Bax Inactivation in Lurcher Mutants Rescues Cerebellar Granule Cells But Not Purkinje Cells or Inferior Olivary Neurons

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Lurcher is a gain-of-function mutation in the δ2 glutamate receptor gene (*Grid2*) that turns the receptor into a leaky ion channel. The expression of the Lurcher gene in heterozygous (*Grid2^{Lc/+}*) mutants induces the death of almost all Purkinje cells starting from the second postnatal week. Ninety percent of the granule cells and 60–75% of the inferior olivary neurons die because of the loss of their target neurons, the Purkinje cells. The apoptotic nature of the neurodegeneration has been demonstrated previously by the presence of activated caspase-3 and DNA fragmentation. *Bax*, a pro-apoptotic gene of the Bcl-2 family, has been shown to be involved in developmental neuronal death. To study the role of *Bax* in *Grid2^{Lc/+}* neurodegeneration, double mutants with *Grid2^{Lc/+}* mice and *Bax* knock-out mice (*Bax−/−*) were generated. *Bax* deletion had no effect on the death of Purkinje cells and inferior olivary neurons, although a temporary rescue of some Purkinje cells could be detected in P15 *Grid2^{Lc/+}*;Bax−/− animals. From postnatal day 15 (P15) to P60, the number of granule cells in *Grid2^{Lc/+}*;Bax−/−mice did not significantly change and was significantly increased compared with the number found in *Grid2^{Lc/+}*;Bax+/+ mice. Granule cell number in P60 *Grid2^{Lc/+}*;Bax−/− mice corresponded to 70% of the number found in wild-type mice. Our results show that *Bax* inactivation in *Grid2^{Lc/+}* mice does not rescue intrinsic Purkinje cell death or the target-related cell death of olivary neurons, but *Bax* inactivation does inhibit persistently target-related cell death in cerebellar granule cells.

Key words: Lurcher; Bax*; Purkinje cell; granule cell; inferior olive; neuronal death*

The heterozygous Lurcher mutant (Grid2^{Lc/+}) has been studied extensively as a model for understanding the mechanisms of cell autonomous and target-related neuronal cell degeneration. In the *Grid2^{Lc/+}* mutant, almost all cerebellar Purkinje cells (PCs), 60–75% of the olivary neurons, and 90% of the granule cells degenerate starting after the first week of postnatal development (Phillips, 1960; Caddy and Biscoe, 1979). Studies of *Grid2^{Lc/+}* ↔ wild type chimeras established that *Grid2^{Lc/+}* Purkinje cell death is cell autonomous, whereas granule cell and olivary neuron cell death is secondary to the loss of their primary target, the Purkinje cells (Wetts and Herrup, 1982a,b). More recent molecular studies have established that the Lurcher mutation is caused by a base pair change in the δ2 glutamate receptor gene (*Grid2*) that greatly increases its conductance (Zuo et al., 1997). Given the nature of *Grid2* gene mutation, it appears likely that *Grid2^{Lc/+}* Purkinje cells are dying by an excitotoxic mechanism. The molecular mechanisms of *Grid2^{Lc/+}* Purkinje cell death have not yet been determined. However, the presence of activated caspase-3 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) in dying Purkinje cells, granule cells, and olivary neurons of Lurcher mice suggest that these neuronal deaths are apoptotic (Norman et al., 1995; Wullner et al., 1995; Selimi et al., 2000).

The purpose of this study was to determine the effects of deleting the pro-apoptotic gene *Bax* on Purkinje, granule, and olivary neuron survival in the *Grid2^{Lc/+}* mutant. We have shown previously that *Grid2^{Lc/+}* Purkinje cell death can be delayed and *Grid2^{Lc/+}* olivary neurons rescued by the overexpression of the anti-apoptotic gene *bcl-2* (Zanjani et al., 1998a,b). The *bcl-2* proto-oncogene encodes an integral membrane protein that inhibits apoptosis in many cell types, although its mechanism of action is still not completely understood (Adams and Cory, 1998). One important function of BCL-2 may be to counteract the pro-apoptotic effects of BAX (Gross et al., 1999). BCL-2 will bind to BAX, and cell death may be regulated by the ratio of BCL-2 to BAX (Oltvai et al., 1993). BAX is involved in developmental cell death because deletion of the *Bax* gene reduces the incidence of naturally occurring neuronal death *in vivo* (White et al., 1998). *Bax−/−* neurons also survive trophic factor withdrawal *in vitro* or after *in vivo* axotomy (Deckwerth et al., 1996). The results of our study show that deletion of *Bax* in the *Grid2^{Lc/+}* mutant does not prevent the excitotoxic Purkinje cell death or the target-related olivary neuron death. However, *Bax* inactivation increases survival of granule cells, supporting a role for BAX in target-related cell death of granule cells. The dichotomy between granule cell rescue and olivary neuron death in the *Grid2^{Lc/+}*;Bax−/− mutant suggests that different cell death mechanisms are at work, even in two neuronal populations that are dying because of the loss of the same target.

MATERIALS AND METHODS

Animals and genotyping. *Grid2^{Lc/+}*;Bax−/− double mutants were generated by heterozygous matings of *Grid2^{Lc/+}* and *Bax+/−* mutants in the animal facilities at the Université Pierre et Marie Curie. *Grid2^{Lc/+}* mutants (B6AKR strain) are maintained in a colony at the Université Pierre et Marie Curie, and heterozygous *Bax+/−* mutants were obtained from Dr. Stanley Korsmeyer (Harvard Medical School, Boston, MA). The *Bax−/−* knock-out mutant was generated by homologous recombination to delete exons 2 through 5 to make a nonfunctional protein (C57BL6/RW-4 strain) (Knudson et al., 1995). In the mating scheme to generate homozygous and heterozygous double mutants, *Grid2^{Lc/+}* males were first mated with *Bax* heterozygous knock-out females. The *Grid2^{Lc/+}*;Bax+/− animals were then intercrossed. Litters were killed at postnatal day 15 (P15), P30, and P60, using the guidelines established by Le Comité National d'Éthique pour les Sciences de la Vie et de la Santé. The *Grid2^{Lc/+}*;Bax+/− and *Grid2^{Lc/+}*;Bax+/+ control animals used in this study were obtained in the same litters as the double mutants.

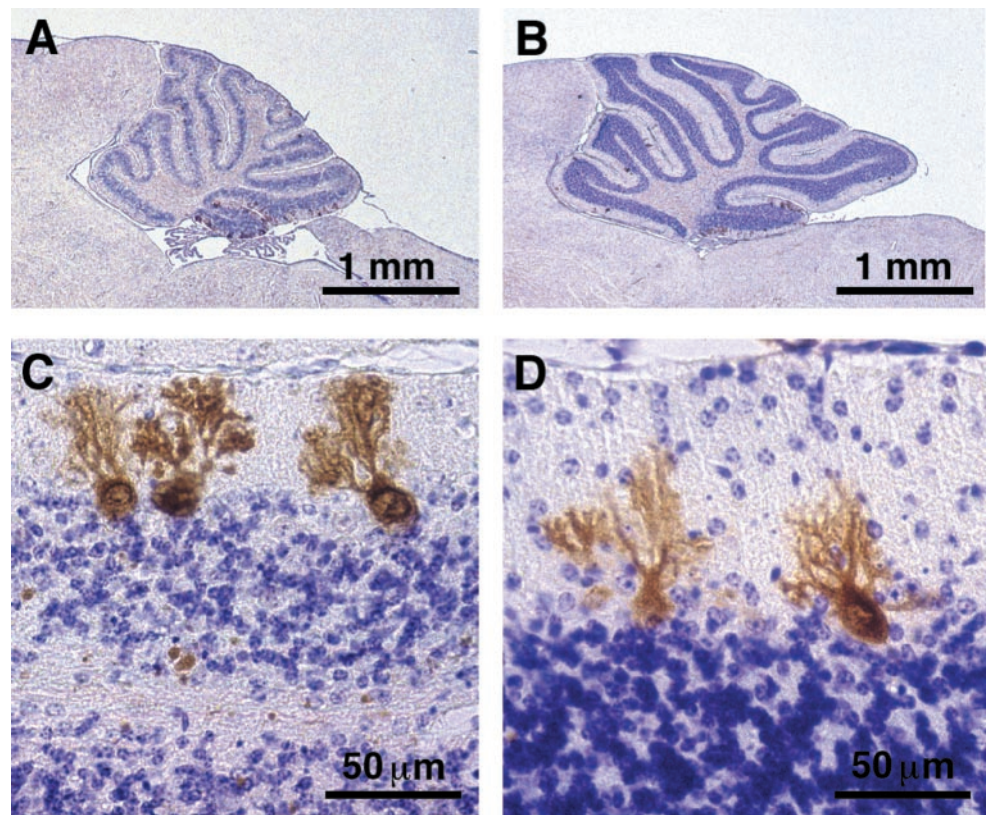Genotyping for *Bax* was performed by PCR using a set of three primers:

Figure 1. *Bax* inactivation in Lurcher mice rescues granule cells but not Purkinje cells. Midsagittal cerebellar sections (10-μm-thick) of 1-month-old animals were immunostained using an anti-calbindin antibody specifically labeling Purkinje cells and counterstained with cresyl violet-thionin. In $Grid2^{Lc/+}$; Bax+/+ control mice (*A*), a massive loss of Purkinje cells and granule cells is detected. Purkinje cells have degenerated as well in $Grid2^{Lc/+}$; Bax−/− double mutant mice (*B*), but an increased number of cresyl violet-stained granule cells are observed. Purkinje cells are atrophic with thicker dendrites in both $Grid2^{Lc/+}$; Bax+/+ control (*C*) and $Grid2^{Lc/+}$;Bax−/− (*D*) double mutant mice.

Bax exon 5 forward primer (5′GAGCTGATCAGAACCATCATG3′), *Bax* intron 5 reverse primer (5′GTTGACCAGAGTGGCGTAGG3′), and *Neo* reverse primer (5′CCGCTTCCATTGCTCAGCGG3′). Cycling parameters were 5 min at 94°C for one cycle, 1 min at 94°C, 1 min at 62°C, and 1 min 30 at 72°C for a total of 30 cycles. PCR products were resolved on a 1.5% agarose gel.

The $Grid2^{Lc}$ allele was detected by PCR followed by single strand chain Polymorphism (SSCP) as described previously (Zuo et al., 1997). Briefly, 200 ng of genomic DNA were used for a PCR with the two following primers: 5′TAAAAGCATATTGATGTTGTTG3′ and 5′CAGCATTT-GTCAGGTTTGGTGAC3′. Cycling parameters were 2 min at 94°C for one cycle, 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for a total of 30 cycles. PCR products were resolved by SSCP using GeneGel Excel 12.5/24 Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and revealed by silver staining of the gel.

Histology. Animals were anesthetized using 0.1 mg/ml chloral hydrate and perfused with 0.9% sodium chloride, followed by 95% ethanol. Brains were dissected, fixed overnight in Clarke's fixative, and processed for paraffin-embedding.

Parasagittal sections (10-μm-thick) of the cerebellum were processed for calbindin immunohistochemistry. Sections were incubated overnight at 4°C with CL-300 monoclonal antibody (dilution, 1:200; Sigma, St. Louis MO). Immunocomplexes were revealed using a peroxidase-conjugated anti-mouse antibody (dilution, 1:500; Jackson ImmunoResearch, West Grove, PA) and diaminobenzidine tetrahydrochloride substrate (DAB Sigmafast; Sigma). Sections were then counterstained with cresyl violet-thionin.

Coronal sections (10-μm-thick) of the brainstem were stained with cresyl violet-thionin to locate the inferior olive and analyze its morphology. Sections from 10 different rostrocaudal levels of the inferior olive were observed in each animal to compare the morphology of all the subnuclei of the olive.

Quantitative analysis. The total numbers of Purkinje cells and granule cells per half-cerebellum were counted in mutant and control cerebella. Cerebellar sections were stained for calbindin immunohistochemistry, and the number of calbindin-positive Purkinje cells was counted in each 40th section at 1000× magnification. The total number of Purkinje cells was calculated from a graph of the number of Purkinje cells in each counted section plotted against the distance of the section from the midline. Corrections were made for double counting Purkinje cells based on the method of Hendry (1976). We chose to use this traditional correction factor for our cell counts instead of more recently developed stereological techniques so that our results would be directly comparable with previously published counts. The total number of granule cells per half-cerebellum was estimated from the volume of the internal granule cell layer (IGL) multiplied by the average density of granule cells in the IGL. The granule cell density was estimated by counting at 1000× magnification the number

of granule cells contained in an area of 25000 μm³. These counts were done in six different regions in four sections from each half-cerebellum. Thus, the number of granule cells in 24 grids were counted to obtain the average density of granule cells. The volume of the IGL was calculated from a graph of granule cell layer area plotted against distance from the midline. The area of the IGL in each 40th section was measured using a CCD camera and NIH Image software. Corrections for double counting errors were made using the methods of Hendry (1976). Three to five animals of each genotype were used at each age: at P15, three animals of each genotype; at P30, four $Grid2^{Lc/+}$;Bax+/+, four $Grid2^{Lc/+}$;Bax+/−, and five $Grid2^{Lc/+}$;Bax−/−; at P60, three $Grid2^{Lc/+}$;Bax+/+ and four $Grid2^{Lc/+}$;Bax−/−. Statistical comparisons were made using ANOVA followed by Newman–Keuls *post hoc* test (significant when $p < 0.05$).

RESULTS

Bax deletion does not rescue the Lurcher phenotype

Lurcher heterozygous ($Grid2^{Lc/+}$) mice were crossed with Bax knock-out ($Bax−/−$) mice to determine whether *Bax* inactivation rescued the Lurcher phenotype. Lurcher homozygotes were never found in the litters, suggesting that *Bax* deletion does not rescue the Lurcher homozygotes and is not sufficient to inhibit the death of brainstem neurons in these animals. The $Grid2^{Lc/+}$;Bax−/− mice were ataxic as the $Grid2^{Lc/+}$;Bax+/+ controls. However, the volume of their cerebellum was greater than that of $Grid2^{Lc/+}$;Bax+/+ mice, suggesting that *Bax* deletion does rescue some of the cells normally degenerating in Lurcher mice.

Bax deletion inhibits granule cell death in Lurcher mice

Purkinje cell death in $Grid2^{Lc/+}$ mice begins at approximately P8, and this neurodegeneration is accompanied by the loss of 90% of the granule cells. By P30, 90% of Purkinje cells have already disappeared (Caddy and Biscoe, 1979), as well as ~85% of granule cells (Doughty et al., 1999).

A qualitative observation of cerebellar sections taken from P30 animals showed that *Bax* deletion in $Grid2^{Lc/+}$ mice inhibited granule cell but not Purkinje cell death. Immunohistochemistry using an anti-calbindin antibody to specifically stain Purkinje cells in the cerebellum showed that almost all Purkinje cells had degenerated in $Grid2^{Lc/+}$;Bax+/+ controls (Fig. 1*A*) and $Grid2^{Lc/+}$; Bax−/− double mutants (Fig. 1*B*) by P30. The morphology of

$Grid2^{Lc/+}$;$Bax+/-$ cerebella (data not shown) was similar to $Grid2^{Lc/+}$;$Bax+/+$ cerebella. Only a few Purkinje cells remained in P30 animals of all three genotypes, primarily in one lobule of the cerebellum, the nodulus. The morphology of Purkinje cells in $Grid2^{Lc/+}$;$Bax-/-$ double mutants (Fig. 1*D*) and $Grid2^{Lc/+}$; $Bax+/-$ cerebella was identical to the morphology of Purkinje cells in $Grid2^{Lc/+}$;$Bax+/+$ control cerebellum (Fig. 1*C*). The dendrites of these cells were atrophic, thicker, and less branched than in normal mice as described previously (Dumesnil-Bousez and Sotelo, 1992; Doughty et al., 1999). Quantitative analysis of the number of Purkinje cells per hemi-cerebellum at P30 showed that there was no significant difference between the numbers of Purkinje cells found in $Grid2^{Lc/+}$;$Bax+/-$ (3208 ± 723) and $Grid2^{Lc/+}$;$Bax+/+$ (3162 ± 298) control mice. The number of Purkinje cells in $Grid2^{Lc/+}$; $Bax-/-$ double mutants (6364 ± 520) was very low, confirming that Bax inactivation does not rescue Purkinje cells. However, this number was significantly higher than the number found in $Grid2^{Lc/+}$; $Bax+/-$ and $Grid2^{Lc/+}$;$Bax+/+$ mice, suggesting that Bax inactivation might delay Lurcher Purkinje cell death (see Fig. 4*A*).

Nuclear staining of the P30 sections using cresyl violet-thionin showed a clear increase of the area of the internal granule cell layer in double mutant cerebella when compared with $Grid2^{Lc/+}$; $Bax+/+$ and $Grid2^{Lc/+}$;$Bax+/-$ control cerebella. The number of granule cells per hemi-cerebellum was significantly higher in $Grid2^{Lc/+}$;$Bax-/-$ cerebella ($10.80 \pm 0.78 \times 10^6$) compared with $Grid2^{Lc/+}$;$Bax+/+$ ($3.16 \pm 0.35 \times 10^6$) and $Grid2^{Lc/+}$;$Bax+/-$ ($2.87 \pm 0.13 - 10^6$) cerebella (see Fig. 4B; ANOVA followed by *post hoc* Newman–Keuls test analysis, $p < 0.01$), confirming that *Bax* inactivation in $Grid2^{Lc/+}$ mice rescues granule cells. The number of granule cells per hemi-cerebellum of $Grid2^{Lc/+}$;$Bax+/-$ animals was not statistically different from the number of granule cells found in $Grid2^{Lc/+}$;$Bax+/+$ animals, showing that inactivation of only one allele of *Bax* is not sufficient to inhibit cell death in $Grid2^{Lc/+}$ mice.

Sixty to 75% of the inferior olivary neurons normally degenerate in $Grid2^{Lc/+}$ mice following the death of their Purkinje cell targets (Caddy et al., 1979; Heckroth et al., 1991; Herrup et al., 1996). Fifty-four percent of these neurons have disappeared by P26 (Caddy and Biscoe, 1979). To determine whether *Bax* deletion was able to rescue the target-related cell death of olivary neurons in Lurcher mice, we analyzed the morphology of the inferior olive in P30 $Grid2^{Lc/+}$;$Bax-/-$ double mutant animals. Coronal sections of the brainstem from 10 different rostrocaudal levels of the inferior olive were stained with cresyl violet-thionin. No obvious difference was detected between the inferior olive of $Grid2^{Lc/+}$;$Bax-/-$ double mutant and $Grid2^{Lc/+}$;$Bax+/+$ control mice (Fig. 2*B*,*C*). A massive loss of olivary neurons was observed in all the subnuclei of the inferior olive in both $Grid2^{Lc/+}$;$Bax-/-$ double mutants and $Grid2^{Lc/+}$;$Bax+/+$ control mice when compared with the inferior olive of a wild-type mouse (Fig. 2*A*). The rostrocaudal extension of the inferior olive was ~1100 µm in both $Grid2^{Lc/+}$;$Bax-/-$ double mutant and $Grid2^{Lc/+}$;$Bax+/+$ control mice. This value is reduced compared with the wild-type one in accordance with the results of Heckroth and Eisenman (1991). This result suggests that *Bax* inactivation does not inhibit the target-related cell death of inferior olivary neurons occurring in $Grid2^{Lc/+}$ mice.

The results of the double $Grid2^{Lc/+}$ and $Bax-/-$ cross at P30 show that *Bax* inactivation in Lurcher mice inhibits target-related granule cell death but not the target-related death of inferior olivary neurons or intrinsic Purkinje cell death.

Purkinje cell death is temporarily delayed by *Bax* inactivation in Lurcher mice

Cerebellar sections from P15 mice were analyzed to look for an early effect of *Bax* inactivation on Lurcher Purkinje cell death. At P15, numerous Purkinje cells were still present in $Grid2^{Lc/+}$; $Bax+/+$ control and $Grid2^{Lc/+}$;$Bax-/-$ double mutant mice (Fig. 3). The presence of gaps in the Purkinje cell layer showed that Purkinje cell degeneration has already begun at this age in animals
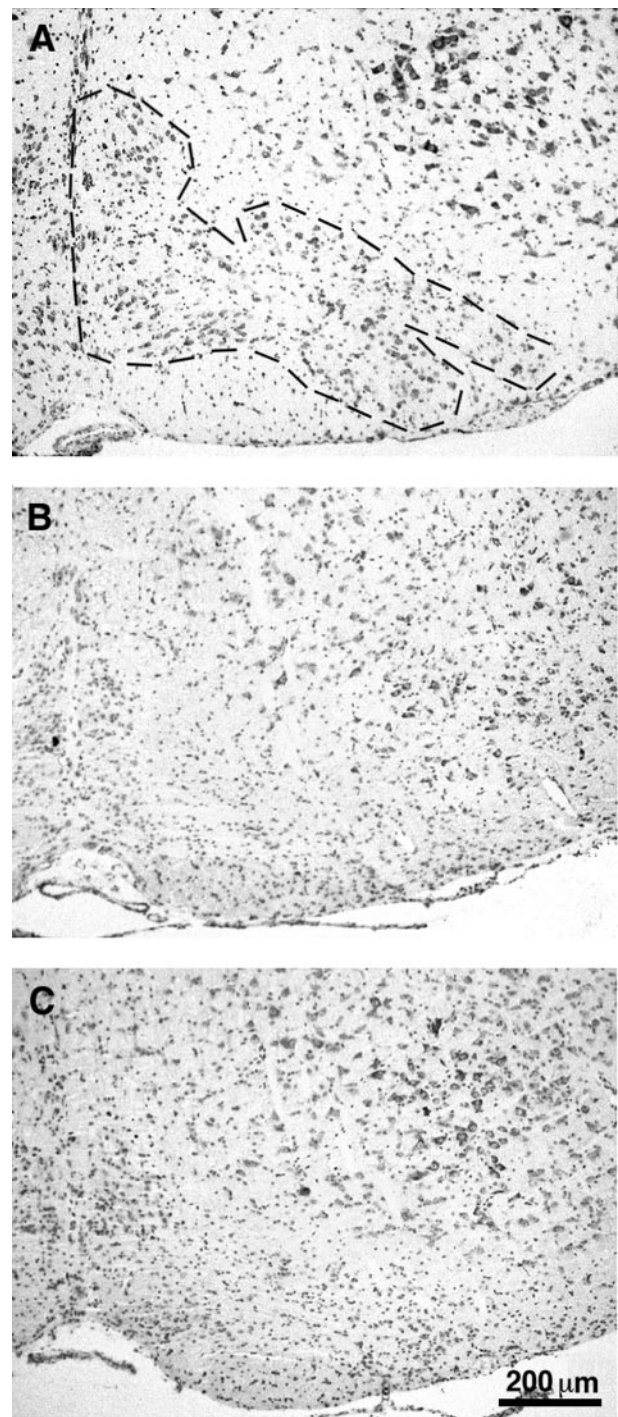

Figure 2. Neurodegeneration in the inferior olive of Lurcher mice is not inhibited by *Bax* inactivation. Coronal sections (10-µm-thick) of the brainstem were stained using cresyl violet-thionin. Comparison of the inferior olive in wild-type mice (*A*, delineated by a *dotted line*) and $Grid2^{Lc/+}$; $Bax+/+$ (*B*) control mice shows a massive degeneration of neurons in the mutant. This neurodegeneration is also observed in $Grid2^{Lc/+}$; $Bax-/-$ double mutants (*C*) and makes the subnuclei of the inferior olive hardly recognizable in contrast to wild-type.

of both genotypes (Fig. 3*A*,*C*), in concordance with the observation that progressive neurodegeneration begins at approximately P8. Quantification of the Purkinje cell number per hemi-cerebellum (Fig. 4*A*) showed that their number was significantly higher in $Grid2^{Lc/+}$;$Bax-/-$ double mutant cerebella (62,680 ± 2152) compared with $Grid2^{Lc/+}$;$Bax+/+$ cerebella (45,261 ± 1813; ANOVA followed by *post hoc* Newman–Keuls test analysis, $p < 0.05$). The analysis of the laterolateral distribution of Purkinje cells
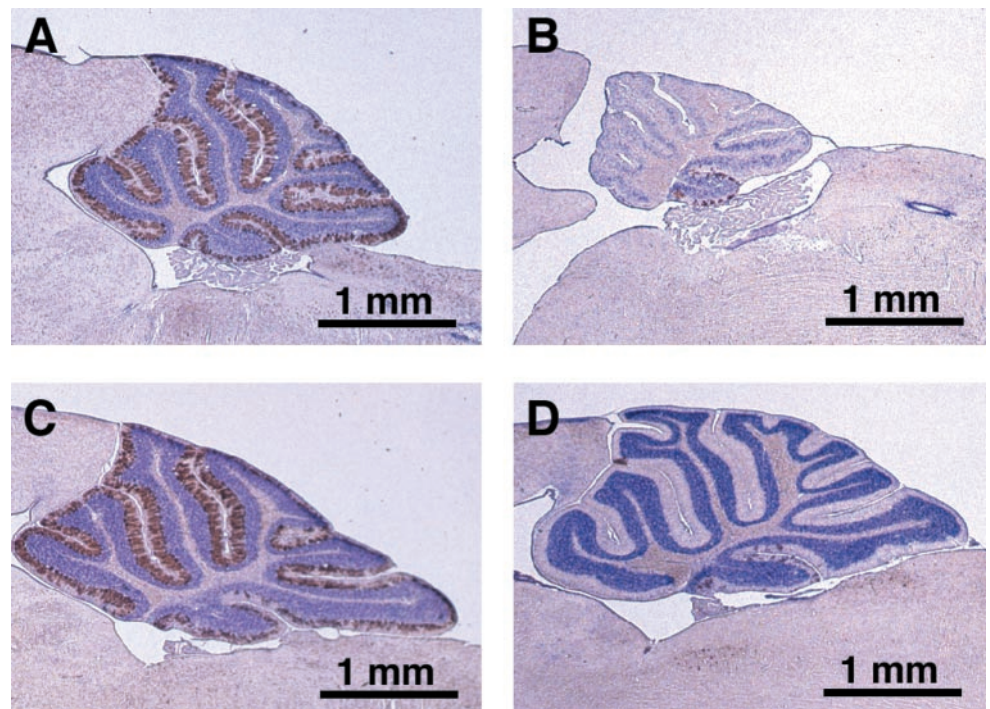
Figure 3. Morphology of the cerebellum of control Lurcher and *Bax* knock-out Lurcher mice at P15 and 2 months. Purkinje cell death has already begun at P15 from $Grid2^{Lc/+}$;Bax+/+ controls (*A*) as well as $Grid2^{Lc/+}$;Bax−/− double mutant mice (*C*), as shown by the presence of gaps in the Purkinje cell layer. The surface of the internal granule cell layer is persistently increased at 2 months in $Grid2^{Lc/+}$; Bax−/− double mutant mice (*D*) when compared with $Grid2^{Lc/+}$;Bax+/+ control animals (*B*).

in both $Grid2^{Lc/+}$;Bax−/− and $Grid2^{Lc/+}$;Bax+/+ cerebella showed that the number of Purkinje cells was increased throughout all the regions of the cerebellum in double mutants, indicating that Bax inactivation does not rescue a particular subpopulation of Purkinje cells (Fig. 4*C*). The number of granule cells in $Grid2^{Lc/+}$;Bax−/− animals was already significantly higher than in $Grid2^{Lc/+}$;Bax+/+ mice ($9.09 \pm 0.38 \times 10^6$ vs $6.50 \pm 0.06 \times 10^6$, respectively; ANOVA, followed by *post hoc* Newman–Keuls test analysis, $p < 0.05$) and was not significantly different from the one found in P30 animals (Fig. 4*B*).

These results show that *Bax* inactivation does not inhibit but temporarily delays the degeneration of some Purkinje cells in Lurcher mice until P30.

Granule cell rescue by *Bax* inactivation is persistent

The effect of *Bax* inactivation in P60 animals was analyzed to determine whether granule cell rescue was a lasting effect in $Grid2^{Lc/+}$ double mutants (Fig. 3*B,D*). In $Grid2^{Lc/+}$;Bax+/+ control mice as well as in $Grid2^{Lc/+}$;Bax−/− double mutant mice, Purkinje cell degeneration was almost complete at this age, except for few cells still remaining in the nodulus (525 ± 97 vs 1268 ± 218, respectively; ANOVA followed by *post hoc* Newman–Keuls test analysis, $p > 0.05$) (Fig. 4*A*). The number of granule cells per $Grid2^{Lc/+}$;Bax+/+ half-cerebellum was $1.39 \pm 0.06 \times 10^6$, ~10% of the normal number of granule cells. Thus, the degeneration of granule cells is almost complete at this age in $Grid2^{Lc/+}$;Bax+/+ control mice as 10% of these cells still remain at P730 (Caddy and Biscoe, 1979). The analysis of cerebellar sections from P60 $Grid2^{Lc/+}$;Bax−/− double mutant mice (Fig. 3*D*) revealed that the area of the internal granule cell layer was still increased when compared with $Grid2^{Lc/+}$;Bax+/+ control mice (Fig. 3*B*). The number of granule cells per hemi-cerebellum in $Grid2^{Lc/+}$;Bax−/− mice ($11.05 \pm 0.41 \times 10^6$) was significantly higher compared with $Grid2^{Lc/+}$;Bax+/+ controls (ANOVA followed by the Newman–Keuls *post hoc* test analysis, $p < 0.05$). This number was not significantly different from the numbers of granule cells found in $Grid2^{Lc/+}$;Bax−/− mice at P15 and P30 (ANOVA followed by the Newman–Keuls *post hoc* test analysis, $p > 0.05$). The number of rescued granule cells is ~70% of the number of granule cells found in wild-type animals (Vogel et al., 1991). These results indicate that granule cell rescue by *Bax* inactivation in Lurcher is persistent until at least 2 months.

DISCUSSION

The Lurcher mutant presents a unique opportunity to study two distinct neuronal cell death mechanisms in the same *in vivo* model in which the primary cause of Purkinje cell death has been identified as a gain of function mutation in a glutamate receptor channel and the secondary cause of granule and olivary neuron cell death is loss of target trophic support. In this study, we show that deletion of *Bax* expression in the $Grid2^{Lc/+}$ mutant does not prevent Purkinje cell or olivary neuron cell death, but it does rescue granule cells.

Purkinje cell death in the $Grid2^{Lc/+}$ mutant

All but a few cerebellar Purkinje cells, 90% of the granule cells, and 60–75% of the olivary neurons degenerate postnatally in the $Grid2^{Lc/+}$ mutant (Phillips, 1960; Caddy and Biscoe, 1979). Whereas $Grid2^{Lc/+}$ PC loss is attributable to a cell autonomous genetic defect involving a gain of function mutation in the δ2 glutamate receptor (*Grid2*) (Zuo et al., 1997), the mechanisms of PC death in the $Grid2^{Lc/+}$ mutant have not yet been definitively characterized. $Grid2^{Lc/+}$ PC death has been described as necrotic based on morphological criteria (Dumesnil-Bousez and Sotelo, 1992). However, dying $Grid2^{Lc/+}$ PCs have been labeled with TUNEL and contain increased levels of apoptosis-related proteins, such as BAX, BCL-x, and both pro-caspase-3 and activated caspase-3, suggesting an apoptotic mechanism (Norman et al., 1995; Wullner et al., 1995, 1998; Selimi et al., 2000).

The involvement of apoptosis-related proteins in $Grid2^{Lc/+}$ Purkinje cell death is also shown by the ability of *bcl-2* transgene overexpression to delay $Grid2^{Lc/+}$ PC death (Zanjani et al., 1998a,b). One of the mechanisms by which BCL-2 may exert its neuroprotective action is through inhibition of the pro-apoptotic protein BAX (Oltvai et al., 1993; Antonsson et al., 1997; Mahajan, 1998). BAX mRNA is expressed at high levels in the postnatal mouse brain (De Bilbao et al., 1999). BAX protein expression levels may gradually decline in the cerebellum through the first 21 d of development, although expression levels of BAX remain high in PCs in the adult (Vekrellis et al., 1997). BAX plays an important role in naturally occurring cell death because neuronal cell death is reduced in Bax−/− mice; for example, the number of facial nucleus motoneurons is increased by 51% (Deckwerth et al., 1996; White et al., 1998).
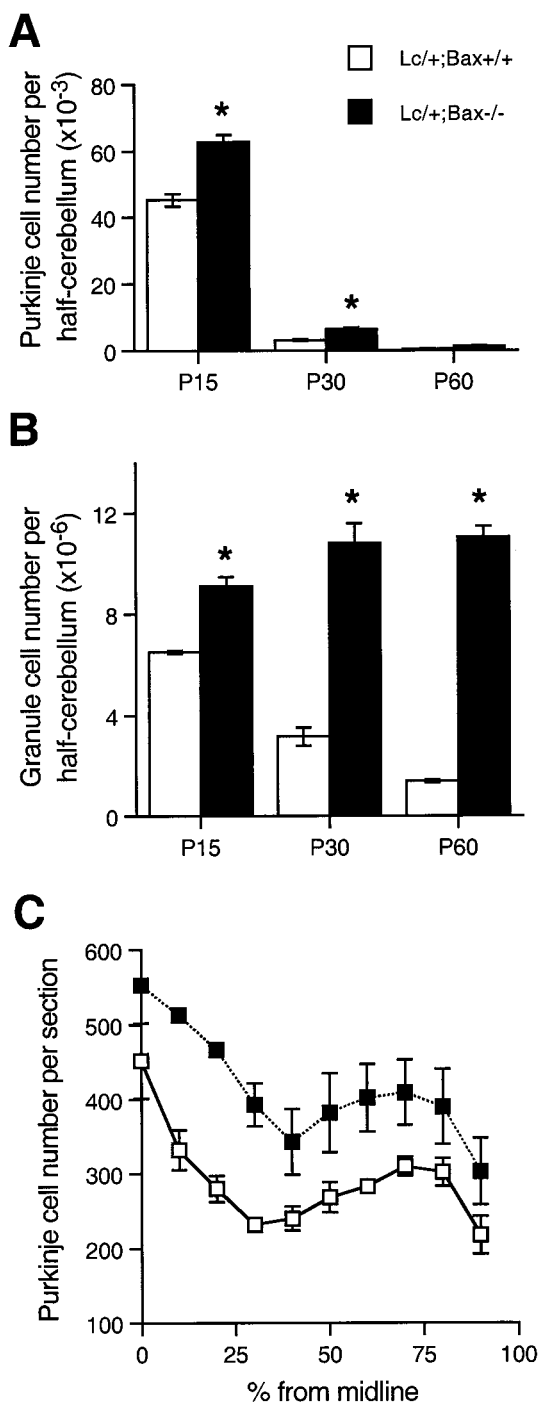
Figure 4. Quantitative analysis of neuronal death in the cerebellum of *Bax* knock-out Lurcher mice. *A*, Purkinje cell number per half-cerebellum was assessed using anti-calbindin immunostained cerebellar sections. *B*, Granule cell number per half-cerebellum was estimated multiplying the area of the internal granule cell layer by the average density of granule cells. *Asterisks* indicate that numbers found in *Grid2^{Lc/+}*;Bax−/− double mutant mice are statistically different from numbers found in *Grid2^{Lc/+}*;Bax+/+ mice (*p* < 0.05, ANOVA followed by *post hoc* Newman–Keuls test analysis). Error bars indicate SEM (*n* = 3–5). *C*, Averaged laterolateral distribution of the Purkinje cell population in P15 aged mutants of both genotypes. The mean Purkinje cell counts per homologous parasagittal section are plotted against the distance from the midline (percentage). Cell numbers in *Grid2^{Lc/+}*;Bax−/− double mutants are significantly higher than in *Grid2^{Lc/+}*;Bax+/+ mice without any effect of the laterolateral position (two-factor ANOVA). Each *point* represents mean ± SEM (for some points, the error bar is smaller than the dot).

BAX expression is increased in dying *Grid2^{Lc/+}* PCs, suggesting it may play a role in their death (Wullner et al., 1998). Yet, it is surprising that deletion of BAX expression only slightly delays *Grid2^{Lc/+}* PC death. The number of *Grid2^{Lc/+}* Purkinje cells is significantly increased at P15 in the *Grid2^{Lc/+}* double mutant, but by P30 this number has returned to a low level, and at P60 is not significantly different to the one found in the *Grid2^{Lc/+}* mutant. The available evidence suggests that *Grid2^{Lc/+}* PCs may die by an excitotoxic mechanism induced by the accumulation of leaky GRID2 subunits at developing PC–parallel fiber synapses (Zuo et al., 1997; De Jager and Heintz, 1998). The Lurcher gain of function mutation in *Grid2* results in a large, constitutive inward sodium current in the cells that express the subunit and might induce a defect in Ca^{2+} homeostasis (Zuo et al., 1997). Although we cannot rule out a role for BAX-induced apoptosis in *Grid2^{Lc/+}* PCs, their death in *Bax* knock-out Lurcher mutants indicates that BAX is not necessary for their demise. One likely hypothesis is that there are multiple pathways and/or other BCL-2 family members capable of inducing cell death in stressed cells, including Purkinje cells. The slight delay in *Grid2^{Lc/+}* PC death seen at P15 may represent the difference between BAX cell death usually induced in *Grid2^{Lc/+}* mutants and a different mechanism induced in *Grid2^{Lc/+};Bax−/−* double mutants. BCL-2 overexpression may be able to temporarily rescue *Grid2^{Lc/+}* Purkinje cells (Zanjani et al., 1998a) because of its ability to bind to BAX and prevent the formation of BAX channels (Antonsson et al., 1997; Mahajan, 1998) and/or its ability to regulate intracellular Ca^{2+} homeostasis (Murphy et al., 1996; Ichimiya et al., 1998; Kuo et al., 1998). However, in the end, the *Grid2^{Lc/+}* mutation is lethal and neither *Bax* inactivation or *bcl-2* overexpression can rescue Lurcher Purkinje cells.

Granule cell survival and olivary neuron death

In the olivocerebellar system, surgical and genetic lesions have shown that the survival of olivary neurons and granule cells is dependent on interactions with their PC targets (Harkmark, 1956; Sotelo and Changeux, 1974; Caddy and Biscoe, 1979; Wetts and Herrup, 1982a,b; Zanjani et al., 1990; Vogel et al., 1991; Herrup et al., 1996). Many olivary neurons and granule cells undergo apoptotic cell death when deprived of their target (Chu and Oberdick, 1995; Smeyne et al., 1995). In the *Grid2^{Lc/+}* mutant, activated caspase-3 is found in dying granule cells and olivary neurons (Selimi et al., 2000). There are multiple lines of evidence that support a role for BCL-2-related proteins, including BAX, in target-related cell death. We have shown previously that overexpression of *bcl-2* in *Grid2^{Lc/+}* olivary neurons will rescue most of the olivary neurons from target-related cell death (Zanjani et al., 1998b). This result is consistent with previous studies showing *in vitro* rescue of neurons from trophic factor withdrawal (Garcia et al., 1992; Allsopp et al., 1993; Batistatou et al., 1993) and *in vivo* rescue of neurons from axotomy or ischemia by *bcl-2* overexpression (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Farlie et al., 1995; Bonfanti et al., 1996; De Bilbao and Dubois-Dauphin, 1996) or deletion of *Bax* expression (Deckwerth et al., 1996; White et al., 1998).

However, our results reveal differences in the pathway leading to this target-related cell death *in vivo*; deletion of *Bax* expression in *Grid2^{Lc/+}* mutants rescues granule cells but does not prevent olivary neuron death. Both populations are dying because of loss of their target, but granule cell death appears to be dependent on *Bax* expression, whereas olivary neuron cell death can be independent of *Bax*. Granule cell rescue could have been a consequence of the delay in Purkinje cell death. For example, in *pcd* mutant mice, Purkinje cell death occurs between the third and sixth postnatal week, and the number of granule cells is not different from controls in P30 animals. However, this number is significantly decreased in 3-month-old *pcd* mutants and follows an exponential decay, suggesting that a transient trophic support from Purkinje cells is not sufficient to inhibit persistently target-related granule cell death (Triarhou, 1998). Not even a slight decrease in granule cell numbers was observed in Bax knock-out Lurcher mice from P15 to P60,

suggesting that the delay in Purkinje cell death is unlikely to be the only cause of granule cell rescue. Our estimates of granule cell numbers indicate that there is an eightfold increase in the number of surviving granule cells in the $Grid2^{Lc/+};Bax-/-$ double mutants compared with $Grid2^{Lc/+};Bax+/+$ controls. Granule cell numbers in our *Bax* knock-out Lurcher mutants are only reduced by 30% compared with wild-type numbers (Vogel et al., 1991). The number of granule cells in $Grid2^{Lc/+};Bax-/-$ double mutants is comparable with the maximum of granule cells detected in $Grid2^{Lc/+};Bax+/+$ cerebella (8×10^6) (Caddy and Biscoe, 1979). Thus, Bax inactivation does not significantly affect the deficit in granule cell genesis detected in Lurcher mutants. It seems more likely that the primary cause of granule cell rescue in Bax knock-out Lurcher mice is the loss of Bax expression. Wild-type and Lurcher granule cells have been shown to express Bax during the period of degeneration in Lurcher cerebellum (Vekrellis et al., 1997; Wullner et al., 1998), and our results suggest that Bax plays an important role in regulating granule cell death after removal of target-related trophic support.

The molecular pathways that link trophic factor deprivation with cell death have not yet been fully characterized. The binding of trophic factors with their receptors triggers a variety of signaling responses that promote cell survival, in some cases through interactions with BCL-2-related proteins (Kaplan and Miller, 1997; Pettmann and Henderson, 1998). For example, NGF or IGF-1 can induce the phosphorylation of BAD, a non-membrane-bound BCL-2 relative, by activating Akt, a serine-threonine protein kinase. In the absence of trophic factor, nonphosphorylated BAD will bind to Bcl-x and prevent the anti-apoptotic activity of BCL-x. The anti-apoptotic activity of BCL-x may involve binding to BAX to prevent it from opening a pore in mitochondrial membranes (Gross et al., 1999). Recently, NGF has also been shown to promote Bcl-2 expression in NGF-responsive neurons (Riccio et al., 1999). It is yet not clear what pathway or pathways are involved in cell death in granule cells and olivary neurons. There are a large variety of BCL-2-related cell death proteins that promote or block cell death (Gross et al., 1999). For example, the loss of *Bax* expression in *Bcl-x* deficient mice prevents most, but not all, cell death so there must be other cell death-promoting proteins (Shindler et al., 1997). The rescue of granule cells, but not olivary neurons, by *Bax* inactivation after target loss indicates that, although BAX is required for target-related cell death in granule cells, there may be other cell death-promoting proteins functioning in olivary neurons.

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