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Dynorphin neurotoxicity induced nitric oxide synthase expression in ventral horn cells of rat spinal cord

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Abstract

Nitric oxide (NO) mediation in the spinal cord injury induced by intrathecal (i.t.) dynorphin (Dyn) administration was studied with NADPH-diaphorase (Nd) histochemistry. Normally, there was rarely NO synthase (NOS) activity in spinal cord motoneurons, and Dyn A(1–17) 10 nmol, which produced only transient paralysis, did not induce Nd/NOS expression in ventral horn cells. After a paralyzing dose of i.t. Dyn A(1–17) 20 nmol, which definitely produced permanent paraplegia and neuronal death, Nd/NOS began to express in motoneurons at 30 min, increased in numbers and intensities at 2–4 h and persisted up to 8 h. Most of Nd/NOS motoneurons disappeared at 24 h coincident with the neuronal death. Quite a few intensively-stained Nd-positive small cells and swollen varicosities became visible only in rats with permanent paraplegia and neuronal death, beginning at 2 h, maximizing at 3–4 h and remaining up to 24 h. These results suggest that NOS expression was induced in the ventral horn of spinal cord, including small cells and varicosities as well as motoneurons closely correlated in time and degree with pathological changes in motoneurons caused by spinal Dyn neurotoxicity.

Keywords: Nitric oxide; Nitric oxide synthase; Neurotoxicity; Dynorphin; Spinal cord injury

NADPH-diaphorase (Nd) histochemical staining reveals the architecture of nitric oxide (NO)-producing cells and cellular processes in great detail [18] and has been universally adopted as a specific marker for NO synthase (NOS) cells [5,18,19]. Recently, experimental studies regarding the role of NO in the central nervous system (CNS) injury have been very controversial. Most experiments in vitro have clearly demonstrated that NO mediates the neural injury induced by excitatory amino acid (EAA) overstimulation and calcium overload [6]. NO donor, given in vitro [6] or in vivo [11], directly induced neuronal degeneration. NO/NOS has been reported to be involved in the acute and chronic CNS injury [2]. Inhibition of NOS has been reported to have a neuroprotective role in focal cerebral ischemia [2,21,22], spinal root avulsion [19] and glutamate neurotoxicity [6]. Quite recently, however, NOS inhibition was proved to aggravate cerebral ischemic damage [4,15], and infusion of L-arginine [13] or NO donors [21] ameliorated the ischemic infarction after middle cerebral artery occlusion in rats. Nd/NOS activities in motoneurons of spinal cord began to express only 1–2 weeks after ventral root avulsion and appeared to be related to the neural regenerative processes following injury [19].

Dynorphin (Dyn) is an endogenous agonist of κ-opioid receptor and participates in spinal nociceptive processes [16]. But, intrathecal (i.t.) administration of Dyn at higher doses has been widely reported to induce dose-dependent hindlimb paralysis [8,12]. We have demonstrated that this is an EAA- and calcium-mediated phenomenon, since both *N*-methyl-D-aspartate (NMDA) receptor antagonists and calcium channel blocker prevent the spinal cord from Dyn neurotoxicity [17]. The purpose of this study is to investigate whether NO mediates Dyn neurotoxicity.

Experiments were performed on Wistar rats (250–300 g). Under pentobarbital sodium (40 mg/kg) anesthesia, an intrathecal line was implanted to the lumbar enlargement according to Yaksh and Rudy (1976). Twenty-four hours later, Dyn A(1–17) (from Peninsula, lot num-

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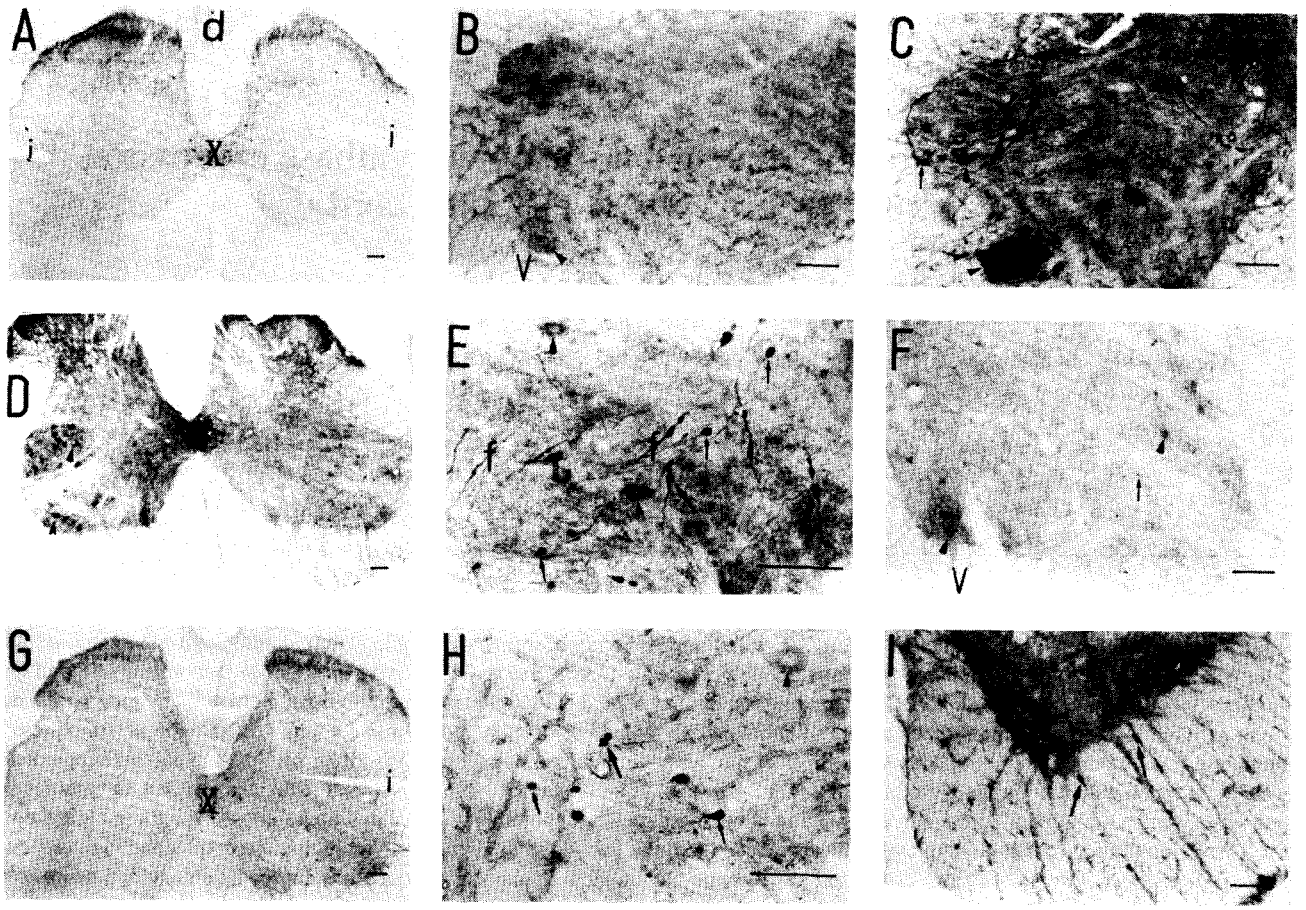

Fig. 1. Nd/NOS staining of spinal sections from normal control rats and under different experimental conditions. (A) Most motoneurons were Nd/NOS-negative in saline-treated rats. (B) A few motoneurons were slightly Nd/NOS-stained at 30 min after Dyn 20 nmol. (C) Several intensively stained cells were circling the lateral motoneuron group and an isolated Nd-positive large cell had rich long processes (big arrow) at 2 h after Dyn. (D) A lot of Nd/NOS-positive small cells, neurites, varicosities (right) and motoneurons (left) became clearly visible 3 h after Dyn. (E) Magnification from (D). (F) Some motoneurons and small cells are still Nd/NOS-positive at 4 h after Dyn. (G) Nd/NOS-positive motoneurons were scarcely seen but a few small cells and varicosities remained intensively Nd-stained at 24 h after Dyn. (H) Magnification from (G). (I) A few intensively-stained Nd/NOS-positive small cells and swollen varicosities were seen in the ventral horn and root at 3 h after Dyn. x, laminae X; v, ventral; d, dorsal; i, lateral incision for labelling; f, neurites; arrows, varicosity; arrowheads, motoneurons; fine arrows, small cells. Scale bars = 100 μm.

ber 22260) or saline was infused in a volume of 10 μl followed by 10 μl of saline to flush the catheter within 1 min. Hindlimb neurological function was evaluated over a 48 h period after infusion. Neurological function was scaled by the eight-point method of Faden (1987).

At 30 min, 2, 3, 4, 8 and 24 h after i.t. Dyn A(1–17) 10 nmol and 20 nmol, respectively, rats were deeply anaesthetized again with pentobarbital sodium and perfused transcardially with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M PBS. The spinal cord 2 cm rostral and caudal to the tip of the implanted catheter were dissected and postfixed for 2–3 h in the same fixative at 4°C, then cryoprotected in 30% sucrose overnight.

Sequential vibratome transverse sections (40 μm) were collected in 0.1 M PBS. Then, freefloating sections were incubated in a freshly-prepared reaction solution containing 0.1 M PBS (pH 7.4), 0.4% Triton X-100, 0.25 mg/ml nitroblue tetrazolium and 1.0 mg/ml β-NADPH (Sigma) at 37°C for 1–2 h. Some of the Nd-stained sections were counterstained with neutral red for their cytoarchitecture. To control variability in histochemical procedure, all the sections went through the same staining run for the same period of time. Furthermore, the saline-controlled sections labelled by incision were mixed with sections from Dyn-treated rats and stained in the same staining tube. The numbers of Nd-positive cells (including motoneurons and small cells) and the total numbers of motoneurons containing a clearly visible nucleus in the ventral horn were counted in 10–20 random sections per animal using a 10× objective and brightfield illumination. Differences between saline control and various experimental treatments were analyzed with one-way ANOVA and FLSD comparison. Statistical significance was determined at $P < 0.05$.

In the saline-treated control rats, isolated Nd-stained

Table 1

Numbers of motoneurons (Mn) and Nd-positive cells (Nd) per section (bilateral) in medial (MV) and lateral (LV) ventral horn following i.t. Dyn A(1–17) 20 nmol

Group	Saline (80)	Dyn A(1–17) 20 nmol					
		30 min (60)	2 h (60)	3 h (40)	4 h (60)	8 h (40)	24 h (40)
MV Mn	47 ± 8	42 ± 12	31 ± 2*	9 ± 1*	35 ± 2*	20 ± 2*	6 ± 1*
MV Nd	1 ± 0.2	4 ± 0.5*	7 ± 0.6*	5 ± 0.4*	5 ± 0.7*	5 ± 0.7*	1 ± 0.3*
LV Mn	82 ± 18	64 ± 10	49 ± 2*	28 ± 2*	47 ± 4*	33 ± 2*	7 ± 1*
LV Nd	2 ± 0.4	14 ± 1.4*	18 ± 1.2*	17 ± 1.2*	16 ± 1.4*	10 ± 1.1*	5 ± 1

Values represent mean ± SEM. Numbers of sections are in parentheses, and 20–30 sections per animal were counted.
*$P < 0.05$, significantly different from saline-treatment.

cells were rarely seen in the ventral horn, usually in the medial portion (Fig. 1A). In Dyn-treated rats, the effect varied with the dose of Dyn used. At 10 nmol, which produced only transient paralysis with the present batch of Dyn, no significant changes in the morphology and distribution of Nd-stained neurons were noticed at all time points after i.t. Dyn A(1–17). There were neither any significant changes of total motoneurons nor pathological findings.

Abnormal Nd/NOS expression and pathological changes became visible after i.t. Dyn A(1–17) 20 nmol, which consistently produced permanent paraplegia. Larger population and stronger staining of Nd-positive cells and neurites were observed. The cell bodies of a few motoneuron began to be lightly stained at 30 min after injection (Fig. 1B). The number and intensity of Nd-positive cells in the ventral horn, especially its lateral portion, increased significantly at 2–4 h and persisted up to 8 h (see Table 1, Fig. 1C–F). While the Nd/NOS-negative motoneurons appeared purely red in saline control rats, the Nd/NOS-positive motoneurons stained purplish as a mixture of blue (Nd-stained) and red (Neutral red counterstained). The Nd/NOS staining of motoneurons varied in intensity and site. The nucleus and most distal processes of the Nd-positive motoneuron were not stained. Quite a few unipolar or bipolar Nd-positive small cells (4–18 μm) and their fibers were most deeply stained in the region of the ventral horn where the motoneurons were lost, in sharp contrast to the contralateral ventral horn (Fig. 1D). They were most significant at 3–4 h. Some varicosities were also swollen. Some of the intensively-stained Nd-positive neurites and swollen varicosities were found to extend into the ventral root or reach the pia surface (Fig. 1I). The maximal numbers of Nd-positive small cells and swollen varicosities per section at 2, 3, 4 and 8 h were 4, 18, 19 and 15, respectively. At 24 h, the majority of the stained motoneurons disappeared, while a few Nd-positive small cells and swollen varicosities were still visible (Fig. 1G,H, Table 1).

Both Nissl staining with thionin and counterstaining with neutral red revealed no histopathological changes in motoneurons at 30 min after Dyn A(1–17) 20 nmol. Significant and progressive degeneration (chromatolysis, nucleus dislocation, nucleolus disappearances, etc.) and even cell death were shown at 2–4 and 8 h (Table 1). At 24 h, most motoneurons disappeared, leaving vacuoles and remnants of dead neurons.

The abnormal Nd/NOS expression and pathological changes were most apparent within the first segment of the spinal cord 0.5 cm from the tip of the catheter, and less so within the second 0.5 cm segment, varying with the severity of Dyn neurotoxicity.

Increasing evidences indicated that Dyn-induced motor dysfunction might involve both opioid and non-opioid mechanisms [8]. Activation of NMDA receptor and calcium overload were also implicated [8,12,16,17]. The present study proved morphologically that spinal Dyn neurotoxicity induced Nd/NOS expression in ventral horn cells in close correlation to motor dysfunction and motoneuronal death.

The source of NO production after CNS injury was unclear. A few in vitro studies have demonstrated that NO produced from neurons [6], astrocytes [9] and microglia [1] might all mediate neurotoxic injury. Neuronal NO production in vivo has been recently reported to exacerbate brain ischemic injury [10]. Nd/NOS expressions were induced in astrocyte by brain ischemia [7]. In this study, the intensively Nd-stained small cells and swollen varicosities appeared only in those rats with irreversible permanent paraplegia and severe motoneuron loss. This implied that NO generated by the small cells and swollen varicosities could be the source of NO that initiated neuronal death. The type and nature of these small cells and swollen varicosities need further investigation. Some of the small cells were more than 10 μm in length and had unipolar or bipolar processes, which appeared to implicate themselves as small interneurons. The others, about 4–10 μm in size, might be glial cells. The enlarged varicosities and neurites might suggest the abnormal changes of axonal transport.

The induction of Nd/NOS expression in motoneurons is a significant but unexplained phenomenon. Nd/NOS-positive cells in vivo have been reported to be selectively resistant to NMDA neurotoxicity and spared in Hunting-

ton's and Alzheimer's diseases [14]. Here in the spinal cord, the induction of normally dormant and unexpressed NOS in motoneurons might help in their survival, but ultimately does not protect them from being killed by i.t. Dyn. We speculate that the expression of NOS in motoneurons could be a part of the body defense mechanism as Wu and Li [19] have suggested.

In conclusion, this is the first report on the induction of Nd/NOS expression by neurotoxic agents in motoneurons, and especially the Nd-positive small cells as well as swollen varicosities in the ventral horn of the spinal cord. They were coincident with Dyn spinal neurotoxicity and closely correlated in time and degree with motor dysfunction and neuronal death.

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