Differences in Cyclin D2 and D1 Protein Expression Distinguish Forebrain Progenitor Subsets

Regulation of neural proliferation is an essential component of brain formation and is driven by both intrinsic cell cycle and extrinsic growth and trophic molecules. Among the cell cycle proteins, understanding of the relative roles of the G1-phase active cyclins D2 and D1 (cD2 and cD1) has been hampered by lack of data regarding their expression patterns. In this study, cD2 immunoreactivity was examined in the neocortex, ganglionic eminences/ striatum, and hippocampal formation from embryonic day 12.5 until postnatal day 60 to more precisely characterize the expression of this protein during forebrain development. The localization of cD1 was also immunohistologically mapped for comparison. Throughout forebrain development, both overlapping and nonoverlapping protein expression of these cyclins suggests the presence of shared and unique cell cycle requirements for neurogenesis that distinguish progenitor pools.

Keywords: Ccnd1, Ccnd2, cerebral cortex, ganglionic eminence, hippocampus, knockout mice

Introduction

Brain histogenesis requires the coordinated regulation of neural precursor proliferation, specification of neuron and glial subtypes, and apoptotic removal of damaged or excess cells. Growth and trophic factors are certainly important for the regulation of proliferation, impacting the cell cycle proteins at several levels. A number of these factors, including sonic hedgehog (Shh), bone morphogenetic proteins (BMPs), and wingless proteins (Wnts), have profound influences on neural specification at the same time that they promote progenitor cell division (Cremisi and others 2003). Moreover, how a neural progenitor divides, whether distinguished by spindle orientation and cleavage plane during cytokinesis or by symmetric or asymmetric segregation of proteins to daughter cells, has a significant impact on cell fate outcomes (Chenn and McConnell 1995; Haydar and others 2003; Fishell and Kriegstein 2005). Although attention is more often focused on the external influences on proliferation, there are indications that the components of the cell cycle machinery may themselves have important roles in regulation of central nervous system progenitor proliferation behavior, though these roles are not well understood. For example, the need in mammalian brain for 3 cyclins D, all of which are capable of controlling the mid-G1 cell cycle checkpoint, is unknown. Loss of 1 or 2 of the 3 cyclins D produces upregulation of the other, so that brain can still develop (Cierny and others 2002). Nevertheless, loss of cyclin D1 (cD1) can be only incompletely compensated for by knock in of cD2 into the cD1 locus, indicating nonredundant functions of these proteins (Carthon and others 2005). Thus, how a progenitor divides is likely to be directed by the cooperation of external and intrinsic regulators to integrate cell divisions with differentiation.

Several lines of evidence indicate that cD1, cD2, and cD3 have unique roles during brain formation. In situ hybridization (ISH) reveals that cD1 and cD2 are differentially expressed during early embryogenesis and neurulation, forming distinct segmentally restricted expression patterns in hindbrain (Wianey and others 1998). Later in embryonic telencephalon, cD1 is the most widely expressed of the three with cD2 mRNA found in more restricted anatomical and temporal patterns in cerebellum and forebrain (Ross and others 1996), whereas cD3 is more important than cD2 to postnatal retinal development (Dyer and Cepko 2001).

Strikingly, total inactivation of cD2 during embryogenesis results in loss of approximately half of cerebellar granule neurons and virtually all stellate interneurons of the cerebellar molecular layer, while sparing basket and Golgi interneurons and Purkinje projection neurons (Huard and others 1999). This indicates cell subtype–specific requirements for cD2 in brain formation. One significant limitation to understanding the roles of cD2 and cD1 during brain development has been a lack of information regarding the differential expression of these proteins during brain histogenesis. We have devised a protocol for immunohistochemical staining of cD2 and cD1 using paraffin-embedded brain tissue sections. The present study maps the differential expression of cD2 and cD1 proteins during histogenesis of the neocortex, ganglionic eminences (GEs)/striatum, and hippocampal formation. The overlapping and unique features of cD1 and cD2 protein expression suggest that cD2 has a role in the late-stage of neurogenesis. Localization in the postnatal and adult brain is consistent with a proposed role of cD2 in the promotion of ongoing neurogenesis in the rostral migratory stream and in the mature hippocampal formation (Kowalczyk and others 2004), though cD1 may have a larger role than suggested by studies using neurospheres from adult brain.

Materials and Methods

Mouse Breeding

Mice were housed in Thorens units under climate-controlled conditions with a 12-h light/dark cycle. Heterozygous cD2+/− × cD2+/− or cD1+/− × cD1+/− breeding pairs were placed in mating cages at 5 PM and separated the next morning, designated embryonic day 0 (E0). Litters were harvested on the designated gestational, postnatal, or adult ages.

Genotyping

Tissues from embryonic or postnatal siblings were used to extract DNA according to the manufacturer’s protocol (Qiagen [Valencia, CA] DNeasy). For genotyping reactions, 100 ng of DNA was used with
oligonucleotide primers for wild-type (WT) sequences and null alleles as previously published (Sicinski and others 1995, 1996).

**Immunohistochemistry**

All postnatal brains were transcardially perfused with 4% paraformaldehyde. Embryonic brains were dissected free of the cranium and were drop fixed in 4% paraformaldehyde overnight at 4 °C, and tissue was processed (Tissue Tech 2000, 2.5-h protocol for embryonic brain) for paraffin embedding in the coronal plane. Postnatal pups and adult mice were transcardially perfused with 4% paraformaldehyde and processed (5- or 11-h protocol) for paraffin embedding in the coronal plane. Brains were sectioned at 6 μm (embryos and postnatal tissues) or 10 μm (adult brain), mounted on adhesive-coated capillary gap slides (Fisher Scientific, Pittsburgh, PA), and immunostained on a TechMate 500 semiautomated stainer after antigen retrieval by steam (30 min). Primary antibodies for immunohistochemistry (IHC) included anti-cyclin D2 (α-cD2) (1:1000, LabVision [Fremont, CA], mouse monoclonal Ab-4, #MS-221), anti-cyclin D1 (α-cD1) (1:5000, LabVision, rabbit polyclonal SP4, #RM-9104), anti-Ki67 (1:2000, Dako, Calpinteria, CA), and anti-BrdU (1:50, Amersham, Piscataway, NJ). Western blot analysis used Ab-4 and a rabbit antibody from Santa Cruz (Santa Cruz, CA) (#sc-593). Anatomical structures were indicated according to established nomenclature (Altman and Bayer 1990a, 1990b, 1990c; Schambra and others 1992).

**Dual Labeling**

Tissue sections (paraffin embedded, 6 μm) were deparaffinized and steamed for 30 min in Reveal (Biocare Medical) followed by 10 min cooling. Sections were pretreated with 3% H2O2 for 10 min, blocked in Sniper (Biocare Medical, Concord, CA) for 30 min, and then incubated with 2 primary antibodies at 4 °C. Antibodies were used at the following dilutions for dual-labeling immunofluorescence: α-D2 (Lab Vision) 1:500, α-D1 (Lab Vision) 1:500, anti-Olig2 (Gift of Dr JA Alberta) 1:1K, anti-Thr1 (Gift of Dr RF Hevner) 1:2K, and anti-BrdU (Amersham) 1:50. Following a 16-h incubation, sections were washed in phosphate-buffered saline and incubated in Alexa Fluor-conjugated secondary antibodies (1:500) for 1 h, washed, and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA).

**Results**

Cyclins D1 and D2 are the primary mid-G1 phase cyclins used in the developing brain (Ross and others 1996; Wianny and others 1998). A previous study used a digoxigenin-labeled antisense riboprobe to map cD2 expression during neurogenesis and observed transient and regionally restricted expression of this cyclin transcript in the telencephalon and cerebellum (Ross and others 1996). In the present study, cD2 protein expression was examined using the more sensitive immunodetection in the neocortex, GE/striatum, and hippocampal formation from E12.5 until postnatal day 60 (P60) to more precisely characterize the anatomic localization of this protein during forebrain development. The expression of cD1 immunoreactivity was also compared with cD2.

Because protein localization of cD1 and especially cD2 has been elusive in brain, it was important to establish the specificity of their immunolabeling using several criteria. Figure 1

**Figure 1.** Specificity controls for cD2 (α-cD2) and cD1 (α-cD1) antibodies in E13 tissues. (A) Though it is well represented in the lens, cD2-immunoreactive cells are confined to the anterior margin of the retina. (B) Western blot analysis of P6 cerebellar protein lysates shows a single cD2 band with either monoclonal (mAb) or polyclonal (rAb) antibody. (C) In contrast, α-cD1 labels the entire retina. (D) Western blot analysis of α-cD1 similarly identifies a single band of appropriate molecular weight. (E–P) Panels show α-cD2 immunoreactivity in cerebral cortex (E), GE (I), and hippocampal formation (M); cD2/– tissues provide a negative control (F, J, N); cD1-immunoreactive cells are shown in cortex (G), GE (K), and hippocampal formation (O), and no α-cD1 labeling is seen in the same regions of cD1 null brain (H, L, P). CTX, cerebral cortex; HP, hippocampus.
compares the staining patterns of anti-cyclin D2 (α-cD2) and anti-cyclin D1 (α-cD1) antibodies in retina and several brain regions at E13.5. Although both were well represented in the embryonic lens, cD1 was found throughout the retina, whereas cD2 was confined to the exit zone of optic nerve and the anterior margin of the retina (Fig. 1A-C). Interestingly, this position in the anterior retina is the location of progenitors in the adult retina (Tropepe and others 2000). Western blot analysis of the α-cD2 (Fig. 1B) and α-cD1 (Fig. 1D) antibodies used revealed single protein bands of the expected molecular weight. Samples of 3 brain regions revealed overlapping and nonoverlapping expression of cD2 and cD1 in cortex (CTX, Fig. 1E-G), GE, (Fig. 1I,K), and hippocampal formation (HP, Fig. 1M,O). In cortex (Fig. 1E,G), cD2 expression was highest at the top of the cortical ventricular zone (VZ) and in the subventricular zone (SVZ), whereas cD1 was seen throughout the cortical VZ. In the GE (Fig. 1I,K), cD2 was again highest in the SVZ, whereas cD1 predominated in the VZ. In the E13.5 hippocampal formation (Fig. 1M,O), cD2 and cD1 proteins were both expressed in the VZ. Importantly, α-cD2 did not label any cells in cD2 nulls (Fig. 1F,J,N), and α-cD1 failed to immunolabel cells in cD1–/– controls (Fig. 1H,L,P). Thus, the antibodies and immunostaining protocols used in this study were highly specific and capable of distinguishing the overlapping and nonoverlapping expression patterns of these 2 cyclins.

Expression of cD2 in the Developing and Adult Neocortex

Cortical neurons are produced from the proliferation of cells in the VZ that largely comprises the pseudostratified ventricular epithelium (PVE), a cell layer adjacent to the ventricular wall, and the SVZ or secondary precursor population (SPP), which borders the PVE (Takahashi and others 1994). For simplicity, these populations will be referred to as the VZ and SVZ. Whereas projection neurons and cortical stellate interneurons are generated from the VZ and SVZ of the neocortex, inhibitory interneurons are produced in the VZ/SVZ of the GIs and then migrate tangentially to enter the cortex. Specific events occurring in the VZ and SVZ may affect the rate of neurogenesis, neuronal differentiation, and consequently, regional patterning. Spatial and temporal differences in cD2 and cD1 expression could result in variations among progenitors in the length of G1-phase and cell cycle duration. This in turn could affect regional patterning within the forebrain by altering subset numbers and/or specification of neocortical projection and stellate interneurons.

The cD1 and cD2 protein expression patterns were examined at E12.5, E14.5, and E17.5 (Fig. 2). At E12.5, cD2 and cD1 immunoreactivities were primarily localized in the cortical VZ. Fewer nuclei were immunolabeled with cD2 antibody compared with cD1, and cD2-labeled cells were primarily distributed in the subventricular VZ removed from the ventricular surface (Fig. 2A). cD1 immunolabeling was robust in the VZ of WT and cD2 null mice (cD2–/–) (Fig. 2B,C), though there seemed to be fewer cD1-positive cells compared with cD2 in the outer VZ, away from the ventricular surface (Fig. 2B).

At E14.5, the numbers and density of cD1- and cD2-immunopositive cells appeared decreased from that observed at E12.5. cD2 immunolabeling was found in the emerging SVZ but is absent at the ventricular surface (Fig. 2D). Processes in the cortical plate that were reminiscent of glial fibers with end-feet at the pial surface in the cortical plate also immunolabeled for cD2 (Fig. 2A,D). cD1-immunoreactive cells were homogeneously distributed in the VZ and in contrast to cD2, at the ventricular surface in WT and cD2 nulls (Fig. 2E,F). No cD1-immunolabeled processes were observed, and, like cD2 nulls at E12.5, cD1 immunoreactivity at the ventricular wall of cD2–/– brains appeared decreased compared with WT.

Unlike earlier ages, VZ expression of cD2 was relatively higher than cD1 at E17.5, a time point when neurogenesis in the cortex is nearly complete (Fig. 2G,H) (Takahashi and others 1995). cD1 immunoreactivity was equivalent to cD2 in the dorsal VZ but substantially lower than cD2 in more medial regions (Fig. 2G,H brackets). Moreover, whereas cD1-labeled cells were found in the cortical plate (Fig. 2H), no cD2-positive cells were seen there (Fig. 2G). The number of cells immunolabeled with nuclear antigen Ki67, a marker expressed from the onset of S-phase through M-phase (Lopez and others 1991), suggested that many but not all cD1-positive cells were actively proliferative (Fig. 2I).
In order to examine whether some of the embryonic cD2-expressing cells were young neurons, E14.5 brain was dual labeled for fluorescence IHC with α-cD2 and α-Tbr1 antibodies (Supplementary Fig. 1). Tbr1 is a transcription factor that is turned on in early born neurons of layer 6 and preplate that have just exited the cell cycle (Hevner and others 2001). For the most part, cD2 and Tbr1 were expressed in distinct cells. However, rare cells were seen to label with both antibodies. Presumably those dual-expressing cells were in a transitional phase between cell cycle exit and neuronal differentiation.

In postnatal cortex, a few cD2-immunoreactive nuclei were found in the P1 cortical gray matter, whereas cD2 was readily found in processes in the superficial layers (Supplementary Fig. 2A,B). Only rare scattered cD2-positive cells could be found in the P7 cortex, and processes were no longer detected (Supplementary Fig. 2E). In contrast, cD1 immunolabeling was found in scattered cortical plate cells at P1 and became much more prominent between cell cycle exit and neuronal differentiation. Presumably those dual-expressing cells were in a transitional phase between cell cycle exit and neuronal differentiation.

Expression of cD2 in the GEs and Striatum

In the embryonic brain, the GE is a transient structure that generates interneurons that migrate to final positions throughout the forebrain (Anderson and others 1997). The lateral portion of the GE is also the incipient corpus striatum and accordingly produces striatal neurons. Rostrally, the GE is divided into the medial ganglionic eminence (MGE) and lateral ganglionic eminence (LGE), which are 2 separate evaginations of the lateral wall of the lateral ventricles. Caudally, only a single swelling, the caudal ganglionic eminence (CGE), is present. Within the GE, the size and spatial distribution of nuclei, the length of the cell cycle, and the pattern of interkinetic nuclear migration vary across the dorsoventral and rostrocaudal axes (Bhide 1996; Sheth and Bhide 1997). Furthermore, more recent studies suggest that separate pools of ganglionic progenitors produce distinct subclasses of cortical interneurons (Xu and others 2001). As in the neocortex, the GE consists of 2 partially overlapping progenitor populations, the VZ (also known as PVE) and the SVZ (a.k.a., SPP) (Bhide 1996), and different events occurring in the VZ and SVZ may affect regional patterning within the striatum and the specification of striatal neurons and cortical interneurons.

The expression of cD1 and cD2 immunoreactivities was examined in the MGE and LGE (Fig. 3). At E12.5, cD2 immunoreactivity was prevalent in the SVZ and nonhomogeneously present in nuclei scattered in the VZ (Fig. 3A) of both the MGE and LGE. In the VZ, cD2 expression was lowest in the ventral LGE and in the dorsal MGE. cD1 immunolabeling was robust throughout the VZ of both the MGE and LGE and also labeled some nuclei and processes in the SVZ of WT (Fig. 3B) and cD2 null embryos (Fig. 3C). Similarly, at E14.5, the expression of cD2 (Fig. 3D) was robust in the SVZ, scattered in the VZ, and lowest in tissue bordering the interganglionic sulcus. The expression pattern of cD1-immunolabeled nuclei at E14.5 in WT (Fig. 3E) and cD2 nulls (Fig. 3F) also resembled that observed at E12.5, that is, robust and uniform in both the MGE and LGE VZs, and labeled processes were apparent, especially in the LGE SVZ. Scattered cD1-immunolabeled nuclei were also found in the SVZ, though far fewer than were labeled by cD2 antibody (compare Fig. 3D and 3E). Dual fluorescence immunolabeling was used to determine whether cD2 and cD1 expression overlapped in the E14.5 MGE (Fig. 3G–L). In the SVZ, most of the progenitors expressed either cD1 (green) or cD2 (red) alone. However, a few VZ cells expressed both cyclins (yellow).

The nature of the cD2 expression in the E12.5 MGE was further examined by dual labeling (Supplementary Fig. 4). After a 60-min bromo-deoxyuridine (BrdU) (50 mg/kg) pulse, sections were immunostained for the S-phase marker and either α-cD2 or α-cD1. This confirmed that the majority of cD2-expressing cells were in the SVZ (Supplementary Fig. 4D–F). Interestingly, although many cD1-expressing progenitors were double labeled for the cyclin and BrdU (Supplementary Fig. 4G–I), only a few cD2 cells were dual labeled, suggesting that cD1-expressing cells moved more rapidly into the S-phase than did cD2-positive progenitors. Following a 16-h BrdU pulse, nearly all MGE progenitors were positive for both BrdU and either cD1 or cD2, indicating that the cyclin-positive cells were proliferating over the 16-h post injection (not shown). The MGE was examined by dual labeling of Olig2 and cD2 antigens at E12.5 and E14.5 (Supplementary Fig. 4J–O). Olig2 is expressed sequentially in neuron precursors and then in oligodendrocyte progenitors (Lu and others 2000; Kessaris and others 2001; Jakovcevski and Zecevic 2005). Nearly all MGE VZ cells were labeled with α-Olig2, and so the antigen must be expressed early in cells giving rise to both neurons and glia. Interestingly, Olig2 and cD2 colocalized in cells within the MGE VZ, whereas cells in the SVZ expressed only Olig2 or cD2 alone, suggesting that cD2+ progenitors diverged from the Olig2+ lineage as they entered the SVZ.

The expression of cD2 in the CGE (Supplementary Fig. 5) was absent in the medial region ( bracket in Supplementary Fig. 5A) and localized mainly in the VZ/SVZ in the lateral CGE at E12.5. However, cD1 was homogeneously distributed in the VZ and also labeled scattered neurons in the SVZ (Supplementary Fig. 5B). Both cD1 and cD2 labeled processes in the lateral CGE. At E14.5, cD2 immunolabeling was still reduced in the central CGE but was visible in the most medial VZ (Supplementary Fig. 5C). cD2 immunoreactivity was greatest in the lateral CGE, and labeled neurons were found in the VZ and SVZ. cD1-immunoreactive nuclei were evenly packed in the VZ, and scattered cD1-immunopositive cells were found in the SVZ (Supplementary Fig. 5D). As at E12.5, processes immunoreactive for both cD2 and cD1 were found in the lateral CGE (arrowheads).

Timed with the final neurogenic divisions around E19, cD2 immunoreactivity appeared to predominate in the striatal VZ (Fig. 4A) compared with cD1-immunoreactive cells (Fig. 4B). Labeled nuclei were distributed in partially overlapping and also complementary zones, so that cD2-immunolabeled cells were pale or absent at the ventricular surface, and labeling...
was greater in the neighboring SVZ (Fig. 4A,C), whereas cD1-immunoreactive nuclei were located immediately adjacent to the ventricular wall (Fig. 4B,D). Both cD1- and cD2-immunoreactive neurons were scattered in the striatum; however, cD1 immunoreactivity identified the greater numbers of cells and also striatal processes in the caudato-putamen.

In the postnatal striatum, cD2-immunolabeled nuclei were abundant along the ventricular wall and most robust at the corticostriatal junction (Supplementary Fig. 6A). Immunolabeling for cD1 was present in the strip of tissue immediately adjacent to the ventricle and in scattered neurons in the striatum (Supplementary Fig. 6B). At P7, the VZ/SVZ expression of cD2 and cD1 proteins (Supplementary Fig. 6C,D) decreased but remained complementary, and cD2 expression was still predominant. In the adult striatum, cD2-immunoreactive nuclei were infrequently observed at the ventricular surface, whereas cD1-immunopositive cells were scattered in the corpus striatum (not shown).

Expression of cD2 in the Hippocampal and Dentate Neuroepithelium and Mature Hippocampus

The hippocampal primordium is identifiable by E12.5 as a swelling of the VZ on the medial wall of the lateral ventricle. This region subsequently differentiates into distinct subicular, ammonic (precursor to CA1, 2, and 3), and dentate neuroepithelial zones. Only a small number of cD2-immunoreactive neurons were present in the ammonic VZ at E12.5 (Fig. 5A). More cD1-immunoreactive cells were found in the same cortical...
hemisphere region of the ammonic neuroepithelium (Fig. 5B). No immunolabeled cells were observed in the marginal zone (MZ) or in the region just rostral (*) to the fimbrial glioepithelium. This latter unlabeled region overlaps the presumptive dentate neuroepithelium, a region in which neurogenesis lags relative to the ammonic and subicular hippocampal epithelia. The numbers and distribution pattern of cD1-immunoreactive cells in cD2 null mice were not different from WT at this age (Fig. 5C). Thus, cD1, but not cD2, is employed for the earliest cell divisions in the hippocampal primordia.

At E14.5, cD2 immunoreactivity was higher than at E12.5 (Fig. 5D). At that age, cD2-immunoreactive cells were scattered in the VZs of both ammonic and dentate neuroepithelia including the cortical hemisphere, delimited by arrowheads (Altman and Bayer 1990a,b; Grove and Tole 1999). cD1 immunoreactivity was also observed in the ammonic VZ but not in the cortical hemisphere of WT mice (between arrowheads) (Fig. 5E). Only a few cD1-immunoreactive neurons occupied the ammonic MZ. In mice lacking cD2, cD1 immunoreactivity in the ammonic neuroepithelium had similar distribution patterns, but cD1 was now apparent in the hemisphere in contrast to WT (Fig. 5F).

At E14.5, cD2 was preferentially used by the cortical hemisphere for which cD1 served as proxy in cD2 nulls. The more abundant expression of cD2 in the E14.5 ammonic neuroepithelium also implicated a prominent role for this cyclin in the later neurogenesis of hippocampal pyramidal neurons.

By E17.5, the hippocampal formation proper has nearly completed neurogenesis, and the migratory streams (secondary dentate matrix [dms] of Altman and Bayer [1990a,b,c]) from the primary dentate matrix are visible. At this age, the dentate gyrus anlage (tertiary dentate matrix of Altman and Bayer [1990a,b,c]) consists of a loose collection of proliferating neuroblasts. At E17.5, both cyclins D were observed in the VZ, though cD1-immunoreactive cells predominated in the secondary and tertiary matrices. cD2 immunoreactivity was found in a more modest number of cells in the dentate migratory stream and in the dentate anlage but was more prominent in the hippocampal fissure (hf), where immunolabeling appeared punctate, perhaps representing fibers in the region (Supplementary Fig. 7). cD1 and cD2 immunolabeling in the hippocampal formation at E19 (Fig. 6) had a similar pattern to that observed at E17.5 with 2 notable changes. cD1 immunolabeling was dramatically reduced in the VZ and in the dms, in both the septal (Fig. 6B) and temporal (Fig. 6E) poles. Furthermore, the migrating neuroblasts of the dms were now primarily cD2 immunoreactive (Fig. 6C,D), though more robust cD1 immunolabeling persisted in the tertiary dentate matrix (Fig. 6B,E). The hf still displayed strong cD2 immunolabeling (Fig. 6D). Therefore, hippocampal neuroblasts in the VZ and dms primarily used cD2 at later stages of hippocampal development.

The rate of granule cell neurogenesis peaks at the end of the first postnatal week. The expression of cD1 and cD2 in the hippocampal formation at P7 (Fig. 7) was restricted to the
tertiary dentate matrix and subgranular zone (SGZ) and a few scattered immunopositive nuclei in the hippocampus. The number of cD2-immunolabeled hilar cells (Fig. 7A,C) was increased compared with that seen at E19 (Fig. 6) and P1 (not shown). The numbers of cD1- (Fig. 7B,D) and cD2-immunolabeled cells were equivalent, but their anatomic distribution differed. Whereas cD2-immunolabeled cells filled the entire hilar region (tertiary dentate matrix), cD1-immunoreactive cells were more restricted to the SGZ (Fig. 7B). The immunolabeling for cD2 in cD1 null mice, and vice versa, resembled WT (Fig. 7E,F). This pattern suggested that cD2 may be utilized for the final division of neuroblasts in the tertiary dentate matrix.

In the adult hippocampal formation (Fig. 8), cD2 was largely confined to the SGZ of the dentate gyrus (Fig. 8A), and no α-cD2 immunoreactivity was seen in the dentate of cD2−/− hippocampus (Fig. 8B, hematoxylin counterstained), whereas an increased number of SGZ cells were cD2 positive in the cD1−/− hippocampal formation (Fig. 8C). In contrast, cD1-immunoreactive cells were scattered throughout the hippocampal formation (Fig. 8D). In WT hippocampus, cD1 protein was also expressed in the SGZ (Fig. 8D,G), and α-cD1 immunolabeling did not significantly increase in the hippocampal formation in mice lacking cD2 (cD2−/−) (Fig. 8E,H), including in the SGZ (Fig. 8G,H). Interestingly, postmitotic pyramidal neurons of CA1 were heavily immunolabeled with α-cD1 (Fig. 8D,E) but not α-cD2 (Fig. 8F) antibody, indicating that cD1 alone was expressed in these CA1 neurons. This α-cD1 labeling appeared to be specific because these cells were not detected with α-cD1 antibody in cD1 null tissue (Fig. 8F). At least some of the cD1- (Fig. 8J) and cD2- (Fig. 8K) immunoreactive cells found in the SGZ were proliferating, as they double labeled with the cyclin and anti-BrdU after a 3-h pulse with BrdU (50 mg/kg), and so were likely to be adult neural progenitors.

**Discussion**

Examination of the temporal and anatomical expression of these D type cyclins in developing brain is a necessary prelude to functional investigations at the molecular level. The expression of cyclins D is tightly regulated not only at the transcriptional level but also at the translational level by their highly conserved 3′ untranslated region sequences and at the post-translational level by protein degradation through ubiquitin pathways (Diehl and others 1998; Pines 1999). Previous reports analyzed only cD2 and/or cD1 mRNA expression during brain development (Ross and others 1996; Sicinski and others 1996; Wianny and others 1998). However, several studies have shown that D cyclin proteins are translationally and posttranslationally regulated so that mRNA levels may not always reflect the peak protein levels (Pines 1999). In forebrain, previous ISH studies using a relatively insensitive digoxigenin system detected little or no cD2 mRNA in the VZ of embryonic neocortex or GE (Ross and others 1996), whereas low levels of cD2 mRNA were detected there by 5′ labeled probes (Sicinski and others 1996). However, the present study reveals that cD2 protein is immunolabeled in cortex, GE, and hippocampal formation. Therefore, it is crucial to establish expression profiles of cD2 and cD1 proteins to understand their relative roles in brain development.
The observed progression of cD1 to cD2 expression in neuroprogenitors as the brain develops is compatible with a competence model of cell fate specification in which the potential of progenitors becomes progressively restricted with developmental age (Cepko and others 1996; Frantz and McConnell 1996). The cD1 and cD2 localization presented here suggests that shifts in components that promote cell cycle progression work along with extrinsic cues to orchestrate histogenesis. External differentiation cues that also regulate progenitor proliferation in forebrain include n-myc and Shh, BMPs, Wnts, and Notch (reviewed in Grove and Tole 1999; Ragsdale and Grove 2001; Donovan and Dyer 2005; Li and Pleasure 2005). These effectors could conceivably have molecular preferences for action on cD2 or cD1. In this regard, it is of interest that cD2 was selectively expressed in the cortical hem, a zone that is particularly rich in Wnt and BMP expression (Shimogori and others 2004).

The temporal pattern suggested that neural progenitors in neocortex, hippocampus, and GE rely on cD1 or cD2, with a tendency to become cD2 “dependent” as progenitors make their late-stage divisions. In the neocortex, cD1 predominated in the VZ at E12.5-E14.5, but cD1 immunolabeling was progressively reduced in the VZ from E17.5 onward. In contrast, cD2 was localized selectively to the SVZ at all embryonic time points in the neocortex and GE, and the numbers of cells labeled with cD2 appeared to increase during late cortical neurogenesis (E17.5), at a time when numbers of cD1-immunoreactive cells were declining. Subsequently, cD1 and cD2 immunoreactivities were localized in the white matter/SVZ and the rostral migratory stream in the postnatal brain. cD2 expression waned in the neocortical layers during the first postnatal week and was transiently localized to fiber processes or scattered neurons in the superficial cortical plate at P1 and P7, respectively.

The D cyclin expression in some fiber processes is interesting and suggests that these proteins can be localized either to the cytoplasm or to the nucleus, according to the division cycle status of the cell. There is precedent for this both in yeast and in mammalian cells. In *Saccharomyces cerevisiae*, G1 cyclins cln2 and cln3 shuttle between the cytoplasm and nucleus (Edgington and Futcher 2001). Several cln2 functions require cytoplasmic localization (e.g., polarization needed for budding), whereas other functions require transport into the nucleus. Similarly, in mammalian cells cyclin A- and cyclin E-cdk complexes have been shown to shuttle between the cytoplasm and nucleus (Jackman and others 2002). It is possible that cyclins D have similar functional requirements for dynamic subcellular localization.

In the GE, cD2 expression was predominant in the SVZ and in the medial VZ of the MGE and the dorsal VZ of the LGE at E12.5 but spared cells in the medial region of the CGE. This pattern of VZ expression may be important for the regional specification of interneuron subtypes. Consistent with this hypothesis, costaining in the E12.5 and E14.5 MGE showed that most progenitors expressed either cD1 or cD2 alone,
although a few cells in the VZ expressed both cyclins. Moreover, the expression of Olig2 in cD2+ cells of the MGE VZ but not in cD2+ cells once they were in the SVZ further suggested that cD1/cD2 expression is regulated as progenitors become progressively determined. From E14.5 until E17.5, cD2 became increasingly restricted to the ganglionic SVZ. cD1 was robust in the VZ at all stages of development examined, though the numbers of cD1-immunolabeled cells in the VZ decreased from E14.5 onward, and scattered striatal cD1-immunolabeled cells (possibly mature neurons) became progressively apparent. At later embryonic and early postnatal ages, cD2 was the predominant cyclin D expressed in germinal cells along the striatal ventricular wall.

Although cD1 predominated in the VZ of the early hippocampal primordium, by E14, cD1 and cD2 were roughly equally represented in the ammonic VZ, while the cortical hemisphere was dependent on cD2. Later, around E17 and E19, the expression of both cyclins was reduced in the ammonic VZ, where neurogenesis has or will soon be completed. Although both cyclins were expressed in the developing dentate gyrus, cD2 was more heavily expressed than cD1 in the hilus and the dentate gyrus. By E17 and continuing into the early postnatal period, cD2 was more robustly expressed than cD1 in the hippocampal VZ, whereas more cD1-immunoreactive cells were found in the secondary and tertiary dentate matrices at E19 and P1. Examined at P7, both cD2 and cD1 were seen in scattered cells around the hippocampal pyramidal layer. However, in the dentate gyrus, cD1-expressing cells became progressively restricted to the margins of the SGZ, and cD2-immunolabeled cells were concentrated in the central hilar region (tertiary dentate matrix) of the SGZ. This segregation of cD1- and cD2-immunoreactive cells in the dentate gyrus was more evident by P10 (not shown).

The adult forebrain displayed several unique features with regard to cyclin expression. First, cD1 was found in scattered cells throughout the cortex, hippocampus, and striatum, presumably among glial elements that continue to turnover throughout life. Surprisingly, cD1 was also expressed in subsets of undoubtedly mature, postmitotic neurons such as in the CA1 hippocampal field and pyramidal neuron nuclei in layers 3 and 5 of the neocortex. The fact that no α-cD1 labeling was seen in the cD1−/− brain indicated that this immunostaining was specific to cD1. It is unlikely that the cyclin supports a cell cycle function in these postmitotic cells. Isolated upregulation of cD1 (or cD1, cB1, and proliferating cell nuclear antigen) has been associated with neuronal apoptosis in other mature systems like dorsal root ganglia and aged adult human hippocampal

Figure 8. cD2 and cD1 protein expression in the hippocampus at P60. (A, B, C) cD2-immunoreactive cells are confined to the SGZ of WT dentate gyrus (A) It is absent in cD2 nulls (B, counterstained with hematoxylin) and is increased in cD1−/− dentate (C). (D, E, F) cD1-immunolabeled cells are scattered throughout the adult hippocampal formation, as seen in WT (D) and cD2−/− tissue. Postmitotic pyramidal neurons of the CA1 region are heavily labeled with α-cD1 antibody in both WT (D) and cD2 null hippocampal formation (E, counterstained with hematoxylin) but are absent in cD1 null hippocampus (F). (G, H) show higher magnification of α-cD1 immunolabeling in dentate gyrus, where cD1 is in cells scattered in all layers of WT (G) and cD2−/− (H) counterstained with hematoxylin) brains. Dashed line in (G) delimits the SGZ. Arrows point to cells located appropriately for dentate neuroprogenitors. (J) Low magnification of WT hippocampus shows that α-cD2 does not immunolabel CA1 pyramidal neurons. Arrows point to cD2-positive cells in dentate (dentate gyrus) and CA3 regions. (J, K) Colocalization of cD1 (J, green) or cD2 (K, green) and BrdU (red) in dentate gyrus of an adult WT following a 3-h BrdU pulse. (J, K) Hematoxylin stain of the P60 temporal hippocampus.
formation (Freeman and others 1994; Yang and others 2003). However, because the CA1 labeling seen here was extensive and was found in normal, early adult brain, cD1 expression there would be less likely to be proapoptotic.

Another intriguing aspect of cyclin expression was the presence of cD1 both in presumably postmitotic neurons and in the region of adult neurogenesis in dentate gyrus and the rostral migratory stream. A recent study has suggested that adult neurogenesis does not occur in mice lacking cD2 (Kowalczyk and others 2004). In their experiments, neurospheres derived from adult hippocampal formation expressed cD2 mRNA by reverse transcription–polymerase chain reaction but not cD1 message. However, that study did not examine cD1 immuno- histochemical labeling (Kowalczyk and others 2004). Data presented here indicate that cD1 is expressed in adult hippocampal formation, including the dentate gyrus, in both WT and cD2−/− brain. Although the cD1-immunolabeled cells in the dentate could be postmitotic, colocalization in some cD1-immunonegative SGZ cells with BrdU following a 3-h pulse suggests that these are indeed proliferating cells. These seemingly disparate observations could result from selection conditions of the neurospheres that could create effects not found in vivo (Reynolds and Rietze 2005), whereas the cells immuno- labeled in the adult dentate gyrus may be proliferating astroglia and not true neuroprogenitors. Nevertheless, the present observed cD1 protein expression suggests that further investigation of the relative roles of cD1 and cD2 in adult olfactory bulb and hippocampal neurogenesis is warranted.

The picture that arises from this study is that cD2 and cD1 are differentially used by neural progenitors and this could contribute to the specification of neuronal subsets. Detailed information regarding the identities of cell subsets expressing cD2 and cD1 awaits further investigation. However, localization reported here suggests that cD2 is most often expressed in late-stage progenitor divisions, and expression, if any, in post- mitotic cells is transient. This is evidenced by 1) the more numerous cD2+ cells in the later dividing SVZ pool compared with VZ of the GE and later embryonic forebrain germinal epithelium, 2) the presence of only rare dual-labeled cD2+ Tbr1+ neurons in the E14.5 cortex, and 3) virtual absence of cD2-labeled cells in the neocortical gray matter posterior. In contrast, cD1 appears to be expressed in certain postmitotic neurons, demonstrated by cD1 immunolabeling in CA1 pyramidal neurons and by adult brain cD1 expression in many cortical cells, some with large nuclei and morphology of pyramidal neurons. Although this postmitotic expression of cD1 has been recognized in aging brain (Yang and others 2003), the present study suggests that this expression is not necessarily pathologic. Of particular interest are the adult neurogenesis niches of the dentate nuclear SGZ and forebrain SVZ that appear to differentially use cD2 and cD1. A basis for dissecting molecular mechanisms in progenitor subpools served by cD2 and cD1 is now established.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

Notes
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