CELL DEATH OF MOTONEURONS IN THE CHICK EMBRYO SPINAL CORD

IX. The Loss of Motoneurons following Removal of Afferent Inputs¹

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Abstract

The primary aim of this study was to clarify the role of supraspinal, propriospinal, and primary sensory afferents in motoneuron (MN) development in the lateral motor column (LMC) of the lumbar spinal cord of the chick embryo. For this purpose three types of operations were carried out on embryonic day (E) 2. (1) The spinal cord was transected at the cervical (C-gap) or at the thoracic (T-gap) level so as to eliminate supraspinal and/or propriospinal inputs to the lumbar cord. (2) The entire lumbar neural crest was removed (NCR) in order to eliminate primary sensory inputs arising from the dorsal root ganglia (DRG). (3) A combined operation of T-gap and lumbar NCR was performed. The numbers of MNs in the LMC of the lumbar spinal cord were counted in embryos sacrificed between E10 and E18.

The number of MNs on E10, when naturally occurring neuron death is almost complete, was not changed following either operation 1 or 2 described above. However, by E16, when naturally occurring neuron death is over, these same deafferented groups had 20 to 25% fewer MNs than did controls. Thus, the removal of *either* descending or sensory (DRG) afferents results in a significant increased loss of MNs that appears to take place only during the final stages of natural neuronal death or later. By contrast, the removal of *both* sources of input (T-gap + NCR) results in an additional 37% loss of MNs by E10 compared to controls. Thus, in this group deafferentation significantly increases cell loss during the major period of naturally occurring MN death (E5 to E10). No further loss of MNs occurs in this group after E10.

Chronic treatment of deafferented embryos with curare from E6 to E9 or from E10 to E14 prevented the naturally occurring MN loss during these stages but was without effect on the increased cell loss induced by deafferentation. These results imply that the cellular mechanisms involved in target- versus afferent-regulated cell death are different.

Collectively, these results indicate that the regulation of MN numbers is more complicated than previously thought. Both targets and afferents appear to be involved in controlling the survival of this population of neurons during the period of naturally occurring MN death.

Naturally occurring cell death is a well known phenomenon that has been shown repeatedly to occur in many parts of the developing nervous system (for review, see

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Oppenheim, 1981a; Hamburger and Oppenheim, 1982). In the lateral motor column (LMC)⁴ of the chick embryo spinal cord, cell death occurs mainly between embryonic day (E) 5 and E10 (Hamburger, 1975) and appears to be regulated primarily, if not entirely, by interactions between motoneuron (MN) axons and their targets (Hamburger, 1934; Hollyday and Hamburger, 1976). It has also been shown that cell death is regulated by some aspect

⁴ The abbreviations used are: CA, catecholamines; C-gap, cervical gap; DRG, dorsal root ganglia; E, embryonic day; HRP, horseradish peroxidase; ION, isthmo-optic nucleus; LMC, lateral motor column; MN, motoneuron; NCR, neural crest removal; SG, substantia gelatinosa; T-gap, thoracic gap.

of synaptic transmission at the neuromuscular junction (Pittman and Oppenheim, 1978, 1979); however, beyond showing that nerve-muscle instructions are important, the precise mechanisms involved in this phenomenon are still obscure (Oppenheim and Chu-Wang, 1983). In addition to the role of the target, it is also conceivable that *afferent* input to the MNs may play a role in the regulation of cell death. Deafferentation has in fact been clearly shown to exacerbate cell death in at least one population of brainstem neurons during the period of naturally occurring cell death (Levi-Montalcini, 1949; Parks, 1979; see also Cunningham, 1982). Although the lack of influence of dorsal root ganglia (DRG), as well as of spinal and supraspinal afferents on certain aspects of the developing LMC have been reported previously in the chick embryo (Bueker, 1943; Levi-Montalcini, 1945; Hamburger, 1946; see Oppenheim, 1981a for review), the number of surviving MNs was not systematically evaluated within the context of naturally occurring MN death in those studies. The present study was undertaken with the major purpose of examining numerical and other changes of MNs in the lumbar LMC following the removal of supraspinal, propriospinal, and/or primary sensory afferents. Portions of this study have been published previously in abstract form (Okado and Oppenheim, 1981).

Materials and Methods

Fertilized eggs of White Leghorn chickens were maintained in a turning incubator with a forced draft at a temperature of 37°C and 70% relative humidity.

Microsurgery

Following candling to locate the position of the embryo, a small lateral window was made in the shell over the embryo. Embryos were stained with a strip of neutral red-agar in order to visualize the site of operation and for *in ovo* staging according to the method of Hamburger and Hamilton (1951). Under the binocular microscope the vitelline membrane over the operation site was torn with fine-tipped forceps, thereby exposing the embryo and spinal cord. Three kinds of operations (Fig. 1) were performed, using a fine tungsten needle and a vibrating microsurgery apparatus (Wenger, 1968; Narayanan, 1970).

(1) "Gap" operations were performed to eliminate supraspinal and/or spinal inputs to the lumbar spinal cord. A part of the spinal cord at levels between the 12th and 15th somite (cervical, C-gap) was removed from embryos at stage 13 (48 to 52 hr of incubation), leaving a "gap" between rostral and caudal segments. A similar operation was done at the level of somites 19 to 22 (thoracic, T-gap) at stage 15 (50 to 55 hr of incubation). In one group

Figure 1. Schematic illustration of the four types of operations performed. See the text for details. N, neural tube; NC, neural crest; NT, notochord; S, somite.

of T-gap animals, a considerably longer piece of spinal cord, comprising six to eight segments (T1 to L1 or L2), was removed at stage 15; these have been designated long-thoracic gaps (L-T-gaps). The C- and T-gap operations resulted in spinal cord ablations that included segments 9 to 12 (C-gap) or segments 17 to 19 (T-gap) when examined on E10 and E18.

(2) The *neural crest* in the region of the lumbar spinal cord (caudal to somite 22) was removed or destroyed (NCR) at stage 16 (51 to 56 hr of incubation), to eliminate the precursor cells of the spinal ganglion. At this stage the neural crest cells destined to form the spinal ganglion of the lumbar spinal cord have not yet migrated ventrally but remain adjacent to the dorsal neural tube (Hamburger et al., 1966). In addition to the vibrating needle, an electrocautery method was also used to destroy the neural crest (Eide et al., 1982), but we found that it was difficult to control the depth of the lesion with this procedure, and thus damage sometimes extended into the ventral half of the spinal cord.

(3) A combined operation of *T-gap and NCR* was performed on embryos at stage 16. Following all of the operations, the lateral window was covered with a small glass coverslip and sealed around the edges with melted paraffin. Operated eggs were returned to a non-turning incubator with a temperature of 37°C and 70% relative humidity and were allowed to develop for another 8 to 16 days. Survival rate following the operations was greater than 80% in the spring and early summer but was very low, sometimes as low as 1%, in winter. Gap animals were sacrificed at E10, E12, E13, E16, and E18 and staged according to the Hamburger-Hamilton series. Animals of NCR and combined gap + NCR operations were sacrificed at E10 and E16. Embryos which had obvious malformations were not used in this study. Furthermore, only embryos with completely disconnected spinal cords, as determined by dissection (T- and C-gaps), or with virtually no DRG cells bilaterally throughout the eight lumbar segments (NCR), as determined histologically, were included in the data analysis.

Pharmacological experiments

In a previous study (Pittman and Oppenheim, 1979) it was shown that most, if not all, of the naturally occurring MN death in the lumbar LMC could be prevented by a chronic, pharmacologic neuromuscular blockade (e.g., curare) during the cell death period. In order to determine whether this phenomenon requires the presence of afferents from the DRG, 2 mg of curare in 0.2 ml of saline were injected daily onto the chorioallantoic membrane between E6 and E9 in both control embryos and embryos which had been subjected to the NCR operation; these animals were sacrificed on E10.

One group of animals with a T-gap was also injected with 2 mg of curare on days E10 to E14 (sacrificed on E15 to E16) in order to determine whether neuromuscular blockade would alter the induced MN loss occurring during these later stages.

Reserpine (40 μ g/40 μ l of Serpasil, CIBA) was injected in the yolk sac on E0, while 40 μ l of the vehicle alone (Serpasil placebo, CIBA) was injected into controls (Pittman et al., 1978). Embryos were incubated until E16 for

the evaluation of a reserpine effect. This experiment was designed to determine the effects of depletion of spinal cord catecholamine (CA) levels on MN survival during the period *after* E10 (i.e., can the transection-induced MN loss be attributed selectively to the loss of CA or the absence of CA-containing axons in the isolated lumbar spinal cord?).

Histological preparations and morphometric analysis

Following appropriate survival times, embryos were sacrificed by decapitation and the lumbar spinal cords were removed. The spinal cords were then fixed in Carnoy's solution, and the entire trunks were fixed in 10% formalin for later examination and dissection. The operated region (gap or NCR) was dissected and examined with the binocular microscope, and unsuccessful cases, that is, those with an incomplete gap or containing obvious DRG, were not processed beyond this step. Lumbar spinal cords were embedded in Paraplast and serially sectioned at either 10 μ m (E10) or 12 μ m (E12 and older), and the sections were stained with thionin. The success of the NCR operation was further checked histologically, and only the cases with a virtually *complete* bilateral depletion of the DRG in the region of the lumbar spinal cord (L1 to L8) were used. To avoid double counting of MNs, only large cells which contained at least one clear nucleolus and which were located in the LMC of the lumbar spinal cord were counted (Chu-Wang and Oppenheim, 1978; Oppenheim et al., 1982a). Counts were made on every tenth section at a magnification of either $\times 400$ (E10) or $\times 250$ (E12 and older), and the total number of MNs was multiplied by 10. The identification of MNs in the LMC was unambiguous, even in those cases involving a slight cellular hypotrophy (see "Results"). Pyknotic nuclei (degenerating cells) were identified and counted as previously described (Chu-Wang and Oppenheim, 1978).

In a few embryos from the various groups, spinal cord volume (cubic millimeters) was measured by drawing the outline of the spinal cord from every tenth section through the entire lumbar region (L1 to L8) on a graphics tablet of an Apple computer; volumes were automatically computed by an appropriate morphometric program. As a measure of cell size, nuclear diameters were determined as previously described (Oppenheim, et al., 1982b).

Horseradish peroxidase (HRP) tracer experiments

HRP was injected into the cervical spinal cord (segment 10) of E8 and E10 embryos *in ovo* in an attempt to retrogradely label any "motoneurons" in the lumbar LMC with ascending axonal projections. Such projections have been found in adult cats (e.g., Matsushita and Hosoya, 1979), and if they are also present in chicks, their loss by retrograde degeneration following spinal transection could confuse the issue concerning the role of *descending* afferents on MN survival. Following 12 to 16 hr survival time, embryos were perfused with saline followed by a solution of 0.5% paraformaldehyde and 2% glutaraldehyde in a 0.1 M phosphate buffer. The whole lumbar spinal cord was serially sectioned at 60 μ m on a freezing microtome. All of the sections were reacted with the tetramethylbenzidine method (Mesulam et al., 1980)

and were counterstained with neutral red. Detailed information about the histochemical and other procedures used here have been described in more detail in a recent report (Okado and Oppenheim, 1984). Retrogradely labeled cells in the lumbar spinal cord were located, and those found in, as well as in close proximity to, the LMC were counted as described above.

Results

The body size of many of the NCR embryos on E12, E16, and E18 was smaller (one to two stages retarded), and the lower extremities were often slightly atrophic relative to age-matched controls. By contrast, NCR embryos on E10 and all embryos with gap operations were macroscopically indistinguishable from control animals. In most cases, however, the gap operations resulted in a small depression over the site of operation; usually skin and feathers were present over the gap site. Most of the NCR embryos on E10 and E16 exhibited a "spina bifida" due to the absence of the dorsal part of the vertebral column (Fig. 2, NCR).

It can be seen in Figures 2 and 3 that the spinal cords of the operated embryos were somewhat reduced in size relative to controls. In the gap animals, this reduction appeared to involve all regions to about the same extent, whereas the dorsal spinal cord was especially reduced in the NCR embryos, particularly on E16. This differential effect on dorsal versus ventral spinal cord was undoubtedly due largely to inadvertent damage to the dorsal cord during NCR (e.g., Hamburger et al., 1966). The combined operations (NCR + T-gap) often resulted in an even greater reduction in the area of the spinal cord, including both dorsal and ventral parts. However, the shape of the LMC was not appreciably changed by any of the operations. Nuclear diameter, which is proportional to cell size (Oppenheim et al., 1982a b), was significantly reduced in the T-gap and NCR + T-gap groups on E16, but not in the NCR group (Fig. 4).

With the exception of the NCR + gap group (combined operation), which had significantly fewer dying cells than did controls, the number of degenerating MNs in the LMC on E10 did not differ between any of the experimental groups and the controls (Table I). However, as expected, both the T- and C-gap groups had significantly more degenerating MNs on E12 and E14 relative to the control group. Degenerating MNs were not found in any experimental or control embryos on E16.

The data on MN numbers in control and experimental cases are shown in Figure 5 and Table II (see also Table IV). At E10 no differences were found following either gap or NCR operations. All groups had 11,000 to 13,000 MNs in the LMC, which is comparable to the values reported in previous publications (e.g., Hamburger, 1975; Chu-Wang and Oppenheim, 1978). Following the combined operation (NCR + T-gap), however, the number of MNs was reduced by approximately 36% on E10. There was no further change in MN number in this group between E10 and E16. By contrast, on E16, MN numbers were reduced by 20 to 36% in the other three operated groups (NCR, T-gap, C-gap). Although the differences between the cervical group and controls did not quite reach statistical significance on E16 ($p < 0.10 > 0.05$), this group did differ significantly from

controls on E18. The magnitude of MN depletion in individual cases in all groups (NCR, T-gap, C-gap) varied considerably, ranging from 10 to 36%. Although the cause of this variability is unknown, it could be related to differing amounts of compensatory input from remaining afferents in individual cases (e.g., see Jackson and Parks, 1983).

The size of the spinal cord was reduced one-third to one-fourth of control values in the NCR group. Although only a small number of cases were examined, the magnitude of MN loss following NCR operation does not appear to be correlated with the extent of reduction of spinal cord size (Fig. 6, Table III).

A detailed re-analysis of four cases from a previous experiment with combined NCR + gap operation (Hamburger et al., 1966) also supports the present findings in that these animals had an approximate 30% reduction in the number of MNs on E15 (Table III). Furthermore, this reduction did not appear to be correlated with the change in spinal cord size.

Pharmacological experiments. Histological examination of serial sections of all of the NCR embryos used here revealed that all DRG cells (L1 to L8) had been removed. Curare injections between E6 and E9 into both normal and NCR embryos resulted in a significant reduction of naturally occurring motoneuron death on E10 (Table IV). Daily curare injections in normal embryos between E10 and E15 also saved the MNs (~2000 cells) normally destined to undergo natural cell death after E10 (Fig. 7).

Whereas curare-treated (E10 to E14), T-gap embryos also had approximately 2000 more neurons than T-gap controls (saline only), the additional 20% induced loss of MNs following T-gap was not altered by curare (Fig. 7).

Depletion of spinal cord CAs by 70 to 80% following reserpine treatment (Pittman et al., 1978) did not lead to a reduction in the number of MNs on E16 (*reserpine* = $12,930 \pm 946$, $n = 5$; *vehicle* = $11,871 \pm 719$, $n = 5$).

HRP study. Figures 8 and 9 are sections of the lumbar enlargement of the spinal cord following HRP injections into the cervical spinal cord on E8 and E10, respectively. Four major cell groups were found to be retrogradely labeled with HRP in the 8-day embryos (Fig. 8). One cluster of labeled cells is found in the region (Fig. 9, *small asterisk*) ventrolateral to the substantia gelatinosa (Fig. 8, SG). This cell group consists of a large number of labeled cells and is found in all segments of the lumbar spinal cord. A second group of labeled cells is found in the area dorsal to the central canal in the midline region of the spinal cord (Figs. 8 and 9, *double arrowheads*). The large aggregation of labeled cells located in the area lateral to the central canal is restricted to the middle and rostral segments of the lumbar spinal cord. From this latter group, labeled cells extend both laterally and dorsally. The dorsal extension is composed of small cells located just medial to the SG. There is a fourth group of labeled cells in the area just medial to the LMC (Fig. 8, *large arrowheads*). In addition, a small number of labeled cells are found within the SG (Fig. 8, *small arrow* in SG). On E10, three additional labeled cell groups can be found (Fig. 9, *small* and *large arrows*, *large arrowheads*, and *double arrowheads*). The first of these is a group of labeled cells within the LMC (Fig. 9, *large arrows*). These

Figure 2. Photomicrographs of representative cross-sections through the lumbar spinal cord (*left*) and the LMC (*right*) on E10 following embryonic microsurgery, indicating that the development of gross morphological features is relatively normal. Magnification: *left*, $\times 56$; *right*, $\times 253$.

cells are generally located in the rostral segments of the lumbar spinal cord. Some of these cells with large, round somata resemble MNs. Although they are relatively rare, a second group is found in the area dorsal to the LMC (Fig. 9, *small arrows*). The third group (Fig. 9, *small*

arrowheads), which is particularly prominent in the rostral segments of the lumbar spinal cord, is located at the ventrolateral margin of the LMC. In addition, a few labeled cells (Fig. 9, *small arrows*) are found in the peripheral margin of the ventrolateral marginal layer.

Figure 3. Photomicrographs of cross-sections through the lumbar spinal cord (*left*) and the LMC (*right*) on E16 following embryonic microsurgery, indicating that the development of gross morphological features is relatively normal. Magnification: *left*, $\times 23$; *right*, $\times 253$.

The total number of labeled cells on E10 in the LMC (L1 to L8) per se was 155. The number of labeled cells which are located in areas closely bordering the LMC, and which might also be misconstrued as MNs (i.e., those

in the medial and ventral border regions of the LMC) is 81 and 196, respectively (L1 to L8). Thus, there are only about 430 neurons on E10 (out of a total of 12,000) located within or close to the lumbar LMC that have

Figure 4. The nuclear diameter (mean $\pm$ SD) of MNs in the lumbar LMC on E16. Sample size is indicated in the bars. *, $p < 0.01$, t -test, two tailed.

TABLE I
The total number of degenerating motoneurons in the lumbar LMC following deafferentation^a

Type of Operation	Age			
	E10	E12	E14	E16
Control	173	76	5	0
T-gap	164	140 ^b	30 ^b	0
C-gap	159	93 ^b	57 ^b	0
NCR	160			0
NCR + gap	84 ^b			0

^a Sample size was 3 for all groups.

^b $p < 0.05$, Mann-Whitney test, one-tailed, compared to controls of the same age.

axons ascending into or through the cervical level and which could conceivably be included in quantitative analysis of MNs in the LMC. Furthermore, the numbers of such cells found at later stages (E14 and E15) following cervical injections of HRP are not substantially increased (Okado and Oppenheim, 1984).

Discussion

Considerable evidence now exists indicating that the regulation of naturally occurring MN death in the chick embryo spinal cord occurs primarily, if not exclusively, by interactions between these neurons and their muscular targets (Oppenheim, 1981a, review). Early limb bud ablation (target removal) results in the death of virtually all MNs during approximately the same time frame as naturally occurring cell death (Hamburger, 1934; Oppenheim et al., 1978); the addition of a supernumerary limb

(increased target size) prevents a significant amount of the naturally occurring loss of MNs (Hollyday and Hamburger, 1976). However, both of these manipulations also induce significant numerical and other changes in the associated DRG (e.g., see Hamburger and Levi-Montalcini, 1949; Hamburger, 1939), which very likely leads to changes in the extent of primary afferent input to interneurons and MNs. Thus, it is not entirely clear from such studies that the only critical factor being altered that could affect MN survival is target size; changes in afferents may also be involved. However, against this is the fact that treatment of chick embryos with nerve growth factor, which prevents most cell death in the DRG (Hamburger et al., 1981), thereby *increasing* primary afferent input to the spinal cord, does not alter MN numbers (Oppenheim et al., 1982a, b). It is also possible to increase or decrease naturally occurring MN death selectively by manipulations that do not alter the number of neurons in the DRG. The blockade of neuromuscular transmission during the period of normal cell death, for example, can rescue virtually all of the MNs that normally die (Pittman and Oppenheim, 1979; Oppenheim, 1981b), and electrical stimulation of the limb bud during this period can induce an *increased* loss of neurons (Oppenheim and Nunez, 1982). However, neither of these manipulations affects the survival or histological development of the DRG neurons. It is, of course, conceivable (albeit rather unlikely) that the *physiological* activity of DRG neurons, and thus the amount of afferent synaptic input to MNs, could have been altered in the studies using neuromuscular blockers.

Figure 5. The number of MNs (mean $\pm$ SD) in the lumbar LMC (L1 to L8) at different ages following embryonic microsurgery.

TABLE II
Cell counts of motoneurons in the lumbar LMC following deafferentation

Type of Operation	10 Days		12 Days		16 Days		18 Days	
	Mean $\pm$ SD	(n) ^a	Mean $\pm$ SD	(n)	Mean $\pm$ SD	(n)	Mean $\pm$ SD	(n)
T-gap ^b	12,984 $\pm$ 1,262	(10)	10,127 $\pm$ 1,627	(6)	7,900 $\pm$ 1,232 ^c	(14)	7,932 $\pm$ 560 ^d	(10)
L-T-gap ^e					7,960 $\pm$ 1,129 ^c	(6)		
C-Gap ^f	12,338 $\pm$ 1,565	(6)			8,364 $\pm$ 1,226	(8)	7,993 $\pm$ 736 ^c	(8)
T- + C-gap ^g	12,714 $\pm$ 1,370	(16)			8,045 $\pm$ 1,183 ^c	(28)	7,959 $\pm$ 625 ^d	(18)
NCR	11,736 $\pm$ 1,988	(6)			6,484 $\pm$ 1,262 ^d	(8)		
NCR + T-gap	8,125 $\pm$ 925 ^c	(4)			7,714 $\pm$ 917 ^d	(9)		
Control	12,824 $\pm$ 1,126	(5)	12,150 $\pm$ 283	(4)	10,187 $\pm$ 412	(6)	10,520 $\pm$ 824	(4)

^a (n), Number of embryos.

^b Four somites long.

^c $p < 0.05$, Mann-Whitney test, one-tailed.

^d $p < 0.01$.

^e L-T-gap, long thoracic gap; eight somites.

^f Four somites long.

^g Data summed for these two operations.

In addition to the possible role of primary DRG afferents in the regulation of MN survival, one must also consider the role of intrinsic afferents that arise from spinal or supraspinal sources and which provide synaptic input to MNs. Previous attempts to evaluate the influence of these intrinsic afferents on MN survival in the chick have been largely negative (Oppenheim, 1981a, review), in that MN numbers were unchanged following the elimination of apparently significant amounts of such afferent input.⁵

⁵ One of these studies (Levi-Montalcini, 1945) did examine a single embryo on E19 that had received a spinal transection on E2. Although it was reported that there was not a depletion of MNs in the lumbar LMC, it is not possible to draw any conclusions from this single case. Furthermore, cell counts were

An important assumption, and one that is usually unstated in all of these deafferentation studies, is that the afferents in question (DRG and intrinsic) normally make synaptic contact with or are in close apposition to MNs during the period of naturally occurring cell death. Synapses do, in fact, begin to form on MNs during the cell death period (Oppenheim et al., 1975), and neurons in the brain, rostral spinal cord, and DRG can be retrogradely labeled following HRP injections into the lumbar spinal cord at any time from E5.5 to hatching (Okado and Oppenheim, 1984). Consequently, it seems plausible that afferent terminals from a variety of sources may be

not done through the entire lumbar region, and the silver stain that was used is not ideal for making reliable cell counts.

Figure 6. The relationship between spinal cord volume and the number of surviving MNs following NCR or NCR + T-gap.

TABLE III

The number of motoneurons in the lumbar LMC on E15 following a combined NCR + Gap operation^a

Specimen No.	Motoneurons	Sensory (DRG) Neurons (Bilateral)	Approximate Reduction in Spinal Cord Size
437	8820	None	+
767	6170	5073	++++
411	6970	None	++
479	7840	None	+++

Mean = 7450; SD = 1140

^aSee the text for details. The reduction in spinal cord size is arbitrarily based on a four-point scale in which one is the least and four the greatest amount of observed reduction; embryo 437 was barely distinguishable from control. Data are from Hamburger et al. (1966).

in a position to exert an influence upon MNs during the major period of cell death (i.e., between E5 and E10). Nonetheless, at present we do not know either the extent to which afferents form synapses on MNs at these stages or the relative contributions of afferents from various sources to the total population of synapses on either developing or adult MNs.

In the present study, we have found that spinal gap operations in which *either* supraspinal afferents (C-gap) or *both* supraspinal and propriospinal afferents (T-gap) were eliminated several days prior to the onset of naturally occurring death of lumbar MNs are without effect on the survival of these cells up to E10 to E12. Similar results were obtained following the removal of virtually all primary afferent input from the DRG (NCR group). By contrast, all of these operations resulted in a significant depletion of MNs at *later* stages (i.e., by E16 to E18). Since a small amount of cell death occurs normally in this population *after* E10 (8% versus 43% *before* E10), one might argue that afferents are primarily involved in maintaining MNs only during the late or terminal stages of naturally occurring cell death. The data on degenerating neurons tend to support this, in that there were

TABLE IV

The effect of curare treatment (E5 to E9) on Motoneuron Survival on E10

Treatment	Survival
Control (<i>n</i> = 5)	12,824 ± 1,126
Curare (<i>n</i> = 5)	17,597 ± 1,400 ^a
NCR + Curare (<i>n</i> = 2)	18,085 ± 799

^a*p* < 0.01 compared to control, Mann-Whitney test, two-tailed.

significantly more dying neurons on E13 and E14, but not E10, in the T- and C-gap groups. Alternatively, afferents may only influence the later *maintenance* of MNs that have competed successfully for targets (or afferents) and thus have survived the entire period of massive cell death. Another alternative possibility, i.e., that the enhanced cell loss at late stages following spinal transection reflects the retrograde degeneration of neurons with ascending axons whose soma is located within or in close proximity to the LMC, has been ruled out here. Although we have shown that such neurons exist (also see Matusushita et al., 1979; Matusushita and Ho-soya, 1979), they are so few in number, even at stages later than E10 (Okado and Oppenheim, 1984), that they cannot account for more than a fraction of the cell loss following either type of gap operation.

The results of the *combined* T-gap + NCR operation indicate, however, that it may not only be the late stages of cell death that are affected by deafferentation. In these preparations, a significant depletion of MNs was seen as early as E10. Although earlier stages were not examined, the fact that these animals had *fewer* degenerating MNs on E10 compared to control values indicates that the enhanced cell death did not occur only between E9 and E10. If this were the case, one would have expected to see more, not fewer, dying cells on E10. Thus, these results imply that the age of onset of a response to deafferentation may be related to the *extent* of the deaf-

Figure 7. The effects of curare treatment from E10 to E14 on the survival of MNs. See the text for details.

Figures 8 and 9. Photomicrographs of cross-sections of one-half of the lumbar spinal cord on E8 (Fig. 8) and E10 (Fig. 9) following the injection of HRP into the cervical spinal cord in order to illustrate the location of MNs backfilled with HRP following cervical injection. CC, central canal. Magnification: Figure 8, $\times 121$; Figure 9, $\times 140$.

ferentiation. At stages earlier than E10, gaps or NCR alone may not remove enough afferents to influence MN survival. If both are removed simultaneously, however, then many MNs may be so deprived of a putative orthograde trophic factor that even at these early stages they can no longer be sustained solely by retrograde influences from their muscular targets.

The number of MNs in a population that fall victim

to deafferentation appears to be relatively fixed. The number of surviving MNs in the lumbar LMC on E16 or E18, following any of the single or combined operations, always ranged between ~ 6500 and 8000 (i.e., a 20 to 35% decrease from control values), and none of these experimental values were statistically different from one another. Consequently, since the combined T-gap + NCR operation probably removes considerably more (although

by no means all) of the MN afferents than do the single NCR or C-gap operations, one would argue that there are a large number of MNs (~65%) that are normally sustained largely by target influences. However, until we know the actual proportion of afferents that are removed by the various operations used here, it is not possible to determine the extent to which these remaining MNs are, in fact, deafferented. Even if the operations used here do remove most of the *normal* afferents to MNs, it is still possible that *new* sources of afferents arise (e.g., from axonal sprouting of segmental interneurons) that compensate for the removed inputs (e.g., Jackson and Parks, 1983). Thus, the proportion of neurons that survive following deafferentation (~65%) may do so by virtue of having received enough compensating inputs.

The additional neurons that die after E10 following the T-gap (or C-gap) operation could not be saved by chronic curare treatment from E10 to E14, even though the neurons that *normally* die during this period were, in fact, saved. This result strongly implies that the induced cell loss following deafferentation is regulated differently than the normally occurring late cell loss. The late induced cell loss following spinal transection also does not appear to be associated with the release of CA from descending CA neurons, since a substantial pharmacological reduction in spinal cord CA activity did not influence MN survival. However, it is conceivable that the small residual CA activity in these preparations was sufficient to sustain MNs. Since reserpine treatment does not destroy the CA cells or their processes, it is also possible that even a total reduction of CA in the descending terminals of these cells would not compromise their possible trophic influence.

The present results would appear to be incompatible with a model of naturally occurring MN death in which the target is thought to be the major source of information for regulating neuronal survival. Although experimental manipulations that seemingly only alter target-related factors can be shown to save virtually all neurons that would normally die (e.g., Creazzo and Sohal, 1979; Nishi and Berg, 1979; Pittman and Oppenheim, 1979), the situation *in vivo* during *normal* development may be considerably more complex. In this regard, the recent ideas of Cunningham (1982) are of particular interest.

Cunningham has extended the traditional competition model of cell death, in which neurons are thought to die by virtue of a failure to compete successfully for the appropriate signals from *either* targets *or* afferents, so as to include another (albeit a related) reason for the failure of neurons to survive the cell death period. He has argued that once neurons compete adequately for either targets or afferents, they then encounter another "critical period" in which they must successfully *balance* afferent input with target contact. Neurons that are either unable to gain target contact which is quantitatively appropriate to their afferent input, or are unable to attain adequate afferent input relative to the amount of target contact, would be less likely to survive. Although considerable more information is needed before one can conclude that this "balance" model is, in fact, applicable to the survival of chick spinal MNs, it provides a plausible and testable hypothesis for explaining the present findings.

Finally, it is of some interest to briefly consider the question of *how* the afferent regulation of neuronal survival is mediated. As discussed in more detail below, afferents may also help regulate the survival of ciliary neurons in the chick embryo, and in this case ganglionic transmission appears to be one means by which this regulation is mediated; chronic blockade of ganglionic transmission during the cell death period leads to increased cell loss (Wright, 1981). This result is similar, in principle, to the well known effects of neuromuscular blockade on muscle differentiation and survival (e.g., Gutmann, 1976). However, it is also the case that in the neuromuscular system trophic proteins have been purified from peripheral nerves and have been shown to regulate certain aspects of muscle development (e.g., Markelonis et al., 1982). Thus, synaptic activity and trophic factors may, either independently or together, dually mediate the effects of afferent or orthograde influences on the differentiation of some types of postsynaptic neurons. In the present situation, however, MN activity following deafferentation (as indicated by hindlimb movements; data not presented) did not differ from controls, a result also found in several previous studies (e.g., Hamburger et al., 1966; Narayanan and Malloy, 1974; Oppenheim, 1975). Although by no means conclusive, these results cast some doubt on the role of afferent synaptic activity, *per se*, in the regulation of MN survival in the chick embryo.

Other attempts to examine the role of afferents in the survival of developing neurons. In one of the earliest reports on the effects of target (limb bud) removal on MN survival in the chick embryo, Hamburger (1934) discussed the possibility that the signal for regulating survival in this situation may be mediated via a "reflex-arc" involving the DRG. However, because Hamburger found that MN survival was quantitatively unrelated to the extent of hypoplasia in the DRG, this idea was tentatively rejected. Later studies from the 1940's, 1950's, and 1960's involving the selective removal of ascending and/or descending spinal and supraspinal input (or removal of the DRG) also failed to detect any changes in the number of surviving MNs (see Hamburger, 1946, and Oppenheim, 1981a for reviews). Consequently, in virtually all subsequent discussions of MN survival during the embryonic, larval, or fetal periods, the target was assumed to be the major, if not the sole, source of "trophic" regulation. However, in practically all of the early chick studies involving deafferentation, embryos were not examined after E8 to E10; thus, the later-occurring loss of MNs that we report here was missed.

The earliest study in which afferents were suggested to play a critical role in neuron survival involved the ciliary ganglion of the chick embryo. Levi-Montalcini (1947) reported that early (E3) removal of the accessory oculomotor nucleus, the avian homologue of the Edinger-Westphal nucleus and the sole source of afferents to the ciliary ganglion, resulted in a near total loss of ciliary neurons by E17.⁶ Naturally occurring neuron death in

⁶ There is some confusion in the literature concerning the outcome of this experiment. In the paper by Levi-Montalcini (1947, p. 145), the author reports that, "In embryos taken for

the ciliary ganglion extends from E8 to E15, and, as with spinal motoneurons, this cell loss can also be greatly accentuated by target (eye) removal or significantly reduced by enlargement of the target (Landmesser and Pilar, 1974; Narayanan and Narayanan, 1978; Pilar et al., 1980). However, whereas the changes in the survival of ciliary neurons following *target* manipulation occur throughout the normal period of cell loss, deafferentation apparently acts primarily during the late stages (i.e., after E11 to E12) of the cell death period. If correct, then this late effect of deafferentation is similar to some of the findings in the present report and to the work of Clarke (1983). Clarke has also reported that there is an accentuated cell death in the chick isthmo-optic nucleus (ION) induced by deafferentation, which occurs during the latter part (E14 to E16) of the normal cell death period for the ION (i.e., E11 to E16) (see also Sohal, 1976).

Perhaps the most oft-cited example of the control of neuron survival by afferents is the study by Levi-Montalcini (1949) on the development of cochlear nuclei in the chick following deafferentation by early removal of the otic placode. She found that the neurons in the deafferented nuclei developed normally, both quantitatively and qualitatively, up to E11, after which there was a cell loss whose magnitude differed for different nuclei. These results have recently been replicated, and it has also been shown that there is a naturally occurring cell loss in these nuclei (Rubel et al., 1976; Parks, 1979). The induced cell loss following deafferentation coincides temporally with the normal cell loss. Although Levi-Montalcini (1949) suggested that the magnitude of cell loss in different cochlear nuclei following otocyst removal was related to the amount of afferents provided by the cochlea to each nucleus (i.e., that the induced cell loss is proportional to the completeness of deafferentation), this may not be entirely correct; there is actually less induced cell loss in a nucleus (magnocellularis) that probably receives all of its input from the cochlea than in one (angularis) that receives only a portion of its input from this source (Parks, 1979). No information is available on the role of the *targets* of the avian cochlear nuclei on neuronal survival.

In a recent study of motoneuron survival in the lumbar spinal cord of *Rana pipiens* tadpoles following removal of the DRG, it was found that MN numbers were signif-

icantly reduced (Davis et al., 1983). However, because the animals in that study were always sacrificed long after the period of normal cell loss (Beaudoin, 1955; Pollack, 1969), and because similar results were also obtained even when the deafferentation was performed *after* the normal period of cell loss, these results cannot be easily related to the role of afferents in naturally occurring MN death. In the present study, for instance, we have found that the total removal of *all* primary afferents from lumbar DRG results in little, if any, increased MN death during the major period of normal cell loss in the chick (i.e., E5 to E10). Increased cell loss only occurs in these preparations during (or following) the terminal stages of normal cell death.

In summary, the results of the present set of experiments indicate that the regulation of naturally occurring MN death may be more complex than previously suspected. In addition to the well proven role ascribed to the MN targets, these data imply that afferents are also involved in this process. As indicated from the reports reviewed above, many other types of neurons may also require trophic or other influences from both their targets and afferents in order to survive during an early period of differentiation.

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study at the 17th to 21st day of incubation, the ganglion is much reduced in volume (1/10 of control) despite the fact that the number of cells are not much reduced from normal." However, when discussing this experiment in a subsequent paper, Levi-Montalcini (1949, p. 230) states that, "The extirpation of the mesencephalon deprives the ciliary neurons of all of their synaptic connections. It was found that a regression starts at 11 days; the hypoplasia progresses during the subsequent period and results in the disappearance of a majority of the cells at hatching, and regressive changes in the few surviving cells." Although this last statement apparently reflects the actual results of the 1947 study (R. Levi-Montalcini, personal communication), we are presently attempting to confirm this independently in our laboratory by early bilateral removal of the accessory oculomotor nucleus.

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