Cerebellar histogenesis is disturbed in mice lacking cyclin D2

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SUMMARY

Formation of brain requires deftly balancing primary genesis of neurons and glia, detection of when sufficient cells of each type have been produced, shutdown of proliferation and removal of excess cells. The region and cell type-specific expression of cell cycle regulatory proteins, such as demonstrated for cyclin D2, may contribute to these processes. If so, regional brain development should be affected by alteration of cyclin expression. To test this hypothesis, the representation of specific cell types was examined in the cerebellum of animals lacking cyclin D2. The loss of this cyclin primarily affected two neuronal populations: granule cell number was reduced and stellate interneurons were nearly absent. Differences between null and wild-type siblings were obvious by the second postnatal week. Decreases in granule cell number arose from both reduction in primary neurogenesis and increase in apoptosis of cells that fail to differentiate. The dearth of stellate cells in the molecular layer indicates that emergence of this subpopulation requires cyclin D2 expression. Surprisingly, Golgi and basket interneurons, thought to originate from the same precursor pool as stellate cells, appear unaffected. These results suggest that cyclin D2 is required in cerebellum not only for proliferation of the granule cell precursors but also for proper differentiation of granule and stellate interneurons.

Key words: Cerebellum, Cell cycle, Cyclin D2, Apoptosis, Brain, Mouse

INTRODUCTION

The control of cell proliferation is a fundamental underpinning of brain development. Formation of brain must integrate three processes: primary genesis of neurons and glia, signaling from differentiated cells that enough neurons or glia have been made, and the disposal of excess cells in a manner that is not injurious to the organism. Observations in both vertebrate and invertebrate systems indicate a role for cell cycle regulation in developmental patterning that likely involves cellular responses to both intrinsic programming and environmental cues (reviewed in Edgar and Lehner, 1996; Ross, 1996; Follette and O’Farrell, 1997). Key cell division regulators include the cyclins, which are activating subunits of specific cyclin-dependent kinases (cdks). Cyclins A, B1 and B2 control the advance through G2; cyclins D1, D2 and D3 control progression through the mid G1 restriction point; and cyclin E regulates the G1/S transition. The redundancy of D and B cyclins has long been assumed to protect the cell against loss of one form. However, this notion of molecular ‘safeguarding’ is probably simplistic since the expression patterns of the cyclins do not entirely overlap. We previously reported that cyclin D2 expression during brain development is anatomically restricted to particular brain regions such as cerebellum (Ross et al., 1996). Furthermore, this cyclin is most heavily expressed either in neural precursors making their final divisions or just after exiting the cell cycle as they differentiate to assume neuronal morphology. This implies that proteins known to control progression through the cell cycle may have an additional role in regional patterning and differentiation of selected neural cells.

The role of cyclin D2 in brain development was investigated using homologous recombinant mice (Sicinski et al., 1996). We reasoned that brain development might be selectively altered since cyclin D2 is regionally expressed in brain (Ross et al., 1996). Although both cerebral and cerebellar cortex are likely to be affected in cyclin D2 nulls, initial studies have focused on cerebellum, where a complete analysis of cell classes and their connections is more readily obtained. In the present study, neuronal populations have been examined in greatest detail.

Cerebellar cortex is composed of three layers comprising 5 neuronal and 3 glial cell types: Purkinje neurons, granule, stellate, basket and Golgi interneurons, and glia, of which radial glia are the most prominent. Cerebellar granule cells are by far the most abundant of any neuronal type in the CNS and occupy the adult internal granular layer (IGL). Golgi interneurons also reside in the IGL. Purkinje cell bodies form a monolayer between the IGL and molecular layer and are the only neurons that project out of the cerebellar cortex. The
molecular layer is made of dendritic arbors of the Purkinje cells, parallel fibers of granule cells, radially oriented glial fibers and the stellate and basket interneurons. In addition, a few astrocytes and oligodendroglia are found scattered in the molecular layer. Its relative simplicity makes cerebellar cortex an excellent subject for structural analyses.

The primary events in cerebellar patterning are also well known. Granule cells originate in a region of the ventricular zone (vz) termed the rhombic lip, starting around E11, and migrate to cover the surface of the cerebellar anlage where they form a secondary germinal zone called the external germinal layer (EGL). In the postnatal EGL, precursors make their final divisions, exit the cycle and migrate inward to form the IGL. In contrast, precursors of the cerebellar vz give rise to the deep cerebellar nuclei and Purkinje cells (reviewed in Altman and Bayer, 1997). The remaining interneurons – stellate, Golgi and basket cells – were demonstrated in recent lineage tracing studies to derive postnatally from precursors near the IVth ventricle that migrate through the white matter to the cerebellar cortex (Hallonet et al., 1990; Zhang and Goldman, 1996).

The present study tested the hypothesis that cyclin D2 participates in patterning of cerebellum. Loss of cyclin D2 expression affected two populations in adult cerebellum, manifested by reduced granule cell numbers and nearly absent stellate interneurons. Differences between null and wild-type siblings were obvious by the second postnatal week and arose not only from defects in primary neurogenesis but also from increased programmed cell death within the EGL. Moreover, the death of stellate cells in the postnatal molecular layer occurred despite preserved representation of basket and Golgi cells, all three of which are thought to derive from a common precursor pool (Hallonet et al., 1990; Zhang and Goldman, 1996). These data suggest that emergence of a significant proportion of granule neurons and the stellate subpopulation requires cyclin D2 expression.

**MATERIALS AND METHODS**

**Animal breeding and genotyping**

Since homozygous cyclin D2 null mice are infertile, the line was maintained in heterozygotes on a C57Bl/6J background. Mice lacking cyclin D2 gene function were obtained from crosses of cyclin D2+/- lacking cyclin D2 gene function were obtained from crosses of cyclin D2+/- males and D2 +/+ females. The day of birth was designated postnatal day 0 (P0). Tailsnips were collected for DNA extraction and PCR-based genotyping using standard methods. Primers used for genotyping were: (1) genomic cyclin D2, (5'GCT GGC CTC CAA TTC TAA TC-3'), (2) wild-type-specific, (5'-CCA GAT TGC AGC TGC TTG 3'), (3) knockout-specific, (5'CTA GTG AGA CGT GCT ACT TC-3'). Typical thermocycle parameters are: 94°C x5 minutes, then 94°C x1 minutes, 66°C x1 minutes, 72°C x6 minutes for 30 cycles, and finally extension @ 73°C x15 minutes.

**In situ hybridization**

The expression of cyclin D2 mRNA in developing cerebellum was examined by in situ hybridization using digoxigenin-labeled cRNA probe transcribed in vitro from a fragment taken from the 3' UTR as previously described (Ross et al., 1996). Wild-type animals were collected at E12.5, 14.5 and P6.

**Histology**

The gross appearance and histological organization of cerebellar cortex in mice lacking cyclin D2 was compared with wild-type littermate controls on P3 (n=6), P14 (n=6) and adult animals (P60 or older, n=5). Animals were deeply anesthetized with i.p. nembutal and transcardially perfused with 4% paraformaldehyde. Brains were dissected from the skull and wild-type, and cyclin D2 null specimens were examined side by side under a dissecting stereomicroscope (Stemi 1000, Zeiss). Brains were cut into sagittal or coronal blocks for overnight postfixation followed by paraffin embedding.

Sections of 4 μm each were cut and mounted on Vectabond™ coated glass slides, paraffin was removed and tissue processed for histochemical staining with cresyl violet or hematoxylin and eosin (H and E). Alternatively, sections were processed for immunohistochemical localization of antigens specific for cell populations present in cerebellum: calbindin for Purkinje cells, phosphorylated neurofilament (NF200) for fibers of basket interneurons (Riederer et al., 1996), the cell surface glycoprotein Human Natural Killer antigen-1 (HNK-1) for Golgi interneurons (Eisenman and Hawkes, 1993), and Glial Fibrillary Acidic Protein (GFAP) for fibers of astrocytic and Bergmann type glia. Anti-calbindin antibody (Sigma) was used at a dilution of 1:10,000. Anti-NF200 antibody (Sigma) was used at a dilution of 1:2,000. Anti-HNK-1 hybridoma supernatant (ATCC) was used at a dilution of 1:50. Anti-GFAP antibody (Boehringer) was used at a dilution of 1:10,000. Following an overnight incubation with primary antibody, immunohistochemical localization was carried out using the appropriate secondary antibody according to manufacturer's recommendations (avidin-biotin detection kit, DAKO). Selected sections were counterstained to demonstrate histological localization and to identify granule neurons.

Sections were examined on a compound microscope (Optiphot, Nikon) fitted with DIC objectives. Care was taken to ensure that sections compared between D2−/− and D2+/+ siblings were appropriately matched. Area measurements and cell counts were obtained from 4 μm thick sagittal sections taken from within 2 mm on either side of the midline and the corresponding sections were matched with respect to the identity of the lobules present. The area of the IGL or of the underlying white matter were compared by first tracing the boundaries of these regions on camera-captured sections and areas were computed using the NIH Image software program (Scion Image, 1.62, public domain). That the compared sections were appropriately matched was supported by the fact that the area of underlying white matter (which lacks any contribution from granule cell processes) was identical in null and wild-type siblings. Cells were counted either from the entire section (when populations were small, as for Golgi interneurons) or along a length from three identical folia when cell numbers were larger (Purkinje cell layer, molecular layer, EGL cells). Photomicrographic images were captured using either a CCD video camera (Nikon FX 350x) or digital spot camera (Diagnostic Instruments Inc.) and transferred into Adobe Photoshop™ for production of figures. Confocal images were obtained using an Olympus AX-70 microscope with Biorad MRC 1024 laserhead.

**BrdU labeling**

In order to examine the fraction of cells in the cerebellar EGL that are actively proliferating within 1 hour, littermates were injected (95 mg/kg, i.p.) with the thymidine analog 5'-bromo-2'-deoxyuridine (BrdU), which was incorporated into nuclei of cells in or entering S-phase during the pulse period. Animals were anesthetized 1 hour post BrdU injection and immediately perfused transcardially with 4% paraformaldehyde. Fixed brain tissues were paraffin embedded for sagittal sectioning at 4 μm. Incorporated BrdU was immunohistochemically detected in cells using a mouse monoclonal anti-BrdU antibody (Amersham) at 1:10,000 dilution. Tissue sections were lightly counterstained with hematoxylin to facilitate cell counting. Using sections taken at the same anatomic levels, the proliferative index was determined for three animals of each age and
genotype, as the fraction of BrdU-positive cells relative to the total number of cells per length of EGL over 4 separate sectors from identical folia of D2+/+ and D2−/− sibs and reported as a mean±s.e.m (%).

**Apoptosis assay**

The relative abundance of cells undergoing programmed cell death was compared between cyclin D2−/− and D2+/+ littermates at postnatal ages P3, P7 and P14. Cells undergoing apoptosis were identified by digoxigenin-labeling of free-3'OH ends of fragmented DNA by terminal deoxynucleotidyl transferase (TdT) (TUNEL assay, ApopTag™ Plus, Oncor). Assays were performed on sections from the same tissue blocks from which sections for BrdU immunohistochemistry were obtained. In situ detection of apoptotic cells was evaluated by fluorescence confocal microscopy using FITC filters (excitation peak 488 nm, emission peak 522 nm). Tissues were counterstained with propidium iodide and detected using a Texas Red filter (excitation peak 568 nm, emission peak 605 nm). The fraction

![Fig. 1. Gross and low-power views of cyclin D2−/− versus D2+/+ littermate brain. One sibling pair is shown. (A) Brains from adult female wild-type (left) and D2 null (right) animals. The mouse lacking cyclin D2 is only 10% smaller in body weight than its D2+/+ sibling (29.6 gm D2+/+ versus 26.4 gm D2−/−). However, the D2−/− cerebral cortex and cerebellum are strikingly smaller, so that the colliculi are more exposed (arrow). (B,C) Hematoxylin- and eosin-stained parasagittal sections through cerebellum of cyclin D2+/+ (B) and D2−/− (C) sibs. D2−/− folia are stunted with shallow interfolial clefts particularly in central (c) and posterior (p) lobes. Arrow shows the internal granular layer (IGL). (D) Area of the IGL and white matter (WM), compared between cyclin D2−/− and D2+/+ siblings. Areas were measured in 4 µm sections at the same anatomic levels in 3 siblings using NIH Image and expressed in pixels (×10^4) (n=6, *P<0.001).

![Fig. 2. Histological appearance of cyclin D2−/− versus D2+/+ littermate cerebellum. (A,B) The hematoxylin and eosin stains reveal a striking reduction in cell number within the molecular layer (ML) of the cyclin D2 null (B) compared to its wild-type sibling (A). (C,D) Calbindin immunohistochemical labeling of Purkinje neurons (brown) reveals relatively normal dendritic arborization in D2+/+ (D) compared to D2+/+ (C) animals despite the reduced cellularity of the ML. Hematoxylin counterstain (blue) reveals the relative preservation of presumed basket interneurons (arrowheads) and absent presumptive stellate cells (arrows in C). (E,F) Calbindin labeling at postnatal day 3 cerebellum shows similarity of neonatal D2−/− (F) and D2+/+ (E) Purkinje neurons. (G) Comparison of at postnatal day 3 Purkinje cell numbers per length of the PCL (n=5, P>0.1) and the crosssectional area (n=6, P>0.1) of the cerebellum in 2 sibling pairs. Area measurements were made in matched sections from the same anatomic level and expressed in pixels (×10^3). PCL, Purkinje cell layer; IGL, internal granular layer. Magnification, ×20.
of cells undergoing apoptosis in the EGL was estimated as the number of ApopTag-positive cells per total number of cells, counted manually in sectors along the length of the EGL, again taken from the corresponding folia in D2−/− and D2+/+ littermates. Data are reported as a mean±s.e.m (%).

RESULTS

Lack of cyclin D2 results in loss of granule cells and stellate interneurons

The appearance and organization of adult cerebellum in mice lacking cyclin D2 expression was compared with gender- and age-matched littermates. The cerebella of cyclin D2−/− animals were significantly smaller than sibling controls (Fig. 1). Folia were foreshortened or stunted compared to the wild-type littermate (Fig. 1B,C). The abundance of granule cells in the internal granular layer (IGL) rendered accurate cell counting impractical. Therefore, as a first approximation of the comparative size of adult granule cell populations, the area of the IGL was measured in 4 µm sections of cyclin D2−/− and D2+/+ cerebella using NIH Image software. A sampling of comparable levels in 3 sibling pairs revealed a 50.8% decrease in the area of the IGL of cyclin D2 nulls (3.43±0.23×10^4 pixels versus 6.98±0.06×10^4 pixels, n=6, P<0.001) (Fig. 1D). This reduction in the area of the IGL compared to no significant change in the area of the underlying white matter (4.25±0.18×10^4 pixels versus 4.18±0.06×10^4 pixels, n=6, P>0.1), which contains no contribution from granule interneurons. The cerebellar white matter contains only axon projections of Purkinje neurons, afferent neuron fibers from brainstem and the deep cerebellar nuclei.

Strikingly, a selected cell population in the molecular layer was all but absent in the cyclin D2 nulls (Fig. 2B). The corresponding cells in cyclin D2+/+ siblings (Fig. 2A), were of a morphology and position consistent with stellate interneurons (Montiero, 1989; Palay and Chan-Palay, 1974). Despite the paucity of cells in the molecular layer, the overall thickness of the layer was comparable to that found in cyclin D2+/+ siblings and the appearance of Purkinje neurons was relatively unchanged (Fig. 2). Purkinje cells labeled with calbindin antibody were more closely ‘packed’ in cyclin D2 nulls, though still arranged in a monolayer in the adult (Fig. 2C,D). The dendritic arborization of Purkinje cells was robust in cyclin D2−/− cerebella, though the primary branches appeared somewhat thicker and secondary arborization in the outer molecular layer was simplified compared to normal siblings (Fig. 2C,D). The number and morphology of Purkinje cells in
the neonatal period were compared (Fig. 2E-G). P3 was chosen because, at this age, Purkinje cell numbers are established and express calbindin, while secondary effects due to postnatal granule cell loss should not yet be significant. Indeed, at P3, the cyclin D2 \textsuperscript{-/-} cerebellum is comparable in size to wild-type sibs (cross-sectional area 36.5±1.49 \times 10^3 pixels versus 33.9±1.19 \times 10^3 pixels, \( n=7, P>0.1 \)). At this age, immature Purkinje neurons in cyclin D2 \textsuperscript{-/-} cerebellum were comparable in morphology to those of D2\textsuperscript{+/+} siblings (Fig. 2E,F). Moreover, the number of Purkinje cell bodies per mm of the layer was the same in P3 cyclin D2\textsuperscript{+/+} and D2\textsuperscript{-/-} sib pairs (107±8 versus 115±6, \( n=5, P>0.1 \)) (Fig. 2G).

Cerebellar cell types were further examined to determine which were affected in animals lacking cyclin D2 (Fig. 3). Again, on H and E staining the molecular layer contained fewer cells (Fig. 3B). Larger, pale, Nissl-positive cells consistent with the morphology of basket interneurons were present in the characteristic position in the deep cyclin D2\textsuperscript{-/-} molecular layer (Figs 2B,D, 3B). Moreover, antibody directed against phosphorylated neurofilament (NF200) selectively labeled fibers of basket cells in cerebellum of both cyclin D2\textsuperscript{-/-} and D2\textsuperscript{+/+} animals, suggesting that basket interneurons were well represented in cyclin D2 nulls (Fig. 3D). Although there is no specific antigenic marker for stellate interneurons, an approximation of the relative representation of cells was obtained from counts of cell nuclei in the outer and inner aspects of the molecular layer (Fig. 3I). Comparing adult sibs, there was no significant difference in the numbers of cells counted in the outer molecular layer (86±7 cells/mm versus 96±6 cells/mm, \( n=6, P>0.1 \)).
However, there was a dramatic difference in the number of nuclei in the outer molecular layer of D2+/+ versus D2-/- pairs (85±5 cells/mm versus 9±3 cells/mm, n=6, P<0.001). Unlike the Purkinje neurons that could be counted in neonates, comparisons of Golgi interneuron numbers were made in adult siblings, since these neurons do not begin to appear in the IGL until the second postnatal week. Antibody directed against the cell surface glycoprotein, HNK-1, revealed Golgi interneurons in the cerebellar IGL of cyclin D2-/- animals (Fig. 3F). Despite the reduction in cross-sectional area of the adult D2-/- IGL, the total number of Golgi interneurons per matched sagittal section was no different in null and wild-type siblings (126±6 versus 113±8, n=4, P>0.1) (Fig. 3J). Counterstain of cyclin D2-/- sections consistently revealed that granule cells were loosely packed in the IGL compared to controls (Fig. 3E,F). Anti-GFAP antibody readily detected glia in cyclin D2-/- nulls (Fig. 3F). Despite the normal timecourse of cyclin D2 mRNA expression in granule precursors (Fig. 5), No cyclin D2 expression was detected in granule precursors originating in the rhombic lip or as these still dividing cells migrated over the surface of the cerebellar anlage to form the EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL. The discrepancy between cyclin D2+/+ and D2-/- siblings was just appreciable at postnatal day 3 (P3) and was clearly apparent by P14 (Fig. 4). At P3, the EGL of cyclin D2 nulls began to appear thin at the tips of forming folia, compared to wild-type littermates (Fig. 4, top panel). The EGL of null animals lacked the well-defined, palisading appearance of the wild type (Fig. 4) in which cells of the superficial EGL are rounded and in a random distribution while, in the deep EGL, cells take on the elongated morphology of migrating neurons. At P14 (Fig. 4, bottom panel), cyclin D2-/- folia were significantly smaller and stunted compared to wild type. The P14 EGL was markedly thinner at both the folial tips and clefts, with most of the remaining cells apparently in the postmitotic early migratory phase. Moreover, the sparseness of cells in the molecular layer was appreciable by P14.

**Temporal pattern of changes in cyclin D2-/- cerebella**

The timing of changes in null cerebella coincided with the normal timecourse of cyclin D2 mRNA expression in granule precursors (Fig. 5). No cyclin D2 expression was detected in granule precursors originating in the rhombic lip or as these still dividing cells migrated over the surface of the cerebellar anlage to form the EGL. Cyclin D2 message was detected in granule precursors only once established in the postnatal EGL (Fig. 5E), where cells in the outer stratum proliferate and in the deep EGL become postmitotic (Fig. 5F; Fujita, 1967). This expression pattern correlated well with the postnatal onset of recognizable differences in the cerebellum of cyclin D2-/- compared to wild-type littermates.

**Reduced granule cell number is associated with decreased proliferation in cyclin D2 nulls**

In the early postnatal animals, 1 hour pulse labeling of proliferating cells with BrdU revealed a reduction in the...
Cyclin D2 in cerebellar development

The proportion of EGL cells in S-phase (Fig. 6A,B). The proliferative index of cells labeled with BrdU within the postnatal EGL was decreased by 41% in cyclin D2+/− pups compared to D2+/+ siblings at P14 (10.3±1.2% versus 17.5±1.5%, P<0.001, n=24 in 3 sibling pairs) (Fig. 6E). Though the thickness of the EGL was not yet strikingly reduced at P3, the proliferative index of P3 EGL was similarly decreased by 36% (24.3±1.1% versus 38.1±1.6%, P<0.001, n=24 in 3 sibling pairs). Thus, over the 1 hour BrdU pulse, a smaller proportion of the EGL from cyclin D2−/− animals was in S-phase than in their wild-type siblings. The relative proliferative indices in wild-type and null cerebella could be skewed if there were a decrease in the apoptotic cell loss of early granule neurons of the deep EGL. Therefore, programmed cell death was examined by TUNEL assay.

**Apoptosis is increased in the EGL of cyclin D2−/− animals**

Previous in vitro data suggest that apoptotic cell loss can be associated with a selective increase in cyclin D expression (Freeman et al., 1994). Therefore, a decrease in apoptosis might be anticipated in the absence of cyclin D2 expression. Interestingly, TUNEL labeling of cerebellar tissue revealed increased apoptosis in nulls that was restricted to the EGL, where cyclin D2 is normally robustly expressed (Fig. 6C,D). Although this increase was apparent at all ages examined, the greater cellularity of the EGL at P7 made quantitation more cumbersome, so that cell counts were performed at P3 and P14. There was no apparent increase in apoptosis once D2−/− granule cells established themselves in the IGL, at which stage cyclin D2 expression is virtually shut down in normals. Strikingly, the rate of apoptosis in the cyclin D2−/− EGL was doubled (Fig. 6F). At P3, the percentage of EGL cells that were TUNEL-positive was increased by 122% of cyclin D2+/+ littermate controls (1.36±0.25% versus 0.64±0.11%, P<0.01, n=34 in 3 sibling pairs). Similarly, at P14, the rate of apoptosis in the EGL was increased 92% in cyclin D2 nulls (2.77±0.2% versus 1.44±0.1% P<0.001, n=43 in 3 sibling pairs). This suggests that there may be a defect in terminal differentiation of granule precursors lacking cyclin D2 that impairs their survival and movement away from the EGL.

**DISCUSSION**

Examination of postnatal cerebellar development in mice lacking cyclin D2 reveals a marked reduction primarily in granule neuron and stellate interneuron numbers, based on IGL area measurements and comparative cell counts of all cerebellar neuronal cell bodies that can be identified with the antigenic markers currently available. Granule cell number is reduced in cyclin D2−/− cerebella by an estimated 51%; cell bodies in a region consistent with stellate interneurons are all but absent; calbindin-stained Purkinje cell numbers are identical in cyclin D2−/− and D2+/+ siblings; Golgi interneuron numbers (labeled with HNK-1) are unchanged; cell nuclei of the morphology and position consistent with basket interneurons are preserved in the inner molecular layer and NF200-positive basket fibers are present in their typical morphology on Purkinje cell bodies. These data support the assertion that loss of cyclin D2 expression selectively affects cerebellar granule and stellate interneurons.

The cyclin D2 null cerebellum is smaller and folia are relatively stunted. This decreased volume is associated with a selective 51% reduction in the cross-sectional area of the IGL, with preservation of the underlying white matter area that contains no granule cell elements. The onset of visible differences in the cyclin D2−/− cerebellum coincides with the postnatal timing and location of cyclin D2 expression in normal animals. This reduction in granule cell number in null animals is due in part to a reduction in primary neurogenesis, reflected in the 36-41% decrease from wild-type controls in the BrdU labeling index of the EGL. Surprisingly, a selective increase in the rate of apoptosis in the EGL also contributes to the reduced granule cell number. This increased programmed cell death is confined to the EGL, suggesting that cells lacking cyclin D2 are impaired in their ability to differentiate and, therefore, die. Finally, cyclin D2 is important for the emergence of the stellate interneurons, but apparently not Golgi or basket cells, in the postnatal period. This is of interest since these three interneuron types are thought to arise from a common precursor pool, suggesting a selective role for cyclin D2 in the differentiation of certain neuronal sublineages.

**Cyclin D2 expression, cerebellar granule cell number and formation of folia**

Loss of cyclin D2 has highly selective consequences for cerebellar granule cells and provides insight into its role in regional development. Cyclin D2 is expressed in granule precursors only in the postnatal period, so that the initial precursor population should be and is established appropriately in the null animal. The 51% decrease in the area of the adult IGL in cyclin D2−/− cerebellum provides only a first approximation of reduced granule cell number. Indeed, granule cell number is anticipated to be reduced even further, since the packing of IGL cells in the null animal appears to be less dense than in wild type. This substantial effect indicates that the functions of D cyclins are not completely redundant, since other D cyclins -- likely D1 -- only partially compensate for the loss of D2. A number of granule cells continue to be generated from the cyclin D2−/− EGL, raising the interesting possibility that there may be a granule cell subpopulation that depends on cyclin D2 expression and it is the cyclin D2-dependent cells that are missing in the null mice. Additional markers of granule cell subtypes will be required to examine this possibility.

The cyclin D2+/− strain also provides insight into the role of granule neurons in formation of cerebellar folia. Although each folium can be accounted for in the cyclin D2+/− nulls, they are ‘stunted’ with shallower inter-folial clefts. There are, at present, two primary hypotheses of cerebellar foliation. In one view, foliation comes about as Purkinje cells assume a ‘stunted’ with shallower inter-folial clefts. There are, at present, two primary hypotheses of cerebellar foliation. In one view, foliation comes about as Purkinje cells assume a 'stunted' with shallower inter-folial clefts. There are, at present, two primary hypotheses of cerebellar foliation. In one view, foliation comes about as Purkinje cells assume a ‘stunted’ with shallower inter-folial clefts. There are, at present, two primary hypotheses of cerebellar foliation. In one view, foliation comes about as Purkinje cells assume a...
produces secondary effects on the survival of granule precursors and young neurons as well (Smyene et al., 1995). In another model, derived from studies of growth rates and evidence in transgenic experiments that manipulate position information from segmentation genes, the expansion of the EGL and/or migration of granule cells into the IGL also participate in folding of the anlage (Mares and Lodin, 1970; Millen et al., 1995; Baader et al., 1998). Indeed, when the entire granule cell population is eliminated, as in the Math1 null mouse, the cerebellum fails to foliate (Ben-Arie et al., 1997). However, in several of these models, the primary molecular defect was either directed to Purkinje cells (Baader et al., 1998), or included primary effects in Purkinje cells (Ben-Arie et al., 1997), or created such complete reduction in granule cell number that subsequent Purkinje cell positioning and elaboration of dendrites would also be secondarily affected (Ben-Arie et al., 1997). Therefore, a pure isolation of effects is difficult to achieve. The appearance of the D2−/− cerebellum supports the view that both events, e.g., EGL cell proliferation/migration and Purkinje cell maturation, contribute to foliation. In this cyclin D2-deficient model, enough granule cells are present to support nearly normal maturation of Purkinje cells, and the cerebellar lobules and folia form. Nevertheless, the granule population is sufficiently reduced to stunt the elongation of folia. Thus, the cyclin D2−/− mouse supports a model in which Purkinje alignment and arborization promote orthogonal folding of the anlage while granule proliferation and/or migration lengthens the folia.

Recent studies clearly implicate regional influences on proliferation, exerted by segmentation genes and neurotrophic factors such as BDNF, in cerebellar patterning (Baader et al., 1998; Schwartz et al., 1997). It is likely that certain growth and trophic factors as well as homeotic gene products will have transcriptional and/or translational effects on cell cycle genes. The present studies suggest that such factors may modulate cell cycle progression or exit in particular neuronal precursors through differential influences on G1 cyclins D2 versus D3 or D1.

**Cyclin D2 in the regulation of neural proliferation and differentiation**

The under-representation of granule neurons could be accomplished if cyclin D2−/− granule precursors dropped out of the cell cycle sooner and so went through fewer rounds of division than their D2+/+ siblings. Alternatively, the length of G1 phase could be prolonged, so that fewer cells would enter S-phase over a one hour period. These possibilities will be addressed in cultured cerebellar granule precursors. The results of TUNEL staining indicate that there is increased apoptotic cell loss in the cyclin D2−/− EGL, in addition to a decreased index of BrdU labeling in the nulls. Thus, results indicate a mixed picture in which EGL cell proliferation and viability are both affected by loss of cyclin D2 expression.

More cells in the EGL undergo programmed cell death in the cyclin D2−/− animals than their +/+ siblings. There is no a priori reason to anticipate such a result. The redundancy of the D cyclins should ensure the continued ability of cells to proliferate when expression of one is lost. Indeed, it is likely that the continued expression of D1 cyclin is responsible for the emergence of granule cells from the postnatal EGL in cyclin D2−/− animals. Moreover, several studies have demonstrated that neural progenitor cells exposed to mitotic inhibitors will cease dividing and differentiate into neurons and glia (Harris and Hartenstein, 1991; Barres and Raff, 1994). Therefore, the premature halting of division would not be expected to result in death. The present results suggest that certain neural cells have specific requirements for the composition of their cyclin D gene expression to remain viable. This composition may serve to fine-regulate cell cycle progression. Alternatively, cyclin D2 may have an additional role in the differentiation of particular neurons, beyond its known function to advance the cell cycle. Cyclin D2 is expressed in postnatal granule progenitor cells as they enter their final rounds of division and prepare to differentiate, but not in the rhombic lip and embryonic EGL. It is in the postnatal EGL that committed precursors are made competent to become granule neurons (Alder et al., 1996). In the absence of cyclin D2 expression, an increased number of precursors fail to differentiate and die in the EGL. Therefore, cyclin D2 appears to be required at the interface between neural cell proliferation and terminal differentiation.

**Hypocellularity of the molecular layer in cyclin D2 nulls is due to loss of stellate interneurons**

The lack of antigenic markers specific for stellate interneurons poses a challenge to the analysis of cell loss in the cyclin D2−/− molecular layer. The contention that the reduced cellularity in this layer is due to selective loss of stellate cells is based on the weight of four observations: (1) the cell numbers in the outer molecular layer are severely reduced (10-fold) and this portion of the molecular layer contains largely stellate neurons, (2) cell numbers in the inner molecular layer are not statistically different between cyclin D2−/− and D2+/+ siblings, suggesting that basket interneurons are at least relatively spared, (3) basket neuron fibers, identified by phosphorylated neurofilament antibody, are clearly present, again supporting the notion that basket interneurons are spared, and (4) the number of Golgi interneurons per matched thin section is the same in knockouts and wild-type cerebella. One cannot rule out the possibility that the molecular layer is sparse because cells have had difficulty migrating out from the periventricular white matter. However, cyclin D2 loss affects granule cell proliferation and survival in the EGL, not migration. By analogy, therefore, it seems more likely that genesis rather than cell motility of stellate interneurons would be affected in D2 nulls. Moreover, the fact that the number of presumptive basket interneurons is unchanged (e.g., cells have not ‘piled up’ in the inner molecular layer) and that the number and position of Golgi interneurons is no different, suggests a primary deficit in stellate neurons rather than a significant migration defect.

**Cyclin D2 and stellate interneuron differentiation**

The absence of stellate cells in the postnatal outer molecular layer suggests that the appearance of this sublineage in the cerebellar cortex requires cyclin D2 expression. Although the origin of these cells has yet to be precisely defined, present data point to a common origin of stellate, basket and Golgi cells from precursors in the periventricular white matter beginning in the first postnatal week. Stellate, basket and Golgi cells were once thought to arise from precursors in the EGL (Ramon y Cajal, 1911; Altman and Bayer, 1997).
However, several recent studies have shown that the EGL gives rise only to granule neurons and that the other cerebellar interneurons arise from germinal cells near the IVth ventricle. Transplantation of isolated EGL cells has demonstrated that they are committed and exclusively give rise to granule neurons (Gao and Hatten, 1994; Alder et al., 1996). In addition, lineage studies using chick-quail chimeras have shown that cerebellar stellate, basket and Golgi interneurons arise from ventricular germinal epithelium (Hallonet et al., 1990). Finally, cell lineage studies using retroviral tags indicate that stellate, basket and Golgi cells are immunohistologically accounted for suggest that cyclin D2 is required for the emergence of this neuronal sublineage. This is consistent with observations in Drosophila that determination of certain neuronal sublineages can depend on cell cycle progression or cytokinesis (Cui and Doe, 1995; Weigmann and Lehner, 1995). Thus, stellate interneurons may provide an example in a mammalian system of such a sublineage requirement for cell cycle cooperation.

Based on observations presented here, it is proposed that cyclin D2 is important for establishing full granule cell number and for successful early differentiation and survival of young granule neurons. In contrast, there is a more stringent requirement for cyclin D2 in the emergence of stellate neurons. Additional studies will be required to determine whether stellate interneurons are lacking in the molecular layer because of an inability of determined precursors to expand their number in response to a growth factor or because the ability of stellate cells to differentiate from their progenitor pool is compromised.

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