Received: 27 July 2022 | Revised: 26 September 2022 | Accepted: 30 September 2022

DOI: 10.1002/hipo.23475

REVIEW

WILEY

# Cell population dynamics in the course of adult hippocampal neurogenesis: Remaining unknowns

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**Funding information**
Novartis Stiftung für Medizinisch-Biologische Forschung; University of Zurich, Grant/Award Numbers: FK-22-054, Suslowa-Postdoc-Fellowship

## Abstract

Neural stem cells (NSCs) generate new neurons throughout life in the mammalian hippocampus. The distinct developmental steps in the course of adult neurogenesis, including NSC activation, expansion, and neuronal integration, are increasingly well characterized down to the molecular level. However, substantial gaps remain in our knowledge about regulators and mechanisms involved in this biological process. This review highlights three long-standing unknowns. First, we discuss potency and identity of NSCs and the quest for a unifying model of short- and long-term self-renewal dynamics. Next, we examine cell death, specifically focusing on the early demise of newborn cells. Then, we outline the current knowledge on cell integration dynamics, discussing which (if any) neurons are replaced by newly added neurons in the hippocampal circuits. For each of these unknowns, we summarize the trajectory of studies leading to the current state of knowledge. Finally, we offer suggestions on how to fill the remaining gaps by taking advantage of novel technology to reveal currently hidden secrets in the course of adult hippocampal neurogenesis.

**KEYWORDS**
adult neurogenesis, cell death, dentate gyrus, neural stem cell, neuronal integration

## 1 | INTRODUCTION

The ability of somatic stem cells to proliferate and differentiate into mature cell types enables them to orchestrate development as well as to support homeostasis and repair of adult tissues. In the central nervous system, the production of new neurons from stem- or progenitor cells was assumed to end after early postnatal development. Therefore, the 1960s observation of the incorporation of radioactive nucleotides into cells within the adult rat hippocampus (Altman & Das, 1965), suggestive of the existence of neural stem cells (NSCs) that produce new neurons throughout adulthood, was met with substantial skepticism. Fast forward to the 1990s, in vitro cultures of NSCs isolated from rodent brains as well as progress in immunohistochemistry and microscopy (Gross, 2000; Kuhn et al., 2018; Palmer et al., 1995, 1997; Reynolds & Weiss, 1992) confirmed the presence of neurogenic precursors within distinct regions of the adult mammalian brain. More recently, advances in transgenic mouse models and in vivo microscopy have provided unequivocal evidence of adult neurogenesis in two zones of the rodent brain: the ventricular-subventricular zone (V-SVZ) lining the lateral ventricles from where newborn cells migrate into the olfactory bulb (OB) (Obernier & Alvarez-Buylla, 2019) and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Denoth-Lippuner & Jessberger, 2021).

Mechanisms and regulators of adult hippocampal neurogenesis have been reviewed recently (Denoth-Lippuner & Jessberger, 2021; Kuhn et al., 2018; Mira & Morante, 2020; Urbán et al., 2019). Therefore, only a brief account is given here (Figure 1), after which we will instead highlight gaps in our understanding of the cellular dynamics in the adult hippocampus. Considering that adult hippocampal neurogenesis remains partially controversial in the human brain (Duque et al., 2022; Kempermann et al., 2018; Moreno-Jiménez et al., 2019; Sorrells et al., 2018; Terreros-Roncal et al., 2021), we here focus on the rodent hippocampus.

  

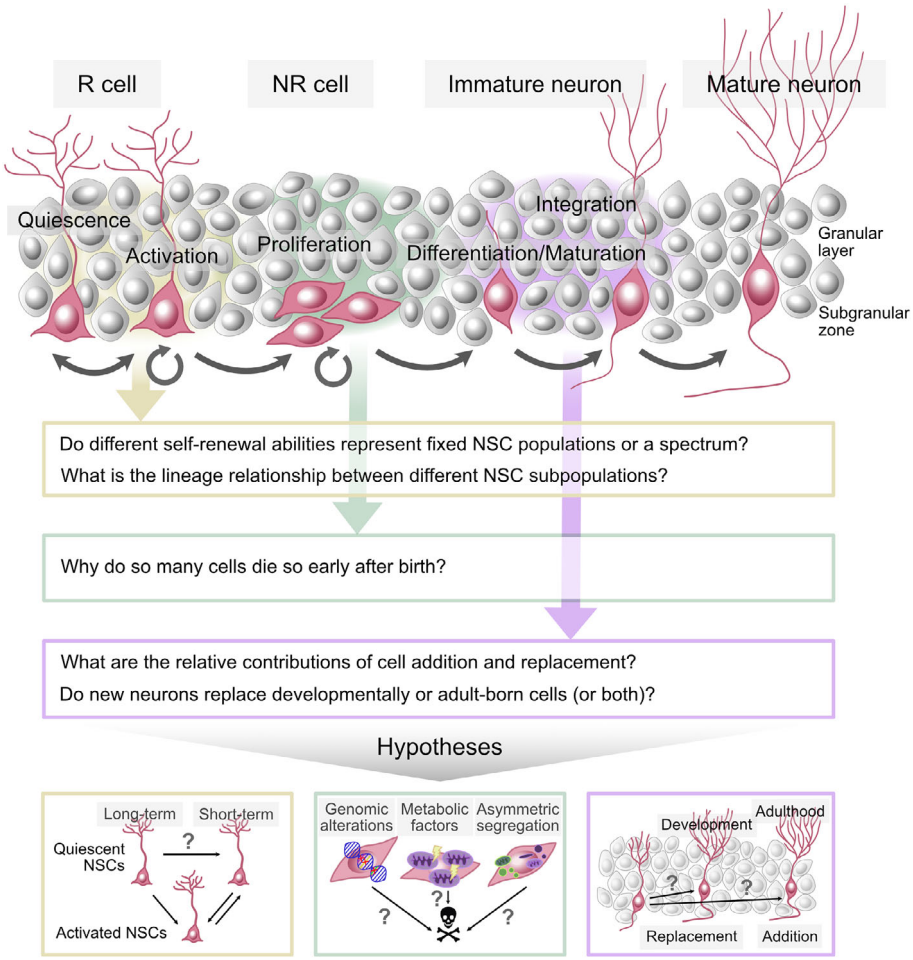


**FIGURE 1** Remaining unknowns during the course of adult hippocampal neurogenesis. A graphical summary of the cellular trajectory during adult hippocampal neurogenesis is provided: Activation of quiescent neural stem cells (R cells) in the subgranular zone of the dentate gyrus generates proliferating NR cells which exit the cell cycle and differentiate into mature neurons that integrate into the preexisting circuitry. This review discusses three gaps in our understanding of this process: Neural stem cell dynamics, early cell death, and neuronal integration dynamics. Shading and arrows indicate which cellular stage each of these unknowns relates to, with colored boxes highlighting the main unanswered questions. Small boxes below the questions illustrate current hypotheses for each of the three topics.

NSCs in the rodent DG resemble radial glia cells (the neural progenitor cells in the embryonic brain, Malatesta et al., 2003) in that they extend a radial process into the granule cell layer. They also display certain morphological as well as gene expression features similar to astrocytes (Filippov et al., 2003; Llorens-Bobadilla et al., 2015; Seri et al., 2001; Shin et al., 2015; Suh et al., 2007). Due to these characteristics, hippocampal NSCs are aptly named radial glia-like cells (R cells or type I cells). R cells are largely found in a quiescent state (Urbán et al., 2016). Upon activation, they give rise to nonradial glia-like progenitor cells (NR cells or type II cells) which, after further rounds of cell division, exit the cell cycle and commit to neuronal differentiation (Bottes et al., 2021; Kempermann et al., 2004; Pilz et al., 2018; Urbán et al., 2019). While immature, newborn neurons migrate tangentially and then radially into the granule cell layer, whereby extensive neurite growth enables integration into the existing network (Sun et al., 2015; Wang et al., 2019; Zhao et al., 2006). Around 2 weeks after birth, the first synapses are formed and cells receive extrasynaptic and synaptic GABAergic and glutamatergic inputs (Denoth-Lippuner & Jessberger, 2021). This is key to survival, as it drives a selection process in which failure to receive input via the NMDA receptor 1 (NR1) results in cell death (Tashiro et al., 2006). The subsequent maturation stage, which lasts for 4–6 weeks, is characterized by transient high excitability and plasticity as well as further dendritic and axonal changes (Gonçalves et al., 2016; Huckleberry & Shansky, 2021). At around 6–8 weeks of cellular age, adult-born neurons are considered fully mature; however, adult-born neurons have been shown to mature way past that age (Beining et al., 2017; Cole et al., 2020). Functionally, newly integrated neurons have been implicated in a range of hippocampus-related processes including spatial memory, behavioral pattern separation (the encoding of very similar experiences into discrete representations), cognitive flexibility, and forgetting of previous experiences (Akers et al., 2014; Anacker & Hen, 2017; Deng et al., 2010; Gao et al., 2018; Ko & Frankland, 2021; Lieberwirth et al., 2016; Miller & Sahay, 2019; Sahay et al., 2011). Within this framework of knowledge, several important gaps remain. Here, following the course of neurogenesis from NSCs to mature neurons, we discuss three fundamental such unknowns, concerning the dynamics of NSC activity, early cell death, and neuronal integration (Figure 1).

## 2 | NSC POPULATION DYNAMICS

Stem cells are defined by two properties: self-renewal (the ability to divide and produce another stem cell) and multilineage differentiation (the capacity to differentiate into at least two different cell types)

(Weissman, 2000). A key area of stem cell biology pertains to describing, for each adult system, how the balance between stem cell self-renewal and differentiation maintains homeostasis. While differentiation is relatively straightforward to quantify, self-renewal is more complex. It can be achieved at the cellular level by asymmetric division (producing one stem and one differentiating cell) or at the population level by the combination of symmetric divisions that either generate more stem cells or consume them by differentiation. Long-term self-renewal may also be achieved when stem cells shuttle between active and quiescent states. The interest in DG stem cell dynamics has largely been motivated by the reproducible observation that adult neurogenesis drops with age (Kuhn et al., 1996). This could be due to adult neurogenesis being driven fully or partially by a stem or progenitor cell pool which depletes by differentiation. Alternatively, NSCs could enter a more quiescent state with age. This could include the whole DG NSC population or only a subset of cells. Early studies aimed at investigating NSC dynamics took advantage of nucleotide analog labeling and retrovirus-based cell lineage tracing. However, these methodologies are population-based and cannot resolve NSC dynamics to the single clone level (Ming & Song, 2005).

A decade ago, two seminal studies attempted to describe the modes of activation of putative DG stem cells by utilizing Cre recombinase-based reporter mouse lines. Encinas and colleagues performed nucleotide analog labeling in a Nestin-Cre-GFP mouse line, whereby Nestin is an intermediate filament expressed by neural stem and progenitor cells (Encinas et al., 2011). By quantifying the pools of quiescent and activated NSCs at different times after labeling on a population level they concluded that upon leaving the baseline state of quiescence, activated NSCs undergo several rapid rounds of asymmetric divisions before terminally differentiating into astrocytes and thus leaving the pool of stem cells. Such a "disposable stem cell" model could partially explain the observations of age-related increase in astrocytes as well as decrease in neurogenesis (Encinas et al., 2011). However, a study published contemporaneously by Bonaguidi and colleagues came to a different conclusion (Bonaguidi et al., 2011). By using clonal labeling achieved by low dose tamoxifen injection into an inducible Nestin-CreER$^{T2}$-driven GFP reporter line, they were able to trace the fate of individual NSC clones. Their data showed that NSCs in the DG display a range of behaviors with R cells exhibiting multiple modes of activation, which includes symmetric and asymmetric division resulting in self-renewal as well as production of neurons and astrocytes. Furthermore, long-term lineage tracing of clones suggested occasional return to quiescence after activation, indicative of long-term self-renewal (Bonaguidi et al., 2011).

The latter finding was also supported by a subsequent study based on genetic manipulation of HECT, UBA, and WWE Domain Containing 1 (HUWE1), an E3 ubiquitin ligase that targets the neural stem- and progenitor marker Achaete-Scute Family BHLH Transcription Factor 1 (Ascl1) for degradation, in neurogenic NSCs (Urbán et al., 2016). Urban and colleagues observed BrdU retention indicative of return to quiescence of previously cycling NSCs (Urbán et al., 2016). Evidence for the existence of long-term renewal of mouse DG NSCs was thus amounting.

It took a further technological advance, intravital imaging, to start reconciling conflicting results by direct observation of individual stem cell behavior within the intact hippocampal niche over extended periods of time. First, chronic in vivo imaging was performed in an Ascl1-CreER$^{T2}$-driven tdTom reporter line. This study by Pilz and colleagues found that NSC activation results in a burst of neurogenic activity which is followed by terminal neuronal differentiation. Their observation was in line with Encinas and colleagues and supported a model whereby NSC activation initiates a developmental-like program that resembles cellular principles observed during the formation of the mouse cortex during embryogenesis (Gao et al., 2014; Pilz et al., 2018). Notably, a second in vivo imaging study, conducted by Bottes and colleagues, provided a more comprehensive view by complementing the Ascl1-CreER$^{T2}$-tdTom data with a Gli1-CreER$^{T2}$-tdTom mouse line, which, by driving Cre recombinase expression from the endogenous upstream promoter/enhancer elements of GLI family zinc finger 1 (Gli1), enables labeling of mostly quiescent NSCs (Ahn & Joyner, 2005). In this study, both return to quiescence after division as well as prolonged self-renewal of up to 100 days was observed. Together with single cell RNA-sequencing analyses, the data suggested a high diversity of NSC behaviors with Ascl1- and Gli1-targeted cells displaying short- and long-term self-renewal, respectively (Bottes et al., 2021). This picture of a range of NSC behaviors is supported by a recent study using clonal analysis in 2–12 month-old Nestin-CreER$^{T2}$-GFP and Ascl1-CreER$^{T2}$-GFP mice which suggests that Ascl1 NSCs act as short-term NSCs that deplete rather quickly following several cycles of division while Nestin-expressing NSCs retain their self-renewal capabilities with age, thereby acting as long-term NSCs (Ibrayeva et al., 2021). Slightly different conclusions were drawn from a study using nucleotide analog labeling combined with immunostaining for cell cycle markers in mice up to 18 months old. The data by Harris and colleagues (Harris et al., 2021) suggest that NSCs in juvenile mice undergo fast-depleting cycles of activation as postulated by the disposable stem cell model (Encinas et al., 2011; Pilz et al., 2018). With advancing age, however, heterogeneity emerges, whereby some NSCs acquire the capacity to return to quiescence, thereby achieving long-term self-renewal (Bonaguidi et al., 2011; Bottes et al., 2021; Urbán et al., 2016). A strength of this work is that it was largely carried out without using NSC marker reporter lines, meaning that NSC behavior was assessed purely on the basis of nucleotide analog incorporation and cell cycle markers. In this way, a representative image of the whole NSC population may have been captured (Harris et al., 2021). Further, considering the age of the mice used in previous studies—young adults (8–10 weeks) supporting disposable stem cell behavior (Encinas et al., 2011; Pilz et al., 2018) and more mature mice when reporting return quiescence (3–4 months and up to 1 year, respectively) (Bonaguidi et al., 2011; Urbán et al., 2016), this study may render conflicting models compatible.

Taken together these data confirm that the rodent DG contains bona fide stem cells that self-renew, but that their behavior is heterogeneous: upon activation some NSC clones produce a burst of progeny before depleting by differentiation. Others can divide and

thereafter return to quiescence, thereby providing long-term self-renewal that may support lifelong neurogenesis. A number of important questions remain. First, the majority of studies discussed here took advantage of transgenic lines enabling labeling of specific subsets of NSCs (e.g., those expressing Ascl1, Nestin, Gli1, etc.). Therefore, it remains to be seen if the acquired data is representative of the whole NSC pool. In this regard, unbiased studies like that of Harris and colleagues are important (Harris et al., 2021). Furthermore, it will be important to establish whether the heterogeneity in behaviors is driven by specific fixed populations that are established during development or whether it arises from a population of NSCs that sit on a quiescence-activity spectrum. Indeed, recent work suggests that all hippocampal progenitor cells share a common, HOPX-expressing embryonic progenitor cell (Berg et al., 2019). In addition, a lineage relationship between different subpopulations is conceivable, whereby certain NSC populations with long-term self-renewal abilities give rise to those with only short-term self-renewal capacity (e.g., Gli1 cells producing Ascl1 cells). Transgenic inducible Cre mouse models allowing for an intersectional genetic approach will be the key to answering some of these questions. Progressing down the course of neurogenesis we will next discuss a well-documented process that occurs shortly after NSC activation but that is still veiled in mystery: the early wave of cell death.

## 3 | CELL DEATH DYNAMICS

A number of adult stem cell systems prominently feature a quality control step during which aberrant immature cells are removed. This includes the adult testis where 75% of germ cells die during differentiation (Dunkel et al., 1997) and the hematopoietic system where autoreactive B-cells are eliminated (Lu & Osmond, 1997).

Programmed cell death, or apoptosis, as this deliberate killing of a cell within a multicellular organism is called, was first described in the 19th century and coined in the 1970s (Kerr et al., 1972; Vogt & Vogt, 1842). Apoptosis encompasses two pathways, extrinsic and instrinsic, respectively. The former is triggered by death receptor ligands while the latter is initiated by the formation of mitochondrial pores by the pro-apoptotic protein Bax. Both pathways ultimately converge on the activation of the effector caspases (cysteine-dependent aspartate-directed proteases, which ensure that cellular components are degraded in a controlled fashion (Fan et al., 2005). Caspase activation is also required for the exposure of phosphatidylserine on the cellular surface, which acts as an "eat me" signal for immune cells that then phagocytose affected cells (Cowan et al., 1984).

Early observations of neural development suggested that a large fraction of newly generated neurons die by apoptosis, likely as a consequence of competition among innervating neurons (Oppenheim, 1991). This finding prompted researchers to investigate whether cell death played a role in the related process of adult hippocampal neurogenesis. Indeed, studies on cellular morphology confirmed the presence of dying cells in the postnatal rat DG (Gould et al., 1991). These findings were supported by studies showing a decrease in the number of new cells quantified between 1 and 2 weeks after radioactive nucleotide labeling. However, it was initially not possible to determine whether this effect was caused by cellular demise or label dilution (Cameron et al., 1993). More detailed nucleotide analog labeling experiments performed in the 2000s confirmed that cell death indeed occurs during adult hippocampal neurogenesis, whereby a large fraction of newborn cells in the SGZ die within the first weeks of their life (Biebl et al., 2000; Cameron & McKay, 2001; Dayer et al., 2003; Kempermann et al., 2003, 2004; Mandyam et al., 2007). Contemporaneously, BrdU labeling as well as staining for apoptosis markers performed in a constitutive Bax knockout mouse model suggested that Bax, and therefore apoptosis, may play an important role in eliminating postmitotic adult-born cells (Sun et al., 2004). Furthermore, it was shown by retrovirus-mediated genetic deletion of the NMDA receptor 1 that the survival of new neurons is competitively regulated during the third week after neuronal birth, whereby relative levels of NR1 activation determine if a cell survives or dies (Tashiro et al., 2006). Taken together these studies suggest that during adult hippocampal neurogenesis, apoptosis occurs for some newborn cells at the immature neuronal stage, most likely due to a lack of synaptic input (Ma et al., 2009; Tashiro et al., 2006). This model was expanded by Sierra et al. (2010). Their study included BrdU labeling performed as cumulative and pulse-chase paradigms combined with immunofluorescence staining of apoptotic cells as well as microglia (the resident macrophages of the brain). It revealed that, in addition to the third week of life, there is also a period of cell death very early in the neurogenic cascade, about 1–4 days after cell birth. Notably, their data revealed that apoptotic cells are removed very rapidly (Sierra et al., 2010). Intriguingly, a contemporary study suggested that immature neurons may also act as phagocytes engulfing neural progenitor cells (Lu et al., 2011).

The description of an early wave of cell death from static data was later corroborated by live imaging in Ascl1-CreER$^{T2}$-tdTom mice, whereby it was found that a large fraction of cells undergoes death around 1–4 days after their birth (Pilz et al., 2018). Interestingly, death seemed to be unevenly distributed between lineages, suggestive of the involvement of hereditary factors. Dead cells were also interspersed between live cells, further hinting at an intrinsic mechanism as opposed to selection mechanisms that may purely depend on diffusible pro-survival factors (Pilz et al., 2018).

In parallel to descriptions of timing, the functional roles of apoptosis during adult hippocampal neurogenesis were also investigated. Using nucleotide analog labeling and scoring apoptosis by markers such as nuclear changes it was found that spatial learning may require both the addition as well as the removal of new hippocampal neurons (Dupret et al., 2007). Reduction of apoptosis has also been explored as a means of improving hippocampal function. Conditional genetic ablation of the pro-apoptotic protein Bax using a Nestin-CreER$^{T2}$-Bax$^{fl/fl}$ mouse line markedly increased the survival of newborn cells. This resulted in enhanced contextual fear discrimination learning, suggestive of improved behavioral pattern separation (Sahay et al., 2011).

In summary, it is now clear that adult hippocampal neurogenesis includes two prominent waves of cell death: an early one occurring

only a few days after cell birth and a later one around the third week of cellular age. While it seems clear that the latter serves to remove immature neurons that have failed to receive inputs from the existing network, the former is veiled in mystery. Findings by Pilz and colleagues hint at the involvement of intrinsic factors (Pilz et al., 2018). However, their identity remains unknown. Genetic alterations, which can be associated with a variety of mechanisms including retrotransposon activity (Baillie et al., 2011; Muotri et al., 2005) or transcription (Suberbielle et al., 2013), are readily detected in the adult DG (Lodato et al., 2015, 2018; McConnell et al., 2013, 2017). This genetic diversity has been linked to functional neuronal diversity (McConnell et al., 2017). However, the integration of cells carrying excess mutations could be harmful. It is thus tempting to speculate that NSC activation causes an upregulation of mechanisms generating genetic alterations with the early death wave removing any overly mutated cells. Equally, asymmetric segregation of damage during neural stem- and progenitor cell division has been reported (Bin Imtiaz et al., 2021; Moore et al., 2015). It is thus possible that the daughter cells receiving the bulk of such factors would manifest as the early cell death wave. Finally, apoptosis may be related to metabolic factors. For example, neuronal differentiation requires cells to switch from a predominantly glycolytic metabolism to oxidative phosphorylation (Iwata et al., 2020; Zheng et al., 2016). Future work using single cell genomics, detailed metabolic measures, and intravital imaging with high temporal and spatial resolution will be needed to test if these hypothetical mechanisms underlie the early wave of cell death during adult hippocampal neurogenesis. For our third and final knowledge gap, we progress down the neurogenic lineage toward the more mature cellular stages to discuss which aspects of neuronal integration await further clarification.

## 4 | DYNAMICS OF NEURONAL ADDITION AND REPLACEMENT

A fundamental challenge faced by adult stem cell systems is the appropriate integration of newly generated cells into the preexisting tissue. In some organs, such as the gastrointestinal tract, this occurs as constant cellular turnover, whereby old cells die at the tip of glands and are replaced by new cells that are shuttled upwards in a conveyor-belt like fashion (Quastler & Sherman, 1959). In others, such as the muscle, adult stem cells can also operate in growth mode, adding more cells to the system (Seale et al., 2001). Considering these two different scenarios, adult hippocampal neurogenesis could cause the DG to grow over time or it could be counterbalanced by coordinated removal of older cells in the same areas. Such turnover could replace developmentally-born neurons, adult-born cells, or both. Of note, for the purpose of this review, developmental neurogenesis in the mouse includes embryonic (starting around embryonic days 10–14) as well as early postnatal neurogenesis (around postnatal day 6–10) (Nicola et al., 2015; Overstreet-Wadiche et al., 2006). Early attempts at elucidating the dynamics of new neuron integration focused on DG size. Volumetric measurements performed in Wistar rats showed that the DG grows linearly over the first year of life (Bayer et al., 1982; Boss et al., 1985). Furthermore, radioactive nucleotide labeling in rats allowed to survive for up to 450 days suggested that new cells are added to the deep aspect of the granule cell layer but that there is no concomitant loss of earlier-generated cells. Together, these studies dismissed the notion of adult granule cell turnover (Crespo et al., 1986). After a period of drought, the subject was taken up again in the 2000s, with new technology enabling more detailed analyses. Kempermann and colleagues used BrdU labeling and immunofluorescence staining in mice to demonstrate that newborn neuron numbers stabilize at 4 weeks after cell birth and that adult born neurons persist for at least 11 months (Kempermann et al., 2003). A contemporaneous study, also based on BrdU labeling, came to similar conclusions with the additional finding that around 26% of newborn neurons labeled at postnatal day 6, so during the peak of dentate gyrus development, seemed to die over the course of the first 6 months of a rat's life (Dayer et al., 2003). This corroborated the idea of continuous addition of adult-born neurons but also hinted at potential replacement of developmentally-born neurons. At the same time, computational modeling suggested that in absence of compensatory mechanisms, continuous addition of excitatory neurons could be detrimental to hippocampal circuit function and cause seizures (Becker, 2005; Meltzer et al., 2005). The models suggested that turnover of neurons may have a positive effect on hippocampal function in that it may minimize interference between highly similar items during recall (Becker, 2005) and reduce the rate of false positive neuronal activation, albeit at the cost of loss of older memories (Meltzer et al., 2005).

Support for neuronal turnover within adult-generated granule cells came from population labeling using inducible Cre mouse lines with quantification of NSC progeny up to 9 months after reporter induction (Lagace et al., 2007; Ninkovic et al., 2007). Neurons born in GLAST-CreER$^{T2}$ adult mice seemed to persist for 9 months; however, following an initial increase in population size, a plateau of labeled cell numbers was observed. This indicated an equilibrium between dying and integrating adult-born neurons (Ninkovic et al., 2007). Similar results were also obtained using the Nestin-CreER$^{T2}$ line, in which an increase in adult-born neurons in the first few months after reporter induction was followed by a plateau at 6 months. The early increase was abolished by ablation of adult neurogenesis with a diphtheria toxin allele (Imayoshi et al., 2008). These results support both the notion of some degree of addition of extra neurons early during rodent life as well as turnover of adult-born granule cells at later stages.

This body of work is complemented by two recent studies. Cahill and colleagues used BrdU injection into rats at postnatal day 6, thereby labeling developmentally-born new neurons. Quantification of cell numbers failed to detect appreciable death between 1–8 weeks post BrdU injection. However, a significant loss of labeled cells between the 2 and 6 month time points suggested delayed death of developmentally born neurons, supporting some level of replacement of developmentally born granule cells (Cahill et al., 2017). Imura and colleagues used a GFAP-Cre mouse line to investigate spatial as well

as temporal aspects of postnatal new DG neuron addition. Importantly, as glial fibrillary acidic protein (GFAP)-expressing progenitors seem absent during embryonic development but become predominant during the postnatal period (Imura et al., 2003; Seki et al., 2014), this model enables the study of postnatal neurogenesis. Quantification of labeled neurons in mice aged 8 days up to 20 months revealed that the majority of DG neurons are derived from progenitors arising early in life. Importantly, however, a steady increase in cell numbers was observed in the inner granule cell layer (in line with previous studies; Crespo et al., 1986; Kempermann et al., 2003; Mathews et al., 2010), while numbers seemed to plateau in the outer layer (Imura et al., 2019). In conjunction with the finding of an age-related increase in labeled cells, this study supports a model whereby early born cells predominate in the mouse DG, but adult-born neurons accumulate in the inner granule cell layer throughout life (Imura et al., 2019).

In summary, there are three models of adult granule cell integration dynamics and studies have generated evidence for each of them. Continuous new neuron addition resulting in DG growth is well supported by rat studies but the mouse DG seems to accumulate granule cells with age, too (Bayer et al., 1982; Boss et al., 1985; Imura et al., 2019; Kempermann et al., 2003). The finding that many neurons generated during development die in adulthood hints at their replacement by adult-born cells (Cahill et al., 2017; Dayer et al., 2003). Finally, quantification of adult-born neuron numbers suggests that after an initial phase of neuronal addition, adult-born cells replace each other, resulting in a steady population size (Imayoshi et al., 2008; Ninkovic et al., 2007).

Several explanations for these seemingly conflicting results are plausible and should be investigated. Firstly, there are substantial differences in cell maturation and death dynamics as well as responses to environment between rats and mice (Snyder et al., 2009). Secondly, as neurogenesis drops significantly with age, any hypothetical increase in the labeled adult-born neuron population in models such as the Nestin-CreER$^{T2}$ mouse is very challenging to quantify as it may be so small that it may be "lost in the noise" and therefore misinterpreted as a plateau. The same holds true for cell death (e.g., of developmentally- or adult-born cells), which is very hard to quantify due to fast removal of apoptotic cells.

Novel experimental strategies are thus required to paint a full picture of adult hippocampal cell integration dynamics. Specifically, individual cells have to be followed over time within the intact hippocampus. For example, live imaging could be performed after differential labeling of developmental- and adult-born cells by retroviral (as used by [Laplagne et al., 2006]), or genetic (e.g., combining Flp-FRT and Cre-lox systems) means. Regardless of which cells are found to be replacing each other, it will then also be interesting to determine whether cell turnover represents simple conveyor-belt like substitution of cells that have died (such as for example in the intestine; Quastler & Sherman, 1959) or active cellular competition (Wagstaff et al., 2016).

## 5 | CONCLUDING REMARKS

As an intriguing feature of brain plasticity, adult hippocampal neurogenesis has fascinated several generations of neuroscientists and stem cell biologists. Driven by curiosity about a fundamental biological process as well as the hope of future therapeutic applications (for example in the context of neurodegenerative diseases), the field has made tremendous progress in the last decades. Largely this has been fuelled by technological advances, whereby from initial nucleotide labeling and autoradiography, researchers can now take advantage of immunofluorescence staining, transgenic mouse models and most recently single cell RNA-sequencing and intravital imaging. This ever-increasing sophistication in methodology allows for more complex biological questions to be answered. Indeed, fledging technologies with high cellular resolution such as spatial transcriptomics as well as various single cell-omics such as epigenomics, metabolomics, and proteomics are likely to push the field forward in the coming years. Addressing the three unresolved questions discussed in this review will heavily rely on new methodologies, especially live imaging in transgenic mice. Long- and short-term NSC populations and their lineage relationships may be resolved using intravital imaging in mouse intersectional genetic models (combining several separate recombinase transgenes driven by different promoters). The cause of the early cell death wave may be investigated by assessing the transcriptional, metabolic, and genomic profile of single cells in Bax$^{fl/fl}$ reporter mice. Finally, the dynamics of neuronal integration may be resolved by chronic intravital imaging in mice in which developmentally- and adult-born neurons are differentially labeled. Of course, while pushing the boundaries of our knowledge, such studies are, as is always the case in the scientific endeavor, bound to generate a multitude of intriguing findings and with them, novel questions.

## ACKNOWLEDGMENTS

We thank Annina Denoth-Lippuner for comments on the manuscript.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## DATA AVAILABILITY STATEMENT

We hereby confirm the absence of shared data associated with this manuscript.

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**How to cite this article:** Olpe, C., & Jessberger, S. (2022). Cell population dynamics in the course of adult hippocampal neurogenesis: Remaining unknowns. *Hippocampus*, 1–10. https://doi.org/10.1002/hipo.23475