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Marked increase in nitric oxide synthase mRNA in rat dorsal root ganglia after peripheral axotomy: *In situ* hybridization and functional studies

(plasticity/neuropeptides/endothelium-derived relaxing factor/coexistence/pain)

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ABSTRACT **Using *in situ* hybridization, we studied nitric oxide (NO) synthase (EC 1.14.23.-) mRNA in lumbar dorsal root ganglia after peripheral transection of the sciatic nerve in rats. The effect of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester on the nociceptive flexor reflex was also studied in axotomized rats. Nerve section induced a dramatic increase in number of NO synthase mRNA-positive cells in the ipsilateral dorsal root ganglia. In some of these cells the peptides galanin and/or vasoactive intestinal polypeptide and/or neuropeptide Y were also strongly up-regulated. Intravenous administration of nitro-L-arginine methyl ester blocked spinal hyperexcitability at much lower dosages in axotomized than in normal animals. The results suggest involvement of NO in the function of lumbar sensory neurons, especially after axotomy, perhaps preferentially at peripheral sites.**

Since the discovery that nitric oxide (NO) is identical to the endothelium-derived relaxing factor (1) in blood vessels (2, 3), the role of NO as an intercellular messenger in the nervous system has received increasing attention (4, 5). NO is formed from L-arginine by the enzyme NO synthase (NOS) (EC 1.14.23.-) which has been purified (6), and the gene for rat brain NOS has been cloned (7), permitting immunohistochemical and *in situ* hybridization analysis of the cellular localization of the enzyme and its site of synthesis (8, 9). Moreover, NOS is identical to neuronal NADPH diaphorase (10, 11), thus enabling localization of NOS with a specific histochemical method (12, 13). Among the neuronal systems positive for NADPH diaphorase are primary sensory neurons (14), the highest number of which are found at the thoracic level, with only few neurons in the lumbar ganglia (14). We have used a synthetic oligonucleotide probe against NOS mRNA to analyze sensory ganglia under normal and experimental conditions with *in situ* hybridization. Our results demonstrate a dramatic up-regulation of NOS mRNA after a peripheral nerve lesion. The possible functional significance of this up-regulation has been analyzed by using a well-established spinal reflex model (15, 16).

MATERIALS AND METHODS

Animals. The experiments were carried out with the approval of the Stockholms norra djurförsöksetiska nämnd, the local animal experimentation and ethics committee.

***In Situ* Hybridization.** Male Sprague–Dawley rats (200 g) were deeply anesthetized, the right sciatic nerve was transected in 22 rats at its origin from the L4, L5, and L6 spinal nerves, and a 5-mm portion of the nerve was resected. After different periods (2, 3, 7, 9, 14, 21, 28, or 72 days), the animals were deeply anesthetized and perfused with Tyrode's solution, and the right and left L4 and L5 dorsal root ganglia (DRG) were rapidly dissected and frozen. In six control rats the L4 and L5 DRGs, trigeminal, nodose, and superior cervical ganglia were dissected out. Chucks containing both experimental and control ganglia were cut (5 μm) in a cryostat (Microm) and thaw-mounted onto the same slide.

Our procedure followed previously published protocols (17, 18). The sequences for the oligonucleotide probes were complementary to the mRNA coding for amino acids 151–164 of rat brain NOS (7), nucleotides 152–199 of rat galanin (GAL) (19), nucleotides 347–394 of rat vasoactive intestinal polypeptide (VIP) (20), nucleotides 1671–1714 of rat neuropeptide Y (NPY) (21), and nucleotides 664–698 of rat α-calcitonin gene-related peptide (CGRP) (22). The oligonucleotide probes were labeled at the 3′ end with deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (NEN) by using terminal deoxynucleotidyltransferase (IBI). The labeled probes were purified and dithiothreitol was added to a final concentration of 10 mM. Specific activities obtained were 1–4 × 10^6 dpm/ng of oligonucleotide. Slides with sections were hybridized for 16–18 hr at 42°C with 10^6 cpm of labeled probe per 100 μl of a hybridization cocktail (17, 18). After hybridization the slides were rinsed, dehydrated, dried, and dipped in NTB2 nuclear track emulsion (Kodak) diluted 1:1 with distilled water, exposed at −20°C for 14 days, developed, fixed, rinsed, and mounted with glycerol for analysis with a dark-field condenser or stained with toluidine blue for viewing under bright-field illumination. Control hybridizations were carried out on ipsilateral ganglia, 7 days after axotomy with labeled probe plus either a 400-fold excess of unlabeled probe or a 400-fold excess of another, dissimilar, unlabeled probe (CGRP).

The percentage of labeled neurons before and after injury was determined by using a 100× objective lens on two sections stained for each of five time intervals (0, 2, 7, 9, 28, and 71 days) after resection from two to four animals per time point. Neurons containing 3 times more grains than mean background grain densities were counted, and the number

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Abbreviations: NOS, nitric oxide synthase; VIP, vasoactive intestinal polypeptide; NPY, neuropeptide Y; CGRP, calcitonin gene-related peptide; GAL, galanin; DRG, dorsal root ganglia; L-NAME, N^{ω}-nitro-L-arginine methyl ester; i.t., intrathecal(ly); CS, conditioning stimulus.

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was divided by the total number of toluidine blue-stained neurons in that section. Mean background grain densities were determined by averaging grain counts over defined areas of the neuropil devoid of positively labeled cell bodies.

To correlate NOS mRNA hybridization with peptide expression after injury approximately 150 individual neurons were identified on photomontages (×400) prepared from serial 5-μm sections of 7-day injured L5 DRG processed for *in situ* hybridization in the sequence NOS/GAL/NOS/NPY/NOS/VIP.

Quantification. Quantitative analysis of volume and NOS hybridization signal was performed for 288 neurons from a 7-day axotomized L5 DRG. Under 100× immersion light microscopy with an interactive image analysis system the percentages of cytoplasmic area covered by silver grains were measured for all neurons with a visible nucleus in the preparation as previously described (23). Volumes were calculated from cross-sectional areas with the assumption that neurons are spherical.

Functional Studies. Acute electrophysiological experiments were carried out on decerebrate, spinalized, unanesthetized female Sprague–Dawley rats (200–250 g) with intact sciatic nerves or 11–15 days after unilateral sciatic nerve section according to previously described methods (16). The NO synthesis inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma) was injected intrathecally (i.t.) onto the lumbar enlargement in a 10-μl volume or intravenously (i.v.) via a catheter in the jugular vein. The effect of L-NAME on the baseline flexor reflex, evoked 1 per min, and sensitization of the flexor reflex after a conditioning electrical stimulus (CS) train of 20 shocks at 0.9 Hz that activated A and C fibers in the intact or axotomized sural nerve was examined.

RESULTS

Normal rat L4 and L5 DRG contained only a few detectable NOS mRNA-positive cells (Fig. 1*A*); trigeminal ganglia, only single cells (Fig. 1*B*); nodose ganglia, many heavily labeled neurons (Fig. 1*C*); and superior cervical ganglia, none (Fig. 1*D*). After transection of the sciatic nerve, there was a marked increase in the numbers of lumbar ganglion cells containing NOS mRNA. Two days after peripheral axotomy approximately 35% of all remaining neurons expressed NOS mRNA (Fig. 1*E*), and a somewhat higher number was de-

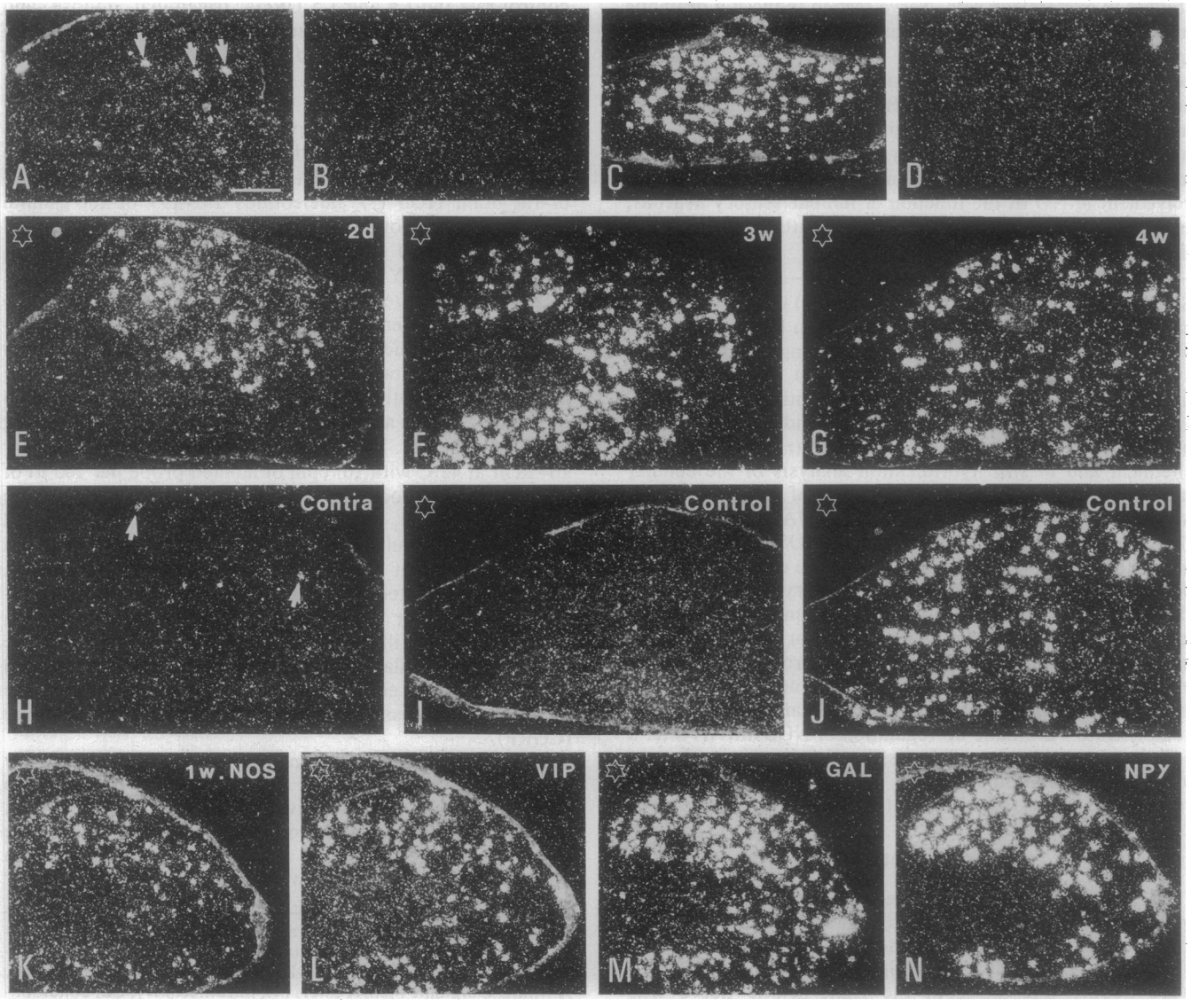

FIG. 1. *In situ* hybridization dark-field autoradiographs of lumbar (*A*, *E–N*), trigeminal (*B*), nodose (*C*), and superior cervical (*D*) ganglia after hybridization with a probe complementary to NOS mRNA (*A–H*, *K*), NOS probe plus an excess of unlabeled NOS probe (*I*), NOS probe plus an excess of unlabeled CGRP probe (*J*), and probes complementary to VIP (*L*), GAL (*M*), and NPY (*N*) of normal (*A–D*) or axotomized (*E–N*) ganglia (*E*, 2 days; *F*, 3 weeks; *G*, 4 weeks; *H–J*, 7 days; *K–N*, 7 days). Arrows point to NOS mRNA-positive neurons. (Six-pointed stars indicate lesion site. Bar indicates 50 μm.)

tected at the following intervals: 45% at 7 days, 40.5% at 9 days, and 45.8% at 28 days (Fig. 1*G*), and 27.5% at 71 days. Quantification of NOS mRNA hybridization signal in 15 DRG sections 7 days after axotomy revealed a bimodal distribution of labeling indices compatible with existence of two populations of neurons—those that express detectable NOS mRNA in response to injury and those that do not (Fig. 2*A*). Little correlation was observed between NOS mRNA expression and cell volume in injured neurons, although there was a tendency for little or no detectable expression of NOS in larger neurons (Fig. 2 *B* and *C*). In contralateral DRG only single cells with detectable NOS mRNA were seen at all time intervals examined (Fig. 1*H*). Analysis of consecutive sections revealed that in parallel to the up-regulation of NOS mRNA (Fig. 1*K*), large numbers of VIP (Fig. 1*L*), GAL (Fig. 1*M*), and NPY (Fig. 1*N*) mRNA-positive cells were seen. GAL and especially NPY mRNA-positive cells were more numerous than NOS mRNA-expressing cells. Only a few GAL and no VIP or NPY mRNA-positive cells were seen on the contralateral side. A majority of NOS mRNA-positive neurons were small and medium-sized (Fig. 3 *A* and *B*), similar to the size of VIP and GAL mRNA-expressing neurons, as opposed to NPY, which labeled predominantly larger neurons. There was a degree of colocalization with all three peptides, the highest incidence of which occurred with GAL, followed by VIP (*cf.* Fig. 3 *C* and *B*) and then NPY. In addition, some small NOS neurons did not express detectable levels of any of the three peptides. The control experiments revealed that excess of unlabeled probe abolished the NOS or peptide mRNA hybridization signal (Fig. 1*I*), while an excess of a dissimilar unlabeled probe did not influence the pattern of hybridization (Fig. 1*J*).

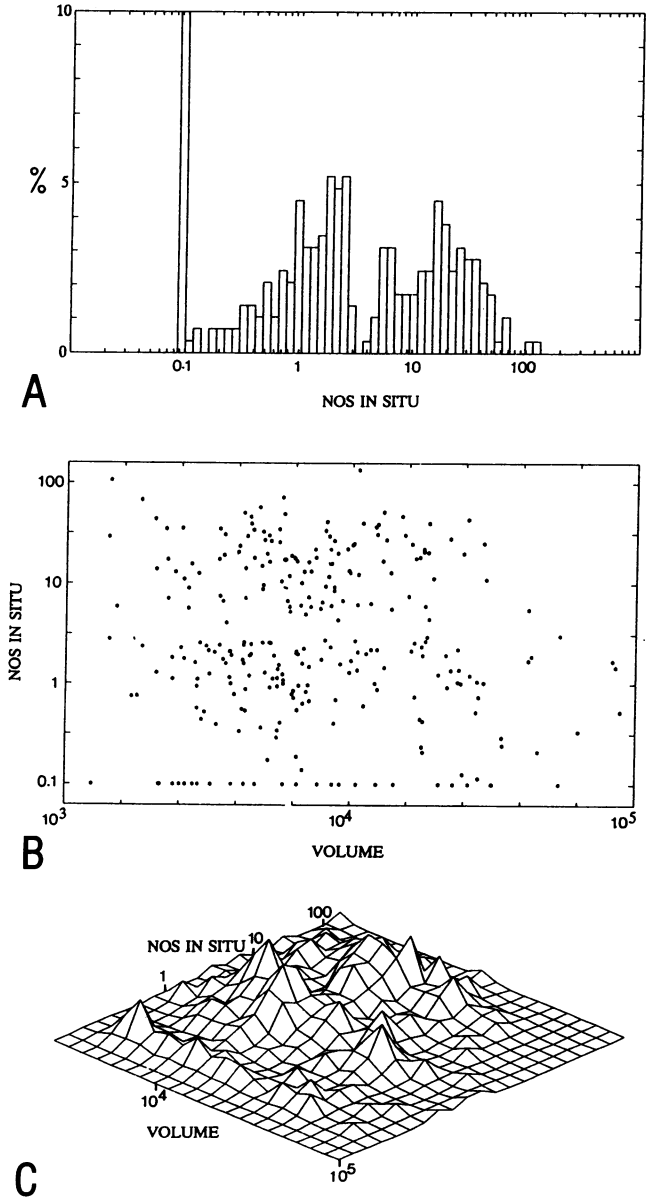

FIG. 2. Quantification of NOS mRNA labeling in a L5 DRG section 7 days after injury. (*A*) Frequency histogram showing the percentage of neurons versus NOS mRNA labeling indices. Note the bimodal distribution compatible with the existence of two populations. (*B*) Scatterplot with volume (μm^3) plotted against NOS hybridization labeling indices. Little correlation is observed between presence of NOS mRNA and cell volume, although there is a tendency for large neurons to be devoid of label. (*C*) Three-dimensional histogram of scatterplot *B* with frequency on the *z*-axis (n = 288 neurons). Note once again the low abundance of large NOS mRNA-positive neurons in the right quadrant.

Intrathecal L-NAME at 0.1 nmol (27 ng) and 1 nmol (270 ng) did not influence the baseline flexor reflex in rats with intact and sectioned sciatic nerves. One, but not 0.1 nmol, of L-NAME similarly blocked facilitation of the flexor reflex induced by the C-fiber CS in rats with intact and sectioned sciatic nerves (Table 1). The baseline reflex was similarly depressed dose-dependently by 10 and 100 nmol i.t. L-NAME in rats with intact and sectioned nerves, 36.2% ± 4.4% (n = 6) after 10 nmol and by 75.3% ± 3.8% (n = 3) after 100 nmol of i.t. L-NAME.

Intravenous L-NAME did not influence baseline flexor reflex excitability up to 1000 μmol/kg. In rats with intact sciatic nerves, L-NAME at 10 and 100 μmol/kg produced no effect upon the CS-induced reflex facilitation, whereas L-NAME at 400 and 1000 μmol/kg partially blocked facilitation of the flexor reflex induced by the C-fiber CS. After sciatic nerve section L-NAME at 100 but not 10 μmol/kg effectively blocked facilitation of the flexor reflex induced by C-fiber CS.

DISCUSSION

Previous histochemical studies (14, 24) have demonstrated that sensory ganglia contain varying amounts of NADPH diaphorase/NOS-positive cell bodies, with only few cells at lumbar levels. The present results are in good agreement with these findings. In contrast, there was a dramatic increase (up to 45% of all neurons) in the number of NOS mRNA-positive cells in the L4 and L5 ganglia after peripheral axotomy, the increase remaining for at least 2 months. This pattern of plasticity for NOS mRNA is very similar to the effect of peripheral axotomy on several peptides. Indeed, a strong increase in number of VIP (25), GAL (26), and NPY (27) -positive primary sensory neurons has been reported and was observed here. In fact, our results show that after axotomy NOS mRNA coexists most often with GAL mRNA, followed by VIP then NPY mRNA, although none of the peptides proved to be an absolute marker of NOS neurons.

The functional significance of plastic changes in messenger molecules in primary sensory neurons is not well understood. In general terms, it is assumed that the compounds that are down-regulated, as shown, for example, for substance P (28), are involved in transmission of nerve impulses at the central branches in the dorsal horn. The down-regulation of these compounds could suppress axotomy-induced hyperexcitability in the somatosensory system responsible for neuropathic pain (29), but it could also serve to direct energy mainly to reparative processes. In contrast, peptides such as VIP, GAL, and NPY are hardly expressed under normal conditions, and their up-regulation after injury may serve to counteract the deleterious effects of axotomy and promote restitution of function and survival. For example, VIP may

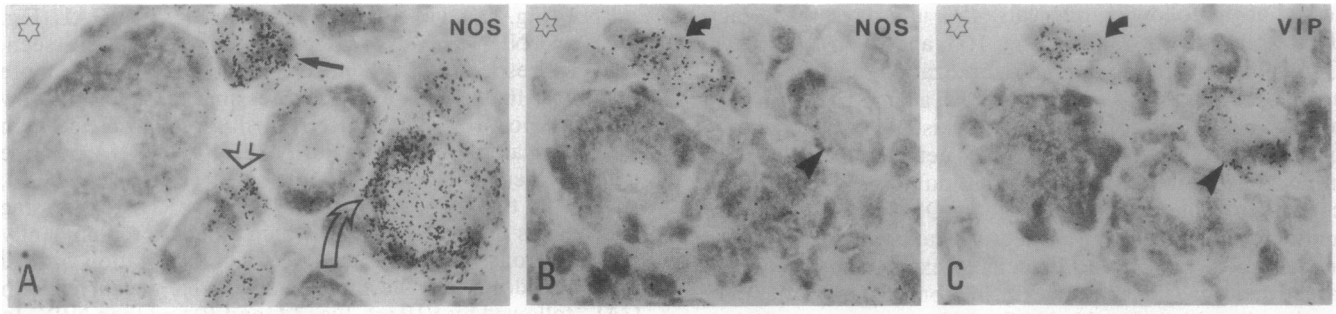

FIG. 3. *In situ* hybridization bright-field autoradiographs showing the cellular localization of NOS (*A* and *B*) and VIP (*C*) mRNA. Small (solid arrow in *A*) and medium-sized (curved arrow in *A*) and sometimes very small cells (open arrow in *A*) are labeled. One cell in 5-μm-thick adjacent sections *B* and *C* contains both NOS and VIP labeling (curved arrows), while another cell (arrowheads) is positive only for VIP mRNA. (Bar indicates 50 μm.)

have trophic effects, and increase blood flow and glucogenolysis (30); GAL, on the other hand, may represent an endogenous analgesic compound counteracting the above-mentioned hyperexcitability (16).

In analogy, up-regulation of NOS and a presumable increase in NO release could enhance regeneration by increasing blood flow. However, the present physiological results also support a role of NO in suppressing neuronal activity in damaged neurons, perhaps at the ganglionic level after axotomy, in addition to confirming previous evidence for NO involvement in nociceptive transmission in the spinal cord of normal rats (31). In the present study L-NAME had a differential effect on spinal cord excitability in normal and axotomized rats dependent on route of administration. Intrathecal L-NAME depressed the baseline flexor reflex and blocked spinal hyperexcitability after a CS train with similar potency in rats with normal and sectioned sciatic nerves. In contrast, i.v. L-NAME blocked spinal hyperexcitability at much lower doses in axotomized than normal animals, indicating that its effect after nerve section may not be in the dorsal horn but in the periphery. Thus, after axotomy NO synthesis could be activated by pathological events associated with primary afferent lesions. For example, after peripheral nerve injury, some Aβ, Aδ, and C afferents develop ongoing activity, originating in the neuroma formed in the central stump of injured afferents (29, 32), as well as in the DRG (33). The present results suggest that blockade of NO synthesis in axotomized primary afferents peripheral to their intraspinal terminals may alter primary afferent transmission, especially in C fibers, since spinal hyperexcitability is mediated by activation of unmyelinated afferents (15).

In this context it is interesting that Morris *et al.* (24) have suggested that NO may act as messenger between DRG neurons and their satellite cells, by inducing a marked up-regulation of cyclic GMP in the satellite cells. NO could also act on macrophages and Schwann cells at the site of injury and in the distal denervated part of the nerve; these sites have been shown to exhibit an increase in transferrin binding and uptake of iron (34), the element associated with the enzyme guanylyl cyclase, which serves as the receptor for NO to activate conversion of GTP to cyclic GMP (35). Thus, NO may act as a messenger molecule *within* or *peripheral* to injured sensory ganglia and not necessarily only at the level of the nerve terminals in the dorsal horn of the spinal cord.

Table 1. Effects of i.t. or i.v. L-NAME on facilitation of the flexor reflex

L-NAME		Nerve intact		Nerve sectioned	
Route	Dose	*n*	% control response	*n*	% control response
i.t.	0.1 nmol	3	109.7 ± 15.0	3	90.0 ± 9.6
	1 nmol	4	36.3 ± 12.6	3	38.3 ± 8.0
	10 nmol	3	4.3 ± 2.7	3	11.7 ± 11.7
i.v.	10 μmol/kg	3	82.7 ± 9.6	3	104.3 ± 16.0
	100 μmol/kg	4	92.3 ± 12.3*	4	17.0 ± 5.7
	400 or 1000 μmol/kg	4	55.5 ± 15.9*		

The reflex was measured after a 20-s 0.9-Hz CS train to the sural nerve innervation territory in rats with intact sciatic nerves or the sural nerve proximal to axotomy in the nerve-sectioned rats. The blocking effect of i.t. L-NAME was the same in rats with intact and sectioned nerves, whereas i.v. L-NAME at 100 μmol/kg inhibited CS-induced reflex facilitation significantly more in axotomized than in normal rats (Mann–Whitney *U* test; *, $P < 0.05$).

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