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Math1 Is Expressed in Temporally Discrete Pools of Cerebellar Rhombic-Lip Neural Progenitors

Report

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Summary

We have utilized an in vivo-inducible genetic-fate-mapping strategy to permanently label cohorts of Math1-positive cells and their progeny that arise in the rhombic lip of the cerebellar primordium during embryogenesis. At stages prior to E12.5, with the exception of the deep cerebellar nuclei, we find that Math1 cells migrate out of the cerebellar primordium into the rostral hindbrain to populate specific nuclei that include cholinergic neurons of the mesopontine tegmental system. Moreover, analysis of *Math1*-null embryos shows that this gene is required for the formation of some of these nuclei. Around E12.5, granule cell precursors begin to be labeled: first, ones that give rise to granule cells that predominantly populate the anterior lobes of the adult cerebellum and later, those that populate progressing more caudally lobes until labeling of all granule cell precursors is complete by E17. Thus, we demonstrate that the cerebellar rhombic lip gives rise to multiple cell types within rhombomere 1.

Introduction

The cerebellum develops from an embryonic primordium within dorsal rhombomere 1 (r1) that contains two germinal zones: the ventricular neuroepithelium and the cerebellar rhombic lip (Altman and Bayer, 1997; Wingate and Hatten, 1999; Zervas et al., 2004). Granule cells of the cerebellum, the most abundant neuronal type in the adult brain, arise from a precursor population that originates in the cerebellar rhombic lip (herein shortened to "rhombic lip") (Alder et al., 1996), whereas all other cerebellar neurons are thought to arise from the ventricular zone (Hallonet et al., 1990). Specification of granule cell precursors requires the basic helix-loop-helix (bHLH) transcription factor Math1 (Ben-Arie et al., 1997), a mouse homolog of *Drosophila* atonal (Akazawa et al., 1995). Once specified, granule cell precursors (GCP) migrate rostrally over the dorsal surface of the cerebellar anlage to form the external granule layer (EGL). GCP maintain Math1 expression throughout their postnatal proliferation in the EGL until their inward radial migration to form the internal granule layer (IGL) (Hatten and Heintz, 1995; Helms et al., 2001).

A number of recent fate-mapping studies have proposed that in addition to GCP, the cerebellar rhombic lip also produces other neuronal lineages that migrate ventrally out of the cerebellar anlage to populate the anterior hindbrain (Wingate, 2001). By microsurgically constructing quail-chick chimeras at E2 (stage 10), with HoxA2 expression to mark the r1/r2 boundary, it was found that in addition to granule cells, grafts of dorsal r1 also gave rise to a ventral population of cells that contributed to the lateral pontine nucleus (Wingate and Hatten, 1999). An in vivo retroviral lineage analysis of chick embryos infected at stages 10–12 found that clones of progenitor cells in the cerebellar primordium that gave rise to granule cells could also give rise to noradrenergic (TH^+) neurons in the locus coeruleus as well as cells of the pontine reticular formation, vestibular nuclei, and parvocellular isthmic nucleus (Lin et al., 2001). Ventral migration of rhombic-lip derivatives along the mid-hindbrain boundary has been directly observed in vivo in zebrafish embryos mosaic for a GFP transgene (Koster and Fraser, 2001). Although these fate-mapping studies clearly suggest that the cerebellar rhombic lip is the germinal origin of specific rostral hindbrain nuclei in addition to granule cells, they were limited by an inability to define either the initial cell populations that are labeled or the precise structures and cell types that arise later in development.

Recently, genetic methods of fate mapping with Cre- or Flp-mediated recombination have been developed that enable permanent marking of cells within embryonic regions defined by gene expression (Dymecki and Tomasiewicz, 1998; Zinyk et al., 1998). Using this approach, it was shown that Wnt1-positive cells of the dorsal hindbrain give rise to the precerebellar afferent nuclei that project mossy fibers to the granule cells of the cerebellum (Rodriguez and Dymecki, 2000). Furthermore, ligand-inducible Cre recombinases have been developed that allow for cohorts of cells to be labeled at specific embryonic stages in transgenic mice (Danielian et al., 1998; Feil et al., 1996). This approach has been utilized to construct temporal fate maps of the Wnt1 and Engrailed-1/Engrailed-2 embryonic territories in the midhindbrain region (Sgaier et al., 2005; Zervas et al., 2004).

In this study, we have generated a temporal fate map of rhombic-lip neural progenitors by expressing an inducible Cre recombinase under the control of the 3′ Math1 enhancer (Helms et al., 2000). Although Math1 is expressed in the entire dorsal neural tube from posterior r1 to the spinal cord (Akazawa et al., 1995), we have focused exclusively on the Math1 neural progenitors of the cerebellar rhombic lip by using En1-Cre cumulative fate mapping (Kimmel et al., 2000; Zervas et al., 2004) to distinguish the Math1 rhombic-lip cells that arise in r1 from those derived from r2–r8. Surprisingly, we find that early cohorts of $Math1^+$ cells (labeled prior to E12.5) give rise to the deep cerebellar nuclei as well as specific nuclei in the rostral hindbrain that include components of the mesopontine cholinergic system. After E12.5, $Math1^+$ cells develop primarily into granule cells, with a progressively rostral to caudal distribution over a period of induction that continues up to E17.

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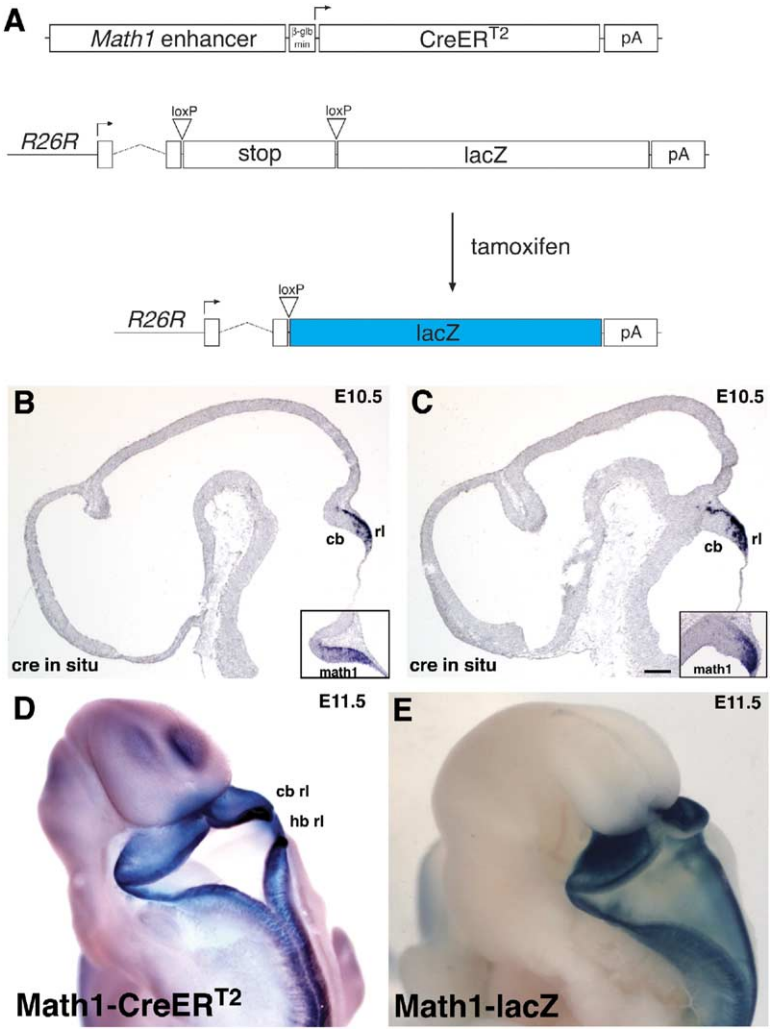

Figure 1. Math1-CreER^{T2} Fate-Mapping Strategy and Transgene Expression

(A) Structure of the Math1-CreER^{T2} transgene. Transgenic animals were crossed into the R26R-stop-lacZ reporter line such that after transient activation of the CreER^{T2} recombinase activity by tamoxifen administration, the R26R stop cassette flanked by loxP sites is excised, resulting in permanent β-galactosidase expression.

(B and C) In situ hybridization for *cre* mRNA on E10.5 Math1-CreER^{T2} medial (B) and medial-lateral (C) sagittal sections. In situ hybridizations for *Math1* at comparable positions are shown in the insets.

(D) Posterior side view of a whole-mount in situ hybridization for *cre* mRNA in an E11.5 Math1-CreER^{T2} transgenic embryo. The perdurance of the transgenic mRNA is similar to that of the β-galactosidase activity detected by whole-mount β-galactosidase staining of an E11.5 Math1-lacZ embryo (E). The purple background signal rostral to the cerebellar primordium that is visible in (D) was present in nontransgenic embryos and, thus, is not due to ectopic expression of the transgene. Abbreviations: cb, cerebellum; hb, hindbrain; rl, rhombic lip. Scale bar in (C) represents 300 μm.

Thus, we present the first temporal fate map of the widely disparate neuronal populations that originate in the cerebellar rhombic lip throughout embryogenesis.

Results

To fate map Math1^{+} cerebellar rhombic-lip progenitors generated throughout embryogenesis, we engineered a line of transgenic mice that express an inducible form of the Cre recombinase (CreER^{T2}) (Feil et al., 1997) under control of the *Math1* enhancer (Helms et al., 2000). We then crossed this transgenic mouse (Math1-CreER^{T2}) with the R26R-stop-lacZ reporter line (Soriano, 1999), in which Cre-mediated excision of a stop cassette flanked by loxP sites results in permanent β-galactosidase expression (Figure 1A). By activating the CreER^{T2} with a single dose of tamoxifen at different embryonic stages, we were able to label cohorts of Math1^{+} cells and determine their subsequent fates in the adult mouse brain.

We verified that the Math1-CreER^{T2} transgene was expressed appropriately by performing section and whole-mount in situ hybridization for *Cre* mRNA on transgenic embryos (Figures 1B–1D) and comparing with age-matched embryos from the Math1-lacZ knockin mouse line (Figure 1E) that expresses β-galactosidase from the endogenous *Math1* locus (Ben-Arie et al., 2000). Although the expression of *Math1* mRNA is restricted to the rhombic lip at early embryonic stages (insets in Figures 1B and 1C), both the transgenic mRNA and β-galactosidase staining in the Math1 knockin showed similar perdurances. We did not observe ectopic expression of the transgene at early embryonic stages, although by E12.5 a limited amount of ectopic expression could be detected in the primordium of the hippocampus. Although this is in part consistent with previous work using this enhancer to express GFP in transgenic mice (Lumpkin et al., 2003), unlike this previous study, we did not detect any ectopic expression in the cortex. In addition to the rhombic-lip expression detailed above, we also observed expression of the transgene in other known regions of Math1 expression, including the dorsal hindbrain and spinal cord as well as in the inner-ear primordia (data not shown). We did not observe any substantial recombination of the reporter locus in the absence of tamoxifen (no labeled

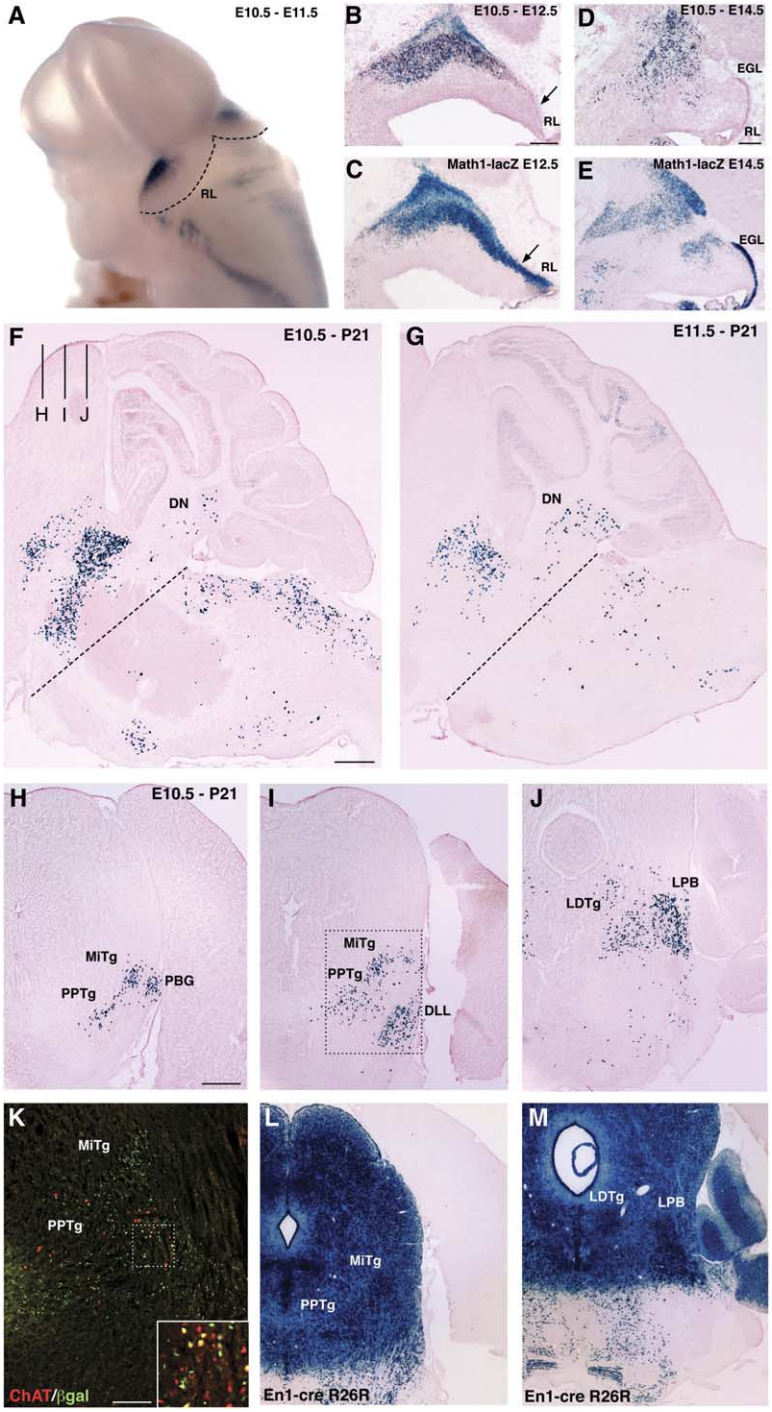

Figure 2. Early Math1$^+$ Populations Migrate into the Rostrolateral Hindbrain

(A) Whole-mount β-galactosidase staining of Math1-CreER^{T2} transgenic embryos gavaged with tamoxifen at E10.5 and analyzed at E11.5.

(B–E) β-galactosidase staining of sagittal tissue sections from E12.5 (B) and E14.5 (D) Math1-CreER^{T2} transgenic embryos gavaged with tamoxifen at E10.5, compared with E12.5 (C) and E14.5 (E) sagittal sections from Math1-lacZ embryos. Note that the perdurance of β-galactosidase staining in the Math1-lacZ embryonic tissue effectively labels earlier Math1$^+$ cohorts of cells that have migrated away from the cerebellar rhombic lip, in addition to later populations in the rhombic lip and emerging EGL (see arrows in [B] and [C]).

(F–G) β-galactosidase staining of lateral sagittal sections of P21 Math1-CreER^{T2} brains from animals administered tamoxifen at E10.5 (F) or E11.5 (G). The dotted line indicates the rhombomeric boundary defined by En1-Cre fate mapping (Zervas et al., 2004).

(H–J) Coronal sections of P21 Math1-CreER^{T2} brains from animals administered tamoxifen at E10.5, stained for β-galactosidase.

(K) Immunohistochemistry for β-galactosidase and ChAT on brain sections from P21 Math1-CreER^{T2} animals gavaged at E10.5 reveals double-labeled cells in the pedunculopontine tegmental nuclei. The field shown in (K) corresponds approximately to the region outlined by the dashed rectangular boxes in (I).

(L–M) Coronal sections from En1-Cre; R26R-stop-lacZ P21 brains stained for β-galactosidase. The coronal planes in (L) and (M) are comparable with those shown in (I) and (J), respectively. Abbreviations: RL, rhombic lip; EGL, external granule layer; DN, deep nuclei; PPTg, pedunculopontine tegmental nuclei; MiTg, microcellular tegmental nuclei; LDTg, laterodorsal tegmental nuclei; DLL, dorsal nuclei of lateral lemniscus; LPB, lateral parabrachial nuclei. Scale bars in (B) and (D) represent 300 μm, those in (F) and (H) represent 1 mm, and that in (K) represent 100 μm.

hindbrain cells, <15–20 labeled granule cells per adult brain).

Previous studies in chick and zebrafish have observed that early in embryogenesis, there are waves of rapidly migratory cells that transit from the cerebellar rhombic lip to occupy positions in the ventrolateral hindbrain. We sought to characterize these early migratory populations in greater detail by labeling the Math1$^+$ rhombic-lip cells generated between E10 and E12. It has been shown previously that the CreER fusion protein translocates to the cell nucleus within 6 hr of tamoxifen administration and that peak marking occurs over a subsequent period of 12–24 hr (Danielian et al., 1998; Zervas et al., 2004). Since administration of tamoxifen at E9.5 or E10.5 resulted in similar populations of labeled cells in the adult (data not shown), we chose to focus on the E10.5 gavage time point for our analyses. To identify the initial marked population, we gavaged female mice pregnant with E10.5 embryos with one dose of tamoxifen (typically 4 mg) and collected

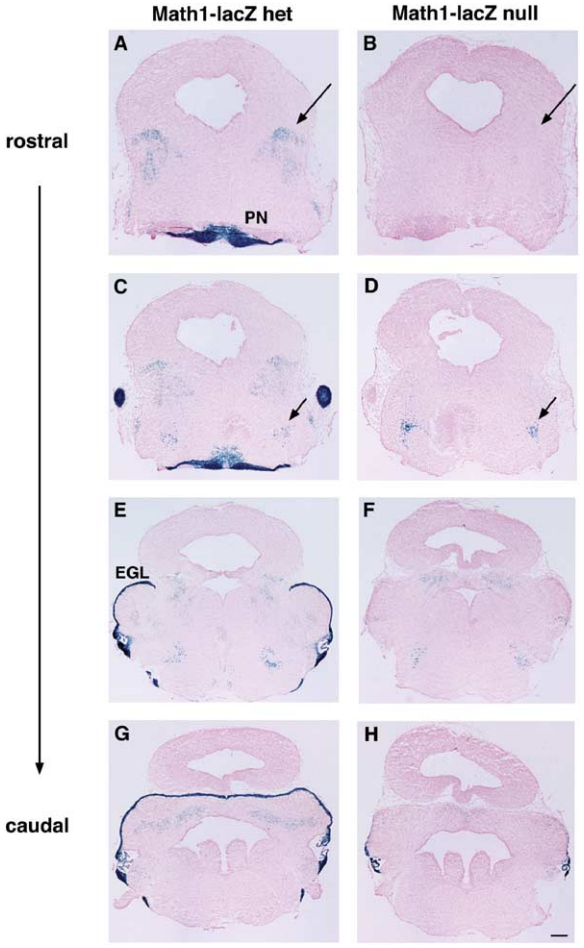

Figure 3. Math1 Is Required for the Formation of the Rostrolateral Hindbrain Nuclei that Originate in the Cerebellar Rhombic Lip

Coronal sections of E17 Math1-lacZ heterozygous (A, C, E, and G) or homozygous (null) embryos (B, D, F, and H) were stained for residual β-galactosidase activity to detect cells that had expressed Math1 at earlier developmental stages. Note that in comparison to the heterozygous embryo, there is no labeling in the rostrolateral hindbrain in the Math1 mutant (long arrows in [A] and [B]). At more caudal positions, some hindbrain nuclei are still present in the Math1-null embryo (short arrows in [C] and [D]). Abbreviations: EGL, external granule layer; PN, pontine nuclei. Scale bar in (H) represents 300 μm.

the embryos 1 day later for whole-mount β-galactosidase staining. As shown in Figure 2A, Math1$^+$ cells labeled by tamoxifen administration at E10.5 are located rostrally and laterally away from the midline by E11.5. Labeling of mitotic progenitor cells in the cerebellar primordium with a single pulse of BrdU at E10.5 revealed that by E12.5, cells that had become postmitotic shortly after the BrdU pulse (i.e., had retained the BrdU label) had migrated rostrolaterally and overlapped with the Math1 population fate mapped by tamoxifen administration at E10.5 (Figure S1). A comparison at E12.5 (2 days after tamoxifen administration) (Figure 2B) with lateral tissue sections from the Math1-lacZ mouse (Figure 2C) shows that although in both cases, the rostral migratory populations are similar, no Math1-CreER^{T2} recombined cells remain in the rhombic lip (see arrows in Figures 2B and 2C). By E14.5, the recombined cells are clearly distinct from the granule cell precursors that have formed the EGL, and most have migrated out of the cerebellar primordium (Figures 2D and 2E).

We fate mapped this cohort of Math1$^+$ cells into the adult and found that the cells that had migrated out of the cerebellar primordium had populated specific nuclei in the lateral anterior hindbrain (Figure 2F). Within the cerebellum, we observe robust labeling of the deep nuclei, particularly with tamoxifen administration at E11.5. Almost no labeled granule cells could be detected in the cerebellum of animals gavaged at E10.5, and gavaging 1 day later at E11.5 only labeled small numbers of granule cells (Figure 2G).

Previous fate mapping of the hindbrain rhombic lip with Wnt1 has shown that the precerebellar nuclei originate in this neuroepithelium (Rodriguez and Dymecki, 2000). However, since Wnt1 does not mark neural progenitors within the cerebellar rhombic-lip populations at least up to E11.5 (Zervas et al., 2004), we have chosen to focus on this region exclusively because these r1-derived hindbrain nuclei were not labeled in earlier studies. To characterize the rostral hindbrain populations labeled by the Math1-CreER^{T2} transgene in more detail, we examined coronal sections from adult (P21) transgenic mice that were gavaged with tamoxifen at E10.5 (Figures 2H–2J). We observed labeled cells in the parabigeminal (PBG), pedunculopontine tegmental (PPTg), and microcellular tegmental (MiTg) nuclei at the most rostral positions (Figure 2H). Progressing caudally, we also observe labeling in the laterodorsal tegmental nuclei (LDTg), dorsal nuclei of the lateral lemniscus (DLL), and lateral parabrachial nuclei (LPB, Figures 2I and 2J). Because the PPTg and LDTg are part of the hindbrain cholinergic system (Woolf, 1991), we performed immunohistochemistry for choline acetyltransferase (ChAT) and β-galactosidase on adjacent sections to those shown in Figures 2H–2J and found double-labeled cells in both nuclei (Figure 2K and data not shown). To demonstrate that these neuronal populations arose in rhombomere 1, we examined whether these nuclei express Engrailed-1 during their development. Cumulative En1-Cre fate mapping has been shown to label the entire mes-r1 territory (Zervas et al., 2004), and because Math1 is not expressed in the mesencephalon (Figure 1), we can infer that nuclei labeled by both fate-mapping approaches originated in rhombomere 1. The β-galactosidase staining of the PBG, PPTg, MiTg, LDTg, and LPB nuclei in En1-Cre; R26R-stop-lacZ sections (Figures 2L and 2M) clearly indicated that the Math1-CreER^{T2} fate-mapped cells in these nuclei originated in rhombomere 1 and not the hindbrain rhombic lip. Other hindbrain nuclei such as the DLL may also arise from the cerebellar rhombic lip but were not unambiguously labeled by En1-Cre fate mapping.

To determine whether the specific hindbrain nuclei that originate in the cerebellar rhombic lip require Math1 for their development, we generated homozygous Math1-LacZ knock in embryos and compared the β-galactosidase staining in the rostral hindbrain of these Math1-null embryos with heterozygous siblings (Figure 3). Because the Math1-null animals die at birth (Ben-Arie et al., 1997), our analysis was restricted to late embryogenesis, but nevertheless even by E17, the

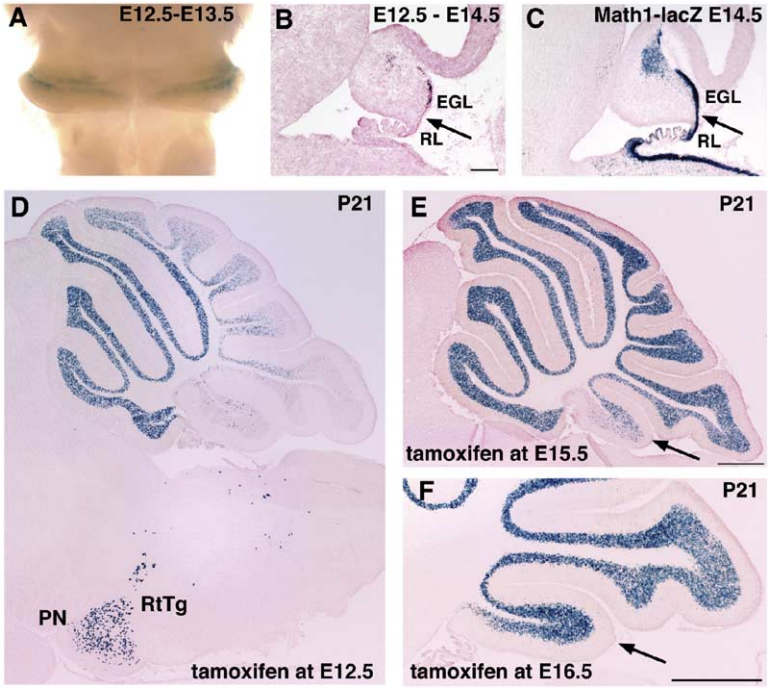

Figure 4. Granule Cell Precursors Are Generated in the Rhombic Lip from E12.5 to E17

(A) Whole-mount β-galactosidase staining of an E13.5 Math1-CreER^{T2} embryo that received tamoxifen at E12.5. The dorsal midbrain has been dissected away to reveal the labeled cells in the cerebellar primordium.

(B and C) Sagittal sections of the E14.5 cerebella from Math1-CreER^{T2} (B) (tamoxifen at E12.5) or Math1-lacZ embryos (C) stained for β-galactosidase. Note that labeled cells are absent in the caudal EGL (arrows in [B] and [C]) in (B).

(D–F) β-galactosidase staining of sagittal sections of P21 Math1-CreER^{T2} cerebella from animals administered tamoxifen at E12.5 (D), E15.5 (E), or E16.5 (F). Note that tamoxifen labeling of granule cell precursors that populate the most posterior lobe (arrows in [E] and [F]) is not complete until after E16.5. Abbreviations: EGL, external granule layer; RL, rhombic lip; PN, pontine nuclei; RtTg, reticulotegmental nuclei of pons. Scale bars in (B) represent 300 mm, and those in (E) and (F) represent 1 mm.

Math1 cells that contribute to the anterior hindbrain nuclei described above could be detected in heterozygous animals by staining for residual β-galactosidase activity (Figures 3A, 3C, 3E, and 3G). Interestingly, although the r1-derived anterior Math1 populations are absent in the Math1 null (compare regions indicated by long arrows in Figures 3A and 3B), some of the more caudally located nuclei are still present (e.g., short arrows in Figures 3C and 3D), implying that the requirement for Math1 is variable among the different hindbrain nuclei that express Math1 during their development.

Granule cell precursors are known to originate from the cerebellar rhombic lip and express Math1 from the time of their embryonic induction throughout their postnatal maturation in the external granule layer. However, because these cells do not become postmitotic until after P3, determining the period of granule-cell precursor induction by embryonic labeling of dividing cells has been difficult. We administered tamoxifen at E12.5 in the Math1-CreER^{T2} transgenics and in contrast to earlier gavage time points found substantial labeling of cells in the EGL by E13.5 (Figure 4A). Comparing labeled cells in Cre transgenic and Math1-lacZ sections at E14.5, we observed that tamoxifen administered at E12.5 in the transgenic embryos primarily labeled granule cell precursors in the rostral EGL, whereas Math1-lacZ labeled the entire EGL (Figures 4B and 4C). By P21, the rostral distribution of early born granule cells is evident in the internal granule layer, with labeled cells biased to the anterior lobes particularly in the vermis (Figure 4D), consistent with a previous fate-mapping study with En1/En2-CreER (Sgaier et al., 2005). Posterior lobes (9–10) are sparsely labeled by E12.5 tamoxifen administration across all medial to lateral positions.

To determine when the induction of progenitors that will give rise to granule cells of the posterior cerebellar lobes occurs, we gavaged transgenic animals with tamoxifen at E14.5 (not shown), E15.5, and E16.5 and fate mapped the labeled cells into the P21 animal. After tamoxifen administration at E15.5, the entire IGL is evenly labeled in the adult, with the exception of the most posterior lobe (arrow in Figure 4E), which is strongly labeled 1 day later (Figure 4F). Thus, we estimate that granule cell precursors are generated in the rhombic lip from approximately E13 to as late as E17 in a rostral to caudal sequence.

Discussion

Our genetic fate mapping demonstrates that rather than representing a single population, Math1 cells within the cerebellar rhombic lip are highly dynamic, with different progenitors expressing Math1 at early versus late stages of neurogenesis giving rise to distinct neuronal populations (Figure 5). We find that prior to E12.5, Math1 is transiently expressed in cohorts of cerebellar rhombic-lip neural precursors that populate the anterior hindbrain and deep cerebellar nuclei in the adult. Short-term fate mapping reveals that Math1 cells labeled at E11–E12, and their progeny do not remain in the rhombic lip but rapidly migrate ventrolaterally, consistent with previous observations in chick and zebrafish (Gilthorpe et al., 2002; Koster and Fraser, 2001). By combining the En1-Cre cumulative fate mapping of the mes/r1 with our Math1-CreER^{T2} transgenic fate mapping, we have characterized the rostral hindbrain nuclei that originate specifically in the cerebellar rhombic lip. Future fate-mapping strategies with combinatorial methods to precisely mark cells at the intersection of genetically defined territories (Awatramani et al., 2003) will ulti-

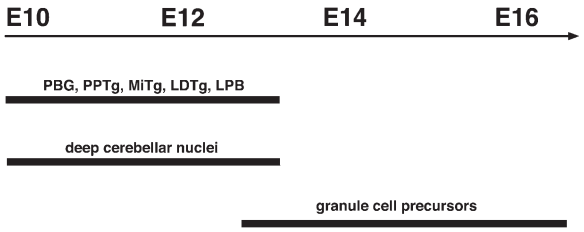

Figure 5. Math1-Expressing Progenitors in the Rhombic Lip Sequentially Produce Different Populations of Neurons within the Hindbrain

Between E10 and E12.5, Math1-expressing rhombic-lip progenitors contribute to a series of nuclei within the hindbrain and mesencephalon including the PBG (parabigeminal nucleus), the PPTg (pendunculopontine tegmental nucleus), the MiTg (microcellular tegmental nucleus), the LDTg (laterodorsal tegmental nucleus), and the LPB (lateral parabrachial nucleus) as well as the deep nuclei within the cerebellum (fastigial, interposed, and dentate nuclei). Subsequent to E12.5, virtually all Math1-expressing cells in the rhombic lip become granule cells. Interestingly, the cerebellar granule cells are produced in sequential cohorts with those ultimately residing in the anterior cerebellum being generated around E12.5, whereas those in the posterior cerebellum are generated at times closer to E15.5 to E16.5.

mately allow for greater resolution of the diverse cell types that are generated in this unique germinal region.

To date, although the cerebellar rhombic lip has been argued to contribute to certain hindbrain nuclei, both the precise structures they contribute to and the cell types they comprise within these structures have remained unclear. We observe contributions of Math1-CreER^{T2}-labeled cells from early embryonic stages to the parabigeminal nuclei, pedunculopontine, microcellular tegmental and laterodorsal tegmental nuclei, and lateral parabrachial nuclei, based on the anatomical position of labeled cells. Analysis of *Math1-LacZ*-null mice shows that several of these nuclei fail to form in the absence of *Math1*-gene function (Figure 3). Although additional hindbrain nuclei such as the dorsal lateral lemniscus and lateral pontine nuclei may also receive contributions from the cerebellar rhombic lip, we could not unambiguously identify the rhombomeric origins of labeled cells in these regions with En1-cre fate mapping. Interestingly, the pedunculopontine and laterodorsal tegmental nuclei are components of the cholinergic system (Woolf, 1991), and accordingly, we observe that some of the Math1 fate-mapped cells in these regions express choline acetyltransferase (Figure 2). These mesopontine tegmental nuclei project primarily to the thalamus but also to many other brain regions, including the substantia nigra and ventral reticular formation, where they provide the only known cholinergic input and, thus, may regulate the activity of dopaminergic neurons in these regions (Yeomans, 1995). To our knowledge, this is the first evidence demonstrating that hindbrain cholinergic nuclei originate in the cerebellar rhombic lip.

The idea that specific bHLH transcription-factor expression in subsets of neural progenitors may define their later integration into a common circuit is intriguing and has been studied in a number of contexts. Math1 has been previously shown to be essential for the development of multiple components of the proprioceptive system, including inner-ear hair cells, the pontine nuclei, cerebellar granule cells, and the D1 interneurons of the spinal cord (Bermingham et al., 2001). In this study, we show that an additional component of the vestibular system—the lateral parabrachial nuclei—also develops from a Math1$^+$ precursor. Interestingly, the lateral parabrachial nucleus has reciprocal connections to the vestibular nuclei in addition to reciprocal connections with the amygdala, hypothalamus, and prefrontal cortex (Balaban, 2004). Thus, the lateral parabrachial nucleus may provide an interface between the vestibular system and telencephalic structures involved in conditioned aversion and fear responses (LeDoux et al., 1988).

The ephemeral expression of Math1 in early cerebellar rhombic-lip progenitors contrasts with that observed in granule cell precursors, whose Math1 expression persists from induction throughout the first two postnatal weeks of development. Considering that we do not label substantial numbers of granule cells until after E12.5, we conclude that the early Math1$^+$ rhombic-lip population is distinct from later Math1$^+$ populations and, therefore, that Math1 is induced de novo in naive cerebellar progenitors throughout embryogenesis. The ongoing induction of Math1 is consistent with previous studies that have implicated BMP signaling in granule cell induction (Alder et al., 1999) because BMPs are expressed in the roof plate relatively late into development. It will be interesting to determine the source of progenitors that are induced to enter the Math1$^+$ rhombic-lip population at later stages when granule cell precursors first emerge.

Experimental Procedures

Transgenic and Reporter Mice

The Math1-CreER^{T2} transgene was generated by subcloning the cDNA encoding the T2 mutant form of a Cre recombinase-estrogen receptor fusion (Feil et al., 1997) (kind gift of P. Chambon) downstream of the 1.4 kb *Math1* 3′ enhancer (Helms et al., 2000) (kindly provided by J. Johnson) and a β-globin minimal promoter. Transgenic mice were generated as previously described (Nagy et al., 2003). Of five founder lines generated, two exhibited appropriate expression levels and patterns for further studies. Transgenic mice were genotyped by PCR for Cre with the following primers: forward primer 5′-TAAAGATATCTCACGTACTG-3′ and reverse 5′-TCTCTG ACCAGAGTCATCCT-3′. Math1-lacZ knockin mice were kindly provided by H. Zoghbi and genotyped as previously described (Ben-Arie et al., 2000). Engrailed-1 Cre mice were kindly provided by A. Joyner and were genotyped as previously described (Kimmel et al., 2000). Rosa26 reporter mice were the generous gift of P. Soriano and genotyped as described (Soriano, 1999). Mice were maintained and sacrificed in accordance with the protocols approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine.

Fate Mapping

Tamoxifen (T-5648, Sigma) was prepared as a 20 mg/ml stock solution in corn oil (C-8267, Sigma). For all labeling experiments, 4 mg tamoxifen/35 g mouse was administered by oral gavage to the pregnant mouse at midday of the appropriate embryonic stage (midday of the observed plug was considered 0.5 days postcoitus). Embryos collected for analysis were briefly fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBS (15′–30′), washed in PBS, and stained directly for β-galactosidase activity or equilibrated in 30% sucrose/PBS prior to mounting in TissueTek (VWR). P21 animals were transcardially perfused with 4% PFA in PBS, and the dissected brains washed in PBS and directly equilibrated in sucrose

prior to mounting. Frozen sections (12–20 μm) were obtained with a Leica CM 3050S (embryonic sections) or Zeiss HM500 OM (adult) cryostat.

β-Galactosidase Histochemistry, RNA In Situ Hybridization, and Immmunohistochemistry

Tissue sections and whole embryos were processed for β-galactosidase histochemistry by two washes (5′, 25′) in 0.1 M phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 5 mM EGTA followed by two brief washes (5′ each) in 0.1 M phosphate buffer, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 prior to staining overnight in the latter buffer supplemented with 5 mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, and 1 mg/ml X-gal (Fisher) at 37°C. Stained tissue sections were washed three times in PBS, counterstained with Fast Red, dehydrated, and coverslipped with Permount prior to bright-field photography on a Zeiss Axioskop. RNA in situ hybridization with digoxigenin antisense probes was performed as described previously (Wilkinson and Nieto, 1993). The *Math1* in situ probe was kindly provided by J. Johnson. The Cre recombinase antisense probe construct was kindly provided by M. Zervas and A. Joyner. For immunohistochemistry with rabbit anti-choline acetyltransferase (AB143, Chemicon, 1:500) and goat anti-β-galactosidase (4600-1409, Biogenesis, 1:400), tissue sections were blocked for 1 hr with 5% normal donkey serum in PBS/0.2% Triton X-100 and incubated overnight with primary antibodies at 4°C. Secondary antibodies used were donkey anti-rabbit IgG Cy3 conjugated (1:400, Jackson Laboratories) and donkey anti-goat IgG Alexa488 conjugated (1:500, Molecular Probes). Assignment and nomenclature of labeled hindbrain nuclei was in accordance with (Paxinos and Franklin, 2001).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/48/1/17/DC1/.

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