

Time Course and Age Dependence of Motor Neuron Death Following Facial Nerve Crush Injury: Role of Fibroblast Growth Factor

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Peripheral nerve crush injury (PNCI) has been used for many years in adult animals to study central and peripheral changes related to regeneration across the injury site. While these adult animals experience full recovery with no neuronal cell loss following PNCI, it has been noted that the injury in perinatal animals is followed by retrograde neuronal cell death. The present study determines, in mice of different postnatal ages, the degree to which motor neurons are vulnerable to PNCI induced cell death and examines the rate of neuronal loss. Animals of 4 days of age and younger were found to be significantly more vulnerable to motor neuron cell death following PNCI. There also was a proportional relationship between age at injury and final motor neuronal survival and an inverse relationship between age at injury and rate of neuronal cell death following injury. In addition a proportional relationship was observed between the expression level of acidic fibroblast growth factor within motor neurons and the resistance to PNCI induced neuronal death. It was also found that PNCI in an environment that contained higher levels of FGFs (either in mice treated with acidic FGF or in transgenic mice that overexpress basic FGF) significantly decreases neuronal cell death following early postnatal injury. © 1999 Academic Press

Key Words: nerve; injury; cell death; FGF; treatment.

INTRODUCTION

For many years peripheral nerve crush injury (PNCI) has been used in adult animals to study central and peripheral changes related to regeneration across the injury site. The complete survival and regeneration of motor neurons seen in injured adult animals are in stark contrast to the significant retrograde neuronal

cell death seen in animals injured at less than 1 week of age (1, 12, 25, 28, 36). These divergent results provide an opportunity to explore the factors that influence the survival observed in the adult animals. The present study provides a detailed description of the time course and rate of neuronal loss following PNCI in mice of different postnatal ages.

Age-related survival appears correlated with the availability of neurotrophic factors. The complex cellular environment of the motor neuron offers many potential sources and molecules for neurotrophic support during the period when a crushed axon is attempting to regenerate. For motor neurons, such as those of the facial nucleus, important factors are derived from the Schwann cells at the injury site (ciliary neurotrophic factor, CNTF (44), neurotrophins (15, 29), and glial cell-line-derived neurotrophic factor, GDNF (13, 31)).

Motor neurons themselves contain extremely high levels of acidic FGF (aFGF) (9, 43). Acidic FGF is a member of the fibroblast growth factor family, which consists of over 15 structurally related proteins (7). FGFs are characterized by their selective binding to the sulfated glycosaminoglycan heparin and by their overlapping mitogenic or neurotrophic actions on a variety of mesodermal, ectodermal, and neuroectodermal cell types (2, 7). The biological effects of the FGFs are mediated by a family of at least four transmembrane receptors that contain intracellular tyrosine kinase domains. All four FGF-receptor genes are expressed in a cell type-specific pattern in the CNS, which includes expression of FGFR-1 by motor neurons (14, 50). Acidic FGF and basic FGF (bFGF) share about 55% amino acid sequence homology, and both can act as mitogens for nonneuronal cells, including astrocytes (34), Schwann cells (5), and oligodendrocytes (6), and they can act as neurotrophic factors for a large variety of peripheral (8, 38, 47) and central (10, 30, 48) neuronal populations. Both aFGF and bFGF appear to be able to bind and stimulate the FGF receptor variants most common in the CNS (see 7, 18, 20 for reviews). In

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contrast to several other members of the FGF family, both aFGF and bFGF lack an amino-terminal hydrophobic signal sequence, which appears to be required for the sorting of proteins into the secretory pathway (49).

Acidic FGF is present in large quantities in the cytoplasmic compartment of select neuronal populations, including motor neurons, where it may represent nearly 0.1% of soluble protein (9, 37, 43). These extremely high tissue concentrations of aFGF and bFGF contrast with the high affinity of FGF receptors, which mediate the full range of FGF biological effects at FGF concentrations of a few nanograms per milliliter. We propose that the high intracellular stores of aFGF present in motor neurons are not normally available to the high-affinity surface receptors, but will become available after damage to the motor neuron, possibly through a simple breach of the plasma membrane. In order to determine whether endogenous aFGF expression in motor neurons is correlated with the degree of motor neuron vulnerability to injury, we determine here the age dependence of the extent to which mice are vulnerable to PNCI induced facial motor neuron loss and examine the developmental expression of aFGF within the motor neurons of the mouse facial nucleus. The effects of treating PNCI lesioned mice with recombinant aFGF and of transgenic overexpression of bFGF are also reported.

EXPERIMENTAL PROCEDURES

Facial Nerve Anatomy

The facial motor nucleus innervates the musculature of the face. The motor neuron cell bodies reside in the ventral lateral portion of the hindbrain just rostral of the pons. In an adult mouse the nucleus extends over a distance of approximately 800 μm and has well-defined boundaries. The axons of these motor neurons leave the CNS at the caudal margin of the pons via cranial nerve VII. The facial nerve exits the cranial cavity via the stylomastoid foramen. The main trunk courses under the external ear and out onto the face musculature until it furcates into its primary divisions as it passes under the external jugular vein (Fig. 1A). The main trunk of the nerve is easily accessible by a simple surgical procedure (Fig. 1B). An injury at this point damages approximately 90% of the motor neuronal fibers that originate within the borders of the facial motor nucleus. The remaining 10% whose cell bodies map to the ventral medial subnucleus within the facial nucleus are accounted for by the retroauricular branch (22), which leaves the main trunk at the stylomastoid foramen prior to the site of injury (see Fig. 1A).

Facial Nerve Injury and Tissue Processing

BALB/c mice of selected ages (days after birth; 0, 2, 4, 7, 14, 21, 28, or 45) were anesthetized and the facial

nerve on the right side was exposed through a 2- to 5-mm incision dorsal and caudal to the external ear. All operations were performed under a stereo dissecting microscope (Leitz 0.7–3 \times zoom). The nerve trunk was released from the surrounding connective tissue by gentle dissection and then injured. The nerve trunk was crushed distal to the auricular branch of the nerve (Fig. 1) with a slight shearing motion, between the tips of jeweler's forceps (Dumont Model PP/45 selected for their size and resistance to deformation) for a count of 5 s, resulting in an approximately 3-mm gap between the nerve stumps. The crush site distal to the auricular branch can readily be examined at all postnatal ages for clear separation of the nerve stumps and retention of the overlying epineurium. If the site was not visible to inspection, for example, due to bleeding, or the stumps were freely separated with no epineurial connection, the animal was excluded from the study. The skin incision was closed with cyanoacrylate adhesive. The animals were monitored in a temperature-controlled environment until they regained consciousness and then were returned to their mother. The surgical protocols used followed approved animal care guidelines.

After the selected survival interval (1, 2, 3, 5, 7, 14, or 30 days after injury) the animals were sacrificed by an overdose of anesthetic, followed by perfusion through the heart with 0.5–5 ml (depending on age) of phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.2) and then by 5–20 ml (depending on age) of phosphate-buffered formalin (5% formalin, 100 mM sodium phosphate, pH 7.2). The brain was rapidly dissected and postfixed in the same fixative at room temperature for 30 min. The tissue was soaked in PBS overnight at 4°C and then cryoprotected by sinking in 15% then 30% sucrose in PBS at 4°C. Serial 25- μm cryostat sections were cut from the rostral pyramids to the inferior colliculus and every fourth section was collected onto the same slide. This resulted in four sets of slides, each set containing sections representing the complete facial nucleus. Each slide set is separated from the adjacent sets by 25 μm and each section on a single slide is separated by 75 μm from the adjacent sections on that slide. The slides were dried flat overnight at room temperature and then stored in desiccated slide boxes at –70°C until staining was performed.

Cellular Identification and Counting

A single set of slides from each animal was stained by standard methods for Nissl substance with toluidine blue. The slides were examined to determine the location of the facial nucleus within the set. These sections were labeled, with the first and last sections labeled

being the sections directly before or after the facial nucleus where no motor neurons were found. Motor neurons were identified as cellular profiles with a diameter larger than 10 μm (all other cells within the facial nucleus have smaller profiles). Motor neuron diameters are also smaller than the 75- μm separation between sections analyzed; thus double counts of the same neuron are avoided. The motor neuron profiles within the boundaries of the facial nucleus were counted at 160 \times using an etched grid reticle (grid squares are 60 μm on a side at 160 \times). As the average neuronal profile area was found to change by less than 5% following PNCI, no correction for cell shrinkage was applied (Kuzis, unpublished results). Total counts recorded for each side of the brain stem were adjusted to account for the neurons from the retroauricular branch of the facial nerve whose axons were not injured. Retroauricular neurons were taken as 10% of the total count from the uninjured side. For each animal, the percentage of injured motor neurons surviving the crush (survival percentage) was calculated by dividing the adjusted motor neuronal profiles from the injured side by the adjusted motor neuronal profiles from the uninjured side. From these individual survival percentages, mean survivals for each data point (by age at injury and postinjury interval) were calculated. Statistical significance was assessed using *t* test and ANOVA as calculated using the "Statistica Mac" software tool. Significance was tested using a *P* value of 0.05 as the upper limit. All counts were performed blinded to the animal's age at injury and survival time.

Acidic Fibroblast Growth Factor Immunohistochemistry

Tissue was collected from BALB/c mice, raised in our breeding colony, of the selected ages (days after birth; 0, 3, 5, 7, 11, 14, 21, and 30) as described above. Coronal 50- μm -thick frozen sections were cut from the rostral pyramids to the inferior colliculus and processed for immunohistochemical staining of aFGF.

For aFGF immunohistochemistry, selected sections were incubated for 30 min in 30.0% (v/v) ethanol and 5.0% (v/v) hydrogen peroxide in 65 mM Tris, pH 7.3, to inactivate endogenous peroxidases, followed by two 2-min washes in Tris-buffered saline (TBS: 100 mM Tris, 150 mM NaCl, pH 8.2). The sections were then incubated for 90 min in blocking solution (100 mM sodium phosphate, 10.0% (v/v) horse serum, 0.5% (v/v) Triton X-100, 0.2% (w/v) sodium azide), followed by overnight incubation (minimum of 12 h) in rabbit antiserum to aFGF or rabbit preimmune serum (both diluted 1:1000 in blocking solution). Sections were washed three times for 10 min each in TBS, incubated for 1 h in goat anti-rabbit antiserum (Sternberger-Meyer) diluted 1:250 in blocking solution containing 5% (v/v) mouse serum), washed as described above,

incubated for 1 h in rabbit peroxidase anti-peroxidase (Sternberger-Meyer) diluted 1:100 in blocking solution containing 5% (v/v) mouse serum and no sodium azide, washed, and reacted with 0.5 mg/ml 3,3'-diaminobenzidine and 0.03% (v/v) hydrogen peroxide in PBS. The reaction was stopped by washing twice for 5 min each with PBS. The stained sections were mounted on gelatin-coated glass slides and coverslipped according to standard procedures. The rabbit polyclonal anti-aFGF serum used here for immunohistochemistry has been characterized previously (43). For anatomical identification of stained cells, the atlas by Sidman was used (41).

Acidic Fibroblast Growth Factor Treatments

Human recombinant acidic fibroblast growth factor was expressed and purified in our laboratory (43). The biologically active purified factor was diluted to a concentration of 25 ng/ μl . Treated animals were provided with a 2-mm cube of gel foam that was soaked in 5 μl of the aFGF solution. The soaked gel foam was placed upon the crush site immediately following verification of the lesion. This resulted in a maximal application of 125 ng per lesion site. Control animals received gel foam soaked with either PBS alone or PBS containing 25 ng/ μl of bovine serum albumin. The neuronal survival observed following these two control treatments was found to be equivalent with that observed in untreated animals.

Basic Fibroblast Growth Factor Transgenic Animals

Mice that carry the human bFGF gene under the control of the phosphoglycerokinase (pgk) promoter have been developed (3). These animals were created in the FVB/N strain, due to its suitability for transgenic manipulation (45).

The transgenic line used to generate the offspring for this study contains a stably integrated construct that, driven by the pgk promoter, produces all four forms of human basic FGF. Basic FGF is normally expressed in four molecular weight forms, arising from alternative initiation sites for translation (11). Use of the pgk promoter leads to constitutive expression in all cells throughout development. Western blot analysis of tissue extracts indicates that the central nervous system of the transgenic mice contains approximately 40-fold more bFGF immunoreactivity than that of their nontransgenic littermates. The normal appearance of the central nervous system in these bFGF transgenic animals lends evidence to the hypothesis of limited bFGF release (16). Injuries were performed as described above in transgenic animals and their nontransgenic littermates. Transgene status was determined as described (3).

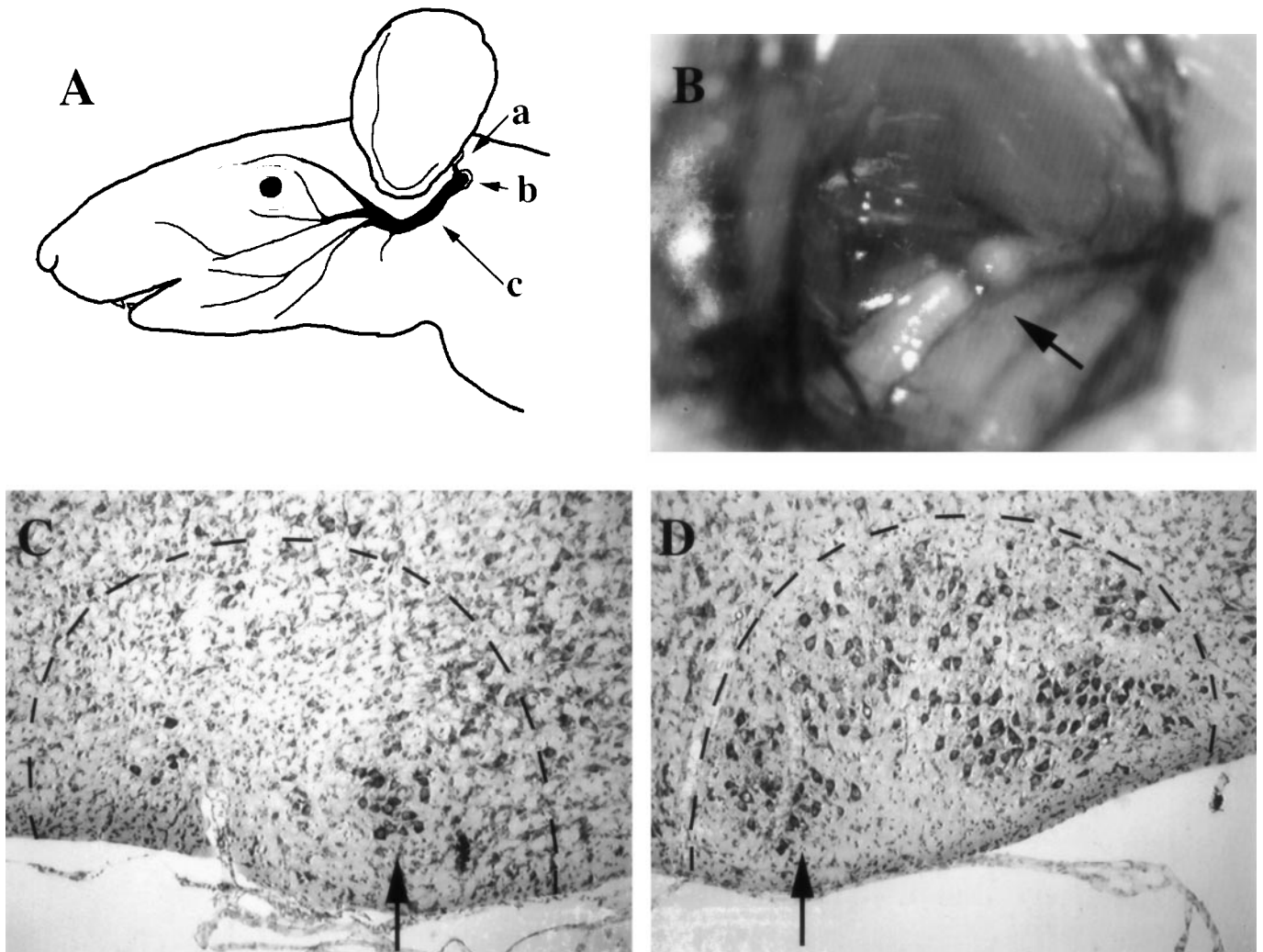


FIG. 1. (A) Line drawing of the anatomical positioning of the mouse facial nerve. Note the path of the nerve trunk, the retroauricular branch, and the site of lesion (a, retroauricular branch of the facial nerve; b, stylomastoid foramen; c, site of facial nerve crush). Drawing adapted from (35). (B) Photograph of a peripheral nerve crush injury of the facial nerve in a 21-day-old mouse. Note the gap between the nerve stumps, which is bridged by a barely visible thin epineurial connection. (C and D) Photomicrographs of the left and right facial nuclei, taken from the same coronal, toluidine blue stained section through the brain stem of an animal that suffered facial nerve crush at 2 days of age and was harvested 14 days after the operation. The broken line defines the approximate extent of the facial nucleus, and the arrows point to the ventral medial facial subnucleus that supplies axons to the retroauricular branch of the facial nerve, which is not lesioned in this model. Note the significant decrease in the number of motor neurons in the facial nucleus ipsilateral to the lesion (C), compared to the number of neurons present in the contralateral control nucleus (D).

RESULTS

Age Dependence of Motor Neuron Survival Following Peripheral Nerve Crush Injury: End-Point Analysis

The first of our objectives was to determine the ages at which postnatal mice are vulnerable to cell death following facial PNCI. Animals of 0, 2, 4, 7, 10, 14, 21, 28, and 45 days of age underwent facial nerve crush (Fig. 1) and motor neuron survival in the facial nucleus was evaluated after a survival interval of 30 days. This 30-day survival interval most likely represents an end-point analysis as there is no evidence in the

literature for late changes in neuronal survival following crush injury, in contrast to what has been reported for the transection injury model (23, 46). The neuronal survival percentage from these animals ranged from a maximum of 101.1 ± 1.1 seen in the 45-day-old animals to a minimum of 12.2 ± 4.6 for the animals injured on the day of birth (Fig. 2). The surviving neurons seen in these animals were evenly distributed throughout the facial nucleus, appearing in no apparent rostral-caudal, dorsal-ventral, or medial-lateral pattern. The only exception to this was the ventral medial subnucleus, which was represented in every animal. As

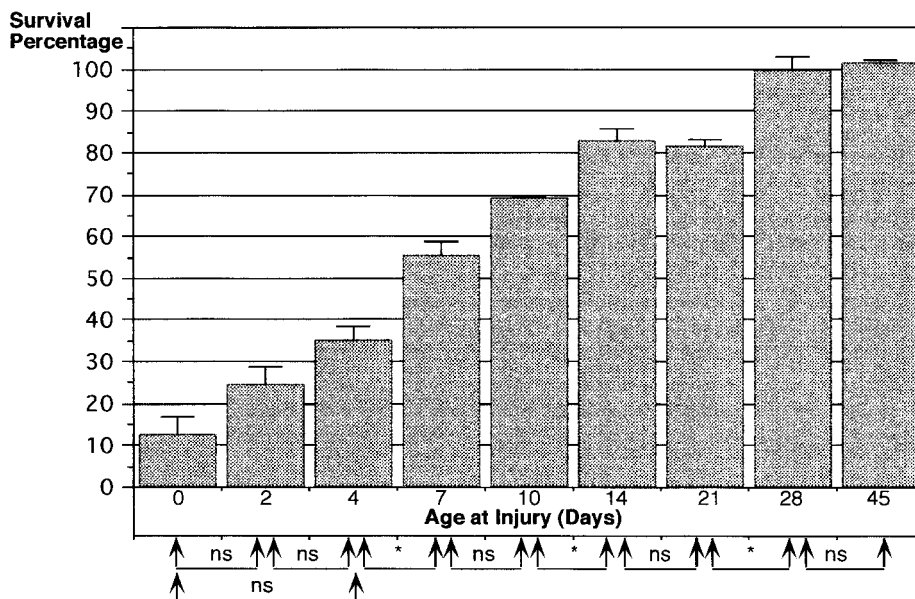


FIG. 2. End-point analysis of the extent of neuronal survival after PNCI performed at different postnatal ages. Neuronal profiles were counted after a 30-day survival interval. Survival percentages are calculated by dividing the number of motor neurons in the facial nucleus on the injured side by the number on the contralateral, noninjured side (see Experimental Procedures for details). Note the significant differences in survival seen between ages of 4 days and younger vs 7 days of age. Age indicated are days after birth (day of birth = 0). *Statistically significant at $P \leq 0.05$ as evaluated by *T* test.

this area normally contains the motor neuron cell bodies whose axons travel in the uninjured retroauricular branch of the facial nerve, these cells provided a convenient localization point during cell counting (Fig. 1).

There is no statistically significant difference between the neuronal survival seen in animals injured at ages of 0, 2, or 4 days ($P \leq 0.05$; Fig. 2). The first significant difference in survival is found between the injuries performed at 4 days and those at 7 days. The comparisons between 10/14 days of age and 21/28 days were also calculated as being significantly different from one another. These observations suggest that the motor neurons of animals injured before the fourth day of age are similarly unequipped to survive the injury. The series of significant differences between older injury ages suggests that after 4 days of age mice are in an active transition to an adult-like injury response.

Time Course of Motor Neuron Death at Different Ages

Animals at 0, 2, 4, 7, and 14 days of age were used, and the extent of neuronal survival was evaluated at 1, 2, 3, 5, 7, 14, and 30 days following PNCI. As expected from the end-point analysis presented above, there were large differences in neuronal loss between different ages at injury. Consistent with the determination that ultimate motor neuronal survival is proportional to age at injury, it can be seen that younger animals have lost a significantly higher percentage of their

facial motor neurons than older animals at every survival interval analyzed (Table 1). In addition, motor neuron loss appeared to proceed more quickly in younger animals than in older animals. For example, when the maximum of motor neuron loss is defined as the loss at 30 days after the injury for any given age group, animals injured on the day of birth will reach 85% of maximal loss within 3 days after the injury. In contrast, 14-day-old animals reach only about 25% of maximal loss within the first 3 days, while the remaining 75% of motor neurons destined to die will more slowly disappear over the next 10 days (Table 2 and Fig. 3A). The following procedure was used to quantify this difference in the relative rates of neuronal loss at different ages: First, the percentage of maximal loss at survival intervals of less than 30 days was calculated by dividing the loss at the shorter survival time by the maximal loss seen at 30 days in this age group. Second, the rate to reach maximum loss was calculated by determining the percentage of maximal loss per day (Fig. 3B). This analysis revealed a distinct difference in the time course of motor neuron death between newborn and 14-day-old animals. A single, early peak in the rate of cell death is characteristic for the newborn. The beginning phase of this early peak is shared in common between the newborn and the 14-day-old animals. However, neuronal loss then is almost not noticeable for the next 3 days in the 14-day-old animal, after which a second, slower peak of motor neuron loss

TABLE 1

Summary of the Extent of Facial Motor Neuron Survival at Different Post-PNCI Survival Times in Mice of Different Ages

Age (days)	Postoperative (days)	<i>n</i>	Mean % survival	Standard error	Mean % loss	% Maximal loss
0	1	3	72.2	4.1	27.8	31.9
0	2	3	36.2	2.4	63.8	73.3
0	3	4	23.4	2.9	76.6	88.0
0	5	2	09.3	3.0	90.7	104.2
0	7	3	06.4	1.9	93.6	107.5
0	14	4	10.3	1.7	89.7	103.0
0	30	2	12.2	4.6	87.8	100.0
2	1	2	67.2	6.6	32.8	43.3
2	2	3	56.9	1.4	43.1	56.8
2	3	4	25.9	4.1	74.1	97.7
2	5	3	12.2	3.8	87.8	115.7
2	7	3	14.0	5.0	86.0	113.3
2	14	3	24.3	2.6	75.7	99.7
2	30	3	24.1	4.8	75.9	100.0
4	1	2	76.5	1.3	23.5	36.2
4	2	4	63.3	1.3	36.7	56.5
4	3	4	58.3	2.3	41.7	64.2
4	5	4	44.7	1.0	55.3	85.1
4	7	4	40.3	1.3	59.7	91.8
4	14	4	43.6	1.6	56.4	86.8
4	30	2	35.0	3.3	65.0	100.0
7	1	3	96.0	1.6	04.0	8.9
7	2	3	73.7	3.0	26.3	58.8
7	3	5	72.4	5.0	27.6	61.8
7	5	4	61.8	1.6	38.2	85.5
7	7	3	54.2	2.1	45.8	102.5
7	14	3	59.1	2.4	40.9	91.5
7	30	2	55.3	3.2	44.7	100.0
14	1	3	96.4	1.8	3.6	20.6
14	2	2	96.4	1.9	3.6	20.6
14	3	3	95.9	1.4	4.1	23.4
14	5	3	93.6	3.2	6.4	36.6
14	7	3	88.9	0.8	11.1	63.5
14	14	3	80.1	2.2	19.9	113.8
14	30	3	82.5	3.2	17.5	100.0

Note. The time course of the extent of motor neuron survival after PNCI in mice of different postnatal ages is shown. Mean percentage survival is calculated by dividing the number of identifiable facial motor neurons on the injured side by the number of neurons on the contralateral uninjured side and then multiplying by 100. Mean percentage loss is calculated by subtracting the mean percentage survival from 100. Maximal loss is defined for each age group to equal the mean loss of motor neurons 30 days after injury. Percentage maximal neuronal loss at post-PNCI survival times shorter than 30 days is defined as the extent of neuronal death observed at that time, divided by the maximal death characteristic for the appropriate age group and multiplied by 100. Note that the total extent of cell loss after 30 days of survival is about five times lower in 14-day-old animals than in newborn animals and that this reduction in vulnerability occurs in a graded fashion during this period of postnatal development.

TABLE 2

Percentage Maximal Neuronal Loss Observed at 3 Days Post-PNCI and Postoperative Days Required to Reach 85% of Maximal Loss

Postnatal age at injury (days)	% Maximal loss at 3 days postoperative	Days required to reach 85% of maximal loss
0	88.0	3
2	97.7	3
4	64.2	5
7	61.8	5
14	23.4	14

Note. Values are presented by age at facial nerve crush. Percentage maximal facial motor neuron death in mice of different postnatal ages in the first 3 days versus the next 27 days post-PNCI. Note that the vast majority of maximal cell death in 0- and 2-day-old animals occurs within the first 3 days after PNCI, while most of cell death in 14-day-old animals occurs between 4 and 30 days post-PNCI in 14-day-old animals. Animals that were injured at a postnatal age of 4 and 7 days show significant cell death both before and after 3 days post-PNCI. Numbers given in Table 1 were used to calculate the data shown.

becomes apparent. Animals between 0 and 14 days of age show varying degrees of transition between these two distinct patterns.

Correlation of Motor Neuron Survival with aFGF Expression

In previous work we had determined the cellular localization and developmental time course of FGF expression in rats (24). In order to confirm the temporal expression pattern in mice we performed immunohistochemical staining of brain-stem sections containing the motor nucleus of cranial nerve VII from mice of different ages (days after birth: 0, 3, 5, 7, 11, 14, 21, and 30). Most facial motor neurons begin to show very faint aFGF immunoreactivity on the day of birth. The staining at 3 days of age is of weak intensity and increases only slightly until 7 days of age. Beginning at day 11 the immunoreactivity gradually increases to reach adult levels by 21 days after birth (Fig. 4). This gradual increase in aFGF immunoreactivity appears to proceed simultaneously in all facial motor neurons. These observations demonstrate that aFGF is present at high concentrations within motor neurons only at developmental ages when they are no longer highly vulnerable to death by PNCI.

Acidic FGF Treatment

The ability of exogenously supplied biologically active recombinant aFGF to increase the neuronal survival after PNCI was tested in animals of 4 and 7 days of age. At 4 days of age (when endogenous aFGF levels are low), the observed motor neuron survival after PNCI is of sufficient extent to detect either a positive or a negative influence of the treatments. When injured at

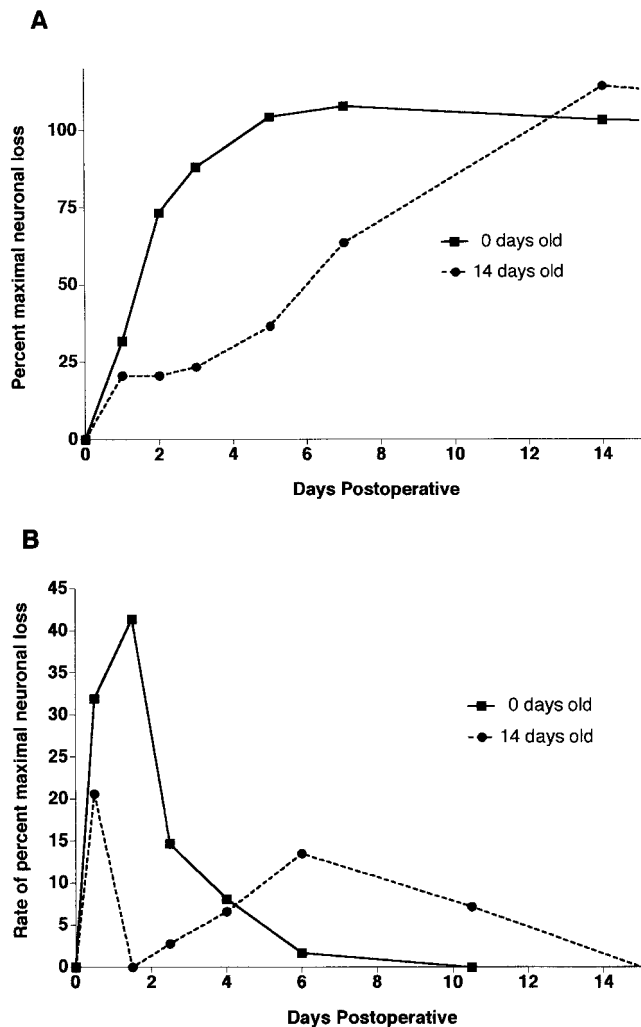


FIG. 3. Analysis of the time course and rate of maximal facial motor neuron death at different postoperative times after PNCCI in newborn and 14 day-old-postnatal mice. Maximal neuronal loss is defined as total neuronal death observed at 30 days post-PNCCI in either newborn or 14-day-old animals. Percentage maximal neuronal loss at post-PNCCI survival times shorter than 30 days is defined as the extent of neuronal death observed at that time, divided by the maximal death characteristic for the appropriate age group and multiplied by 100. The time course of percentage maximal neuronal death is plotted in A, and the rate of change in percentage maximal death per day is plotted in B. Note that motor neuron death in 14-day-old animals proceeds significantly more slowly than in newborns and that the rate of change in maximal cell loss shows a single early peak in newborns, while an additional second peak is observed in 14-day-old animals. The numbers given in Table 1 were used to calculate the data shown in this figure.

this age the normal neuronal survival percentage seen after a postinjury interval of 7 days is 40.3% (Table 1). We found that in treated animals the motor neuron survival percentage rose to 56.5%, which represents an increase of 16.2% compared to the untreated animals. To see whether the effects were altered with age we also treated animals injured at 7 days of age, an age outside

of the most vulnerable period for neuronal cell death stimulated by PNCCI, when endogenous aFGF levels are moderate. In these animals we observed that neuronal survival in the treated animals rose to 67.7% as opposed to the untreated survival rate of 52.3%. This increase in motor neuron survival of 9.4%, while smaller than the increase seen in the 4-day-old animals, was still statistically significant ($P \leq 0.05$, Table 3).

PNCCI in Transgenic Animals Expressing a Basic Fibroblast Growth Factor Transgene

FVB/N transgenic mice that overexpress bFGF were used to test the ability of endogenous, misexpressed FGF to reduce PNCCI induced neuronal loss.

PNCCIs were performed in 4-day-old pups from transgenic mothers bred with nontransgenic FVB/N males, and it was observed that the transgenic offspring showed a small (9%) but statistically significant increase in motor neuron survival when compared to their nontransgenic littermates (Table 3). In addition, transgenic animals injured at 7 days of age had survival rates 7.8% higher than those of their nontransgenic littermates. These results indicate that endogenous FGF neurotrophic activity can support to some extent the survival of injured motor neurons. It is possible that the relatively small increase in survival observed is due to the fact that bFGF may not be able to fully replace aFGF *in vivo* or that the global overexpression of the bFGF transgene by the pgk promoter is not entirely appropriate for obtaining maximum responses. Interestingly, the nontransgenic FVB/N animals showed somewhat lower overall neuronal survival than the Balb/c mice used in the development of the PNCCI model. A likely explanation for this observation is that individual strains of mice may vary slightly in their reactions to PNCCI.

DISCUSSION

The decision to investigate the developmental dynamics of motor neuron cell death following axotomy in a PNCCI model, instead of the somewhat more commonly used transection model, is based on the following two observations: First, while adult motor neurons survive an initial transection, a second slower phase of degeneration leads to the elimination of a significant number of neurons later on (23). Thus, it is possible that in younger animals this second phase of cell death may overlap with earlier phases of injury induced cell death and confound results. This potential problem is of less concern in the PNCCI model, where adult neurons (which successfully regenerate) demonstrate minimal cell death. Second, partial axonal regeneration can occur in some transection models. This additionally would confound efforts to quantify neuronal survival after the injury.

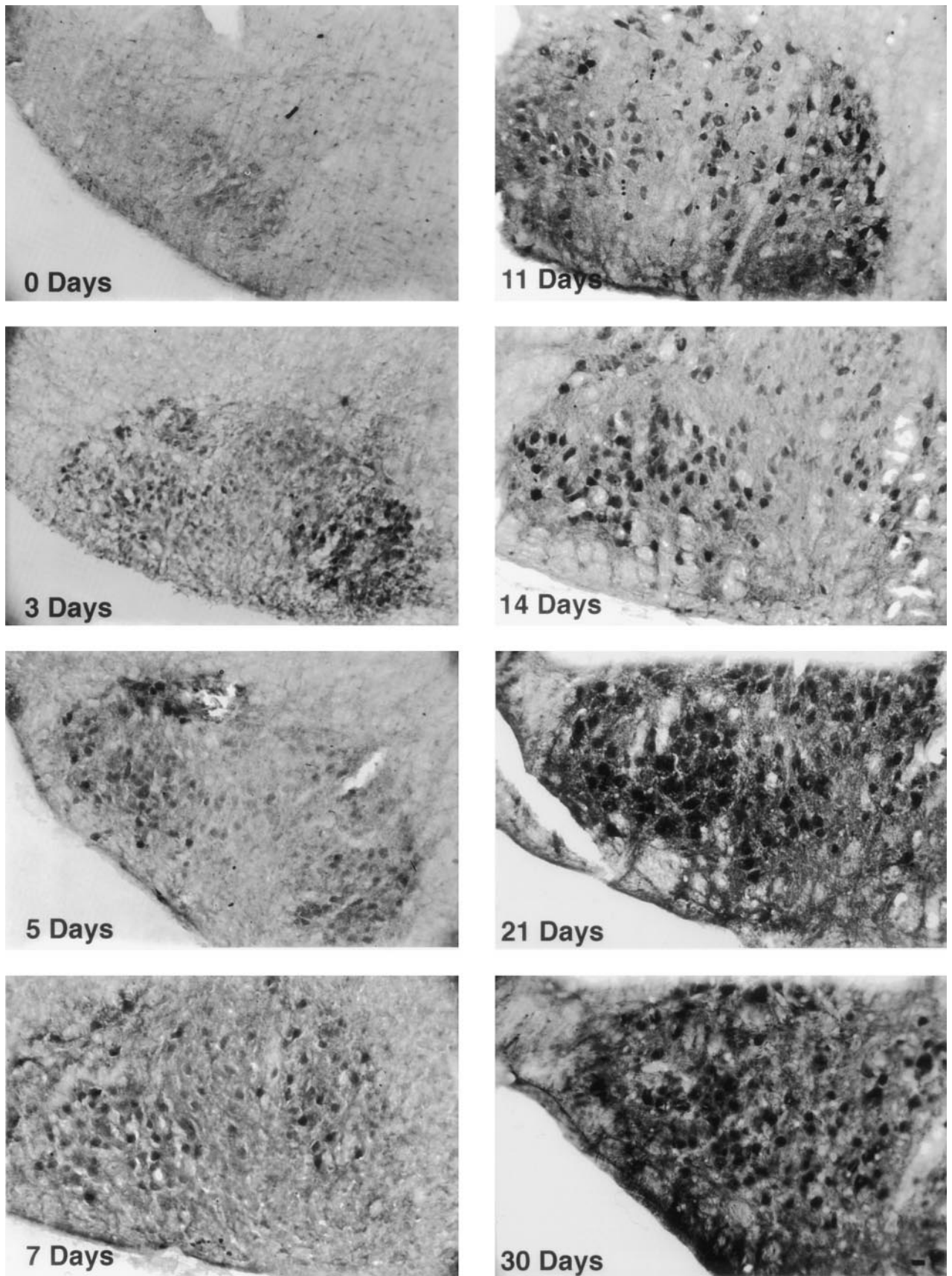


FIG. 4. Time course of the intensity of aFGF immunoreactivity in facial motor neurons in animals of different postnatal ages. Sections through the facial nucleus of 0-, 3-, 5-, 7-, 11-, 14-, 21-, and 30-day-old mice were stained for aFGF. Note the very faint immunoreactivity visible on the day of birth. The staining intensity of motor neurons shows a gradual increase to adult levels by day 21. Bar, 20 μ m.

TABLE 3

The Effect of aFGF Application and Transgenic Expression of bFGF on Facial Motor Neuron Survival after PNCI

Age (days)	Calculated condition	<i>n</i>	Mean % survival	Difference in % survival	<i>P</i> Value
4	Untreated	5	40.45	16.03	0.0002
4	Treated	2	56.48		
7	Untreated	5	52.30	9.42	0.015
7	Treated	2	61.72		
4	Nontransgenic	9	30.77	9.05	0.0038
4	Transgenic	7	39.82		
7	Nontransgenic	2	43.82	7.82	0.023
7	Transgenic	6	51.64		

Note. The effect of exogenous application of aFGF and of transgenic overexpression of bFGF on the extent of facial motor neuron death induced by PNCI. Note that application of exogenous (25 ng) FGF-1 to the lesion site and global overexpression of bFGF lead to a similar extent of moderate increase in facial motor neuron survival after PNCI in 4-day-old animals, while a smaller effect is observed in 7-day-old animals.

The present study demonstrates that the total extent of motor neuron survival following PNCI is directly related to the postnatal age at which injury occurs (Fig. 2). These observations are in good agreement with previous studies of the extent of cell death following PNCI in adult and newborn animals (25–28, 42, 46). In addition, it is shown here that the rate at which motor neurons die following PNCI is characteristically different between motor neurons of newborn and 14-day-old animals: Death occurs rapidly and almost completely within the first 3 days postinjury in newborns, while a bimodal distribution of death is observed in 14-day-old animals, with a very rapid death of about 25% of all the neurons destined to die within the first day after injury, no more additional significant death for the next 2 days, followed by a slower disappearance over the next 10 days of the remaining 75% of neurons destined to die (Fig. 3). Motor neurons of animals between 1 and 14 days of age show a gradual transition between these two patterns, while cell death in animals older than 14 days is minimal. It is currently unclear whether the mechanisms of cell death are apoptotic or necrotic and whether the fast and slow phases of death are regulated by distinct or different mechanisms. The present study also demonstrates that the gradual developmental increase of expression of the motor neuron trophic factor aFGF within motor neurons parallels the development of resilience to PNCI induced motor neuron cell death. Similarly, it confirms that application of exogenous aFGF after PNCI leads to a moderate increase in motor neuron survival (4) and that global overexpression of a bFGF transgene has a like effect. The relatively modest extent of this effect may be due to the fact that adult peripheral nerves (where PNCI does

not induce motor neuron death) normally contain exceedingly high levels of aFGF, which may only partially be mimicked by the application of limited amounts of exogenous FGFs or by transgenic expression of FGFs. Together, these data suggest that aFGF, derived from injured or dying motor neurons, promotes the survival of motor neurons affected by the injury. It is important to integrate this conclusion into the current understanding of how other neurotrophic factors regulate motor neuron survival.

Historically, it has been thought that developing motor neurons, like most other neurons, depend among other influences on the supply of trophic factors from their target (32). These target derived factors likely include members of the family of neurotrophins (21), GDNF (13), cardiotrophin-1 (33), and FGF-5 (17). It is also known that cells present in the adult peripheral nerve begin to express neurotrophic factors after injury. These induced factors include members of the family of neurotrophins (15, 29) and GDNF (31). More recently, it has become clear that additional trophic factors are constantly present in the mature motor neuron (aFGF, (9, 43)) or in neighboring Schwann cells (CNTF, (44)). The levels of aFGF and CNTF present in mature peripheral nerves are significantly more abundant (from 100 to 10,000 times higher in concentration) than those of neurotrophins present in the target tissues or inducible by injury even though all these factors are fully active at concentrations of a few nanograms per milliliter. In addition CNTF and aFGF lack hydrophobic signal peptides and are largely present in intracellular, nonsecretory compartments.

How might the role of aFGF relate to the role played by neurotrophins and other factors in promoting the successful survival and regeneration of motor neurons? The long-term survival of both adult and developing motor neurons is ultimately dependent on access to target derived secreted factors. In adult animals, trophic factors induced in injured nerve likely act to rescue motor neurons from death after nerve crush. However, significant time is required for the *de novo* expression of trophic factors and to allow for their secretion and action. We propose that aFGF is released in sufficient quantities immediately after injury from lesioned axons to provide a rapid neurotrophic and mitogenic signal in the vicinity of the injury. In this model, the aFGF signal is necessarily transient, as the source of the factor disappears, after the axon has either sealed or degenerated. If this view is correct, the main physiological role of aFGF in the peripheral nerve is to promote neuronal survival for a short period after injury and to initiate Schwann cell responses to the injury, until other, induced factors can perform these functions. A similar role may be proposed for CNTF released from injured Schwann cells (39).

The data presented here support this model. The

developmental time course of the ability of motor neurons to survive PNCI parallels the rise in levels of aFGF and also of CNTF in Schwann cells (44). Thus, the action of these preexisting factors, which are stored in high levels in nonsecretory compartments, may be crucial in providing a window of time for the induction of secreted trophic factors. A detailed analysis of the rate of survival of motor neurons after PNCI in 14-day-old animals agrees with this conclusion: The very first (24 h postinjury) increase in cell death is similar between 14- and 1-day-old animals, but apparent cell death then ceases in the 14-day-old animals for a few days, while it continues strongly in the 1-day-old animals. This is consistent with the view that the initial damage in the 14-day-old releases a moderate amount of aFGF and CNTF, which rescues motor neurons for about 2 days, while no such aFGF or CNTF is available in the newborn. Additional support comes from observations that both STAT3 and ERK phosphorylation (which mediate CNTF and FGF action) are observed in nerves within 30 min after the injury (40). The second phase of cell death, seen after 3 days postinjury in the 14-day-old animal, may be due to the fact that aFGF and CNTF are present at 14 days of age at moderate levels, but not yet at the very high adult levels. It is also known that aFGF and CNTF, in addition to exhibiting neurotrophic activity, can act on a large number of different nonneuronal cell types, and the action of aFGF or CNTF may include providing an early stimulus for the induction of other neurotrophic factors by Schwann cells. It is of interest in this regard that cells that are probable targets for injury released aFGF in the nerve, such as nonmyelinating Schwann cells, continue their proliferation and migration into the second month after birth (19). Thus it is possible that the 14-day-old nerve is not yet mature enough to fully respond to aFGF and CNTF. Obviously, many aspects of this model of trophic factor action after peripheral nerve injury remain to be tested. The PNCI model provides a stable, well-characterized tool for the examination of the regulation of neuronal survival and neuronal death following injury.

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