

Neuronal Differentiation in the Adult Brain: CDK6 as the Molecular Regulator

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Nicolas Caron, Emmanuelle C. Genin,
Renaud Vandenbosch, Pierre Beukelaers,
Laurent Nguyen, and Brigitte Malgrange

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Abstract

The occurrence of adult neurogenesis within the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and the sub-ventricular zone (SVZ) of the lateral ventricles is now well established. However the molecular mechanisms involved in proliferation, differentiation, migration and functional integration newborn neurons in pre-existing neural network remain largely unknown. The cyclin-dependent kinases (Cdks) belong to a family of serine/threonine kinases, which control the progression of cells through several phases of the cell cycle. Cdk4 and Cdk6, and then Cdk2, with their specific cyclins (cyclins D for Cdk4/6, cyclins E for Cdk2), control the G1/S phase transition by phosphorylating retinoblastoma family proteins ultimately releasing E2F transcription factors, which are essential for cell cycle progression. In the adult brain, Cdk6 also controls the length of G1 phase, which is a critical step for balancing proliferation with neuronal differentiation.

Adult Neurogenesis

Introduction

For a century, it was established that brain ceased to grow “new nerve cells after birth”. However, in the late 1960s, thanks to a new method developed to label dividing cells with H³-thymidine, Altman

N. Caron • E.C. Genin • R. Vandenbosch
P. Beukelaers • L. Nguyen • B. Malgrange (✉)
GIGA-Neurosciences, Developmental Neurobiology
Unit, University of Liege, Avenue de l’Hopital,
1 BatB36, 1er etage, Liège 4000, Belgium
e-mail: bmalgrange@ulg.ac.be

showed the production of new cells in the rat adult brain (Altman and Das 1965). Subsequently, new techniques have been developed to study cell proliferation and highlight the birth of new neurons during adulthood. In the 1990s, the use of bromodeoxyuridine (BrdU) and immunohistochemical techniques coupled with confocal microscopy allowed the definite identification of adult neurogenesis in mammals including human beings (Reynolds and Weiss 1992). Indeed, neural stem cells (NSCs) were isolated from the adult brain and cultured. These cells could be expanded and showed self-renewal ability and multipotentiality *in vitro*, two characteristics of stemness. NSCs were the likely source of new neurons *in vivo* as they were able to differentiate in culture into all cell types of the brain, including neurons. Neurogenesis takes place throughout life in two discrete brain areas: the dentate gyrus of the hippocampus (DG) and the subventricular zone of the lateral ventricle (SVZ). The process of adult neurogenesis encompasses the proliferation of resident neural stem and progenitor cells and their subsequent differentiation, migration, and functional integration into the pre-existing synaptic network. In the DG, excitatory granule neurons are generated throughout life from stem and progenitor cells in the subgranular zone (SGZ). While in the SVZ, highly proliferative progenitors give rise to new neurons that migrate through the rostral migratory stream (RMS) to reach the olfactory bulb (OB) where they predominantly differentiate into GABAergic inhibitory interneurons.

Adult Neurogenesis in the Dentate Gyrus

The hippocampus is a cortical structure that belongs to the limbic system. It is folded onto itself and it locates in the medial temporal lobe of the brain. It is composed of two major structures: the Ammon's horn (CA1, CA2, and CA3), the DG divided into three main parts: the hilus, the granular zone (GCL) and the SGZ. The process of adult hippocampal neurogenesis encompasses the slow proliferation of early stem or progenitor

cells located in the SGZ, the subsequent faster proliferation of more restricted progenitors (expansion phase), the selection for survival or elimination of young neurons, the integration of the surviving neurons into the pre-existing neuronal network of the GCL. The last phases of postmitotic development include gradually increasing neuronal connectivity and changes in physiological neuronal properties. Newly integrated granule neurons project their axons to CA3 and are electrophysiologically functional.

Type-1 Cells: Radial Glia-Like Stem Cells

The development of new neurons in the adult DG is now well defined. Indeed, several cell stages have been described and can be differentiated by antigenic and morphological criteria. Hippocampal adult neurogenesis originates in the SGZ from astrocyte-like type-1 cells whose soma is triangular-shaped and. These cells possess a radial glia-like morphology as they send a long apical extension into the molecular layer that contacts blood vessels through vascular end feet. Numerically, type-1 cells represent the most abundant precursor cell type but rarely divide. These quiescent astrocyte-like type-1 cells express glial fibrillary acidic protein (GFAP), as well as nestin, an intermediate filament protein, and the transcription factor, Sox2 (Fig. 2.1). However, these cells do not express S100B, a protein specifically present in the postmitotic astrocytic population.

Type-2 Cells: Progenitors Cells

Type-1 cells divide asymmetrically and give rise to type-2 precursor cells, also known as transit amplifying precursors (TAP) or intermediate progenitor cells (IPC). Indeed, these cells possess an intense mitotic activity, thereby amplifying the pool of highly proliferating progenitors. Type-2 cells are morphologically distinct from type-1 cells: their processes are short and horizontally oriented. Type-2 cells are often subdivided in early progenitors, i.e. type-2a cells, and more mature type-2b cells. Type-2a cells still express markers of radial glia including nestin and Sox2 but no longer GFAP, a specific marker for type-1

cells. Type-2b cells show the first antigenic signs of neuronal differentiation and express several neuroblast markers such as doublecortin (DCX), a microtubule-associated protein involved in differentiation and neuronal migration, and the transcription factors NeuroD and Tbr2. The first synaptic inputs are GABAergic and recorded in type-2 cells, which suggest that these cells are wired to the pre-existing hippocampal network.

Type-3 Cells: Neuroblasts

Type-3 precursor cells correspond to a transition from a proliferative stage (type-2b cells) to postmitotic immature neurons. These cells are the most neuronally committed precursors and possess restricted proliferative capacity as compared to type-2b cells. Type-3 cells express markers of the neuronal lineage such as DCX, PSA-NCAM, NeuroD and Prox1 but lack the expression of glial markers. They exit the cell cycle and migrate on a short distance into the GCL, where they give rise to immature DCX neurons expressing calretinin, a calcium-binding protein, and neuronal nuclei (NeuN), a postmitotic neuronal marker. Most immature neurons produced will not survive and only few newborn granule cells will be stably integrated into the synaptic network of the DG.

Stem Cells or Not in the Dentate Gyrus?

Initially, hippocampal precursor cells were found to be multipotent *in vitro* and able to differentiate into electrophysiologically functional granule excitatory neurons. This persistent neuronal production in the adult DG arise from type-1 radial glial precursors (Doetsch et al. 1999), however whether these cells are able to self-renew by symmetric and/or asymmetric division remains under debate.

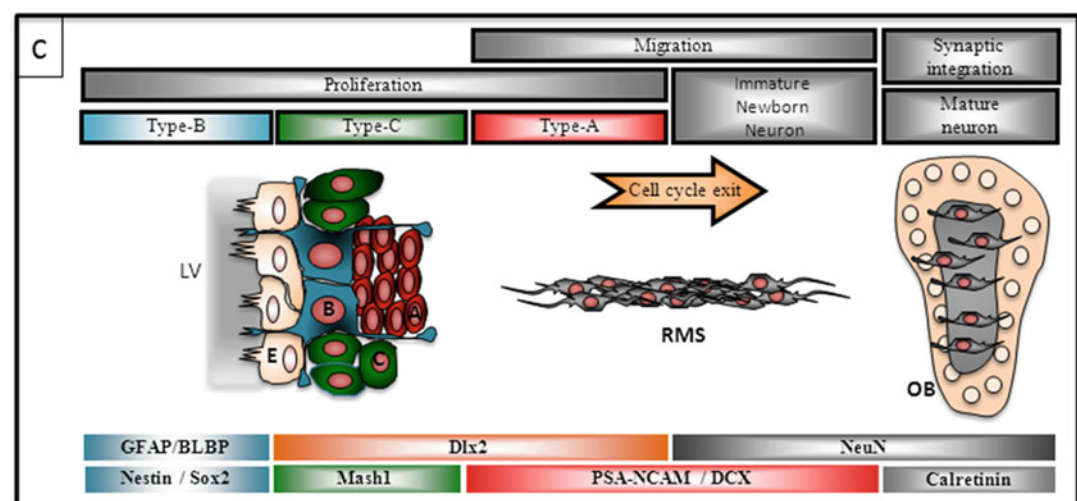
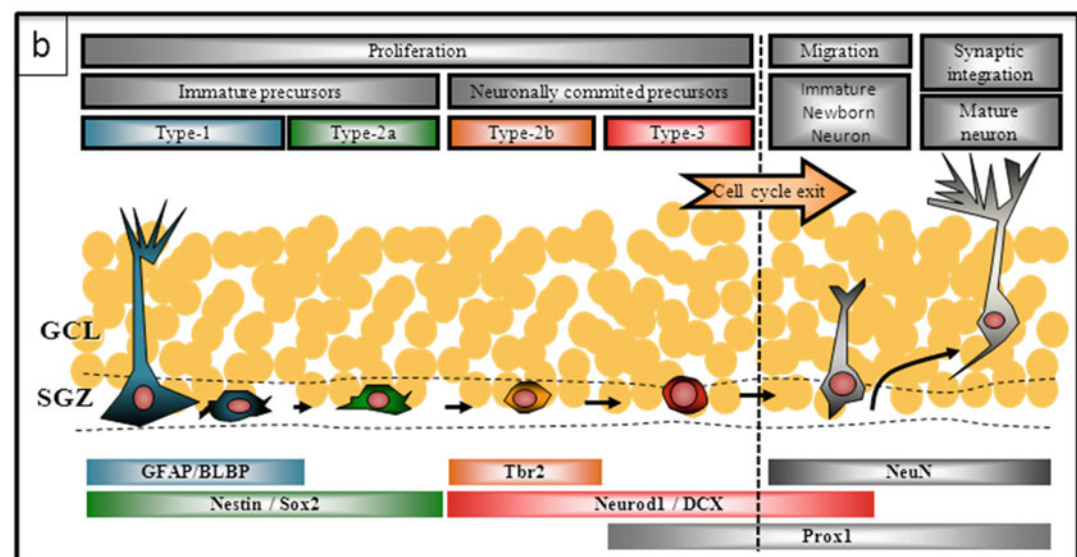
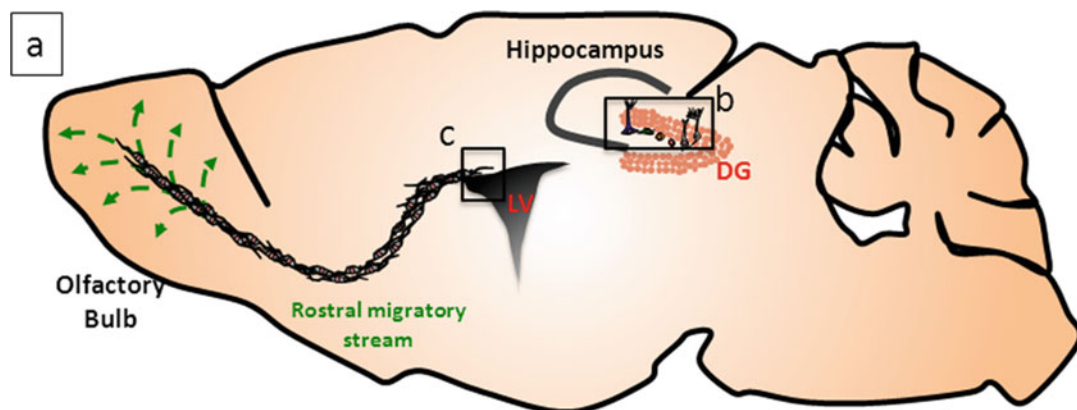
Indeed, it has been demonstrated, both *in vitro* and *in vivo*, that type-1 cells enter the cell cycle to generate, through asymmetric divisions, type-2 cells that in turn become neuroblasts, which then differentiate into mature neurons. Nevertheless, several teams were unsuccessful in identifying true stem cells in the SGZ. Seaberg and van der Kooy (2002) followed by Bull and Bartlett (2005) failed to isolate cells with

self-renewal ability and multipotentiality in the adult DG (Bull and Bartlett 2005; Seaberg and Van Der Kooy 2002). More recently, Clarke et al. used *in vitro* clonal assay to challenge the stem cell properties (self-renewal and multipotentiality) of postnatal DG precursors. They found that type-1 cells are lineage-restricted progenitors with limited proliferative capacity and never detected colonies with abilities of self-renewing and multipotentiality (Clarke and Van Der Kooy 2011). Likewise, Encinas et al. showed *in vivo* that, after a limited number of asymmetric divisions, type-1 cells exit the cell cycle and start acquiring the astrocytic morphology, therefore leading to exhaustion of the “stem cell” pool (Encinas et al. 2011). However, among type-1 cells, some have a long-term ability to generate neurons and glia as well as to undergo self-renewing symmetric divisions, and therefore are true stem cells (Bonaguidi et al. 2011).

Adult Neurogenesis in the SVZ

The SVZ is composed of three main cell types: neuroblasts (Type-A cells), radial glia-like cells (Type-B cells), and TAPs (IPCs, Type-C cells) (Fig. 2.1). This heterogeneous population of cells exists in a dedicated environment, mainly composed of multiciliated ependymal cells (type-E cells) lining the ventricular surface as well as specialized vascular network (Ming and Song 2011). Arrangement of this “niche” is spatially defined, in that ependymal cells are organized in a pinwheel-like fashion surrounding monociliated type-B cells touching the ventricular surface (Mirzadeh et al. 2010). This «pinwheel structure» is only present in the ventricular wall of the neurogenic SVZ region suggesting an important role for intercellular interactions in neurogenesis (Mirzadeh et al. 2010). In addition, SVZ NSCs (i.e. type-B cells) extend basal processes that terminate on blood vessels that lie beneath the ependymal layer.

During lineage progression, astrocytic-like type-B stem cells produce TAPs that in turn give rise to type-A neuroblasts. These cells are immature proliferating neurons that migrate



over long distances (several millimetres) from the SVZ bordering the lateral ventricles of the brain via the rostral migratory stream (RMS) toward the olfactory bulb (OB) through a tube formed by astrocytes. In the OB, type-A cells detach from the RMS and migrate radially toward glomeruli where they differentiate and becoming GABAergic granule or periglomerular interneurons.

Type-B Cells: Radial Glia-Like Stem Cells

Type-B cells extend an apical ending that contact the ventricle and a long basal process ending on blood vessels. The number of contacts in the ventricle appears to increase when SVZ proliferation is stimulated (Mirzadeh et al. 2010). Type-B cells are considered to be the NSCs with ultrastructural characteristics of astroglial cells such as irregular contours that profusely fill the spaces between neighbouring cells, a long basal process that terminates on blood vessels and a single primary cilium that reaches the ventricle lumen. The function of the single primary cilium remains to be understood. This cilium can have a mechanosensory function, suggesting that the force of CSF flow itself may exert an influence on the proliferative state of the stem cell (Singla and Reiter 2006). This organelle has also been implicated in Sonic Hedgehog (Shh), Wnt, and PDGF signaling pathways, which are important for the stemness maintenance (Ihrle and Alvarez-Buylla 2011).

Type-B cells express, like type-1 cells in the DG, GFAP, nestin and sox2 but, importantly, not S100B an astrocytic marker also expressed by ependymal cells (Ming and Song 2011). In the RMS, all type-B cells ensheath migrating

neuroblasts. Type-B cells divide to generate by successive asymmetric or symmetric division TAPs (type-C cells).

Type-C Cells: Transient Amplifying Cells

Type-C cells represent the most important pool of proliferating progenitors within the SVZ, as type-2 cells in the DG. These cells are larger and more spherical than type-B cells and are intimately connected to blood vessels. Type-B cells form a tubular sheath surrounding type-C cells. Initial observations revealed that in vitro “neurospheres” were derived from type-B NSCs (Doetsch et al. 2002), however, a new study indicates that this neurosphere-forming ability is also an intrinsic property of type-C cells (Pastrana et al. 2009). In vivo, these cells still lack a specific clear-cut molecular marker that differentiate them from other cells (Doetsch et al. 1999). It was reported recently that type-C cells express specifically transcription factor Mash1 and a homeobox transcription factor, Distal less 2 (Dlx2), which is also expressed in type-A cells (Ming and Song 2011). Type-C cells will give rise to neuroblasts also named type-A cells.

Type-A Cells: Neuroblasts

The ultimate precursor type in the lineage progression of the adult SVZ is type-A cell. These cells express Dlx2 and are also characterized by the expression of proteins related to migration as DCX and PSA-NCAM (PolySialic Acid – Neuronal Cell Adhesion Molecule) – the embryonic form of NCAM. In the RMS, type-A cells form chains and migrate toward the OB through glial tube formed by astrocytes.

Fig. 2.1 Schematic representation of adult rodent neurogenesis. (a) Illustration of a sagittal section through the mouse brain showing location of neurogenic zones: the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone of the lateral ventricles (LV) where neurons are produced and migrate to the olfactory bulb (OB) via the rostral migratory stream (RMS). (b) Schematic summary of the neuronal differentiation cascade in the adult dentate gyrus. Each cell type is character-

ized by the expression of specific markers. (c) Representation of progenitor cell types and neurons in the SVZ/RMS/olfactory system based on the expression of localization and expression of specific markers. *GFAP* Glial Fibrillary acid protein, *BLBP* Brain lipid-binding protein, *Thr2* T-box brain protein 2, *DCX* Doublecortin, *Prox1* prospero homeobox 1, *NeuN* Neuronal Nuclei, *Dlx2* distal-less homeobox2, *Mash1* mammalian achaete scute homolog 1, *PSA-NCAM* Poly-Sialated Neural Cell Adhesion Molecule, *E* ependymal cells

Six days after their birth in the SVZ, the first neuroblasts arrive in the OB and migrate radially into the layers where they differentiate into mature interneurons. A majority of neuroblasts differentiate into GABAergic granule neurons while a minority differentiate into GABAergic periglomerular neurons and a small portion into dopaminergic neurons. Finally, new granule cells with mature morphologies display spontaneous synaptic potentials (Carleton et al. 2003).

Cell Cycle

Introduction

The cell cycle consists in a series of sequential phases that takes place in a cell leading to its division and replication. The duration of the cell cycle varies from 2 to 3 h in unicellular organisms (such as *Saccharomyces cerevisiae*) to 24 h in eukaryotic cells. In prokaryotic cells, which lack a nucleus, the cell cycle occurs through binary fission: circular DNA is replicated, and then the cell splits into two identical cells which contain an exact copy of the original DNA. In eukaryotic cells, the cell cycle is an elaborate replication process divided in two periods: interphase and mitosis (M). Interphase leads to DNA duplication and comprises three distinct phases: G1 (Gap 1), S phase (DNA synthesis) and G2 (Gap 2). During G1, the expression of genes required for G1 progression and S phase entry results mostly from the initial Rb phosphorylation. After DNA replication that occurs in S phase, the cell enters in G2 where significant proteic biosynthesis occurs, mainly involving in the generation of microtubules necessary for mitosis. The process of mitosis comprises five phases: prophase, metaphase, anaphase, telophase and cytokinesis (a cytoplasmic division). Errors in mitosis often lead to cell cycle arrest and apoptotic cell death to avoid genomic instability and accumulation of mutations. Dependent of environmental and development signals, cells in G1 may stop their division and, temporarily or permanently, leave the cell cycle to enter in a quiescence or senescence state, i.e. G0 phase (Gap 0). During this

stage, cells may remain quiescent for long periods of time (like skin fibroblasts or liver hepatic cells), possibly permanently (i.e. neurons or cardiac cells); or cells may enter in senescence in response to DNA damage or degradation.

The progress through the cell cycle requires the assembly of complexes of cyclin-dependent kinase (CDK, catalytic subunit) and a cyclin (regulator subunit) (Fig. 2.2). Functional heterodimer enters into the cell nucleus where it gets phosphorylated by a CDK-activating kinase (CAK) to activate or inhibit its target proteins and allows the entry into the next phase of the cell cycle. In mammals, they are at least 15 different cyclins known from Cyclin A to Cyclin Y, and 21 genes encoding CDKs among which a few (CDK1, CDK2, CDK4, CDK6) are exclusively associated with cell cycle regulation (Malumbres and Barbacid 2009). Each cyclin can bind one or two CDKs and each CDK can be associated with one or two cyclins. CDKs are constitutively expressed in cells whereas cyclins are synthesized in a cyclic manner in response to various molecular signals at specific cell cycle stages.

When quiescent cells (G0) enter into the cell cycle (in G1), the D-type cyclins (i.e. cyclin D1, D2 and D3) are expressed in response to mitogenic signals and bind to their catalytic partners, CDK4 or CDK6. The kinase activity of these complexes results in the expression of genes whose products are involved in G1/S transition and S phase progression. The G1/S transition requires the binding of CDK2 to cyclin E. CDK2 activity is also implicated in S phase progression through binding with cyclin A. Cyclin A is also important for G2/M transition through its association with CDK1. Finally, during M phase, cyclin B/CDK1 phosphorylates several important structural components of the cell that are responsible for nuclear envelope breakdown and reorganization of the microtubule and actin filaments.

Redundancy exists between cyclins: in the absence of cyclin E-Cdk2, cyclin A-Cdk2 can promote G1/S transition while cyclin D-CDK4/6 and cyclin E-CDK2 play redundant roles during G1/S progression. The degradation of cyclins A

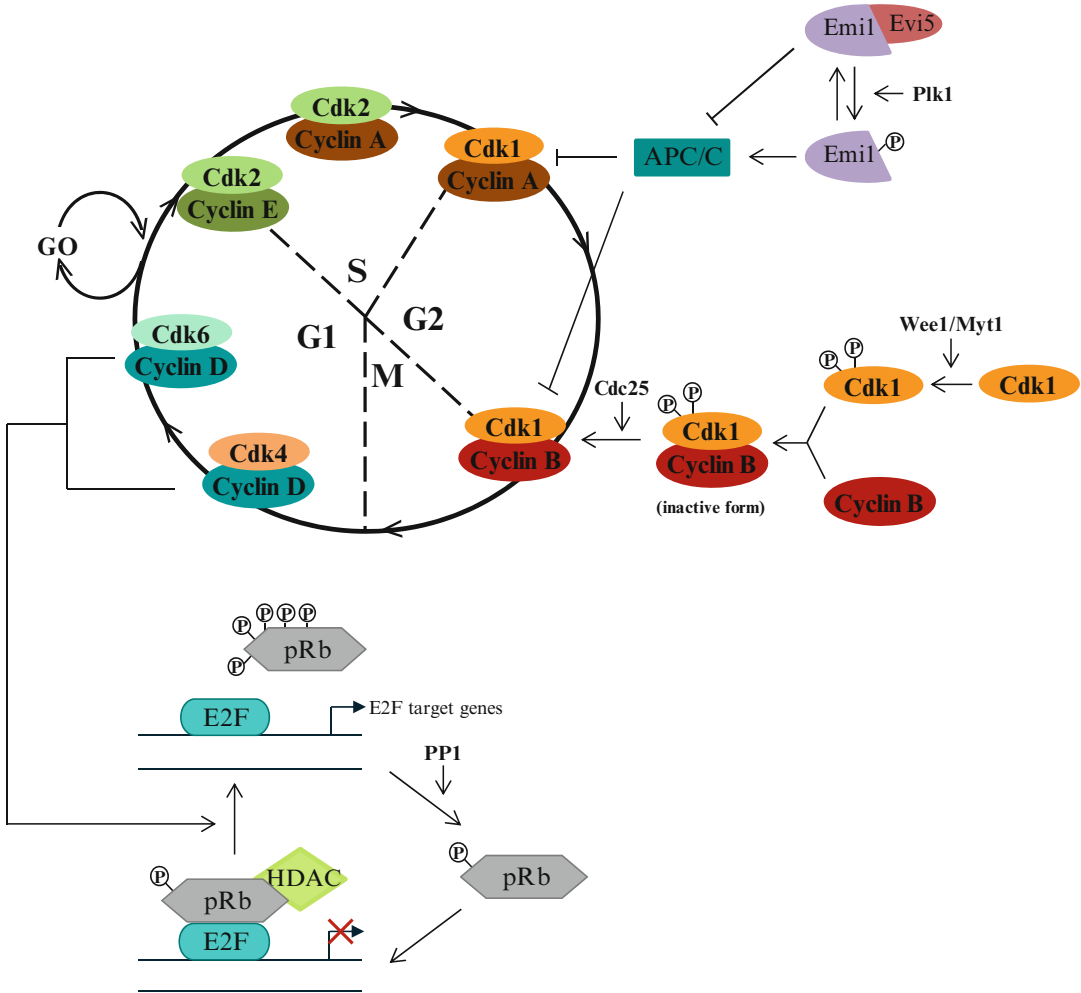


Fig. 2.2 Different aspects of cell cycle regulation. Modulation of cell cycle progression by CDK/cyclins, Rb/E2F pathway and M-phase promoting factor (MPF, i.e. CDK1/CyclinB) modulators

and B is regulated by the APC/C complex (Anaphase-Promoting Complex/Cyclosome) and the APC/C inhibitor Emi1 (Early Mitotic Inhibitor 1). The APC/C complex is an ubiquitin multisubunit E3 ligase, which contribute to the degradation of cyclins A and B by promoting poly-ubiquitinylation. Expression of these cyclins in S phase and G2 requires the inactivation of APC/C by Emi1 whose transcription is induced by E2F transcription factors. Emi1 is stabilized through binding to Evi5, while its degradation occurs after phosphorylation by Plk1 (Polo-like kinase 1).

CDKs Modulators

The activity of CDKs is regulated by both activating and inhibitory phosphorylations due to their association with cyclins, but also with “non-cyclin” CDK activators or CDK inhibitors.

CDK Inhibitors

The cell cycle is negatively regulated by two families of CDK inhibitors (CKIs): INK4 and CIP/KIP (Sherr and Roberts 1999). CKIs inhibit CAK phosphorylation of CDK by conformational changes, but without binding to CAK.

- The INK4/ARF Family

The INK4/ARF (Inhibitor of Kinase 4/Alternative Reading Frame) family exclusively binds and inhibits CDK4 and CDK6 by blocking their binding to D-type cyclins during G1 phase progression. This family contains four members: INK4A (also named p16), INK4B (p15 or CDKN2B), INK4C (p18 or CDKN2C) and INK4D (p19 or CDKN2D). A unique gene encodes all INK4 proteins, which are 15- to 19-kDa polypeptides containing several ankyrin repeats implicated in their interaction with CDK4 and CDK6. These proteins show poor sequence homology (approximately 40 %), however they are highly conserved among species (for example, each of the INK4 proteins in the mouse shares 90 % identity with the corresponding proteins in human). INK4 proteins are equally potent as inhibitors, but are differentially expressed during mouse development and show diversity in their pattern of expression suggesting cell-lineage specificity or tissue-specific functions.

- The CIP/KIP Family

Members of CIP/KIP (CDK Interacting Protein/Kinase Inhibitory Protein) family are negative regulators of the cell cycle progression. The CIP/KIP family is composed of three members: p21^{Cip1} (CDKN1A), p27^{Kip1} (p27, CDKN1B) and p57^{Kip2} (CDKN1C), which are negative regulators of cyclin E/CDK2, cyclin A/CDK2 and cyclin B/CDK1, and negative or positive regulators of cyclin D/CDK4-6 (Besson et al. 2008). Contrary to the INK4/ARF family, the action of Cip/Kip members depends on their binding to both cyclins and Cdk subunits, that occurs on a specific N-terminal domain conserved in all CIP/KIP members, the “LFG” domain. The remainder of their sequence is poorly conserved, suggesting that each of these proteins present specific functions in the regulation of the cell cycle. Under physiological conditions, p27 is highly expressed during G0 and early G1 phase, and then rapidly decrease in late G1 and S phase. p27 blocks S phase progression through inhibition of cyclin E/CDK2 and cyclin A/CDK2. p21 is less specific, it inhibits the activity of cyclin E-A/CDK2 and cyclin B/CDK1 complexes, and also cyclin D/CDK4 that controls G1 checkpoint.

Moreover, p21 can bind PCNA (Proliferating Cell Nuclear Antigen) subunit of δ DNA polymerase and inhibits this enzyme activity to block DNA replication. p57 seems more implicated in the regulation of the cell cycle during the embryonic development, and it present a tissue-specificity expression.

Cip/Kip proteins could also act as non-inhibitory or positive modulators of cell cycle progression by facilitating the transition between the G1 and the S phase. Their progressive binding to increasing amount of cyclin D/CDK4 dimers releases their inhibitory activity on cyclin E/CDK2 and thus favors cell cycle progression. The balance between inhibitory and non-inhibitory functions of CIP/KIP could be linked to their phosphorylation state within specific domains. For example, p27 is an inhibitor of cyclin D/Cdk4 complexes in cell cycle-arrested cells, while it is associated with active cyclin D/Cdk4 complexes in proliferating cells. Indeed, p27 is preferentially tyrosine phosphorylated (Tyr88 and 89) in proliferating cells, causing it to bind cyclin D/Cdk4 in a non-inhibitory mode. In quiescent cells, p27 is dephosphorylated while bound to cyclin D/Cdk4 and therefore inhibits complexes activity (James et al. 2008). It is also possible that CIP/KIP role as inhibitor or non-inhibitor could depend directly on their expression level (Labaer et al. 1997).

Other CDK Modulators

In addition to activation by cyclins and inhibition by CKIs, CDKs can also be modulated by other proteins, such as CDK activating kinase (CAK), RINGO/Speedy family of proteins, Wee1/Myt1 kinases and Cdc25 phosphatases.

- CDK Activating Kinase (CAK)

The CDK/cyclin complexes are activated by phosphorylation at specific sites on the CDKs by CDK activating kinase (CAK). CAK is formed by the association of CDK7, cyclin H and the assembly factor MAT1. It activates CDK1, CDK2, CDK4 and CDK6 by phosphorylation on a conserved threonine residue. This phosphorylation is responsible for an increase binding of CDK to its substrate. The activation of CAK requires the binding of CDK7 to cyclin H, which

allows the phosphorylation of CDK7 on a conserved Thr170 residue in the activation loop. But, contrary to the others CDKs, which need to be phosphorylated to be active, the phosphorylation of CDK7 on its activation segment is not essential for CAK activity. Indeed, MAT1 can substitute Thr170 phosphorylation and is sufficient to activate CDK7/cyclin H complex. CDK7 presents a second site of phosphorylation at Ser164 in Human, whose activation by phosphorylation due to CDK7/cyclin H complex enhances activity and cyclin binding. CAK is also a subunit of the transcription factor TFIIF, which predominantly phosphorylates proteins involved in transcription, including the RNA polymerase II large subunit C-terminal domain involved in the transition to transcriptional pre-initiation from transcriptional initiation stage. TFIIF can phosphorylate CDK2, but less efficiently than free CAK.

- **RINGO/Speedy Family**

Speedy or RINGO (Rapid Induced of G2/M progression in Oocytes) proteins are direct activators of CDK1 and CDK2. They also bind CDK5, but not CDK4 or CDK6. To date, five mammalian RINGO/Speedy family members have been identified, RINGO A to E. These proteins contain a conserved central region of about 100 residues called the ‘Speedy-box’ essential for CDK binding and activation, although with different affinities: the binding and the activation of CDK2 is more efficient than CDK1. Unlike cyclins, RINGO/Speedy proteins do not phosphorylate CDKs in the activation loop of the kinase domain. Therefore, unconventional not yet identified roles for RINGO/Speedy proteins in the regulation of cell cycle are suggested.

- **M-phase Promoting Factor (MPF) Modulators:**

Wee1/Myt1 Kinases and Cdc25 Phosphatases
The entry of cell in mitosis is controlled by the M-phase promoting factor (MPF, Nobel Price of Physiology and Medicine 2001 shared by Paul Nurse, Tim Hunt and Leland Hartwell). MPF is a complex of two proteins, cyclin B and CDK1. This complex is necessary for G2/M transition but must be rapidly inactivated during mitosis to avoid several mitosis cycles. CDK1 is expressed at constant level during the whole cell cycle, whereas cyclin B has a cyclic expression with an

initiation of synthesis at the end of S phase. Cyclin B accumulates during G1, S, G2, M phases but decreases rapidly at the end of mitosis (at the checkpoint). The increase of cyclin B concentration allows the formation of CDK1/cyclin B complex, whose activity is determined by the phosphorylation state of CDK1 (Fig. 2.2). To be active, CDK1 must be phosphorylated on Thr161 by CAK. In interphase, after its association with cyclin B, CDK1 is held in a relatively inactive state by simultaneous phosphorylation of Thr14 and Tyr15 residues located within the ATP-binding site by Wee1 and Myt1. These two residues must be dephosphorylated for the activation of the cyclin B/CDK1 complex and thus to promote the initiation of mitosis. Dephosphorylation is ensured by the Cdc25 phosphatases (CDC25 A, CDC25 B and CDC25 C, in humans). Thus, the active heterodimer cyclin B/CDK1 (phosphorylated on Thr161) inhibits Wee1 and Myt1 kinases and activates Cdc25 phosphatases, therefore increasing CDK1 activity, and favors the entry of cell in mitosis by phosphorylation of its target proteins such as histone H1, lamin or microtubule-associated proteins, responsible for chromosome condensation during mitosis, fragmentation and solubilization of nuclear lamina or microtubules dynamic stability, respectively. When mitosis is initiated, CDK1 activates the degradation of cyclin B by poly-ubiquitinylation, and then CDK1 is dephosphorylated on Thr161 by a phosphatase: a new cycle can begin.

Rb/E2F Pathway

The primary function of cyclin D-dependent kinases – CDK4 and CDK6 – is phosphorylation of a retinoblastoma tumor suppressor protein (pRb), encoded by the retinoblastoma susceptibility gene (RB1). The retinoblastoma tumor suppressor family (also named “pocket proteins”), pRb, together with the related proteins p107 and p130, plays a critical role in cell cycle regulation. pRb proteins have approximately 50 % of sequence homology, and contain a specific and conserved domain named the “pocket domain” that is responsible for their repressor function. pRb are transcriptional repressors that regulates the cell cycle through their ability to bind and

sequester E2F transcription factors (Swiss and Casaccia 2010). pRb interacts and inhibits E2F through its specific “pocket domain” to coordinate the initiation of S phase by mitogenic signaling molecules (McClellan and Slack 2007). To this end, pRb activity is modulated by a balance of phosphorylation/dephosphorylation that arises from kinase and phosphatase activities in a cell cycle-dependent manner (Fig. 2.2). Indeed, at G0 and early G1, Rb is hypophosphorylated allowing its binding to E2F factors blocking their transactivation domain and inhibiting the recruitment of co-repressors (as histone deacetylase 1 (HDAC1) or Brg1/hBRM, members of the SWI/SNF nucleosome remodeling complex) as well as the expression of E2F target genes. The cyclin/CDK complexes responsible for the cell cycle progression hyperphosphorylate pRb from late G1 to mitosis, thus relieving the constraints on E2F proteins and allowing the expression of genes required for DNA synthesis and cell cycle progression. Towards the end of M phase, the protein phosphatase 1 (PP1), which is also necessary for mitotic exit, dephosphorylates pRb. E2Fs are required during cell cycle progression, as they contribute to irreversibility of G1/S transition via a positive feedback loop: E2Fs activate the overexpression of cyclin E, which interacts with Cdk2, resulting in activation of pRb phosphorylation.

“Cell Cycle” CDKs in Neurogenesis

The mechanisms involved in proliferation, neuronal differentiation, migration and functional integration in the adult brain remain largely unknown. Initially identified as core cell cycle regulators, some Cdk s have emerged as multifaceted proteins with functions beyond cell cycle regulation. Recently, accumulating evidence ascribed crucial roles to regulators of Cdk4/6 activity in controlling adult neurogenesis.

Cdk1

Contrary to other “cell cycle” Cdk s, the lack of Cdk1 expression in mice leads the death at morula stage (E2.5) (Santamaria et al. 2007). Thus,

Cdk1 seems to be the only cell cycle Cdk whose loss is not compensated by the others Cdk s during early stages of embryonic development. In absence of the other cell cycle Cdk s, such as Cdk2, Cdk4 and/or Cdk6, Cdk1 seems to be able to form functional complexes with all cyclins implicated in the embryonic cell cycle division, resulting in the activation of the pRb/E2F pathway and therefore in the cell cycle progression (Santamaria et al. 2007). A possible role of Cdk1 in adult neurogenesis remains to be elucidated.

Cdk2

Cdk2 was thought to be indispensable to drive cells through the transition from G1 to S phases and for the progression in S phase. The generation of Cdk2^{-/-} mice has changed this theory. Indeed, Cdk2^{-/-} mice are viable and develop normally with a minor body weight reduction (Ortega et al. 2003), therefore suggesting that Cdk2 is not essential for proliferation in embryonic or adult mice. However Cdk2^{-/-} mice are sterile, shedding light on Cdk2 importance in germ cells.

Regarding brain cells, Cdk2 is required for oligodendrocyte progenitor cells (OPCs) proliferation in vitro (Belachew et al. 2002), suggesting that Cdk2 may have a similar role in adult neurogenesis. In the absence of Cdk2 in adult mice, a decrease of density and proliferation of NSCs is observed in the SVZ (Jablonska et al. 2007) while no significant difference is detected in the DG (Vandenbosch et al. 2007). Under pathological conditions, such as demyelinating lesions, the absence of Cdk2 in adult mice is associated with increase OPCs renewal and differentiation, thus resulting in promotion of remyelination (Caillava et al. 2011). In numerous organs, the absence of Cdk2 can be compensated by other Cdk s. In Cdk2^{-/-} cells, Cdk1 binds to Cyclin E, and this active complex regulates efficiently G1/S transition. Indeed, the absence of Cdk2 in perinatal mice is associated with Cdk4 overexpression in SVZ cells, suggesting that CDK4 compensate for the loss of CDK2 expression (Jablonska et al. 2007). This compensatory effect is absent at later postnatal stages, leading to impaired neurogenesis.

Cdk4

Knockout mice for Cdk4 are viable but infertile and show growth retardation. Moreover, these mice develop insulin-deficient diabetes due to a degeneration of beta-islet pancreatic cells (Rane et al. 1999). In adult mice, Cdk4 expression is exclusively restricted to dividing NSCs in the DG and SVZ (Beukelaers et al. 2011). Cdk4, in association with its cyclin partner (i.e. cyclin D1), play a critical role in adult neurogenesis by modulating the length of G1 phase. Indeed, overexpression of Cdk4 and cyclin D1 is associated with a reduction of G1 length, which results in inhibition of adult neurogenesis and increases of NSCs population (Artegiani et al. 2011; Lange et al. 2009). However, the absence of Cdk4 does not impact on adult neurogenesis in adult mice (Beukelaers et al. 2011).

Cdk5

Although Cdk5 does not act as a checkpoint kinase during cell cycle progression, it can regulate several proteins implicated in the cell cycle, such as pRb. Mice lacking Cdk5 die in utero around E16.5 (Ohshima et al. 1996). In the brain, Cdk5 plays a critical role in corticogenesis and neuronal migration during embryonic development (Ohshima et al. 1996). Cdk5 plays also a critical role in adult neurogenesis where it is implicated in the migration of neuroblasts in the SVZ, in the migration and maturation of newborn neurons, and also in the regulation of adult-generated neuron survival in the hippocampus (Lagace et al. 2008). However, the molecular mechanisms of its role in adult neurogenesis remain unknown.

Cdk6 a Key Molecular Regulator for Adult Neurogenesis

Cdk6-deficient mice are viable, but present a reduced proliferation of erythroid lineage and a decrease in the size of thymus and spleen. In the adult brain, the expression pattern of Cdk6 is restricted to the two zones of adult neurogenesis and more precisely to the proliferating cells

(i.e. Ki67+ cells) (Beukelaers et al. 2011). Our laboratory showed that the lack of Cdk6 results in reduced number of proliferating cells in both the adult DG and SVZ, suggesting that Cdk6 is essential to control the biology of progenitor cell in these neurogenic areas. More precisely, the quantification of the different subpopulations of proliferating cells revealed that only neuronally committed progenitor cell population, i.e. type-2b and type-3 in the DG and Type C and A in the SVZ, are impaired in the absence of Cdk6. The immature population of progenitor cells, i.e. type-B (SVZ) and type-1 and 2a (DG) cells remains unaffected in both wild type and Cdk6^{-/-} mice. The decrease of neuronally committed cells observed in Cdk6^{-/-} neurogenic areas could affect neuronal cell production. Indeed, the authors showed a reduction of the number of young postmitotic neurons (DCX+/NeuN+) in the two neurogenic areas, consistent with the decreased proliferation.

It has been shown that neuronally committed precursors undergo a define number of cell divisions prior exiting the cell cycle and differentiating into neurons. Thus, the selective reduction of neuronal committed precursors likely arises from a premature exhaustion of progenitor through increased rate of cell cycle exit. Beukelaers et al. (2011) showed that the absence of Cdk6 increased the cell cycle exit in the DG and in the anterior part of the RMS of Cdk6^{-/-} animals. Altogether, Cdk6 controls the proliferation and cell cycle exit of neuronally committed precursors.

Recently, Lange et al. demonstrated that a lengthening of G1 by Cdk4/cyclin D1 down-regulation is sufficient to promote the exit of the cell cycle of cortical progenitors and their differentiation into projection neurons (Lange et al. 2009). In addition, Malumbres et al. demonstrated a lengthening of G1 in Cdk6^{-/-} T lymphocytes (Malumbres et al. 2004). Using cumulative S-phase labelling with BrdU, Beukelaers et al. (2011) demonstrated that the overall duration of the cell cycle (T_c) in DG cycling precursors was increased in Cdk6^{-/-} (22 h32' in the DG ; 12 h34' in the SVZ) compared to WT (19 h55' in the DG ; 9 h58' in the SVZ). Importantly, this increase was linked to a specific increase of G1 length in neuronally committed precursors (+28.08 % in the DG and +82.21 % in the SVZ).

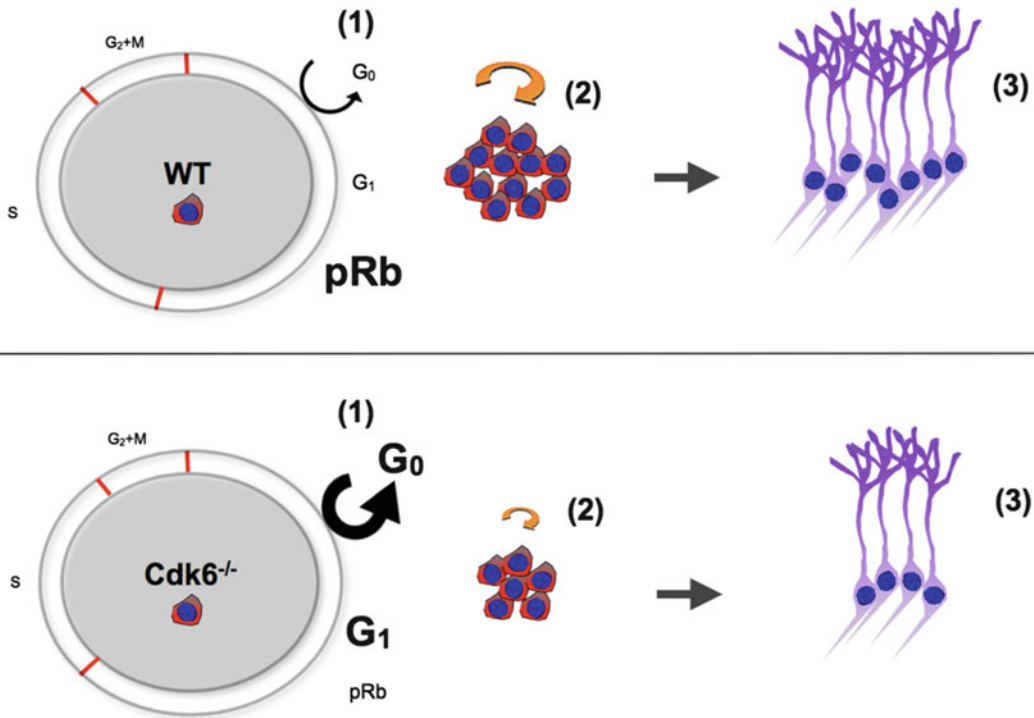


Fig. 2.3 Schematic representation of the phenotype of adult neurogenesis in *Cdk6*^{-/-} mice. *Cdk6*^{-/-} adult neural precursors display increased G1 phase length concomitant with pRb hypophosphorylation. Lengthening

of G1 leads to premature cell cycle exit into G0 (1), reduces precursor expansion (2) and, finally, neurogenesis (3) (from Beukelaers et al. 2011)

During the G1 phase, Cdk6 phosphorylates pRb on Ser780, leading to the release of E2F and S phase entry. In the absence of Cdk6 a decrease of phosphorylation of Rb was observed in the DG and the SVZ (Beukelaers et al. 2011). Therefore, Cdk6 controls the expansion of neuronally committed precursors by controlling the hyperphosphorylation of pRb and the G1 phase length (Fig. 2.3).

Conclusion

It has been experimentally demonstrated that lengthening of G1 is sufficient to induce neurogenesis and that shortening G1 inhibits neurogenesis and increases the formation and proliferation of NSCs (Artegiani et al. 2011; Beukelaers et al. 2011). Moreover, the switch between proliferation and differentiation of neuronal production

in the adult brain is partly controlled by CDK6 via the modulation of G1 phase duration. Therefore, the manipulation of Cdk/Cyclin complexes could be used as a new cell-based therapeutic approach to restore neuronal loss in several neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disorder or multiple sclerosis. The aim of this strategy would be to induce endogenous neural cells in the adult nervous system to form new neurons, by controlling their proliferation versus differentiation.

The crucial advantage of this therapeutic strategy is to enhance the proliferation of endogenous adult NSCs to avoid the danger of immunological graft rejection. Indeed, until now, several therapeutic approaches using neural cells have been studied, such as transplantation of oligodendrocyte progenitor cells or phenotypically restricted neuronal progenitor cells or mixed progenitor

pools, but all of these present high risks of immunological graft rejection. Moreover, a part of the challenge in cell replacement therapy is to produce new cells (i.e. neurons) with the precise characteristics needed to restore brain function that has been compromised during the disease (e.g. the specific loss of GABAergic neurons in Huntington's disease or the progressive decrease of dopaminergic neurons in Parkinson's disorder). The nervous system has an intrinsic capacity to produce new neural progenitor cells, which can restore the connectivity with existing networks in the patient's brain. This capacity can be used as therapeutic approach for a multiple of neurodegenerative disorders.

It is known that genetic modifications, in particular cell cycle modifications, enhance the risk of tumor formation. However, the balance between proliferation and differentiation of neural cells can be temporarily manipulated to avoid the deregulation of the cell cycle and formation of cancer stem cells. Indeed, after stroke, it has been shown that G1 phase is dynamically and transiently modified, with an early expansion of the SVZ neural progenitor pool due to a decrease of G1 length and an increase of neurogenesis that is not associated with tumorigenic effect.

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