Constitutive Expression of Functionally Active Cyclin-Dependent Kinases and Their Binding Partners Suggests Noncanonical Functions of Cell Cycle Regulators in Differentiated Neurons

Neurodegeneration in Alzheimer’s disease and various experimental lesion paradigms are associated with an unscheduled upregulation of cell cycle–related proteins, indicating a link between cell cycle reactivation and neuronal death. Recent evidence, however, suggests that at least some of the canonical cell cycle regulators are constitutively expressed in differentiated neurons of the adult brain. Systematic investigations on the constitutive expression of cell cycle regulators in differentiated neurons in vivo, providing the basis for further insights into their potential role under pathological conditions, however, have not been carried out. Here, we demonstrate a constitutive neuronal expression of Cdk1, 2, and 4; their activators cyclins D, A, B, and E; and their inhibitors p57Kip2 within the neocortex of adult mice by western blot and immunocytochemistry. Expression was verified by single-cell reverse transcriptase–polymerase chain reaction applied to individual microscopically identified neurons captured with laser dissection. Immunoprecipitation and in vitro kinase assays revealed that Cdk1, 2, and 4 are properly complexed to cyclins and exhibit kinase activity. This physiological expression of positive cell cycle regulators in adult neurons is clearly not related to neuronal proliferation. Taken together, our findings demonstrate a constitutive expression of functionally active cyclin-dependent kinases and their regulators in differentiated neurons suggesting a noncanonical role of cell cycle regulators potentially linked to neuronal plasticity and/or stability.

Keywords: cyclin-dependent kinase inhibitors, cyclins, mice, neocortex, plasticity

Introduction

Once neurons are terminally differentiated, they lose their ability to proliferate. Maintenance of mitotic quiescence is essential for the functional stability of the complexly wired neuronal system. At the same time, however, terminally differentiated neurons retain a remarkable dynamic plasticity, which provides the basis for a lifelong structural adaptation of neuronal networks to meet functional needs. It had traditionally been assumed that the majority of molecules that regulate activation and orderly progression through the cell cycle are repressed in the mitotically quiescent G0 state. Although neurons are terminally differentiated and lack the ability to divide, the expression of potential cell cycle regulators such as cyclins D, A, B, and E as well as Cdk1, 2, and 4 in the adult brain has repeatedly been reported (see Fig. 1 for the role of cell cycle regulatory proteins in cell division cycle) (Tamaru et al. 1993; Miyajima et al. 1995; Li, Chopp, and Powers 1997; Small et al. 1999, 2001; Timsit et al. 1999; Fischer et al. 2001; Ino and Chiba 2001; Katchanov et al. 2001; Liu and Greene 2001; Becker and Bonni 2004). Several studies, however, have reported the expression of cyclins, Cdk1, and Cdk2 under normal conditions in mature neurons, and an involvement in physiological functions beyond cell cycle regulation, including transcription, neuronal morphogenesis, and plasticity, has been proposed (Ross and Risken 1994; Yan and Ziff 1995; Ross et al. 1996; Xiong et al. 1997; Huard et al. 1999; Richter 2001; Becker and Bonni 2005). Still, it remains obscure whether Cdk1 and cyclins form active complexes in differentiated neurons giving rise to kinase activity under normal conditions. Therefore, in the present study, we analyzed the expression of cyclins, Cdk1, and Cdk2 by both the protein and mRNA level by immunocytochemistry and single-cell reverse transcriptase–polymerase chain reaction (RT-PCR) applied to individual microscopically identified neurons captured with laser dissection. Immunoprecipitation and in vitro kinase assays were applied to analyze whether Cdk1 is properly complexed to cyclins and exhibit kinase activity.

Materials and Methods

Animals

Brains were obtained from 6 adult NMRI mice. In addition, 3 mouse embryonic brains (embryonal day 14, E14) were used for immunoprecipitation-based kinase assays. The animals were strictly treated following the guidelines set by the Panel on Euthanasia and the Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Ethical Committee of Leipzig University. We made all efforts to minimize the number of animals used and their suffering.

Tissue Preparation and Immunocytochemistry

Mice were overdosed with carbon dioxide and perfused transcardially with ice-cold saline. Animals were then fixed by perfusion with 0.1 M phosphate-buffered saline containing 4% formaldehyde (pH 7.4). The removed brains were postfixed in the same solution for 3 days at 4 °C and cryoprotected in 30% sucrose. Blocks of mouse brain were cut in the coronal plane at 30 μm on a freezing microtome. Sections were incubated in 0.1 M Tris-buffered saline (TBS, pH 7.4) containing 1% H2O2 (30 min) to quench endogenous peroxidase activity. For antigen retrieval, sections were pretreated with citrate buffer (pH 6.0) for 10 min in a microwave oven. Nonspecific binding sites were masked by incubation with blocking solution containing 0.3% fat-free dried milk and 0.1% gelatine. Subsequently, tissue was immunoreacted with one of Stefanie Schmetsdorf, Ulrich Gärtner and Thomas Arendt

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Stefanie Schmetsdorf and Ulrich Gärtner contributed equally to this work

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the primary antisera listed in Table 1 overnight at 4°C. Section-bound antibodies were revealed with biotinylated donkey anti-rabbit antiserum (1:1000, 1 h, Amersham; Buckinghamshire, UK) or donkey anti-goat antiserum (1:1000, 1 h, Jackson, Suffolk, UK), ExtrAvidin®-peroxidase conjugate (1:1000, 1 h, Sigma, Munich, Germany) and 3,3'-diaminobenzidine (DAB)/NiNH₄SO₄ as chromogen. All incubation steps were separated by intense washing in TBS. For double-fluorescence immunostaining, sections were reacted first with anti-Cdk antisera, followed by visualization with Cy2-tagged donkey anti-rabbit antiserum (1:500, 1 h, Jackson ImmunoResearch Inc.). In a second step, after reapplying blocking, tissue was incubated with anticyclin antisera, and fluorescent detection was performed by using Cy3-conjugated donkey anti-rabbit antiserum (1:500, 1 h, Jackson ImmunoResearch Inc.). The specificity of labeling was tested by omitting primary antisera. After such incubation, tissue was devoid of immunoreactivity as expected (Fig. 4). Finally, the sections were coverslipped with Entellan® (Merck, Darmstadt, Germany). Images were captured with the digital microscope camera AxioCam HRc running on the AxioVision 3.1 Software (both Carl Zeiss Vision) and processed with Adobe® Photoshop™ 7.0 (Adobe Systems, Mountain View, CA).

Electrophoresis and Immunoblotting
The total protein fraction (50 μg per lane, Bradford assay) of neocortical homogenates was separated by 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and subsequently transferred onto polyvinylidene fluoride membrane (Polsyscreen, DuPont-New England Nuclear, Boston, MA), using a semidry blot system (TransBlot™, BioRad, Hercules, Canada). Equal amount of the protein loaded on the gel was controlled by Coomassie staining. For immunodetection of cyclins, Cdk, and Cdkis, membranes were incubated in 2% bovine serum albumin (in TBS) and probed with primary antisera (Table 1; 1 h, dilution 1:500) at room temperature. After washing in TBS/0.1% Tween-20, blots were incubated with biotinylated donkey anti-rabbit antiserum (1 h, 1:1000, Amersham) or anti-goat antiserum (1 h, 1:1000, Jackson), respectively. Immunodetection was visualized with ExtrAvidin®-peroxidase conjugate (1 h, 1:1000, Sigma) and DAB/NiCl₂/0.015% H₂O₂.

RNA Isolation and Reverse Transcription
Single pyramidal neurons of layer V (200 cells per trial) were dissected from freshly prepared Nissl-stained frozen sections (30 μm) of adult mouse somatosensory cortex (Fig. 2) using laser microdissection and pressure catapulting technology (PALM® MicroBeam system, PALM, Microlaser Technologies AG, Bernried, Germany). RNA of collected cells was extracted with RNeasy® Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer’s instructions. First strand synthesis was conducted by incubating 8 μl RNA, 1 μl Random Hexamer, and 1 μl 2'-deoxynucleoside 5'-triphosphate for 5 min at 65°C followed by rapid cooling on ice. To each mixture, the following was added: 2 μl 10× reverse transcriptase (RT) buffer, 1 μl 25 mM MgCl₂, 2 μl 0.1 M dithiothreitol (DTT), and 1 μl RNase inhibitor. This cocktail was incubated for 2 min at 25°C before 1 μl SuperScript II RT was added. The mixture was then incubated for 10 min at 25°C and subsequently heated for a further 50 min at 42°C. The reaction was stopped by heating at 70°C for 15 min and terminated by incubation with 1 μl RNase H for 20 min at 37°C. All cDNA samples were stored at −20°C until analysis.

Real-Time RT-PCR
Real-time RT-PCR was performed on Rotor-Gene™ 2000 (Corbett Research, Sydney, Australia). For each marker, 3 independent experiments were performed. In each experiment, duplicates of a standard dilution series of specific polymerase chain reaction (PCR) fragments for each cell cycle protein and cDNA (total RNA equivalent) of unknown

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**Table 1**

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Dilution</th>
<th>Antibody specificity</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Cyclin A (H-432)</td>
<td>1:800</td>
<td>Cdk2 (M2)</td>
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<tr>
<td>Cyclin A (C-19)</td>
<td>1:800</td>
<td>Cdk4 (H-303)</td>
<td>1:800</td>
</tr>
<tr>
<td>Cyclin B1 (H-433)</td>
<td>1:800</td>
<td>Cdk4 (C-19)</td>
<td>1:800</td>
</tr>
<tr>
<td>Cyclin B1 (H-20)</td>
<td>1:800</td>
<td>p15Ink4 (C-20)</td>
<td>1:500</td>
</tr>
<tr>
<td>Cyclin D3 (H-292)</td>
<td>1:500</td>
<td>p16Ink4 (M-156)</td>
<td>1:500</td>
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<tr>
<td>Cyclin D3 (C-16)</td>
<td>1:500</td>
<td>p16Ink4 (C-19)</td>
<td>1:500</td>
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<tr>
<td>Cyclin E (C-20)</td>
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<td>p19Ink4 (M-19)</td>
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<tr>
<td>Cdk1 (C-297)</td>
<td>1:500</td>
<td>p27Kip1 (C-19)</td>
<td>1:500</td>
</tr>
<tr>
<td>Cdk2 (C-298)</td>
<td>1:500</td>
<td>p57Kip2 (C-19)</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Note: Host species of all antisera is rabbit, except p18Ink4c (C19) and p57Kip2 (C-19), which are from goat. All antisera were obtained from Santa Cruz Biotechnology, Inc., Heidelberg, Germany.

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![Figure 1](https://example.com/figure1.png)

**Figure 1.** Schematic illustration of the involvement of cell cycle regulatory proteins in cell division. During the cell division cycle, expression and activity of Cdns and cyclins, the positive regulatory subunits of Cdns, are regulated in a phase-dependent manner. The activity of cyclin-Cdk complexes is negatively controlled by inhibitors of the Ink4 family (p15Ink4, p16Ink4, p18Ink4, p19Ink4) and of the Cip/Kip family (p21Waf1/Cip1, p27Kip1, p57Kip2), G0, stationary phase; G1, first gap phase; R, restriction point; S, DNA synthesis phase; G2, second gap phase; M, mitosis phase.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Photomicrographs of a coronal frozen Nissl-stained section before (A) and after (B) laser microdissection of single layer V pyramidal neurons. Corresponding cell locations are indicated by arrow heads. Bar in (B) (also refers to A): 50 μm.
samples were amplified in a 25-µl reaction mixture containing AccuPrime™ Taq Polymerase (Invitrogen™ Life Technologies, Karlsruhe, Germany) in 1× PCR buffer, 50 µM forward and reverse primers (sequences of primers are given in Table 2), SYBR® Green I nucleic acid stain (1:2000; Cambrex Bio Science Rockland, Inc., Verviers, Belgium), and nuclease-free water. The thermal profile consisted of 1 cycle at 95 °C for 5 min followed by 45 cycles at 95 °C for 30 s then 60-64 °C for 1 min. Product specificity was confirmed in initial experiments by agarose gel electrophoresis and routinely by melting curve analysis. For each run, data acquisition and analysis were done by RotorGene™ 2000 system software. The relative number of molecules of each transcript was determined by interpolating the cycle threshold values of the unknown samples to each standard curve.

PCR products were separated by flatbed electrophoresis in 2% agarose gels (100 V for 30–40 min), stained with ethidium bromide, and visualized under an UV transilluminator. Gel images were captured using Phoretix™ Grabber software version 2.0 (NonLinear Dynamics, Newcastle upon Tyne, UK).

Preparation of Cell Extracts and Immunoprecipitation
Freshly prepared neocortical tissue was homogenized in NP-40 lysis buffer (0.5% Nonidet P-40 in 20 mM Tris-HCL, pH 7.2, containing 100 mM NaCl, 2 mM MgCl2, 5 mM NaF, 1 mM Na3VO4, 1 mM DTT, and 100 µg/mL phenylmethylsulphonylfluoride [PMSF] and 2 µg/mL Leupeptin). After a 1-h incubation at 4 °C, lysates were cleared by centrifugation at 15 000 × g for 30 min and the supernatant was frozen.

For immunoprecipitation, undiluted primary antisera (anti-Cyclin D3, Cyclin A, Cyclin B, and Cyclin E; anti-Cdk1, -Cdk2, and -Cdk4) or normal rabbit antisera (control) were preincubated for 1 h with 10 µL Dynabeads® protein A solution (Dynal, Hamburg, Germany) at 4 °C. Dynabeads® protein A-antibody complexes were washed 3 times with bead buffer (20 mM Tris-HCL, pH 7.2, 2 mM MgCl2, 0.5% NP-40, 0.15 M NaCl, 1 mM DTT, 100 µg/mL PMSF, 2 µg/mL Leupeptin) and resuspended in bead buffer. Aliquots of NP-40 extracted proteins were incubated with Dynabeads® protein A-antibody complexes and shaken gently overnight at 4 °C. Immunoprecipitates (IPs) were washed 3 times with bead buffer.

To test for coimmunoprecipitation, the precipitated pellets were separated by SDS-PAGE and immunoblotted using antisera against relevant binding partners.

Immunoprecipitation-Based In Vitro Kinase Assays
IPs (anti-Cyclin D3,-Cyclin A,-Cyclin B,- and Cyclin E) were washed in kinase buffer (20 mM Tris-HCL, pH 7.2, 10 mM MgCl2, 1 mM DTT, 60 mM β-Glycerophosphate, and 100 µg/mL PMSF and 2 µg/mL Leupeptin) and resuspended in 50 mM kinase buffer containing 0.5 mg of histone H1, 10 µg of [γ-32P]-ATP, and 20 µM ATP. All reaction mixtures were incubated for 1 h at 30 °C and stopped by boiling for 5 min in 4× Laemmli sample buffer. Then, equal amounts of protein (10 µg per lane) were resolved on 12% SDS gels. Histone H1 was separated from free [γ-32P]-ATP on 12% polyacrylamide gels. Phosphorylated histone H1 was detected by autoradiography (Kodak X-Omat film, Rochester, NY). Control incubations were performed in the absence of histone. In addition, IPs from incubation with normal rabbit serum (NRS) were used as a control further.

Results
Neuronal expression of cell cycle regulatory proteins in the neocortex of adult mice was analyzed both at the protein and mRNA level. Immunocytochemical detection of cell cycle markers was verified by single-cell RT-PCR applied to individual microscopically identified neurons captured with laser dissection. Immunoprecipitation and in vitro kinase assays were used to analyze whether cyclin-dependent kinases are properly complexed to cyclins and exhibit kinase activity.

Western Blotting and Immunocytochemistry
A panel of antibodies was used both on western blots and immunocytochemical preparations to detect Cdk1, 2, and 4 and their binding partners such as cyclins D, A, B, and E as well as the inhibitors p15Ink4b, p16Ink4a, p18Ink4c, p19Ink4d, p21Waf1/Cip1, p27Kip1, and p57Kip2 (Figs 3 and 5). On western blots, signals at the appropriate molecular weight were obtained with at least 2 different antibodies for each protein (Fig. 3).

Immunocytochemical detection of each protein was performed with at least 2 different antisera that gave rise to identical staining patterns. Control sections that were not incubated with primary antibody remained unstained (Fig. 4).

In general, there was no definite evidence for glial expression of any of the analyzed cell cycle-related proteins (Fig. 5). A

![Figure 3](https://academic.oup.com/cercor/article-abstract/17/8/1821/316074)

**Table 2**

<table>
<thead>
<tr>
<th>Transcripts to be detected</th>
<th>Primer forward</th>
<th>Primer reverse</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>Cyclin D3</td>
<td>5’gtcacaaccctgcgaacgc3’</td>
<td>5’agctgacagaaagcaagc3’</td>
<td>208</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>5’acctgctccagctgccgaac3’</td>
<td>5’ttgagctgagggactgtg3’</td>
<td>176</td>
</tr>
<tr>
<td>Cyclin B</td>
<td>5’catgctggcagctgacagc3’</td>
<td>5’tgctgacagaaagcaagc3’</td>
<td>216</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>5’caccacagtaccctgcgaac3’</td>
<td>5’tccaccctgctacaaacc3’</td>
<td>176</td>
</tr>
<tr>
<td>Cdk1</td>
<td>5’tcctgcgtctgtagctacac3’</td>
<td>5’tccaccctgctacaaacc3’</td>
<td>203</td>
</tr>
<tr>
<td>Cdk4</td>
<td>5’tggctcagcagctgacagc3’</td>
<td>5‘ttgccagctgagggactgtg3’</td>
<td>357</td>
</tr>
<tr>
<td>Neurofilament H</td>
<td>5’agcgaccagacacagtccga3’</td>
<td>5’gcggctgctctgccgtc3’</td>
<td>176</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein</td>
<td>5’ctcagcacaacatcactcga3’</td>
<td>5’tccaccctgctacaaacc3’</td>
<td>184</td>
</tr>
</tbody>
</table>
synopsis of characteristics of neuronal immunostaining is given in Table 3. Immunoreactivity for cyclins D, A, B, and E was clearly detectable in neocortical neurons. It was most prominent in pyramidal cells that were most intensely stained in layer V. Apart from nuclear localization, cyclins D and A were also present in the perinuclear cytoplasm and in dendrites of these neurons. In contrast to this, immunostaining of cyclins E and B was more pronounced in dendrites but present in nuclei to a lesser extent (Fig. 5).

Similar to cyclins, immunoreactivity for Cdk1 and Cdk4 is found in pyramidal neurons within layers II/III and V (Fig. 5). The expression of these proteins is preferentially detected in the nuclear compartment. In contrast to these kinases, Cdk2 is present only in the perinuclear cytoplasm of neurons (Fig. 5). Cdk5 and their corresponding cyclins are colocalized in neurons (Fig. 6).

The expression of Cdk inhibitors is distributed equally throughout cortical layers. The Ink4 inhibitors p15Ink4b and p18Ink4c are present in dendrites but not in nuclei. P16Ink4a immunoreactivity, on the other hand, is found only in neuronal nuclei (Fig. 5). Staining for p21Waf1/Cip1 and p27Kip1 proteins is present both in the nuclear and in the cytoplasmic compartment. Moreover, p21Waf1/Cip1 immunoreactivity is also detectable in pyramidal cell dendrites. The inhibitor p57Kip2 is exclusively seen in the cytoplasmic compartment of pyramidal cells (Fig. 5).

**Single-Cell mRNA Analysis by Laser Microdissection and Real-Time RT-PCR**

Immunohistochemical findings indicate the occurrence of a variety of cell cycle markers in layer V pyramidal cells of adult mice. To rule out any false-positive results arising through potential cross-reactivity of antibodies, we verified these findings with an additional independent method and analyzed the expression of corresponding transcripts at the single-cell level by PCR after laser microdissection of individual neurons identified under the microscope.

We were able to obtain PCR products specific for cyclins D3, A, B, and E and Cdks 1 and 4 as well as p27Kip1, amplified from cDNA templates prepared from individual layer V neurons. Simultaneous detection of neurofilament H message but not of glial fibrillary acidic protein mRNA ensured that only neurons but not glial cells were included in the analysis. The presence of single products of the expected size was verified by gel electrophoresis (Fig. 7).

**Physical and Functional Interactions between Cyclins and Cdks**

The pattern of immunohistochemical labeling (see Figs 5 and 6) suggests the neuronal coexpression of cell cycle-related proteins. In order to determine whether Cdks and corresponding cyclins interact physically, IPs of cyclin D3, cyclin A, cyclin B, and E as well as Cdk1, Cdk2, and Cdk4 were immunoblotted with antisera to the physiologically relevant binding partners. The following proteins have been shown to be reciprocally communoprecipitated, strongly indicating their physical association: cyclin D3 with Cdk4, cyclins A and E with Cdk2, and cyclin B with Cdk1 (Fig. 8).

Beyond the characterization of physical interactions, the functional relevance of cyclin-Cdk complexes at different developmental stages was tested by an immunoprecipitation-based in vitro kinase assay. Incubation of histone H1 with anti-Cyclin D3, -Cyclin A, -Cyclin B, or -Cyclin E IPs clearly resulted in phosphorylation of the substrate, although to a lesser extent compared with incubation with IPs obtained from embryonic lysates. No activity was found when NRS IPs were assayed (Fig. 9).

**Discussion**

The findings of the present study demonstrate the constitutive neuronal expression of numerous cell cycle–related proteins in the adult mouse neocortex. Cell cycle regulators were found in the perikaryal cytoplasm, in dendrites, and also in nuclei of differentiatied neurons. As a potential cross-reactivity of antisera with other proteins cannot entirely be ruled out, neuronal expression, detected on immunohistochemical preparations and western blot, for a selected number of cell cycle regulators was verified at the single-cell mRNA level. In addition, biochemical data demonstrate that cyclins are adequately complexed with Cdks, resulting in catalytic kinase activity. Because immunohistochemical data do not suggest the presence of significant cyclin and Cdk levels in the glial population, biochemical activity is likely attributed to neurons.

Present data provide direct evidence for the constitutive expression of cyclins D, A, B, and E in postmitotic neurons within the cerebral cortex suggesting a function for these proteins beyond the regulation of activation and progression of the cell cycle. These observations are consistent with previous evidence from several groups, including our own, to indicate that cyclins D, A, B, and E in particular are expressed in mature neurons of the neocortex and hippocampus under...
physiological conditions (Tamaru et al. 1993; Miyajima et al. 1995; Oka et al. 1996; Van Lookeren Campagne and Gill 1998a; Small et al. 1999, 2001; Timsit et al. 1999; Matsumaga 2000; Hoozemans et al. 2002; Gärnert et al. 2003; De Falco et al. 2004; Schmetsdorf et al. 2005). This constitutive expression of cyclins in differentiated neurons, however, has not been investigated systematically, and their functional binding to cyclin-dependent kinases had not been reported previously. In proliferative cells, cyclins localized to the cytoplasm are proteolytically degraded by ubiquination and destruction in proteasomes (Sudakin et al. 1995; Clurman et al. 1996; Klotzbucher et al. 1996; Udvary 1996; Diehl et al. 1997, 1998). However, cytoplasmic sequestration

**Figure 5.** Immunohistochemical localization of cyclins, Cdns, and Cdkis in the somatosensory cortex of adult mouse brain at 2 different magnifications thereby zooming in on layer V neurons. Bar at bottom of third column (also refers to first column): 200 µm. Bar at bottom of fourth column (also refers to second column): 25 µm.
of cyclins, for example, of cyclin E and cyclin D1, might be characteristic for postmitotic neurons and may be associated with a physiological role other than regulation of the cell cycle (Matsunaga 2000; Sumrejkanchanakij et al. 2003; De Falco et al. 2004). As has been shown for cyclin D1, cytoplasmic compartmentalization is determined by interaction with proteins such as glycogen synthase kinase-3β and specific exportins (Diehl et al. 1998). It is thus tempting to speculate that in differentiated neurons, cyclins might have alternative binding partners that prevent degradation and assist in mediating non-cell cycle-related functions.

In addition to cyclin expression, present results also clearly showed the presence of Cdk1, 2, and 4 in postmitotic neurons of the neocortex. Immunohistochemical findings illustrate the localization of Cdk5 both in the cytoplasmic compartment and in the nuclei of neurons, so extending recent data on mouse hippocampus (Schmetsdorf et al. 2005). In addition to immunohistochemical detection, we also provide evidence for expression of Cdk1 and Cdk4 at the single-cell mRNA level of identified neurons and demonstrate a physical interaction between Cdk1, 2, and 4 and the relevant cyclins, which results in adequate levels of kinase activity to suggest a catalytic function. Expression of cyclin-dependent kinases has previously been observed in a variety of differentiated neurons. In postmitotic sympathetic neurons of cervical ganglia, for example, mRNAs encoding for Cdk4 and Cdk5 have been detected (Freeman et al. 1994), whereas other studies have described expression of Cdns 1 and 2 in postmitotic differentiating cells, including neocortical neurons (Okano et al. 1993; Wood and Zinsmeister 1993; Delalle et al. 1999; Watanabe et al. 1999). Constitutive expression of Cdk2 and Cdk4 has also been found in differentiated muscle and PC12 cells (Jahn et al. 1994; Dobashi et al. 1996).

Cellular functions of active cyclin–Cdk complexes have previously been attributed to additional physiological effects, such as metabolic regulation or basal transcription (Kaffman et al. 1994; Roy et al. 1994; Rickert et al. 1996). If the dendritic localization of cyclins is considered, the expression of Cdns and cyclins in differentiated neurons may reflect a function in network stabilization and/or neuroplastic mechanisms. In neurons, Cdk1 phosphorylates microtubule-associated proteins, including tau protein, and neurofilaments (Hisanaga et al. 1991; Mawal-Dewan et al. 1992; Scott et al. 1993). Very recently, it was found that Cdk2 can be activated by neuregulin, and knockdown or inhibition of Cdk2 prevents neuregulin-induced differentiation and expression of the acetylcholine receptor ε subunit in myotubes (Lu et al. 2005). Thus, we speculate that Cdk activity might be associated with the regulation of synaptogenesis and cytoskeletal dynamics during neuronal morphogenesis and differentiation in the adult brain.

The present immunohistochemical analysis further demonstrates the expression of Ink4 and Cip/Kip inhibitors of

### Table 3

Synopsis of subcellular distribution of cell cycle–related proteins in layer V neurons

<table>
<thead>
<tr>
<th>Marker</th>
<th>Localization</th>
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<tbody>
<tr>
<td></td>
<td>Nuc</td>
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<tr>
<td>CycD</td>
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<td>CycE</td>
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<td>CycA</td>
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<td>CycB</td>
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<td>p27</td>
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<tr>
<td>p57</td>
<td>–</td>
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</tbody>
</table>

Note: The number of plus symbols indicates grading of staining intensity: {++++} > {++} > {+}. Minus symbol denotes negligible staining. nuc, nucleus; cyt, cytoplasm.

### Figure 6

Double-fluorescence immunolabeling showing the cellular colocalization of cyclin D–Cdk4 (H-292, H-303), cyclin A–Cdk2 (H-432, H-298), cyclin B–Cdk1 (H-433, H-297), and cyclin E–Cdk2 (M-20, H-298). Corresponding images are arranged in columns. Bar at bottom of fourth column: 20 μm.
cyclin-dependent kinases in mature cortical neurons, which confirms and extents previous findings on mouse hippocampal neurons (Schmetsdorf et al. 2005). Neuronal expression of Cdk inhibitors may indirectly indicate the presence of cyclins/Cdks and might be involved in establishing and maintaining the differentiated state of neurons and prevents them from cell cycle reentry.

The intensity of p19Ink4d expression, for example, increases during neural ascent from the ventricular zone to the cortical plate along with progressive differentiation of neurons. Therefore, this inhibitor has been suggested to play a role in maintaining neurons in the postmitotic state (Zindy et al. 1999). p19Ink4d cooperates with the Cip/Kip inhibitor p27Kip1, and both are required to control cell cycle exit and to repress neuronal proliferation (Lee et al. 1996; Zindy et al. 1997, 1999; Löwenheim et al. 1999; Miyazawa et al. 2000). Accordingly, p27Kip1 expression is high in cells exiting the proliferative phase (Lee et al. 1996; Van Lookeren Campagne and Gill 1998b; Delalle et al. 1999). Cortical postmitotic neurons accumulate high levels of p27Kip1 to bind and inactivate cyclin A/E-Cdk2 and cyclin D-Cdk4 complexes (Lee et al. 1996; Cunningham and Roussel 2001; Sumrejkanchanakij et al. 2003). Suppression of Cdk activity by p27Kip1 is regarded as a key determinant for neuronal differentiation and stability (Kranenburg et al. 1995; Cunningham and Roussel 2001).

Recently, hypoxia-ischemia-induced DNA synthesis preceding neuronal cell death was shown to be associated with a reduction in p27Kip1 and p16Ink4a levels and, in parallel, with an increase in Cdk2 activity (Kuan et al. 2004).

Also p21Waf1/Cip1 is induced during neuronal differentiation and occurs in fully differentiated neurons (Van Grunsven et al. 1996; Xiong et al. 1997; Van Lookeren Campagne and Gill 1998b). Whether p21Waf1/Cip1 might be necessary per se for neuronal differentiation and quiescence or not is controversial (Parker et al. 1995; Van Grunsven et al. 1996). However, there is increasing evidence that at least some Cip/Kip inhibitors may operate in postmitotic neurons to support differentiation and specification of distinct neuron types by regulating nuclear transport, cell motility, and transcriptional activation (LaBaer et al. 1997; Ohnuma et al. 1999, 2001; Dyer and Cepko 2000; Coqueret 2003).

The present results on a constitutive expression of cell cycle regulators in the healthy adult brain do not negate their presumed pathogenetic role in Alzheimer’s disease. Comparing Alzheimer’s disease brains with age-matched normal brain, a variety of cell cycle proteins has been found at higher expression levels, which has been conceptually linked to cell death (Arendt et al. 1996; Vincent et al. 1996; Nagy et al. 1997). Of note, cell cycle proteins might show alterations in their subcellular distribution in Alzheimer’s disease where they are found predominantly associated with neurofibillary degeneration. Differences in compartmentation and segregation through binding to other proteins might not only affect their function but also modify their degradation. Potential differential effects through postmortem autolysis, therefore, need to be evaluated.

Conclusions

Complex wiring of the mature nervous system, which in general does not allow the effective integration of new neurons, and the extreme morphological polarity of nerve cells themselves may be contributing factors regarding the withdrawal
of differentiated neurons from cell cycle activity. On the other hand, neurons are very able to undergo functional and morphological adaptations in response to changes in network activity. The concomitance of both irreversible withdrawal from cell cycle and a high potential for plasticity is unique to neurons in comparison with all other cell populations. It has therefore been hypothesized that proteins normally involved in cell cycle machinery may assume functions in neuronal plasticity (Arendt 2003). Interestingly, in Alzheimer’s disease, suggested to be a neuroplastic disorder, several cell cycle–related proteins have been found to be ectopically expressed in neocortical and hippocampal pyramidal neurons: populations with a high degree of neuronal plastic capacity (Arendt, Holzer, and Gärtnert 1998; Nagy and Esiri 1998; Herrup and Arendt 2002). These facts support the idea that the constitutive expression of cell cycle proteins occurring predominantly in cortical pyramidal neurons might be related to mechanisms of neuroplasticity and/or to processes that maintain neuronal stability. Taken together, a complex pattern of cyclins, Cdk, and Cdkis is constitutively expressed in postmitotic neurons where it results in functionally active Cdk-cyclin complexes suggesting a noncanonical function of these cell cycle regulators.

Notes

This work was supported by the Interdisziplinäres Zentrum für Klinische Forschung Leipzig at the Faculty of Medicine of the Universität Leipzig (C1). We thank Dr Cuong Hoang-Vu and Mrs Kathrin Hamme (Experimental and Oncological Surgery Research Group, University Department of General, Visceral, and Vascular Surgery, Martin-Luther-University, Halle, Germany) for the opportunity to use the PALM® laser microdissection and pressure catapulting system and for excellent technical support. The authors also wish to thank Dr Zoe McIvor for linguistic revision of the manuscript. Conflict of Interest. None declared.

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