sealed onto coverglass with silicon grease. Explants were covered with a layer of collagen, secured in a Leiden microperfusion chamber (Harvard Apparatus). Explants were cultured in complete Neurobasal medium at 37°C gassed by the passage of 5% CO_2/95% air over the surface of the medium. GFP-labelled explants were imaged for periods of up to 24 hours on an inverted compound microscope (Nikon Diaphot 200) equipped with a laser scanning confocal microscope (BioRad MRC 600) at 10 to 15 minute intervals (30% laser power, 10-15 optical sections). Projections of optical sections were compiled into time-lapse animations (n=10) using Adobe Premiere v.6.0 (Adobe Systems Incorporated).

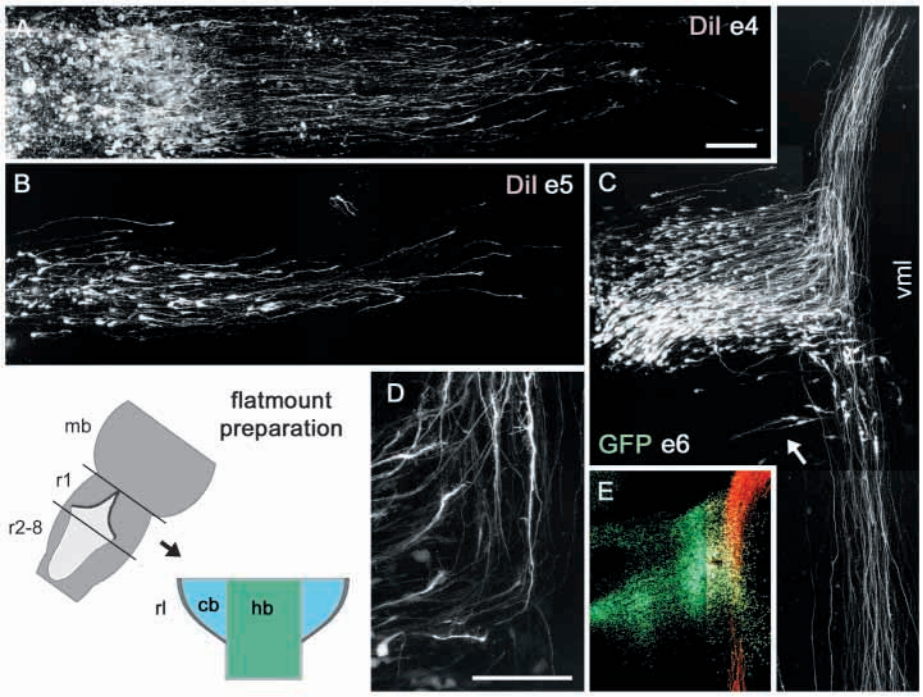

Fig. 1. Rhombic lip derivatives at E4 and E5 project to the ventral midline. The schematic diagram (bottom left) shows the preparation of flatmount explants with the rhombic lip of r1 (rl) highlighted in dark grey. Dorsal r1 is the presumptive cerebellum (cb), while ventral r1 and r2-8 comprise the hindbrain (hb). Rhombic lip derivatives were labelled, either acutely by DiI application to the rhombic lip, or cumulatively by isochronic grafting of GFP-electroporated dorsal r1 into host neural tube at E2. Acute labelling reveals that at both (A) E4 and (B) E5, the rhombic lip generates unipolar migratory cells that project leading processes directly to the ventral midline. (C) In an E6 chimaera, GFP-labelled leading processes of cells that have migrated into ventral r1 turn either rostrally or caudally close to the ventral midline (vml). An arrow indicates a neurone with rudimentary dendrites. The rhombic lip (to the left) lies outside the field of view. (D) At higher magnification, turning axons display increased growth cone complexity and short interstitial branches, but do not seem to bifurcate.
(E) Immunohistochemical staining of a quail/chick chimaera constructed in a similar manner and with the same field of view shown to that in C. Cell bodies were labelled using the Q¢PN antibody (green). The monoclonal antibody QNTAN (red) reveals the presence of an unspecified neuronal membrane-bound epitope (Tanaka et al., 1990) and identifies longitudinal processes as definitive axons [for experimental details, see Wingate and Hatten (Wingate and Hatten, 1999)]. In these and all other flatmount micrographs, rostral is towards the top and the dorsoventral axis runs from left (the rhombic lip) to right (the ventral midline). Plates show the pialward surface of flatmounted brains. Scale bars: in A, 100 μm for A,B,C,E; in D, 100 μm for D. mb, midbrain.

RESULTS

From a previous study of avian cerebellar development, it is known that while at least two different cell populations are generated at the cerebellar rhombic lip, this heterogeneity is not reflected in the morphology of cells at a given time point (Wingate and Hatten, 1999). To examine the migration of rhombic lip derivatives at different stages in the chick, we contrasted the results of acute labelling by DiI in explant culture with the product of cumulative labelling in microsurgically constructed GFP-expressing chimaeras. Co-culture techniques were then used to assess candidate guidance factors and gauge the differing responses of migrating cells in early and late generated cohorts.

Early born cohorts extend processes into ventral r1

From E4 onwards, streams of labelled migrating cells were observed 24 hours after the application of DiI to the rhombic lip of cultured cerebellar explants. Migrants at both E4 (Fig. 1A) and E5 (Fig. 1B) display a distinctive, unipolar form with a single process that extends almost to the ventral midline. Over 24 hours in culture, cell bodies migrate approximately 150 μm with leading processes up to 500 μm in length. To follow the fate of cells beyond this period, we adapted a fate-mapping approach that had previously been shown to selectively label rhombic lip derivatives (Wingate and Hatten, 1999). The anterior neural tube of donor embryos at E2 was labelled by electroporation of RCASBP(B)-*egfp* and the dorsal segment of r1 microsurgically grafted into dorsal r1 of a host E2 chick embryo in ovo. GFP-labelled cells in chimaeras

resulting from such isochronic and isotopic transplants represent the cumulative product of proliferation within the rhombic lip.

Examination of GFP-chimaeras at E6 revealed rhombic lip derivatives that have migrated into ventral rhombomere 1 (Fig. 1C). The leading processes of cells turn either rostrally or caudally as they approach the midline, displaying complex growth cone-like structures and short interstitial branches (Fig. 1D). No bifurcating processes were seen and some cell bodies are able to follow their leading processes for short distances longitudinally. A small number of these dispersed cells exhibit rudimentary dendrites (Fig. 1C, arrow). Processes extend long distances along the ventral midline suggesting that they form permanent structures. Immunolabelling of E6 quail-chick chimaeras with quail-specific antibodies reveals that the longitudinal segments of these processes are indeed axons (Fig. 1E). As no cells in ventral r1 appear to have retracted a process, we conclude that these axons form directly from the leading processes of ventrally migrating cells.

Late-born cohorts are restricted to the cerebellum

At E6, examination of dorsal r1 in GFP-chimaeras (Fig. 2A) reveals that cells emerging from the rhombic lip at later ages retain a unipolar morphology but display significantly shorter leading processes (20 μm). The absence of cells with long leading processes in dorsal r1 suggests either that the entire population has migrated ventrally, or that early born cells (E4-E5) have retracted their processes. The absence of differentiated derivatives within dorsal r1 (in contrast to ventral r1) supports the former conclusion and indicates a chronotopic distribution of rhombic lip derivatives with the youngest born cells closest to their point of origin.

At the boundary between dorsal, presumptive cerebellum and ventral territory (Fig. 2A, arrows), leading processes of migrating cells are directed rostrally. This turning point can also be distinguished by an accumulation of cell bodies. Acute labelling with DiI at E6 (Fig. 2B) confirms that late-born migratory rhombic lip derivatives have short processes, which are deflected rostrally at the cerebellum/hindbrain interface (arrow). No cells migrate beyond this boundary (data not shown). Cells generated at E7 have a similar morphology but display a more variable process orientation (Fig. 2C). The restriction of rhombic lip derivatives to the cerebellar territory after E6 suggests that they are granule cell precursors. Comparing the distribution of *Pax6* (Fig. 2D) and *erbB4* (Fig. 2E) transcripts, which are expressed in granule cells (Engelkamp et al., 1999; Dixon and Lumsden, 1999), with acute DiI labelling at E6 (Fig. 2F) confirms that the accumulation of these cells corresponds with the formation of the EGL, which is initiated at its ventrolateral edge in chick (Hanaway, 1967; Wingate and Hatten, 1999).

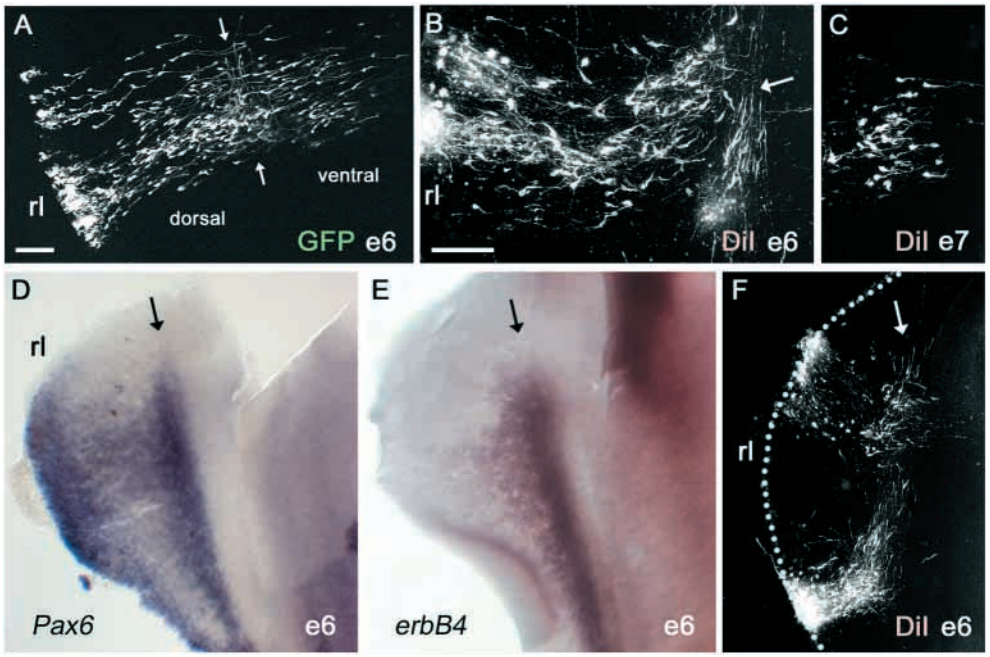

Fig. 2. Rhombic lip derivatives generated after E6 are restricted to the cerebellum. (A) GFP label in dorsal r1 at E6 shows the cumulative distribution of migratory cells generated from the rhombic lip. Close to their origin, cells that are presumably younger exhibit short processes. Cell bodies have accumulated at the interface between dorsal and ventral territory (arrows) where leading processes are aligned longitudinally. The field of view does not include the ventral midline. The rhombic lip (rl) is indicated towards the left. (B) Acute labelling at E6 demonstrates that the leading processes of all later born cells are deflected rostrally at the boundary between dorsal and ventral r1 (arrow). The bright fluorescence to the left of the panel is a site of DiI application at the rhombic lip (rl). (C) Cells generated at E7 within dorsal r1 do not migrate as far as the boundary within 24 hours. The rhombic lip lies outside the panel towards the left. (D,E) At E6, in situ hybridisation for *Pax6* and *erbB*, respectively, reveals that granule cell markers are concentrated at the ventrolateral edge of the cerebellum (arrows). (F) This corresponds with the boundary encountered by acutely labelled rhombic lip derivatives at E6 (arrow). The edge of the explant is highlighted (dotted line). Scale bars: in A, 100 μm for A,D,E,F; in B, 100 μm for B,C.

Time-lapse confocal microscopy reveals the mode of tangential migration

From the onset of migration, rhombic lip derivatives move in the direction in which they extend a leading process. This suggests two alternative modes of migration: either, a cell body moves within an established process only once it has made contact with a given target region (perikaryal translocation); or the leading process navigates a trajectory but maintains a constant length in front of the following soma. In chimaeras, the constitutive expression of GFP allowed cells to be filmed over 24 hours in intact flatmounted E6 cerebellum. Analysis of movie sequences shows that rhombic lip derivatives employ the latter mode of migration.

Fig. 3A shows the starting frame for a sequence of 71 confocal stacks taken at 10 minute intervals (see Movie 1

at http://dev.biologists.org/supplemental/). As cell bodies migrate, leading processes maintain an approximately constant length with growth-cone-like structures actively exploring the substrate. Although migration is not a bimodal mechanism of process extension followed by perikaryal translocation, the movement of the cell body is clearly saltatory. At the beginning of each migration step, cell bodies elongate as if the soma were being actively pulled away from a substrate anchor. The movement of five distinct cells (a-e) was plotted with respect to a differentiating neurone (n) (see Movie 2 at http://dev.biologists.org/supplemental/). Superimposition of successive frames in a single composite (Fig. 3B) reveals gaps in the generated trails that correspond to rapid movement (of up to 20 μm in 10 minutes). By contrast, slower movement is indicated by a lighter colour of dot at a given position, such that a white dot (representing the cumulative addition of several frames) indicates no movement for at least 30 minutes. Migrating cells (a-e) show a succession of pauses followed by rapid movement.

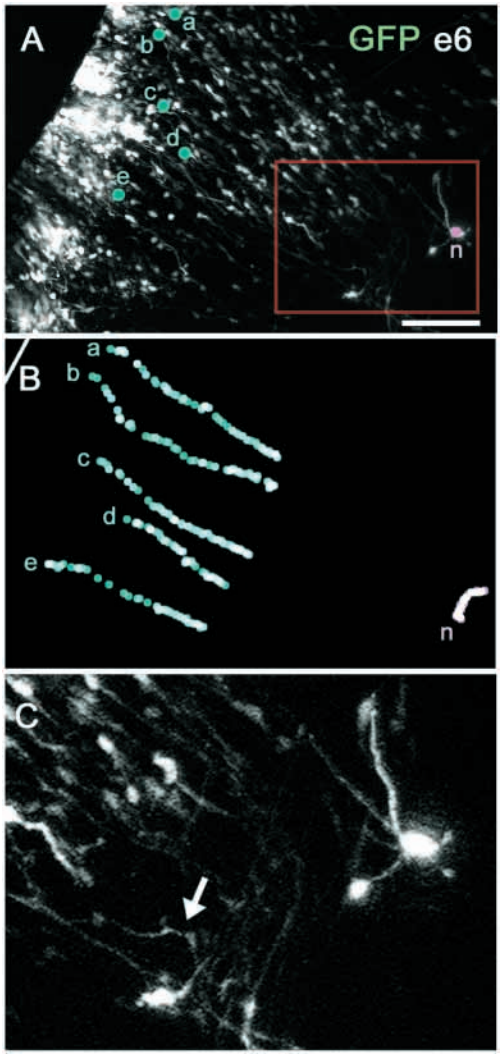

Fig. 3. Time-lapse confocal microscopy in GFP-chimaeras reveals the mode of cell migration. (A) Opening frame of a time-lapse movie showing cell movement over 24 hours (see Movie 1 at http://dev.biologists.org/supplemental/). Green dots identify cell bodies (a-e) whose progress was monitored from frame to frame (composite). For reference, the cell highlighted in pink is a static neurone (n) just ventral to the cerebellum. The ventral midline is outside the field of view (towards the bottom right). The boxed area highlights the boundary between dorsal and ventral r1 (see C). (B) Superimposing the positions of cells a-e in successive frames reveals the path and speed of migrating cells. The lighter the colour of the trail, the lower the rate of cell body movement (white indicates stationary). The position of the rhombic lip is indicated by a white line (top left). (C) Opening frame of a higher magnification movie (see Movie 3 at http://dev.biologists.org/supplemental/) of the region bounded by the red box in A. An arrow indicates a single leading process approaching the boundary of the cerebellum which fasciculates with rostrally projecting processes. Scale bar: 100 μm.

The behaviour of processes at the distal cerebellar boundary was examined at higher magnification (see Movie 3 at http://dev.biologists.org/supplemental/). The rostrad turning of an identified leading process (Fig. 3C, arrow) appears to involve a phase of searching and subsequent fasciculation with underlying rostrally extending fibres. The turning response of late-born derivatives at this boundary revealed by DiI labelling may therefore be mediated in part by cell-cell interactions.

Temporal changes in the responses of rhombic lip cells to migration cues

Fig. 4 summarises the migration of rhombic lip derivatives as revealed by the results of acute and cumulative labelling strategies. To assess the factors that might guide early and late cells from the rhombic lip to their alternate destinations, dye-labelled fragments of rhombic lip were cultured on the surface of whole hindbrain and cerebellar explants under collagen. Fig. 5A contrasts the migration of endogenous DiI-labelled cells (red) within an E4 r1 explant with the migration of cells derived from a labelled fragment (green) placed on its pialward surface. Fragments produce streams of unipolar migrating cells, which can follow the same axis of migration as underlying rhombic lip derivatives migrating within intact tissue. We exploited this property to assay guidance cues at different distances from rhombic lip or floor plate.

The micrographs in Fig. 5B-E compare the behaviour of rhombic lip cells from E4 and E6 fragments when placed on identical E6 cerebellum and hindbrain explants (as defined in Fig. 1: cb and hb, respectively). On cerebellar substrates, the processes of E4 cells display a uniform orientation away from

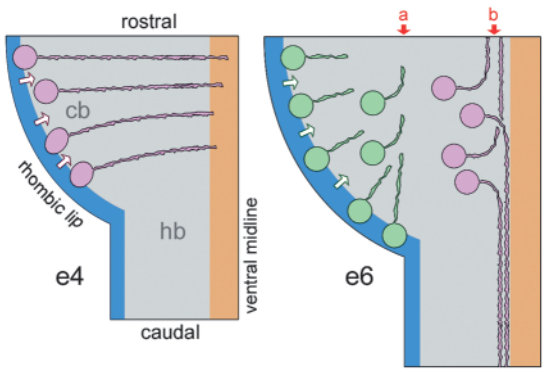

Fig. 4. The behaviour of rhombic lip derivatives in r1. GFP-chimaeras demonstrate that cells born at E4 and E5 (pink) migrate into ventral r1. Their leading processes turn at the ventral midline (b), while later born migrants (green) turn rostrally at the edge of the cerebellar anlage (a).

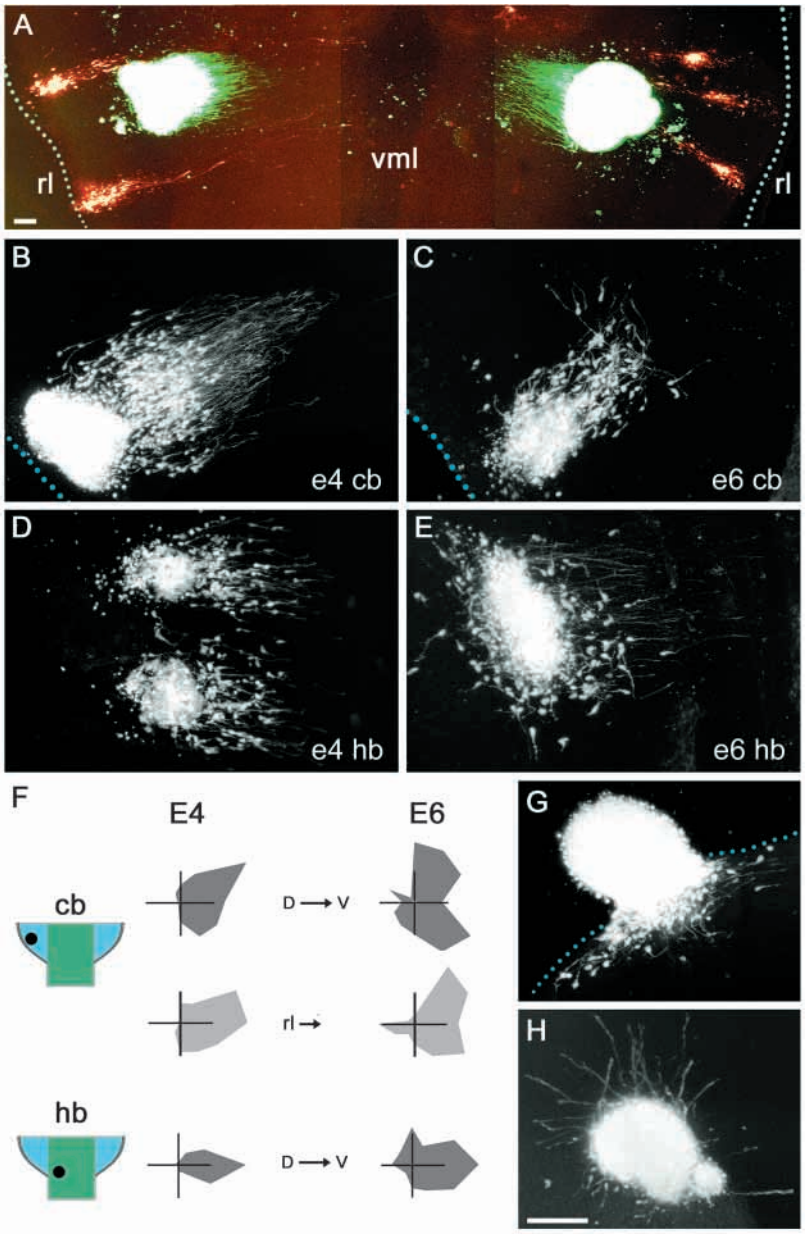

Fig. 5. Co-cultures reveal migration cues within r1. (A) Cell migration can be induced from rhombic lip fragments cultured directly on the pialward surface of flatmounted explants. Cells from E4 rhombic lip fragments (green) align themselves along the same axis as endogenous migratory cells labelled with DiI (red) in the underlying E4 explant. The full bilateral dorsoventral axis is shown in the confocal montage with the ventral midline (vml) at its centre. (B) E4 fragment on E6 cerebellum. (C) E6 fragment on E6 cerebellum. (D) E4 fragment on E6 hindbrain. (E) E6 fragment on E6 hindbrain. (F) Radial plots compare the orientation of the leading process of 1683 clearly identified cells from E4 or E6 rhombic lip fragments (n=110) with respect to an axis perpendicular to the ventral midline. For each plot, orientations were scored in 15° bins and plotted as a percentage of the mode. The orientation on E6 cerebellar substrates (top) is contrasted with that on E6 hindbrain (bottom), as indicated in the accompanying schematic diagrams (left). In light grey (middle), the orientation on cerebellar territory is re-plotted with respect to an axis perpendicular to the closest segment of rhombic lip. (G) Cells only exit a fragment of rhombic lip where it contacts the explant substrate (the boundary of the explanted hindbrain is denoted by a broken line). (H) An isolated rhombic lip fragment will produce processes but few cells emerge. As no migrating cells were observed, these fragments were excluded from analysis. Scale bars: in A, 100 μm for A; in H, 100 μm for B-E,G,H.

the rhombic lip and exit the labelled fragment unilaterally (Fig. 5B). Cells from E6 rhombic lip show a broader range of trajectories and may exit from any part of the fragment (Fig. 5C). Hindbrain substrates were permissive for the migration of both E4 and E6 cells, despite the normal restriction of the latter, late-born rhombic lip derivatives to the cerebellum. It therefore seems unlikely that a diffusible signal from the midline is responsible for the normal exclusion of E6 derivatives from ventral regions. On hindbrain, cells from E4 fragments again show a more uniform migration (Fig. 5D), while the processes of E6 cells placed on the same E6 substrate are demonstrably less sensitive to underlying migration cues (Fig. 5E). Close to the midline, the preferred orientation of leading processes is aligned to the dorsoventral axis.

The cumulative orientation of individual cell processes from a large number of assays (n=110) was plotted for each of the four conditions (Fig. 5F). On cerebellum (cb), orientation is less sharply tuned when plotted on axes perpendicular to the ventral midline (DV axis) than to the rhombic lip (rl). This is perhaps indicative of a role for the rhombic lip in organising the initial trajectory of migration. By contrast, on hindbrain substrates (hb), the orientation of leading processes was sharply perpendicular to the midline. In general, the trajectory of cells from E4 fragments was more uniform than that of cells at E6. This indicates a lower sensitivity of E6 cells to guidance cues available from identical substrates.

Although diffusible cues may orientate migration, the egress of cells from a fragment is dependent on physical contact with the explant surface. When a labelled fragment partially abuts the substrate, cells will only emerge from the tissue interface (Fig. 5G), suggesting that contact with basal lamina is an essential requirement for migration (Hausmann and Sievers, 1985). Rhombic lip fragments cultured in collagen alone extend long processes but few, if any, cell bodies are visible (Fig. 5H).

Responsiveness to netrin 1 changes over time

To determine whether the temporal changes in migratory cell behaviour can be attributed to a changing responsiveness to netrin1 (Alcántara et al., 2000), we placed small aggregates of netrin1-expressing 293-EBNA cells close to the rhombic lip in DiI-labelled explants at E4 and E6 (n=24). Over this period, netrin 1 is exclusively expressed on either side of the floorplate (data not shown). At E4, cell processes project directly towards the midline without any rostrocaudal deviation (Fig. 6A).

When confronted with a piece of explanted floorplate placed over the path of E4 migrating cells (Fig. 6B), leading processes stall beneath the source of ectopic netrin1 due to turning and apparent localised exploration. At a higher magnification (Fig. 6C), netrin 1-expressing cells can induce both rostral and caudal turning in leading processes of E4 cells that pass close by. Deviations from a strictly ventral trajectory are never seen in controls at E4. Neither netrin 1 nor floorplate induces branching or increased growth in leading processes.

Turning is normally observed in cells generated at E6, whose processes are deflected rostrally (but never caudally) at the cerebellar boundary (Fig. 6D). In contrast to the responses of cells at E4, ectopic netrin1 placed close to the rhombic lip at E6 neither attenuates cell migration nor disrupts rostral turning (Fig. 6E). This implies that later born rhombic lip derivatives lose their responsiveness to netrin 1, correlating with changes in their morphology, behaviour (Fig. 4) and general sensitivity to dorsoventral guidance cues (Fig. 5).

Cell migration is inhibited by Slit2

Although not attracted by netrin 1, E6 migratory cells still show a preference for dorsoventral migration when cultured on explanted substrates (Fig. 5D). This suggests the involvement of other diffusible guidance cues such as chemorepulsion from the rhombic lip. Strong candidates as diffusible migration cues are proteins of the slit family (Wu et al., 1999), which are expressed in the rhombic lip in the mouse (Yuan et al., 1999). Using degenerate PCR primers, we obtained cDNA clones for chicken *Slit1* and *Slit2* and examined their expression together with that of the known cognate receptors *Robo1*/*Robo2* from E4 to E7. Of these, *Slit2* and *Robo2* showed expression patterns consistent with a role in chemorepulsion at the rhombic lip. The putative ligand *Slit2* is expressed at both the ventral midline and rhombic lip from E4 through to at least E7 (Fig. 7A). Weak *Robo2* expression is detectable in rhombic lip derivatives as early as E4 (data not shown). By E7, it is strongly expressed throughout the EGL (Fig. 7B) in a pattern that is complementary to the strong expression of *Slit2* at the rhombic lip (Fig. 7C).

To assess whether rhombic lip cells are susceptible to slit activity, we placed a source of Slit2 (Neuro2a cells) in the path of their migration. We were unable to see an effect in DiI labelled explants at either E4 or E6 suggesting either an absence of response or poor diffusion of Slit2. We therefore confronted rhombic lip fragments, cultured on an explant substrate (as in Fig. 5A) with control 293-T cells (Fig. 7D,G) or Neuro2a cells (n=40). At E4, the migration of cells is not affected when Neuro2a cells are placed at a distance to the labelled rhombic lip fragment (Fig. 7E). However migration is blocked when Neuro2A cells and labelled rhombic lip are directly juxtaposed (Fig. 7F). By comparison, migrating cells pass directly under cells that do not secrete Slit2 (293-T: Fig. 7D,G). At E6, cell process orientation is more disorganised and, as at E4, Neuro2a cells exert little influence when placed at a distance (Fig. 7H). However, when Neuro2a cells are juxtaposed to an E6 rhombic lip fragment, the latter appears to be polarised: cells only emerge from the side of the fragment opposed to the source of Slit2 (Fig. 7I). These data are consistent with a role for Slit-Robo signalling in organising cell migration away from the rhombic lip and in the deflection of the leading processes of early-born cells at the ventral midline.

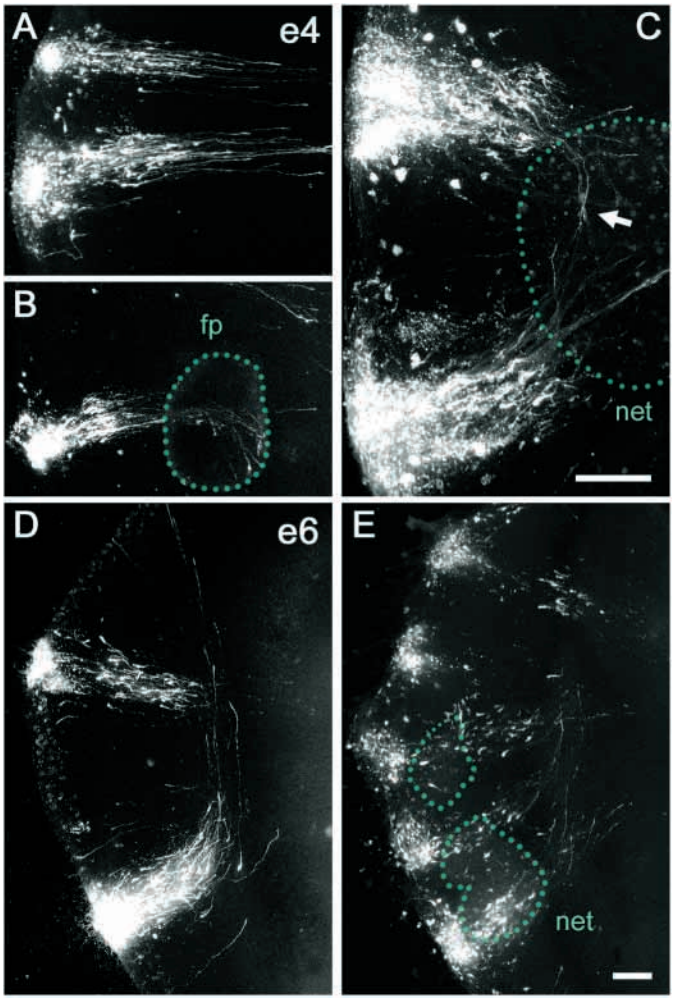

Fig. 6. Later born cells lose their response to an exogenous source of netrin 1. DiI-labelled rhombic lip cells were challenged with ectopically positioned netrin 1-expressing cells or floorplate (indicated by green dotted lines). (A) Leading processes of E4 migrants extend directly to the midline. (B) Leading processes at E4 stall beneath an ectopic floorplate (fp) fragment placed on the surface of the explant. (C) An exogenous source of netrin 1 can induce both rostral and caudal (arrow) turning in E4 labelled cells. (D) At E6, cell processes turn rostrally at the cerebellar boundary (first seen in caudally labelled migrants). (E) The normal extension and turning of leading processes at E6 cannot be deflected by an exogenous source of netrin 1. Scale bars: in E, 100 μm for A,B,D,E; in C, 100 μm for C.

DISCUSSION

Using a variety of labelling approaches we have examined the migration of rhombic lip derivatives in rhombomere 1 of the chick embryo. A strict temporal organisation underlies the generation of two distinct populations. From E4, an early-born cohort of ventrally migrating neurones condenses outside the cerebellum. From E6, the migration of a larger mass of granule cell precursors is blocked at a point that demarcates the ventrolateral boundary of the EGL, as defined by the expression of characteristic molecular markers. Successive cohorts of rhombic lip derivatives share a uniformly unipolar morphology; however, the leading processes of newly

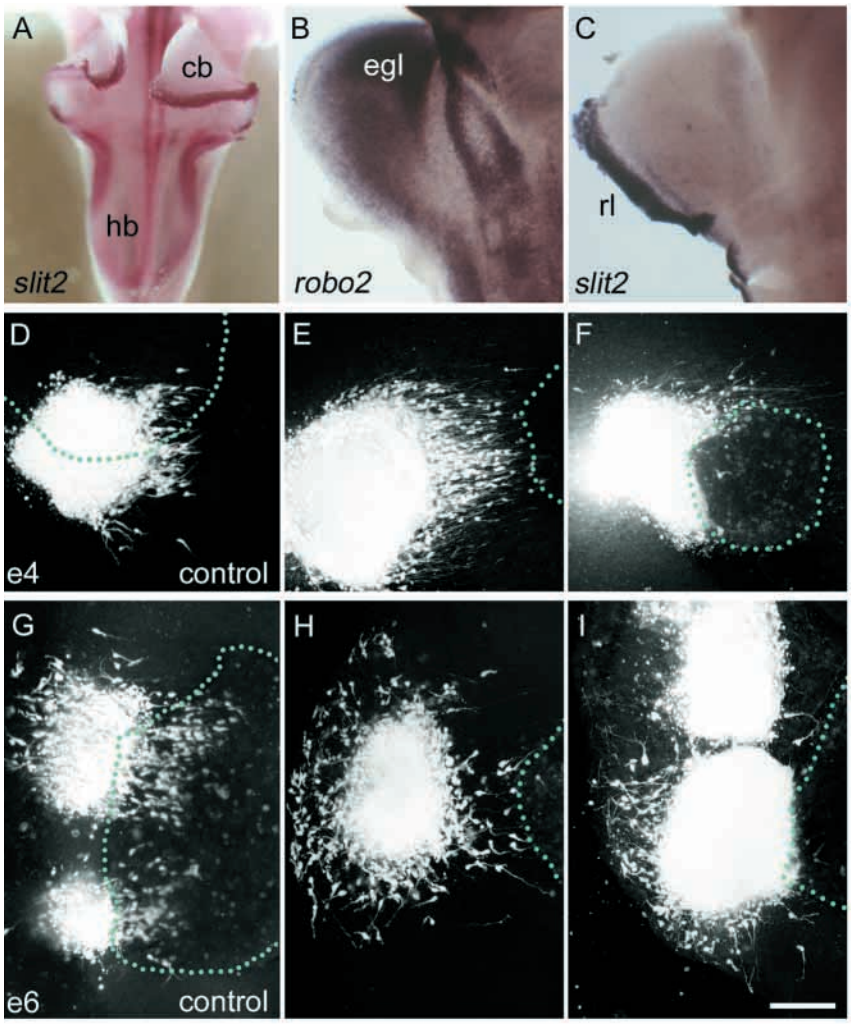

Fig. 7. Expression patterns and co-culture assays suggest a role for slit-Robo signalling at the rhombic lip. At E7, the expression patterns of *Slit2* and *Robo2* are complementary in the rhombic lip and EGL, respectively. (A) Whole-mount brain showing *Slit2* expression in rhombic lip of the hindbrain (hb) and cerebellum (cb) and at the ventral midline. (B) *Robo2* expression in the EGL (egl) of a flatmounted cerebellum. (C) Complementary expression of *Slit2* in the rhombic lip (rl) at the same stage. (D) For E4 rhombic lip fragments, cell migration along the dorsoventral axis (left to right) is unaffected by the close proximity of an overlying pellet of control 293-T cells (whose boundary is indicated by green dotted lines). (E) Similarly, migration is unaffected by a pellet of Neuro2a cells placed at a distance to the labelled rhombic lip explant. (F) Migration is attenuated only in close proximity to a source of Slit2. (G) For E6 rhombic lip fragments, migration of labelled cells is again unaffected by control 293-T cells. (H) As at E4, Slit2-secreting Neuro2a cells are unable to affect migration at distance. (I) However, close proximity of Neuro2a cells polarises migration from rhombic lip explants such that cells only exit from the side of the fragment opposed to the source of Slit2. Scale bar: in I, 100 μm for D-I.

emerging cells become progressively shorter as development proceeds. Time-lapse imaging of GFP-labelled cells reveals that leading processes navigate a circumferential (dorsoventral) migration path, maintaining a constant length as they do so. Cell bodies follow by saltatory translocation. Migration and co-culture assays reveal that rhombic lip derivatives become less attracted to the ventral midline and netrin 1 as development proceeds corresponding to their behaviour in vivo. Orderly migration away from the rhombic lip may be orchestrated in part by chemorepulsion. At relevant developmental stages, *Slit2* is expressed at both the ventral midline and rhombic lip while a source of Slit2 (Neuro2a cells) can inhibit migration of cells at early and late stages.

A balance of cues directs dorsoventral migration

Our observations of co-cultured explants suggest that migrating cells recognise a framework of dorsoventral guidance cues, which include netrin 1 and Slit2. That netrin 1 can attract early cerebellar rhombic lip derivatives is unsurprising given its well-characterised chemotropic role in axon guidance towards the ventral midline (reviewed by Culotti and Merz, 1998). Moreover rhombic lip derived neurones produced in the caudal hindbrain (r2-8) are attracted by netrin 1 (Alcántara et al., 2000; Yee et al., 1999) and fail to migrate correctly in its absence (Bloch-Gallego et al., 1999). Similarly, the chemorepellent activity of Slit2 has been shown to play a widespread role in guiding axon growth (Brose et al., 1999; Nguyen Ba-Charvet et al., 1999) and cell migration (Hu, 1999; Kramer et al., 2001; Li et al., 1999; Zhu et al., 1999). For rhombic lip derivatives, netrin 1 might only be crucial for the migration of the relatively small population of derivatives born from E4-E5 and destined for ventral r1. Our observations confirm, in chick, results from studies in mouse, which indicate that rhombic lip derivatives destined for a cerebellar fate are insensitive to netrin 1 (Alcántara et al., 2000). The activity of Slit2 may provide a necessary cue for the initial orientation of cells exiting the rhombic lip and ensure that there is little accumulation of derivatives close to the rhombic lip until a confluent EGL is formed at E7.

Changes in sensitivity to netrin1 parallel a switch in migrant cell fate

Acute labelling strategies allowed us to identify the point at which rhombic lip derivatives become restricted to the cerebellum and form the EGL. The onset of the turning of leading processes in E6 rhombic lip derivatives at the ventrolateral boundary of cerebellar territory is spatiotemporally precise. The observation that ventral r1 at E6 is permissive for migration in co-culture assays, suggests that this boundary is not established by long-range diffusible signals from the ventral midline. Rather, the consistent rostrad turning of leading processes at the cerebellar boundary point to an interaction between migrating cells and a polarised substrate at E6. Our time-lapse microscopy observations reveal the presence of an underlying population of rostrally projecting processes at this boundary. Fasciculation with such processes

may result in the characteristic rostral turning that first defines the cerebellar boundary.

While the origin of these rostrally projecting processes is unclear, a parsimonious model is that the loss of sensitivity to netrin1 in caudally originating rhombic lip derivatives in r1 results in their leading processes projecting rostrally (perpendicular to the rhombic lip; Fig. 4). As leading processes cross paths with those of more rostrally derived (and hence ventrally orientated) cells, they form the ventrolateral cerebellar boundary. The accumulation of the EGL in chick is initiated at its ventrolateral boundary, distal to the lip, as assessed by molecular markers (Fig. 2D,E), fate-mapping (Wingate and Hatten, 1999) and cytoarchitecture (Hanaway, 1967). This strongly suggests that the first granule cell precursors to halt at the cerebellar boundary homotypically inhibit the migration of succeeding cohorts. In this way, a loss of sensitivity to netrin 1 in E6 rhombic lip derivatives may trigger the self-organisation of the EGL by mutual inhibition. Recent insights into the role of the Robo receptor in silencing responses to netrin (Stein and Tessier-Lavigne, 2001) and promoting fasciculation (Rajagopalan et al., 2000; Simpson et al., 2000) raise the intriguing prospect that Robo receptor regulation and activation may underlie these changes in behaviour.

The leading processes of migrating cells are axons

Using a novel approach of constructing GFP-labelled chimaeras by microsurgery, rhombic lip precursors were specifically identified and filmed by time-lapse confocal microscopy. We show that the leading processes of ventrally migrating cells turn at the ventral midline and extend longitudinally to become axons. This suggests that the leading process of migrants is itself a rudimentary axon and that the mechanism of migration is that of normal axon growth. Caudal rhombic lip-derived migratory populations appear to share this unipolar morphology (Kyriakopoulou et al., 2002; Yee et al., 1999). Direct time-lapse observation of living cells clarifies the mode of migration. Rather than comprising a bimodal process of axon extension followed by perikaryal translocation ‘through’ this process, as suggested in other systems (Bloch-Gallego et al., 1999; Bourrat and Sotelo, 1988), overall cell length remains approximately constant: axons navigate while cell bodies follow in a series of jumps which can be less or more pronounced.

If tangential migration is an elaboration of axon growth, it points to a pivotal role for the cell body in initiating and halting cell movement (Bourrat and Sotelo, 1990). Migration starts as the cell body loses adhesion with the substrate and ceases when a suitable substrate affinity is re-established. The decision to stop migration is likely to be made autonomously by the cell body when it reaches its target, rather than by the leading process. For ventrally migrating rhombic lip derivatives, the leading process may have extended through such a target nucleus well before the cell body arrives. Such a model predicts the use of different guidance receptors at the cell body and leading process, or the differential sublocalisation of elements of a single guidance system within the cell.

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