Genetic lineage tracing defines distinct neurogenic and gliogenic stages of ventral telencephalic radial glial development
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Abstract

Background: Radial glia comprise a molecularly defined neural progenitor population but their role in neurogenesis has remained contested due to the lack of a single universally accepted genetic tool for tracing their progeny and the inability to distinguish functionally distinct developmental stages.

Results: By direct comparisons of Cre/loxP lineage tracing results obtained using three different radial glial promoters (Blbp, Glast, and hGFAP), we show that most neurons in the brain are derived from radial glia. Further, we show that hGFAP promoter induction occurs in ventral telencephalic radial glia only after they have largely completed neurogenesis.

Conclusion: These data establish the major neurogenic role of radial glia in the developing central nervous system and genetically distinguish an early neurogenic Blbp+Glast+ stage from a later gliogenic Blbp+Glast+hGFAP+ stage in the ventral telencephalon.

Background

Radial glia (RG) comprise a molecularly defined cellular population, playing critical roles during central nervous system (CNS) development as both neural progenitors and as a scaffolding for migrating neurons [1,2]. Despite their established role as the major neuronal precursors in the cerebral cortex [3-8], two fundamental aspects of RG development and function remain contested. The first regards the question of whether, similar to the situation in cortex, RG function as the principal neuronal progenitors in all CNS regions. This question has been addressed with Cre/loxP lineage tracing using two different RG-specific promoters, the mouse brain lipid binding protein (Blbp) promoter and the human glial fibrillary acidic protein (hGFAP) promoter. However, sharply different patterns of recombination were driven by each promoter, prompting two distinct models for the RG role in neurogenesis. As significant neuronal recombination was detected in only a subset of brain regions when the hGFAP promoter was used to drive Cre [5,9], it was proposed that RG differ regionally in terms of their neurogenic potential and generate neurons only in certain brain regions [5,10,11]. In contrast, the extensive recombination driven in all brain regions by the Blbp promoter supported an alternative model in which essentially all RG populations are neurogenic and generate most neurons in the CNS [8]. Each model has different implications not only for RG function but also the larger issue of the mechanisms used to generate neurons during development. An accurate picture of lineal relationships within the developing brain is, therefore, essential but the controversy surrounding the RG contribution to neurogenesis has remained unresolved.
A second related issue of contention concerns the question of when cells with the molecular properties of RG first appear in the CNS. Resolution of this question is necessary as elucidation of the mechanisms responsible for driving RG differentiation requires analysis of the relevant pathways when they first become active, not at later stages when the end products of these programs become morphologically evident. Two different estimates for the time point when induction of RG differentiation occurs in the murine forebrain have been proposed based on Cre/loxP lineage tracing results (used to indicate onset of transcription). Whereas the mouse Blbp promoter drove recombination from embryonic day (E)10 [8], the hGFAP promoter is not active until after E12 [10]. These contrasting results have been alternatively proposed to reflect either precocious or delayed activity of the Blbp or hGFAP promoters, respectively [8,10,11], but neither direct evidence supporting either model nor data obtained using an independent RG promoter has yet been reported.

A third deficiency in the current understanding of RG development is the inability to distinguish RG at distinct stages of maturation. Multiple lines of evidence indicate that RG populations progress stepwise through distinct developmental stages during which particular classes of neurons or glia are generated, and that progression to successively later stages is accompanied by restricted potential to produce earlier cell types. For example, cortical RG stop generating neurons at late stages of embryogenesis and transform into astrocytes postnatally [1]. Determination of the precise time points when such transitions occur using in vivo genetic lineage tracing is a prerequisite to the identification of mechanisms regulating shifts in neurogenic potential; to date, few such studies have been conducted focusing on RG.

We report here that: similar to what was observed using the Blbp promoter for lineage tracing, RG expressing the glial high affinity glutamate transporter (Glast, a universally accepted RG marker [10-12]) are molecularly detectable in the forebrain by E10 and generate most neurons in the brain; hGFAP promoter activity is poorly correlated with RG differentiation in multiple brain regions, failing to drive recombination until days after RG first appear; and in ventral telencephalon, hGFAP activity commences after RG are predominantly gliogenic, genetically distinguishing an early neurogenic Blbp+Glast+hGFAP+ stage from a later gliogenic Blbp+Glast+hGFAP+ stage in this region.

Results

Endogenous GLAST expression and recombination in Glast::Cre;R26R mice are detected in the forebrain from E10.5

Due to the discrepant results previously obtained using the hGFAP and Blbp promoters, we performed Cre/loxP lineage tracing using the Glast promoter to drive Cre expression. Like Blbp, Glast expression is restricted to RG and astrocytes and molecularly distinguishes them from developmentally earlier neuroepithelial stem cells [5,12,13]. To generate mice expressing Cre under the regulation of the Glast promoter, we inserted a Cre-polyA cassette into the second exon of the Glast gene in a bacterial artificial chromosome (BAC) and generated BAC transgenic mice. Five separate Glast::Cre founder lines were obtained, all of which yielded similar results when used for lineage tracing; representative results from one line are presented.

Specificity of the Glast BAC was confirmed using in situ hybridization (ISH) and immunofluorescent staining. Cre mRNA was detected in ventricular zone (VZ) cells throughout the embryonic CNS and closely paralleled endogenous Glast expression (Figure 1a–d). Importantly, all Glast-expressing regions contained Cre mRNA and regions known to differentially express GLAST showed similar Cre mRNA gradients (Figure 1c,r). Postnatally, Cre protein was absolutely restricted to astroglial cells in all brain regions examined; no colocalization with neuronal markers was observed (Figure 1e–i). To confirm that the Glast BAC drives specific expression in all GLAST+ RG, Glast::eGFP transgens using the same BAC vector and marker insertion locus were analyzed. Immunostaining demonstrated specific enhanced green fluorescent protein (eGFP) expression in the majority of GLAST+ RG and astrocytes, endogenous GLAST gradients were faithfully recapitulated, and no neuronal expression was detectable (Figure 1j–v). Taken together, these data establish that the Glast::Cre transgene drives RG- and astroglial-specific expression and can be used to trace the neuronal progeny of all Glast-expressing progenitors in the brain. It should be noted however that BAC-driven expression was absent from the spinal cord floorplate (Figure 1r–t); as such, progeny derived from floorplate Glast* progenitors can not be traced in these mice.

As either precocious or delayed Cre activity could obscure the true contribution of Glast* RG to neurogenesis, details on when and where Cre recombination first occurs embryonically is required. This information is also relevant for defining the timing of the neuroepithelial stem cell (NE) to RG developmental transition, marked by the presence in the latter of ‘astroglial’ characteristics. These have been defined as molecular features unique to astroglial cells in the postnatal brain and are proposed to
Figure 1 (see legend on next page)
include BLBP and GLAST [5, 12, 13]. We therefore stained embryonic sections to determine when these three ‘astroglial’ molecules first become detectable in the forebrain. At E9.5, neither GLAST nor BLBP could be detected in the forebrain whereas both were highly expressed in the hindbrain at this age (Figure 2a,c). By E10.5, both molecules were present in the forebrain and both exhibited similar higher-rostral, lower-caudal gradients of expression (Figure 2b,d). Furthermore, double labeling confirmed that both molecules are induced in the same cellular population (Figure 2e,f). These results demonstrate that cells with the molecular characteristics of RG are present in the forebrain by E10.5.

To determine when recombination occurs and how well correlated it is with endogenous GLAST, we crossed Glast::Cre mice to the Rosa26[loxP]Z[14] and Z/EG [15] Cre reporter lines, which express β-galactosidase (R26R) or eGFP (Z/EG) after Cre-mediated recombination. Histochecmical staining of Glast::Cre;R26R embryos demonstrated a tight concordance between the onset of recombination and onset of endogenous GLAST expression, with β-galactosidase activity and GLAST protein both becoming detectable in the forebrain at E10.5 (Figure 2a,b,g–i). Importantly, this spatiotemporal pattern of recombination also matches what was observed in Blbp::Cre;R26R embryos [8]. The fact that both endogenous expression and recombination driven by the promoters of two different RG genes are so similar strongly suggests that the NE→RG transition occurs between E9.5 and E10.5 in the forebrain. It should also be noted that the timing of recombination in Glast::Cre;R26R and Blbp::Cre;R26R embryos sharply contrasts with that driven by the neuroepithelial cell marker Nestin, as recombination in Nestin::Cre;R26R embryos is readily detectable throughout the CNS by E8.5 [16]. Thus, the data together provide both molecular and genetic evidence for the existence of a developmental transition that occurs by E10.5 in the forebrain, consisting of an earlier Nestin+Blbp+Glast+ NE stage and a later Nestin+Blbp+Glast+ RG stage. Analysis of recombination in Glast::Cre;R26R embryos at later time points showed a similarly tight concordance between GLAST expression and recombination (Figure 2k–m), providing further support for the specificity of the Glast::Cre transgene and for the validity of the lineage tracing results.

Glast+ RG generate neurons throughout the brain
Adult Glast::Cre;R26R mice were analyzed to identify neurons derived from Glast+ RG. For comparative purposes, we also examined recombination in hGFAP::Cre;R26R mice using the identical hGFAP::Cre transgenic line [9] as the Malatesta study [5]. Similar to previous reports [5, 9], very few recombined neurons were observed in hGFAP::Cre;R26R mice in the ventral telencephalon, diencephalon or midbrain, and cerebellar Purkinje neurons were unreccombined (Figure 3a,f,k). In contrast, recombination in Glast::Cre;R26R mice was extensive in all of these brain areas (Figure 3b,g,l). Moreover, NeuN/β gal double labeling demonstrated that most neurons in these regions were recombined and, therefore, radial glial-derived (Figure 3c,d,h,i,m,o). As the Glast::Cre;R26R and Blbp::Cre;R26R tracing results [8] are essentially the same, these data establish that RG in all brain regions transit through a neurogenic stage of development and generate most neurons in the brain.

hGFAP promoter activity is poorly correlated with RG differentiation in multiple brain regions
Why are the hGFAP lineage tracing results different from those using the Blbp and Glast promoters if all three promoters are cell-specifically expressed in RG? As neurogenesis precedes gliogenesis during development, one possibility is that the hGFAP promoter does not become active in some brain regions until after RG neurogenesis is complete. This idea is supported by two observations: eGFP expression in hGFAP::eGFP mice is undetectable in the GE until E14.5 [5]; whereas most Blbp+ RG isolated from the E11.5 GE generated neurons in clonal cultures, most clones generated from cells isolated from E14.5 GE
Figure 2 (see legend on next page)
lacked neurons [8]. This latter result indicated that GE RG neurogenesis may be largely completed by E14.5.

To test the hypothesis that onset of hGFAP promoter activity is delayed with respect to the onset of RG differentiation, we determined when recombination first occurs in hGFAP::Cre;Z/EG and hGFAP::Cre;R26R embryos. Prior to E12.5, no recombination in the forebrain was observed in either reporter line (data not shown). By E12.5, extensive recombination had occurred in the cortex but was completely absent in the GE (Figure 4a–c). This result was striking as the GE VZ at this age is composed of fully morphologically differentiated BLBP+GLAST+ RG (Figure 4a–f; note that all BLBP+ and GLAST+ cells in the E12 GE are RC2+ [17], establishing that they are RG and not astrocytes). Similar results were observed in the thalamus (Figure 4h,i) and cortical hem (Figure 4j,l).

Analysis of the GE at E14.5 showed that some recombination had occurred medially, but the vast majority of VZ cells remained unrecombined (Figure 4m,n) and appreciable recombination could not be observed here until E15.5 (Figure 4o). Taken together, these results establish that hGFAP promoter activity is poorly correlated with RG differentiation in multiple brain regions, requiring several days after endogenous RG markers are first induced before becoming active. Direct examination of hGFAP promoter activity further illustrated this point: even though some expression can be detected in the E14.5 GE, significantly higher expression was observed in the cortex (Figure 4p,q). Moreover, weak ventral expression persisted as late as E16.5 (Figure 4r). This is the opposite of the endogenous RG markers BLBP and GLAST, which are more highly expressed ventrally at E14.5 and roughly equivalent at E16.5 [8] and further demonstrates that the hGFAP promoter does not accurately reflect normal characteristics of RG development in mice. Lastly, we note that previous work showed that all GE hGFAPα RG are also GLAST+ [5] and that at E14.5 (when GE hGFAP-mediated recombination first occurs), all BLBP+ RG are also GLAST+ [17]. Thus, hGFAP does not define a unique BLBP-GLAST- population of radial glia.

Discussion

In this study we have clarified and defined two major aspects of RG development and function. First, we have shown that cells with the molecular features of RG are present in the forebrain from E10.5, establishing that the NE→RG transition commences in this region between E9.5 and E10.5. Second, we have solidified the concept that RG are the primary neuronal progenitors in the developing brain by showing that: most neurons in the brain are derived from Glast+ RG; and in multiple brain regions, the heterologous hGFAP promoter is not active until several days after morphologically and molecularly defined RG first appear. Thus, the limited neuronal recombination driven by the hGFAP promoter is not indicative of regionally restricted potential as proposed [5,10,11] but rather a result of delayed hGFAP activity in certain RG populations.

As the recombination observed in Cre/loxP tracing reflects transcriptional activity of the promoter driving Cre, lineages can be traced from the time points when the molecular programs responsible for inducing promoter activity first become active. Results using endogenous mouse RG promoters such as Blbp and Glast therefore record lineages from the time when cells first become molecularly distinguishable as RG. The validity of this approach has been questioned by the suggestion that the presence of both molecular and morphological features are required in order to define cell types [11]. However, this idea does not take into account the fact that molecular changes necessarily precede and drive subsequent morphological
Glast+ radial glia generate the majority of neurons in the brain. (a-o) X-gal histochemical staining for β-gal activity (a,b,f,g,k,l) and immunofluorescent staining for NeuN (red) (c,e,h,j), β-galactosidase (green; d,e,i,j,n,o) and Calbindin (red) (m,o) in hGFAP::Cre;R26R (a,f,k) or Glast::Cre;R26R (b,e,g,j,l-o) adult mice. Regions immunofluorescently stained are striatum (c-e), thalamus (h-j) and cerebellum (m-o). In hGFAP::Cre;R26R mice, very few recombined neurons are present in the ventral telencephalon, diencephalon or midbrain, and cerebellar Purkinje neurons are unrecombined (a,f,k). PCL, Purkinje cell layer. In contrast, neuronal recombination was extensive throughout the brains of Glast::Cre;R26R mice (b,g,l) and double labeling confirmed that most neurons were recombined (c-e,h-j,m-o). The similarity of the Glast::Cre;R26R and Blbp::Cre;R26R lineage tracing results establishes that radial glia are the primary neuronal progenitors throughout the developing brain. Scale bars: 500 μm (a,b,f,g); 50 μm (k,l); 35 μm (m-o); 25 μm (c-e,h-j).
Figure 4 (see legend on next page)
therefore, induction of type [21,22] and time. Second, Notch signaling promotes the RG phenotype [23]; thus, two different RG genes are induced at the same time points.

Finally, mice lacking ErbB2 does not exclude a given identity during development since many morphological characteristics of differentiated cells appear only after the required molecular changes take place.

In addition to such reasoning, several lines of evidence support the idea that the NE→RG transition occurs in the forebrain by E10. First, expression of both Blbp and Glast becomes detectable in the forebrain by this time (Figure 2); thus, two different RG genes are induced at the same time. Second, Notch signaling promotes the RG phenotype [21,22] and Blbp is a direct Notch target gene [23]; therefore, induction of Blbp expression indicates that Notch-dependent RG differentiation has occurred. Finally, mice lacking ErbB2, a gene required for RG differentiation, begin showing impaired RC2 labeling in the telencephalon from as early as E9.5 [24] (some recombination restricted to the most rostral portion of the forebrain was detectable in E9.5 Blbp::Cre;R26R embryos [8]). Taken together, these data demonstrate that the molecular programs responsible for triggering RG differentiation are already active in some forebrain regions by E9.5 and induce broad expression of RG target genes by E10.5. They further suggest that an understanding of the mechanisms regulating the NE→RG transition will require analysis of molecular events occurring between these time points.

An important technical issue regarding Cre/loxP lineage tracing is that recombination efficiency is directly dependent upon cellular recombinase concentrations; this point must be considered when comparing results obtained using distinct transgenic or targeted lines. For instance, when four different promoters of varying strengths were used to drive Cre and compared in embryonic stem cells, recombination efficiency was directly correlated with promoter activity: the strongest promoter drove the highest levels of recombination [25]. Furthermore, even when the identical promoter is used, recombination efficiency can vary dramatically according to copy number. For example, merely doubling the copy number of a β-actin::Cre transgene (from one to two copies) resulted in a 10-fold increase in the number of recombined cells in the brain [26]. For these reasons, caution must be used when interpreting negative results in lineage tracing experiments as a lack of recombination could reflect weak promoter activity rather than restricted progenitor potential. Indeed, differences in copy number likely explain why subsequently generated hGFAP::Cre transgenic lines yielded higher levels of neuronal recombination [27] than observed using the original line [5,9]. It will be important to keep these issues in mind when comparing lineage tracing results in which the same genomic locus was either targeted (that is, knockin) or used as a transgene. Whereas knockins can, at most, provide two targeted alleles, tens to hundreds of copies are typical for transgenics [28]. Therefore, in many cases, transgenics may be more likely than knockins to drive recombination at the time when transcription commences and consequently should give a more accurate picture of the progeny derived from a given molecularly defined progenitor population.

Although the onset of Blbp and Glast transcription distinguishes RG from nestin+Blbp:Glast NE, the available evidence indicates that, as a population, Blbp+Glast+ RG themselves transit through multiple distinct developmental stages. For example, the hGFAP promoter does not drive any recombination in cortex prior to E12.5 (data not shown). As rodent Gfap is not normally expressed so early in RG neurogenesis, it is possible that the timing of hGFAP

Figure 4 (see previous page) The hGFAP promoter fails to drive recombination in fully morphologically differentiated radial glia (RG) of the ventral telencephalon and thalamus. Immunostaining for eGFP (green) (a,c,g,i,l,p,q) and GLAST (red) (d,f,h,k,l), X-gal staining for β-gal (m-o) and Cre ISH (r) on hGFAP::Cre;Z/EG (a-l), hGFAP::Cre:R26R (m–o,r) or hGFAP::eGFP (p,q) embryos, at E12.5 (a–l), E14.5 (m,n,p,q), E15.5 (o), and E16.5 (r). Panels (b,c,e,f) show cortex and GE in (a,d) at higher magnification; panels (h,i) show thalami in (g) at higher magnification, arrows in (g–i) mark identical spots in all three panels; panels (n,q) show higher magnification images of cortex and GE in (m,p). In contrast to the Blbp and Glast promoters, the hGFAP promoter is not active in forebrain prior to E12.5 (not shown). By E12.5, recombination is detected in cortical RG but absent from GE (a–c). thalamus (g–i) and cortical hem (j–l). Importantly, both BLBP (not shown) and GLAST (d–f,h,k,l) are highly expressed in these regions. Moreover, these unrecombined cells have molecular and morphological properties of fully differentiated RG cells (e.g. long radial processes coursing from VZ to pia). Even as late as E14.5, few GE RG are recombined (m,n), and significant recombination is not observed until E15.5 (o) when most RG in the region have completed neurogenesis. hGFAP promoter activity itself is not only delayed with respect to RG differentiation, but also weak ventrally compared to its dorsal activity (p,q,r). Scale bars: 300 μm (m,o,r); 200 μm (a,d,p); 100 μm (c,f,g,n,q); 60 μm (b,e,h,l).
activity in mice merely reflects the peculiarities of a heterologous promoter. However, it is alternatively possible that a fundamental transition occurring at E12.5 upregulates hGFAP activity; if so, the latter stage might represent the terminal phase of RG generation of layer Vb neurons as only about 30% of these neurons were recombined in hGFAP::Cre;R26R mice [5]. It is also well known that RG ultimately cease generating neurons and switch to production of glia; although several pathways have been implicated in promoting gliogenesis (for example, Jak-STAT signaling [29] and NFI genes [30]), a comprehensive description of the mechanisms involved is lacking and requires transcriptional profiling and comparisons between neurogenic and gliogenic RG. Steps in this direction have been taken by transcriptome analyses of FACS purified RG whose neurogenic potential was determined by in vitro clonal analyses [31]. However, given that progenitor neurogenic potential in vivo can differ from that observed in vitro [32], subsequent studies might yield additional insights by defining potential on the basis of either in vivo transplantation or genetic lineage tracing. Accordingly, our lineage tracing data provide direct evidence that hGFAP promoter activity commences in ventral telencephalic RG only after neurogenesis is largely completed and thus distinguishes a later Blbp::Glast::hGFAP–gliogenic stage from an earlier Blbp::Glast::hGFAP–neurogenic stage in this region. This is significant as it enables direct in vivo comparisons of neurogenic and gliogenic ventral telencephalic RG via FACS purification and microarray analyses using hGFAP::eGFP and Blbp::eGFP or Glast::eGFP transgenic mice. Such studies should facilitate efforts to more thoroughly determine the molecular mechanisms that control progenitor potential and fate specification.

Conclusion
These data establish the major neurogenic role of RG in the developing CNS and genetically distinguish an early neurogenic Blbp::Glast::hGFAP–stage from a later gliogenic Blbp::Glast::hGFAP–stage in the ventral telencephalon.

Materials and methods
Mice and BAC transgenesis
All protocols were approved by the Rockefeller University IACUC. Glast::Cre BAC transgenic mice were generated using homologous recombination to insert a Cre-SV40polyA cassette at the site of the endogenous ATG (exon II, BAC clone RP23-63O21). Transgenic FVB/N mice were generated using standard procedures [28]. R26RlacZ, Z/EG, hGFAP::Cre, and hGFAP::eGFP mice were obtained from JAX (Bar Harbor, Maine, USA). Glast::eGFP mice were obtained from GENSAT (New York, NY, USA). The BAC clone used to generate Glast::eGFP mice is the same as that used for the Glast::Cre mice and the eGFP-polyA cassette was targeted to the identical locus in the BAC as the Cre-polyA cassette. Analysis of endogenous gene expression was done using C57BL/6J (JAX).

Tissue processing and analysis
Tissues were either immersion fixed (embryos) or perfused (postnatal/adult) with 4% paraformaldehyde, cryoprotected in 15% sucrose in phosphate-buffered saline (PBS), and cut on a cryostat (15 μm) or vibratome (75 μm). Cryosections were dried at room temperature for 2 hours, a step that improved signal (insufficient drying was responsible for the previous inability to detect BLBP protein in the E10.5 forebrain [8]). Primary antibodies used were: rabbit α-BLB (1: 1500); rabbit α-β gal (1:500; ICN Santa Ana, CA, USA)); mouse α-Calbindin (1:500; Swant, Bellinzona (Switzerland)); rabbit α-Cre (1:500 and TSA amplified; Novagen, San Diego, CA, USA); goat α-GFP (1:500; US Biological, Swamscott, MA, USA); guinea pig α-GLAST (1:5000; Millipore, Billerica, MA, USA); mouse α-NeuN (1:200; Millipore, Billerica, MA, USA); rabbit α-phosphorylated histone H3 (PH3, 1:250; Millipore, Billerica, MA, USA). Alexa 488-coupled secondary antibodies were obtained from Molecular Probes, and Cy3-coupled secondary antibodies were from Jackson Immunoresearch (West Grove, PA, USA); both were used at 1:750. Sections were preblocked in 5% donkey serum, 0.1% Triton X-100 in PBS and incubated with primary antibodies overnight at 4 °C. After washing, sections were incubated with secondary antibodies (generated in donkey) for 2 hours in the same solution at room temperature. Confocal imaging was done on an LSM 510 Axioplan (Zeiss). For X-gal histochemical staining, tissues were incubated at 37 °C in X-Gal solution: 1 mg/ml X-gal (Sigma, St. Louis, MO, USA), 20 mM K3Fe(CN)6, 20 mM K4Fe(CN)6, 2 mM MgCl2 and 0.02% NP-40 in PBS.

Non-radioactive ISH
Riboprobes to detect Cre mRNA were generated using T7 polymerase off PCR amplified template DNA generated with the following primers: forward, CAAGCTCGAAITACCTACTAAAAGGtccattact-gacctaccc; reverse, AGAGACGGGTAAATACGACTCACA-TATAGGGCctaatcgccatcttccagcag. Probe hybridization and development was done using standard protocols.

Abbreviations
BAC: bacterial artificial chromosome; BLBP: brain lipid binding protein; CNS: central nervous system; E: embryonic day; eGFP: enhanced green fluorescent protein; GLAST: glial high affinity glutamate transporter; hGFAP: human glial fibrillary acidic protein; ISH: in situ hybridization; NE: neuroepithelial stem cell; PBS: phosphate-buffered saline; PH3: phosphorylated histone H3; RG: radial glia; VZ: ventricular zone.
**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

TEA performed all experiments and wrote the manuscript with input from NH.

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