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.,...
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 have used such a treatment to see whether or not it may induce neurogenesis in the brain of cyclin D2 KO animals. 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 brain neurons. 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 $\sim 25\%$, 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-
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 not 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; Cieumerych 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
animals; 3-d-old animals injected with BrdU and analyzed 2 d later. with selective alteration of adult neurogenesis, aiming at ad-
moral neuronal precursors. Further exploration of such mech-
discriminating between proliferation of adult versus develop-
mice in functional tests on physiological and pathological sig-
Hence, we may suggest that the less pronounced effect of cyclin 
derived from 5-d-old hippocampi express all three cyclins D. 
sors proliferating in response to a brain injury. This dramatic 
not affect proliferation of neuronal precursors in olfactory epi-
ment (Fig. 2 a), BrdU-positive cells were counted under a light microscope 
488; Molecular Probes; green). Confocal images were acquired using a 

different sizes were selected and pooled together for mRNA extraction by 
was replaced every 3 d. On the seventh day in culture, 15–20 spheres of 
ence of 20 ng/ml of human recombinant EGF (Sigma-Aldrich). Medium 
ated mechanically and enzymatically to obtain single cell solution. Cells 
Neurospheres were cultured as described previously (Kukekov et al., 

cDNA, subcloned into pBluescript SK plasmid, was used (Sicinski et al., 
As a template for riboprobe synthesis, a fragment of mouse cyclin D2 
gated tyramide (New England Nuclear) and Alexa 488–conjugated 
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 

tunnels, ladders, hiding places, and a running wheel, receiving a novel 

tours and beehives, which provide a complex and multi-

smaller. This deficit is specific for the brain neurons, as it does not affect proliferation of neuronal precursors in olfactory ep-
ithelium of the nasal turbinates as well as nonneuronal precu-
sors 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 ex-
plained by compensation exerted by other cyclins D during de-
velopmental neurogenesis in cyclin D2–deficient animals. 
In conclusion, we would like to suggest using D2 KO mice in functional tests on physiological and pathological sig-
nificance of the newly born neurons in the adult brain. Further-
more, our results reveal the existence of molecular mechanisms discriminating between proliferation of adult versus develop-
mental neuronal precursors. Further exploration of such mech-

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/6 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 other-
wise) −/− (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 suf-
ferring, 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 injec-
tions 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 cere-
bral cortex (anteroposterior − 2.12 mm; lateral = ± 1.5 mm; and ven-
tral = 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 to-
gether 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 af-
ter the last injection, mice were processed for immunocytochemistry.

Immunocytochemistry
Determination of BrdU labeling was done as described previously (Mal-
berg et al., 2000) with modifications. Anti-mouse BrdU [1:1,000; Boeh-
ringer] was followed by biotinylated goat anti–mouse (Extra 2 kit; Sigma-
Aldrich), extravadin (Extra 2 kit; Sigma-Aldrich), and Ni-enhanced DAB 
(ABC kit; Vector Laboratories). For double labeling, sections were incu-
bated overnight with pooled primary antibodies: rat-anti-BrdU [1:100; Ac-
curate] 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-Fluar 
488; Molecular Probes; green]. Confocal images were acquired using a 

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

Immunochemistry 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 biotinyl-
ated tyramide (New England Nuclear) and Alexa 488–conjugated streptavidin. The in situ hybridization procedure using digoxigenin-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 dissoci-
ated mechanically and enzymatically to obtain single cell solution. Cells 
were plated at the concentration 5–7.5 × 10⁴/ml and cultured in the pres-
ence 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).

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 hippo-
campal 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 confo,stalk, 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.
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°C (cyclin D1), 53°C (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′-GGGCGGTATGAAACAAGCAGA-3′, 5′-ACCAGCCTTCTCCTCACATCT-3′; cyclin D2, 5′-GTCTTCGACAACTCGTGATGAC-3′, 5′-ACAGCTTCCTCTTTGCTG-3′; cyclin D3, 5′-CCGAATTCGACGTCTTAGTG-3′, 5′-GAATGCTGACATCTTG-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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References
Altman, J., and G.D. Das. 1964. Autoradiographic examination of the effects of enriched environment on the rate of glial multiplication in the adult rat brain. Nature. 204:1161–1163.

Cameron, H.A., C.S. Woolley, B.S. McEwen, and E. Gould. 1993. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience. 56:337–344.

Cierny, M.A., A.M. Kenney, E. Sicinska, I. Kalaszczynska, R.T. Bronson, D.A. Peterson, and F.H. Gage. 1998. Neurogenesis in the adult human hippocampus. Nat. Med. 4:1313–1317.

Eriksson, P.S., E. Perfilieva, T. Bjork-Eriksson, A.M. Alborn, C. Nordborg, J.E. Malberg, J.E. Nestler, and R.S. Duman. 2000. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J. Neurosci. 20:9104–9110.

Reynolds, B.A., and S. Weiss. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. 255:1707–1710.

Robker, R.L., and J.S. Richards. 1998. Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. Mol. Endocrinol. 12:924–940.

Rosenzweig, M.R., and E.L. Bennett. 1996. Psychobiology of plasticity: effects of training and experience on brain and behavior. Behav. Brain Res. 78:57–65.

Santarelli, L., M. Saxe, C. Gross, A. Surget, F. Battaglia, S. Dulawa, N. Weiss-taubb, J. Lee, R. Duman, O. Arancio, et al. 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science. 301:805–809.

Shi, Y., D. Chichung Lie, P. Taupin, K. Nakashima, J. Ray, R.T. Yu, F.H. Gage, and R.M. Evans. 2004. Expression and function of orphan nuclear receptor TLX in adult neural stem cells. Nature. 427:78–83.

Shors, T.J., G. Miesegaes, A. Beylin, M. Zhao, T. Rydel, and E. Gould. 2001. Neurogenesis in the adult is involved in the formation of trace memories. Nature. 410:372–376.

Sicinska, E., I. Altfasits, L. Le Cam, W. Swat, C. Borowski, Q. Yu, A.A. Ferrando, S.D. Levin, Y. Geng, H. von Boehmer, and P. Sicinski. 2003. Requirement for cyclin D3 in lymphocyte development and T cell leukemia. Cancer Cell. 4:451–461.

Sicinski, P., J.L. Donaher, S.B. Parker, T. Li, A. Fazeli, H. Gardner, S.Z. Haslam, R.T. Bronson, S.J. Elledge, and R.A. Weinberg. 1995. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell. 82:621–630.

Sicinski, P., J.L. Donaher, Y. Geng, S.B. Parker, H. Gardner, M.Y. Park, R.L. Robker, J.S. Richards, L.K. McGinnis, J.D. Biggers, et al. 1996. Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature. 384:470–474.

Solvason, N., W.W. Wu, D. Parry, D. Mahony, E.W. Lam, J. Glassford, G.G. Klaus, P. Sicinski, R. Weinberg, Y.J. Liu, et al. 2000. Cyclin D2 is essential for BCR-mediated proliferation and CD5 B cell development. Int. Immunol. 12:631–638.

Suslov, O.N., V.G. Kukekov, E.D. Laywell, B. Scheffler, and D.A. Steindler. 2000. RT-PCR amplification of mRNA from single brain neurons. J. Neurosci. Methods. 96:57–61.

Takahashi, T., R.S. Nowakowski, and V.S. Caviness Jr. 1992. BUDR as an S-phase marker for quantitative studies of cytokinetic behaviour in the murine cerebral ventricular zone. J. Neurocytol. 21:185–197.

van Praag, H., G. Kempermann, and F.H. Gage. 2000. Neural consequences of environmental enrichment. Nat. Rev. Neurosci. 1:191–198.

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