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Axotomy increases NADPH-diaphorase activity in the dorsal root ganglia and lumbar spinal cord of the turtle *Trachemys dorbigni*

W.A. Partata[1], A.M.R. Krepsky[1], M. Marques[1] and M. Achaval[2]

Departamentos de [1]Fisiologia and [2]Ciências Morfológicas, Instituto de Ciências Básicas e da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil

Abstract

Seven days after transection of the sciatic nerve NADPH-diaphorase activity increased in the small and medium neurons of the dorsal root ganglia of the turtle. However, this increase was observed only in medium neurons for up to 90 days. At this time a bilateral increase of NADPH-diaphorase staining was observed in all areas and neuronal types of the dorsal horn, and in positive motoneurons in the lumbar spinal cord, ipsilateral to the lesion. A similar increase was also demonstrable in spinal glial and endothelial cells. These findings are discussed in relation to the role of nitric oxide in hyperalgesia and neuronal regeneration or degeneration.

Key words
- NADPH-diaphorase
- Axotomy
- Turtle

Correspondence
W.A. Partata
Departamento de Fisiologia
ICBS, UFRGS
Rua Sarmento Leite, 500
90050-170 Porto Alegre, RS
Brasil
Fax: +55-51-316-3166

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Introduction

Nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) is considered to be equivalent to nitric oxide synthase (NOS), which is the enzyme responsible for nitric oxide (NO) synthesis (1). Hence, the simple histochemical method for NADPH-d detection has been used to investigate the distribution of neurons containing NOS activity.

Several studies in mammals have demonstrated that peripheral axotomy raises NADPH-d activity in the motor and sensory nuclei of the vagus nerve (2), cranial motor neurons (3), intermediolateral cell column of the lumbosacral spinal cord (4) and neurons of the dorsal root ganglia (5). This enzyme was also found in many neurons throughout the turtle central nervous system (6,7). However, no studies on the changes of NADPH-diaphorase activity in turtle spinal cord have been done. Turtles are interesting experimental models because they are thought to be most closely related to the stem reptilian and mammalian ancestors. Therefore, the aim of the present study was to determine the effect of sciatic nerve transection on the NADPH-d activity in neurons of the dorsal root ganglia and in the lumbar spinal cord.

Material and Methods

Adult turtles *Trachemys dorbigni* of both sexes weighing 900 to 1,200 g were collected from a local population in the southern region of Brazil with permission from the authorities of the Instituto Brasileiro de Meio Ambiente e dos Recursos Renováveis (IBAMA) (License 031/95 DEVIS). The

turtles were housed in a large tank with a mound of dry sand in the center, continuously flushed with tap water at room temperature and fed fresh meat *ad libitum* three times a week. The animals were used 60 days after capture.

For peripheral axotomy the turtles were anesthetized with ether by the method of Belló and Belló-Klein (8). Under anesthesia and sterile conditions, the right sciatic nerve was exposed and transected approximately 5 mm distal to the sciatic notch. In this nerve transection, a 2-mm segment of the nerve was removed to ensure that the transection was complete. The wound was closed with surgical thread and the animals were sacrificed 7, 15, 30, 60 and 90 days later (N = 3 for each time interval). On the final day of the experiment, both operated and control turtles were deeply anesthetized with 25 mg/kg Thionembutal administered intraperitoneally and perfused through the heart with cold saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The dorsal root ganglia corresponding to the sciatic nerve emergence were removed bilaterally as also was the lumbar spinal cord, postfixed in the same solution for 2 h, and cryoprotected by immersion in 15 and 30% sucrose solution in PB at 4°C, with continuous shaking, until they sank. Serial coronal sections (50 µm) were obtained with a cryostat and collected in cold phosphate buffer. The sections were treated with 0.1 M PB containing 12 µl Triton X-100 for 10 min and transferred to fresh histochemical medium containing 0.5 mg/ml ß-NADPH, 0.2 mg/ml nitro blue tetrazolium and 0.2 M PB containing 12 µl Triton X-100. After pre-incubation at room temperature for 5 min under continuous shaking, they were incubated at 37°C for 4 h. The reaction was stopped by the addition of excess 0.1 M PB, and the material was mounted onto gelatinized slides and coverslipped with Entellan. Control sections were incubated in a reaction medium without substrate. Sections were examined by different observers, some of

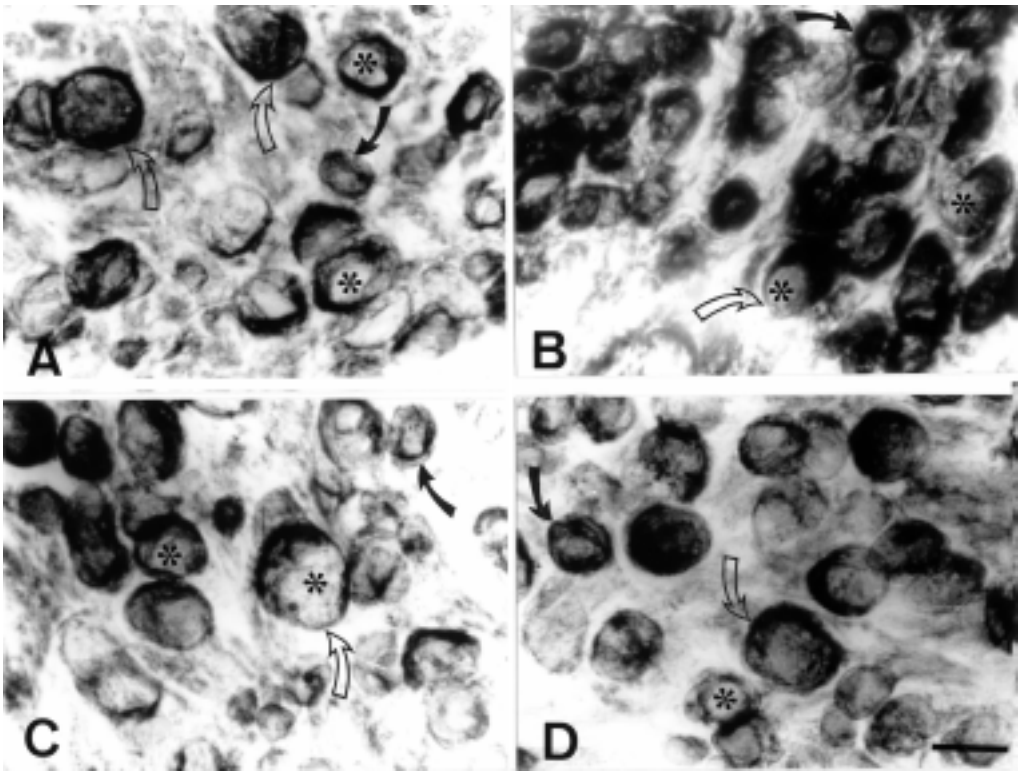

Figure 1 - NADPH-d activity in medium (open arrows) and small (filled arrows) neurons of the turtle dorsal root ganglia after transection of the sciatic nerve. Material from untreated turtles (A) and material obtained 15 (B), 30 (C) and 90 days (D) after peripheral axotomy. Asterisks indicate negative activity in the nucleus. Scale bar = 150 µm.

whom did not know the treatments. The results were photographed with a Nikon Optiphot-2 microscope equipped with a Nikon FX-35DX camera.

Results

After histochemistry, the dorsal root ganglia from control animals showed strong NADPH-d activity in small (16 µm) and medium (24 µm) neurons (Figure 1A). On the 7th day following axotomy, these neurons showed increased NADPH-d staining, which persisted to the 15th day (Figure 1B), with NADPH-d activity returning to the control level in small cells by 30 days (Figure 1C). In the medium cells, NADPH-d staining continued to be elevated up to 90 days (Figure 1D). This increase was bilateral.

For the dorsal horn description we followed the pattern described for turtles by Fernández et al. (9). In the lumbar spinal cord from control turtles moderate NADPH-d reaction occurred in the Ia and Ib areas, and intense reaction was observed in the II and III areas (Figure 2A). Strongly labelled neurons were found in the dorsal commissure and in the lateral column of the dorsal horn. Two subtypes of giant neurons were easily distinguished in the lateral column: 1) neurons with dendritic branches on the ipsilateral side and 2) neurons with one or several dendritic branches crossing to the opposite side. Bitufted neurons were observed in the dorsal commissure (Figure 2A,C,D). In addition, intensely labelled neurons were radially oriented towards the dorsal horn surface. Transection of the sciatic nerve resulted in a bilateral increase of NADPH-d staining in all areas and neuronal types of the lumbar spinal cord. This increase was observed at 7, 15 and 30 days (Figure 2B,C). However, there was a small reduction at 60 and 90 days after axotomy, but this activity was still higher than in the control animals. No reaction product was detected in the motoneurons of control turtles. However, peripheral transection induced an intense

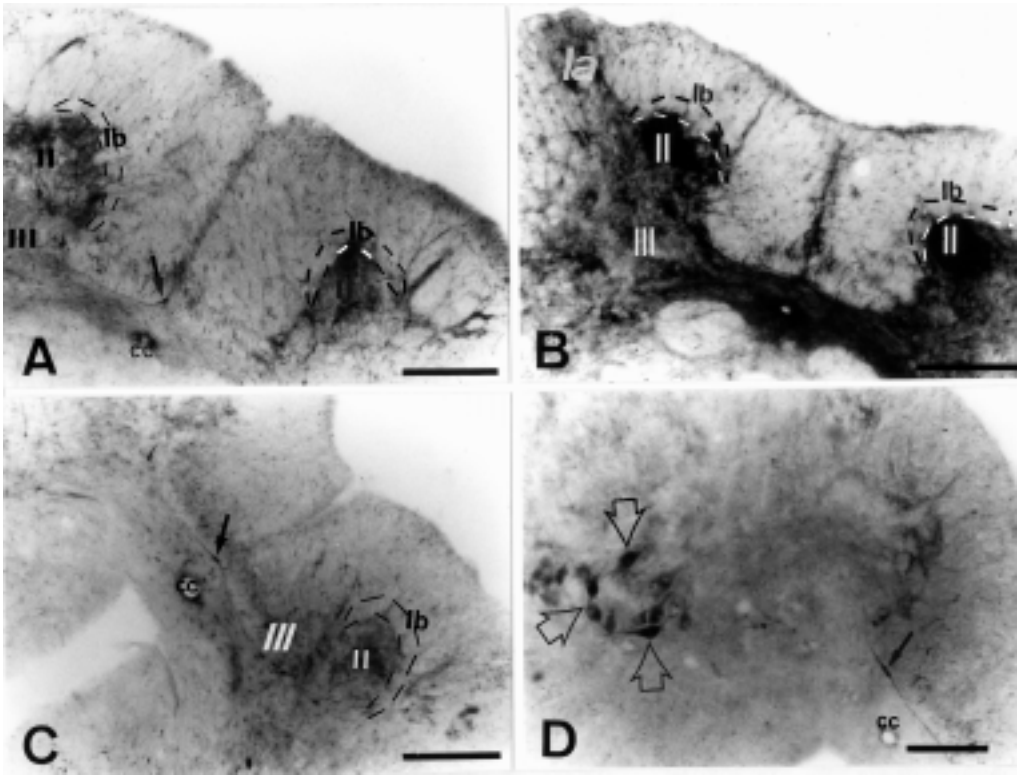

Figure 2 - NADPH-d activity in the turtle spinal cord after transection of the sciatic nerve. Material from an untreated turtle (A) and material obtained 15 (B) and 30 days (C) after peripheral axotomy. Open arrows in D indicate positive motor neurons 90 days after sciatic nerve transection. Filled arrows indicate bitufted neurons. CC, Central canal. Ia, Ib, II and III correspond to dorsal horn areas. Scale bar = 300 µm (A, B and C) and 150 µm (D).

NADPH-d expression in motoneurons on the side ipsilateral to the lesion. After 7 and 15 days this reaction was granular, but exhibited a homogeneous distribution at 30, 60 and 90 days (Figure 2D). Parallel to these changes, there was a strong histochemical staining in glial and endothelial cells, which occurred at 7 days and persisted up to 90 days.

Discussion

This study demonstrates that transection of peripheral nerve fibers increases the NADPH-d reaction in neurons of the dorsal root ganglia and in the dorsal horn of the lumbar spinal cord of the turtle, as also demonstrated in the rat (4,5). However, in turtles NADPH-d activity decreased in small cells of the dorsal root ganglia 30 days after axotomy, while in rats this activity persisted up to 50 days (5). The increase in NADPH-d staining in area Ia of the dorsal horn suggests a role for NO in the turtle nociceptive pathway, since this area appears to be analogous to the Lissauer tract of mammals (9). A recent study proposed a role for NO as a spinal mediator of thermal hyperalgesia in mammals (10). However, the distribution of c-fos-immunoreactive nuclei differed from the NADPH-d-stained neurons in rat spinal cord following unilateral hindpaw inflammation (11). At the moment, the c-fos immunoreaction of the turtle spinal cord remains to be determined. Thus, it is difficult to formulate a hypothesis about this question. Nevertheless, a previous study on mammals has suggested that the bilateral NADPH-d activity after unilateral hindpaw inflammation appears to be associated with neuronal activity, which probably contributes to the overall response to a focal injury and to the subsequent "experience" of pain (11).

Sciatic nerve transection induced the appearance of the NADPH-d reaction in the spinal motoneurons of the turtle. In rats these cells only exhibited this activity after neonatal peripheral axotomy (12) or following ventral root avulsion in adult rats (13). We can offer no explanation for these differences because the role played by NO in the outcome of lesioned mature motoneurons is still unclear. It has been speculated that NO may protect neurons, since this molecule is involved in regeneration mechanisms in rats (14) and its expression is not coincident with neuronal cell death in this animal (15,16). In addition, the increase in NOS activity after axotomy may mediate a vasodilating effect, possibly promoting survival and/or regenerative processes, since NO has been shown to produce highly potent vasodilator effects (17). On the other hand, NO may exert toxic effects after neuronal injury. It has been suggested that the increase in NOS activity in rat motoneurons following ventral root avulsion is involved in the subsequent death of the injured neurons, since pretreatment with a NOS inhibitor significantly increased the number of surviving motoneurons (13). In contrast, previous experiments demonstrated that the failure of neural regeneration after axotomy in mammals may be due to removal of target-derived trophic factors (13,18,19). These studies suggested that in this situation certain neurotrophic factors produced by non-neural cells associated with the remaining axons protected the injured motoneurons from death, and the neurotrophic factors available for the injured motoneurons may be decreased when axotomy is performed very close to the spinal cord. Thus, it might be suggested that NADPH-d staining present in turtle and neonatal rat motoneurons after peripheral axotomy or following strong axonal injury in adult rats may result in significant neurotrophic deficiencies. If this occurs, the adult turtle neurons need the same trophic factors as those of neonatal rats. Further research is necessary in order to elucidate this hypothesis.

Nevertheless, several studies in mammals have demonstrated that glial cell line-derived neurotrophic factor (GDNF) is a potent inhibitor of NOS expression in axotomized motoneurons, and its expres-

sion, normally low in the sciatic nerve, was significantly increased after nerve transection (20,21). Another factor with a role in this situation is c-Jun. In mammals this factor is one of the earliest events in the nerve cell body response to nerve injury, which can mediate cell death, survival or regeneration (22). The activity of these factors is unknown in turtles. Thus, further studies of these neurotrophic factors are necessary to elucidate the mechanisms which determine the role of NO in axotomy. Within this context, data from the present study do not indicate a causal relationship of NADPH-d expression with neuronal cell death in turtles. But they represent the first evidence that the effects of axotomy in these animals show some similarities with those observed in rats. Thus, the turtle seems to be a good model to study the effects of peripheral axotomy. In addition, further studies in turtles under these experimental conditions can contribute to elucidating whether NO functions correspond to a neurodestructive or neuroprotective signal in injured cells.

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