The Journal of Neuroscience, January 1, 1998, *18*(1):319–327

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

H. S. Zanjani,[1,2] M. W. Vogel,[4] J. C. Martinou,[3] N. Delhaye-Bouchaud,[1] and J. Mariani[1]

[1]*Laboratoire de Neurobiologie du Développement, Institut des Neurosciences et Unité de Recherche Associée, Centre National de la Recherche Scientifique 1488, Université Pierre et Marie Curie, 75005 Paris, France,* [2]*Physiological Science Department, University of California at Los Angeles, Los Angeles, California 90095-1527,* [3]*Geneva Biomedical Research Institute, Glaxo Wellcome Research and Development SA, 1228 Plan-les-Ouates, Geneva, Switzerland, and* [4]*Maryland Psychiatric Research Center, University of Maryland Medical School, Baltimore, Maryland 21228*

The *Lurcher* mutant has been extensively studied as a model for cell-autonomous and target-related cell death, yet there are still many unknowns concerning the mechanisms of neuronal degeneration in this mutant. As a key regulator of apoptosis, a *bcl-2* transgene has been overexpressed in the heterozygous *Lurcher* mutant to investigate the effects of BCL-2 on two types of *in vivo* neuronal cell loss in *Lurcher*: cell-autonomous Purkinje cell degeneration and target-related olivary neuron death. Six adult +/*Lc* mutants expressing a human *bcl-2* transgene (*Hu-bcl-2*) were generated by crossing +/*Lc* mutants with NSE71 *Hu-bcl-2* transgenic mice. Analysis of these brains showed that *bcl-2* overexpression did not prevent +/*Lc* Purkinje cell degeneration, but it did rescue most olivary neurons from target-related cell death. Although the number of olivary neurons was equivalent to wild-type numbers, the inferior olive nucleus was significantly shorter in its rostrocaudal extent, suggesting that olivary neurons are atrophied. We propose that *Lurcher* gene action causes Purkinje cell degeneration independently of a BCL-2-mediated pathway. Furthermore, although *bcl-2* overexpression rescues olivary neurons from target-related cell death, it does not prevent the atrophy associated with the loss of target-related trophic support.

Key words: olivary neurons; Purkinje cells; BCL-2; Lurcher *mutant; programmed cell death; cerebellar mutants*

Many genes have been identified that promote or inhibit cell death pathways, but their relative functions in various types of programmed and pathological cell death have yet to be established. The purpose of this study is to determine whether one of the key regulators of apoptotic cell death, BCL-2, can regulate two distinct types of cell death: cell-autonomous neuronal degeneration and target-related cell death as occurs in the heterozygous *Lurcher* (+/*Lc*) mutant. Virtually all cerebellar Purkinje cells, 90% of the granule cells, and 60–75% of the olivary neurons degenerate postnatally in the +/*Lc* mutant (Phillips, 1960; Caddy and Biscoe, 1979). Although +/*Lc* Purkinje cell loss is attributable to a cell-autonomous genetic defect involving a gain of function mutation in the δ2 glutamate receptor (Zuo et al., 1997), olivary neuron and granule cell death is secondary to the loss of their primary target, the Purkinje cells (Wetts and Herrup, 1982a,b). Many olivary neurons and granule cells undergo apoptotic cell death when deprived of their target (Chu and Oberdick, 1995; Smeyne et al., 1995), but the mechanisms of Purkinje cell death in the +/*Lc* mutant have not yet been characterized definitively. Both necrotic (Dumesnil-Bousez and Sotelo, 1992) and apoptotic (Norman et al., 1995; Wullner et al., 1995) mechanisms have been hypothesized on the basis of morphological and molecular criteria.

The *bcl-2* proto-oncogene produces an integral membrane protein that inhibits apoptosis in many cell types, including neuronal cell lines after the withdrawal of growth factors (Hockenberry et al., 1990; Garcia et al., 1992; Allsopp et al., 1993; Batistatou et al., 1993). Overexpression of BCL-2 during embryonic or postnatal development *in vivo* can significantly reduce the extent of naturally occurring cell death in a wide variety of neurons (including Purkinje cells and olivary neurons) and can rescue neurons from external injuries (e.g., axotomy or ischemia) or genetic lesions (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Farlie et al., 1995; Sagot et al., 1995; Bonfanti et al., 1996; Chen et al., 1996; De Bilbao and Dubois-Dauphin, 1996; Lawrence et al., 1996; Zanjani et al., 1996, 1997). The purpose of this investigation is to determine whether overexpression of a human *bcl-2* transgene in the *Lurcher* mutant will rescue +/*Lc* Purkinje cells from their genetically programmed cell death and olivary neurons from target-related cell death. BCL-2 was overexpressed in the *Lurcher* mutant by crossing +/*Lc* mutants with a line of transgenics (NSE71) that overexpresses the human *bcl-2* (*Hu-bcl-2*) proto-oncogene using a neuron-specific enolase promoter (Dubois-Dauphin et al., 1994; Martinou et al., 1994). *Hu-bcl-2* expression in NSE71 transgenics begins around birth, and the transgene is expressed in most neurons throughout the nervous system, including Purkinje cells and olivary neurons (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Zanjani et al., 1996, 1997). Analysis of *Hu-bcl-2*–+/*Lc* mutants shows that transgene expres-

Received May 21, 1997; revised Oct. 17, 1997; accepted Oct. 22, 1997.

This work was supported by Grants given to J.M. from Fondation de la Recherche Médicale Grant "vieillissement" to J.M., a grant from Fondation de France AFM to J.M., European Community Biotech Grant BIO4CT96 0774 to J.M. and J.C.M., and National Institutes of Health Grants NS 29277 and NS 34309 and a Special Research Initiative Support University of Maryland Medical School grant to M.W.V. We thank Ms. P. Bouquet for technical assistance and Ms. Sharon Candeloro for editorial assistance.

Correspondence should be addressed to Dr. Michael W. Vogel, Maryland Psychiatric Research Center, University of Maryland Medical School, P.O. Box 21247, Baltimore, MD 21228.

sion fails to prevent +/*Lc* Purkinje cell degeneration, but does rescue most olivary neurons.

MATERIALS AND METHODS

Animals. Male heterozygous *Lurcher* mutants (+/*Lc*) were crossed with female NSE71 transgenics to create +/*Lc* mutants (and +/+ controls) that overexpress the *Hu-bcl-2* transgene. The *Lurcher* mutant mice used in this study (genotype, ++/*LcMi*^{wh}) were kindly provided by Dr. K. W. Caddy (University College London). In this strain the *Lurcher* gene (*Lc*) is on a 129 Sv genetic background, and it is closely linked to the gene microphthalmia and white coat color (Mi^{wh}), with a 10% frequency of crossover (Doughty et al., 1995). +/*Lc* mutants were distinguished from their +/+ littermates by the presence of white spots on their coat and their smaller eyes compared with controls. NSE71 transgenic mice were provided by Dr. J. C. Martinou (Biomedical Research Institute, Glaxo Wellcome Research and Development SA) (Dubois-Dauphin et al., 1994; Martinou et al., 1994). Tail samples from weanling pups were used to identify mice overexpressing the *Hu-bcl-2* transgene using previously described PCR protocols (Martinou et al., 1994, Zanjani et al., 1996). The crossing experiments were performed in the animal colonies at the Université Pierre et Marie Curie. Mice were provided food and water *ad libitum* and maintained on a 12 hr light/dark cycle.

The crossing experiments produced 22 adult animals. Ten of these mice were identified as +/*Lc* in genotype by the appearance of white spots at birth and later by their microphthalmia. All of these mice later showed the ataxia symptoms characteristic of +/*Lc* mutants. Of the 10 +/*Lc* mutants, 6 were genotyped as *Hu-bcl-2* transgenics by PCR. The 12 remaining mice were identified as wild type at the *Lurcher* locus, because they did not show the *Mi*^{wh} phenotype, and they displayed normal locomotory behaviors. Five of these mice tested positive as *Hu-bcl-2* transgenics. In addition to the adult mice, two litters of mice were killed at 18 d of postnatal development. Based on the same criteria, four of the pups were identified as +/*Lc* mutants that also expressed the *Hu-bcl-2* transgene.

Histology. Adult and postnatal day 18 (P18) mice were killed by cardiac perfusion with 4% paraformaldehyde in phosphate buffer (0.1 M) while deeply anesthetized with Avertin. All of the adult mice were between 5 and 6 months old when they were killed. Brains were post-fixed overnight in the same fixative and then dehydrated through graded alcohol series and embedded in paraffin. The cerebellum was sectioned sagittally, whereas the brain stem was sectioned coronally, both at 10 μm. Sections were stained with cresyl violet.

Cell counts. Neurons in the inferior olivary nucleus (ION) were counted using previously described techniques (Shojaeian et al., 1985a,b; Zanjani et al., 1990, 1994). In brief, olivary neurons were counted in each olivary subnuclei on both sides of the brain in every fifth section at 60× with either a Zeiss or Olympus microscope. Only cells with a clear nucleus and a distinct nucleolus or nucleolar fragment were counted. The correction factor of Floderus (1944) was used for correcting olivary neuron double-counting errors. The area and circular diameter of >300 olivary neurons were measured in each brain to calculate the correction factor for that brain. We chose to use this traditional correction factor for our cell counts instead of more recently developed stereological techniques (e.g., Williams and Rakic, 1988; Andersen et al., 1992), so that our results would be directly comparable with previously published olivary neuron counts, including our own (Shojaeian et al., 1985a,b; Zanjani et al., 1990, 1994, 1997; Herrup et al., 1996). In addition to the cell counts, in coronal sections of each brain we measured the area of olivary neuron cell body profiles (>90 cells measured per brain) and the rostrocaudal extent of the ION. The rostrocaudal extent of the ION was determined by multiplying the number of sections in which olivary neurons were identified by the average thickness of the sections (10 μm). Quantitative data obtained from the two sides of each animal were averaged, and mean values were calculated for each group. Statistical comparisons between wild-type mice, *Hu-bcl-2* transgenics, and *Hu-bcl-2*–+/*Lc* mutants were made using a one-factor ANOVA. *Post hoc* comparisons were made using a Bonferroni–Dunn test (StatView 4.5). Measurements of nucleolar diameter and size of cell body profiles were made using a Macintosh IIci computer and the NIH Image program.

Immunocytochemistry. Immunostaining was used to detect expression of the human BCL-2 protein in olivocerebellar neurons. Animals were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was removed, post-fixed for 1 hr in the same fixative, and cryoprotected in a 30% sucrose/PBS solution at 4°C overnight. Brains were cut at 40 μm on a cryostat and collected in PBS. Sections were incubated for 1 hr in normal horse serum (3%) diluted in PBS containing 0.3% Triton X-100 and then incubated overnight at 4°C in mouse monoclonal antibody to human Bcl-2 (Cambridge Research Biochemicals) diluted in the same buffer. The primary antibody was revealed using the avidin–biotin–peroxidase technique. Calbindin immunostaining in the cerebellum was performed on paraffin sections using an antiserum to calbindin (calbindin-binding protein) from Sigma (St. Louis, MO) and visualized using the avidin–biotin–peroxidase technique using standard protocols. Color photomicrographs of immunolabeled sections were digitized on an Epson ES-1200C scanner and assembled into color plates in Adobe Photoshop. The images were contrast-enhanced but otherwise unretouched.

RESULTS

In this study *Hu-bcl-2* transgenic mice (line NSE71; Dubois-Dauphin et al., 1994) were crossed with ++/*Lc* Mi^{wh} mutants to overexpress the *Hu-bcl-2* transgene in +/*Lc* Purkinje cells and olivary neurons. In the ++/*Lc* Mi^{wh} mutant strain, the *Lurcher* gene is closely linked to microphthalmic–white. The microphthalmic–white mutation causes a white coat spot in neonates and a microphthalmia that can be recognized within a few weeks. Both phenotypes were used to identify +/*Lc* mutants. Six adult mice (*Hu-bcl-2*–+/*Lc* mutants) were prospectively identified as +/*Lc* mutants that overexpress *Hu-bcl-2* on the basis of a white coat spot, indicating linkage to *Lurcher* and by PCR for *Hu-bcl-2* expression. In retrospect, the +/*Lc* genotype of the animals was confirmed histologically, because virtually all of the Purkinje cells degenerated in these mice.

Qualitative observations

Cerebellum

The foliation and laminar organization of the cerebellum in wild-type and *Hu-bcl-2* transgenic cerebella (Fig. 1*A*) are similar and well preserved, as described previously (Zanjani et al., 1996). In contrast, the degeneration of +/*Lc* Purkinje cells during the first postnatal month development dramatically alters cerebellar cytoarchitecture in the +/*Lc* mutant, as revealed by Nissl staining (Fig. 1*B*). The +/*Lc* cerebellum is visibly atrophied because of the prominent loss of granule cells associated with the disappearance of their Purkinje cell targets. Overexpression of the *Hu-bcl-2* gene in the +/*Lc* mutant mice was clearly insufficient to prevent +/*Lc* Purkinje cells from degenerating; the cerebella of the *Hu-bcl-2*–+/*Lc* mutants (Fig. 1*C*) were similar in size to those of +/*Lc* mutants (Fig. 1*B*). Cerebellar atrophy was already apparent in the mutants when their brains were removed under a dissecting microscope (results not shown). Examination of serial sections at the light microscope level showed that +/*Lc* Purkinje cell somas had virtually disappeared from the *Hu-bcl-2*–+/*Lc* cerebellum, and the number of cerebellar granule cells was also dramatically reduced. The *Hu-bcl-2* transgene is not expressed in cerebellar granule cells, so their loss is consistent with the loss of their Purkinje cell targets.

To verify the virtually complete degeneration of Purkinje cells in *Hu-bcl-2*–+/*Lc*–mutants, cerebellar sections were stained with a monoclonal antibody against calbindin. Calbindin is heavily expressed in Purkinje cell bodies and dendrites in wild-type animals (Fig. 2*A*) and NSE71 *Hu-bcl-2* transgenic mice (Fig. 2*B*), making it easy to identify Purkinje cells. Only a very few Purkinje cells with small cell bodies and poorly developed dendrites are detected in the *Hu-bcl-2*–+/*Lc* mutant with anti-calbindin antibody labeling (Fig. 2*C*). The number, abnormal shape, and location (mostly in the nodulus) of +/*Lc* Purkinje cells in the *Hu-bcl-2*–+/*Lc* mutant (Fig. 2*D*) were indistinguishable from those of the rare Purkinje cells detected in the +/*Lc* mutant cerebellum.

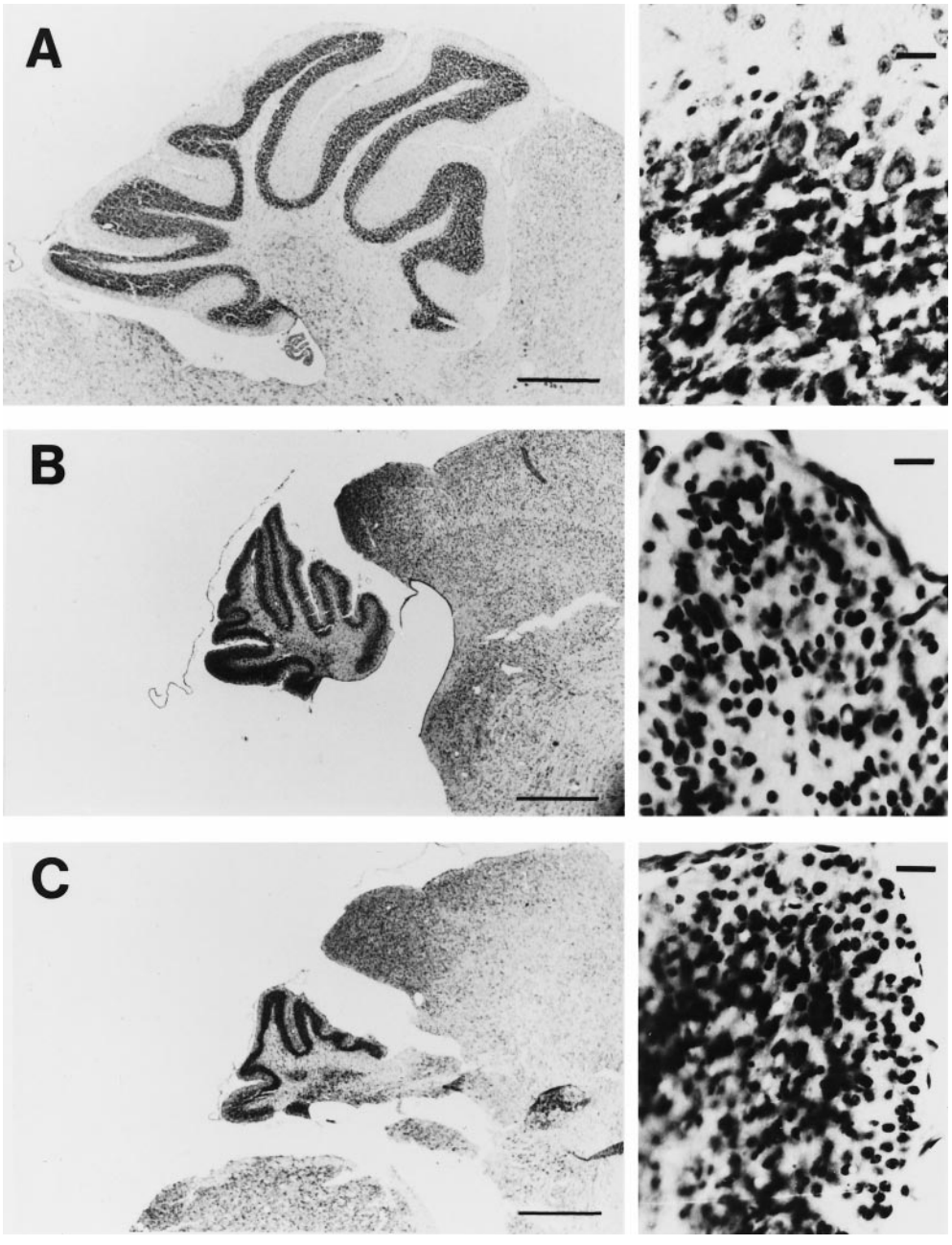

Figure 1. Photomicrographs of midsagittal sections of cerebella from a wild-type mouse (*A*), a +/*Lc* mutant (*B*), and an *Hu-bcl-2*–+/*Lc* mutant (*C*) stained with cresyl violet. The low-power photographs on the *left* show the folial organization of each cerebellum, and the higher-magnification *insets* on the *right* illustrate the cytoarchitecture of each type of animal. Both the +/*Lc* and the *Hu-bcl-2*–+/*Lc* mutants are characterized by a conspicuous reduction in cerebellar size attributable to the loss of almost all Purkinje cells and most granule cells. Scale bars: low-magnification photographs, 500 μm; high-magnification photographs, 20 μm.

Immunolabeling for human BCL-2 protein was detected in the olivary neurons of both *Hu-bcl-2* transgenic mice (Fig. 2*E*) and *Hu-bcl-2*–+/*Lc* mutants (Fig. 2*F*). Human BCL-2 labeling in the *Hu-bcl-2*–+/*Lc* mutants is not as robust as in the Hu-bcl-2 transgenics, but immunostained olivary neurons are apparent throughout the ION in the *Hu-bcl-2*–+/*Lc* mutants, as shown in the lower-magnification photomicrograph in Figure 2*F* (*arrows* indicate individual neurons). Figure 2*F, inset*, shows human BCL-2 immunolabeling in *Hu-bcl-2*–+/*Lc* mutant olivary neurons at a higher magnification.

In addition to looking for calbindin-stained +/*Lc* Purkinje cells and BCL-2-labeled olivary neurons in *Hu-bcl-2*–+/*Lc* adults, four *Hu-bcl-2*–+/*Lc* mutants were killed during the period of +/*Lc* Purkinje cell death (P18) to look for human BCL-2 expression in +/*Lc* Purkinje cells before their degeneration. Selected sections were stained with antibodies to human BCL-2, and positive staining showed that the *Hu-bcl-2* transgene is expressed in +/*Lc* Purkinje cells before they degenerate (Fig. 3). Human BCL-2 immunoreactivity is concentrated in the molecular layer and Purkinje cell layer, as shown in a low-power photomicrograph of a posterior lobule of a P18 *Hu-bcl-2*–+/*Lc* mutant (Fig. 3*A*). Human BCL-2 immunoreactivity in the transgenics is not as robust as the Golgi-like calbindin labeling (compare Figs. 2*A,B*, 3), but labeled Purkinje cells are apparent. Most +/*Lc* Purkinje cells at P18 have abnormal dendritic trees, and in the *Hu-bcl-2*–+/*Lc* mutants their cell bodies and dendrites are filled with BCL-2 protein, as shown by human BCL-2 immunoreactivity in a higher-power photomicrograph from an anterior lobule (Fig. 3*B, arrows*). The Purkinje cells shown in Figure 3*B* are located in an anterior lobule, so they are destined to die; all Purkinje cells in anterior lobules will have degenerated by 5 months postnatally, the age when the adult mutants were analyzed.

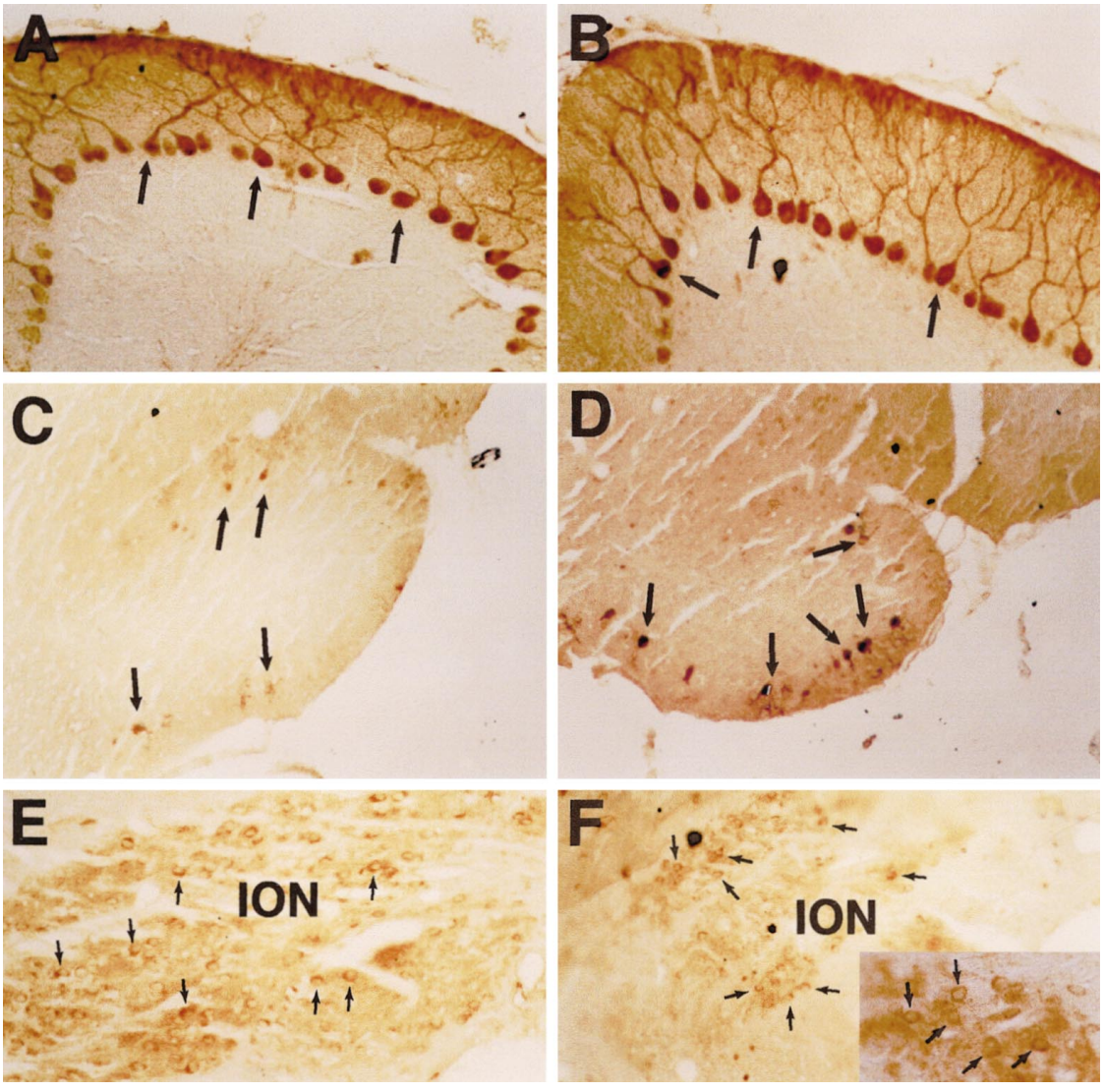

Figure 2. Immunostaining for calbindin or human BCL-2 antigen in the olivocerebellar system of wild-type, transgenic, and mutant mice. *A, B*, Photomicrographs show the selective labeling of all Purkinje cell somas and dendrites by anti-calbindin antibodies in wild-type (*A*) and *Hu-bcl-2* transgenic (*B*) mice. A few Purkinje cells are indicated by *arrows*. In contrast, only a few Purkinje cells immunopositive for calbindin (*arrows*) survive in +/*Lc* (*C*) and *Hu-bcl-2*–+/*Lc* mutant (*D*) cerebella, primarily in the nodulus lobule. *E, F*, Photomicrographs illustrate immunolabeling for the human BCL-2 protein in inferior olivary neurons in coronal sections at low magnification of an adult *Hu-bcl-2* transgenic (*E*) and an adult *Hu-bcl-2*–+/*Lc* mutant (*F*). *Inset* in *F*, Anti-human BCL-2-labeled olivary neurons at a higher magnification. Immunopositive olivary neurons are indicated by *small arrows*.

Inferior olive

Overexpression of the *Hu-bcl-2* transgene in NSE71 mice increases the number of olivary neurons but does not appreciably alter the cytoarchitecture of the inferior olive subnuclei (Fig. 4*A*) (Zanjani et al., 1997). As shown in a coronal section in Figure 4*A*, the four main subnuclei of the inferior olive, medial accessory olive, the dorsal accessory olive, the principal olive, and the dorsomedial cellular column are clearly delineated in NSE71 transgenics. In contrast, there is relatively poor definition of the subnuclei of the inferior olive in the adult *Lurcher* mutant (Fig. 4*B*) compared with wild-type or *Hu-bcl-2* transgenics. Overexpression of the Hu-bcl-2 transgene restores the anatomy and cytoarchitecture of the inferior olive subnuclei in *Hu-bcl-2*–+/*Lc* transgenic mice (Fig. 4*C*). The distribution of olivary subnuclei is almost identical to that in *Hu-bcl-2* transgenic (Fig. 4*A*) or wild-type littermates (data not shown). At a higher magnification, there are no distinct morphological differences in the appearance of olivary cells in *Hu-bcl-2* transgenics, +/*Lc* mutants, and *Hu-bcl-2*–+/*Lc* mutants (Fig. 4*A–C, insets*), although the cell bodies of olivary neurons in the +/*Lc* and *Hu-bcl-2*–+/*Lc* mutants are slightly smaller compared with Hu-bcl-2 transgenics (see next section).

Quantitative descriptions

Visual inspection of sections from *Hu-bcl-2*–+/*Lc* mutants demonstrated that overexpression of the *Hu-bcl-2* transgene in +/*Lc* mutant mice does not prevent Purkinje cell degeneration, but it does rescue most olivary neurons from target-related cell death. To quantitate the amount of olivary neuron rescue in *Hu-bcl-2*–+/*Lc* mutants compared with NSE71 transgenics, wild-type mice, and +/*Lc* mutants, we counted the mean number of olivary neurons per hemisphere in a subsample of animals from all four groups of mice. To compare the anatomy of the inferior olive nucleus among the four groups, we calculated the rostrocaudal

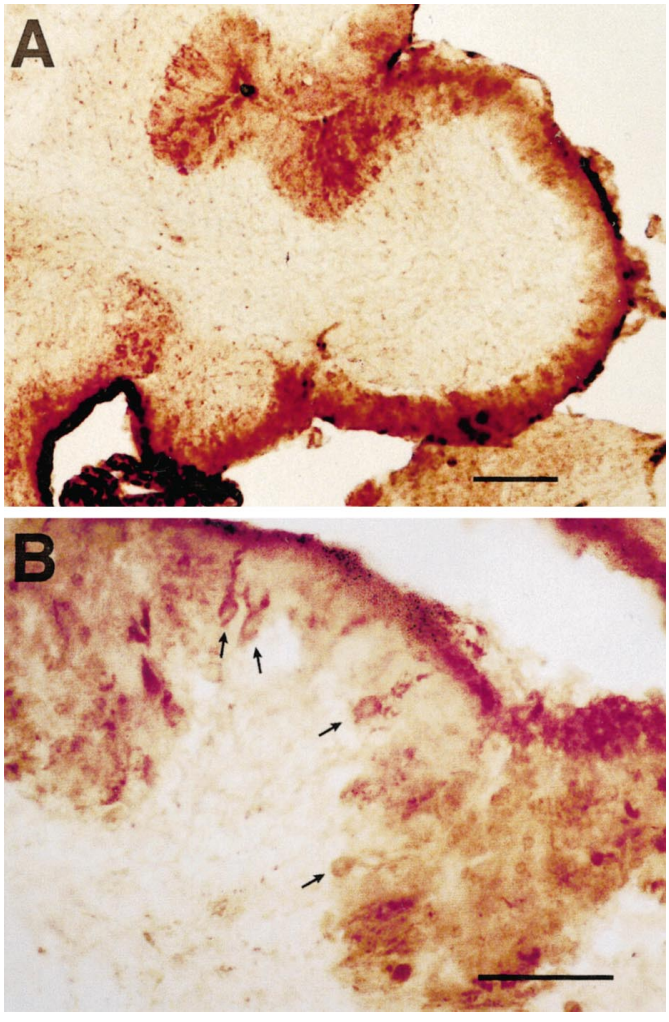

Figure 3. Immunostaining for human BCL-2 antigen in the cerebellum of a P18 +/*Lc*–*Hu-bcl-2* mutant mice. Immunoreactivity is confined largely to the molecular and Purkinje cell layers, as shown in the photomicrograph of a posterior lobule of the +/*Lc*–*Hu-bcl-2* mutant (*A*). At a higher magnification (*B*), individual +/*Lc* Purkinje cells can be seen with stunted dendritic trees. B, The photomicrograph is taken from an anterior lobule, so these BCL-2-immunostained +/*Lc* Purkinje cells are destined to die. Scale bars: *A*, 100 μm; *B*, 50 μm.

extent of the inferior olive and measured the area of olivary neuron cell body profiles in the same coronal sections that were used for the cell counts.

Confirming the qualitative observations, the mean number of olivary neurons per hemicerebellum in the *Hu-bcl-2*–+/*Lc* mutants (Table 1, Fig. 4) is indistinguishable from the number of olivary neurons in wild-type controls and increased by 166% relative to olivary neuron numbers in +/*Lc* mutant mice. Somewhat surprisingly, olivary neuron numbers in the *Hu-bcl-2*–+/*Lc* mutants are 20% lower than the number of olivary neurons in NSE71 *Hu-bcl-2* transgenic mice. The mean differences between olivary neuron cell counts in *Hu-bcl-2*–+/*Lc* mutants, *Hu-bcl-2* transgenics, and +/*Lc* mutants are highly significant (ANOVA, $F_{(3,10)} = 115.012$; $p < 0.001$). Olivary neuron counts are significantly higher in *Hu-bcl-2*–+/*Lc* mutants compared with +/*Lc* mutants (Bonferroni–Dunn *post hoc* test, $p < 0.0001$) but significantly lower compared with *Hu-bcl-2* transgenics (Bonferroni–Dunn *post hoc* test, $p < 0.0005$).

Although olivary neurons numbers are comparable in wild-type and *Hu-bcl-2*–+/*Lc* mutants, quantitative analysis of the rostrocaudal extent of the inferior olive suggests that the region is atrophied in *Hu-bcl-2*–+/*Lc* mutants. The rostrocaudal length of the olive nucleus is significantly shorter in *Hu-bcl-2*–+/*Lc* mutant mice compared with *Hu-bcl-2* transgenics and wild-type mice (Table 1, Fig. 4B) (ANOVA, $F_{(3,10)} = 37.074$; $p < 0.0001$). The mean rostrocaudal extent is significantly reduced by >30% in the *Hu-bcl-2*–+/*Lc* mutants compared with both *Hu-bcl-2* transgenics and wild-type mice (Bonferroni–Dunn *post hoc* test, $p < 0.0001$). Furthermore, despite the 166% increase in the number of olivary neurons in *Hu-bcl-2*–+/*Lc* mutants compared with +/*Lc* mutants, there are no significant differences between the rostrocaudal extent of the inferior olive in the two mutant groups (Bonferroni–Dunn *post hoc* test, $p > 0.1$).

The decrease in the rostrocaudal extent of the inferior olive nucleus in *Hu-bcl-2*–+/*Lc* mutants and +/*Lc* mutants may reflect decreases in cell body size and/or neuropil volume. As a first approximation of cell body size, we measured the area of olivary neuron cell body profiles in coronal sections and found significant differences between *Hu-bcl-2* transgenics and *Hu-bcl-2*–+/*Lc* mutants, +/*Lc* mutants, and wild-type mice (Table 1, Fig. 5) (ANOVA, $F_{(3,9)} = 31.5$; $p < 0.0001$). The average area of olivary neuron cell body profiles in *Hu-bcl-2*–+/*Lc* and +/*Lc* mutants is decreased compared with wild-type values by 12 and 19%, respectively; however, the differences only approach significance for the +/*Lc* mutant data ($p = 0.068$). In contrast, average olivary neuron profile areas are >50% higher in *Hu-bcl-2* transgenics compared with all other groups (Bonferroni–Dunn *post hoc* test, $p < 0.001$).

DISCUSSION

The results of this study demonstrate that overexpression of BCL-2 in the +/*Lc* mutant does not prevent Purkinje cell death, but it does rescue most olivary neurons. The simplest explanation for these results is that +/*Lc* Purkinje and olivary neuron cell death is mediated by two distinct BCL-2-independent and BCL-2-dependent pathways. The implications of these results for deciphering the mechanisms of +/*Lc* Purkinje cell death and olivary neuron death are discussed in turn.

+/*Lc* Purkinje cell death

Cell-autonomous neuronal degeneration in the *Lurcher* mutant is apparently caused by a gain of function mutation in the δ2 glutamate receptor (GluR) subunit that results in a large, constitutive inward current in the cells that express the subunit (Zuo et al., 1997). The GluR δ2 subunit is preferentially expressed in cerebellar Purkinje cells (Takayama et al., 1996) and perhaps in selected hindbrain regions, because there is massive cellular degeneration in these regions in homozygous *Lurcher* mutant embryos (Cheng and Heintz, 1997; Resibois et al., 1997). The timing of +/*Lc* Purkinje cell death coincides with the translocation of GluR δ2 immunoreactivity from Purkinje cell dendritic shafts to dendritic spines, especially to the postsynaptic membrane (Takayama et al., 1996). In the *Lurcher* heterozygote, Purkinje cells undergo cell-autonomous degeneration, whereas olivary neuron and granule cell death is secondary to Purkinje cell loss (Wetts and Herrup, 1982a,b). Neuronal degeneration caused directly by the leaky GluR δ2 subunit is presumably attributable to the induction of excitotoxic mechanisms, and Purkinje cells must be especially sensitive to this mutation in the heterozygote (Zuo et al., 1997). The available evidence suggests that +/*Lc* Purkinje

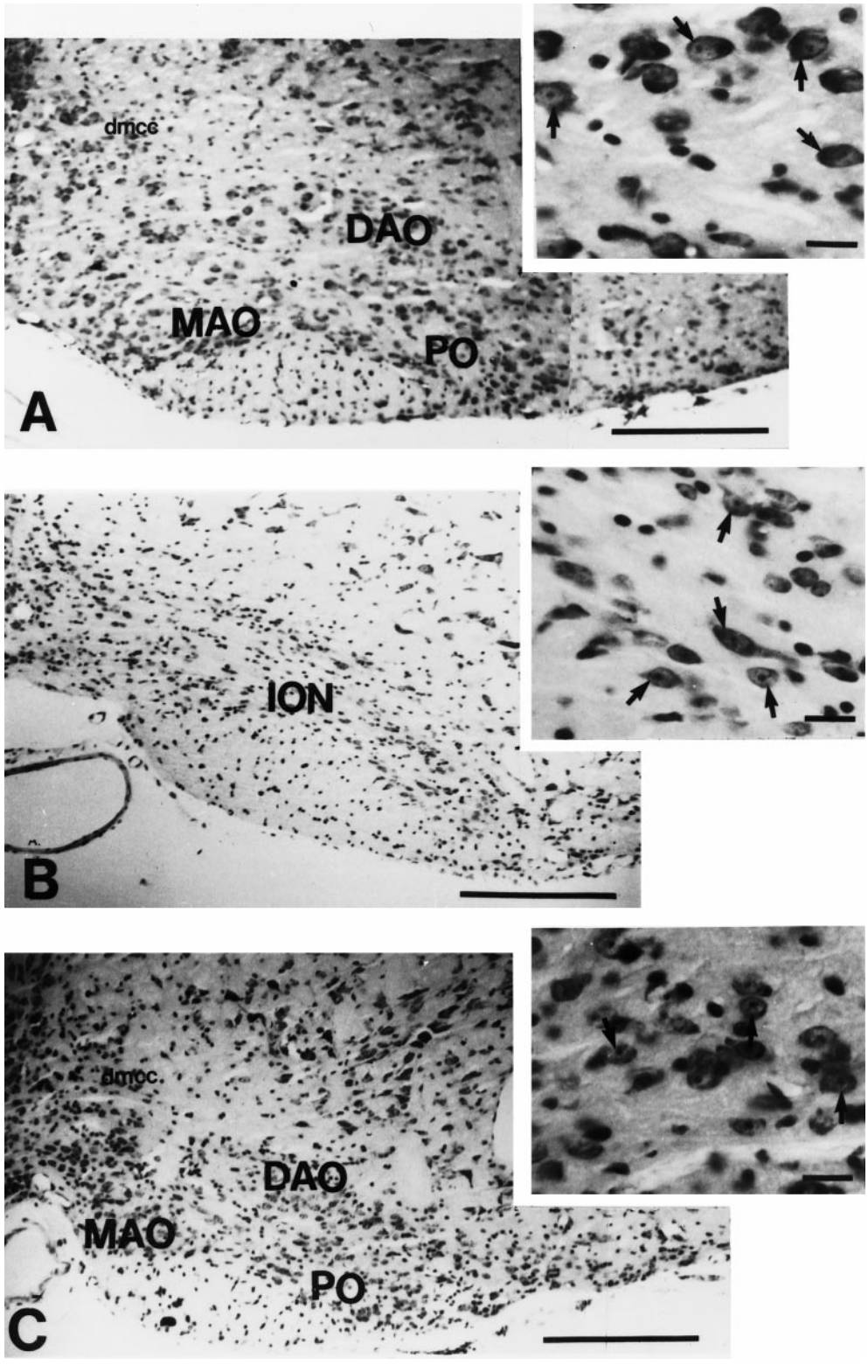

Figure 4. Coronal sections through the inferior olivary complex of an *Hu-bcl-2* transgenic (*A*), a +/*Lc* mutant (*B*), and an *Hu-bcl-2*–+/*Lc* mutant (*C*). Distinct subnuclei are apparent in the organization of the inferior olivary complex in both the *Hu-bcl-2* transgenic and the *Hu-bcl-2*–+/*Lc* mutant. In contrast, the inferior olivary nucleus appears atrophic in the +/*Lc* mutant (*B*). The *insets* show the somas of olivary neurons at higher magnification (*arrows*); olivary neuron cell bodies appear shrunken in the +/*Lc* and *Hu-bcl-2*–+/*Lc* mutants compared with controls. *MAO*, Medial accessory olive; *DAO*, dorsal accessory olive; *PO*, principal olive; *dmcc*, dorsomedial cell column. Scale bars: low-power photomicrographs, 200 μm; *insets*, 40 μm.

Table 1. Corrected numbers for inferior olivary neurons per hemicerebellum, the mean rostrocaudal extent of the inferior olive nuclei, and the mean area and long diameter of olivary neuron cell body profiles for wild-type, NSE 71 *Hu-bcl-2* transgenic, *Hu-bcl-2*–+/*Lc* mutant, and +/*Lc* mutant mice

	Olivary neurons ± SE	Rostrocaudal extent ± SE (μm)	Olivary neuron cell body area ± SE (μm²)
Wild type ($n = 2$)	13,800 ± 140	1200 ± 35	52.5 ± 3
Hu-bcl-2 ($n = 4$)	17,600 ± 790*	1250 ± 27	81.4 ± 4*
Hu-bcl-2–+/*Lc* ($n = 4$)	14,100 ± 360	830 ± 31**	46.3 ± 2
+/*Lc* ($n = 4$)	5300 ± 170**	820 ± 48**	42.3 ± 3 ($n = 5$)

* Significantly increased above wild-type values (Bonferroni–Dunn *post hoc* test, $p < 0.001$).

** Significantly decreased below wild-type values (Bonferroni–Dunn *post hoc* test, $p < 0.001$).

cells may die by an excitotoxic mechanism induced by the accumulation of leaky GluR δ2 subunits at developing Purkinje cell→parallel fiber synapses (Zuo et al., 1997). This hypothesis is consistent with previous studies showing that +/*Lc* Purkinje cells must differentiate before they degenerate (Wuenschell et al., 1990; Messer et al., 1991; Norman et al., 1995). The rescue of +/*Lc* Purkinje cells in a homozygous *staggerer* background (Messer et al., 1991), for example, could be attributable to a block in the developmental expression of the GluR δ2 receptor.

Although +/*Lc* Purkinje cells are likely to be dying by a mechanism that is initiated by an excitotoxic lesion, the sequence of molecular events leading to +/*Lc* Purkinje cell death still remains to be determined. Dumesnil-Bousez and Sotelo (1992) argue on morphological criteria that +/*Lc* Purkinje cells degenerate by a necrotic process; however, fragmented DNA can be detected in degenerating +/*Lc* Purkinje cells, suggesting that +/*Lc* Purkinje cells may degenerate by an apoptotic mechanism (Norman et al., 1995; Wullner et al., 1995). Our results suggest that whatever mechanisms are involved, the overexpression of *bcl-2* in the +/*Lc* Purkinje cells is not sufficient to prevent their degeneration. Positive BCL-2 immunolabeling in the P18 +/*Lc*–*Bcl-2* mutant shows that the human *bcl-2* transgene is present in +/*Lc* Purkinje cells before their death, so their degeneration is not attributable to a failure of transgene expression in the afflicted cells. Ectopic expression of BCL-2 has been shown to prevent excitotoxic lesions in other systems (Lawrence et al., 1996). BCL-2 is not normally expressed in adult Purkinje cells (Castren et al., 1994), but it appears to be expressed in Purkinje cells during early embryonic and postnatal development, so it may play a role in regulating Purkinje cell survival early in development (Merry et al., 1994). BCL-2 overexpression, for example, may rescue Purkinje cells from naturally occurring cell death (Zanjani et al., 1996). It is not clear why BCL-2 overexpression fails to rescue +/*Lc* Purkinje cells (Zhong et al., 1993a,b; Jia et al., 1996; Lawrence et al., 1996). A likely explanation is that the aberrant ion current induced by the mutant GluR δ2 receptor induces a cell death pathway that is independent of or downstream from BCL-2 regulation. Both *bcl-2*-dependent and -independent cell death pathways have been described in other neurons (Allsopp et al., 1993, 1995; Coulpier et al., 1996). For example, in the +/*Lc* mutant, the ICE-like proteases that execute neuronal cell death programs (Martinou and Sadoul, 1996; Schwartz and Milligan, 1996) may be activated directly in chronically depolarized +/*Lc* Purkinje cells.

+/Lc olivary neuron cell 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 Purkinje cell 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). The hypothesis for target-related cell death is well established; if target cells are removed during a critical period during development, the afferent neurons will undergo retrograde cell death (Oppenheim, 1991). We have shown previously that overexpression of the *Hu-bcl-2* transgene rescues olivary neurons from naturally occurring cell death (Zanjani et al., 1997). In this study, we show that overexpression of

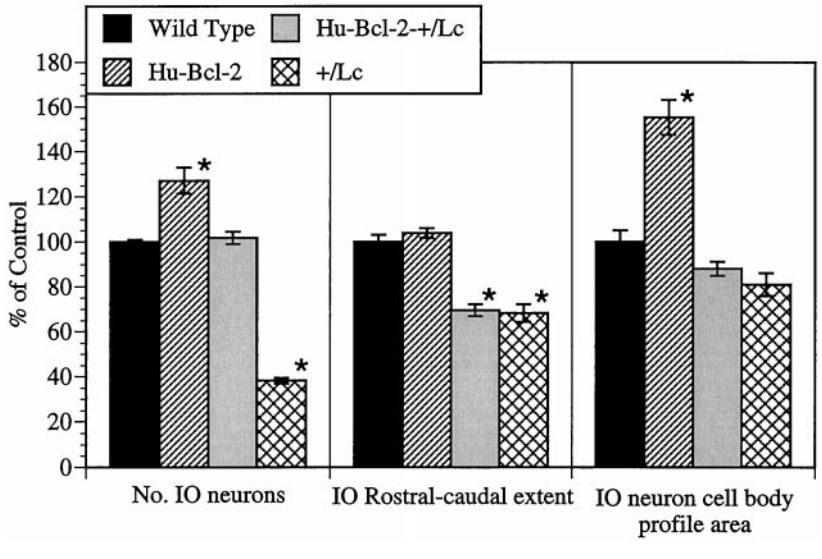

Figure 5. Quantitative analysis of inferior olive numbers and size in wild-type mice (*black bars*), *Hu-bcl-2* transgenics (*striped bars*), NSe71–+/*Lc* mutants (*gray bars*), and +/*Lc* mutants (*cross-hatched bars*). Values are expressed as percentage of wild-type values for the number of olivary neurons, rostrocaudal extent of the inferior olive, and inferior olive cell body profile area. Results that are significantly different from wild-type values are indicated by *asterisks* (Bonferroni–Dunn *post hoc* test, $p < 0.0083$).

Hu-bcl-2 in the +/*Lc* mutant rescues most olivary neurons from target-related cell death. 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). A surprising result, however, is that the number of olivary neurons in the NSE71 line of *Hu-bcl-2* transgenics is increased by 26% compared with *Hu-bcl-2*–+/*Lc* mutant mice. This disparity in survival suggests either that the transgene is not expressed equally in all ION neurons or that not all olivary neurons can be rescued by BCL-2 overexpression. There is indirect evidence that olivary neurons may die by different mechanisms after target removal. Degenerating olivary neurons in the caudal olive can be detected with terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end-labeling (TUNEL) labeling at P4 in transgenic mice with neonatal Purkinje cell death (Chu and Oberdick, 1995). However, TUNEL-positive olivary neurons are not detected after P7, although olivary neuron cell death appears to proceed at least through P15. However, it is not yet apparent why olivary neurons may die by different mechanisms after target removal and/or destruction.

Despite the increase in the number of olivary neurons to wild-type levels in *Hu-bcl-2*–+/*Lc* mutants, the inferior olive complex appears atrophic. The rostrocaudal extent of the inferior olive in *Hu-bcl-2*–+/*Lc* mutants is significantly reduced just as in the +/*Lc* mutant, and there is a trend toward smaller average cell body profile areas in coronal sections of *Hu-bcl-2*–+/*Lc* and +/*Lc* mutants. The rostrocaudal extent of the inferior olive and olivary neuron cell body size are similarly reduced in the *staggerer* mutant, suggesting that olivary neurons atrophy in the absence of their Purkinje cell targets (Zanjani et al., 1994). One class of olivary neurons with large, simple, and unramified dendrites may be predominantly lost in both mutants (Caddy and Biscoe, 1979; Zanjani et al., 1994). Although the loss of this cell type could account for the smaller olive in the *sg*/*sg* and +/*Lc* mutants, it is likely that these cells are rescued in *Hu-bcl-2*–+/*Lc* mutants, and there is a general reduction in the size of olivary neurons and the olivary neuropil in the *Hu-bcl-2*–+/*Lc* mutants as a consequence of Purkinje cell loss.

The atrophy of inferior olive neurons rescued by *Hu-bcl-2* overexpression supports a hypothesis for BCL-2-mediated rescue of olivary neurons from trophic factor deprivation after Purkinje cell degeneration. *In vitro* and *in vivo* experiments suggest that the survival functions of BCL-2 can be separated from growth-promoting activities. Sympathetic neurons transfected with *bcl-2 in vitro* and deprived of growth factors will survive, but they suffer the same decrease in protein synthesis rates after NGF withdrawal as wild-type cells (Greenlund et al., 1995). $BAX^{-/-}$ neurons also survive trophic factor withdrawal *in vitro* or after *in vivo* axotomy, but their cell bodies are atrophied (Deckwerth et al., 1996). In contrast, motoneurons rescued with exogenous glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, neurotrophin 3, and insulin-like growth factor 1 appear normal in size, indicating that the neurotrophic factors can prevent the atrophy associated with axotomy (Li et al., 1994; Oppenheim et al., 1995; Yan et al., 1995) (but see Yan et al., 1993). The implication is that although *bcl-2* expression (or the lack of BAX) can block the cell death pathway in models of target-related cell death, the lack of appropriate trophic factors prevents the rescued cells from growing normally. Conversely, the profiles of inferior olive neurons in the *Hu-bcl-2* transgenics are significantly increased above wild-type values. It seems unlikely that the increase is a direct effect of *Hu-bcl-2* transgene expression on olivary neurons, because olivary neurons in the *Hu-bcl-2*–+/*Lc* mutants are not similarly affected. Instead, the increase in olivary cell body size in *Hu-bcl-2* transgenics may reflect a hypertrophy attributable to increased target-derived trophic support from the increased numbers of Purkinje cells in the transgenics. These results illustrate the key roles neurotrophic factors play in supporting neuronal survival and growth.

REFERENCES

Allsopp TE, Wyatt S, Paterson HF, Davies AM (1993) The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. Cell 73:295–307.

Allsopp TE, Kiselev S, Wyatt S, Davies AM (1995) Role of bcl-2 in the brain-derived neurotrophic factor survival response. Eur J Neurosci 7:1266–1272.

Andersen BB, Korbo L, Pakkenberg B (1992) A quantitative study of the human cerebellum with unbiased stereological techniques. J Comp Neurol 326:549–560.

Batistatou A, Merry DE, Korsmeyer SJ, Greene LA (1993) Bcl-2 affects survival but not neuronal differentiation of PC12 cells. J Neurosci 13:4422–4428.

Bonfanti L, Strettoi E, Cheirzi S, Cenni MC, Liu X-H, Martinou J-C, Maffei L, Rabacchi SA (1996) Protection of retinal ganglion cells from natural and axotomy-induced cell death in neonatal transgenic mice overexpressing bcl-2. J Neurosci 16:4186–4194.

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

Castren E, Ohga Y, Berzaghi MP, Tzimagiorgis G, Thoenen H, Lindholm D (1994) Bcl-2 messenger RNA is localized in neurons of the developing and adult rat brain. Neuroscience 61:165–177.

Chen J, Flannery JG, LaVail MM, Steinberg RH, Xu J, Simon MI (1996) bcl-2 overexpression reduces apoptotic photoreceptor cell death in three different retinal degenerations. Proc Natl Acad Sci USA 93:7042–7047.

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

Chu T, Oberdick J (1995) An early critical period in olivocerebellar interactions defined by targeted ablation of Purkinje cells. Soc Neurosci Abstr 21:916.

Coulpier M, Junier M-P, Peschanski M, Dreyfus PA (1996) Bcl-2 sensitivity differentiates two pathways for motoneuronal death in the wobbler mutant mouse. J Neurosci 16:5897–5904.

De Bilbao F, Dubois-Dauphin M (1996) Time course of axotomy-induced apoptotic cell death in facial motoneurons of neonatal wile type and bcl-2 transgenic mice. Neuroscience 71:1111–1119.

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

Doughty ML, Patterson L, Caddy KWT (1995) Cerebellar Purkinje cells from the *Lurcher* mutant and wild-type mouse grown in vitro: a light and electron microscope study. J Comp Neurol 357:161–179.

Dubois-Dauphin M, Frankowski H, Tsujimoto Y, Huarte J, Martinou J-C (1994) Neonatal motoneurons overexpressing the bcl-2 protooncogene in transgenic mice are protected from axotomy-induced cell death. Proc Natl Acad Sci USA 91:3309–3313.

Dumesnil-Bousez N, Sotelo C (1992) Early development of the *Lurcher* cerebellum: purkinje cell alterations and impairment of synaptogenesis. J Neurocytol 21:506–529.

Farlie PG, Dringen R, Rees SM, Kannourakis G, Bernard O (1995) bcl-2 transgene expression can protect neurons against developmental and induced cell death. Proc Natl Acad Sci USA 92:4397–4401.

Floderus S (1944) Untersuchungen uber den Bau der menschlichen Hypophyse mit besonderer Berucksichtigung der quantitativen mikromorphologische Verhaltnisse. Acta Pathol Microbiol Scand 53:1–276.

Garcia I, Martinou I, Tsujimoto Y, Martinou JC (1992) Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. Science 258:302–304.

Greenlund LJS, Korsmeyer SJ, Johnson Jr EM (1995) Role of BCL-2 in

the survival and function of developing and mature sympathetic neurons. Neuron 15:649–661.
Harkmark W (1956) The influence of the cerebellum on development and maintenance of the inferior olive and the pons. J Exp Zool 131:333–372.
Herrup K, Shojaeian-Zanjani H, Panzini L, Sunter K, Mariani J (1996) The numerical matching of source and target populations in the CNS: the inferior olive to Purkinje cell projection. Dev Brain Res 96:28–35.
Hockenberry D, Nuñez G, Milliman C, Schreiber RD, Korsmeyer SJ (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334–336.
Jia W, Wang Y, Qiang D, Tufaro F, Remington R, Cynader M (1996) A bcl-2 expressing viral vector protects cortical neurons from excitotocity even when administered several hours after the toxic insult. Mol Brain Res 42:350–353.
Lawrence MS, Ho DY, Sun GH, Steinberg GK, Sapolsky RM (1996) Overexpression of Bcl-2 with herpes simplex virus vectors protects CNS neurons against neurological insults in vitro and in vivo. J Neurosci 16:486–496.
Li L, Oppenheim RW, Lei M, Houenou LJ (1994) Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. J Neurobiol 25:759–766.
Martinou JC, Sadoul R (1996) ICE-like proteases execute the neuronal death program. Curr Opin Neurobiol 6:609–614.
Martinou JC, Dubois-Dauphin M, Staple J, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C, Huarte J (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13:1017–1030.
Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ (1994) bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. Development 120:301–311.
Messer A, Eisenberg B, Plummer J (1991) The *lurcher* cerebellar mutant phenotype is not expressed on a *staggerer* mutant background. J Neurosci 11:2295–2302.
Norman D, Feng L, Gubbay J, Chan E, Heintz N (1995) The *Lurcher* gene induces apoptotic death in cerebellar Purkinje cells. Development 121:1183–1193.
Oppenheim RW (1991) Cell death during development of the nervous system. Annu Rev Neurosci 14:453–581.
Oppenheim RW, Houenou LJ, Johnson JE, Lin L-FH, Li L, Lo AC, Newsome AL, Prevette DM, Wang S (1995) Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. Nature 373:344–346.
Phillips RJS (1960) "*Lurcher*," a new gene in linkage group XI of the house mouse. J Genet 57:35–42.
Resibois A, Cuvelier L, Goffinet AM (1997) Abnormalities in the cerebellum and brainstem in homozygous *Lurcher* mice. Neuroscience 80:175–190.
Sagot Y, Dubois-Dauphin M, Tan SA, de Bilbao F, Aebischer P, Martinou JC, Kato AC (1995) Bcl-2 overexpression prevents motoneuron cell body loss but not axonal degeneration in a mouse model of a neurodegenerative disease. J Neurosci 15:7727–7733.
Schwartz LM, Milligan CE (1996) Cold thoughts of death: the role of ICE proteases in neuronal cell death. Trends Neurosci 19:555–562.
Shojaeian H, Delhaye-Bouchaud N, Mariani J (1985a) Decreased number of cells in the inferior olivary nucleus of the developing *staggerer* mouse. Dev Brain Res 21:141–146.
Shojaeian H, Delhaye-Bouchaud N, Mariani J (1985b) Neuronal death and synapse elimination in the olivocerebellar system. II. Cell counts in the inferior olive of adult X-irradiated rats and weaver and reeler mutant mice. J Comp Neurol 232:309–318.
Smeyne RJ, Chu T, Lewin A, Bian F, Crisman SS, Kunsch C, Lira SA, Oberdick J (1995) Local control of granule cell generation by cerebellar Purkinje cells. Mech Cell Neurosci 6:230–251.
Sotelo C, Changeux J-P (1974) Transsynaptic degeneration "en cascade" in the cerebellar cortex of *staggerer* mutant mice. Brain Res 67:519–526.
Takayama C, Nakagawa S, Watanabe M, Mishina M, Inoue Y (1996) Developmental changes in expression and distribution of the glutamate receptor channel d2 subunit according to the Purkinje cell maturation. Dev Brain Res 92:147–155.
Vogel MW, McInnes M, Zanjani HS, Herrup K (1991) Cerebellar Purkinje cells provide target support over a limited spatial range: evidence from *Lurcher* chimeric mice. Dev Brain Res 64:87–94.
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 250:358–363.
Williams RW, Rakic P (1988) Three-dimensional counting: an accurate and direct method to estimate numbers of cells in sectioned material. J Comp Neurol 278:344–352.
Wuenschell CW, Messer A, Tobin AJ (1990) Lurcher Purkinje cells express glutamic acid decarboxylase and calbindin mRNAs. J Neurosci Res 27:65–70.
Wullner U, Loschmann P-A, Weller M, Klockgether T (1995) Apoptotic cell death in the cerebellum of mutant *weaver* and *Lurcher* mice. Neurosci Lett 200:109–112.
Yan Q, Elliott JL, Matheson C, Sun J, Zhang L, Mu X, Rex KL, Snider WD (1993) Influences of neurotrophins on mammalian motoneurons in vivo. J Neurobiol 24:1555–1577.
Yan Q, Matheson C, Lopez OT (1995) In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature 373:341–344.
Zanjani HS, Mariani J, Herrup K (1990) Cell loss in the inferior olive of the *staggerer* mutant mouse is an indirect effect of the gene. J Neurogenet 6:229–241.
Zanjani HS, Herrup K, Guastavino J-M, Delhaye-Bouchaud N, Mariani J (1994) Developmental studies of the inferior olivary nucleus in *staggerer* mutant mice. Dev Brain Res 82:18–28.
Zanjani HS, Vogel MW, Delhaye-Bouchaud N, Martinou JC, Mariani J (1996) Increased cerebellar Purkinje cell numbers in mice overexpressing a human bcl-2 transgene. J Comp Neurol 374:332–341.
Zanjani HS, Vogel MW, Delhaye-Bouchaud N, Martinou JC, Mariani J (1997) Increased olivary neuron and cerebellar granule cell numbers in transgenic mice overexpressing the human *bcl-2* gene. J Neurobiol 32:502–516.
Zhong LT, Kane DJ, Bredesen DE (1993a) BCL-2 blocks glutamate toxicity in neural cell lines. Mol Brain Res 19:353–355.
Zhong LT, Sarafian T, Kane DJ, Charles AC, Mah SP, Edwards RH, Bredesen DE (1993b) bcl-2 inhibits death of central neural cells induced by multiple agents. Proc Natl Acad Sci USA 90:453–4537.
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. Nature 388:769–773.