The role of the rhombic lip in avian cerebellum development

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SUMMARY

We have used a combination of quail-chick fate-mapping techniques and dye labelling to investigate the development of the avian cerebellum. Using Hoxa2 as a guide for the microsurgical construction of quail-chick chimaeras, we show that the caudal boundary of the presumptive cerebellum at E6 maps to the caudal boundary of rhombomere 1. By fate mapping the dorsoventral axis of rhombomere 1, we demonstrate that granule cell precursors are generated at the rhombic lip together with neurons of the lateral pontine nucleus. DiI-labelling of cerebellum explants reveals that external germinal layer precursors have a characteristic unipolar morphology and undergo an orientated, active migration away from the rhombic lip, which is apparently independent of either glial or axon guidance or ‘chain’ formation.

Key words: Granule cell, Lateral pontine nucleus, Quail-chick chimaera, Cell migration, Hoxa2

INTRODUCTION

The vertebrate neuraxis is patterned by dorsoventral and anteroposterior molecular cues into regionally distinct subdivisions each characterised by a different repertoire of neuronal types. In the early neural tube, these complementary patterning systems can be interpreted in terms of the orthogonal axes of a Cartesian co-ordinate system (Lumsden and Krumlauf, 1996). However, for CNS structures generated later in development, these basic motifs are often obscured by gross morphogenic changes. We have re-examined the development of one such structure, the cerebellum, in the light of recent molecular evidence indicating that its development may be far simpler than previously presumed. In particular, we have investigated the role of the rhombic lip, which lies at the interface between the cerebellum and roofplate, in the induction of cerebellar granule neurons. Over half the adult brain consists of cerebellar granule cells, which receive mossy fibre input chiefly from the pons and participate in the relays that govern Purkinje cell activity and hence cerebellar output. Granule cell development involves two distinct phases of proliferation and migration (Cajal, 1911). Of these, the second phase of cell division in the external granule cell layer (EGL) and subsequent, radial, inward migration to form the internal granule cell layer has been extensively investigated (Hatten and Heintz, 1995). By comparison, the first phase of precursor induction leading to the formation of the EGL is poorly understood. The precise anteroposterior and dorsoventral origins of EGL precursors and their mode of migration over the pial surface of the cerebellum remain key questions in developmental neurobiology.

The cerebellum arises in a region that initially encompasses the boundary between the mesencephalon and the metencephalon (Hallonet et al., 1990; Millet et al., 1996). The constriction, or isthmus, that separates these two embryonic vesicles is an important signalling centre and a number of studies have demonstrated that genes induced at the isthmus regulate both cerebellum and midbrain development (Lumsden and Krumlauf, 1996). However, it is only recently that the boundaries of the anlage have been investigated. By accurately constructing a fate map according to molecular anatomy, Millet et al. (1996) showed that the posterior boundary of Otx2 expression indelibly marks the caudal limit of the presumptive midbrain and hence, by inference, the rostral boundary of the cerebellar anlage. Accordingly, targeted mutation of this gene leads to a rostral expansion of cerebellum (Acampora et al., 1997). Conversely, a targeted mutation of Hoxa2, the most anterior Hox gene in the hindbrain, results in a caudal enlargement of the cerebellum (Gavalas et al., 1997). Does Hoxa2 define the caudal limit of presumptive cerebellum? At early embryonic stages, there are no structural landmarks delimiting the region that lies between the limits of Hoxa2 and Otx2 expression (Prince and Lumsden, 1994; Millet et al., 1996). However, in the later embryo, this domain resolves to a single defined neurorome designated as rhombomere (r) 1.

The role of dorsoventral patterning in cerebellar development, and in particularly the induction of granule cells, is currently unclear: recent molecular data indicate that granule cells are a dorsally derived cell population, but this conclusion is contradicted by fate-map data and classical neuroanatomy. Key to understanding this apparent anomaly is determining the derivatives and dorsoventral origins of the rhombic lip. Various authors have argued that granule cells are born exclusively at the rhombic lip and subsequently migrate over the surface of the cerebellum to form the EGL (Schaper, 1897, adapted by Harkmark, 1954a; Miale and Sidman, 1961; Altman and Bayer, 1978, 1987, 1997; Hynes et al., 1986; Ryder and Cepko, 1994; Hatten and Heintz, 1995). This is supported by recent studies...
showing that a number of granule neuron markers are initially also expressed at the rhombic lip (Hatten et al., 1997). A targeted mutation of one of these genes, the pro-neural atonal homologue, Math-1, leads to a complete loss of granule cells (Ben-Arie et al., 1997). Similarly, dissociation studies show that all rhombic lip derivatives are apparently positive for a granule cell lineage-specific marker, RU49 (Alder et al., 1996). Both Math-1 and RU49 can be induced in presumptive cerebellum by the dorsalising factor, BMP (Alder et al., 1999), indicating a role for dorsoventral patterning in granule cell precursor determination. However, nearly all anatomical models suggest that the rhombic lip is a caudal structure and hence under the influence of a very different set of anteroposterior patterning cues (Altman and Bayer, 1978, 1985; Alvarez Otero et al., 1993; Ryder and Cepko, 1994). Moreover, a precise fate map of the dorsoventral axis of the metencephalon at E2 shows that granule cells are derived from all but the most ventral neural tube (Hallonet and Le Douarin, 1993). Therefore, whether the rhombic lip is the sole source of granule cells remains unclear and, significantly, the proposal that rhombic lip is a dorsally derived structure has yet to be confirmed.

We have used a combination of techniques to assess the precise caudal boundary of the cerebellar anlage at E6 and the dorsoventral origins of the rhombic lip. These show that the rhombic lip is a dorsally derived structure and the source of EGL precursors. The caudal limit of presumptive cerebellar territory corresponds to the boundaries between r1 and r2, suggesting that, at E6, the cerebellar anlage is derived from r1. EGL precursors have a distinctive morphology and mode of early migration. Perhaps surprisingly, granule cell precursors are generated alongside a population of ventrally migrating neurons that give rise to the lateral pontine nucleus. This suggests a possible lineage relationship between two functionally connected, but topographically and phenotypically distinct neuronal populations.

Fig. 1. (A) Dorsal view of the mesencephalon/metencephalon (mes/met) region of an E2 (stage 10) embryo triple-labelled by in situ hybridisation for Otx2 (blue), Hoxa2 (blue) and Gbx2 (red). Dark-grey shading in the accompanying schematic diagram shows the area from which r1 grafts were derived. (B) Four types of unilateral graft, of varying dorsoventral extent, are represented schematically as transverse sections of r1.

Fig. 2. The relationship between r1, r2 and the E6 cerebellum. (A) Ventricular view of an E3 chimaeric hindbrain showing the distribution of cells derived from an r2 graft (green) with respect to rhombomere boundaries (enhanced by Nomarski optics). Scale bars in A,C, 200 µm. (B) Lateral view a whole stage 19 chimaera showing r1 derivatives (brown) in the CNS and cranial nerves. Quail neural crest cells from r1 can be seen in the ophthalmic lobe of the trigeminal ganglion (Vo) and ensheathing the oculomotor (III) and trochlear (IV) nerves. (C) Ventricular view of an E6 chimaeric hindbrain showing the distribution of cells derived from an r2 graft relative to Hoxa2 expression. (D) Schematic diagrams explaining the dissection and orientation of the flat-mounted cerebellum (cb) and hindbrain (hb): rhombic lip is shaded blue. In all subsequent figures, anterior is to the top of the page. (E) At E6, the caudal limit of an r1 graft maps to the caudal limit of the cerebellum. Quail cells can be seen in the trigeminal (V) but not the facial/vestibulocochlear (VII/VIII) ganglia. (F) A ventricular view shows negligible caudal spread from r1. Ventral midline, (vml) is left, while the rhombic lip (rl), fringe (f) and roofplate (rp) are to the right. (G) At E7, Hoxa2 is excluded from the cerebellum. (H) In the ventricular layer at E6, the anterior limit Hoxa2 matches the r1/r2 boundary identified by grafting. Scale bar for F, H, 300 µm.

MATERIALS AND METHODS

Quail-chick chimaeras

The metencephalon of E2 (Hamburger and Hamilton stages 10 to 11: Hamburger and Hamilton, 1951) Japanese quail embryos (Treslow Farms, Chester Town, Maryland) was isolated in vitro and treated with dispase (1 mg/ml in L15 for 15 minutes). Mesoderm and ectoderm.
were removed and grafts prepared from either the left or right side of r1 (Fig. 1A) using flame-sharpened tungsten needles. Different graft types were prepared according to their dorsoventral extent (Fig. 1B): whole (0°-180°), dorsal (0°-10° to -50°), subdorsal (50°-180°) and ventral (120°-180°). Host chicken eggs (Spafas Inc., Preston, Connecticut) were then windowed using sharp scissors and the prepared quail tissue orthotopically grafted into the neural tube of stage-matched hosts. Eggs were re-sealed with ‘Scotch’ tape and incubated for a further 1-6 days at 37°C, before fixation in either 3.5% paraformaldehyde in 0.1 M PBS (PF) or Dent’s fixative (1:4 dimethylsulphoxide:methanol) overnight at 4°C.

**Proliferation assay**

Chicken eggs were windowed at E3 and re-sealed to prevent the formation of blood vessels over the roof of the egg. At E6, the window was re-opened and approximately 6 μl of bromodeoxyuridine (BrdU: 15 mg/ml in sterile water) was injected via a glass micropipette into a vein within the chorioallantoic membrane. Eggs were re-sealed and re-incubated at 37°C for 30 minutes prior to fixation in PF.

**DiI-labelling of cerebellar explants**

Chicken or quail embryos were decapitated at E3-6 and the cerebellar plate rapidly dissected away from surrounding tissue. A fine glass rod coated with either 1,1’-didodecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate (DiIC12) or FM-DiI (Molecular Probes Inc., Eugene, Oregon) was used to focally label the rhombic lip by briefly applying its tip (1-2 seconds) to the ventricular surface. The isolated cerebellum was embedded in collagen (Vitrogen 100, Collagen Bio-Materials, Palo Alto, California), pial side downwards, in a culture dish with glass coverslip base. The gel was submerged in a physiological medium adapted from Ard et al. (1985) and maintained at 37°C and 5% CO₂ for 1-2 days prior to fixation in PF.

**Histochemistry**

Brains and cerebellar explants were processed for immunohistochemistry using a standard whole-mount protocol (Lumsden and Keynes, 1989). Chimaeras fixed in PF were labelled using the quail-cell-specific, perinuclear marker QePN (Developmental Studies Hybridoma Bank, Iowa City, Iowa) and FITC- or peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania). Some were vibratome-sectioned at 100 μm. Brains fixed in Dent’s fixative were double-labelled with QePN and the quail-specific neuronal antibody, QN (Tanaka et al., 1990), and FITC-conjugated and Cy3-conjugated secondary antibodies, respectively. BrdU-labelled cells were identified by a monoclonal antibody (Bio-Science Products AG, Emmenbrücke, CH) and a Cy3-conjugated secondary antibody. Quail explants were labelled with the endothelial and quail cell-specific monoclonal antibody, QH1 (Hybridoma Bank) and a FITC-conjugated secondary antibody. PF fixed tissue was cleared in PBS/glycerol (1:10) with 2.5% 1,4-diazabicyclo[2.2.2]octane and 0.02% sodium azide. Tissue treated in Dent’s fixative was dehydrated through a methanol series (50%, 70%, 100%, 100%) and cleared in 1:2 benzyl alcohol/benzyl benzoate. Some brains were processed for in situ hybridisation using *Hoxa2* digoxigenin-labelled riboprobes as previously described (Wingate and Lumsden, 1996) or triple-labelled with *Hoxa2* (digoxigenin), *Otx2* (digoxigenin) and *Gbx2* (FITC)-labelled riboprobes.

**Photomicrography and time-lapse imaging**

Images were captured using a SPOT camera (Diagnostic instruments Inc., Sterling Heights, Michigan) or a confocal, inverted microscope.
(MRC 600: BioRad, Welwyn, UK). Some images were enhanced by ‘seed-fill’ filtering for contiguous structures using VoxelView software (Vital Images Inc., Minneapolis, Minnesota) running on a Silicon Graphics Indigo workstation. For time-lapse confocal photomicroscopy, laser output was set at 10% of maximum. Sequential photomicrographs were assembled into films using TweenMachine software (Nick Didkovsky, Rockefeller University) and Voxel Animator (Vital Images).

RESULTS

Determination of the caudal boundary of the cerebellum

To assess the caudal limit of the cerebellum in terms of the segmental organisation of the early neural tube, we fate mapped the r1/2 boundary to E6. Quail-chick chimaeras were constructed at E2 by the orthotopic transplantation of sections of neural tube from quail donor to chick host in ovo. At this age, r1 and r2 are still fused into a single vesicle, the metencephalon, and are not divided by a recognisable segmental constriction. Hence, we used gene expression to define r1 territory (Fig. 1A). From at least stage 5 (E. Bell and R. J. T. W., unpublished observations), r1 is visible as a region that is positive for Gbx2 (Shamim and Mason, 1998) lying between the expression domains of Otx2 (Millet et al., 1996) and Hoxa2 (Prince and Lumsden, 1994). By these criteria, we estimated that r1 occupies the rostral two thirds of the metencephalon at stage 10-11. All grafts were unilateral and chimaeras that showed developmental asymmetry were discarded (n=30/107). The distribution of quail cells was assessed using the quail-specific nuclear marker, QePN.

We first confirmed that the chosen caudal boundary of presumptive r1 grafts and the anterior boundary of r2 grafts mapped to the r1/2 boundary. A number of chimaeras were hence examined at E3 or E4 (n=13) prior to the loss of hindbrain segmentation. Fig. 2A shows a flat-mounted hindbrain in which the anterior limit of an r2 graft, derived from Hoxa2 expression, corresponds to the emergent r1/2 boundary. Rostral to this boundary, r1 gives rise to neural crest derivatives in the ophthalmic lobe of the trigeminal ganglion and surrounding the trochlear and oculomotor nerves (Fig. 2B).

By the time that the presumptive cerebellum is distinct (E6), overt rhombomeric boundaries have disappeared. Hence to assess the r1/2 boundary, a number of chimaeras were processed for in situ hybridisation for Hoxa2 (n=13). At E6, the r1/2 graft boundary continues to map to the anterior limit of Hoxa2 expression (Fig. 2C). There appears to be no significant rostral migration of r2 cells into r1. Unilateral, whole r1 grafts (n=7) also indicate that the r1/2 boundary maps to the caudal edge of the cerebellar plate (Fig. 2E) and reveal that there is no contralateral cell migration between presumptive cerebellar hemispheres. Within the proliferative, ventricular layer (Fig. 2F), r1-derived territory has a sharp caudal boundary at the lateral angle of the fourth ventricle (the embryonic ‘rautenbreite’: His, 1890). The anterior limit of Hoxa2 expression lies at the caudal boundary of the cerebellum in whole mounts (Fig. 2G) and, in the ventricular layer (Fig. 2H), appears complementary to the caudal boundary of r1-derived precursors (Fig. 2F).

These grafts enabled us to define the regional origin of the cerebellar rhombic lip within an E2 embryo for subsequent grafts of dorsoventrally defined quail neural tube sections. The rhombic lip was designated as comprising the lateral edge of the flattened cerebellum and hindbrain (blue shading in Fig. 2D). The cerebellar rhombic lip is distinguished by a fringe of densely packed cells that lie in close contact to the roofplate (Fig. 2F). At high power (Fig. 3A), a decreasing packing density of progressively more dorsal quail cells can be seen to determine the transition from neural tube precursor cells in the rhombic lip through the fringe population to the epithelial cells of the roofplate. Analysis of short-term BrdU incorporation in E6 cerebellum revealed that both the lip and the fringe contain a high density of cells in s-phase (Fig. 3B).

Rhombic lip gives rise to migratory EGL precursors

Orthotopic grafts were used to determine both the dorsoventral origin of the cerebellar rhombic lip and its derivatives. Restricted grafts (of variable rostrocaudal extent within r1 territory) were made of the dorsalmost neural tube at E2 (Fig. 1B) and analysed at E6 (stage 27-30). The range of derivatives varied systematically with the extent of graft (Table 1). All dorsal grafts gave rise to both roofplate cells and neural crest. In a few cases (n=3/20), there were no other derivatives apart from these two populations. These chimaeras were characterised by donor cells that lay within the roofplate (Fig. 4A), failing to spread into the neural tube (Fig. 4B) and contributing only to neural crest derivatives (Fig. 4C).

If a dorsal graft was sufficiently large to encompass both roofplate and rhombic lip (n=17/20) then two, distinct additional populations of neural derivatives were always generated: a dorsal pial layer constituting the EGL and a population of ventral cells close to, but not abutting the midline (Table 1). In both cases, these derivatives must reach their final positions by migration from a pool of precursors lying at the interface between neural tube and roofplate; the rhombic lip. These migrations take place outside the ventricular layer. A high-magnification view of the pial side of the graft boundary reveals streams of cell nuclei that appear to bud from the edge of the graft (Fig. 4E). By contrast, within the ventricular layer of the same graft, neuroepithelial precursors break away from the body of the quail graft and move ventrally in a far more restricted manner (Fig. 4F), consistent with mixing as opposed to active migration (Wingate and Lumsden, 1996). Larger grafts reveal an orderly organisation of migratory streams reflected in their fasciculated appearance (Fig. 4G,H).

Table 1. Fates of quail cells from different types of r1 graft (Fig. 1) in E6 chimaeras

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Whole</th>
<th>Dorsal</th>
<th>Subdorsal</th>
<th>Ventral</th>
</tr>
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<tbody>
<tr>
<td>graft type</td>
<td>Roofplate</td>
<td>Neural crest</td>
<td>EGL</td>
<td>Ventral cells</td>
</tr>
<tr>
<td>Whole</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Dorsal</td>
<td>+</td>
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<td>Subdorsal</td>
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<td>Ventral</td>
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Graft types were scored for the presence (+) or absence (−) of derivatives in four different tissues within r1; roofplate of the fourth ventricle, neural crest (in cranial nerve roots), EGL and ventral postmitotic neurons. Number of chimaeras = n.
Despite only a narrow isthmus of roofplate separating hemispheres, graft-derived cells never crossed the dorsal midline (Fig. 4H). Streams emerge from all points along the curved edge of the cerebellar rhombic lip, perpendicular to its interface with the roofplate and merge into a distal accumulation of cells at the rostroventral edge of the anlage. In Fig. 4I (arrow), this accumulation of candidate EGL precursor cells can clearly be seen when the chimæra in Fig. 4D is divided at the dorsal midline and flat-mounted (see Fig. 2D). No EGL was observed in embryos at stage 19 or younger and distinctive pial migrations were first observed at stage 20.

Rhombic lip also gives rise to ventral neurons

In addition to contributing to EGL, a small number of cells follow a circumferential path and condense ventrally. In every chimæra where a dorsal graft gave rise to EGL precursors, ventral derivatives were also found (Fig. 4I, asterisk; Table 1). The identity of these cells was investigated using a quail-specific neuronal marker QN (Tanaka et al., 1990). Fig. 5 shows a confocal micrographic montage of a chimæra in which quail cells derived from a dorsal graft were fluorescently double-labelled with QN (red) and Q¢PN (green). The perinuclear marker, Q¢PN, labels all graft derivatives. These consist principally of a carpet of EGL precursors overlying the cerebellum and a population of ventral cells that spread rostrocaudally to form a longitudinal nucleus with a pronounced, medially displaced, caudal tail. The nucleus lies lateral to, but not abutting, the ventral midline. In addition, QN reveals at least three populations of graft-derived axons. Ventral cells give rise to a longitudinal axon tract extending rostrally into the midbrain and caudally into the hindbrain (Fig. 5a). Weakly labelled contralaterally projecting fibres are visible at the midline (Fig. 5b). Arising from the body of the graft itself, ventral to the rhombic lip, a group of fasciculated axons project first ventrally and then caudally towards the hindbrain (Fig. 5c). At higher magnification (inset), no specific association between graft-derived dorsoventrally projecting axons and pial migratory precursor cells could be seen.

Contact with rhombic lip correlates with generation of migratory streams

We transplanted sections of r1 that excluded the most dorsal segment of the neural tube (subdorsal grafts: Fig. 1B) to determine whether contact with the rhombic lip is required to generate EGL. In half of chimæras receiving subdorsal r1 grafts (n=5/10), quail precursor cells failed to spread far enough dorsally to contact the roofplate (Fig. 6A). In these cases, no migratory, graft-derived EGL precursor cells were visible on the pial cerebellar surface in either flatmounts or coronal sections (Fig. 6B). By contrast, if quail cells mixed sufficiently with host tissue to come into contact with the rhombic lip (Fig. 6C, asterisk), then labelled cells were also present in the EGL (Fig. 6C, arrow). Significantly, even where graft-derived cells abutted the roofplate, no neural crest was generated from any subdorsal graft (Table 1).

Precursor cells derived from grafts of the ventral third of r1 never spread to contact rhombic lip and do not give rise to EGL (n=9; Table 1). Donor and host cells in ventral neural tube (Fig. 6D, arrow) intermingled less readily than at graft interfaces in dorsal neural tube (Fig. 6E, arrow). In Fig. 6D, the distribution of ventral quail neurons (blue dots) is superimposed on the distribution of grafted precursor cells (brown label) from which they arose. Postmitotic cells have spread rostrocaudally to form a longitudinal domain directly adjacent to the floorplate in r1. Ventrally derived postmitotic cells were never observed in dorsal neural tube.

Rhombic lip derivatives have a distinct morphology and migratory behaviour

We focally DiI-labelled the rhombic lip of E5 cerebellar explants to investigate the morphological heterogeneity of migrating cells and their mode of migration. In particular, we wished to determine whether the orderly and directed streaming of precursors away from the lip reflected either a structured glial or axonal substratum, or the ‘chain’ migration exhibited in other granule cell populations (Wichterle et al., 1997). Explants were cultured for 24-48 hours and then cell dispersal was analysed by confocal microscopy. In all cases, a variable number of small unipolar cells with a single leading process could be observed streaming away from the injection site on the pial surface of the explant. In Fig. 7A, a labelled population of cells has been reconstructed from a series of optical sections. Shown, inset, a single migrating cell has been digitally isolated from background label by a seed-fill algorithm. The majority of cells were oriented such that their single process projected directly away from the rhombic lip. We failed to label any underlying scaffold of orientated glial or axonal processes in any of the explants. Nor did we see any evidence of the homotypic binding that characterises chain migration. To confirm that unipolar cell profiles were unique to rhombic lip-derivatives, we compared the pattern of labelling following dye application to the rhombic lip (Fig. 7B) and to slightly more ventrally located precursors (Fig. 7C) within a number of explants. The latter never gave rise to migratory, unipolar cells. The dorsoventral specificity of their origin, orderly directional migration and restriction to the pial surface made these cells strong candidates for EGL precursors identified in quail-chick chimæras.

DiI injections also revealed a second population of larger, rounded cells deeper within the mantle layer (Fig. 7A, asterisk), which readily disperse rostrocaudally and exhibit an amoeboid appearance. Differences in the mode of migration of labelled cells was revealed by confocal time-lapse photomicroscopy. Larger, amoeboid cells show rapid movement in various directions and apparent phagocytic behaviour reminiscent of CNS macrophages (Fig. 7D). To confirm their identity, we therefore labelled quail explants with the quail-specific antibody, QH1, which specifically binds to cells with an endothelial and haemopoetic lineage (Pardanaud et al., 1987) and identifies macrophages and microglia (Cuadros et al., 1993, 1997). In an explant cultured for 24 hours, QH1 reveals blood vessels and two distinct, rostrocaudally aligned ranks of macrophages (arrows). Close to the rhombic lip, the outer rank of cells is disrupted at the point where applications of DiI have been made (asterisks). When rhombic lip derivatives labelled with a fixable form of DiI (FM-DiI) are counter-stained with QH1 (Fig. 7F), it is clear that a number of macrophages (initially outside the rhombic lip) have incorporated the dye (which, by contrast to DiI, is retained in vesicles rather than cell membranes). The size and distribution of double-labelled cells are reminiscent of the amoeboid population labelled by DiI injection in chick
explants. Double-labelled cells displaying a primitive, dendritic, microglial morphology (Cuadros et al., 1997) were also observed distant to the injection site (Fig. 7F, inset). This suggests that amoeboid macrophages phagocytose damaged cells within the injection site and subsequently develop into cerebellar microglia.

**DISCUSSION**

We have investigated the origins of precursors of cerebellar granule cells in the chick using a combination of fate-mapping techniques and direct cell-labelling in cerebellar explants. We have demonstrated that the caudal limit of the cerebellar anlage, at the time of EGL precursor generation (E6), maps to the r1/2 boundary as defined by the expression of Hoxa2. We also show that precursors of the EGL arise at the rhombic lip apparently in response to local, lineage-independent factors that operate at the interface between neural tube and roofplate. Generated alongside EGL precursors are neurons that migrate to ventral neural tube and which extend axons to the hindbrain, spinal cord and contralateral ventral r1. Finally, Dil-labelling in explants reveals that the rhombic lip-derived EGL precursors have a distinct, unipolar morphology and undergo an orderly migration diametrically away from the lip. This migration appears to be independent of glial or axonal substratum guidance or chain migration.

**Induction of EGL precursors**

Our grafting experiments reveal that EGL precursors are generated initially at all points along the cerebellar rhombic lip which itself is derived from dorsal rather than caudal cerebellar anlage (Miale and Sidman, 1961; Altman and Bayer, 1978, 1985, 1997; Hallonet and Le Douarin, 1993; Ryder and Cepko, 1994). Moreover, the EGL originates at the interface between roofplate and neural tube: roofplate precursors (dorsal to the rhombic lip) give rise to neural crest but not EGL (Fig. 4B), while neuroepithelium ventral to the rhombic lip (Fig. 6A) produces neither neural crest nor EGL precursors.

Despite apparent similarities in the origin and behaviour of neural crest and EGL precursors, we demonstrate that these two temporally distinct populations of dorsally derived migratory precursor cells do not share a common lineage. Following subdorsal grafts, in half of the resulting chimaeras, quail precursors reach the rhombic lip by random mixing with host cells where they generate EGL precursors (Fig. 6C), but never neural crest (Table 1). From this, we conclude that quail precursor cells only come into contact with the roofplate after the temporal window for neural crest production has closed. This suggests that granule cell precursors may be induced by local, lineage-independent mechanisms at the rhombic lip subsequent to E3. Such a model is consistent with the ongoing involvement of dorsalising factors such as BMPs in granule cell induction (Alder et al., 1999) well beyond their role in early CNS patterning (Lumsden and Krumlauf, 1996) and neural crest production (Baker and Bronner-Fraser, 1997).
The identification of the rhombic lip as the source of granule cells is consistent with in vitro (Alder et al., 1996) and descriptive studies that identify a number of rhombic lip markers as characteristic of a granule cell lineage (Zic-1; Aruga et al., 1994; Math-1: Akazawa, 1995; Ben-Arie et al., 1996; RU49; Yang et al., 1996). Targeted mutation of such rhombic lip markers leads to either a partial (Zic-1: Aruga et al., 1998) or complete loss of granule cells (Math-1: Ben-Arie et al., 1997). However, this simple model is apparently contradicted by a previous dorsoventral fate map of E2 cerebellar territory, which demonstrated that EGL precursors arise from a broad dorsoventral extent of neural tube at E2 (Hallonet and Le Douarin, 1993). Only grafts of the entire dorsal two thirds of the neural tube were sufficient to subsequently label all granule cells at E15, suggesting that there is no spatially defined dorsal origin for EGL precursors. We suggest that this anomaly can be explained by the large degree of mixing between precursor cells of the dorsal neural tube after grafts are performed at E2 (Fig. 6E; also, Clarke et al., 1998). Such mixing implies that mitotic progeny of even a relatively ventrally located precursor cell at E2 might reach the rhombic lip by E5. Hence, although the rhombic lip is a dorsally induced structure, at E2 only the ventral third of r1 is definitively excluded from contributing cells to the EGL (Fig. 6D; also, Hallonet and Le Douarin, 1993). This map of potential fates is shown schematically in Fig. 8A. It is unclear...
is whether such de facto lineage restriction from E2 reflects the early specification of ventral r1 to a non-cerebellar fate.

The migration of rhombic lip derivatives

We have shown that EGL precursors display a highly ordered migration directed away from the rhombic lip (Fig. 8B) and accumulate, initially, at a point furthest away from their origin. The initial direction of migration is perpendicular to the axis of the rhombic lip and hence, in terms of major CNS axes, varies systematically with the curve of the edge of the cerebellar plate. EGL precursors undergo active migration (Hausmann and Sievers, 1985; Ryder and Cepko, 1994) as opposed to a ‘passive sprawl’ (Altman and Bayer, 1997). Candidate granule precursor cells have a characteristic unipolar morphology reminiscent of both migrating cells visualised deep in the EGL considerably later in development (Ryder and Cepko, 1994) and tangentially migrating cells in other CNS regions (O’Rourke et al., 1997). A second labelled population of larger, highly motile, amoeoboid and apparently phagocytic cells are positive for the glial marker, QH1, suggesting that they are macrophages scavenging dying cells from around the labelling site (Cuadros et al., 1993, 1997).

Our direct observations of precursor migration help to resolve the question of how the EGL is initially assembled. Previous models have attempted to reconcile the proposed origin of its precursors at the rhombic lip (a caudal and medial structure) with the classical observation that the EGL appears first laterally (Herrick, 1894; Hanaway, 1967), ‘creeping [rostro-medially] over the cerebellar surface like a veil’ (Bonnevie and Brodal, 1946). The majority of migration was hence assumed to be caudolateral to rostromedial, in parallel with the development of the layer as a whole. This model required that all cells were induced at a single point, the ‘caudolateral angle’ of the rhombic lip (Fig. 8C; Miale and Sidman, 1961). Other models have proposed that coverage is achieved by a caudostral migration sometimes coupled with a tangential, predominantly lateromedial dispersion (Fig. 8D; Hynes et al., 1986; Ryder and Cepko, 1994; Hatten and Heintz, 1995; Altman and Bayer, 1997). We show here that two different axes of dispersal are not required to explain the initial lateromedial development of the EGL. Indeed, the origin of granule cells most closely conforms to their predicted point of production in the earliest model described – that of Schaper’s (1897, adapted by Harkmark, 1954a; Fig. 8E). The orientation of the ‘creep’ of the EGL is almost diametrically opposed to the actual direction of precursor migration and appears to result from the initial preferential accumulation of actively migrating cells distal (lateroventral) to their origin at the rhombic lip. We predict that subsequent waves of migrating cells stop at successively more proximal points on the surface of the anlage, perhaps inhibited from further migration by the accumulation of older precursors.

The orderly migration of rhombic lip derivatives suggests a precise guidance system that is apparently independent of structural substratum cues. Direct Dil-labelling of the rhombic lip fails to reveal an underlying glial or axonal (Hynes et al., 1986) fibre scaffold that might direct migration. Similarly, bundles of axons arising from neurons generated close to the rhombic lip (possibly of the fastigial nucleus) did not appear to be closely associated with migratory precursors. The fasciculated appearance of migratory streams (Fig. 4G,H) suggests an affinity between precursors (Hausmann and Sievers, 1985) and might also be indicative of ‘chain’ migration. However, Dil-labelling did not reveal specific homotypic binding between migrating granule cells, correlating with the lack of such behaviour in vitro (Wichterle et al., 1997).

In the absence of a structural scaffold underlying the orientated migration of EGL precursor cells, a molecular guidance system might be involved in the form of either diffusible or substratum-bound cues. Of the various possibilities, a roofplate-derived chemorepellant, possibly BMP itself, would provide a parsimonious explanation for the initial direction of cell migration but would not explain the long-range, ventral targeting of derivatives. By contrast, a diffusible, ventrally derived chemoeffectant seems unlikely as a source of guidance at later stages, given the geometry of precursor migration (perpendicular to a curving rhombic lip), the changing surface of the cerebellum over time and the considerable distances involved. Similarly, a co-ordinate system of substratum molecular cues (either membrane-bound or secreted) would have to be maintained against a background of considerable cell mixing, rapid growth and morphogenetic transformation. Alternatively, substratum guidance cues might be generated by precursors themselves (Hatten et al., 1982), although such a mechanism would seem unlikely to account for the apparent precision of migration and would not account for the initial trajectory of migration. Ultimately, a combination of such systems might be required, possibly varying in their contributions as migration paths are established and then maintained during cerebellar morphogenesis.

Identity and significance of ventrally migrating cells

Large-scale ventral migration of neurons from the dorsal rhombencephalon was first proposed by His (1890) and has recently been directly demonstrated in chick using quail-chimaera fate maps (Tan and Le Douarin, 1991) and lineage analysis by retrovirus (Hemond and Glover, 1993) or dye injection (Clarke et al., 1998). However, only indirect evidence, through ablation (Harkmark, 1954b) or birthdating studies (Altman and Bayer, 1987), supports His’ proposal that the rhombic lip itself is the origin of streams of migratory cells. Our observations provide the first direct evidence that the induction of a population of ventrally migrating neurons occurs precisely at the rhombic lip. These neurons are always generated alongside EGL precursors suggesting either an unusual lineage relationship between two very distinct neuronal phenotypes or the coexistence of separate precursor pools within the cerebellar rhombic lip.

Ventral derivatives of the rhombic lip appear to condense in the position of the lateral pontine nucleus (Brodal et al., 1950; Harkmark, 1954b; Armstrong and Clarke, 1979; Tan and Le Douarin, 1991). Their position is consistent with the classical description of the nucleus as a subpial condensation, which is lateral to the midline, becoming more medial at its caudal pole where it may merge with the medial pontine nucleus (Brodal et al., 1950). Longitudinal axonal projections from the neurons both rostrally and caudally confirm this identification (Fig. 5). The lateral pontine nucleus also sends mossy fibres ipsilaterally to the cerebellum (Brodal et al., 1950; Harkmark, 1954b), although we failed to immunolabel...
these projections at these stages. Ipsilaterally projecting mossy fibres synapse on granule cells throughout both hemispheres (Armstrong and Clarke, 1979). Surprisingly, r1 rhombic lip derivatives never contributed contralaterally to the medial pontine nucleus whose origins from dorsal neural tube have been described in chick (Harkmark, 1954b; Tan and Le Douarin, 1991). Possibly, medial pontine neurons migrate both circumferentially and anteriorly into r1 territory from more caudal rhombomeres.

The shared origin of both granule cells and precerebellar nuclei at the rhombic lip might help to explain mechanisms underlying subsequent development of connectivity. We show that lateral pontine neurons, which form long range synaptic connections with cerebellar granule neurons, are derived from the same spatially defined neuroepithelium as their axonal target population. A common lineage within such a neuroepithelium might generate a shared repertoire of cell surface molecules that facilitate the subsequent development of appropriate long-range connectivity.

The r1/2 boundary and the caudal cerebellum

The first fate maps of the avian cerebellum revealed that the anlage spans the midbrain/hindbrain isthmus at E2 (Hallonet et al., 1990; Alvarez Otero et al., 1993). This indicated, perhaps surprisingly, that cerebellar boundaries did not map to any definitive embryonic structures. Recently, a very precise fate map of the isthmus revealed that the constriction itself migrates rostrally, after E2, only coinciding with the caudal limit of expression of the midbrain marker, Otx2, after E3 (Millet et al., 1996). Hence the rostral limit of the cerebellum lies at the caudal edge of Otx2 expression: but the structural isthmic constriction, taken as a grafting landmark in previous fate maps, does not assume its definitive position until later in development. In a complementary experiment, we have used molecular data to define the initially cryptic anterior boundary of r2 and show, by fate mapping to E6, that cells from this segment do not migrate rostrally into r1. At this age, the r1/2 boundary maps to the caudal boundary of the cerebellum. A previous fate map has indicated that r2 contributes to the cerebellum at E10 (Marín and Puelles, 1996) suggesting either that an anterior migration takes place from E6-10, or that our derivation of r2 territory by Hoxa2 expression (confirmed at E3 and E6) is more accurate than previous estimates. The latter explanation is supported by previous results indicating that Hoxa2 is not expressed in the cerebellum at E10 (Wingate and Lumsden, 1996). However, an absence of r2 neurons from the definitive avian cerebellum can only be confirmed by detailed analysis of older chimaeras. Considered alongside the published isthmic fate map (to stage 20: Millet et al., 1996), our observations raise the possibility that the cerebellum is derived entirely from r1.

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