Objectives: To develop a mouse model for measuring facial nerve injury and recovery and to test the hypothesis that overexpression of the antiapoptotic gene, bcl2, enhances recovery of facial nerve function after peripheral crush injury.

Design: Prospective analysis of recovery of function after facial nerve crush injury in mice at juvenile (postnatal day 7) and adult (postnatal day 30) ages with blind comparison of wild-type and transgenic bcl2 overexpression littermates at both ages and immunohistologic confirmation of overexpression of bcl2 in facial motoneurons in transgenic animals.

Results: Adult wild-type mice demonstrated full recovery of facial nerve function (measured as eye blink and whisker movement) within 3 weeks of injury. Juvenile wild-type mice demonstrated diminished recovery of function. Juvenile transgenic bcl2 overexpression mice demonstrated more rapid and complete recovery of eye blink but not whisker movement in comparison with wild-type littermates.

Conclusions: Measurement of facial nerve function in mice after injury is feasible. Enhanced recovery of facial nerve function in adult mice and mice overexpressing bcl2 indicates that preservation of central motoneurons after injury may improve function after peripheral nerve injury.

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Facial nerve injuries are an important cause of morbidity after trauma or extirpative surgery. Axonal degeneration distal to a peripheral nerve injury is a well-described phenomenon and is most directly responsible for target (eg, muscle) paralysis and atrophy. However, muscle function after nerve regeneration may be affected not only by preservation of the target muscle, but by the number of surviving motoneurons (and thus axons) present. Loss of central motoneurons after peripheral lesions is well described and may ultimately affect muscle function after a period of recovery and reinnervation. In the case of the facial nerve, cut or crush injuries to the nerve result in loss of neurons in the facial motor nucleus and thus affect the extent of recovery of movement achieved after reinnervation of the facial musculature.

The facial nerve has been used as a model for study of the central effects of peripheral injury in a variety of animals.1-4 Interestingly, this is an age-dependent phenomenon.5,3 In mice, peripheral facial nerve crush injury in juvenile animals (aged postnatal day 7 [P7] or less) results in loss of more than 50% of facial nucleus neurons.6 Moreover, neuronal loss occurs through an apoptotic process, the time course of which has been delineated.4 In adult mice, the same injury results in no loss of central motoneurons. The molecular mechanism for this loss of sensitivity to peripheral injury remains unclear, but some important clues exist.

In the rat, age-dependent differential activation of the nitric oxide pathway has been demonstrated after facial nerve axotomy.7 In that study, decreased activity of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase was noted in juvenile animals, and apoptotic changes were noted in facial motoneurons after axotomy.7 Overexpression of the antiapoptotic gene, bcl2, has been shown to decrease susceptibility to cell death in facial nucleus motoneurons after facial nerve injury.8,9 This finding has been supported by in vitro studies of motoneuron survival, in which the effects of brain-derived neurotrophic factor withdrawal are mitigated by manipulation of cell death regulators, such as bcl2.9 These studies sug-
gest that axotomy causes a series of events that ultimately lead to apoptosis of central motoneurons in juvenile animals. In none of these studies, however, was an attempt made to examine the recovery of facial nerve function after injury. Indeed, no model exists for examination of facial nerve recovery in the mouse.

The goals of this study were 2-fold. The first was to develop a mouse model for examination of facial nerve recovery after facial nerve injury. To do so, we examined wild-type (C57/Bl6) 30-day-old mice after facial nerve cut or crush injury to the extratemporal facial nerve. Facial nerve function was monitored by examining eye closure and whisker movement. This study presents a standard scale for measurement of both eye closure and whisker movement.

The second goal was to use this model to test functional recovery after facial nerve injury in genetically manipulated mice. The first candidate gene was bcl2, a well-conserved, prototypical antiapoptotic gene. Transgenic mice in which the human bcl2 transgene had been placed under a neuron-specific enolase (NSE) promoter was used to examine the effect of neuroprotection on recovery of facial movement after crush injury. A previous study demonstrated the overexpression of bcl2 in the central nervous system in this mouse.

METHODS

ANIMALS

Transgenic mice overexpressing the human bcl2 gene under the control of an NSE promoter (NSE73a line) were the generous gift of Dong-Feng Chen, MD, PhD. The NSE73a male breeders were bred to wild-type C57/Bl6 females. Litters were thus mixed of approximately equal numbers of transgenic and wild-type (control) mice. Mouse genotype was identified by tail clip DNA analysis using polymerase chain reaction. All mouse cages were checked daily for new litters; new pups were considered as described. Adult wild-type mice demonstrated recovery and whisker movement were graded for each mouse as described. Adult wild-type mice demonstrated recovery of both whisker movement and eye closure by approximately the 10th day postoperatively, and this level of function remained stable at 20 days postoperatively (Figure 1 and Figure 2).

SURGICAL PROCEDURES

Litters born to heterozygous NSE73a breeders underwent surgery at age P7 or postnatal day 30 (P30). Mice of all ages were anesthetized by means of inhaled methoxyflurane until they were areflexic; this level of anesthesia was maintained throughout the surgical procedure. An incision was made inferior to the external auditory canal. The skin incision was closed with cyanoacrylic glue. Litters were returned to parents within 1 hour.

MEASUREMENT OF FACIAL NERVE FUNCTION

Facial nerve function was examined at a variety of intervals postoperatively. Mice were examined individually for eye closure and whisker function according to the following scale: a grade of 0 was given for no detectable movement, 1 for detectable motion, 2 for significant (but asymmetric) voluntary motion, and 3 for symmetric voluntary motion. Eye closure was detected by gentle stimulation of the lid margin with a cotton swab. Whisker movement was detected by gentle stimulation of the ipsilateral whiskers with a cotton swab. Mice were immediately replaced in cages after examination. Statistical comparisons were made by means of unpaired t test.

HISTOLOGIC EXAMINATION

Animals were killed with carbon dioxide intoxication and transcardially perfused with phosphate-buffered 4% paraformaldehyde (pH, 7.4). After perfusion, the brain was immediately dissected from the skull and placed in fresh fixative for 24 to 48 hours. Brains were serially dehydrated in graded alcohols, embedded in paraffin, and serially sectioned at 10 µm in the coronal plane. A 1-in-5 series of sections was mounted on gelatin-coated slides, dewaxed in xylene, thionin-stained, and coverslipped with DPX (distrene, plasticiser, and cylene) mounting medium.

IMMUNOCYTOCHEMISTRY

Litters of mice used for immunocytochemical studies were killed as described in the preceding paragraph. Brains were fixed, embedded in paraffin, sectioned, and mounted as noted previously, with the exception that the duration of fixation was 18 hours. Antigen retrieval was performed on the brainstem sections in preparation for immunohistochemistry. Slides were placed in a Coplin jar containing 10mM citric acid solution, pH 6, and steamed in a rice cooker for 25 minutes, then cooled in ice for 10 minutes. Slides were then rinsed in buffered saline, incubated in 0.6% hydrogen peroxide in isotonic sodium chloride solution for 30 minutes, and rinsed again. Tissue was blocked for 1 hour with 5% normal goat serum and 1% Triton-X in phosphate-buffered saline. Sections were then incubated overnight with primary antibody: anti-bcl2 ΔC-21 (rabbit polyclonal, 1:2000; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). Tissue was then rinsed and incubated for 30 minutes with secondary antibody (Vector biotinylated goat anti-rabbit, 1:200; Vector Laboratories, Burlingame, Calif) followed by rinsing and incubation in avidin biotinylated complex (Vector ABC; Vector Laboratories) solution for 30 minutes at room temperature. Horseradish peroxidase reaction product was visualized with the use of diaminobenzidine as the chromagen. Photomicrographs were acquired by digital photography (SPOT II E camera, Digital Instruments, Sterling Heights, Mich) and transferred to Adobe Photoshp 5.5 software (Adobe Systems Inc, San Jose, Calif).

RESULTS

TIMING OF RECOVERY AFTER FACIAL NERVE CRUSH IN WILD-TYPE ANIMALS

Wild-type (C57/Bl6) mice were examined at 5-day intervals after unilateral facial nerve crush injury. Eye closure and whisker movement were graded for each mouse as described. Adult wild-type mice demonstrated recovery of both whisker movement and eye closure by approximately the 10th day postoperatively, and this level of function remained stable at 20 days postoperatively (Figure 1 and Figure 2).

AGE DEPENDENCE OF RECOVERY AFTER FACIAL NERVE CRUSH IN WILD-TYPE ANIMALS

Previous studies have demonstrated that facial nerve crush injury in juvenile mice results in loss of central motoneurons in the facial nucleus. To examine facial nerve recovery after loss of central motoneurons in juvenile
mice, facial nerve crush injury was performed on age P7 wild-type mice and recovery was examined. As shown in Table 1, animals that underwent facial nerve crush at age P7 demonstrated much lower whisker movement scores on postoperative days 5, 10, and 20 than animals that underwent the same manipulation at age P30. No age-dependent difference was found in comparison of whisker movement on postoperative day 5 (Table 1; P = .34). However, by day 10, lower whisker movement scores were observed in P7 animals (0.9 ± 0.1 [n = 8] and 2.8 ± 0.2 [n = 10] for P7 and P30, respectively; P < .01). Although P7 animals did demonstrate some movement by day 20, this was still much less than that observed in P30 animals (Table 1). Full recovery of whisker movement did not occur in animals aged P7 when examined as long as 90 days after injury (Table 1).

Measurement of eye closure showed lower scores in animals aged P7 vs P30 at the time of facial nerve crush (Table 2). Although no difference in movement scores were noted on postoperative day 5, by postoperative day 10, P30 animals demonstrated more rapid recovery than their juvenile counterparts (0.8 ± 0.3 [n = 8] and 2.8 ± 0.2 [n = 10] for P7 and P30 animals, respectively; P < .01). Recovery of function occurred slowly in P7 mice, and movement scores continued to be lower in these mice when compared with P30 mice on postoperative day 20 (1.4 ± 0.2 [n = 8] and 2.8 ± 0.2 [n = 10] for P7 and P30 mice, respectively; P < .01). Although the trend of diminished recovery of function continued as long as 90 days postoperatively, this did not achieve statistical significance when compared with P30 animals (Table 2).

### FACIAL NERVE RECOVERY AFTER CRUSH INJURY IN bcl2 OVEREXPRESSION ANIMALS

The antiapoptotic gene, bcl2, has been shown to protect facial motoneurons from cell death after peripheral injury. To study the possible enhancement of recovery after injury, litters of mixed wild-type and bcl2 overexpression mice underwent facial nerve crush and were examined. Mice are phenotypically indistinguishable at these ages and were not genotyped until facial nerve recovery data were recorded. All genotyping was done in blind fashion. The expression of the human bcl2 transgene in the facial motor nucleus was confirmed via immunohistochemistry (Figure 3). Minimal staining was observed in facial motor nucleus neurons in wild-type mice (data not shown).

As noted before, P30 mice demonstrated rapid recovery of both eye blink and whisker movement by post-

<table>
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<tr>
<th>Table 1. Age Dependence of Recovery of Whisker Movement After Facial Nerve Crush Injury in Wild-Type Animals*</th>
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<td><strong>Age at Surgery</strong></td>
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Abbreviation: NA, not applicable.

*Data shown are mean ± SEM (number of mice). P = .34 for comparison of age groups on day 5; P < .01 for comparison of age groups on days 10 and 20; P < .01 for comparison of postnatal day 7 animals surviving 90 days and postnatal day 30 animals surviving 20 days. See the “Measurement of Facial Nerve Function” subsection of the “Methods” section for movement rating scale.
operative day 10. To test the hypothesis that \textit{bcl2} enhanced the speed of recovery during this period, age-matched wild-type and \textit{bcl2} overexpression mice underwent unilateral facial nerve crush injury as described herein. In mice aged P30 at the time of injury, the speed of recovery after facial nerve injury was virtually identical in wild-type and \textit{bcl2} overexpression mice (data not shown).

To test the hypothesis that \textit{bcl2} might enhance recovery after peripheral facial nerve injury in juvenile mice, litters of mixed wild-type and transgenic mice were tested. Transgenic mice are phenotypically indistinguishable, and genotyping was performed in blind fashion after data were collected. In mice aged P7 at the time of surgery, \textit{bcl2} did not affect speed or extent of recovery of whisker movement after facial nerve crush (Figure 4). By postoperative day 30, wild-type and \textit{bcl2} overexpression mice demonstrated similar whisker function (Figure 4).

Eye closure, however, was enhanced in \textit{bcl2} overexpression mice (Figure 5). A trend toward improved function in \textit{bcl2} overexpression mice was observed by postoperative day 10. This trend reached statistical significance by postoperative day 15, when \textit{bcl2} overexpression mice demonstrated mean eye closure scores of 1.8 ± 0.3 (mean ± SEM; n = 4) and wild-type mice from the same litters scored 1.1 ± 0.1 (n = 8; P < .05 compared with \textit{bcl2} overexpression littermates). This statistical difference continued through postoperative day 30 (Figure 5).

**COMMENT**

Age-dependent changes in central neuronal sensitivity to removal of afferent input (in the case of sensory neurons) or removal of target organs (in the case of motoneurons) are well described. In the facial motor nucleus, neurons in juvenile animals are more susceptible to retrograde cell death after axonal injury. Previous studies have shown that, after facial nerve crush injury in P7 mice, 45% of neurons are lost within days of the injury, while mice P28 or older demonstrate no loss of neurons. Whereas no previous study sought to correlate these well-known central effects with function, the present study demonstrates differential recovery of facial nerve function in juvenile vs adult mice after peripheral injury.

The molecular mechanism of decreased sensitivity of adult neurons to axonal injury remains unclear. Several lines of evidence point to the retrograde neuronal loss in juvenile mice as an apoptotic event. First, axotomized facial motoneurons in P2 mice demonstrated TUNEL (TdT-mediated dUTP digoxigenin nick-end labeling) within hours of injury, peaking at approximately 28 hours. Second, mice overexpressing the antiapoptotic gene \textit{bcl2} demonstrate little or no TUNEL labeling after the same injury. Overexpression of \textit{bcl2} or \textit{bcl-xL}, another antiapoptotic gene, protects facial motoneurons from axotomy-induced cell death. Furthermore, \textit{bcl2} not only prevents morphologic
apoptotic death of axotomized neonatal transgenic motoneurons but also permits motoneurons to conserve functional electrophysiologic properties. The final common pathway for neuronal cell death in this circumstance is likely caspase-3. The influence of \textit{bcl2} on this process indicates that decreased sensitivity of adult neurons may be a result of altered expression of genes of the apoptotic regulatory pathway.

The trigger for these apoptotic events is neurotrophin withdrawal. Neurotrophic factors are known to play a role in target deprivation–induced neuronal cell death. Acidic fibroblast growth factor expression has been correlated with survival in facial motoneurons after crush injury and is likely acting as a neurotrophin rather than a mitogen in this scenario. Apoptosis and neurotrophin withdrawal are linked, as demonstrated by...
in vitro studies of motoneuron survival, in which the effects of brain-derived neurotrophic factor withdrawal are mitigated by manipulation of cell death regulators, such as bcl2. Further linking neurotrophins and apoptosis is evidence that target-dependent (eg, neurotrophin-dependent) neuronal cell death can be regulated by well-conserved cell death pathways, supported by the dual role of the p75NTR receptor.

Correlates of central neuronal loss and peripheral nerve function have been examined in other systems. For example, in the adult monkey, quantitative electrophysiologic measurements showed greater loss of muscle response when compared with greater central neuronal loss. While electrophysiologic examination of facial muscle function in neonatal mice presents technical challenges, such a study may ultimately be of value for more quantitative examination of nerve recovery. In another study, nimodipine was found to enhance speed of recovery after facial nerve injury but did not affect retrograde neuronal cell loss. No studies of recovery of facial nerve function after injury exist in the mouse. The development of such a model provides a platform for examination of the molecular mechanisms behind this process by examination of genetically manipulated mice.

The evidence presented herein that bcl2 overexpression in facial motoneurons enhances recovery of some aspects of facial nerve function is the first demonstration of improved functional recovery after facial nerve injury using a genetic manipulation. Interestingly, recovery of function was notably different when measured with eye closure or whisker movement. This may be a result of lower sensitivity or decreased reliability of the latter measurement and is one reason why 2 measures of facial nerve function were used during this study. A less likely, though possible, explanation is that the subset of motoneurons innervating the whiskers differs in survival characteristics from those innervating the orbicularis oculi. Electrophysiologic measurement of muscle activity during the regenerative period would provide more quantitative data in this regard.

The development of a mouse model of facial nerve injury sets the stage for future studies of the effects of manipulation of cell death regulators on facial nerve recovery after peripheral injury. The present study indicates that the central effects of facial nerve injury have profound implications for rehabilitation in many patients, particularly those in the pediatric age group. However, the finding that manipulation of genes in the apoptotic pathway can prevent long-term dysfunction after nerve injury provides hope that future studies may lead to improved functional rehabilitation of patients after facial nerve injuries.

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REFERENCES