A Cell Cycle Alteration Precedes Apoptosis of Granule Cell Precursors in the *weaver* Mouse Cerebellum

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A missense mutation in the gene coding for the G-protein-activated inwardly rectifying potassium (GIRK) channel, GIRK2, is responsible for apoptosis in the external germinal layer (EGL) of the cerebellum and a nonapoptotic death of midbrain dopaminergic neurons in the *weaver* (*wv*) mouse. Failure of axonogenesis and migration are considered to be the primary consequences of GIRK2 channel malfunction in the cerebellum. We investigated whether a disruption of the cell cycle precedes the failure of migration and axonogenesis and leads to massive apoptosis. To this end, immunohistochemistry and immunoblotting for PCNA, Cdk4, cyclin D, cyclin A, and the Cdk inhibitor p27/kip1, as well as *in situ* end-labeling for apoptotic DNA fragmentation, were applied to cerebella of P7-P21+/+, *wv*/+, and *wv/wv* mice. In +/+ and *wv*/+ mice, the expression of cell cycle proteins was limited to the outer, premigratory zone of the EGL. Antibodies to p27, a marker of cell differentiation, gave a reverse staining pattern. Due to migration delay, patches of p27-positive cells persisted in the outer EGL in P21 *wv*/+ mice. On the contrary, marked cell cycle up-regulation and absence of p27 occurred throughout the EGL at all ages in *wv/wv* mice, indicating an inability to switch off the cell cycle. Mitotic index evaluation showed that cell cycle activation was unrelated to proliferative events. Cell cycle proteins were not expressed in the substantia nigra, suggesting that nonapoptotic death of mature dopaminergic neurons is not preceded by abortive cell cycle re-entry. Our data show that abnormalities of the cell cycle in *wv/wv* cerebellum represent a major and early consequence of GIRK2 channel malfunction and may strongly influence the susceptibility of EGL cells to apoptosis. These observations may help in understanding the pathogenesis of human neurological channelopathies. (*Am J Pathol* 1999, 155:365–373)

The *weaver* (*wv*) mutation in the homozygous (*wv/wv*) mouse is associated with a loss of granule cells in the cerebellum, dopaminergic neurons in the midbrain, and germinal epithelium in the testis.^{1–3} The same regions are less severely affected in heterozygous (*wv*/+) mice. *weaver* has been identified as a missense mutation in the *girk2* gene, which encodes the G-protein-activated inwardly rectifying potassium (GIRK) channel GIRK2.⁴ *In vitro*, the mutation leads to a complex series of changes in channel activity, ranging from a loss of G-protein-dependent inward rectifier current regulation^{5,6} to reduced selectivity for potassium over sodium and calcium ions.^{7–12} This alteration results in chronic depolarization and cell death. *In vivo* studies show that the granule cell precursors of the external germinal layer (EGL) of the cerebellum die massively via apoptosis within the first 3 postnatal weeks.^{13–16} At variance with the cerebellum, mature dopaminergic neurons of the *weaver* midbrain degenerate at a later time through a nonapoptotic mechanism.^{16,17}

Why EGL cells are selectively prone to undergoing massive apoptosis is unclear. Based on similarities in timing and genetic regulation between *girk2wv*-dependent cell death and naturally occurring cell death in the developing cerebellum,^{16,18} it is likely that a disruption of selective developmental features of EGL cells caused by GIRK2 channel malfunction may lead to an increased susceptibility to apoptosis. It has been hypothesized that the *weaver* gene acts on EGL cells after their exit from cell cycle and before their migration from the EGL;¹⁹ accordingly, failure of neuronal migration and axonogenesis would be the primary consequences of the mutation.^{1,19–23} EGL cells would thus be prevented from responding to appropriate environmental cues, a situation that may eventually lead to apoptosis.^{24,25}

In this paper we have considered an alternative hypothesis, namely, that the *girk2wv* gene mutation produces abnormalities of control of the cell cycle in EGL

Supported in part by PHS grants P01 NS 27613 and R01 NS14426 and by European Community Copernicus program grant CIPA-CT93–0210.

Accepted for publication April 11, 1999.

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cells. This abnormality may be of fundamental importance for the onset of neurodegeneration and apoptosis and may precede further developmental events such as migration and axonogenesis. Cell proliferation and apoptosis are closely related phenomena, as they share several regulatory mechanisms, and any perturbation of the cell cycle control may greatly influence the cell's susceptibility to apoptosis.^{26,27} For example, increased expression of cell cycle genes and proteins is found during apoptosis^{28–31} and rescue from death occurs if expression of cell cycle proteins is inhibited.^{31–34} Furthermore, apoptosis-related proteins have been recently shown to modulate cell cycle protein activation during apoptosis.³¹

To clarify whether abnormalities of the cell cycle regulation are present in the *weaver* cerebellum, we have studied the expression of the proliferating cell nuclear antigen (PCNA), a DNA polymerase subunit which is required for S-phase DNA synthesis and is widely expressed during the cell cycle from late G1 to late G2/early M phase.³⁵ To define more precisely any perturbation of cell cycle progression, we have investigated the expression of cyclin D (a marker of early and mid-G1 phase), cyclin-dependent kinase (Cdk) -4 (involved in G1-to-S progression but expressed throughout the cell cycle), cyclin A (a marker of G2-to-M transition),³⁶ and the Cdk inhibitor p27/kip1 (a negative regulator of G1 progression).³⁷ Finally, data related to expression of cell cycle proteins have been compared with those obtained using the *in situ* end-labeling (ISEL) technique for detecting DNA damage and apoptosis-related DNA fragmentation.³⁸

Materials and Methods

Animals and Experimental Design

weaver mutant and wild-type mice were obtained from a colony established at Indiana University Medical Center from heterozygous *weaver* mice that originated from Jackson Laboratory (Bar Harbor, ME). Mutant and wild-type mice are maintained on a B6CBA-A^w-J/A hybrid stock. Genotype analysis was performed as described.³⁹ Twenty-four mice were used for morphological studies, eight each of three genotypes (+/+, *wv*/+, *wv*/*wv*) were divided into four age groups (P7, P11, P14, P21). For biochemical studies, eight mice, four each from the homozygous genotypes +/+ and *wv*/*wv*, were divided into two age groups, P7 and P14. We did not study mice younger than P7, because until this age active proliferation occurs throughout the entire thickness of the EGL in all three genotypes,¹⁹ and differences in the expression of proliferation markers would have been negligible.

Tissue Preparation for Morphology Studies

Mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with 10 ml of normal saline and subsequently with 60–120 ml of 4%

paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.2. Brains were removed and postfixed in the same fixative for 30–60 minutes. Tissues were dehydrated in graded ethanols, cleared in xylene, and embedded in paraffin. Midsagittal sections of cerebrum and cerebellum and transverse sections of midbrain were cut at 6 μ m and mounted on poly-L-lysine-coated slides.

Antibodies

Anti-PCNA is a mouse monoclonal antibody against rat PCNA (clone PC10, Dako, Glostrup, Denmark) that reacts with PCNA from yeast, insects, and vertebrates. Anti-cyclin D1 is a rabbit polyclonal antibody against mouse cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA) that is also reactive, to a lesser extent, with cyclin D2. Anti-Cdk4 is a rabbit polyclonal antibody against mouse Cdk4 (Santa Cruz). Anti-cyclin A is a rabbit polyclonal antibody against mouse cyclin A (Santa Cruz). Anti-p27 is a rabbit polyclonal antibody against human p27 (Santa Cruz) that is also mouse and rat reactive.

Recognition of dopaminergic neurons in the substantia nigra was accomplished using an antiserum raised in rabbits against bovine tyrosine hydroxylase (Eugene Tech, Allendale, NJ). Whenever feasible, specificity of the immune reaction was tested by incubating the antibodies with the respective immunizing peptides before immunostaining.

Immunohistochemistry

Deparaffinized sections were soaked in 3% hydrogen peroxide to block endogenous peroxidase activity. Primary antibodies were applied overnight at 4°C at the following dilutions: anti-PCNA, 1/30,000; anti-cyclin D and -Cdk4, 1/2000; anti-cyclin A and -p27, 1/200. Before immunostaining, the sections were placed in 0.01 mol/L citrate buffer, pH 6, and heated in a microwave oven (Whirlpool) at 750 W until boiling and then at 350 W for 15 minutes. Immune reactions were revealed with the streptavidin biotin complex technique, except for PCNA, which was revealed with the peroxidase-antiperoxidase technique. Cobalt chloride-intensified diaminobenzidine was used as peroxidase substrate. Sections were counterstained with hematoxylin, dehydrated, and mounted in Permount.

In all experiments, the immunoreactivity of proliferating cells in the periventricular matrix served as an internal control of the reaction.

Western Blot Analysis

Cerebella from P7 and P14 +/+ and *wv*/*wv* mice were snap-frozen and homogenized at 4°C by loose dounce in 200 μ l of RIPA buffer (20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 5 mmol/L EDTA) in the presence of a protease

inhibitor cocktail. The homogenates were cleared by centrifugation for 15 minutes at 15,000 rpm, sonicated for 15 seconds, and total protein in the extracts was determined. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by loading equal protein amounts (25 μ g) for each sample into 12% acrylamide/bis-acrylamide gel.⁴⁰ The proteins were transferred to nitro-cellulose and blots were incubated with the primary antibodies. Immune complexes were visualized by the enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia Biotech).

To quantify cell cycle protein immunoreactivities, films were scanned using a Scanjet 4C (Hewlett-Packard, Tokyo) scanner. Densitometric analysis was performed using Image ProPlus (Media Cybernetics, Silver Spring, MD) software. Protein levels were expressed as relative optical density measurements, determined by comparing the density of the immunoreactive bands in the same blot.

ISEL

ISEL was performed as previously described.¹⁶ Briefly, deparaffinized sections were treated with 0.5–2.5 μ g/ml of proteinase K (Sigma) in Tris-buffered saline (TBS) for 15 minutes at room temperature. After rinsing in terminal deoxynucleotidyl transferase (TdT) buffer (25 mmol/L Tris/HCl, 200 mmol/L sodium cacodylate, 5 mmol/L cobalt chloride), the sections were incubated with the labeling mix (20 U of TdT (Boehringer Mannheim) and 1 nmol of fluorescein-11-dUTP (Boehringer Mannheim) in 100 μ l of TdT buffer) for 120 minutes at 37°C. After rinsing in 300 mmol/L sodium chloride/30 mmol/L sodium citrate, the sections were incubated with anti-fluorescein sheep antibody conjugated with peroxidase (Boehringer Mannheim), diluted 1/500 in TBS, for 30 minutes at 37°C. The reaction was revealed with cobalt chloride-intensified DAB. Sections were counterstained with hematoxylin. Control of the reaction was performed by omitting TdT from the labeling mix.

Quantification of Mitotic Cells

Cell counts were made from 5 to 8 cerebellar sections for each animal, using an oil-immersion objective at 1000 $\times$ magnification. On each section, six areas (0.02 mm² each) containing the whole thickness of the EGL were chosen, so that the anterior-to-posterior axis of the cerebellum was equally sampled. The number of EGL cells per area and the number of mitotic figures per area were counted. The area values were added together and a mean value/section was determined. For each section, a mitotic index (MI) was expressed as the percentage of mitotic cells/number of EGL cells, and a mean MI was calculated for each genotype and age group. Statistical analysis was performed using one-way analysis of variance and a Student's *t*-test.

Results

Cell Proliferation in the Cerebellum

PCNA

In all preparations studied, the immunoreactivity for PCNA was exclusively located in the nucleus. In the EGL, the staining appeared most frequently as diffuse or less frequently as punctate, without a clear difference in the proportion of the two patterns among the three genotypes. In the subependymal matrix, the diffuse pattern was predominant, whereas a punctate pattern was less frequently seen. Differences in the pattern of staining among the genotypes are described below.

In *+/+* mice at P7, the EGL was 6 to 10 cells thick and could be grossly divided into a superficial zone, formed by undifferentiated round cells, and a deeper zone, formed by elongated cells about to migrate toward the internal granule cell layer.⁴¹ Mitoses and rare pyknotic nuclei were mainly found in the outer EGL. Strong PCNA immunoreactivity was found in nuclei of the superficial zone, with a progressive reduction of the labeling in the deeper zone (Figure 1A). Cells at the interface between EGL and Purkinje cell layer were PCNA-negative. In the Purkinje and internal granule cell layers, numerous cells, that we interpreted as proliferating astrocytes on the basis of previous studies,^{30,41} were PCNA-positive. At P11, the EGL was reduced to 2–3 layers of PCNA-positive cells, while most migrating cells across the developing molecular layer and mature granule cells were PCNA-negative. At P14, the EGL was reduced to a thin, almost single cell layer, while the granule cell layer was already well developed. Most EGL cells were still PCNA-positive (Figure 1D), indicating their proliferative status, in agreement with previous autoradiography data.⁴¹ At P21, the EGL had completely disappeared, while the granule cell layer had reached its full thickness and contained occasional PCNA-positive glial cell. (Figure 1G).

In *wv/+* mice at P7, the EGL was 8–14 cells thick, due to a cell migration delay.¹⁹ Compared to *+/+* mice, the demarcation between PCNA-positive cells in *wv/+* mice, located in the outer zone of the EGL and PCNA-negative cells, located in the deeper zone, tended to be sharper (Figure 1B). Two well demarcated zones were still present at P11 and to a minor extent at P14 (Figure 1E). No PCNA-labeled granule cells were found in the hypercellular molecular layer or in the granule cell layer. At P21, the EGL had largely disappeared along the cerebellar surface except for occasional patches of 3–4 layers of cells. In these areas, PCNA immunoreactivity was completely absent, indicating that these cells had already exited the cell cycle (Figure 1H).

In *wv/wv* mice at P7, the EGL was primarily formed by round, undifferentiated cells.¹ Mitotic figures were scattered in the whole EGL, whereas clusters of pyknotic nuclei^{16,19} were mostly located in the inner portion of the EGL. By PCNA immunohistochemistry, virtually all cells throughout the entire EGL were strongly stained (Figure 1C). Progressive reduction in thickness of the EGL occurred in P11 and P14 mice; however, PCNA immunore-

Figure 1. PCNA immunostaining in the EGL from $+/+$ (A, D, G), $wv/+$ (B, E, H), and wv/wv (C, F, I) mice at P7 (A-C), P14 (D-F), and P21 (G-I). Note the progressive dilution of labeling in migratory EGL cells in P7-P14 $+/+$ (A, D) and $wv/+$ (B, E) mice. In P21 $+/+$ mice (G), the EGL has disappeared, and no PCNA staining is seen in either molecular or granule cell layer. Patches of cells persist in the EGL of P21 $wv/+$ mice (H), but they have become PCNA-negative. On the contrary, EGL cells remain labeled at all ages in wv/wv mice (C, F, I). In addition to proliferating cells, strongly labeled apoptotic cells can be seen in A and C (arrows). All pictures were taken at 1000 $\times$ except G (400 $\times$).

activity was still present in all cells of the EGL (Figure 1F). At P21, the EGL was composed of 1–3 layers of cells. Contrary to $wv/+$ mice, cells of the EGL in wv/wv mice were still PCNA-immunoreactive, indicating that these cells had never exited the cell cycle (Figure 1I).

Other Cell Cycle Markers

An immunostaining pattern consistent with that described for PCNA was found with the antibody to Cdk4; as a matter of fact, most cells in the PCNA-labeled compartments were also Cdk4-positive. Antibodies to cyclin D and cyclin A stained a major subset of cells in these compartments. The specificities in the pattern of expression for each marker are likely due to the different temporal appearance of the proteins during the cell cycle: Cdk4 and PCNA levels remain elevated for most of the cell cycle, whereas cyclin D and cyclin A have a short half-life.³⁶ At P7-P11, immunoreactive cells in $+/+$ mice (Figure 2, A and D) and in $wv/+$ mice (Figure 2, B and E)

were found in the outer proliferative zone of the EGL, while the inner postproliferative zone was free of labeling. On the contrary, labeled cells in wv/wv mice were distributed throughout the entire EGL (Figure 2, C and F). At P14, labeled cells were rare in $+/+$ mice and limited to the outermost zone of the EGL in $wv/+$ mice, while they were numerous in wv/wv mice. At P21, all cells forming patches in the EGL of $wv/+$ mice were unlabeled (Figure 3A), whereas the majority of EGL cells in wv/wv mice were strongly labeled (Figure 3B).

We also studied the expression of Cdk inhibitor p27. Because its levels are inversely related to the proliferative state of cells,³⁷ a reverse pattern of staining was expected in comparison to that of markers of cell cycle progression. As a matter of fact, areas showing increased expression of PCNA, cyclins and Cdk4 were p27-negative, whereas cells in the inner zone of the EGL of $+/+$ and $wv/+$ mice, negative for proliferation markers, became p27-positive (Figure 2, G and H). In wv/wv mice, all cells of the EGL were constantly p27-negative at all

Figure 2. Immunostaining for Cdk4 (A-C), cyclin D (D-F), and p27/kip1 (G-I) in $+/+$ (A, D, G), $wv/+$ (B, E, H), and wv/wv (C, F, I) mice at P7. The labeling pattern of Cdk4 and cyclin D is similar and corresponds to that of PCNA (see Figure 1, A-C). In particular, only premigratory EGL cells are labeled in $+/+$ (A, D) and $wv/+$ (B, E) mice, while migratory cells of the innermost EGL zone are no longer stained. On the contrary, diffuse staining of the whole EGL is present in wv/wv mice (C, F). Antibodies to p27 produce a reverse staining pattern, with migratory EGL cells in $+/+$ (G) and $wv/+$ (H) mice becoming progressively labeled. On the contrary, all EGL cells in wv/wv mouse remain p27-negative. All pictures were taken at 1000 $\times$.

ages, further proof that these cells had never exited the cell cycle (Figure 2I). Differences in the staining pattern were particularly impressive at P21. The p27 labeling was diffusely present in the internal granule cell layer of $+/+$ and $wv/+$ mice and in the patches of residual EGL in wv/wv mice (Figure 3D). In contrast, the EGL cells of wv/wv mice remained p27-negative (Figure 3E).

Western Blot Analysis

The expression of PCNA and p27 was evaluated on frozen samples of whole cerebellum from $+/+$ and wv/wv mice at P7 and P14 (Figure 4). Immunoblotting for PCNA revealed in all cases a band of 36 kd. At P7, PCNA was present in both $+/+$ and wv/wv mice, with a four-fold increase in the latter. At P14, PCNA was virtually absent in $+/+$ mice, while it was still detectable in wv/wv mice, although its levels were reduced to 10% of those of P7 wv/wv mice. Therefore, results from Western blot analysis

of PCNA expression showed a close correlation with those of immunohistochemistry.

In the case of p27, a band of 27 kd was found in all cases. A faint band was present at P7 in $+/+$ mice, while an even weaker band was present in wv/wv mice. At P14, p27 levels in $+/+$ mice increased by six-fold compared to P7 $+/+$ mice. A band became also evident in P14 wv/wv mice, but its intensity was three times less than that seen in P14 $+/+$ mice. The presence of detectable levels of p27 in wv/wv mice is due to the fact that the tissue homogenate included the whole cerebellum and not just the EGL. Nevertheless, comparison with $+/+$ mice at both ages clearly showed greatly reduced p27 levels in wv/wv mice.

Evaluation of the Mitotic Index

The percentage of mitotic cells in the EGL was evaluated on H&E sections from $+/+$, $wv/+$, and wv/wv mice

Figure 3. Immunostaining for cyclin A (A, B) and p27 (D, E) in P21 *wv/+* (A, D) and *wv/wv* (B, E) mice. Note that patches of cells in the EGL have an opposite expression of cell cycle markers in the two genotypes. Absence of cyclin and presence of p27 in *wv/+* cells indicates the postproliferative, differentiated nature of these cells, which are about to migrate from the EGL. On the contrary, persistence of cyclin expression and lack of p27 in *wv/wv* mouse indicates the proliferative, undifferentiated nature of EGL cells. C shows ISEL labeling in the EGL of P7 *wv/wv* mice. Note that most apoptotic cells are concentrated at the boundary with the Purkinje's cell layer. F: Double immunostaining for tyrosine hydroxylase and PCNA in the substantia nigra of P14 *wv/wv* mice. PCNA strongly labels an apoptotic cell (arrow), while dopaminergic neurons are unstained. All pictures were taken at 1000 $\times$.

at P7, P11, P14, and P21. Despite the massive activation of cell cycle proteins in *wv/wv* EGL, no change in the mitotic index was found among the genotypes at all ages studied (Figure 5).

Cell Death in the Cerebellum

Cell death in the EGL was examined by ISEL staining as well as by immunohistochemistry for cell cycle-related

proteins on P7-P21 mice of all three genotypes. ISEL staining was always limited to nuclei with evident apoptotic features (nuclear pyknosis, fragmentation into apoptotic bodies). In *+/+* and *wv/+* mice, scattered (less than 2% of total EGL cells) ISEL-labeled nuclei were found at P7, extremely rare at P14, and absent at P21. In *wv/wv* mice, ISEL-labeled nuclei were found at all ages and amounted to nearly 10% of all EGL cells. Most apoptotic nuclei were located in clusters in the proximity of the Purkinje cell layer (Figure 3C).

On sections adjacent to ISEL-stained ones, we investigated whether cell cycle markers were also expressed by cells showing morphological changes of apoptosis, a likely reflection of the involvement of cell cycle proteins to the final stage of the apoptotic cascade. The pattern of PCNA immunoreactivity in the apoptotic cells closely

Figure 4. Immunoblot analysis of PCNA (top) and p27/kip1 (bottom) protein expression in P7-P14 *+/+* and *wv/wv* mice. PCNA is upregulated in P7 *wv/wv* mice and is still detectable at P14. Compared to PCNA, p27 is constantly more expressed in *+/+* mice and becomes more evident in older mice.

Figure 5. Evaluation of the mitotic index in the EGL of *+/+*, *wv/+*, and *wv/wv* mice between P7 and P21. No statistically significant difference was found between the three genotypes at any age. No value is given for P21 *+/+* mice because the EGL is no longer represented.

matched that of ISEL, revealing a distinct labeling of most apoptotic nuclei in all genotypes (Figure 1, A and C). The intensity of PCNA staining in the apoptotic cells was constantly higher than that of surrounding nonapoptotic cells. On the contrary, we could not detect any staining of apoptotic nuclei using the antibodies to the other cell cycle markers.

Substantia Nigra

Activation of cell cycle genes occurs during apoptosis of postmitotic neurons as an abortive attempt to reenter the cell cycle.^{26,28,30,32–34} We investigated whether postmitotic dopaminergic neurons, which are a major target of *girk2^{wv}* action, showed aberrant expression of cell cycle markers during the period of maximal cell loss. Sections of midbrain were double immunostained for tyrosine hydroxylase and cell cycle markers. However, no staining of dopaminergic neurons could be detected with any of the proliferation markers tested. Only occasional ISEL-labeled, PCNA-positive apoptotic nuclei (1–2/section) were found, with no differences among the three genotypes (Figure 3F). This apoptosis is a likely evidence of developmentally programmed cell death, as reported previously.^{16,42}

Discussion

Since the *weaver* mutation was identified,⁴ considerable advancement has been made in understanding the functional alterations of mutated GIRK2 channels.^{5–12} It is generally assumed that target cells in *weaver* mice die as a result of chronic depolarization; however, the pathogenetic mechanisms that lead to selective activation of apoptosis in cerebellar granule cell precursors but not in dopaminergic neurons of midbrain are not completely understood. Here we show that abnormalities in the control of cell cycle in *wv/wv* cerebellum represent a major and early consequence of the altered GIRK2 channel function that may strongly influence the susceptibility of EGL cells to undergo apoptosis.

Developmental Changes in weaver Cerebellum

In earlier studies, the development of the EGL in *weaver* mutant mouse had been examined by means of autoradiography²⁰ and mitotic index evaluation.¹⁹ These studies showed that the mitotic activity in P1–P8 *weaver* cerebellum was unchanged in comparison to wild-type mice, leading to the hypothesis that postproliferative events such as failure of neuronal migration and of neurite extension might be the primary consequences of the *weaver* mutation.^{1,19–22} After identification of the mutation, the same hypothesis was still supported by Maricich et al.,²³ who suggested that the *girk2* gene might be necessary for proper migration of neurons during development.

Cell Cycle Abnormalities in weaver Cerebellum

Although failure of neuronal migration and axonogenesis may indeed induce an apoptotic response,²⁴ we reasoned that perhaps some earlier event related to regulation of the cell cycle might be disrupted by the GIRK2 malfunction, leading to massive apoptosis. In normal conditions, the cell cycle represents a sequence of highly ordered processes that result in the duplication of a cell. Cell cycle proteins are sequentially activated and inactivated to move the cell through a series of checkpoints,³⁶ and any perturbation of this sequence may lead to growth arrest and death through activation of apoptosis.^{26,28}

Whereas the present evaluation of the mitotic index in mice up to P21 confirms that the *weaver* mutation apparently does not perturb cellular proliferation, analysis of cell cycle events reveals striking changes in the cerebellum of *wv/wv* mice as compared to *+/+* and *wv/+* animals. In wild-type and heterozygous mice, cell cycle proteins are up-regulated in proliferating cells located in the outer zone of the EGL, whereas migrating cells of the inner zone are no longer immunoreactive. These results are in line with previous autoradiography data showing that normal granule cell precursors cease DNA synthesis before differentiating and starting their migration.⁴¹ A recent report on PCNA and cyclin D1 expression in the developing rat cerebellum also reached similar conclusions.⁴³

In contrast, several cell cycle abnormalities are present in homozygous *weaver* mice. First, marked up-regulation of cell cycle proteins occurs throughout the entire EGL at all ages studied, suggesting that *weaver* EGL cells cannot switch off the cell cycle. This hypothesis is further supported by the observation that p27, a cell-intrinsic timer that arrests the cell cycle and initiates differentiation,³⁷ is not expressed in the *weaver* EGL. Second, the mitotic activity in the EGL is unchanged, suggesting that persistent activation of cell cycle machinery is unrelated to proliferative events. Third, continuous activation of cell cycle proteins appears to be a prolonged phenomenon, as *weaver* EGL cells are known to die 2 to 4 days after being generated.²⁰ Fourth, continuous cell cycle activation in *wv/wv* mice is not due merely to migration delay or to a position effect on cells within the EGL, but is rather an early consequence of the altered GIRK2 channel function. As a matter of fact, EGL cells in *wv/+* mice have a delay in migration but exit the cell cycle normally, as shown by the complete absence of cell cycle marker expression and by the strong p27 immunostaining in the cell patches at P21.

Cell Cycle Abnormalities and Apoptosis

Abnormalities in the cell cycle machinery may directly favor the activation of apoptosis.^{26,27} Furthermore, inability to switch off the cell cycle in *wv/wv* mice affects cells that are receiving at the same time developmental cues to stop proliferating and start migration/differentiation, as suggested by the observation that neurotrophin and neurotrophin receptor expression is unchanged in the *weaver*

cerebellum.⁴⁴ Thus, *weaver* EGL cells would at the same time receive conflicting signals for proliferation and growth arrest/differentiation, a situation that has been postulated to promote apoptosis in neurons.²⁸

In the present model, PCNA was strongly expressed in apoptotic cells as a likely reflection of its other activity in nucleotide excision repair of damaged DNA.⁴⁵ Lack of staining of apoptotic cells with the other cell cycle markers may be due to the short half-life of these proteins;³⁶ alternatively, it may indicate that they are necessary for the preparation phase, but not for the execution phase of apoptosis.^{28,30}

Ion Channel Malfunction and Cell Cycle Abnormalities

The mechanism through which regulation of the cell cycle is altered by the GIRK2^{wv} channel activity is at present only a matter of speculation. Cell cycle deregulation is unlikely to be a direct consequence of chronic depolarization, as the latter event usually arrests cell cycle progression and promotes differentiation.^{46,47} Moreover, chronically depolarized cerebellar granule cells show an overall decrease of cell cycle-related proteins before undergoing apoptosis.⁴⁸ On the other hand, changes in voltage-dependent ion currents occur during the cell cycle, suggesting a close interdependence between the two events.⁴⁹ Abnormal permeability of the mutated channel to calcium ions might be important, since calcium fluxes are known to modulate progression throughout the cell cycle.⁵⁰ *In vitro* studies should better clarify this point.

Lack of Cell Cycle Abnormalities in weaver Substantia Nigra

Dopaminergic neurons in the *weaver* substantia nigra die through a nonapoptotic mechanism of programmed cell death.^{16,17} Here we show that this neuronal population does not express cell cycle proteins during the period of maximal neurodegeneration, a finding that has some interesting implications. At the time of degeneration, dopaminergic neurons are postmitotic, fully differentiated cells that have already made synaptic connections with the striatal target cells. Lack of cell cycle protein expression rules out the presence of an abortive mitotic attempt, as has been shown during death of postmitotic neurons in several experimental paradigms *in vitro*^{28,32–34} and *in vivo*,³⁰ as well as in human neurodegenerative disorders.⁵¹ Lack of cell cycle reactivation in *weaver* dopaminergic neurons might simply depend on the nonapoptotic nature of the death process, although cell cycle-independent pathways of neuronal apoptosis have been also described.³⁴ The present observations suggest that the susceptibility to undergo apoptotic *versus* nonapoptotic cell death might greatly depend on the degree of maturation of a neuron.⁵² This fact may be relevant for the pathogenesis of neurodegenerative diseases, in which the role of apoptosis has been questioned.⁵³

Conclusion

In conclusion, our data point to a severe alteration of cell cycle regulation in *wv/wv* EGL cells, a situation that highly increases their susceptibility to undergo massive apoptosis. We suggest that cell cycle perturbation, rather than failure of axonogenesis and migration, is the primary consequence of the mutated GIRK2 channel activity in cerebellum. These observations extend our knowledge on mechanisms of neuronal degeneration and may help in understanding the pathogenesis of human neurological disorders characterized by defective ion channel functioning.^{54–56}

Acknowledgments

We thank Debra Lucas, Rose Richardson, and Yue Feng for technical help and Bradley S. Glazier for editorial assistance.

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