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**Brief Communication**

# Motoneuron-Derived Neurotrophin-3 Is a Survival Factor for PAX2-Expressing Spinal Interneurons

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Rat spinal cord interneurons undergo programmed cell death shortly after birth. We investigated here whether cell death of interneurons could be regulated by trophic factors produced by motoneurons, one of their main targets. To test this hypothesis, we studied the effect of the selective destruction of motoneurons on the survival of interneurons in organotypic cultures of embryonic rat spinal cords. Motoneurons were eliminated by an anti-p75$^{NTR}$-specific immunotoxin (192 IgG-saporin). We then observed a decrease of 28% in the number of ventral spinal interneurons immunoreactive (IR) for the homeoprotein PAX2. This was correlated with an increase in the number of apoptotic nuclei in the same area. Because neurotrophin-3 (NT-3) is specifically produced by motoneurons and because interneurons express the NT-3 high-affinity receptor trkC, we examined the role of NT-3 in the survival of PAX2-IR interneurons. Addition of NT-3 to 192 IgG-saporin-treated explants rescued ventral PAX2-IR interneurons. Depletion of secreted NT-3 by anti-NT-3 antibodies induced 66% loss of ventral PAX2-IR interneurons. We conclude that motoneuron-derived NT-3 is a trophic factor for ventral PAX2-IR interneurons.

*Key words: programmed cell death; spinal interneuron; motoneuron; 192 IgG-saporin; neurotrophin-3; PAX2*

In the spinal cord, developmental cell death has been studied extensively for motoneurons. In rat, approximately half of motoneurons die between embryonic day 15 (E15) and postnatal day (P1) (Oppenheim, 1986). Although interneurons constitute the majority of neurons within the spinal cord, there are few data on their developmental cell death. A first study in chick, based on the classic Nissl stain, found no evidence for developmental cell death of interneurons (McKay and Oppenheim, 1991). However, in rat, apoptosis-specific methods have shown that spinal interneurons also undergo programmed cell death (Lawson et al., 1997). Other studies have also reported apoptotic cells throughout the spinal cord in neonatal mice and rat (Oliveira et al., 1997; Grieshammer et al., 1998; White et al., 1998). In rat, the first apoptotic nuclei located outside the motor column appear after E16. At E20, the distribution of apoptotic nuclei extends into the intermediate gray matter, and, by P2, most of the apoptotic cells are detected in the dorsal horns (Lawson et al., 1997). The peak of interneuron apoptosis occurs between E20 and P2 and, after that, of motoneurons. Because motoneurons represent the principal target of ventral interneurons, we investigated whether the death of the latter could be regulated by motoneuron-derived trophic factors.

This was tested by analyzing the effect of the selective destruction of motoneurons on the survival of spinal interneurons using embryonic rat spinal cord explants. In this system, three-dimensional organization and connectivity are conserved, and motoneurons as well as interneurons undergo apoptosis as they do *in vivo* (Sedel et al., 1999). Motoneurons were selectively killed with a monoclonal antibody (IgG-192), raised against the low-affinity neurotrophin receptor p75$^{NTR}$, which is coupled to the ribosome-inactivating protein saporin (Wiley and Kline, 2000). In the developing rat spinal cord, only motoneurons express p75$^{NTR}$ (Yan and Johnson, 1988) and thus specifically bind this immunotoxin (192 IgG-saporin).

Using this approach, we show that elimination of motoneurons results in the death of ventral spinal interneurons expressing the homeoprotein PAX2. Neurotrophin-3 (NT-3) is specifically expressed by spinal motoneurons during the period of interneuron cell death (Henderson et al., 1993; Buck et al., 2000), and interneurons express trkC, the high-affinity NT-3 receptor (Henderson et al., 1993). Thus, we hypothesized that NT-3 exerts a trophic effect on PAX2-expressing interneurons. Such a function is supported by our experiments.

## MATERIALS AND METHODS

*Explant cultures.* The rostral part of brachial neural tubes from E13 rat embryos was dissected in PBS–glucose (33 mM). Explants (4 mm in length) corresponding to the neural tubes were opened dorsally and flattened on Biopore membranes (Millipore, Bedford, MA) as described previously (Sedel et al., 1999). The culture medium contained Neurobasal medium completed with B27, penicillin–streptomycin (100 U/ml), 200 mM L-glutamine, and 5% horse serum (reagents from Invitrogen). Explants were cultured in the absence (control) or presence of the following molecules diluted in culture medium: 192 IgG-saporin (200 ng/ml; Advanced Targeting Systems, San Diego, CA), NT-3 (200 ng/ml; Peprotech, London, UK), and rabbit anti-NT-3 (100 $\mu$g/ml, AB1780SP; Chemicon, Temecula, CA).

*Rat motoneuron and spinal interneuron cultures.* Motoneurons were purified from E14 embryos as described previously (Arce et al., 1999), plated at $2 \times 10^3$ cells/cm$^2$ in four-well dishes, and cultured in Neurobasal–B27 supplemented with 2% horse serum, 0.5 mM L-glutamine, 12.5 $\mu$M $\beta$-mercaptoethanol, ciliary neurotrophic factor (1 ng/ml), and glial cell line-derived neurotrophic factor (100 pg/ml) (Peprotech).

Primary cultures of spinal cord neurons were prepared from E14 embryos as described previously (Béchade et al., 1996). Neurons were

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plated at $10^5$ cells/cm$^2$ in four-well culture plates and maintained in Neurobasal–B27 medium.

*Antibodies and immunochemistry.* Primary antibodies used were as follows: rabbit anti-PAX2 (1:200; Zymed, San Francisco, CA), polyclonal goat anti-choline acetyltransferase (AB144, 1:1000; Chemicon), monoclonal anti-Islet-1 (1:100, clone 4D5; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Secondary antibodies were carboxymethyl indocyanine-3 (CY3)-goat anti-mouse IgG (1:200), Texas Red-donkey anti-goat IgG (1:200), and FITC-goat anti-rabbit IgG (1:200) (Jackson ImmunoResearch, West Grove, PA). Explants were fixed by immersion in 4% paraformaldehyde overnight at 4°C, transferred to PBS–30% sucrose for 24 hr at 4°C, and frozen in Tissue-Tek OCT. One transverse cryostat section 16-$\mu$m-thick from every 10 sections was mounted on Superfrost plus glass slides, incubated for 15 min in PBS with 0.1% Triton and 0.2% gelatin (PBGT), and incubated overnight at 4°C with the primary antibody diluted in PBGT. Slides were rinsed in PBS–Tween 20 (0.1%) (PBT) and incubated with the secondary antibody diluted in PBGT for 2 hr at room temperature. After washes in PBS, slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) supplemented with 4′,6′-diamidino-2-phenylindole (DAPI) (100 ng/ml; Roche Diagnostics, Hertforshire, UK). Controls without the primary antibody were negative for all immunostainings.

*Double terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling staining and immunochemistry.* Explants were fixed as above. Cryostat sections were permeabilized in PBS–Triton X-100 (0.1%) for 5 min, preincubated 15 min with terminal deoxynucleotidyl transferase buffer (Amersham Biosciences, Little Chalfont, UK), followed by incubation for 4 hr at 37°C in the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) reaction solution as described previously (Sedel et al., 1999). Then, they were washed three times in PBT and incubated overnight at 4°C with the anti-PAX2 antibody and streptavidin-FITC (1:200; Amersham Biosciences) diluted in PBT. After three washes in PBT, sections were incubated for 2 hr at room temperature with a donkey anti-rabbit IgG coupled with CY3 (Jackson ImmunoResearch) diluted 1:500 in PBT. Sections were washed three times in PBS and mounted in Vectashield containing DAPI.

*Confocal microscopy and quantification.* Sections were analyzed under a Leica (Nussloch, Germany) confocal microscope (objective 40×). The quantification of PAX2-immunoreactive (IR) interneurons and apoptotic nuclei were performed on digitized images. For each explant, the mean number of PAX2-IR interneurons and apoptotic nuclei per section were computed within the entire ventral horn. In the dorsal horn, the mean number of PAX2-IR interneurons was determined, in each experimental condition, from two squares ($88 \times 88$ $\mu$m$^2$) sampled at random. At least three independent experiments, using two to four explants, were done for each experimental condition. To evaluate the effects of the treatments, the means of PAX2-IR interneurons and apoptotic nuclei were expressed as percentages of control values. The SD for the control experiments derives from a normalization of each control value by the average of the number of PAX2-IR interneurons or apoptotic nuclei obtained in control explants. Comparisons between the mean values of control and treated explants were performed by statistical analysis using ANOVA, followed by a Scheffe's $F$ test.

## RESULTS

### Expression of PAX2 in spinal cord explants

During development, groups of spinal interneurons can be identified by expression of homeodomain transcription factors (Matise and Joyner, 1997), including PAX2, which is expressed in multiple spinal interneuron populations in chick and mouse (Nornes et al., 1990; Burrill et al., 1997). We used explants of E13 rat brachial neural tubes disconnected from supra-medullary afferents and peripheral target tissues as described previously (Sedel et al., 1999). After 6 d *in vitro* (DIV), PAX2 immunoreactivity was detected in many neurons of the ventral and dorsal horns (Fig. 1*A*), and the distribution pattern resembled that observed *in vivo* at E19, a stage equivalent to that analyzed in explants (E13 plus 6 DIV) (Fig. 1*B*). Double immunolabeling with choline acetyl transferase (ChAT) antibody, a specific motoneuronal marker (Phelps et al., 1991), showed that PAX2 antibodies did not label motoneurons (see Fig. 3*C*).

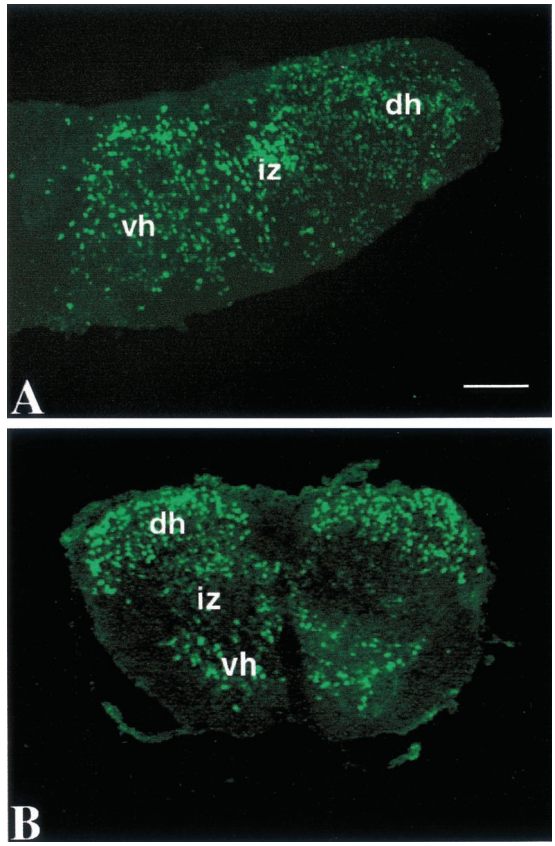


*Figure 1.* PAX2-IR neurons are detected in the dorsal and ventral horns in spinal cord explants after 6 DIV (*A*) and *in vivo* at E19 (*B*). *vh*, Ventral horn; *dh*, dorsal horn; *iz*, intermediate zone. Scale bar, 100 $\mu$m.

### Destruction of motoneurons in explants treated with 192 IgG-saporin results in death of ventral PAX2 interneurons

To check its motoneuronal specificity, 192 IgG-saporin was added after 2 DIV to cultures of spinal interneurons, as well as to cultures of purified motoneurons. In the latter, at 6 DIV, Islet-1-IR motoneurons (Tsuchida et al., 1994) were counted. Comparison of treated and control cultures (Fig. 2) indicated that motoneurons (Fig. 2*A*) but not interneurons (Fig. 2*B*) were killed by the immunotoxin.

We next examined whether 192 IgG-saporin could also selectively kill motoneurons in spinal cord explants. We found that, after 6 DIV, ChAT-IR motoneurons disappeared completely in the ventral horns of 192 IgG-saporin-treated explants (Fig. 3*A*), whereas they were still numerous in control explants (Fig. 3*C*). The death of motoneurons was accompanied by a visible decrease in the number of PAX2-IR interneurons within the ventral horns (Fig. 3*A*) quantified to be 28 ± 3% (mean ± SEM; $n = 8$). In these explants, PAX2 immunoreactivity-associated fluorescence of the remaining interneurons was as bright as that found in control explants, suggesting that the diminution of the number of PAX2-IR interneurons was not attributable to a decrease in PAX2 immunoreactivity (Fig. 3*A*). In the dorsal horns of treated explants, comparison with controls showed that the percentage of surviving PAX2-IR interneurons was 91.3 ± 2.6% (mean ± SEM; $n = 6$) and that the difference was not significant, empha-

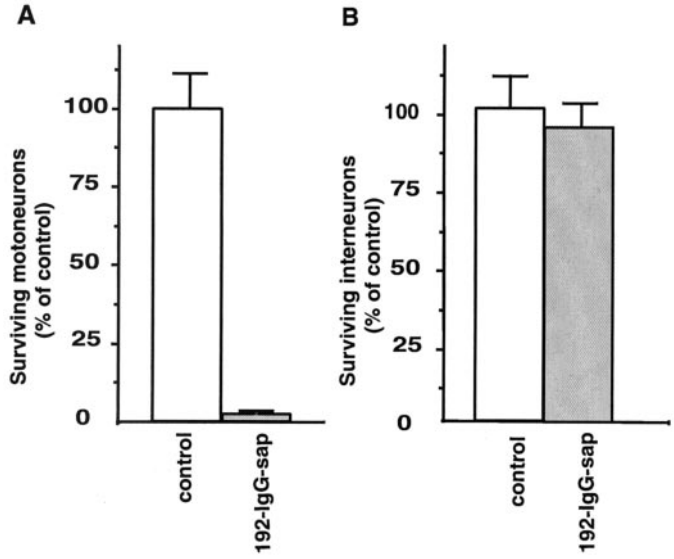


*Figure 2.* 192 IgG-saporin kills motoneurons (*A*) but not interneurons (*B*) in spinal cord primary cultures. Spinal interneurons and purified motoneurons were cultured for 2 DIV in culture medium and then for 4 d in the same medium without (control) or with 192 IgG-saporin. The number of motoneurons per well was 103 ± 11 and 2 ± 0.11 in control and treated cultures, respectively. The number of interneurons counted in a field with a 40× objective was 111 ± 11 and 104 ± 7.5 in control and treated cultures, respectively. Data are expressed as the percentage of surviving neurons compared with that of untreated neurons. Results from two independent experiments were pooled (mean ± SEM; $n = 4$ wells).

sizing the lack of toxicity of 192 IgG-saporin (Fig. 4*A*). TUNEL staining (Gavrieli et al., 1992) was used to investigate whether this loss resulted from apoptotic cell death. As seen from direct observations, the number of apoptotic nuclei was higher in the treated ventral horns (Fig. 3*D*) compared with control cultures and displayed a 105 ± 43% (mean ± SEM; $n = 6$) increase in treated explants compared with control explants (Fig. 4*B*). This increase did not correspond to dying motoneurons because, at 6 DIV, motoneurons were already dead in 192 IgG-saporin-treated explants (Fig. 3*A*). The increase in the number of apoptotic nuclei was greater than expected from the decrease in number of PAX2-IR interneurons. This could result from the fact that some PAX2-negative cells (glial cells or neurons) also underwent apoptosis. Altogether, our results established that the selective elimination of motoneurons resulted in the death of PAX2-IR interneurons. This suggested that motoneurons produced trophic factors required for the survival of these interneurons.

### NT-3 rescues PAX2-IR interneurons in 192 IgG-saporin-treated explants

If ventral PAX2-IR interneurons depend on NT-3 produced by motoneurons for their survival, addition of exogenous NT-3 to 192 IgG-saporin-treated explants should prevent them from dying. Indeed, in spinal cord explants cultured with both 192 IgG-saporin and NT-3, we found that the number of PAX2-IR ventral interneurons (Fig. 4*A*) and the number of TUNEL-positive nuclei (Fig. 4*B*) were comparable with that of controls (untreated explants) after 6 DIV. This indicates that the effect of NT-3 was not attributable to an increase in PAX2 immunoreactivity but rather results from a diminution of cell death. Thus, addition of exogenous NT-3 to 192 IgG-saporin-treated explants resulted in the rescue of virtually all PAX2-IR interneurons. Motoneurons were not rescued from death because ChAT immunoreactivity was still undetectable in the treated explants (data not shown). Moreover, we showed previously that addition of NT-3 to spinal cord explants does not promote motoneuronal survival (Sedel et al., 1999).

### Anti-NT-3 treatment results in death of PAX2-IR ventral interneurons

NT-3 is expressed by motoneurons in spinal cord explants (Sedel et al., 1999). We therefore treated explants with an antiserum that specifically blocks the biological activity of NT-3 (Zhou and Rush, 1995). After this treatment, very few PAX2-IR interneurons were observed in the ventral horn area (Fig. 3*C*): the decrease was 66 ± 4% (mean ± SEM; $n = 5$) compared with controls (Fig. 4*A*). This diminution of PAX2-IR ventral interneurons was associated with a 140 ± 15% (mean ± SEM; $n = 3$) increase in the number of apoptotic nuclei in the same area (Figs. 3*F*, 4*B*). Again, the increase in the number of apoptotic nuclei was higher than that expected given the decrease in the number of PAX2 interneurons. This suggests that anti-NT-3 most likely causes death of cells not expressing PAX2. In the dorsal horn, anti-NT-3 antibody had no effect on the number of PAX2-IR neurons (91 ± 2.6% of the control; mean ± SEM; $n = 8$) (Fig. 4*A*). Therefore, the survival effect of NT-3 is specific to ventral PAX2-IR interneurons.

In anti-NT-3-treated explants, labeling for anti-PAX2 and anti-ChAT antibodies resulted in yellow labeling of motoneurons (Fig. 3*C*). The FITC-conjugated anti-rabbit antibody used to reveal the anti-PAX2 antibody also binds to the rabbit anti-NT-3 antibody associated with ChAT-IR motoneurons visualized by a CY3 secondary antibody. This double labeling shows that the anti-NT-3 antibody is selectively bound to motoneurons. This is in agreement with the finding that NT-3 transcripts are specifically synthesized by motoneurons in spinal cord explants (Sedel et al., 1999). For additional confirmation, anti-NT-3 explants cultured for 6 DIV were immunostained with the anti-ChAT antibody revealed with a CY3 secondary antibody and a FITC secondary antibody to visualize the anti-NT-3 binding sites: we observed that ChAT-IR motoneurons, but not ChAT-negative neurons, were NT-3-IR (Fig. 3*B′*).

## DISCUSSION

192 IgG-saporin has been used extensively *in vitro* and *in vivo* as a powerful tool to eliminate $p75^{NTR}$-expressing neurons (Wiley and Kline, 2000). We found that the selective destruction of motoneurons by 192 IgG-saporin caused the death of ventral PAX2-IR interneurons. This death does not result from a direct killing effect of 192 IgG-saporin because (1) 192 IgG-saporin had no effect on the survival of spinal interneurons in culture nor on that of dorsal spinal interneurons in the explants, and (2) only $p75^{NTR}$-IR neurons (i.e., motoneurons) were killed by 192 IgG-saporin. We checked by immunochemistry that interneurons did not express $p75^{NTR}$ in treated explants (data not shown). An indirect toxic effect attributable to the liberation of toxic substances by dying motoneurons is unlikely because, in other *in vitro* systems, in cocultures containing basal forebrain and cortical neurons, elimination of basal forebrain neurons with 192 IgG-saporin induced death of a specific population of cortical neurons but did not damage other neurons (Ha et al., 1998). Therefore, the survival of PAX2-IR interneurons is most likely regulated by trophic factors produced by motoneurons.

We show here that the supply of NT-3 in the absence of motoneurons rescued PAX2-IR ventral interneurons from death

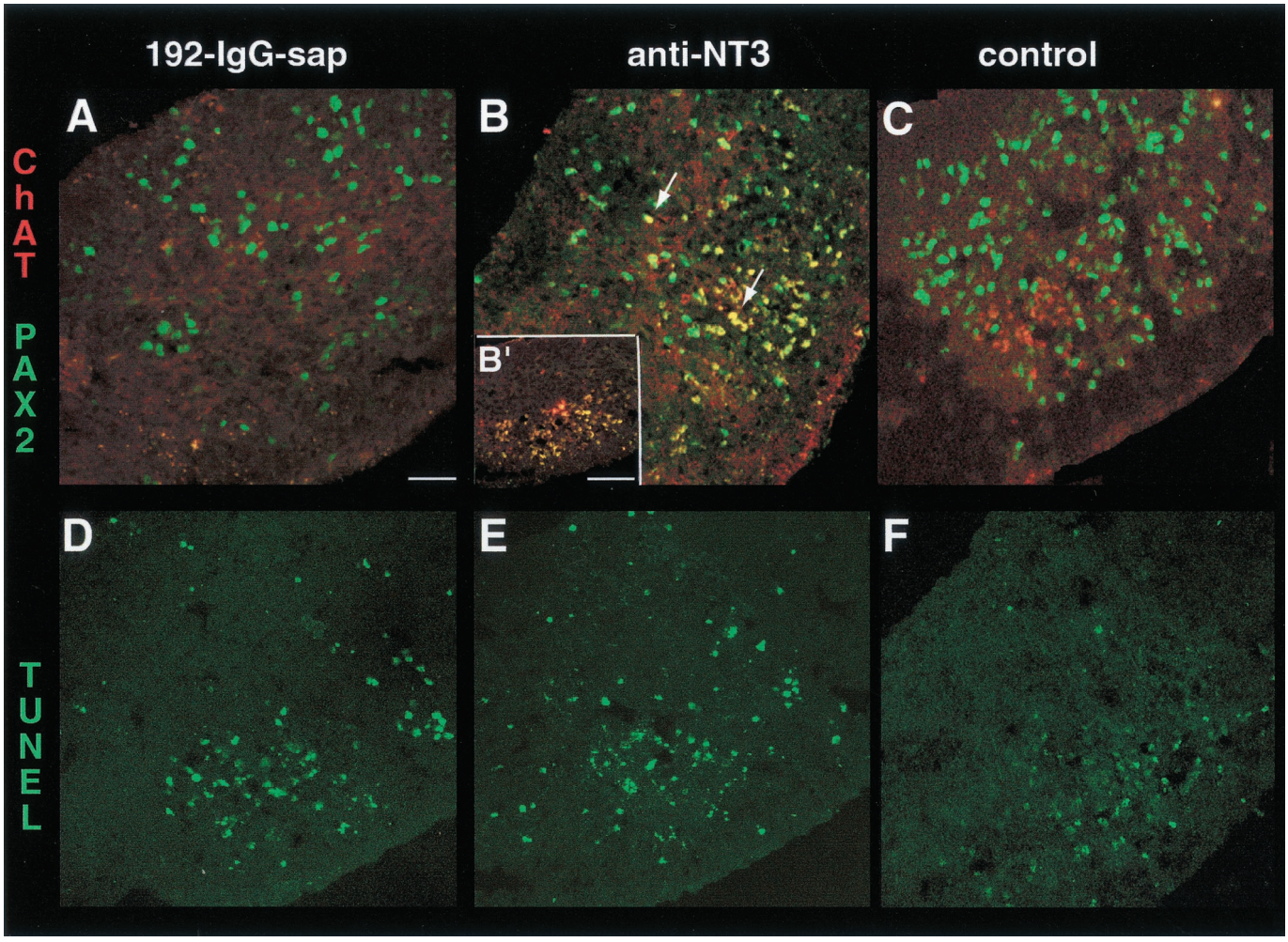


*Figure 3.* Effects of 192 IgG-saporin and anti-NT-3 antiserum on the survival of PAX2-IR interneurons (*A–C*) and on the presence of apoptotic nuclei (*D–F*) within the ventral horns of spinal cord explants. *A–C*, Double immunostaining using anti-ChaT (*red*) and anti-PAX2 (*green*) antibodies on transverse sections of explants cultured for 6 DIV with 192 IgG-saporin (*A*), with anti-NT-3 antibody (*B*), or in controls (*C*). ChaT-IR motoneurons were absent in 192 IgG-saporin-treated explants (*A*) but were present in control (*C*) and anti-NT-3-treated (*B*) explants. In *B*, motoneurons are in *yellow* (see *arrows*) because the FITC-conjugated antibody used to detect the PAX2 antiserum revealed the presence of the anti-NT-3 bound to ChaT-IR (*red*) motoneurons. Note the reduction in the number of PAX2-IR interneurons in 192 IgG-saporin (*A*) and anti-NT-3-treated explants (*B*). *B′*, ChaT-IR motoneurons express NT-3. Anti-NT-3-treated explants were immunostained using an anti-ChaT antibody revealed with a CY3-conjugated antibody (*red*), and anti-NT-3 binding sites were visualized using an FITC-conjugated antibody (*green*). *D–F*, TUNEL-stained nuclei of transverse sections of explants cultured for 6 DIV with 192 IgG-saporin (*A*), with anti-NT-3 (*B*), or in controls (*C*). Note the increased number of apoptotic nuclei compared with control in both 192 IgG-saporin- and anti-NT-3-treated explants. Scale bar: *A–F*, 50 μm; *B′*, 25 μm.

and that depletion of endogenous NT-3 caused death of PAX2-IR ventral interneurons. Altogether, these data are compatible with the notion that NT-3 produced by motoneurons is a survival factor for PAX2-IR ventral interneurons. Anti-NT-3 treatment leads to a 66% decrease in PAX2-IR ventral interneurons, whereas 192 IgG-saporin had a weaker effect resulting in a decrease of only 26%. This difference may be accounted for by their mechanisms of action. The effect of anti-NT-3 is immediate because it blocks the trophic effect of NT-3 by an antibody-antigen reaction. In contrast, 192-IgG-saporin induces a progressive death of motoneurons, as indicated by results of immunolabeling with anti-ChAT antibody (data not shown) and thus a continuous and progressive decrease in NT-3 availability.

NT-3 knock-out mutant mice or *in vivo* administration of blocking antibodies against NT-3 indicated that NT-3 impairment induces a severe loss in sensory and sympathetic neurons but has no effect on the survival of CNS neurons (Ernfors et al., 1994; Tessarollo et al., 1994; Oakley et al., 1995). We quantified the number of PAX2-IR ventral interneurons in the spinal cord of $NT3^{-}/^{-}$, $NT\text{-}3^{+}/^{-}$, and wild-type mice at P2 and found no statistical difference between the NT-3 knock-out and the control mice (data not shown). A straightforward interpretation for the discrepancy between these quantifications and the trophic effect of NT-3 found *in vitro* is that *in vivo* spinal interneurons have access to additional factors with overlapping or redundant functions with regard to that of NT-3. These survival factors could be associated with sources such as supraspinal or dorsal root afferents that are absent in the explants after their dissection out of the embryos. In rat, the peak of naturally occurring cell death for DRG occurs at E17–E19 just before interneuronal death (Coggeshall et al., 1994). BDNF and NT-4/5 are synthesized in DRG during development, and trkB, their specific receptor, is expressed throughout the spinal cord (Henderson et al., 1993). Therefore, these neurotrophins could act as survival factors for

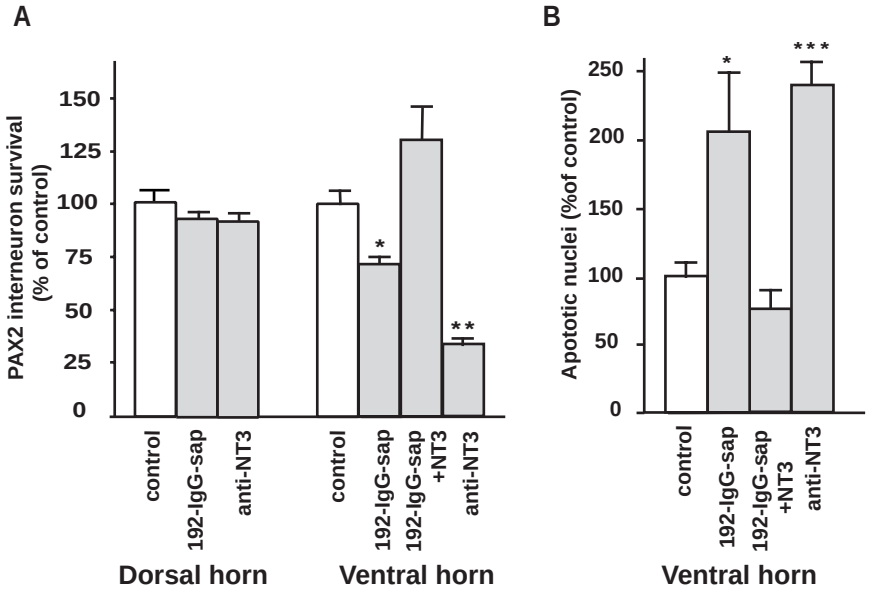


*Figure 4.* Quantification of PAX2-IR interneurons (*A*) and apoptotic nuclei (*B*) within the spinal cord explants. Explants were incubated for 10 min before culture and then cultured for 6 DIV in culture medium without (control) or with 192 IgG-saporin (200 ng/ml), NT-3 (200 ng/ml), or anti-NT-3 (100 $\mu$g/ml). *A*, Percentages (treated vs control) of PAX2-IR interneurons present in the explants were treated as indicated. The number of experiments for each condition were as follows: control, $n = 14$; 192 IgG-saporin, $n = 8$; 192 IgG-saporin and NT-3, $n = 3$; and anti-NT-3, $n = 5$. *B*, Percentages of TUNEL-positive nuclei present in the ventral horns of spinal cord explants (treated vs control as in *A*). The number of experiments were as follows: control, $n = 7$; 192 IgG-saporin, $n = 6$; 192 IgG-saporin and NT-3, $n = 3$; and anti-NT-3, $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Mean ± SEM; Scheffe's *F* test (ANOVA).

spinal interneurons acting in an anterograde manner as they innervate interneurons. Indeed, there is evidence for anterograde transport of BDNF (Kohara et al., 2001). Another possibility derives from the timing of interneuron death in mouse. Apoptotic nuclei outside motor pools are found at P4 in mouse (White et al., 1998). Unfortunately, PAX2-IR is progressively lost after P2 and cannot be detected at P4 in the ventral horns. Therefore, our quantification of PAX2-IR ventral interneurons performed at P2 might have missed interneuron death.

Two studies analyzing the survival of spinal interneurons in mouse mutants characterized by the absence of motoneurons are in apparent contradiction with our results (Grieshammer et al., 1998; Kablar and Rudnicki, 1999). These mutant embryos lack skeletal muscle as a consequence of either deletion of transcription factors *Myf5* and *MyoD* or expression of the gene encoding the diphtheria toxin A fragment in myoblasts. In both mutants, elimination of skeletal muscle resulted in the loss of almost all spinal motoneurons by E18.5 or by birth. In contrast, the number of spinal interneurons was unaffected. However, in both studies, spinal interneurons were quantified at the early stages (E17.5 or E18.5) just at the end of motoneuronal death, before the timing of programmed interneuronal death (White et al., 1998). Therefore, the interval between motoneuron loss and the counting of interneurons was too short to allow interneurons to die.

In our study, only ventral interneurons died, whereas dorsal interneurons were spared. This suggests that only interneurons close to motoneurons require NT-3 to survive. Therefore, NT-3 may act as a target-derived neurotrophic factor. Recent studies have analyzed the synaptic connectivity of PAX2-IR spinal interneurons with motoneurons. Ventral populations of PAX2-IR interneurons can be subdivided into three groups based on the expression of the transcription factors Engrailed-1 (EN1) and EVX1/2 (Burrill et al., 1997). Many if not all EN1-expressing interneurons establish direct synaptic connections with motoneurons. Moreover, expression of EVX1/2 delineates a class of intersegmental commissural interneurons that accumulate in a domain that will form the lamina VIII in the adult spinal cord (Moran-Rivard et al., 2001). The connectivity between interneurons located in lamina VIII and motoneurons has not been studied in rat, but, in cat, they synapse directly with contralateral motoneurons (Harrison et al., 1986). Thus, two subpopulations of PAX2-IR ventral interneurons establish direct synaptic connections with motoneurons. However, these two populations could not be followed in spinal cord explants because the expression of EN1 and EVX1/2 is lost in explants (data not shown).

In conclusion, our results indicate that motoneuron-derived NT-3 is a potential trophic factor for spinal interneurons during development.

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