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Newborn cortical neurons: only for neonates?

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Abstract

Despite a century of debate over the existence of adult cortical neurogenesis, a consensus has not yet been reached. Here, we review evidence of the existence, origin, migration, and integration of neurons into the adult and neonatal cerebral cortex. We find that the lack of consensus likely stems from the low rate of postnatal cortical neurogenesis that has been observed, the fact that it may be limited to sub-types of interneurons, and variability in other conditions, both physiological and environmental. We emphasize that neurogenesis occurs in the neonatal cortex and neural stem cells exist into adulthood; perhaps these progenitors are dormant, but they may be reactivated, for example, with injury.

Keywords

neural stem cell; neurogenesis; hypoxia; Tuberous Sclerosis Complex; neural progenitor cell; subventricular zone

Introduction

The rapid and robust expansion of the cerebral cortex relative to other brain areas has been proposed as the crowning achievement of human evolution. Indeed, the cerebral cortex plays a critical role in all perceptions, thoughts, and behaviors that distinguish humans from other animals. Do these complex cognitive phenomena require a stable brain structure? Early experimental evidence suggested that cortical neurogenesis (**Glossary**) in primates occurred only during embryonic development[1]. These findings, coupled with the complexity of primate behavior, led to the development of a “central dogma”: that the postnatal (and thus, adult) cerebral cortex possesses a stable number of neurons, all generated prior to birth; and the lack of new neurons or other regenerative capacity was a trade-off for the complexity and diversity of cognitive functions performed by the cerebral cortex. However, a substantial number of recent studies have challenged these central concepts.

The central dogma was first called into serious question in 1999 by a study showing evidence for adult-born neurons in the neocortex of macaques[2]. This work was quickly followed by several other studies in different mammalian classes reporting conflicting data on adult neurogenesis in part due to the limitations of the labeling techniques. Work in adults has also called attention to the neonatal period, which was recently shown to display

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protracted neurogenesis to different cortical regions, including the prefrontal cortex (PFC) in infant humans[3].

The occurrence of newborn neurons in the postnatal cortex implies the existence of neural progenitor cells (NPCs) or neural stem cells (NSCs). The brain contains two well-accepted postnatal NSC niches, the subventricular zone (SVZ) and hippocampal subgranular zone (SGZ), contributing to persistent neurogenesis in the olfactory bulb and hippocampus[4]. Additional niches close to the SVZ in the neonatal and adult brain have been reported, but the evidence of a neurogenic niche in the cortex is lacking. Nevertheless, several studies provided evidence of dormant bipotential progenitor cells in the cortical parenchyma. These different pools of progenitor cells are amenable to manipulated for generating neurons following brain repair.

Many studies have found postnatal neurogenesis in pathological conditions[5] that arise from NPCs in the SVZ and/or the parenchyma. Two conditions, hypoxic insult and mTOR hyperactivity as observed in Tuberous Sclerosis Complex (TSC), will be discussed.

Neonatal and adult cortical neurogenesis has been the subject of several thorough reviews [1,4,6–8]. Here, we focus mainly on recent work in adult and neonates related to neurogenesis and the sites of NPCs. In the interest of brevity, we have essentially limited our assessments to studies of neurogenesis in the neocortex and piriform cortex (Pir).

Evidence for and against adult-born cortical neurons

A widespread method to label dividing cells is the use of exogenous nucleotide analogs that are incorporated into DNA during its synthesis and thus cells in S-phase of the cell cycle. These nucleotide analogs include tritiated thymidine and the thymidine analog bromodeoxyuridine (BrdU)[9]. Use of BrdU, which can be visualized immunohistochemically, allows co-staining for neuronal markers. Advantages and limitations of the labeling methods are summarized in Table 1. We discuss studies reporting adult neurogenesis in the neocortex and Pir and several studies that did not detect it. The neocortex, which is the most evolutionary recent part of the cerebral cortex, is organized as six layers while the Pir is phylogenetically old and organized as three layers.

The use of tritiated thymidine aided the identification of the two brain regions with the most prominent postnatal neurogenesis: the SVZ-olfactory bulb and the SGZ-hippocampal granule cell layer[10,11]. Tritiated thymidine also labeled a sparse population of proliferative cells in the neocortex of adult rats[10,12] of presumed neuronal identity based on electron microscopy but at exceedingly low rates (0.011%)[12].

More recently, supporting evidence for neocortical neurogenesis, albeit at an extremely low rate, has been published using BrdU labeling and co-immunostaining for the neuronal marker NeuN (Table 2 and Figure 1A)[13–18]. Though use of BrdU is widespread, the field lacks standard experimental paradigms and very few studies quantified the number of adult-born BrdU/NeuN+ cells[13,17]. Quantification revealed that the incidence of adult-born neurons was extremely low (between 0.0026% and 0.012% in Monkeys; 3 newborn neurons/mm^3 in rats[17]). From these studies, two important characteristics of adult-born neurons emerged. First, their existence is likely transient[13]. Second, they are small, GABAergic interneurons, and not large, glutamatergic projection neurons[17,19].

There are studies that did not find evidence of adult-born neurons in the neocortex[20–24] (Table S1 in the Supplementary material online). One study, performed in humans, used a clever approach: the use of radioactive (14)C taken up during nuclear testing[20]. The biggest caveat to this study is the relatively low detection sensitivity of 1%, which is below the

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reported rate of adult cortical neurogenesis. They also calculated that they would miss neurons that survive for <4.2 months[20] and may thus have missed short-lived adult-born neurons. Another study in mice analyzed only a few cells (50) and, thus, may have missed BrdU/NeuN+ cells[22]. A study in rats focused on pyramidal neurons elegantly showed that pyramidal neurons are not born during the postnatal period[23], though not excluding the possibility that interneurons are born.

In addition to the neocortex, there are conflicting reports on neurogenesis in the adult Pir[25]. Such studies were motivated by the identification of layer II cells with a neuronal morphology expressing the immature neuronal markers, doublecortin (DCX) and the polysialylated form of the neural cell adhesion molecule PSA-NCAM[26]. However, labeled cells are now known to be post-mitotic and embryonic born[26–28], suggesting that some of the early studies may have erroneously identified BrdU expression in PSA-NCAM and NeuN cells in adults[15,29,30]. Nevertheless, a stream of PSA-NCAM+ cells has been reported suggesting migration of immature neurons towards the cortex despite the lack of integration[15,29–31]. Genetic fate mapping studies using reporter mice [e.g. expressing yellow fluorescent protein (YFP) upon tamoxifen injection] expressed an inducible Cre (CreERT2) under a specific cell type promoter also reported the generation of YFP/NeuN+ neurons[32–34] except in one study[35]. In this latter study, YFP was expressed in a few Pir neurons 1.5 days following tamoxifen injection suggesting that the promoter was abnormally turned on in neurons[35]. The most recent study clearly identified YFP/NeuN+ cells in the Pir, but these cells were not generated following proliferation (no staining for the BrdU analog EdU)[36]. This finding needs to be replicated in other lines of transgenic mice and perhaps using other approaches.

In conclusion, the majority of studies reported the presence of adult-born neurons, likely GABAergic interneurons, using BrdU and neuronal markers in the neocortex and neurons of unclear identity using BrdU and genetic mapping in the Pir. However, the rate was extremely low and the limitations of the labeling techniques have contributed to the controversy. Therefore, we discuss some of the technical parameters that may have affected the reported numbers.

Limitations of non-invasive birthdating techniques and immunostaining

Tritiated thymidine and BrdU likely underestimate the number of newborn neurons since they only label cells in S-phase. This may underlie false-negative reports. However, increasing dosage is not a solution because high doses of BrdU can lead to false-positive reports of neurogenesis[37,38]; BrdU can be taken up by neurons undergoing DNA repair or non-proliferative DNA synthesis after brain injury[39] and BrdU labeling in NeuN+ cells has been found following transplantation of dead cells[40]. Thus, just because a cell is labeled with BrdU doesn't necessarily mean that it is proliferative.

Any study using immunostaining has limitations. NeuN is thought to be a "pan-neuronal" marker, but it is not expressed in all neuronal populations[41] and has been found in adult neocortical nestin-GFP/NG2+ cells[42]. The widely used marker of immature neurons DCX has been found in cells that differentiated into glial cells[43] and in post-mitotic neurons thought to undergo structural plasticity[26]; similarly, some PDGFR+ and NG2+ oligodendroglial progenitor cells (OPCs) express DCX[44–45–48]; NG2 also labels pericytes on capillaries[49] and neonatal NPCs[48,50]. Finally, PSA-NCAM is not a neuroblast marker, but is perhaps best described as a marker for migrating cells[51]. These examples emphasize the need for staining cells with multiple markers[17]. Identification of co-labeling by BrdU and NeuN is difficult. Satellite glia cells are tightly juxtaposed to neurons; thus demonstration of

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co-labeling requires optimizing the optical Z-section thickness, imaging entire cells for examining Z-projections; this was not routinely performed in earlier studies (e.g.[2,14]).

Several other factors could influence the number of adult-born neurons including gender, environment, health, stress levels, hormonal states (*e.g.* during pregnancy or mating seasons)[16], as well as injury and disease. For example, one study in macaques was performed using individually housed animals exposed to a daily schedule of enrichment[13]. It is also intriguing that large variability in terms of animals exhibiting newborn neurons in the same study was reported[15,52]. This emphasizes the need for examining neurogenesis in different conditions and, in particular, in animals in a more "normal" environment than a cage such as adult feral rodents.

In conclusion, BrdU staining should be paired with staining for DNA repair markers and several neuronal markers to confirm that BrdU/NeuN+ cells were healthy, newborn neurons.

Sites of NSCs in the postnatal brain

In the early twentieth century, Allen identified mitotic cells along the lateral ventricle (LV) in adult rats[53]. Three decades later, the existence of NPCs in a germinal zone along the LV was proposed based on the observation of tumors near the ventricle[54]. Yet, it is only in the 1990's that a direct proof of the existence of NSCs in the SVZ along the LV was provided for all adult mammalian species examined, including humans[4,55–58]. These NSCs result from the transformation of radial glia during the neonatal period and display astrocytic properties[59,60]. NSCs rarely divide, self-renew, and generate both neurons and glia in vivo. Evidence was also reported for the existence of radial glia-like NSCs in the hippocampal SGZ[61,62]. The SVZ and SGZ are the main postnatal neurogenic niches[4]. Although no neurogenic niche has been found in the neocortex, one has been reported in the white matter beneath the neocortex in rats[63], named the temporal germinal layer (TGL). The TGL is close to the posterior part of the SVZ, but DiI injection into the LV suggested that the SVZ and TGL are disconnected. Despite co-staining of BrdU+ cells with the NPC transcription factor Pax6 or Olig2[64–66], and with DCX and NeuN, no BrdU/NeuN+ were found in the adjacent cortex; the majority of the newborn cells is thought to undergo apoptosis.

Several studies reported that NSCs of the SVZ contributed to anterior and posterior/temporal streams of migrating neuroblasts (Figure 1C and D). Streams containing BrdU/PSA-NCAM + or β3 Tubulin+ (TuJ1) cells have been reported in the subcortical white matter of rabbits[15,52]. The anterior streams project beneath the frontal cortex and disintegrate upon entry into the cortex. Finding a few cortical BrdU/NeuN+ cells adjacent to the stream suggested that PSA-NCAM+ cells in the stream generated neurons. A temporal stream from the ventral SVZ along the LV inferior horn to the amygdala and Pir has also been reported in monkeys, rats, and bats using staining for immature neuronal markers, BrdU at different time points post-injection, or DiI labeling[14,29,31]. Despite the evidence of these streams, additional approaches (e.g. viral tracing) are required to show that BrdU/NeuN+ cells originated from SVZ cells.

Intriguingly, induction of the tyrosine kinase receptor ErbB2 in 6-week-old mature astrocytes using inducible transgenic mice led to re-expression of radial glial identity in NSCs of the dorsal SVZ but not in cortical astrocytes. This was accompanied with SVZ cell proliferation *in vivo*, and generation of GABAergic and glutamatergic neurons *in vitro* and NeuN+ cells in the cortex[67]. This study highlights a remarkable plasticity of NSCs in the SVZ that can be harvested upon genetic manipulation.

Despite the absence of a NSC niche in the cortex, the following studies suggest the existence of NPCs in the brain parenchyma. Indeed, NPCs were isolated from the adult human

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subcortical white matter by labeling dissociates with OPC markers (CNP2 or A2B5)[68] (Figure 1C). Isolated cells formed neurospheres and generated functionally competent neurons and glia *in vitro* and after xenograft into fetal rat brain[69]. The same group reported the absence of cells with neurogenic potential from the temporal lobe of epileptic patients[70]. But in this study, culturing cells without bFGF may be an important difference because cells isolated from the adult rat neocortex generated neurons *in vitro* only after FGF exposure bFGF[71]. These studies suggest the existence of dormant NPCs in the white matter and neocortex that have the potential to generate neurons *in vitro*.

Based on these findings, the potential of OPCs to act as NPCs was investigated in vivo using genetic fate-mapping strategies in Rosa26R mice carrying CreERT2 under the Pdgfra or NG2 promoter[34–36,72]. YFP+ neurons were found in the anterior Pir[34,36], but EdU expression in every OPC but not in YFP+ neurons suggested that these neurons were not generated from OPCs[36]. It remains to be examined whether an unidentified population of *Pdgfr*a-expressing NPCs was labeled by YFP or whether YFP expression results from abnormal Cre expression. Of interest is the potential of reactive astrocytes to reacquire NSC features and generate neurons at least in vitro[73]. Indeed, using genetic fate mapping and cell type-specific viral targeting, a study showed that proliferating astrocytes (GFAP+) but not NG2 cells following a stab wound injury displayed multipotency and self-renewal *in vitro* although they generated only astrocytes in vivo[73]. Consistent with this finding, they subsequently showed that expression of neurogenic transcription factors in proliferative astrocytes *in vitro* led to neuron generation[74–76]. Collectively, these studies suggest that the adult cortex possesses anti-neurogenic properties preventing NPCs and proliferative astrocytes following insult from generating neurons.

The neonatal period displays protracted cortical neurogenesis and offers clues to NSC identity

The neonatal rat brain continues to grow by nearly 6 times in weight and in size from birth to adulthood[77]. An increase in the number of neocortical neurons has been reported in different animal species, including humans[78–81]. As a quick note, neonatal rodents (P7–P12) are the age-equivalents of *in utero* humans[82]. The increase in brain weight and size was hypothesized to be partly due to embryonically born neuroblasts remaining along the LV and then migrating through the cortical plate[83]. Although these descriptive studies rely on extrapolations of neuron numbers based on density and brain size, they highlight the possibility that some neurons are born during the neonatal period. Consistent with this idea, a recent finding is the identification of a stream of DCX+ cells in infants up to 8 month old[3] that is reminiscent of that identified in adult rabbits[15,52] (Figure 2A). The stream in humans emanates from cells migrating to the olfactory bulb and culminates within the ventromedial PFC. Whether neuroblasts detach from the stream, migrate into the cortex, and integrate into the circuitry are important questions to address in future studies.

The following studies reported the presence of protracted neurogenesis during the neonatal period from NCSs in the SVZ, in the white matter, and the parenchyma (Table 3). Only one elegant study showed that neocortical pyramidal neurons are not generated from NSCs in the SVZ during neonatal life[23]. Although they identified BrdU/NeuN+ cells expressing GFP following intraventricular GFP retroviral labeling, they showed that GFP expression in pyramidal neurons resulted from fusion with infected microglia (Table 1). This study does not contradict those reporting neurogenesis of neocortical interneurons.

Two recent studies reported the generation of neurons (~20,000) during the late neonatal period using BrdU in mice[84,85]. This is a relatively high number that was based on extrapolations. It was proposed that these newborn neurons originate from the SVZ based on

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the presence of DCX+ and Mash1+ cells[84]. It remains unclear whether these migrating neuroblasts contribute to the 20,000 neurons identified. Nevertheless, additional studies identified the neonatal SVZ as a source of cortical neurons. Mice expressing the $5HT_3$ receptor fused to GFP allowed the identification of GFP+ cells migrating to the cortex in neonates[18]. Retroviral labeling in or above the SVZ together with BrdU showed that a cohort of DCX+ cells migrates from the SVZ to frontal cortical structures and become GABAergic interneurons (Figure 2E–F). Considering the large difference between the number of migrating DCX+ cells and BrdU/NeuN+ neurons, it is possible that many of the newborn neurons have a transient life. Further investigation revealed two GABAergic neuronal populations and provided the first functional evidence that these neurons displayed action potentials and received synaptic inputs[86]. A small population of multipolar GABAergic neurons spread across all frontal cortical layers and corresponded to the tail end of embryonic neurogenesis. A second population of small axon-less GABAergic neurons integrated in the olfactory and orbital cortices with a peak generation around P1 in mice.

Another study recently reported the generation of interneurons during the neonatal period, but with a twist[87]. Using transgenic mice, they identified a pool of GABAergic and 5HT3+ precursors in the dorsal white matter (Figure 2G–I). These progenitors appear around embryonic day 19 and peak at P7. Some of these precursors expressed Pax6 and were proliferative. Importantly, they generated migrating DCX+ cells and GABAergic neurons in the anterior cingulate cortex. This finding questions whether some of the virally infected cells reported to generated GABAergic interneurons in the two previous studies were these transient white matter precursors.

One study explored the presence of neurogenesis from parenchymal OPCs. Using genetic fate mapping in Rosa26R-YFP mice carrying CreERT2 under the proteolipid promoter (in some OPCs and oligodendrocytes), they observed YFP+ cells expressing TuJ1, NeuN, and the vesicular glutamate transporter 1 identifying them as glutamatergic pyramidal neurons in the dorsal and ventral cortex, but at very low incidence[44]. It remains unknown whether these neurons would accumulate with time after tamoxifen, as one would predict if the permanently labeled progenitors persisted beyond the neonatal period.

Collectively, these studies identify three sources of newborn neurons in neonates: a transient pool of white matter progenitors, bipotential cortical progenitors, and NSCs in the SVZ. The function of these neonatally-born interneurons and their duration of life remain to be investigated.

Pathophysiological conditions that impact postnatal cortical neurogenesis

Our comparison of neonatal and adult neurogenesis has suggested that adult neurogenesis may be actively suppressed by strong anti-neurogenic molecules mediating a non-cell autonomous effect, in addition to a lack of supportive molecules. A pioneering study showed that cortical neurogenesis can be induced in adult mice by triggering cell death[88]. Here, we focus on two pathological conditions, hypoxia and TSC that are associated with increased and dysregulated neonatal neurogenesis, respectively. These studies emphasize that neonatal cortical neurogenesis can be manipulated and may impact neurological behavior.

Hypoxia

An estimated 1–8/1000 births are complicated by hypoxic ischemia, a condition in which oxygen levels drop below optimal levels. Approximately half of the neonates exposed to hypoxia have prolonged neurological delays. In perinatal mice, chronic hypoxia is associated with massive apoptosis of cortical neurons, decreased cortical volume, and, in some strains, neurological dysfunction[84,89]. In other strains, the decreased cell number and

cortical volume recovered[84,85]. Part of this recovery seems to involve a doubling of newborn cortical neurons following hypoxia. This was assessed using stereology (see caveats in Table 1) and BrdU, which could incorporate into neurons during non-proliferative DNA synthesis following hypoxia. The source of these newborn BrdU+ neurons was originally speculated to be SVZ cells[84], but new evidence using lineage analysis suggest that some are generated from neonatal parenchymal astrocytes[85]. This report is in agreement with a previous study showing that neonatal radial glia or immature parenchymal astrocytes retain neurogenic potential *in vitro*[90]. Collectively, these studies emphasize the presence of neonatal neurogenesis that is enhanced following a hypoxic insult and may contribute to neurological improvement.

Tuberous Sclerosis Complex

TSC is a monogenic, developmental disorder characterized by abnormal brain development associated with seizures, hydrocephalus, and severe neurological symptoms[91–93]. TSC is caused by mutations in *TSC1* or *TSC2* leading to hyperactivity of the mTOR pathway[94,95]. TSC patients display cortical malformations that are essentially generated during embryonic life and have been modeled in mice[96,97]. It has been reported that *Tsc1* deletion in neonatal SVZ cells using inducible nestin-Cre mice generated SVZ nodules[98,99]. Intriguingly, ectopic *Tsc1*^{null} neurons were also found in the cortex. Using targeted electroporation to delete *Tsc1* selectively in NSCs of the SVZ (see caveats in Table 1)[100], a study reported the presence of heterotopia along the migratory path to olfactory structures and ectopic cells in the nucleus accumbens and in the neocortex[99]. The identity and function of these *Tsc1*^{null} cortical neurons remain to be examined. Collectively, these findings show that the production of newborn neurons from the neonatal SVZ can be enhanced or neurons can be re-routed to reach cortical structures upon mTOR activation. It remains to be examined whether such abnormal infiltration of neurons to forebrain structures contributes to network malfunction in TSC.

Concluding Remarks

In the adult cortex, one point of consensus is that the numbers of adult-born neurons are low and their lives are short. Thus, we need to search for them, like needles in a haystack, perhaps in many “haystacks” subject to different conditions. In addition, supplemental approaches need to be developed since BrdU may label neurons repairing their DNA; alternatively, markers of DNA repair need to be routinely performed. Due to their low number, it is difficult to conclude whether the identified BrdU/NeuN+ cells are the result of progenitor proliferation or neuronal repair. Another important finding is the presence of anterior and posterior streams of presumably migrating neuroblasts going towards the frontal cortex and Pir. Based on these studies, it seems that some neuroblasts are made and reach white matter, but die since the adult brain is not permissive for newborn neuron survival under normal conditions. These studies - as well as studies in injury and disease models - suggest that the adult brain may be actively suppressing neurogenesis. This suggests that these mechanisms can potentially be exploited to make new neurons for brain repair. Thus, the more critical question is whether one, or more, of the NPC populations would be amenable to manipulation and enhancement of their production of cells; or if, once we have more molecular details, de-differentiation of mature astrocytes into NPCs will be a more promising strategy (see Box 1 for additional questions).

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Box 1

Outstanding questions

- Are BrdU/NeuN+ cortical neurons undergoing DNA repair? In other words, do the few adult-born neurons stain for markers of DNA repair?
- What new approaches can be applied to study postnatal neurogenesis? For example, non-invasive *in vivo* imaging, in combination with transgenic mice in which specific neural populations are labeled, would be a valuable approach to take to track NSCs and newborn neurons longitudinally, for weeks or months.
- Are neonate-born cortical neurons derived from diverse progenitor pools including those from the SVZ, the parenchyma, or white matter transient progenitors?
- What is the identity and/or fate specification of SVZ stem cells, parenchymal stem cells and bipotential progenitors? Can the fate be altered by microenvironmental cues or it is predetermined?
- What is (are) the source(s) of variability in the number of newborn neurons in the postnatal cortex?
- What are the molecular pathways controlling neonatal neurogenesis from NSCs to neuronal integration? This is a critical question to be able to enhance or limit neuronal production when necessary.
- What disorders/pathological states are associated with altered postnatal cortical neurogenesis?
- What is the contribution of the newborn cortical neurons on network function in health and disease during postnatal life?

During the neonatal period, there is a clear generation of a subpopulation of GABAergic neurons in rodents. In this case, perhaps more controversial is the identity of the pool of progenitors that generate these GABAergic neurons. There is evidence for contributions from the SVZ, from transient white matter progenitors, and possibly from parenchymal progenitors. However, though it seems like it might be critical to generate the sheer numbers of cortical neurons, the function of neonatal neurogenesis isn't much more defined than that of adult neurogenesis. Yet, two facts are undeniable: neural stem cells exist, perhaps they are latent or dormant, but they can be reactivated, for example, with injury. Identifying these cells and defining the molecules keeping them dormant is at the key of brain repair and tumor prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Glossary

Bipotential/ Neural progenitor cells	cells generated from NSCs that are bipotential (*i.e.* generate neurons and glia) and have self-renewal capacity but more limited than NSCs
Neonatal electroporation	this technique allows cDNA to enter cells by applying voltage, which creates small pores in the cell membranes and pushes charged cDNA to enter cells. In neonatal mice, following pressure ejection of a cDNA plasmid into the LV, voltages are applied across the head of anesthetized pups allowing plasmid entry into NSCs lining the ventricle
Neurogenesis	a process leading to the generation of neurons through several stages, rare NSC assymetric division and generation of bipotential progenitor cells; proliferation of progenitor cells leading to pool amplification and generation of neuroblasts, neuroblast proliferation, post-mitotic differentiation, migration, maturation, and synaptic integration
NSCs	neural stem cells, which can generate both neurons and glia and self-renew indefinitely. In the adult, NSCs have a more restricted fate than embryonic NSCs and generate only certain types of neurons
Transgenic floxed, inducible Cre (CreERT2), and Rosa26R reporter mice	floxed is used to describe the bordering of a DNA sequence between two LoxP sites and is abbreviated "flanked by LoxP." Cre recombinase expression in cells expressing a floxed gene leads to gene sequence excision and deletion. In reporter Rosa26R mice, a Stop sequence is inserted in the Rosa26 locus between two LoxP sites preceding a reporter gene (e.g. YFP). Upon Cre expression, the Stop sequence is excised leading to the reporter expression. CreERT2 mice express a CreERT2 under a specific promoter that requires tamoxifen to be active. Upon tamoxifen injection, Cre is translated to the nucleus and excises any floxed gene in CreERT2 mice crossed with floxed mice. Using CreERT2-Rosa26R mice allows to label cells expressing a specific promoter driving CreERT2 and track their progeny

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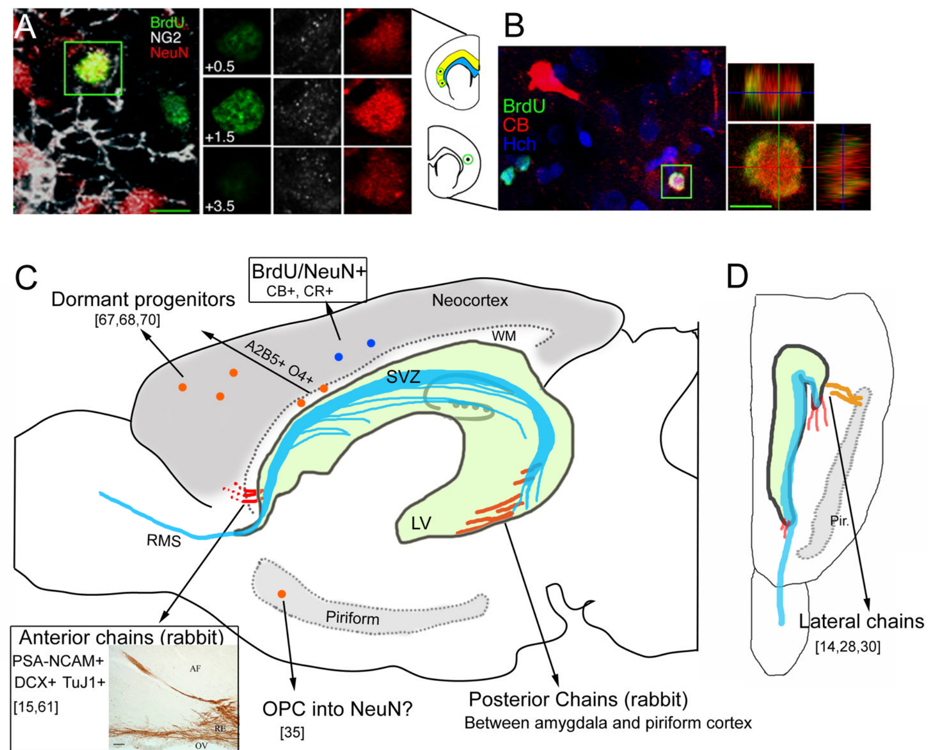

Figure 1. Sites of adult progenitor cells and adult born neuroblasts in cortical regions

(A) A 4–5-wk-old cell in rat cortex labeled with BrdU and NeuN. Sample z-planes through the green boxed area are shown with color separation on the right, showing colocalization of BrdU and NeuN but not NG2 [17]. The location of the cell shown in A is circled in the diagram of a coronal section; the analyzed region of cortex is shown in yellow on the diagram, and the subcortical white matter, used as a boundary for the analysis, is shown in blue. **(B)** Image of an 11–12-wk-old BrdU/Calbindin+ CB) cell in a coronal section of rat brain. Higher magnification orthogonal views are shown on the right [17]. Hch: hoetsch (blue). **(C and D)** Diagram of a sagittal (C) and a horizontal (D) section of an adult mouse brain illustrating the main sites of NPCs. The neocortex and the piriform cortex are highlighted in grey. The blue line illustrates migratory chains of neuroblasts throughout the SVZ located along the lateral ventricle (green, LV). The LV is underrepresented as it folds until the level of the piriform cortex (Pir). The red line illustrates anterior and posterior chains of DCX and PSA-NCAM+ cells that have been observed in the rabbit. The anterior chains are immersed into the white matter (WM) of the anterior forcep of the WM. The inset depicts newborn cells labeled with PSA-NCAM in anterior chains [15]. Abbreviations: CB, calbindin; CR, calretinin; DCX, doublecortin; OPC, oligodendrocyte progenitor cells; RMS, rostral migratory stream. Adapted, with permission, from [17] (A and B), [15] (C, inset).

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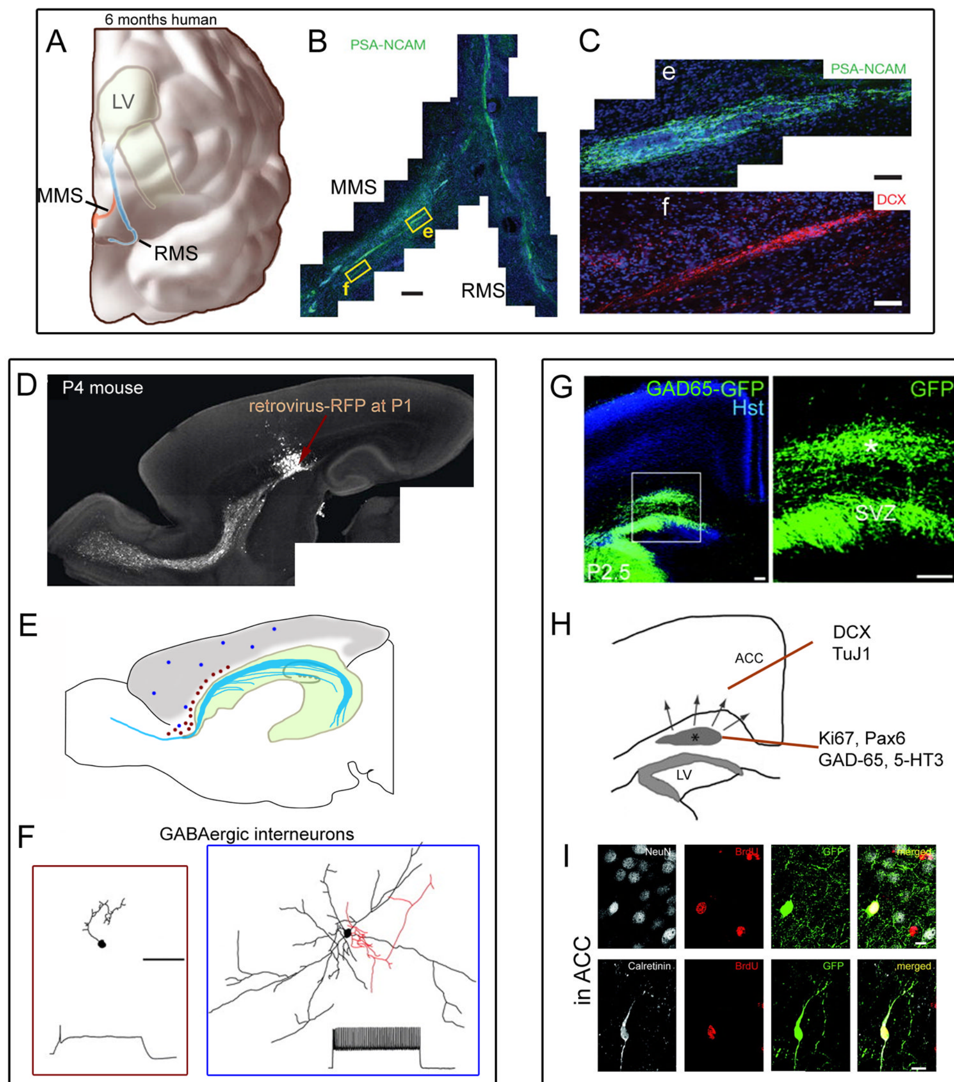

Figure 2. Major sites of neonatal neurogenesis in the cortex

(A) Diagram of a hemisected immature (6 months old) human cortex containing the rostral migratory stream (RMS) and the medial migratory stream (MMS) originating from the SVZ along the lateral ventricle (LV) [3]. **(B)** Coronal reconstruction of a 6-month specimen showing PSA-NCAM$^+$ cells in the MMS diverging from the RMS to reach the ventro-medial prefrontal cortex [3]. (C) Chains of PSA-NCAM$^+$ cells (i) and DCX+ cells (ii) in the MMS [3]. The images correspond to the yellow boxes in (B). Scale bars: 150 μm (B) and 20 μm (C). **(D–F)** Generation of newborn neurons from the SVZ. (D) Red fluorescent protein (RFP)-labeled cells in a P4 mouse injected with a retrovirus encoding RFP at P1. The arrow points to the injection site [85]. (E) Location of newborn neurons (red and blue circles) in the cortex above the lateral ventricle (LV, green) and streams of migrating SVZ neuroblasts (blue). The red versus blue colors refer to two different categories of GABAergic newborn neurons (as described in F). (F) Newborn GABAergic neurons were divided in two categories, small axonless neurons (left, red square) and multipolar neurons (right, blue square) exhibiting different spiking patterns [85]. Scale bar 50 μm.**(G–I)** Generation of anterior cingulate neurons (ACC) in mice from a transient progenitor pool in the white matter above the SVZ. (G) Images at low (left) and higher (right) magnification showing at

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P2.5 a pool of GAD65-GFP+ cells in the white matter progenitor pool (white star) and dorsal SVZ [87]. Abbreviation: Hst, Hoechst. (H) Schematic coronal section depicting during the first postnatal week migration of GAD65-GFP+ cells from the white matter progenitor population (indicated with a star) toward cortical regions. A fraction of progenitor cells were Ki67+, Pax6+, and all were GAD-65 and 5-HT3+. Migrating cells were DCX and TuJ1+ [86]. Scale bars: 100 μm. (I) Confocal images of ACC layer VI at P10 showing GAD65-GFP+ interneurons labeled for NeuN or calretinin and BrdU. BrdU was injected postnatally at P0.5 (3× 20 mg/kg intraperitioneal) [86]. Scale bar: 10 μm. Adapted, with permission, from [3] (A–C), [85] (D, F) and [86] (G–I).

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Table 1

Advantages and disadvantages of the different approaches used to identify newborn neurons

Technique	Specific approach	Advantages	Disadvantages
Anatomical	Cellular morphometry, Nissl and Golgi stainings, and electron microscopy	• Can be performed on existing tissues of any species, including humans	• Unable to determine the birth date
	Cell Count	• *in vivo* labeling of cells not necessary	• Often limited to a few regions as it is difficult to count all regions • Stereology is used to extrapolate to other sections, but extrapolations don't account for regional differences
Birthdating		• Labels cells in S phase	• Labels only a fraction of cells • Incorporation during DNA repair and none-productive mitosis • Presence of BrdU labeling in NeuN+ cells following transplantion of BrdU+ dead cells
	Tritiated Thymidine	• Linear kinetics for integration and emission	• Radioactive • Induces DNA damage • Indirect visualization
	Halogenated Thymidine Analogs (i.e. BrdU)	• Multiplexing possible	• Toxic • Can induce neuronal differentiation or fate changes Rapid dilution during proliferation • Indirect visualization by fluorescent antibodies
	(14)C	• Applicable to human tissues	• Used based on population analysis with resolution limited to <1% of total cell population
Fluorescent Tracing		• Live imaging possible	• Injection can lead to tissue damage
	Lentiviral Adenoviral	• Widespread integration	• Labels mitotic and postmitotic cells • Large genomic insertion
	Retroviral	• Integrated into proliferating cells	• Not specific for progenitor cells (e.g. glia, microglia) • Can induce microglial fusion • Site of genomic integration can lead to pathological defect
	Postnatal electroporation	• Amenable to use with most plasmids	• Labels mitotic and possibly postmitotic cells

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Technique	Specific approach	Advantages	Disadvantages
			• Rapid dilution if not used with genomic recombination • Precise birthdating not possible • Application of large amounts of voltage with unknown outcome
Transgenic Mice		• Live imaging possible • Restricted expression possible • Appropriate with inducible systems	• Transgene may not distinguish birth date • May be expressed in many cell types • Transcriptional activity may be altered with injury

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Table 2

Reports of adult neurogenesis in the neocortex of mammals[a]

Species	Age with respect to BrdU labeling	BrdU labeling (and additional neuronal markers)	Additional details	Conclusions	Regions	Refs
Monkeys						
Macaca fascicularis (male)	5–6 years	BrdU/NeuN+ (at 9 wk: 12–36% of BrdU+ cells)	• Housed individually and were exposed to daily enrichment • NeuN: nuclear staining	Transient generation of small neurons	Frontal and temporal cortex	13
Macaca fascicularis (female)	2–5 years	26 days survival: 18 BrdU/NeuN + cells in >500 cortical slices	• NeuN: small cells, nuclear staining	Very few. Presumably small interneurons	Principal sulcus and ventral part of area 14	63
Macaca	6–12 years	BrdU/TuJ1+ cells in a stream	• DiI intraventricular injection 3wks post-injection • Temporal migratory stream of DiI+ cells from SVZ to amygdala	Very few NeuN/BrdU+ cells	Inferior temporal cortex and Pir	14
Squirrel monkeys	3–6 years	28 days survival NeuN in 27% of BrdU+ cells in the piriform cortex.				
Rabbits						
New Zealand White rabbits	4–6 month 1–2 year	• 30 days survival: BrdU/PSA-NCAM+ cells in migratory chains • 60 days survival: some BrdU/NeuN+ detected in the cortex	• PSA-NCAM staining: identification of anterior and posterior chains of neuroblasts in the mature parenchyma. • Chain frequency: 20% of young 50% of adults	Very few BrdU/NeuN+ cells in the cortex.		15,52
Rodents						
Male golden hamster	4–5 months	7 weeks post- injection: BrdU/ NeuN+ cells occasionally found in cortex	• Nuclear NeuN staining • Photoperiod changed the number of Brdu+ cells (but unknown whether NeuN+)	• Very few in cortex • Presumably small interneurons	Regions are unknown	16
Male Sprague- Dawley rats	9–10 weeks	• 32 days survival: 33/7624 BrdU+ also NeuN+ • 3/10 brains: no NeuN/BrdU+	• Nuclear NeuN staining	• Very few GABAergic neurons: 3 cells/ mm^3	Infralimbic, cingulate, somatosen sory and secondary motor V–VI cortex	17

Species	Age with respect to BrdU labeling	BrdU labeling (and additional neuronal markers)	Additional details	Conclusions	Regions	Refs
		• BrdU+ cells were CR +, CB+, or GABA+		• Variability among rats		
Male rats	12–13 weeks	• Thymidine; 30 days survival * Neuronal identity based on morphology, including synapses along their bodies and dendrites	Frequency: 0.011% (1 in 10,000) 38 sections: 10 neurons	• Few neurons	• Visual cortex (layer IV)	12
Mice (5HT$_3$- EGFP transgenics)	1–3 months	BrdU/NeuN+ cells		Small axonless GABAergic interneurons	Preferentially deep cortical layers, frontal cortex, AOC and orbital cortex	86

[a]Abbreviations: AOC, anterior olfactory cortex; CB: calbindin; CR: calretinin

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Table 3

Reports of neonatal neurogenesis in the neocortex [a]

Species	Age with respect to BrdU labeling	BrdU labeling (except human study) with additional neuronal labeling	Viral labeling or fate-mapping	Conclusions	Regions	Refs
Humans						
Infants	< 8 months	DCX+ and PSA-NCAM+ cells	No	Existence of a stream of presumably migrating neuroblasts	Ventromedial prefrontal cortex	3
Mice						
Wild-type	P10;P49	• P49: 20,000 BrdU/NeuN+ cells • 6.7% BrdU+ are NeuN+	None	• 20,000 neurons based on stereology	Neocortex and cingulate cortex	84,85
5HT$_3$-EGFP transgenic	P1–4; P30	• 72 hr survival: BrdU/ EGFP close to SVZ • 10 days survival: BrdU/ EGFP/DCX in frontal cortical and subcortical structures • 4weeks:some NeuN+, CR +	EGFP retrovirus injection in dorsal SVZ at P3-P4	• Small GABAergic axonless • Action potentials and synaptic inputs • Multi polar GABAergic neurons: tail of embryonic neurogenesis	Deep layers (VI) of mostly AON and orbital cortex In cortical layer II–VI	18 86
GAD65-GFP and 5HT$_3$-GFP transgenics (and other lines)	Different neonatal ages	• Different injection and survival times: • Staining for Pax6, DCX, Ki67, NeuN	No	• Transient pool of proliferative Pax6, GAD65, and 5HT3+ progenitors in the dorsal white matter • Peaks at P 7 • Generates migrating DCX + cells and BrdU/NeuN/CR + neurons	ACC	87
Plp-Cre x R 26R- YFP transgenics	P8 (tamoxifen); P60	• EdU (BrdU equivalent)	Post-tamoxifen: P15; P60	• TuJ1+ cells and a few NeuN+ cells generated from endogenous	Dorsal and ventral forebrain cortex	44

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Species	Age with respect to BrdU labeling	BrdU labeling (except human study) with additional neuronal labeling	Viral labeling or fate-mapping	Conclusions	Regions	Refs
				• Progenitors		

[a]Abbreviations: ACC: anterior cingulate cortex; AON: anterior olfactory nucleus; CR: calretinin; EGFP, enhanced green fluorescent protein; GAD65: glutamic acid decarboxylase 65; Plp, proteolipid protein.