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Cell Death of Spinal Motoneurons in the Chick Embryo following Deafferentation: Rescue Effects of Tissue Extracts, Soluble Proteins, and Neurotrophic Agents

Yin Qin-Wei,[a] James Johnson, David Prevette, and Ronald W. Oppenheim

Department of Neurobiology and Anatomy, and the Neuroscience Program, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27157

In the absence of descending spinal and supraspinal afferent inputs, neurons in the developing lumbar spinal cord of the chick embryo undergo regressive changes including cellular atrophy and degeneration between embryonic days 10 and 16. There are significant decreases in the number of motoneurons, interneurons, and sensory (dorsal root ganglion) neurons. Although there are several possible explanations for how afferents might regulate the maintenance of neuronal viability, we have focused attention on the putative role of neurotrophic agents in these events. Previous studies have shown that specific tissue extracts (e.g., muscle, brain), soluble proteins, growth factors, and trophic agents can promote the *in vitro* and *in vivo* survival of avian motoneurons during the period of natural cell death (embryonic days 6–10). Several of these agents were also effective following deafferentation. These included brain extract (BEX), muscle extract (MEX), conditioned medium from astrocyte cultures (ACM), as well as the following neurotrophic agents: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), S-100, insulin-like growth factor-I (IGF-I), ciliary neurotrophic factor (CNTF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (CDF/LIF). Both transforming growth factor-β (TGF-β) and acidic fibroblast growth factor (aFGF) were ineffective. Although considerable more work is needed to determine which (and how) specific CNS-derived trophic agents regulate motoneuron survival, the present results are consistent with the notion that neurotrophic agents released from or modulated by synaptic inputs to target neurons promote neuronal differentiation and survival in the CNS.

[Key words: development, spinal, motor neurons, neurotrophic, cell death, chick embryo, afferents]

During neuronal development large numbers of immature neurons are lost by a process of programmed or naturally occurring cell death (Oppenheim, 1991). In the spinal cord of the chick embryo approximately one-half of all postmitotic motoneurons undergo cell death over a period of several days between embryonic day (E) 6 and E12 (Hamburger, 1975; Chu-Wang and Oppenheim, 1978). Although previous studies have suggested that the skeletal muscle targets of motoneurons are the major, if not the sole, source of the signals that regulate the extent of cell loss, it is becoming increasingly clear for motoneurons, as well as for many other types of neurons, that there are very likely other sources of trophic signals besides those derived from targets (Cunningham, 1982; Oppenheim, 1991; Galli-Resta and Resta, 1992; Korsching, 1993). In addition to the possible role of trophic agents derived from or associated with afferent terminals, glia cells, the extracellular matrix, and from other non-neuronal cells (e.g., macrophages, connective tissue, etc.), signals mediated by neuronal activity are also receiving increasing attention as putative sources of trophic influence on developing neurons (Born and Rubel, 1988; Maderdrut et al., 1988; Collins et al., 1991; Rubel et al., 1991; Catsicas et al., 1992; Galli-Resta et al., 1993; Schmidt and Kater, 1993).

Following a previous observation showing that the removal of substantial amounts of descending afferent input to developing spinal motoneurons in the chick embryo results in enhanced cell death (Okado and Oppenheim, 1984), we have begun to explore the possible mechanisms for this induced cell loss. In this situation, deafferentation of lumbar motoneurons was achieved by the removal of two or three segments of thoracic neural tube on E2, thereby preventing the later projection of descending spinal and supraspinal axons to the lumbar region. Beginning on E10 and continuing until E15–16, 20–30% of the surviving motoneurons undergo degeneration and death. The total cell loss between E6 and E15 is 70–80% in deafferented embryos versus 50% for unoperated or sham-operated control embryos (with all of the loss, in this case, occurring between E6 and E11–12). Because local segmental and sensory afferents remain intact in this model, it has not been possible to determine whether complete deafferentation would result in the loss of virtually all lumbar motoneurons, as occurs following the total loss of motoneuron targets (Hamburger, 1958; Oppenheim et al., 1978). However, if one can extrapolate from the situation with avian ciliary ganglion (CG) neurons, this would seem a likely outcome. Total deafferentation of the CG results in as much induced cell death (> 90%) as is observed following target deletion (Furber et al., 1987).

Although there are several possible mechanisms that may account for the deafferentation-induced loss of spinal motoneurons, in the present article we focus mainly on the possible role

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Correspondence and reprint requests should be addressed to Ronald W. Oppenheim at the above address.

[a]Present address: Laboratory for Molecular Neuroscience, Mailman Research Center, McLean Hospital, 115 Mill Street, Belmont, MA 02178.

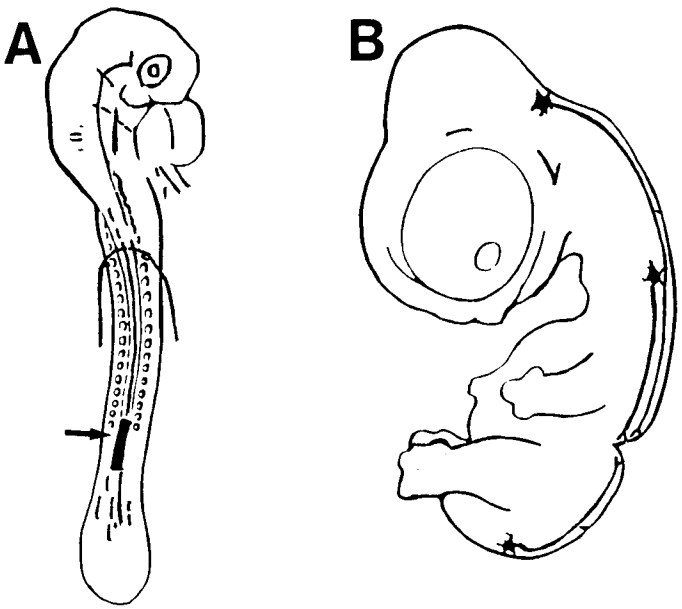

Figure 1. A spinal gap 2–4 somites long (*arrow*) was made in the presumptive thoracic neural tube on E2 (*A*). As shown in *B*, this serves to prevent the ingrowth of descending spinal and supraspinal afferent axons to the lumbar region (deafferentation).

of brain or spinal cord–derived neurotrophic signals that may be lost or perturbed following deafferentation. The rationale for this stems from observations made both *in vitro* and *in vivo*, indicating that soluble CNS-derived proteins can promote the survival of developing motoneurons during the period of naturally occurring cell death (Dohrmann et al., 1987; Yin et al., 1992; Johnson et al., 1994) and that they do so as effectively as proteins and tissue extracts derived from target muscle (Dohrmann et al., 1986, 1987; Oppenheim et al., 1988a, 1993; Bloch-Gallego et al., 1991). Accordingly, our strategy has been to determine whether tissue extracts, soluble proteins, and purified growth factors that effectively rescue motoneurons from *naturally occurring* cell death *in vitro* and *in vivo* can also prevent the later *induced* cell loss following deafferentation. Together with the biochemical observations in the report by Johnson et al. (1994), these studies represent a beginning attempt to define the biological and biochemical properties of putative novel and known CNS-derived neurotrophic factors that serve as motoneuron survival agents during normal avian development.

Materials and Methods

Eggs. Fertilized eggs were obtained as a generous gift from Hubbard Farms, Statesville, NC, and were incubated in the laboratory at 37°C and 60% relative humidity until they reached the age required for experimentation.

Deafferentation. Prevention of the formation of descending spinal and supraspinal afferent inputs to the lumbar spinal cord was accomplished by removing three or four segments of the thoracic neural tube (somites 21–24) just rostral to the last thoracic segment (see Okado and Oppenheim, 1984). This created a "gap" in the neural tube that prevents axons from crossing in either direction (Fig. 1). Because the gap was created on E2 (stage 13–14; Hamburger and Hamilton, 1951), whereas the first descending afferents to the lumbar region do not appear until 2 d later (Oppenheim et al., 1988b), lumbar neurons develop in the absence of a significant portion of their normal descending afferent input. In all successful cases that were used for further analysis, a vertebral canal was formed in the operated (gap) region and the vasculature and other surrounding tissues appeared normal rostral and caudal to the gap (Fig. 2). Controls consisted of both unoperated and sham-operated embryos. The sham operations involved removal and replacement of three or four segments of thoracic neuronal tube. Approximately one-third to one-half of controls were sham operated. There were no differences between sham versus unoperated controls, so the results have been pooled. Following surgery (or sham), the window in the shell was sealed with transparent adhesive tape and the eggs placed in an incubator where they remained unturned until embryos were killed for histology or until the initiation of treatment with tissue extracts or trophic agents.

Administration of trophic agents. Tissue extracts, conditioned medium, and trophic molecules were delivered (dropped) onto the highly vascularized chorio-allantoic (CAM) membrane through the window in the shell beginning on E9 and continuing daily until E15. Several previous studies have confirmed that this is an efficient and reliable method for the delivery of trophic agents, drugs, and neurotoxins to the avian embryo (Oppenheim et al., 1978, 1988a; Pittman and Oppenheim, 1979). Typically, the agents were administered in volumes of 50–100 μl. Control embryos received 50–100 μl of avian Ringers solution. Both aFGF and bFGF were always administered together with 25 μg of heparin present in the injection solution. Tissue extracts and conditioned medium were prepared as described below. The purified or recombinant growth factors and trophic agents used here include: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), S-100β, transforming growth factor-β (TGFβ), ciliary neurotrophic factor (CNTF), insulin-like growth factor I (IGF-I), acidic and basic fibroblast growth factor (aFGF, bFGF), cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) and platelet-derived growth factor (PDGF-AB). All of these agents were administered in doses of 5–10 μg/d from E9 to E15. Human recombinant BDNF, NT-3, PDGF, TGF-β, bFGF, and CDF/LIF were gifts from Amgen, Inc. Human recombinant CNTF was a gift from Synergen, Inc., and human recombinant IGF-I, a gift from Cephalon, Inc. Recombinant S-100 was a gift from Linda Van Eldik. Purified murine NGF was provided by Eugene Johnson, Jr.

Preparation of brain and muscle extracts. Chick brain extract (BEX) was prepared from whole brains of E16 embryos, whereas muscle extracts (MEX) were prepared from skinned and deboned hindlimb muscles of E16 embryos. Both brains and muscles were homogenized in a modified PBS solution containing 1 mM EDTA, EGTA, BEN, NEM, and 0.1 mM PMSF to minimize proteolysis. Homogenates were centrifuged (1 hr at 23,000 × *g*) and the supernatant collected for 0–25%, 25–75%, and 100% $AmSO_4$ precipitation fractions. In each case, the $AmSO_4$ was added dropwise, agitated for 2 hr, and precipitated protein recovered by centrifugation (30 min at 15,000 × *g*). Complete saturation is achieved by addition of $AmSO_4$ to the 75% saturated supernatant, followed by equilibration for 18 hr and centrifugation. The 0–25%, 25–75%, and 75–100% fractions are resuspended in 0.9% NaCl to final volumes equal to one-fourth to one-half the original volume (protein concentration of 1.5–2.0 mg/ml), dialyzed, and frozen. Crude BEX and the $AmSO_4$ fractions were always dialyzed prior to use through a 1000 MWCO Spectrophor 1 membranes for 36–48 hr against two changes of 0.9% NaCl. After dialysis, samples were centrifuged for 15 min at 1000 × g and any pellet that formed was discarded. Protein concentrations were determined using bicinchoninic acid (Smith et al., 1985). All the above procedures was carried out at 4°C and fractions were either used immediately or stored at −70°C for no longer than 1–2 weeks prior to use. Heat and trypsin treatment of crude BEX was as described (Oppenheim et al., 1988a, 1993) and was always carried out just prior to the use of BEX in an experiment.

Astrocyte-conditioned medium (CM). Astrocytes were purified from the cerebral cortices of neonatal mice according to the method of McCarthy and deVellis (1980). The brain tissue was dissected, the meninges removed, after which the tissue was minced and the cells dissociated. The dissociated cells were plated into 75 cm^2 flasks on plastic (three to five cortices/flask) in medium containing 10% fetal calf serum. The first medium change is after 1 d. After 1–2 additional d, ara-c (5×10^{-5} M) and fluorodeoxyuridine (10^{-5} M) were added to inhibit fibroblast division and left for 2 d. After this time, the medium only contains 10% fetal calf serum without mitotic inhibitors. Collection of the CM began when the cells were nearly confluent, which is approximately 6 d after the initial plating. The CM was passed through 0.8 μm and 0.45 μm filters, frozen (−80°C) for 48 hr and then lyophilized completely over 48 hr. The lyophilized material was later resuspended in distilled water, dialyzed against 0.9% NaCl for 72 hr, and centrifuged and the supernatant was stored at −80°C. Prior to use for *in vivo* treatment of chick embryos, the CM was reconcentrated to 1–2 mg/ml protein in 0.9%

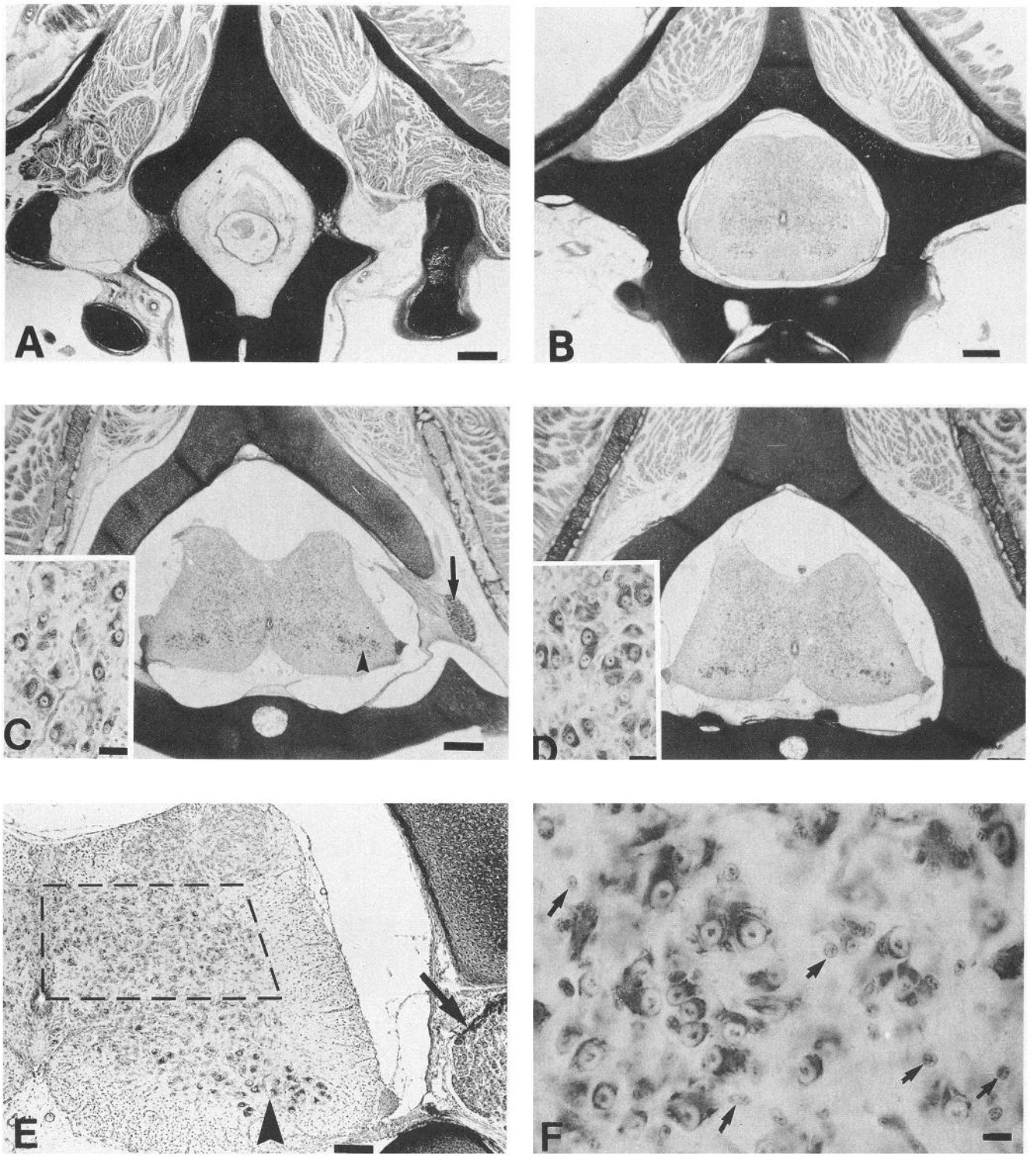

Figure 2. Photomicrographs of transverse sections of the spinal cord, and of spinal motoneurons and interneurons, in control and deafferented embryos on E16. *A,* A section through the thoracic gap region in which neural tissue is absent. *B,* A section through thoracic spinal cord just caudal to the gap. *C* and *D,* Sections through the lumbar region (L3) of a control (*C*) and deafferented (*D*) embryo. *Insets* illustrate motoneurons in the lateral motor column. The *arrow* in *C* (and *E*) indicates the dorsal root ganglion and the *arrowheads* in *C* and *E* the lateral motor column (ventral horn). *E,* Deafferented embryo indicating the region where interneurons were counted (*dashed lines*). *F,* Higher magnification of interneurons. *Arrows* in *F* indicate some of the non-neuronal cells that were excluded from the counts of interneurons. Scale bars: *A–D,* 250 μm; *E,* 100 μm; *F,* 12 μm; *insets,* 22 μm.

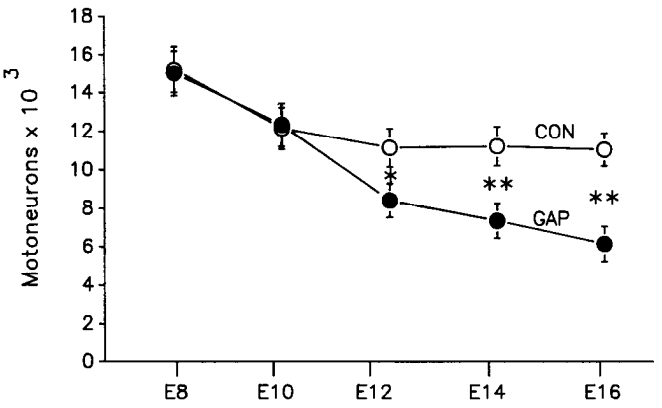

Figure 3. Motoneuron numbers (mean ± SD) in the lumbar spinal cord of control and deafferented embryos (n = 10–20 for each data point). *, $P < 0.01$; **, $P < 0.005$; control versus gap (t tests). Naturally occurring motoneuron death begins on E6, when there are approximately 22,000 motoneurons present, and continues until E12, although 90% of the cell loss occurs between E6 and E10.

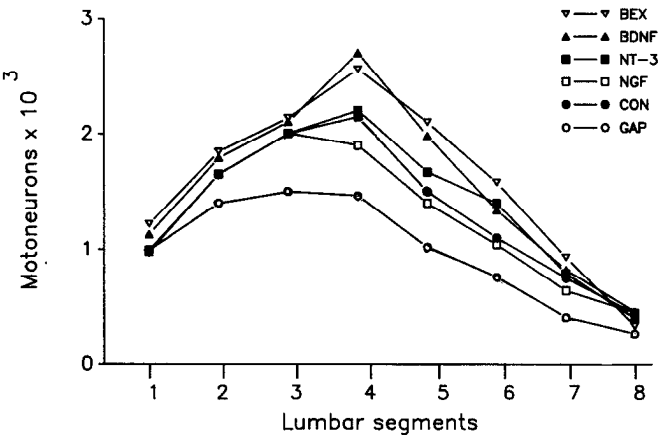

Figure 4. The distribution of motoneurons in each of the eight lumbar segments of control, gap, and treated embryos on E16. Segments were defined by adjacent dorsal root ganglia as described previously (Oppenheim et al., 1993).

saline and filter sterilized. Control embryos were treated with tissue culture medium containing 10% fetal calf serum.

Histology and morphometric analysis. Control and operated (gap) embryos were killed at various times between E8 and E16. The entire thoracolumbar spinal cord was dissected with vertebra and dorsal root ganglia (DRG) attached, immersion fixed in Carnoy's solution for 10–20 hr, and processed and embedded in paraffin. Serial transverse sections (12 μm) were cut on a rotary microtome through the entire block, placed on slides, and stained with thionin. As shown in Figure 2, the success of the surgery could be easily ascertained from examination of sections through the thoracic gap region. Embryos that contained any neural tissue bridging the two sides of the gap were discarded.

Motoneurons in the lumbar lateral motor column (LMC, Fig. 2) were identified (Chu-Wang and Oppenheim, 1978) and counted in every 10th section using criteria that effectively exclude double counting of the same cell in adjacent sections (Oppenheim et al., 1989; Clark and Oppenheim, 1994). The values obtained were multiplied by 10 to give an estimate of total motoneuron numbers. Using the same criteria, interneurons in every 20th section in the region of the gray matter delineated in Figure 2 and DRG cells in the L3 ganglion were counted in every 5th section. The values obtained for interneurons and DRG cells were multiplied by 20 and 5, respectively, to generate estimates of total cell numbers. The histological slides were coded so that the person doing the cell counts was blinded as to whether a given embryo was a control or "factor"-treated animal. The region indicated in Figure 2 that was used to count interneurons excludes primarily laminae 1–4 (dorsal horn) and laminae 8, 9, and a small part of 7, in the ventral region (Martin, 1979). The remaining area between the dashed lines comprises approximately 50% of the gray matter in both control and gap embryos on E16 (McKay and Oppenheim, 1991).

The length of the lumbar spinal cord was estimated by counting the total number of sections in which the LMC was detected (L1–L8), and this number was then multiplied by the section thickness (12 μm). Nuclear size (diameter) of motoneurons, which is roughly proportional to soma size (Oppenheim et al., 1982, 1992a), was measured as described (Oppenheim et al., 1988a).

Table 1. Motoneuron size (mean ± SD) and spinal cord length (mean ± SD) on E16 in control and gap embryos and in gap embryos following treatment with exogenous agents

	Nuclear diameter of MN (μm)		Length of lumbar cord (μm)	
	Mean ± SD	N	Mean ± SD	N
Control	12.8 ± 0.16*	(50)	6410 ± 381**	(10)
Gap	11.5 ± 0.10	(50)	6057 ± 230	(11)
BEX	12.9 ± 0.14*	(50)	6400 ± 414	(8)
MEX	13.0 ± 0.13*	(50)	6387 ± 490	(9)
bFGF	12.8 ± 0.11*	(50)	6400 ± 336	(7)
aFGF	11.3 ± 0.13	(50)	5916 ± 478	(5)
S100	12.8 ± 0.12*	(50)	6521 ± 405**	(7)
PDGF	12.6 ± 0.15*	(50)	6180 ± 155	(5)
CNTF	12.6 ± 0.13*	(50)	6460 ± 393**	(7)
BDNF	12.8 ± 0.14*	(50)	6456 ± 186**	(6)
NT-3	12.7 ± 0.13*	(50)	6432 ± 484**	(6)
NGF	12.1 ± 0.17	(50)	6225 ± 503	(10)
CDF/LIF	12.1 ± 0.18	(50)	6360 ± 147	(6)
IGF	12.7 ± 0.13*	(50)	6456 ± 157**	(6)
TGF-β	11.9 ± 0.14	(50)	6192 ± 276	(6)

* $p < 0.01$ versus gap.

** $p < 0.05$ verus gap.

Results

Effects of deafferentation

In agreement with our original report describing the effects of deafferentation on motoneuron survival (Okado and Oppenheim, 1984), we have confirmed that in this situation motoneuron numbers in the lumbar spinal cord initially die at the normal rate up to E10 at which time cell numbers are comparable in control and deafferented embryos (Fig. 3). However, between E10 and E16 there is a gradual increased loss of motoneurons such that by E16 deafferented embryos have 20–35% fewer surviving cells. After E16, motoneuron numbers appear stabilized. In our earlier report, we also demonstrated that there were increased numbers of pyknotic (degenerating) motoneurons after E10 in deafferented embryos, indicating that the increased cell loss was owing to active cell death. This was also the case in the present study. On E13, there were virtually no pyknotic motoneurons observed in control embryos (1.3 ± 0.3, n = 5), whereas deafferented embryos exhibited an average of 43 ± 11 (n = 5) pyknotic cells in lumbar segments L1–L8. The motoneuron loss that occurs following deafferentation involves virtually all regions (segments) of the lumbar cord (Fig. 4). Although we have also shown here that surviving deafferented motoneurons on E16 are slightly smaller than control motoneurons (Table 1), whether this slight (but statistically significant) difference is biologically meaningful is not clear. Histologically, motoneurons and interneurons in the deafferented cord appear healthy and fully differentiated and exhibit no obvious

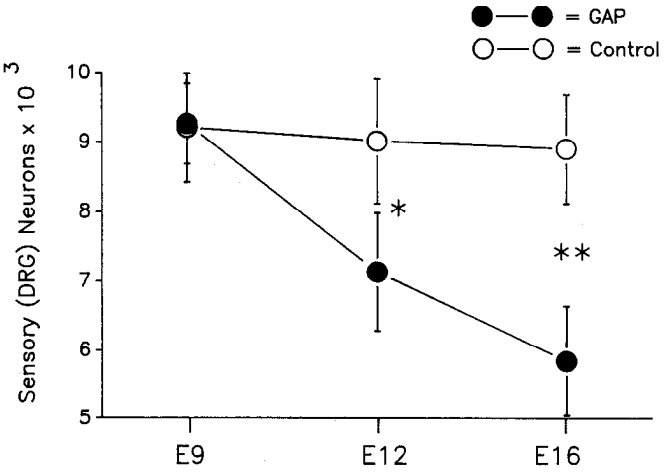

Figure 5. The number of sensory neurons (mean ± SD) in the L3 DRG on E9, E12, and E16 following deafferentation. *, $P < 0.01$; **, $P < 0.0005$ (t tests).

signs of atrophy or chromatolysis (Fig. 2). Again, however, these data on reduced cell size are consistent with the findings reported previously (Okado and Oppenheim, 1984).

Deafferentation of the lumbar spinal cord results in an overall reduction in the size of this region of the spinal cord. In our previous report, this was only an impression based on subjective criteria, whereas we have now confirmed this observation using lumbar spinal cord length as index of size. As shown in Table 1, there was a small (~ 6%), but statistically significant, reduction in spinal cord size on E16 following deafferentation. A similar reduction in lumbar spinal cord size was found on E10 (data not shown). Despite the reduction in spinal cord and motoneuron size, the identification of the boundaries of the lateral motor column (ventral horn) and of motoneurons was unambiguous.

Following the creation of a gap in the thoracic neural tube, sensory neurons in the lumbar DRG with ascending projections would be prevented from reaching their more rostral targets. This situation could result in retrograde changes, including the death of DRG cells, which could, in turn, indirectly (e.g., transneuronally) influence the development and survival of neurons in the lumbar region. For this reason, we have examined cell numbers in one representative lumbar DRG (L3) on E9, E12, and E16 following deafferentation. Cell numbers in the deafferented embryos were normal on E9, but were reduced by 23% on E12 and by 35% on E16 (Fig. 5, Table 2). Preliminary observations indicate that there are similar reductions in DRG cells in other lumbar ganglia following deafferentation. The creation of a gap in the thoracic neural tube results in deafferentation of both motoneurons *and* interneurons in the lumbar cord, and also prevents rostrally projecting interneurons in the lumbar region from contacting their target neurons in the upper spinal cord and brain. Either or both events could directly affect the survival of interneurons and indirectly affect motoneuron survival. As shown in Figure 6, following deafferentation there is, in fact, a significant (25%) reduction in the number of interneurons in the intermediate gray region of lumbar cord on E16.

Effects of treatment with tissue extracts and astrocyte-conditioned medium

In our initial experiments, in which deafferented embryos were treated daily from E9 to E15 with 200 μg of protein of unfractionated (crude) BEX, we found that this treatment was sufficient to prevent the expected loss of motoneurons following deafferentation (Fig. 7). Furthermore, pretreatment of the crude BEX with heat or trypsin completely abolished this survival-promoting activity (Fig. 7). Biologically active crude BEX was able to rescue motoneurons following deafferentation in virtually all segments of the lumbar cord (Fig. 4). Because studies of the effect of BEX on naturally occurring motoneuron death in normal embryos between E6 and E10 indicated that a 25–75% $AmSO_4$ fraction of BEX was most effective in rescuing cells (Johnson et al., 1994), we have also examined the effect of different $AmSO_4$ fractions following deafferentation. Neither the 0–25% nor the 75–100% $AmSO_4$ BEX fractions were effective in this situation (Fig. 7). By contrast, the 25–75% BEX fraction was as effective in preventing cell death following deafferentation as the crude, unfractionated BEX. In a beginning attempt to identify the cellular source of the survival activity in BEX, we have examined whether CM from astrocyte cultures (ACM) is effective in preventing cell death following deafferentation.

Table 2. Sensory neuron (L3 DRG) numbers (mean ± SD) on E16 in control and gap embryos and in gap embryos following treatment with exogenous agents

Treatment	Cell numbers	*N*
Control	9207 ± 979	6
Gap	5840 ± 926*	12
BEX	5997 ± 1123*	9
MEX	6312 ± 985*	5
bFGF	6071 ± 970*	5
aFGF	6195 ± 1122**	5
S100	8215 ± 952	5
PDGF	8028 ± 1200	4
CNTF	8364 ± 649	6
BDNF	10055 ± 953	5
NT-3	9092 ± 1130	5
NGF	8239 ± 876	5
CDF/LIF	6110 ± 1068*	5
IGF-I	6114 ± 1209*	5
TGF-β	6788 ± 1041*	4

* $p < 0.01$ versus control.

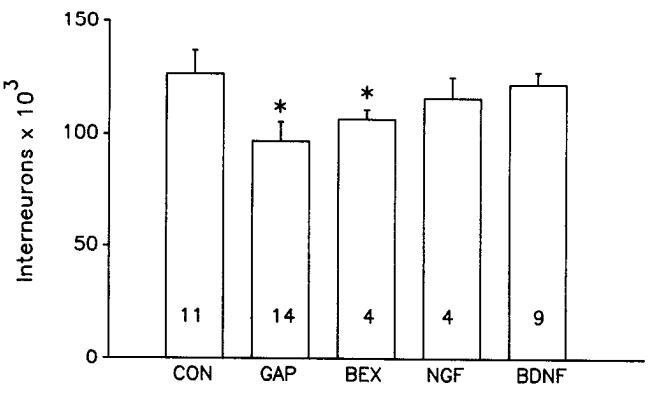

Figure 6. The number of lumbar interneurons (mean ± SD) on E16 in the region indicated in Figure 2 of control and deafferented (*GAP*) embryos and of deafferented embryos treated with brain extract (*BEX*), nerve growth factor (*NGF*), or brain-derived neurotrophic factor (*BDNF*). *, $P < 0.01$ versus control (t tests). Both the *NGF* and *BDNF* groups are also significantly different from *Gap* ($P < 0.01$).

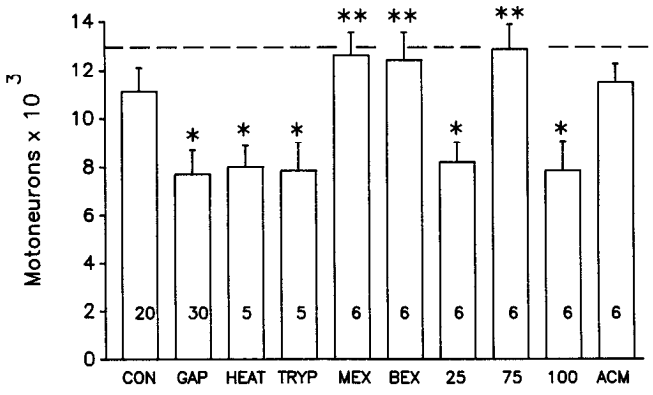

Figure 7. The number of lumbar motoneurons (mean ± SD) on E16 in control and deafferented (*GAP*) embryos and in deafferented embryos treated with crude brain extract (*BEX*), heat- or trypsin-inactivated BEX, muscle extract (*MEX*), $AmSO_4$ fractions of BEX (25%, 75%, 100%, see text), and astrocyte-conditioned medium (*ACM*). The *dashed line* indicates the number of motoneurons present in control and gap embryos on E9, the age when treatment with exogenous agents began. As indicated in Figure 2, naturally occurring cell death continues until E12. *, $P < 0.001$ versus control; **, $P < 0.01$ versus control (*t* tests).

As shown in Figure 7, ACM was, in fact, able to prevent the cell loss in this situation. Although we initially included MEX in this study as a negative control (not expecting that a putative muscle-derived trophic agent would prevent cell death following CNS deafferentation), in fact, as shown in Figure 7, MEX (25–75% $AmSO_4$ fraction) was as effective as BEX. Although BEX and MEX were also effective in preventing the reductions in both motoneuron and spinal cord size following deafferentation (Table 1), neither agent reversed the loss of DRG cells (Table 2). Furthermore, BEX was also ineffective in preventing the loss of interneurons in the lumbar cord (Fig. 6).

Because lumbar motoneurons in normal control embryos continue to undergo naturally occurring cell death between E9 and E12 (see Fig. 3), it was of interest to examine whether this later *normal* cell death could also be prevented by BEX, MEX, and ACM. As shown in Table 3, treatment with BEX, MEX, and ACM from E9 to E12 prevented most of the normal motoneuron death during this period. Thus, treatment of deafferented embryos with these and other agents from E9 to E15 has the potential to prevent both natural and deafferentation-induced motoneuron death. This is the reason why in some cases trophic agent-treated deafferented embryos have significantly greater numbers of motoneurons on E16 than unoperated controls (e.g., see Fig. 7).

Table 3. Motoneuron numbers (mean ± SD) on E13 in control embryos and in embryos treated with exogenous agents

Treatment	Cell numbers	*N*
Control	10791 ± 855	18
MEX	13016 ± 917*	6
BEX	13270 ± 1020*	5
25	10223 ± 1115	5
75	13334 ± 912*	7
100	9931 ± 1203	5
ACM	11501 ± 616**	5
bFGF	10575 ± 959	5
S100	12413 ± 970*	6
PDGF	10309 ± 861	5
CNTF	12871 ± 890*	8
BDNF	12511 ± 767*	6
NT-3	10608 ± 795	5
CDF/LIF	10199 ± 1003	5
IGF-I	12409 ± 660*	7
TGF-β	10103 ± 810	5

* $p < 0.01$ versus control.
** $p < 0.05$ versus control.

Table 4. Motoneuron numbers (mean ± SD) on E16 in control and gap embryos and in gap embyros treated with trophic agents

Treatment	Cell numbers	*N*	% Difference versus gap
Gap	7715 ± 977*	30	0
Control	11108 ± 1005	20	30
MEX[a]	12591 ± 1077	6	39
BEX[a]	12413 ± 1105	6	38
BDNF	12041 ± 9926	6	37
S100	11616 ± 1000	6	33
CNTF	11511 ± 9127	7	33
PDGF	11378 ± 8076	6	32
NT-3	11216 ± 8166	6	31
CDF/LIF	11050 ± 9296	6	30
bFGF	10405 ± 1223	6	26
IGF-I	10283 ± 8057	7	25
NGF	10195 ± 1007	10	23
aFGF	8129 ± 1217*	5	5
TGF-β	7810 ± 956*	6	1

[a] Data from Figure 7 for comparison.
* $p < 0.01$ versus control.

Effects of treatment with growth factors and trophic agents

We have recently shown that a number of previously identified proteins with neurotrophic activity are able to rescue developing avian motoneurons from naturally occurring cell death when administered *in vivo* (Oppenheim et al., 1993), and several of these also promote the survival of purified populations of motoneurons *in vitro* (Arakawa et al., 1990; Martinou et al., 1990, 1992; Gouin et al., 1993; Henderson et al., 1993). Accordingly, it was of interest to determine whether any of these same agents are also effective in preventing motoneuron loss following deafferentation. Of the 11 agents tested, only two, TGFβ and aFGF, were ineffective (Table 4). The other agents tested varied somewhat in their ability to prevent cell death, with BDNF, CNTF, and S100 being the most effective, followed by NT-3, PDGF, and CDF/LIF (intermediate group), whereas IGF-1, NGF, and bFGF were the least effective (Table 4). However, it is important to point out that the differences in effectiveness between these various agents can be almost entirely accounted for by their differing abilities to prevent naturally occurring cell death between E9 and E12. For example, BDNF, S100, CNTF, IGF, MEX, and BEX effectively prevent motoneuron death during this period in unoperated embryos, whereas the other agents are either ineffective or less effective (Table 3). By contrast, all the agents tested (except for aFGF and TGF-β) were more or less comparable in their ability to prevent the *specific* cell loss in-

duced by deafferentation (i.e., motoneuron numbers on E16 were similar to that of untreated control embryos). Although not all treatment groups were examined regarding the rostral–caudal distribution of rescued motoneurons, in those that were (NGF, BDNF, NT-3), it was apparent that treatment rescued deafferented motoneurons throughout the lumbar cord (Fig. 4).

With the exception of CDF/LIF, NGF, PDGF, aFGF, and TGF-β, all of the other agents that were examined for their effect on motoneuron and spinal cord size following deafferentation were found to prevent the reductions in either one or both of these two measures (Table 1).

As reported above, neither BEX nor MEX were able to prevent the loss of sensory neurons in the DRG following deafferentation. Similarly, bFGF, aFGF, TGF-β, IGF-1, and CDF/LIF were also ineffective on sensory neurons, whereas NGF, BDNF, NT-3, S-100, PDGF, and CNTF all promoted survival in the DRG following deafferentation, with BDNF and NT-3 being the most effective (Table 2). Although the survival of interneurons following deafferentation has only been examined in the NGF- and BDNF-treated groups, it is of interest that both agents were able to rescue interneurons in this situation (Fig. 6).

Discussion

The removal of several segments of thoracic neural tube on E2 in the chick embryo creates a situation in which the lumbar region is isolated from descending influences from both spinal cord and supraspinal afferents. Axons from these rostral regions normally begin to innervate the lumbar cord between E4 and E5 (Singer et al., 1978; Oppenheim et al., 1988b; Shiga et al., 1991) with the major period of synaptogenesis being after E10 (Oppenheim et al., 1975). Although it is not possible to estimate quantitatively the precise extent to which lumbar motoneurons are deafferented by this procedure, it seems likely that a substantial amount of the normal input is derived from these rostral sources and that much of this input develops during later stages, after E10. The fact that deafferentation does not affect motoneuron numbers until after E10 is consistent with this idea. Whereas motoneuron death up to E10 occurs at the normal rate in both control and deafferented embryos, between E10 and E16 deafferented embryos lose an additional 20–30% of lumbar motoneurons. Because this loss is accompanied by an increase in the number of degenerating (pyknotic) motoneurons, we conclude that the reduction in cell number is a result of active motoneuron death. A similar loss of lumbar motoneurons has been reported following thoracic spinal transection in adult rats (Eidelberg et al., 1989) and there are many reports of increased cell death in other neuronal populations following deafferentation (for reviews, see Oppenheim, 1991; Rubel et al., 1991). Therefore, afferent regulation of neuronal survival is a widespread phenomenon occurring in different species and types of neurons.

Deafferentation also results in other changes in the lumbar cord, including reduced motoneuron and spinal cord size, as well as a reduction in the number of surviving sensory (DRG) neurons and interneurons. Deafferentation of neurons is known to result in cellular atrophy and other regressive changes (Globus, 1975; Deitch and Rubel, 1984). The reduction in spinal cord size can very likely be accounted for by a combination of factors, including reduced cell numbers (motoneurons, interneurons, and possibly glia) and the loss of descending fiber tracts. In any event, the small ($\sim$ 6%) reduction in spinal cord size cannot by itself account for (i.e., be the cause of) the much greater reduction in numbers of motoneurons or interneurons. Additional evidence against this possibility comes from our observation that the spinal cord is already reduced in size on E10 *prior* to the increased loss of motoneurons in deafferented embryos. Additionally, the occurrence of increased numbers of dying cells on E13 following deafferentation also indicates that the loss of motoneurons is due to cell death and is not an artifact of reduced spinal cord or motoneuron size. Moreover, as noted previously (Pittman and Oppenheim, 1979; Okado and Oppenheim, 1984), motoneuron numbers can be increased, decreased, or unchanged in the face of decreased spinal cord and motoneuron size. Finally, it is also important to point out that the reduction in motoneurons cannot account for the loss of interneurons since even a virtually complete loss of motoneurons following limb bud removal does not alter the survival of spinal interneurons (McKay and Oppenheim, 1991).

We have previously found that spinal interneurons do not appear to undergo naturally occurring cell death. Rather, following their generation, interneuron numbers remain stable (McKay and Oppenheim, 1991). Therefore, it is unlikely that the loss of interneurons observed here reflects enhanced naturally occurring cell death. Instead, this loss appears to be a *de novo* event induced by the loss of afferents. It is interesting that this induced loss is restricted to subpopulations of interneurons. As reported previously (and as confirmed in the present study, data not shown), we failed to observe a reduction of interneurons in either the dorsal horn or in the ventral gray matter in proximity to the lateral motor column (McKay and Oppenheim, 1991). Rather, only interneurons in the gray matter in between these regions, which comprises about 50% of the total gray matter, were reduced in number following deafferentation. Perhaps the interneurons in this region are more severely deafferented by the surgery employed in creating a gap in the neural tube.

Because some sensory neurons in the DRG, as well as some types of spinal interneurons, make direct synaptic contact with motoneurons, the reduction in survival of motoneurons in the present situation (gap) could be due either to reduced inputs from these local (lumbar) sources of input, or to the removal of descending (nonlumbar) inputs, or to some combination of these. Because treatment with some of the exogenous agents tested here (e.g., BEX) rescues deafferented motoneurons, but is without affect on interneuron survival, it seems unlikely that motoneuron death is secondary to the loss of interneurons. Similarly, because some of the agents tested (e.g., bFGF) prevent motoneuron death following deafferentation without affecting the survival of sensory neurons, it is also unlikely that the loss of motoneurons in this situation (in the absence of treatment) is secondary to the loss of inputs from sensory afferents. Additionally, in previous studies, treatment with NGF during the period of naturally occurring cell death rescued significant numbers of sensory neurons in the DRG, but was completely ineffective in promoting motoneuron survival (Oppenheim et al., 1982). Finally, because some of the effective agents examined by us (e.g., FGF, PDGF) are reported to be involved in myogenesis (Burgess and Macaig, 1989; Bowen-Pope et al., 1991), their mode of action in preventing cell death after deafferentation could be indirect via a direct effect on muscle differentiation. One argument against this is the fact that some of these agents can promote motoneuron survival *in vitro* in the complete absence of muscle cells (Dohrmann et al., 1986; Arakawa et al., 1990; Bloch-Gallego et al., 1991). In addition, we have previ-

ously shown that MEX rescues motoneurons *in vivo* without affecting muscle development (Oppenheim et al., 1993).

An alternative hypothesis is that the agents that prevent the death of motoneurons following deafferentation do so by acting directly on motoneurons. If this idea is correct, then there are at least two ways in which this direct effect on motoneurons may be mediated. Exogenous agents may either compensate for the loss of endogenous orthograde signals associated with descending afferents (e.g., Schecterson and Bothwell, 1992), or they may compensate for a loss of motoneuron access to target-derived neurotrophic agents, a loss that is somehow induced by the reduction in descending afferent inputs; that is, deafferented motoneurons may be at a disadvantage in gaining access to target-derived factors (Li et al., 1992). Although at present, the data do not allow us to distinguish between these two possibilities, the fact that MEX is effective in rescuing motoneurons from deafferentation could be taken as supporting the "target" hypothesis. However, in this case we cannot exclude the possibility that MEX contains similar neurotrophic agents to those that are present in the CNS and which normally directly mediate survival via CNS afferents. For example, BDNF is expressed in both muscle and the CNS (Henderson et al., 1993; Koliatos et al., 1993; McKay et al., 1993). MEX may also contain factors different from those in the CNS, but which can, nonetheless, compensate for the loss of CNS-derived molecules.

A final possibility that we also cannot exclude is that the exogenous agents compensate for the loss of trophic support from non-neuronal (glial) cells, a loss that is somehow associated with deafferentation. Although some of the effective agents examined by us can promote the survival of purified populations of motoneurons in the absence of non-neuronal cells (Dohrmann et al., 1986, 1987; Arakawa et al., 1990; Gouin et al., 1993; Henderson et al., 1993), some of these agents (e.g., IGF, CNTF, NT-3) are also known to prevent the normal death of developing glial cells (Barres et al., 1992, 1993) and glial cells are known to be a rich source of neurotrophic agents (Barde et al., 1978; Lindsay, 1979; McCaffery et al., 1984; Eagleson et al., 1985; Manthrope et al., 1986; Bosch et al., 1988; Giulian et al., 1988; Eccleston et al., 1990; Acheson et al., 1991; Engele and Bohn, 1991; Matsuoka et al., 1991; Cunha and Vitkovic, 1992; Friedman et al., 1992). Therefore, following deafferentation some of the effective agents examined by us may either promote the survival of glial cells or stimulate the release of trophic agents from surviving glial cells.

It is of interest that some of the agents tested here that were effective in preventing the death of motoneurons following deafferentation (NGF, bFGF) have previously been shown to be *ineffective* in rescuing motoneurons from naturally occurring or programmed cell death (Oppenheim et al., 1982, 1988a, 1992a). One explanation for this difference is that deafferented neurons may express receptors for trophic factors that are not normally expressed or that are normally expressed only at low levels. For example, whereas developing motoneurons are known to express the low-affinity (P75) NGF receptor (Yan et al., 1988; Heuer et al., 1990), they are not thought to express the high-affinity biologically active *trk*A receptor (Henderson et al., 1993; Koliatsos et al., 1993; Yan et al., 1993) and, as predicted, NGF does not prevent normal motoneuron cell death (Oppenheim et al., 1982, 1988a, 1992b). Nonetheless, as shown here, NGF is effective in rescuing deafferented motoneurons from cell death and it is also effective in preventing motoneuron death following peripheral axotomy in avian embryos and neonatal rats (Houenou et al., 1993; Yan et al., 1993). Although in these situations NGF may be either acting indirectly (e.g., via sensory afferents, but see above), or by activating other *trk* receptors that *are* expressed on motoneurons (Henderson et al., 1993; McKay et al., 1993; Tessarollo et al., 1993; Yan et al., 1993), it is also possible that following perturbation, *trk*A receptors are upregulated on avian motoneurons (e.g., Cheng and Mattson, 1991; Miwa et al., 1993), or on other neuronal or non-neuronal cells in the CNS that may then indirectly influence motoneuron survival by an NGF-induced release of other trophic agents. We are presently attempting to examine the expression of the mRNA for these receptors in the spinal cord following axotomy and deafferentation.

It is also conceivable that developing motoneurons normally change their trophic requirements, such that they depend upon different trophic agents before, during, and after the period of naturally occurring cell death (Barde et al., 1980; Eagleson and Bennett, 1986; Lindsay, 1988; Larmet et al., 1992; Wright et al., 1992; Buchman and Davies, 1993; Masu et al., 1993). For example, it is known that developing avian and mammalian motoneurons become gradually less dependent upon target-derived factors for their survival (Snider et al., 1992; Houenou et al., 1993) and that, in the case of avian embryos, this transition occurs at about the time that motoneurons become vulnerable to deafferentation. Thus, the normal transition in survival requirements from target dependency to afferent dependency may reflect the need for different trophic factors.

Because a wide variety of neurotrophic agents can also promote normal motoneuron survival both *in vitro* and *in vivo* (Arakawa et al., 1990; Gouin et al., 1993; Hughes et al., 1993a; Neff et al., 1993; Oppenheim et al., 1993), it is unlikely that the extensive responsiveness observed here is peculiar to the deafferentation situation. Furthermore, a similar widespread responsiveness to a variety of neurotrophic factors occurs following injury of other types of neurons (e.g., LaVail et al., 1992; Isacson, 1993). Thus, there is a growing recognition that developing motoneurons, as well as other types of neurons, may normally depend upon the combined action of multiple trophic factors (as well as on other types of signals) for optimum survival (Oppenheim, 1991; Hughes et al., 1993b; Korsching, 1993).

Although the survival of many types of developing neurons has been shown to depend upon signals associated with afferent inputs (Parks, 1979; Giordano and Cunningham, 1982; Davis et al., 1983; Okado and Oppenheim, 1984; Clarke, 1985; Friedman and Price, 1986; Furber et al., 1987; Linden and Pinon, 1987; Crewther et al., 1988; Hashisaki and Rubel, 1989; Sohal et al., 1992; Johnson and Bottjer, 1984), in only a few cases has the nature of these signals been examined in any detail. The synaptic activity of afferent inputs is one putative trophic signal that has been implicated in neuronal survival. The *in vivo* survival of sympathetic and parasympathetic neurons (Wright, 1981; Maderdrut et al., 1988), of neurons in the optic tectum and superior colliculus (Catsicas et al., 1992; Galli-Resta et al., 1993), and of neurons in the cochlear nuclei (Rubel et al., 1991) have all been shown to depend upon normal levels of synaptic transmission by afferents. In all of these cases, activity blockade results in *reduced* survival of postsynaptic neurons. Blockade of electrical activity *in vitro* also reduces neuronal survival (Brenneman and Eiden, 1986; Lipton, 1986; Ramakers and Boer, 1991; Ramakers et al., 1991). By contrast, the chronic depolarization of neurons *in vitro promotes* survival in many cell types (Gallo et al., 1987; Collins et al., 1991; Ling et al., 1991;

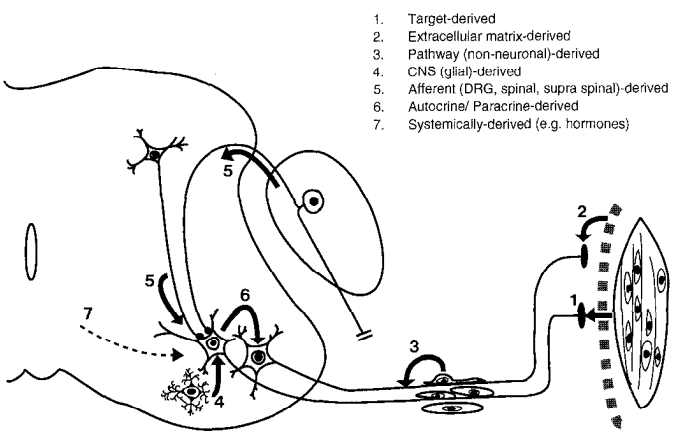

Figure 8. Schematic illustration of known and putative sources of trophic signals acting on spinal motoneurons. The *dashed line* adjacent to the muscle on the right represents extracellular matrix.

Franklin and Johnson, 1992). Depolarization-induced changes in neuronal survival appear to be mediated by the modulation of cytoplasmic calcium levels (Cheng and Mattson, 1991; Collins et al., 1991; Johnson et al., 1992). Therefore, it is possible that calcium regulates endogenous neurotrophic agents that would act in an autocrine or paracrine fashion (e.g., Lu et al., 1989; Elde et al., 1991; Ghosh et al., 1994). Another possibility is that activity may induce an upregulation of neurotrophic factor receptors in postsynaptic cells (Cohen-Cory et al., 1991, 1993; Isackson et al., 1991; Lu et al., 1991; Zafra et al., 1991; Birrin et al., 1992; Castrén et al., 1992; Gwag et al., 1993). It is also possible that depolarization-induced changes in cytoplasmic calcium may act by directly modulating the expression of genes involved in programmed cell death (e.g., Johnson and Deckwerth, 1993). One possibility that is clearly not supported by these data is that normal motoneuron death is a result of afferent excitotoxicity. If this were the case, one would expect decreased rather than increased death following deafferentation (see Fournier LeRay et al., 1993).

Although spinal motoneurons in the chick embryo are synaptically driven by afferent inputs during (and after) the period of naturally occurring cell death (Provine, 1972; Bekoff, 1976; Landmesser and O'Donovan, 1984; O'Donovan et al., 1992), in a previous study in the chick embryo, it was found that the loss of descending afferents did not alter the activity of lumbar motoneurons as measured by the amount of hindlimb motility (Okado and Oppenheim, 1984). Furthermore, direct perturbations of *spinal cord* electrical activity do not affect motoneuron survival in the chick (Okado and Oppenheim, 1984; Landmesser and Szente, 1987; Fournier Le Ray et al., 1993) or frog embryo (Olek and Edwards, 1980). Accordingly, although further studies are needed, it seems reasonable to argue that activity per se is not acting as a trophic signal in the afferent regulation of motoneuron survival.

Regardless of the specific mechanisms involved, our data provide support for the idea that afferent regulation of motoneuron survival during development may be mediated by neurotrophic agents. To this extent, our results are consistent with a modified version of the neurotrophic theory (e.g., Oppenheim, 1991; Thoenen, 1991; Korsching, 1993), one in which targets, afferents, and perhaps even non-neuronal cells are considered as normal sources of trophic support (Fig. 8). A more complete understanding of how afferents act to promote motoneuron survival will require the identification of the specific trophic molecules (and their receptors) that are involved (e.g., Johnson et al., 1994).

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