The External Granule Layer of the Developing Chick Cerebellum Generates Granule Cells and Cells of the Isthmus and Rostral Hindbrain

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The external granule layer (EGL) on the dorsal surface of the developing cerebellum consists of neural progenitors originating from the rostral rhombic lip (RRL). The RRL and the EGL were thought to give rise exclusively to the granule neurons of the cerebellum (Alder et al., 1996). To study the fate of individual RRL cells, we used a retroviral library to mark clones in the chick embryo at Hamburger–Hamilton stages 10–12. RRL clones comprised the EGL and cerebellar granule cells, as expected. Surprisingly, however, as many as 50% of the RRL clones also contained cells ventral to the cerebellum proper. Ventral derivatives were found in clones with a medial origin, as well as in those with a lateral origin along the RRL. Some of the ventral progeny appeared to be in the process of migration, whereas others appeared to be differentiating neurons in the isthmus and the rostral hindbrain region, including the locus

coeruleus (LC) and pontine reticular formation. Furthermore, the Phox2a marker of LC precursors was detected in the EGL within the anterior aspect of the cerebellum. A stream of cells originating in the EGL and expressing Phox2a was observed to terminate ventrally in the LC. These data demonstrate that single RRL progenitor cells are not restricted to producing only cerebellar granule cells; they produce both cerebellar granule cells and ventral derivatives, some of which become hindbrain neurons. They also suggest that some progeny of the EGL escape the cerebellum via the anterior aspect of the cerebellar peduncles, to contribute to the generation of ventral structures such as the LC.

Key words: cerebellum; hindbrain; retrovirus; lineage; migration; rhombic lip

Cells of the vertebrate CNS are generally produced by multipotent progenitor cells in the ventricular zone (Cepko et al., 1997). A notable exception to this rule was thought to be external granule layer (EGL) progenitor cells of the cerebellum, which were thought to produce only cerebellar granule neurons. EGL cells originate from the rostral portion of the rhombic lip, the free margin of the hindbrain surrounding the dorsal opening of the fourth ventricle (Hatten and Heintz, 1995), and reside over the dorsal surface of the developing cerebellum (Ramon y Cajal, 1911; Miale and Sidman, 1961; Hanaway, 1967).

The developmental potential of the EGL and rhombic lip cells has been investigated using transplantation and lineage-tracing studies. Both the murine embryonic rhombic lip cells and the postnatal EGL cells differentiated exclusively into granule cells when implanted into the postnatal murine cerebellar EGL (Gao and Hatten, 1994; Alder et al., 1996). Likewise, retrovirally labeled clones originating from the chick rostral rhombic lip (RRL) were found to contain EGL cells and granule cells (Ryder and Cepko, 1994), and postnatal murine EGL cells were found to generate granule cells but not other cerebellar cell types (Zhang and Goldman, 1996). It was thus thought that the RRL progeni-

tors were restricted to producing only cerebellar granule cells. However, the lineage-tracing methods used in these studies precluded identification of clonally related cells that traveled outside of the cerebellum.

When transplanted into the hippocampal dentate gyrus, EGL cells were found to acquire the biochemical and morphological properties of the hippocampal granule neurons (Vicario-Abejo et al., 1996). In a recent chick–quail chimera experiment, grafts of the dorsal portion of rhombomere 1 were found to contribute to both the cerebellar EGL and a specific ventral structure, the lateral pontine nucleus, but not to other ventral regions (Wingate and Hatten, 1999). In a previous chick–quail chimera study (Hallonet and Le Douarin, 1993) using dorsal grafts that included the rhombomere 1, it was found that the grafts gave rise to many other ventral neural structures, along with the cerebellar EGL and granule neurons (Hallonet and Le Douarin, 1993). It could not be determined from these studies whether the EGL cells and the ventral neurons were derived from a common progenitor pool or from separate progenitor pools intermixed in the RRL.

To address the issues raised in these previous studies concerning the fate of the progeny of individual RRL cells, we used a retroviral library, CHAPOL (Golden et al., 1995), to analyze chick RRL clones. This library allows for the definition of clonal relationships regardless of the location of the sibling cells. We found that cerebellar EGL cells as well as some cells in the isthmus and rostral hindbrain region were the progeny of individual RRL cells infected at stages 10–12. The chick RRL is thus not restricted to generating cerebellar granule cells at these stages. In addition, Phox-2a, a homeobox gene essential for the development of the locus coeruleus (LC) (Morin et al., 1997), was

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transiently expressed in a dorsal–ventral stream of LC precursors. Interestingly, the dorsal end of the Phox-2a stream overlapped with the Pax-6-expressing cells in the anterior aspect of the EGL. The lineage and gene expression data together suggest a novel pathway along which some EGL progeny migrate ventrally to contribute to the neuronal population of the rostral hindbrain region.

MATERIALS AND METHODS

Lineage analysis using retroviral library CHAPOL. Fertilized White Leghorn chick eggs were purchased from SPAFAS (Norwich, CT). A detailed description of the CHAPOL library has been given (Golden et al., 1995; Cepko et al., 1998). Briefly, the CHAPOL library is a mixture of replication-defective retroviruses, each of which encodes the human placental alkaline phosphatase (AP). Each member in the library also carries a distinct 24 bp insert. Concentrated CHAPOL viral stocks were injected into the neural tube of chick embryos between stages 10 and 12. Eggs were sealed and returned to the incubator until the day of harvest, from embryonic day 8 (E8) through E18 (Table 1). The morphology of retrovirally labeled cells was revealed by the standard procedure of AP histochemistry, which was extended for 24–48 hr to increase the sensitivity of detection. Each minimal tissue fragment encompassing the individual AP+ cells or AP+ cellular clusters was picked from the frozen tissue sections (each called a “pick”) and was subjected to clonal identification by PCR sequencing. The distinct 24 bp oligonucleotide insert in each viral genome was amplified and sequenced for clonal assignment as previously described (Golden et al., 1995). The extent of clonal dispersion defined in this way was probably an underestimate for the following reasons: (1) not all virally infected cells could be visualized equally well by AP histochemistry; and (2) the oligonucleotide tag could not always be amplified from individual AP+ cells (Golden and Cepko, 1996; Lin and Cepko, 1999). We noted that some virally infected EGL cells and granule cells became visible only after a longer period of AP histochemical staining than was used to visualize others (data not shown).

To minimize underestimating the clonal boundaries caused by the loss or an undetectable level of AP expression within infected cells, tissue fragments without AP+ cells were routinely sampled and analyzed for potential nonexpressing, or “silent,” viral genomes (Golden and Cepko, 1996; Lin and Cepko, 1999). Some embryos had a yield of a PCR product as high as 30–50% for the tissue picks without AP expression throughout the cerebellum. Often the same viral genome insert was found throughout the cerebellum, indicating the presence of a large, silent clone, most likely a silent granule cell clone (Lin and Cepko, 1999). The high prevalence and ubiquitous distribution of infected cells that did not express detectable AP within such brains greatly confounded the clonal analysis in these cerebella. Therefore, only cerebella that had a 0–30% PCR+ rate for the areas without AP expression were included for analysis in this study.

Immunohistochemistry. Twenty-five micrometer coronal cryosections of the developing chick midbrain–hindbrain region were prepared for immunohistochemistry. The primary antibodies used in this study included monoclonal anti-Pax6 (Developmental Studies Hybridoma Bank), anti-serotonin (Sigma, St. Louis, MO), anti-tyrosine hydroxylase (Sigma), and anti-Phox2a (Pattyn et al., 1997). The procedure of immunohistochemistry using DAB detection was as previously described (Lin and Cepko, 1998). Double-label immunostaining was performed by using Cyanine (Cy)-2- and Cy-3-conjugated secondary antibodies, and the color images were taken and merged using a Hamamatsu (Hamamatsu City, Japan) digital camera and OpenLab software.

Nonradioactive *in situ* hybridization. Six micrometer coronal paraffin sections of the developing chick cerebellum were prepared for *in situ* hybridization. The procedure of *in situ* hybridization of the granule cell marker *Zic-1* using a digoxigenin-labeled riboprobe was as previously described (Lin and Cepko, 1998).

RESULTS

Retroviral lineage analysis of clones containing cerebellar EGL cells

To label RRL clones in the chick cerebellum, we injected a concentrated CHAPOL stock into the chick neural tube between Hamburger–Hamilton stages 10 and 12. The brains of infected embryos were harvested from E8 to E18. Virally labeled cells

were visualized by AP histochemistry first on whole-mount specimens and then on frozen tissue sections. The AP+ cells observed in the cerebellar anlage were analyzed for their clonal identity by PCR sequencing of the retroviral genome (see Materials and Methods).

Clones within the embryonic chick cerebellum that contained EGL cells were chosen for analysis. These clones will be referred to as RRL/EGL clones because they had the following characteristics: (1) one or more closely associated cellular clusters (presumably the clonal origin) along the RRL, i.e., the posterior margin of the cerebellar anlage; (2) tangentially migrating cells in the EGL; (3) inwardly migrating cells in the presumptive molecular layer; and (4) differentiating and differentiated granule cells in the internal granule layer (IGL) (Ryder and Cepko, 1994). On the basis of these criteria, 20 RRL/EGL clones were identified in 15 cerebella after PCR and sequencing analysis (Table 1).

Consistent with previous studies (Hallonet et al., 1990; Ryder and Cepko, 1994), the RRL/EGL clones at E8–E12 generally exhibited a spatial pattern, which suggested that they migrated rostrally from the RRL and then transversely within the EGL, often traversing the midline of the cerebellum (Figs. 1, 2). Clones with a medial origin and those with a lateral origin were found (Table 1). For the clones analyzed at E15–E18, no distinct clonal origin adjacent to the posterior margin of the cerebellum was visible; instead, many more differentiated granule cells in the IGL were observed (data not shown). None of the RRL/EGL clones contained cerebellar cells other than those of the EGL and granule cells (Table 1), consistent with an early separation of granule cell fate from the fates of other cerebellar cell types.

While analyzing the RRL/EGL clones in the cerebellum, we noticed on several occasions that a few AP+ cells ventral to the cerebellar anlage were in the vicinity of the labeled EGL cells (e.g., Figs. 1–4). Curiously, there was not a separate ventral clonal origin [i.e., labeled radial glia or ventricular zone (VZ) cells] associated with these ventral cells as would be expected for other ventral VZ clones (e.g., Fig. 2C–F, *black arrows* indicate a hindbrain clone with a ventral clonal origin). These ventral cells were analyzed for their clonal relationships by PCR and sequencing to determine whether they were lineally related to the neighboring, labeled EGL cells. Interestingly, 11 of the 20 RRL/EGL clones so analyzed were found to have sibling cells ventral to the cerebellum proper (Table 1).

Earlier stages of cerebellar development were examined to learn how the ventral derivatives of the RRL could have arisen. Between E8 and E12, some of the sibling cells outside of the cerebellar cortex were found to have a bipolar morphology, consistent with active migration, whereas others displayed a multipolar morphology, consistent with differentiation as neurons (Figs. 2B–G, 3F–H, L–M). Because the isthmus and the rostral hindbrain region were still undergoing substantial reorganization during this period, the anatomical localization of these ventral cells could not be precisely determined. Approximately, these cells were found in the presumptive areas of the cerebellar peduncles, the vestibular nuclei, the LC, and the pontine reticular formation (Table 1, Figs. 2–4). At E18, two RRL/EGL clones with ectocerebellar members were found to have these members in the parvocellular isthmus nucleus (Table 1, Fig. 5). On the basis of the morphology delineated by the AP histochemistry, these cells were most likely neurons (Fig. 5). No glial cell siblings were observed in these clones.

Table 1. List of the RRL/EGL clones

Embryo ^a	Inject (stage) ^b	Harvest ^c	RRL/EGL clone ^d	Origin ^e	No. Hb sib (cell type) ^f	Hb sib location ^g	No. unrelated clones ^h
JZ-2	10	E8	JZ2-10-3	Medial	3 picks (u)	LC, PR	Nil
JZ-10	10	E8	JZ10-4	Medial	6 picks (n, m, u)	LC, PR	4 Hb
		E8	JZ10-19	Medial	2 picks (n, m, u)	LC, PR	
JZ-5	10	E8	JZ5-1	NF	3 picks (n, u) and – area	PR	1 Hb
		E8	JZ5-2	NF	3 picks (n, u)	PR, other	
LC5-5	11	E8–E9	LC5-5-9-1	Midline	2 picks (n)	Isth	7Hb, 1Hb + DCN, 1 DCN, 4Cb
JL36-13	10	E9	JL36-13-6-3	Lateral	8 picks (n, m, u)	VN, LC?	Nil
JL39-2	12	E9.5	JL39-2-9D-13	Lateral	None	NA	3 Cb
JL32-26	8	E10	JL32-26-11	Lateral	1 pick & – area	PR	5 Hb
JL33-30	10	E11–E12	JL33-30-3-8	Lateral	3 picks (n, u)	LC, PR	1 Isth, 1 Hb
JL33-42	10	E11–E12	JL33-42-4-16	Lateral	None	NA	1 Hb + DCN
JL40-12	12	E15	JL40-12-6B-7	NF	None	NA	2 Hb, 1 Cb glia
			JL40-12-6B-17	NF	None	NA	
JG24-4	12	E18	JG24-4-3-8	NF	None	NA	8 Pkj, 2 P + G, 1 DCN, 2 –
		E18	JG24-4-4/5-27	NF	None	NA	
JG24-8	12	E18	JG24-8-4-2	NF	None	NA	1 P + G, 2 Cb glia
JL32-7	10	E18	JL32-7-9-2	NF	None	NA	2 Hb
JL35-4	10	E18	JL35-4-3-1	NF	1 pick (n)	PIN	4 Hb, 2 (–)
JL35-17	10	E18	JL35-17-9-1	NF	1 pick (n)	PIN	1 Isth, 1 Hb, 1 Cb glia
			JL35-17-9-20	NF	None	NA	

The list shows all of the RRL/EGL clones in this study, as defined by PCR sequencing of the CHAPOL retroviral genome (see Materials and Methods).

^aNumber assigned to the embryo.

^bHH stage of retroviral injection.

^cEmbryonic day of harvest.

^dNumber of the RRL/EGL clone. The RRL/EGL clones that contained progeny outside the cerebellum proper are shown in bold type.

^eThe origin of a given clone is designated according to the relative position along the width of the RRL, as midline, medial, or lateral. When no discernible origin in the RRL or the posterior margin of the cerebellum was observed, either because of a loss of tissue or because of maturity of the clone, the origin of the clone is designated NF (not found).

^fNumber of cell picks in the hindbrain or isthmus region that were derived from an RRL/EGL clone with the tentative identity of the cell type(s) in the parenthesis. n, Neuron; m, migrating cell; u, unidentified; –, AP-negative pick.

^gTentative localization of the RRL/EGL progeny in the hindbrain or isthmus region. Isth, Isthmus; LC, locus ceruleus; PIN, parvocellular isthmus nucleus; PR, pontine reticular formation; VN, vestibular nuclei; NA, not applicable.

^hClones not lineally related to the RRL/EGL clone. Cb, Cerebellar VZ clone; Cb glia, cerebellar glial clone; P, Purkinje cell clone; P + G, Purkinje cell and glial clone; DCN, clone in the deep cerebellar nuclei; Hb, hindbrain-restricted clone; Isth, isthmus-restricted clone; –, AP-negative tissue pick. It should be noted that two clones were found to contain both neurons in the deep cerebellar nuclei and neurons in the vestibular nuclei in the hindbrain, thus designated Hb + DCN.

Figure 1. An RRL/EGL clone (JZ2-10-3, harvested at E8; see Table 1) with several sibling cells in the rostral hind-brain region ventral to the cerebellum. *A*, Dorsal view of the whole mount of the cerebellum of embryo JZ2, with two sets of black lines bracketing the levels of sections shown in *B–F* and an arrow showing the clonal origin in the medial rhombic lip. *B–F*, Six micrometer coronal sections of embryo JZ2 at the rostral portion of the cerebellum in rostral-to-caudal order. Red arrows indicate the clonally related cells defined by PCR sequencing analysis. Dorsal is at the top, and the midline is to the left in *B–F*. This clone contained a few cells in the hindbrain close to the presumptive locus coeruleus (*B, D, F*, bottom red arrows), as well as numerous migrating EGL cells in the cerebellum (*C, D, F*, top red arrows). No AP+ hindbrain siblings were found in the more caudal levels (data not shown). Cb, Cerebellum; Cp, cerebellar peduncle; Hb, hindbrain.

Models for the ventral siblings of the RRL/EGL clones

Several distinct, but not mutually exclusive, models can be proposed that describe the origin of ectocerebellar cells in the

RRL/EGL clones. First, it is possible that an early progenitor in the midbrain–hindbrain junction proliferates and splits into two distinct VZ clusters, with one giving rise to the cerebellar EGL–

Figure 2. Two RRL/EGL clones (JZ10-5-4 and JZ10-5-19; see Table 1) contained several cells in the region ventral to the cerebellum at E8. *A*, Dorsal view of the cerebellar area of embryo JZ10 shown as a whole mount, with a series of *black lines* indicating the levels of sections shown in *B–G*. The rostral aspect of the cerebellum is at the *top*, and the midline is indicated by a *black arrow* in *A*. *B–G*, Serial 60 μ m coronal sections of embryo JZ10 at the levels indicated in *A*. Dorsal is at the *top*, and the midline is to the *left*. The PCR-defined sibling cells in clone JZ10-5-4 are indicated by *red arrows*, and those in clone JZ10-5-19 are indicated by *blue arrows*. *Black arrows* in *C–F* indicate a distinct, unrelated hindbrain clone with radial glia in the VZ of the fourth ventricle (*IV*). Both clones JZ10-5-4 and JZ10-5-19 contained sibling cells in the hindbrain area (probably the prospective pontine reticular formation and the locus coeruleus; *B–E*, *red, blue arrows*; *F*, *bottom-most blue arrow*) and many cells in the cerebellar anlage (*F, G, arrows*). Some siblings in the rostral hindbrain region appeared to be migrating cells (*C, arrows*), and others appeared to be differentiating neurons (*B, D–F, arrows*) in the hindbrain. In addition, many cellular processes, probably those of the migrating cells, were observed in the cerebellar peduncle (*B–G*). Considerably more AP+ hindbrain siblings of clones JZ10-5-4 and JZ10-5-19 were observed in the rostral than in the caudal sections (compare *B–E* with *F–G*; some data not shown). Two separate clusters of AP+ cells, most likely the clonal origins, were found in the medial portion of the posterior margin of the RRL (the midline indicated in *A*, *black arrow*). The *red arrow* in *A* indicates the origin of JZ10-5-4 as confirmed by PCR sequencing, whereas the origin of JZ10-5-19 cannot be labeled because of the failure of PCR amplification of the presumptive origin.

granule cells and the other populating the ventral region (Fig. 6*A*). In other words, the progeny within each of these two regions are derived from distinct subclones. This model predicts a clonal origin in the RRL and a second origin in the VZ of the ventral neural tube. We did not find a separate VZ origin in the isthmus or the hindbrain region associated with ventral progeny as early as E8 (e.g., Figs. 1–3), despite the fact that many hindbrain clones still retained their clonal origins within the VZ at this time (e.g., Fig. 2*C–F*, *black arrows*). This model remains a formal possibility because the distinct VZ origins for the ventral sibling cells might have completely disappeared before E8 and hence escaped detection. We, nonetheless, do not favor this model, because the observation of cells that appeared to be migrating through the cerebellar peduncles in the anterior region is not consistent with this model (Fig. 2*D,G*).

The second model is based on the fact that the caudal portion of the rhombic lip gives rise to cells that migrate over the ventral surface of the neural tube to populate the pontine and the inferior olivary nuclei (Fig. 6*A*, *green arrows*) (Harkmark, 1954). It is

conceivable that the junction between the rostral and the caudal rhombic lips consists of cells with a dual potential, such that they give rise both to the EGL cells and to cells that migrate ventrally (Fig. 6*B*). This model predicts that RRL/EGL clones with ventral siblings should have a lateral origin. In contrast, we found ventral sibling cells in RRL/EGL clones with a medial origin (Table 1; Figs. 1, 2) as well as in those with a lateral origin (Table 1; Figs. 3, 4). In addition, regardless of the location of the clonal origin along the RRL, the ventral siblings of the EGL clones were never found in the posterior hindbrain where the inferior olive nuclei reside (e.g., Figs. 1–5). These observations argue against the model that the RRL/EGL clones with ventral siblings originate only from a transitional zone between the rostral and the caudal rhombic lips.

All aspects of the observations presented here are most consistent with a third model. In this model, EGL cells, originating at any mediolateral position along the RRL, first migrate rostrally on the surface of the cerebellum. A small subset of cells then migrates ventrally from the anterior aspect of the cerebellar

Figure 3. An RRL/EGL clone (JL36-13-6-3; see Table 1) contained several sibling cells in the region ventral to the cerebellum at E9. *A, B*, Dorsal oblique view of the whole-mount cerebellar area of embryo JL36-13 in two distinct focal planes, with a series of white lines indicating the levels of sections shown in *C–K*. The anterior aspect of the cerebellum is at the top. The midline is not in view but is to the right in *A* and *B*. *C–K*, Sixty micrometer coronal sections of the same embryo at each level indicated in panel *A*. *F–H*, *L–N*, High-power views of the framed areas in *C–E* and *I–K*, respectively. Dorsal is at the top in *C–N*. Red arrows and arrowheads indicate the PCR-defined sibling cells in this clone (JL36-13-6-3). This clone contained cells in the rostral hindbrain area (corresponding approximately to the prospective vestibular nuclei; *F–H*, red arrows), cells in the cerebellar peduncle (*L, M*, red arrows), and cells in the EGL (*E, M*, red arrowheads; also see *A, B*, black arrows, for whole-mount views). Some of the siblings ventral to the cerebellar anlage appeared to be in the process of migration with a typical bipolar morphology (*G, H, L, M*, red arrows), whereas others appeared to be differentiating neurons (*F, M*, red arrows). No AP⁺ hindbrain siblings were observed in the more caudal sections (*K*; some data not shown). This clone originated from the lateral edge of the RRL (*B*, black arrowhead; also see *K, N* for sections).

anlage into the isthmus and the rostral hindbrain region by E9. This model is directly supported by the observations shown in Figures 2 and 3 (and Fig. 7*C,D*, as discussed below), in which cells that appeared to be migrating in this direction can be seen.

Molecular marker expression of EGL and LC neurons

To gain further insight into the identity of the ventral siblings of the RRL/EGL clones, we noted that some monoaminergic neurons of the LC and the pontine reticular formation project axons into the cerebellar cortex in an unusual manner (Fig. 7*A*). Rather than forming the classic climbing fibers or the mossy fibers in the cerebellum, these monoaminergic axons bifurcate at a T formation in the molecular layer, resembling the axons of granule cells (Chan-Palay, 1975; Mugnaini and Dahl, 1975). This similarity, combined with the fact the T-shaped axonal morphology of the cerebellar granule cells reflects their migration from the EGL to

the IGL, suggested the possibility that a subset of the monoaminergic neurons was also derived from the EGL via a dorsal-to-ventral migratory route.

As would be predicted by this hypothesis, we observed a few tyrosine hydroxylase (TH)-positive cells in the cerebellar anlage and the anterior aspect of the cerebellar peduncles at E7–E8 (Fig. 7*C,D*, arrows). These TH-positive cells dorsal to the presumptive LC were located very close to the cerebellar EGL marked by Zic1 (Fig. 7*B*), and they exhibited a bipolar morphology consistent with migrating cells. No TH-positive cells were observed within the cerebellum proper or in the cerebellar peduncles later during development (data not shown).

We labeled several additional RRL/EGL clones with the retroviral library and processed the tissues with double immunohistochemistry using anti-TH and anti-AP. Despite an extensive

Figure 4. An RRL/EGL clone (JL33-30-3-8; see Table 1) contained several sibling cells in the region ventral to the cerebellum at E11–E12. *A–D*, Sixty micrometer coronal sections of embryo JL33-30 in a rostral-to-caudal order. The *blue arrows* indicate the clonally related cells defined by PCR sequencing. Dorsal is at the *top*, and midline is to the *right*. *E*, Dorsal oblique view of the cerebellar region of this embryo stained by whole-mount AP histochemistry, with a series of *black lines* indicating the levels of sections shown in *A–D*. The orientation in *E* is indicated by a frame of reference (*a*, anterior; *p*, posterior; *m*, medial; *l*, lateral; *d*, dorsal). This clone contained cells in the hindbrain area (possibly the prospective vestibular nuclei and locus coeruleus; *A*, *blue arrow*, *B*, two *bottom blue arrows*) and many cells in the EGL and the IGL (*B–D*, *top blue arrows*). No AP+ hindbrain siblings were observed in the more caudal sections (*D*; some data not shown). This clone originated from the lateral edge of the posterior margin of the RRL (*E*, *black arrow*).

series of such experiments using several different anti-AP antibodies, we were not able to co-label the RRL/EGL clones and the hindbrain TH-positive cells because of the lack of consistent anti-AP staining. It is likely that the use of a retroviral library with a different reporter gene, such as the green fluorescent protein, will facilitate the detection of clonal members with additional cellular markers in the future.

Expression of the early LC marker Phox2a overlaps with EGL marker Pax6

We further examined molecular markers expressed in LC neurons earlier than TH. Two paired-type homeodomain transcription factors, Phox2a and Phox2b, are known to be expressed in LC precursors before the onset of TH expression in the mouse and zebra fish (Pattyn et al., 1997; Guo et al., 1999). Indeed, Phox2a is absolutely required for the differentiation of LC neurons (Morin et al., 1997; Guo et al., 1999). In keeping with the idea that some LC neurons undergo a dorsal-to-ventral migration, the Phox2a-expressing LC precursors were found in a dorsal–ventral stream around the lateral aspect of the rostral hindbrain at E7 (Fig. 8). Interestingly, the dorsal end of such a Phox2a-expressing cellular stream coincided with the Pax6-expressing cerebellar EGL, with individual cells expressing both genes (Fig. 8*B,C*). This provides additional support to the notion that some EGL progenitors can give rise to sibling cells that migrate out of the cerebellum proper into the ventral region. In the process of ventral migration, these EGL siblings appear to cease expression of the standard granule cell markers, such as Pax6 and Zic1, and concomitantly acquire novel noncerebellar characteristics, such as the expression of Phox2a and, perhaps, TH.

DISCUSSION

Over the past decade, cerebellar granule neurons were thought to be the only progeny of the RRL and EGL progenitors (Hallonet et al., 1990; Otero et al., 1993; Ryder and Cepko, 1994; Alder et al., 1996; Zhang and Goldman, 1996). Our data are consistent with those of the earlier studies in that no other cerebellar cell types were found in RRL/EGL clones. We were able to extend the search for RRL/EGL progeny to regions outside of the

Figure 5. An RRL/EGL clone (JL35-17-9-12; see Table 1) at E18 contained a sibling cell in the isthmus region. This is a 60 μ m parasagittal section of the midbrain and cerebellar region of embryo JL35-17. Rostral is to the *left*, and dorsal is at the *top*. Clone JL35-17-9-12, indicated by the *red arrows*, contained migrating EGL cells in the cerebellum and a neuron in the isthmus region, specifically in the parvocellular isthmus nucleus (*bottom, red arrow within the framed area*). The tentative neuronal morphology of this isthmic sibling cell is better appreciated at the high-power view in the *top left inset*. The *blue arrows* indicate a distinct, unrelated clone containing many glial cells in the cerebellar cortex. *Isth*, Isthmus; *Mb*, midbrain.

Figure 6. Several models are proposed for the development of RRL/EGL clones with ventral progeny in the isthmus and the rostral hindbrain. Each panel is a three-dimensional schematic diagram of the midbrain–hindbrain area. The orientation in *A–C* is indicated by a frame of reference in *C* (*A*, anterior; *D*, dorsal; *L*, lateral). *A*, It has been well established that the cells from the RRL migrate rostrally to give rise to EGL cells in the cerebellum (solid red arrows), whereas the cells of the caudal rhombic lip migrate ventrally to give rise to the pontine and inferior olivary nuclei (green arrows). Model 1 (*A*) suggests that a progenitor splits early in development into two separate clusters of cells, with one giving rise to EGL cells of the cerebellum (solid red arrows) and the other producing hindbrain cells (dotted red arrows). This model does not invoke any ventral migration of the cells from the RRL, and it predicts the existence of a separate clonal origin in the VZ of the fourth ventricle. Model 2 (*B*) suggests that cells originating from the transitional zone between the rostral and the caudal rhombic lip migrate rostrally as well as ventrally (blue arrows). This model predicts that the origins of these clones cluster around the lateral aspect of the RRL. Model 3 (*C*) suggests that EGL progenitor cells produce some progeny that migrate ventrally into the isthmus and the rostral hindbrain via the anterior aspect of the cerebellar peduncle. These clones can originate from either the medial (red arrows) or the lateral (blue arrows) portion of the RRL. *D*, Frontal perspective of model 3, with its orientation indicated by the frame of reference at the bottom right corner. EGL cells migrate rostrally away from the rhombic lip and transversely across the midline (solid red arrows). A subset of the progeny of an EGL clone migrates ventrally to the hindbrain and the isthmus only via the anterior aspect of the cerebellar peduncle (dotted red arrows). CRL, Caudal rhombic lip.

cerebellum thanks to the high complexity of the CHAPOL retroviral library (Golden et al., 1995). We demonstrated that the RRL/EGL clones generate not only granule neurons of the cerebellum but also some cells in the isthmus and the rostral hindbrain region of the chick embryo. Because the RRL/EGL clones had origins only at the RRL, the result also demonstrates that the progeny of RRL cells are not restricted to the cerebellar granule cell fate in the chick embryo at stages 10 and 11.

Several lines of evidence show that the pontine and inferior olivary nuclei in the ventral hindbrain derive from the rostral and caudal rhombic lip, respectively (Harkmark, 1954; Wingate and Hatten, 1999; Yee et al., 1999; Alcantara et al., 2000). Hallonet and Le Douarin (1993) showed by chick–quail grafts that dorsally derived cells, which give rise to the rostral rhombal lip and EGL, also could give rise to ventral hindbrain cells. More recently, Wingate and Hatten (1999) reported that grafts of the dorsal portion of rhombomere 1 often gave rise to the cerebellar EGL and the lateral pontine nucleus, on the basis of their location and morphology. It was unclear whether the EGL and the lateral pontine nuclei (or other ventral cells in the studies of Hallonet and Le Douarin, 1993) actually shared a set of common progenitors, or whether distinct progenitor pools within the grafts gave rise to the EGL and ventral derivatives. Our results establish unequivocally that there is a common clonal origin within the RRL for the EGL, the cerebellar granule cells, and some ventral hindbrain cells. The ventral hindbrain progeny of the retrovirally marked RRL/EGL clones reside within or in the vicinity of the

LC, pontine reticular formation, vestibular nuclei, and parvocellular isthmic nucleus. These locations are in keeping with the observations of Hallonet and Le Douarin (1993), who saw graft-derived cells in these same locations. Curiously, Wingate and Hatten (1999) did not observe graft-derived cells in these ventral locations. The reason for this difference is currently unknown.

We further explored the possible EGL origin for some LC noradrenergic neurons. We showed that the Phox2a-expressing LC precursors migrate ventrally toward the presumptive LC, forming an arc extending from the dorsal to the ventral hindbrain. A few differentiating neurons that began to express TH were transiently found in the cerebellar peduncles connecting the cerebellum and the rostral hindbrain. Because no such TH-expressing cells were observed in the mature cerebellar peduncles or within the cerebellum proper, these TH-expressing cells were likely en route to a ventral location, i.e., the LC. Recently it was shown that, in zebra fish, Phox2a-expressing LC precursors first appear in the dorsal hindbrain and then move ventrally (Guo et al., 1999). Our data are consistent with this finding in confirming a dorsal origin for Phox2a-expressing cells in the chick embryo. Furthermore, our data represent the first demonstration of the overlapping expression of an LC property by cells in the rostral EGL. It suggests that some EGL cells, when reaching the rostral end of the cerebellar territory, turn on Phox2a and begin a ventral migration, during which they eventually lose the expression of EGL markers such as Zic-1 and Pax-6. It will be interesting to

Figure 7. Some monoaminergic neurons in the ventral region share a similar axonal morphology with cerebellar granule cells, and they may originate from the EGL. *A*, Schematic diagram depicting the axonal morphologies of the cerebellar granule cells (black), the mossy fibers (light blue), the climbing fibers (dark blue), and the monoaminergic neurons in the ventral regions (red). A subset of the serotonergic neurons in the pontine reticular formation and the noradrenergic neurons in the locus coeruleus project axons to the cerebellar cortex. The monoaminergic cerebellopetal axons generally exhibit morphology distinct from that of the mossy fibers or of the climbing fibers. Some of them terminate diffusely in the cerebellar cortex (red dotted lines), whereas others assume a T-shaped bifurcation in the molecular layer (red solid line), very similar to the granule cell axons. Adopted and modified from Chan-Palay (1975). *B*, *In situ* hybridization of the Zic-1 probe on a 10 μ m coronal section of the E8 chick brain at the level of the LC. Zic-1 is a cerebellar granule cell marker, and a high level of Zic-1 mRNA expression was observed in the EGL but not the cerebellar peduncle. *C*, *D*, Anti-TH antibody staining on 25 μ m coronal sections of E7–E8 chick cerebellar and hindbrain regions. Although *B–D* were derived from different embryos of the same age, coronal sections at a similar level of the rostral hindbrain were used for comparison. TH is a marker for catecholamine-producing cells, including the noradrenergic neurons in the locus coeruleus. In addition to the cluster of TH-positive cells in the presumptive LC, some scattered TH-positive cells were observed in the cerebellar anlage and the cerebellar peduncles (*C*, *D*, arrows). A few TH-positive cells in the cerebellar peduncle exhibited a morphology consistent with migrating cells. No TH-positive cells were observed in the EGL. ML, Molecular layer; PCL, Purkinje cell layer.

learn whether the signal(s) of the isthmus organizer, such as Fgf-8 and Wnt-1, are responsible for this transition.

Both the LC precursors and the EGL cells develop in response to dorsal bone morphogenetic protein (BMP) signals. The BMPs are expressed in the roof plate and the dorsal ectoderm (Lee et al., 1998). A proper level of BMP-2 and -7 signaling has been shown to be required for the expression of Phox2a in the LC precursors and for LC formation (Guo et al., 1999). Interestingly, BMP-6, BMP-7, and growth differentiation factor-7 can also induce the ventral mes- and met-enkephalic cells to express the EGL markers Math-1 and Zic-1 (Alder et al., 1999). Our model suggests a simple explanation for the common involvement of BMP signals in the EGL and the LC development; i.e., BMP signals act on a common progenitor pool that produces both cerebellar granule cells and LC cells.

The T-shaped axonal bifurcation in the molecular layer of the cerebellum is another shared feature of cerebellar granule cells and LC cells (Mugnaini and Dahl, 1975). It should be noted that, in the vertebrate CNS, the T-shaped axonal branching morphol-

ogy is found in several neuronal classes. As a classic example, the axons of the spinal cord commissural neurons typically exhibit a T-shaped bifurcation after crossing the floor plate to the contralateral side. The commissural neurons are descendants of the dorsal Math-1-expressing progenitor cells (Helms and Johnson, 1998). Their specific axonal branching pattern is generated during the process of axonal outgrowth and pathfinding under the influence of axonal branching factor(s) (Wang et al., 1999), apparently independent of the movement of the commissural neuronal cell body. On the other hand, the T-bifurcation of the cerebellar granule cell axons is a natural consequence of granule cell migration (Ramon y Cajal, 1911). Most axonal inputs into the cerebellar cortex, in contrast, assume the morphology of climbing fibers or that of mossy fibers (Fig. 7*A*) (Ramon y Cajal, 1911). It is thus interesting, within the context of the cerebellar cortex, to speculate about the possibility of a connection between the axonal morphology of the LC cells and their cellular origin. Perhaps the T-shaped axonal bifurcation of some LC cerebellar projections is also a direct consequence of their origin and migration from the EGL just like the cerebellar granule cells. For technical reasons,

Figure 8. Expression of LC markers Phox2a and TH visualized by antibody staining supports the idea of the ventral migration of EGL cells in model 3 (*A*, same as Fig. 6*C*). *B*, *C*, Co-localization of LC precursor marker Phox2a (green) and EGL marker Pax6 (red) at the rostral aspect of the cerebellum. The relative position of the areas shown in *B* and *C* is indicated by the top left frame in *A*. It is interesting to note that the medial EGL expressed only Pax6, and the intermediate EGL expressed both Phox2a and Pax6, whereas the lateral EGL expressed only Phox2a. *D*, Phox2a (green)-expressing LC precursors stretching from the dorsal region to the ventral region close to the TH-expressing LC neurons (red). Most if not all TH-expressing cells in the LC ceased to express Phox2a. The *D* corresponds to the right framed area in *A*. The staining was done with the 30 μm coronal sections of E7–E8 chick embryos.

we have not yet shown that the ventral members of the EGL clones indeed exhibit the T-shaped axonal bifurcation in the cerebellum. This intriguing hypothesis thus remains to be formally established.

A previous birth-dating study showed that the LC neurons become postmitotic from E2 to E6 (their “birthdays”) in the chick embryo (Yurkewicz et al., 1981). Although most of the cerebellar granule neurons do not become postmitotic until much later during development, the chick EGL was observed to approach the anterior aspect of the cerebellar anlage as early as E5–E6 (Hanaway, 1967; Feirabend, 1990; Wingate and Hatten, 1999). Thus it is likely that only a subset of LC neurons can be derived from the EGL progenitors, i.e., those LC neurons with the latest birthdays. In this regard, it is notable that only a subset of the LC afferents exhibit the T bifurcation morphology in the molecular layer of the chick cerebellum as well (Mugnaini and Dahl, 1975).

In summary, retroviral lineage and molecular marker analysis in the developing chick cerebellum led us to the conclusion that some EGL progeny migrate ventrally via the anterior aspect of the cerebellar peduncles to populate the ventral hindbrain region, including the LC. Whether this phenomenon is conserved across vertebrate species is currently unknown. In primates, for example, the LC neurons are generated between E27 and E36 (Levitt and Rakic, 1982), but cerebellar granule cells begin to migrate into the IGL only after E80 (Rakic, 1971). Although it is possible that some EGL cells migrate early to form the LC in primates, these temporal differences might suggest that primate LC neurons are not directly derived from the EGL. Nevertheless, because we observed ventral EGL siblings in areas other than the LC in the

chick embryo, the general notion of some EGL progeny migrating ventrally remains possible in primates and other vertebrate species.

Finally, the extracortical origin for the GABAergic interneurons of the cerebral cortex provides an interesting precedent for the data reported here. Many of the cortical GABAergic interneurons originate in the lateral ganglionic eminence and migrate dorsally to the cerebral cortex (Anderson et al., 1997; Tamamaki et al., 1997), similar to the ventral migratory streams of the LC precursors originating from the EGL. An interesting theme that might be emerging is that some CNS progenitors give rise to restricted groups of cells with shared properties. This type of clone is not what was predicted, and conversely, the types of clones that were more predictable have not been found. For example, clones that are restricted to the same functional domain or that are the same basic cell type (e.g., neuron vs glia) are not the rule (for review, see Cepko et al., 1997). Now we see that some clones comprise very restricted types of cells in particular locations, such as the cerebellar granule cells and the LC neurons, but these same clones do not have, for example, other types of cerebellar neurons or glia. Although the cerebellum and LC are in distinct functional regions and are some distance apart, the granule cells and some LC cells may derive from a common response of their progenitors to BMP and may also share a T-shaped axonal bifurcation. Elucidating the mechanisms that underlie the production of these various types of progeny from specific CNS progenitors and the mechanisms of tangential neuronal migration will be important for a deeper understanding of neural development.

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