

generated), subsequently declining to 45 ± 3 cells/100 μm at P15 (that is, just after the end of neuronogenesis) and even less at P18 (Figure 4D). Remarkably, this time course mirrored that of murine Tbr2⁺ cells (Figure 4E), which, however, were more numerous and reached their peak linear density slightly later, close to the end of neuronogenesis. Finally, as for radial distribution, modal distance between Tbr2⁺ cells and ventricular surface varied in the opossum from about 120 to 140 μm at P1 to P8, to 60 μm at P15. In synthesis, Tbr2⁺ cells were detectable throughout the neuronogenetic window, prevalently clustered around the border between the VZ and IZ, but also scattered within the VZ, exactly like placental basal progenitors [33] (Figure 4F).

So, based on pH3 data, a basal proliferative compartment does not seem to exist within the neuronogenic neocortical field (the frequency of abventricular mitoses starts to increase just around the end of neuronogenesis). Based on Tbr2 expression, a compartment molecularly similar to the basal one of placentals can be distinguished, interposed between ventricular precursors and abventricular neurons. How to solve this conundrum? Rather than being due to the absence of proliferative activity, the paucity of abventricular pH3⁺ cells might reflect a very slow cell cycle progression in front of a short duration of M phase [34,35] (our unpublished observations), possibly leading to an underestimation of proliferative population. To rule out this possibility, we looked for presumptive basal cells in S phase, which would reasonably be several times more frequent than the corresponding M phase ones [34,35]. So, we pulsed P4 to P10 opossum pups with BrdU, fixed their brains and looked for cortical Tbr2⁺ cells also immunoreactive for BrdU. Such cells were extremely rare, ($N_{(\text{Tbr2}^+ + \text{BrdU}^+)}/N_{(\text{Tbr2}^+)} = 0$ at P4, 0 at P6, $3.3 \pm 1.6 \times 10^{-3}$ at P10), and the vast majority of Tbr2⁺ cells lay above the BrdU⁺ ones (Additional file 3A). Moreover, as expected, Tbr2⁺ cells were even more rarely positive for the mitotic pH3 marker ($N_{(\text{Tbr2}^+ + \text{pH3}^+)}/N_{(\text{Tbr2}^+)} = 0$ at P4, 0 at P6, $4.4 \pm 2.9 \times 10^{-4}$ at P10, $5.4 \pm 2.7 \times 10^{-4}$ at P14; Additional file 3B). These data strongly suggest that, in marsupials, Tbr2⁺ cells are not proper basal progenitors, but mainly represent a subventricular postmitotic compartment, interposed between intermitotic apical progenitors and postmitotic abventricular neurons.

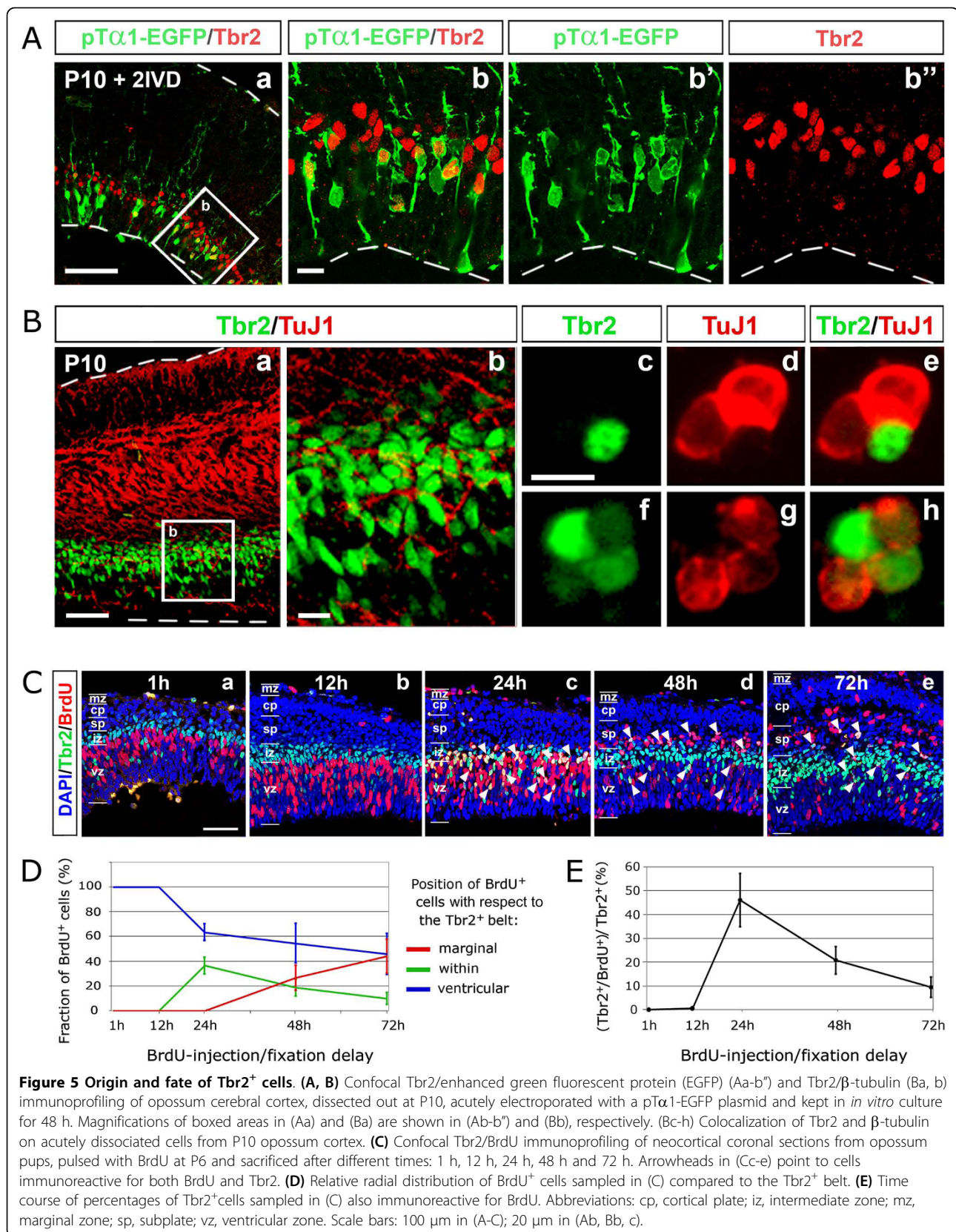
To corroborate this interpretation, we further investigated the origin and fate of Tbr2⁺ cells. In rodents, Tbr2⁺ cells are generated by a subset of neuronally committed apical progenitors, called 'pin-like' cells or short neural precursors, which, facing the ventricular cavity and not contacting the pial surface, can be distinguished from radial glial cells for specific firing of the α 1-tubulin promoter (pT α 1⁺ cells) [36,37]. We electroporated a pT α 1-enhanced green fluorescent protein (EGFP) construct into the P10 opossum cortex and fixed

electroporated brains 2 days later, after a final pulse (60 minutes) of BrdU. After verifying their vitality and cytoarchitectonic integrity (by scoring radial distribution of BrdU⁺ and Tbr2⁺ cells; Additional file 4), we finally looked for GFP⁺/Tbr2⁺ cells (Figure 5A). We found many of them (Figure 5Ab-b"), confirming that opossum Tbr2⁺ cells derive from apical progenitors, as in rodents. We then compared the distribution of Tbr2 and the neuronal marker β -tubulin on coronal slices of P10 opossum brains (Figure 5B). Tbr2⁺ cells lay below the main β -tubulin domain, but a substantial overlap between the two antigens was detectable as well (Figure 5Ba,b): in particular, around 30% of acutely dissociated neocortical cells expressing Tbr2 were also positive for β -tubulin (Figure 5Bc-h), consistent with the hypothesis that Tbr2 is transiently expressed before the activation of neuron-specific markers.

Finally, to get a better temporal resolution of the developmental process under examination, we pulsed P6 pups with BrdU, fixed their brains after different times (from 6 h up to 72 h), and immunoprofiled BrdU⁺ cells, performing time-course analysis of their radial distribution (Figure 5C, D). Comparing profiles obtained at different times, BrdU⁺ cells seemed to move along a wave, from the ventricular side towards the marginal aspect of the cortical wall. In particular, up to 12 h, all BrdU⁺ cells lay deep to the Tbr2⁺ belt (Figure 5Cb); starting from 24 h, some of them were detectable within this belt (Figure 5Cc); at 48 h, about one-third of them were above it (Figure 5Cd); finally, at 72 h, most had overcome the Tbr2⁺ belt, so that only a few remained near the ventricle (Figure 5Ce). Remarkably, the percentage of Tbr2 cells also immunoreactive for BrdU displayed a biphasic trend, arising from about 0% at 12 h up 46% at 24 h and subsequently declining to less than 10% at 3 days (Figure 5E). In synthesis, neural progenitors exiting the cell cycle leave the VZ and activate Tbr2 18 ± 6 h after the last DNA synthesis; 1 day later, the same cells massively move to more marginal positions, where they downregulate Tbr2, while activating neuron-specific markers (Figure 5Bc-h).

The apical proliferative compartment and its dynamics

Previous results indicate that the apical progenitor compartment is the place where pallial projection neurons are generated. To reconstruct the dynamics of this compartment, we performed time-course analysis of its hallmark, Pax6 [33]. As in placentals, this homeoprotein was specifically expressed in the pallial VZ throughout neurogenesis (Figure 6A). On 10- μm -thick sections, linear frequencies of Pax6⁺ cells were 160 ± 16 cells/100 μm at P4, 132 ± 4 cells/100 μm at P8, 92 ± 8 cells/100 μm at P10, and 66 ± 11 cells/100 μm at P15 (Figure 6B). These values were slightly lower than the corresponding mouse



ones (185 ± 16 cells/100 μm at embryonic day (E)12.5, 156 ± 13 cells/100 μm at E14.5, and 96 ± 13 cells/100 μm at E16.5; data not shown), although the two temporal progressions were basically very similar. These results suggests that decreased neuronal density and reduced thickness of the opossum cortex [38], rather than reflecting a change of the apical proliferative compartment size, mainly originates from the absence of a basal, transient amplifying population.

Moreover, in placental neocortex, the Pax6⁺ compartment is not homogeneous. It includes neural stem cells with the morphology of radial glial cells and pT α 1⁺ neuronally committed progenitors, having lost their contact with the pial surface. It is noteworthy that, upon electroporation of the pT α 1-EGFP transgene into the opossum cortex, fluorescent cells displayed morphology of proper radial glial cells, extending from the ventricular edge to the pial surface (Figure 6Ca). Moreover, despite the time elapsed after electroporation, pial processes and end-feet of many of these cells were still not immunoreactive for neuron-specific β -tubulin, ruling out that they were neurons (Figure 6Cb-b"). This means that, in marsupials, either pT α 1 fires in radial glial cells, or retraction of the pial process by neuronally committed progenitors is delayed compared to rodents.

Distribution and generation of cortical GABAergic cells

In the mouse, GABAergic neurons are generated within ventral forebrain and reach the cortex by tangential migration, according to a well characterized spatio-temporal pattern [39,40]. First, interneurons enter the cortex at around E12.5, and the migratory wave reaches the mid-neocortex at E13.5 and then the hippocampus by E15.5. Early migration is mainly superficial; by E13.5 a robust periventricular migratory route appears, becoming predominant at later stages. In order to establish developmental correspondences between mouse and opossum, we systematically scored the distribution of GABA immunoreactive cells in the developing postnatal telencephalon of the marsupial. From P1 up to P14, a huge number of GABA⁺ cells were detected within the ventral telencephalon. Here, they were early confined to abventricular layers, and later also detectable near the ventricle (Figure 7Aa-d). Cortical distribution was more complex. At P1 only a diffuse and light immunoreactivity was detectable throughout the marginal pallium (Figure 7Aa'), and rare GABA⁺ somata were localized in presumptive paleocortex (Figure 7Aa and data not shown). At P4 a substantial number of GABA⁺ cells were present in the abventricular half of both paleo- and neocortex, but not in the archicortex

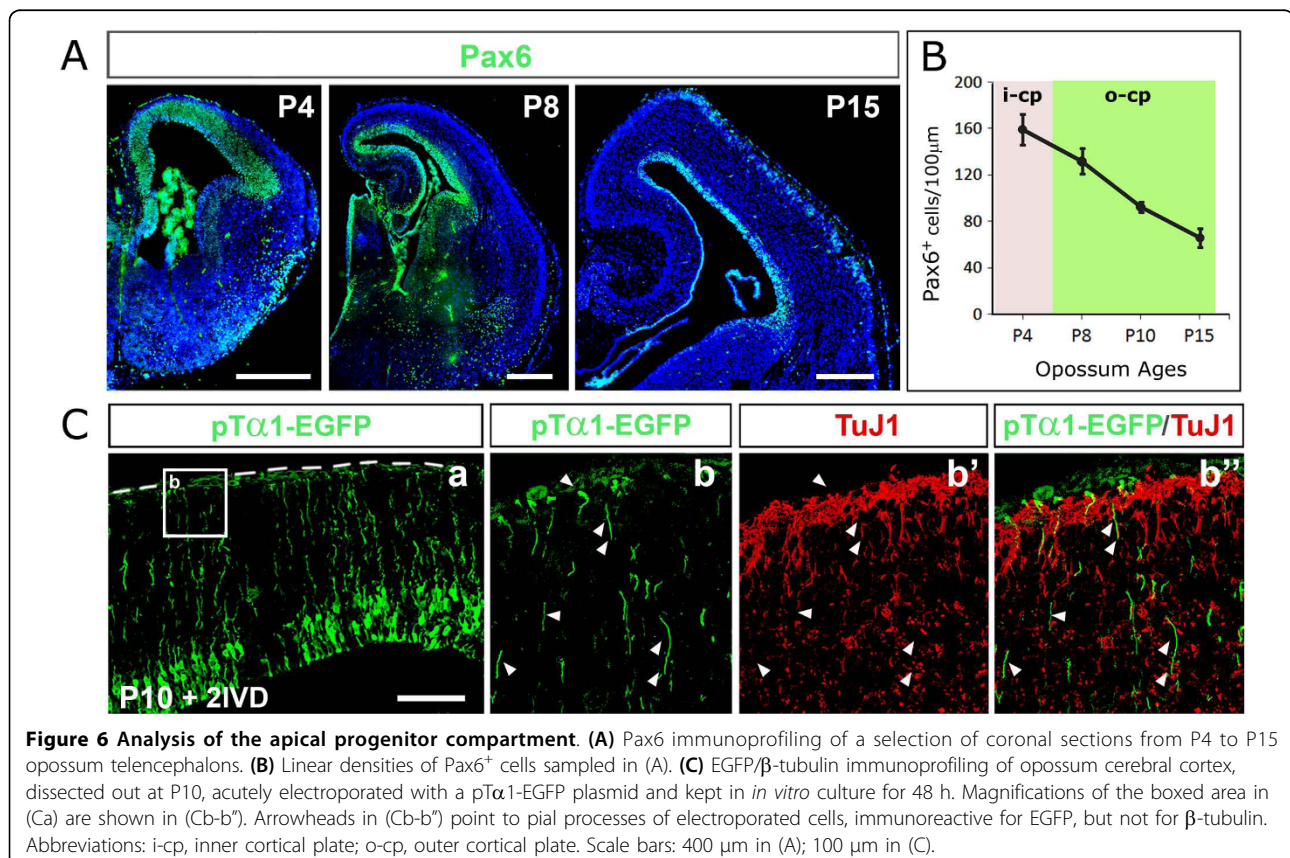
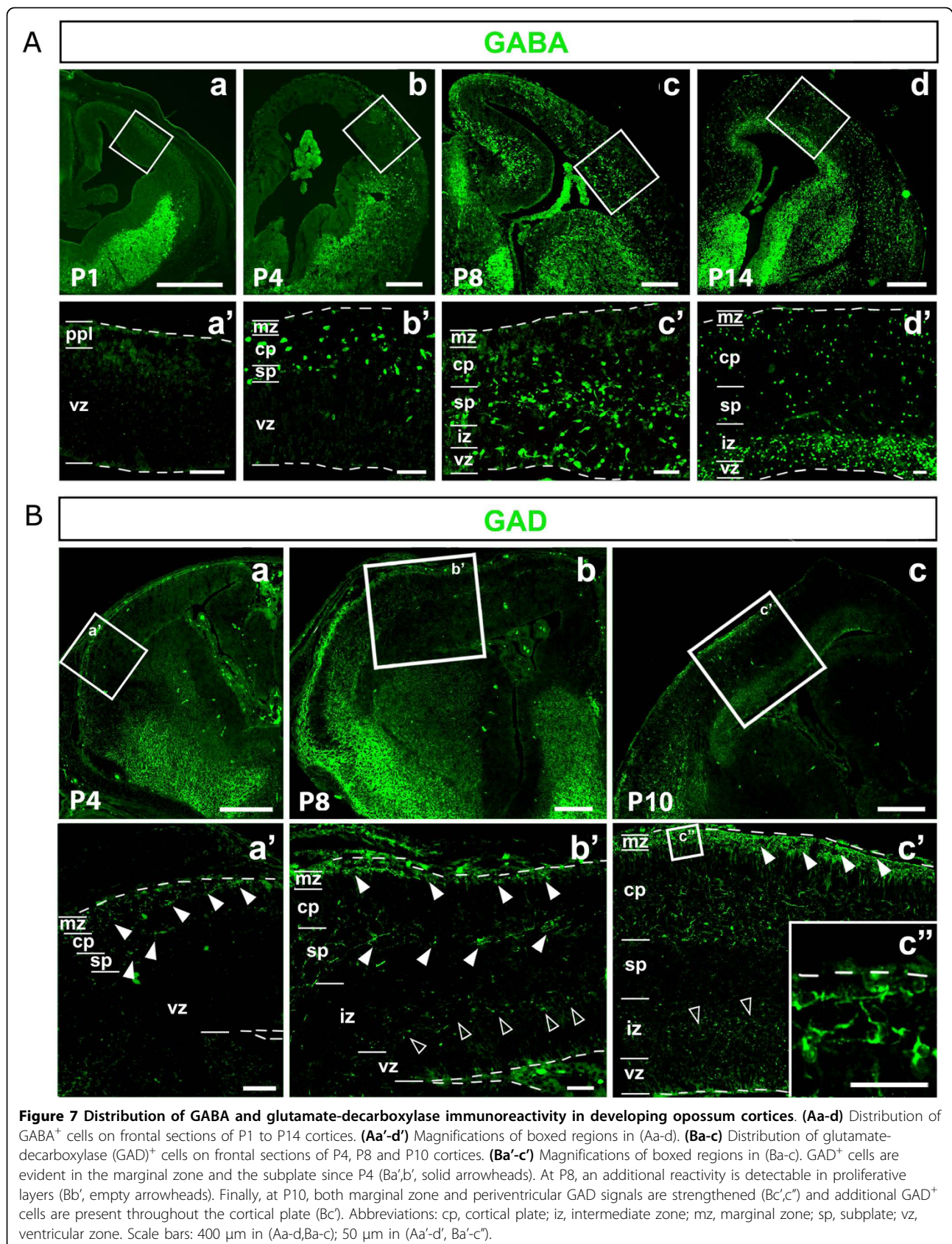


Figure 6 Analysis of the apical progenitor compartment. (A) Pax6 immunoprofiling of a selection of coronal sections from P4 to P15 opossum telencephalons. **(B)** Linear densities of Pax6⁺ cells sampled in (A). **(C)** EGFP/ β -tubulin immunoprofiling of opossum cerebral cortex, dissected out at P10, acutely electroporated with a pT α 1-EGFP plasmid and kept in *in vitro* culture for 48 h. Magnifications of the boxed area in (Ca) are shown in (Cb-b"). Arrowheads in (Cb-b") point to pial processes of electroporated cells, immunoreactive for EGFP, but not for β -tubulin. Abbreviations: i-cp, inner cortical plate; o-cp, outer cortical plate. Scale bars: 400 μm in (A); 100 μm in (C).



(Figure 7Ab,b'). At P8, adventricular GABA⁺ cells were present throughout the cortex, including the marginal hippocampus; ventricular GABA⁺ cells were conversely limited to paleo- and neocortex (Figure 7Ac,c'). Finally, at P14, GABA⁺ cells were detectable throughout the cortex, clustered in a narrow periventricular belt and more loosely distributed elsewhere (Figure 7Ad,d'). Remarkably, the spatio-temporal distribution of glutamate-decarboxylases 65 and 67 (GAD65 and GAD67) was consistent with the GABA pattern (Figure 7B).

The apparently higher frequency of GABA⁺ cells within the opossum cortical VZ, compared to the mouse, suggested that, in marsupials, at least some of these cells might be locally generated. To test this hypothesis, we assayed the capability of early cortical tissue, not containing any GABA⁺ cells and isolated from ventral forebrain, to generate such cells *in vitro*. We dissected out cerebral cortices from P1 opossum brains, cultured them *in vitro* for 8 days in the presence of saturating iododeoxyuridine (IdU), exposed then to a final pulse of BrdU before fixation (90 minutes), and finally immunoprofiled them for GABA, IdU and BrdU (Additional file 5Aa). We found cells expressing the three antigens in different combinations. Numerous BrdU⁺ cells were detectable, suggesting that, before fixation, cultures were still healthy (Additional file 5Ab). Among GABA⁺ cells, some were IdU⁻ (Additional file 5Ac-c", empty arrowheads). These cells were reasonably generated before the dissection and acquired their mature GABA⁺ phenotype in culture. However, a large fraction of GABA⁺ cells was also IdU⁺ (Additional file 5Ac-c", solid arrowheads), suggesting that they could have been locally generated by cortical progenitors that underwent their final DNA synthesis during *in vitro* culturing.

To confirm such a conclusion, we re-addressed this issue *in vivo*. We pulsed P5 pups with BrdU, fixed their brains 24 h later and finally looked for neocortical BrdU⁺/GAD⁺ or BrdU⁺/GABA⁺ cells as an index of local interneuron generation. We reasoned that 24 h should be a sufficiently long time delay to allow newborn neurons of the GABAergic lineage to switch robust GAD and GABA immunoreactivities on and sufficiently short to avoid ventrally generated interneurons entering the cortex. As expected, we found many BrdU⁺/GAD⁺ or BrdU⁺/GABA⁺ cells in basal ganglia. However, only very few of them were detectable in neocortex (Additional file 5B). All this suggests that, in marsupials, as in rodents, most interneurons are generated outside the cortex, in the basal telencephalon. The relatively high frequency of IdU⁺/GABA⁺ cells we found in cortical explants may have two explanations. The few interneurons generated in cultured cortical explants accumulated over as many as 8 days and, due to experimental detachment of cortex from basal ganglia,

were poorly diluted by those coming from the subpallium. Moreover, in a large fraction of cases, IdU was probably not uptaken by GABAergic-lineage pallial precursors during pre-neuronogenic DNA synthesis, but by progenies of subpallial precursors (already present in the cortical explant at the time of its removal), during post-mitotic DNA repair. These two mechanisms are not mutually exclusive and may largely account for apparent inconsistencies between the results of the *in vitro* and *in vivo* approaches.

Post-neuronal histogenesis: generation of astrocytes and oligodendrocytes

In rodents, gliogenesis follows neuronogenesis and that happens in the developing opossum cortex as well. To assess astrocytogenesis progression, we looked at the distribution of the Glial fibrillary acid protein antigen (Gfap) from P8 (that is, the middle of the neuronogenic window) onward. A periventricular signal detected from the beginning was restricted to hippocampus at P8 (Additional file 6Aa), and spread throughout the cortical field at P12 (Additional file 6Ab). This signal presumptively corresponds to the soma of radial glial cells, which, in primates, share this marker with true astrocytes [41,42]. An adventricular Gfap signal associated with cells with the morphology of astrocytes could be found only later, starting from P18 to P20 (data not shown; Additional file 6Ac-f). These cells were detectable in the MZ, the grey matter and white matter. A subset of them coexpressed the astrocyte marker S100 β (Additional file 6Ag-i").

The differentiation of oligodendrocytes was studied by monitoring immunoreactivity for the marker O4 [43]. A strong signal was found at both analyzed ages, P40 and P60, restricted to white matter, internal capsule and hippocampal commissure (Additional file 6Ba,b; *M. domestica* has no corpus callosum [44]).

Discussion

In the present study we found that the cortico-cerebral neuronal complement is generated in the opossum pup between P1 and P16, molecular diversification of neurons belonging to distinct laminae largely resembles that of placentals, and migration of cortical plate neurons follows the 'inside-out' rule. We demonstrated that opossum neocortical projection neurons are almost entirely generated by apical progenitors, and that Tbr2, the hallmark of placental basal progenitors, is only transiently expressed by opossum post-mitotic elements, prior to the activation of neuron-specific genes. Moreover, we showed that such absence of a basal transient amplifying population is the main reason for the reduced thickness and decreased neuronal density, distinguishing the marsupial from the placental cortex

[38,45] despite conserved intracolumnar upper/lower layer neuron ratios [19]. As for GABAergic neurons, we found that whereas many are born in the subpallium, as in rodents [39,40], and invade the cortex by P4, a small fraction of them is also generated within cortical periventricular layers. Finally, as in placentals, cortical histogenesis continues with astrocytogenesis (from P18 onward) and ends up with oligodendrocytogenesis (around P40 and later).

We found that molecular diversification of the cortical plate is highly conserved between marsupials and placentals, with *Foxp2* and *Tle4* restricted to deeper layers and *Brn1* and *Cux1* mainly confined to upper layers. This suggests that the neocortical hexalaminar profile arose before the branching between these two subclasses, about 180 My ago [46]. Minor differences were observed in derivatives of PPL, namely the phylogenetically most ancient component of our cortex [47]. Based on Calretinin immunostaining, the opossum SP appeared quite prominent, as previously assessed by simple histological inspection [8], far thicker than in rodents [48]. Cells expressing *Reln*, a hallmark of Cajal-Retzius neurons, were detectable beneath the pia mater, in both neo- and archicortex, like in placentals. However, their appearance did not predate the splitting of the preplate, as happens in rodents [48]. We reconstructed rules governing marsupial neocortical lamination by following two complementary experimental approaches. First, we compared the mature distribution of neocortical laminar markers with the radial settling profile of neurons generated at different developmental times, as assessed by BrdU pulse-chase analysis. Second, we performed systematic time-course expression analysis of a selection of these markers, from their appearance to the end of the lamination process. Both approaches indicated that, in *M. domestica*, radial neuronal migration takes place in a way similar to placentals. Like in rodents [27-29], such a process is reasonably promoted by the glycoprotein Reelin, released by Cajal-Retzius cells and, later, by some CP neurons. This protein seems, however, to be dispensable for PPL splitting, which apparently occurs in the absence of detectable expression of its mRNA.

Beyond the study of laminar differentiation and radial migration, we paid special attention to the origin of cortical neurons, both glutamatergic and GABAergic. In placentals, glutamatergic neurons are generated within the dorsal telencephalon by two periventricular proliferative compartments, the apical and the basal, the former confined to the VZ, the latter mainly lying in the subventricular zone [17,49]. The existence of a basal compartment in the opossum was controversial [18]. A recent study reports that such a compartment is actually present in developing marsupial brains, suggesting that it emerged in the eutherian-metatherian lineage [19]. In

the opossum, however, basal proliferative activity arises only around the end of cortical neuronogenesis (Figure 3B) and thus contributes little to the generation of the neocortical neuronal complement. We reanalyzed this topic by an integrated approach, including scoring of pH3⁺ mitoses and Tbr2⁺ cells, immunoprofiling of acutely administered BrdU and pTα1-driven EGFP, as well as BrdU pulse-chase analysis. We found that, in the opossum, cells expressing Tbr2 do not form a transient amplifying population, but represent a postmitotic transitional compartment, passed through by neuroblasts in the process of switching Pax6 off and activating neuron-specific β-tubulin. Interestingly, among sporadic abventricular pH3⁺ precursors detectable in the cortex at P14, more than four-fifths do not express Tbr2 (Additional file 3B), suggesting that such precursors may belong not to the neuronogenic but to the gliogenic lineage. This scenario is not surprising. Tbr2 has been detected in the developing pallium of other vertebrates missing a basal proliferative compartment, such as Anamnia and birds [50-52]. Moreover, besides corticogenesis, Tbr2 is also expressed in the gastrulating embryo, where it promotes epithelium-to-mesenchyme transition [53]. So this transcription factor, rather than specifying basal progenitor identity in the developing cortex, might play a more general morphogenetic role, inducing apical precursors to leave embryonic epithelia they belong to [16].

Anyway, it has to be recalled that some abventricular pH3⁺ mitoses can be found in the lateral-most opossum pallium. It is tempting to speculate that such basal proliferative activity might increase the final neuronal output of the small ventricular sector in between neopallial and striatal fields, in charge of generating paleocortex and other latero-ventral derivatives of the amniote telencephalon [54].

Time-course GABA/GAD immunoprofiling of the developing cortex, immuno-characterization of early cortical explants as well as *in vivo* short-term BrdU pulse-chase of GABAergic neurons showed that generation of cortical interneurons in the opossum is mainly confined to ventral telencephalon, like in other Amniota [20-22]. Actually, there is some local cortical interneurogenesis; however, the amplitude of this phenomenon is modest as in rodents [55], far smaller than in primates [56-58].

Conclusions

This study provides three main results on the developing opossum cortex. First, projection neurons and interneurons are generated by pallial and subpallial precursors, respectively, as in eutherians. Second, a neocortex with a eutherian-like molecular profile is laid down, following the inside-out rule, suggesting that the hexalaminar blueprint emerged prior to eutherian-metatherian branching.

Third, differently from eutherians, neocortical projection neurons are almost entirely generated by apical neural precursors and basal progenitors hardly contribute to their genesis; *Tbr2*, the hallmark of eutherian basal progenitors, is transiently expressed by postmitotic progenies of apical precursors prior to the activation of more mature neuronal markers.

Based on temporal progression of the morphogenetic subroutines that were the subject of analysis, a comparative opossum/mouse cortical developmental timetable is sketched in Figure 8.

Materials and methods

Animals

Opossums (*M. domestica*) at different postnatal ages were obtained from the colony maintained at the animal house facility of the University of Trieste, Italy. Mice (*Mus musculus*, strain CD1) were purchased from Harlan (San Pietro al Natisone, UD, Italy). Opossums were staged by systematic daily inspection of the colony for newborn litters, P0 corresponding to the day of birth. Mice were staged by timed breeding and vaginal plug inspection. Animal handling and subsequent procedures were in accordance with European laws (European Communities Council Directive of November 24, 1986 (86/609/EEC)) and with National Institutes of Health

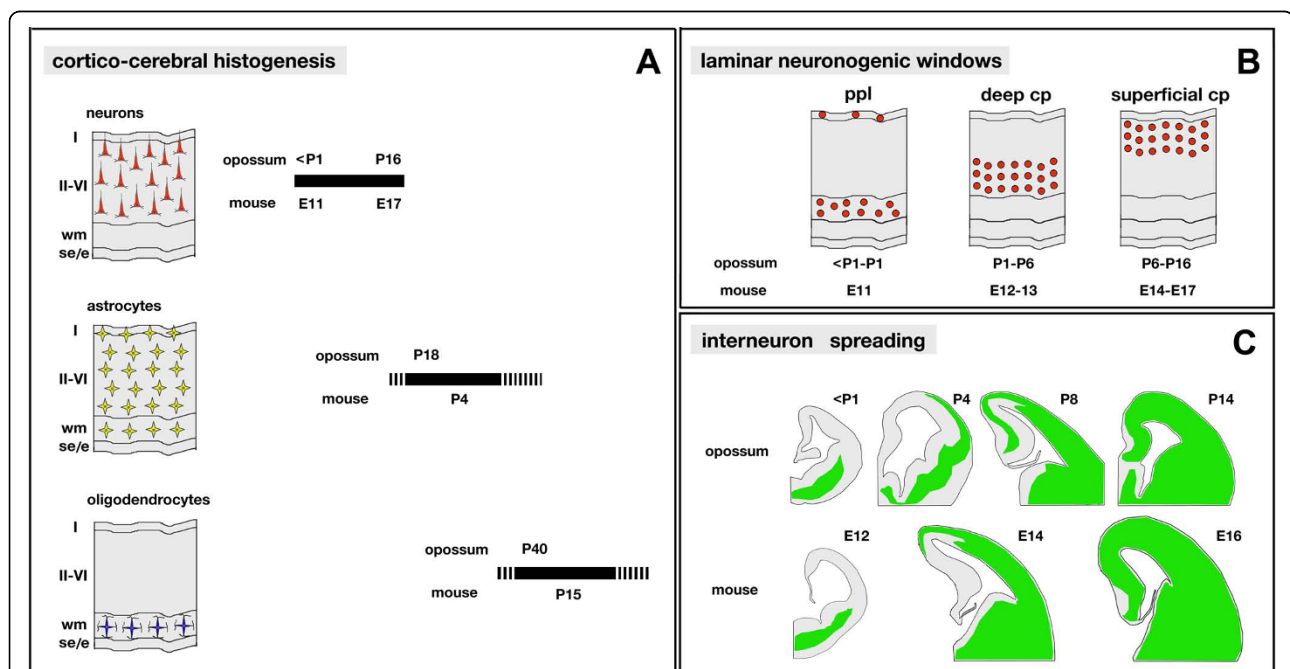
guidelines. In particular, to harvest opossum brains (from P1 to P40), young animals were killed by decapitation, after hypothermia. When the entire CNS had to be recovered, the animals were alternatively killed by aorta resection, again after hypothermia. In the case of P60 animals, they were terminally anesthetized by urethane and transcardially perfused with 4% paraformaldehyde. Mouse embryos (E10.5 to E18.5) were harvested from pregnant dames killed by cervical dislocation.

Bromodeoxyuridine administration

BrdU was administered to P1 to P18 opossum pups at a dose of 200 µg/g body weight in 0.9% NaCl by subcutaneous injections. During administration, pups were left attached to the mother, previously anesthetized with isoflurane. All injected animals were sacrificed at the age of P30 and their brains used for kinetic studies.

Organotypic cultures

Two kinds of organotypic brain cultures were used for this study, adherent and floating. Adherent cultures were set up according to the classical Stoppini method [59], with minor modifications. Briefly, P1 opossum neocortices and basal ganglia were dissected, placed on distinct falcon cyclopore PET membranes and kept at the atmosphere-medium interface as follows. After priming



(1 h at 32°C in DMEM-F12-Glutamax, 10% FBS/N2, 0.6% glucose, under 95% air/5% CO₂ atmosphere), membranes were transferred into 12-multiwell plates, each well containing 1.2 ml of Neurobasal-B57-Glutamax, 0.6% glucose (supplemented with Fungizone/Pen/Strept mix), and kept for 8 days at 32°C at the interface between medium and a 95% air/5% CO₂ atmosphere. For floating cultures, P10 opossum CNSs were dissected and repeatedly washed in DMEM-F12-Glutamax/0.6% glucose/Fungizone/Pen/Strept, paying particular attention to avoid any damage. After further manipulations (DNA injection and electroporation), CNSs were transferred to 50 ml Falcon tubes (one per tube), each containing 15 ml of DMEM-F12-Glutamax/0.6% glucose/N2, 1%FBS/Fungizone/Pen/Strept. Cultures were maintained at 32°C for two days, in the presence of bubbling 95%O₂/5%CO₂. In cases of both adherent and floating cultures, to verify brain vitality, a final pulse of BrdU (5 µg/ml of medium) was given 90 minutes before explant fixation.

Brain electroporation

Electroporation was performed on acutely dissected P10 opossum CNSs, put in a petri dish filled with 0.6% glucose, 1× PBS. An aqueous 1 µg/µl solution of plasmid DNA (1 µl; containing 0.01% fast green dye) was injected into a lateral ventricle using glass capillaries 1B100-3 (World Precision Instruments, Sarasota, FL, USA) prepared using the micropipette puller P-97 (Sutter Instrument Company, Novato, CA, USA). Two parallel, 5-cm spaced, rectangular electrode plates (4 cm × 6 cm) were placed on both sides of the telencephalon and three 100 V pulses (each 100 ms long; interval between consecutive pulses 450 ms) were delivered using an electro-square-porator (BTX 830). Plasmids pTα1-EGFP (kindly provided by E Ruthazer) and pDsRed2-N1 (Clontech, Mountain View, CA, USA) were used for electroporation.

Histology

CNS specimens (both mouse and opossum, whole brains and explants) were fixed in 4% paraformaldehyde-PBS overnight at 4°C, cryoprotected in 30% sucrose/PBS and cut coronally at 10 µm. Cryosections were mounted on Fischer SuperFrost Plus slides, and subsequently processed for *in situ* hybridization or immunohistochemistry.

To accurately determine the fraction of Tbr2⁺ cells also expressing β-tubulin, six freshly dissected P10 opossum cortices were pooled and dissociated to single cells by gentle trituration. Cells were resuspended in DMEM-F12, 1% serum, plated onto slides previously covered with 20 µg/ml poly-D-lysine, and left to attach for 1 h at room temperature. Slides were processed for immunofluorescence, as described elsewhere.

Immunofluorescence

Immunofluorescence was performed as previously described [60], with minor modifications. Generally, prior to use, sections were boiled for 5 minutes in 10 mM pH6 citrate buffer. This step was omitted in the case of β-tubulin, β-tubulin/GFP, GAD and GABA stainings. In the case of BrdU detection, sections were also treated for DNA depurination (2 M HCl, for 15 minutes at room temperature) and then neutralized (in 0.1 M borate buffer, pH 8.5, for 15 minutes at room temperature). For other combined immunofluorescences (BrdU/Tle4, BrdU/Cux1, BrdU/Tbr2, IdU/GABA, BrdU/GAD and BrdU/GABA), the HCl concentration was reduced to 0.2 M. Sections were incubated for 1 h at room temperature under blocking mix (1× PBS, 10% FBS, 1 mg/ml bovine serum albumin, 0.1% Triton X100) and then incubated at 4°C overnight with primary antibody in blocking mix.

Primary antibodies used were as follows: mouse monoclonal anti-BrdU (clone B44, Becton Dickinson, Franklin Lakes, New Jersey, USA), 1:50; rat anti-BrdU (clone ICR1, Abcam, Cambridge, UK), 1:500; goat anti-Brn1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), 1:30; mouse monoclonal anti-Calretinin (clone M7245, Dako, Glostrup, Denmark), 1:50; rabbit anti-Cux1 (Santa Cruz), 1:30; rabbit anti-Foxp2 (Abcam), 1:1,500; rabbit anti-Gaba (Sigma-Aldrich Corp., St. Louis, MO, USA), 1:8,000; rabbit anti-GAD (Sigma), 1:1,000; rabbit anti-Gfap (Dako), 1:500; chicken anti-GFP (Abcam), 1:800; mouse monoclonal anti-O4 (clone O4, R&D Systems Inc., Minneapolis, MN, USA), 1:600; rabbit anti-Pax6 (Abcam); 1:300; rabbit anti-pH3 (Upstate Biotechnology Inc, Lake Placid, NY, USA), 1:600; mouse anti-pH3 (Cell Signaling Technologies Inc, Danvers, MA, USA), 1:100 (limited to pH3/Tbr2 colocalization assays); rabbit anti-S100β (Dako), 1:200; rabbit anti-Tbr1 (kindly provided by Robert Hevner), rabbit anti-Tbr2 (Abcam), 1:500; rabbit anti-Tle4 (Santa Cruz), 1:30; mouse anti-neuron-specific class III β-tubulin (clone Tuj1, Covance, Emeryville, CA, USA), 1:500.

Finally, immunoreactivity was revealed after 2-h incubation with secondary Alexa antibodies, 488 and 594 (Molecular Probes, Emeryville, CA, USA), 1:400.

In situ hybridization

Non-radioactive *in situ* hybridization was performed as previously described [61], with minor modifications. The probe used corresponded to exons 1 to 12 of the *M. domestica Reelin (Reln)* coding region (Ensembl mdo-chr.8: nt 155064879-154744337).

Imaging and confocal microscopy

Fluorescent labeled sections were imaged and analyzed using a fluorescent Nikon (Tokyo, Japan) Eclipse 80i

microscope and a DS-2MBWC digital microscope camera. Confocal photos were taken by a TCS SP2 Leica confocal microscope; they were generally collected as 1.0- μm -thick Z-stacks, and as 3.0- μm -thick Z-stacks in the case of pT α 1-EGFP electroporated cells. All images were processed using Adobe Photoshop CS3 software.

Sample sizing

Unless otherwise stated, each experiment was performed at least in triplicate. In cases of laminar birthdating (Figure 3), per each BrdU pulsing time, three mid-frontal 800- μm -wide neocortical sectors, from four cortices were profiled. pH3⁺ cell counting was performed in similarly, but throughout the neocortical field (Figure 4A, B). The same applies to Tbr2⁺ (Figure 4C-F), Tbr2⁺/BrdU⁺ (Figure 5C) and Pax6⁺ (Figure 6A) cell counting, but restricted to 200- μm -wide parietal sectors. Error bars in Figures 4, 5 and 6 represent standard deviations. For Tbr2⁺/BrdU(1 h)⁺ and Tbr2⁺/pH3⁺ counting (Additional file 3), at least three animals per age were analyzed (only two for Tbr2⁺/pH3⁺ counting at P6) and not less than 600 (at P4), 1,600 (at P6), 3,000 (at P10) and 3,000 (at P14) Tbr2⁺ cells were scored for each brain; Tbr2⁺/BrdU(1 h)⁺ and Tbr2⁺/pH3⁺ data are reported in the main text as average \pm standard error of the mean. Finally, the prevalence of Tbr2⁺ elements among abventricular pH3⁺ cells at P14 was calculated by collecting and scoring about 15 abventricular pH3⁺ cells per brain, from three distinct brains.

Additional file 1: Late expression of Calretinin in the opossum cortex. (A-F) Calretinin (Calb2) immunoprofiling of coronal sections from P18 to P30 opossum cortices. Solid arrowheads point to immunopositive cells in the outer cortical plate at P18 (A), and double arrowheads demarcate the deeper, areally restricted expression domain visible starting from P20 onward (C-F). Empty arrowheads highlight the absence of Calb2⁺ cells in more superficial rows of the CP at P20 and later (B, C, F). Abbreviations: e, ependyma; hi, hippocampus; mz, marginal zone; se, subependymal zone; wm, white matter; II, III, IV, V, VI are cortical layers. Scale bar: 100 μm .

Additional file 2: Abventricular mitotic pH3⁺ cells in the lateral opossum pallium. (A-D) Opossum telencephalons from P4 to P30. (A'-D') Magnifications of boxed areas in (A-D). Solid arrowheads point to abventricular mitotic pH3⁺ cells within the lateral-most cortex. Scale bar: 300 μm (A-D); 100 μm (A'-D').

Additional file 3: Mutual periventricular distribution of Tbr2 and proliferation markers in the developing opossum cortex. (A) Comparative Tbr2/BrdU immunoprofiling of P4, P6 and P10 opossum neocortices, pulsed by BrdU 1 h before fixation. (B) Comparative Tbr2/pH3 immunoprofiling of selected P4, P6, P10 and P14 opossum neocortical sectors, harboring rare abventricular pH3⁺ precursors. Empty arrows in (B) point to places occupied by Tbr2/pH3⁺ elements. Scale bar: 50 μm .

Additional file 4: Cytoarchitectonic integrity of opossum cortical explants. (A) Distribution of BrdU and Tbr2 immunoreactivity in the cortex of an opossum brain explanted at P10, electroporated by pT α 1-EGFP, kept in floating culture for 48 h and terminally administered with a 60-minute pulse of BrdU. Scale bar: 100 μm .

Additional file 5: Generation of opossum cortical GABA⁺ and GAD⁺ cells. (Aa) Strategy for assessing birthplaces of cortical GABA⁺ cells *in vitro*, on cortical explants. (Ab-c'') BrdU/IdU/GABA immunoprofiling of

radial sections of neo-/archi-cortical explants, dissected out at P1, kept in culture for 8 days in the presence of IdU and terminally administered with BrdU. Empty arrowheads in (Ac-c'') point to GABA⁺/IdU⁺ cells, presumably born before tissue dissection; solid arrowheads point to GABA⁺/IdU⁺ cells, presumably generated by cortical progenitors that underwent their final DNA synthesis *in vitro*. (Ba, Bd) GAD/BrdU and GABA/BrdU immunoprofiling of P6 telencephalons, from opossum brains pulsed by BrdU 24 h before fixation. (Bb-b'', Bc-c'') Magnifications of boxed regions in (Ba); (Be-e'', Bf-f'') magnifications of boxed regions in (Bd). Arrowheads in (Bb'', Bc'') point to cortical and basal GAD⁺/BrdU⁺ cells, respectively. (Bb''', Bc''') Magnifications of boxed regions in (Bb'', Bc''), where large immunoreactivity spots are detectable around the nucleus of GAD⁺ cells, from both cortex and ganglia. Arrowheads in (Be'', Bf'') point to double positive GABA⁺/BrdU⁺ cells. (Be''', Bf''') Magnifications of boxed regions in (Be'', Bf''). Abbreviations: BG, basal ganglia; CX, cortex. Scale bars: 200 μm in (Ba, Bd); 50 μm in (Ab-c'', Bb-b'', Bc-c'', Be-e'', Bf-f'').

Additional file 6: Gliogenesis in the opossum cortex. (Aa-f) Gfap immunoprofiling of coronal sections from P8 to P60 cortices. Arrowheads in (Aa) and in (Ab) point to Gfap⁺ presumptive radial glial cells, within P8 hippocampus and P12 cortical periventricular layers, respectively. Arrows in (Ac-f) indicate Gfap⁺ cells with astrocyte morphology. (Ag-i'') Combined Gfap/S100b immunoprofiling of P20 to P30 cortices, showing specific colocalization of these two antigens within more mature astrocytes, in cortical plate (CP) and marginal zone (MZ). (Ba, b) Immunoprofiling of P40 to P60 mid-frontal cortical sections for the oligodendrocyte-specific marker O4: an intense staining may be found in white matter (WM), internal capsule (ic) and hippocampal commissure (hc). Scale bars: 100 μm in (Aa-f); 50 μm in (Ag-i); 200 μm in (B).

Abbreviations

BrdU: bromodeoxyuridine; CNS: central nervous system; CP: cortical plate; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; GABA: gamma-aminobutyric acid; GAD: glutamate-decarboxylase; Gfap: Glial fibrillary acid protein antigen; E: embryonic day; EGFP: enhanced green fluorescent protein; IdU: iododeoxyuridine; IZ: intermediate zone; MZ: marginal zone; My: million years; P: postnatal day; PBS: phosphate-buffered saline; pH3: phosphohistone 3; PPL: preplate; SP: subplate; VZ: ventricular zone.

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Authors' contributions

EP performed the experiments. EP and AM designed the experiments, analyzed data and wrote the manuscript.

Competing interests

The authors declare that they have no competing interests.

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