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

Catherine Béchade, Catherine Mallecourt, Frédéric Sedel, Sheela Vyas, and Antoine Triller

Laboratoire de Biologie Cellulaire de la Synapse Normale et Pathologique, Institut National de la Santé et de la Recherche Médicale U497, Ecole Normale Supérieure, 75005 Paris, France

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-p75NTR-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 nuclei 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 p75NTR, which is coupled to the ribosome-inactivating protein saporin (Wiley and Kline, 2000). In the developing rat spinal cord, only motoneurons express p75NTR (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 μM 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 μg/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 × 10⁴ cells/cm² in four-well dishes, and cultured in Neurobasal–B27 supplemented with 2% horse serum, 0.5 mM L-glutamine, 12.5 μM β-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
with 0.1% Triton and 0.2% gelatin (PBGT), and incubated overnight at 4°C. Explants were mounted on Superfrost plus glass slides, incubated for 15 min in PBS 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-μm-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%) (PBGT) 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 immunohistochemistry.** 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 antibody coupled with CY3 (Jackson ImmunoResearch) diluted 1:500 in PBT. Sections were washed three times in PBST 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 × 88 μm²) 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 determined 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 (Matile 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. 1A), and the distribution pattern resembled that observed in vivo at E19, a stage equivalent to that analyzed in explants (E13 plus 6 DIV) (Fig. 1B). Double immunolabeling with choline acetyltransferase (ChAT) antibody, a specific motoneuronal marker (Phelps et al., 1991), showed that PAX2 antibodies did not label motoneurons (see Fig. 3C).

**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 (Tsukida et al., 1994) were counted. Comparison of treated and control cultures (Fig. 2) indicated that motoneurons (Fig. 2A) but not interneurons (Fig. 2B) 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. 3A), whereas they were still numerous in control explants (Fig. 3C). The death of motoneurons was accompanied by a visible decrease in the number of PAX2-IR interneurons within the ventral horns (Fig. 3A) 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. 3A). 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-
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. 4A) and the number of TUNEL-positive nuclei (Fig. 4B) were comparable with that of controls (untreated explants). 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 antiseraum 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. 3C): the decrease was 66 ± 4% (mean ± SEM; n = 5) compared with controls (Fig. 4A). 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. 3F, 4B). 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. 4A). 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. 3C). 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. 3B').

DISCUSSION

192 IgG-saporin has been used extensively in vitro and in vivo as a powerful tool to eliminate p75NTR-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 p75NTR-IR neurons (i.e., motoneurons) were killed by 192 IgG-saporin. We checked by immunohistochemistry that interneurons did not express p75NTR 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.
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<sup>-/-</sup>, NT-3<sup>-/-</sup>, 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 (Coggshall 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 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.
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., 1996; 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 the diphtheria toxin A fragment in myoblasts. In both mutants, percentages (treated vs control) of PAX2-IR interneurons present in the ventral horns of spinal cord explants (treated vs control as in A). The number of experiments 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).

REFERENCES


