Review

Continuous neurogenesis in the adult brain

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Neurogenesis occurs throughout adulthood in the mammalian brain by coordinated proliferation and differentiation of adult neural stem cells. Newborn neurons are incorporated into the functional networks of both the olfactory bulb and the hippocampal dentate gyrus, suggesting significant roles of adult neurogenesis in brain functions. In this review, we discuss the recent findings about the integration mode of new neurons into the existing neural circuits. We further address the potential significance of adult neurogenesis in higher brain functions such as olfactory and spatial memory.

Key words: adult neurogenesis, Cre, dentate gyrus, neural stem cell, olfactory bulb.

Introduction

It is now widely accepted that neurogenesis occurs continuously in the forebrain of adult mammals, including humans (McKay 1997; Gage 2000; Alvarez-Buylla et al. 2001; Temple 2001; Doetsch 2003; Ming & Song 2005). The generation of new neurons in the adult brain was first reported by Altman and colleagues using the [H3]-thymidine-incorporation labeling method in the dentate gyrus of the rat hippocampus (Altman & Das 1965). They reported a series of papers about the neurogenesis in various regions of adult rats, including neocortex and olfactory bulb (Altman 1966, 1969). Long-term survival of newborn neurons in the hippocampus was also demonstrated (Kaplan & Hinds 1977). The development of a bromodeoxyuridine (BrdU)-incorporation labeling method enabled us to analyze the characteristics of newborn neurons by combination with immunohistochemistry. Adult neurogenesis was observed with BrdU incorporation in all mammals examined, including humans (Eriksson et al. 1998). In this review, we focus on neurogenesis in the rodent brain.

In normal conditions, neurogenesis occurs in two brain regions of adult rodents, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus. A huge number of neurons born in the SVZ migrate through the rostral migratory stream, and become local interneurons (granule cells and periglomerular cells) in the olfactory bulb, while neurons born in the SGZ migrate into the granule cell layer and become granule cells of the dentate gyrus. It has been shown that newly formed neurons are incorporated into the functional networks of both the olfactory bulb and the dentate gyrus, suggesting a significant impact of adult neurogenesis on brain functions (van Praag et al. 2002; Carleton et al. 2003; Kee et al. 2007).

Genetic labeling of neural stem cells

To study adult neurogenesis in vivo, it is necessary to identify newly generated neurons among billions of pre-existing neurons in the adult brain. Three major approaches have been frequently adapted to label newborn neurons (Ming & Song 2005; Gould 2007). One is based on the incorporation of exogenous nucleotide analogs, such as [H3]-thymidine and BrdU, into dividing cells. During DNA replication, these nucleotide analogs are incorporated into newly synthesized DNA of neural stem cells (NSCs)/neural progenitor cells and inherited to their progeny, such as new neurons. Another one is genetic marking with retroviruses, which are only infectious to dividing cells. The retroviral genome is integrated into the host genome during the M-phase of the cell cycle. Retroviruses carrying reporter genes,
such as green fluorescent protein (GFP) and LacZ, can be used for specific labeling of dividing NSCs/progenitor cells. However, these methods have only limited efficiency, because [H3]-thymidine and BrdU can be administered only for restricted periods, and because administration of retroviruses into the brain using injection needles can infect only small populations. A third one is a genetic method using transgenic mice, in which reporter proteins are expressed under the control of promoters of immature neuron-specific genes such as doublecortin (DCX) and proopiomelanocortin (POMC) (Overstreet et al. 2004). However, these promoters are active only transiently during neuronal differentiation, and it is impossible to label newborn neurons permanently. Therefore, we need more efficient methods that allow selective and permanent marking of newborn neurons in a temporally controlled manner.

Recently, a new method, the Cre/loxP system, has been extensively used for fate-mapping analysis of progenitor cells in various tissues (Nagy 2000; Kessaris et al. 2005) (Fig. 1A). The bacteriophage P1 Cre recombinase efficiently excises DNA, which is flanked by two directly repeated loxP recognition sites, in mammalian cells. By crossing transgenic mice expressing Cre in a cell type-specific manner with reporter mice, we can trace the lineage of the progeny of Cre expressing cells with reporter gene expression such as GFP and LacZ. In reporter mice, a reporter gene is under the control of an ubiquitous promoter such as CAG (CMV enhancer/chicken β-actin promoter) and Rosa26 promoter, but the expression is interrupted by a loxP-flanked transcriptional STOP cassette. In these mice, recombination by Cre results in the removal of the STOP cassette and permanent expression of reporter protein. Temporal control of Cre-mediated recombination can be achieved by using the ligand-dependent chimeric recombinase CreERT2. CreERT2 is constructed by fusing Cre to the mutated ligand-binding domain (LBD) of estrogen receptor (Metzger et al. 1995; Feil et al. 1997). It has been shown that in transgenic mice, CreERT2 is activated by administration of tamoxifen, a synthetic estrogen antagonist, but not by natural ligands of LBD such as 17β-estradiol (Indra et al. 2000; Li et al. 2000; Leone et al. 2003).

In order to mark NSCs and their progeny in the adult brain, we generated Nes-CreERT2 transgenic mice (Imayoshi et al. 2006), in which CreERT2 is expressed under the control of the neural progenitor-specific nestin promoter and enhancer (Zimmerman et al. 1994) (Fig. 1B). Nestin is expressed in the SVZ of the adult brain (Doetsch et al. 1997), and in Nes-CreERT2 transgenic mice, NSCs and transit-amplifying cells expressed CreERT2. In the presence of tamoxifen, Cre-mediated recombination occurred efficiently in NSCs, and the majority of newborn neurons generated from such recombined NSCs were labeled with reporter gene expression after tamoxifen treatment (Imayoshi et al. 2008).

**Replacement of granule cells in the olfactory bulb**

The SVZ is a layer extending along the lateral wall of the lateral ventricle and contains many dividing cells
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It was previously shown that a subset of glial fibrillary acidic protein (GFAP)+ cells (Type B cells) function as neural stem cells in the adult SVZ (Doetsch et al. 1999). Type B cells have the ultrastructural features of astrocytes and express GFAP, a canonical astrocyte marker protein. Type C and Type A cells can be identified as GFAP+Mash1+Dlx2+ and GFAP+Dlx2+DCX+PSA-NCAM+ populations, respectively (Doetsch et al. 1997; Parras et al. 2004). It has been reported that the ependymal cells that surround the lateral ventricle (Type E cells) may also be NSCs (Johansson et al. 1999), but several studies have shown that ependymal cells are quiescent and do not have the potentials of NSCs (Doetsch et al. 1999; Capela & Temple 2002).

Neuroblasts originating from the SVZ of adult rodents migrate into the olfactory bulb, where they differentiate into local interneurons (Lledo et al. 2006) (Fig. 2B). Neuroblasts migrate through a path known as the rostral migratory stream. This long distance migration is not supported by radial glia or axon fibers as in the embryonic brain, but through tubular structures formed by specialized astrocytes (Lois et al. 1996). After neuroblasts reach the core region of the olfactory bulb, they turn to migrate radially to granule cell layers and periglomerular layers, where they differentiate into mature interneurons. Adhesion molecules (such as Poly-Sialated Neural Cell Adhesion Molecule [PSA-NCAM] and tenascin-R) are required for the proper organization of the rostral migratory stream. Various chemoattractant and repellent signals have been shown to regulate the direction of neuroblast migration and the final destination of new neurons (Lledo et al. 2006). Interestingly, the flow of cerebrospinal fluid generated by beating of ependymal cilia appears to generate gradients of Slit proteins for directing the migration of neuroblasts (Sawamoto et al. 2006).

A huge number of neurons born in the SVZ continue to migrate into the olfactory bulb. It has been suggested that in the olfactory bulb old neurons are replaced by new neurons, because the volume of the olfactory bulb does not significantly change over the lifespan (Roselli-Austin & Altman 1979; Kaplan et al. 1985; Petreanu & Alvarez-Buylla 2002) and a relatively high-rate of apoptosis is observed in the granule cell layers (Biebl et al. 2000). However, it has not been clear to what extent old granule cells are replaced by new neurons, and whether the majority is replaced or only subsets of granule cells are repeatedly replaced in the olfactory bulb (Lledo et al. 2006).

Granule cells are axonless inhibitory interneurons that have both a basal dendrite and an apical dendrite. The apical dendrites make bidirectional dendродendritic synapses on their spines with the secondary dendrites of mitral cells. These bidirectional synapses receive glutamatergic input from the lateral dendrites of the mitral or tufted cells (projection neurons of the olfactory bulb) and release GABA back onto these projection neurons (Mori & Shepherd 1994). These dendrodendritic synapses (Fig. 2A).
synapses are thought to be responsible for lateral inhibition of the projection neurons in the olfactory bulb (Yokoi et al. 1995). Previous reports showed that olfactory discrimination learning increases the survival of new neurons, and that sensory experience during a critical period is important for the survival of new neurons (Yamaguchi & Mori 2005; Alonso et al. 2006). These observations led to the hypothesis that continual integration of new neurons has important roles in the olfactory function, such as olfactory discrimination and plasticity of olfactory circuits. Several studies suggested that a supply of new neurons is useful for plasticity and olfactory discrimination (Gheusi et al. 2000; Lledo et al. 2006). However, the definite roles of olfactory interneuron replacement have not been unveiled.

We crossed Nes-CreERT2 mice with Rosa26-stop-LacZ and Rosa26-stop-CFP reporter mice and induced Cre recombinase activity in adults with tamoxifen and thereby efficiently labeled neural stem cells and their progeny (Imayoshi et al. 2008). After tamoxifen treatment, neuronal differentiation from recombined NSCs, and migration and maturation of labeled new neurons into the olfactory bulb were observed, as reported previously (Petreanu & Alvarez-Buylla 2002). Long-term analysis revealed that almost the entire granule cell population was replaced by new neurons over a 12-month period in the deep region of the olfactory bulb (Fig. 2C). In contrast, in the superficial region, only half of the neurons were replaced. It was reported that granule cells born during an early postnatal stage predominantly settle in the superficial region of the granule cell layer and survive longer (Lemasson et al. 2005). Therefore, deep and superficial granule cells have different turnover rates, and the replacement mode is different between superficial and deep regions.

Very little is known about synaptic outputs of new granule cells. It remains to be determined whether a new granule cell makes the synaptic contacts to the same targets as the eliminated old granule cell, or to the completely different targets. Further engineering and application of in vivo multiphoton confocal imaging might help to solve this issue (Mizrahi 2007).

We next asked whether death of old neurons is induced by a supply of new neurons or old neurons die even in the absence of new neurons. To address this question, the majority of new neurons were ablated by crossing Nes-CreERT2 mice with neuron-specific enolase (NSE)-stop-DTA mice. In this double transgenic mice, tamoxifen treatment in adults induces the DTA (diphtheria toxin fragment A) expression from neuron-specific enolase locus, and results in specific ablation of newborn neurons (Imayoshi et al. 2008). Blockade of the supply of new neurons led to gradual decrease of the granule cell number in the olfactory bulb (Fig. 2D). Thus, granule cells have an intrinsic short lifetime, and cell death occurs irrespective of a supply of new neurons, indicating that continuous neurogenesis is essential for maintenance of the granule cell number in the olfactory bulb. Recent study demonstrated that neuronal elimination is an active process, rather than a simple consequence of non-use, providing the vacancy for coming new neurons (Mouret et al. 2008). To assess whether the supply of new neurons is required for discrimination and memory of odors, we carried out olfactory discrimination tests using tamoxifen treated Nes-CreERT2; NSE-stop-DTA mice (Imayoshi et al. 2008). Surprisingly, blocking olfactory neurogenesis had no effect on simple olfactory discrimination and memory tests, although more difficult tasks about odor-associated memory in different contexts could depend on newborn neurons.

Periglomerular cells in the glomerular layer as well as granule cells in the olfactory bulb are replaced by newborn neurons (Kohwi et al. 2007). Periglomerular cells were subdivided into at least three subtypes based on immunoreactivity to tyrosine hydroxylase, calbindin and calretinin (Kosaka et al. 1998), which show different turnover rates (Kohwi et al. 2007; Ninkovic et al. 2007). In mice, all three periglomerular cell subtypes seem to be GABA-expressing inhibitory neurons, but the physiological characteristics and functional roles of each neuronal subtype have not been well determined. Future studies using more sophisticated ablation methods, such as interneuron subtype-specific ablation, may unveil the functional significance of replacement of each interneuron subtype in the adult olfactory circuits.

Addition of granule cells in the dentate gyrus
In the SGZ of the adult brain, a subset of GFAP-positive astrocytes has been shown to be progenitors for new granule neurons (Seri et al. 2001, 2004) (Fig. 3A). These GFAP-positive cells (Type I cells) have radial processes extending through the entire granule cell layer and short tangential processes in the molecular layer. GFAP-negative progenitors, which have only short processes (Type II cells), were also observed in the SGZ (Suh et al. 2007). Definitive lineage relationship between these two progenitors remains to be determined, but it seems that Type I cells give rise to Type II cells (Fukuda et al. 2003). Nestin and Sox2 are expressed by both progenitors (Suh et al. 2007). New neurons in the SGZ migrate only a short distance into the inner granule cell layer, and extend axons projecting through the hilus region into the CA3 region (Zhao et al. 2006). Recent studies showed that Reelin signaling and DISC1
regulate migration and positioning of newborn granule cells (Duan et al. 2007; Gong et al. 2007). Granule cells receive excitatory synaptic input from ascending fiber tract originating from the entorhinal cortex.

Previous studies showed that few pre-existing neurons die in the dentate gyrus, and that the total number of granule cells of the dentate gyrus increases during adulthood. Therefore, it has been suggested that neurogenesis contributes to the increase in neuronal number in the dentate gyrus (Bayer et al. 1982; Boss et al. 1985; Crespo et al. 1986; Dayer et al. 2003; Kempermann et al. 2003). Tamoxifen treatment efficiently induced the recombination in the SGZ progenitor cells of adult Nes-CreERT2; Rosa26-stop-CFP mice, and labeled granule cells gradually increased in number and expanded into the granular cell layer, mostly in its inner side (Imayoshi et al. 2008) (Fig. 3B). Labeled new neurons increased to about 10% of the total granule cell number within 6 months after tamoxifen treatment, but this increase stopped thereafter. This saturation is probably due to decrease of neurogenesis in the SGZ of aged mice (Kuhn et al. 1996). We also examined the effect of ablation of neurogenesis in the dentate gyrus. Blockade of neurogenesis inhibits the increase in the total number of granule cells (Fig. 3C). This contrasts with control mice, in which the number of granule cells increases over the same period of time. Thus, neurogenesis in the dentate gyrus led to addition of neurons to the existing system, and without neurogenesis, the structure was maintained but the increase in the granule cell number was inhibited (Fig. 3). The pre-existing neuronal circuit does not seem to be significantly affected by blockade of neurogenesis. This is a sharp contrast to the olfactory bulb (Fig. 2), where inhibition of neurogenesis resulted in substantial reduction of the granule cell number. These results suggest different roles and integration modes for adult neurogenesis: maintenance and reorganization of the whole interneuron system in the olfactory bulb; modulation and refinement of existing neuronal circuits in the dentate gyrus.

To assess whether neurogenesis in the dentate gyrus is required for hippocampus-dependent memory, tamoxifen-treated Nes-CreERT2; NSE-stop-DTA mice were trained for spatial and fear learning tasks. Blockade of neurogenesis was found to impair retention of spatial memory and formation of contextual memory. These results agreed well with the previous reports (Snyder et al. 2005; Saxe et al. 2006). However, the precise mechanism of how new neurons contribute to the hippocampus-dependent memory is still unclear. As in the case of the olfactory bulb, very little is known about synaptic inputs and outputs of new dentate granule cells. A possible hypothesis is that newborn granule cells are preferentially integrated into special hippocampal circuits. Place cells in hippocampal CA1 area probably receive spatial information from the entorhinal cortex and CA3 area. Interestingly, direct input from the entorhinal cortex is sufficient for establishing fundamental properties of place cells in the CA1 area, and the input from the dentate gyrus-CA3 system is required for the efficient associative recall of spatial memory (Brun et al. 2002; Nakazawa et al. 2002). Blockade of neurogenesis impaired recall, but not acquisition, of spatial memory after a certain time passed (Imayoshi et al. 2008). Detailed analysis of the effect of continuous neuronal addition on the plasticity of hippocampal circuits by electrophysiological studies will define how neurogenesis contributes to the hippocampus-dependent memory.
Concluding remarks

Since the discovery of adult neurogenesis, many extensive studies have been carried out on various aspects of this phenomenon, including proliferation, fate-specification of adult neural stem cells, and migration, maturation and synaptic integration of new neurons. Postdevelopmental neurogenesis is found to be an evolutionarily conserved phenomenon, and functional importance on brain activities has just begun to be unveiled. Further understanding of adult neurogenesis will help the application of cell replacement therapy to the damaged brain after injury or neurological diseases. Especially, it is very important to understand what is happening on existing neural circuits and brain functions when exogenous neurons are added. Full understanding of the regulatory mechanism and functional roles of endogenous adult neurogenesis will help us develop the sophisticated strategy of regenerative medicine for neurological diseases in humans.

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Conflict of Interest

No conflict of interest has been declared by I. Imayoshi, M. Sakamoto, T. Ohitsuaka or R. Kageyama.

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