Subset of early radial glial progenitors that contribute to the development of callosal neurons is absent from avian brain

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The classical view of mammalian cortical development suggests that pyramidal neurons are generated in a temporal sequence, with all radial glial cells (RGCs) contributing to both lower and upper neocortical layers. A recent opposing proposal suggests there is a subgroup of fate-restricted RGCs in the early neocortex, which generates only upper-layer neurons. Little is known about the existence of fate restriction of homologous progenitors in other vertebrate species. We investigated the lineage of selected Emx2$^+$ [vertebrate homeobox gene related to *Drosophila empty spiracles* (*ems*)] RGCs in mouse neocortex and chick forebrain and found evidence for both sequential and fate-restricted programs only in mouse, indicating that these complementary populations coexist in the developing mammalian but not avian brain. Among a large population of sequentially programmed RGCs in the mouse brain, a subset of self-renewing progenitors lack neurogenic potential during the earliest phase of corticogenesis. After a considerable delay, these progenitors generate callosal upper-layer neurons and glia. On the other hand, we found no homologous delayed population in any sectors of the chick forebrain. This finding suggests that neurogenic delay of selected RGCs may be unique to mammals and possibly associated with the evolution of the corpus callosum.

neocortex | Emx2 | cortical development | neurogenesis | chick

Several neural progenitor subtypes have been identified in the developing mammalian neocortex (1–4). At the onset of neurogenesis, multipotent proliferative radial glial cells (RGCs) reside at the ventricular surface. RGC divisions can be "self-renewing," a symmetrical division in which two identical RGCs are generated; "direct neurogenic," generating a neuroblast and a multipotent progenitor; or "indirect neurogenic," which gives rise to an intermediate progenitor that migrates to divide in the subventricular zone. The timing of these events is crucial for cell number and fate determination (5–7) and vary among different mammalian species. Importantly, very little is known about the homologous populations of RGCs in other vertebrate species and about their role in cortical evolution.

Observations made from Golgi-stained material (4, 8), birth-dating (5, 9), and lineage-tracing analysis (10–14) contributed to the formation of the "sequential" hypothesis. Accordingly, each RGC in the early brain generates most cortical cell types by sequential mitoses (15). Long-range subcortical projection neurons of the deep layers are generated first, followed by the callosal projection neurons of the upper layers, and finally the glial cells (16).

However, recent genetic fate mapping of selected lineages has revealed a subpopulation of early RGCs expressing *Cux2*, which only gives rise to Satb2-positive upper-layer cortical neurons (17). This finding suggests that the early neurogenic brain may contain several types of cortical progenitors, each of which constrained to generating certain subpopulations of neurons, known as the "restricted progenitor hypothesis" (18). The existence of these restricted progenitors has been challenged (1–4, 19). Early clonal analysis performed by retroviral-mediated gene transfer also led to differing interpretations (12, 14, 20).

Interestingly, these alternative interpretations diverge in the origin of callosal upper-layer cortical neurons. The appearance of neurons with callosal projections in the upper layers is considered an important event in the evolution of the mammalian neocortex, as there is no corpus callosum in sauropsid brains (21). Based on selected gene expression patterns, Suzuki et al. suggested that upper-layer–like cortical neurons are situated in a different sector of chick pallium with respect to the position of lower-layer–like neurons (22). Their birth-dating studies demonstrated that upper-layer–like neurons are generated in a biased fashion from spatially restricted progenitors (15). They also suggest that the potential to adopt the sequential pattern of neurogenesis remains, but is suppressed in vivo. To resolve these issues, further comparative clonal studies of single progenitors are needed (23, 24).

Here we investigate the lineage of selected Emx2$^+$ [vertebrate homeobox gene related to *Drosophila empty spiracles* (*ems*)] RGCs in the mammalian pallium. We describe a subset of progenitors that lack neurogenic potential during early neocortical development. After self-renewing and transit-amplifying mitoses, these RGC progenitors exclusively give rise to callosal upper-layer neurons and glia. We therefore suggest the temporal and spatial coexistence of restricted and sequential RGC progenitors in the mammalian cortical ventricular zone. Additionally, we observed no homologous heterochronic behavior in this population within chick forebrain, suggesting the neurogenic delay of some RGCs is a mammal-specific attribute, possibly related to the evolution of neurons involved in interhemispheric dorsal pallial communications. Our study establishes the existence, time frame, and lineage

Significance

Understanding development and evolution of the neocortex has important implications. We describe here a major difference between avian and mammalian dorsal pallial progenitors regarding their fate restrictions. In mouse cortex we identified an early population of radial glial cells that are delayed in the generation of neurons. After self-renewal and transit-amplifying mitoses, these murine progenitors become committed to the genesis of upper-layer callosal pyramidal neurons and glia. In the chick embryonic pallium we identified a homologous population of progenitors; however, these have no delayed neurogenesis and their lineage is therefore not segregated from that of the other pallial progenitors. We hypothesize on the relation between the early neurogenic delay of progenitors and the origin of the corpus callosum.

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of these early Emx2^{+} RGC progenitor population fate restricted to the upper layers of mammalian cortex but absent in the avian brain.

Results

Mitotic Dynamics of *Emx2*-Expressing Progenitors. To study the lineage of subsets of cortical progenitors, we used in utero coelectroporation of arrested fluorophores together with Cre-expressing constructs driven by selective promoters (Fig. 1*A*). Only cells expressing Cre can recombine the arresting STOP signal and consequently activate the expression of nuclear-tagged EGFP. We selected various transcription factors with expression restricted within a sector of the telencephalon to target expression of Cre. RGCs of the dorsal, but not ventral, pallium express homeobox transcription factor Emx2 (25–27). Two different enhancer sequences for this gene (Fig. 1*B*) have been described (28, 29). We examined whether the activity levels of these two independent enhancer sequences could reflect different timing, frequency of divisions, and progeny in ventricular zone (VZ) progenitors. We used these two sequences, separately or in the same construct, to label cortical RGCs through Cre expression. The triggering recombination event can only happen at the progenitor stage when the *Emx2* locus is active (30). We alternatively used the CAG promoter that drives expression in an unspecific and ubiquitous fashion for our control experiments. CAG-Cre constructs labeled any VZ progenitor present at the time of electroporation.

Early cortical progenitors were labeled at embryonic day (E)12, aided by the CAG promoter. We performed all our quantitative analysis on electroporations to the lateral neocortex at E12. By E14, the derivatives of this unspecific labeling were found in all developmental compartments of the neocortex (Fig. 1 *C* and *J*), from the germinative zones in the VZ and subventricular zone (SVZ) to the cortical plate (CP; 17.9 ± 2.3% of all labeled cells). By contrast, the three different constructs driven by Emx2 enhancers (3′, 5′, and 3′–5′) showed a variety of other lineages (Fig. 1 *D–F* and *J*). Progenitors targeted by either the 3′ or the 5′ Emx2 enhancer produced a lineage characterized by a significant reduction in the number of EGFP-labeled cells in the cortical plate (Fig. 1 *E* and *J*; 3′ enhancer lineage in CP 6.6 ± 0.8%, $n = 4$; 5′ enhancer lineage in CP 6.5 ± 1%). This reduction was further emphasized when labeling progenitors with the construct containing both enhancers simultaneously (3′–5′ Emx2-Cre; Fig. 1 *F* and *J*). Virtually no cells generated by 3′–5′ Emx2-Cre–labeled progenitors were found in the CP at E14 (0.7 ± 0.3%, 5 cells in a total of 813 studied cells). Remarkably, the RGCs labeled by both enhancers (Fig. 1*F*) were less neurogenic than those labeled with CAG, as the former did not generate neurons during early cortical development. For the sake of clarity, we shall refer to these 3′–5′ Emx2-Cre–labeled progenitor cells as "Emx2 progenitors." Such neurogenically delayed progenitors, embedded in a broader population of neurogenic RGCs (Fig. 1*G*), were defined as cortical RGCs by their bipolar morphology (Fig. 1*G*) and expression of Pax6 and Nestin (Fig. 1 *H* and *I*). Importantly, the differential lineage described cannot be due to differential efficiencies of the Cre-expressing constructs. CAG-Cre labels more cells than Emx2-Cre, but if the latter were not selective for a subpopulation of progenitors, its reduced labeled progeny would occupy all embryonic cortical layers (germinative zone, GZ; intermediate zone, IZ; and CP) in similar proportions to CAG-Cre. The constructs used

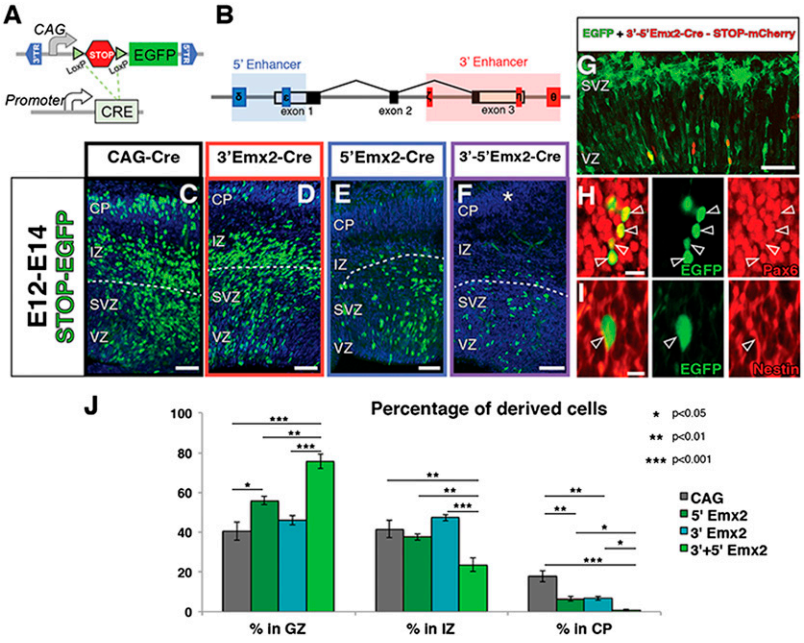

Fig. 1. Different *Emx2* enhancers selected several subsets of RGCs. (*A*) Schematic diagram of the labeling paradigm. Arrested fluorophores were electroporated and became active after Cre recombination of the STOP signal, therefore only Cre-expressing cells became fluorescent. (*B*) Schematic structure of the *Emx2* gene and its enhancer sequences. An enhancer appears upstream of the gene, represented in blue and named 5′ enhancer, whereas other enhancer sequences are located between exons 2 and 3 and downstream of the gene, represented here in red and named 3′ enhancer. These two enhancers were used to drive expression of Cre, therefore triggering the label. (*C–J*) Cortical distribution of labeled cells at E14 after electroporation of arrested nuclear EGFP at E12. The labeling was driven by different promoter sequences. (*C*) CAG promoter labeled any progenitors in the VZ. Their progeny spanned all embryonic cortical layers, from germinative areas to the cortical plate. (*D*) The 3′ Emx2 enhancer labeled a population of progenitors whose lineage was similar to that for CAG. (*E*) The 5′ Emx2 enhancer restricted the labeling to some progenitors only, their lineage only weakly colonized the cortical plate. (*F*) When both Emx2 enhancers were used together in the same Cre-expressing construct, the label was reduced to only a subset of RGCs. The progeny of these RGCs remained locally in the germinative zones 2 d after electroporation. The asterisk indicates the lack of derived neuroblasts in the cortical plate. (*G–I*) Cortical progenitors labeled through both enhancers simultaneously (red) were RGCs, as noted by their morphology revealed with coelectroporated membrane-tagged EGFP (*G*) and their expression of Pax6 (*H*) and Nestin (*I*). (*J*) Distribution of EGFP-labeled cells in the E14 cortex after electroporation at E12. $n = 4$ per experiment; DAPI counterstain in blue. Data are represented as mean ± SEM. (Scale bars, 50 μm in *C–F*, 25 μm in *G*, 10 μm in *H*, and 5 μm in *I*.)

express either Cre or EGFP, but do not modify the transcriptional profile of the transfected progenitor.

Although we focused our study on the early progenitors of the lateral cortex, which we tend to electroporate more efficiently and repeatedly at E12, we also checked the presence of neurogenic delay in the dorsal cortex and in other regions of the pallium. Although early electroporations at E12 on the dorsal cortex showed similar delay as we describe in the lateral cortex, the medial-most region of the pallium (primordium of the cingular cortex and hippocampus) did not show a similar neurogenic delay to that found on the lateral side (Fig. S1). Therefore, the ability of Emx2 enhancers to identify neurogenically delayed progenitors appeared restricted to at least the entire rostrocaudal extension of the lateral and dorsal, but not to the medial pallium. Also, as expected from previous studies (28), we also detected a low activity of Emx2 enhancers in the ganglionic eminences.

If Cre constructs were able to differentially drive recombination of the reporter gene in other non-RGC populations, it could explain the presence or absence of neuroblasts in the early cortical plate. We specifically examined this by means of birth-dating assay. We found that most recombination-mediated activation of the fluorophore expression happens in RGCs (Fig. S2). RGCs exposed to the lateral ventricle were electroporated with CAG-Cre and immediately injected with BrdU at E12. After 24 h, most EGFP-labeled cells were $BrdU^+$, demonstrating that they derive from a mitotic cell (Fig. S2). We found nearly no $EGFP^+$-derived cells in the cortical plate and only the minority of the cells in the intermediate zone were $BrdU^-$ (Fig. S2*B*, empty arrowheads). These very occasional $BrdU^-$-labeled cells could represent the small cohort of progenitors that were still at the VZ just after completing mitosis, at the moment of electroporation (therefore $EGFP^+$), which necessarily precedes BrdU injection (so $BrdU^-$). A vast majority of EGFP cells derived from a mitotic RGC, as intermediate precursors of the SVZ lack contact with the ventricular surface and therefore are not transfected. We show that a ubiquitously active construct as CAG-Cre did not recombine the reporter gene in postmitotic cells. Accordingly, Emx2-Cre was also incapable of such recombination, as its enhancer activation sequences only showed activity in RGCs (28, 29).

At subsequent stages of development, Emx2 progenitors maintained their heterochronic neurogenic behavior (Fig. 2). At E14 and E15, Emx2 progenitors remained premitotic, with their derived cells mostly located in cortical germinative zones. Cells remained in areas labeled by phosphohistone 3 and Ki67 immunohistochemistry (Fig. 2 *A*, *B*, and *E*), in both proliferative compartments as shown by $Pax6^+$ (VZ) and $Tbr2^+$ (SVZ) (Fig. 2 *C*–*D*). Many Emx2-derived cells at E14 were still mitotic as shown by Ki67 and Tbr2 expression (Fig. S3 *A* and *B*). A reduced number of cells derived from Emx2 progenitors settled in the CP at E15, as shown by Tbr1 immunohistochemistry (Fig. 2*F*). The distribution of the Emx2 progenitor lineage differed widely from that of the unspecified progenitors lineage (Fig. 2 *A′*–*F′*), where many cells at E14 and E15 had migrated out of the germinative zones and reached the CP, shown by Tbr1 expression (Fig. 2*F′*).

Interestingly, progenitors labeled through either Emx2-Cre or CAG-Cre at the later stage of E14, were not immediately neurogenic (Fig. S3 *C* and *C′*). Two days postelectroporation, their progeny were still limited to the germinative zones only. If CAG-Cre could recombine in other non-RGC populations, we would expect a broader lineage at E16 (similar to that observed from E12 to E14, shown in Fig. 1*C*), but only germinative zone cells were labeled. However, by E18, 4 d after electroporation, both sets of progenitors had generated neurons that migrated radially toward the CP (Fig. S3 *D* and *D′*). These experiments performed at E12 and E14 demonstrate that the Emx2-Cre construct selectively labeled nonneurogenic early RGCs of the lateral neocortex.

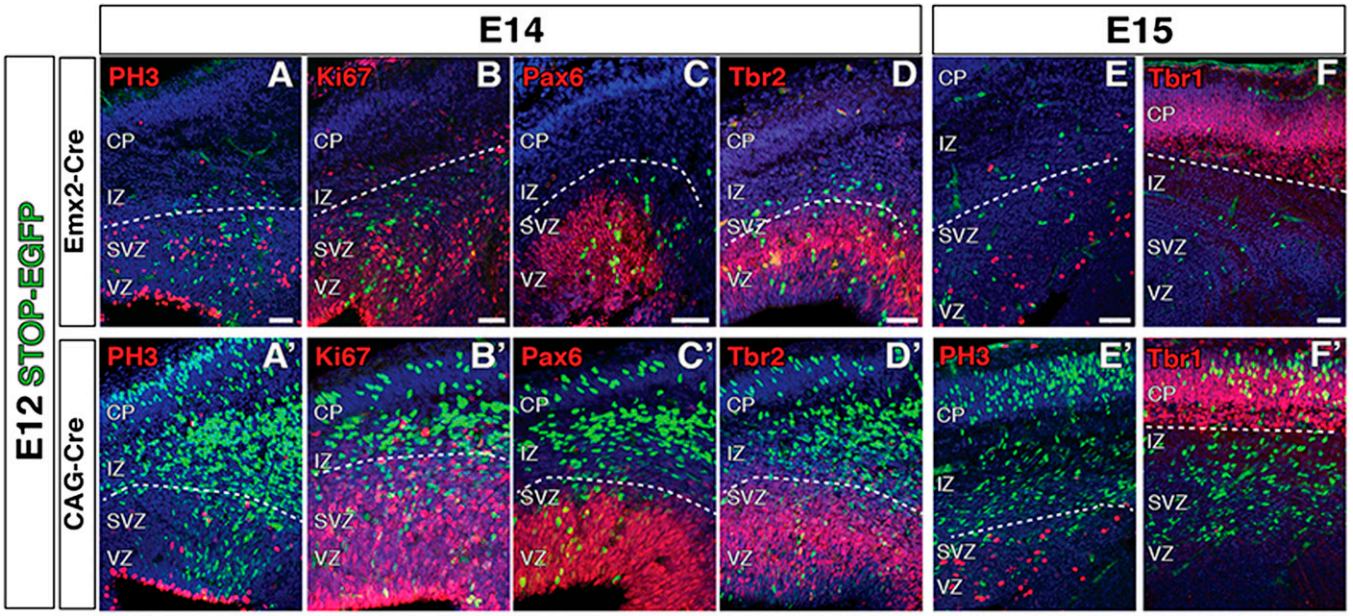

Fig. 2. Delayed neurogenesis led to a late colonization of the cortical plate by Emx2 progeny. (*A*–*D* and *A′*–*D′*) Location of EGFP-labeled cells (green) at E14 lateral neocortex (dorsal *Left*, ventral *Right*), transfected at E12, and stained for immunoreactivity with PH3, Ki67, Pax6, and Tbr2 (red). DAPI staining is shown in blue. Nuclear-tagged EGFP labeling conducted with both Emx2 enhancers (*A*–*D*) mostly revealed cells in VZ and SVZ as noted by the expression of mitotic markers and transcription factors expressed in cortical progenitors. CAG promoter drove the labeling to more RGCs (*A′*–*D′*), many derived cells were located outside the germinative zones by E14. (*E* and *F* and *E′* and *F′*) Distribution of labeled lineage 3 d after electroporation at E15. Emx2 lineage remained in the germinative areas, marked by PH3 immunoreactivity (*E*), and only a few cells settled in the cortical plate marked with Tbr1 immunostaining (*F*). The broad population of progenitors labeled with CAG produced numerous neuroblasts and neurons that have already migrated toward or into the cortical plate (*F′*). $n = 3$ per experiment. CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; SP, subplate; SVZ, subventricular zone; uCP, upper cortical plate; VZ, ventricular zone. (Scale bars, 50 μm.)

Emx2-Labeled Progeny Populated Upper Cortical Layers. As Emx2 progenitors are yet to initiate differentiating divisions during early cortical neurogenesis, we expected their lineage to be different from that of other RGCs that do not undergo such heterochronic delay. Therefore, we studied the long-term lineage of these RGCs, aided by transposition of the reporter gene through *piggyBac*-mediated recombination (31, 32). This lineage-tracing method enabled us to study total progenies over time (Fig. 3 and Fig. S4). At E15, 3 d after electroporation, the majority of labeled cells remained in the germinal and intermediate zones below the CP (Figs. 2 *E* and *F* and 3*B*). By postnatal day 0 (P0), Emx2 progenitors had generated pyramidal neurons that settled in the upper CP after radial migration (Fig. 3*C*). This location was permanent; most of these neurons remained in layers II to IV at P10 and P16 (Fig. 3 *D* and *E*). Glial cells derived from Emx2 progenitors, present at these later time points, did not restrict their location to any particular layer (Fig. 3*E*, arrowheads). It is important to point out that fluorescence was detected in multiple cell types. Because the genomic integration of the reporter gene occurs randomly, it could take place into low transcriptional genomic areas. As a result, some cell types could show no expression of the fluorescent reporter despite gene integration. This silencing problem, common in single events of integration (such as after viral particle infection) was not apparent in our electroporations. The electroporated *pigyyBac* randomly integrated multiple copies of the reporter gene in each transfected progenitor, and as a result, EGFP was expressed in all types of neural cells. Because the *piggyBac* integration system is common to both our experimental conditions (CAG and Emx2 progenitors), the silencing cannot explain the segregated lineage found from Emx2 progenitors.

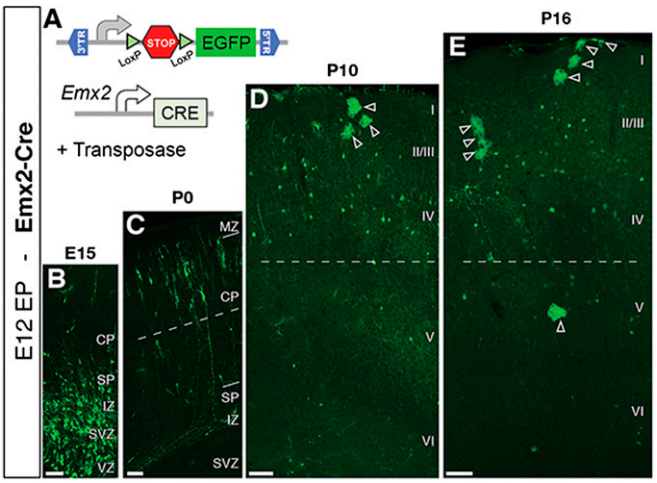

Fig. 3. Location of Emx2-derived population throughout embryonic and postnatal development (E15, P0, P10, and P16). (*A*) A mixture of Emx2-Cre, stop-floxed EGFP, and transposase constructs were used for the selective labeling of lineage from E12 electroporation. Both Emx2 enhancers were used simultaneously for selectively driving the expression of Cre and therefore for labeling the Emx2 progenitors. A transposase enzyme includes the active EGFP sequence into the genome of the progenitor thus labeling the entire progeny. (*B*–*E*) Examples of the distribution of progeny at subsequent developmental stages in coronal sections through the lateral neocortex of mice electroporated at E12 (*B*, E15, $n = 3$; *C*, P0, $n = 7$; *D*, P10; and *E*, P16, $n = 5$). (*B*) At E15, most labeled cells remained in VZ and SVZ. (*C*) By P0, the neuronal lineage settled in the upper cortical plate (uCP). (*D* and *E*) At midpostnatal stages P10 (*D*) and P16 (*E*), the Emx2-derived neurons located mostly the upper layers II–IV. The Emx2-derived glial cells colonized all cortical layers (arrowheads). CP, cortical plate; I–VI, cortical layers 1–6; IZ, intermediate zone; MZ, marginal zone; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone. (Scale bars, 100 μm in *D* and *E*, 50 μm in *C*, and 25 μm in *B*.)

As expected from the late developmental time point at which Emx2 progenitors become neurogenic (E14–E15), all neuronal progeny colonized only the upper cortical layers. This is in line with the inside-out pattern of cortical neurogenesis. Accordingly, most Emx2 progeny were generated through SVZ transit-amplifying mitoses (Fig. S3), the most common form of neurogenesis after midgestation. The heterochronic delay in onset of neuronal differentiation was responsible for the lack of deep layer neurons in the Emx2 progeny.

We wondered whether the restricted pattern in upper cortical layers could result from an artifact related to the expression of two different proteins. We compared the lineages of progenitors observed with one single protein (underlined) (CAG-EGFP) versus those that needed two proteins (CAG/Emx2-Cre + CAG-STOP-mCherry; Fig. S4*A*). When Emx2 promoter sequences drove the red labeling (Fig. S4 *B*–*D*), the same upper cortical plate-restricted pattern was observed at P0 (Fig. S4*C*), as opposed to the whole cortical plate pattern observed from simultaneous unspecific green labeling (Fig. S4*B*). In addition, when the CAG promoter was used to drive both labelings (EGFP and Cre-mediated mCherry), there were labeled cells in all regions of the cortical plate, with no difference between them (Fig. S4 *E*–*G*). These experiments demonstrate that the neurogenic quiescence observed in Emx2 progenitors cannot be explained by a delay in expression of two proteins.

To better characterize the progeny of the Emx2 progenitors in the cortex, we used several laminar markers at P5 aided by *piggyBac* transposase. At this time, most of the derived cells are neurons, preceding the subsequent waves of gliogenesis (Fig. 4). Glial cells do not relate their cortical location to their birthdate so glia in the deeper cortex could mask the differential lineage. Therefore, glial cells were excluded from quantification. In addition we used selected neuronal markers for upper (Cux1 and Brn2) and lower (Ctip2 and Otx1) cortical layers.

Postnatal progeny of E12 Emx2 progenitors from the lateral cortex consisted mainly of neurons located in upper layers of cortex, as shown by Cux1 and Brn2 expression (Fig. 4 *A*, *B*, *E*, and *G*). A total of 80.3 ± 3.2% of Emx2 progenitor-derived neurons were located in cortical layers II–IV (657 of 804 counted neurons). In addition, these neurons showed a tendency to express these markers, given that 49.8 ± 7.1% of $EGFP^+$ neurons were positive for Cux1. These data contrast with the near absence of Emx2-derived neurons in Ctip2- or Otx1-expressing deep cortical layers (Fig. 4 *C*, *D*, *F*, and *G*). Only 4.2 ± 3.1% of Emx2-derived neurons expressed Ctip2 at P5.

As expected, neurons derived from CAG-labeled unspecified progenitors (which included Emx2 progenitors) were found in all cortical layers defined by the selected markers (Fig. 4 *A′*–*D′* and *G*). The proportion of neurons in layers II–IV and V–VI was 27.8% and 72.2 ± 0.6%, respectively (upper/deeper ratio of 0.384), significantly different from that of Emx2-derived neurons (upper/deeper ratio of 4.08). Emx2 progenitors were over 10 times more likely to generate an upper-layer neuron than the average unspecified progenitor.

The unspecific lineage covering all cortical layers supports the existence of sequential progenitors. However, as a control experiment, it also validated the neuronal distribution labeled through the Emx2 enhancers and the presence of restricted progenitors. The data presented above support both hypotheses for the development of cortical pyramidal neurons, as it shows the temporal and spatial coexistence of both restricted and sequential RGC progenitors in mouse cortex.

Cell Fate in the Emx2-Labeled Population. Because Emx2 progenitors missed the early neurogenic phase toward the deep cortical layers, we asked whether these were multipotent progenitors or composed of subsets restricted to neuronal or glial cell fate. Over the course of development, Emx2 progenitors of the lateral neocortex permanently labeled from E12 by the *piggyBac* transposition of the

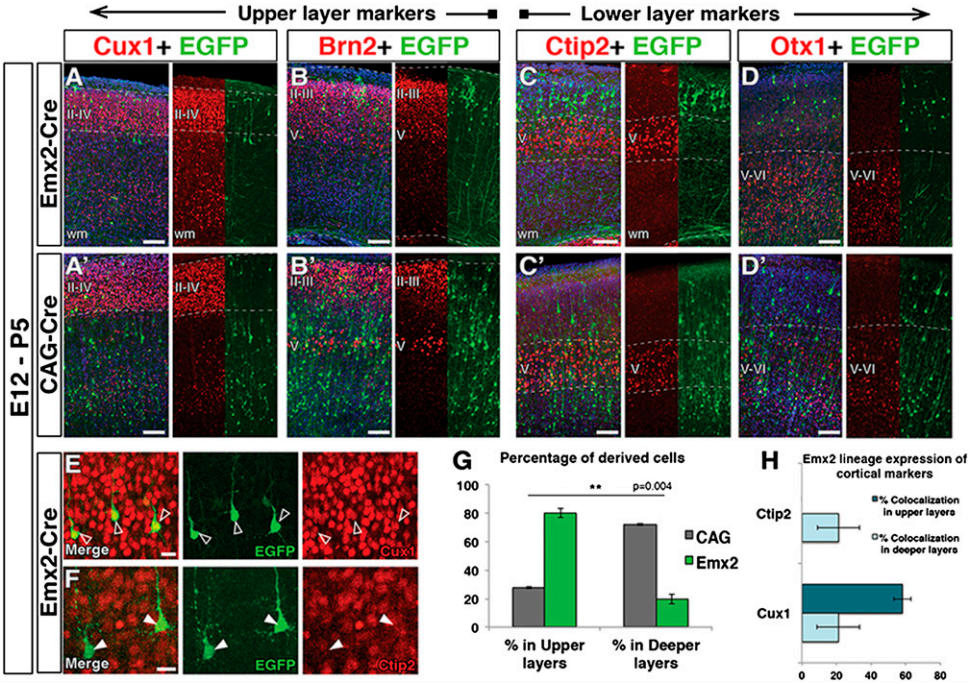

Fig. 4. Selective distribution of Emx2-derived neurons in the upper layers of the postnatal neocortex. Coronal sections through the lateral cortex of P5 mice electroporated at E12. Either Emx2 progenitors (*A*–*F*) or unspecified progenitors through CAG (*A′*–*D′*) were labeled and their postnatal neuronal progeny was compared with upper cortical layer markers Cux1 (*A* and *A′*) and Brn2 (*B* and *B′*) or deep cortical layer markers Ctip2 (*C* and *C′*) and Otx1 (*D* and *D′*). (*Left*) Merged image of DAPI counterstain in blue, EGFP expression in green, and immunohistochemistry for the layer-specific marker in red. (*Right*) Divided into two sectors, to display EGFP expression (*Left*) and immunohistochemistry (*Right*) separately to indicate the layer distribution of the labeled cells. (*E* and *F*) High magnification of Emx2-derived neurons expressing Cux1 (empty arrowheads; *E*) but not Ctip2 (filled arrowheads; *F*). (*G*) Quantification of neurons located in upper (II–IV) or deeper layers (V and VI) of the P5 neocortex from each population of progenitors. (*H*) Percentage of colocalization of the Emx2-derived neurons with Cux1 and Ctip2 in upper or deeper cortical layers. II–VI, cortical layers 2–6. $n = 3$ animals quantified per experiment. Data are represented as mean ± SEM. (Scale bars, 100 μm in *A*–*D* and *A′*–*D′* and 20 μm in *E* and *F*.)

fluoroprotein cassette were found to generate all three major neural types present in the postnatal brain: neurons, astrocytes, and oligodendrocytes (Fig. 5). As gliogenesis persists long after birth in mouse cortical development, the proportion of each cellular type varies with time. We selected P15 to study the cell fate of the Emx2-derived lineage. By this stage, neurogenesis is finished, both types of glia have been generated for several days or weeks, and the brain resembles the mature adult brain. The Emx2 lineage consisted of a total of 75.3 ± 2.8% neurons, 19.4 ± 2.1% astrocytes, and 5.4 ± 0.7% oligodendrocytes, denoted by cellular morphology and expression of NeuN, GFAP, and Olig2 markers, respectively (Fig. 5 *A*–*F*). We could confidently identify neuronal morphology within the Emx2 lineage because we used a membrane-tagged version of EGFP, which preferentially marks axonal branching and efferences (23, 33). As expected from their late birthdate (Fig. 2), upper position in the neocortex (Figs. 3 and 4), and immunoreactivity for Cux1 and Brn2 (Fig. 4), the Emx2-derived neurons mostly extended their axons contralaterally through the corpus callosum (Fig. 5 *G* and *H*). Their contralateral phenotype was confirmed by their expression of Satb2 (Fig. 5*J*, 88.2% of all labeled neurons were Satb2$^+$), a well-known regulator of contralateral pyramidal neuronal fate (34). However, some other neurons of the lineage eventually projected subcortically, with a small population entering the cerebral peduncle.

Emx2-Labeled Population in the Chick Forebrain. Sauropsids lack a homologous structure to the corpus callosum (21). In addition, the number of upper-layer–like cortical homologous neurons is reduced. These two features have led to the suggestion that the expansion of upper-layer callosal projection neurons was a major event in the early origin of the neocortex. Based on this idea, we hypothesized that sauropsid embryonic forebrains do not host a homologous population of neurogenic-delayed progenitors.

To test this hypothesis, we labeled targeted subsets of RGCs in embryonic chick forebrain and compared their lineage to that of unspecifically labeled RGCs (Fig. 6). We used the same constructs, including the Emx2-Cre construct, which contained genomic murine Emx2 sequences highly conserved across vertebrate evolution (28). Chick pallial progenitors were labeled at E4 stage (Hamburger and Hamilton stages HH23 and HH23–HH24), the onset of telencephalic neurogenesis, comparable to mouse E12, and the derived lineages were studied. According to the high conservation of these sequences in tetrapods (28), the murine Emx2 enhancers were able to trigger fluorophore expression in pallial progenitors, which suggests a conserved activation mechanism in the *Emx2* locus. Several pallial subdomains were analyzed (Fig. 6*A*) for two reasons: the homologous structure to the mammalian neocortex remains controversial in sauropsid brains (35, 36), and due to spatially restricted (but not temporally restricted) dynamics underlying generation of the different glutamatergic neuron subtypes in the avian pallium (22).

In contrast to our findings in mice, the lineage of Emx2 progenitors at E6 (approximately HH29; Fig. 6 *B*–*E*) showed no differences compared with the lineage of unspecific progenitor labeling (Fig. 6 *B′*–*D′*, and *E*). Moreover, this similarity was consistent from medial to ventral pallia. Regardless of the promoter that drove the labeling (either CAG or both Emx2 enhancers simultaneously), all labeled progenitors derived neuroblasts that migrated radially toward the pallial mantle zones as early as 48 h after transfection. Therefore, chick Emx2 progenitors did not delay neurogenic divisions in the way that the homologous murine population did. The wide distribution of the Emx2 progeny

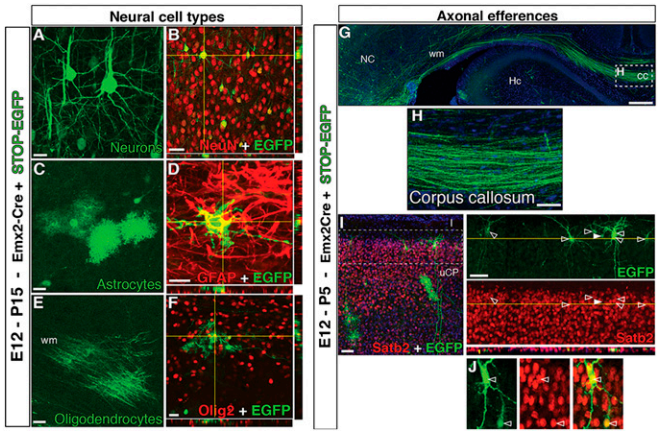

Fig. 5. Multipotent Emx2 progenitors gave rise to callosal neurons, astrocytes, and oligodendrocytes. Coronal sections through the lateral cortex of P15 (*A–F*, $n = 3$) or P5 (*G–I*, $n = 3$) mice showing the derived EGFP$^+$ lineage of multipotent Emx2 progenitors (green) electroporated at E12. DAPI staining is shown in blue, immunohistochemistry in red, and orthogonal views acquired using confocal microscopy from yellow lines demonstrate colocalization. (*A*) Morphology of upper layer cells showing somatodendritic morphology and axonal arborizations characteristic of neurons. (*B*) The neuronal identity of some EGFP-expressing Emx2 progenitor derived cells was further confirmed with NeuN immunostaining. (*C*) EGFP label revealed postnatal cortical astrocytes in the Emx2 progeny at P15. (*D*) Example demonstrating that some of these morphologically assigned astrocytes also expressed GFAP. (*E*) Emx2-derived oligodendrocytes in the cortical white matter (wm). (*F*) Oligodendrocyte fate was confirmed by immunohistochemistry against Olig2. (*G*) Axonal efferent projections from the Emx2 progenitor-derived neuronal lineage preferentially travel contralaterally through the corpus callosum (cc). (*H*) High power view of the axonal bundle at the cc. (*I* and *J*) Most derived neurons expressed the protein Satb2 (empty arrowheads). White arrowheads point to a Satb2$^-$ neuron. Confocal orthogonal view and (*J*) high magnifications are shown to demonstrate colocalization. HC, hippocampus; LI, cortical layer I; NC, neocortex; uCP, upper cortical plate. (Scale bars, 250 μm in *G*, 50 μm in *B*, *H*, and *I*, and 20 μm in *A*, *C–F*, and *J*.)

at E6 showed that the observed differences in neurogenesis in mouse neocortical development were not caused by a displaced recombination of the reporter gene in differential populations of embryonic cells.

As both Emx2 and unspecific RGCs became direct-neurogenic early during chick pallial development, we postulated that their long-term lineages would also be equivalent. We labeled both E4 populations simultaneously using a combination of *piggyBac*-transposable constructs, by which a nonarrested EGFP labels all progenitors indiscriminately and an arrested mCherry plasmid vector only labels Cre-expressing Emx2 progenitors (Fig. 6*F*). Similar to the short-term lineage experiment, we investigated several pallial subdivisions (Fig. 6*G*). As expected, both EGFP$^+$- and mCherry$^+$-derived cells settled in the same telencephalic areas up to E11, when neurogenesis has largely ended (Fig. 6 *H–L* and Fig. S5). We found slow-dividing progenitors that generated a low number of neurons within the mantle zone in the medial pallium (Fig. 6*H*). The number of derived neurons increased in the dorsal pallium-derived hyperpallium (Fig. 6*I*) and peaked in the lateral and ventral pallia-derived dorsal ventricular ridge nuclei (Fig. 6 *J–L*). However, despite the variable mitotic dynamics within the pallium, we did not find any differences between the lineages generated from Emx2 and unspecific progenitors. The lack of neurogenic delay of Emx2 progenitors in chick might be associated with the absence of an intradorsal pallial connecting population homologous to the mammalian corpus callosum.

Discussion

The identification of neural progenitor subtypes and their specific contribution to neocortex is important for understanding how neocortical architecture, connectivity, and function arise during development and evolution (16). We show here that, during early cortical neurogenesis, a subset of RGCs remains committed to the expansion of the progenitor pool without generating the early-generated deep layer cortical neurons. Due to this absence of early neurogenic potential, such progenitors lack the sequential ability to generate all types of projection neurons in the cortex and therefore only contribute to the later-born cortical populations of upper-layer callosal neurons and glia. In addition, we demonstrate that a homologous population of pallial avian RGCs does not present this neurogenic delay and consequently these do not give rise to a segregated subset of pallial neurons (Fig. 7).

Fate-Restricted Progenitors Are Selected by Emx2 Activity. The homeobox gene *Emx2* encodes a transcription factor known to play key roles in cortical development by mediating neuroblast proliferation, migration and differentiation (37, 38), astrogenesis (39), and cortical areal patterning and specification (40–42). This gene is regulated by two independent enhancer sequences, which present striking differences in activity levels across the forebrain (28, 29). Our experiments suggest that the activation of these enhancers could be characteristic of self-renewal and transit-amplifying early RGCs. In addition, the employment of both enhancers simultaneously revealed a population of RGCs initially programmed exclusively for self-renewal, suggesting an important interaction between these regulatory regions. The cellular correlation of high *Emx2* activity levels and the commitment to self-expanding divisions was not unexpected, as the Emx2 protein has been shown to act as a powerful inhibitor of neurogenesis. In vitro cultures of Emx2-overexpressing cortical progenitors generate larger cellular clones (38), and *Emx2*$^{-/-}$ mutant RGCs slow down DNA synthesis and leave the cell cycle prematurely (43). Altogether, these data support the selectivity of the Emx2-Cre construct for nonneurogenic early cortical progenitors.

Emx2 Progenitors Support Both the Restricted and the Sequential Hypotheses of Cortical Neurogenesis. The existence of multipotent sequential progenitors among the RGC population of the developing mouse neocortex has gathered considerable evidence. From classical staining to modern clonal labeling analyses, a wide number of early RGCs have been shown to demonstrate a sequential proliferative behavior both in vivo and in vitro (12, 44–47). Despite such comprehensive evidence, the existence of fate-restricted progenitors has also been uncovered (17, 48). Some authors consider all RGCs to be multipotent and sequential (10, 19). Others oppose this idea and suggest that all upper cortical layer neurons derive from fate-restricted *Cux2*-expressing progenitors, precluding the existence of sequential progenitors that would give rise to both upper and deeper cortical layer neurons (17). Our experiments on the other hand, support the complementary existence of both types of progenitors. We propose the concept of an embryonic cortical neuroepithelium inhabited by an assortment of different RGCs, both sequential and fate restricted. Whereas our selective labeling of Emx2 progenitors clearly targeted a population of neurogenic-delayed, fate-restricted progenitors, we also consider that our experiments with the nonselective promoter support the existence of sequential RGCs. These findings restore McConnell's previous idea that "the ventricular zone is a mosaic of proliferating cells with distinct developmental potentials ranging from a self-renewing, multipotent stem cell to cells restricted to the production of neurons, glia, or even distinct subtypes of neurons" (49). As such, not all RGCs are progenitors under the sequential program, just as not all upper-layer cortical neurons derive from early fate-restricted progenitors. Strong support for

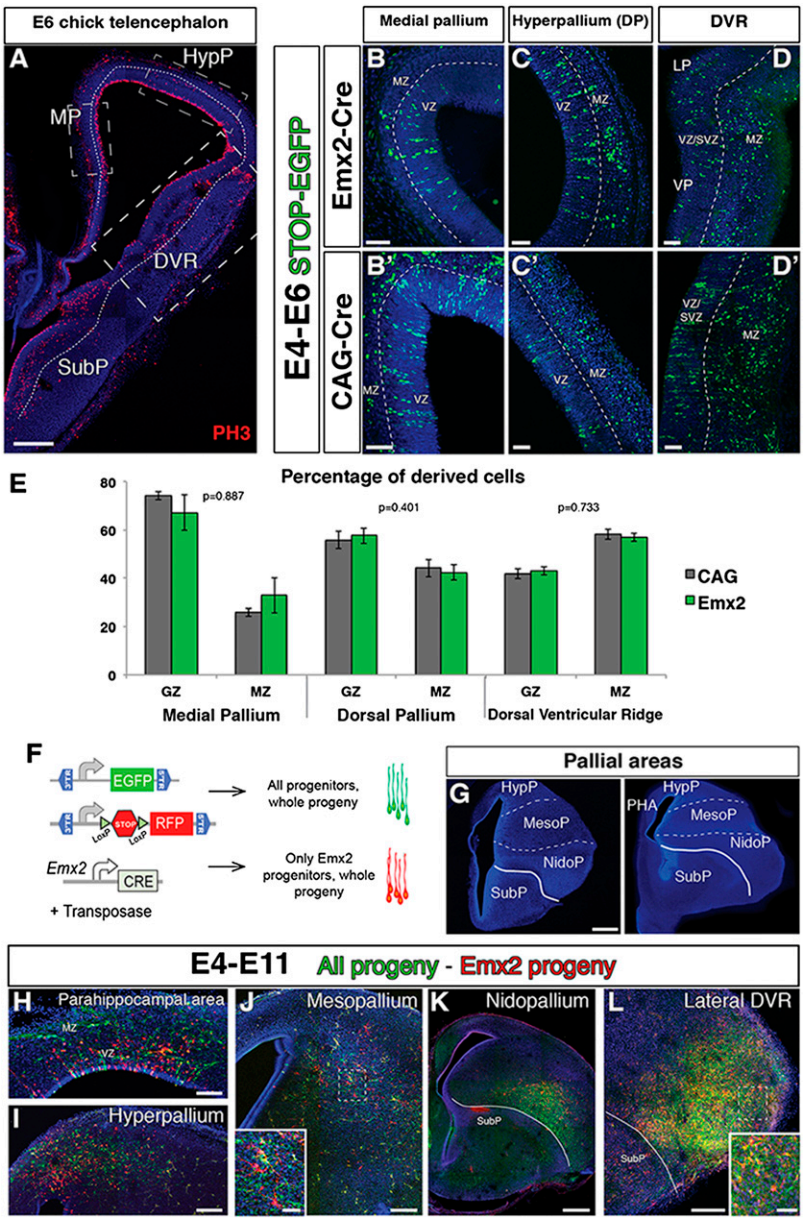

Fig. 6. Emx2-labeled population in the chick forebrain did not segregate a separate neuronal population from the unspecified progenitors through CAG. (*A*) Composite image of coronal section through middle forebrain of an E6 chicken embryo depicting the different pallial areas (MP, medial pallium; HypP, hyperpallium; DVR, dorsal ventricular ridge, SubP, subpallium). PH3 immunostaining in red depicts the mitotic compartments at the germinative ventricular (VZ) and subventricular (SVZ) zones. DAPI is shown in blue. (*B–D*, *B′–D′*) Transfection of nuclear-tagged EGFP by in ovo electroporation was performed at early neurogenesis, E4, at the medial pallium (*B* and *B′*), the dorsal pallium/hyperpallium (HypP; *C* and *C′*), or the ventral and lateral pallia/dorsal ventricular ridge (DVR; *D* and *D′*). Either Emx2-Cre (*B–D*) or CAG-Cre (*B′–D′*) drove the labeling of progenitors and generated a very similar pattern. Both constructs labeled equivalent progenitors that immediately started to colonize the postmitotic compartment of the mantle zone (MZ) and showed no signs of delayed neurogenesis. (*E*) Quantification of derived cells in either the MZ or the germinative zone (GZ) in the different pallial zones showed similar proportions to Emx2 or unspecified progenitors ($n = 3$ animals minimum per experiment). Data are represented as mean ± SEM. (*F*) Constructs electroporated at E4 for simultaneous labeling to compare the labeled lineage at E11 of Emx2 and unspecified progenitors. CAG-EGFP labeled all progenitors reaching the ventricular zone, whereas arrested CAG-STOP-mCherry only labeled cells after Cre recombination mediated by Emx2 enhancers. Fluorescent protein cassettes were integrated into the genome of chick neural stem cells by piggyBac transposition. (*G*) Coronal sections of E11 chick forebrain depicting the different pallial derivatives at rostral and middle telencephalic levels at the end of embryonic neurogenesis. (*H–L*) Examples of derived lineages at E11 from both Emx2 promoter-driven (red) and unspecified CAG promoter-driven progenitors (green) in the different pallial areas studied. Box in *L* depicts an area of lateral DVR. See also Fig. S3 for separate fluorescent channel images. DVR, dorsal ventricular ridge; HypP, hyperpallium; MesoP, mesopallium; MP, medial pallium; MZ, mantle zone; NidoP, nidopallium; PHA, parahippocampal area, SubP, subpallium. (Scale bars, 500 μm in *G* and *K*; 250 μm in *A* and *L*; 200 μm in *H–J*; 50 μm in *B–D*, *B′–D′*, and *Insets* in *J* and *I*; and 20 μm in *A*, *C–F*, and *J*.)

this heterogeneity of cortical progenitors can be found in a previous murine clonal analysis of early progenitors (E11) that described a variety of clustered cells at E14 (48). Among a variety of other clones, the authors classified many clones (termed by the authors, "P clones"), which comprised all of the clonally related cells within the germinative zone of the neocortex. We consider these P clones to be examples of early clones derived from neurogenic-delayed RGCs.

It is interesting to establish the relationship of early Emx2 progenitors with other previously described early fate-restricted

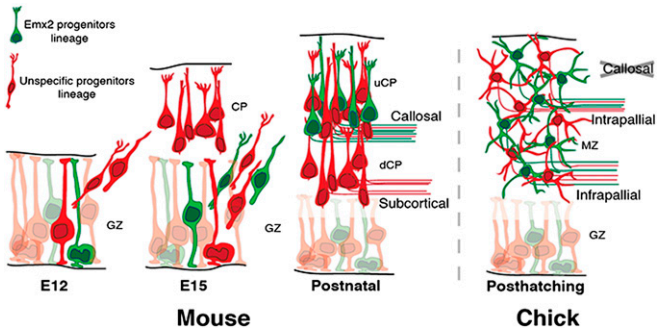

Fig. 7. Summary. In mouse, sequential (red cells) and fate-restricted (green cells) progenitors inhabit the early cortical neuroepithelium at E12. Whereas sequential progenitors contribute to the development of all cortical layers, fate-restricted Emx2 progenitors miss the first neurogenic stages and only contribute to the genesis of the upper layer callosal cortical neurons. In chick, Emx2 progenitors are not delayed, their lineage is not segregated from that of other resident progenitors, and a homologous of the corpus callosum is not formed.

RGCs (17). We have shown our Emx2 construct is able to find self-renewing progenitors, and therefore we consider the Emx2 population encompassing the more specific Cux2-expressing population of cortical progenitors. As Emx2 progenitors are also gliogenic, the almost complete absence of glial cells in the lineage of Cux2-expressing progenitors, a feature of a more restricted cell lineage, supports our view. However, as shown in recent articles, the Cux2-expressing traced lineages occupy a wider thickness of the cortical plate depending on the genetic background of the mouse strain (50, 51). This fact makes it very difficult to establish the direct relationship between Emx2 progenitors that we observed in wild type and Cux2-expressing RGCs that were always described in transgenic mouse lines.

A crucial difference between our study and previous publications is the stages of embryonic and postnatal development under investigation (17, 19). Most of our experiments were performed at E12, the earliest time point when most progenitors stop self-renewing and the progenitor pool becomes neurogenic (10). In other cases, the labeling of progenitors relies on tamoxifen supply (17, 19), which permits targeting of progenitors as early as E10. Whereas our experiments cannot exclude the possibility of a multipotent sequential RGC at E10 later becoming fate restricted at E12, we strongly believe this not to be the case for two reasons: one of the main studies on the subject has shown that progenitors at E10 can be fate restricted (17); and as shown in our control experiments labeling unspecified RGCs at E12, the major neuronal production populates the deep (V and VI) layers at this stage. Furthermore, most of our analysis was performed at P5, at the onset of a vast gliogenesis period. In other investigations, the analysis was carried out at later stages of postnatal development, after a substantial reorganization of the cortical neuroepithelium through widespread cellular death (52). Therefore, the final lineages in our research are not directly comparable to those described in other studies (10, 19).

We highlight previous demonstrations of heterogenic early VZ progenitors. Among these demonstrations, we highlight the existence of clusters of clonally related pyramidal neurons arranged horizontally in the upper cortical layers of mouse (53), rat (46), and macaque (12). Independent of the embryonic time point at which stem progenitors were labeled (early neurogenesis in rat and mouse; midneurogenesis in macaque), derived neurons followed the inside-out pattern of neurogenesis. Accordingly, to occupy the same upper laminar position, these neurons must have been generated in a late short time window, incompatible with an origin from a single sequential progenitor cell.

Much of the support for the homogenous sequential nature of the early ventricular zone comes from in vitro studies (44, 47). However, extrapolation of findings from in vitro assays to the behavior of progenitors in the living brain should be taken with caution. For example, early cortical progenitors cultured in vitro first generate Cajal-Retzius cells, although in the living animal these neurons are generated from other progenitors, outside the dorsal pallium (54, 55).

The Role of Heterochronic Neurogenic Delay in Forebrain Evolution. Given heterochrony refers to a change in the timing of developmental events, leading to changes in size and shape (56), we consider that Emx2 progenitors have evolved heterochronicity with respect to the rest of the RGC population. As these early progenitors are actively mitotic but not neurogenic, the Emx2 progenitor pool expands before the onset of upper-layer neurogenesis. This pool increase is a well-known cause of brain expansion (57).

Heterochrony is a powerful source of evolutionary change. As such, we suggest that the neurogenic delay and associated fate restriction of a subset of RGCs could have played two independent roles in cortical evolution.

First, Emx2 progenitors' heterochrony may underlie the origin of the mammalian eutherian corpus callosum. We found a homologous population of pallial progenitors in the chick brain using the same methodology and selective enhancer sequences (28). However, the avian population did not delay onset of neurogenesis and consequently did not produce a fate-restricted lineage at any sector of the pallium. Interestingly, chick Emx2 progenitors are not committed to the generation of only upper-layer–like neurons as these were not circumscribed to the lateral wall of the avian pallium, where upper-layer–like neurons are generated (22). One of the most characteristic features of mammalian supragranular neurons is the projection of contralateral efferences through the corpus callosum. Sauropsid brains lack a homologous dorsal pallial interhemispheric tract, and it is possible that the appearance of delayed progenitors committed to the generation of novel interhemispheric connections emerged as a functional advantage through positive selection (21). This appearance could be a major cause of brain divergence in the evolutionary roots of the eutherian branch of mammals. Because other mammalian lineages, like marsupials, also lack a corpus callosum (58, 59), it becomes interesting to study the presence and lineage of heterochronic-delayed progenitors in the marsupial dorsal pallia.

A second suggested role of neurogenic delay of RGCs in cortical evolution is related to the expansion of supragranular cortices in mammals. It is known that cortical cytoarchitecture varies greatly between orders over mammalian eutherian evolution (60). This variability can be accounted for in developmental neurogenic terms. We suggest that a potential further expansion of the Emx2 progenitor pool within the mammalian lineage could be the cause of described enlargement of supragranular cortices of carnivore, and specially primate, brains (61). An examination of early neurogenesis in primate brains may reveal a higher proportion of Emx2 progenitors among the RGC ventricular pool.

In summary, we have shown the presence of fate-restricted early neural progenitors in murine neocortex, but not in chick dorsal pallium. An evolutionary emphasis on the heterochronic delay of these neural progenitors might have played a role in both the origin of the mammalian corpus callosum and the expansion of upper cortical layers within the mammalian phylogenetic tree.

Materials and Methods

Animals. All animal experiments were approved by University of Oxford's ethical review committee and conducted in accordance with personal and project licenses under the UK Animals (Scientific Procedures) Act (1986). Adult C57BL/6 mice were obtained from a local breeding colony at the

University of Oxford [based on the Harlan (UK) strain]. These were maintained on a 12/12-h light/dark cycle (7:00 AM, lights on) and provided with ad libitum access to food and water. The day when vaginal plug was detected was referred to as E0.

Fertile hens' eggs, obtained from Winter Egg Farm (UK), were incubated at 38 °C in a humidified atmosphere until required stages. The day when eggs were incubated was considered E0.

Plasmid Constructs. All of the plasmid constructs used in this study were built for a previous study (23), which describes these constructs in detail. The concentration of different plasmids was kept constant among the different experiments in mouse and chicken embryos. Labeling constructs (pPB-CAG-STOP-EGFP; pPB-CAG-STOP-mCherry; pPB-CAG-STOP-H2BEGFP; pPB-CAG-STOP-MbEGFP; pPB-CAG-EGFP) were transfected at a final concentration ranging from/between 200–500 ng/μL; Cre expressing constructs under CAG or Emx2 enhancers (pCAG-Cre; p5′Emx2-Cre; p3′Emx2-Cre; pEmx2-Cre) were electroporated at 100 ng/μL; the transposase enzyme expressing-construct (mPB) was consistently transfected at 300 ng/μL. Cre-expressing constructs were the less concentrated in the mixture of plasmids. This fact implied that over 95% of transfected cells had at least one copy of each of the other labeling and transposition constructs (23, 62). It is described that *piggyBac* integrates an average of nine copies per cell (63). Under these previously described circumstances, we consider that virtually all progenitors transfected with the Cre construct (the one that triggers the labeling) had integrated at least one copy of the reporter gene (no more than one is needed for the detection of the cell even at postnatal stages) and many more than one copy in the majority of the cases. If any cells contained Cre but not labeling constructs, these were undetectable, did not mask any result, and were not taken into consideration in the study. In addition, episomal copies of the labeling cassettes that were not included onto the genome only added brighter fluorescence.

In Utero Electroporation. Transfection by electroporation of embryonic neural progenitors was performed as described previously (64). Briefly, E12–E14 pregnant mice were anesthetized by inhalation of isoflurane administered in conjunction with 100% oxygen. After midline laparotomy, the uterine horns were exposed out of the abdominal cavity and constantly warmed and hydrated with prewarmed sterile saline. The heads of embryos were transilluminated and injected with a glass electrode to fill up the lateral ventricle specifically. Each embryo was injected with a volume less than 1 μL comprising the mixture of plasmids. The embryos were then electroporated. Forebrain ventricular zone cells were transfected by means of a BTX Electroporator ECM 830 (Harvard Apparatus). At E12, the electrodes gently pressed the forebrain, setting them parallel to the brain midline. Therefore, all E12 electroporations transfected lateral neocortical progenitors. Electroporation was accomplished by five pulses (50 ms) discharging a 500-μF capacitor charged to 35–45 V with a sequencing power supply. The voltage pulse was discharged using a pair of platinum round plates (3 mm in diameter). Buprenorhpine (vetergesic) was administered to the pregnant mice before surgery (0.05 mg/kg) and the injected embryos were examined at different embryonic and postnatal stages.

In Ovo Electroporation. Electroporation of chicken embryos was performed as previously described (23). Briefly, eggs were incubated in vertical position at 38 °C. Plasmids were injected with a volume of less than 1 μL into the lateral ventricles of E4 chicken embryos using a fine pulled glass needle. Four electric pulses (14–17 V, 15-ms pulses with a 950-ms interval; BTX electroporator ECM) were then applied to the brain between insulated silver 40 mm × 0.8 mm wire electrodes with flattened pole (Intracel). Drops of Ringer's solution supplemented with antibiotics (penicillin/streptamycin; Sigma) were added to the egg. Embryos were incubated until E6–E11.

Tissue Processing. Embryonic murine brains and E6 chicken embryonic brains were fixed by immersion in 4% (wt/vol) paraformaldehyde (PFA), whereas postnatal mice and E11 chicken embryos were transcardially perfused with PBS followed by PFA. All of the brains were sectioned in the coronal plane at 50–70 μm thickness in a vibrating microtome (Leica VT1000S). For BrdU birthdating analysis, pregnant dams were intraperitoneally injected with BrdU 100 mg/kg body weight in sterile saline (BD Biosciences) after suturing the wound (minutes after transfection by electroporation). Brains were collected after 24 h, fixed by immersion in PFA for 4 h, and then transferred to PBS.

Immunohistochemistry. Single and dual immunohistochemical reactions were performed as described previously (65) using the following primary antibodies: rabbit antibody to Pax6 (Covance; PRB-278P, 1:200), mouse antibody to Nestin (Chemicon; MAB353, 1:1,000), chick antibody to EGFP (Aves; GFP 1020, 1:10,000), rabbit antibody to Ki67 (Abcam; ab15580, 1:1,000), rabbit antibody to PH3 (Chemicon; 06–570, 1:1,000), rabbit antibody to Tbr2 (Abcam; ab23345, 1:500), rabbit antibody to Tbr1 (Chemicon; AB9616, 1:1,000), goat antibody to Cux1 (Santa Cruz; sc-28185, 1:500), goat antibody to Brn2 (Santa Cruz; sc-6029, 1:200), rat antibody to Ctip2 (Abcam; ab18465, 1:500), mouse antibody to Otx1 (Chemicon; MAB5602, 1:1,000), mouse antibody to Satb2 (Abcam; ab51502, 1:500), mouse antibody to NeuN (Chemicon; MAB377, 1:100), rabbit antibody to GFAP (Sigma; G9269, 1:500), mouse antibody to BrdU (Progen; 11200, 1:500), and rabbit antibody to Olig2 (Millipore; AB9610, 1:1,000). Immunostaining for BrdU required DNA denaturalization in 1 M HCl (Sigma) at 39 °C for 50 min prior to blocking with serum.

For secondary antibodies, we used Alexa 568 goat antibody to rabbit IgG (Molecular Probes; A11011, 1:1,000), Alexa 568 antibody to mouse IgG (Molecular Probes; A11004, 1:1,000), Alexa 568 goat antibody to rat IgG (Molecular Probes; A11077, 1:1,000), Alexa 568 donkey antibody to goat IgG (Molecular Probes; A11057, 1:1,000) and Alexa 488 goat antibody to chicken (Invitrogen; A11039, 1:1,000).

Imaging and Analysis. Images were captured using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microimaging). Similar image parameters (laser power, gain, pinhole, and wavelengths) were maintained for images from each brain and adjusted for new specimens. The used fluorophores were DAPI, EGFP, Alexa 488, mCherry, and Alexa 568. Z stacks were taken individually for each channel and then collapsed to get maximum intensity projections. Images were adjusted and analyzed using ImageJ (Image Analysis in Java, NIH) and Adobe Photoshop CS6 (Adobe Systems).

Cell Counting and Statistical Analysis. Cells were counted on confocal images aided by ImageJ. In some cases nuclear-tagged EGFP was used to label the cells, making the counting more reliable. DAPI staining along with the staining pattern of well-known immunohistochemical markers were used to define anatomical landmarks and boundaries relevant to each analysis.

It is difficult to find equivalent and comparable electroporations at the level of fluorescence brightness and anatomical region electroporated. We based a reliable and repeatable quantification by following strict inclusion criteria according to the age, site of electroporation, quality of the electroporations, etc. Therefore, our analysis was performed in at least three perfectly matched electroporations, but many more animals were also studied and imaged, showing the same differential cortical lineage. We counted a minimum of three representative sections of each electroporated brain. Due to the variability in the number of cells labeled in each electroporation, we compared percentages of cells positioned in each of the regions of interest. One-way ANOVA and nonparametric least significant difference and Bonferroni tests compared these percentages.

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1. Fietz SA, et al. (2010) OSVZ progenitors of human and ferret neocortex are epithelial-like and expand by integrin signaling. *Nat Neurosci* 13(6):690–699.
2. Hansen DV, Lui JH, Parker PRL, Kriegstein AR (2010) Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature* 464(7288):554–561.
3. Kriegstein AR, Noctor SC (2004) Patterns of neuronal migration in the embryonic cortex. *Trends Neurosci* 27(7):392–399.
4. Rakic P (1971) Guidance of neurons migrating to the fetal monkey neocortex. *Brain Res* 33(2):471–476.
5. Angevine JB, Jr, Sidman RL (1961) Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* 192:766–768.
6. McConnell SK (1992) The control of neuronal identity in the developing cerebral cortex. *Curr Opin Neurobiol* 2(1):23–27.
7. Imayoshi I, Kageyama R (2014) bHLH factors in self-renewal, multipotency, and fate choice of neural progenitor cells. *Neuron* 82(1):9–23.
8. Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. *J Comp Neurol* 145(1):61–83.

9. McConnell SK (1988) Development and decision-making in the mammalian cerebral cortex. *Brain Res* 472(1):1–23.
10. Gao P, et al. (2014) Deterministic progenitor behavior and unitary production of neurons in the neocortex. *Cell* 159(4):775–788.
11. Grove EA, et al. (1993) Multiple restricted lineages in the embryonic rat cerebral cortex. *Development* 117(2):553–561.
12. Kornack DRD, Rakic P (1995) Radial and horizontal deployment of clonally related cells in the primate neocortex: Relationship to distinct mitotic lineages. *Neuron* 15(2): 311–321.
13. Luskin MB, Parnavelas JG, Barfield JA (1993) Neurons, astrocytes, and oligodendrocytes of the rat cerebral cortex originate from separate progenitor cells: An ultrastructural analysis of clonally related cells. *J Neurosci* 13(4):1730–1750.
14. Walsh C, Cepko CL (1992) Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. *Science* 255(5043):434–440.
15. Lai T, et al. (2008) SOX5 controls the sequential generation of distinct corticofugal neuron subtypes. *Neuron* 57(2):232–247.
16. Rakic P, Ayoub AE, Breunig JJ, Dominguez MH (2009) Decision by division: Making cortical maps. *Trends Neurosci* 32(5):291–301.
17. Franco SJ, et al. (2012) Fate-restricted neural progenitors in the mammalian cerebral cortex. *Science* 337(6095):746–749.
18. Franco SJ, Müller U (2013) Shaping our minds: Stem and progenitor cell diversity in the mammalian neocortex. *Neuron* 77(1):19–34.
19. Guo C, et al. (2013) Fezf2 expression identifies a multipotent progenitor for neocortical projection neurons, astrocytes, and oligodendrocytes. *Neuron* 80(5): 1167–1174.
20. O'Rourke NA, Sullivan DP, Kaznowski CE, Jacobs AA, McConnell SK (1995) Tangential migration of neurons in the developing cerebral cortex. *Development* 121(7):2165–2176.
21. Suárez R, Richards LJ (2014) Evolution and development of interhemispheric connections in the vertebrate forebrain. *Front Hum Neurosci* 8:497.
22. Suzuki IK, Kawasaki T, Gojobori T, Hirata T (2012) The temporal sequence of the mammalian neocortical neurogenetic program drives mediolateral pattern in the chick pallium. *Dev Cell* 22(4):863–870.
23. García-Moreno F, Vasistha NA, Begbie J, Molnár Z (2014) CLoNe is a new method to target single progenitors and study their progeny in mouse and chick. *Development* 141(7):1589–1598.
24. Vasistha NA, et al. (June 13, 2014) Cortical and clonal contribution of Tbr2 expressing progenitors in the developing mouse brain. *Cereb Cortex,* 10.1093/cercor/bhu125.
25. Gulisano M, Broccoli V, Pardini C, Boncinelli E (1996) Emx1 and Emx2 show different patterns of expression during proliferation and differentiation of the developing cerebral cortex in the mouse. *Eur J Neurosci* 8(5):1037–1050.
26. Puelles L, et al. (2000) Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. *J Comp Neurol* 424(3):409–438.
27. Simeone A, et al. (1992) Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex. *EMBO J* 11(7): 2541–2550.
28. Suda Y, et al. (2010) The same enhancer regulates the earliest Emx2 expression in caudal forebrain primordium, subsequent expression in dorsal telencephalon and later expression in the cortical ventricular zone. *Development* 137(17):2939–2949.
29. Theil T, Aydin S, Koch S, Grotewold L, Rüther U (2002) Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon. *Development* 129(13):3045–3054.
30. Cecchi C (2002) Emx2: A gene responsible for cortical development, regionalization and area specification. *Gene* 291(1-2):1–9.
31. García-Marqués J, López-Mascaraque L (2013) Clonal identity determines astrocyte cortical heterogeneity. *Cereb Cortex* 23(6):1463–1472.
32. Siddiqi F, et al. (2014) Fate mapping by piggyBac transposase reveals that neocortical GLAST+ progenitors generate more astrocytes than Nestin+ progenitors in rat neocortex. *Cereb Cortex* 24(2):508–520.
33. Livet J, et al. (2007) Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* 450(7166):56–62.
34. Alcamo EA, et al. (2008) Satb2 regulates callosal projection neuron identity in the developing cerebral cortex. *Neuron* 57(3):364–377.
35. Belgard TG, et al. (2013) Adult pallium transcriptomes surprise in not reflecting predicted homologies across diverse chicken and mouse pallial sectors. *Proc Natl Acad Sci USA* 110(32):13150–13155.
36. Dugas-Ford J, Rowell JJ, Ragsdale CW (2012) Cell-type homologies and the origins of the neocortex. *Proc Natl Acad Sci USA* 109(42):16974–16979.
37. Faiella A, et al. (1997) A number of schizencephaly patients including 2 brothers are heterozygous for germline mutations in the homeobox gene EMX2. *Eur J Hum Genet* 5(4):186–190.
38. Heins N, et al. (2001) Emx2 promotes symmetric cell divisions and a multipotential fate in precursors from the cerebral cortex. *Mol Cell Neurosci* 18(5):485–502.
39. Falcone C, Filippis C, Granzotto M, Mallamaci A (2014) Emx2expression levels in NSCs modulate astrogenesis rates by regulating EgfRand Fgf9. Glia 63(3):412–422.
40. Mallamaci A, Muzio L, Chan CH, Parnavelas J, Boncinelli E (2000) Area identity shifts in the early cerebral cortex of Emx2-/- mutant mice. *Nat Neurosci* 3(7):679–686.
41. Muzio L, et al. (2002) Conversion of cerebral cortex into basal ganglia in Emx2(-/-) Pax6(Sey/Sey) double-mutant mice. *Nat Neurosci* 5(8):737–745.
42. O'Leary DD, Chou SJ, Sahara S (2007) Area patterning of the mammalian cortex. *Neuron* 56(2):252–269.
43. Muzio L, Soria JM, Pannese M, Piccolo S, Mallamaci A (2005) A mutually stimulating loop involving emx2 and canonical wnt signalling specifically promotes expansion of occipital cortex and hippocampus. *Cereb Cortex* 15(12):2021–2028.
44. Gaspard N, et al. (2008) An intrinsic mechanism of corticogenesis from embryonic stem cells. *Nature* 455(7211):351–357.
45. Luskin MB, Pearlman AL, Sanes JR (1988) Cell lineage in the cerebral cortex of the mouse studied in vivo and in vitro with a recombinant retrovirus. *Neuron* 1(8): 635–647.
46. Reid CB, Liang I, Walsh C (1995) Systematic widespread clonal organization in cerebral cortex. *Neuron* 15(2):299–310.
47. Shen Q, et al. (2006) The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. *Nat Neurosci* 9(6):743–751.
48. Cai L, Hayes NLN, Nowakowski RSR (1997) Synchrony of clonal cell proliferation and contiguity of clonally related cells: Production of mosaicism in the ventricular zone of developing mouse neocortex. *J Neurosci* 17(6):2088–2100.
49. McConnell SK (1995) Constructing the cerebral cortex: Neurogenesis and fate determination. *Neuron* 15(4):761–768.
50. Eckler MJ, et al. (2015) Cux2-positive radial glial cells generate diverse subtypes of neocortical projection neurons and macroglia. *Neuron* 86(4):1100–1108.
51. Gil-Sanz C, et al. (2015) Lineage tracing using Cux2-Cre and Cux2-CreERT2 mice. *Neuron* 86(4):1091–1099.
52. Finlay BL, Slattery M (1983) Local differences in the amount of early cell death in neocortex predict adult local specializations. *Science* 219(4590):1349–1351.
53. Brown KN, et al. (2011) Clonal production and organization of inhibitory interneurons in the neocortex. *Science* 334(6055):480–486.
54. Bielle F, et al. (2005) Multiple origins of Cajal-Retzius cells at the borders of the developing pallium. *Nat Neurosci* 8(8):1002–1012.
55. García-Moreno F, López-Mascaraque L, De Carlos JA (2007) Origins and migratory routes of murine Cajal-Retzius cells. *J Comp Neurol* 500(3):419–432.
56. Gould SJ (1977) *Ontogeny and Phylogeny* (Harvard University Press, Cambridge, MA).
57. Caviness VS, Jr, Takahashi T, Nowakowski RS (1995) Numbers, time and neocortical neuronogenesis: A general developmental and evolutionary model. *Trends Neurosci* 18(9):379–383.
58. Ashwell KW, Waite PM, Marotte L (1996) Ontogeny of the projection tracts and commissural fibres in the forebrain of the tammar wallaby (Macropus eugenii): Timing in comparison with other mammals. *Brain Behav Evol* 47(1):8–22.
59. Mark RF, Marotte LR (1992) Australian marsupials as models for the developing mammalian visual system. *Trends Neurosci* 15(2):51–57.
60. Krubitzer L, Kaas J (2005) The evolution of the neocortex in mammals: How is phenotypic diversity generated? *Curr Opin Neurobiol* 15(4):444–453.
61. Hutsler JJ, Lee D-G, Porter KK (2005) Comparative analysis of cortical layering and supragranular layer enlargement in rodent carnivore and primate species. *Brain Res* 1052(1):71–81.
62. Rana ZA, Ekmark M, Gundersen K (2004) Coexpression after electroporation of plasmid mixtures into muscle in vivo. *Acta Physiol Scand* 181(2):233–238.
63. Woltjen K, et al. (2009) piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 458(7239):766–770.
64. García-Moreno F, et al. (2010) A neuronal migratory pathway crossing from diencephalon to telencephalon populates amygdala nuclei. *Nat Neurosci* 13(6):680–689.
65. García-Moreno F, Vasistha NA, Trevia N, Bourne JA, Molnár Z (2012) Compartmentalization of cerebral cortical germinal zones in a lissencephalic primate and gyrencephalic rodent. *Cereb Cortex* 22(2):482–492.