p27kip1 Is Required for Functionally Relevant Adult Hippocampal Neurogenesis in Mice

Henrik Hörster, Alexander Garthe, Tara L. Walker, Muhammad Ichwan, Barbara Steiner, Muhammad Amir Khan, Dieter Chichung lie, Zeina Nicola, Gerardo Ramirez-Rodriguez, Gerd Kempermann

STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

p27kip1 Is Required for Functionally Relevant Adult Hippocampal Neurogenesis in Mice

HENRIK HÖRSTER,[a] ALEXANDER GARTHE,[b] TARA L. WALKER,[a] MUHAMMAD ICHWAN,[a] BARBARA STEINER,[c] MUHAMMAD AMIR KHAN,[d] DIETER CHICHUNG LIE,[e] ZEINA NICOLA,[a,b] GERARDO RAMIREZ-RODRIGUEZ,[f] GERD KEMPERMANN[b]

Key Words. Stem cells • Hippocampus • Learning • Cell cycle • Granule cells • Plasticity

ABSTRACT

We asked whether cell-cycle associated protein p27kip1 might be involved in the transition of precursor cells to postmitotic maturation in adult hippocampal neurogenesis. p27kip1 was expressed throughout the dentate gyrus with a strong nuclear expression in early postmitotic, calretinin-positive neurons and neuronally determined progenitor cells (type-3 and some type-2b), lower or absent expression in radial glia-like precursor cells (type-1) and type-2a cells and essentially no expression in granule cells. This suggested a transitory role in late proliferative and early postmitotic phases of neurogenesis. Inconsistent with a role limited to cell cycle arrest the acute stimuli, voluntary wheel running (RUN), environmental enrichment (ENR) and kainate-induced seizures increased p27kip1 expressing cells. Sequential short-term combination of RUN and ENR yielded more p27kip1 cells than either stimulus alone, indicating an additive effect. In vitro, p27kip1 was lowly expressed by proliferating precursor cells but increased upon differentiation. In p27kip1−/− mice neurogenesis was reduced in vivo, whereas the number of proliferating cells was increased. Accordingly, the microdissected dentate gyrus of p27kip1−/− mice generated more colonies in the neurosphere assay and an increased number of larger spheres with the differentiation potential unchanged. In p27kip1−/− monolayer cultures, proliferation was increased and cell cycle genes were upregulated. In the Morris water maze p27kip1−/− mice learned the task but were specifically impaired in the reversal phase explainable by the decrease in adult neurogenesis. We conclude that p27kip1 is involved in the decisive step around cell-cycle exit and plays an important role in activity-regulated and functionally relevant adult hippocampal neurogenesis. STEM CELLS *2017;35:787–799*

SIGNIFICANCE STATEMENT

The cell cycle associated factor p27kip1 has multiple roles in adult hippocampal neurogenesis, including the control of cell cycle exit. A p27kip1 knockout mouse shows increased proliferation of precursor cells, but (as shown in the study) reduced net neurogenesis: p27kip1 is required for controlled cell cycle exit.

INTRODUCTION

Adult hippocampal neurogenesis consists of a series of consecutive developmental steps, many of which show a distinct and partly independent regulation. While large numbers of genes have been described that directly or indirectly affect cell proliferation, information about effects on other stages of development is scarce [1]. We were interested in key molecules at the transition point between the proliferative precursor cell stage and the postmitotic differentiation stages.

Central to our interests are the differential effects of behavioral interventions on cells in the course of adult neurogenesis. Physical activity (i.e., here voluntary wheel running of rodents, RUN) is a strong inducer of adult hippocampal neurogenesis with a prominent, but not exclusive, effect on precursor proliferation, especially at the level of intermediate type-2 progenitor cells [2]. Exposure to an enriched environment (ENR) in contrast primarily (but again not exclusively) promotes the survival of early postmitotic cells [2]. Five weeks after only 10 days of exercise, neurogenesis from cells that had been labeled during the last three days of the exercise period was also increased [3], suggesting that exercise would also exert a pro-neurogenic effect beyond the induction of proliferation. Brandt and coworkers discovered that exercise indeed promotes cell cycle exit [4].

[a]CRTD – Center for Regenerative Therapies Dresden, Technische Universität Dresden, Dresden, Germany; [b]German Center for Neurodegenerative Diseases (DZNE) Dresden, Dresden, Germany; [c]Department of Neurology, Charité University Medicine Berlin, Berlin, Germany; [d]Adult Neurogenesis Group, Institute of Developmental Genetics, Helmholtz Center Munich, Oberschleißheim, Germany; [e]Institut für Biochemie, Friedrich-Alexander Universität Erlangen-Nürnberg, Erlangen, Germany; [f]Laboratory of Neurogenesis, Division of Clinical Investigations, National Institute of Psychiatry "Ramón de la Fuente Muñiz", México, D.F, México

Correspondence: Gerd Kempermann, M.D., German Center for Neurodegenerative Diseases (DZNE) Dresden, CRTD — DFG Center for Regenerative Therapies Dresden, Dresden, Germany, Fetscherstraße 105, 01307 Dresden, Germany. Telephone: +49 (0) 351 458 82201; Fax: +49 (0) 351 458 82209; e-mail: gerd.kempermann@crt-dresden.de; gerd.kempermann@dzne.de

Received December 5, 2013; accepted for publication October 10, 2016; first published online in STEM CELLS *EXPRESS* October 28, 2016.

1066-5099/2016/$30.00/0

http://dx.doi.org/10.1002/stem.2536

Searching for specific regulatory factors that might mediate this effect, we here focused on p27kip1 (Cdkn1b) because of its proposed role in cell cycle exit [5]. Together with p16(Ink4A), p18(Ink4C), p19(Ink4D), and p21(Waf1/Cip1), p27kip1 acts as inhibitor of cyclin-dependent kinases. P27kip1 has already been studied in the context of adult neurogenesis in the subventricular zone (SVZ)/olfactory bulb, where p27kip1 deletion resulted in decreased neurogenesis, presumably via increasing the number of intermediate progenitor cells [6, 7]. Essentially the same effect has also been found at the peak of olfactory neurogenesis early postnatally [8]. Relatedly, Mairet-Coello and coworkers reported that while p57Kip^2 affects both radial glial cells and intermediate progenitor cells during corticogenesis, p27kip1 exclusively regulates the intermediate progenitor cells [9]. Andreu and coworkers finally showed, in what might appear as partial conflict with that observation, that p27kip1 is critical for maintaining quiescence of radial glia-like type 1 precursor cells in the adult hippocampus. They described that the cyclin-CDK interaction domain is necessary for these stem-cell related effects. But Andreu et al. also described that p27kip1 remains expressed beyond that initial stage and provided arguments that p27kip1 might be also involved in cell-cycle exit [10]. Our present study addresses issues related to that second aspect but extends on their finding, especially by placing it in the context of activity-dependent regulation and function. Their study and ours are thus complementary, together drawing a complex picture of p27kip1 function in adult neurogenesis.

There are additional previous reports on p27kip1 in the context of adult hippocampal neurogenesis. Ramos and coworkers reported that decreased proliferation after treatment with Diethylstilbestrol was associated with increased p27kip1 in type-2 progenitor cells [11]. In a model of status epilepticus, Varodayan et al. described shortened cell cycle with unaltered p27kip1 expression but decreased phosphorylation of p27kip1 and increased p27kip1 expression with increasing differentiation [12].

In the context of cannabinoid receptor-dependent regulation of adult hippocampal neurogenesis, Palazuelos and coworkers showed that inhibition of p27kip1 as a downstream target of the mTOR pathway resulted in increased proliferation [13]. Finally, in human hippocampal precursor cells the antidepressant sertraline increased differentiation and inhibited proliferation, an effect that was associated with increased expression of p27kip1 [14].

Regulation through p27kip1 would not be limited to neurogenesis per se. For example, in spinal cord injury FOXO3a expression as potential upstream regulator was decreased leading to reduced p27kip1 expression in astrocytes and increased proliferation [15]. Similar findings have been made for traumatic brain injury [16]. This might speak in favor of a conserved yet manifold p27kip1-dependent mechanism in neural precursor cell biology.

The aim of the present study was to provide evidence that besides its established function in stem cell function [10], p27kip1 is an important molecule at the critical step of adult hippocampal neurogenesis between the proliferative and the postmitotic stage of neuronal development, especially in the context of activity-dependent regulation.

Materials and Methods

Animals and Housing Conditions

The experiments on the acute expression of p27kip1 after exposure to ENR, RUN and kainic acid (KA) was done on tissue from our previously published study [17]. For the study of additive effects, female C57BL/6 mice ($N = 6$ per group) were obtained from Charles River (Sulzfeld, Germany). The age of the animals was 8-10 weeks at the beginning of the experiments. Mice with p27kip1 +/+, p27kip1 +/− and p27kip1 −/− genotypes ($N = 5$ per group) were a kind gift of Helmut Kettenmann, Berlin. All animals were kept in the animal facility of the Max Delbrück Center for Molecular Medicine (MDC) Berlin-Buch and appropriate permission was obtained from the appropriate local authority at the time of the experiment (LaGetSi). The behavioral experiments and all cell culture studies were done at the CRTD in Dresden and here the approval was granted by Regierungspräsidium Dresden. All applicable national and European rules for animal welfare were followed. Dependent on the experiment, the mice were kept either under standard housing conditions, or under experimental housing conditions as described below, with a 12 hours light/dark cycle and food and water ad libitum.

Experimental Design

Mice were injected intraperitoneally with a single dose of the proliferation marker 5-bromo-2′-deoxyuridine (BrdU, Sigma, Steinheim, Germany) and 1 hour later exposed to RUN, ENR, or KA conditions. Twenty-four hours later the animals were killed and their brains processed. Details about conditions of this short-term experiment, the application of BrdU and kainic acid (KA) has been described elsewhere [17]. Importantly, in that experiment BrdU was injected before the animals were exposed to either of the three experimental or control conditions!

The experiment on additive effects (Fig. 2) was designed after our previous study [3] but with much shorter exposure times (3 + 4 days as opposed to 10 + 35 days). Cells were labeled with BrdU on the last day of the initial 3-day period.

In the experiment with the p27kip1 knockout mice, BrdU was injected 2 hours before perfusion.

Tissue Preparation

Under deep anesthesia with ketamine (50 mg/kg body weight, Sigma, Germany), animals were perfused transcardially with 4% paraformaldehyde (PFA, Merck, Darmstadt, Germany) in 0.1 M phosphate buffer, pH 7.4. After having been removed from the skulls, postfixed in 4% PFA overnight and transferred into a 30% sucrose solution in 0.1 M phosphate buffer (pH 7.4), brains were cut in 40 μm thick coronal sections on a dry-ice-cooled copper block on a sliding microtome (SM 200R, Leica, Bensheim, Germany) and cryoprotected.

Immunohistochemistry

To detect dividing cells, we used the proliferation marker BrdU visualized with the 3,3′-diaminobenzidine (DAB) peroxidase method (ABC, Vectastain Elite, Vector Laboratories, Biozol, Eching, Germany). Pretreatment for BrdU staining was done with 2 N hydrochloric acid for 30 minutes in order to denature DNA. As primary antibody we used monoclonal rat

anti-BrdU (1:500; Biozol, Eching, Germany), as secondary biotinylated donkey anti-rat (1:500;z Jackson ImmunoResearch Europe, Suffolk, U.K.). For further details of BrdU staining procedure see [17]. Sections were mounted on slides and coverslipped with Neomount.

Immunofluorescent staining was done as described elsewhere [18]. The primary antibodies were applied in the following concentrations: goat anti-Doublecortin (DCX; 1:200; Santa Cruz Biotechnologies, Heidelberg, Germany), guinea pig anti-glial fibrillary acidic protein (anti-GFAP, 1:1,000, Advanced Immunochemistry, California, U.S.A.), rabbit anti-GFP (1:400, Abcam, Cambridge, U.K.), goat anti-GFP (1:1,000, Santa Cruz Biotechnologies), rabbit anti-Calretinin (1:2,000; Swant, Marly, Switzerland), mouse anti-p27 (1:1,000; BD Transduction Laboratories, Erembodegem, Belgium) for DAB-peroxidase staining and rabbit anti-p27 (1:500; Santa Cruz Biotechnologies) for immunofluorescent labeling. As secondary antibodies for immunofluorescent staining, we used: donkey anti-goat, donkey anti-guinea pig, and donkey anti-rat, donkey anti-rabbit and donkey anti-mouse conjugated to different fluorophores (Cy5, FITC, Rhod-X, all 1:250, Jackson Immunoresearch). Fluorescent sections were mounted in polyvinyl alcohol with diazabicyclo-octane (DABCO, Biozol, Eching, Germany) to avoid fading.

Quantification

Our quantification protocol of cells labeled with the DAB-peroxidase method, in this study BrdU-, Ki67, and p27kip1-positive cells, has been described elsewhere [18] and has been used in numerous later studies. Sections of 40 μm thickness were counted in one-in-six series through a light-microscope (40x objective; Leica, Bensheim) and the numbers multiplied by 6 to assess the total number of cells per dentate gyrus (SGZ and granule cell layer, left hemisphere). Ki67 numbers shown are the mean for both hemispheres.

Triple-labeled cells were analyzed using spectral confocal microscopy (Leica TCS SP2 or SP5).

Adherent Adult Hippocampal Precursor Cells

Adult hippocampal neural precursor cells were isolated and cultured as previously reported [19]. Precursor cells were plated on laminin precoated coverslips or dishes and cultured with 20 ng/ml of human epidermal growth factor (EGF) and 20 ng/ml of human Fibroblast Growth Factor-2 (FGF2; both from PeproTech, Hamburg, Germany) in Neurobasal medium supplemented with B27 (Gibco, Darmstadt, Germany), for 24 hours. After this time, we replaced the medium for medium without added growth factors to induce precursor cell differentiation. Individual monolayers of precursor cells were fixed or lysed at different time points to perform immunocytochemistry or Western blot.

Western Blot

Precursor cells were lysed as reported [20]. Total lysate from the precursor cell cultures was obtained with radioimmunoprecipitation assay buffer (RIPA) buffer (150 mM sodium chloride, 10% glycerol, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml leupeptin, 25 μg/ml aprotinin and 1 mM sodium ortho-vanadate in 50 mM Tris-HCl, pH 7.6) and homogenized with an ultrasonic homogenizer for 30 seconds. Total protein was obtained by centrifugation at 14,000 rpm and the content was quantified using Bradford reagent (Bio-Rad, Munich, Germany). Proteins were separated and transferred to nitrocellulose paper. Membranes were blocked with 5% skim milk in 0.05% Tween 20 tris-buffered saline (TTBS) and incubated with the rabbit anti-p27 (1:1,000; Santa-Cruz, Heidelberg, Germany) and with the mouse anti-GAPDH antibody 1:5,000 (Chemicon, Hampshire, England). Blots were washed three times with TTBS and incubated for 1 hour in a 1:1,000 dilution of horseradish peroxidase-conjugated donkey anti-mouse or donkey anti-rabbit antibodies. Proteins were visualized with the enhanced chemiluminescence detection system (Immobilon; Millipore, Billerica, MA). Autoradiograms were scanned and analyzed with the ImageJ program (NIH).

Neurosphere Culture

Eight week-old p27kip1+/+ or p27kip1−/− mice were killed, their brains immediately removed, and the dentate gyri were microdissected [21, 22]. The tissue was enzymatically digested using the Neural Tissue Dissociation Kit (Miltenyi, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Following a final wash in Hank's balanced salt solution (HBSS, PAA, Laboratories, Cölbe, Germany) the pellet was resuspended in 1 ml of HBSS and filtered through a 40 μm cell sieve (Falcon). Cells were plated at a density of one dentate gyrus per 96-well plate, which from our experience yields 0 to 1 spheres per well in wild-type C57Bl/6 mice of the indicated age. We did not assess the fraction of spheres per number of cells isolated but the yield from the tissue. The neurosphere growth medium consisted of Neurobasal medium (Gibco, Life Technologies, Darmstadt, Germany), supplemented with 2% B27 (Thermo Scientific, Waltham, MA, USA), 1× GlutaMAX (Thermo Scientific, Waltham, MA, USA) and 50 units per millilter Penicillin/Streptomycin (Life Technologies). The following growth factors were also included: 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml fibroblast growth factor 2 (FGF2). Potassium chloride concentration was added to half of the wells at a final concentration of 15 mM. Cells were incubated in humidified 5% CO_2 for 7 days for SVZ or 12 days for dentate gyrus to permit neurosphere formation. All experiments were done in triplicates.

For differentiation, neurospheres were plated onto poly-D-lysine (PDL, Sigma, Steinheim, Germany) and laminin-coated coverslips in the neurosphere medium without growth factors. The neurospheres were allowed to differentiate for 7 days in humidified 5% CO_2 until flattened and adherent. The differentiated neurospheres were then fixed with 4% PFA in 0.1 M phosphate buffered saline (PBS) at room temperature for 30 minutes. After washing with PBS, they were stained for either the neuronal markers βIII-tubulin, the astrocytic marker GFAP, the oligodendrocyte marker O4, with a 4,6-diamidino-2-phenylindole (DAPI) counterstain to visualize the nuclei. Per condition, 4 coverslips were seeded with multiple neurospheres each and differentiated as described. For quantification, at least four random fields of view (based on Dapi) were chosen and phenotyped, resulting in a total of 12 counts per condition.

Morris Water Maze Task

As described elsewhere [23], mice were trained in the reference memory version of the Morris water maze task [24] to locate a hidden escape platform in a circular pool (1.89 m diameter). Water was made opaque with nontoxic white paint and kept at a temperature of 19°C–20°C. Each mouse was

given 6 trials a day for 5 consecutive days with an intertrial interval of 30 minutes. The platform position was changed after day 3 (Fig. 6). Mice were released from one of four possible starting points and allowed to search up to 120 seconds for the platform. During each day the starting position remained constant. Irrespective of trial performance mice were guided to the platform and allowed to remain there for at least 15 seconds. Swim paths were recorded using Ethovision (Noldus) and further analyzed using Matlab (The Mathworks).

For testing the effects of the respective genotypes on path length, number of goal crossings and spatial strategy use, we applied analysis of variance (ANOVA) models. Testing of main and interaction effects was done using F-test statistics. Because the identity of individual mice could not be traced back, we averaged daily measurements of path length and number of spatial strategies to avoid inflated error rates due to pseudo replicates.

Parameters of the ANOVA models were estimated by a maximum likelihood approach. Appropriateness of models was confirmed by analyzing the residuals. For the behavioral data, statistical analyses were done using R.

Results

Type-2b/3 Cells in the SGZ and Postmitotic Neurons Express p27kip1

We first investigated the expression patterns of p27kip1 in the adult hippocampus. p27kip1 was detectable throughout the dentate gyrus but showed a condensation along the SGZ (Fig. 1A), suggesting an enrichment in precursor cells. In addition, some radial Nestin-GFP-positive precursor cells (type-1 cells; Fig. 1B) and nonradial type-2a cells coexpressed nuclear p27kip1, mostly at a lower intensity. The observation of p27kip1 in some radial glia-like cells is in accordance with the idea, proposed by Andreu et al. that p27kip1 is important for maintaining stem cell quiescence [10].

In contrast, most DCX-positive cells (including nonradial Nestin-coexpressing 2b cells), strongly expressed p27kip1 in the nucleus. This implied that p27kip1 is expressed by precursor cells of the SGZ with a certain emphasis on the late precursor cell stages in the course of adult neurogenesis (Fig. 1B). Quantitative statements regarding p27kip1 expressing cells in the following text relate to the strongly expressing cells, unless otherwise noted.

As DCX expression spans from precursor cell to early postmitotic stages, we stained for Calretinin (CR), which is expressed during the postmitotic maturation phase of the newborn granule cells [25]. We found that essentially all CR-positive cells also expressed p27kip1 (Fig. 1D). Based on the immunofluorescent intensity, the expression levels varied. We did not, however, perform an objective quantitative assessment of this variation. All CR-positive cells are also positive for NeuN [25]. We found that p27kip1 is essentially absent from CR-negative more mature granule cells. Besides, Figure 1C highlights that most of the weak p27kip1 is in neurons (of the inner granule cell layer), whereas (highlighted in the other panels) a strong nuclear p27kip1-expression is found in precursor cells of the SGZ.

In summary, almost every CR-positive cell, most of the DCX-positive cells, only few Nestin-GFP-positive cells and no mature granule cells of the outer granule cell layer expressed nuclear p27kip1. DCX-positive cells show strong nuclear expression of p27kip1. Later stages show a more diffuse and weak staining extending into the cytoplasm or no staining at all. This suggested to us that besides a function in some radial glia-like cells [10], p27kip1-expression initially increases with advanced neuronal development but might be redistributed with respect to the sub-cellular localization. A certain level of p27kip1-expression might be required for the maintenance of the initial maturation stage and this function might be independent of the role in cell cycle exit.

Behavioral Activity Increases p27kip1 Expressing Cells

We have previously shown that the acute (24 hours) exposure to the experimental conditions of voluntary wheel RUN and ENR, as well as the induction of seizures by systemic application of Kainate (KA) all increased precursor cell proliferation in the dentate gyrus [17]. We used sections from that experiment to now study how p27kip1-positive cells would respond to these conditions. We quantified cells with the strong nuclear expression as we had seen in the intermediate precursor cells.

The total number of strongly expressing p27kip1-positive cells significantly increased after acute RUN, ENR, and KA compared to CTR (ANOVA, $p < .001$), but did not show any paradigm specificity (Fig. 2). This indicated that 24 hours of exposure to pro-neurogenic stimuli acutely upregulate p27kip1-expression in the sense that more cells express p27kip1. Given that the behavioral stimuli affect precursor cells type-2 and later, this effect is presumably independent of an effect of p27kip1 on stem cell quiescence [10].

To further corroborate our result under the condition of longer exposure to the stimuli, we studied the effect of a combination of RUN and ENR on p27kip1-expression at 3 to 7 days, that is, when according to our previous analyses the peak in the pro-proliferative response to RUN has been reached [26]. We made use of an experimental design that we had used in the past to demonstrate that effects of RUN and ENR are additive, but used a condensed version [3].

We found that both 3 and 7 days of RUN increased the number of p27kip1-expressing cells in the dentate gyrus but that there was no difference between the two time-points (Fig. 2B). Compared to 7 days CTR conditions, both 7 days of RUN and 7 days of ENR significantly increased the number of p27kip1-expressing cells. The increase in RUN even significantly differed from ENR. Four days of ENR after 3 days of CTR housing increased the number of p27kip1-positive cells compared to CTR and was not different from 7 days of ENR.

The combination of both stimuli, 3 days of RUN plus 4 days of ENR, led to a significantly greater number of cells strongly expressing p27kip1 compared to both the priming stimulus alone (3 days of RUN plus 4 days of CTR conditions) but also, more importantly, 7 days of RUN, further strengthening the idea that RUN and ENR are additive also with respect to the number of p27kip1-expressing cells (Fig. 2B). This implies, however, that p27kip1 will have functions beyond cell cycle inhibition and is more generally associated with neurogenesis. This, again, is in line with previous reports [27, 28].

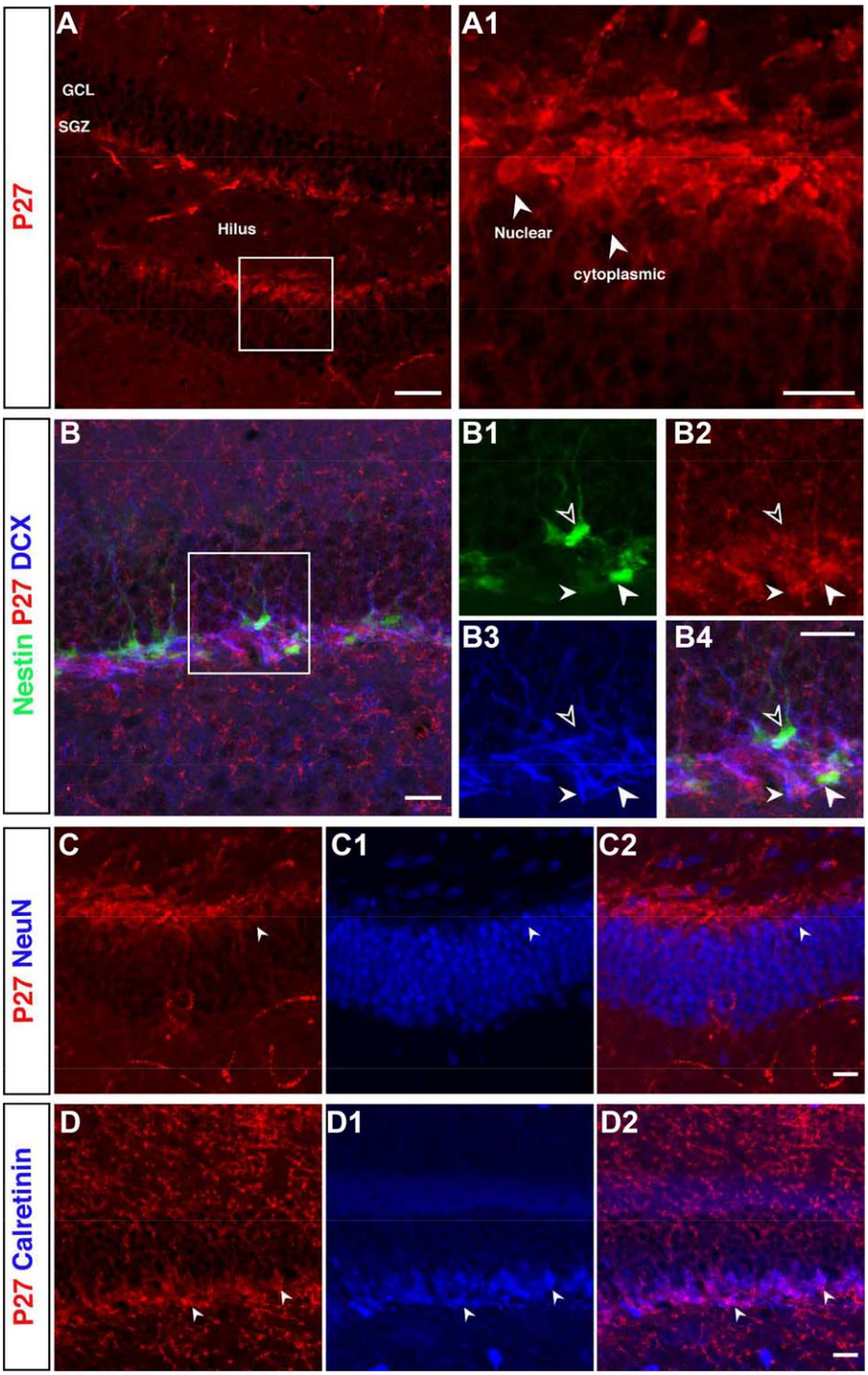


Figure 1. Confocal microscopic images of p27kip1 stain and biomarkers representing several stages of neuronal development. **(A)**: Expression-pattern of p27kip1 (red) in the dentate gyrus with nuclear condensation along the SGZ. **(A1)**: High power magnification from marked area in A to highlight the nuclear versus cytoplasmic localization of p27kip1 (arrowheads). **(B)**: Costaining of p27kip1 (red) and DCX (blue) in Nestin-GFP-positive precursor cells (green). Only few Nestin-GFP-positive cells coexpress p27kip1: type-1 cells (open large arrowhead) are usually negative for p27kip1, type-2a cells (Nestin-GFP-positive and DCX-negative) are often positive, and type-2b and type-3 cells (DCX-positive) show coexpression of p27kip1. **(C)**: Colocalization of p27kip1 (red) with NeuN (blue). **(D)**: Double-staining for Calretinin (blue) and p27kip1 (red). All CR-positive cells also express p27kip1. Scale bars, 50 μm for A and 20 μm for the other panels. Abbreviations: DCX, doublecortin; GCL, granule cell layer; SGZ, subgranular zone.

During the normal course of adult neurogenesis, most newborn cells are eliminated within about a week after division. This process is apoptotic and increasing anti-apoptotic factors increase survival [29, 30]. Exposure to an enriched environment also promotes survival by reducing cell death [31]. In the present experiment we could not yet address the

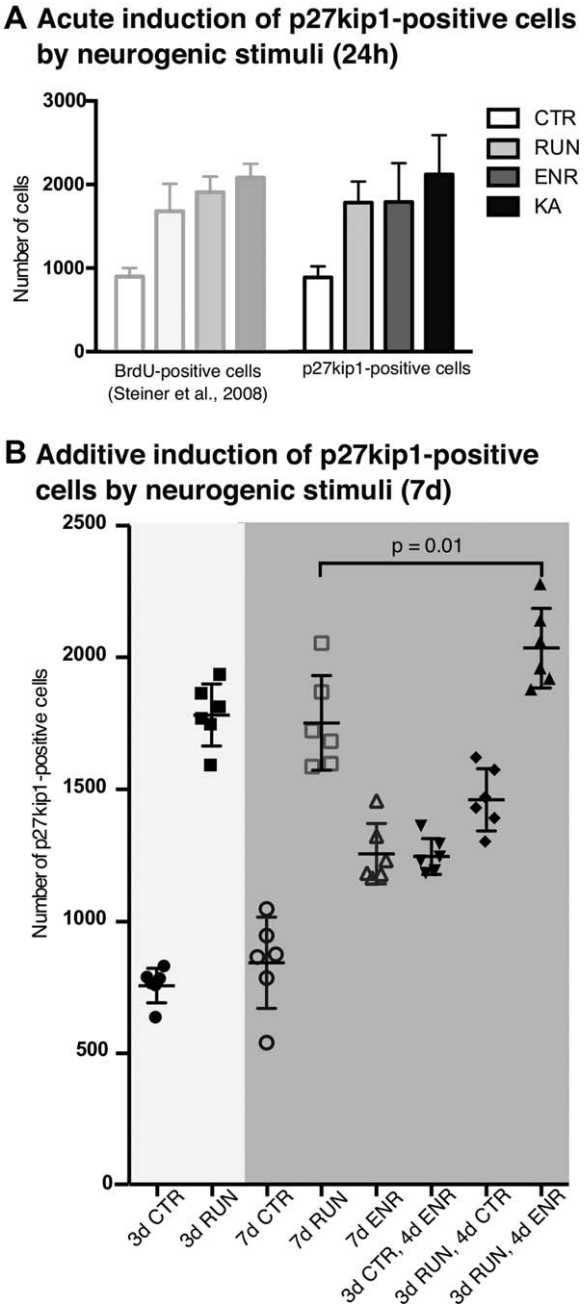


Figure 2. Quantitative assessment of p27kip1 expression under neurogenic stimulation conditions. **(A)**: Upregulation of the number of p27kip1-positive cells (strong nuclear expression) after short-term stimulation. All stimulated animals (RUN, ENR, KA) showed increased levels of p27kip1 compared to the CTR. There were no significant differences between the stimulation groups. **(B)**: Highly increased induction of p27kip1 after 3 and 7 days RUN without significant differences between the two time-points. ENR has less impact on p27kip1 expression, whereas the combination of RUN an ENR shows even significantly increased p27kip1 compared to 7 days RUN ($p = .01$). Abbreviations: CTR, control group; ENR, environmental enrichment; KA, kainate; RUN, running.

question, how p27kip1 might be involved in survival mechanisms. It is possible that knockout of p27kip1 not only kept cells in cell cycle but also increased cell death of cells at this stage. A likely contribution of p27kip1 to survival is related to mediating autophagy [32]. For our context this question will have to be addressed in specifically designed experiments.

In Vitro, p27kip1 Is Increased upon Differentiation

We next used adherent monolayer cultures of adult hippocampal precursor cells to further analyze this pattern (Fig. 3). These cultures allow studies at the level of individual cells and are very homogenous with up to 95%-98% representing precursor cells. In some contrast to our in vivo results we found that under proliferation conditions (Fig. 3A, 3D), there was a cytoplasmic immunofluorescent signal for p27kip1 in essentially all cells. Upon withdrawal of growth factors EGF and FGF2, cell proliferation (Fig. 3A) and the cells with immunofluorescence-detectable p27kip1 decreased (Fig. 3B). However, total p27kip1 expression protein levels showed a twofold and fourfold increase under proliferation conditions at 24 and 48 until 96 hours after differentiation, respectively, (Fig. 3C). After 96 hours of differentiation, the localization of p27kip1 was nuclear and in some cells the staining was weaker than that observed at earlier time of differentiation (Fig. 3D3, [3]D4) and p27kip1 was expressed by both neurons and astrocytes (Fig. 3D3, [3]D4, Fig. 3E). Whereas the overall expression pattern thus reflected the sequence observed in vivo, the initial strong cytoplasmic staining of p27kip1 in the precursor cells did not (compare Fig. 1).

Increased Precursor Cell Proliferation and Reduced Neurogenesis in p27kip1−/−

In order to gain more insight into the functional relevance of p27kip1 in adult hippocampal neurogenesis in vivo, we studied p27kip1 knockout and heterozygous mice. The p27kip1−/− mice showed significantly higher levels of cell proliferation in the dentate gyrus compared to wild type controls (ANOVA: $F(2, 30) = 272.5$, $p < .0001$; Tukey post hoc test: $p < .0001$; Fig. 4). This result based on BrdU-immunohistochemistry was also confirmed with a second set of animals stained for Ki67 (Fig. 4A, quantification in Fig. 4B; ANOVA: $F(2, 12) = 4.453$, $p = .0358$; Tukey post hoc test: $p = .0351$). The total number of early postmitotic neurons as expressed by the number of CR-positive cells, in contrast, was very low and significantly reduced compared to controls ($p < .0001$). The characteristic band CR-immunoreaction in the inner third of the molecular layer was conspicuously reduced in intensity in the knockout mice (Fig. 4C).

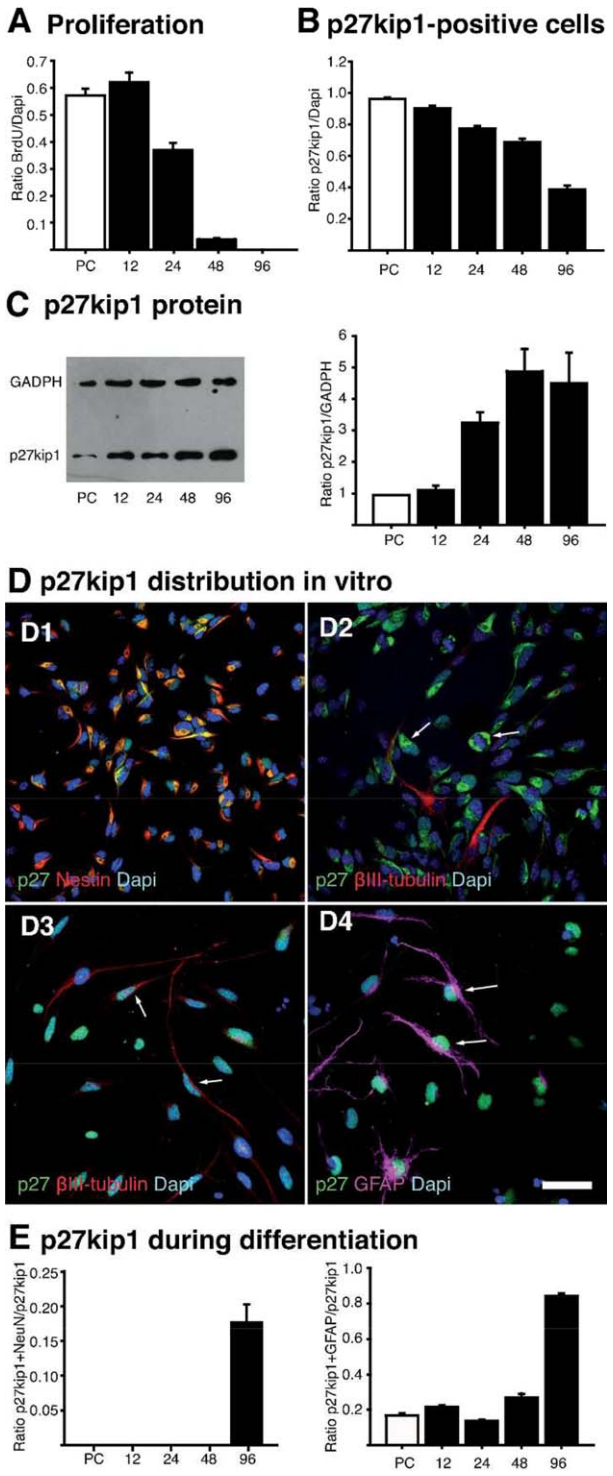
There were no statistically significant differences between the heterozygous p27kip1+/− and wild type animals ($p = .2449$ for BrdU, $p = .1620$ for Ki67, and $p = .9342$ for CR).

We used a version of the neurosphere assay to obtain additional information on precursor cell behavior ex vivo [33]. Acutely after isolation the number of spheres that form, when cells are freely floating in the media at very low density, gives a rough estimation of precursor cell activity in the sample as does the size of the neurosphere that forms in a given time.

Precursor cells are responsive to "activation" by depolarization that can be elicited by the addition of KCl to the media [20, 34]. We found that dissections from the dentate gyrus of p27kip1−/− mice yielded a greater number of neurospheres than those of wildtype controls and in both conditions the yield was increased by the addition of KCl (Fig. 5A). Among the neurospheres from the SGZ of p27kip1−/−, more had a

large diameter, whereas the number of small neurospheres was unchanged (Fig. 5B). When the neurospheres of p27kip1−/− mice or wildtype controls were seeded into culture dishes and exposed to differentiation conditions the relative distribution of phenotypes was identical (Fig. 5C-5D).

Consistent with these observations were our findings in monolayer cultures. Proliferation, as assessed by BrdU incorporation was increased in cultured precursor cells from p27kip1−/− mice (*t* test: $p < .0001$; Fig. 5E). Western blot analysis revealed not only the absence of p27kip1, but also unaltered levels of p21 and increased expression of cell cycle genes Cdk4 (*t* test: $p = .054$) and possibly Cyclin D1 (*t* test: $p = .10$; Fig. 5 F).



In the Water Maze p27kip1−/− Mice Show a "Neurogenesis Phenotype" with Impaired Reversal Learning

Our general hypothesis on the function of adult-generated hippocampal neurons is that adult hippocampal neurogenesis allows the flexible integration of novel pieces of information into established contexts [23], resulting in increased adaptability [35]. We trained mice on a modified type of the reference memory version of the Morris water maze task with 6 trials per day for 5 days and a new platform position (reversal) on the beginning of day 4 [23].

Because an increase of adult neurogenesis was shown to improve the flexible integration of novel information into preexisting contexts, we asked whether a greater number of precursor cells and reduced numbers of postmitotic new neurons due to a constitutive lack of p27kip1 would also result in an altered spatial learning performance. As indicated by decreasing path length and probe trial performance (Fig. 6A and 6C), all genotypes generally learned to find the hidden platform. Compared to wildtype controls, p27kip1−/− mice showed significantly longer path lengths ($p = .049$), indicating an impaired spatial learning ability in task acquisition. However, assessing memory for the correct platform position by measuring the number of former goal crossings in the probe trial revealed no differences between genotypes (p27kip1+/−: $p = .522$, p27−/−: $p = .61$, Fig. 6B).

We also analyzed the search strategies used by the mice to locate the hidden platform in the water maze pool (Fig. 6D). Assessing the number of animals using a hippocampus-dependent search strategy in each trial revealed that compared to wild types p27kip1−/− mice relied significantly less on efficient spatial search strategies ($p = .037$, Fig. 6D). Because we had previously found that mice with suppressed adult neurogenesis showed massively increased perseverance in searching at the old goal position after platform reversal, we asked whether a greater number of precursor cells would

Figure 3. Expression changes of p27kip1 during precursor cell differentiation in vitro. **(A)**: Proliferation of precursor cells during a differentiation time-course (12, 24, 48, or 96 hours) shows a decrease in the ratio of BrdU incorporation from 24 hours after differentiation in comparison to PC. **(B)**: Also the proportion of p27kip1-positive cells shows a decrease during the differentiation time course. **(C)**: Immunoblot and the histogram show that protein level of p27kip1 increase during differentiation (lower bands). GAPDH was used as loading control (Immunoblot upper bands). **(D)**: Precursor nestin-positive cells show cytoplasmic p27kip1 expression (Panel **D1-D2**). Arrows in panel D2 indicate dividing and nondividing cells with expression of p27kip1 in the cytoplasm (green). Nestin and β-III tubulin are shown in red (D1 and D2, respectively). Differentiated precursor cells show nuclear expression of p27kip1 (green) in neurons (**D3**, β-III tubulin in red) and astrocytes (**D4**, GFAP in purple). Proportion of neurons (NeuN) or astrocytes (GFAP) coexpressing nuclear p27kip1 is shown in panel **(E).** Error bars represent SEM. $p < .001$ (A; PC vs. 24, 48, and 96 hours, respectively); $p < .001$ (B; PC vs. 24, 48 and 96 hours, respectively), $p < .001$ (C**,** PC vs. 48 and 96 hours); $p < .001$ (E, neurons; E, astrocytes; PC vs. 96 hours). Scale bar (in D4 for all D panels), 40 μm. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole4,6-diamidino-2-phenylindole; PC, proliferative cells.

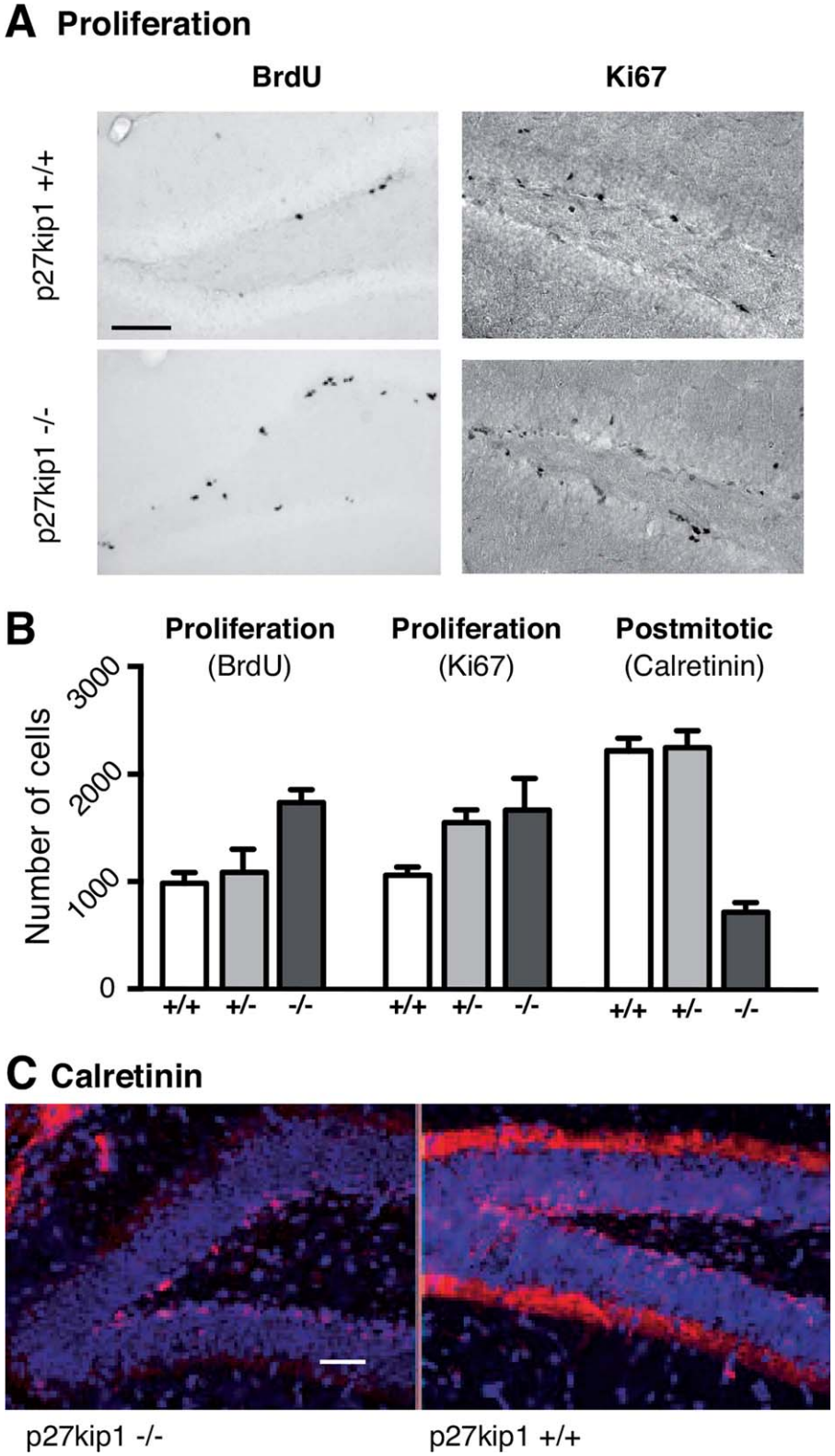


Figure 4. Quantitative assessment of proliferating cells (BrdU and Ki67) and young postmitotic neurons that express Calretinin (CR) in p27kip1 −/−, +/− and +/+ ($N = 5$ per group). **(A)**: The images depict exemplary BrdU- (left) or Ki67-labeled (right) hippocampal sections showing increased proliferation in the knockouts compared to wildtype controls ($p = .0032$), whereas the difference between p27kip1 +/− and wildtype was not statistically significant ($p > .05$). The same was true for a second series of sections from a new set of animals (−/−, $N = 6$; +/−, $N = 5$; −/− $N = 4$). CR-expressing cells were strongly decreased in p27kip1−/− compared to the control ($p < .0005$), whereas p27kip1 +/− and wildtype were nearly on the same level. Scale bar, 200 μm for BrdU and 150 μm for Ki67. Panel **(B)** gives the quantitative results. **(C)**: confocal microscopic images highlighting the massive reduction of CR-positive cells in the subgranular zone. Many of the remaining cells are interneurons. The characteristic staining pattern of CR in the inner molecular layer is absent in the p27kip1 −/− mice. Scale bar, 150 μm. Abbreviation: BrdU, 5-bromo-2′-deoxyuridine.

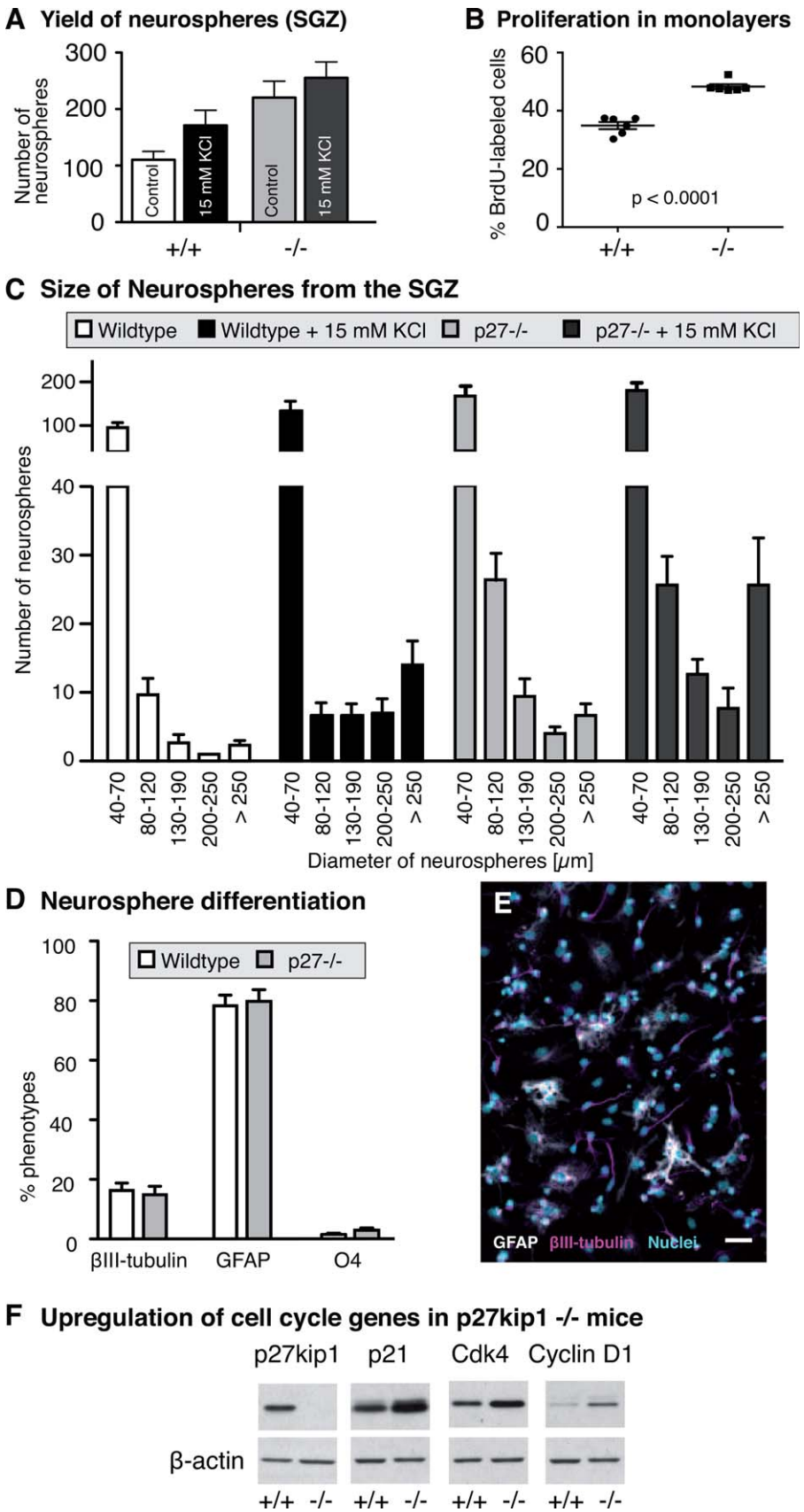


Figure 5. Knockout of p27kip1 increased precursor proliferation but not differentiation potential ex vivo. **(A)**: Histogram depicting the number of neurospheres derived from the SGZ of p27kip1−/− and p27kip1+/+ mice ($n = 3$ mice per group, one-way ANOVA $p = .017$). **(B)**: Histogram depicting the size distribution of the neurospheres generated from the p27kip1−/− and p27kip1+/+ mice. **(C)**: Histogram depicting the differentiation potential of the neurospheres generated from the p27kip1−/− and p27kip1+/+ mice. **(D)**: Representative image of a differentiated p27kip1−/− neurosphere is shown (Scale bar, 40 μm). **(E)**: 5-bromo-2′-deoxyuridine incorporation in monolayers of precursor cells obtained from p27kip1−/− mice. **(F)**: immunodetection of proteins (Western blot) related to cell cycle control revealed upregulation in p27kip1 −/− mice. Abbreviations: GFAP, glial fibrillary acidic protein; KCl, potassium chloride; SGZ, subgranular zone.

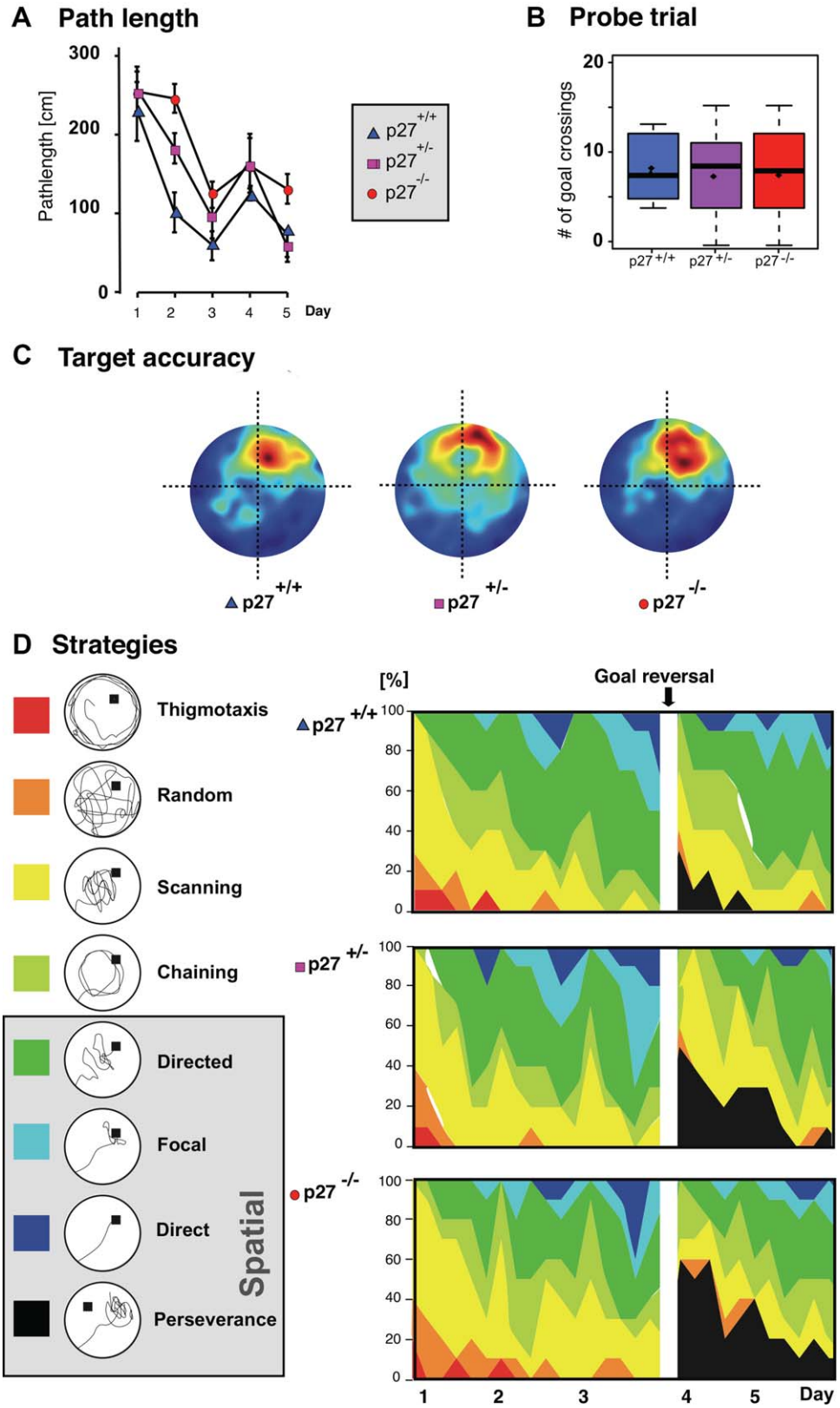


Figure 6. Differences in spatial learning abilities of p27kip1 −/−, +/− and +/+ mice in the water maze. **(A)**: Path length to reach the hidden platform. Shown are means ± SD. **(B)**: Probe trial performance indicated by number of crossings of former goal positions. **(C)**: Target accuracy in probe trial indicated by heat maps. Dark-red zones represent a sixfold presence probability. **(D)**: Algorithm-based strategy classification. Left: Examples of search strategies used for classification. Right: Contribution of respective search strategies to group performance. Color-code as indicated in (A).

also result in an overall improved goal-related plasticity. To assess functional plasticity, we measured how many trials it took the mice after goal reversal to regain an average path length that was at least equal to or shorter than the one reached on the last day (i.e., day 3) of the first acquisition period. Analysis of variance revealed differences between genotypes in the number of trials needed to regain performance levels of the first acquisition: p27kip1−/− mice needed significantly more time to regain initial acquisition performance, indicating a reduced goal-related plasticity ($p = .0431$).

In summary, p27kip1−/− mice showed longer path lengths, a less frequent use of hippocampus-dependent search strategies to navigate toward the hidden goal, and impaired goal-related plasticity after platform reversal. No differences between genotypes were found regarding their preference for the correct goal position in the probe trial.

Discussion

In this study we found that p27kip1 is upregulated upon behavioral activity in the neurogenic zone of the adult hippocampus. p27kip1 binds to and inhibits the CyclinE/CDK2 and CyclinD/CDK4 complexes during late G1 phase, which are important for cell cycle progression [36]. However, the pattern of an activity-induced increase in the number of neuronally determined precursor cells with nuclear expression of p27kip1 as we describe it here is not consistent with the idea of p27kip1 being only a cell cycle inhibitor. That p27kip1 has such function is undisputed and convincing evidence has been provided that it also plays such role in adult neurogenesis: maintaining radial glia-like precursor cells (type-1) in quiescence [10]. Our findings are not in contradiction to those observations, but we provide new data on an additional function later in the course of neuronal development and within the context of activity-dependent regulation.

Our findings rather support a function both in relation to cell cycle exit and differentiation. Indeed, a function of p27kip1 in cytokinesis has also been implicated [37].

Nevertheless, our ex vivo data (Fig. 5D and E) suggest that lack of p27kip1 does not impair differentiation, even though p27kip1 expression increases in the course of differentiation (Fig. 3C). This is consistent with the observation that as the only representative of the Cip/Kip family, p27kip1 has been related to roles in neural differentiation and migration [27, 28, 38]. In the olfactory epithelium, a site of adult neurogenesis in the peripheral nervous system, p27kip1 knockout mice also had increased precursor cell proliferation and reduced neuronal differentiation [39].

Both in vitro and in vivo, the subcellular distribution of p27kip1 in differentiated cells was distinct from its localization at the precursor cell stage. The strong nuclear localization of p27kip1 in precursor cells gave way to a more diffuse presence in neurons. This pattern might indicate differential and potentially independent functions, similar to Tis21, for which we have found a biphasic expression in the course of neurogenesis [40]. These results are also in line with other reports about subcellular distribution and function of p27kip1 that have been described by others [41–43]. The switch in distribution is almost dichotomous and no gradient of expression is found. This facilitated quantification of the strongly expressing precursor cell stages. There are, however, general caveats related to such cut-off decisions in that the experimental manipulations might alter expression without the hypothesized change in functional state. From the overall picture our results draw and the available literature, we do not think that this is the case here.

We predicted that p27kip1 staining in postmitotic neurons should also be more cytoplasmic rather than nuclear as in the precursor cell stages. However, as granule cells have only a very narrow cytoplasmic rim around the nucleus and NeuN tends to show some overlapping perinuclear staining, this question could not be sufficiently resolved. It has been suggested that cytoplasmic expression of p27kip1 in neurons is associated with neuronal maturation and function and independent of cell-cycle-related events [41] (but see also: Ref. [8]). Although we could only distinguish the clear nuclear signal at the precursor cell stage from a more diffuse and weaker immunoreaction at postmitotic and mature stages, our findings are consistent with a view that there are distinct functions of p27kip1 in the course of neuronal development, which are dependent on the subcellular localization. The diffuse more cytoplasmatic expression pattern of p27kip1 is thought to play a role in stabilizing intracellular functions [41] but other authors propose that after cell cycle exit, p27kip1 is merely channeled out of the nucleus and then degraded [44–46]. This latter position is at some discrepancy with the lifelong expression of p27kip1 in postmitotic neurons. In fact, most of these propositions have been made in relation to the hematopoietic system, where cytoplasmic p27kip1 has been linked to oncogenesis. But p27kip1 can directly interact with complexes that regulate transcription of various classes of genes, not only related to cell cycle [47].

We have hardly ever seen nuclear p27kip1 in radial glia-like type-1 cells and only rarely in nonradial glia-like intermediate precursor cells (type-2a). This distribution is in line with reports on p57kip^2/p27kip1-expression patterns in progenitor cells during corticogenesis [9], suggesting that strong nuclear p27kip1 is typical for intermediate progenitor cells. p27kip1-expression appeared to be linked to the neuronal lineage from the type-2b cells onward reaching a hundred percent expression-rate in CR-positive cells, which represent the earliest stage of postmitotic neurons in the dentate gyrus [25].

In some apparent contrast to the finding that chronic stress, which decreases adult neurogenesis, increased p27kip1 [48], we saw that the positive regulation of adult neurogenesis by behavioral activity [17] was also associated with greater p27kip1-expression. The strongest pro-proliferative stimuli also showed the largest response in the number of p27kip1-expressing cells.

We here modified our previously published study design to corroborate the potential interaction of the two behavioral stimuli RUN and ENR in a more acute time setting [3]. Intriguingly, we saw that also with respect to the absolute number of p27kip1-positive cells the combination of RUN and ENR surpassed the consequences of either stimulus alone (Fig. 2B). This suggests that p27kip1 might be involved in the activation of programs controlling the transition from precursor cell stages to the postmitotic survival and differentiation phase in adult hippocampal neurogenesis. Both the data on the intracellular distribution, especially in the ENR group, and the

information from the literature indicate that these functions might be distinct and possibly independent. Cytoplasmic p27kip1 is thought to play roles in migration (which in adult neurogenesis occurs during the type-3 and early postmitotic stage) and cell-cell interactions as required for structural and functional integration [43, 49, 50]. In a cancer-cell model, experimental reduction of cytosolic p27kip1 lowered cell motility and survival [42]. In the absence of p27kip1, however, neuronal differentiation was not impaired in vitro (Fig. 5D and 5E). The exact role of cytosolic p27kip1 in neurons remains to be unraveled. This research would go beyond the scope of the present study.

Nevertheless, the proposed function of p27kip1 in the regulation of adult neurogenesis is consistent with our observation that in p27kip1−/− mice precursor cell proliferation was increased and greater precursor cell activity could be detected ex vivo. At the same time neurogenesis in terms of CR-positive cells in vivo was reduced (Fig. 4).

We have proposed a specific function of adult-generated neurons for allowing the flexible integration of novel information into previously established contexts [23, 51], which is also consistent with related concepts by others [52]. In line with the idea that that function is dependent on the production of new neurons rather than activity of the precursor cells (which are the targets of the ablation strategies used to abolish adult neurogenesis in functional studies), we found the previously reported neurogenesis-dependent impairment in the reversal test of the Morris water maze (Fig. 6). The p27kip1−/− mice showed signs of additional deficits not related to adult neurogenesis, potentially explainable by the role of p27kip1 in mature neurons but not the subject of the present study.

Conclusion

In summary, we describe a role of p27kip1 in adult neurogenesis that is necessary for successful cell cycle exit under baseline conditions and increased in the context of behavior-induced regulation of adult neurogenesis. Future studies will benefit from a conditional cell type-specific manipulation of p27kip1 in order to rule out developmental influences and separate the presumably different functions that p27kip1 has in controlling and regulating adult neurogenesis.

Acknowledgments

H.H. and A.G. were supported by graduate program 429 funded by DFG, Deutsche Forschungsgemeinschaft. The project was partly financed through a grant from the German Federal Ministry of Education and Research, BMBF (01GN0974). Additional basic funding was provided by Volkswagenstiftung. The project was initiated at the Max Delbrück Center for Molecular Medicine (MDC) Berlin-Buch and later continued in Dresden.

Author Contributions

H.H. and G.K.: Designed the study; H.H., A.G., T.W., M.I., A.K., Z.N., and G.R.: Performed the experiments; B.S. and C.L.: Provided material and tools; H.H., A.G., T.W., M.I., B.S., A.K., C.L., Z.N., G.R., and G.K.: Analyzed the data; H.H., T.W., and G.K.: Wrote the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Overall RW, Paszkowski-Rogacz M, Kempermann G. The mammalian adult neurogenesis gene ontology (MANGO) provides a structural framework for published information on genes regulating adult hippocampal neurogenesis. PLoS One 2012;7:e48527.

2 Kronenberg G, Reuter K, Steiner B et al. Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. J Comp Neurol 2003;467:455–463.

3 Fabel K, Wolf SA, Ehninger D et al. Additive effects of physical exercise and environmental enrichment on adult hippocampal neurogenesis in mice. Front Neurosci 2009;3:50.

4 Brandt MD, Maass A, Kempermann G et al. Physical exercise increases Notch activity, proliferation and cell cycle exit of type-3 progenitor cells in adult hippocampal neurogenesis. Eur J Neurosci 2010;32:1256–1264.

5 Cunningham JJ, Levine EM, Zindy F et al. The cyclin-dependent kinase inhibitors p19(Ink4d) and p27(Kip1) are coexpressed in select retinal cells and act cooperatively to control cell cycle exit. Mol Cell Neurosci 2002;19:359–374.

6 Doetsch F, Verdugo JM, Caille I et al. Lack of the cell-cycle inhibitor p27Kip1 results in selective increase of transit-amplifying cells for adult neurogenesis. J Neurosci 2002;22:2255–2264.

7 Gil-Perotin S, Haines JD, Kaur J et al. Roles of p53 and p27(Kip1) in the regulation of neurogenesis in the murine adult subventricular zone. Eur J Neurosci 2011;34:1040–1052.

8 Li X, Tang X, Jablonska B et al. p27(KIP1) regulates neurogenesis in the rostral migratory stream and olfactory bulb of the postnatal mouse. J Neurosci 2009;29:2902–2914.

9 Mairet-Coello G, Tury A, Van Buskirk E et al. p57(KIP2) regulates radial glia and intermediate precursor cell cycle dynamics and lower layer neurogenesis in developing cerebral cortex. Development 2012;139:475–487.

10 Andreu Z, Khan MA, Gonzalez-Gomez P et al. The cyclin-dependent kinase inhibitor p27 kip1 regulates radial stem cell quiescence and neurogenesis in the adult hippocampus. Stem Cells 2015;33:219–229.

11 Ramos JG, Varayoud J, Monje L et al. Diethylstilbestrol alters the population dynamic of neural precursor cells in the neonatal male rat dentate gyrus. Brain Res Bull 2007;71:619–627.

12 Varodayan FP, Zhu XJ, Cui XN et al. Seizures increase cell proliferation in the dentate gyrus by shortening progenitor cell-cycle length. Epilepsia 2009;50:2638–2647.

13 Palazuelos J, Ortega Z, Diaz-Alonso J et al. CB2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling. J Biol Chem 2012;287:1198–1209.

14 Anacker C, Zunszain PA, Cattaneo A et al. Antidepressants increase human hippocampal neurogenesis by activating the glucocorticoid receptor. Mol Psychiatry 2011;16:738–750.

15 Zhang S, Huan W, Wei H et al. FOXO3a/p27kip1 expression and essential role after acute spinal cord injury in adult rat. J Cell Biochem 2013;114:354–365.

16 Zhao W, Wang Y, Shi W et al. The expression of FBP1 after traumatic brain injury and its role in astrocyte proliferation. J Mol Neurosci 2013;51:687–694.

17 Steiner B, Zurborg S, Horster H et al. Differential 24 h responsiveness of Prox1-expressing precursor cells in adult hippocampal neurogenesis to physical activity, environmental enrichment, and kainic acid-induced seizures. Neuroscience 2008;154:521–529.

18 Kempermann G, Gast D, Kronenberg G et al. Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development 2003;130:391–399.

19 Babu H, Claasen JH, Kannan S et al. A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. Front Neurosci 2011;5:89.
20 Babu H, Ramirez-Rodriguez G, Fabel K et al. Synaptic network activity induces neuronal differentiation of adult hippocampal precursor cells through BDNF signaling. Front Neurosci 2009;3:49.
21 Hagihara H, Toyama K, Yamasaki N et al. Dissection of hippocampal dentate gyrus from adult mouse. J Vis Exp 2009;pii:1543.
22 Walker TL, Kempermann G. One mouse, two cultures: Isolation and culture of adult neural stem cells from the two neurogenic zones of individual mice. J Vis Exp 2014;84:: e51225.
23 Garthe A, Behr J, Kempermann G. Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PLoS One 2009;4:e5464.
24 Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47–60.
25 Brandt MD, Jessberger S, Steiner B et al. Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. Mol Cell Neurosci 2003;24:603–613.
26 Kronenberg G, Bick-Sander A, Bunk E et al. Physical exercise prevents age-related decline in precursor cell activity in the mouse dentate gyrus. Neurobiol Aging 2006;27: 1505–1513.
27 Nguyen L, Besson A, Roberts JM et al. Coupling cell cycle exit, neuronal differentiation and migration in cortical neurogenesis. Cell Cycle 2006;5:2314–2318.
28 Itoh Y, Masuyama N, Nakayama K et al. The cyclin-dependent kinase inhibitors p57 and p27 regulate neuronal migration in the developing mouse neocortex. J Biol Chem 2007;282:390–396.
29 Sun W, Winseck A, Vinsant S et al. Programmed cell death of adult-generated hippocampal neurons is mediated by the proapoptotic gene Bax. J Neurosci 2004;24: 11205–11213.
30 Sahay A, Scobie KN, Hill AS et al. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature 2011;472:466–470.
31 Young D, Lawlor PA, Leone P et al. Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat Med 1999;5:448–453.
32 Sun X, Momen A, Wu J et al. p27 protein protects metabolically stressed cardiomyocytes from apoptosis by promoting autophagy. J Biol Chem 2014;289:16924–16935.
33 Walker TL, Wierick A, Sykes AM et al. Prominin-1 allows prospective isolation of neural stem cells from the adult murine hippocampus. J Neurosci 2013;33:3010–3024.
34 Walker TL, White A, Black DM et al. Latent stem and progenitor cells in the hippocampus are activated by neural excitation. J Neurosci 2008;28:5240–5247.
35 Kempermann G. New neurons for 'survival of the fittest'. Nat Rev Neurosci 2012; 13:727–736.
36 Ibanez IL, Policastro LL, Tropper I et al. H2O2 scavenging inhibits G1/S transition by increasing nuclear levels of p27KIP1. Cancer Lett 2011;305:58–68.
37 Serres MP, Kossatz U, Chi Y et al. p27(Kip1) controls cytokinesis via the regulation of citron kinase activation. J Clin Invest 2012;122:844–858.
38 Godin JD, Thomas N, Laguesse S et al. p27(Kip1) is a microtubule-associated protein that promotes microtubule polymerization during neuron migration. Dev Cell 2012;23: 729–744.
39 Legrier ME, Ducray A, Propper A et al. Cell cycle regulation during mouse olfactory neurogenesis. Cell Growth Differ 2001;12: 591–601.
40 Attardo A, Fabel K, Krebs J et al. Tis21 expression marks not only populations of neurogenic precursor cells but also new postmitotic neurons in adult hippocampal neurogenesis. Cereb Cortex 2009;20:304–314.
41 Paris M, Wang WH, Shin MH et al. Homeodomain transcription factor Phox2a, via cyclic AMP-mediated activation, induces p27Kip1 transcription, coordinating neural progenitor cell cycle exit and differentiation. Mol Cell Biol 2006;26:8826–8839.
42 Wu FY, Wang SE, Sanders ME et al. Reduction of cytosolic p27(Kip1) inhibits cancer cell motility, survival, and tumorigenicity. Cancer Res 2006;66:2162–2172.
43 Besson A, Gurian-West M, Chen X et al. A pathway in quiescent cells that controls p27Kip1 stability, subcellular localization, and tumor suppression. Genes Dev 2006;20:47–64.
44 Serres MP, Zlotek-Zlotkiewicz E, Concha C et al. Cytoplasmic p27 is oncogenic and cooperates with Ras both in vivo and in vitro. Oncogene 2011;30:2846–2858.
45 Katagiri K, Ueda Y, Tomiyama T et al. Deficiency of Rap1-binding protein RAPL causes lymphoproliferative disorders through mislocalization of p27kip1. Immunity 2011; 34:24–38.
46 Min YH, Cheong JW, Kim JY et al. Cytoplasmic mislocalization of p27Kip1 protein is associated with constitutive phosphorylation of Akt or protein kinase B and poor prognosis in acute myelogenous leukemia. Cancer Res 2004;64:5225–5231.
47 Pippa R, Espinosa L, Gundem G et al. p27Kip1 represses transcription by direct interaction with p130/E2F4 at the promoters of target genes. Oncogene 2012;31:4207–4220.
48 Heine VM, Maslam S, Joels M et al. Increased P27KIP1 protein expression in the dentate gyrus of chronically stressed rats indicates G1 arrest involvement. Neuroscience 2004;129:593–601.
49 McAllister SS, Becker-Hapak M, Pintucci G et al. Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions. Mol Cell Biol 2003;23:216–228.
50 Assoian RK. Stopping and going with p27kip1. Dev Cell 2004;6:458–459.
51 Garthe A, Roeder I, Kempermann G. Mice in an enriched environment learn more flexibly because of adult hippocampal neurogenesis. Hippocampus 2016;26:261–271.
52 Aimone JB, Wiles J, Gage FH. Potential role for adult neurogenesis in the encoding of time in new memories. Nat Neurosci 2006;9:723–727.

