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p27kip1 Is Required For Functionally Relevant Adult Hippocampal Neurogenesis In Mice

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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 (KA) 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 *2016; 00:000–000*

SIGNIFICANCE STATEMENT

In the course of adult hippocampal neurogenesis, the step from the precursor cell stage to the differentiating new neurons that become integrated is a crucial one. It is also regulated by behavioral activity. In this paper we demonstrate that the cell cycle associated protein p27kip1 that seems to play several different roles in neurogenesis is also necessary for this regulated transition. In p27kip1 knockout mice cell proliferation is upregulated but the normally following step to differentiation and integration is missing. Ac-

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cordingly, the mice also showed impaired performance in a learning task that specifically targets the functional contribution of new neurons.

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 ten 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 colleagues discovered that exercise indeed promotes cell cycle exit [4].

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 colleagues 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 colleagues, 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 colleagues 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.

MATERIAL & METHODS

Animals and housing conditions

The experiments on the acute expression of p27kip1 after exposure to ENR, RUN and 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 h 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) and 1 h 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) 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) and cryoprotected.

Immunohistochemistry

To detect dividing cells, we used the proliferation marker BrdU visualized with the DAB-peroxidase method (ABC, Vectastain Elite, Vector Laboratories). 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), as secondary biotinylated donkey anti-rat (1:500; Dianova). 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 (1:200; Santa Cruz Biotechnologies), guinea pig anti-GFAP (1:1000, Advanced Immunochemistry), rabbit anti-GFP (1:400, Abcam, Cambridge, UK), goat anti-GFP (1:1000, Santa Cruz Biotechnologies), rabbit anti-Calretinin (1:2000; Swant), mouse anti-p27 (1:1000; BD Transduction Laboratories) 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, Dianova). Fluorescent sections were mounted in polyvinyl alcohol with diazabicyclo-octane (DABCO) 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 pre-coated 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, Germany), for 24 hours. After this time, we replaced the medium for withdrawal-growth factors medium to induce precursor cells 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 RIPA buffer (150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.5% triton X-100, 1 mM 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 14000 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-TBS (TTBS) and incubated with the rabbit anti-p27 (1:1000; Santa-Cruz) and with the mouse anti-GAPDH antibody

1:5000 (Chemicon, Hampshire, England). Blots were washed 3 times with TTBS and incubated for 1 hour in a 1:1000 dilution of HRP-conjugated donkey anti-mouse or donkey anti-rabbit antibodies. Proteins were visualized with the enhanced chemiluminescence detection system (Immobilon; Millipore, Billerica, MA, USA). 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) according to the manufacturer's instructions. Following a final wash in Hank's balanced salt solution (HBSS, PAA) 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), supplemented with 2% B27 (Invitrogen), 1× GlutaMAX (Life Technologies) and 50 units/ml Penicillin/Streptomycin (Life Technologies). The following growth factors were also included: 20 ng/ml EGF and 20 ng/ml FGF-2. KCl 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 PDL 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 PBS at room temperature for 30 minutes. After washing with PBS, they were stained for either the neuronal markers βIII-tubulin, the astrocytic marker glial fibrillary acidic protein (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 4 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 non-toxic white paint and kept at a temperature of 19–20°C. Each mouse was given 6 trials a day for 5 consecutive days with an inter-trial 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 s. Swim paths were recorded using Ethovision (Noldus) and further analyzed using Matlab (The Mathworks, USA).

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 non-radial type-2a cells co-expressed 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 doublecortin (DCX)-positive cells (including non-radial 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. 1C). 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, Fig. 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 h) exposure to the experimental conditions of voluntary wheel running (RUN) and environmental enrichment (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, $p < 0.001$), but did not show any paradigm specificity (Fig. 2). This indicated that 24 h of exposure to pro-neurogenic stimuli acutely up-regulate 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, i.e. 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].

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 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 and D), 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 2 and 4-fold 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, D4) and p27kip1 was expressed by both neurons and astrocytes (Fig. 3D3, D4 and 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: $F(2, 30) = 272.5$, $p < 0.0001$; Tukey post hoc test: $p < 0.0001$; 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: $F(2, 12) = 4.453$, $p = 0.0358$; Tukey post hoc test: $p = 0.0351$). 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 ($p < 0.0001$). 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 ($p = 0.2449$ for BrdU, $p = 0.1620$ for Ki67, and $p = 0.9342$ 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-D).

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: $p < 0.0001$; 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: $p=0.054$) and possibly Cyclin D1 (t-test: $p = 0.10$; 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 pre-existing 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 ($p = 0.049$), 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+/-: $p = 0.522$, $p27^{-/-}$: $p=0.61$, 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 ($p = 0.037$, 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 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 ($p = 0.0431$).

In summary, p27kip1-/- mice showed longer path lengths, a less frequent use of hippocampus-dependent search strategies to navigate towards 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 up-regulated 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. 5E) 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 not 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 non-radial 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 onwards reaching a hundred percent expression-rate in CR-positive cells, which represent the earliest stage of post-mitotic 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 mo-

tility and survival [42]. In the absence of p27kip1, however, neuronal differentiation was not impaired in vitro (Fig. 5D and E). The exact role of cytosolic p27kip1 in neurons remains to be unraveled. This research went 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.

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.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

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

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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 subgranular zone (SGZ). GCL, granule cell layer. **A1**, high power magnification from marked area in A to highlight the nuclear vs. cytoplasmic localization of p27kip1 (arrowheads). **B**, co-staining of p27kip1 (red) and doublecortin (DCX, blue) in Nestin-GFP-positive precursor cells (green). Only few Nestin-GFP-positive cells co-express 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 co-expression of p27kip1. **C**, co-localization 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.

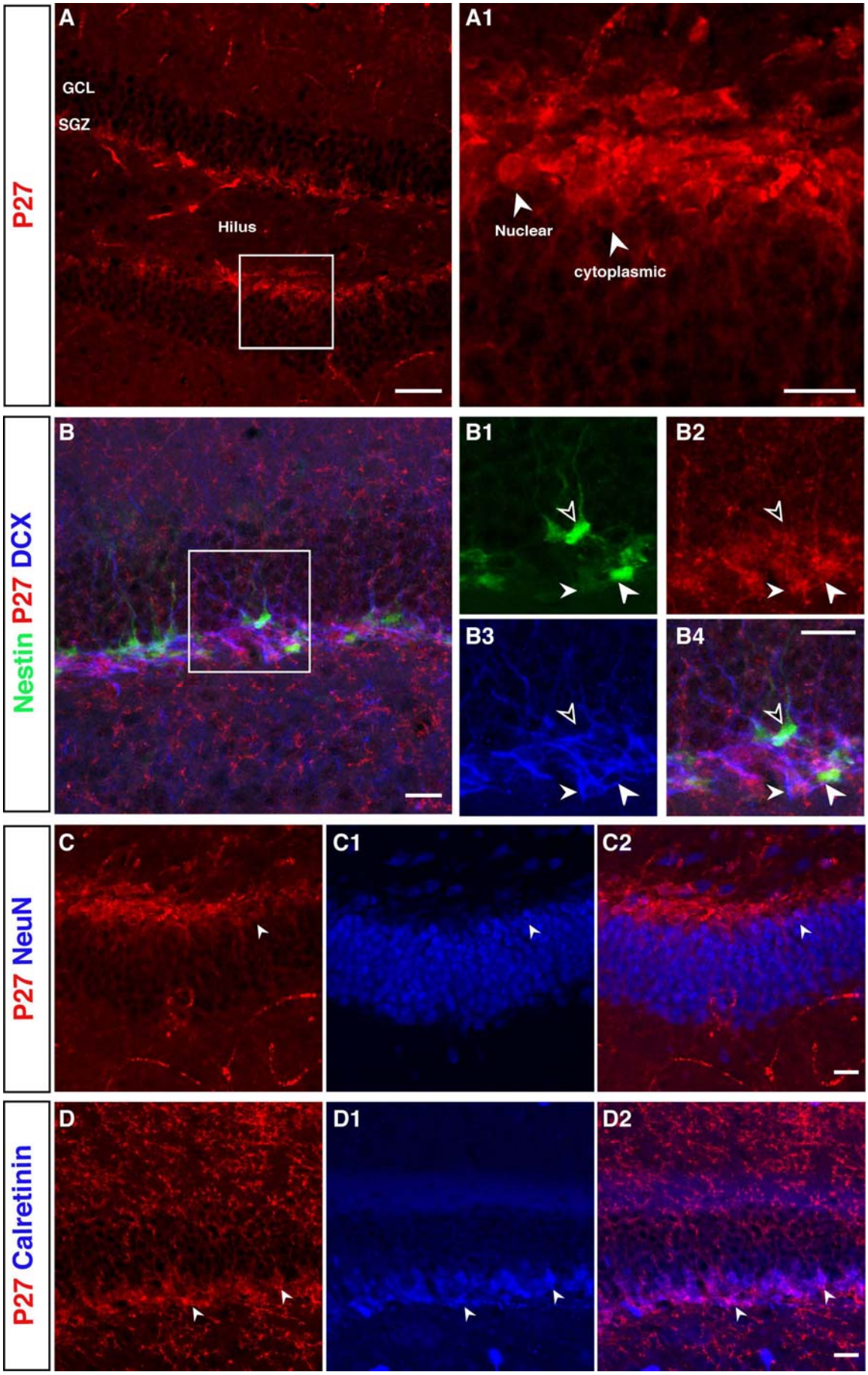
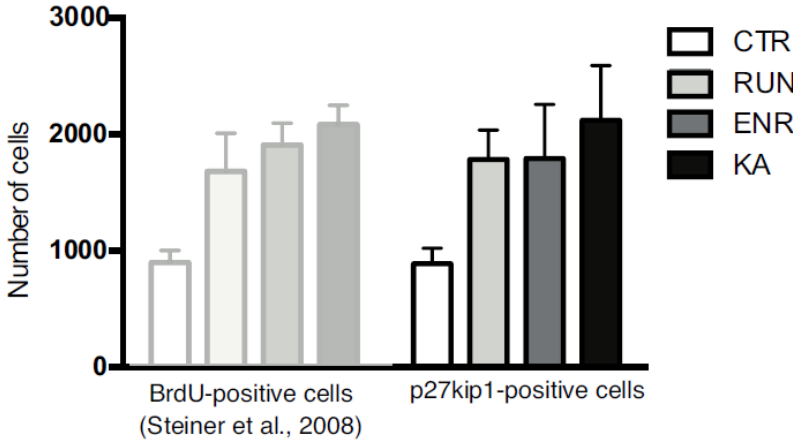


Figure 2. Quantitative assessment of p27kip1 expression under neurogenic stimulation conditions. A, up-regulation of the number of p27kip1-positive cells (strong nuclear expression) after short-term stimulation. All stimulated animals (RUN, ENR, KA) showed increased levels of p27kip1 compared to the control group (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 ($p = 0.01$).

A **Acute induction of p27kip1-positive cells by neurogenic stimuli (24h)**



B **Additive induction of p27kip1-positive cells by neurogenic stimuli (7d)**

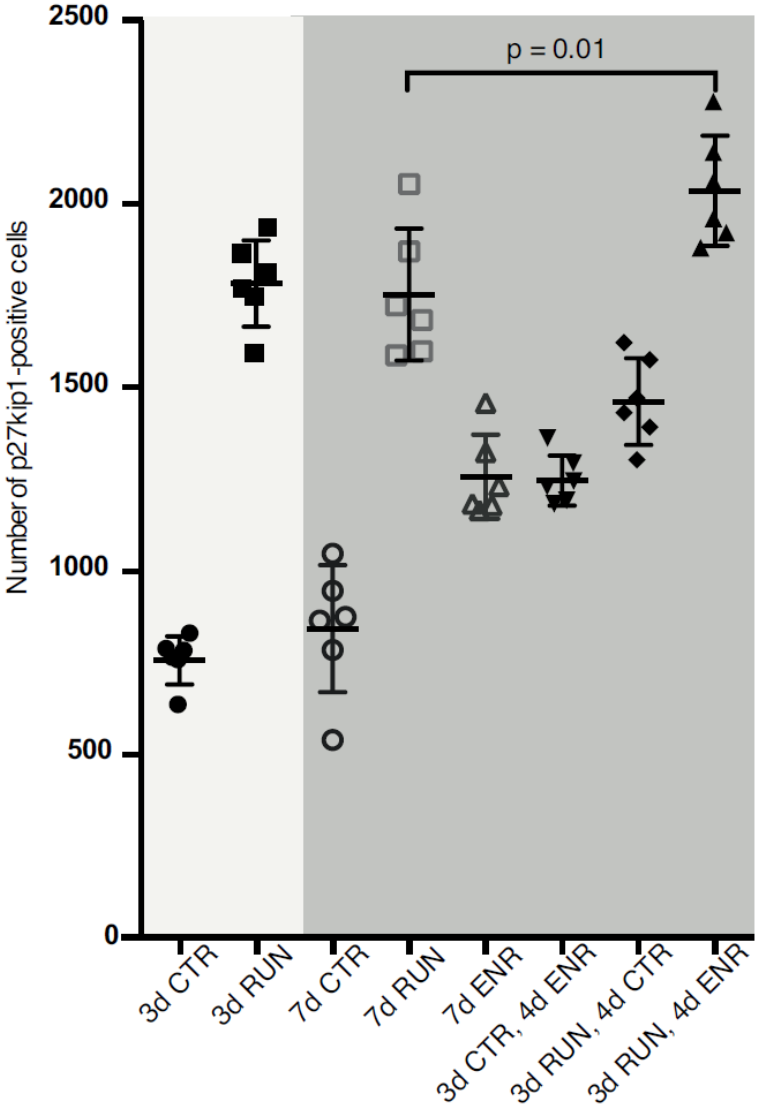


Figure 3. Expression changes of p27kip1 during precursor cell differentiation *in vitro.* **A**, Proliferation of precursor cells during a differentiation time-course (12, 24, 48 or 96 hours) shows a decrease in the ratio of BrdU incorporation from 24 h after differentiation in comparison to proliferative cells (PC). **B**, Also the proportion of p27kip1-positive cells shows a decrease during the differentiation time course. **C,** Immmunoblot 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 non-dividing 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) co-expressing nuclear p27kip1 is shown in panel ***E***. Error bars represent SEM. $p < 0.001$ (***A***; PC vs 24, 48 and 96 h, respectively); $p < 0.001$ (***B***; PC vs 24, 48 and 96 h, respectively), $p < 0.001$ (***C,*** PC vs 48 and 96 h); $p < 0.001$ (***E***, neurons; ***E***, astrocytes; PC vs 96 h). Scale bar (in D4 for all D panels), 40 μm.

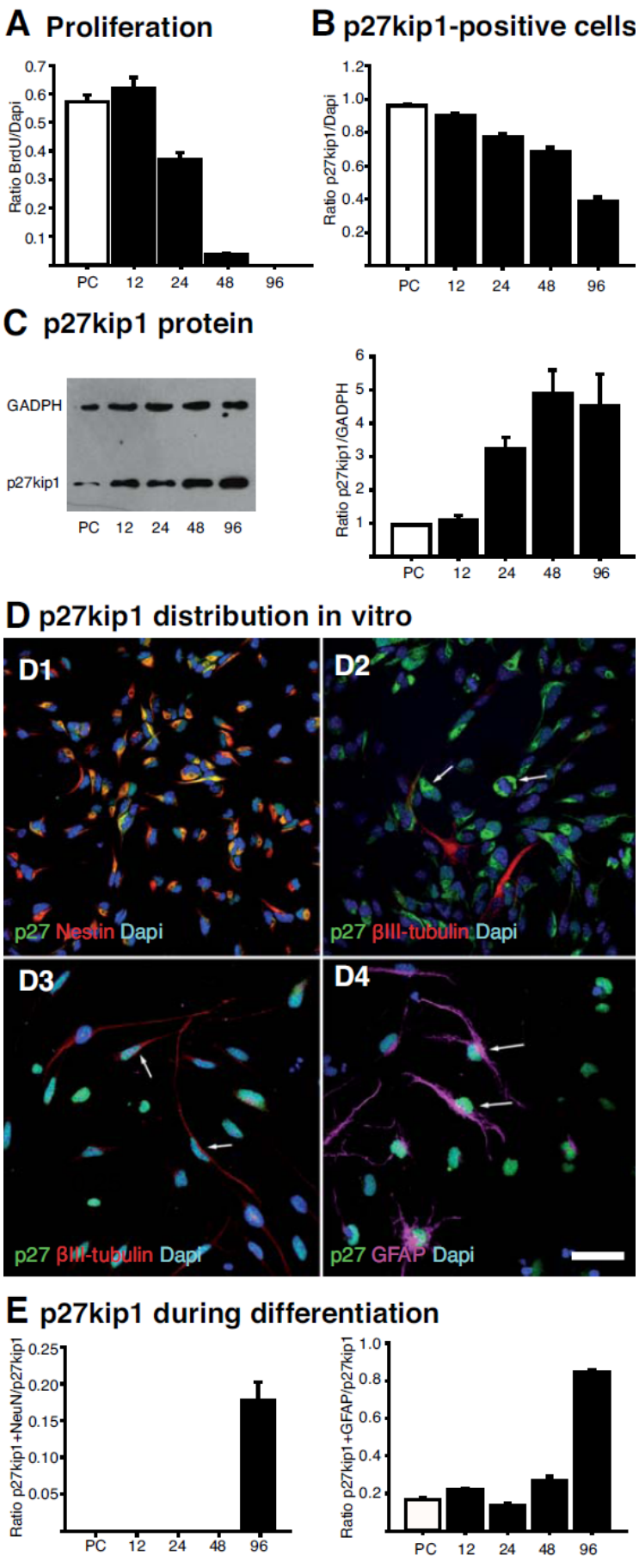
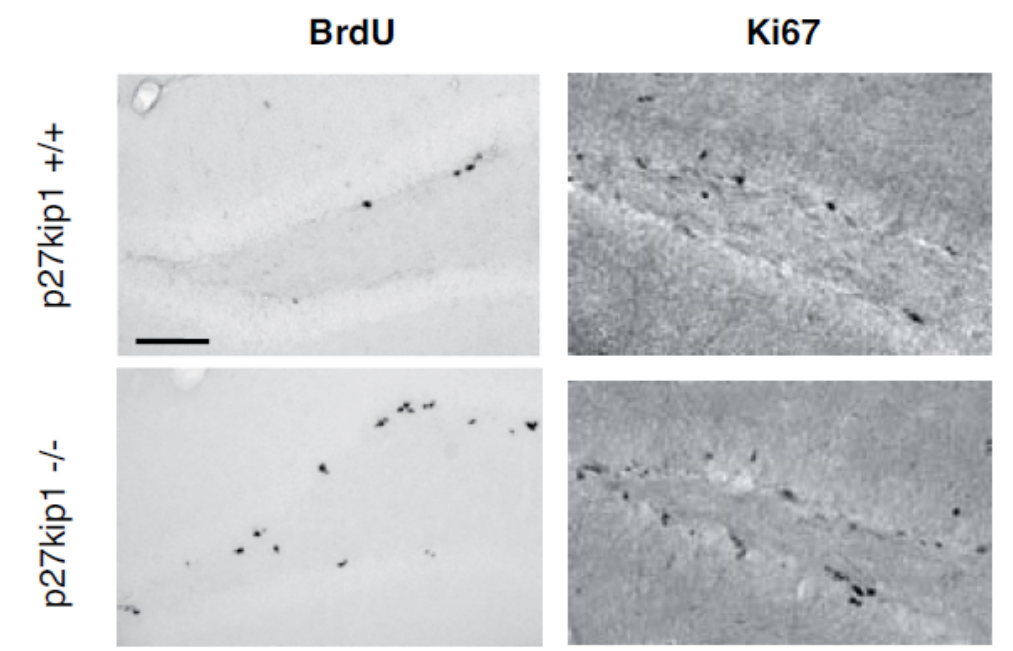
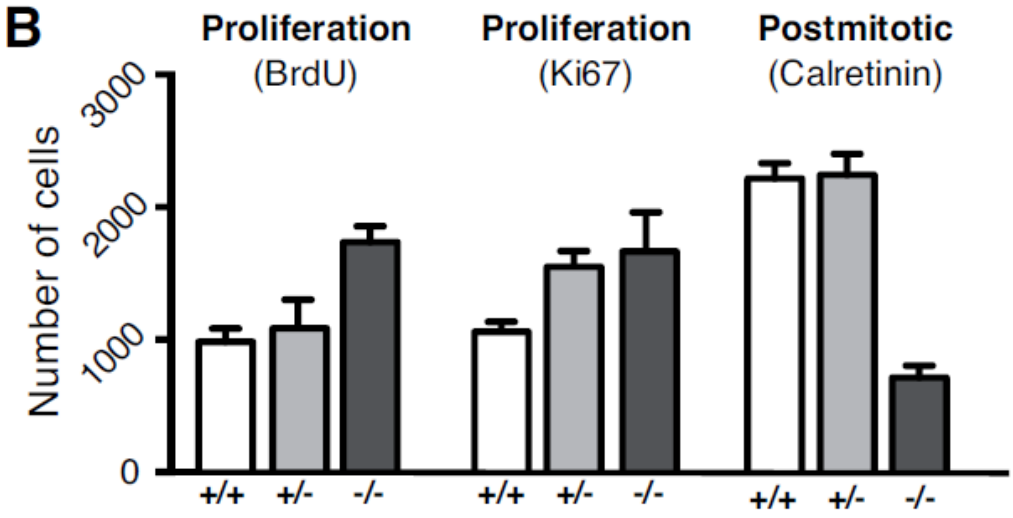


Figure 4. Quantitative assessment of proliferating cells (BrdU and Ki67) and young postmitotic neurons that express Calretinin (CR) in p27kip1 -/-, +/- and +/+ (*N* = 5 per group). A, the images depict exemplary BrdU- (left) or Ki67-labeled (right) hippocampal sections showing increased proliferation in the knockouts compared to wildtype controls ($p = 0.0032$), whereas the difference between p27kip1 +/- and wildtype was not statistically significant ($p > 0.05$). 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 ($p < 0{,}0005$), 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 Calretinin-positive cells in the subgranular zone. Many of the remaining cells are interneurons. The characteristic staining pattern of Calretinin in the inner molecular layer is absent in the p27kip1 -/- mice. Scale bar, 150 µm.





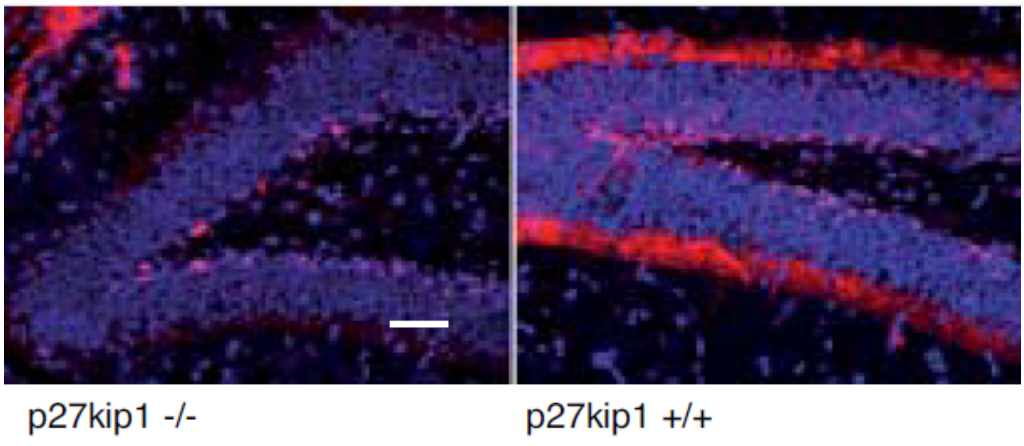


Figure 5. Knock-out of p27kip1 increased precursor proliferation but not differentiation potential ex vivo. A, Histogram depicting the number of neurospheres derived from the SGZ of p27kip1-/- and p27kip1+/+ mice (n = 3 mice per group, One-Way ANOVA p = 0.017). **B**, Histogram depicting the size distribution 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**, BrdU incorporation in monolayers of precursor cells obtained from p27kip1-/- mice. **F**, immunodetection of proteins (Western blot) related to cell cycle control revealed up-regulation in p27kip1 -/- mice.

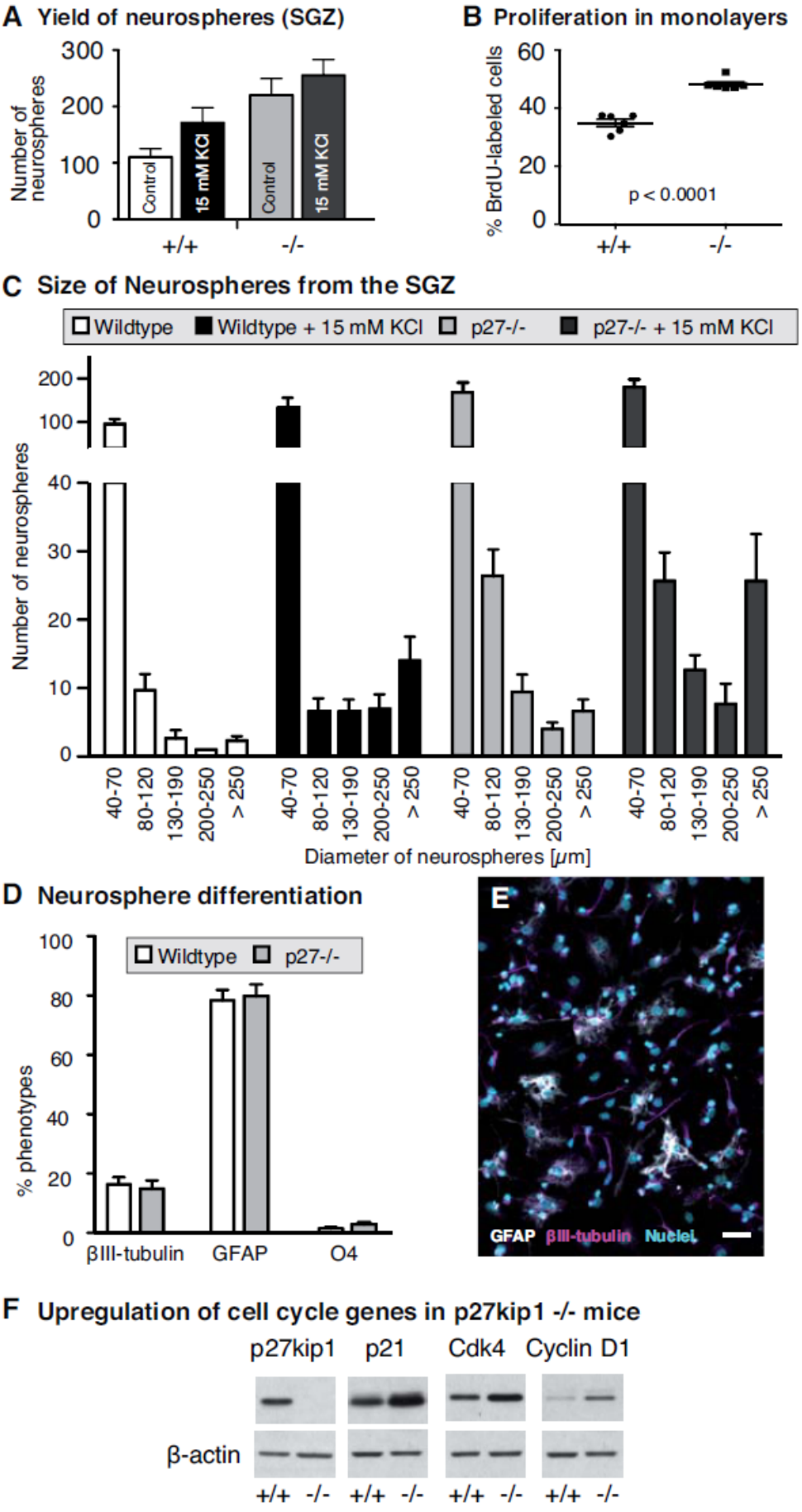
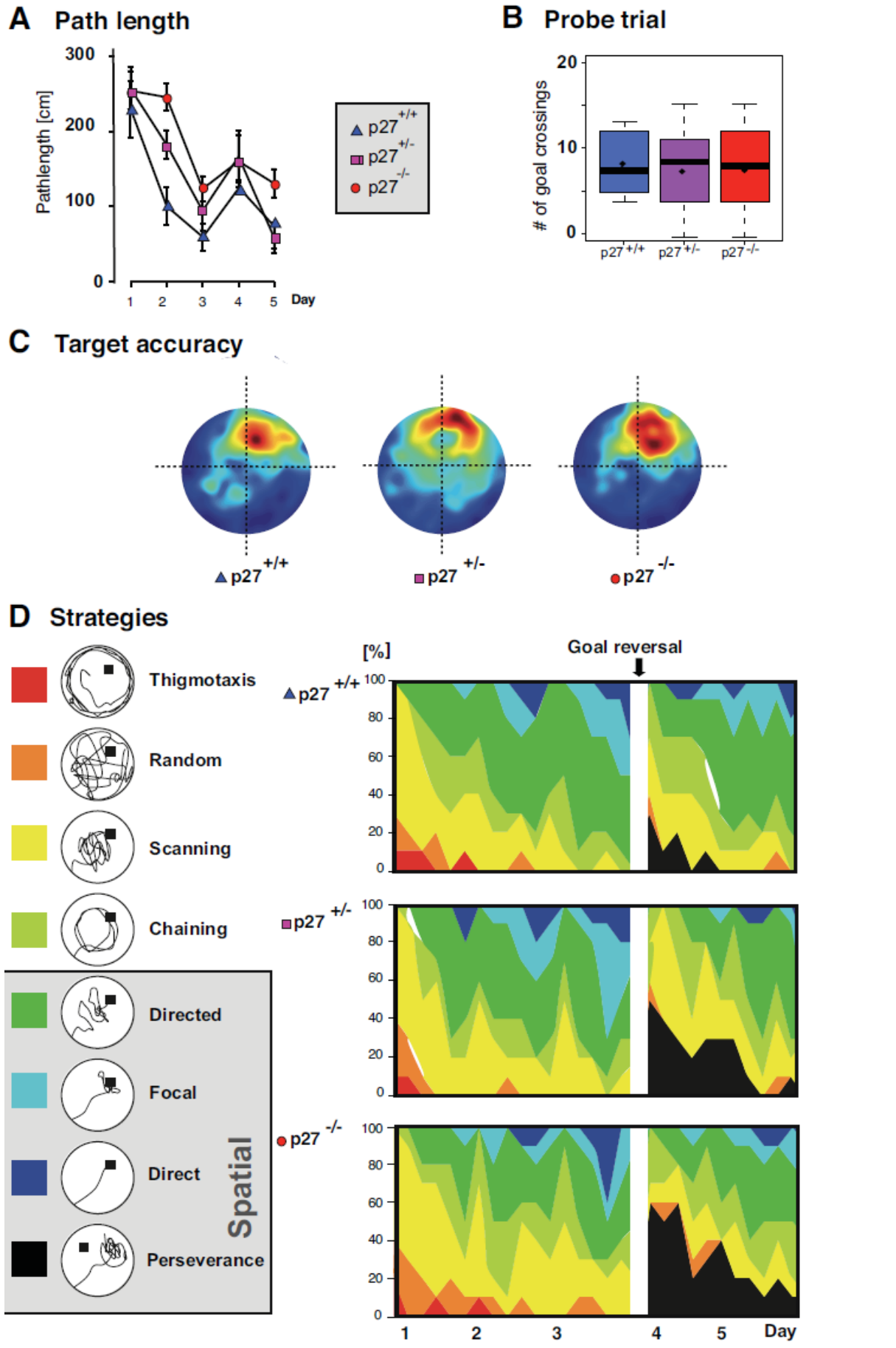
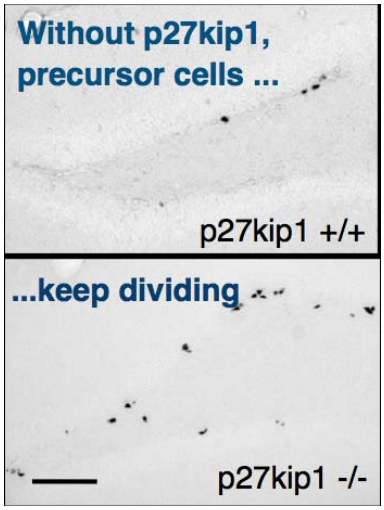


Figure 6. Differences in spatial learning abilities of p27kip1 -/-, +/- and +/+ mice in the water maze. **(A)** Path length to reach the hidden platform. Shown are means ± standard deviations. **(B)** Probe trial performance indicated by number of crossings of former goal positions. **(C)** Target accuracy in probe trial indicated by heat maps. Dark-red zones represent a 6-fold 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).





Graphical abstract

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. The images show precursor cells in the adult murine hippocampus, labeled with the bromodeoxyuridine (BrdU) method in wildtype mice (top) and p27kip1 knockout mouse (bottom). Loss of p27kip1 results in an increase in the number of BrdU-positive cells. Scale bar, 150 μm.

