The Journal of Neuroscience, May 15, 2000, *20*(10):3687–3694

Neurodegeneration in *Lurcher* Mice Occurs via Multiple Cell Death Pathways

Martin L. Doughty,[1] **Philip L. De Jager,**[1] **Stanley J. Korsmeyer,**[3] **and Nathaniel Heintz**[1,2]

[1]*Laboratory of Molecular Biology and* [2]*Howard Hughes Medical Institute, The Rockefeller University, New York, New York 10021, and* [3]*Harvard Medical School, Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts 02115*

Lurcher (*Lc*) is a gain-of-function mutation in the δ2 glutamate receptor (GRID2) that results in the cell-autonomous death of cerebellar Purkinje cells in heterozygous *lurcher* (+/*Lc*) mice. This in turn triggers the massive loss of afferent granule cells during the first few postnatal weeks. Evidence suggests that the death of Purkinje cells as a direct consequence of GRID2^{Lc} activation and the secondary death of granule cells because of target deprivation occur by apoptosis. We have used mice carrying *null* mutations of both the *Bax* and *p53* genes to examine the roles of these genes in cell loss in *lurcher* animals. The absence of *Bax* delayed Purkinje cell death in response to the *GRID2^{Lc}* mutation and permanently rescued the secondary death of granule cells. In contrast, the p53 deletion had no effect on either cell death pathway. Our results demonstrate that target deprivation induces a *Bax*-dependent, *p53*-independent cell death response in cerebellar granule cells *in vivo.* In contrast, *Bax* plays a minor role in GRID2^{Lc}-mediated Purkinje cell death.

Key words: lurcher*; cerebellum;* Bax*;* p53*; caspase-3; apoptosis*

Evidence supporting a role for apoptosis in mammalian neurodegenerative diseases has arisen from cell culture models of neuronal cell death [for review, see Lee et al. (1999)] and from descriptive studies documenting the activation of developmental cell death genes in neurological disorders [for review, see Heintz and Zoghbi (2000)]. Several issues have arisen from these studies that require direct investigation in well characterized models of neurodegeneration in vivo. For example, studies of cell death in a variety of *in vitro* culture systems (Choi, 1994; Deshmukh and Johnson, 1998; Lee et al., 1999), as well as the investigation of developmental cell death in the nervous system *in vivo* (Pettmann and Henderson, 1998), have established both that different molecules can participate in apoptosis in distinct cell types and that disparate stimuli can activate distinct cell death pathways in a single cell type. Descriptive studies of both human and mouse neurodegeneration *in vivo* reflect the complex picture that has arisen from these investigations and support the general idea that a wide variety of abnormal stimuli can result in the activation of the effector phase of apoptosis in neurons (Green, 1998; Los et al., 1999; Vaux and Korsmeyer, 1999). These studies have also demonstrated a direct role for specific proteins in neuronal cell death *in vitro* or *in vivo*. Thus, analysis of cell death in *Bax null* mutant mice has established a direct role in neuronal cell death in response to trophic factor withdrawal (Deckwerth et al., 1996) and during the normal development of a variety of brain structures (White et al., 1998). A role for *p53* in both radiation-induced neuronal cell death *in vivo* (Woods and Youle, 1995) and excitotoxic cell death *in vitro* (Xiang et al., 1998) has also been demonstrated. These studies provide a foundation for the investigation of inherited neurodegenerative disease.

Lurcher (*Lc*) is a semidominant mouse mutation that results in ataxia because of massive loss of cerebellar neurons during the first 4 postnatal weeks (Caddy and Biscoe, 1979). The *lurcher* mutation results in the activation of the δ2 glutamate receptor (GRID2) in cerebellar Purkinje cells, leading to a constitutive, inward current (Zuo et al., 1997). Death of Purkinje cells in *lurcher* animals is cell autonomous (Wetts and Herrup, 1982a,b) and arises as a direct consequence of the increased activity of the receptor, because *null* mutations of *GRID2* do not result in Purkinje cell death (Kashiwabuchi et al., 1995). Descriptive studies of cerebellar degeneration in *lurcher* mice suggest that Purkinje cell death occurs by apoptosis (Norman et al., 1995; Wullner et al., 1995). This is consistent with the elevated expression of *Bax*, *Bcl-X* (De Jager, 1998; Wullner et al., 1998), and *procaspase-3* (Selimi et al., 2000) in postnatal *lurcher* Purkinje cells and with the increased levels of active caspase-3 (Heintz and De Jager, 1999) and DNA nicking (Norman et al., 1995) in the small percentage of these cells that have entered the effector phase of the cell death pathway. The death of granule cells and inferior olivary neurons in *lurcher* animals is secondary to the death of Purkinje cells and is presumably caused by the loss of Purkinje cells as targets for these two neuronal populations.

Because of the elevated level of *Bax* expression observed in *lurcher* animals (Wullner et al., 1995; De Jager, 1998) and the evidence supporting a role for *p53* in *Bax*-mediated neuronal cell death in response to both genotoxic and excitotoxic activity in neurons (Xiang et al., 1998), we have examined the roles of these genes in neurodegeneration in the *lurcher* cerebellum. We report here that Purkinje cell death in +/*Lc:Bax*$^{-/-}$ mice is delayed relative to that in *Bax*-expressing +/*Lc* littermates and that granule cell death is permanently rescued in these animals. Examina-

---

Received Dec. 15, 1999; revised Feb. 15, 2000; accepted March 2, 2000.

This work was supported by the Howard Hughes Medical Institute (N.H.), the National Institutes of Health (NIH)—National Institute of Neurological Disorders and Stroke Program Project PHS NS 30532 (N.H. and M.L.D.), and the NIH—National Institute of General Medical Sciences Grant GM 07739 (P.L.D.J.).

Correspondence should be addressed to Dr. Nathaniel Heintz, Laboratory of Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021. E-mail: heintz@rockvax.rockefeller.edu.

Dr. De Jager's present address: Department of Medicine, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215.

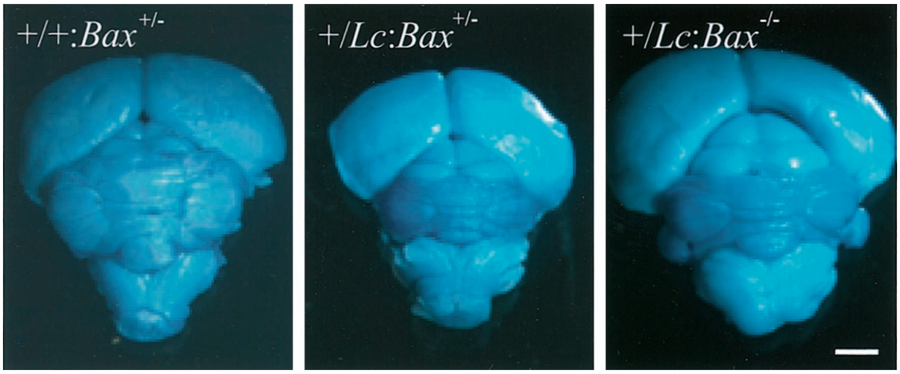

Figure 1. *Bax* deletion reduces +/*Lc* cerebellar atrophy. Posterior view of the whole cerebellum lightly stained with cresyl violet. Note the larger lobules and more pronounced fissure of the +/*Lc*: *Bax*$^{-/-}$ compared with the +/*Lc*:*Bax*$^{+/-}$ cerebellum. Scale bar, 1 mm.

tion of +/*Lc*:*p53*$^{-/-}$ mice revealed no alteration in cell death. These results demonstrate that granule cell death as a result of target deprivation *in vivo* occurs via a *Bax*-dependent, *p53*-independent pathway and that *Bax* plays a minor role in cell death induced by the constitutive activation of the δ2 glutamate receptor in cerebellar Purkinje cells. Finally, in contrast to *Bax*-expressing +/*Lc* littermates, we observed an absence of active caspase-3 in the Purkinje cells of +/*Lc*:*Bax*$^{-/-}$, indicating the activation of a *Bax*-independent, caspase-3-independent death pathway in these cells.

MATERIALS AND METHODS

Breeding and genotyping of mice. Mice (C57BL/6) heterozygous for *Bax* (Knudson et al., 1995) were mated with Balb/C *lurcher*^{J} (+/*Lc*^{J}) mice to obtain F1 +/*Lc*:*Bax*$^{+/-}$ offspring. +/*Lc*:*Bax*$^{+/-}$ mice were crossed to generate +/*Lc* and wild-type (+/+) mice that were either *Bax*$^{-/-}$, *Bax*$^{+/-}$, or *Bax*$^{+/+}$. The mice were genotyped for *Bax* by single PCR from the wild-type *Bax* allele and from the *neo* cassette using DNA prepared from tails (Qiagen, Hilden, Germany). A region of the wild-type *Bax* allele was amplified using an exon 2 forward primer (5′-CTTGG-GAGAAGAACAACACTGC-3′) and an intron 3 reverse primer (5′-CTGAACAGATCATGAAGACAGG-3′). A region of the *neo* cassette was amplified using the forward 5′-GATTGCACGCAGGTTCTCCG-3′ and reverse 5′-CCTGGCGAACAGTTCGGCTGG-3′ primers. The mice were identified as +/*Lc* by their ataxia. The +/*Lc* genotype was sometimes confirmed by single PCR from the *Lc* allele of *GRID2* (*GRID2*^{Lc}) using an intron 2 forward primer (5′-GCACTGAATGTGTATGACTTCAG-3′) and an exon B reverse primer (5′-GGTGATAGTGAGGAAAGT-3′).

Mice (129S3) heterozygous for *p53* (*Trp53*^{tm1Tyj}; The Jackson Laboratory, Bar Harbor, ME) were mated with Balb/C +/*Lc*^{J} mice to obtain F1 +/*Lc*:*p53*$^{+/-}$ offspring. +/*Lc*:*p53*$^{+/-}$ mice were crossed to generate +/*Lc* and +/+ mice that were either *p53*$^{-/-}$, *p53*$^{+/-}$, or *p53*$^{+/+}$. The mice were genotyped for *p53* by single PCR from the wild-type *p53* allele and from the *neo* cassette using DNA prepared from tails (Qiagen). A region of the wild-type *p53* allele was amplified using an exon 6 forward primer (5′-CCCGAGTATCTGGAAGACAG-3′) and an exon 7 reverse primer (5′-ATAGGTCGCCGGTTCAT-3′). The *neo* cassette was amplified using the same *neo* primers used for the *Bax* genotyping. The mice were identified as +/*Lc* as described above.

Histology and immunocytochemistry. Mice killed for cresyl violet staining and calbindin immunocytochemistry were deeply anesthetized with sodium pentobarbital and perfused intracardially with 0.9% NaCl followed by 95% ethanol. The brains were post-fixed in 75% ethanol and 25% acetic acid, dehydrated, and embedded in paraffin. Midsagittal cerebellar sections 10 μm thick were cut, mounted on slides, and stained with cresyl violet or incubated overnight at 4°C in a 1:400 dilution of mouse monoclonal anti-calbindin antibodies (Sigma, St. Louis, MO). The immunolabeling was revealed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and DAB (Sigma).

Mice killed for anti-active caspase-3 immunocytochemistry were deeply anesthetized with sodium pentobarbital and decapitated, and the brain was frozen in ornithine carbamyl transferase compound (Sakura) in an acetone bath at −20°C. The brains were stored at −80°C until use. Sagittal cerebellar sections 10 μm thick were then cut, mounted on slides, fixed in acetone at −20°C, washed in 0.03% H_2O_2 to block endogenous peroxidases, and incubated overnight at 4°C in a 1:500 dilution of polyclonal anti-active caspase-3 antibodies (PharMingen, San Diego, CA). Immunolabeling was revealed as described above.

Cell counts. The number of granule cells per midsagittal cerebellar section was estimated from the total area and granule cell density of the internal granule cell layer (IGL). The area of the IGL was measured from an image captured on a video graphics card using NIH Image software and a CCD video camera attached to a Nikon microscope at 20× magnification. The IGL of the captured image was outlined freehand, and the area enclosed was measured using NIH Image software. The IGL cell density was estimated from the number of granule cells enclosed in a 3600 μm^2 area defined by an ocular graticule at 1000× magnification. Granule cell counts were taken from the posterior, mid, and anterior cerebellum and used to calculate an average density.

Purkinje cell counts were performed on calbindin-immunostained midsagittal cerebellar sections. The total number of immunostained cell bodies in the Purkinje cell layer (PCL) and molecular layer (ML) of the section was counted at 200× magnification using Nomarski optics.

All percentages of adult wild-type cell numbers quoted in the text are calculated from the +/+:*Bax*$^{+/-}$ value.

RESULTS

Bax deletion reduces *lurcher* cerebellar atrophy

The increased expression of the proapoptotic *Bcl*-2 family member *Bax* in the dying cerebellar Purkinje cells of *lurcher* (+/*Lc*) mice (Wullner et al., 1995; De Jager, 1998) strongly favors a role for this protein in the putative excitotoxic death of the cell. To determine whether *Bax* expression is required for +/*Lc* Purkinje cell death, we generated +/*Lc* mice deficient for *Bax* (+/*Lc*: *Bax*$^{-/-}$) by crossing +/*Lc* heterozygous *Bax* mice (+/*Lc*:*Bax*$^{+/-}$). +/*Lc*:*Bax*$^{+/-}$ crosses resulted in the death of on average one in four of the neonates, consistent with the neonatal lethality noted in homozygous *lurcher* pups (Cheng and Heintz, 1997). The remaining pups developed normally into the third postnatal week when a majority of the litter began to exhibit ataxia characteristic of the +/*Lc* mutation (a swaying of the body with a tendency to fall from side to side). Genotyping revealed that these ataxic mice included +/*Lc*:*Bax*$^{-/-}$ as well as +/*Lc*:*Bax*$^{+/-}$ and +/*Lc*:*Bax*$^{+/+}$ offspring. No difference was observed in the timing of the onset of ataxia between the three +/*Lc*:*Bax* genotypes. All these mice remained ataxic into adulthood and were killed for morphological analysis at postnatal day 30 (P30; $n = 5$ mice). After dissection it was evident that the cerebella of +/*Lc*:*Bax*$^{-/-}$ mice were noticeably larger with deeper, more-pronounced fissures than that of their +/*Lc*:*Bax*$^{+/-}$ and +/*Lc*:*Bax*$^{+/+}$ littermates (Fig. 1). This observation indicated a reduction of the cerebellar atrophy associated with the +/*Lc* mutation in mice deficient for *Bax*. A similar effect was observed in +/*Lc*:*Bax*$^{-/-}$ mice analyzed at later ages (P60, $n = 2$ mice, and P300, $n = 3$ mice).

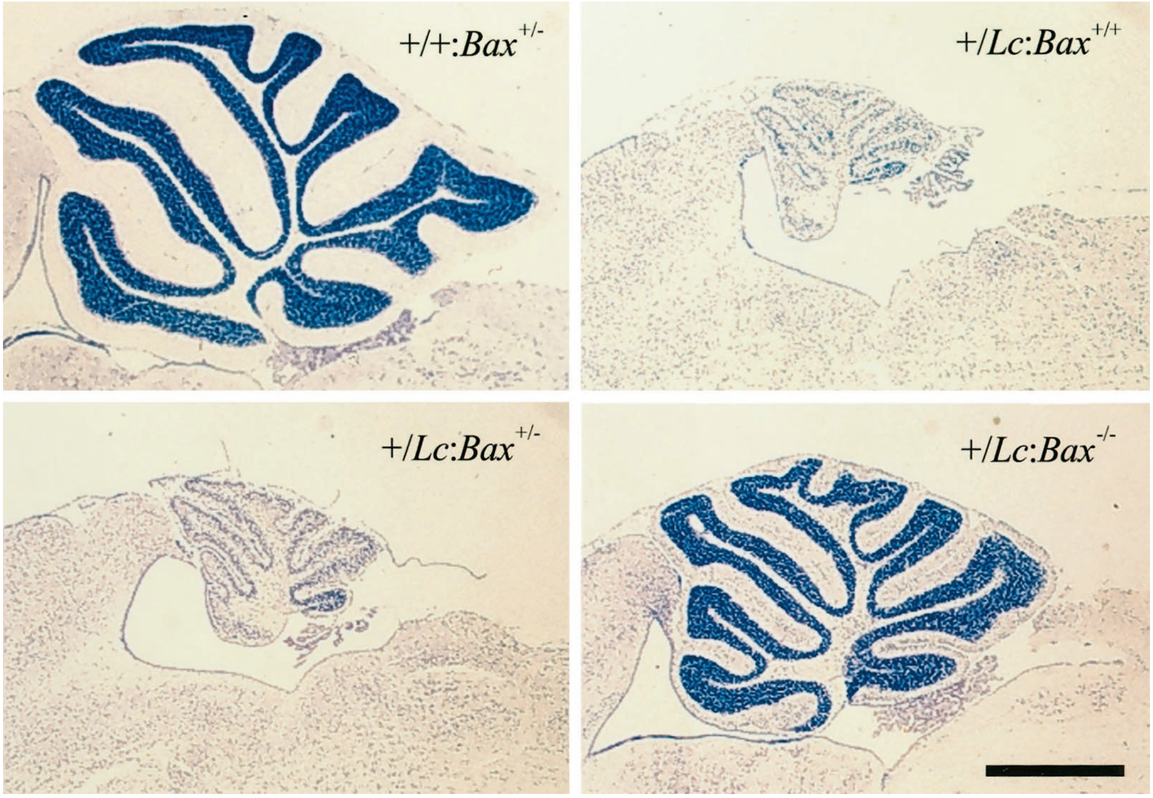

Figure 2. *Bax* deletion rescues granule cells from secondary cell death in +/*Lc* mice. Midsagittal cerebellar sections of postnatal day 30 mice from +/*Lc*:*Bax*$^{+/-}$ crosses stained with cresyl violet. Note the large numbers of granule cells in the well lobulated cerebellum of +/*Lc*:*Bax*$^{-/-}$ mice compared with the cell-sparse, atrophic lobules of +/*Lc* mice containing either one or both alleles of the *Bax* gene. Scale bar, 1 mm.

Bax deletion rescues granule cells and delays Purkinje cell death in *lurcher* mice

Granule cells make up the bulk of the cerebellar mass, and cresyl violet staining of cerebella sections from +/*Lc*:*Bax*$^{-/-}$ mice revealed the presence of a large, cell-dense IGL. The numerous granule cells and normal lobular pattern of the cerebellar cortex in +/*Lc*:*Bax*$^{-/-}$ mice were in stark contrast to the granule cell-sparse, atrophic lobules of the +/*Lc*:*Bax*$^{+/+}$ and +/*Lc*:*Bax*$^{+/-}$ littermates (Fig. 2). Despite the abundant presence of granule cells, the examination of +/*Lc*:*Bax*$^{-/-}$ sections under high-powered objectives revealed the presence of few Purkinje cells at the interface of the IGL and ML. The large-scale absence of Purkinje cells in +/*Lc*:*Bax*$^{-/-}$ mice was confirmed by immunolabeling P30 cerebellar sections with antibodies to calbindin (Fig. 3). Calbindin immunolabeling also revealed abnormal Purkinje cell morphology in +/*Lc*:*Bax*$^{-/-}$ mice: the Purkinje cells were not aligned in a monolayer and had stunted, poorly developed dendritic trees that failed to reach the pial surface (Fig. 3*b*), a morphology that is characteristic of +/*Lc* mice.

We estimated the number of granule cells per midsagittal cerebellar section at P30 and P300 using morphometric analysis and cell counts. The data revealed the presence of 60% of the wild-type (+/+:*Bax*$^{+/-}$) number of granule cells in +/*Lc*:*Bax*$^{-/-}$ mice at both ages (~32,000 granule cells per section), compared with a value of 10% or less in the +/*Lc*:*Bax*$^{+/+}$ and +/*Lc*:*Bax*$^{+/-}$ mice at P30 (Fig. 4*a*). The persistence of 60% of the wild-type number of granule cells in the +/*Lc*:*Bax*$^{-/-}$ mice indicates that the deletion of *Bax* permanently rescues these cells from target-related cell death. In spite of the evidence of widespread Purkinje cell death in the calbindin-immunolabeled sections of +/*Lc*:*Bax*$^{-/-}$ mice, Purkinje cell counts revealed significantly increased numbers of Purkinje cells in the *Bax*-deficient +/*Lc* mice at P30. At this age there was 15% of the wild-type (+/+:*Bax*$^{+/-}$) number of Purkinje cells in +/*Lc*:*Bax*$^{-/-}$ mice compared with 6% or less in +/*Lc* mice with either one or both *Bax* alleles (Fig. 4*b*). However, the examination +/*Lc*:*Bax*$^{-/-}$ mice at P300 demonstrated that +/*Lc* Purkinje cell death was delayed but not prevented by the deletion of *Bax*, because the number of Purkinje cells was reduced to 1% of the wild-type value in these older mice (Fig. 4*b*). The delay but eventual death of Purkinje cells in +/*Lc*:*Bax*$^{-/-}$ mice indicates the existence of a *Bax*-independent cell death pathway in Purkinje cells.

We next examined +/*Lc* mice deficient for *Bax* at the younger age of P15, the approximate midpoint in the granule cell death response in *lurcher* mice (see Doughty et al., 1999). By examining these younger mice, we hoped to establish whether (1) the presence of 60% of the wild-type number of granule cells at P30 and older was the result of the partial rescue of the cell population or the prevention of all target-related granule cell death in the mutant, (2) Purkinje cell death was delayed in all or only a regional subset of cells, and (3) +/*Lc* Purkinje cell development was ameliorated in the absence of *Bax* expression. We conducted the same analysis as before using cresyl violet staining and calbindin immunocytochemistry of midsagittal cerebellar sections ($n = 5$ mice). At P15 there was already a clear increase in the size and density of the IGL of +/*Lc*:*Bax*$^{-/-}$ compared with +/*Lc*:*Bax*$^{+/-}$ littermates (Fig. 5*a*). Cell counts and morphometric analysis of the IGL confirmed a significant increase in granule cell numbers in the +/*Lc*:*Bax*$^{-/-}$ mice (Fig. 5*c*). The estimated number of granule cells per section at P15 was very similar to the

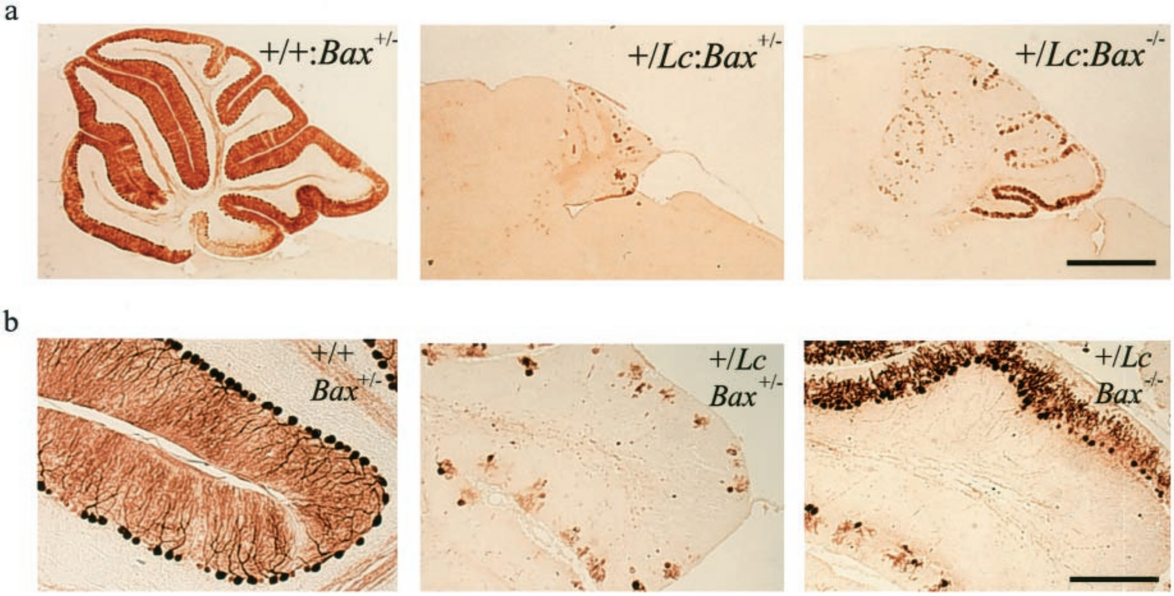

Figure 3. *Bax* deletion partially rescues +/*Lc* Purkinje cells from cell-autonomous death. Calbindin-immunolabeled midsagittal cerebellar sections of postnatal day 30 mice from +/*Lc*:*Bax*$^{+/-}$ crosses. *a,* Whole cerebellum view. Note the increased numbers of immunolabeled Purkinje cells in the posterior cerebellum of +/*Lc*:*Bax*$^{-/-}$compared with the +/*Lc*:*Bax*$^{+/-}$ mice. *b,* Higher-powered view of the posterior cerebellum showing the stunted Purkinje cell morphology characteristic of the *Lc* mutation in the +/*Lc*:*Bax*$^{+/-}$and +/*Lc*:*Bax*$^{-/-}$ mice. Scale bars: *a*, 1 mm; *b*, 100 μm.

value obtained for +/*Lc*:*Bax*$^{-/-}$ mice at P30 and P300 (33,000 cells per section at P15 compared with on average 32,000 cells per section in the older animals). The presence of equal numbers of granule cells at P15, P30, and P300 in +/*Lc*:*Bax*$^{-/-}$ mice, in addition to the absence of any pyknotic profiles in the IGL at P15 (in contrast to the numerous profiles observed in +/*Lc*:*Bax*$^{+/-}$ littermates), suggests that target-related granule cell death in +/*Lc* mice is prevented by the deletion of *Bax*. If granule cell death is completely rescued by *Bax* deletion, then the decrease in total granule cell numbers in +/*Lc*:*Bax*$^{-/-}$ mice is presumably attributable to reduced production of granule cells in response to local signals from Purkinje cells (Smeyne et al., 1995; Wechsler-Reya and Scott, 1999). Cell counts revealed greater numbers of Purkinje cells in the +/*Lc*:*Bax*$^{-/-}$ at P15 (62% of the adult wild-type value) compared with P30 (15% of the adult wild-type value) (Fig. 5*d*). Calbindin immunolabeling of P15 cerebellar sections revealed a similar pattern of Purkinje cell distribution in +/*Lc*:*Bax*$^{-/-}$ and +/*Lc*:*Bax*$^{+/-}$ littermates (Fig. 5*b*), although all of the remaining cells had poorly developed dendrites. The facts that the number of surviving Purkinje cells in early postnatal +/*Lc*:*Bax*$^{-/-}$ animals is directly proportional to the number of granule cells present in the adult, that Purkinje cell loss in these animals is eventually complete, that no further granule cell loss is noted after P15 in +/*Lc*:*Bax*$^{-/-}$ mice, and that granule cell production is known to be controlled locally by Purkinje cells (Smeyne et al., 1995; Wechsler-Reya and Scott, 1999) are all consistent with the complete rescue of secondary granule cell death in response to loss of *Bax* expression. However, we cannot exclude the possibility of a *Bax*-independent cell death pathway as the cause for the lower number of granule cells in +/*Lc*:*Bax*$^{-/-}$ mice.

Bax-mediated neuronal death in *lurcher* mice is *p53* independent

The expression of the tumor suppressor gene *p53* has been shown to be required for many *Bax*-mediated cell death pathways (Aloyz et al., 1998; Xiang et al., 1998; Cregan et al., 1999). To examine the possible requirement of *p53* expression for the *Bax*-mediated neuronal cell death in +/*Lc* mice, we generated +/*Lc* mice deficient for *p53* (+/*Lc*:*p53*$^{-/-}$) by crossing +/*Lc* mice heterozygous for *p53* (+/*Lc*:*p53*$^{+/-}$). We hypothesized that a rescue of cerebellar cell death in +/*Lc*:*p53*$^{-/-}$ mice comparable with that observed in +/*Lc*:*Bax*$^{-/-}$ mice would indicate the need for *p53* expression to activate the *Bax* cell death pathway in the +/*Lc* cerebellum. As is the case for *Bax*, the deletion of *p53* failed to prevent the development of ataxia in the third postnatal week in +/*Lc*:*p53*$^{-/-}$ mice. However in contrast to the +/*Lc*:*Bax*$^{-/-}$ mice, +/*Lc*:*p53*$^{-/-}$ mice showed no sign of amelioration in the cerebellar atrophy associated with the +/*Lc* mutation when killed at P30 (n = 4 mice). Histological analysis confirmed that the cerebella of +/*Lc*:*p53*$^{-/-}$ mice were indistinguishable from that of their +/*Lc*:*p53*$^{+/-}$ littermates (data not shown). The meager presence of Purkinje cells and granule cells in the cerebella of +/*Lc*:*p53*$^{-/-}$ mice demonstrates that the *Bax*-mediated death of these cells is independent of *p53* expression.

Caspase-3 activation in *lurcher* mice is *Bax* dependent

Caspase-3, a widespread effector protease in apoptotic cell death, has been shown to be activated in the dying Purkinje cells and granule cells of +/*Lc* mice (Selimi et al., 2000). Interestingly, the activation of caspase-3 in granule cell cultures switched from medium containing high to low concentrations of potassium requires the expression of *Bax* (Miller et al., 1997). We therefore examined the expression of active caspase-3 in +/*Lc*:*Bax*$^{-/-}$ and +/*Lc*:*Bax*$^{+/-}$ mice during the period of Purkinje cell death (P15–P20) using an antibody that only recognizes the active subunit of the protease. The immunocytochemical labeling of cerebellar sections from +/*Lc*:*Bax*$^{+/-}$ mice confirmed the expression of active casapse-3 in dying Purkinje cells and granule cells (data not shown). Consistent with the results of Selimi et al. (2000), few Purkinje cells were immunolabeled with the antiserum (on aver-

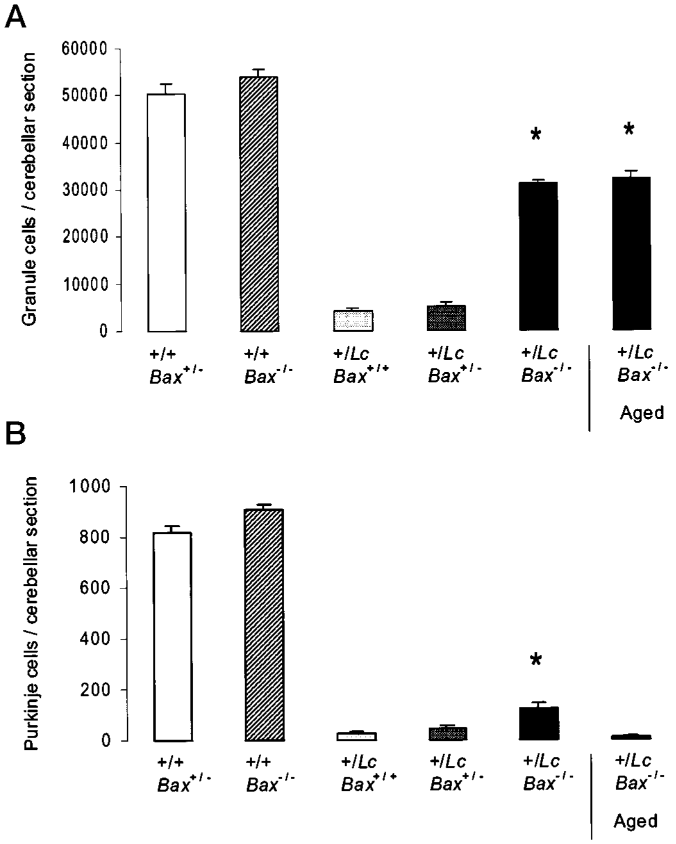

Figure 4. Quantification of granule cell and Purkinje cell survival in postnatal day 30 and postnatal day 300 (*Aged*) mice from +/*Lc*:*Bax*$^{+/-}$ crosses. *a, Bax* deletion permanently rescues granule cells from target-related cell death in +/*Lc* mice. The number of granule cells per midsagittal cerebellar section was estimated from the area and cell density of the internal granule cell layer. *b, Bax* deletion delays but does not prevent cell-autonomous Purkinje cell death in +/*Lc* mice. The number of Purkinje cells per midsagittal cerebellar section was counted from sections processed for calbindin immunocytochemistry. The data are presented as the mean value ± SEM (n = 3–5 mice). *Asterisks* denote statistically significant differences: *a*, $^*p < 0.000001$; *b*, $^*p < 0.05$ (unpaired two-tailed Student's t test).

age, three per sagittal section of the cerebellar vermis; $n = 2$ mice from 2 litters, 5 sections per animal), indicating the rapid death of the cell after cleavage and activation of the caspase. In contrast, the simultaneous labeling of cerebellar sections from +/*Lc*:*Bax*$^{-/-}$ mice failed to detect any active caspase-3 expression in Purkinje cells or granule cells ($n = 3$ mice from 3 litters, 5 sections per animal). These contrasting observations suggest that caspase-3 is not activated in the dying Purkinje cells of +/*Lc*:*Bax*$^{-/-}$ mice and suggest the existence of a caspase-3-independent, *Bax*-independent cell death pathway in the cell.

DISCUSSION

Previous studies of +/*Lc* mice suggest an apoptotic mechanism of Purkinje cell death involving *Bax* expression and induction of caspase-3 activity (Norman et al., 1995; De Jager, 1998; Wullner et al., 1998; Selimi et al., 2000). By applying a genetic approach, we have demonstrated the involvement of *Bax* in both the primary, cell-autonomous death of Purkinje cells and the secondary loss of granule cells in +/*Lc* mice. The deletion of *Bax* expression in +/*Lc* mice permanently rescued granule cells from target-related cell death and delayed but did not prevent Purkinje cell death. From these loss-of-function experiments, we conclude there is an absolute requirement for *Bax* expression in target-related granule cell death, despite the presence of only 60% of the +/+ number in adult and aged +/*Lc*:*Bax*$^{-/-}$ mice. This conclusion is based on the strong evidence of a positive mitogenic role of Purkinje cells in granule cell production (Smeyne et al., 1995; Wechsler-Reya and Scott, 1999). Thus the retarded development of Purkinje cells in +/*Lc*:*Bax*$^{-/-}$ mice is likely to reduce granule cell production in these mice. The delay but failure to prevent +/*Lc* Purkinje cell death by the deletion of *Bax* expression indicates the presence of both *Bax*-dependent and *Bax*-independent death pathways in these cells. Next, we examined the role of *p53*, a molecule widely implicated in the regulation of *Bax*-mediated neuronal cell death (Aloyz et al., 1998; Xiang et al., 1998; Cregan et al., 1999). Our analysis showed that the deletion of *p53* expression in +/*Lc* mice did not prevent target-related granule cell death, demonstrating that *Bax* is expressed independently of *p53* in these cells. Finally, we suggest that the activation of caspase-3 in +/*Lc* cerebella (Selimi et al., 2000) requires the expression of *Bax*. Because Purkinje cells continue to die in +/*Lc*:*Bax*$^{-/-}$ mice, this observation suggests the activation of a *Bax*-independent, capase-3-independent death pathway in the cell.

Bax activity is essential for many programmed cell death (PCD) events in CNS development (Deckwerth et al., 1996; White et al., 1998), and the protein has been shown to regulate neuronal sensitivity to both excitotoxic and genotoxic injury (Xiang et al., 1998; Cregan et al., 1999). Therefore the observation that *Bax* expression is increased in +/*Lc* Purkinje cells (De Jager, 1998; Wullner et al., 1998) suggested a role for this protein in the cell-autonomous death of this cell. Interestingly, increased *Bax* expression was not reported in the cerebellar granule cells of +/*Lc* mice by these authors, despite the large-scale death of these neurons after the loss of target Purkinje cells. Contrary to these observations, our loss-of-function experiments clearly demonstrate that the target-related death of granule cells is dependent on *Bax* expression, whereas *Bax* deletion does not prevent +/*Lc* Purkinje cell death. Nevertheless, the rate of +/*Lc* Purkinje cell death was slowed in the absence of *Bax*. This indicates the involvement of *Bax* in Purkinje cell death, but it also reveals the activation of a *Bax*-independent pathway in the cell.

The presence of a *Bax*-independent cell death pathway in Purkinje cells is not surprising considering the strong activation of the δ2 glutamate receptor in response to the *lurcher* mutation. Thus, induction of both *Bax* and *Bcl-X* has been demonstrated in +/*Lc* Purkinje cells (De Jager, 1998; Wullner et al., 1998), suggesting a possible role for the proapoptotic isoform of *Bcl-X* in a parallel pathway for activation of the effector phase of apoptotic cell death in these neurons. This is consistent with the demonstration that granule cells from *Bax*$^{-/-}$ mice will undergo excitotoxic death in response to NMDA application in culture (Miller et al., 1997). Alternatively, the removal of *Bax* as a rate-limiting component of the cell death pathway active in +/*Lc* Purkinje cells may provoke a less well defined pathway. The observation that active caspase-3 is not detected in +/*Lc*:*Bax*$^{-/-}$ Purkinje cells demonstrates that this alternative cell death pathway does not involve the activation of this protease.

The permanent rescue of secondary granule cell loss in +/*Lc*:*Bax*$^{-/-}$ mice is consistent with previous studies of apoptotic cell

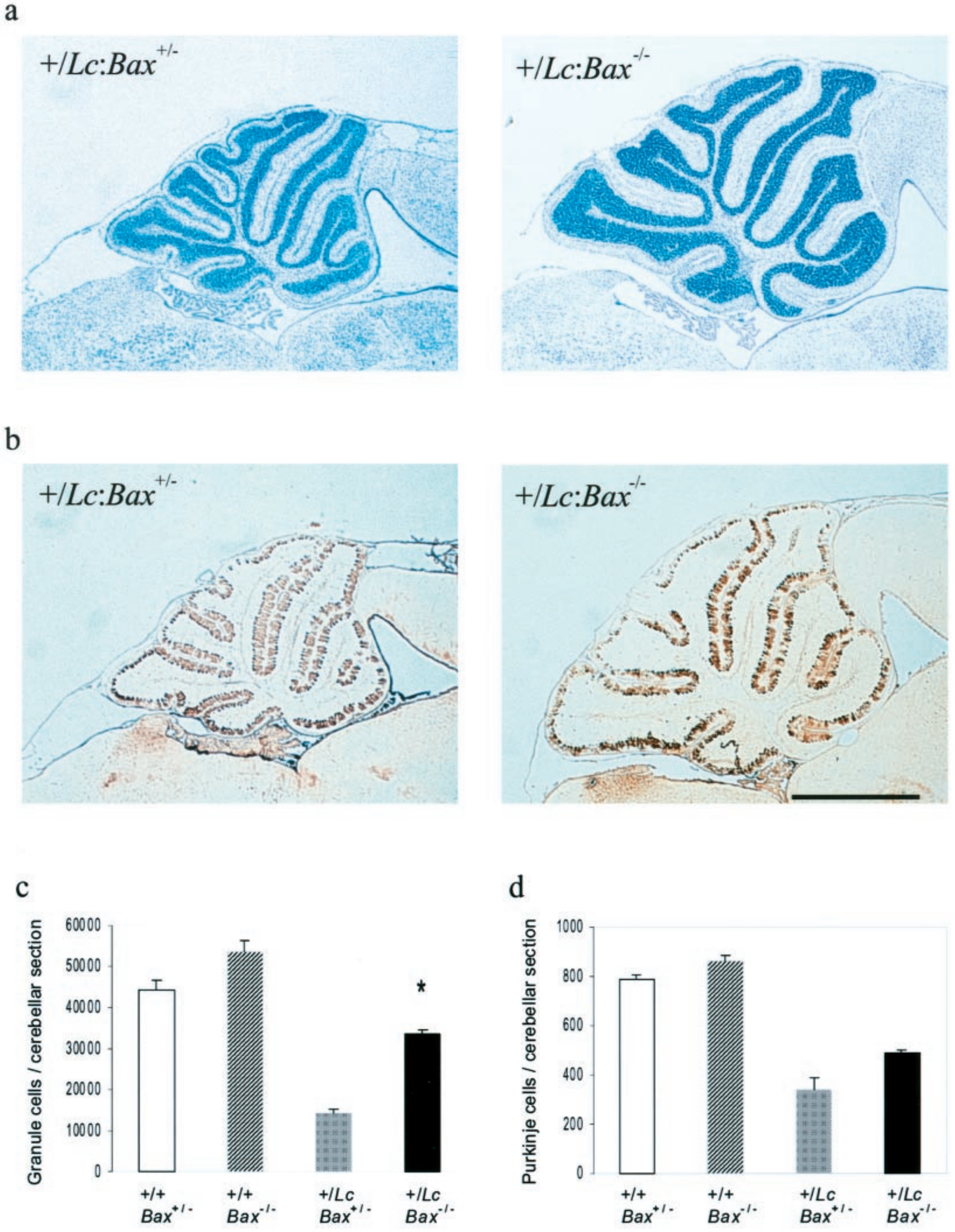

Figure 5. The effect of *Bax* deletion on granule cell and Purkinje cell development in +/*Lc* mice. *a,* Midsagittal cerebellar sections of postnatal day 15 mice from +/*Lc*:*Bax* $^{+/-}$ crosses stained with cresyl violet. Note the already increased cerebellar size and greater area and cell density of the internal granule cell layer in the +/*Lc*:*Bax* $^{-/-}$ mice. *b,* Calbindin-immunolabeled midsagittal cerebellar sections of postnatal day 15 mice from +/*Lc*:*Bax* $^{+/-}$ crosses. Gaps in the Purkinje cell layer reveal cell death in the developing +/*Lc*:*Bax* $^{+/-}$ and +/*Lc*:*Bax* $^{-/-}$ mice. Scale bar: *a, b,* 1 mm. *c,* Quantification of granule cell survival in postnatal day 15 mice from +/*Lc*:*Bax* $^{+/-}$ crosses. The data are the mean estimated number of granule cells per midsagittal cerebellar section ± SEM (n = 3–5 mice). The *asterisk* indicates a statistically significant difference ($p < 0.00001$; unpaired two-tailed Student's t test). *d*, Quantification of Purkinje cell survival in postnatal day 15 mice from +/*Lc*:*Bax* $^{+/-}$ crosses. The data are the mean number of calbindin-immunolabeled Purkinje cells per midsagittal cerebellar section ± SEM (n = 3–5 mice).

death in response to target deprivation. Thus, a requirement for *Bax* in cell death attributable to trophic factor withdrawal for sympathetic and motor neurons has been demonstrated using *Bax* $^{-/-}$ mice (Deckwerth et al., 1996). Furthermore, partial rescue of inferior olivary neurons from target-related cell death by the overexpression of *Bcl-2* in +/*Lc* mice has been reported (Zanjani et al., 1998) (it should be noted that *Bcl-2* was not overexpressed in granule cells in these mice). These examples support the activation of a common, *Bcl-2* family-mediated cell death response to the loss of target support. In spite of experiments indicating a role for *p53* in cell death models induced by DNA damage (Woods and Youle, 1995), excitotoxicity (Xiang et al., 1998), PCD, and trophic factor withdrawal (Aloyz et al., 1998) and the expression of *p53* in developing cerebellar granule cells (van Lookeren Campagne and Gill, 1998), our results failed to reveal a role for *p53* in granule cell death in response to target deprivation. This is interesting considering the reported induction of a *Bax*-dependent cell death pathway in response to adenovirus-mediated delivery of *p53* in granule cells *in vitro* (Cregan et al., 1999). We conclude that the activation of *Bax* in response to target deprivation *in vivo* occurs by an alternative *p53*-independent mechanism, indicating the presence of multiple pathways for the activation of *Bax*-dependent cell death in cerebellar granule neurons. Although we have not tested directly the requirement for *caspase-3* in granule cell death in +/*Lc*:$Bax^{-/-}$ mice, the fact that active caspase-3 was not observed in these cells strongly supports an important role for this protease.

The data presented here suggest that the constitutive activation of GRID2^{Lc} induces multiple death pathways in the Purkinje cell that involve apoptotic (*Bax* expression and caspase-3 activation) and unknown, possibly necrotic, mechanisms. The complete rescue of granule cells in +/*Lc*:$Bax^{-/-}$ mice, on the other hand, confirms that these cells die by a purely apoptotic mechanism. Recently, a number of studies (Martinou et al., 1994; Ankarcrona et al., 1995; Krajewski et al., 1995; Chen et al., 1998; Namura et al., 1998) have indicated that apoptosis is a key component of ischemic brain injury [for a review of current perspectives, see Lee et al. (1999)]. Thus, the features of +/*Lc* cell death in many ways mirror the events of brain injury caused by transient cerebral ischemia (Heintz and Zoghbi, 2000). In both cases, the primary insult is the activation of glutamate receptors, and this leads first to the expression of neuroprotective *Bcl-2* family members that, once overwhelmed, results in the activation of cell death pathways that include *Bax* expression and caspase activation, as well as unknown molecules. In turn, the primary lesion leads to the secondary loss of afferent neurons by apoptosis. However, unlike models of cerebral ischemia, the site of the primary lesion is well characterized in +/*Lc* mice, and this advantage should prove invaluable in the use of this mutant as a model to study cell death mechanisms in ischemic brain injury.

REFERENCES

Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR, Miller FD (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J Cell Biol 143:1691–1703.

Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15:961–973.

Caddy KW, Biscoe TJ (1979) Structural and quantitative studies on the normal C3H and Lurcher mutant mouse. Philos Trans R Soc Lond [Biol] 287:167–201.

Chen J, Nagayama T, Jin K, Stetler RA, Zhu RL, Graham SH, Simon RP (1998) Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J Neurosci 18:4914–4928.

Cheng SS, Heintz N (1997) Massive loss of mid- and hindbrain neurons during embryonic development of homozygous lurcher mice. J Neurosci 17:2400–2407.

Choi DW (1994) Calcium and excitotoxic neuronal injury. Ann NY Acad Sci 747:162–171.

Cregan SP, MacLaurin JG, Craig CG, Robertson GS, Nicholson DW, Park DS, Slack RS (1999) Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. J Neurosci 19:7860–7869.

Deckwerth TL, Elliott JL, Knudson CM, Johnson Jr EM, Snider WD, Korsmeyer SJ (1996) BAX is required for neuronal death after trophic factor deprivation and during development. Neuron 17:401–411.

De Jager PL (1998) Cloning and characterization of the lurcher allele of glutamate receptor δ2: aberrant activation of a receptor complex results in neuronal death. PhD thesis, The Rockefeller University.

Deshmukh M, Johnson Jr EM (1998) Evidence of a novel event during neuronal death: development of competence-to-die in response to cytoplasmic cytochrome *c*. Neuron 21:695–705.

Doughty ML, Lohof A, Selimi F, Dumesnil-Bousez N, Mariani J (1999) Afferent-target cell interactions in the cerebellum: negative effect of granule cells on Purkinje cell development in lurcher. J Neurosci 19:3448–3456.

Green DR (1998) Apoptotic pathways: the roads to ruin. Cell 94:695–698.

Heintz N, De Jager PL (1999) GluRδ2 and the development and death of cerebellar Purkinje neurons in lurcher mice. Ann NY Acad Sci 868:502–514.

Heintz N, Zoghbi HY (2000) Insights from mouse models into the molecular basis of neurodegeneration. Annu Rev Physiol 62:779–802.

Kashiwabuchi N, Ikeda K, Araki K, Hirano T, Shibuki K, Takayama C, Inoue Y, Kutsuwada T, Yagi T, Kang Y (1995) Impairment of motor coordination, Purkinje cell synapse formation, and cerebellar long-term depression in GluR delta 2 mutant mice. Cell 81:245–252.

Knudson CM, Tung KSK, Tourtellotte WG, Brown GAJ, Korsmeyer SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270:96–99.

Krajewski S, Mai JK, Krajewska M, Sikorska M, Mossakowski MJ, Reed JC (1995) Upregulation of bax protein levels in neurons following cerebral ischemia. J Neurosci 15:6364–6376.

Lee J-M, Zipfel GJ, Choi DW (1999) The changing landscape of ischaemic brain injury mechanisms. Nature [Suppl] 399:A7–A14.

Los M, Wesselborg S, Schulze-Osthoff K (1999) The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. Immunity 10:629–639.

Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13:1017–1030.

Miller TM, Moulder KL, Knudson CM, Creedon DJ, Deshmukh M, Korsmeyer SJ, Johnson Jr EM (1997) Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. J Cell Biol 139:205–217.

Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J, Moskowitz MA (1998) Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci 18:3659–3668.

Norman DJ, Feng L, Cheng SS, Gubbay J, Chan E, Heintz N (1995) The lurcher gene induces apoptotic death in cerebellar Purkinje cells. Development 121:1183–1193.

Pettmann B, Henderson CE (1998) Neuronal cell death. Neuron 20:633–647.

Selimi F, Doughty ML, Delhaye-Bouchaud N, Mariani J (2000) Target-related and intrinsic neuronal deaths in Lurcher mutant mice involve different apoptotic pathways but are both mediated by caspase-3 activation. J Neurosci 20:992–1000.

Smeyne RJ, Chu T, Lewin A, Bian F, Crisman S, Kunsch C, Lira SA, Oberdick J (1995) Local control of granule cell generation by cerebellar Purkinje cells. Mol Cell Neurosci 6:230–251.

van Lookeren Campagne M, Gill R (1998) Tumor-suppressor p53 is expressed in proliferating and newly formed neurons of the embryonic and postnatal rat brain: comparison with expression of the cell cycle regulators p21Waf1/Cip1, p27Kip1, p57Kip2, p16Ink4a, Cyclin G1, and the proto-oncogene Bax. J Comp Neurol 397:181–198.

Vaux DL, Korsmeyer SJ (1999) Cell death in development. Cell 96:245–254.

Wechsler-Reya RJ, Scott MP (1999) Control of neuronal precursor proliferation in the cerebellum by sonic hedgehog. Neuron 22:103–114.

Wetts R, Herrup K (1982a) Interaction of granule, Purkinje and inferior olivary neurons in lurcher chimeric mice. I. Qualitative studies. J Embryol Exp Morphol 68:87–98.

Wetts R, Herrup K (1982b) Interaction of granule, Purkinje and inferior olivary neurons in lurcher chimeric mice. II. Granule cell death. Brain Res 250:358–362.

White AF, Keller-Peck CR, Knudson CM, Korsmeyer SJ, Snider WD (1998) Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. J Neurosci 18:1428–1439.

Woods KA, Youle RJ (1995) The role of free radicals and p53 in neuron apoptosis *in vivo*. J Neurosci 15:5851–5857.

Wullner U, Loschmann PA, Weller M, Klockgether T (1995) Apoptotic cell death in the cerebellum of mutant weaver and lurcher mice. Neurosci Lett 200:109–112.

Wullner U, Schulz JB, Krajewski S, Reed JC, Klockgether T (1998) Bcl-2, Bax and Bcl-x expression in neuronal apoptosis: a study of mutant weaver and lurcher mice. Acta Neuropathol (Berl) 96:233–238.

Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, Morrison RS (1998) Bax involvement in p53-mediated neuronal cell death. J Neurosci 18:1363–1373.

Zanjani HS, Vogel MW, Martinou JC, Delhaye-Bouchaud N, Mariani J (1998) Postnatal expression of *Hu-Bcl-2* gene in *Lurcher* mutant mice fails to rescue Purkinje cells but protects inferior olivary neurons from target-related cell death. J Neurosci 18:319–327.

Zuo J, De Jager PL, Takahashi KA, Jiang W, Linden DJ, Heintz N (1997) Neurodegeneration in Lurcher mice caused by mutation in δ2 glutamate receptor gene. Nature 388:769–773.