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Embryonic Precursor Cells from the Rhombic Lip Are Specified to a Cerebellar Granule Neuron Identity

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Summary

The specification of diverse classes of neurons is critical to the development of the cerebellar cortex. Here, we describe the purification of early embryonic precursors of cerebellar granule neurons from the rhombic lip, the dorsal aspect of the midbrain/hindbrain region. Isolation of rhombic lip cells reveals a homogenous population of precursor cells that express general neuronal markers and the granule cell marker RU49, but fail to extend neurites or express differentiation markers. Differentiation is induced by coculture with external germinal layer (EGL) cells, or their membranes, suggesting that a local inducing factor acts after formation of the EGL. Thus, proliferating precursors within the rhombic lip are specified to be granule cells very early, with the availability of an inducing factor increasing over the course of development.

Introduction

The generation of the diverse array of neurons in the mature brain is one of the most remarkable features of vertebrate development (McConnell, 1995). This process begins as cells are specified to appropriate fates and instructed to differentiate from an immature and often multipotent precursor (Anderson, 1989) to a morphologically distinct neuron (Ramon y Cajal, 1911). Studies have shown that environmental cues that are spatially (Jessell and Melton, 1992; Stemple and Anderson, 1993; Smith, 1993; Brustle et al., 1995; Dickinson et al., 1995; Fishell, 1995) or temporally restricted (or both) (McConnell and Kaznowski, 1991; Altshuler and Cepko, 1992; Dehay et al., 1993; Vogel and Davies, 1993) play important roles in these processes. These external cues appear to initiate a pattern of gene expression within precursor populations that maintains cell specification and drives differentiation to the mature phenotype. By analyzing patterns of gene expression, it appears that the acquisition of cell fate (Lo and Anderson, 1995) and differentiation to the mature phenotype (Kuhar et al., 1993; Sommer et al., 1995) is a stepwise process. The cerebellar granule cell provides a convenient system for studying the molecular basis of cell specification and differentiation, as precursor populations can be identified and isolated at different stages of development (reviewed by Hatten and Heintz, 1995).

In the postnatal cerebellum, the granule neuron precursors are located in a distinct ventricular zone at the surface of the cerebellum termed the external germinal layer (EGL) (Ramon y Cajal, 1889; Miale and Sidman, 1961). After birth, rapid proliferation in the EGL expands the zone from a single cell layer to a layer about eight cells in thickness (Fujita et al., 1966). During the second postnatal week, the migration of postmitotic granule cell precursors from the EGL into the underlying cerebellar cortex results in the disappearance of the EGL. A feature of granule cell development, revealed by fate mapping of the cerebellar cell population (Hallonet et al., 1990), is that the EGL gives rise to only one class of neuron, the granule cell. Evidence to support this conclusion has been provided by implantation (Gao and Hatten, 1994) and retroviral labeling studies (Zhang and Goldman, 1996). Furthermore, the postnatal granule cell precursor is able to proliferate and then differentiate into a neurite-bearing granule cell in vitro (Gao et al., 1991). These early stages of differentiation require close apposition of granule neuron precursors and are characterized by the expression of several proteins, including TAG-1 (Dodd et al., 1988) and astrotactin (Zheng et al., 1996; Gao et al., 1991). Therefore, by the early postnatal stage, EGL cells are normally specified to a granule cell identity and can undergo early stages of differentiation. Here, we examine when that specification occurs in the course of EGL cell development.

The precursors of the cells in the early postnatal EGL arise from the dorsal portion of the metencephalon within a structure called the rhombic lip (Ramon y Cajal, 1889, 1911; Miale and Sidman, 1961; Altman and Bayer, 1985a, 1985b; Hallonet et al., 1990; Hallonet and LeDouarin, 1993; Otero et al., 1993; Ryder and Cepko, 1994). The position of the rhombic lip results from incomplete closure of the dorsal neural tube and the subsequent formation of the pontine flexure that displaces the gapped edges laterally (see Hatten and Heintz, 1995). Around embryonic day 13 (E13) in the mouse, cells arising from the rhombic lip stream up over the edge of the tissue in a rostromedial direction onto the roof of the emerging cerebellar anlage to form the EGL (Ramon y Cajal, 1889; Hatten and Heintz, 1995). By E16, the murine EGL extends across the whole roof of the cerebellar anlage as a thin layer of proliferating cells (Miale and Sidman, 1961). In contrast, the Purkinje cells, and other cerebellar cell types, are derived from a pool of medial precursors that become postmitotic prior to E13 and migrate out through the wall of the anlage (Miale and Sidman, 1961; Altman and Bayer, 1985a, 1985b). The fact that the rhombic lip constitutes a displaced zone that can be dissected from the rest of the cerebellar anlage provides a means of exploring the early mechanisms of granule cell specification.

The present study examines the characteristics of isolated rhombic lip cells. Our results demonstrate that rhombic lip cells express general neuronal markers and the granule cell marker RU49, but not other cell type markers. Isolated rhombic lip cells proliferate, but fail to extend neurites or express TAG-1 or astrotactin, markers of granule cell differentiation. Differentiation of rhombic lip cells into granule neurons is induced after

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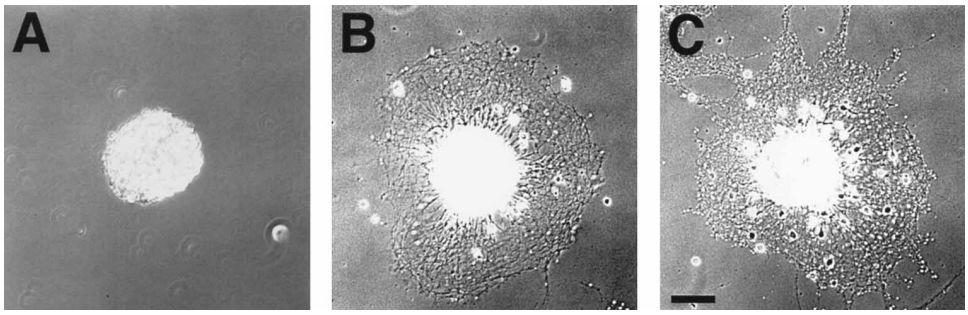

Figure 1. Purified Rhombic Lip Cells Do Not Extend Neurites In Vitro

Cells were dissociated and allowed to reaggregate for 24 hr before being transferred to poly-D-lysine-coated dishes. After an additional 24 hr in culture, the cells were fixed. While no neurites are visible in the E14 rhombic lip cell reaggregates (A), E17 cells from the nascent EGL are already capable of extending a halo of neurites (B), which are comparable with the neurites present on a reaggregate of P6 EGL cells (C). Scale bar, 100 μm.

implantation into the EGL of postnatal cerebella (Gao and Hatten, 1994) or by coculture with cells isolated from the EGL (Gao et al., 1991). The differentiation-inducing factor acts locally and its activity increases over the course of development. Thus, cells along the dorsal aspect of the closing neural tube are specified to a granule cell identity by E14, which is early in the course of cerebellar histogenesis. While some of the rhombic lip cells are already competent to respond to a differentiation-inducing activity at E14, as the first cells leave the rhombic lip to establish the EGL, the ability to induce differentiation of other granule cell precursors is achieved only after formation of the EGL is complete, in the late embryonic period.

Results

Rhombic Lip Cells Fail to Differentiate

Previous studies demonstrated that precursor cells purified from the early postnatal EGL form neurites and express markers of later stages of differentiation in an in vitro assay system (Gao et al., 1991). In the present study, we purified progenitor cells from the rhombic lip of the E14 cerebellar anlage and examined the expression of cellular markers and formation of neurites by reaggregates of this cell population. Whereas cells purified from the early postnatal EGL extended a dense halo of neurites after 24 hr in vitro (Figure 1C), rhombic lip cells failed to extend processes (Figure 1A) even after 1 week in vitro. When cells from the nascent EGL were purified from the E17 anlage and cultured as reaggregates, they extended a halo of neurites with a length and density similar to that observed for reaggregated P6 EGL cells (Figure 1B). This result suggests that the progeny of rhombic lip cells can differentiate, as measured by neurite extension, at about E17, the stage at which they have formed a thin layer across the roof of the cerebellar anlage.

To examine in more detail whether time in vitro would influence differentiation, we plated rhombic lip cells on an uncoated substratum for 3 days before testing their ability to extend neurites. Under these conditions, ~30% of the rhombic lip cells were labeled during an overnight pulse of BrdU (data not shown). This data indicates that, like P6 EGL cells (Gao et al., 1991), rhombic lip cells continue to proliferate in vitro. However, once cells are moved to a poly-D-lysine-coated substratum, proliferation ceases (X. L. Liu, C. Gallagher, and M. E. H., unpublished data) and P6 EGL cells extend neurites. In contrast with P6 EGL cells, rhombic lip cells did not extend neurites for assay periods of up to 48 hr after transfer to a poly-D-lysine-coated substratum. By vital dye labeling, the plating efficiency of purified rhombic lip cells was equivalent to that of early postnatal EGL cells, with a 70% survival rate after 48 hr on a coated surface. Thus, prolonged culture of purified rhombic lip cells did not induce neurite extension.

Rhombic Lip Cells Express Granule Cell Markers, but Not Differentiation Markers

To characterize the state of specification of rhombic lip cells, we examined the expression of cell markers. We first examined the expression of nestin, which labels undifferentiated precursor cells (Hockfield and McKay, 1985). While all the rhombic lip cells express nestin, fewer of the E17 and P0 cells express this marker and almost none of the P6 EGL cells express nestin in vitro. Those P6 EGL cells that continue to express nestin are either small round cells or elongated cells that do not have a neuronal morphology (Figures 2C and 2D). This temporal expression pattern suggests that nestin is down-regulated in differentiated cells and that a higher percentage of postnatal EGL cells differentiate in vitro compared with embryonic EGL cells.

We next investigated expression of the zinc finger protein RU49, which has recently been shown by Yang et al. (1996) to be a granule cell marker. In situ hybridization has revealed expression of RU49 in the rhombic lip of E14.5 mice and in the EGL and internal granule layer (IGL) of older mice. We found that all of the rhombic lip cells in culture express RU49 and that it continues to be expressed in differentiated P6 EGL cells in vitro, suggesting that while the rhombic lip cells express precursor markers, they are already specified at an early stage to a granule cell identity (Figures 2A and 2B). These data also demonstrate that the culture conditions do not alter the expression of cell markers observed in vivo.

To determine whether rhombic lip cells express markers of differentiated EGL cells, we stained for the neu-

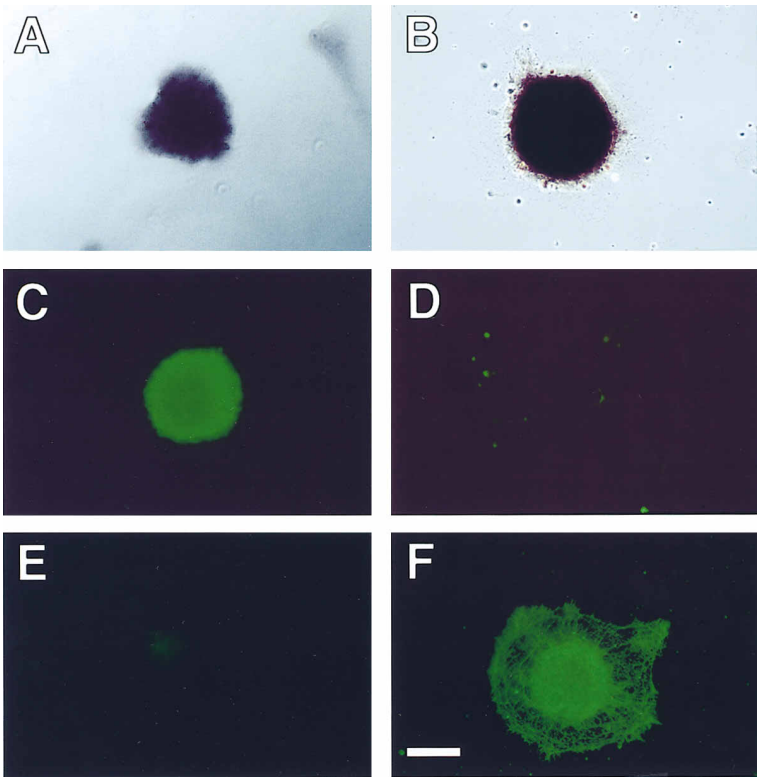

Figure 2. Rhombic Lip Cells Express Granule Cell Markers but Not Differentiation Markers

E14 rhombic lip cell reaggregates (A, C, and E) or P6 EGL reaggregates (B, D, and F) were fixed after 24 hr on poly-D-lysine and processed for in situ hybridization or immunocytochemistry. The zinc finger protein RU49 (A and B), which is a granule cell marker, is expressed in rhombic lip and EGL cells. Nestin (C and D), which labels precursor cells, is expressed in all of the rhombic lip cells but in only a few of the EGL cells. TAG-1 (E and F), which is a neuronal cell surface glycoprotein, is not yet expressed in any of the rhombic lip cells but is expressed highly on the surface of differentiated EGL cells. Scale bar, 100 μm.

ronal cell surface glycoprotein TAG-1 and the neuron–glial adhesion molecule astrotactin. Rhombic lip cells in vitro did not express either of these markers, but both of these proteins are expressed in migrating and differentiating granule cells in vivo and in E17, P0, and P6 granule cells that have extended neurites in vitro (Figures 2E and 2F; Table 1) (Dodd et al., 1988; Gao et al., 1992; Kuhar et al., 1993; Zheng et al., 1996; Kofuji et al., 1996).

The expression of other cell-specific markers was also examined. The rhombic lip cells in vitro express the general neural marker neural cell adhesion molecule (NCAM), and the neuronal markers neuron-specific enolase (NSE) and neurofilament 160 (NF 160). However, none of the rhombic lip cells express the Purkinje cell marker calbindin, or the glial-specific markers RC2 and glial fibrillary acidic protein (GFAP) (Table 1). Taken together, these data suggest that the rhombic lip is a homogenous population of progenitor cells that express general neuronal markers and the granule cell marker RU49.

Rhombic Lip Cells Differentiate when Implanted into Neonatal Cerebellum

Previous studies have shown that purified postnatal EGL granule cell precursors are competent to migrate into the cerebellar cortex and differentiate into mature granule cells within the internal granule cell layer after im-

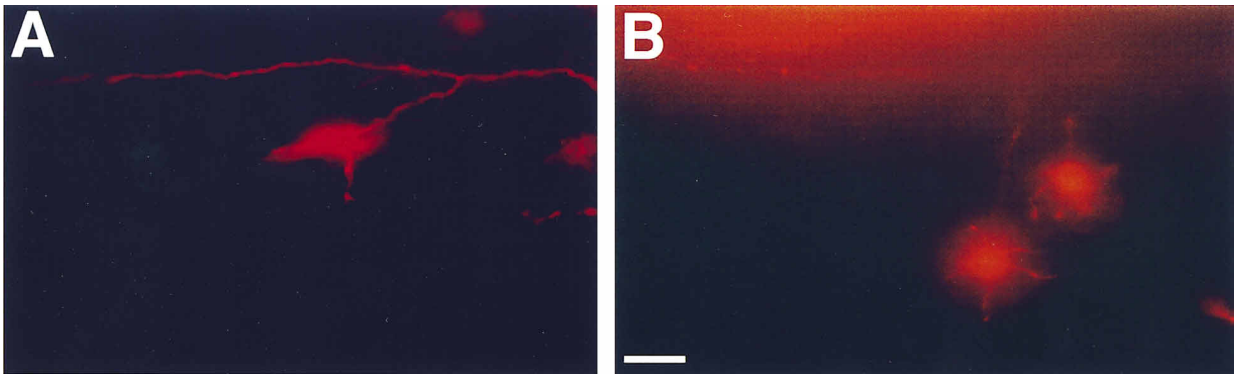

Figure 3. Rhombic Lip Cells Can Differentiate into Mature Granule Cells when Implanted into the EGL of a Host P6 Cerebellum

(A) and (B) E14 rhombic lip cells were triturated to a single cell suspension and labeled with the lipophilic dye PKH-26 before being implanted into the EGL of P6 mice. The animals were sacrificed 3–5 days later, and their brains examined for the location and morphology of the labeled cells. The labeled cells migrated past the Purkinje cell layer to the IGL. The ascending axon of the labeled neuron formed a "T-shape" characteristic of mature granule cells. Within the IGL, the cell extended several short dendritic processes. Scale bar, 50 μm.

Table 1. Characterization of Rhombic Lip Cells In Vitro

Marker	E14 Rhombic Lip Cells	E17, P0, P6 EGL Cells
Granule cell antigen		
RU49	+	+
Precursor antigen		
Nestin	+	±
Neuronal antigens (early)		
NCAM	+	+
NF 160	+	+
NSE	+	+
Calbindin	−	−
Neuronal antigens (late)		
TAG-1	−	+
Astrotactin	−	+
Glial antigens		
RC2	−	−
GFAP	−	−

Cells were plated as reaggregates as described in Experimental Procedures and then processed for in situ hybridization or immunocytochemistry with the markers listed above. + indicates that all of the cells in the reaggregate were positive for a given marker. n = 2.

Table 2. Summary of Induction Experiments

Inducer	Rhombic Lip Neurite Outgrowth
E14 Rhombic lip monolayer	−
E17 EGL monolayer	±
P0 EGL monolayer	+
P6 EGL monolayer	+
Glial monolayer	−
Purkinje monolayer	−
Cortical monolayer	±
3T3/COS Cell monolayer	−
P6 EGL conditioned medium	−
P6 Cerebellar membranes	+

5000 PKH-26-labeled rhombic lip cells were plated on a monolayer of 1.5×10^6 inducing cells. After 48 hr, the percent of rhombic lip cells with neurites was quantitated in three fields. + indicates that more than 30% of the cells had neurites. ± indicates that 2%–30% of the cells had neurites. n = 5.

plantation into the EGL of isochronic mice (Gao et al., 1992). In the present study, heterochronic experiments were carried out by implanting E14 rhombic lip cells labeled with the lipophilic dye PKH-26 into the P6 murine EGL. After survival times of 3–5 days, the fate of implanted cells was assayed by their morphology (Gao and Hatten, 1994). After implantation into the early postnatal EGL, rhombic lip cells migrated and differentiated into granule cells with the characteristic "T-shaped" morphology of a mature granule neuron, including parallel fibers in the molecular layer and dendritic processes in the IGL (Figures 3A and 3B). Similar results were seen when E17 nascent EGL cells were implanted into the P6 EGL (data not shown). Granule cells were the only class of differentiated labeled cells detected in the cerebellar cortex following rhombic lip implantation, as was observed following implantation of P6 EGL cells (Gao and Hatten, 1994). These results suggest that the rhombic lip cells are already specified to become granule cells when transplanted into the early postnatal cerebellum.

Preliminary experiments (n = 4) have been performed to examine the commitment of rhombic lip cells to a granule cell identity. We heterotopically implanted purified labeled rhombic lip cells into the lateral ventricles of E14 mouse cortices. We observed that 2 days following implantation, most labeled rhombic lip cells had formed small reaggregates that were attached to the ventricular surface. Very few of the labeled cells, ~0.02% (n = 8), projected processes into the wall of the cortical anlage. These cells expressed the bipolar morphology characteristic of developing granule cells in the cerebellum (data not shown). Very few cells were visible 4 days after implantation, but some had short processes (M. Li, J. A., C. Gallagher, and M. E. H., unpublished data).

Rhombic Lip Cells Can Be Induced to Differentiate by EGL Cells

To identify whether a single cell class within the P6 environment would induce rhombic lip cell differentiation, rhombic lip cells were cocultured with cell types purified from P6 cerebella and rhombic lip cell neurite extension was assayed in vitro. In these experiments, rhombic lip cells were labeled with the dye PKH-26 and plated on a monolayer of unlabeled cells. After 48 hr in culture, the labeled cells were assayed for neurite extension. In isochronic experiments, when labeled rhombic lip cells were plated on a monolayer of unlabeled rhombic lip cells, less than 0.1% (n = 5) of the labeled cells extended neurites (Figures 4A and 5A). In contrast, when rhombic lip cells were plated on a monolayer of P6 EGL cells, a subpopulation of the labeled cells extended neurites (Figures 4B and 5A).

To examine whether neurite extension by rhombic lip cells was accompanied by the down-regulation of general CNS precursor cell markers, we stained the rhombic lip cells on the P6 EGL monolayer for nestin, and found that the rhombic lip cells with neurites were now nestin negative and that only the round rhombic lip cells and a few elongated cells in the P6 EGL monolayer were still positive for the marker (Figures 4C and 4D).

The effect of developmental age of the cells on neurite induction was examined by plating labeled rhombic lip cells on monolayers of EGL cells purified from different developmental stages. On a monolayer of E17 EGL cells, 17.1% ± 1.7% (averaged and normalized to the percent of P6 EGL cells with neurites on a P6 monolayer ± SEM; n = 5) of the rhombic lip cells had neurites after 48 hr. Monolayers of older EGL cells from P0 and P6 mice induced the differentiation of 32.0% ± 4.0% and 37.3% ± 5.7% (SEM; n = 5) of the rhombic lip cells, respectively (Figure 5A). The number of rhombic lip cells that differentiated did not increase if the cultures were maintained for up to 1 week or if a second layer of P6 EGL cells was plated on top. Monolayers of postnatal EGL cells were thus significantly ($p < 0.01$, *t* test) more effective at inducing differentiation of the rhombic lip cells than monolayers of embryonic cells. However, the difference

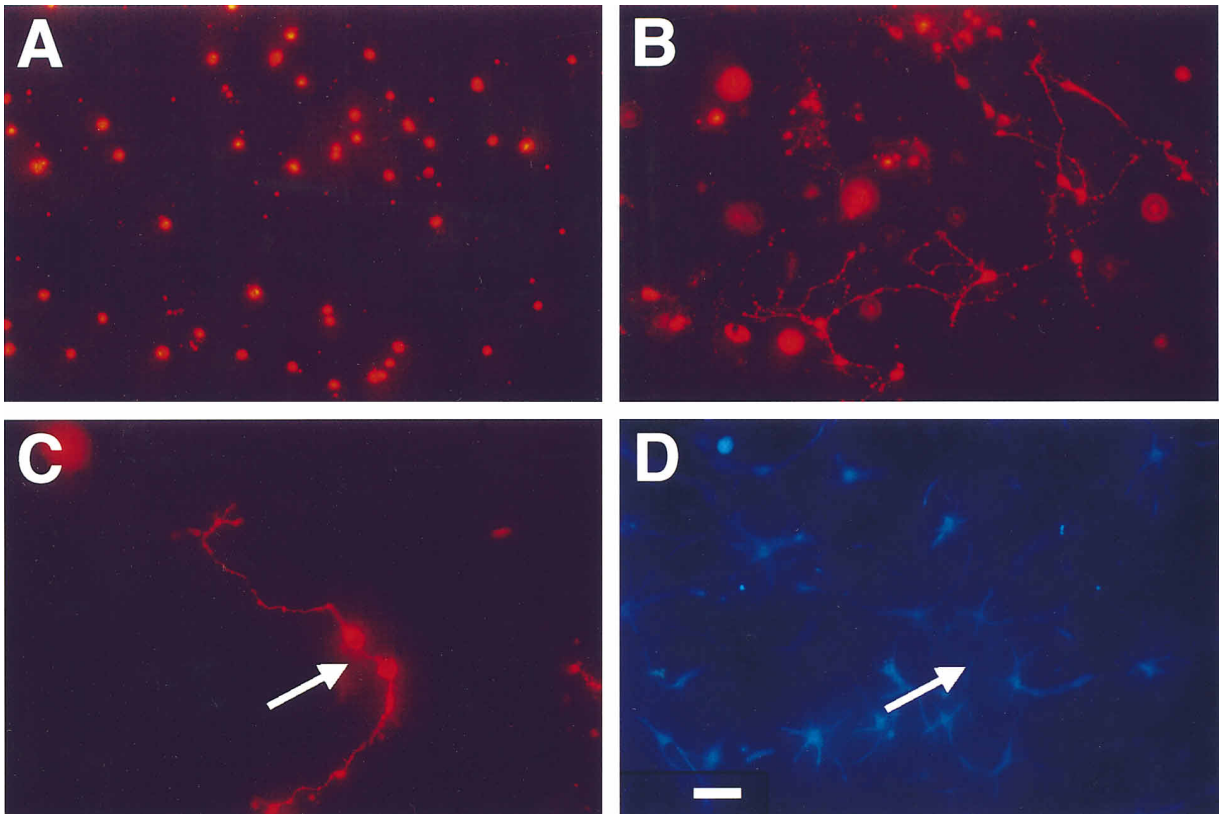

Figure 4. Rhombic Lip Cells Can Extend Neurites when Cultured on a Monolayer of P6 EGL Cells

Isolated E14 rhombic lip cells were labeled with the dye PKH-26 and plated at a low density on a monolayer of unlabeled E14 rhombic lip cells (A) or P6 EGL cells (B). While none of the labeled cells differentiated on the rhombic lip cell monolayer after 48 hr, many of those plated on the EGL cells extended neurites. A pair of dye-labeled E14 rhombic lip cells (marked by the arrow) on a P6 EGL monolayer (C) were double labeled with an antibody to the precursor marker nestin using an AMCA-conjugated secondary antibody (D). While some of the cells in the P6 EGL monolayer stained positive for nestin, the rhombic lip cells with neurites no longer expressed the marker. Scale bar, 50 μm in (A) and (B); 25 μm in (C) and (D).

between older and younger postnatal EGL cell monolayers was not significantly different ($p > 0.05$, *t* test). Therefore, there is a positive correlation between the maturity of the EGL cells in the monolayer and the percent of rhombic lip cells that were induced to differentiate.

To examine whether the competence to respond to the inducing factor changes with developmental age, P6 EGL cells were plated as monolayers, and cells from different stages were dye labeled and plated on the P6 EGL monolayers ($n = 4$). We observed that the postnatal EGL cells were more likely to extend neurites on a P6 EGL monolayer than the embryonic cells on a P6 EGL monolayer (Figure 5B). Once again, there was a significant difference between the embryonic and postnatal ages, but not among the postnatal ages. The failure of all rhombic lip cells to differentiate, even in coculture with older cells, suggests that only a subpopulation of cells within the rhombic lip is competent to respond to local inducing signals in the EGL required for neurite extension. Furthermore, these experiments indicate that the competence to respond to the inducing factor provided by the P6 EGL cells also increases during development.

Since granule cells are only one of the several classes of cells in the cerebellum, monolayers were prepared from other cell types to determine their effect on rhombic lip cells (Table 2). Monolayers of purified glial, Purkinje, or forebrain cortical cells caused the majority of the rhombic lip cells to assume a neuroepithelial appearance (data not shown), resembling drawings of rhombic lip cells proliferating and moving over the surface of the cerebellar anlage in vivo (Ramon y Cajal, 1911; Ryder and Cepko, 1994). The labeled rhombic lip cells on a glial monolayer were positive for nestin, NCAM, and NF 160, but negative for RC2 and GFAP (data not shown), indicating that they retained neuronal identity. Interestingly, monolayers of cortical cells did cause a few (8.6% ± 1.9% [SEM; $n = 4$]) of the rhombic lip cells to extend neurites that resembled those of granule cells. Non-neuronal cells, including NIH 3T3 and COS cell monolayers, had no effect on rhombic lip cell neurite formation.

The Inducing Factor Acts Locally

To examine whether the induction of rhombic lip cell neurites by EGL cells was mediated by a freely diffusible factor or by local mechanisms, rhombic lip cells were cocultured with medium conditioned by P6 EGL cells. In this system, the EGL cells were plated onto a Millipore

filter that allowed diffusion of soluble factors onto the rhombic lip cells plated underneath the culture insert (Hunter and Hatten, 1995). Even after 1 week in culture, no rhombic lip cell neurite extension was observed (Figure 6A). In addition, conditioned media from cultures of cells isolated from the whole P6 cerebellum, EGL cells of different ages, or choroid plexus cells did not cause rhombic lip cells to extend neurites. These data are consistent with the conclusions that the inducing activity we assayed is not a freely diffusible factor and is either highly unstable or acts at a very short range.

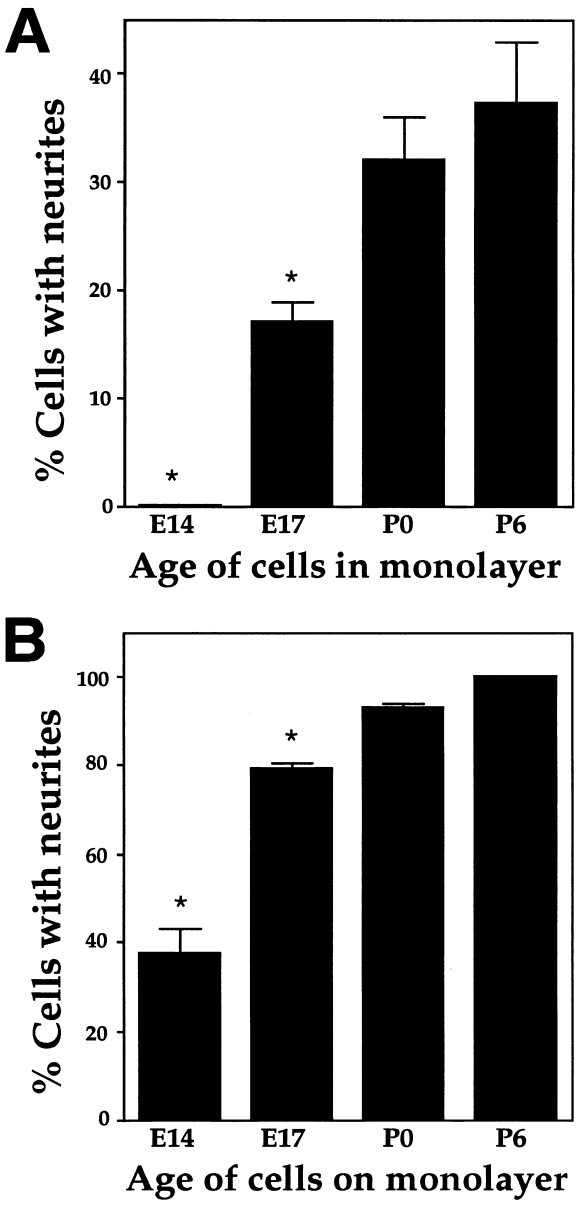

Figure 5. Both the Inducing Activity and the Competency to Respond to the Inducing Factor Increase with Age of the Cells

(A) E14 rhombic lip cells or EGL cells from different aged mice were purified and plated in a monolyer. After the cells had settled, E14 rhombic lip cells labeled with PKH-26 were plated at a low density on top (n = 5).

(B) P6 EGL cells were plated in monolayers, and labeled rhombic lip or EGL cells purified from different aged mice were plated at a low density on top (n = 4). In both (A) and (B), the cultures were fixed 48 hr later and the percent of labeled cells with neurites was quantitated in three separate fields. All data were averaged and normalized to the percent of P6 EGL cells that differentiate on a P6 EGL monolayer ± SEM. The asterisk indicates that the data are significantly different from the rightmost bar of each graph ($p < 0.05$, *t* test).

To examine whether factors or activities associated with the plasma membrane were sufficient to induce neurite formation by rhombic lip cells, we tested the effect of plasma membranes prepared from P6 cerebella. In contrast with results obtained by coculture of P6 EGL cells in a Millipore filter system, membranes from the cerebellum induced rhombic lip cells to form neurites (Figure 6B). The effect of the membranes was dose dependent and saturated at 100 μg/ml (58.6% ± 14.5%, averaged and normalized to the maximal response of P6 EGL cells ± SEM; n = 4) (Figure 6C). Heart membranes had no significant effect on rhombic lip cell neurite extension compared with cerebellar membranes on rhombic lip cells at 100 μg/ml ($p < 0.05$, *t* test), with minor levels of activity in membranes prepared from lung or liver. Furthermore, no effect has previously been observed with membranes from 3T3 or glial cells (Gao et al., 1992). In summary, these experiments suggest that the differentiation-inducing factor acts locally on rhombic lip cells.

To test whether differentiation is the result of an increase in the levels of an inducing factor or a decrease in the levels of an inhibitory factor during development, we studied the effect of rhombic lip cells on the differentiation of P6 EGL cells. P6 EGL cells were dye labeled and mixed with unlabeled rhombic lip cells at ratios of 1:100, 1:10, 1:1, 10:1, and 100:1. Both labeled and unlabeled neurites were visible in all of these cultures (data not shown), suggesting that the rhombic lip cells do not contain an activity that inhibits the differentiation of P6 EGL cells. Therefore, the concentration or potency of a differentiation-inducing factor increases during development.

Discussion

The purification of rhombic lip cells provides an approach for examining the mechanism of granule cell specification and differentiation. The current studies demonstrate that cells from the rhombic lip region, the diversity of which has not been previously determined, express RU49, a granule cell marker, and have a single differentiated morphology in vitro. These data suggest that the rhombic lip cells are a homogenous population of precursors already specified to granule cell identity at E14, not multipotent progenitors capable of generating a diverse number of cell types in the cerebellum. The failure of purified rhombic lip cells to form neurites or express markers of differentiated granule cells, including TAG-1 and astrotactin, suggests that they are not yet competent to differentiate when cultured in isolation. Local signals appear to control this step in development, since coculture with older precursor cells or implantation into the postnatal cerebellum induces differentiation of a subpopulation of rhombic lip cells. Thus, at E14, when the EGL is emerging, a subpopulation of cells is already competent to respond to local signals that

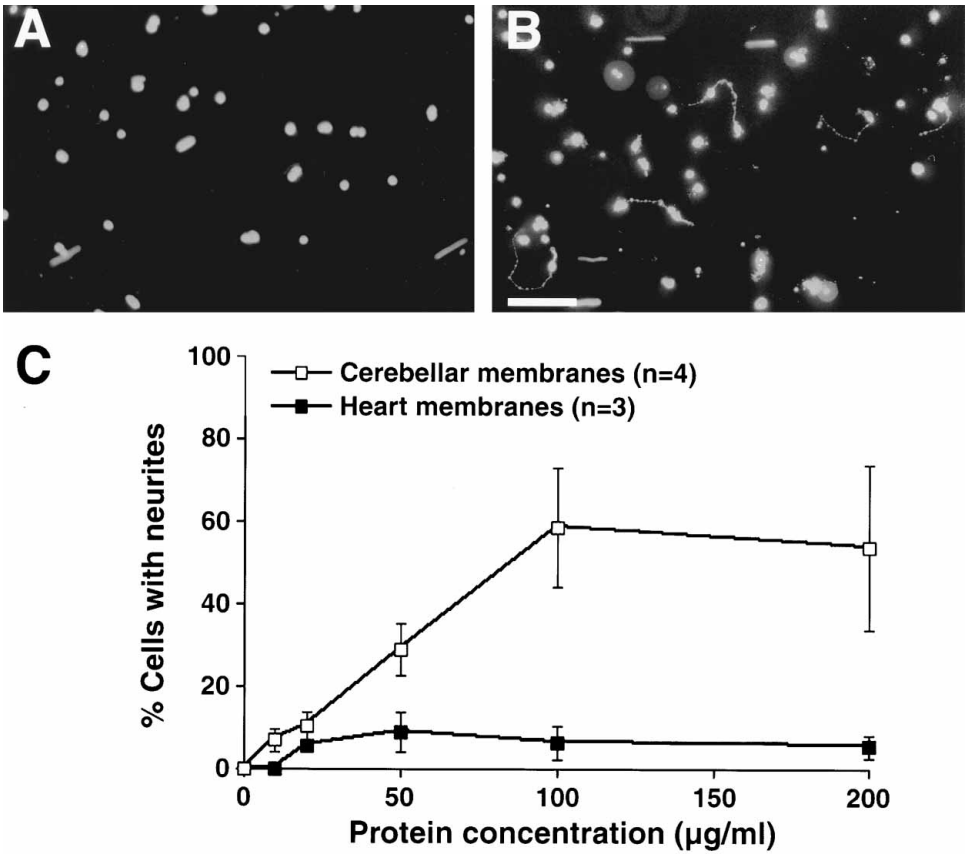

Figure 6. The Inducing Factor Acts Locally

E14 rhombic lip cells were labeled with PKH-26 and plated at a low density on a poly-D-lysine-coated dish.

(A) P6 EGL cells were plated in tissue culture inserts above the rhombic lip cells, and the cultures were allowed to grow for up to 1 week, but no neurites were observed on the labeled rhombic lip cells.

(B) Plasma membranes isolated from P6 cerebella were added to the rhombic lip cells at 100 μg/ml and 48 hr later the cultures were fixed. Neurites are apparent on ~58% of the rhombic lip cells. Scale bar, 100 μm.

(C) Dose dependence of the effect of plasma membranes on rhombic lip cell neurite outgrowth. Membranes isolated either from cerebellum (open squares, n = 4) or heart (closed squares, n = 3) were added at varying concentrations to low density cultures of labeled rhombic lip cells. After 48 hr, the percent of cells with neurites was quantitated for three different fields. All data was averaged and normalized to the percent of P6 EGL cells with neurites in the presence of 50 μg/ml cerebellar membranes ± SEM. The effect of the membranes on rhombic lip cells saturates at 100 μg/ml, at which point the cerebellar membranes are significantly more effective at inducing neurite outgrowth than the heart membranes ($p < 0.05$, *t* test).

regulate differentiation, whereas the ability to induce differentiation of other granule cell precursors is acquired after completion of EGL formation, in the late embryonic period.

Our conclusion that rhombic lip cells are specified to a granule neuron identity is consistent with studies demonstrating that the rhombic lip is the origin of the EGL (Altman and Bayer, 1985a, 1985b; Hallonet and LeDouarin, 1993; Otero et al., 1993; Ryder and Cepko, 1994), and that the progeny of the EGL are specified to a granule neuron identity (Hallonet et al., 1990; Gao and Hatten, 1994; Zhang and Goldman, 1996). The present work extends previous work by isolating granule cell precursors at an earlier stage and demonstrating directly that these rhombic lip cells differentiate into granule cells.

Previous studies have shown that EGL is not inhibitory for the differentiation of other cerebellar cell types, including interneurons and glia (Gao and Hatten, 1994). The finding that the transplantation of purified rhombic lip precursors into the P6 EGL generates exclusively granule neurons indicates that these cells are normally specified to a granule cell identity. This conclusion is supported by our finding that rhombic lip cells are positive for RU49, a zinc finger protein that marks granule cells. It is important to note that the present experiments do not fully examine whether rhombic lip cells are committed to this fate. Preliminary experiments have been performed to examine the commitment of rhombic lip cells to a granule cell identity. Our preliminary data obtained by implanting purified rhombic lip cells into fetal cortex (M. Li, J. A., C. Gallagher, and M. E. H., unpublished data) and coculturing rhombic lip cells with cortical cells support the conclusion that rhombic lip cells are committed. However, a more complete study of marker expression following heterotopic transplantation needs to be performed to confirm these conclusions. From our current data, we propose that, within the context of cerebellar development, rhombic lip cells are specified to a granule cell identity.

The results obtained in this study are consistent with the general conclusion that precursor cells in the dorsal aspect of the neuroepithelium express markers of granule cell identity very early within the program of histogenesis of the cerebellar anlage. The timing of their specification is similar to that of the other cerebellar progenitor cell populations within the more medial aspect of the neuroepithelium. The striking difference between progenitors in the dorsal rhombic lip domain is that they are specified long before their penultimate cell division. Thus, whereas immature Purkinje cells exit the cell cycle within the ventricular germinal zone (Miale and Sidman, 1961), precursors along the rhombic lip continue to proliferate, moving onto the roof of the anlage to form a displaced germinal zone.

Although specified to become granule neurons, precursors within the rhombic lip are not all competent to differentiate into process-bearing granule neurons and none of them has yet obtained the ability to induce differentiation of other granule cell precursors. This finding indicates that multiple steps are required for acquisition of the mature phenotype. The failure of isolated rhombic lip cells to undergo differentiation after prolonged cell culture, equivalent to the time required for cells to begin to enter a differentiation program in vivo, suggests that the tempo of differentiation is not set by an intrinsic "clock" exclusively. Alternatively, a "clock" may normally regulate differentiation in vivo, but the isolated precursor cells have been removed from the signal required to activate it. The initiation of differentiation, as assayed by neurite extension in vitro, appears to require local signals provided by other cells within the cerebellum. Heterotypic interactions between rhombic lip cells and Purkinje or glial cells do not seem to provide the correct signal for rhombic lip cells to differentiate into granule neurons, which is consistent with previous studies of postnatal granule cell precursors (Gao et al., 1991, 1992). Rather, it appears to be more mature granule cell precursors that provide the signal for rhombic lip cell differentiation. The simplest interpretation of our heterochronic cell mixing experiments is that competence to induce EGL cell differentiation is attained sometime after completion of the formation of a single cell layer across the roof of the anlage in the late embryonic period. As the density of the cells in the EGL increases, starting at E16 and peaking after P6, so does the competence of the granule cell precursors to respond to the inducing factor and to induce differentiation. The fact that the first differentiated granule neurons appear within the internal granule cell layer as early as E19 in vivo lends credence to our observations that embryonic EGL cells are competent to differentiate in vitro.

Our results suggest that the rhombic lip cells go through a series of steps to become competent to differentiate into granule cells. A possible model that incorporates our observations is illustrated (Figure 7). During the early stages of development, precursor cells proliferate under the influence of homotypic precursor cell interactions. By E14, about half of these cells have acquired the competence to receive a signal to differentiate. At a later stage of development, approximately E17, more of the cells are competent to respond to the differentiation signal, and the ability of precursor cells to induce differentiation of other progenitors within this zone commences. As development continues, the number of cells competent to respond to the inducing activity, as well as the levels of the signal, rise. In the illustrated model, competence to respond to the differentiation factor (i.e., competence to differentiate) is indicated by expression of a cell surface receptor, while the ability to induce differentiation is indicated by the expression of the ligand for this receptor. This receptor–ligand model is based on our observation that the granule cell requires cell–cell contact to advance through the various stages of differentiation; however, we cannot eliminate the possibility that the inducing factor is a diffusible factor that is either unstable or requires a high local concentration and so acts at a very short range. We have shown, however, that differentiation is not caused by a decrease in the levels of an inhibitory factor over the course of development, since embryonic rhombic lip cells do not inhibit the differentiation of postnatal EGL cells.

The specification of granule cell identity within the rhombic lip precursor population suggests that the signals responsible for establishing the identity of the granule cell act early in nervous system development. In this regard, it is interesting to consider the possibility that the specification of these progenitors may result from their dorsal positioning within the developing neuroepithelium (Hallonet and LeDouarin, 1993). Thus, factors that influence dorsoventral polarity of the neural tube (Lumsden and Graham, 1995; Liem et al., 1995) may also induce rhombic lip cell specification. The precursor population of the rhombic lip therefore provides an experimental approach to identify the factors that dorsalize the granule cell precursors and control the expression of cellular markers, such as RU49, that are critical to the patterning and histogenesis of the CNS.

Experimental Procedures

Isolation of Progenitor Cells from the Rhombic Lip of the Cerebellar Anlage

The present experiments were carried out with cells isolated from C57BL/6_J mice. To isolate rhombic lip cells, pregnant dams were anesthetized with nembutal, embryos were removed from the uterus, and the midbrain/hindbrain region was removed by dissection. The region containing the cerebellar anlage and the brainstem was isolated by two incisions: across the colliculus anteriorly and the spinal cord posteriorly using a Beaver 4310 scalpel (#65 blade). After removal of the meninges and the choroid plexus covering ventricle IV, the cerebellum was separated from the pons as described (Hatten and Sidman, 1978). The rhombic lip, clearly visible as a thin skirt of cells at the dorsal aspect of the anlage was removed with a castroviejo–wheeler discission knife (18 mm) (Weck Opthalmics, Xomed-Treace, Inc.). The rhombic lip tissue was transferred to a 15 ml tube containing chilled Ca^{2+}- and Mg^{2+}-free PBS (CMF-PBS), and washed by centrifugation at 750 × g for 5 min at 4°C. To prepare a single cell suspension, tissue fragments were incubated in 0.08% trypsin (Worthington) in CMF-PBS containing 0.02% EGTA for 15 min at 37°C. After resuspension in 2 ml of CMF-PBS containing 0.05 mg/ml DNAse (Worthington), the tissue was dissociated into single cells by gentle trituration. In some experiments, isolated rhombic lip cells were labeled with PKH-26 (10 μl/ml, Sigma) as described (Gao et al., 1992; Gao and Hatten, 1994).

EGL cells were purified from E17, P0, and P6 cerebella as described (Gao et al., 1991; Hatten, 1985). Astroglial cells were purified from the same preparation used to harvest EGL cells as described by Hatten (1985). Purkinje cells were purified from P0 or E17 mice as described by Baptista et al. (1994), with the exception that ovomucoid was omitted in the trituration step. Cortical cells were obtained

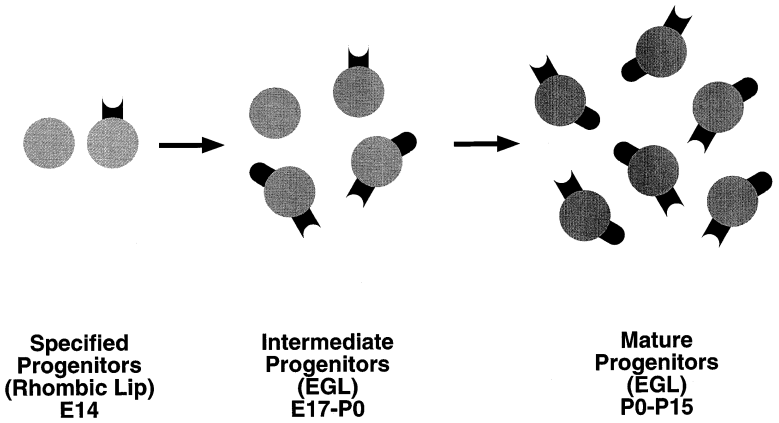

Figure 7. A Model of Rhombic Lip Cell Differentiation

In this model, competence to respond to a differentiation-inducing factor is indicated by expression of a cell surface receptor, while the ability to induce differentiation is indicated by the expression of the ligand for this receptor. At E14, rhombic lip cells proliferate under the influence of homotypic precursor cell interactions, and about half of them are competent to receive a signal to differentiate into granule cells. This cell population is already specified to granule cell identity at this early stage. The ability of precursor cells to induce differention of other progenitors within this zone commences somewhat later, by E17. From that point on, the number of cells competent to respond to the inducing activity, as well as the levels of the signal, rise.

from E14 mouse forebrain as described (Hunter and Hatten, 1995). All cultures were maintained in poly-D-lysine-coated (0.5 mg/ml) Nunc 16 well Lab Tek culture slides in Eagle's basal medium with Earle's salts (BME), supplemented with 2 mM glutamine, 32 mM glucose, 20 U/ml penicillin–streptomycin, 10% horse serum, and 5% fetal bovine serum.

For reaggregate cultures, cells were plated at 3×10^5 cells/well in uncoated wells and maintained at 37°C for 24 hr. For neurite extension assays (Gao et al., 1992), the reaggregates were transferred into serum-free medium (DMEM, B27 and N2 supplements [Gibco], 1 mM sodium pyruvate, 2 mM glutamine, 32 mM glucose, and 20 U/ml penicillin–streptomycin) by centrifugation at 250 × g and plated in poly-D-lysine-coated wells for a minimum of 24 hr at 37°C. The cultures were then rinsed in phosphate-buffered saline (PBS) (pH 7.4), fixed in 4% paraformaldehyde in PBS for 15 min, washed in PBS, and mounted in Gel/Mount (Bio-Meda Corp.).

BrdU Labeling of Rhombic Lip Cells In Vitro

Cells were plated as reaggregates and incubated overnight at 37°C with BrdU (1:1000, Amersham). The reaggregates were then transferred to poly-D-lysine-coated wells in serum-free medium and allowed to attach overnight at 37°C. They were then fixed as above and treated with 0.1% Triton X-100 in PBS for 20 min. Staining was performed according to the directions of the manufacturer and the slides were mounted in Gel/Mount. The mitotic index was measured by microscopy, a Zeiss Axiophot microscope fitted with Nomarski optics to count the percentage of labeled cells in five different optical planes within the reaggregate. At least five reaggregates were counted per well, providing a sample of ~3000 cells.

In Situ Hybridization of RU49 Expression

The protocol followed those of Schaeren-Wiemers and Gerfin-Moser (1993) and Yang et al. (1996) with the following minor modifications. Reaggregate cultures were fixed in 4% paraformaldehyde and permeabilized at −20°C in 100% methanol. The cells were then rehydrated through a series of methanol and finally put into PBS with 0.1% Tween-20. The slides were then treated with 10 μg/ml proteinase K, washed, and refixed in 4% paraformaldehyde. After acetylation, and prehybridization, the digoxigenin (DIG)-labeled probes (Yang et al., 1996) were added at 1 μg/ml overnight at 72°C.

Immunocytochemistry of Cellular Markers and Markers of Granule Cell Differentiation

The cells were fixed in 4% paraformaldehyde, rinsed in PBS, and incubated in 1 M glycine (pH 7.5) for 15 min. They were then washed again in PBS and, for cytoplasmic antigens, permeabilized in 0.1% Triton X-100 with 2% milk in PBS for 15 min. All slides were blocked in 20% NGS in PBS for 3 hr. The cultures were then incubated in primary antibody in 1% NGS overnight at 4°C: TAG-1 (1:1, provided by J. Dodd and T. Jessell) (Dodd et al., 1988); NCAM (1:100, Sigma) (Thiery et al., 1977); GFAP (1:1000, Dako) (Hatten and Liem, 1981); astrotactin (1:1000) (Zheng et al., 1996); RC2 (Misson et al., 1988); nestin (1:50, provided by R. McKay) (Hockfield and McKay, 1985); calbindin (1:10,000, SWant) (Celio et al., 1990); NSE (1:100, BioGenex), NF 160 (1:50, provided by R. Liem); and preimmune serum (1:1000). The antibody was washed off by four rinses in PBS with 2% milk and the cultures were incubated in either goat anti-mouse or anti-rabbit FITC-conjugated secondary antibodies (1:100, Cappel) for 30 min. Following washes as described above, the cultures were mounted in 1,4-diazabicyclo-[2.2.2]octane (DABCO, 0.22 M, Sigma) in PBS. Labeling was recorded with a Zeiss Axiophot microsope fitted with epifluorescent illumination, plan-neofluor 20× and 40× objectives, and an Axiophot camera module.

For double-labeling experiments involving antibody staining of PKH-26 stained cells, the cells were permeabilized by freezing them in 30% sucrose in PBS at −20°C for 1 hr followed by thawing at 37°C and rinsing in PBS. Secondary antibodies (goat anti-rabbit IgG and goat anti-mouse AMCA, ICN Biomedicals) were used at 1:10 for 2 hr.

Implantation of Rhombic Lip Cells and Embryonic EGL Cells

For implantation into the P6 cerebellar EGL, rhombic lip and E17 EGL cells were purified as described above and labeled with PKH-26. Single cell suspensions (10,000 labeled cells) were implanted into each cerebellar hemisphere as described by Gao and Hatten (1994). The incorporation of labeled cells into the EGL and differentiation into cells expressing morphological features of granule neurons, including the signature "T-shaped" parallel fiber axon and small cell body with radiating short dendrites (see Gao and Hatten, 1994 for details of cell identification), were examined 3–5 days postimplantation by fluorescence microscopy of serial coronal sections.

Heterotopic implantation of PKH-26-labeled rhombic lip cells into the lateral ventricles of E14 mouse embryos was performed using Isoflurane inhalation as an anesthetic for the pregnant dam. A midline laparatomy was performed and 10,000 cells were injected per embryo by hand using a Hamilton syringe. Embryos were perfused 2 or 4 days following implantation and 100 μm thick vibratome sections of the brains were examined as above.

Coculture of Rhombic Lip Cells with Other Cell Types

For coculture experiments, 1.5×10^6 unlabeled cells were plated on a poly-D-lysine-coated well and the cells were allowed to settle for at least 30 min before PKH-26-labeled rhombic lip cells were added on top of the culture at low density (5000 cells/well). After 48 hr, the cultures were fixed with 4% paraformaldehyde for 15 min and mounted in DABCO. For double monolayer experiments, 1.5×10^6 P6 EGL cells were added 30 min after the labeled rhombic lip cells were plated onto the first monolayer.

Coculture of Rhombic Lip Cells with Conditioned Medium or Membrane Fractions of Cerebellar Cells

For experiments with conditioned medium, reaggregates of rhombic lip cells were plated on uncoated wells for 24 hr, after which they were transferred to poly-D-lysine-coated slides in serum-free medium. Alternatively, PKH-26-labeled rhombic lip cells were plated at a low density (5000 cells/well) directly onto a poly-D-lysine-coated well. EGL cells from different-aged mice or other cell types were plated in separate tissue culture inserts (Nunc) in serum-free medium (4×10^5 cells/insert) and placed on top of the rhombic lip cells (Hunter and Hatten, 1995). In some cases, conditioned medium from P6 EGL cultures was added directly to rhombic lip cell cultures. Rhombic lip cells were examined every 24 hr for up to 1 week and then fixed in 4% paraformaldehyde.

Membranes were prepared from mouse P6 cerebella and liver, as well as from adult heart and lung, according to Stitt and Hatten (1990) with the following modifications. Tissue was collected in a buffer containing protease inhibitors (50 mM Tris [pH 7.2], 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 mM pepstatin, and 1 mM aprotinin) and homogenized with a Dounce homogenizer. After the two-phase polyethylene glycol–Dextran gradient (Brunette and Till, 1971), the plasma membrane pellet was resuspended in CMF-PBS and centrifuged again at 40,000 × g for 50 min to eliminate polymer contamination. The final pellet was resuspended in 20 mM Tris (pH 7.2) and 1 mM $MgCl_2$ buffer and rehomogenized. Protein concentration was determined by bicinchoninic acid (BCA, Pierce) analysis. PKH-26-labeled rhombic lip cells were plated at a very low density (5000 cells/well) in poly-D-lysine-coated Nunc 16-well Lab Tek culture slides. Membrane preparations were immediately sonicated and added to the cultures at final protein concentrations of 10–200 μg/ml. Cultures were fixed after 48 hr with 4% paraformaldehyde and mounted in DABCO.

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