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RESEARCH ARTICLE

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# Delayed loss of spinal motoneurons after peripheral nerve injury in adult rats: a quantitative morphological study

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**Abstract** The existence of retrograde cell death in sensory dorsal root ganglion (DRG) cells after peripheral nerve injury is well established. However, with respect to retrograde motoneuron death after peripheral nerve injury, available data are conflicting. This may partly be due to the cell counting techniques used. In the present study, quantitative morphometric methods have been used to analyse retrograde motoneuron death induced by spinal nerve injury in adult rats. For comparison, DRG cells were also included in the study. The C7 spinal nerve was transected about 10 mm distal to the DRG and exposed to the fluorescent tracer fast blue in order to retrogradely label the spinal motoneurons and DRG cells of the C7 segment. At 1–16 weeks postoperatively, the nuclei of fast-blue-labelled C7 motoneurons and DRG cells were counted in consecutive 50-µm-thick serial sections. For comparison, the physical disector technique and measurements of neuronal density were also used to calculate motoneuron number. The counts of fast-blue-labelled motoneurons revealed a delayed motoneuron loss amounting to 21% and 31% after 8 and 16 weeks, respectively ($P$<0.001). The remaining motoneurons exhibited 20% ($P$<0.05) soma atrophy. Using the physical disector technique, the motoneuron loss was 23% ($P$<0.001) after 16 weeks. Calculations of neuronal density in Nissl-stained sections failed to reveal any motoneuron loss, although after correction for shrinkage of the ventral horn a 14% ($P$<0.001) motoneuron loss was found. The fast-blue-labelled DRG neurons displayed 51% ($P$<0.001) cell loss after 16 weeks, and the remaining cells showed 22% ($P$<0.001) soma atrophy. In summary, cervical spinal nerve injury induces retrograde degeneration of both motoneurons and DRG cells. However, to demonstrate the motoneuron loss adequate techniques for cell counts have to be employed.

**Keywords** Axon reaction · Retrograde cell death · Atrophy · DRG neurons · Brachial plexus injury

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## Introduction

Lesions to the cervical spinal nerves and roots are common injuries affecting mainly young individuals involved in traffic accidents. The functional restoration is poor and usually results in severely disabling motor and sensory deficits (Kay 1998; Terzis et al. 1999). This may at least in part be due to retrograde cell death among the injured neurons, which will restrict the capacity for peripheral nerve regeneration (Thanos et al. 1998).

Following peripheral nerve injury, the axotomized motor and sensory neurons undergo a series of retrograde degenerative changes, which may result in neuronal death (for reviews see Aldskogius et al. 1992; Bisby and Tetzlaff 1992; Lowrie and Vrbová 1992; Snider et al. 1992; Aldskogius and Svensson 1993; Kreutzberg 1995; De la Cruz et al. 1996; Aldskogius and Kozlova 1998). It is generally believed that interruption of intra-axonal transport of neurotrophic factors from the periphery to the cell bodies plays a role in this degenerative reaction (Lindsay 1994; Kreutzberg 1995; Sendtner 1996; Gillen et al. 1997; Hefti 1997; Terenghi 1999), since the motoneurons and dorsal root ganglion (DRG) cells seem to depend for their integrity upon trophic factors derived from their peripheral targets (reviewed in Davies 1996; Lindsay 1996) and from the Schwann cells surrounding the peripheral axons (Terenghi 1999).

In previous studies, the amount of cell loss observed after peripheral nerve injury has shown considerable variation. This may be explained by differences in age and species of the animal, type of neuron, proximodistal level of the lesion, postoperative survival time and type of injury. For example, it seems that in newborn animals

almost any type of peripheral nerve injury induces cell death among the axotomized motor and sensory neurons (reviewed in Lowrie and Vrbová 1992; Snider et al. 1992). In adult animals, the extent of cell loss varies between different studies, but it is generally agreed that both DRG neurons (for reviews see Aldskogius et al. 1992; Tandrup et al. 2000) and cranial motoneurons degenerate after peripheral nerve *transection* (Törnqvist and Aldskogius 1994; Yu 1997; Johnson and Duberley 1998; Mattsson et al. 1999) and that spinal motoneurons are lost after ventral root *avulsion* (Hoffmann et al. 1993; Wu 1993; Koliatsos et al. 1994; Novikov et al. 1995, 1997; Martin et al. 1999).

In adult spinal motoneurons subjected to distal *crush injury* followed by regeneration, no cell loss was found (Arvidsson and Aldskogius 1982; Crews and Wigston 1990; Johnson et al. 1991; Swett et al. 1991; Yu 1997; Kuzis et al. 1999). After permanent peripheral nerve *transection* or limb *amputation*, complete motoneuron survival has been reported in several studies (Carlson et al. 1979; Schmalbruch 1984; Johnson et al. 1991; Vanden Noven et al. 1993; Piehl et al. 1995; Anneser et al. 2000), although retrograde motoneuron death has also been described (Casanovas et al. 1996), especially after long postoperative periods (Kawamura and Dyck 1981; Suzuki et al. 1995). It has also been reported that ventral root axotomy within a distance of 2 mm from the spinal cord induces 40–70% motoneuron loss in rats (Gu et al. 1997), while no cell death follows more distal transection of the ventral roots in either rats (Wu 1993; Gu et al. 1997) or cats (Hoffmann et al. 1993).

Since the variability in results between previous studies may at least partly depend on the techniques used for neuronal counts, the present morphometric investigation was undertaken to analyse quantitatively the extent of retrograde motoneuron death induced by peripheral nerve injury in adult rats. The results indicate that spinal motoneurons undergo delayed degeneration after spinal nerve transection, but that the extent of observed cell loss is highly dependent on the technique used for cell counting.

## Materials and methods

### Experimental animals

The experiments were performed on adult (age 10–12 weeks; *n*=41) female Sprague-Dawley rats (Möllegaard Breeding Centre, Denmark). The animal care and experimental procedures were carried out in accordance with the standards established by the NIH Guide for Care and Use of Laboratory Animals (National Institutes of Health Publications No. 86–23, revised 1985) and the European Communities Council Directive (86/609/EEC). This study was also approved by the Northern Swedish Committee for Ethics in Animal Experiments. All surgical procedures were carried out with the animals under general anaesthesia using a mixture of ketamine (Ketalar, Parke-Davis; 100 mg/kg i.v.) and xylazine (Rompun, Bayer; 10 mg/kg i.v.).

### Fast blue labelling of spinal motoneurons and DRG cells

In order to retrogradely label the C7 spinal motoneurons and DRG cells, an incision was made along the right clavicle. After splitting the major pectoral muscle, the brachial plexus was exposed. The ventral branch of the C7 spinal nerve was identified and transected about 10 mm distal to the C7 DRG and at a distance of 15–20 mm from the C7 spinal cord surface. The proximal end of the cut nerve was inserted into a small cup filled with a 2% aqueous solution of the fluorescent dye fast blue (Sigma). The cup was sealed with a mixture of silicone grease and Vaseline and maintained in situ for 2 h. After removal of the cup, the nerve was rinsed in saline and covered with a thin sheet of Parafilm to prevent dye leakage into the surrounding tissue. The muscles and skin were closed in layers and the rats were given saline (2 ml s.c.) and benzylpenicillin (Boehringer Ingelheim; 60 mg i.m.). The survival times after fast blue labeling were 1 week (*n*=11), 4 weeks (*n*=6), 6 weeks (*n*=6), 8 weeks (*n*=6) and 16 weeks (*n*=12). The animals surviving for 1 week after axotomy and fast blue labelling served as controls. In a previous study, we have shown that fast blue produces a very efficient retrograde labelling of spinal motoneurons at 1 week after tracer application and that the staining remains constant for at least 6 months (Novikova et al. 1997b). Another advantage is that the fast-blue-labelled motoneurons can be easily distinguished in the spinal cord sections and, therefore, other cells in the ventral horn will not be included in the motoneuron counts. In addition, since the number of labelled motoneurons is independent of changes in neuronal density, the counts will not be affected by tissue shrinkage secondary to loss or atrophy of motoneurons.

### Processing of tissue

After 1–16 weeks survival, the rats were deeply anaesthetized with an overdose of sodium pentobarbital (240 mg/kg, Apoteksbolaget, Sweden) and transcardially perfused with Tyrode's solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The C6–C8 spinal cord segments and the C7 DRGs were removed and postfixed overnight. The DRGs were cryoprotected in 20–30% sucrose for 2–3 days at 4°C, embedded in Tissue-Tek (OCT, Miles Inc., Elkhart, IN) and frozen at –80°C. The spinal cord block was cut serially into 50-µm-thick parasagittal sections (for counts of fast-blue-labelled motoneurons at 1, 4, 6, 8 and 16 weeks postoperatively) or transverse sections (for disector method and profile counts at 1 and 16 weeks postoperatively) on a Vibratome (Leica Instruments, Germany). The DRGs were cut into serial 40-µm-thick sections on a cryomicrotome.

All sections were mounted on gelatin-coated slides, dried overnight, briefly cleared in xylene, embedded in DPX and stored at 4°C. In each transverse spinal cord section at 1 and 16 weeks postoperatively, the area occupied by fast-blue-labelled motoneurons was outlined (see below). The sections were then counterstained with cresyl violet for calculation of neuronal density and physical disector measurements (see below).

### Counts of fast-blue-labelled neurons

At 1, 4, 6, 8 and 16 weeks postoperatively, fast-blue-labelled motoneurons and DRG cells were identified under a Leitz Aristoplan microscope using Leitz filter block A. The nuclear profiles of the labelled cells were counted throughout the entire series of sections at ×250 magnification (Novikova et al. 1997b; Novikov et al. 2000). The numbers of nuclear profiles were not corrected for split nuclei, since no significant difference in nuclear size between the operated animals at 1 week and 16 weeks postoperatively was found with regard to either motoneurons (14.51±0.33 µm and 14.00±0.36 µm, respectively; mean ± SEM; $P>0.05$) or DRG neurons (14.31±0.22 µm and 13.59±0.36 µm, respectively; mean ± SEM; $P>0.05$) and since the nuclear diameters were small in comparison with the section thickness used (Coggeshall and Lekan 1996). Also, when comparing counts of nuclear profiles in a pool of fast-blue-labelled motoneurons with the data obtained after

complete serial reconstructions of the same neurons, the recorded cell numbers differ by less than 3%, which indicates that the former counting method is reliable (Novikov et al. 1995, 1997, 2000). The cross-sectional soma area of the labelled neurons was measured with a Eutectic Neuron Tracing System (Raleigh, NC).

### Physical disector

The C7 motoneurons at 1 and 16 weeks after axotomy were counted in randomly selected sections (ten pairs per rat) using a modified physical disector method (Coggeshall and Lekan 1996; West 1999). The disector area was defined in each section with the aid of the Eutectic Neuron Tracing System at ×250 final magnification and corresponded to the cross-sectional area occupied by the retrogradely fast-blue-labelled motoneurons. In transverse reconstructions of the ventral horn, the disector area was mapped and printed out. With the aid of a drawing tube attached to a Leitz Dialux 22 microscope, an image of a reference section counterstained with cresyl violet was superimposed on the printout of the disector area. Multipolar profiles with clearly visible Nissl bodies and diameters exceeding 10 µm were considered to be neurons, while small (5–8 µm) profiles with dark nuclei and a light rim of cytoplasm were classified as glial cells. After outlining all the neuronal profiles within the borders of the disector area, the reference section was replaced by the adjacent look-up section. If a neuronal profile was present in the reference section but not in the look-up section, it was included in the counts. The total number of neurons was calculated for a length of 1 mm of the C7 spinal cord segment using the formula: 20× (counted number of neurons/number of counted pairs of sections).

### Profile counts and estimation of neuronal density

Most previous studies of retrograde motoneuron degeneration have operated with total number of neuronal profiles or neuronal density (= number of neurons per unit area). Also in the present study, neuronal density was calculated to allow comparisons with previously reported data on motoneuron survival. In 50-µm-thick cresyl-violet-stained sections of the very same animals as those used for physical disector measurements, an unbiased sampling frame (West 1993, 1999) with a size of 250×250 µm (i.e. 25–30% of the total area occupied by the FB-labelled motoneurons; see comments on sampling frame size by Benes and Lange 2001) was randomly superimposed on an optical section through the most ventrolateral part of the ventral horn. Motoneurons were distinguished from glial cells as described above. Cell counts were made both on the control and operated sides of the spinal cord. Neuronal profiles located within the frame or being in contact with either of two adjacent borders of the frame were included in the counts. The data were sampled from 16–20 randomly selected sections and presented as mean neuronal density.

### Microphotography and image processing

Fast-blue-labelled spinal motoneurons were photographed under a Leitz Aristoplan microscope using Kodak 64T color reversal film. The film images were captured on computer with the aid of a Nikon LS-4500AF film scanner using 1800 dpi output resolution. Images of motoneurons stained with cresyl violet were captured with a Spot RT Color CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI) attached to a Nikon Eclipse E800 light microscope at 1520×1080 dpi optical resolution. The captured images were resized, grouped into a single canvas and labelled using Adobe Photoshop 5.5 software. The contrast and brightness were adjusted to provide optimal clarity.

### Statistical analyses

A one-way analysis of variance (ANOVA) followed by the post hoc Newman-Keuls test (counts of fast-blue-labelled motoneurons) and the unpaired *t*-test (neuronal density, physical disector and DRG neurons) were used to compare different experimental groups (Prism, GraphPad Software Inc.; San Diego, CA).

## Results

In control rats at 1 week after unilateral C7 spinal nerve transection, the C7 spinal cord segment contained 1086±37 (mean ± SEM) fast-blue-labelled motoneurons, which agrees with previously published data (Wu et al. 1994).

Counts of the total number of fast-blue-labelled C7 motoneurons at 4, 6, 8 and 16 weeks after the injury indicated that no cell loss occurred during the first 6 postoperative weeks. However, at 8 and 16 weeks postoperatively, 21% and 31%, respectively, of the labelled neurons had been lost ($P<0.001$; Fig. 1). At 4 weeks after the injury, the labelled motoneurons displayed a 12% increase in soma size ($P<0.05$), which gradually turned into 17–18% atrophy at 8–16 weeks postlesion ($P<0.05$; Fig. 1). In addition to the labelled motoneurons, fast blue was also observed in small cells with microglial appearance (Fig. 2A, B) and in small fragments probably representing disintegrated neurons (Rinaman et al. 1991; Angelov et al. 1995; Novikova et al. 2000).

The loss of C7 motoneurons at 16 weeks postoperatively resulted in 14% shrinkage ($P<0.0001$) of the area of the C7 ventral horn which was occupied by fast-blue-labelled cells (Fig. 3) and, consequently, a significant reduction of the reference space.

To compare the results obtained by counting nuclei of fast-blue-labelled motoneurons, the retrograde motoneuron loss was in addition calculated by means of the physical disector technique and by estimating neuronal density (see “Materials and methods”). Using the physical disector, it was found in the control animals at 1 week postoperatively that a 1 mm length of the C7 spinal cord

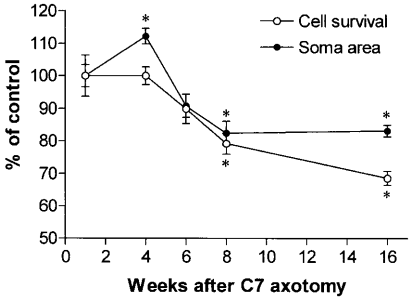


**Fig. 1** Time course of degeneration and atrophy of motoneurons after C7 spinal nerve transection ($n = 6$ animals in each group). *Error bars* indicate ± SEM. At 1 week, SEM for survival and cell atrophy is 3.4% and 6.3%, respectively. *$P<0.001$ (cell survival, experiment vs control) and $P<0.05$ (soma area, experiment vs control)

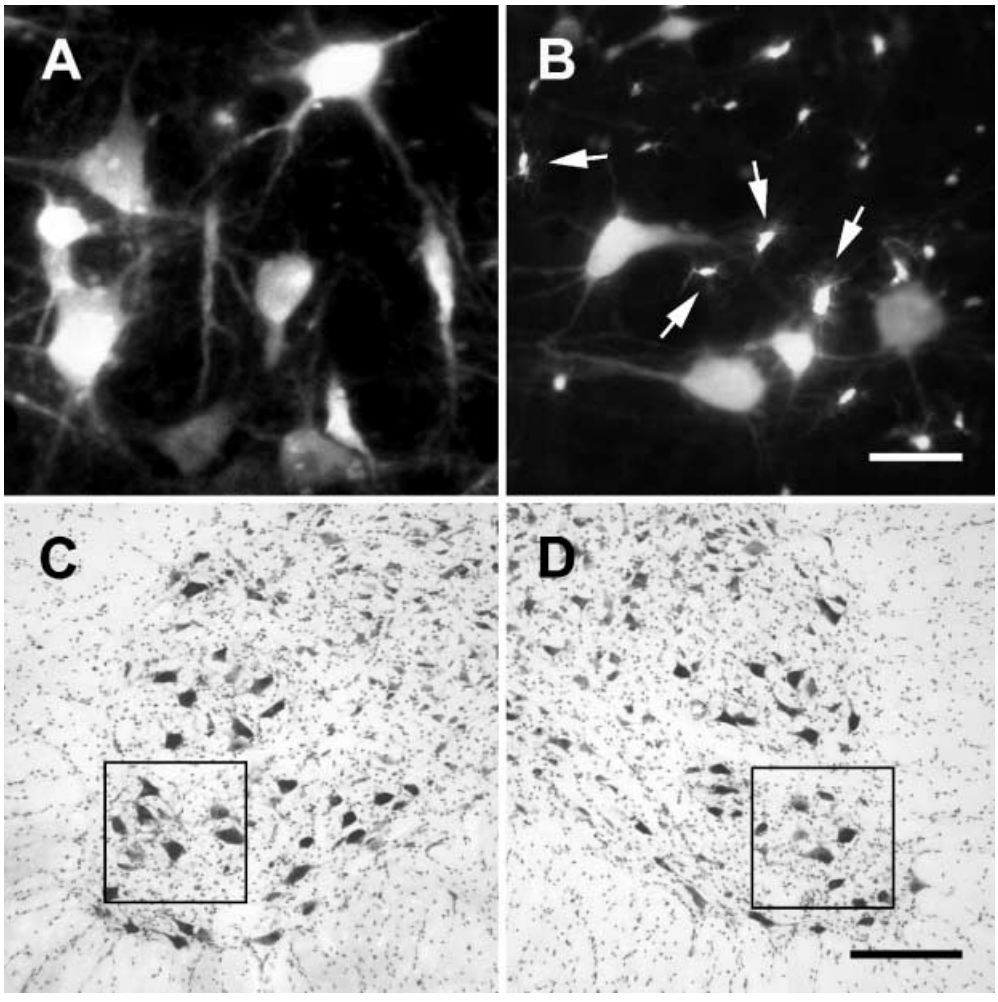


**Fig. 2** Fast-blue-labelled C7 spinal motoneurons at 1 week **(A)** and 16 weeks **(B)** after C7 spinal nerve transection. Examples of small fast-blue-labelled cells with microglial appearance are indicated by *arrows*. Cresyl-violet-stained transverse sections through the ventral horn of the intact **(C)** and operated **(D)** side of the C7 spinal cord segment at 16 weeks after C7 spinal nerve injury. The sampling frame was randomly applied to the most ventrolateral part of the ventral horn. Note that the numbers of neuronal profiles are similar on both sides, and that it is difficult to determine the exact border of the motor nucleus and to distinguish atrophic motoneurons from interneurons in Nissl-stained preparations **(D)**. *Scale bars* 50 μm **(A, B)**, 200 μm **(C, D)**

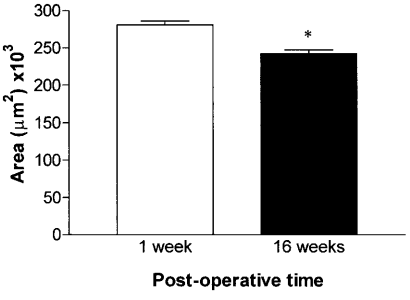


**Fig. 3** Ventral horn area occupied by fast-blue-labelled motoneurons at 1 and 16 weeks after C7 spinal nerve transection. *Error bars* indicate SEM **P*<0.05 (1 week vs 16 weeks postoperatively)

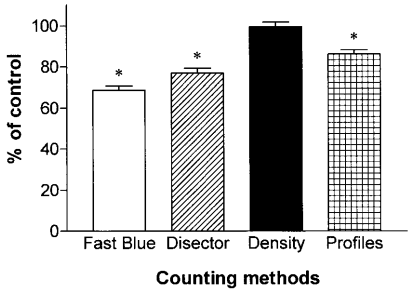


**Fig. 4** Number of surviving motoneurons at 16 weeks after C7 spinal nerve transection estimated by different counting techniques (*Fast Blue* counts of small nuclear profiles of fast-blue-labelled motoneurons in thick serial sections; *Disector* counts of Nissl-stained motoneurons using the physical disector technique; reference space, *V(ref)*, was calculated from the area occupied by fast-blue-labelled motoneurons; *Density* estimation of numerical density of neuronal profiles in Nissl-stained sections through the ventral horns using a sampling frame; *Profiles* neuronal density corrected for shrinkage of the ventral horn, see "Materials and methods"). *Error bars* indicate SEM. **P*<0.001 (experiment vs control for fast blue and disector; operated side vs control side for density and profiles)

segment contained 703±30 (mean ± SEM) neurons within the reference space defined by the distribution of fast-blue-labelled motoneurons. At 16 weeks postoperatively, the neuronal loss amounted to 23% ($P$<0.001; Fig. 4), which was significantly different from the value (31%) obtained using counts of fast-blue-labelled motoneurons (normalized data sets; $t$-test, $P$<0.05).

In contrast, counts of Nissl-stained profiles located within the sampling frame (Fig. 2C, D) did not show *any*

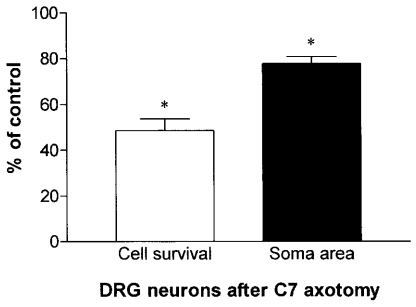


**Fig. 5** Survival and soma area of C7 DRG neurons at 16 weeks after C7 spinal nerve transection ($n$ = 6 animals in each group). *Error bars* indicate SEM. *$P$<0.001 (experiment vs control)

change in neuronal density between 1 week (11.74±0.26 profiles per frame) and 16 weeks (11.68±0.26 profiles per frame; $P$>0.05; Fig. 4) postoperatively. However, when compensating for the postoperative shrinkage of the ventral horn by instead calculating the neuronal density of the reference space outlined by fast-blue-labelled motoneurons (Fig. 3), a 14% cell loss was found ($P$<0.001).

In the fast-blue-labelled DRG cells, the counts of nuclear profiles revealed a 51% cell loss ($P$<0.001) between 1 week (9323±209) and 16 weeks (4533±237) postoperatively. In the latter group, the remaining cells exhibited 22% atrophy ($P$<0.01; Fig. 5).

## Discussion

The present study shows that axotomy of a cervical spinal nerve in adult rats induces not only a retrograde loss of DRG neurons (Aldskogius et al. 1992; Tandrup et al. 2000), but also delayed degeneration of spinal motoneurons. With respect to the different methods for calculating cell number, the counts of fast-blue-labelled neurons and the physical disector technique demonstrated a delayed motoneuron loss. In contrast, neuronal density measurements were unable to reveal the 20–30% cell loss found with previous methods, although a 14% reduction in motoneuron number was found after correction for postoperative shrinkage of the ventral horn.

### Methodological aspects on cell counts

In studies of neuronal degeneration, the selection of appropriate methods for cell counting is of crucial importance since biased estimates and low precision will otherwise lead to erroneous conclusions about the extent of neuronal loss.

Three methods for cell counts were employed in the present study to assess motoneuronal loss, namely counts of fast-blue-labelled neurons, a modified physical disector method, and profile counts for calculation of neuronal density. Although the latter method has been regarded as "biased" (Gundersen et al. 1988; West 1993; Oorschot 1994; Coggeshall and Lekan 1996; Hedreen 1998; West 1999), it was included for comparison since the vast majority of previous reports on retrograde degeneration of adult spinal motoneurons have been based on estimates of neuronal density (see "Introduction").

Retrograde neuronal tracers have been widely used to determine post-traumatic cell death in the nervous system (Mori et al. 1997; Bradbury et al. 1998; Houle and Ye 1999; Wang et al. 1999). With respect to motoneuron degeneration, several tracers have been successfully employed, including horseradish peroxidase (HRP) (Schmalbruch 1984; Johnson et al. 1991; Casanovas et al. 1996), fluoro-gold (Crews and Wigston 1990; Koliatsos et al. 1994) and fast blue (Wu et al. 1994; Novikov et al. 1995; Novikova et al. 1997a). For example, pre-labelling with fluoro-gold allows precise estimates of motoneuron loss after ventral root avulsion, as confirmed by comparisons with the optical disector method (Koliatsos et al. 1994). However, since neuronal labelling with fluoro-gold is stable for only about 4 weeks compared to at least 6 months for fast blue (Novikova et al. 1997b), the former tracer will be of limited value in long-term experiments. Similarly, HRP labelling persists only for a limited time and has been reported not to include all axotomized neurons (Peyronnard et al. 1986), although this has since been questioned (Swett et al. 1991).

An important advantage of using fast blue labelling *in combination* with the disector method is that any change in reference volume, *V(ref)* (Gundersen et al. 1988; West 1993), caused by tissue shrinkage and neuronal atrophy can be precisely estimated. It is virtually impossible to determine *V(ref)* in sections where only three to four neuronal profiles remain which, in addition, may exhibit pronounced atrophy (see also Fig. 2 in Novikov et al. 1997). The decrease in *V(ref)* after peripheral nerve injury is most likely related to degenerative changes in the motoneuron dendrites (Standler and Bernstein 1982; Brännström et al. 1992; O'Hanlon and Lowrie 1995) and will, if defined inaccurately, result in biased estimates of motoneuron number even with the disector technique. Yet another proposed stereological method, the fractionator (Gundersen et al. 1988; West 1993; Oorschot 1994; Coggeshall and Lekan 1996), which does not require estimation of *V(ref)*, will probably not be effective in counts of spinal motoneurons, since it will estimate the *total* number of neurons in the area rather than the number of neurons belonging to a specific neuronal population, e.g. axotomized motoneurons. In the present study, the difference in cell number obtained between the counts of fast-blue-labelled motoneurons, on one hand, and the physical disector counts of neurones located within the reference volume, on the other, might be explained similarly. Thus, in the former case only the motoneurons belonging to the transected ventral branch of the C7 spinal nerve were labelled and counted, while in the latter case the reference space also included the motoneurons of the uninjured dorsal branch of the C7 spinal nerve. Therefore, in the present experiments the physical disector method will underestimate the number

of axotomized motoneurons lost after spinal nerve injury. Moreover, the physical disector method may not distinguish between atrophic motoneurons and normal interneurons in the ventral horn (see, e.g. Fig. 2D). In the present study, a correction factor compensating for postoperative shrinkage of *V(ref)* resulted in a significant motoneuron loss after peripheral nerve transection.

In summary, therefore, it seems advantageous to combine prelabelling of neurons with stereological methods in order to accurately determine nuclear borders and to increase the precision of neuronal counts in both the intact and injured nervous system.

## Factors influencing retrograde degeneration of adult spinal motoneurons

Several factors have been described to influence the extent of retrograde motoneuron death after peripheral nerve injury in adults, such as the distance from injury site to the cell body, the type of nerve lesion and the postoperative survival time.

With respect to cranial motoneurons, transection of the trochlear (Book et al. 1996) and facial nerves (Mattsson et al. 1999) close to the brainstem results in more extensive cell loss than distal injury of the same nerves. On the other hand, axotomy of the hypoglossal nerve at a distance of 8 mm from the brainstem has been reported to produce motoneuron loss similar to a more distal axotomy (Törnqvist and Aldskogius 1994).

With respect to spinal motoneurons, previous studies have reported that no significant cell death occurs at 6–16 weeks after transection of the C7 motor axons at a distance exceeding 2 mm from the spinal cord surface (Hoffmann et al. 1993; Wu et al. 1994; Gu et al. 1997). However, in these investigations profile counts or estimates of neuronal density were used to determine neuronal survival and, furthermore, shrinkage of the ventral horn secondary to motoneuron degeneration was not taken into account. Although the latter methods failed to reveal any motoneuron loss in the present study, it should be noted that in the case of a massive 70–100% motoneuronal loss following, e.g. ventral root avulsion, all counting methods including profile counts and estimates of neuronal density (Hoffmann et al. 1993; Wu 1993; Li et al. 1995; Piehl et al. 1995; Kishino et al. 1997; Martin et al. 1999), fast blue labelling with serial reconstructions (Novikov et al. 1995; Novikov et al. 1997), counts of fast blue/fluorogold-stained neurons (Koliatsos et al. 1994; Novikova et al. 1997a) and the optical disector (Koliatsos et al. 1994) demonstrate a significant reduction in motoneuron number. It seems, therefore, that the profile counting technique is capable of revealing massive cell death, but fails to detect small or moderate cell losses. The low sensitivity of the technique is probably not only related to the issue of "split profiles" in profile-based sampling methods (Coggeshall and Lekan 1996; Guillery and Herrup 1997; Hedreen 1998; West 1999), but also to the failure to observe and compensate for changes in reference volume when applying the technique to, e.g. Nissl-stained preparations (see also review by Gahr 1997).

In human patients with extremely long survival times after proximal limb amputation (Kawamura and Dyck 1981) or shoulder amputation (Suzuki et al. 1995), a substantial motoneuron loss has been described. Although the latter reports used the "insensitive" profile counting method, the retrograde degeneration was apparently extensive enough to overcome the relative increase in neuronal density caused by shrinkage of the ventral horn.

## Delayed loss of motoneurons after spinal nerve injury

Delayed onset of retrograde degeneration has been described in axotomized adult rubrospinal neurons after cervical spinal cord injury (Houle and Ye 1999; Novikova et al. 2000), in DRG neurons after peripheral nerve transection (Tandrup et al. 2000) and in various cortical and hippocampal regions after traumatic brain injury (Smith et al. 1997). Although the mechanism underlying the delay in retrograde degeneration after axotomy remains unclear, at least two factors may contribute to prolonged survival of axotomized adult motoneurons, namely intramedullary sprouting of "supernumerary motoraxons" and/or "dendraxons" (Lindå et al. 1985; Havton and Kellerth 1987) and an increased discharge of neurotrophic molecules from the non-neuronal cells surrounding the remaining proximal motor axons (reviewed in DiStefano and Curtis 1994; Sendtner et al. 1994; Friedman et al. 1995b; Gillen et al. 1997; Bartlett et al. 1998; Terenghi 1999).

In accordance with the latter hypothesis, a number of studies have demonstrated that different neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) (Yan et al. 1994; Novikov et al. 1995; Wang et al. 1997), neurotrophin-3 (Fernandes et al. 1998) and neurotrophin-4/5 (Friedman et al. 1995a), can rescue adult spinal motoneurons from retrograde degeneration after ventral root avulsion and, also, restore their cholinergic phenotype after peripheral nerve transection and stimulate motor axonal regeneration (Kishino et al. 1997; Novikov et al. 1997; Novikova et al. 1997a).

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