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Neonatal motoneurons overexpressing the *bcl-2* protooncogene in transgenic mice are protected from axotomy-induced cell death

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ABSTRACT **In vitro, the overexpression of the *bcl-2* protooncogene in cultured neurons has been shown to prevent apoptosis induced by neurotrophic factor deprivation. We have generated transgenic mice overexpressing the Bcl-2 protein in neurons, including motoneurons of the facial nucleus. We have tested whether Bcl-2 could protect these motoneurons from experimentally induced cell death in new born mice. To address this question, we performed unilateral lesion of the facial nerve of wild-type and transgenic 2-day-old mice. In wild-type mice, the lesioned nerve and the corresponding motoneuron cell bodies in the facial nucleus underwent rapid degeneration. In contrast, in transgenic mice, facial motoneurons survived axotomy. Not only their cell bodies but also their axons were protected up to the lesion site. These results demonstrate that *in vivo* Bcl-2 protects neonatal motoneurons from degeneration after axonal injury. A better understanding of the mechanisms by which Bcl-2 prevents neuronal cell death *in vivo* could lead to the development of strategies for the treatment of motoneuron degenerative diseases.**

Apoptotic neuronal cell death that occurs during development of the nervous system requires protein synthesis (1, 2) and is regulated by epigenetic factors such as retrograde neurotrophic factors (3, 4). Apoptosis probably results from an unbalance between positive and negative regulators of cell survival (5). One positive regulator identified in vertebrates is the Bcl-2 oncoprotein (6–8). *In vitro*, this 25-kDa membrane-associated protein (9–11) is capable of rescuing neurons from apoptosis induced by neurotrophic factor deprivation (12–14). These observations led to the hope that Bcl-2 could be a tool for the treatment of neurodegenerative diseases although there was no evidence that the protein could block neuronal death under pathological circumstances *in vivo*. One of the reasons to challenge this hope was that the type of cell death occurring *in vivo* (15) could be different from classical apoptosis described *in vitro* (2, 16). We therefore decided to test the effects of Bcl-2 *in vivo*, on transgenic mice, in a model of nerve injury that leads to neuronal cell death. In rodents, during the early postnatal period, lesion of the facial or sciatic nerve leads to a rapid degeneration of the axotomized motoneurons (17–19). This model has been exploited to investigate the effect of neurotrophic factors on motoneuron survival (20–23). To determine whether overexpression of Bcl-2 protects axotomized motoneurons, we generated transgenic mice in which neurons overexpress Bcl-2. Unilateral section of the facial nerve was performed on 2-day-old transgenic mice, in which facial motoneurons overexpress the Bcl-2 protein, and on wild-type pups from the same litter. Seven days after the lesion, motoneuron cell bodies were always present in the ipsilateral facial nucleus in transgenic mice but had degenerated and disappeared from the ipsilateral facial nucleus in the wild-type animals.

MATERIALS AND METHODS

Production of Transgenic Mice. The DNA insert from pB4 containing the human *bcl-2* coding region was excised with *Eco*RI, blunt-ended, and subcloned into the blunt-ended *Sph* I site of pNSE-LacZ that contains a 1.8-kb 5′ flanking DNA sequence from the rat neuron-specific enolase (*NSE*) gene fused to the *lacZ* gene (24, 25). A 2.7-kb fragment containing the 1.8-kb 5′ flanking region of the *NSE* gene fused to *bcl-2* was excised from the plasmid with *Sac* I (partial digestion followed by blunt-ending) and *Hin*dIII. This fragment was finally subcloned in front of the simian virus 40 tumor intron and polyadenylylation signal already inserted into the Bluescript cloning vector pSK+ (Stratagene). This construct was called EB-2 (12). Transgenic mice were prepared by pronuclear injections (26) using linear DNA prepared from *Kpn* I/*Not* I-digested EB-2. Embryos for injections were obtained by crossing B6D2F1 mice. Integration of the transgene into the mouse genome was assessed by Southern blot analysis of genomic DNA isolated from the mouse tail.

Two lines, lines 57 and 71, were used in this study. Males heterozygous for the transgene were crossed with C57BL/6 females. We confirmed the presence of the *bcl-2* transgene in the litter obtained by PCR. In the transgenic mice used in this study, the number of motoneurons in the facial nucleus was the same as the number in wild-type animals.

Unilateral Lesion of the Facial Nerve. We unilaterally transected the facial nerve of 2-day-old wild-type and transgenic mice from the same litter. Pups were anesthetized by hypothermia and the two main branches of the nerve that correspond to motoneurons in the intermediate and lateral parts of the facial nucleus (27) were sectioned distal (0.5 mm) to the division from the common trunk under the ear. The branch that innervates the caudal auricular muscle and corresponds to motoneurons in the ventro-medial part of the nucleus (27) was not sectioned. The lesion was restricted to reduce the size of the skin wound and thus minimize cannibalization of operated pups by their mothers. Pups were allowed to survive 7 days. Thereafter, they were reanesthetized with sodium pentobarbital (5 mg/100 g) and fixed by intracardiac perfusion with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS, pH 7.35). The presence of the facial nerves was examined on both sides of the head. The brain was removed, postfixed for 1 h in the same fixative at 4°C, and cryoprotected in 30% (wt/vol) sucrose in PBS at 4°C. Then two sets of 20-μm-thick alternating coronal sections of the brainstem were cut in a cryostat and stained with cresyl violet. Some alternating sections were stained for

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Abbreviation: GFAP, glial fibrillary acidic protein.

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Bcl-2 and glial fibrillary acidic protein (GFAP) immunoreactivity.

The facial nuclei were examined on every section, and the number of motoneurons was counted. Data are the mean ± SEM of seven wild-type and seven transgenic mice. The unpaired Student *t* test was used for the statistics, comparing transgenic mice ($n = 7$) to wild-type mice ($n = 7$).

We confirmed the presence and expression of the *bcl-2* transgene in mice by PCR analysis (data not shown) and Bcl-2 immunostaining at the end of the experiment.

Immunocytochemistry. Bcl-2 immunocytochemistry was performed on intact 2-day-old pups to assess the presence of the protein at the time of lesioning. GFAP immunocytochemistry in the facial nuclei was performed in mice lesioned at postnatal day 2 and sacrificed 7 days later.

The brain of 2-day-old intact wild-type and transgenic mice was dissected and fixed by immersion in 4% paraformaldehyde in PBS. The brain of 9-day-old lesioned transgenic mice was removed after fixation by intracardial perfusion. Twenty-micrometer-thick alternating sections were cut in a cryostat, laid on chromalun/gelatin-coated slides, permeabilized in PBS/0.2% Triton X-100 for 1 h, incubated for 2 h at room temperature with a mouse monoclonal antibody specific to the human Bcl-2 (28), followed by a 90-min incubation with a fluorescein-conjugated goat antibody raised against mouse IgG (1:1000 dilution, Antibodies Inc.). To detect GFAP immunoreactivity, sections were permeabilized in 0.2% Triton X-100 and incubated overnight with rabbit polyclonal antibodies to GFAP (1:100 dilution, Sigma). Immunofluorescence detection was performed with fluorescein-conjugated goat antibody raised against rabbit immunoglobulin (1:75 dilution, Vector Laboratories) and Texas red-conjugated goat antibodies to rabbit immunoglobulin (1:75 dilution, Vector Laboratories) for double immunohistological staining of Bcl-2 and GFAP on the same section detected using a confocal microscope.

Chemicals. Antibodies were as follows: anti-GFAP developed in rabbit from Sigma (G-9269), highly fluorescent anti-mouse γ-globulin (Antibodies Inc.), fluorescein- and Texas red-conjugated goat anti-rabbit antibodies (Vector Laboratories).

RESULTS

We have produced transgenic mice whose neurons overexpress the human Bcl-2 under control of the *NSE* promoter. Our study was performed on two lines of transgenic mice, lines 57 and 71, that contain, respectively, 16 and 20 transgene copies in their genome (data not shown). We have crossed wild-type C57BL/6 mice with these two lines of transgenic mice. In the litters obtained, half of the pups were transgenic, as confirmed by the presence of the *bcl-2* transgene by PCR (data not shown). The presence of Bcl-2 immunoreactivity at postnatal day 2 was assessed by immunostaining (Fig. 1*A*). No human Bcl-2 protein immunoreactivity was detected in the wild-type mice (Fig. 1*B*).

Effects of the Facial Nerve Lesion. At postnatal day 2, the facial nerve was unilaterally transected in both wild-type and transgenic mice. Seven days later, inspection of the lesion site revealed that, in wild-type mice, the proximal part of the nerve had degenerated (Fig. 2*A*). The total number of motoneurons in the facial nucleus at the lesioned side (mean for seven mice: 682 number not corrected) was reduced by 75% compared to the number found in the control contralateral nucleus (mean for seven mice: 2940 number not corrected) (Figs. 3 *A* and *B* and 4). The caudal auricular branch of the facial nerve was not affected by the surgery; the corresponding motoneuron cell bodies located in the ventro-medial part of the facial nucleus survived. In contrast, in transgenic mice, the proximal part of the nerve was still visible at the lesion site

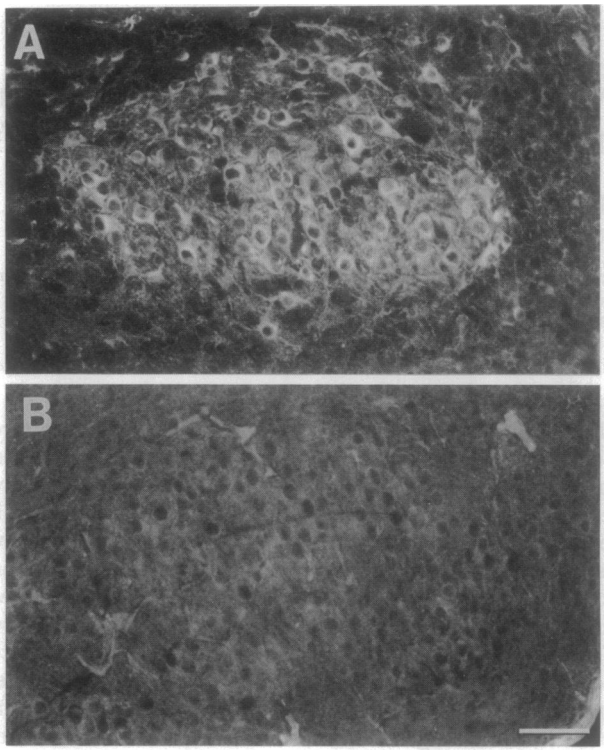

FIG. 1. Immunostaining of human Bcl-2 protein in facial motoneurons of 2-day-old mice. (*A*) Transgenic mouse. (*B*) Wild-type mouse. (Bar = 100 μm.)

(Fig. 2*B*) and facial motoneuron cell bodies were present in all subdivisions of the nucleus. They were smaller but retained good Nissl staining and the morphology of their nucleus was normal (Figs. 3 *D* and *E* and 5 *B* and *C*). The

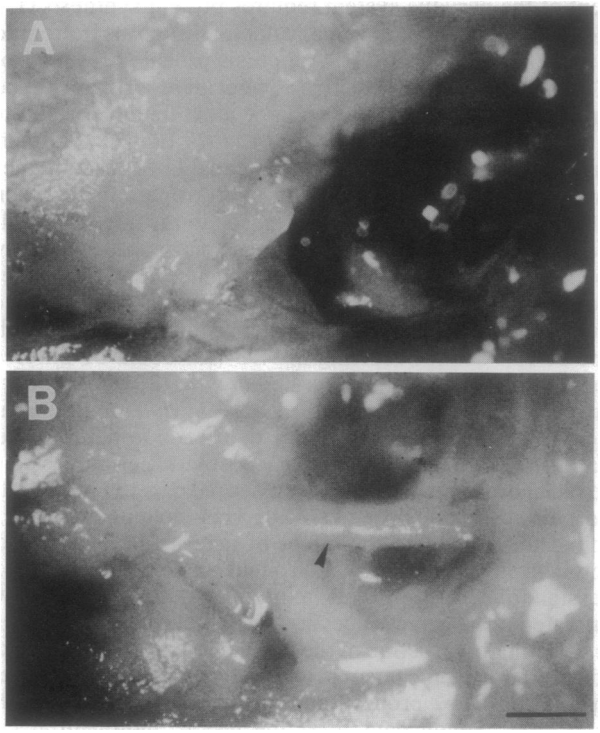

FIG. 2. Lesioned facial nerves of wild-type and transgenic mice. The facial nerve was observed 7 days after axotomy. (*A*) In wild-type mice, both the distal and proximal parts of the nerve had degenerated. (*B*) In contrast, in transgenic mice, the proximal portion of the nerve was still present (arrowhead). (Bar = 500 μm.)

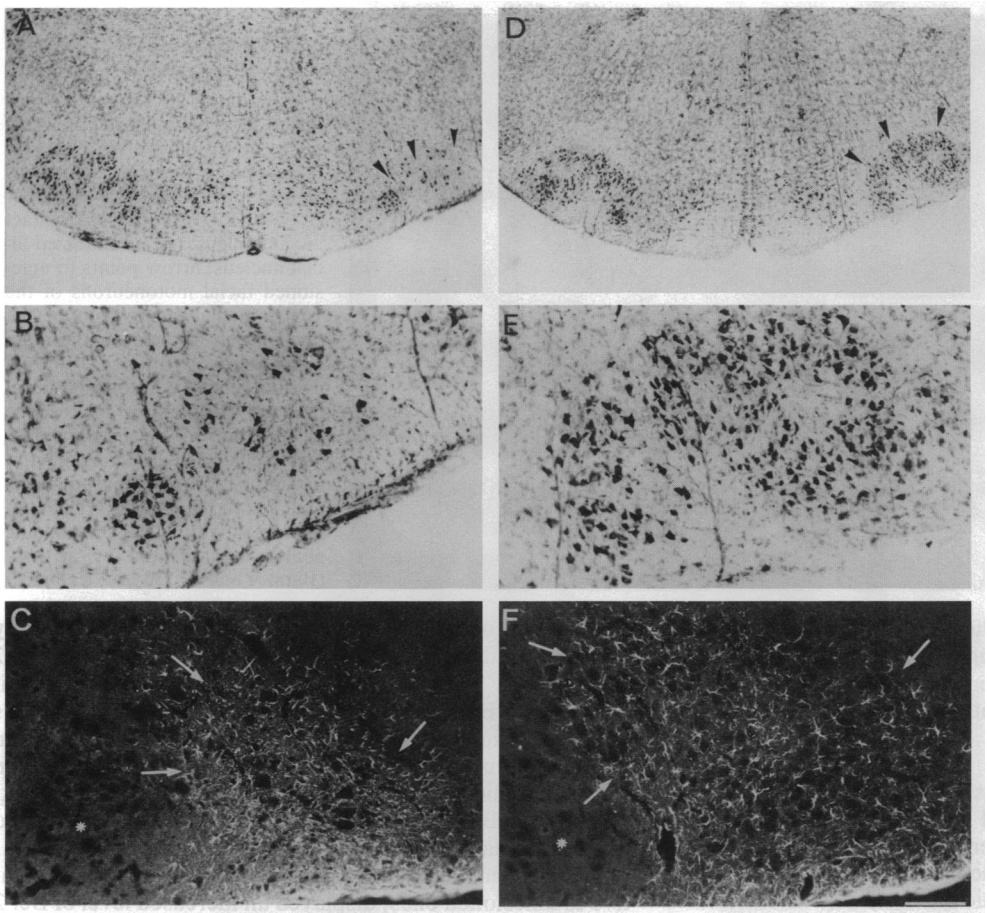

FIG. 3. Morphological analysis of the facial nucleus in brainstems of wild-type (*A–C*) and transgenic (*D–F*) mice 7 days after unilateral section of the facial nerve. (*A*, *B*, *D*, and *E*) Cresyl violet staining. The lesioned facial nucleus is located on the right side and indicated by arrowheads. (*C* and *F*) Immunostaining for GFAP. Arrows indicate the perimeter of the GFAP immunoreactivity domain. An asterisk indicates the intact ventro-medial subdivision of the facial nucleus corresponding to the unlesioned caudal auricular branch of the facial nerve. (Bars: *A* and *D*, 400 μm; *B*, *C*, *E*, and *F*, 130 μm.)

number of motoneurons in the nucleus ipsilateral to the lesion (mean for seven mice: 3296 number not corrected) was not significantly different from the number of motoneurons in the contralateral nucleus (mean for seven mice: 3120 number not corrected) (Fig. 4). Finally, we observed that the level of Bcl-2 immunoreactivity was higher in axotomized motoneurons (Fig. 5*B*) than in noninjured motoneurons (Fig. 5*A*). Motoneurons in the ventro-medial subdivision of the lesioned facial nucleus, and corresponding to the caudal auricular branch of the facial nerve, were spared from axotomy and displayed a lower level of Bcl-2 immunoreactivity (Fig. 5*B*).

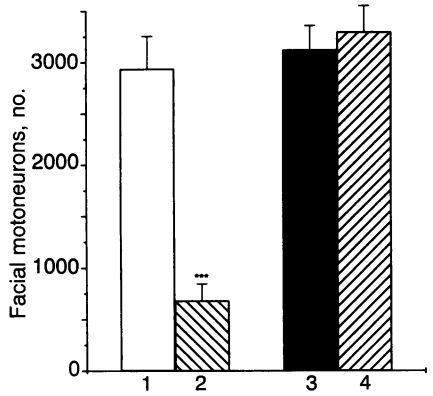

FIG. 4. Motoneuron survival in the facial nuclei in wild-type and transgenic mice 7 days after unilateral lesioning of the facial nerve. Data are the mean ± SEM from seven wild-type and seven transgenic mice. Bars: 1, wild-type mice, nucleus on the unoperated side; 2, wild-type mice, nucleus on the operated side; 3, transgenic mice, nucleus on the unoperated side; 4, transgenic mice, nucleus on the operated side. ***, $P < 0.001$, when comparing wild-type and transgenic mice.

Similar results were obtained on mice from lines 57 and 71, suggesting that the survival effects are independent of the transgene insertion in the genome.

GFAP Immunoreactivity. We observed GFAP immunoreactivity surrounding the surviving motoneurons in the lesioned facial nucleus of the transgenic animals (Fig. 3*F*). The level of astroglial immunoreactivity did not appear to differ from that seen in wild-type axotomized animals where most motoneurons had degenerated (Fig. 3*C*). The ventro-medial subdivision of the facial nucleus corresponding to the unlesioned caudal auricular branch of the nerve was devoided of GFAP immunoreactivity in both types of mice (Fig. 3 *C* and *F*). In 9-day-old mice that underwent unilateral facial nerve section at the age of 2 days, confocal microscope examination of brainstem section processed for double immunostaining of Bcl-2 and GFAP revealed reactive astrocytes close to surviving motoneurons strongly immunoreactive for Bcl-2 (Fig. 5*C*). No GFAP immunoreactivity was observed in the contralateral unlesioned facial nucleus in both wild-type and transgenic mice.

DISCUSSION

In the present work, we have shown that in transgenic neonatal mice, motoneurons overexpressing Bcl-2 do not degenerate after axotomy, whereas in wild-type neonatal mice, axotomized facial motoneurons degenerate. One of the many hypotheses to explain motoneuron degeneration after axotomy in wild-type newborn animals is that motoneurons lack specific retrogradely transported neurotrophic factors and, therefore, execute an autolytic program (3, 4, 29–31). In support of this hypothesis, it has been shown that local application of ciliary neurotrophic factor or brain-derived neurotrophic factor to the cut nerve prevents the death of axotomized motoneurons in newborn rats (20–23). Our pres-

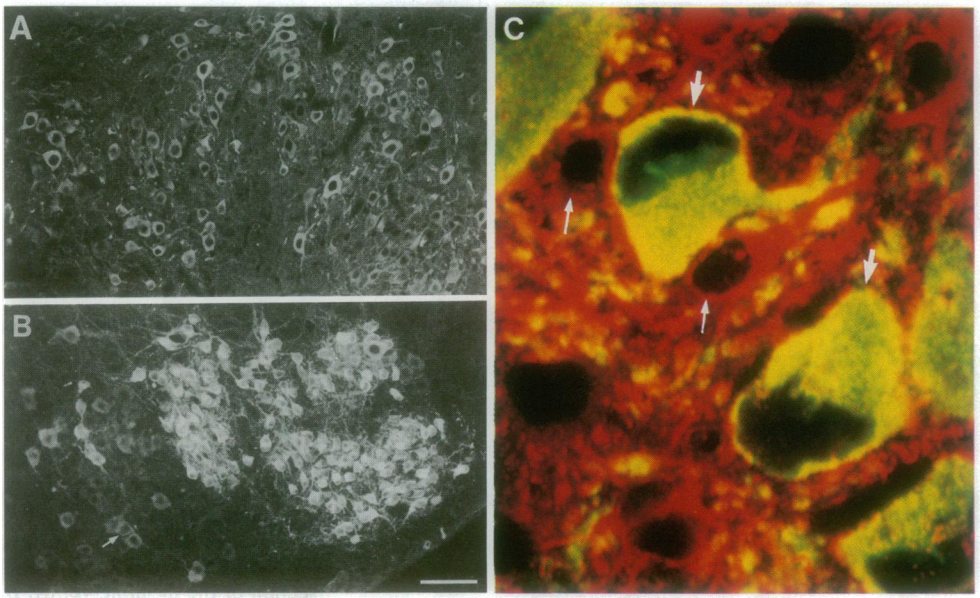

FIG. 5. Bcl-2 and GFAP immunostaining of intact and axotomized motoneurons of transgenic mice. Seven days after axotomy, brainstem sections were immunostained for Bcl-2 alone (*A* and *B*) or Bcl-2 and GFAP (*C*). (*A*) Intact facial nucleus. (*B*) Axotomized facial nucleus; arrow points to unlesioned facial motoneurons of the ventro-medial subdivision of the nucleus corresponding to the intact caudal auricular branch of the facial nerve. (*C*) Axotomized facial nucleus. The confocal microscope examination shows strong fluorescein-labeled Bcl-2 immunoreactivity in axotomized motoneurons and Texas red-labeled GFAP immunoreactivity in the surrounding glial cells indicated by arrows. (Bars: *A* and *B*, 130 μm; *C*, 8 μm.)

ent data on transgenic mice and the previous results obtained on cultured neurons (12–14) suggest that Bcl-2 could thus mediate the survival effects of neurotrophic factors. Consistent with this interpretation, both Bcl-2 and neurotrophic factors (20) rescue axotomized motoneurons but do not prevent the glial reaction.

The numbers of motoneurons in the facial nucleus of transgenic mice of lines 57 and 71 were not significantly different and were pooled. Furthermore, these numbers were not significantly different from the number in wild-type mice (means: 3120 neurons in transgenic mice and 2940 neurons in wild-type mice). This allows a relevant comparison of motoneuron number among various mice used in the present experiments. Thus, in these two lines, the overexpression of Bcl-2 has not prevented developmental neuronal death (1). This is possibly due to the fact that the necessary overexpression of Bcl-2 might have occurred after the naturally occurring cell death period. However, in a different line of transgenic mice, line 73 not used in the present experiments, a 30–40% excess of facial motoneurons was observed, suggesting that, in line 73, overexpression of Bcl-2 is initiated earlier in development and thus may have prevented, at least partially, developmental motoneuronal death (32).

One of the earliest reactions observed in the facial nucleus after motoneuron axotomy is an intense synthesis of GFAP by astrocytes. This reaction occurs within 24 h after nerve injury and could be a response to a signal provided by the lesioned motoneurons (33, 34). In the present work, we observed GFAP immunoreactivity surrounding the surviving motoneurons in the lesioned facial nucleus of the transgenic animals but not in the ventro-medial subdivision of the nucleus corresponding to motoneurons sending axons in the unlesioned caudal auricular branch of the facial nerve. This later observation suggests that the GFAP immunoreactivity observed in other subdivisions of the nucleus is effectively a consequence of facial motoneuron axotomy. Bcl-2 is thus capable of rescuing injured motoneurons but does not prevent glial reaction in response to the lesion.

In human fetal nervous system, human Bcl-2 immunoreactivity was detected in ventral spinal cord neurons but was not investigated in the facial nucleus (35). Recently, a short report described a limited distribution of Bcl-2 mRNA in the brain of adult and developing rats but did not mention an autoradiographic signal in central motoneurons (36). A low level of mouse Bcl-2 expression was detected in whole brain preparations of 6- or 7-day-old mice (37). No immunoreactivity for the human Bcl-2 protein was detected in our experiments on wild-type mice, confirming that the antibody we have used does not detect the murine form of the Bcl-2 protein (28). Thus, it seems reasonable to assume that if endogenous Bcl-2 is present in neonatal facial motoneurons in wild-type mice, it is not able to protect these neurons from axotomy-induced cell death. In contrast, our observation on transgenic mice suggests strongly that the induced overexpression of Bcl-2 is related to the survival of axotomized neonatal facial motoneurons.

In transgenic animals, lesioned facial motoneurons, relative to unlesioned ones, displayed an increased level of Bcl-2 immunoreactivity. This suggests that in these motoneurons protein synthesis has not been interrupted. This result is reminiscent of observations in injured adult rodent neurons, describing modifications of gene expression and overexpression of peptides such as calcitonin-gene-related peptide, vasoactive intestinal peptide, and galanin (33, 34). However, it remains uncertain whether this augmentation in Bcl-2 immunoreactivity results from an increase of the *NSE* promoter activity in response to axotomy (24) or from an accretion of Bcl-2.

The mechanisms by which Bcl-2 protects neurons and other cell types (38–42) from normally and experimentally induced cell death are not elucidated. However, evidence exists that Bcl-2 may act in regulating an antioxidant pathway at sites of free-radical generation *in vitro*. Indeed, recent data indicate that Bcl-2 can prevent cellular damage including lipid peroxidation (43) by inhibiting the conversion of $O_2^{\cdot-}$ (44, 45). It is known that neurons are at particular risk from free-radical damage. Polyunsaturated fatty acids, a major constituent of neuronal membranes, are an ideal substrate for lipid peroxidation. A role for oxidants has been suggested in Parkinson and Huntington diseases (46) and recently in neuronal death in familial amyotrophic sclerosis (47). Indeed, mutations within Cu/Zn superoxide dismutase argue strongly for an oxygen-radical-mediated loss of motoneurons (47). These results suggest that the cause of motoneuronal cell death after axotomy is an increase in reactive oxidants. Alternatively, to prevent cell death, Bcl-2 could impair the cleavage of pro-interleukin β by the interleukin 1β-converting enzyme, and thus the production of interleukin 1β (48, 49), a cytokine whose level is augmented in Alzheimer disease (50). However, it cannot be excluded that Bcl-2 could prevent cleavage, by the interleukin 1β-converting enzyme of other proteins that are essential for cell viability (49). Further investigations of our *in vivo* system should add significantly to our under-

standing of Bcl-2 function and allow testing of the effects of Bcl-2 in various types of neurodegenerative diseases.

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