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Journal Title: Journal of Neuroscience Nursing
Volume: Volume 29, Number 9
Publisher: Lippincott, Williams & Wilkins | 2009-03-04, Pages 2902-2914
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1523/JNEUROSCI.4051-08.2009
Permanent URL: https://pid.emory.edu/ark:/25593/tv4qv

Final published version: http://dx.doi.org/10.1523/JNEUROSCI.4051-08.2009

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Accessed August 27, 2019 1:23 AM EDT
p27KIP1 Regulates Neurogenesis in the Rostral Migratory Stream and Olfactory Bulb of the Postnatal Mouse

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Neuronal progenitor cells of the anterior subventricular zone (SVZa) migrate along the rostral migratory stream (RMS) to the olfactory bulb, where they exit the cell cycle and differentiate. The molecular mechanisms that regulate SVZa progenitor proliferation and cell-cycle exit are largely undefined. We investigated the role of p27KIP1 in regulating cell proliferation and survival in the RMS and olfactory bulb between postnatal day 1 (P1) and P14, the peak period of olfactory bulb neuron generation. A large proportion of cells in the RMS and the olfactory bulb express cytoplasmic p27KIP1, but a small percentage display high nuclear p27KIP1 immunostaining, which exhibit a caudal low–rostral high gradient: lowest in the SVZa and highest in the glomerular layer of the olfactory bulb. p27KIP1 is also present in the nucleus and/or the cytoplasm of neuron-specific type III β-tubulin(+) cells. Cells with strong nuclear p27KIP1 expression are BrdU(−) and Ki67(−). The percentage of BrdU(+) cells in the SVZa, RMS, and olfactory bulb is higher in p27KIP1 null than wild-type (WT) mice at all ages analyzed. Consistent with these findings, p27KIP1 overexpression in cultured p27KIP1 null and WT SVZ cells reduced cell proliferation and self-renewal. Finally, in p27KIP1 null mice, the diameter of the horizontal limb of the RMS is larger than in WT mice, and development of the olfactory bulb granule cell layer is delayed, together with increased apoptotic cell density. Our results indicate that in the postnatal brain, p27KIP1 regulates the proliferation and survival of neuronal cells in the RMS and olfactory bulb.

Introduction

The production of interneurons in the olfactory bulb originates from neuronal progenitor cells derived from the anterior part of subventricular zone (SVZa) (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Menezes et al., 1995). Previous studies demonstrated that in mice most of the granule and periglomerular cells in the olfactory bulb are generated between embryonic days 18 (E18) and postnatal day 5 (P5) (Hinds, 1968). SVZa-derived neuronal progenitors tangentially migrate along the rostral migratory stream (RMS) to the olfactory bulb, where they radially migrate and differentiate into interneurons of the granule cell layer (gcl) and glomerular layer (gl) (Luskin, 1993; Lois and Alvarez-Buylla, 1994). The molecular mechanisms that regulate SVZa neuronal progenitor proliferation and differentiation in the RMS and olfactory bulb are not fully determined.

Progression of the cell cycle from G1 to S phase is positively regulated by cyclin-dependent kinases (CDKs), including CDK2 and CDK4, and negatively modulated by two families of CDK inhibitors (CDKIs), the INK4 and the CDK-inhibitory protein (CIP)/kinase-inhibitory protein (KIP) families (Sherr and Roberts, 1995). INK4 includes p16INK4a (p16), p15INK4b (p15), p18INK4c (p18), and p19INK4d (p19). The CIP/KIP family comprises p21CIP1 (p21), p27KIP1 (p27), and p57KIP2 (p57). p27 null [p27 knock-out (p27KO)] mice display a larger brain size (Fero et al., 1996; Kiyokawa et al., 1996). Several reports demonstrated that p27 regulates many aspects of neurogenesis, including neural progenitor proliferation, migration, and/or differentiation (Zindy et al., 1999; Levine et al., 2000; Miyazawa et al., 2000; Legrier et al., 2001; Cunningham et al., 2002; Doetsch et al., 2002; Goto et al., 2004; Nguyen et al., 2006). All these studies indicate that p27 plays critical roles in the development of the CNS (Cunningham and Roussel, 2001).

The spatial and temporal pattern of p27 expression in the RMS and olfactory bulb during the peak period of neurogenesis is still unknown. Furthermore, a functional role for p27 as a regulator of neuronal cell proliferation and survival in the RMS and olfactory bulb during this developmental process is still undefined. This information would be essential to understand specific aspects of olfactory bulb development during the first 2 postnatal weeks (i.e., during the period of maximal growth of this region in mice).

In the present study, we investigated p27 expression and regulation in cells of the mouse SVZa, RMS, and olfactory bulb during the first 2 weeks of postnatal development. We also studied p27 function in neural cell proliferation and survival in the same brain regions. We demonstrate that the spatiotemporal expression pattern of p27 is consistent with a role of this CDKI as an...
inducer of cell-cycle exit in neuronal progenitor cells of the SVZa and their progeny that migrate along the RMS into the olfactory bulb. We also demonstrate that functional inactivation of p27 in a KO mouse mutant prevents cell-cycle exit of neuronal progenitors, delays development of the gcl in the perinatal olfactory bulb, and promotes apoptotic cell death in this region. Finally, we show that p27KIP1 overexpression in cultured p27KIP1 null and wild-type (WT) SVZ cells reduced cell proliferation and self-renewal. Our data indicate that p27 plays an important functional role in the regulation of neuronal progenitor cell development during crucial phases of olfactory bulb growth.

Materials and Methods

Animals. The colony of WT mice and p27KO mice was generated from the same founders by isolating p27 and WT alleles. Genotypes of mice were determined by PCR analysis of tail DNA. Mice at P1, P7, and P14 were used in the present study. The plug day was regarded as E0. Birth
usually occurred on the 20th day of gestation. To normalize the ages of the animals examined, E20 was considered to be P0.

**Preparation of sections.** To analyze neural cell proliferation, animals were given intraperitoneal injections of 5-bromo-2'-deoxyuridine (BrdU; 200 mg/kg; catalog #BS002; Sigma) dissolved in 0.9% sodium chloride/7 mM NaOH 3 h before perfusion. P1 and P7 mouse pups were anesthetized by hypothermia, and P14 animals were anesthetized with two times the anesthetic dose of chloral hydrate (3% in 0.1 M PBS; catalog #C8383; Sigma). Animals were initially perfused transcardially with cold 0.1 M PBS (0.1 M sodium phosphate, 150 mM NaCl, pH 7.4), followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The brains, including the olfactory bulbs, were removed from the skull, postfixed at 4°C in the same fixative solution overnight, and cryoprotected with 30% sucrose. Tissues were embedded in O.C.T. (catalog #4858; Thermo Fisher Scientific) frozen with liquid nitrogen that was cooled with 2-methyl-butane. The brains were finally sectioned in the sagittal plane on a cryostat at 10 μm and mounted onto Superfrost Plus slides (catalog #48311-703; Thermo Fisher Scientific).

**Immunohistochemistry.** Sections containing the entire RMS were selected, and single- or double-labeled immunohistochemistry was performed. To expose the p27 and Ki67 antigen-binding epitopes (i.e., retrieval), the following procedure described by Tang et al. (2007) was used. Citrate buffer (0.01 M, pH 6.0) was preheated in a coplin jar in a 700 W microwave for 10 min at 30% power. Immediately after this preheating, slides were immersed in the hot (98°C) buffer. After 5 min, the solution (with slides) was reheated for 30 s at 30% power twice with a 5 min interval. After 16 min (5 min plus 30 s plus 5 min plus 30 s plus 5 min), the coplin jar was removed from the microwave, and the solution (with slides still immersed) was allowed to cool down to room temperature for 20 min. For BrdU staining, sections were immersed in 2N HCl at 40°C for 30 min to denature the DNA and rinsed twice with 40 mM borate buffer, pH 8.4, for 15 min to neutralize HCl. For p27/BrdU double labeling, the HCl treatment was first performed, followed by antigen retrieval. Slides were then processed for immunohistochemistry as described below.

**Briefly,** sections were washed in 0.1 M PBS, pH 7.4, for 30 min and kept in the blocking solution (2% normal goat serum in 0.3% Triton X-100/0.1 M PBS, pH 7.4) for 1 h. Subsequently, sections were incubated with primary antibodies, which were exposed at 4°C overnight. If tissues were stained with more than one antibody, this was done simultaneously. The antibodies used were as follows: mouse monoclonal p27 (1:200; catalog #610242; Transduction Laboratories, BD Biosciences) and rabbit monoclonal Ki67 (1:150; catalog #RM-9106-50; NeoMarkers) to identify cells in the cell cycle; and rat monoclonal BrdU (2 μg/ml; catalog #ab6326-250; Abcam) to identify cells in S phase of the cell cycle. BrdU is a thymidine analog that is incorporated into DNA during the S phase of the cell cycle. Ki67 is a protein expressed during all active phases of the cell cycle, late G1 to M phase, but is undetectable in quiescent cells (G0 phase). Therefore, Ki67 represents an ideal marker to determine whether a given cell is in any phase of the cycle. Rabbit polyclonal neuron-specific type III β-tubulin (nTuB; 2.5 μg/ml; catalog #PRB-435P; Covance) and mouse monoclonal nTuB (1.25 μg/ml; catalog #G7121A; Promega) were used to identify neurons, and mouse monoclonal neuronal nuclei (NeuN; 2 μg/ml; catalog #MAB377; Millipore Bioscience Research Reagents) to identify mature-terminally differentiated neurons. The following day, sections were rinsed with 0.1 M PBS and incubated with secondary antibodies, including goat anti-rat 488 (4 μg/ml; catalog #A11006; Invitrogen), goat anti-rat 568 (4 μg/ml; catalog #A11031), goat anti-rabbit 488 (4 μg/ml; catalog #A11008), and goat anti-rabbit 647 (4 μg/ml; catalog #A21245) counterstained with the fluorescent nuclear dye Hoechst 33342 (1 μg/ml; catalog #H3570, Sigma). After a 1 h incubation, sections were rinsed with 0.1 M PBS, pH 7.4, and coverslipped with Vectashield (catalog #H-1000; Vector Laboratories).

In some experiments, the adjacent slides were counterstained with SYTO 24 (0.5 μM; catalog #S-7559; Invitrogen), and the number of SYTO 24-labeled cells was counted to determine the number of total cells.

p27(+) and BrdU(+) cell quantification. Sections containing the entire RMS (two sections per animal; three animals of each genotype at each age) were examined with a Zeiss Axiosplan equipped with an LSM 510 confocal system. Laser scanning at 488 nm to image nsTuB(+) cells, and at 543 nm to image BrdU(+) or p27(+) cells, was performed sequentially to avoid bleed-through of the fluorescent signals. For sections of P1 and P7 mice, three fields were taken of each chosen region (SVZa, vertical limb (vl), horizontal limb (hl), and core of olfactory bulb (OBc) of the RMS, and the gcl and gl of the olfactory bulb) using a 100× oil lens of a confocal microscope. Because the SVZa is reduced in size at P14, two rather than three fields were taken in this region.
Subcellular distribution of p27 in the SVZa at P1. Photomicrographs of an identical section of the SVZa stained with antibodies to p27 (A), p27 and nsTub (B), and p27 and SYTO 24 (C) are shown. A, Photomicrograph showing large numbers of p27(+) cells (red) in the SVZa. B, Photomicrograph of the distribution of p27(+) (red) and nsTub(+) (green) cells. p27 (red) is localized both in the nucleus and/or in the cytoplasm (green; stained by nsTub). C, Distribution of p27(+) cells in relation to SYTO 24(+) nuclei. The p27(+) nuclei (red) overlap with the SYTO 24 (blue) nuclei. Asterisks in the middle of p27(+) nuclei and arrowheads point to examples of p27(+) cytoplasmic cells at P1. Scale bar, 20 μm.

Quantification of positive cells was performed on files obtained from confocal images. To count cells with strong nuclear p27 staining (NUCp27SP), images were processed with Photoshop 6.0, and the number of NUCp27SP cells was counted by an observer who was blind to the ages and genotypes of the animals (Miyazawa et al., 2000). ImageJ software was used to count the SYTO 24(+) cell number (total cells) and to measure the scored area. The density of NUCp27SP cells was calculated from the total number of NUCp27SP cells divided by the total scored area. The total cell density was calculated by the number of SYTO 24(+) cells divided by the scored area. The percentage of NUCp27SP cells was calculated by dividing the density of the positive cells by the total cell density. The quantification of BrdU(+) cells was also based on the focal microscope files, and it was performed following the same procedure as described above. All the data are presented as mean ± SEM. Statistical analysis was performed using two-way repeated ANOVA and the post hoc Tukey’s test.

Measurement of the thickness of RMS. Sections containing the entire RMS were selected and counterstained with the nuclear dye Hoechst. The thickness of hl of the RMS was used to represent the thickness of RMS. The hl displays an even diameter in a short segment immediately after the elbow of the RMS, before merging into the olfactory bulb. Images were taken of the hl using a Zeiss Axioscope fluorescence microscope equipped with a QImaging Retiga 1300 monochrome camera and processed with Canvas software. The thickness was measured using a micrometer by an observer blind to the genotypes and ages of the animals. Statistical analysis of data was performed using the two-way ANOVA and post hoc Tukey’s test.

Programmed cell death. To quantify apoptotic cell number and determine the phenotype of apoptotic cells, nsTub-terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end double labeling (TUNEL) was performed based on protocols described previously by Doetsch et al. (2002) and Bauer et al. (2003), with some modification. Briefly, sections were first immunostained for nsTub. On the second day, sections were washed for 30 min after secondary antibody incubation. Subsequently, sections were incubated in TdT buffer (30 mM Tris-HCl, pH 7.5, containing 140 mM sodium cacodylate (catalog #C-0250; Sigma) and 1 mM CoCl2 (catalog #C-3169; Sigma)) for 5–10 min. Subsequently, 0.06 nmol of biotin-16-dUTP (catalog #1138908910; Roche) and 24 U of TdT (catalog #03333 574001; Roche) were applied to every section and incubated for 1 h at 37°C, followed by a 15 min rinse at room temperature in TB buffer (300 mM NaCl (catalog #S271-3; Thermo Fisher Scientific) 30 mM sodium citrate (catalog #S-4641; Sigma)) to inactivate the enzyme. After a 5 min rinse in 0.1 M PBS, pH 7.4, sections were blocked with 10% normal goat serum in 0.3% Triton X-100/0.1% PBS, pH 7.4, for 1 h and incubated with Alexa 568-conjugated streptavidin (2 μg/ml; catalog #S-11226; Invitrogen) for 1 h at room temperature. After a distilled water wash, sections were coverslipped with Vectashield. Negative control sections were processed for omission of the incubation with TdT. Before TdT buffer incubation, positive control sections were incubated with DNase I (grade II, 500 U/ml in 50 mM Tris-HCl, pH 7.5; catalog #10-104-159-001; Roche) for 10 min at room temperature and rinsed with PBS for 15 min. TUNEL(+) cells in the whole RMS and gel were counted by an observer who was blind to the genotypes and ages of the animals, and the scored areas of the whole RMS and gel were calculated with Canvas software. The density of TUNEL(+) cells was determined by using the total TUNEL(+) cell number divided by the scored area. Statistical analysis of the data was performed using two-way ANOVA and the post hoc Tukey’s test.

Neurosphere cultures. P1 WT and p27KO mice were used to dissect out the SVZ. The SVZ tissue was digested for 30 min at 37°C in HBSS (catalog #14170-161; Invitrogen) containing papain (13 U/ml; catalog #T4762;
Results

tural period, SVZa-derived neuronal progenitor cells are the major type of cells in the neonatal and early postnatal RMS, which can be well labeled by nStub (Menezes and Luskin, 1994; Law et al., 1999; Falls and Luskin, 2006) (Fig. 1B,D). Most intensely labeled cells could be seen in the OBc, in the rostral segment of the RMS, and in neuronal cell layers of the olfactory bulb (Fig. 1C,E). Most, if not all, of the p27(+) cells were double labeled with the neuronal marker nStub (Fig. 1B,D,F). No p27 expression was detected in the SVZa, RMS, or OB of p27KO mice (supplemental Fig. 1, available at www.jneurosci.org as supplemental material, and data not shown).

Based on the visual assessment of nuclear p27 label intensity, we classified cells as nuclear p27 negative (NUCp27-), nuclear p27 weakly positive (NUCp27WP), and nuclear p27 strongly positive (NUCp27SP) (Fig. 2A–F). We found that the vast majority of nStub(+) cells are also p27(+). In the SVZa, few nStub(+) cells are also NUCp27SP (Fig. 2A–C). Compared with the SVZa, more nStub(+) cells are NUCp27SP in the OB (Fig. 2D–F). To quantitatively analyze the expression pattern of p27, we scored the number of NUCp27SP cells in the SVZa, vl, hl, and OB of the RMS and in the gcl and gl of the olfactory bulb. At each age, the percentage of NUCp27SP cells showed a gradient from the SVZa, vl, hl, and OBc of the RMS to the neuronal cell layers of the olfactory bulb, with the lowest percentage in the SVZa and the highest in the gcl (caudal→rostral gradient) (Fig. 2G). When compared with P1, the percentage of NUCp27SP cells in each

p27 expression pattern in the RMS and neuronal cell layers of the olfactory bulb

SVZa-derived neuronal progenitor cells migrate along the RMS toward the olfactory bulb. Figure 1A shows a parasagittal section of a P1 WT mouse stained with the nuclear marker Hoechst to show the high density of cells in the RMS. During the early post-
p27 inhibits cell proliferation in the RMS and olfactory bulb

To assign a role to p27 in the regulation of cell proliferation in the RMS and olfactory bulb, we first performed double labeling with anti-p27 and markers of cell proliferation (i.e., BrdU and Ki67). We observed that from P1 to P14, NUCp27SP cells were consistently negative for BrdU and Ki67 but NUCp27WT cells expressed detectable BrdU and Ki67 (Fig. 4). These data indicate that high levels of nuclear p27 expression likely induce cells to withdraw from the cell cycle.

To directly address whether p27 inhibits cell proliferation, we compared the percentage of BrdU(+) cells between WT mice and p27KO mice. In WT mice, we observed that some BrdU(+) nuclei (Fig. 5B) were surrounded by nsTub-labeled cytoplasm (Fig. 5A), indicating that these proliferating cells are neuronal progenitors. Given the time span after BrdU injection (3 h), these BrdU(+) cells are likely in the So rG2 phase of the cell cycle. Furthermore, in cultured SVZa progenitor cells, some nsTub(+) cells were also labeled with phospho-Histone H3, a marker for M phase (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

We quantitatively scored the percentage of BrdU(+) cells in the RMS and neuronal cell layers of the olfactory bulb. In WT mice, we observed that BrdU(+) cells displayed a caudal high–rostral low gradient at each age analyzed (P1, P7, P14), which was in contrast with the gradient of p27 expression (Fig. 5C–E). We also found that, from P1 to P14, the percentage of BrdU(+) cells was significantly higher in p27KO mice compared with WT mice in all regions analyzed (Fig. 5C–E). At P1, WT mice exhibited a well developed BrdU labeling gradient (Fig. 5C) that was absent in p27KO mice (SVZa vs vl is slight decrease in OBc). Importantly, although proliferation in the SVZa of WT mice declined from P1 to P7 and P14 (≈ 50%), the percentage of BrdU(+) in p27KO mice showed a significant increase at each age analyzed (Fig. 5C–E).
little change. Finally, despite the fact that cell proliferation in p27KO mice remained higher than in WT mice from P1 to P14, the p27KO mice displayed a progressively steeper gradient with increasing age (Fig. 5C–E).

To further assess the role of p27 in inhibiting cell proliferation, we analyzed the ratio of the percentage of BrdU(+) cells between p27KO and WT mice. This ratio displayed a trend toward higher values from SVZa to the OBc (particularly high in the OBc) (Fig. 5F, G), suggesting that the contribution of p27 to inhibition of cell proliferation increases as cells migrate from the SVZa to the OBc. This finding is consistent with the spatial expression pattern of p27 shown in Figure 2. However, even in p27KO mice, the proliferation rate in OBc was much lower than in the SVZa, and proliferation decreased with increasing age, indicating an important role of other factors in controlling the cell proliferation rate. At all ages studied, the proliferation rate was also higher in target layers of the olfactory bulb (gcl, gl) of p27KO mice compared with WT mice. At P14, proliferation persists in these neuronal layers of p27KO mice, but it was almost undetectable in WT mice. Altogether, these data indicate that SVZa-derived neuronal progenitor cells can proliferate in vivo and in vitro and p27 inhibits the proliferation of cells in the RMS and olfactory bulb.

The RMS size is increased in the p27KO mouse

To determine whether the changes in cell proliferation observed in the p27KO mice affected the size of the RMS, we measured the thickness of RMS in the hl of the RMS. Figure 6, A and B, shows fluorescent photomicrographs showing staining of the nuclear dye Hoechst 33342 in parasagittal sections of the RMS in the P1 WT (A) and p27KO (B) mice. Arrowheads point to the location where the thickness of RMS was measured in the hl of the RMS. Scale bar, 400 μm. C, D, Bright-field photomicrographs of NeuN staining showing that at P1 the olfactory bulb gcl is thinner in p27KO mice (D) compared with WT mice (C). Dashed lines outline the gcl. Scale bar, 400 μm. E, F, Quantitative comparison of the thickness of the RMS and gcl of olfactory bulb between WT and p27KO mice. E, The RMS thickness is significantly larger in the p27KO mice compared with WT mice at all ages analyzed. *p < 0.05. F, At P1, the thickness of the gcl is significantly thinner in p27KO mice than in WT mice. *p < 0.05. The number of NeuN(+) cells per 200 μm in the RMS. At P1, the number of NeuN(+) cells per 200 μm length appears to be lower in p27KO mice than in WT mice, although not significantly different. At P7, the number of NeuN(+) cells is significantly higher in p27KO mice than in WT mice. At P14, the number of NeuN(+) cells appears to be higher in p27KO mice than in WT mice, although not significantly different. *p < 0.05. H, The graph shows that there is not a significant difference in the NeuN(+) cell density in the gcl between the genotypes at any of the ages quantitatively analyzed.
the RMS of P1 WT and p27KO mice, respectively. At all ages tested, the diameter of the hl was significantly larger (~20%) in p27KO mice than that in WT mice (Fig. 6E). These data strongly suggest that a higher cell proliferation rate in the RMS contributes to an increase in its size.

Development of the neuronal cell layers of the olfactory bulb is delayed in the p27KO mouse

We also investigated whether the development of the olfactory bulb was affected in p27KO mice by comparing the thickness of the gcl and the number of neurons in WT and p27KO mice. Given that SVZa-derived neuronal progenitor cells will become postmitotic when they reach the gcl, we labeled sections with the mature neuronal marker NeuN to visualize the gcl. Figure 6, C and D, shows a photomontage of the P1 gcl in the WT and p27KO mouse, respectively. By taking the tip of the olfactory bulb as 12:00, we found that at the 11:00 position the thickness of the gcl was significantly decreased (~25%) in p27KO mice compared with WT mice. Conversely, only nonsignificant differences were observed at P7 or P14 (Fig. 6F). We then counted the number of NeuN(+) cells in the gcl in the two genotypes. At P1, the number of NeuN(+) cells was reduced in p27KO versus WT mice, although this difference was not significant. In contrast, at P7 the number was significantly higher in p27KO mice than in WT mice (Fig. 6G). At P14, this trend continued, although differences were not significant (Fig. 6G).

The differences in the number of NeuN(+) cells observed between p27KO and WT mice could be attributable to the decrease in thickness of the gcl, therefore we also quantified the density of NeuN(+) cells in this region. We observed that cell density did not significantly change between genotypes at any age analyzed (Fig. 6H). These data indicate that development of the gcl was delayed at P1, but this was not caused by a delay in neuronal cell differentiation.

p27 plays a role in cell survival in the RMS

To study whether cell survival was affected in p27KO mice, we performed TUNEL labeling as a marker of cells undergoing cell death at different developmental stages. The number of TUNEL(+) cells was counted, and the density of TUNEL(+) cells was determined in the entire RMS and gcl.

Figure 7 and supplemental Figure 3A–D (available at www.jneurosci.org as supplemental material) show that, in both genotypes and at all ages, we observed TUNEL(+) cells distributed randomly and did not display an apparent gradient. No more TUNEL(+) cells were found in the SVZa, where a higher cell proliferation index was found (Fig. 5) compared with other regions (Fig. 7; supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

To determine the phenotype of TUNEL(+) cells, we per-
formed nsTub/TUNEL double labeling. Consistent with previous reports (Doetsch et al., 2002; Moreno-Lopez et al., 2004), at P1 a small percentage of TUNEL(+) cells were also nsTub(+)(data not shown), and the majority of TUNEL(+) cells were not labeled with nsTub. At this age, the density of TUNEL(+) cells was higher in the RMS of p27KO than in WT mice (Fig. 8A). At P7 and P14, the density of TUNEL(+) cells in the RMS did not show significant differences between p27KO and WT mice (Fig. 8A). Finally, at P1, the density of TUNEL(+) cells in the gcl was significantly higher in p27KO mice than in WT mice, but it was not significantly different at P7 and P14 (Fig. 8B). These data indicate that increased apoptosis contributes to the delay in the development of the gcl in P1 p27KO mice. Because the RMS also contains other progenitor cell populations during the first and second postnatal week, we believe that a percentage of the TUNEL(+) cells at P7 and P14 might be NG2-expressing cells or their progeny (Aguirre and Gallo, 2004).

Role of p27 in SVZ progenitor/stem cell proliferation and self-renewal
To determine whether the phenotype observed in p27KO SVZ progenitors in vivo was also reproduced in vitro, we analyzed the proliferation potential of NPCs of the WT and p27KO mouse SVZ by neurosphere formation assays. The number of neurospheres was determined after three successive passages (7 d each) of NPCs from p27KO and WT mice. Throughout all three passages, the number of neurospheres calculated per 10,000 seeded cells was significantly higher in the p27KO than in WT mice (Fig. 9 A, D, G). Infection of a p27 retrovirus in both groups of cells significantly diminished the number of formed neurospheres in the second and third passage compared with cells infected with a mock retrovirus (Fig. 9 B, C, E, F, H, I). The effects of the retrovirus on neurosphere formation were more pronounced in p27KO cells than in WT cells (Fig. 9 C, F, H, I).

To determine the proliferation rate in p27KO and WT NPCs after retroviral infection, we immunolabeled cultured cells or neurospheres with anti-Ki67 antibody. Both in p27KO and WT cultures, the rate of proliferation was significantly reduced after retroviral infection compared with cells infected with a mock retrovirus (Fig. 10 A, B, E, F, J). Caspase 3 immunostaining was also used to determine whether p27 overexpression enhanced cell apoptosis. After infection with the p27 retrovirus, apoptosis increased by ~6-fold in p27KO cells and by 1.5-fold in WT cells (Fig. 10 C, D, G, H, J).

To gain further insight into the molecular mechanism(s) underlying the involvement of p27 in SVZ cell proliferation, we analyzed expression of a number of cell-cycle regulatory proteins, including the following: (1) the cyclin-dependent kinases CDK2, CDK4, and CDK6; (2) their activators cyclin D and cyclin E; (3) the other inhibitor p21Cip1; and (4) their phosphorylated substrates: Rb-total, p-Rb(Ser780), p-Rb(Ser807). Western blot analysis was performed in WT and p27KO SVZ protein extracts from P1 mice. Figure 11 shows that, in p27KO SVZ tissue, expression of CDK2, Rb-total, p-Rb(Ser780), and p-Rb(Ser807) was upregulated compared with WT. Conversely, expression of p21 was reduced in p27KO SVZ compared with WT.

Altogether, these results indicate that p27 plays an important role in regulating NPC proliferation in the SVZ and strongly suggest that the CDK2–Rb pathway is engaged in maintaining proliferation of this progenitor cell population.

Discussion
In this study, we determined the spatiotemporal expression pattern of p27 in the RMS and in neuronal cell layers of the olfactory bulb. Spatially, the expression of p27 increases from the SVZa to the olfactory bulb, similar to the p19 expression pattern (Coskun and Luskin, 2002), although the increase observed in p27 shifted more rostrally. Temporally, the expression of p27 at P1 is higher than that at P7 and P14, consistent with a previous study showing a decreasing mRNA expression of p27 in the RMS with age (van Lookeren Campagne and Gill, 1998). Concerning p27 in the neuronal cell layers of the olfactory bulb, its expression decreases with age in the gcl but increases in the gl. Collectively, these findings support the notion that the interneurons in the gcl and gl may be different neuronal subtypes (Smith and Luskin, 1998; Hack et al., 2005; Kohwi et al., 2005; Lemasson et al., 2005), and that p27, as well as the other cell-cycle inhibitor p19, plays different roles in these interneurons, such as regulation of cell-cycle exit or maintenance of cellular quiescence (Legrier et al., 2001).

SVZa-derived neuronal progenitor cells migrate toward the olfactory bulb along the RMS, and differentiate into mature interneurons when they reach the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1994). These neuronal progenitor cells continue to divide during their migration (Menezes et al., 1995; Smith and Luskin, 1998; Law et al., 1999; Coskun and Luskin, 2002). Recent studies have shown that many factors, such as CDKI p19 (Coskun and Luskin, 2002), slit (Nguyen-Ba-Chari et al., 2004), tenasin-R (Saghatelyan et al., 2004), and prokine
tin 2 (Ng et al., 2005) are involved in the regulation of the proliferation, migration, and differentiation processes in this pathway. It has also been found that p27, a member of the CDKIs, regulates neural cell proliferation, migration, and differentiation in different brain regions, including the cerebellum (Miyazawa et al., 2000), retina (Levine et al., 2000; Cunningham et al., 2002), cerebral cortex (Zindy et al., 1999; Goto et al., 2004; Nguyen et al., 2006), adult subventricular zone (Doetsch et al., 2002), and olfactory epithelium (Legrier et al., 2001).

The function of p27 is closely related with its subcellular localization. The nuclear localization of this protein is a prerequisite for its inhibitory action on cell proliferation (Reynisdottir and Massague, 1997; Orend et al., 1998; Tomoda et al., 1999). In the nucleus, p27 inhibits the activity of cyclin E/A–CDK2 and prevents cell-cycle progression (Sherr and Roberts, 1995). Conversely, p27 is exported to the cytoplasm after phosphorylation in Thr 187 or Ser 10 (Rodier et al., 2001; Boehm et al., 2002). Cytoplasmic p27 decreases the level of nuclear p27, a regulatory step that is required for a cell to reenter the cycle, and promotes cell migration, as demonstrated in HepG2, fibroblasts, lung cancer...
Overexpression of p27 reduces proliferation and self-renewal of NPCs of the SVZ. A–F, SVZ neurospheres were obtained from single cell suspensions prepared from WT (A–C) and p27KO (D–F) mice at P1. To assess neural progenitor self-renewal potential, neurosphere numbers were counted after the first, second, and third passage, respectively. B, C, E, F, SVZ cells from P1 brains were first cultured and then infected with either a mock-retrovirus (B, E) or p27-retrovirus (C, F) to assay proliferation and self-renewal in SVZ cells of the WT and p27KO mice. Scale bars, 500 μm. G, A higher number of neurospheres were found throughout all three passages in p27KO cultures compared with WT. 

Our study also indicates that, in early postnatal SVZ neural progenitors, cell survival is decreased in p27KO mice compared with WT mice. This suggests that apoptosis regulated cell survival and contributes to the delayed development of the gcl. In this study, we did not find that SVZa exhibits higher apoptosis compared with other regions, although this region displays the highest proliferation rate. Therefore, in agreement with previous studies (Moreno-Lopez et al., 2004; Lemasson et al., 2005), apoptosis was not affected when SVZ cell proliferation rate was enhanced.

Consistent with a previous report demonstrating that a loss of p27 in the adult SVZ promotes proliferation of transit amplifying cells (Doetsch et al., 2002), we observed enhanced SVZ cell proliferation in the p27KO mouse, as demonstrated by a higher percentage of Ki67+/cells and a higher rate of neurosphere formation than in WT cells. To establish that p27 only partially contributes to regulate cell proliferation in these brain regions (Miyazawa et al., 2000), and some CDKs (van Lookeren Campagne and Gill, 1998; Padmanabhan et al., 1999) or CDK inhibitors (CDKIs) such as p16 (Molofsky et al., 2006), p19 (Zindy et al., 1999; Coskun and Luskin, 2001; Cunningham et al., 2002), p21 (Siegenthaler and Miller, 2005), and p57 (Dyer and Cepko, 2001) are involved in regulating cell proliferation or maintaining cells in the postmitotic state.
cantly reduced compared with WT cells. This is at variance from previous in situ analysis of the adult SVZ (Doetsch et al., 2002); however, this discrepancy could be explained by our parallel finding that in the early postnatal SVZ of the p27KO mouse p21 is upregulated in NPCs. It has been demonstrated that p21 plays an important role in maintaining self-renewal and survival of neural progenitors (Herzog et al., 2002; Kippin et al., 2005; Shi et al., 2005). Additionally, in agreement with Doetsch et al. (2002), we also observed a significant increase in cell death of Tuj1(+) neuroblasts in the RMS and gel of the olfactory bulb, indicating that p27 is involved in cell survival of committed neuronal progenitors.

CDKs regulate cell proliferation via activation of different molecular targets, including Rb, p107, and p130 (Yoshikawa, 2000). Based on Western blot analysis of SVZ proteins in WT and p27KO mice, we are proposing that CDK2 and Rb play a major role in regulating NPC proliferation in the p27KO mouse SVZ.

It is established that Rb activation occurs through hyperphosphorylation of specific amino acid residues, resulting in the release of E2F transcription factors, which play a crucial role in promoting G1–S transition (Mittnacht, 1998; White et al., 2005; Knudsen and Knudsen, 2006). Indeed, we found that, together with enhanced expression levels of CDK2 and Rb, Rb phosphorylation was also increased in the SVZ of the p27KO mouse, indicating that the CDK2–Rb pathway is engaged in maintaining proliferation of SVZ progenitor cells. Together with an increase in cell proliferation, we also observed lower levels of p21 in the SVZ of p27KO mice, suggesting a reduction in cell differentiation. These data are consistent with studies in which p21, among other CDKIs, was shown to be involved in glial cell differentiation (Durand et al., 1998; Zezula et al., 2001).

Figure 10. Overexpression of p27 reduces cell proliferation in WT and p27KO NPCs and promotes cell death in p27KO SVZ cells. A–H, SVZ cells from P1 WT and p27KO brains were first cultured and then infected with a mock-retrovirus or a p27-retrovirus. Cells were maintained under culture conditions that promoted cell proliferation for 7 d. Cells were fixed, and immunocytochemistry was performed with anti-Ki67 (A, B, E, F) and anti-Caspase 3 (C, D, G, H) antibodies. Scale bars: A, B, E, F, 250 μm; C, D, G, H, 100 μm. I, Quantitation of cell proliferation, as determined by anti-Ki67 immunostaining. In both WT and p27KO cells, a reduction in cell proliferation (percentage of Ki67(+) cells) was observed after p27-retrovirus infection compared with mock-retrovirus. J, Quantitation of Caspase 3(+) cells after p27- and mock-retrovirus infection. Note that only in p27KO cells did p27 overexpression increase the percentage of Caspase 3(+) cells. Data were obtained from second and third passage neurospheres of three independent experiments and are expressed as averages ± SEM. *p < 0.03 (t test).
In summary, by analyzing early postnatal development of the p27KO mouse, we demonstrate that this CDKI plays a crucial role in regulating cell proliferation and survival in major neurogenic regions of the postnatal brain, including the SVZ, RMS, and olfactory bulb. Our results point to p27 as one of the crucial regulators of postnatal neurogenesis and a putative target for strategies aimed at enhancing neuronal cell repair and regeneration that include either manipulation of endogenous progenitors or transplantation of exogenous undifferentiated neural cells.

References


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