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Induction of nitric oxide synthase activity and motoneuronal cell damage

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SUMMARY

Experimental findings obtained in our laboratory on the induction of nitric oxide synthase (NOS), the synthetic enzyme of the free radical nitric oxide, in motoneurons of the rat after different paradigms of injury are summarized. NOS was found to be induced in the mature axotomized facial motoneurons. However, no induction of NADPH-diaphorase histochemical staining, which is a marker of NOS activity, was detected in facial motoneurons after axotomy at birth, when evidence of apoptosis was obtained in the facial nucleus on the side of the lesion. On the other hand, NOS activity was found to be induced in mature facial motoneurons in the absence of degenerative changes, concomitantly with facial muscle paralysis caused by botulin toxin A. Together, our data indicate that the death of motoneurons, at least of the immature ones, can occur in the absence of NOS induction; that NOS induction in mature motoneurons is not necessarily related to their death, and that NOS can be induced in motoneurons by different paradigms of structural and functional injury.

Key words: Axonal injury - Neurotoxicity - Motoneurons - Free radicals.

INTRODUCTION

The recent discovery that nitric oxide (NO), a gas with free radical properties, is a messenger molecule in the central nervous system (Dawson and Snyder, 1994) has modified classical concepts of interneuronal communication. This novel messenger is not stored in synaptic vesicles and is not released by exocytosis upon membrane depolarization but is instead synthesized on demand. NO diffuses from one neuron to another by acting directly on intracellular components. The enzyme that synthesizes NO is constitutively expressed in neuronal subpopulations (Bredt et al., 1991; Vincent and Kimura, 1992), but can also be induced in neurons by paradigms of injury and, in particular, by axon transection.

The constitutive neuronal isoform of the NO synthetic enzyme, nitric oxide synthase (NOS), is a Ca^{2+}/calmodulin- and NADPH-dependent enzyme which produces NO by converting L-arginine into citrulline (Moncada and Higgs, 1993). Increases in intracellular calcium are required to activate NOS, and this increase in intracellular calcium can be mediated by the activation of glutamate N-methyl-D-aspartate (NMDA) receptors (Garthwaite et al., 1989; Garthwaite and Boulton, 1995).

The excess of NO is in part responsible for glutamate neurotoxicity in primary neuronal cell culture and in animal models of stroke (Dawson and Dawson, 1996). It is likely that most of the neurotoxic actions of NO are mediated by peroxynitrite ($ONOO^-$), the reaction product of NO and superoxide anion. In pathological conditions, peroxynitrite and oxigen free radicals can be generated by cells in which severe damage of the antioxidant capacity is present (Dawson and Dawson, 1996).

Moreover, NOS can be induced in neurons by a variety of pathological stimuli. In particular, several studies have reported that NOS can be induced in motoneurons after nerve lesion. Experimental evidence and hypotheses on the

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involvement of oxidative stress in neurodegeneration have recently stimulated debate on the possible role of NO in the reaction of motoneuronal cell groups to nerve injury. An involvement of NO in the maturation of motoneurons has also been implied. For instance, it has been suggested that local and transient production of NO in neurons surrounding motoneuronal pools in the ventral horn of the spinal cord may contribute to motoneuron differentiation (Kalb and Agostini, 1993). Retrograde NO signals may also participate in the regulation of neuromuscular synaptogenesis (Wong et al., 1995). On the other hand, NO may be involved in the death of immature spinal motoneurons which are disconnected from their peripheral targets: in neonatal spinal motoneurons, NOS has been found to be induced after sciatic axotomy and it has been demonstrated that many of these motoneurons die after the nerve trauma (Clowry, 1993). On the other hand, in this latter study NOS was not found to be induced in adult spinal motoneurons, in which degenerative changes were not prominent after sciatic nerve transection. In addition, NOS activity is induced in both immature and mature spinal motoneurons by root avulsion (Wu, 1993; Wu et al., 1995). NOS induction has also been reported in motoneurons of different brainstem nuclei after various types of nerve injury (Kristensson et al., 1994; Ruan et al., 1994, 1995; Wong et al., 1995).

On the basis of the above-mentioned data, it remains to be clarified whether differences in NOS induction between immature and mature motoneurons after axotomy represent a general feature and whether NO production could be related to immature or mature motoneuronal cell death or to a protective response to injury.

NOS ACTIVITY INDUCTION AFTER INJURY OF FACIAL MOTONEURONS

NOS activity is induced in facial motoneurons after nerve lesions in adulthood

NOS is not constitutively expressed in mature and immature facial motoneurons (Vincent and Kimura, 1992). We performed experiments on facial motoneurons because they constitute a relatively homogeneous cell population, subjected to extensive studies after axonal injury and regeneration (Olsson and Kristensson, 1979; Söreide, 1981; Graeber and Kreutzberg, 1988). In our studies we have analyzed the relationship between NOS induction and the damage of facial motoneurons. To this purpose, the facial nerve was transected in adult rats: a segment of the trunk of the facial nerve was cut behind the ear, near the stylomastoid foramen, just before the nerve ramifies into branches.

Induction of NOS was investigated by means of NADPH-diaphorase (NADPH-d) histochemical activity, which is equivalent to NOS in aldehyde-fixed tissue (Matsumoto et al., 1993; Buwalda et al., 1995). In particular, in adult injured motoneurons, NADPH-d was found to correspond to the neuronal NOS isoform (Wu et al., 1994).

In our studies (Mariotti et al., 1997), all surgical procedures were performed under ether or pentobarbital anesthesia. Axotomy of the facial nerve resulted in motor deficits, which were verified in all animals 1 day after the operation. All rats were then perfused via the ascending aorta with saline followed by a fixative solution containing 4% paraformaldeyde in 0.1 M phosphate buffer, pH 7.4 (PB). The brainstems of the rats were dissected out, soaked in 20% sucrose overnight, and sectioned on a freezing microtome into 40-µm-thick transverse sections. The sections were incubated free-floating in a solution of 0.1% ß-NADPH (reduced, from Sigma), 0.05% nitroblue tetrazolium (Sigma), and 0.3% Triton-X-100 in PB for 1-2 h at 37°C.

In keeping with other reports of NOS induction in facial motoneurons after different kinds of axonal injury (Ruan et al., 1994; Wong et al., 1995), we detected NOS induction in these cells after transection of the facial nerve in adult rats (Fig. 1 C). We have also observed NADPH-d staining induction in facial motoneurons after axotomy at different postnatal ages: the histochemical positivity was induced in facial motoneurons when the axotomy was performed from the end of the first postnatal week to adulthood; the nerve cell loss was less severe in the facial nucleus lesioned during postnatal maturation than at birth. Our findings indicated that the postoperative NADPH-d activity induction in these neurons, and its temporal sequence, varied according to the age of the animal (Mariotti et al., 1997). The rats were allowed to survive for 2,4,7,10 or 20 days after axotomy. Intense induction of NADPH-d staining was observed in the axotomixed facial motoneurons of 5 weeks old rats, sacrificed 20 days after nerve lesion (Fig. 1 A,B), but was not evident in these motoneurons 4 days after axotomy. When the facial nerve was transected in 2 or 3 months old rats, NADPH-d positivity was very intense in the axotomized motoneurons 1 week after the lesion (Fig. 1 C); cell loss was not evident in the axotomized adult facial nucleus after 1 week, and the neurons of the lesioned facial nucleus did not display in these cases a marked dissolution of the Nissl substance. On the other hand, a remarkable induction of NADPH-d positivity was detected in the facial motoneurons of 1 week-old rats 4 days after nerve transection, when only a minor proportion of the lesioned motoneurons showed eccentric nuclei and perikaryal shrinkage.

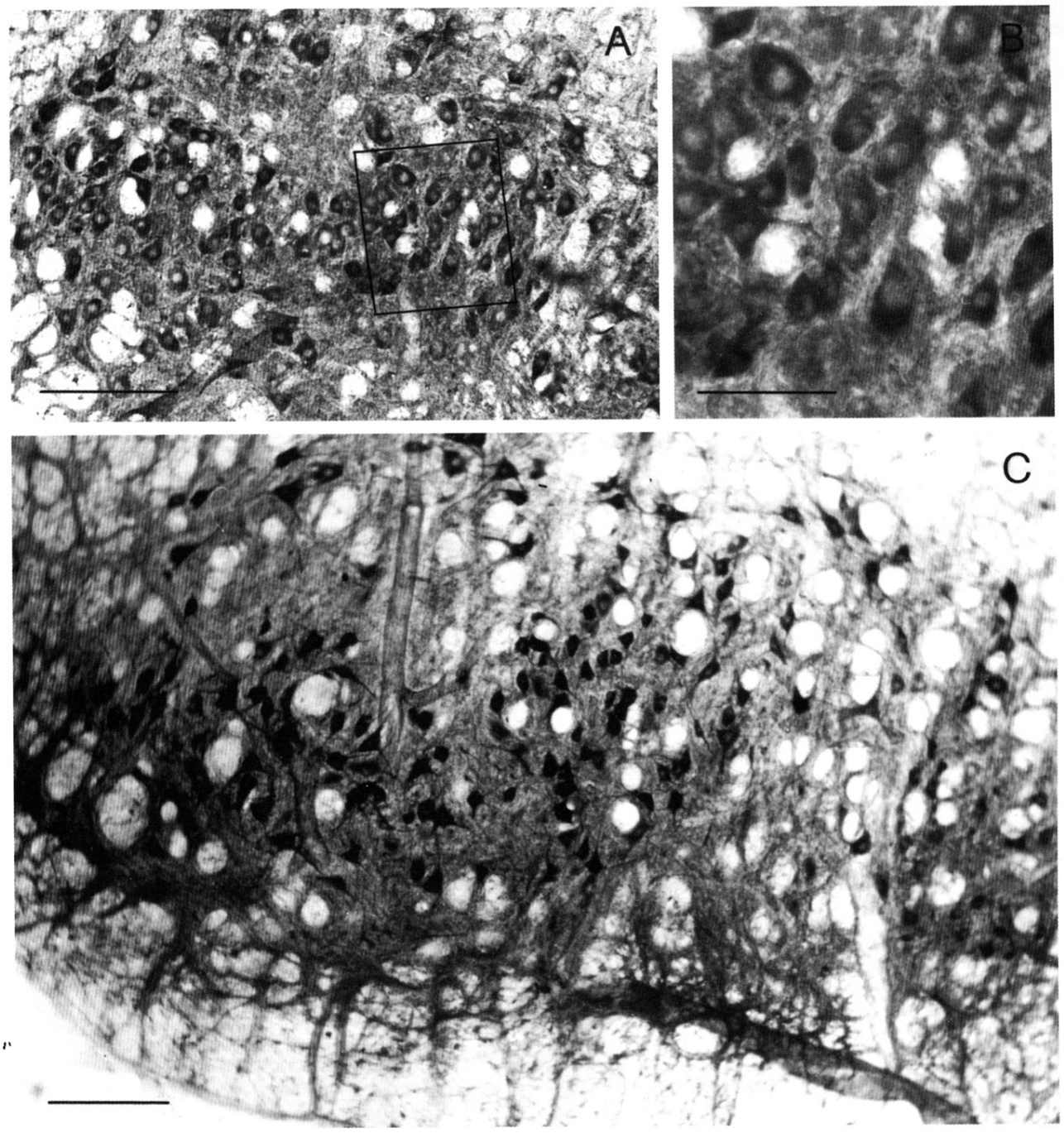

Fig.1.– NADPH-diaphorase histochemical positivity in motoneurons of the facial nucleus after nerve transection in 5 weeks old rat 20 days after axotomy (A; B represents at higher magnification the area boxed in A), and 7 days after axotomy in a 3-month old rat (C). Scale bars: 200 μm in A, 500 μm in B, 120 μm in C.

Lack of NOS activity induction in immature facial motoneurons axotomized at birth

Aiming at revealing a correlation of NOS induction with motoneuronal cell death, we decided to verify the occurrence of this phenomenon in the earliest stage of postnatal development (Mariotti et al., 1997). It is known that at this age an axonal lesion of immature motoneurons results in very severe cell loss (Lowrie and Vrbova, 1992). Following the same surgical procedures used for adult animals, we operated the rats at birth. Postoperative survival times of 24 h, 48 h, or 4 days were selected in these experiments, in which NADPH-d activity was studied in the facial motoneurons. In addition, in order to verify the occurrence of apoptotic phenomena in the axotomized facial motoneurons, after facial transection at birth, brainstems of rats were paraffin-embedded and processed for *in situ* nick-end labeling of DNA fragmentation (TUNEL technique) (Gavrieli et al., 1992).

In the rats in which the facial nerve was cut a few hours after birth, no NADPH-d staining was observed in facial motoneurons 1 or 2 days later (Fig. 2 D). However, 1 day after axotomy at

birth the Nissl staining of adjacent sections revealed degenerative changes in motoneurons, including pyknosis or fragmentation of their nuclei, on the side of the injury. An extensive motoneuron loss was evident on the operated side 2 days after the axotomy. Four days after axotomy the facial motoneurons had completely disappeared and only gliosis was present in the cresyl violet-stained sections.

In the sections processed with the TUNEL technique, positive nuclei showing an intense and selective staining were consistently detected in motoneurons of the facial nucleus 1 and 2 days after axotomies. TUNEL-positive neurons were evident especially 24 h after nerve transection and were visible only ipsilaterally to the lesion (Fig.2 A,B,C).

NOS activity can be induced in facial motoneurons by functional damage

Intramuscular injections of botulin toxin A (BoTx) were made into the snout of 3-month and 3-week old rats, resulting in transient paraly-

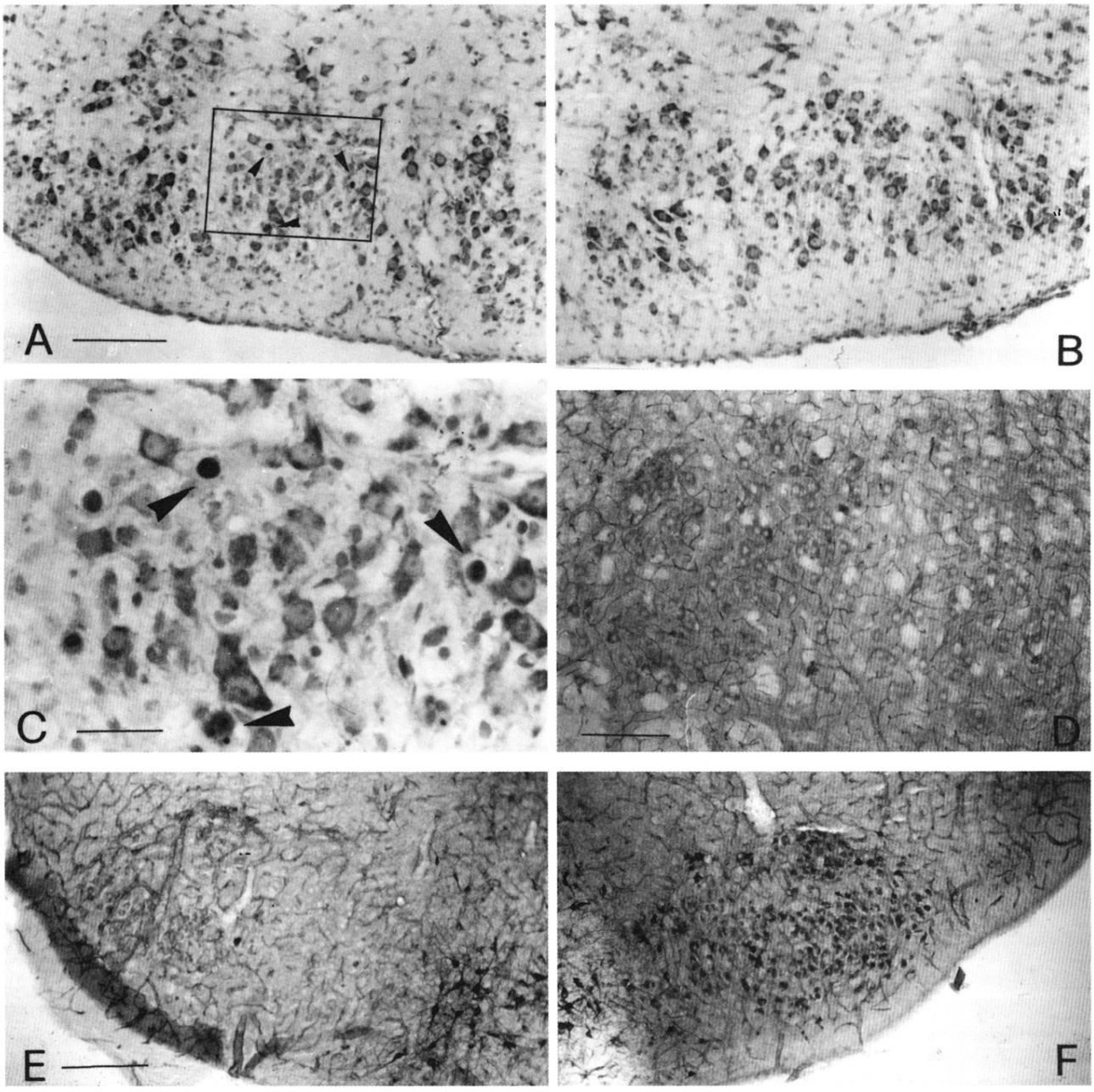

Fig.2.– A,C: *In situ* nick-end labeling of DNA fragmentation (TUNEL-technique) in the facial nuclei 1 day after unilateral transection of the facial nerve at birth (the sections have also been stained with thionin). The photomicrographs in A and C illustrate the side ipsilateral to the axotomy (C represents at higher magnification the area boxed in A), whereas B illustrates the contralateral side. Note in A and C the occurrence of positive neuronal nuclei and apoptotic bodies (arrowheads), which are not detectable in B. As shown in D, NADPH-diaphorase staining is not evident in the axotomized facial motoneurons 1 day after nerve transection at birth. The photomicrographs in E and F illustrate the facial nuclei processed for NADPH-diaphorase histochemistry in a 1-week old rat 4 days after axotomy: E is the side contralateral to the axotomy, and no histochemical staining is evident in the intact facial motoneurons; NADPH-diaphorase positivity is instead evident in the axotomized motoneurons (F). Scale bars: 500 μm in A,B,D, 200 μm in C, 100 μm in E,F.

sis of the facial muscles (Mariotti and Bentivoglio, 1996). This protocol was performed to investigate NOS induction in a paradigm of functional disturbance associated with integrity of the axon and survival of motoneurons. Intramuscular injection of BoTx causes paralysis due to inhibition of acetylcholine release at the neuromuscular junction (Schiavo et al., 1994). Following acute treatment with sublethal doses of the toxin, the motor function of the exposed neurons is restored.

Intramuscular injections of BoTx (Calbiochem; supplied as 1 mg/ml in 50 mM NaOAC and 200 mM NaCl) were made under chloral hydrate anesthesia into the snout of young and adult rats, dividing the total volume into three deposits in different sites. Saline was injected with the same paradigm in control rats. All animals were then deeply anaesthetized with pentobarbital and transcardially perfused, as previously described, after different survival times.

No NADPH-d activity was detected in the facial motoneurons of the control adult and young animals injected with saline. These findings indicated that the mechanical trauma of the injection had not resulted in NADPH-d staining in the facial nucleus. NADPH-d positivity was instead seen in facial motoneurons after BoTx injections, even though no degenerative changes were evident in these motoneurons. In all these experiments, NADPH-d induction was transient and disappeared when muscle function was restored. The positive motoneurons clearly prevailed on the side of the injection, but some stained cells were also seen in many of the cases on the contralateral side, indicating that the relatively large injected volumes could have spread through the snout.

These data suggest that retrograde signaling of functional synaptic disturbances may account for NO production in the parent neuronal cell bodies and that NOS induction in motoneurons may not necessarily be related to structural injury.

Implications of the data and concluding remarks

Our findings and a large body of current literature provide demonstration that NOS can be induced in neurons by different types of pathological stimuli. The evidence of NOS activity in response to various kinds of injury indicates that this enzyme is not only constitutive but can also be induced in neurons which normally do not express it. Current opinion about the role of NO in the reaction of motoneurons to injury, suggested by several authors, is that this free radical exerts a neurotoxic effect (Dawson and Dawson, 1996). Such a hypothesis is also supported by experiments indicating that NOS induction parallels the death of injured motoneurons and that the administration of NOS inhibitors can rescue motoneurons from damage (Wu and Li, 1993).

In our experiments, however, we observed marked apoptotic phenomena in immature facial axotomized motoneurons at a time when no NADPH-d positivity was detectable. This finding suggests that NOS induction may not necessarily be related to motoneuronal cell death after injury. After axotomy at birth, facial motoneurons died very rapidly, probably not allowing NOS induction to become manifest. Thus, our data suggest that the death of immature motoneurons disconnected from their target and NOS induction may be unrelated phenomena.

Our findings do not rule out the possibility that NOS induction could exert a toxic effect on motoneurons at later developmental ages. In agreement with Lowrie (1992), we observed that the mechanisms behind motoneuron response to injury and survival vary considerably according to the stage of maturation, and that motoneuron susceptibility to nerve lesion is higher at early postnatal ages. We also report for the first time a remarkable age dependency in the time course of NOS induction in facial motoneurons axotomized postnatally. Enzyme activity was markedly induced 4 days after the nerve transections performed at the end of the first postnatal week, whereas NADPH-d positivity was moderate 4 days after axotomies performed at the end of the second postnatal week. On the other hand, an intense histochemical staining was detected only after a longer period of time when axotomy was performed in older rats. Therefore, the present data support previous evidence that NOS induction in motoneurons *in vivo* requires a time interval after nerve injury, suggesting that such temporal gradient is age-dependent.

As for the experiments in which we tested the effect of muscle paralysis, we wish to emphasize that BoTx injections into the facial muscles of rats induced a reversible NOS induction in facial motoneurons, whose persistence paralleled that of muscle inactivity. The signalling mechanism for nerve cell body changes are not yet known, but they may involve an effect by retrogradely transported BoTx (Wiegand et al., 1976) or reduced level of retrogradely transported molecules following a block in exocytosis-coupled endocytosis (Enerbäck et al., 1980). However, no chromatolysis was evident in our experiments in motoneuronal cell bodies up to 4 days after BoTx intoxication, indicating that no degenerative changes occurred at the time of NOS induction in these

cells. BoTx injections in the muscle do not prevent the uptake and retrograde axonal transport to motoneurons of extracellular molecules (Kristensson and Olsson, 1978), indicating that retrograde signalling mechanisms are operant in these conditions and could have triggered NOS induction.

Paralysis induced by BoTx could elicit early metabolic changes in the neuronal perikarya innervating the paralyzed muscles, and our data suggest that these changes could involve free radical production. In this respect, it is important to emphasize that the NOS induction in motoneurons after intramuscular BoTx injection was transient, and that the induction disappeared when muscle function was restored.

Thus, the data we obtained in motoneurons demonstrate that NOS induction may be associated with functional derangement of neurons destined to survive. These latter data do not rule out the possibility of an involvement of NO in neuronal cell death, but do indicate that increased NO production may represent a response of the neuronal machinery to an insult against which neurons require protection.

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