Temporal identity transition in the avian cerebellar rhombic lip

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Abstract

The rhombic lip is a discrete strip of neuroepithelium bordering the roofplate of the fourth ventricle, which gives rise to a defined sequence of migratory neuronal derivatives. In rhombomere 1 of the chick, early born cells give rise to post-mitotic hindbrain nuclei, while later derivatives comprise of cerebellar granule cell precursors, a unique proliferative, migratory precursor population that forms the external granule cell layer. We have examined the temporal specification of these two populations using a heterochronic grafting strategy, in ovo. When transplanted into younger neural tube, rhombic lip cells maintain their characteristic molecular markers and migrate into the hindbrain. Granule cell precursor derivatives of late grafts are, in addition, able to exploit neural crest streams to populate the branchial arches. Within the neural tube, derivatives of early and late rhombic lip progenitors display patterns of migration and process extension, characterised by specific trajectories and targets, which are consistent with their temporal origin. However, the normal temporal progression of cell production is disrupted in grafted progenitors: transplanted early rhombic lip fails to subsequently produce granule cell precursors. This indicates that, while the behaviour of derivatives is intrinsically specified at the rhombic lip, the orderly temporal transition in cell type production is dependent on extrinsic cues present only in the later embryo.

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Introduction

Cellular diversity is generated by a combination of positional and temporal cues. Discrete progenitor domains within the ventricular layer are allocated by a Cartesian coordinate system based on the embryonic dorsoventral and rostrocaudal axes (Lumsden and Krumlauf, 1996). Spatially defined progenitor populations then give rise to different neuronal populations over time — a process which adds an extra dimension of complexity and flexibility in the generation of neuronal diversity. This temporal dimension in cell fate determination has proved extremely difficult to investigate in vertebrate systems due to the paucity of suitably simple experimental models. Previous studies have defined an effective experimental approach to unravelling the factors involved in temporal transitions in a defined progenitor pool. Specifically, temporal patterning of cell fate can be understood by calibrating the relative contributions of intrinsic and extrinsic factors to specification. One way of doing this is by in vitro isolation of progenitors: for example in the vertebrate retina, progenitor heterogeneity, microenvironmental interactions and an internal cell division clock have all been shown to contribute to temporal fate determination (Pearson and Doe, 2004). An alternative in vivo approach is heterochronic grafting, whereby progenitors are challenged with a defined older or younger environment. This strategy has been instrumental in demonstrating both the principle of temporal specification in cell fate determination in the cortex (McConnell and Kaznowski, 1991) and in revealing the changing potential of vertebrate neural progenitors (Baker et al., 1997; Frantz and McConnell, 1996; Rapaport et al., 2001).

From these studies, it has also become clear that the ideal experimental model would comprise of an easily isolated homogeneous progenitor pool, which shows a characteristic order in the production of discrete cell populations. In this respect, the embryonic rhombic lip provides an excellent system in which to examine the temporal patterning of neurogenesis. It comprises a spatially discrete precursor pool, which lies at the interface between dorsal neural tube and non-neuronal roofplate of the fourth ventricle (Wingate, 2001). The upper or cerebellar rhombic lip is derived exclusively from rhombomere (r)1 (Wingate and Hatten, 1999) and...
comprises a pool of homogeneous dividing cells that is easily isolated by microdissection (Alder et al., 1996; Alder et al., 1999). It gives rise to a unique population of migratory neural precursors, the cerebellar granule cell precursor, which forms a secondary germinal layer, the external granule cell layer (EGL) over the surface of dorsal r1. Recent studies have shown that the rhombic lip in r1 also gives rise to small populations of post-mitotic derivatives that migrate into the ventral, non-cerebellar neural tube in chick (Wingate and Hatten, 1999), zebrafish (Köster and Fraser, 2001) and mouse (Machold and Fishell, 2005; Wang et al., 2005). These populations comprise a variety of spatially distributed nuclei that are functionally interrelated components of the proprioceptive system (Wang et al., 2005). Furthermore, different populations are generated in a strict sequence with non-overlapping birthdates, where granule precursors are the final population to be generated (Gilthorpe et al., 2002; Machold and Fishell, 2005; Wang et al., 2005). This combination of characteristics makes the rhombic lip in r1 an ideal model for the study of temporal succession in neurogenesis. In particular, the transition from the production of post-mitotic derivatives to a population of committed precursors (Alder et al., 1996), which will subsequently undergo a phase of tangential migration and further rounds of cell division, represents an intriguing and important question in developmental neurobiology.

In chick, the temporal transition that initiates granule cell precursor production takes place between embryonic day (E)5 and 6 (Gilthorpe et al., 2002). In this study, we therefore investigated the differences between the intrinsic patterns of development in rhombic lip derivatives at E4 and E6. These populations are morphologically distinct: E4-derived neurons migrate into ventral r1 and extend axons rostrally and caudally along the ventral midline, E6-derived cells extend short transient leading processes that populate dorsal r1 (Gilthorpe et al., 2002). We transplanted microdissected rhombic lip into E2 dorsal neural tube. This approach allows the behaviour of donor cells from different stages of rhombic lip maturation to be contrasted against a defined host environment: differentiated cells and their associated axon scaffold are absent, but diffusible migration cues are expressed in their characteristic domains. Chimaeras were analysed using molecular markers and a detailed assessment of cell morphology and migration. We find that derivatives of the rhombic lip progenitor pool display temporally specified intrinsic programmes of development. However, extrinsic cues, which are absent in heterochronic chimaeras, are specifically required for the temporal transitions within this progenitor pool that generate neuronal diversity.

Materials and methods

Microsurgical construction of chimaeras

Quail and chick embryos were incubated to provide donor tissue for heterochronic/heterotopic grafts. At E2 chick, eggs were windowed and RCASBP(B)-egfp plasmid (1 mg/ml in water containing 0.0015% Fast Green) was injected into the rostral hindbrain. One to three 50 ms/10 V square waveform electrical pulses were passed between electrodes placed ventral and dorsal to the neural tube. Eggs were resealed and re-incubated. Electroporated donor chick embryos were harvested at either E4 or E6. Quail donor embryos were harvested at E6 only. Rhombomere 1-derived rhombic lip fragments were dissected away from donor hindbrain tissue in Tyrode’s solution using flame-sharpened tungsten wire. A single fragment of quail (Fig. 2A) or GFP labelled chick (Fig. 3A) rhombic lip was then transplanted into the dorsal segment of rhombomeres 1, 2, 3, 4 or 5 of E2 host embryos in ovo (Wingate and Lumsden, 1996). In a number of control embryos, non-rhombic lip donor tissue was used, which comprised fragments from either the cerebellum that lies adjacent to the rhombic lip or from the dorsal midbrain. A separate group of microsurgical chimaeras was constructed in which donor rhombic lip fragments were transplanted into the post-optic mesoderm of st.13 chick host embryos. Chimaeras were incubated for up to a further 8 days before harvesting and fixation in 4% paraformaldehyde in 0.01 M phosphate buffer (PFA) at 4°C.

In situ hybridisation and immunohistochemistry

Quail/chick chimaeras were processed for in situ hybridisation with Dig-labelled riboprobes for a number of genes using standard protocols (Myat et al., 1996): Pax6, ErbB4, Zic1, Sox10, FoxD3, Brn3a, Snail, Slug and Hoxa2. Riboprobes for Ptf1a, ROXz and Cath1 were synthesised from sequences (1028O4, 1033L4 and 496O6, respectively) obtained from a published EST database (www.chick.umist.ac.uk). Using standard immunohistochemistry protocols (Lumsden and Keynes, 1989), chimaeras were counterstained using the Q¢PN antibody (Developmental Studies Hybridoma Bank, University of Iowa), which recognises a species-specific perinuclear antigen and a fluorescent Alexa 488 anti-mouse secondary antibody (Molecular Probes). GFP chimaeras were processed for immunohistochemistry with the polyclonal anti-GFP antibody and the Alexa 488 anti-rabbit secondary antibody. These were double-stained with monoclonal antibodies TUJ-1 (Chemicon) or phosphorylated histone H3 (United States Biological) with either anti-mouse or anti-rabbit Alexa 568 secondary antibodies (Molecular Probes), respectively. Late surviving quail/chick chimaeras were paraffin wax embedded and sectioned parasagitally at 5 μm. Alternate sections were processed for immunohistochemistry with either Q¢PN or an anti-Pax6 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa) and a peroxidase-conjugated secondary antibody. Tissue was subsequently counterstained with haematoxylin and eosin.

Imaging

Wholemount chimaeras were digitally imaged on a Leica stereo photomicroscope equipped with epifluorescence. A proportion of these were further dissected (n = 56). The hindbrain was dissected and cut along the dorsal midline and flattened, pial side uppermost in glycerol/PBS (90%/10%) on a slide under coverglass. A number of chimaeras were embedded in gelatin (20% in 0.01 M PBS) and sectioned transversely at 35 μm on a vibratome (Leica). Confocal micrographs of wholemount and flatmounted chimaeras were collected on either an Olympus Fluoview AX70 microscope or a Nikon Diaphot equipped with a BioRad MRC 600 confocal imaging system.

Results

To examine the degree of intrinsic patterning of rhombic lip precursors, we performed a series of heterochronic/heterotopic grafts of rhombic lip from different ages into younger host chick embryos in ovo at E2 (Hamburger and Hamilton stage (st.) 10
The fate of rhombic lip derivatives was analysed below according to their molecular expression, migration paths and morphology. We focused our analysis on cerebellar rhombic lip fragments (from r1) which had been grafted into more posterior rhombomeres. This strategy removed any possible effects attributable to homotypic interactions between donor cells and host r1 derivatives. Heterochronic grafts into younger embryos allowed donor cells to confront a structurally naive environment. At this point in development, neurons within the host rhombencephalon have yet to develop definitive axons (Moody et al., 1989) and there are no characteristic longitudinal tracts. We determined that the diffusible guidance molecules, Slit2 and Netrin1, are nevertheless present within midline structures at this early stage (Fig. 1).

It has been demonstrated previously that migratory rhombic lip derivatives respond to Slit2 (Figs. 1A, B) at dorsal and ventral midlines, which acts as a chemorepellent and Netrin1 (Figs. 1C, D) at the ventral midline only, which can act as a chemoattractant (Alcántara et al., 2000; Gilthorpe et al., 2002). Therefore, while there are no axon tracts in the hindbrain at E2, molecules that are capable of guiding migration are expressed in the same domains as in the later embryo.

**Rhombic lip grafts retain endogenous marker expression profiles**

Rhombic lip fragments were dissected from E6 quail embryos and grafted into the dorsal hindbrain of E2 chick hosts in ovo (Fig. 2A). Resulting chimaeras were allowed to survive for 2 days before molecular analysis by in situ hybridisation with a panel of riboprobes (Table 1) and processing with the anti-quail antibody Q¢PN to identify the donor cells using a fluorescent secondary antibody. Firstly, we confirmed that rhombic lip donor tissue did not include cerebellar cells outside the rhombic lip and did not become caudalised following heterotopic transplantation. Grafted rhombic lip fragments were negative for Ptf1a (n = 7) (Figs. 2B–D), a marker of dorsal, non-rhombic lip precursors (Hoshino et al., 2005), and RORα (n = 5) (Table 1), a marker of young Purkinje cells (Hamilton et al., 1996). Grafts were also negative for Hoxa2 (n = 7) (Figs. 2E–G), which is expressed in neural tube caudal to r1 (Prince and Lumsden, 1994). Correspondingly, rhombic lip transplants expressed characteristic rhombic lip markers: ErbB4 (n = 7) (Figs. 2H–J), Pax6 (n = 6), Cathl (n = 4) and Zic1 (n = 4) (Table 1). In a number of wholemount chimaeras, quail cells appeared to have either been passively expelled from the neural tube or to have migrated into the adjacent branchial arch in a neural-crest-like manner. We hence examined whether rhombic lip grafts had adopted any of the dorsal markers associated with neural crest: Sox10 (n = 3) (Figs. 2K, L), FoxD3 (n = 6) and Brn3a (n = 4) (Table 1). We also examined the expression of neural crest markers specifically associated with delamination: Slug (n = 5) (Figs. 2M–O) and Snail (n = 6) (Table 1). Transplanted rhombic lip cells were negative for all of these markers.

We also performed heterochronic/heterotopic transplants of rhombic lip fragments into post-optic mesoderm to challenge...
the molecular specification of cells with an overtly non-neural environment. At 2 days post-graft, rhombic lip derivatives still express Cath1 (Figs. 2P, Q) and ErbB4 (Figs. 2R, S). Since the resolution of in situ hybridisation is relatively poor at the single cell level, we also stained chimaeras with an antibody to Pax6 (a marker of granule cell precursors and definitive granule cells), which is still expressed in grafted quail cells up to 8 days after grafting (Figs. 2T, U).

**Rhombic lip can integrate into the neural tube and its derivatives are able to enter the peripheral environment**

To examine the behaviour and axonal projections of donor cells, we electroporated donor chick embryos at E2 with the RCAS(B)-egfp plasmid and dissected rhombic lip fragments from survivors at E6. These were transplanted into st.10 host embryos (Fig. 3A), and resulting chimaeras were examined 24 h later at E3. Detailed examination of GFP label in wholemount embryos (Fig. 3B) appeared to indicate successful integration of graft donor tissue into the host neural tube (n = 12). Transverse sections show cells both within peripheral tissue (Fig. 3C, *) and beneath the pia of the neural tube (Fig. 3D, arrow). In many chimaeras (Fig. 3E, J).

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**Table 1**

Presence or absence of rhombic lip, neural crest, delamination, cerebellar and axial molecular markers in heterochronic/heterotopic grafts (* denotes analysis by both antibody and in situ hybridisation)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Label</th>
<th>Expression in transplanted cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cath1</td>
<td>Rhombic lip</td>
<td>+</td>
</tr>
<tr>
<td>Pax6*</td>
<td>Rhombic lip</td>
<td>+</td>
</tr>
<tr>
<td>ErbB4</td>
<td>Rhombic lip</td>
<td>+</td>
</tr>
<tr>
<td>Zic1</td>
<td>Rhombic lip</td>
<td>+</td>
</tr>
<tr>
<td>Sox10</td>
<td>Neural crest</td>
<td>−</td>
</tr>
<tr>
<td>FoxD3</td>
<td>Neural crest</td>
<td>−</td>
</tr>
<tr>
<td>Brn3a</td>
<td>Neural crest</td>
<td>−</td>
</tr>
<tr>
<td>Snail</td>
<td>Delamination</td>
<td>−</td>
</tr>
<tr>
<td>Slug</td>
<td>Delamination</td>
<td>−</td>
</tr>
<tr>
<td>Ptf1a</td>
<td>Non-rhombic lip dorsal r1</td>
<td>−</td>
</tr>
<tr>
<td>RORα</td>
<td>Cerebellar Purkinje cells</td>
<td>−</td>
</tr>
<tr>
<td>Hoxa2</td>
<td>Hindbrain (r2–r8) and spinal cord</td>
<td>−</td>
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</tbody>
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cells that exit dorsally from the neural tube populate adjacent cranial ganglia (Fig. 3F). The location of both populations of derivatives was confirmed by counterstaining with the TUJ-1 antibody against the early neural marker β-tubulin (Fig. 3G). At high power, cells are clearly located within the body of the ganglion (Fig. 3H, *) and co-express the neuronal marker. Monopolar graft-derived cells within the neural tube integrate with host circumferential axons and extend long leading processes consistent with active neuronal migration. Grafts double-labelled for the proliferation marker phosphohistone H3 revealed that donor cells retain the capacity to divide (Fig. 3I). Optical sectioning shows labelled nuclei in G2 of mitosis within the body of the rhombic lip implant (Fig. 3J, arrow).

The dispersal of donor derivatives within the host environment was examined in lateral views of wholemount embryos at various subsequent time points. At 24 h post-transplantation (Fig. 4A; n = 10), cells move ventrally from the graft and extend short leading processes both rostrally and caudally (Fig. 4A′). At 30 h, cells begin to actively migrate both rostrally and caudally (Fig. 4B; n = 14). By 40+ h, there is a pronounced increase in the lengths and numbers of process extensions accompanied by a greater dispersal of cell bodies from the graft tissue (Fig. 4C; n = 34). Such defined cell movements and projections are consistent with the process of cell migration and reflect active responses to different cues presented in the peripheral and central host environment. Cell movement within the periphery is confined to the axial region adjacent to the graft. By comparison, the long projections and rostrocaudal movement of rhombic lip derivatives take place exclusively within the neural tube.

Peripheral migration is specifically enhanced in E6 rhombic lip derivatives

As a control for the potential passive dispersal of graft derivatives (Bronner-Fraser, 1984), we grafted GFP-labelled fragments of dorsal midbrain, or the cerebellar anlage adjacent to the rhombic lip, from E6 donors into E2 hosts. In contrast to rhombic lip tissue, cells from control tissue grafts remain largely at the graft site or as isolated clusters (n = 5) (Fig. 4D). Cells extend only a small number of irregular processes, and a very small minority adopts the polarised morphology of migrating neurons (Fig. 4E). There was no evidence of either

![Diagram of cell migration](image_url)
active migration or the coordinated extension of processes along the axis of the neural tube. This indicates that the ability to migrate is specific, or specifically enhanced, in rhombic lip derivatives. It also suggests that the final distribution of rhombic lip derivatives after transplantation is unlikely to arise from passive expulsion of donor tissue during embryonic growth.

When E6 rhombic lip cells are transplanted into a non-neural environment, they continue to display a capacity to migrate. Derivatives of heterotopic grafts of rhombic lip into post-optic mesoderm of st.13 hosts are able to integrate into the orbit of the eye at E6 (n = 2/6) (Fig. 4F). Migrating cells exhibit a monopolar migratory profile but with leading processes that are randomly oriented (Fig. 4G). By contrast, rhombic lip derivatives both within the neural tube and entering the periphery from dorsal hindbrain display highly ordered processes suggesting that in both cases they encounter and are able to actively respond to a patterned environment.

The ability of transplanted rhombic lip derivatives to exit the neural tube and populate the branchial arches at E6 (Fig. 4H) is severely curtailed in grafts from E4 donors (Fig. 4I). While E4 rhombic lip fragments produce migratory cells which migrate within the neural tube, they rarely exit the host hindbrain. This implies that the generation of derivatives that can exit the neural tube and populate peripheral tissue is a property of a specific population of E6 rhombic lip precursors, which give rise to proliferative granule cell precursors. Furthermore, this selectivity suggests that this process relies on active migration rather than passive sprawl.

**Rhombic lip cells exploit neural crest migration paths when entering the periphery**

A strong candidate for patterning the movement of rhombic lip derivatives in the periphery is neural crest which streams out of the dorsal neural tube at E2. As neural crest production varies between odd and even numbered rhombomeres, this pattern provides an ideal template for testing its role in facilitating peripheral migration of grafted cells: specifically, neural crest production is attenuated in rhombomeres 3 and 5, while streams from rhombomeres 2, 4 and 6 contribute to the 1st, 2nd and 3rd branchial arches, respectively. We grafted E6 rhombic lip into dorsal rhombomeres 2, 3, 4 and 5 and found that derivatives in even numbered segments (n = 28) follow the same pathway as the associated migratory neural crest populations. For example, derivatives of grafts into r2 (Fig. 5A) populate the ophthalmic (Vo) and maxillo-mandibular (Vm) branches of the trigeminal nerve in a pattern that correlates with neural crest migration (Fig. 5B). By contrast, transplants into odd numbered rhombomeres 3 and 5 (n = 10), where neural crest production is reduced, fail to enter adjacent peripheral tissue directly (Fig. 5C) but instead join the more posterior neural crest stream emanating from r4 (Fig. 5D; arrow). Likewise, grafts into r4 (Fig. 5E) make significant contributions to neural crest migratory pathways associated with the facial (VIIth) and vestibulocochlear (VIIIth) nerves and the second branchial arch (Fig. 5F, arrow). The registration between grafted cell migration and neural crest is very precise and reflects active growth rather than passive dispersal. For example, the leading processes of

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**Fig. 5. Rhombic lip cells exploit neural crest migration paths when exiting the neural tube.** Peripheral migration paths of transplanted rhombic lip cells are coincident with neural crest streams exiting the neural tube. (A) Donor E6 GFP-labelled rhombic lip cells transplanted into r2. (B) At higher power, cells can be seen to populate the ophthalmic (Vo) and maxillo-mandibular (Vm) lobes of the trigeminal ganglia of cranial nerve V, emulating the behaviour of neural crest from r2. (C) Derivatives of rhombic lip transplanted into r3 do not enter the periphery directly. (D) Closer examination reveals that cells exit caudally (arrow) to join the neural crest stream associated with r4 and subsequently enter branchial arch (BA) 2. (E) Graft-derived cells in r4 move in parallel with the neural crest stream populating BA2. (F) High power micrographs show cells exiting directly from r4 (arrow) integrated into the VII/VIIIth ganglion. (G) Fine projections follow the path of the chorda tympani into BA1 (arrow). (H) At the distal extremity of the BA2, scattered GFP-labelled graft derivatives maintain their monopolar form.
migrating graft derivatives follow the small rostral stream of neural crest that traces the path of the chorda tympani (VIIth) from second into first branchial arch (Fig. 5G, arrow). Rhombic lip cells are able to migrate deep into the branchial arches of host tissue and retain their characteristic monopolar morphology even at the extremes of their migration path (Fig. 5H).

These results indicate that E6 rhombic lip cells are not only able to survive outside the neural tube but are also able to respond to specific migration cues. The manner in which rhombic lip cells exit the neural tube and subsequently migrate into the periphery appears to rely on neural crest streams. This reveals that the intrinsic migratory capacity of rhombic lip neurons is coupled with a degree of opportunism in pathway selection moderated by the choice of available substrates.

Donor cells in the neural tube share migration and projection paths that are coincident with other longitudinal tracts of the host hindbrain

Derivatives of transplanted rhombic lip tissue extend long leading processes that integrate within host neural tissue (Fig. 4C). Chimaeras were stained with the TUJ-1 antibody to examine the relation of donor cell processes to the neuronal tracts developing within the host neural tube. At E3 (Fig. 6A), TUJ-1 labels the two prominent longitudinal tracts: the medial longitudinal fasciculus (MLF) descending from the interstitial nucleus of Cajal and the lateral longitudinal fasciculus (LLF) extending from the mesencephalic trigeminal nucleus (MTN). In chimaeras collected 24 h post-transplantation, GFP-labelled rhombic lip cells exit the graft site, migrate a short distance and then project processes either rostrally and caudally along the neural tube (Fig. 6B, arrowheads). Descending axons from the MTN are still some distance from the graft site at this stage. However, TUJ-1 staining suggests that the dorsoventral coordinate of this turn corresponds with the laterality of the prospective LLF, which is pioneered by MTN axons (Fig. 6C).

To confirm this observation, we analysed chimaeras collected after 48 h (Fig. 6D). In these embryos, long processes extend in both rostral and caudal directions (Fig. 6E) and are coincident with the TUJ-1-positive axons in the LLF (Fig. 6F). These results indicate that grafted E6 rhombic lip produces cells that follow the same laterality as the future LLF. However, the timing of rostral or caudal extension suggests that the dorsoventral choice of turning point in migrating cells is independent of interactions with the endogenous axon scaffold.

Rhombic lip cells retain age-specific intrinsic programmes of migration within the neural tube

In heterotopic chimaeras, E6 rhombic lip graft derivatives migrate ventrally within the neural tube before executing a precise turn at a consistent dorsoventral laterality with respect to the ventral midline. This is similar to their behaviour within their normal environment of the E6 chick where cells migrate from the rhombic lip of r1 to the ventrolateral edge of the cerebellar anlage where they turn rostrally. This is different to earlier born cerebellar rhombic lip derivatives at E4, which undergo a more ventral tangential migration reaching the ventral midline before processes turn and project both rostrally and caudally. We therefore performed heterochronic transplantation of E4 rhombic lip cells into E2 hosts to assess whether the migration of graft derivatives reflects an endogenous migratory programme.

![Fig. 6. E6 rhombic lip derivatives within the neural tube display migration and projection paths that are coincident with other longitudinal tracts of the host hindbrain.](image-url)

(A) Staining with TUJ-1 shows the longitudinal tracts in a lateral view of a wholemount chimaera at E2 in relation to the transplanted rhombic lip (box). (B) GFP-labelled cells (arrowheads) turn rostrally on exiting the graft. (C) This point corresponds with the dorsoventral laterality of the prospective lateral longitudinal fasciculus (LLF) descending from the midbrain (arrowhead). (D) At 48 h post-graft, a dorsolateral view of a chimaera shows that longitudinal tracts are established throughout the hindbrain axis. (E) GFP-labelled cells (box in D) have migrated ventrally and extended long leading processes both caudally and rostrally (arrowheads). (F) The laterality of process extension and migration of grafted cells coincides with the LLF. MTN, mesencephalic trigeminal nucleus; MLF, medial longitudinal fasciculus; INC, interstitial nucleus of Cajal; V–IX, cranial nerves.
Fig. 7 contrasts wholemount and flatmount preparations of chimaeric hindbrains following the heterochronic transplantation of E4 and E6 rhombic lip into rhombomere 2. The axial location of the graft did not affect the pattern of migration of its derivatives within the neural tube. We find, instead, that migration phenotype varies with the age of the donor tissue. Thus, E4-derived cells migrate exclusively ventrally and the majority extend leading processes to the midline, which then turn both rostrally and caudally \((n = 26)\) (Figs. 7A, B). By comparison, E6-graft-derived cells make only a short ventral migration and then are able to migrate predominantly rostrally, crossing one or more rhombomere boundaries \((n = 60)\) (Figs. 7C, D). Despite this rostralward bias in migration, E6 cells extend long leading processes in both rostral and caudal directions along a consistently more dorsal longitudinal trajectory than E4 graft derivatives. The rostral processes fan out and terminate within r1 (Fig. 7D, arrowhead). By contrast, the rostral processes of E4 cells bypass the cerebellum and continue on a ventral course through the midbrain and into the forebrain (Fig. 7B, arrowhead). The distinct turning and projections of donor E4 versus E6 cells correspond broadly to their behaviour within their normal environment and suggest that they are committed to a defined migration programme by age-specific, intrinsic factors.

Intrinsic migration programmes are shaped by extrinsic factors in the host hindbrain

Detailed examination reveals that the ventral extent of cell migration and process extension amongst E4 grafts shows a degree of variability. In all cases, rostral projections extend directly into the midbrain. However, the longitudinal tracts formed by E4 grafted cells can form at the MLF \((n = 14)\) or at various distances from the floorplate in the ventral half of the neural tube \((n = 6)\). A number of chimaeras show a biphasic laterality \((n = 8)\). Process extension at the MLF is consistently bilateral, although cell bodies never cross the midline. Occasionally, processes run within the floorplate \((n = 2)\) and exhibit multiple re-crossing (Figs. 8A, B).

Rostral processes of E6 cells \((n = 26)\) have an invariant laterality and extend into prospective cerebellar territory of dorsal r1 (Fig. 8C). On crossing into r1, processes execute frequent dorsal and ventral turns as recorded in their undulating trajectory (Fig. 8D). This suggests that process extensions continue in a consistently rostral direction but are confined to a broader dorsoventral corridor within r1 (Fig. 8E), which is itself dorsally expanded when compared to more caudal hindbrain. Projections from E6 cells never extend into the midbrain and processes that reach the isthmus display a sharp dorsoventral turn (Fig. 8C, inset). Rostrally migrating monopolar cells are able to cross multiple rhombomere boundaries before reaching r1 (Fig. 8F). However, boundaries were a favoured route for occasional ventral projections to the midline from E6-derived cells \((n = 5);\) Fig. 8G). This observation echoes previously published work which indicates that boundaries are preferentially permissive for axon growth (Lumsden and Keynes, 1989).

Stereotyped differences in the behaviour of E4- and E6-graft-derived migrating cells were consistent across the vast majority of chimaeras that we examined as flatmounts \((n = 55)\). Similar phenotypes were generated when cerebellar rhombic lip
fragments were orthotopically transplanted into r1 (n = 8, data not shown). It is particularly noteworthy that grafted E4 rhombic lip progenitors never progressed to produce E6-like derivatives. An exceptional E6 graft (n = 1) showed aspects of both phenotypes (Fig. 8H). Cells display ventral projections along the rhombomere 3/4 boundary (Fig. 8I), which turn at the midline to form a bilateral rostral and caudal longitudinal projection. A second population of grafted cells extend rostral processes towards the cerebellum, but appear to be repelled at the r1/2 boundary (Fig. 8H, inset), with the exception for a single leading process bypassing the cerebellar anlage. This suggests that dorsal r1 represents a non-permissive territory to cells with an essentially E4 character. We interpret this hybrid as representing an intermediate phenotype due to age variation in donor tissue. This single example of a hybrid phenotype illustrates the fine temporal balance between dorsoventral pathway selection and target choice in rhombic lip derivatives of different ages.

Fig. 8. Intrinsic migration programmes are shaped by extrinsic factors in the host hindbrain. Flatmount neural tube preparations of chimaeras show a variety of features that indicate an interplay between intrinsic and extrinsic cues. (A) Cells derived from E4 rhombic lip grafted into r4 extend projections to the midline which populate the floorplate. (B) Single axons meander across the midline. (C) E6 cells grafted into r3 project into r1 but are deflected at the isthmus (inset). (D) Processes extending through the cerebellar anlage exhibit an undulating trajectory. (E) “Looping” is specific to r1 and can be most parsimoniously interpreted as the cumulative record of rostral growth within a zone of equivalent dorsoventral positional values. (F) E6 rhombic lip cells retain a monopolar morphology after crossing into r1. (G) Overlay of confocal and brightfield images shows processes tracking ventrally along rhombomere boundaries. (H) A single example of a hybrid phenotype was displayed by a graft of E6 rhombic lip into r3. Cell processes extend longitudinally ventrally but also at intermediate and dorsal literalities. Dorsally, there is little rostral cell migration (E4-like) and leading processes are repelled by boundaries at the r1/2 border (high power inset), with the exception of a single process. (I) An overlay of fluorescence and brightfield micrographs show that cell processes extend to the midline along rhombomere boundaries (E6-like) but then form bilateral rostral and caudal projections either side of the floorplate (E4-like).
Intrinsic factors govern rhombic lip migration

In their normal context, cells derived from E4 rhombic lip are Netrin1-sensitive (Gilthorpe et al., 2002) and project processes to the floorplate, which turn either rostrally or caudally and extend along the midline (Fig. 9A, “b”). E6 rhombic lip generates granule cell precursors that are relatively insensitive to Netrin1 (Alcántara et al., 2000; Gilthorpe et al., 2002), project short processes ventrally and turn rostrally at the edge of the cerebellar anlage (Fig. 9A, “a”). In both cases, cell bodies initially follow their leading processes and settle either at the ventral midline (E4) or over the surface of cerebellum (E6) where they form the EGL.

When transplanted into E2 hindbrain, both E4 and E6 rhombic lip derivatives choose projection paths that reflect temporal origins (Fig. 9B); E4 derivatives migrate towards the floorplate and extend long processes rostrally and caudally within a ventral corridor. Rostral processes bypass dorsal r1 and project into the forebrain. E6 cells extend processes within a narrow, dorsoventral corridor that corresponds to the laterality of the prospective LLF and target, rostrally, the prospective cerebellum where they terminate in a series of dorsoventral oscillations. Our interpretation of these oscillating trajectories is that the dorsal r1 represents an area of dorsoventral equivalence: processes continue a rostral extension but meander between the boundaries of a wider permissive corridor, giving rise to a characteristic undulating trajectory.

In addition to these intrinsic patterns of growth and migration, rhombic lip derivatives display subtle alterations in their behaviour that can be attributed to extrinsic factors that are absent in E2 host hindbrains. Firstly, E4 derivatives project in a relatively broad ventral domain and consistently cross the midline (Fig. 9B, “e”). E6 rhombic lip derivatives are opportunistic migratory neural cells

Grafts of cerebellar rhombic lip from E6 donors retain their native molecular markers, capacity to divide and generate migratory derivatives when transplanted. However, heterochronic transplantation reveals an unexpected ability in this specific cohort of rhombic lip derivatives to populate cranial ganglia and branchial arches. This behaviour relies on the presence of migrating neural crest in a manner that is reminiscent of the dependence of placodally derived, primary sensory neurons on neural crest streams in establishing central innervation (Begbie and Graham, 2001). This suggests that either neural crest provides a good generic substrate for neural growth or that rhombic-lip-derived granule cell precursors specifically harbour latent, crest-like responses to peripheral cues, perhaps related to their common origins within the Wnt1-positive dorsal progenitor pool (Awatramani et al., 2003; Rodriguez and Dymecki, 2000). While there may be some shared properties between E6 rhombic lip derivatives and neural crest (dorsal neural tube origins, some molecular markers and an ability to proliferate), it is clear that granule cell precursors do not revert to a crest-like phenotype in terms of either early dorsal markers, delamination molecules (Table 1) or morphology. They also exclusively express a definitive neural marker, whereas cranial neural crest gives rise to a range of cell types (Baker et al., 1997). This concurs with previous transplantation assays that suggested that cerebellar rhombic lip is a committed precursor pool (Alder et al., 1996; Lee et al., 2005). However, in contrast to previous studies (Alder et al., 1996; Alder et al., 1999; Carletti et al., 2002), we show that granule cell precursors are able to thrive outside their normal environment. This may have implications for understanding the most common of childhood brain tumours, medulloblastoma. Misregulation of granule cell precursor division is heavily implicated amongst the multi-factorial causes of this condition (Ellison et al., 2003). Our results suggest that mutation of guidance factors that regulate the distribution of this unique migratory precursor population may also be an unexpected contributory factor in the failure of normal proliferative controls.

Discussion

Using a heterochronic grafting strategy, we have examined the patterning of migration in derivatives of rhombic lip in r1. By placing grafts heterotopically into caudal rhombomeres 2–5, we show that graft-derived cells are able to exploit neural crest streams in young host hindbrains to populate the periphery through active growth and migration. Within the neural tube, derivatives of E4 and E6 rhombic lip execute distinct, temporally specified programmes of movement and process extension, indicating a large intrinsic component to the patterning of tangential migration. However, transplanted E4 rhombic lip only ever produces E4-like derivatives, suggesting that temporal identity transition in the progenitor pool is blocked in the absence of appropriate extrinsic signals.

E6 rhombic lip derivatives are opportunistic migratory neural cells

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In addition to these intrinsic patterns of growth and migration, rhombic lip derivatives display subtle alterations in their behaviour that can be attributed to extrinsic factors that are absent in E2 host hindbrains. Firstly, E4 derivatives project in a relatively broad ventral domain and consistently cross the midline (Fig. 9B, “e”). In rare examples, processes occupy the floorplate where they re-cross the midline many times. Secondly, the processes of E6 derivatives are far longer than normal and can project caudally in chimaeras. These results indicate that certain elements governing process extension are regulated by non-autonomous, extrinsic cues that are absent in the host environment of microsurgical chimaeras.

A parsimonious molecular model for our observations is that an age-specific repertoire of intrinsic guidance responses is assigned at the rhombic lip. Environmental interactions refine the precision of decisions taken by growth cones at choice points. For example, the retention of the Unc5H3 receptor in E6 derivatives would prevent processes crossing into the midbrain (Przyborski et al., 1998), while the appropriate intrinsic regulation of Robo receptors would regulate laterality and midline crossing. Robo receptors mediate Slit chemorepulsion and inhibit chemotraction by Netrin (Marillat et al., 2004; Simpson et al., 2000; Stein and Tessier-Lavigne, 2001), and both Netrin1 and Slit2 are expressed in appropriate midline domains in E2 host brains (Fig. 1). By contrast, environmental influences that are absent in host brain are the longitudinal axon scaffolds normally encountered by ventrally migrating cells. Deviations in laterality and unexpected contralateral tracts in E4 migrants may relate to lack of normal fasciculation at the MLF. For E6 migrants, the lengthening of leading processes and loss
of rostrocaudal selectivity in growth cones may reflect the absence of an established LLT and/or the absence of an EGL.

We have previously proposed that interactions between E6 granule cell precursors in forming the EGL are autoinhibitory (Gilthorpe et al., 2002) — a model that is supported by our results. In E2 host brains, when isolated from neighbouring granule cell precursors, E6 cells extend longer and more exploratory processes. This is recorded in their caudal projections and affinity for rhombomere boundaries. By contrast, associated cell bodies only migrate in a rostral direction, indicating that different intrinsic constraints apply to process outgrowth and cell migration. This suggests either that perikarya and growth cones respond independently to guidance cues (Causeret et al., 2004) or that perikaryal translocation is only initiated in a subset of cells that establish an appropriate trajectory.

**Temporal specification and transitions of rhombic lip derivatives**

Sequential production of different neuronal populations is a conserved feature of the vertebrate rhombic lip (Gilthorpe et al.,

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**Fig. 9.** Summary of results and a model of rhombic lip progenitor transition at the cerebellar rhombic lip. (A) The normal migration of rhombic lip derivatives in r1 adapted from Gilthorpe et al. (2002). (B) Summary of heterochronic/heterotopic grafts showing that derivatives of transplanted rhombic lip display modifications of an intrinsic age-specific programme of migration and targeting. (C) A model for progenitor/precursor composition at the r1 rhombic lip drawn from this study and that of Machold and Fishell (2005). A self-renewing rhombic lip progenitor pool is shown in orange. This generates a discrete sequence of committed precursor pools that express the atonal homologue Math1/Atoh1 (green). On initiation of Atoh1 expression, both precursors and their derivatives are fated to leave the rhombic lip (Machold and Fishell, 2005). Younger derivatives (prior to E6 in chick) rapidly down-regulate Atoh1 (purple) while older derivatives form granule cell precursors which maintain both Atoh1 expression and retain the ability to proliferate. The postulated progenitor pool requires an extrinsic cue for temporal transition between producing early and later precursor populations.
2002; Köster and Fraser, 2001; Machold and Fishell, 2005; Wang et al., 2005; Wingate, 2005). Our study demonstrates that migration paths of rhombic lip cells are temporally specified and, within the context of heterochronic graft experiments, temporally committed. This contrasts to the plasticity of earlier neural crest progenitors, where environmental cues dictate the fate and migration paths of their derivatives (Baker et al., 1997). Temporal commitment is hence a property of dorsal progenitor pools that is acquired during development.

There are various possible mechanisms by which cells become temporally specified at the rhombic lip. Firstly, cell fate could be regulated by an endogenous cell division clock within the progenitor pool. Secondly, progenitors might be bipotent or heterogeneous (Alexiades and Cepko, 1997) and respond to a temporally regulated inductive cue from the environment. The fact that E4 grafts do not go on to produce E6-like derivatives indicates that the progression from an E4 to an E6 precursor pool is not regulated by an internal clock. Contrastingly, when transplanted into a new environment, rhombic lip cells retain many aspects of their identity and behave in a manner that is largely independent of extrinsic factors. Thus, despite the apparent absence of an endogenous clock, temporal identity is intrinsic to the transplanted rhombic lip precursors. This apparent paradox is resolved if environmental influences are required solely for the transition between different phases of rhombic lip production. In this model, progenitors would have the capacity to respond to transitional cues external to the rhombic lip, which are only present in the later embryo. Thus, when removed from this environment (as in the heterochronic grafting situation), progenitors are frozen at that specific point in development.

Likely extrinsic candidates for regulating temporal transitions are cues derived from the maturing roofplate of the fourth ventricle, which abuts the rhombic lip. This non-neuronal ectoderm gives rise to the choroid plexus and expresses high levels of the retinoic acid synthetic enzymes in late embryonic development (Zhang et al., 2003). Correspondingly, the rhombic lip initiates specific expression of retinoic acid catabolic enzymes (Wilson and Wingate, unpublished observations) indicating the formation of a local signalling gradient (Swindell et al., 1999). Less likely is the role of feedback mechanisms in regulating the temporal transitions in the rhombic lip precursor pool, as demonstrated for vertebrate retina (Belliveau and Cepko, 1999; Waid and McLoon, 1998). The possibility of feedback between rhombic lip derivatives and their precursors is limited as newly generated neurons rapidly migrate tangentially away from their point of origin and do not retain a connection with the ventricular layer (Gilthorpe et al., 2002; Machold and Fishell, 2005).

Recent fate-mapping experiments using a Math1 reporter mouse raise interesting questions about the nature of the progenitor pool that responds to such extrinsic transitional cues (Machold and Fishell, 2005; Wang et al., 2005; Wingate, 2005). The onset and transient expression of Math1/Atoh1 appears coincident with the initiation of age-specific programmes of rhombic lip migration: all Math1-positive cells exit the rhombic lip (Machold and Fishell, 2005; Wang et al., 2005). However, the location of the progenitors which generate Math1-positive precursors could not be identified in this study. By contrast, our microdissected rhombic lip fragments apparently contain a mixture of both migratory and sedentary proliferative cells. It seems possible that rhombic lip is a heterogeneous assembly of both Math1-positive precursors and an as yet, molecularly unidentified, self-renewing, stem cell population undergoing a sequence of externally regulated, stage-specific, temporal transitions (Fig. 9C).

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