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Apoptosis of Spinal Interneurons Induced by Sciatic Nerve Axotomy in the Neonatal Rat is Counteracted by Nerve Growth Factor and Ciliary Neurotrophic Factor

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ABSTRACT

We have previously shown that not only motoneurons and dorsal root ganglion cells but also small neurons, presumably interneurons in the spinal cord, may undergo apoptotic cell death as a result of neonatal peripheral nerve transection in the rat. With the aid of electron microscopy, we have here demonstrated that apoptosis in the spinal cord is confined to neurons and does not involve glial cells at the survival time studied (24 hours). To define the relative importance of the loss of a potential target (motoneuron) and a potential afferent input (dorsal root ganglion cell) for the induction of apoptosis in interneurons in this situation, we have compared the distributions and time courses for TUNEL labeling, which detects apoptotic cell nuclei, in the L5 segment of the spinal cord and the L5 dorsal root ganglion after sciatic nerve transection in the neonatal (P2) rat. In additional experiments, we studied the effects on TUNEL labeling of interneurons after treatment of the cut sciatic nerve with either ciliary neurotrophic factor (CNTF) to rescue motoneurons or nerve growth factor (NGF) to rescue dorsal root ganglion cells. The time courses of the TUNEL labeling in motoneurons and interneurons induced by the lesion show great similarities (peak at 8–48 hours postoperatively), whereas the labeling in dorsal root ganglion cells occurs later (24–72 hours). Both CNTF and NGF decrease the number of TUNEL-labeled interneurons, but there is a regional difference, in that CNTF preferentially saves interneurons in deep dorsal and ventral parts of the spinal cord, whereas the rescuing effects of NGF are seen mainly in the superficial dorsal horn. The results are interpreted as signs of a trophic dependence on both the target and the afferent input for the survival of interneurons neonatally. J. Comp. Neurol. 447:381–393, 2002.

Indexing terms: TUNEL labeling; motoneuron; dorsal root ganglion; nerve injury; trophism

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During development of the nervous system, neurons are generated in larger numbers than needed. As a consequence, many neurons die, undergoing programmed cell death (PCD) during the period when contact with target cells is established (Oppenheim, 1991; Pettmann and Henderson, 1998). This death is probably, at least in part, the result of a competition among neurons for target-derived neurotrophic factors during this critical period (Hamburger, 1958; Levi-Montalcini, 1987; Oppenheim, 1991). In the rat, the PCD of spinal motoneurons occurs between embryonic days 15 (E15) and 17 (E17; Harris and McCaig, 1984), whereas primary afferent neurons in dorsal root ganglia (DRG) die between E17 and E19 (Coggeshall et al., 1994). Lawson et al. (1997) showed that rat spinal cord interneurons also undergo PCD, but in this case death occurs around birth [E20 to postnatal day (P)4].

It is well established that a peripheral nerve lesion in neonatal rats leads to an extensive death of axotomized neurons (Romanes, 1946; Schmalbruch, 1987, 1988; Lowrie and Vrbová, 1992). One possible explanation for the fatal response to axon injury in neonatal neurons is that the disconnection between the neuron cell body and the target organ leads to a deficit in the neurotrophic supply. This is supported by the fact that delaying the reinnervation can increase the magnitude of neuronal death after axotomy (Kashihara et al., 1987). Also, the administration of different neurotrophic factors to cut axons can rescue specific types of neurons (Ip and Yancopoulos, 1995; Lewin and Barde, 1996). Thus, treatment with nerve growth factor (NGF) after axotomy counteracts cell death among DRG neurons, whereas application of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and neurotrophin-3 (NT-3) separately or in combination rescues axotomized spinal motoneurons (Sendtner et al., 1990, 1992; Oppenheim et al., 1991, 1992; Yan et al., 1992; Vejsada et al., 1995). However, it is still unclear why different neuronal populations and even neurons from the same pool respond differently to injury and do not have the same regenerative capacity (Fawcett, 1992).

Cell death can be induced via necrosis or apoptosis. Necrosis is characterized by rapid cell disintegration, cellular swelling, organelle dysfunction, and passive cell disassembly (Walker et al., 1988). These events activate the immune system, leading to macrophage invasion and local inflammation. In contrast, apoptosis is an active process, accomplished by a specialized and highly conserved cellular machinery, leading to a series of morphological alterations. Thus, it is characterized by early chromatin condensation, followed by internucleosomal DNA cleavage, cell shrinkage, reorganization of the cytoskeleton, organelle relocation, and production of apoptotic bodies (Wyllie et al., 1980). Apoptosis requires the activation of certain genes and is carried out by a family of cysteine proteases called *caspases*. The entire process ends in a silent and quick cell self-destruction without detectable inflammation. Members of the Bcl-2 protein family seem to be key regulators of apoptosis by inhibiting or enhancing activation of the caspases (for references see Adams and Cory, 1998; Ashkenazi and Dixit, 1998; Thornberry and Lazebnik, 1998). In the nervous sytem, cell death may involve aspects of both apoptosis and necrosis (Li et al., 1998; Pettmann and Henderson, 1998). Moreover, as has been shown recently, PCD of motoneurons may occur in the absence of caspases, although the characteristic morphological changes and the time course differ from what is ordinarily found during PCD (Oppenheim et al., 2001).

We have shown that apoptotic death may underlie the loss of spinal motoneurons, DRG neurons, and interneurons in the spinal cord after neonatal sciatic nerve transection (Oliveira et al., 1997). In this situation, the loss of interneurons coincides with the programmed apoptotic death of such neurons, which occurs from birth up to the sixth postnatal day in the rat (Lawson et al., 1997), whereas PCD of motoneurons and DRG neurons takes place prenatally (Harris and McCaig, 1984; Oppenheim, 1991; Coggeshall et al., 1994). Thus, the damage caused by a neonatal peripheral nerve transection involves not only death of directly injured motoneurons and DRG neurons but also, indirectly, a loss of spinal interneurons. The aim of this study was to weigh the roles of motoneurons and DRG neurons, respectively, in the deleterious outcome of the interneurons. This was done by comparing the time courses of the apoptosis, as revealed by use of the TUNEL technique (Gavrieli et al., 1992) among motoneurons, DRG cells, and interneurons after neonatal sciatic nerve transection and by studying the effects of CNTF and NGF, which rescue motoneurons and DRG neurons, respectively, in the TUNEL labeling pattern of interneurons.

MATERIALS AND METHODS

Animals, surgery, and tissue preparation

For this study, 42 neonatal Sprague-Dawley rats were used. The animals were collected in two main groups (operated and unoperated) of 21 animals in each group. In the operated group, 2-day-old pups were anesthetized by use of deep hypothermia, and the left sciatic nerve was exposed and cut at the sciatic foramen level. After survival times of 4, 8, 12, 24, 48, and 72 hours and 7 days, the rats were killed by intraperitoneal administration of an overdose of chloral hydrate (0.6 mg/kg) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. In the unoperated groups, the rats were killed at the same age as the pups in the operated groups. The L4, L5, and L6 spinal cord segments and L5 DRG were dissected out and left in the same fixative for 24 hours, washed in PBS, and kept in PBS with 10% sucrose overnight at 4°C. Cryostat transverse sections of the spinal cords (18 μm) and the DRG (12 μm) were obtained and transferred to chrome alum gelatin-coated slides, dried at room temperature (RT) for 30 minutes, and stored at –20°C until utilization. Three neonatal rats had their sciatic nerves transected and were sacrificed 24 hours after operation. Such animals were used for an ultrastructural study of the spinal cord and DRG. The experimental procedures were approved by the local ethical committee at the Karolinska Institutet (project N57/96).

Electron microscopy

Twenty-four hours after the operation, the rats were sacrificed with an overdose of chloral hydrate and transcardially perfused with 20 ml of a solution containing glutaraldehyde 2% and paraformaldehyde 1% in PBS 0.1 M, pH 7.4. The lumbar intumescence and the L5 DRG were dissected out, postfixed with osmium tetroxide (2%), buffer rinsed, and acetone dehydrated for embedding in

Vestopal. Transverse semithin (0.5 μm) sections were obtained and stained with toluidine blue for light microscopic analysis. Ultrathin sections were then obtained for ultrastructural analysis.

Staining of apoptotic nuclei (TUNEL labeling)

The sections were postfixed in ethanol/acetic acid (2:1) for 5 minutes at –20°C and rinsed twice for 5 minutes in PBS. The slides were transferred to a humidified chamber, and the equilibration buffer solution was applied (Oncor, Gaithersburg, MD; s7110-1) and incubated for 5 min at RT. The equilibration buffer was shaken off, and the TdT enzyme solution (Oncor; s7110-2 and -3) was applied for 60 min at 37°C. The reaction was stopped with the stop/wash solution (Oncor; s7110-4) for 30 min at 37°C. After washing in PBS for 10 minutes, the sections were incubated with the fluorescein solution (Oncor; s7110-5 and -6) for 30 minutes. The slides were rinsed in PBS and mounted with glass coverslips in 30% glycerol solution.

Analysis of TUNEL-positive cells

In each animal, six alternate sections of the L4–L6 spinal cord segments and four sections of the L5 left and right ganglia were stained with the fluorescein-based TUNEL protocol and used for mapping and counting of apoptotic cells. All TUNEL-positive nuclei in each studied section of the spinal cord were examined in a Leitz DM RBE microscope at 400× and plotted in a drawing of a representative section with a digital tablet (Wacon Ultrapad). For each survival time and treatment, TUNEL-labeled profiles in 18 (spinal cord) or 12 (DRG) sections from three separate animals were indicated in a single representative drawing. The mapped profiles were then digitized to Adobe Illustrator 7.0 software for PC. The numerical data are presented as means ± SD. For statistical analysis, the ANOVA and Newmann-Kuels tests with Primer 1.0 software were used at the 5% level to assess differences between means.

Treatment with neurotrophic factors (CNTF, NGF, and CNTF + NGF)

Nine neonatal (P2) Sprague-Dawley rats were subjected to sciatic nerve transection on the left side, as previously described. Immediately after the lesion, a piece of gelfoam soaked with 15 μl of CNTF (human recombinant, 2.5 μg/μl in PBS) or NGF (Sigma, St. Louis, MO; 10 μg/μl; n = 3) or a mixture of NGF plus CNTF (concentrations as above; n = 3) was placed in direct relation to the proximal nerve stump in three animals. After 24 hours, the animals were killed and subjected to tissue fixation, sectioning, and TUNEL labeling as described above. The numerical data were obtained as described above, but, in the case of the interneurons, ratios between the ipsi- and the contralateral sides were calculated. Statistically significant differences between ratios were assessed using the Mann-Whitney test with Statview 4.0 software at the 5% level.

RESULTS

Ultrastructural analysis

The ultrastructural study of the lumbar intumescence 24 hours after the sciatic nerve transection revealed a number of neurons undergoing a degenerative process. In lamina IX, a few dying motoneurons were observed among normal cells. Such neurons were undergoing chromatolysis and showed the basic characteristics of the apoptotic process (Fig. 1). Thus, with regard to the nucleus content, different degrees of chromatin condensation were observed, resulting in an increased electron density. The nuclear envelope was fragmented, allowing contact between the nuclear and the cytoplasmic contents. Another characteristic of the apoptotic motoneurons was the presence of membrane bubbling, resulting in the formation of apoptotic bodies. The cytoplasm displayed different degrees of electron density, and usually organelles such as mitochondria and endoplasmic reticulum could be identified as unusually collected in electron-dense cores that might generate apoptotic bodies. Some of these structures were in close relation to the nuclear contents. In this way, it is possible that, at later stages of degeneration, apoptotic bodies containing chromatin or not could be found in the ventral horn.

Small neurons undergoing degeneration were observed in different locations of the spinal cord, being more abundant on the ipsilateral side of the lesion. We believe that neurons in different degrees of degeneration could be identified, from early to late stages of apoptosis. Those considered to be in the early stage of apoptosis showed slight nuclear and cytoplasm alterations, such as early chromatin condensation, and minor organelle compactness. Also, in some cases, membrane bubbling could be observed, indicating initial loss of membrane integrity.

Small neurons considered to be in an intermediate stage of apoptosis displayed the classical characteristics of a cell death process. In these cases, both the nucleus and the cytoplasm compartments have an altered electron density. Chromatin compactness and membrane bubbling were also observed. Neurons regarded as being in an advanced stage of apoptosis showed lack of nuclear integrity and nuclear envelope. Also, chromatin compactness was very high. Cytoplasm contents displayed different electron density patterns, and apoptotic body formation was frequent. In some cases, cell fragments were identified as being phagocytized by glial cells without presence of inflammation (Fig. 2).

A number of apoptotic sensory neurons, present in the DRG, could be identified after sciatic nerve lesion. This is in agreement with the TUNEL labeling, showing that a rather large number of sensory neurons became apoptotic within the first 24 hours after lesion. In contrast to normal neurons, which showed a large and central nucleus with loose chromatin limited by a delicate bilayer envelope, neurons under degeneration had a rather condensed chromatin peripherally distributed and a very electron-dense core positioned centrally. The nuclear envelope showed a discontinuity, and, in some cases, membrane interdigitation could be identified as part of a process of fragmentation. Such characteristics differed somewhat from those in the spinal cord and may reflect differences in the apoptotic pattern of these neurons. The cytoplasm of the dying sensory neurons showed extensive bubbling of the cell membrane and an augmented electron density. Generation of apoptotic bodies could be observed in cases of advanced degenerative process (Fig. 3).

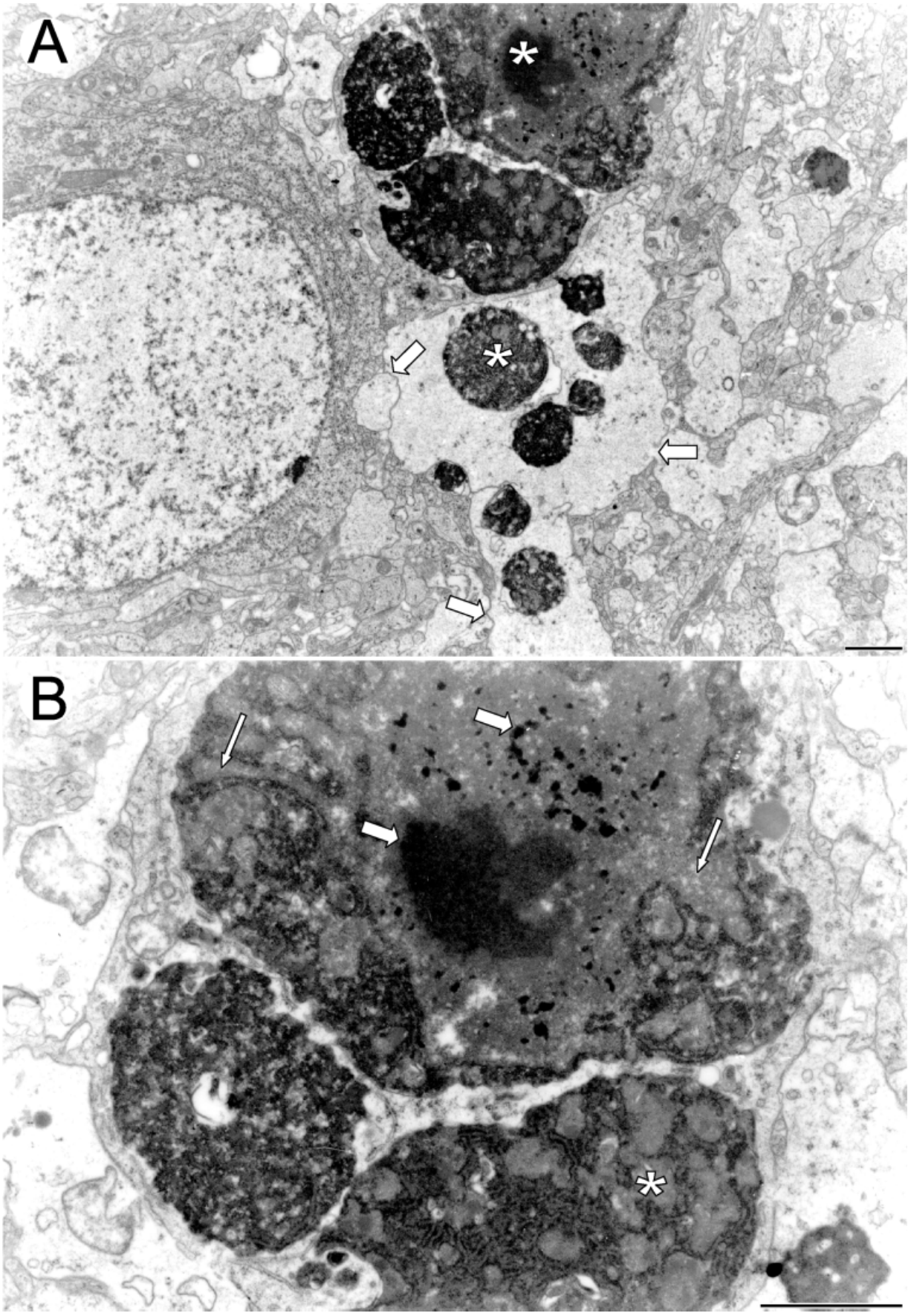
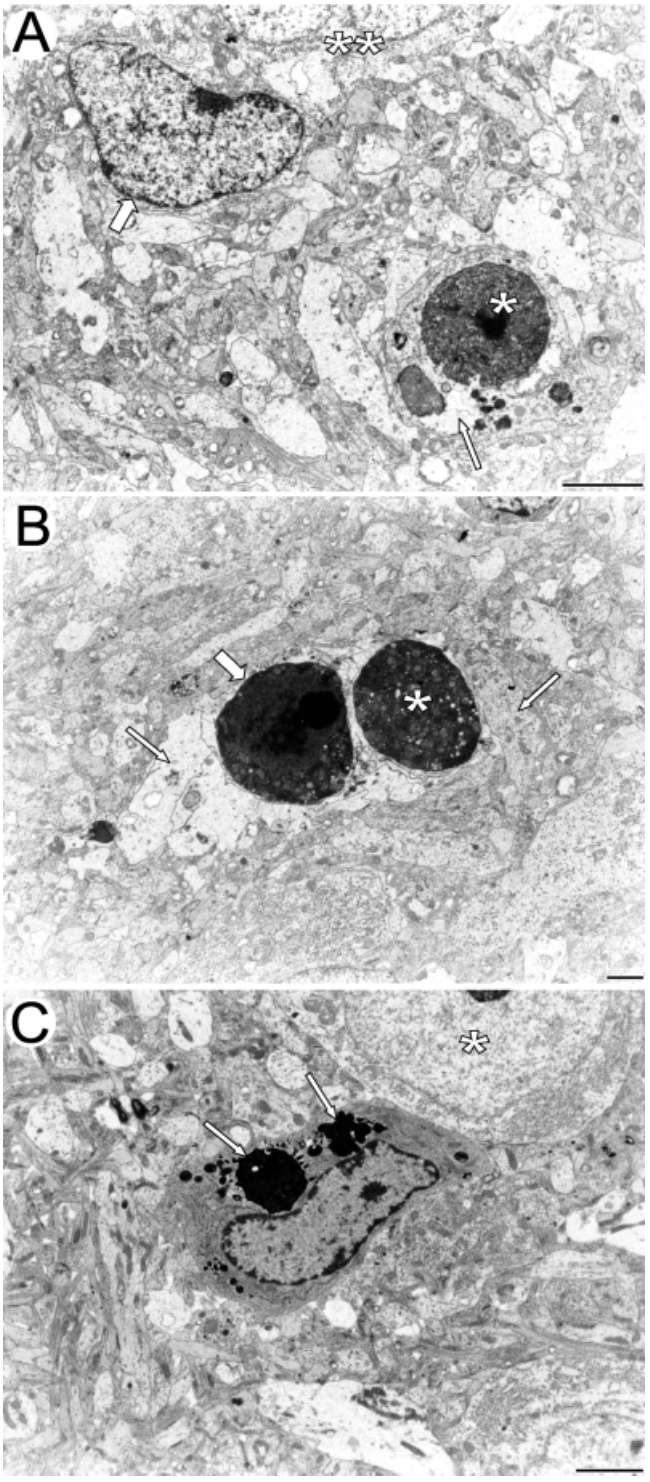


Fig. 1. **A:** Electron micrograph from lamina IX at the level of the lumbar intumescence, showing an apoptotic motoneuron. Nucleus and cytoplasm fragmentation can be observed. Note the presence of membrane bubbling (arrows) and different degrees of augmented electron density, both in the nucleus and in the cytoplasm (asterisks). **B:** Detail of A showing the lack of integrity of the nuclear envelope, leading to contact between the chromatin and cytoplasm contents (thin arrows). Note that the chromatin shows different degrees of compactness (thick arrows). Observe that organelles such as mitochondria and endoplasmic reticulum are packed in an electron-dense core that may generate an apoptotic body without nuclear content (asterisk). Scale bars = 3 μm.

Location and morphology of TUNEL-labeled cells in the normal rat

In normal animals, several TUNEL-labeled nuclei were found in the spinal cords at all ages studied (Figs. 4A,H, 5, 7A, 8). As observed in counterstained sections, such nuclei were located in small cells in both the dorsal and the ventral horns. The numbers of TUNEL-labeled cells in unoperated animals did not differ between sides, but, at 2.5 days after birth, a transient increase in TUNEL labeling among interneurons was observed (Fig. 8). Small numbers of motoneurons were detected as TUNEL positive (Figs. 5, 7A), and the L5 DRG showed very few TUNEL-labeled cells (Figs. 6A,H, 7B). TUNEL-labeled cells showed the basic characteristics of apoptosis, which were chromatin condensation and fragmentation and cell shrinkage and disintegration. Attempts to identify TUNEL-labeled cells as neurons or glial cells by the use of neuron- or glia-specific markers failed; in our hands, the vast majority of TUNEL-labeled cells had lost their neuronal or glial phenotype. In no instance, however, could TUNEL-labeled cells be identified as glial cells in the spinal cord, supporting our ultrastructural data.



Time course of TUNEL labeling of neurons after sciatic nerve lesion

At the first time interval studied after the lesion (4 hours), the number of TUNEL-labeled motoneurons was still normal. However, at 8 hours, there was a sharp increase in the number of labeled motoneurons (Figs. 5, 7A). This increase was maintained up to 48 hours and then decreased to normal levels. No increase in TUNEL labeling of motoneurons could be detected on the unoperated side.

The number of TUNEL-labeled cells on the lesion side of the spinal cord outside the lateral motor column was about twice normal at 8 hours after the lesion. This increase was maintained at high levels up to 24 hours after lesion, then gradually returned to normal at 72 hours postoperatively. As shown in Figures 5 and 8, the lesion-induced pattern of TUNEL labeling among interneurons in the superficial dorsal horn (approximately laminae I–III) and in medial part of deeper laminae (approximately laminae IV–VI) showed a large degree of similarity, with a possible quicker return to normal in the superficial laminae. The remaining part of the spinal gray matter outside the lateral motor column (Fig. 8D) showed only minor differences from normal. This part may also include ventrally located motoneurons. On the contralateral side, there was a 50–100% increase in TUNEL labeling between 8 and 12 hours postoperatively in the superficial dorsal horn, whereas no such increase was seen in the medial part of deeper laminae.

The sciatic nerve transection induced an extensive TUNEL labeling of ipsilateral DRG cells, which was first

Fig. 2. **A:** Electron micrograph from laminae IV–VI showing a neuron suggestive of undergoing apoptosis. Note the chromatin compactness (asterisk) and the cytoplasm alterations, such as membrane bubbling (thin arrow). A normal glial cell can also be observed (thick arrow). **B:** Electron micrograph of a small neuron in laminae IV–VI considered to be in an advanced stage of apoptosis. Note the presence of cell compartments with different electron densities (thin arrows). The nucleus and part of the cytoplasm are packed as a large electron-dense core that may generate an apoptotic body (thick arrow). Another apoptotic body contains only cytoplasm contents (asterisk). **C:** Electron micrograph at the level of laminae IV–VI showing a glial cell containing lysosomes with different degrees of compactness, indicating phagocytosis of apoptotic bodies (arrows). Note the presence of a normal-appearing neuron (asterisk) and that no sign of inflammation is observable. Scale bars = 3 μm.

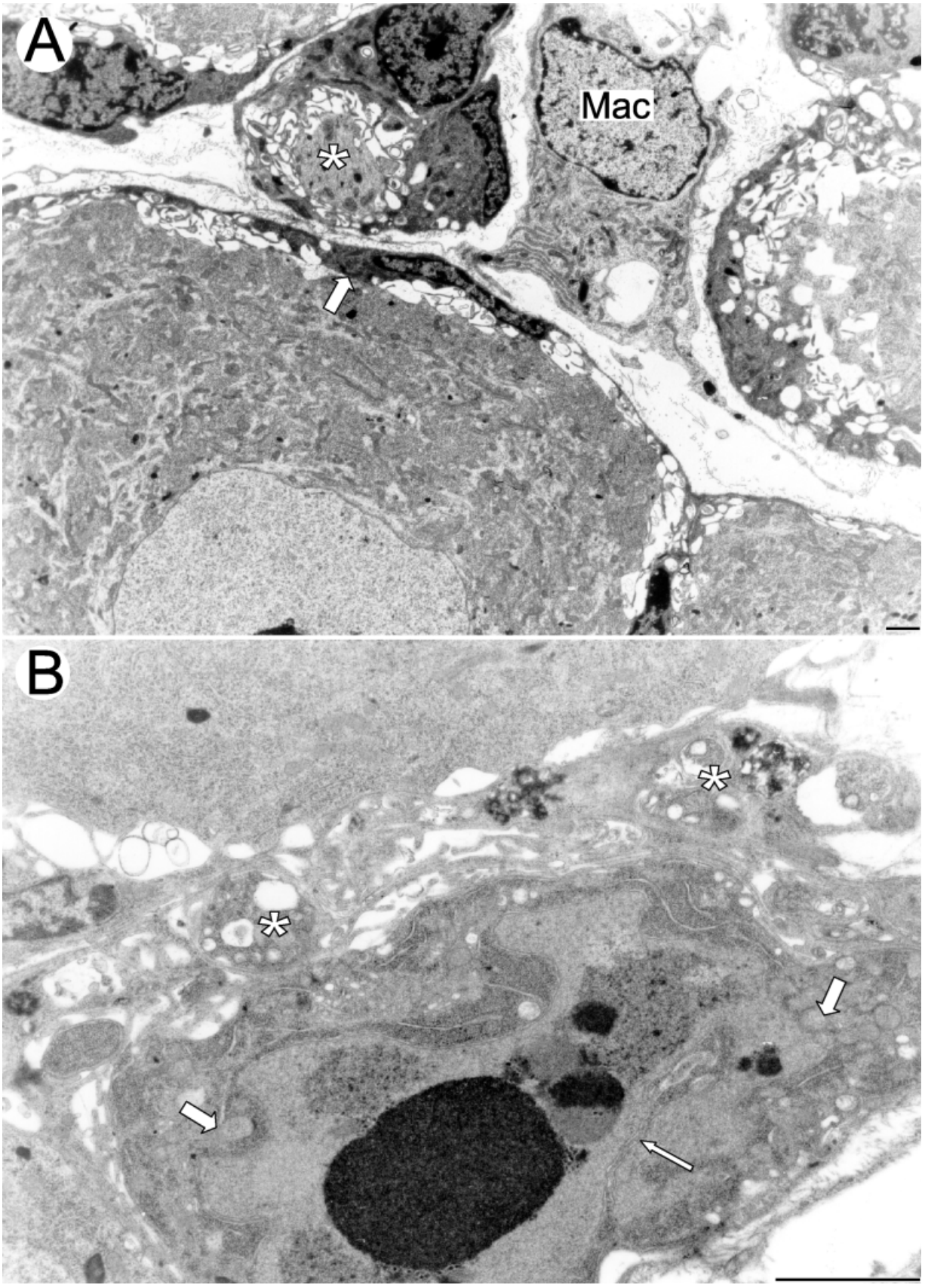


Fig. 3. **A:** Electron micrograph of a DRG, 24 hours after ipsilateral sciatic nerve transection. The asterisk shows an apoptotic body, probably deriving from a neuron, being phagocytized by glial cells. A macrophage-like cell can also be observed in the surroundings (Mac). A large sensory neuron showing a slight enhanced electron density can also be observed. Note that the satellite cell (arrow) related to the neuron shows some signs of degeneration. Other apoptotic bodies can also be observed. **B:** Electron micrograph of a sensory neuron undergoing an advanced stage of apoptotic degeneration. Note the nuclear envelope discontinuity (thin arrow) as well as different degrees of chromatin compaction. Nuclear contents are also in contact with the cytoplasm components (thick arrows). Extensive cytoplasm bubbling can also be observed (asterisks). Scale bars = 3 μm.

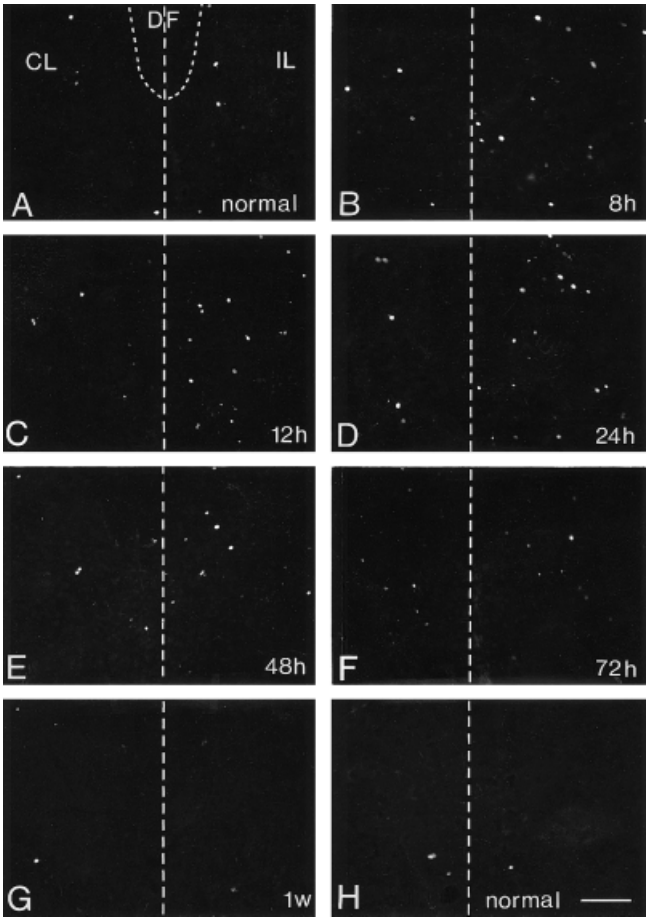


Fig. 4. Photomicrographs of transverse sections through the L5 spinal cord segment showing TUNEL-labeled interneurons in an unoperated P2 rat (**A**) and after sciatic nerve transection in P2 rats at 8 hours (**B**), 12 hours (**C**), 24 hours (**D**), 48 hours (**E**), 72 hours (**F**), and 7 days (**G**) postoperatively. **H** shows the same area in an unoperated P9 rat. DF, dorsal funiculus; CL, contralateral; IL, ipsilateral. Scale bar = 200 μm.

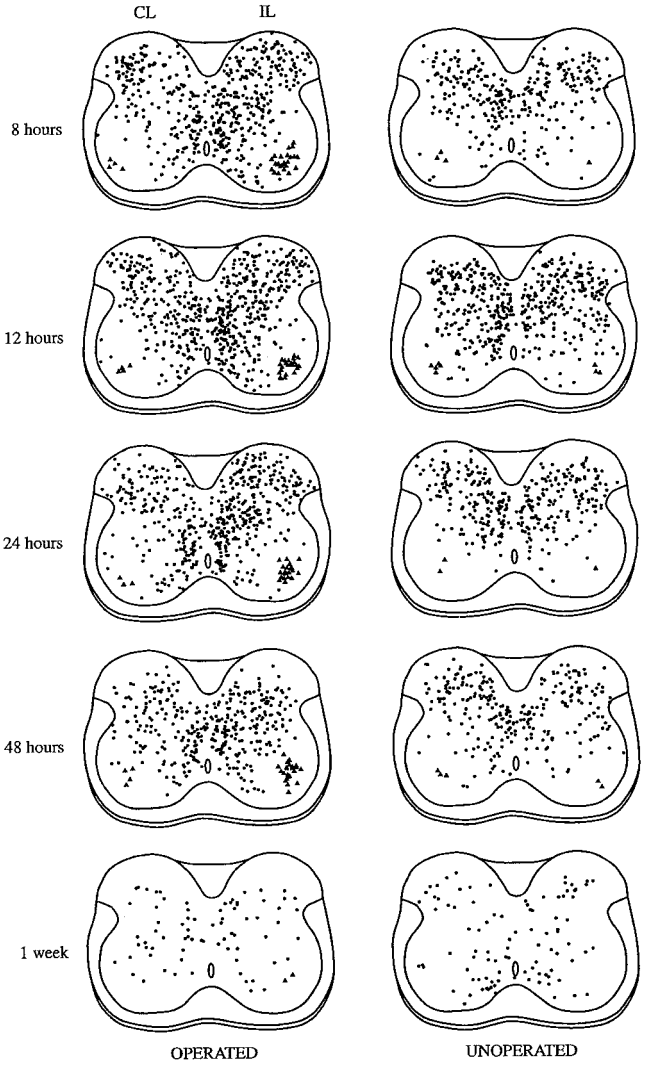


Fig. 5. Distribution of TUNEL-labeled interneurons (circles) and motoneurons (triangles) after sciatic nerve transection in P2 neonatal rats at survival times of 8, 12, 24, and 48 hours and 7 days. All TUNEL-positive neurons found at the lumbar intumescence level (L4–L6) are plotted in a schematic drawing of a representative transverse section. The distribution of TUNEL labeling displayed by unoperated rats of the same ages as the operated animals is also shown. CL, contralateral; IL, ipsilateral.

detected at 12 hours after lesion (Figs. 6, 7B). The labeling frequency peaked at 24 hours, then gradually decreased after 48–72 hours, reaching normal levels after 1 week. No increased labeling was seen on the unoperated side.

Treatment with neurotrophic factors (NGF, CNTF, NGF + CNTF)

The increase in death rate among spinal interneurons after neonatal sciatic nerve lesion, as suggested here, may depend on a loss of afferent input to the interneurons and/or a loss of target cells, such as axotomized motoneurons. To weigh the relative importance of these two possibilities, the proximal stump of the cut nerve was treated with neurotrophic factors with a reported rescuing effect on motoneurons (CNTF) and sensory DRG neurons (NGF). These factors were also used in combination. Twenty-four hours after neurotrophic factor application, the TUNEL method was used to identify the apoptotic neurons.

In control animals that were treated with PBS, there were no differences in the number of apoptotic motoneurons and sensory neurons compared with untreated animals. Similarly, the number and distribution pattern of TUNEL-positive interneurons in the PBS treated animals did not differ from those in the untreated group.

The application of CNTF diminished the number of TUNEL-labeled motoneurons by at least 60% (Fig. 9A). This cytokine was less effective in reducing the number of TUNEL-positive sensory neurons, although some rescuing effect cannot be excluded (Fig. 9B). With regard to the CNTF effect on interneurons, there was a significant reduction ($P < 0.05$) in the ratio between numbers of TUNEL-labeled profiles on the lesion and control sides outside the lateral motor nuclei, with the greatest effects seen in the ventral part of the cord (lateral lamina IV to lamina VII; Fig. 10).

NGF treatment profoundly reduced the number of TUNEL-labeled DRG neurons after lesion but did not have

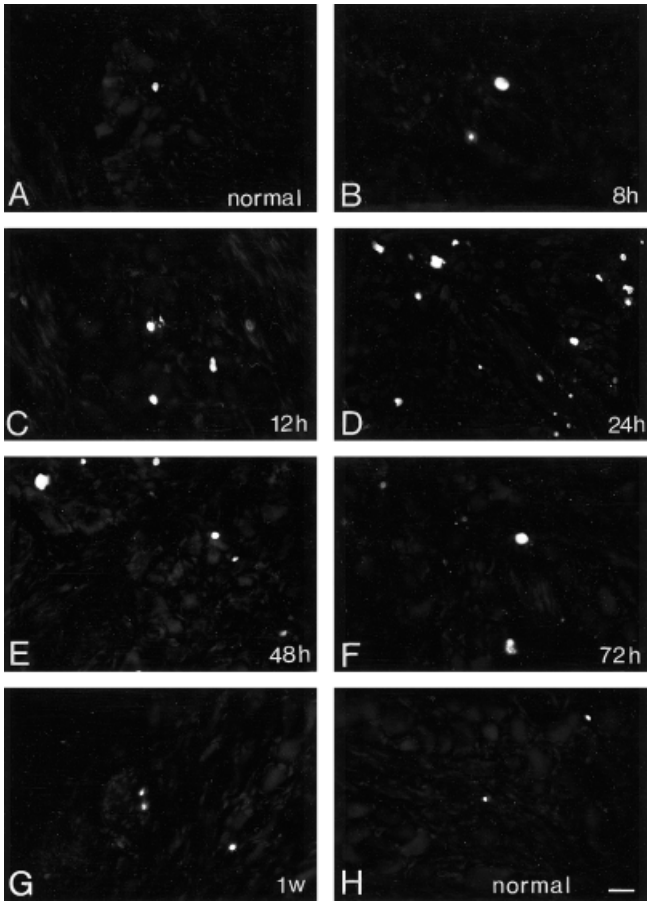


Fig. 6. Photomicrographs of transverse sections through the L5 DRG showing the TUNEL-labeled sensory neurons in an unoperated P2 rat (**A**) and after sciatic nerve transection in P2 rats at 8 hours (**B**), 12 hours (**C**), 24 hours (**D**), 48 hours (**E**), 72 hours (**F**), and 7 days (**G**) postoperatively. **H** shows the same area in an unoperated P9 rat. Scale bar = 50 μm.

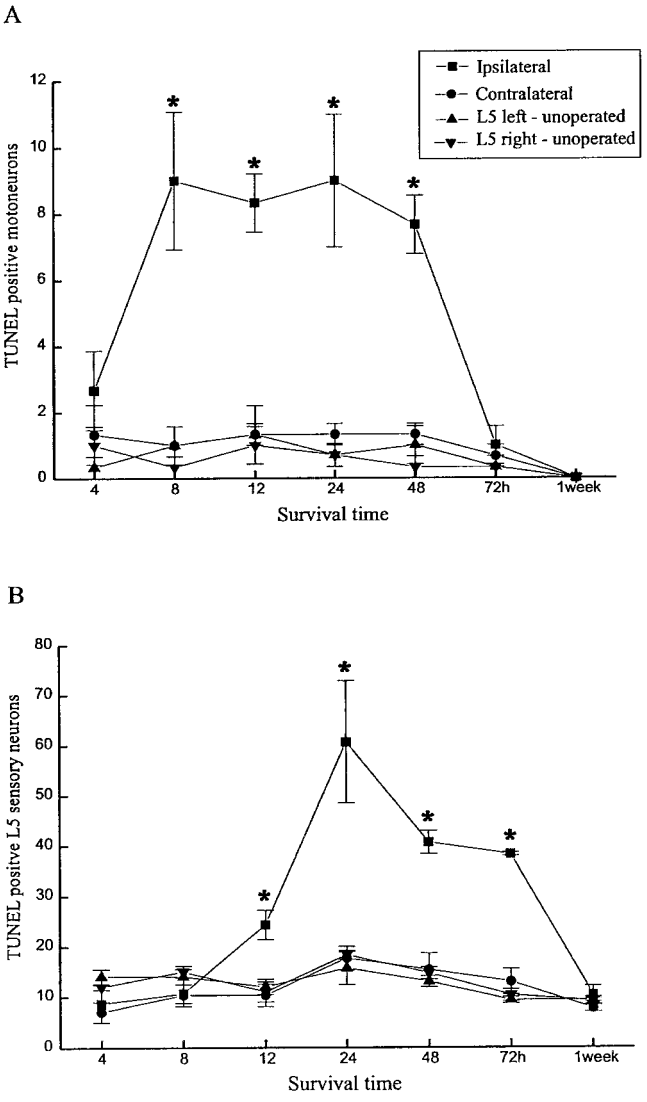


Fig. 7. **A:** Graph showing the time course for TUNEL labeling of motoneurons after neonatal (P2) sciatic nerve transection. The numbers of motoneurons found on the contralateral side of the spinal cord in operated animals and on the left and right sides of unoperated rats are also shown. **B:** Graph showing the time course for TUNEL labeling of DRG sensory neurons after neonatal sciatic nerve transection. The numbers of sensory neurons found on the contralateral side of the spinal cord in operated animals and on the left and right sides of unoperated rats are also plotted. Asterisks indicate values in operated animals that are significantly ($P < 0.05$) different from normal values.

any effects on the labeling of spinal motoneurons (Fig. 9B). Also, NGF exerted effects on the TUNEL labeling of interneurons, with a statistically significant ($P < 0.05$) decrease in the ratio between numbers of labeled cells on the lesion and control sides in treated animals (Fig. 10). In this case, however, there was a tendency toward greater effects in the superficial dorsal horn than in deeper laminae.

The combined application of CNTF and NGF to the proximal nerve stump diminished the number of TUNEL-labeled motoneurons to the same extent as after CNTF alone (Fig. 9A). In the DRG, the numbers of TUNEL-positive cells were almost normalized after this treatment (Fig. 9B). The combination did not seem to affect the labeling of interneurons to any greater extent than was seen for either CNTF or NGF alone, with the exception of the medial part of deep laminae in the dorsal horn, where the combined treatment almost extinguished the lesion-induced labeling (Fig. 10).

The administration of CNTF and NGF did not alter the number of TUNEL-labeled interneurons on the contralateral side of the spinal cord, which should exclude systemic effects of CNTF and NGF administration. It should be noted, though, that the effects of these substances were studied only at one time point (24 hours), when no increase in TUNEL labeling is seen contralaterally after sciatic nerve lesion without treatment.

DISCUSSION

Cell types undergoing apoptosis and labeled with the TUNEL technique

One crucial question regarding the interpretation of the TUNEL labeling data regards the extent to which it rep-

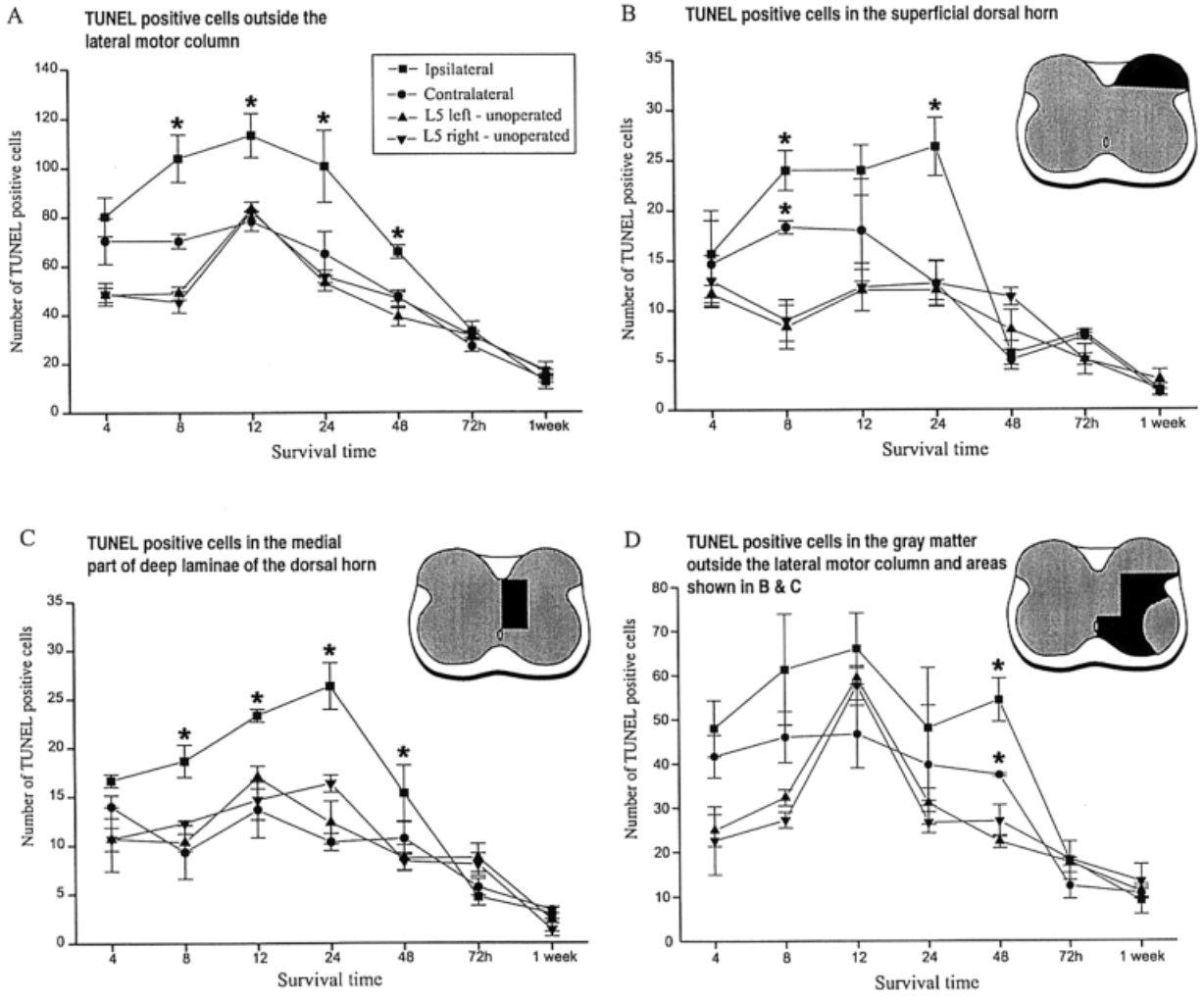


Fig. 8. Graphs showing the time courses for TUNEL labeling of interneurons after neonatal sciatic nerve transection. The numbers of interneurons found on the contralateral side of the spinal cord in operated animals and on the left and right sides of unoperated rats are also plotted. **A** shows all labeled neurons outside the lateral motor column; the other graphs show interneurons in the superficial dorsal horn, approximately covering laminae I–III (**B**); the medial part of the deep dorsal horn, approximately corresponding to medial laminae IV–VI (**C**); and all other parts of the gray matter outside the lateral motor column (**D**). Asterisks denote values that are significantly ($P < 0.05$) different from normal values.

resents labeling of neurons and not supporting cells that could be affected secondarily, as a consequence of the removal of neurons with their axons. As for the DRG, such secondary effects have indeed been described from a recent study in the rat (Whiteside et al., 1998). Our ultrastructural results revealed that motoneurons, sensory neurons, and spinal interneurons displayed the basic characteristics of the apoptotic process, which were described previously by other authors (Kerr et al., 1972; McConkey and Orrenius, 1996; Lawson and Lowrie, 1998). In this sense, chromatin compactness and degeneration of the nuclear envelope were frequently observed in the neurons undergoing apoptosis. Also, nuclear alterations, such as chromatin compactation and, in some cases, contact between nuclear and cytoplasm contents were observed, as previously described by Clarke and Hornung (1989) and Clarke (1990). Events related to chromatolysis, e.g., migration of the nucleus to the periphery and Nissl substance dissolution, were observed in our analysis, in accordance to the classical descriptions (Peters et al., 1991). In a few cases, glial cells were identified phagocytizing apoptotic bodies without any sign of inflammation. This has been described as fundamental when characterizing the apoptotic process (McConkey et al., 1996). In no case could we detect any apoptotic cells resembling glial cells.

In the study by Whiteside et al. (1998), neonatal sciatic nerve transection resulted in a significant increase in TUNEL labeling in the DRG at 1 day postsurgery, which was due predominantly to labeling of neurons, but, at 3 days of survival, there was a further increase in labeling, with a strong dominance of glial cells. Our results differ somewhat, in that the peak of TUNEL labeling frequency had occurred already at 1 day postlesion, which may further support a lower degree of glial labeling in our study. Anyway, the slower increase of TUNEL labeling in the

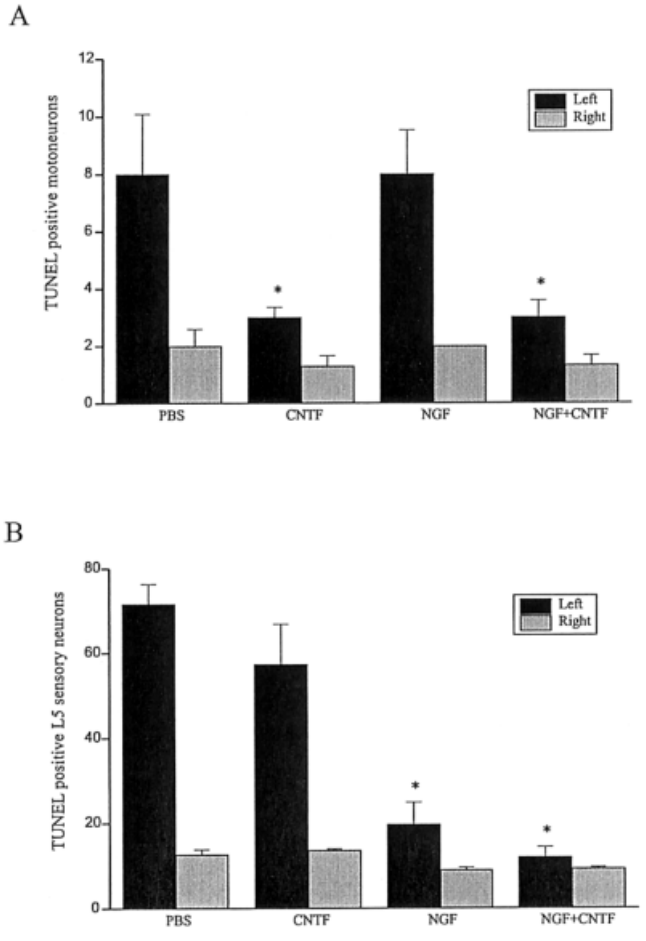


Fig. 9. **A:** Graphs showing the number of TUNEL-positive motoneurons 24 hours after neonatal sciatic nerve transection and treatment with PBS, CNTF, NGF, and a mixture of NGF + CNTF. **B:** Graph showing the number of TUNEL-positive L5 dorsal root ganglia sensory neurons 24 hours after sciatic nerve transection and treatment with PBS, CNTF, NGF, and a mixture of NGF + CNTF.

DRG compared with the spinal cord as demonstrated here should still reflect a true difference in the response of the involved neurons. The possibility that some TUNEL-positive cells in the spinal cord following peripheral nerve lesion could still be secondarily affected glial cells was not confirmed ultrastructurally. Also, if such cells make up a large proportion of the labeled profiles, one would expect a labeling in the dorsal column, which contains large numbers of primary afferent axons that are likely to be degenerating after the lesion. Such labeling has not been detected here, which indicates that the large majority of TUNEL-labeled profiles in the spinal cord are indeed neurons.

In this context, it should be noted that developing neurons may die by a TUNEL-negative pathway, as demonstrated in mice after genetic deletion of caspases (Oppenheim et al., 2001). Still, the temporal delay and different morphological characteristics of dying neurons in such animals were not seen here and could, therefore, represent an alternative process that is not normally seen in PCD in the spinal cord.

Time courses of TUNEL labeling

We have reported previously that, after sciatic nerve transection in the neonatal rat, not only motoneurons and DRG cells die but also interneurons in the spinal cord, which can only indirectly be affected by the peripheral nerve lesion. We have here tried to weigh the relative importance of the afferent input vs. the target for the fate of interneurons in the studied situation. First, we investigated and compared the time courses of the TUNEL labeling of spinal motoneurons, interneurons, and DRG sensory neurons. Our results show a very early response in both motoneurons and interneurons, with the first TUNEL-positive labeling induced by the lesion already at 4 hours postoperatively. This increase in TUNEL labeling was most robust at 8–24 hours after the lesion among the interneurons and at 8–48 hours among the motoneurons. In contrast, DRG cells responded later to the axotomy. Thus, such cells displayed the first signs of an increase in TUNEL labeling at 12 hours after the lesion, with the peak at 24 hours, but still, at 72 hours postlesion, there was a significant increase in TUNEL labeling frequency.

Earlier studies in the neonatal rat have shown that motoneurons start to die off on the third day after peripheral nerve injury, with a peak at 6 days (Lowrie et al., 1994). Our findings suggest a quicker response, but it should be noted that the TUNEL technique used here demonstrates DNA fragmentation in cells that are still existing. Furthermore, a recent study using the TUNEL technique has reported a time course for the labeling of motoneurons similar to that shown here (Lawson and Lowrie, 1998). Thus, the possibility that some motoneurons die later as a consequence of events that are not associated with apoptosis should be considered. In this context, it is of interest that a recent study in the mouse has demonstrated that the morphological characteristics of motoneuron death following neonatal axotomy, although accompanied by TUNEL labeling, exhibit both similarities to and differences from currently accepted definitions of apoptosis (Li et al., 1998).

With regard to the time course of the lesion-induced TUNEL labeling of interneurons, our results differ from another recent study of the apoptosis in the spinal cord after peripheral nerve lesion in the neonatal rat (Lawson and Lowrie, 1998). In this study, was detected an increase in TUNEL labeling outside the motor nuclei that started as late as 4 days postoperatively, with a peak at 6 days and a return to normal at 8 days, which gives a considerably slower time course than was found in our study. The reason for the difference is not clear, but one obvious discrepancy in methodology between the two studies is that, in the previous study, the sciatic nerve was crushed, whereas we transected the nerve. These situations present large differences in the availability of substances confined to the distal nerve segment for the lesioned neurons, which may well have consequences for indirectly affected interneurons. Still, as mentioned above, this difference in methodological approach did not seem to have a major influence on the time course of TUNEL labeling of motoneurons.

The time courses found here may be interpreted as the interneurons being more strongly dependent on the target motoneurons than on the afferent input. It should be noted, however, that cutting the peripheral branch of a primary afferent neuron induces a number of changes in

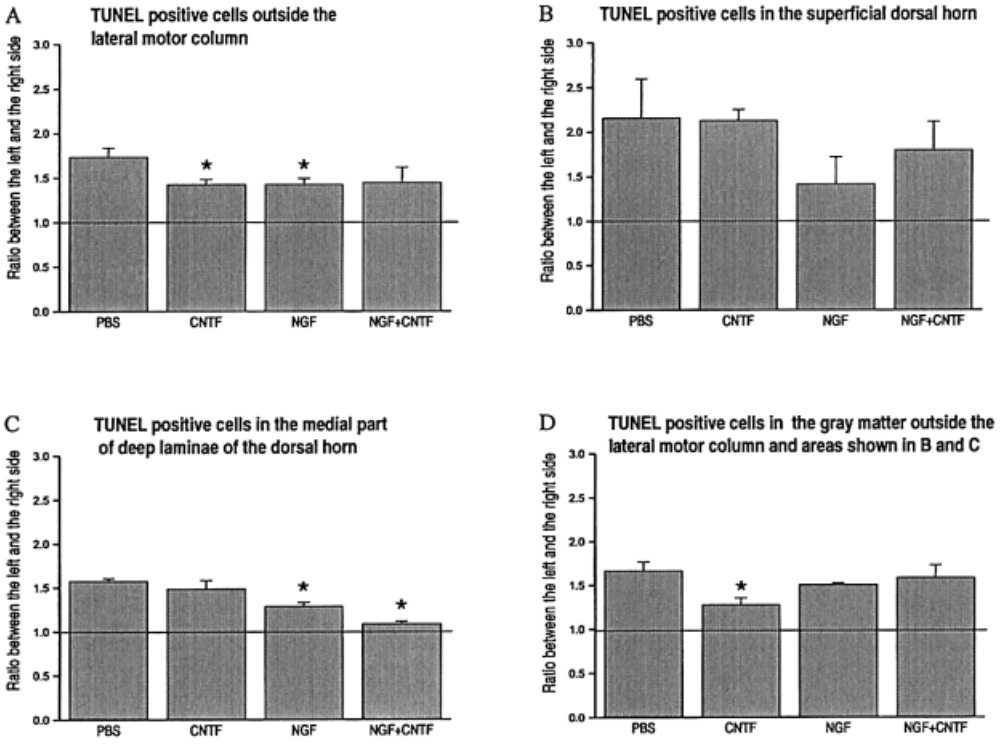


Fig. 10. **A:** Graphs showing the effects of PBS, CNTF, NGF, and a mixture of CNTF and NGF on the induction of TUNEL labeling of interneurons after sciatic nerve lesion. The bars represent ratios between the numbers of interneurons on the ipsi- vs. the contralateral sides of the spinal cord. **B–D:** Graphs as in A, but selecting TUNEL-labeled interneurons with a location in the superficial dorsal horn (B), the medial deep part of the dorsal horn (C), and all other parts of the gray matter outside the lateral motor column (D). The respective areas are shown in Figure 5.

the severed neuron, with a down-regulation of a number of substances, such as calcitonin gene-related peptide, substance P, and other neuropeptides (Jessel et al., 1979; Nielsch et al., 1987; Xu et al., 1990; Dumoulin et al., 1991; Kashiba et al., 1992; Hökfelt et al., 1994), and an up-regulation of neurotrophins and their receptors, such as BDNF and trkB (Ernfors et al., 1993). This may in turn mean that any trophic influences through this route may malfunction and provoke interneurons to die before any death among the afferent neurons themselves is detected.

Effects of CNTF and NGF on axotomized neurons

It is well known that by the application of neurotrophic factors to the proximal stump of the transected nerve in the lesion model studied here, it is possible to rescue directly affected motoneurons and DRG neurons. Among such neurotrophic substances, NGF has been reported to save DRG neurons after axotomy (Yip et al., 1984), although no trophic effects by NGF have been found on axotomized motoneurons in the neonatal rat (Yan et al., 1993), which is consistent with the lack of the high-affinity NGF receptor trkA in these cells. Still, in the neonatal mouse, some beneficial effect of NGF on motoneuron survival after axotomy has been reported (Li et al., 1994). With regard to CNTF, it has been repeatedly reported to prevent death of motoneurons after neonatal axotomy, at least transiently (Sendtner et al., 1991; Oppenheim et al., 1991; Vejsada et al., 1995; Ikeda et al., 1996; Tan et al., 1996; Ulenkate et al., 1996). CNTF has also been claimed to counteract apoptotic death of DRG neurons following neonatal axotomy in the chick and mouse (Lo et al., 1995). In our hands and at the time interval studied (24 hours after lesion), NGF had no effect on the TUNEL labeling frequency of motoneurons, whereas there was a pronounced reduction of TUNEL labeling of DRG neurons. With regard to CNTF, some reduction of TUNEL labeling frequency was indeed seen in the DRG, but the effect on the motoneurons was much more significant. Thus, we have concluded that the use of NGF and CNTF in our lesion model allows us to study in a reasonably selective way the relative contribution of the afferent input and target of the interneurons for their survival.

Contribution of afferent input and target neurons to interneuron survival

After NGF treatment, which rescues a large portion of the sensory DRG neurons, the number of TUNEL-labeled interneurons decreased, particularly in the superficial laminae of the dorsal horn, i.e., in those parts of the spinal cord that are most densely innervated by primary afferent fibers (Brown, 1991). CNTF also rescued interneurons, but in this case the predominant effects were seen in deeper parts of the dorsal horn and in the ventral horn, for which it can be anticipated that premotor interneurons are located (Burke, 1990; Brown, 1991; Jankowska, 1992). The combined effect of CNTF and NGF was most clearly demonstrated in the medial parts of laminae IV–VI, which

is the location for type Ia and type Ib interneurons, mediating disynaptic activity from primary afferent fibers to motoneurons (Jankowska, 1992).

Our findings lend strong support to the idea that the sources for trophic support of a neuron should not be restricted to the target of the neuron or to the environment of its axonal pathway but also include the afferent input to the neuron (see, e.g., Burek and Oppenheim, 1996; Lowrie and Lawson, 2000). This has previously been most clearly demonstrated in studies in which peripheral sense organs have been removed. For example, removal of the eye in newborn mice is followed by a massive degeneration of the superior colliculus (DeLong and Sidman, 1962) and lateral geniculate (Heumann and Rabinowicz, 1980). Thus, when studying the mechanisms underlying cell death among neurons as a consequence of normal development or after early lesions, it seems necessary to consider an interaction between the target and the afferent input in control of the cell death process (see, e.g., Linden, 1994). The impact of the afferent input in this sense should be of particular interest when studying neurons with an elaborately developed dendritic system, which allows a large number of inputs to exert their effects.

LITERATURE CITED

Adams JM, Cory Z. 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281:1322–1326.

Ashkenazi A, Dixit VM. 1998. Death receptors: signaling and modulation. Science 281:1305–1308.

Brown AG. 1991. Functional organization in groups of neurons. In: Nerve cells and nervous systems. An introduction to neuroscience. London: Springer-Verlag. p 159–175.

Burek MJ, Oppenheim RW. 1996. Programmed cell death in the developing nervous system. Brain Pathol 6:427–446.

Burke RE. 1990. Spinal cord: Ventral horn. In: Shepherd GM, editor. The synaptic organization of the brain. New York: Oxford University Press. p 88–132.

Clarke PG. 1990. Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol 181:195–213.

Clarke PG, Hornung JP. 1989. Changes in the nuclei of dying neurons as studied with thymidine autoradiography. J Comp Neurol 283:438–449.

Coggeshall RE, Pover CM, Fitzgerald M. 1994. Dorsal root ganglion cell death and surviving cell numbers in relation to the development of sensory innervation in the rat hindlimb. Dev Brain Res 82:193–212.

DeLong GR, Sidman RL. 1962. Effects of eye removal at birth on histogenesis of the mouse superior colliculus: an autoradiographic analysis with tritiated thymidine. J Comp Neurol 118:205–219.

Dumoulin FL, Raivich G, Streit WJ, Kreutzberg GW. 1991. Differential regulation of calcitonin gene-related peptide (CGRP) in regenerating rat facial nucleus and dorsal root ganglion. Eur J Neurosci 3:338–342.

Ernfors P, Rosario CM, Merlio JP, Grant G, Aldskogius H, Persson H. 1993. Expression of mRNAs for neurotrophin receptors in the dorsal root ganglion and spinal cord during development and following peripheral or central axotomy. Mol Brain Res 17:217–226.

Fawcett JW. 1992. Intrinsic neuronal determinants of regeneration. TINS 15:5–8.

Gavrieli Y, Sherman Y, Bem-Sasson SA. 1992. Identification of cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501.

Hamburger V. 1958. Regression versus peripheral control of differentiation on motor hypoplasia. Am J Anat 102:365–409.

Harris A.J, McCaig CD. 1984. Motoneuron death and motor unit size during embryonic development of the rat. J Neurosci 4:13–24.

Heumann D, Rabinowicz T. 1980. Postnatal development of the dorsal lateral geniculate nucleus in the normal and enucleated albino mouse. Exp Brain Res 38:75–85.

Hökfelt T, Zhang X, Wiesenfeld-Hallin Z. 1994. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. TINS 17:22–30.

Ikeda K, Iwasaki Y, Shiojima T, Kinoshita M. 1996. Neuroprotective effect of various cytokines on developing spinal motoneurons following axotomy. J Neurol Sci 135:109–113.

Ip NY, Yancopulos GD. 1995. Neurotrophic factors and their receptors. Progr Brain Res 105:189–195.

Jankowska E. 1992. Interneuronal relay in spinal pathways from proprioceptors. Progr Neurobiol 38:335–378.

Jessel T, Tsuno A, Kanazawa I, Otsuka M. 1979. Substance P: depletion in the dorsal horn of the rat spinal cord after section of the peripheral process of primary sensory neurons. Brain Res 168:247–259.

Kashiba H, Senba E, Ueda Y, Tohyama M. 1992. Co-localized but target-unrelated expression of vasoactive intestinal polypeptide and galanin in rat dorsal root ganglion neurons after peripheral nerve crush injury. Brain Res 582:47–57.

Kashihara Y, Kuno M, Miyata Y. 1987. Cell death of axotomized motoneurons in neonatal rats and its prevention by peripheral reinervation. J Physiol 386:135–148.

Kerr JFR, Willie AH, Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 26:239–257.

Lawson SJ, Lowrie MB. 1998. The role of apoptosis and excitotoxicity in the death of spinal motoneurons and interneurons after neonatal nerve injury. Neuroscience 87:337–348.

Lawson SJ, Davis HJ, Bennett JP, Lowrie MB. 1997. Evidence that spinal interneurons undergo programmed cell death postnatally in the rat. Eur J Neurosci 9:794–799.

Levi-Montalcini R. 1987. The nerve growth factor 35 years later. Science 237:1154–1162.

Lewin GR, Barde Y-A. 1996. Physiology of the neurotrophins. Annu Rev Neurosci 19:289–317.

Li L, Oppenheim RW, Lei M, Houenou LJ. 1994. Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. J Neurobiol 25:759–766.

Li L, Houenou LJ, Wu W, Lei M, Prevette DM, Oppenheim RW. 1998. Characterization of spinal motoneuron degeneration following different types of peripheral nerve injury in neonatal and adult mice. J Comp Neurol 396:158–168.

Linden R. 1994. The survival of developing neurons: a review of afferent control. Neuroscience 58:671–682.

Lo AC, Li L, Oppenheim RW, Prevette D, Houenou LJ. 1995. Ciliary neurotrophic factor promotes the survival of spinal sensory neurons following axotomy but not during the period of programmed cell death. Exp Neurol 134:49–55.

Lowrie MB, Lawson SJ. 2000. Cell death of spinal interneurones. Progr Neurobiol 61:543–555.

Lowrie MB, Vrbová G. 1992. Dependence of posnatal motoneurons on their targets: review and hypothesis. TINS 15:80–84.

Lowrie MB, Lavalette D, Davis CE. 1994. Time course of motoneurons death after neonatal sciatic nerve crush in the rat. Dev Neurosci 16:279–284.

McConkey DJ, Orrenius S. 1996. Signal transduction pathways in apoptosis. Stem Cells 14:619–631.

Nielsch U, Bisby MA, Keen P. 1987. Effect of cutting or crushing the rat sciatic nerve on synthesis of substance P by isolated L5 dorsal root ganglia. Neuropeptides 10:137–145.

Oliveira ALR, Risling M, Deckner M, Lindholm T, Langone F, Cullheim S. 1997. Neonatal sciatic nerve transection induces TUNEL labeling of neurons in the rat spinal cord and DRG. Neuroreport 8:2837–2840.

Oppenheim RW. 1991. Cell death during the development of the nervous system. Annu Rev Neurosci 14:453–501.

Oppenheim RW, Prevette D, Qin-Wei Y, Collins F, Macdonald J. 1991. Control of embryonic motoneurons survival in vivo by ciliary neurotrophic factor. Science 251:1616–1618.

Oppenheim RW, Qin-Wei Y, Prevette D, Yan Q. 1992. Brain derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature 360:755–757.

Oppenheim RW, Flavell RA, Vinsant S, Prevette D, Kuan CY, Rakic P. 2001. Programmed cell death of developing mammalian neurons after genetic deletion of caspases. J Neurosci 21:4752–4760.

Peters A, Palay SL, Webster HdeF. 1991. The fine structure of the nervous system—neurons and their supporting cells. New York: Oxford University Press. p 494.

Pettmann B, Henderson CE. 1998. Neuronal cell death. Neuron 20:633–647.

Romanes G. 1946. Motor localisation and the effects of nerve injury on the ventral horn cells of the spinal cord. J Anat 80:117–131.

Schmalbruch H. 1987. Loss of sensory neurons after sciatic nerve section in the rat. Anat Rec 219:323–329.

Schmalbruch H. 1988. The effect of peripheral nerve injury on immature motor and sensory neurons and on muscle fibres. Possible relation to the histogenesis of Werdnig-Hoffmann disease. Rev Neurol 144:721–729.

Sendtner M, Kreutzberg GW, Thoenen H. 1990. Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature 345:440–441.

Sendtner M, Holtmann R, Kolbeck R, Thoenen H, Barde Y-A. 1992. Brain derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. Nature 360:757–759.

Tan SA, Deglon N, Zurn AD, Baetge EE, Bamber B, Kato AC, Aebisher P. 1996. Rescue of motoneurons from axotomy-induced cell death by polymer encapsulated cells genetically engineered to release CNTF. Cell Transplant 5:577–587.

Thornberry NA, Lazebnik Y. 1998. Caspases: enemies within. Science 281:1312–1316.

Ulenkate HJLM, Gispen W-H, Jennekens FGI. 1996. Effects of ciliary neurotrohic factor on retrograde cell reaction after facial nerve crush in young adult rats. Brain Res 717:29–37.

Vejsada R, Sagot Y, Kato AC. 1995. Quantitative comparison of the transient rescue effects of neurotrophic factors on axotomized motoneurons in vivo. Eur J Neurosci 7:108–115.

Walker NI, Harmon BV, Gobe GC, Kerr JF. 1988. Patterns of cell death. Methods Achiev Exp Pathol 13:18–54.

Whiteside G, Doyle CA, Hunt SP, Munglani R. 1998. Differential time course of neuronal and glial apoptosis in neonatal rat dorsal root ganglia after sciatic nerve axotomy. Eur J Neurosci 10:3400–3408.

Wyllie AH, Kerr JF, Currie AR. 1980. Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306.

Xu X-J, Wiesenfeld-Hallin Z, Villar MJ, Fahrenkrug J, Hökfelt T. 1990. On the role of galanin, substance P and other neuropeptides in primary sensory neurons of the rat: studies on spinal reflex excitability and peripheral axotomy. Eur J Neurosci 2:733–743.

Yan Q, Elliot JL, Snider WD. 1992. Brain derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell dath. Nature 360:753–755.

Yan Q, Elliott JC, Matheson C, Sun J, Zhang L, Mu X, Rex KL, Snider WD. 1993. Influences of neurotrophins on mammalian motoneurons in vivo. J Neurobiol 24:1555–1577.

Yip HK, Rich KM, Lampe PA, Johnson EM Jr. 1984. The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. J Neurosci 4:2986–2992.