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Co-induction of neuronal interferon-gamma and nitric oxide synthase in rat motor neurons after axotomy: a role in nerve repair or death?

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Summary

Induction of an interferon-gamma-like molecule, previously isolated from neurons (N-IFN-γ), and of the neuronal isoform I of the synthetic enzyme of the free radical nitric oxide, nitric oxide synthase I, as well as of NADPH-diaphorase, were examined in axotomized dorsal motor vagal and hypoglossal neurons. Unilateral transection of the vagal and hypoglossal nerves was performed in the same rat and an induction of N-IFN-γ and nitric oxide synthase I immunostaining as well as NADPH-diaphorase histochemical positivity was observed in the ipsilateral motoneurons after 2–4 days. The immuno- and enzyme- histochemical positivities were much stronger in the dorsal motor vagal neurons than in hypoglossal neurons. Two and 4 weeks after axotomy N-IFN-γ immunoreactivity and NADPH-diaphorase positivity persisted in the former, but started to decrease in the latter neurons. Previous data have shown that 23 weeks after nerve transection the majority of the dorsal motor vagal neurons are lost, while the majority of the hypoglossal neurons survive. The high and persistent expression of N-IFN-γ and nitric oxide synthase I after axotomy in the dorsal motor vagal neurons, that are largely destined to die, indicates that the co-induction of these two molecules may be implicated in the pathogenesis of neuronal degeneration.

Introduction

A neuronal interferon-gamma-like molecule (N-IFN-γ) is constitutively expressed in certain neurons in the CNS and in small neurons in peripheral ganglia in rats both *in vivo* (Ljungdahl *et al.*, 1989) and *in vitro* (Eneroth *et al.*, 1991). Recently, N-IFN-γ has been purified by affinity chromatography from trigeminal ganglia. It differs from lymphocyte-derived IFN-γ in molecular weight, but shares antibody binding epitopes and a number of biological activities with the latter; both molecules have antiviral effects, induce major histocompatibility complex (MHC) class I and II antigens and have similar effects on cell proliferation and differentiation (Olsson *et al.*, 1994). N-IFN-γ can also be induced in neurons. For instance, facial motor neurons, which normally do not express the molecule, show a transient appearance of N-IFN-γ after axotomy (Olsson *et al.*, 1989; Kiefer *et al.*, 1991). Concomitantly, MHC antigens appear in or around the same neurons (Maehlen *et al.*, 1988, 1989; Streit *et al.*, 1989; O'Malley & MacLeish, 1993).

An additional important biological activity of lymphocyte-derived IFN-γ, which is produced by activated T and natural killer cells, has recently been described, namely that in combination with lipopolysaccharide it is a most potent inducer of nitric oxide synthase (NOS), the enzyme synthesizing the free radical nitric oxide (NO). Nitric oxide synthase occurs in different isoforms and IFN-γ induces NOS-II in macrophages, endothelial, microglial and other cells (Ding *et al.*, 1988; Marletta *et al.*, 1988; Chao *et al.*, 1992). The calcium/calmodulin dependent NOS-I is constitutively present in subpopulations of neurons in both the CNS and PNS, where NO has been ascribed both a neurotransmitter function and a role in neurotoxicity (Dawson *et al.*, 1992). Nitric oxide synthase exhibits NADPH-d activity and in fixed brain tissue the enzyme histochemical staining reaction for NADPH-d co-localizes with NOS (Dawson *et al.*, 1991; Hope *et al.*, 1991; Matsumoto *et al.*, 1993). Recent findings indicate that NOS-I may also be induced

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in neurons; in dorsal root ganglia (DRG) the number of NADPH-d and NOS-I positive neurons increases markedly after sciatic nerve transection (Verge *et al.*, 1992; Fiallos-Estrada *et al.*, 1993; Zhang *et al.*, 1993) and NADPH-d appears in motor neurons after peripheral nerve trauma (Gonzales *et al.*, 1987; Wu, 1993). In addition, Herdegen and colleagues (1993) have reported induction of NADPH-d and NOS-I expression in neurons after axotomy within the rat brain.

Since N-IFN-γ and NOS may be involved in determining the fate of neurons after axotomy, it is of interest to compare their appearance in neurons with different responses to nerve transection. We here compared two sets of motor neurons that display such differences, namely those of the dorsal nucleus of the vagus and the hypoglossal nucleus. Previous studies using this experimental model of nerve transection have shown that there is no or only a minimal loss of hypoglossal neurons (Aldskogius *et al.*, 1980; Neiss *et al.*, 1992), but about 30% loss of dorsal motor vagal neurons 14 days after axotomy (Aldskogius *et al.*, 1980). The difference between the two nuclei is also very evident after about 6 months when only 24–35% of the hypoglossal neurons have disappeared (Aldskogius *et al.*, 1980; Snider and Thanedar, 1989), while more than 70% of the vagal neurons are lost (Aldskogius *et al.*, 1980; Laiwand *et al.*, 1987). Using this experimental paradigm, we investigated the induction of N-IFN-γ and NADPH-d or NOS-I and compared the intensity and persistence of the immuno- and enzyme-histochemical positivities of these molecules between the dorsal motor vagal and the hypoglossal neurons.

Materials and methods

Young adult, female Sprague-Dawley rats (Alab, Stockholm), weighing about 200 g, were used. Under anaesthesia with 6% chloral hydrate the hypoglossal nerve on the right side was transected at a level of the posterior part of the digastric muscle. On the left side the vagal nerve was transected at an upper cervical level. The distance from the site of nerve transection to the brain stem was 12–14 mm for both nerves. About 8–10 mm of the distal nerve stumps were resected to impede nerve regeneration. Two unoperated rats served as controls.

On each of day 2, 4, 7, 14 and 28 postoperatively, 3–6 animals were killed, the brain stems dissected and snap-frozen on dry ice. Cryostat sections, 10 and 60 μm thick, were cut and adjacent sections then processed. For immunohistochemistry of N-IFN-γ and NOS-I the 10 μm-thick sections were fixed for 30 sec in ice-cold buffered 4% paraformaldehyde, washed in phosphate-buffered saline (pH 7.4) and then fixed in acetone at −20° C for 30 sec. After washing, the sections were incubated with 2% normal horse serum or 2% normal goat serum (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Sections were then incubated overnight at +4° C with monoclonal mouse anti-IFN-γ antibodies DB1, previously characterized by van der Meide and colleagues (1989), which were diluted in 2% normal horse serum to 5 μg ml^{-1}. The sections were then incubated with biotinylated horse anti-mouse IgG secondary antibodies (Vector Laboratories), which were diluted 1:80, for 1 h at room temperature. Another series of 10 μm-thick sections was processed for the immunohistochemical detection of NOS. A previously characterized anti-NOS-I antiserum raised in rabbit (Bredt *et al.*, 1990), which was diluted 1:400 in 2% normal goat serum, was applied overnight at +4° C. After washing, the sections were incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG secondary antibodies (Vector Laboratories), which were diluted 1:80. For both DB1 and NOS-I immunohistochemistry, the sections were then processed with avidin biotin peroxidase complex (DAKO, Glostrup, Denmark) for 30 min at room temperature and stained with 3-amino-9-ethylcarbazole (Sigma, USA) as substrate. For visualization of NADPH-d the 60 μm-thick sections were fixed in 2.5% glutaraldehyde/0.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 5 min, followed by a thorough wash in the same buffer. After 5 min preincubation in 0.3% Triton X-100 (Sigma), the sections were incubated for 1–3 h at +37° C with 0.1 M phosphate buffer/ 0.3% Triton X-100/0.5 mM Nitro Blue Tetrazolium (Sigma)/ 1 mM β-NADPH (Sigma).

Intensities of the staining reactions in the remaining neurons were graded by two different observers, unaware of the group assignments, comparing the operated to the unoperated sides: 0, no increased reactivity; 1, slight increase; 2, moderate increase; 3, strong increase.

Results

No immunoreaction of N-IFN-γ was observed in nerve cell bodies in the dorsal motor vagal and the hypoglossal nuclei of unoperated rats or in the contralateral sides of the operated rats (Fig. 1D,F). There was no, or only a faint, NOS-I immunoreactivity (Fig. 2D,G) or NADPH-d histochemical staining (Fig. 2A–C) in the same nerve cell bodies.

After transection of the vagal and hypoglossal nerves, induction of immunostaining for N-IFN-γ and NOS-I as well as of NADPH-d histochemical staining occurred in the operated dorsal motor vagal and hypoglossal nuclei (Figs 1,2). The intensity of the staining positivities is graded in Table 1.

Two days after transection N-IFN-γ immunoreactivity appeared in the dorsal motor vagal nucleus, as well as in the hypoglossal nucleus (Table 1). However, N-IFN-γ immunoreactivity in the hypoglossal neurons was very faint. The N-IFN-γ immunoreaction intensity increased 4 and 7 days after transection in the dorsal motor vagal nucleus (Fig. 1A,C,D and Table 1), in which about 90% of the neurons were now N-IFN-γ-immunoreactive. Most (about 80%) of the hypoglossal neurons had also become positive 4 and 7 days after axotomy (Fig. 1B,D and Table 1). However, one week after lesion N-IFN-γ immunoreactivity in the

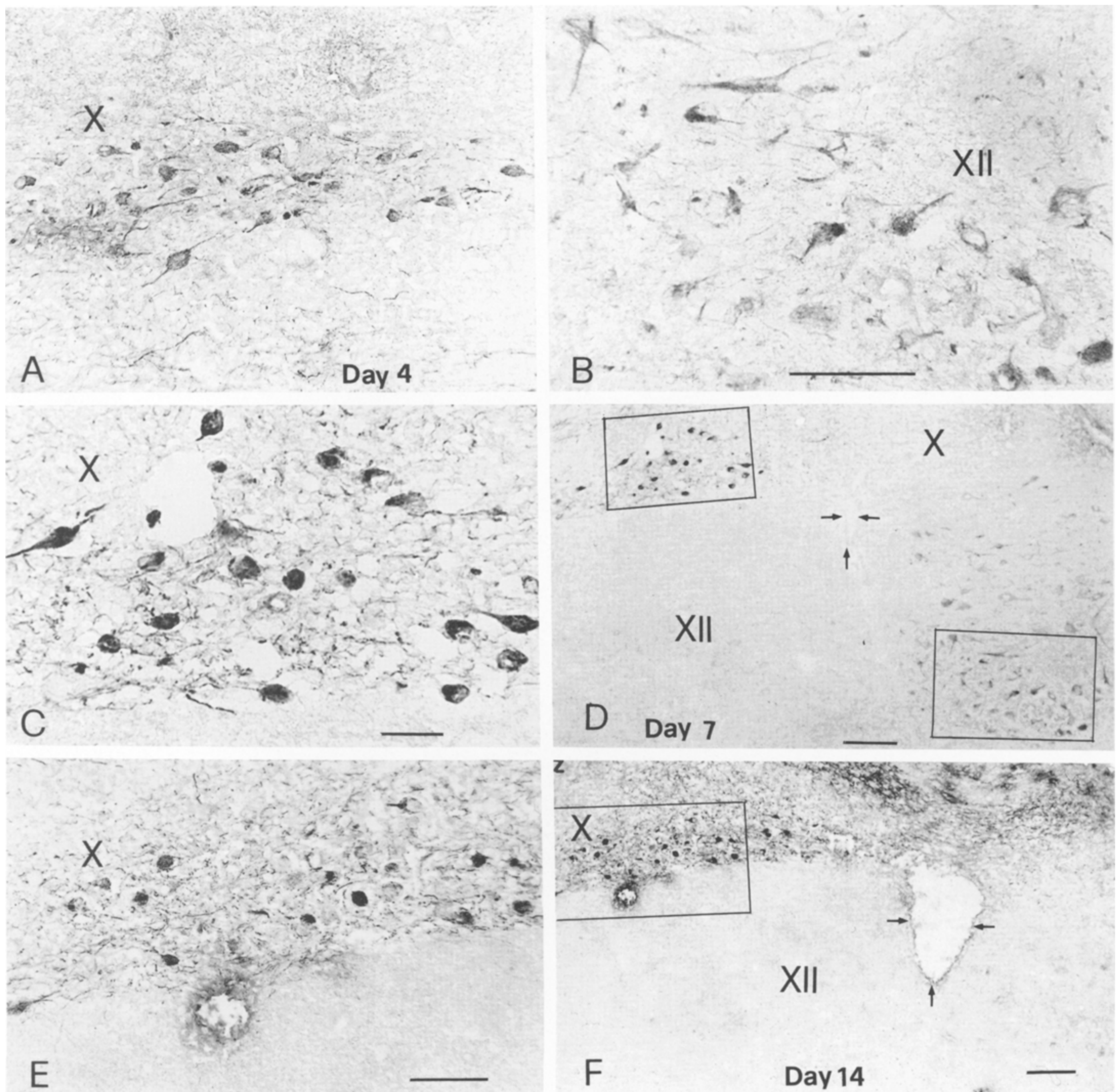

Fig. 1. Photomicrographs of immunoreactivity to DB1 antibodies, that recognize neuronal interferon-γ, in the dorsal motor vagal (X) and hypoglossal (XII) motor neurons 4 (A), 7 (B–D) and 14 (E,F) days after transection of the left vagal nerve and right hypoglossal nerve. Note ipsilateral to the lesion the induction of DB1-immunoreactivity, not detectable on the contralateral sides (D,F). Note also that in the dorsal vagal motoneurons DB1-immunoreactivity is evident 4 days after transection (A), and increases and persists in the following two weeks (C,E). In contrast, one week after the lesion DB1-immunoreactivity in hypoglossal motoneurons is less intense than in the vagal neurons (B,D; compare B and C) and decreases, being hardly detectable at low power, two weeks after transection (F). B and C correspond to the insets in D (B, lower right; C, upper left). E corresponds to the inset in F. Arrows point to the central canal. Scale bars: A, B, E, 80 μm; C, 40 μm; D, F, 100 μm.

hypoglossal neurons was less intense than in the dorsal motor vagal neurons (Fig. 1B,C,D and Table 1). The strong N-IFN-γ immunoreactivity persisted in the vagal neurons 14 (Fig. 1E,F) and 28 days (Table 1) postoperatively. On the other hand, on the second week after nerve transection the N-IFN-γ-immunoreactivity was considerably reduced in the hypoglossal neurons (Table 1) in which it was

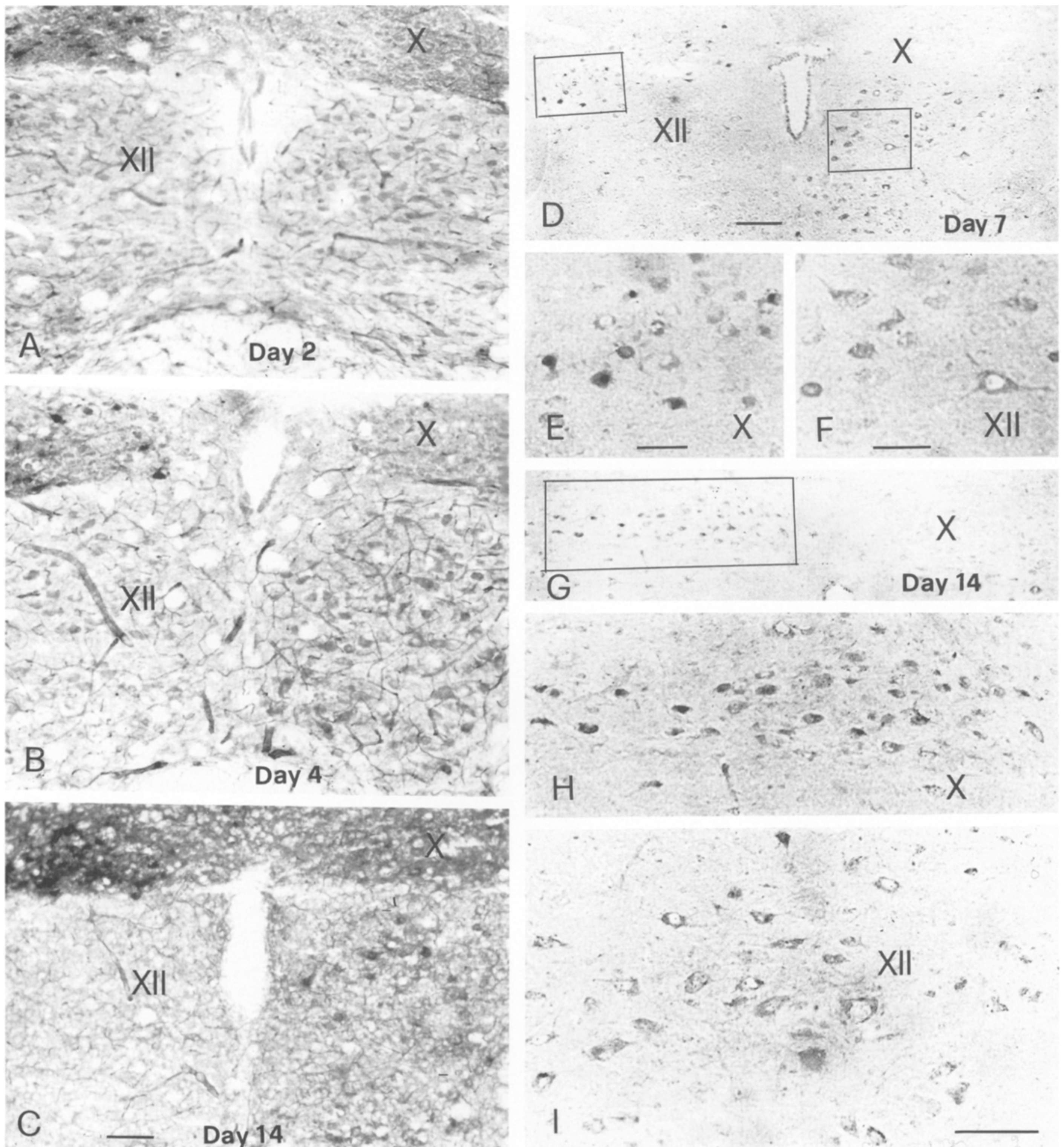

Fig. 2. Photomicrographs of NADPH-d histochemical staining (A–C) and immunoreactivity to NOS-I (D–I) in the dorsal motor vagal (X) and hypoglossal (XII) motor neurons in the same paradigm (i.e. transection of the left vagal and right hypoglossal nerves). Note that NADPH-d staining is induced in the ipsilateral vagal motor neurons on the second day after lesion (A), and increases after 4 days (B); the NADPH-d induction is still clearly evident 2 weeks after the lesion (C). NADPH-diaphorase induction is also evident in the hypoglossal motor neurons ipsilateral to the lesion 4 days (B) and 2 weeks (C) after axotomy, although less intense than in the vagal ones. In both the vagal and hypoglossal motor neurons the NADPH-d induction is clearly shown by the comparison with the sides contralateral to nerve transections. Note that the staining intensities in the vagal motor neurons are heterogeneous, which probably reflects the presence of functional subpopulations of neurons in this nucleus. In agreement with NADPH-d histochemistry, induction of NOS-I immunoreactivity is evident in the ipsilateral vagal motor neurons (D,E,G,H), as well as in the hypoglossal neurons (D,F,I) during the first (D–F) and second (G–I) week after lesion. Nitric oxide synthase-I-immunoreactivity in the hypoglossal neurons shown in I was detected in the same section from which dorsal motor vagal neurons are shown in G and H. E and F correspond to the insets in D and H to the inset in G. Scale bars: A–C, 100 μm; D,G, 125 μm; E,F, 100 μm; H,I, 90 μm.

Table 1. Assessment of staining intensity after nerve transection. The axotomized side is compared to the contralateral side in each case.

	Dorsal motor vagal nucleus		*Hypoglossal nucleus*	
	N-IFN-γ	*NADPH-d*	*N-IFN-γ*	*NADPH-d*
Day 0	–	–	–	–
Day 2	+	++	(+)	–
Day 4	++	+++	++	++
Day 7	+++	+++	++	++
Day 14	+++	+++	+	++
Day 28	+++	+++	+	+

hardly detectable at low power magnification (Fig. 1F).

There was a marked increase in NADPH-d staining in some of the ipsilateral dorsal motor vagal neurons by 2 days after transection, while the hypoglossal neurons displayed no clear positivity at this time (Fig. 2A and Table 1). Four (Fig. 2B) and 7 days after axotomy, an intense histochemical NADPH-d positivity was observed in the majority of the neurons in the dorsal motor nucleus of the vagus nerve (Table 1), while some neurons remained unstained or as lightly stained as on the control side. In the ipsilateral hypoglossal nucleus almost all neurons showed a moderately increased staining intensity 4 (Fig. 2B) and 7 days after transection (Table 1). The staining intensity in the hypoglossal neurons was, however, markedly less than that in the vagal neurons 4 (Fig. 2B) and 7 days postoperatively (Table 1). After 14 (Fig. 2C) and 28 days the strong NADPH-d staining intensity persisted in several neurons in the dorsal motor vagus nucleus, but was reduced in the hypoglossal neurons (Table 1). Immunostaining for NOS, carried out 4, 7 and 14 days after nerve transection, was positive in the ipsilateral vagal and hypoglossal neurons. After 7 days about 90% of the neurons in each nucleus was positive, but the intensity was much higher in the vagal neurons (Fig. 2D–I). The number of labelled neurons was reduced 14 days after axotomy in the hypoglossal nucleus, while the majority of neurons in the vagal nucleus remained intensely positive. No NADPH-d or NOS-I reaction was observed in glial cells within the nuclei (Fig. 2).

Discussion

The present study shows a remarkable difference in the intensity and persistence of N-IFN-γ immunoreactivity between the axotomized hypoglossal and dorsal motor vagal neurons. N-IFN-γ immuno-reaction was much more intense in the latter than in the former neurons, in which it also decreased after 14 days. The partly transient appearance of N-IFN-γ in axotomized hypoglossal neurons is consistent with the temporal pattern observed previously in regenerating facial motor neurons after nerve transection (Olsson *et al.*, 1989). The recently purified N-IFN-γ is a protein with a molecular weight distinct from lymphocyte-derived IFN-γ, but the two molecules share structures allowing for recognition by the same antibodies and for similar biological effects (Olsson *et al.*, 1994). Both molecules can regulate cell proliferation and differentiation as well as the induction of MHC antigens, which in the immune system serve as recognition molecules. Therefore the transiently expressed N-IFN-γ after axotomy may play an important role during nerve regeneration and repair. Although this role has still to be defined, growth-regulating effects of lymphocyte-derived IFN-γ on neurons were shown by Chang and colleagues (1990). In tissue culture experiments, lymphocyte-derived IFN-γ promoted survival of nerve growth factor-deprived sympathetic neurons expressing with IFN-γ receptors. Furthermore, lymphocyte-derived IFN-γ has effects on astrocyte differentiation (Erkman *et al.*, 1989), on Schwann cell proliferation (Eccleston *et al.*, 1989) and on MHC class II expression (Kingston *et al.*, 1989).

However, induction of high or persistent levels of N-IFN-γ may have deleterious effects on neurons, since a strong and persistent N-IFN-γ positivity occurred in the dorsal motor vagal neurons, which are slowly and progressively lost after nerve transection. This nerve cell loss amounts to about 75% at 164 days postoperatively (Aldskogius *et al.*, 1980). An aberrant and persistent expression of lymphocyte-derived IFN-γ had also been associated with cell death. For instance, an abnormal expression of IFN-γ in pancreatic islet cells can cause a slowly progressive loss of these cells. This cell loss was proposed to be mediated by cytotoxic lymphocytes, which can be attracted into a tissue by IFN-γ (Sarvetnick *et al.*, 1990). A similar phenomenon may occur after axotomy, since we have observed that lymphocytes are also attracted to areas of axotomized motor nerve cell bodies (Olsson *et al.*, 1992).

There was also a striking parallelism between nerve cell death and intensity of NADPH-d and NOS-I induction. In the hypoglossal neurons, which largely survive axotomy, the expression was moderate and partly transient. In the dorsal vagal neurons, of which a majority eventually die after axotomy, the intensities of NADPH-d and NOS-I reactivity were much stronger and the strong reaction persisted longer than in the hypoglossal neurons. A strong NADPH-d reaction was similarly reported after avulsion, but not transection, of ventral roots of the rat spinal cord motor neurons, which are prone to die after such a trauma (Wu, 1993). Similarly, sciatic nerve injury in

neonatal rats induces NADPH-d activity in spinal motor neurons, which die to a large extent after such a trauma, while no NADPH-d activity is seen in these motor neurons after a corresponding injury in adult rats when neuron death is absent or minimal (Clowry, 1993).

Dawson and colleagues (1993) have proposed that NO derived from neurons can be a source of neuronotoxicity, since in cultures, elimination of NOS-I expressing neurons diminishes the subsequent toxicity of an excitatory amino acid. A mechanism for neuronal cell death in the present model may therefore also be related to the expression of this calcium-dependent enzyme. The induction of NOS-I in the axotomized neurons coincided with that of N-IFN-γ, and it would be of interest to examine if a causual relationship exists, i.e. if N-IFN-γ by an autocrine mechanism can induce NOS. Although Chao and colleagues (1992) observed that lymphocyte-derived IFN-γ causes an NO-mediated reduction in survival of neurons only when they were co-cultivated with microglial cells (indicating a role of microglia-derived NO in acute nerve cell death), persistently increased levels of NOS-I in neurons *in vivo* may be relevant for the slowly progressive nerve cell death following nerve transection, since it may lead to an increased vulnerability of the neurons to calcium-mediated neurotoxicity (Bredt & Snyder, 1989).

Alternatively, based on observations of induction of persistent expression of NADPH-d and NOS-I in axotomized neurons within the brain Herdegen and colleagues (1993) have suggested a role of these molecules in neuroprotection. This would be in line with the observation that neurons which constitutively express NOS-I are more resistant to a variety of noxious insults (Dawson *et al.*, 1992). Such a protection may be directly offered by a nitric-oxide blockade of NMDA receptor (Manzoni *et al.*, 1992) or to the presence of a more efficient intrinsic scavenger system for free radicals in such neurons. However, the intrinsic properties of neurons with constitutive expression of NOS-I and those in which NOS-I is induced may vary and the observation by Wu and Li (1993) that treatment with a specific NOS inhibitor can significantly reduce motor neuron death after ventral root avulsion favours an association between NOS induction and nerve cell death in this system.

In conclusion, we observed co-induction of the biologically highly active molecules N-IFN-γ, NADPH-d and NOS-I in motor neurons after nerve transections. The parallelism between on one hand the intensity and persistence in the expression of these molecules and on the other hand the fate of the neurons indicates that these molecules may belong to the factors involved in determining whether an axotomized nerve cell will survive and be able to repair its axon or become destined to die.

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