The critical role of cyclin D2 in adult neurogenesis

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Adult neurogenesis (i.e., proliferation and differentiation of neuronal precursors in the adult brain) is responsible for adding new neurons in the dentate gyrus of the hippocampus and in the olfactory bulb. We describe herein that adult mice mutated in the cell cycle regulatory gene *Ccnd2*, encoding cyclin D2, lack newly born neurons in both of these brain structures. In contrast, genetic ablation of cyclin D1 does not affect adult neurogenesis. Furthermore, we show that cyclin D2 is the only D-type cyclin (out of D1, D2, and D3) expressed in dividing cells derived from neuronal precursors present in the adult hippocampus. In contrast, all three cyclin D mRNAs are present in the cultures derived from 5-day-old hippocampi, when developmental neurogenesis in the dentate gyrus takes place. Thus, our results reveal the existence of molecular mechanisms discriminating adult versus developmental neurogeneses.

Introduction

In the adult mammalian brain, there is a limited proliferation and differentiation of neuronal precursors (neurogenesis) within the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) responsible for the formation of new DG neurons as well as subventricular zone of the lateral ventricles and rostral migratory stream, which are the source of new neurons in the olfactory bulb (OB; Cameron et al., 1993; Lois and Alvarez-Buylla, 1993; Eriksson et al., 1998). Originally described in the early sixties (Altman and Das, 1964), the adult neurogenesis has recently received considerable attention. Despite the proposed role of this phenomenon in learning (Feng et al., 2001; Shors et al., 2001) and action of antidepressants (Malberg et al., 2000; Santarelli et al., 2003), its functional significance for the brain function remains poorly elucidated, partially because of lack of appropriate genetic models for study. Furthermore, no molecular mechanisms discriminating between proliferation of adult and developmental neuronal precursors have been reported.

Cyclins D are cell cycle regulatory proteins that control specific cyclin-dependent kinases. Three cyclins D have been described, D1, D2, and D3. In most of the cells, there is an expression of more than one cyclin D. However, in those instances where only one cyclin D is expressed, its mutation produces significant phenotypic abnormalities. In particular, mice lacking cyclin D1 display narrow, tissue-specific abnormalities within the retina and mammary glands (Sicinski et al., 1995; Ma et al., 1998). In contrast, mice lacking cyclin D2 display tissue-specific abnormalities in the ovaries and testes (Sicinski et al., 1996; Robker and Richards, 1998). An additional phenotype of cyclin D2–deficient mice was described in B cells by Solvason et al. (2000), whereas Huard et al. (1999) reported cerebellar abnormalities. Lastly, cyclin D3–deficient mice also have a narrow, cell-type specific phenotype (Sicinska et al., 2003).

In this paper, we checked whether adult neurogenesis is critically dependent on either cyclin D1 and/or D2. Hence, mice mutated in respective genes were analyzed for the adult neurogenesis. We have found that cyclin D2 but not D1 mutation completely abolishes proliferation of neuronal precursors in the adult brain, thus indicating a pivotal role of cyclin D2 in adult neurogenesis.

Results and discussion

Most often the neurogenesis is revealed by means of intense incorporation of DNA precursors, e.g., BrdU into neuronal nuclei that occurs during the S phase of the cell cycle (Takahashi et al., 1992; Luskin, 1993; Kuhn et al., 1996). In the first series of experiments, we investigated BrdU incorporation 1 d after the last of four i.p. injections. Mice with cyclin D2 gene disrupted through homologous recombination (D2 KO; Sicinski et al.,

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Abbreviations used in this paper: DG, dentate gyrus; OB, olfactory bulb; SGZ, subgranular zone; WT, wild-type.

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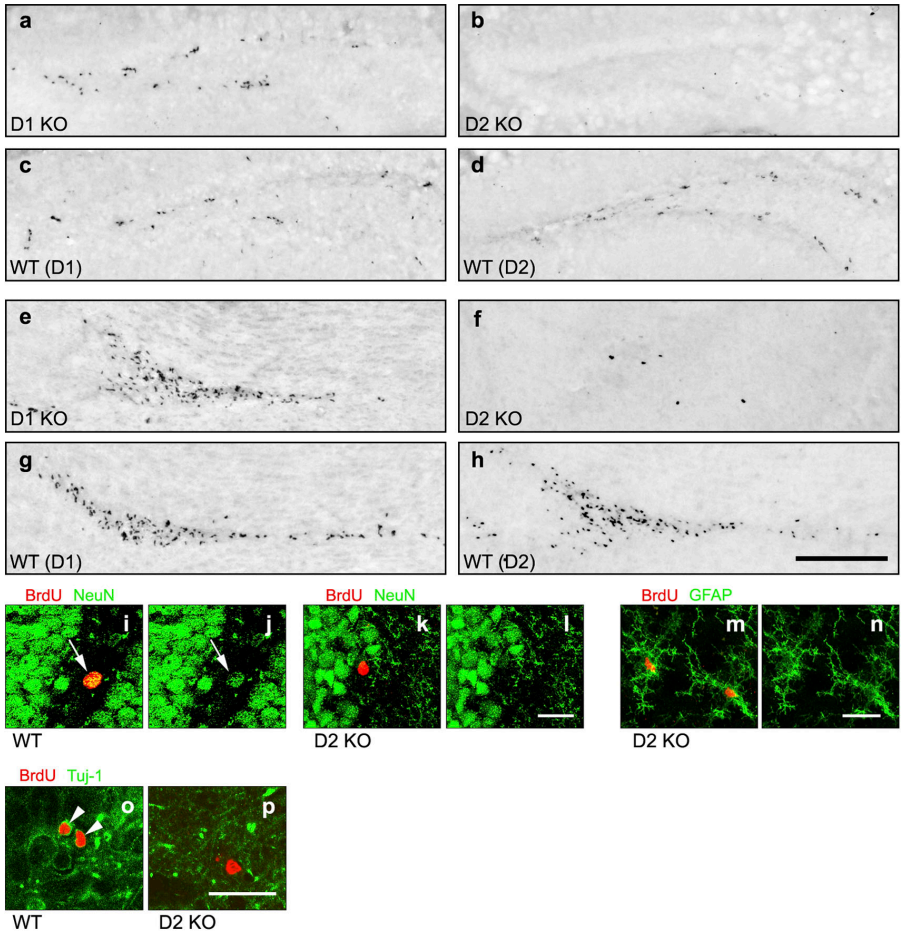

Figure 1. **Adult hippocampus and OB of D2 KO mice deficient in neurogenesis.** (a–d) Extensive labeling in hippocampal DG in controls: wild-type (WT) from D1 KO line (c), WT from D2 KO line (d) and D1 KO mouse (a). Lack of labeling in DG of D2 KO hippocampus (b). (e–h) Similar phenomenon in OB. (i and j) Colocalization (arrows) of BrdU (red) and NeuN (green) in WT hippocampus. (k and l) BrdU-positive nuclei in D2 KO hippocampus never colocalize with NeuN. (o) Similar colocalization (arrowheads indicating green rings) of BrdU (red) and Tuj-1 (green) with no colocalization in D2 KO (p). (m and n) Colabeling of BrdU with glial fibrillary acidic protein (GFAP, green) in D2 KO hippocampus. Confocal images: single-plane (i–l and o and p) and composed (m and n). Bars: (a–h) 200 μm; (i–l, m and n, and o and p) 20 μm.

1996) were virtually deprived of BrdU incorporation in the hippocampus and OB. In contrast, robust labeling of these brain structures could be observed in the brains of their wild-type (WT) littermates (Fig. 1, a–h). In addition, mice deficient in cyclin D1 (Sicinski et al., 1995) displayed BrdU incorporation similar to their control WT littermates.

To identify the phenotype of the very few BrdU-positive cells in DG of cyclin D2 KO mice, we used double immunolabeling with BrdU antibody and either neuronal (NeuN, Tuj-1) or astrocytic (GFAP) marker. We used animals that were allowed to survive 3 d (Tuj-1) or 3 wk (NeuN, GFAP) after the last injection. In the hippocampi of D2 KO animals, we have not found any clearly displaying either NeuN (Fig. 1, k and l) or Tuj-1 (Fig. 1 p), as was often the case with WT mice (Fig. 1, i, j, and o). In contrast, in the hippocampi of D2 KO mice, BrdU labeling could be observed in astroglia (Fig. 1, m and n).

It has been reported that exploration of a novel, enriched environment produces increase in the BrdU labeling in the hippocampus (Rosenzweig and Bennett, 1996; Kempermann et al., 1997; van Praag et al., 2000). We used such a treatment to see whether or not it may induce neurogenesis in the brain of cyclin D2 KO mice. However, no increase in the number of scarce BrdU-positive cells in the hippocampi of the D2 KO animals was observed (Fig. 2 a). In contrast, such a phenomenon was clear in WT mice, with over twofold increase of hippocampal BrdU labeling.

One could consider that D2 KO mice may be deficient in BrdU incorporation into the adult brain cells. To address this possibility, we have used the cerebral cortex mechanical lesion model that evokes extensive proliferation of glial cells around the injury site (Condorelli et al., 1990). We have observed a robust BrdU labeling, especially closely to the lesion site in the brains of both WT and D2 KO mice (Fig. 2, d–g). Again, no specific labeling of SGZ could be noted in the hippocampi of the very same mutant mice, in contrast to their WT littermates (Fig. 2, h and i).

Results of the previous experiment revealed that there is BrdU incorporation in the cyclin D2 KO mice, although not into the brain neurons. To see if the observed deficiency in neurogenesis is limited to the brain, we have looked into neuronal BrdU labeling in nasal mucosa. We have found no abnormalities in BrdU incorporation into the neuronal precursors as well as no apparent differences in the thickness and in neuronal density of olfactory epithelium of the nasal turbinates in WT versus D2 KO mice (Fig. 2, b and c).

We have also investigated the effect of cyclin D2 mutation on the overall brain structure. We have found that the mutant brain is smaller by ~25%, whereas the weight of the whole mice was lower by no more than 10%, when comparing the D2 KO and WT animals (Fig. 3). These size reductions are already evident in the brains of 2-wk-old mice (unpublished data). The overall brain structure of the D2 mutants appeared close to nor-

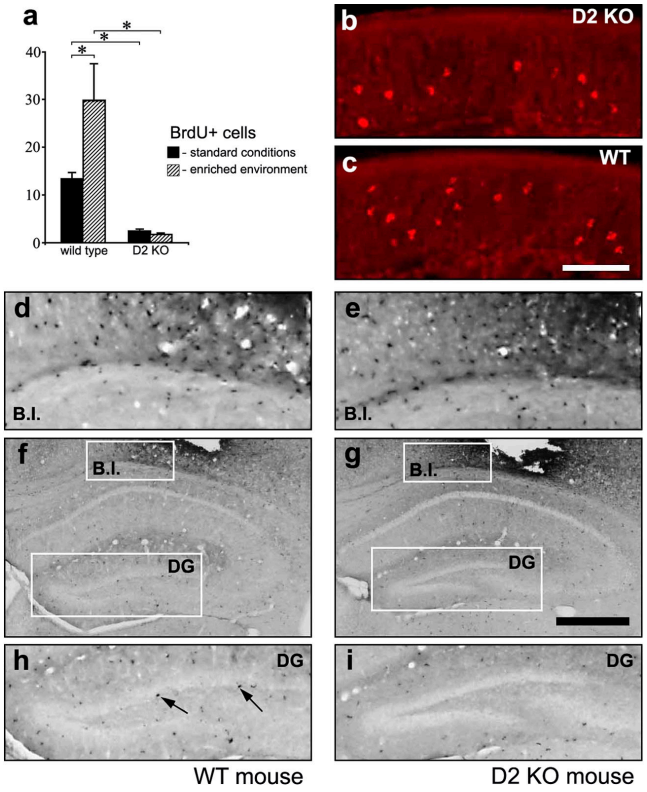

Figure 2. **Specificity of the deficit in BrdU incorporation in D2 KO mouse brain.** (a) The WT mice displayed significant ($P < 0.05$), over twofold, increase in the number of BrdU-positive cells in the hippocampal DG after 2 mo of animal exposure to the enriched environment. No significant change was observed in the D2 KO hippocampi. *, $P < 0.05$. (b and c) Lack of difference in the BrdU-labeled neurons in the olfactory epithelium of the nasal turbinates in WT and D2 KO. (d–i) BrdU incorporation following mechanical injury of the cerebral cortex. BrdU extensive labeling around the site of injury to the cortex in WT (d and f) as well as cyclin D2 KO (e and g) brains. No BrdU labeling in the DG of D2 KO animals (i) observed in WT (h, arrows). DG, dentate gyrus; B.I., brain injury area. Bars: (b and c) 16 μm; (f and g) 500 μm.

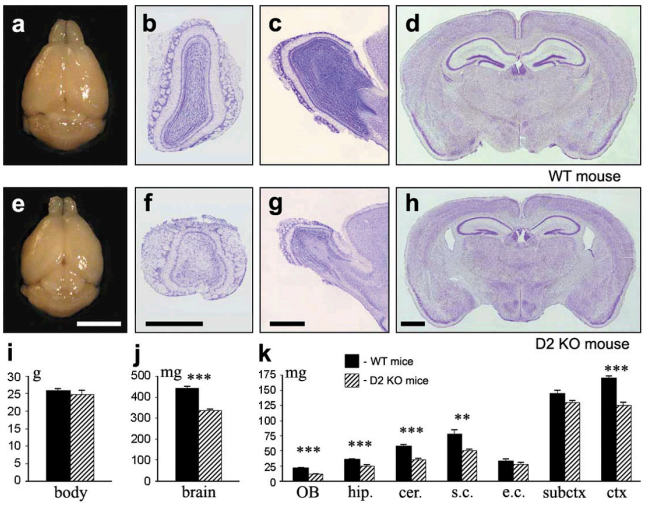

Figure 3. **WT and cyclin D2 KO brains differ in weight and structure.** (a and e) Gross morphology. (b–d and f–h) Nissl staining. (i) WT and cyclin D2 KO animals do not differ substantially in body weight, whereas the brains of the latter are significantly smaller (a and e, d, h, and j). The differences concern OB, hippocampus (hip.), cerebellum (cer.), sensory cortex (s.c.), cortex altogether (ctx), and, to a lesser extent, subcortical structures (subctx) and amygdala/enthorinal cortex (e.c.). The results are derived from 3-mo-old males ($n = 6$). ***, $P < 0.0001$; **, $P < 0.01$. Bars: (a and e) 5 mm; (b and f, c and g, and d and h) 1 mm.

mal, although such brain regions as hippocampus, occipital cortex, cerebellum, and OB in particular were significantly smaller in mutants than in the WT mice (Fig. 3), whereas the lateral ventricles were expanded (Fig. 3, d and h). Notably, this phenotype is strikingly reminiscent of the effects of the orphan nuclear receptor TLX deficiency that is also lacking adult neurogenesis in the brain (Shi et al., 2004). In OB, the size reduction did associate with a well-defined and prominent abnormality in anatomic structure. As evidenced by Nissl-stained preparations (Fig. 3, b, c, f, and g), there was a striking decrease in cell density in the internal granular layer, and, to a lesser extent, a decrease in number of periglomerular granule cells. In the former layer, the characteristic striated aggregations of granule interneurons were clearly disrupted. Other neuronal populations, including mitral cells, appeared not to be disturbed. The abnormalities in OB structure that are evident in adult mice are also present at P5 (unpublished data). However, hippocampal DG of 5-d-old mice was not strikingly different between WT and D2 KO with plentiful neurogenesis also in the latter (Fig. 4, b and d).

Previous work on mice lacking members of the D-cyclin family revealed that specific defects could be observed in the tissues where only a single cyclin D is expressed under physiological conditions (Sicinski et al., 1995; Huard et al., 1999; Ciemerych et al., 2002). Otherwise, apparent compensation from the remaining, intact D-cyclins masked any effects of the mutations. Thus, we set out to investigate whether or not the cyclin D2 is expressed as the only cyclin D in neuronal precursors of WT animals. Because neurospheres are believed to be in vitro expandable progeny of neuronal precursors (Reynolds and Weiss, 1992), we cultured the neurospheres derived from WT adult hippocampal progenitors and tested for the cyclins D expression pattern using the RT-PCR approach. We have found that indeed cyclin D2 mRNA is the only cyclin D transcript expressed in such cultures (Fig. 4). In contrast, the neurospheres derived from WT 5-d-old pups (when endogenous, developmental proliferation of the DG neuronal precursors is very active; Fig. 4 c) expressed all three cyclins D (i.e., D1, D2, and D3; Fig. 4 e). In the context of this finding we have also studied whether or not cyclin D2 is expressed in neuronal hippocampal progenitors. Indeed we identified several cyclin D2–positive cells to express also nestin, the marker of neural progenitors (Hockfield and McKay, 1985). Somehow surprisingly, we were able to develop neurospheres from adult cyclin D2 KO hippocampi. Interestingly, these cultures showed the expression of cyclin D1 mRNA, probably reflecting a compensatory effect observed under the in vitro conditions.

The major findings of this work can be summarized as follows. Lack of functional cyclin D2, resulting from the gene ablation but not the absence of functional cyclin D1, results in virtually complete absence of proliferation of neuronal precursors in the adult brain. In contrast, developmental neurogenesis, although also affected, still allows for formation of the brain, with all the major structures present, albeit, some of them

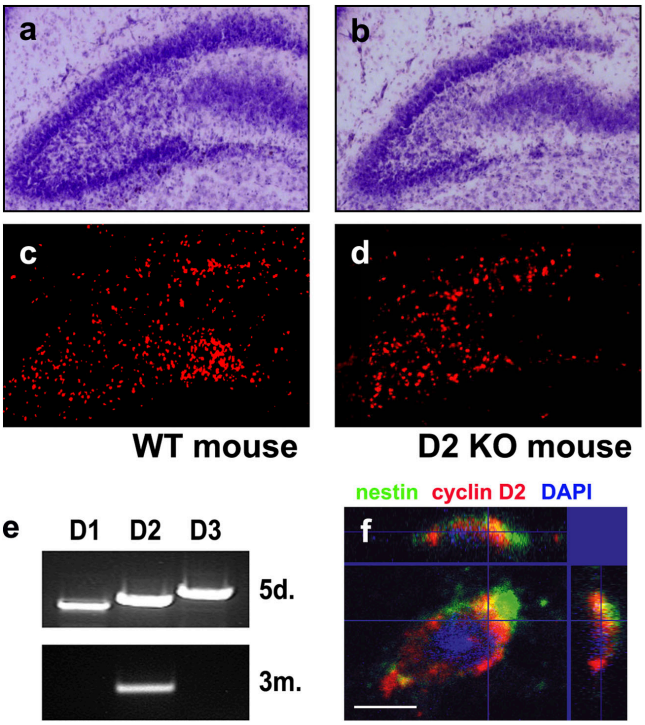

Figure 4. **High level of neurogenesis in DG of 5-d-old cyclin D2–deficient animals; 3-d-old animals injected with BrdU and analyzed 2 d later.** Nissl analysis of mutants (b) and WT controls (a). (c and d) BrdU labeling in the DG. (e) Differential expression of cyclins D in developing and adult hippocampal neuronal precursors. Expression of cyclin D mRNAs in neurosphere cultures derived from hippocampi of 5-d- and 3-mo-old WT mice. In the adult material only the cyclin D2 mRNA is expressed. (f) Colabeling of nestin (green) and cyclin D2 mRNA (red) in WT DG SGZ. A double-positive cell is shown. The image has been scanned at high resolution (100× objective, 1.4 NA) with a high zoom factor together with two additional cross-sections through the entire confocal stack, taken at the levels indicated by vertical and horizontal blue lines that demonstrate overlapping nestin immunoreactivity and cyclin D2 mRNA signals within the whole cell body. Blue shows DAPI staining revealing the cell nucleus. Bar, 5 μm.

smaller. This deficit is specific for the brain neurons, as it does not affect proliferation of neuronal precursors in olfactory epithelium of the nasal turbinates as well as nonneuronal precursors proliferating in response to a brain injury. This dramatic phenotype can be explained by the fact that in vitro expanded neuronal precursors from adult hippocampi, forming so called neurospheres, express solely cyclin D2, whereas neurospheres derived from 5-d-old hippocampi express all three cyclins D. Hence, we may suggest that the less pronounced effect of cyclin D2 KO on developmental versus adult neurogenesis can be explained by compensation exerted by other cyclins D during developmental neurogenesis in cyclin D2–deficient animals.

In conclusion, we would like to suggest using D2 KO mice in functional tests on physiological and pathological significance of the newly born neurons in the adult brain. Furthermore, our results reveal the existence of molecular mechanisms discriminating between proliferation of adult versus developmental neuronal precursors. Further exploration of such mechanisms would be of great importance in creating animal models with selective alteration of adult neurogenesis, aiming at advancing our knowledge on the phenomenon as well as allowing to develop potential therapeutic tools to treat the damaged brain. Future work should also address whether or not cyclin D2 plays a special role in abnormal, e.g., tumorogenic, proliferation of neural precursors in the brain.

Materials and methods

Animals and their treatment

Cyclin D1 and D2 mutant mice were generated before (Sicinski et al., 1995, 1996) and kept under C57Bl background. They were crossed once with Balb/c mice, and the lines were kept as cyclin D2 heterozygotes (+/−). Their homozygous progeny, 3–4-mo-old (if not indicated otherwise) −/− (KO) and +/+ (WT) littermates were used in all experiments. The animals were kept under natural light/dark cycle in the Plexiglas cages with water and food provided ad libitum. To minimize animal suffering, the rules established by the Ethical Committee on Animal Research of Nencki Institute and based on the Animal Protection Act Polish Republic were strictly followed.

The animals were injected with BrdU, i.p., four times over 2 d (two injections 2 h apart, followed by the same treatment 22 h later, always 50 mg/kg in a single dose) and killed either 24 h after the last injection for single anti-BrdU staining or 3 wk later for double staining with anti-NeuN or anti-GFAP and for the staining of the nasal epithelium. In the experiment with cortical injury (Results), the mice received mechanical lesion of the cerebral cortex (anteroposterior = −2.12 mm; lateral = ±1.5 mm; and ventral = 1.0 mm from bregma; Condorelli et al., 1990). It was followed by BrdU injections for the next 2 d and immunodetection on the third day.

Behavioral analysis

In the enriched environment experiment, animals (males) were kept together for 2 mo in a large (45 × 35 × 27 cm) iron-wired cage filled with tunnels, ladders, hiding places, and a running wheel, receiving a novel object daily, while control animals were kept in standard conditions. On the days 53–60, all animals received 50 mg/kg BrdU once daily. 24 h after the last injection, mice were processed for immunocytochemistry.

Immunocytochemistry

Determination of BrdU labeling was done as described previously (Malberg et al., 2000) with modifications. Anti-mouse BrdU (1:1,000; Boehringer) was followed by biotinylated goat anti–mouse (Extra 2 kit; Sigma-Aldrich), extravidin (Extra 2 kit; Sigma-Aldrich), and Ni-enhanced DAB (ABC kit; Vector Laboratories). For double labeling, sections were incubated overnight with pooled primary antibodies: rat-anti-BrdU (1:100; Accurate) together with mouse anti-NeuN (1:500; Chemicon) or mouse anti–Tuj-1 (1:100; R&D Systems), or mouse anti-GFAP (1:500; Chemicon) followed by goat anti–rat Cy3 (Chemicon; red) and goat anti–mouse (Alexa-Fluor 488; Molecular Probes; green). Confocal images were acquired using a microscope (model TCS SP2; Leica). For the enriched environment experiment (Fig. 2 a), BrdU-positive cells were counted under a light microscope in the granular, SGZs, and in the hilus of the DG of five sections per animal (five animals per group).

Combined immunocytochemistry for nestin and in situ hybridization for cyclin D2 mRNA

Immunostaining was performed as described in the previous section with a mouse monoclonal anti-nestin antibody (MAB353; Chemicon), diluted 1:100. The immunoreaction was visualized using HRP-conjugated goat anti–mouse antibody (EnVision; DakoCytomation) followed by biotinylated tyramide (New England Nuclear) and Alexa 488–conjugated streptavidin. The in situ hybridization procedure using digoxygenin-labeled cRNA probes was performed as described by Guzowski et al. (1999). As a template for riboprobe synthesis, a fragment of mouse cyclin D2 cDNA, subcloned into pBluescript SK plasmid, was used (Sicinski et al., 1996).

In vitro culture of neurospheres and RNA extraction

Neurospheres were cultured as described previously (Kukekov et al., 1997; Lobo et al., 2003) with modifications. Hippocampi were dissociated mechanically and enzymatically to obtain single cell solution. Cells were plated at the concentration 5–7.5 × 10^4/ml and cultured in the presence of 20 ng/ml of human recombinant EGF (Sigma-Aldrich). Medium was replaced every 3 d. On the seventh day in culture, 15–20 spheres of different sizes were selected and pooled together for mRNA extraction by Dynabeads mRNA DIRECT kit (Dynal).

RT-PCR

Reverse transcription reaction was conducted using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Thermal profiles were as follow: predenaturation for 3 min at 94°C, denaturation for 30 s at 94°C, annealing for 30 s at 67 (cyclin D1), 53 (cyclin D2), and 55°C (cyclin D3, nestin), elongation for 1 min at 72°C, and final elongation for 10 min at 72°C. For amplification of mouse D-type cyclins, we designed primers that are separated from each other by an intron and located in the sites that are not conserved between particular D-type cyclins. Primer sequences were as follows: cyclin D1, 5′-GGCGGATGAGAACAAGCAGA-3′, 5′-ACCAGCCTCTTCCTCCACTT-3′; cyclin D2, 5′-GTTCTGCAGAACCTGT-TGAC-3′, 5′-ACAGCTTCTCCTTTTGCTGG-3′; cyclin D3, 5′-CGCAATTG-CAGCTTCTAGGT-3′, 5′-GAATGGCTGTGACATCTGTG-3′. Lengths of amplicons were as follow: 376 bp for cyclin D1, 448 bp for cyclin D2, and 582 bp for cyclin D3. The presence of nestin mRNA was confirmed in neurospheres by RT-PCR (Suslov et al., 2000).

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