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The lurcher gene induces apoptotic death in cerebellar Purkinje cells

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SUMMARY

In the neurologically mutant mouse strain lurcher (*Lc*), heterozygous animals display cell autonomous degeneration of cerebellar Purkinje cells beginning in the second postnatal week. During the course of our studies to identify the genetic lesion responsible for this disease (Norman et al., 1991), we have formulated an hypothesis suggesting that in *Lc* Purkinje cells homeostasis is sufficiently perturbed to lead to the activation of programmed cell death, thus resulting in neuronal loss and the consequent neurologic disease (Heintz, 1993). To address this possibility, we have examined the properties of *Lc* Purkinje cells as they die during the second postnatal week. Our light and electron microscopic studies demonstrate that dying *Lc* Purkinje cells exhibit the characteristic morphologic features of apoptosis, including nuclear condensation, axon beading and membrane blebbing. Using an in situ end-labeling method, we have also detected nicked nuclear DNA in these cells. Furthermore, we have examined the expression of the sulfated glycoprotein 2 (SGP2), whose mRNA is induced in both T-cells and prostate epithelial cells undergoing apoptotic death. We show by in situ hybridization that SGP2 is not expressed at detectable levels in normal Purkinje cells, but that its mRNA is present in *Lc* Purkinje cells prior to their death. Also expression of the Kv3.3b potassium channel, which marks the terminal phase of Purkinje cell differentiation, is evident in *Lc* Purkinje cells prior to their death. These data demonstrate that the *Lc* mutation induces apoptosis in cerebellar Purkinje cells following their maturation in postnatal cerebellum. Isolation of the *Lc* mutation and further analysis of its action in eliciting apoptosis can provide an important opportunity for understanding the etiology of neurodegenerative disease.

Key words: programmed cell death, apoptosis, lurcher, neurodegeneration, Purkinje cell, gene expression, mouse

INTRODUCTION

Lurcher, a semi-dominant mutation, was first described and assigned to mouse chromosome six by Phillips (1960). Homozygous lurcher (*Lc*/*Lc*) animals die within a few hours of birth with no gross anatomical abnormalities. Heterozygous lurcher (*Lc*/+) animals, on the other hand, live a normal life span and are fertile, although females tend to have small litters. The obvious behavioral phenotype of lurcher animals is apparent in heterozygotes by postnatal day 14, at which time normal littermates lose their juvenile unsteadiness (Phillips, 1960; Swisher and Wilson, 1977). *Lc*/+ animals display locomotor difficulties and impaired motor control. Affected mice have a wobbly, 'lurching', mild-to-moderate ataxic gait. They also tend to fall from side to side and to walk backward, especially when placed on a flat surface outside the cage.

Histological analysis of the cerebella of *Lc* heterozygotes (Swisher and Wilson, 1977) indicates early postnatal degeneration of several cell types, beginning with the loss of Purkinje cells during the second week of life. Loss of the entire Purkinje cell layer and large percentages of granule and olivary neurons and Bergmann glia is seen later (Caddy and Biscoe, 1976). While the *Lc* gene itself remains to be isolated, studies of *Lc*↔wild-type chimeric mice (Wetts and Herrup, 1982a,b) have proven that the primary site of action of the *Lc* gene is the Purkinje neuron itself, and that delayed degeneration of other cell types occurs secondarily, presumably due to lack of essential contacts with Purkinje cells.

In order to begin to understand the mechanism of action of the *Lc* gene, it is essential to establish the mode of death responsible for the Purkinje cell degeneration. In general, cell death can be thought of in terms of either necrosis or apoptosis. The term apoptosis was coined by Kerr et al. (1972) to describe the morphological changes associated with many types of dying cells (for reviews see Schwartzmann and Cidlowski, 1993; Ucker, 1991; Vaux, 1993; Williams et al., 1992; Williams and Smith, 1993; Wyllie et al., 1980; Wyllie, 1981,1987). Apoptosis is distinct from necrosis. Necrosis is a passive process which can be caused by external influences such as viral infection, toxins or injury, and requires no de novo protein or RNA synthesis. It is characterized by swelling of cellular cytoplasm and organelles, particularly mitochondria, leading ultimately to rupture of the cell. Cells undergoing apoptotic death, in contrast, require metabolic energy and gene expression (reviewed in Fesus et al., 1991) to actively kill themselves through a unique series of morphologically identifiable stages. Nuclear chromatin becomes condensed and nuclei may fragment although most intracellular organelles

(except in some cases the endoplasmic reticulum) remain intact. Blebbing of nuclear and cellular membranes is also common in apoptotic death. Fragments of cells dying in this manner are phagocytosed by macrophages and neighboring cells so no leakage of intracellular macromolecules occurs and the inflammatory response typical of necrotic death is not seen. Another common hallmark of apoptosis is the internucleosomal cleavage of DNA (by an as-yet undiscovered endonuclease) into fragments that are multiples of approximately 200 base pairs, a process often seen as ‘DNA laddering’ through gel electrophoresis. Finally, several cell types dying by apoptosis express specific gene products, such as the sulfated glycoprotein (SGP2), (for reviews see Jenn and Tschopp, 1992; May and Finch, 1992; Michel et al., 1992).

Apoptotic cell death has been shown to occur in many systems. For instance, many (but not all) cells undergoing programmed cell death also exhibit apoptotic characteristics. Programmed cell death describes the stereotypic loss of individual cells at specific times during development. Programmed cell death was first described as a normal feature in the development of multi-cellular organisms by Glucksman (1951), and is considered to be a form of cellular suicide (Glucksman, 1951; Saunders, 1966). This process, like proliferation, differentiation and growth control arrest, is thought to play an essential role in controlling the balance among cell populations throughout animal development. Programmed cell death, which is particularly well-described in the nematode *Caenorhabditis elegans* (for reviews see Chalfie, 1984; Ellis et al., 1991), is well-documented throughout nature. Programmed cell death has been described during insect and amphibian metamorphosis, for example in the intersegmental muscles of silkmoths (Lockshin and William, 1965) and in tail atrophy in *Xenopus laevis* tadpoles (Weber, 1969). Programmed cell death is also prominent embryologically in higher vertebrates, for example during the invagination of the neural plate into the neural tube (Glucksman, 1951) and in interdigital cell death during limb formation (Saunders, 1966). Other examples of the occurrence of programmed cell death in vertebrates include clonal elimination of autoreactive T-cells during negative selection in the thymus (MacDonald and Lees, 1990; Sambhara and Miller, 1991), neuronal death during synaptogenesis (for reviews see Oppenheim, 1985, 1991; Williams and Herrup, 1988), epidermal tissue homeostasis (Budtz, 1985), and normal retinal (Young, 1984), spinal cord (Hamburger and Oppenheim, 1982) and corpus callosum development (Hankin et al., 1988).

Apoptosis has also been described for photoreceptor degeneration in a number of mouse mutants such as retinal degeneration (*rd*), retinal degeneration slow (*rds*) and rhodopsin (Chang et al., 1993) and in pathological situations including regression of prostate tissue postcastration, death of neurons after trophic factor withdrawal and glucocorticoid-induced death of thymocytes (reviewed in Schwartzman and Cidlowski, 1993).

Although apoptosis is well-documented in pathological situations, there is little known about the generality of this phenomenon in neurologic disease. Establishing the mechanism of cellular degeneration in human disease has proved difficult as biopsy material only becomes available after the majority of cells have died. In addition a combination of histochemical, biochemical and molecular techniques are required to usefully define a degenerative process as being due to apoptosis. Consequently, no definitive studies are available. Using the *Lc* mouse as a model for neurodegeneration has allowed us to perform such a study.

We have examined the properties of *Lc* Purkinje cells as they die postnatally. We have used light and electron microscopy, in situ hybridization and an in situ DNA end-labeling technique to demonstrate that, unlike wild-type littermates, the Purkinje cells of lurcher animals show morphology characteristic of apoptotic cell death, contain nicked nuclear DNA at the time of their death, and express a marker gene, SGP2, that is expressed in several cell types undergoing both apoptosis and programmed cell death (Collard and Griswold, 1987). The fact that these properties are only evident in the mutant animals allows us to conclude that the lurcher gene inappropriately induces apoptosis in cerebellar Purkinje cells as they mature during the first few postnatal weeks. Furthermore, analysis of the expression of the Kv3.3b potassium channel is detected in *Lc*/+ Purkinje cells prior to their death, indicating that these cells enter the final phase of their maturation prior to death. Thus, identification of the primary genetic lesion responsible for the lurcher disease and detailed characterization of the molecular mechanisms of apoptosis elicited in response to this lesion will offer the opportunity to achieve a mechanistic understanding of neurodegeneration in this inherited neurodegenerative disease. If, as we have previously argued (Heintz, 1993), activation of a common genetic pathway leading to apoptosis is involved in many instances of neurodegeneration, then definition of the molecular events that can ectopically activate this program in mature neurons is of primary importance.

MATERIALS AND METHODS

Molecular genotyping

Lurcher (B6CBA-A^{w-J}/A-*Lc*) and *Mus mus castaneus* (CAST/Ei) mice were purchased from the Jackson Laboratory and maintained at the Specific Pathogen Free facility at the Rockefeller University Laboratory Animal Research Center. Intercross progeny were generated by mating *M.m. castaneus* with B6CBA-A^{w-J}/A-*Lc* mice, selecting animals expressing the lurcher phenotype and intercrossing these mice. High molecular weight DNA was prepared and Southern blot analysis performed essentially as described (Norman et al., 1991). Restriction fragment length polymorphisms (RFLPs) were detected at the *etl-1* locus by using the restriction enzyme *Rsa*I. The labeled probe used to visualize the *etl-1* RFLP was a 331 bp fragment from the plasmid deltaH100 (kindly provided by A. Gossler) excised using the restriction enzymes *Bam*HI and *Hin*dIII. DNA fragments were gel-purified in 1% low-melting-point agarose and radiolabled directly using the random primer method originally described by Feinberg and Vogelstein (1983).

Immunocytochemistry

To demonstrate the Purkinje cell death in intercross mutant animals, genotyped postnatal day (P) 8 and P12, *Lc*/+ and normal littermates were fixed with 4% paraformaldehyde, sectioned on a cryostat at a thickness of 15 µm and processed for immunohistochemistry with anti-Calbindin antibody. After blocking with 10% normal goat serum, the sections were incubated with a 1:200 dilution of mouse monoclonal anti-Calbindin antibody (clone CL-300, Sigma) for 1 hour, washed and subsequently stained with rhodamine-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories).

Light and electron microscopy

To observe the morphology of dying Purkinje cells in lurcher mice, genotyped P12 *Lc*/+ mice and normal littermates were perfused transcardially with 3% glutaraldehyde (EM Sciences Inc.) in 0.1 M phosphate buffer under the anesthesia of 0.1 mg/g sodium pentobarbital (Abbott Laboratories). Cerebella of perfused animals were quickly dissected out and cut into 1 mm^3 pieces and postfixed in the same fixatives for at least 3 more hours. The tissues were then washed in PBS three times and bathed in 1% osmium tetraoxide (EM Sciences Inc.) in 0.1 M phosphate buffer for 30 minutes. After several washes in PBS, the tissues were dehydrated in an alcohol series and imbedded in Epon. Semi-thin sections of 0.5 µm thickness were cut from the Epon-embedded tissues, stained with methylene blue and subjected to light microscopy to observe the apoptotic bodies, the nuclear condensation, the shrinkage of dying Purkinje cells and the lack of inflammatory response in lurcher cerebellum. Ultrathin sections from selected areas were then sectioned and observed under the electron microscope to confirm the results of the light microscopy and to examine the integrity of cellular organelles.

In situ hybridization analysis

To compare SGP2 and Calbindin gene expression in lurcher mice with normal mice and to look for expression of Kv3.3b in lurcher Purkinje cells by in situ hybridization, genotyped P12 *Lc*/+ mice and their normal littermates were killed by decapitation under anesthesia of 0.1 mg/g sodium pentobarbital (Abbott Laboratories). Their cerebella were quickly dissected out and frozen immediately in OCT (Miles Inc.) on dry ice. The frozen tissues were sectioned to 15 µm on a cryostat, collected onto Vectabond-treated slides (Vector Laboratories), and fixed immediately by immersion in 4% paraformaldehyde (Polysciences Inc.) for 30 minutes. After three washes in PBS, the sections were dehydrated in an alcohol series and stored at −70°C in boxes with desiccant. Sense and anti-sense digoxigenin-labeled riboprobes of SGP2 and Calbindin were transcribed from plasmids pGEM-60HE (kind gift of Dr Steven Sylvester) and pC28B (kind gift of Dr Monique Thomasset) with T7/Sp6 and T3/T7 RNA polymerases (Promega), respectively. The Kv3.3b riboprobes were prepared as described (Goldman-Wohl et al., 1994). The probes were hydrolyzed to 200-300 base pairs by incubation in sodium carbonate buffer (pH 10) at 60°C for the appropriate time. The hybridization was performed according to the Genius kit and as described (Kuhar et al., 1993; Feng et al., 1994) in 0.3 M NaCl, 50% formamide and 10% dextran sulfate at 60°C for 16 hours. The final wash stringency was 0.2× SSC at 60°C for 1 hour. The hybridized sections were incubated for 3 hours with alkaline phosphate-conjugated sheep anti-digoxigenin Fab′ antibody at 1:500, washed and processed for colorimetric detection using NBT and X-phosphate.

In situ labeling for DNA fragmentation

Semithin sagittal sections of fixed cerebella from lurcher and wild-type littermates at P12 were treated with 1 µg/ml Proteinase K (Boehringer Mannheim Biochemicals (BMB)) for 15 minutes at room temperature then washed in PBS for 5 minutes four times. Each section was treated with 1 unit DNA polymerase 1 (BMB) in the presence of digoxigenin-labeled nucleotides (0.5 µl of digoxigenin labeling mixture from BMB) at 37°C for 60 minutes in a humidified chamber. The sections were washed in TB buffer (300 mM NaCl and 30 mM sodium citrate) for 15 minutes, then rinsed briefly in two changes of PBS. Sections were incubated with 10% normal goat serum in PBS for 30 minutes, then in the same solution plus a 1:500 dilution of anti-mouse Calbindin primary antibody (Sigma) for 40 minutes. Sections were washed four times for 5 minutes each with PBS then incubated with FITC-conjugated anti-digoxigenin antibody and rhodamine-conjugated goat-anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) for one hour at room temperature. Sections were washed extensively in PBS and examined by epifluorescence microscopy using a Nikon Optiphot microscope.

RESULTS

Molecular genotyping of neonatal animals

Studying cerebella of young lurcher animals can be problematic because the obvious behavioral phenotype characteristic of this mutant mouse strain does not become apparent until P14, when normal mice lose their typical juvenile unsteadiness. Historically, this problem has been addressed by typing lurcher animals using linkage between *Lc* and another mutation on mouse chromosome six, micropthalmia-white (Mi^{wh}), which shows a distinctive coat color. There are two potential problems with this method of genotyping. First, the *Lc* and Mi^{wh} loci will remain linked in only 85-90% of the animals born, because they are separated by more than 10 centiMorgans (cM). Therefore, as many as 15% of the animals would be mis-typed using this method. Second, there is a chance that introducing a second mutation could interfere with the action of the *Lc* gene, because the Mi^{wh} phenotype is more complex than simply a coat color change (Lyon and Searle, 1989) and includes, for example, micropthalmia, abnormalities of the inner ear and smaller numbers of certain cells derived from the neural crest including spinal ganglia, adrenal medulla and dermis. It is possible that expression of the Mi^{wh} gene in lurcher animals could confound analysis of the lurcher phenotype.

Use of a molecular marker known to be tightly linked to the *Lc* gene, in an intrasubspecific *Lc*/+ × *M.m. castaneus* intercross, enables one to accurately genotype lurcher animals at any age, including embryonically. Thus, an intersubspecific, phenotypic backcross was generated by crossing B6CBA-A^{w-J}/A-*Lc* and *M.m. castaneus* mice, selecting progeny expressing the lurcher phenotype, and intercrossing these F_1 animals. A total of 83 of these animals were collected at different ages and typed by Southern analysis using an RFLP previously identified for the molecular marker *etl-1* (Gossler, personal communication) as shown in Fig. 1. This marker maps very close to *Lc*, as shown by RFLP analysis of more than 500 animals of an intersubspecific, phenotypic backcross (Norman et al., 1991 and our unpublished data). In this previous analysis, two recombination events between *etl-1* and *Lc* were detected in 443 animals studied, giving a calculated genetic distance of 0.45±0.23 cM between these two markers. This marker can

Fig. 1. Representative Southern hybridization analysis of mouse genomic DNA to distinguish between lurcher and wild-type animals. The restriction fragment length polymorphism between *Lc*/+ and +/+ animals, when the DNA is digested with the restriction enzyme *Rsa*I and hybridized to the *Etl-1* probe, is shown. The genotype of all the animals used in this work was determined using this method.

therefore be used to genotype animals at any age with an accuracy of greater than 99%. In the 83 animals collected from this cross and typed molecularly, the normal 1:2:1 ratio of homozygous normal, heterozygous and homozygous mutant animals: (21: 44: 18) was observed. As expected, all genotyped *Lc*/+ animals from this cross exhibited the typical *Lc* phenotype if allowed to mature, or displayed characteristic Purkinje cell loss when examined histologically. Penetrance of the *Lc* phenotype on this intercross, therefore, was 100%.

Calbindin staining of cerebella from intercross animals

To demonstrate the loss of Purkinje cells in lurcher cerebella, we stained P8 and P12 heterozygous lurcher and normal littermate cerebella with a primary antibody directed against the Purkinje cell marker Calbindin D28. As can be seen in Fig. 2, there is little difference between normal and lurcher cerebella up until the end of the first postnatal week. However, 4 days later, obvious gaps in the Purkinje cell layer can be seen (compare Fig. 2C,D). By this time, the secondary dendrites of Purkinje cells in normal mice have elaborated and the molecular layer has grown thicker. In contrast, the dendrites of Purkinje cells in lurcher mice remain stunted and the molecular layer is much thinner compared to their normal littermates (note that Fig. 2C,D are of the same magnification). In addition, numerous swellings on the axons of Purkinje cells in

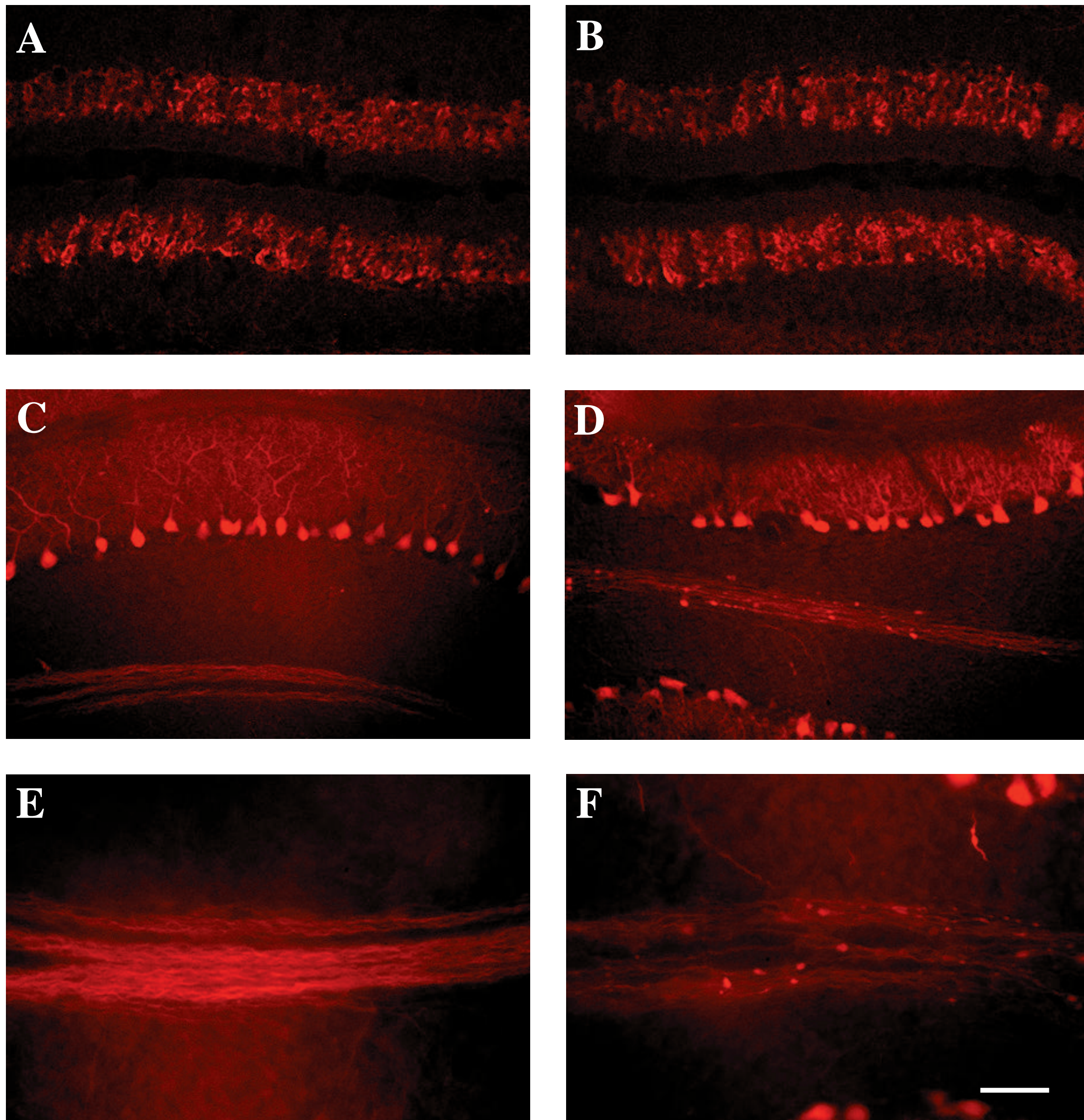

Fig. 2. Immunostaining of sagittal sections of cerebella of lurcher and wild-type littermates with a monoclonal antibody raised against the Purkinje cell marker Calbindin. (AB) P8 normal and mutant cerebella, respectively; at this stage they appear indistinguishable. (D) The extensive degeneration of *Lc*/+ Purkinje cells at P12 as compared to a normal littermate (C). (E,F) An axon tract from a normal and mutant cerebella, respectively, revealing both varicosities and blebbing in Purkinje axons from the mutant animals. The scale bar represents 30 μm in A-D and 15 μm in E and F.

the internal granule layer (IGL) and white matter can be seen in the lurcher cerebellum but are absent in normal mice (Fig. 2E,F). This axon-beading phenomenon was first observed by Cajal (reviewed in DeFelipe and Jones, 1991) in Purkinje cell axons that had lost contact with their targets and undergone self-destruction. Recent studies on PC12 cells after withdrawal of nerve growth factor (NGF), have related this phenomenon to the initial steps of programmed cell death (Pitmann et al., 1993). This immunohistochemical study confirms previous observations that lurcher Purkinje cells die rapidly during the

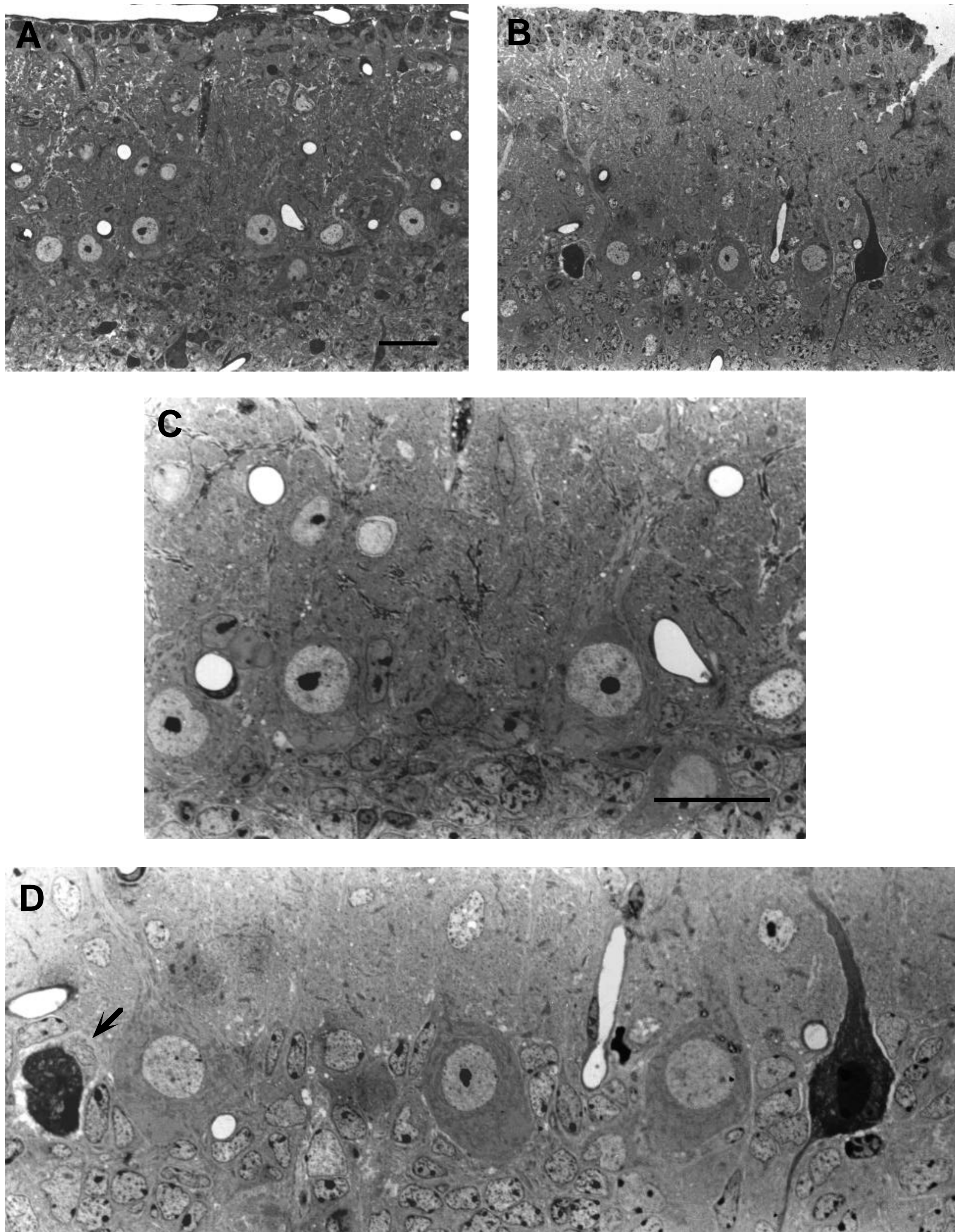

Fig. 3. Methylene-blue-stained light micrographs of P12 cerebella from *Lc* mice and their normal littermates. (A,C) The typical morphology of normal Purkinje cells with lightly stained and round nuclei. In *Lc* cerebella (B), gaps can be seen in the Purkinje cell layer. Some of the remaining Purkinje cells show characteristics of apoptotic cells, i.e., reduced cell size, condensed nuclei and irregular cytoplasmic and nuclear membranes (D). Occasionally, an apoptotic body can be seen in the Purkinje cell layer, engulfed by a cell (arrow) with glial characteristics, such as a lightly staining and convoluted nucleus. Scale bars represent 50 µm (A,B) and 20 µm (C,D).

second postnatal week (Wetts and Herrup, 1982a,b), and that they exhibit axon beading, which is potentially an early sign of active cell suicide.

Morphological analysis of lurcher Purkinje cells

To examine the mode of death of Purkinje cells in lurcher mice, we first looked for the morphological hallmarks of apoptosis: nuclear condensation, membrane blebbing, intact cellular organelles, lack of inflammatory response and apoptotic bodies in phagocytes. P12 lurcher cerebella (n=11) were fixed and processed for microscopy. Semi-thin sections (0.5 μm) were stained with methylene blue and subjected to light microscopy. In the normal controls (n=7), all Purkinje cells appear lightly stained with fine chromatin and smooth nuclear and cytoplasmic membranes (Fig. 3A,C). In contrast, large darkly stained cells with condensed nuclei are detected in the Purkinje cell layer in lurcher animals, separated by several normal-looking Purkinje cells (Fig. 3B,D). Their large size, pyramidal shape and location in the Purkinje cell layer with apical dendrites in the molecular layer definitively identify them as Purkinje cells. A closer examination further demonstrates the irregularity of their cytoplasmic membranes (Fig. 3D). In some cases, remnants of these darkly stained cells, resembling apoptotic bodies, can be seen being engulfed by other cells with the morphological features of glial cells (Fig. 3D, arrow), such as a lightly stained and convoluted nuclear shape. We did not see lysis of tissues in the Purkinje cell layer or infiltration of white blood cells in any of the samples examined. Therefore, at the light microscopic level, we observed characteristic morphological features of apoptosis in lurcher Purkinje cells and saw no indication of necrosis.

To further confirm these observations, we cut ultrathin sections of selected areas and subjected them to electron microscopy. As shown in Fig. 4B, the nuclear chromatin of the dying lurcher Purkinje cells is markedly condensed as compared with the normal cells seen in Fig. 4A, and cell and nuclear membrane blebbing is obvious in the lurcher but not wild-type Purkinje cells. Glial cell processes (Fig. 4B, arrowheads) can be seen wrapping around the dying Purkinje cell, ready to engulf its remnants. In contrast, the cellular organelles, including Golgi apparatus and mitochondria are relatively normal. These ultrastructural characteristics of dying Purkinje cells are consistent with the light microscopic observations, indicating that lurcher Purkinje cells are dying by apoptosis.

DNA fragmentation in lurcher Purkinje cells

A second feature associated with apoptosis is the specific

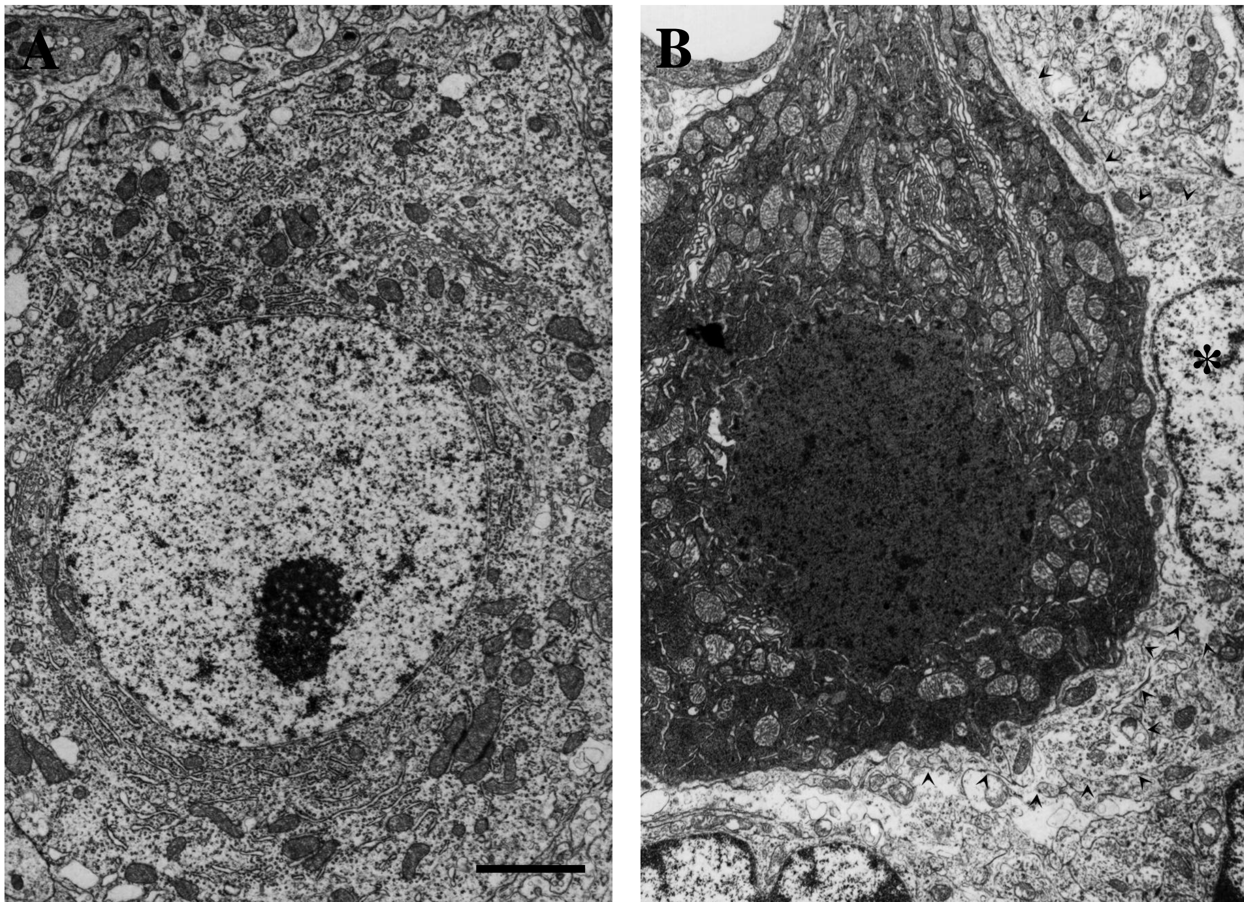

Fig. 4. Electron micrographs of normal and *Lc* Purkinje cells. Comparing normal (A) and dying *Lc* Purkinje cells (B), the apoptotic features of the dying cells are clearly recognizable, including a condensed nucleus, reduced size, membrane blebbing and relatively normal organelles. A glial cell, identified by its light nucleus (asterisk), can be seen wrapping its processes (arrowheads) around the dying Purkinje cell, ready to engulf its remnants. The scale bar represents 3 μm.

degradation of DNA into oligonucleosomal-sized fragments. Generally, this fragmentation is detected by preparation of high molecular weight genomic DNA and visualization by ethidium bromide staining of a DNA ladder, after electrophoresis through an agarose gel. We used this technique to examine the DNA of dying Purkinje neurons in cerebella of lurcher mutant mice, but saw no DNA laddering. This may be due to the relatively small number of Purkinje cells in the cerebellum and the small percentage of these neurons that might be dying at any given time. Several types of more sensitive methodology to detect fragmented DNA have recently been published (Gavrieli et al., 1992; Wood et al., 1993). Fig. 5 shows a representative experiment where the DNA modifying enzyme DNA polymerase I is used to incorporate digoxigenin-labeled dUTP at any sites of cleaved or nicked DNA. The digoxigenin incorporation is visualized using a flourescein-conjugated anti-digoxigenin antibody, and the sections are double-labeled with a monoclonal antibody against the Purkinje cell marker Calbindin, detected using a rhodamine-conjugated secondary antibody. Labeling of the nuclei of a subset of lurcher, but not normal, Purkinje cells is evident. These results demonstrate the presence of nicked DNA in *Lc* Purkinje cells prior to their death as a result of the mutation, again indicating that these cells are dying by apoptosis.

In one of the sections from a wild-type cerebellum (Fig. 5A) some faint staining is visible in a position corresponding to the external granular layer (EGL). This may be due either to the presence of proliferating granule cells labeled during DNA replication, or to the presence of granule cells still undergoing developmental cell death in the EGL. Although in *Lc* massive secondary degeneration of granule and other neurons occurs, we would not expect to see substantial labeling of these cells by this assay at these early time points, because massive secondary granule cell death occurs much later, only after the majority of *Lc* Purkinje cells have died (Caddy and Biscoe, 1976). We have not addressed the issue of whether the secondary neuronal loss in *Lc* animals seen in the internal granule layer and inferior olive occurs by apoptosis because loss of these populations is not a direct effect of the *Lc* mutation (Wetts and Herrup, 1982a,b).

Expression of SGP2 in lurcher Purkinje cells

Apoptosis has generally been defined by its characteristic morphological features and the presence of DNA fragmentation. However, a number of genes have now been described which are thought to regulate or be involved in the genetic pathway(s) of apoptosis. These genes can therefore be used as markers to detect apoptosis and as molecular tools to begin to understand

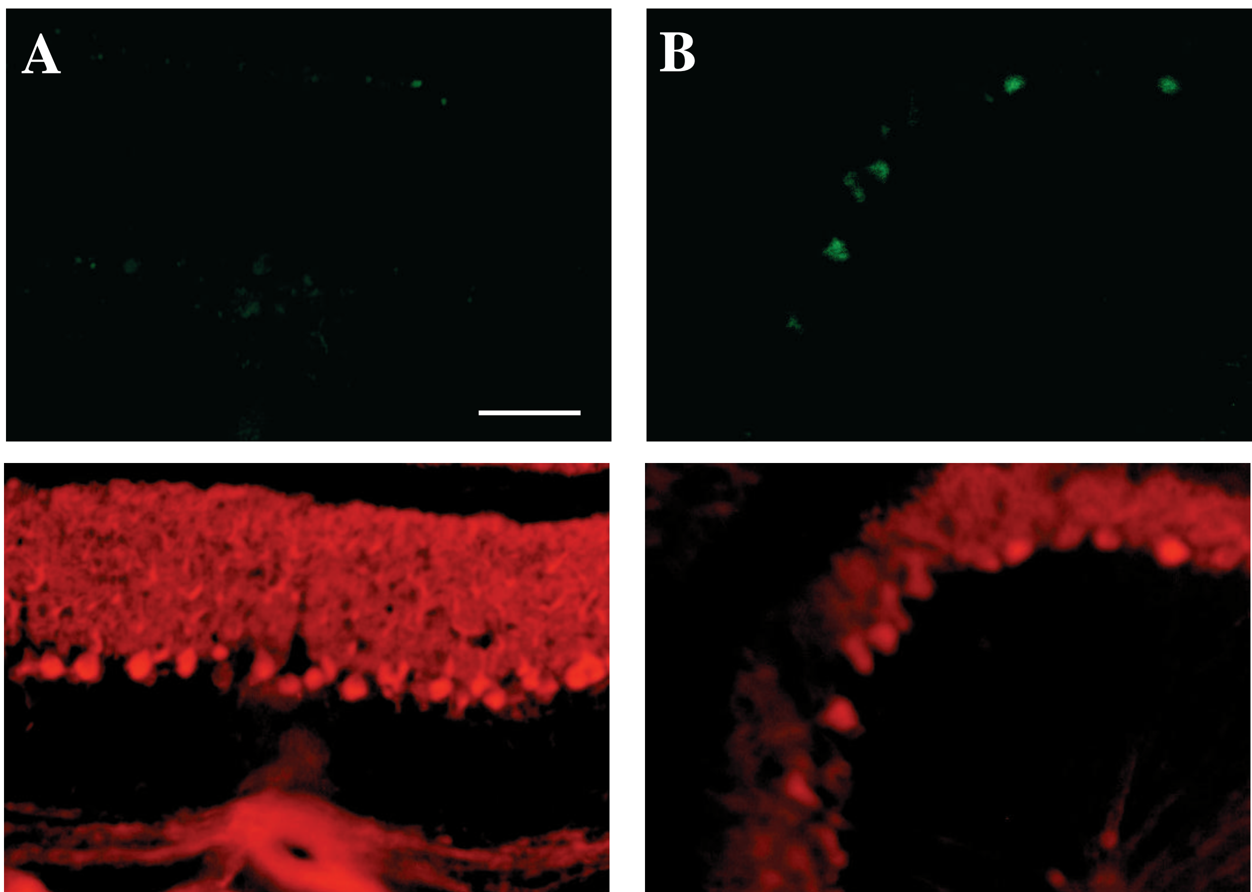

Fig. 5. In situ labeling of free 3′ hydroxyl groups using unmodified DNA Polymerase I. (B) The presence of fragmented DNA in Purkinje cells of a *Lc*/+ cerebellum at P12. (A) A similar section from a normal littermate in which no signal is found in the Purkinje cell layer. D (*Lc*/+) and C (+/+) show double-labeling of the same sections with the Purkinje cell marker Calbindin, showing the positions of the Purkinje cell bodies. The scale bar represents 30 μm.

the process itself. SGP2 is an acidic glycoprotein secreted constitutively by Sertoli cells which has received attention as a molecule shown to be upregulated in some cells undergoing apoptosis and programmed cell death. Thus, SGP2 is induced in regressing prostate after castration, in apoptotic thymocytes induced by glucocorticoid treatment, and in regressing interdigital epithelium during normal limb development (Leger et al., 1987; Bettuzi et al., 1989, 1991). To provide a third and independent line of evidence that *Lc* Purkinje cells are dying by apoptosis, we have assayed for the expression of SGP-2 in *Lc* Purkinje cells. Fig. 6 shows in situ hybridization analysis of cerebellar tissue from lurcher and wild-type littermates at P12, probed with sense or anti-sense riboprobes transcribed from an SGP2 cDNA clone. As is evident from this figure, SGP2 mRNA is expressed at high levels in Purkinje cells of *Lc* mutant animals, but not their wild-type littermates. Since we have not detected SGP2 mRNA in Purkinje cells of wild-type animals at any time postnatally, we conclude that the lurcher gene can activate an ectopic program of gene expression shown to be activated in several non-neuronal systems undergoing apoptosis and programmed cell death.

Expression of Kv3.3b in lurcher Purkinje cells

To determine the extent of maturation attained by Purkinje cells prior to their death, we have examined *Lc* Purkinje cells for expression of a molecular marker for terminal differentiation known as Kv3.3b (Goldman-Wohl et al., 1994). The Kv3.3b gene encodes a *Shaw*-type potassium channel that is first expressed in Purkinje cells between P8 and P10, remaining on throughout adulthood. This Potassium channel is the latest molecular marker for Purkinje cell maturation thus far identified. Transcription of this gene is, therefore, the best molecular marker for Purkinje cell maturation, correlating with synaptogenesis and mature physiologic function (Goldman-Wohl et al., 1994). As shown in Fig. 7, *Lc* Purkinje cells express the Kv3.3b ion channel prior to their death, demonstrating that *Lc* Purkinje cells synthesize the latest known molecular marker in their differentiation pathway prior to death, and suggesting that *Lc* Purkinje cells may attain mature physiologic characteristics prior to the induction of apoptosis as a consequence of the mutation.

DISCUSSION

Apoptosis occurs throughout nature in normal development and disease. In this paper, we present three lines of evidence that the Purkinje cell degeneration that is apparent in the neurologically mutant mouse lurcher occurs through apoptosis: *Lc* Purkinje cells exhibit the morphologic features of cells dying by apoptosis; they contain nicked nuclear DNA prior to their death in the second postnatal week and they express a molecular marker known to be induced in several well-characterized cases of apoptosis in non-neuronal tissues. That these properties are present in *Lc* animals and not their normal littermates, was definitively demonstrated by molecular genotyping of the individual animals examined in this study. We

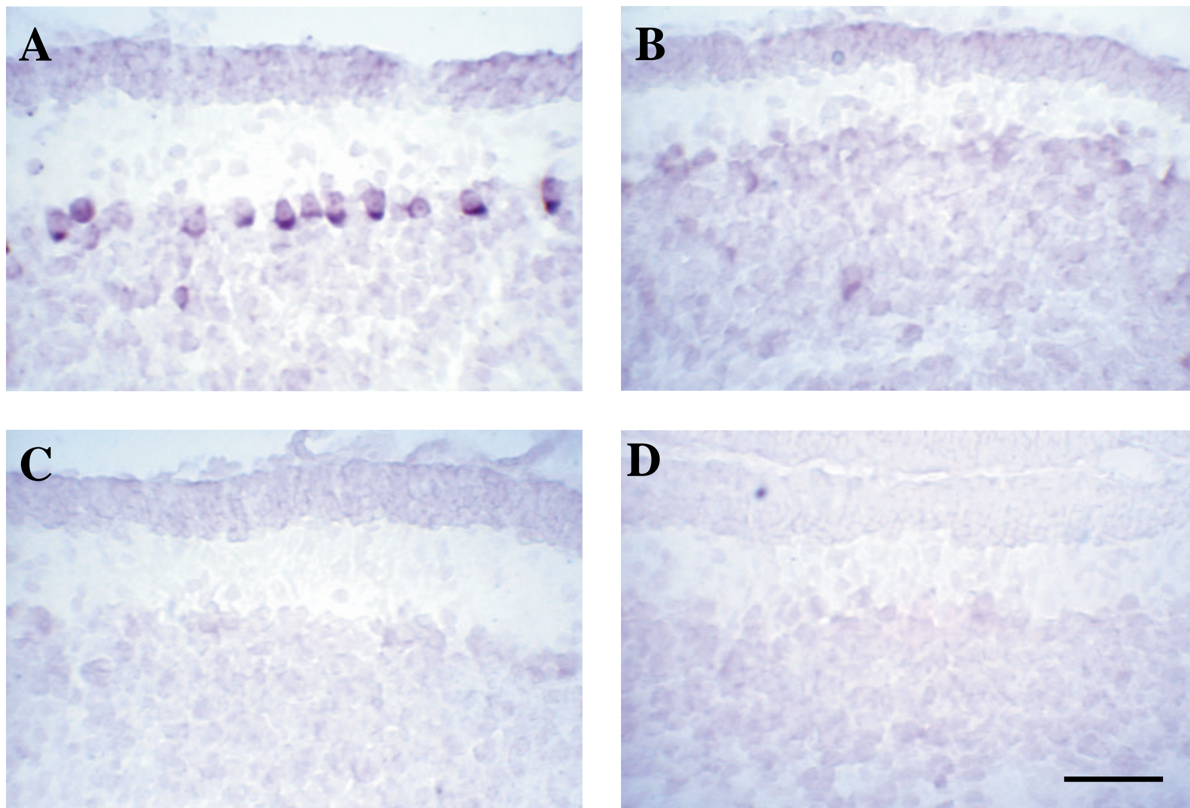

Fig. 6. In situ hybridization of SGP2 to *Lc* and normal cerebella. Sagittal sections from mutant (A, C) and normal (B, D) cerebella at P12 were hybridized to anti-sense (A,B) and sense (C,D) probes for SGP2. The anti-sense probe specifically stains *Lc* Purkinje cells, which are destined to die over the next few days. The scale bar represents 30 μm.

conclude that the *Lc* gene induces apoptosis in mature Purkinje cells in heterozygous animals. The critical issue that remains to be investigated is the nature of the molecular mechanisms that connect the *Lc* gene to activation of the apoptotic pathway. We are currently pursuing two avenues toward addressing this issue. First, we are continuing our efforts to positionally clone the *Lc* mutation and have begun detailed physical mapping of the *Lc* locus and characterization of YAC clones covering the *Lc* genetic interval to achieve that goal. Second, we have begun to analyze DNA sequences necessary for activation of SGP2 transcription in response to the *Lc* mutation. We believe that a combination of these approaches will be instrumental in understanding neurodegeneration in *Lc* mutant mice and that data from these studies can lead to a more complete understanding of the molecular events controlling induction of apoptosis in this neurodegenerative disease.

A reasonable question that remains to be answered concerns the generality of apoptosis as a mechanism for neuronal cell loss in neurodegenerative disease. Although criteria for establishing the mechanism of cell death during neurodegeneration are not universally agreed upon, evidence that induction of apoptosis is the mechanism for cell loss has been presented in several cases. For example, Wong and collaborators have provided convincing evidence that loss of photoreceptor cells in the retina of *rd*, *rds* and Rhodopsin mutant mice occurs through apoptosis. They have also cited preliminary evidence to support the involvement of apoptosis in human retinal degeneration (Chang et al., 1993). In some forms of human macular degeneration, morphologic evidence has been presented that may also implicate apoptosis in the observed cell loss (Burns and Feeney-Burns, 1980).

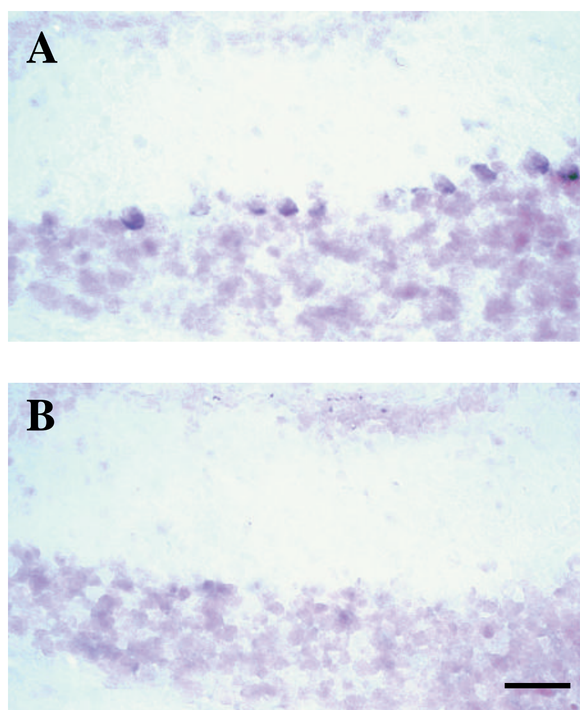

Fig. 7. In situ hybridization of Kv3.3b to a *Lc* cerebellum. Sagittal sections from a *Lc* cerebellum at P12 were hybridized to anti-sense (A) and sense (B) probes for Kv3.3b. Darkly staining Purkinje nuclei are visible in A while the control probe does not hybridize. The scale bar represents 20 μm.

Elevated levels of SGP2 expression in patients with Alzheimers (May et al., 1990) and Pick's (Duguid et al., 1989) diseases may also implicate apoptosis as an operant mechanism in those diseases. These data, taken together with our present findings, strongly suggest that mutations in a range of unrelated genes can result in cell loss by the common mechanism of apoptosis. If these results can be extended to other neurodegenerative disorders, then detailed studies of the mechanisms for activating apoptosis in individual diseases will be crucial for identification of common underlying pathways contributing to pathogenic activation of apoptosis.

While many programmed deaths during development have been shown to occur by apoptosis, it is still not clear whether programmed cell death occurs by similar or different mechanisms to the pathological induction of apoptosis. Thus it is possible that all cases of programmed cell death and apoptosis represent the same genetic pathway merely differing in the nature of the inducing signal, or perhaps at the molecular level many different pathways exist. The finding that SGP-2 expression is a common feature in some cases of apoptosis and programmed cell death may suggest the former model. A knowledge of the genetic pathways involved will be essential to resolve these questions. It may also be possible to identify the points at which aberrant stimuli can activate the cell death program and to determine whether such activations represent reactivation of a mechanism that was previously essential to the development of that cell type.

An important consideration concerning the mechanism of action of the *Lc* gene is the timing of Purkinje cell death. It has been proposed that the lurcher gene acts by causing degeneration of Purkinje cells after they terminally differentiate rather than causing a disruption in this pathway that secondarily induces apoptosis (Messer et al., 1991; Heintz et al., 1993). Messer et al. (1991) have shown that the staggerer mutation, which blocks normal differentiation of Purkinje cells, rescues the lurcher phenotype suggesting that the differentiation of Purkinje cells is in fact essential for the lurcher gene to exert its pathological effect. This result is consistent with the earlier work of Wuenschell et al. (1990) and Messer et al. (1990) who have shown the expression of the genes glutamic acid decarboxylase (GAD), calbindin and calmodulin in lurcher Purkinje cells and with the work of Dumesnil-Bousez and Sotelo (1992), who showed that the early phases of Purkinje cell synaptogenesis are normal in *Lc* mice. In addition, Tano et al. (1992) have shown that the expression pattern of the zebrin antigen develops normally in *Lc* until Purkinje cell death begins. This molecule is expressed by specific subsets of Purkinje cells in a compartmental fashion. Our finding that an even later marker of differentiation, the ion channel Kv3.3b, is also expressed in *Lc* Purkinje cells strengthens the idea that these cells may require maturation to die as a consequence of this mutation. In a separate study (Heckroth et al., 1990), it was shown that olivocerebellar fibers ascend normally in to *Lc* cerebellar until P9, but that they fail to climb away from the Purkinje cells and enter the molecular layer, as they should normally. This data has been interpreted to suggest that the *Lc* gene does indeed disrupt the normal development of Purkinje cells such that they no longer provide appropriate signals for the maturation of the climbing fibers. However, given that this failure occurs just as the Purkinje cells begin to die, it seems probable that this is yet another secondary effect of the *Lc*

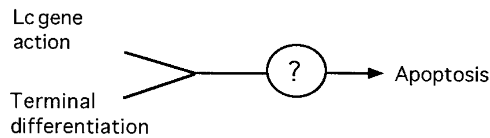

Fig. 8. Summary of the steps involved in neurodegeneration in *Lc*. *Lc* gene action requires prior differentiation of Purkinje cells to elicit apoptosis by an as yet unknown mechanism. Uncovering the details of this process may reveal important common themes for other neurodegenerative disorders that appear to fit into this scheme.

mutation that is observed due to the ensuing Purkinje cell death.

The factors contributing to neurodegeneration in *Lc* mice have been summarized in Fig. 8. We believe that current evidence suggests that a single dose of the *Lc* gene can induce apoptosis in cerebellar Purkinje cells only after their maturation. In this case, the timing of Purkinje cell loss in this disease is both a function of the particular *Lc* molecular lesion and contributions from signals received only by mature Purkinje cells. This type of model is consistent with studies on a number of *Drosophila* retinal degeneration mutants (Steele et al., 1992), where it has been shown that death of the photoreceptors requires prior light stimulation. The *Drosophila* precedent, and the requirement for maturation of *Lc* Purkinje cells prior to the induction of apoptosis, raise the intriguing possibility that the timing of cell loss in inherited neurodegenerative disease may reflect both the nature of the particular genetic lesion and the physiologic activity of the particular neuronal classes affected.

We have previously offered a speculative hypothesis suggesting that induction of apoptosis in terminally differentiated neurons represents the activation of a preexisting pathway of programmed cell death in these cells that could result from rather diverse perturbations in signal transduction akin to those that activate cell growth during the course of transformation (Heintz, 1993). We further speculated that the mechanisms regulating cell growth and programmed cell death might be quite similar, although the effector pathways activated by these mechanisms are obviously very different. The present demonstration that the *Lc* gene induces apoptosis of Purkinje cells provides an opportunity to test these ideas and to evaluate the significance of other regulators of apoptosis (Barinaga, 1994) in the context of a very well characterized and experimentally accessible neurodegenerative disease.

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