REVIEW

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Future NEUROLOGY

Monitoring neurogenesis in the cerebral cortex: an update

Maria Pia Postiglione[1] & Simon Hippenmeyer*[,1]

ABSTRACT: The cerebral cortex, the seat of our cognitive abilities, is composed of an intricate network of billions of excitatory projection and inhibitory interneurons. Postmitotic cortical neurons are generated by a diverse set of neural stem cell progenitors within dedicated zones and defined periods of neurogenesis during embryonic development. Disruptions in neurogenesis can lead to alterations in the neuronal cytoarchitecture, which is thought to represent a major underlying cause for several neurological disorders, including microcephaly, autism and epilepsy. Although a number of signaling pathways regulating neurogenesis have been described, the precise cellular and molecular mechanisms regulating the functional neural stem cell properties in cortical neurogenesis remain unclear. Here, we discuss the most up-to-date strategies to monitor the fundamental mechanistic parameters of neuronal progenitor proliferation, and recent advances deciphering the logic and dynamics of neurogenesis.

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[1]IST Austria (Institute of Science & Technology Austria), Am Campus 1, A-3400 Klosterneuburg, Austria
*Author for correspondence: simon.hippenmeyer@ist.ac.at

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LEARNING OBJECTIVES

Upon completion of this activity, participants should be able to:

- Describe recent techniques for monitoring neurogenesis, based on a review
- Discuss recent advances in understanding the dynamics of neurogenesis
- Assess future directions for research and clinical implications regarding neurogenesis

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Author & credentials: ***Maria Pia Postiglione, PhD,*** *Institute of Science and Technology Austria, Klosterneuburg, Austria.*

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Overview: cerebral cortex development

One of the most striking evolutionary features underlying the unique cognitive human abilities is the emergence of an exceedingly enlarged cerebral cortex. The origin of the neocortex can be traced back to reptiles, which show for the first time a laminar, although simple, organization of telencephalic projection neurons [1,2]. A progressive increase in the numbers of cortical neurons, size and shape of the neocortex resulted in the emergence of the highly folded (gyrencephalic) mammalian brain found in the majority of primates and humans [3–5]. The human cerebral cortex is composed of an extraordinary number of neurons and glia cells that originate from a variety of progenitor stem cells located in the ventricular (VZ) and subventricular (SVZ) zones **(Figure 1)** [5,6]. The identification and classification of progenitor stem cells that produce the different types of neurons in the CNS has a long history that started more than a century ago. In 1887, His introduced the concept of two distinct progenitor types, one each for neurons and glial cells, on the basis of histological observations of mitotic figures in the VZ of human embryos. He suggested that these proliferating cells were germinal 'Keimzellen', producing one daughter cell that remained in the VZ, and a postmitotic 'neuroblast' that migrated away and became a neuron. The nonproliferating or quiescent cells within the VZ on the other hand were believed to constitute a syncytium of spongioblasts that would later give rise to glia cells [7]. The concept of His has been challenged by Vignal, Schaper and Koelliker, who suggested that the neuroblasts and spongioblasts were alternative forms of the same progenitor cell, while moving from the apical to the basal side of the VZ, depending on the phase of the cell cycle [8–10]. Their notion was later supported by Sauer who also described the phenomenon of interkinetic nuclear migration (IKNM) in the VZ. During IKNM, mitotic progenitor cell nuclei display asynchronous but alternate movements depending on their cell cycle phase. While in the G1 phase, the nucleus migrates to the basal end of the progenitor, proceeds through the S phase and moves back to the apical side in G2 to eventually complete mitosis [11–13]. Nevertheless, the concept proposed by His remained prominent until live imaging at the individual progenitor cell level demonstrated that radial glia progenitors (RGPs) account for the generation of all excitatory projection neurons in the cerebral cortex [14,15]. Cortical

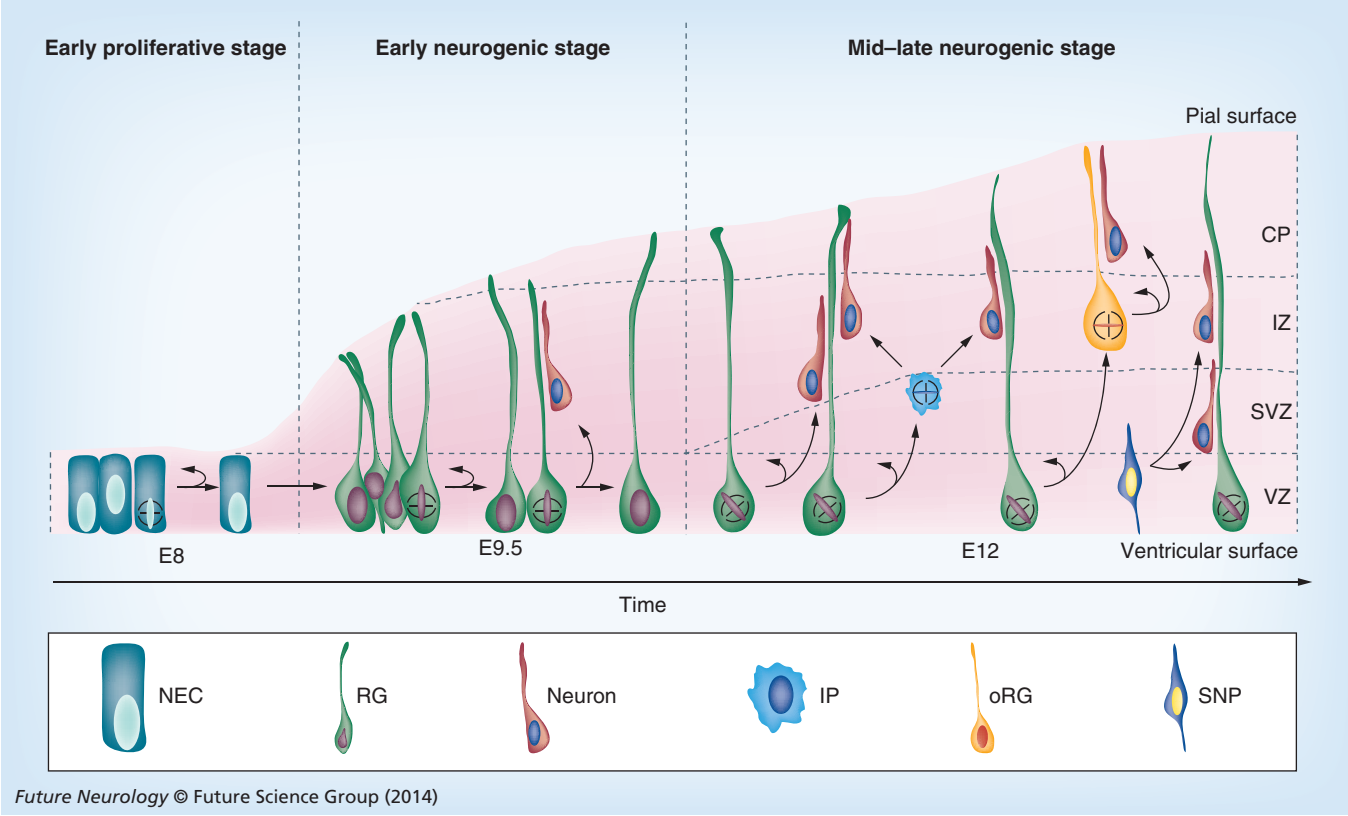

Figure 1. Neurogenesis in the mouse cortex. NECs initially divide symmetrically to expand their pool and progressively transform into radial glia progenitor (RGP) cells. RGPs divide symmetrically, and asymmetrically to produce neurons that migrate radially to form the CP. At earlier stages, RGPs divide asymmetrically to produce neurons directly, then switch to indirect neurogenesis through IPs or oSVZ oRGs. oRGs divide asymmetrically to produce neurons in mice, or transient amplifying cells in humans. IPs mostly populate the SVZ while oRGs translocate into the IZ for neurogenesis. SNPs are located in the VZ, where they divide symmetrically to produce neurons. CP: Cortical plate; IP: Intermediate progenitor; IZ: Intermediate zone; NEC: Neuroepithelial cell; oRG: Outer radial glia; RG: Radial glia; SNP: Short neural precursor; SVZ: Subventricular zone; VZ: Ventricular zone.

projection neurons are produced in consecutive waves during embryogenesis. They assemble in an inside-out fashion whereby earlier generated neurons populate innermost layers, and later generated neurons radially migrate along the elongated basal glial processes of RGPs to settle in more superficial layers in the developing cortex [16,17]. Severe neurological disorders, including megalencephaly, microcephaly and lissencephaly, but also more subtle neurodevelopmental disorders, such as schizophrenia and autism, can result if neurogenesis is impaired or neuronal migration is disrupted in human [18–22]. Excitatory cortical projection neurons represent approximately 80%, and inhibitory interneurons approximately 20% of all neurons in the cerebral cortex [23]. Cortical interneurons are generated by progenitors in the ventral telencephalon, and migrate tangentially to the developing cortical plate [24–26]. Interestingly, interneuron progenitors in the ventral ganglionic eminences also display a morphological RGP fate character with an extended basal process [27–29], albeit dorsal and ventral RGPs express distinct sets of fate determining transcription factors [30,31]. During the last decade, besides RGPs a variety of distinct progenitors **(Figure 1)**, including intermediate progenitors (IPs), short neural precursors (SNPs), transient amplifying progenitors (TAPs) and outer SVZ (oSVZ) progenitors (outer radial glia cells [oRGs]) have been discovered [3,5,6,32]. The broader criteria for a coarse classification of neuronal progenitors include the site of mitosis, cell polarity, molecular makeup, mode of division and proliferative capacity [3,5,6,32]. Although it is currently not clear how many different progenitor types exist at all, and how they holistically orchestrate neurogenesis, several key questions emerge regarding the precise numbers and distinct neuron

classes that are produced by the entire progenitor community but also individually by each progenitor during cortical neurogenesis. For instance, which cellular and molecular programs dictate the quantitative neuron output in distinct progenitor types? On the qualitative level, what are the precise neuronal lineages that are generated from individual progenitor cells? Also, more generally, how do progenitor stem cells shape and influence the fate of distinct lineages, which may on their part contribute to establish specificity of neuronal connectivity in defined cortical circuits? In order to begin to address these fundamental questions it is important to precisely monitor neurogenesis at the individual progenitor level, and to functionally dissect the signaling pathways that regulate neuronal progenitor identity and neurogenic dynamics. This article will include an update on the currently available approaches that enable the tracing of neurogenesis at high resolution and also discuss recent advances that provide novel mechanistic insight into the cellular and molecular principles of neurogenesis.

Monitoring neurogenesis

• Neuron birth dating

Major advances in tracking neurogenesis became possible once tracers like tritiated thymidine ([^{3}H]-thymidine) were introduced. Tritiated thymidine is a radiolabeled deoxynucleoside that is incorporated into the DNA during replication in cell proliferation and remains in the nuclei of dividing cells as a permanent marker [33]. Birth-dating experiments, in which [^{3}H]-thymidine was administrated to rodents at different times during brain development, showed that neurons that are born at the same time occupy the same layer, thereby building up the cortex in an 'inside-first, outside-last' fashion [16]. Tritiated thymidine was substituted over the time by nonradioactive and/or halogenated thymidine analogs like 5-bromo-2′-deoxyuridine, 5-ethynyl-2-deoxyuridine, 2′-deoxy-5-iodouridine or 5-chloro-2′-deoxyuridine, which can be visualized by staining with specific antibodies [34–36]. Thymidine analogs in combination with markers of cell proliferation, such as Ki67, allow quantitative assessment of the postmitotic neuron production rate and numbers of cycling progenitors. In effect, the rate of cell cycle exit and neurogenesis can be determined by quantification of the proportions of progeny from mitoses exiting the cell cycle (quit fraction) or continuing to proliferate across defined temporal windows [37].

A genetic approach has also been developed to selectively detect neurogenic progenitors during cortex development. In particular, expression of GFP from the *Tis21* locus in mice specifically labels neurogenic progenitors and permits dynamic live imaging analysis [38]. *Tis21-GFP* mice have been successfully used to isolate molecular markers for neurogenic progenitors and in combination with cumulative DNA labeling using thymidine analogs to determine temporal cell cycle parameters in distinct progenitor populations [39,40]. An elegant contemporary method allows the direct tracking of cell cycle changes in real time and thus assay cell proliferation in single progenitor cells. This method, called fluorescent ubiquitination-based cell cycle indicator (FUCCI) **(Figure 2A)**, couples dual color imaging with distinct cell cycle progression phases [41]. FUCCI allows the precise distinction between the G1 and the S/G2/M phases by expression of two distinct (red and green) fluorescent probes in living cells. The FUCCI probes mark nuclei in G1 phase in red and those in S/G2/M phases in green. Thus, FUCCI permits in principle the live tracking of the dynamic cell cycle oscillations during IKNM in the ventricular zones of the developing brain and thus the 4D monitoring of neurogenesis [41] **(Figure 2)**. FUCCI technology has recently been further extended and two optimized probes have been introduced in the genomic *Rosa26* locus [42] to also enable conditional and cell type-specific expression of the FUCCI probes by using different Cre recombinase drivers [43]. Altogether, FUCCI technology holds the potential to systematically determine cell cycle parameters and neurogenic dynamics in genetically distinct neuronal progenitors *in vivo* and in real time by using live imaging.

• Monitoring neurogenesis by lineage tracing

Whether different classes of neurons are generated from the same progenitor cell type, or from distinct fate-restricted progenitors is a major open issue [30,44]. In other words, does one type of progenitor sequentially generate a series of different neurons by changing or adjusting its neurogenic potential over time? Or do different progenitors generate only one and always the same type of neurons during their neurogenic phase? This topic has been intensely

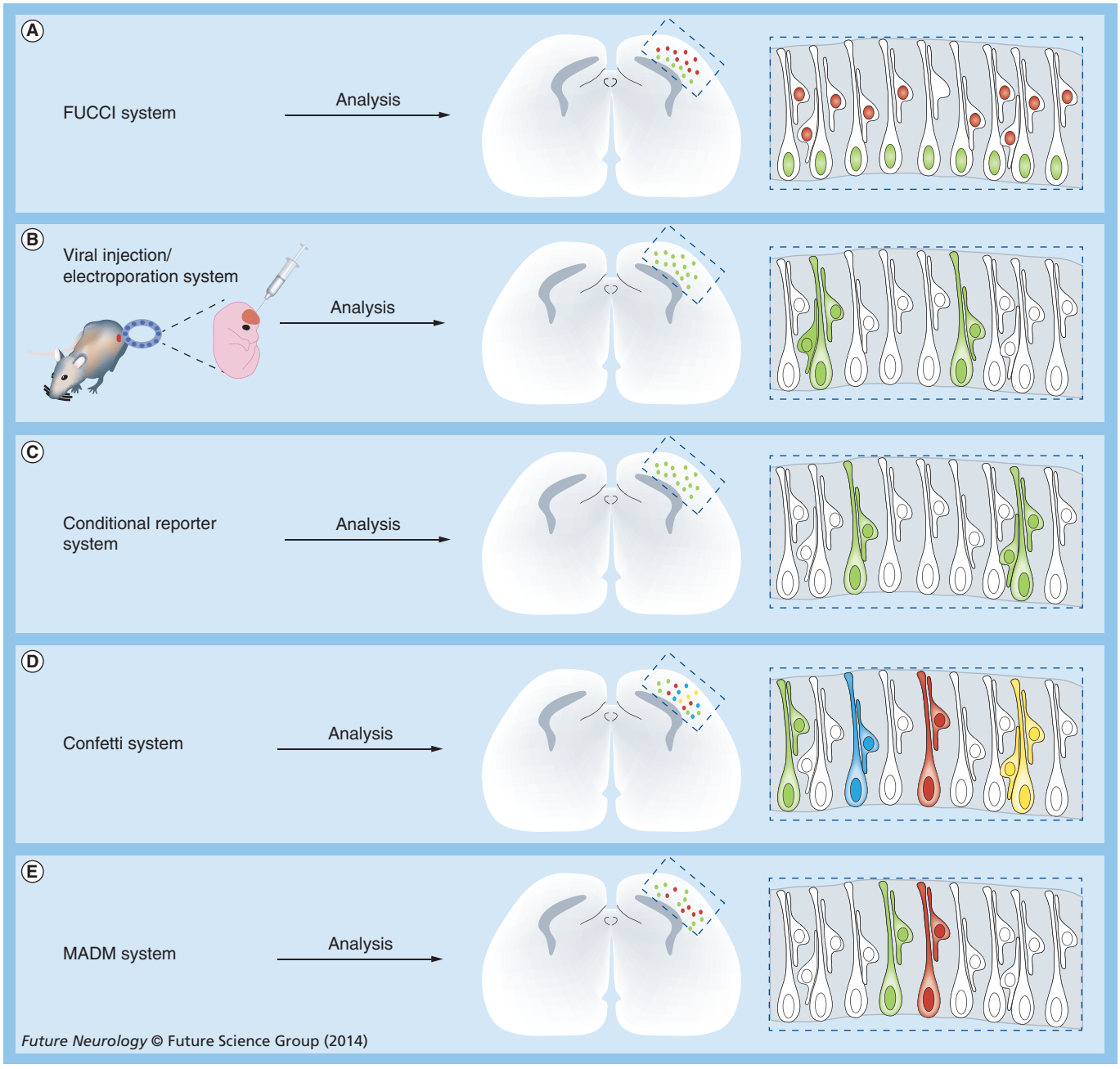

Figure 2. Lineage tracing systems for monitoring neurogenesis. Lineage tracing systems and expected outcomes in assays of cortical neurogenesis. **(A)** FUCCI-mediated tracking of neural progenitor cell cycle dynamics. FUCCI probes can distinctly mark nuclei of dividing progenitors in G1 phase (red) and those in S/G2/M phases (green). The red marker is retained in differentiating neurons as illustrated. **(B)** A virus or a plasmid can be used as vehicles to express a genetic cell marker (e.g., enhanced green fluorescent protein) in neural progenitors via *in utero* injection into the lateral ventricle and/or electroporation in mouse embryos. Low titers of virus (or concentration of electroporated plasmids) allow the labeling of sparse clones. **(C)** Sparse CreER-mediated recombination of a reporter allele in cortical progenitors and their clonal progeny. **(D)** Sparse induction of Confetti by using CreER-mediated recombination in neural progenitors may generate several individual but distinctly labeled (green, red, cyan or yellow) clones. **(E)** MADM-based two-color labeling of cortical clones. FUCCI: Fluorescent, ubiquitination-based cell cycle indicator; MADM: Mosaic analysis with double markers.

studied in the cerebral cortex by using diverse lineage tracing (determination of progenitor cell division pattern and distribution of clonally related neurons that build up the cortex) assays. Historically, lineage tracing methods including transplantation approaches, chimera generation,

retrovirus infection, electroporation or recombination-based fate mapping allowed mostly *post hoc* examination of clones [45–48]. Here, we focus on a set of conditions that also enable the monitoring of neurogenesis in real time by live imaging.

Lineage tracing by using retroviruses

In order to pursue high resolution single cell lineage tracing, progenitor stem cells should be marked in a sparse but permanent manner. In the most optimal case, the marker will be transferred to the whole lineal progeny and even after numerous rounds of cell division still robustly mark all daughter cells. Retroviruses encoding markers such as GFP or LacZ have been widely used to trace the progeny generated by a single neural progenitor cell **(Figure 2B)**. Low titers of retrovirus were used to infect embryonic mouse and rat cortices by *in utero* injection into the ventricles at different developmental stages. Initially, these paradigms were applied to study the composition of neural progenitor derived lineages, and the distribution of clonally related cells at postnatal stages [49–51]. A real breakthrough was achieved when GFP expressing retrovirus infections of neuronal progenitor cells was coupled with live imaging protocols to monitor neurogenesis by individual progenitors in real time [15,52,53]. These studies represent true landmarks since they for the first time demonstrated that RGPs are mitotically active throughout neurogenesis and produce neurons either directly or indirectly via intermediate progenitors (see also below). In addition to *in utero* application of retroviruses into the ventricle of developing embryos, a technically somewhat similar approach can also be used: *in utero* electroporation of plasmids encoding fluorescent markers such as GFP [54] **(Figure 2B)**. If carried out at low concentration and if plasmid DNA is carefully titrated, clonal labeling may be obtained and in combination with live imaging can reveal the dynamics of neurogenesis. *In utero* electroporation also has the advantage that multiple plasmids may be coelectroporated and gene knockdown experiments in dividing progenitors may provide mechanistic insight on a molecular functional level [55].

Lineage tracing by using recombinase-mediated reporter expression

A versatile approach which enables dynamic lineage tracing is based upon the use of the Cre recombinase/LoxP system. Ideally, Cre recombinase expression is controlled by a progenitor cell type-specific promoter. Cre recombinase-mediated excision of a transcriptional *Stop* sequence flanked by *LoxP* sites (commonly denominated as LSL), promotes marker gene expression from distinct reporters within genetically defined cell populations **(Figure 2C & 3A)** [42,56–58]. The modification of the Cre recombinase to render it inducible (CreER versions) greatly improved the analysis of neurogenesis and more generally the study of brain development and function [59]. CreER is a fusion of Cre recombinase with the ligand binding domain of the estrogen receptor (ER) and inducible by the estrogen analog tamoxifen (TM) [60]. The high efficiency of the CreER recombinase combined with conditional reporter alleles can generate large numbers of labeled progenitor cells and their downstream lineages. Thus one can analyze neurogenesis on a population level. However, by titrating the dose of TM, one can, in principle, also sparsely label clonal progenitor lineages in the developing cortex. Neurogenesis of individual progenitors can be assayed by using high resolution live imaging protocols. An important restriction of TM-mediated CreER induction is that the maximum TM dose that can be applied to pregnant female mice is limited (in order to avoid high abortion rate or infanticide). Alternatively, low titer infection with Cre recombinase-expressing retroviruses or electroporation of plasmids encoding Cre recombinase can also be used to sparsely induce marker gene expression and even at the clonal density **(Figure 2B & 3A)** [52,54]. Such efforts allowed, for instance, the unprecedented lineage analysis of RGPs generating cortical interneurons in the medial ganglionic eminence [28,29]. Both studies used the expression of Cre recombinase under the control of a transcription factor, Nkx2.1 expressed in the interneuron progenitor cells. In one case [28], the *Nkx2.1-Cre* driver was first crossed to a reporter mouse conditionally encoding the viral receptor. Next, low-titer retrovirus encoding GFP was applied by *in utero* intraventricular injection. The approach used by Ciceri and colleagues employed similar *in utero* retrovirus application but here the expression of the virally encoded marker was conditional and dependent upon Cre expression driven from the *Nkx2.1* locus [29]. Brown and colleagues then used dynamic live imaging of labeled RGPs generating cortical interneurons and analyzed their neurogenic properties.

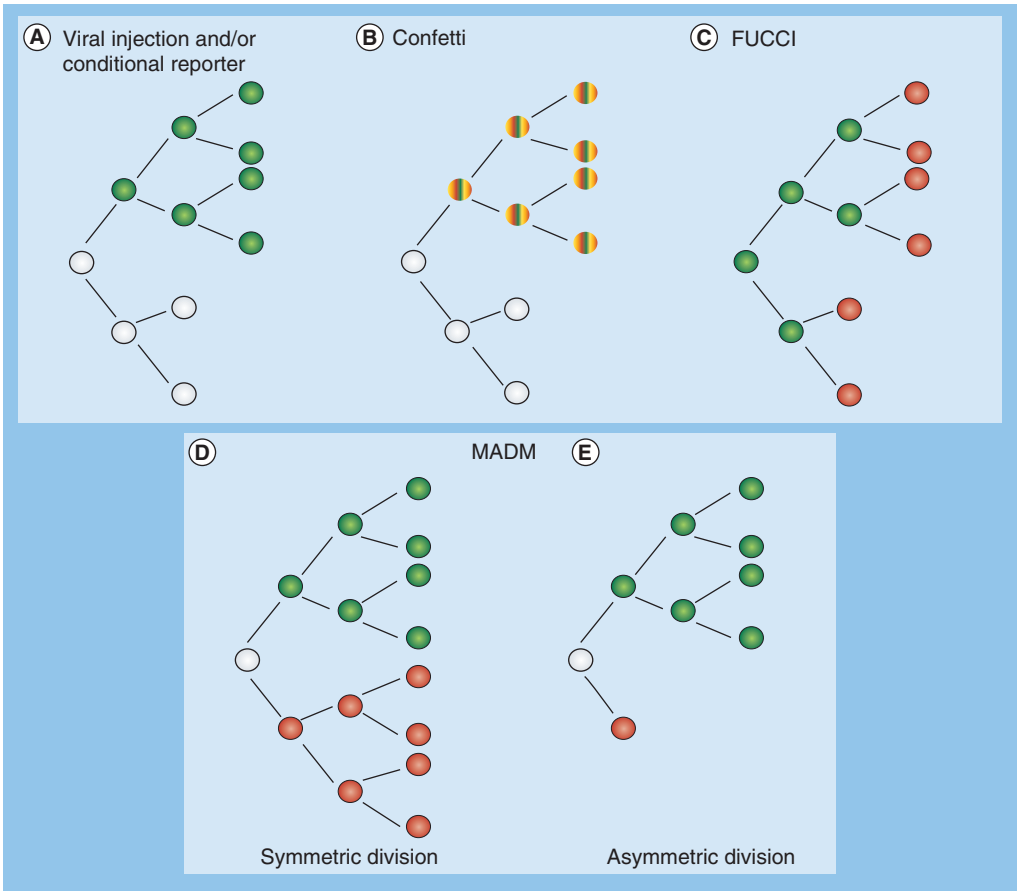

Figure 3. Comparison of expected cortical progenitor lineage trees. (A) Viral injection, plasmid electroporation or CreER-mediated sparse recombination of a reporter allele generates monocolor lineages. **(B)** CreER-mediated sparse recombination of the Confetti cassette can generate individual clones in distinct colors (presented as four color rainbow). **(C)** FUCCI activation generates cells that express green or red markers depending on the cell cycle stage. Progenitors that are in the G1 phase of the cell cycle (and postmitotic neurons) will be marked in red, while cells in the S/G2/M phase will appear in green. **(D & E)** MADM can generate two daughter cells expressing either the green or red marker. The mode of progenitor division can be inferred by comparison of the numbers of neurons in red and green, respectively. **(D)** Upon symmetric division, the green and red progeny of a MADM clone is equal in size (green/red ratio is 1). **(E)** Asymmetric progenitor divisions generate clones with different red and green cell numbers (green/red ratio >1 in this example).
FUCCI: Fluorescent, ubiquitination-based cell cycle indicator; MADM: Mosaic analysis with double markers.

Remarkably, these interneuron-producing RGPs closely resembled the RGPs in the dorsal telencephalic VZ generating excitatory projection neurons. In effect, RGPs located in the medial ganglionic eminence also exhibited IKNM, divided at the surface of the VZ and generated interneurons by asymmetric divisions [28].

The recombinase-based techniques described so far permit the tracing of one or just a few genetically defined progenitor lineages in one color. It would however be ideal to label multiple lineages at the same time and in distinct colors. The Brainbow strategy [61] can in principle accommodate such multicolor labeling of distinct progenitor lineages. Brainbow enables the conditional but stochastic expression of multiple fluorescent proteins from a single transgene, using Cre-mediated excision between pairs of incompatible lox sites. Combination of multiple copies of the Brainbow transgene allows the multiple fluorescent proteins to recombine in different ways and could, in principle, result in cell labeling with up to approximately 100 different fluorescent color hues [61]. Transgenic Brainbow cassettes

were recently inserted into the genomic *Rosa26* locus (also known as Confetti) [62]. Confetti was originally generated to trace intestinal stem cell progeny but can in principle be used to trace any progenitor lineage including the neuronal lineages by using appropriate Cre recombinase drivers **(Figure 2D & 3B)** [63].

Lineage tracing by using mosaic analysis with double markers

One of the most classic approaches for neurogenic lineage tracing involves the creation of genetically mosaic animals where two or more subpopulations of dividing progenitor stem cells have distinct genotypes. Several mosaic labeling systems have been established by using genetic approaches in distinct organisms [46,64–66]. Here, we focus on the mosaic analysis with double markers (MADM) technique in mice [64,67]. For MADM, two reciprocally split marker gene (GFP-tdT) cassettes are introduced at identical genomic loci on homologous chromosomes [67–69]. These split marker genes can be reconstituted in a LoxP/Cre recombinase-dependent manner in mitotically active neuronal progenitor stem cells via interchromosomal recombination. As a consequence of a MADM event, the two progeny of a neuronal progenitor cell are labeled in two distinct colors, GFP and red tdT, respectively. Since interchromosomal (trans) recombination rates are much (up to 100–1000-times) less frequent than intrachromosomal or *cis* recombination, as described in the above sections, MADM can generate very sparse individual progenitor cell clones in combination with temporally TM-inducible CreER drivers. Thus, in the most optimal scenario, a single MADM event can generate one two-colored clone originating from a single neuronal stem cell **(Figure 2E)**. In combination with 4D live imaging, MADM, can in principle provide unambiguous information on the birth dates of individual progenitor clones and their precise cell division patterns (i.e., whether they divide symmetrically or asymmetrically) [69] **(Figure 3D & E)**. An added MADM feature is the possible introduction of gene mutations allowing clonal two-color labeling with concomitant genetic manipulation. As such, these MADM applications lead to genetic mosaics with wild-type daughter cells labeled with one color (e.g., red) and homozygous mutant siblings with the other (e.g., green) in an unlabeled heterozygous environment. In summary, MADM can provide a quantitative optical readout of the proliferation mode (symmetric vs asymmetric) of progenitors at the single cell level **(Figure 3D & E)** and thus permit the determination of the developmental progenitor potential *in situ*. Future functional MADM analysis of candidate genes controlling the balance between expansive symmetric and neurogenic asymmetric progenitor cell division also promise the systematic dissection of molecular pathways regulating the division mode of neuronal progenitor stem cells in time and space.

Cortical stem & progenitor cells dynamically orchestrate cortical neurogenesis

The early embryonic neuroepithelium is composed of neuroepithelial stem cells from which all subsequent neural progenitor stem cells and their neuron lineages derive. In this section we will describe recent advances of how distinct progenitor classes orchestrate neurogenesis and point to open questions that will be discussed in the perspective below.

• Neuroepithelial cells

In all mammals the entire CNS is derived from a single densely packed pseudostratified layer of highly polarized neuroepithelial cells (NECs) **(Figure 1)**. The pseudostratified appearance of the neuroepithelium is a reflection of the IKNM [12]. NEC polarity is essential for neuroepithelial organization, maintenance and homeostasis [70]. Furthermore, polarized distribution of cell fate determinants in NECs appears to control the fine balance between symmetric and asymmetric progenitor divisions [70]. Such a balance is critical for the generation of the appropriate number of the more specialized RGPs and thus represents a fundamental requirement for producing the correct number of neurons in the adult cortex. In the initial stages of neurogenesis, NECs arrange the mitotic spindle parallel (division plane perpendicular) to the VZ and divide mostly symmetrically, thereby expanding the progenitor pool [32]. Perturbation of spindle anchoring to the lateral NEC walls leads to randomization of spindle orientation, precocious generation of neurons and apoptosis [71]. Lineage tracing experiments demonstrated that many NECs are also capable of dividing asymmetrically to produce neurons, before they generate RGPs [72–74]. Starting from E9 in mouse, NECs transform into radial glial cells [75]. It is currently, however, not entirely clear if this process involves an asymmetric NEC

division or merely a cellular and/or molecular rearrangement within the NECs. Thus, it will be important in the future to determine the precise cellular and molecular mechanisms controlling the transition from NECs to RGPs.

• Radial glia progenitor cells

The NEC to RGP transition is associated with changes in morphology, gene expression, and mode of division [75]. At the onset of neurogenesis, RGPs progressively switch from a symmetric proliferative to an asymmetric neurogenic mode of division that give rise not only to neurons, but also to other neural progenitor populations including IPs and oRGs **(Figure 1)** [14,15,27,53,76–78]. The precise molecular mechanisms controlling the fate of the RGP daughter cells are currently unclear but subject of extensive research. Lineage tracing coupled with live imaging and loss of function approaches as described above, hold the greatest potential to unravel the precise principles of asymmetric RGP divisions. Several models of how asymmetric RGP division can be achieved have been proposed. These models include parameters such as cleavage plane orientation, mitotic spindle-size asymmetry, dynamic distribution of polarity cues, differential inheritance of the basal process, cell cycle length and the asymmetric inheritance of the centrosome and/or ciliary membrane [79–82]. Regardless of the precise mechanisms in each model (except possibly the cell cycle length model), either cell fate determinants or entire organelles (e.g., centrosome, basal process) are asymmetrically segregated prior to mitosis and differentially inherited by the two distinct daughter cells. The 'fate determinant' segregation can rely entirely on intrinsic mechanisms or may depend on extrinsic factors that generate local differences inside the receiving cell. Similar to NECs, RGPs divide mostly symmetrically at early stages of neurogenesis (E9–E11 in mouse) with the mitotic spindle oriented in parallel to the VZ [75]. Perturbation of the spindle orientation at these stages by loss of function of for instance the evolutionarily conserved Lis1-complex leads to precocious RGP-mediated neurogenesis and depletion of the RGP pool [22,55,71,83]. Thus, the control of spindle orientation is critical in the first phases of neurogenesis to keep the balance between symmetric proliferative and asymmetric neurogenic radial glial cells divisions. As the rate of neurogenesis increases during mid corticogenesis, the balance shifts towards a predominant asymmetric mode of division with an increase in the production of neurons either by direct or indirect neurogenesis via IPs and oRGs **(Figure 4A)** [5]. Recent evidence suggests that the orientation of the mitotic spindle can have a direct influence on the fate of the daughter cell produced from RGPs [77,84]. In effect, artificial alteration of spindle orientation in dividing RGPs towards a more oblique mode of division by overexpression of the adaptor protein mouse INSC causes an increase in oRG production by RGPs in the IZ/SVZ of the mouse developing cortex **(Figure 4A)** [84]. Albeit the orientation of the mitotic spindle could fulfill a direct and instructive role in determining the cell fate of the daughter cells produced by RGPs, it has been shown that spindle-size asymmetry also coincides with asymmetric RGP division **(Figure 4B)** [79]. Neurons appear to originate preferentially from the larger, and the self-renewing RGP from the smaller spindle pole. Mechanistically, components of the planar cell polarity signaling pathway seem to regulate spindle-size asymmetry [79]. During RGP mitosis, the centrosome (i.e., both of its two centriols) is duplicated and it has been suggested that the asymmetric inheritance of the 'older' mother centrosome versus the 'younger' daughter centrosome could play an important role in asymmetric RGP division **(Figure 4C)** [85]. Although the precise mechanisms remain to be determined, dividing RGPs might employ asymmetric centrosome inheritance as a means of cellular memory whereby the mother centrosome is preferentially inherited by the self-renewing RGP and the daughter centrosome by the differentiating sister cell [85]. Furthermore, the ciliary membrane attached with the mother centriole is endocytosed at the onset of RGP mitosis and inherited by the self-renewing RGP. It has been proposed that the centrosomal association of the ciliary membrane could act in an instructive way to promote expedited ciliogenesis in the nascent RGP and thereby retaining important stem cell fate-promoting signaling from the ventricular cerebrospinal fluid **(Figure 4C)** [81,86].

• IPs & SNPs

IPs are located predominantly in the VZ/SVZ, express the transcription factor Tbr2 (also know as Eomes) and are present throughout cortical neurogenesis [87,88]. Single cell gene expression profiling and lineage tracing indicate that two (morphologically distinct) subtypes

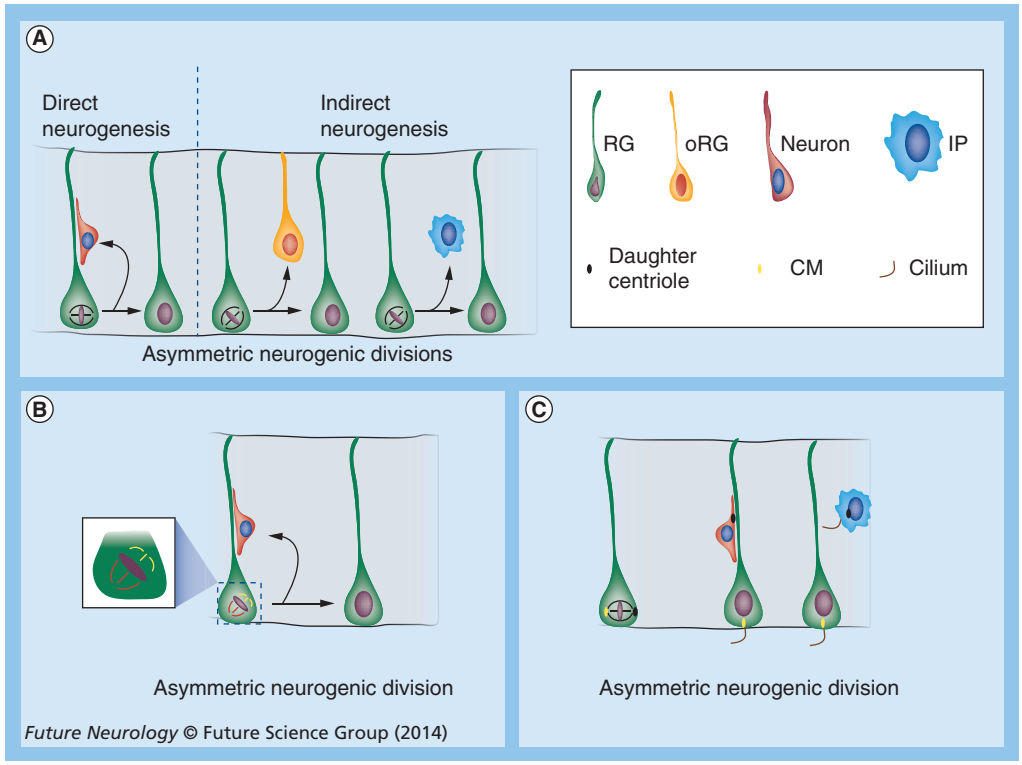

Figure 4. Mechanisms of asymmetric neurogenic progenitor divisions. (A) Orientation of the mitotic spindle appears to mediate direct or indirect (via IPs or oRGs) neurogenesis during asymmetric neurogenic radial glia progenitor (RGP) divisions. Oblique (or vertical, not illustrated) divisions preferentially give rise to neurons in an indirect manner. **(B)** Spindle size asymmetry correlates with the fate outcome of the daughter cells in asymmetric neurogenic RGP divisions. The daughter that originates from the larger spindle pole will become a neuron, while the other adopts a RGP fate. **(C)** Centrosome (i.e., centrioles) and CM inheritance could instruct daughter cell fates in asymmetric neurogenic RGP divisions. The cell that inherits the mother centriole associated with the ciliary membrane remnant becomes RGP, while the other adopts a neuron or IP fate.
CM: Ciliary membrane; IP: Intermediate progenitor; oRG: Outer radial glia; RG: Radial glia.

of IPs are present in the cortex: short radial apical IPs (with an apical process) in the VZ, and multipolar IPs in the SVZ **(Figure 1)** [88,89]. Both IP types derive from mitotic RGPs and their production is regulated by Tbr2 and cyclin D1/2 expression, RGP spindle orientation and cell cycle length [38,53,77,84,90,91]. Time lapse imaging *in vivo* has illustrated that apical IPs divide symmetrically to produce two neuron daughter cells. By contrast, IPs divide mostly symmetrically to generate two neurons but retain a limited capacity for self-renewal and thereby the potential to produce neuron doublets and/or quartets [53,88]. *In vivo* analysis of neurogenic IP divisions using the *Tis21*-reporter, and loss of function of *Tbr2* in knockout mice indicated that IPs may contribute to the generation of pyramidal neurons of all cortical layers [38,92,93].

SNPs are also located in the VZ [94,95] and can be distinguished from RGPs by tubulin α-1 expression. SNPs contact the ventricular surface through a short apical process, which is retracted during mitosis. Interestingly, SNPs and RGPs have different cell cycle kinetics and contribute distinctly to overall cortical neurogenesis. While RGPs often generate neurons through IPs, SNPs produce postmitotic neurons (at least layer IV neurons during mid-neurogenesis) through symmetric neurogenic divisions [94,95].

• Outer subventricular zone progenitors

Cerebral cortex expansion, a trademark of mammalian brain evolution, is associated with an increase in neuron numbers [3,5,96,97]. However, a larger cortex size may not only reflect a higher number of neurons but instead also an extended variety of neuron and/or progenitor cell lineages.

A new type of basally dividing progenitors (oSVZ progenitors or outer radial glia cells [oRGs]) has been recently identified in the oSVZ in humans [76], mice [77,78], ferret [98,99] and marmoset [100,101]. Unlike RGPs, oRGs are located far from the ventricle, with no apical contact to the luminal surface, but they possess a long basal process that extends to the pial surface [76]. Time lapse imaging of mice GFP-infected RGPs demonstrated that mice oRGs originate by asymmetric RGP division [78]. In the developing human neocortex oRGs are generated by oblique RGP divisions [102], consistent with the observation that mitotic spindle orientation alterations in dividing mouse RGPs (towards a more oblique mode of division) caused an increased oRG production [77,84]. Interestingly, dynamic live imaging revealed that oRGs undergo mitotic somal translocation although a functional significance has not yet been established [78]. Neurogenic oRGs produce neurons through asymmetric division but species-specific differences in oRG-mediated neurogenesis have been described. It appears that in the mouse, oRGs produce neurons directly and not via any IP [78]. By contrast, human oRGs typically divide asymmetrically, thereby self-renewing and producing a bipolar daughter cell (TAP cell) capable of proceeding through multiple rounds of symmetric self-renewing divisions before expressing IP markers [76]. These findings may suggest that oRGs and/or TAPs and their unique neurogenic amplifying properties may account for the greater expansion and gyrification of the human neocortex [5,96,97]. This hypothesis was recently addressed by selective artificial amplification of either the IP or oRG progenitor pools in mice and ferrets [103,104]. Interestingly, it was found that IP amplification tends to promote tangential expansion of the cortex while an increased population of oRGs can trigger radial expansion and gyrification [103,104]. Radial expansion at sites of prospective gyra appears to correlate with decreased expression levels of the putative transcriptional regulator Trnp1 in developing human fetuses [104]. It will be intriguing to determine the entire spectrum of species-specific differences in oRG neurogenic potential. Interestingly, in primates (macaque monkey) five distinct types of oRG progenitors were identified in the oSVZ [105]. These different oRG classes may indeed exhibit distinct neurogenic capacities and thus contribute to generating neuronal diversity during neocortical development. Future analysis of species-specific qualitative and quantitative differences in the cortical oRG progenitor pools promises unprecedented insights into the evolutionary mechanisms imposing on neurogenesis.

Conclusion & future perspective

The cerebral cortex is the largest structure of the human brain and composed of a sophisticated network of billions of neurons. It is not well understood how cortical neuronal circuits account for behavior and cognitive activity and how alterations in the cytoarchitecture may lead to neurological and psychiatric disorders or dementia. One approach, towards a more coherent mechanistic understanding, is to decipher the logic of neurogenesis and thus to determine the types and numbers of cortical neurons produced and how they successively build up the cortical entity during development. The cellular and molecular mechanisms controlling neurogenesis remain, however, still largely elusive and we here discuss a number of open key questions. Although several distinct classes of neuronal progenitors have been described [5,6], it is still not clear how many distinct types effectively exist and may contribute qualitatively and quantitatively to cortical neurogenesis. Thus, it will be important to identify all progenitor types and determine the cellular and molecular principles that regulate the establishment of progenitor diversity, and how this in turn can influence the generation of distinct neurons. Along these lines, several models have been proposed regarding the multipotency of RGPs [30,44]. It has been recently suggested that fate-restricted RGPs exist albeit most experimental evidence indicates that RGPs are multipotent (i.e., produce all types of cortical projection neurons) and progressively restrict their developmental potential regarding the type of neuron that they produce at any given time [30,44,63,106,107]. To clarify the above issue it will be revealing to decipher the quantitative and qualitative clonal units on an individual progenitor level. Are the neurogenic potentials of distinct progenitors at any given developmental time predetermined, implicating a rather deterministic mode of neurogenesis? On a cell biological level, how does a progenitor know whether to divide symmetrically to expand its pool or start producing neurons? Once in the neurogenic division mode: what are the signaling pathways that in a progenitor direct the choice whether neurogenesis occurs directly or

indirectly via IP and/or oRG? Lineage tracing approaches coupled with functional candidate gene analyses can help to contribute to our mechanistic understanding of the critical switch from expanding progenitor to asymmetric neurogenic divisions. On the circuit level, how does the lineage relationship of clonally related neurons derived from the same progenitor affect or instruct the specificity of neuronal connectivity in cortical circuits [108,109]? If we further project this question onto the stem and progenitor cells, one might ask whether distinct progenitors, and perhaps distinct progenitor lineages, encode prespecified neuron lineages, and

EXECUTIVE SUMMARY

Overview: cerebral cortex development

- The cerebral cortex is composed of an extraordinary number of projection and interneurons that originate from a diverse variety of neural stem cells, located in the ventricular zone (VZ)/subventricular zone (SVZ), during embryonic development.
- The precise cellular and molecular mechanisms regulating the functional neurogenic neural stem cell properties remain, to a large extent, elusive but disruptions in neurogenesis can lead to severe neurological disorders, including microcephaly, autism and epilepsy.

Monitoring neurogenesis

- Monitoring neurogenesis at high single progenitor cell resolution is essential for the quantitative and qualitative analysis of the neuronal lineages generated from individual classes of progenitors.
- Birth dating approaches using thymidine analogs or fluorescent transgenic probes, including *Tis21* and fluorescent, ubiquitination-based cell cycle indicator mice can reveal the precise cell cycle dynamics in proliferating progenitors.
- Sparse and/or conditional retrovirus-mediated progenitor cell infections can reveal radial glia progenitor (RGP) properties, such as morphology and cell division dynamics.
- Conditional transgenic reporter alleles in combination with TM-inducible CreER recombinases can sparsely mark proliferating progenitors and their lineages in a cell type-specific manner.
- Mosaic analysis with double markers can serve as a tool for lineage tracing experiments in real time and can be coupled to concomitant loss of gene function analysis.

Cortical stem & progenitor cells dynamically orchestrate cortical neurogenesis

- Neuroepithelial cells are the earliest type of neural precursor in the neural tube and divide mostly symmetrically to amplify the progenitor pool.
- RGPs derive from neuroepithelial cells and initially divide mostly symmetrically to expand their pool. As the rate of neurogenesis increases, asymmetric neurogenic divisions dominate. Mitotic spindle orientation, size asymmetry and inheritance of mother centrosome, together with ciliary membrane, may regulate the outcome of asymmetric RGP divisions.
- Short neural precursors are located in the VZ and intermediate progenitors in the SVZ and contribute to neuron production.
- Outer radial glias derive from RGPs in the VZ by asymmetric oblique divisions. Expansion of intermediate progenitors and outer radial glias correlate with the expansion and gyrification of the neocortex during evolution.

Future perspective

- Future efforts shall reveal how many distinct progenitor types exist and how they each contribute qualitatively and quantitatively to cortical neurogenesis.
- Lineage tracing approaches coupled with functional candidate gene analyses will help to contribute to our mechanistic understanding of cortical neurogenesis.
- Determination of the precise mechanisms controlling neurogenesis may provide a basis for prospective future embryonic stem cell-based approaches in the context of directed brain repair.

how different progenitors can, via their specific neuron output, contribute to shape the architecture of neuronal microcircuits in distinct functional cortical areas. Systematic monitoring of neurogenic parameters coupled with spatiotemporal lineage tracing and eventual physiological assessment of neuronal connectivity between clonally related neurons shall promise insights into the above issues. Lastly, how does the relative abundance of distinct progenitor cell types in different species drive the evolution of brain size and gyrencephaly? Progenitor types may have evolved new features and properties along the evolution of species. Thus, the systematic categorizing of progenitor types and their abundance in various species holds the promise of unprecedented insights not only into how progenitors build small or large brains in distinct species, but also into the underlying mechanisms shaping neocortex cytoarchitecture, morphology and gyrencephaly [5,96,97]. Loss-of-function analyses of human specific candidate genes by using cerebral organoids may provide new insights also into the underlying basis of neurodevelopmental diseases including microcephaly [110].

In conclusion, future efforts aimed at indexing progenitor diversity and decoding their individual neurogenic properties should provide a framework to rigorously address the above open questions. In a broader context, these endeavors can also contribute to our knowledge of cortical neuron and microcircuit specification and thus provide a possible foundation for prospective future embryonic stem cell-based approaches in the context of directed brain repair [111,112].

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Monitoring neurogenesis in the cerebral cortex: an update

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Activity evaluation: where 1 is strongly disagree and 5 is strongly agree.					
	1	**2**	**3**	**4**	**5**
The activity supported the learning objectives.					
The material was organized clearly for learning to occur.					
The content learned from this activity will impact my practice.					
The activity was presented objectively and free of commercial bias.					

1. According to the review by Drs. Postiglione and Hippenmeyer, which of the following statements about recent techniques for monitoring neurogenesis is **correct?**

- ☐ **A** Birth-dating approaches using guanidine analogues can reveal precise cell cycle dynamics in proliferating progenitors
- ☐ **B** Sparse and/or conditional progenitor cell infections mediated by DNA viruses can reveal properties of radial glia progenitors (RGPs)
- ☐ **C** Tamoxifen does not affect CreER recombinases
- ☐ **D** Mosaic analysis with double markers can be a tool for lineage-tracing experiments in real time and can be coupled to concomitant loss of gene function analysis

2. According to the review by Drs. Postiglione and Hippenmeyer, which of the following statements about recent advances in understanding the dynamics of neurogenesis is **correct?**

- ☐ **A** Neuroepithelial cells (NECs) appear relatively late in neurogenesis
- ☐ **B** RGPs give rise to NECs
- ☐ **C** Short neural precursors in the ventricular zone contribute to neuron production
- ☐ **D** Outer radial glial cells give rise to RGPs in the subventricular zone

3. According to the review by Drs. Postiglione and Hippenmeyer, which of the following statements about future directions for research and clinical implications regarding neurogenesis would **most** likely be correct?

- ☐ **A** It is well understood how alterations in the cytoarchitecture may lead to neurologic and psychiatric disorders or dementia
- ☐ **B** No evidence to date exists that RGPs are multipotent
- ☐ **C** Functional candidate gene analyses are unlikely to be helpful in understanding neurogenesis
- ☐ **D** Assessing progenitor diversity and individual neurogenic properties could provide a possible foundation for prospective future embryonic stem cell–based approaches to directed brain repair