The Journal of Neuroscience, November 1, 1999, 19(21):9201–9208

Neuronal Nitric Oxide Synthase mRNA Upregulation in Rat Sensory Neurons after Spinal Nerve Ligation: Lack of a Role in Allodynia Development

Z. David Luo,[1] S. R. Chaplan,[1] B. P. Scott,[1] D. Cizkova,[1] N. A. Calcutt,[2] and T. L. Yaksh[1]

Departments of [1]Anesthesiology-0818 and [2]Pathology-0612, University of California, San Diego, La Jolla, California 92093

Pharmacological evidence suggests a functional role for spinal nitric oxide (NO) in the modulation of thermal and/or inflammatory hyperalgesia. To assess the role of NO in nerve injury-induced tactile allodynia, we examined neuronal NO synthase (nNOS) expression in the spinal cord and dorsal root ganglia (DRG) of rats with tactile allodynia because of either tight ligation of the left fifth and sixth lumbar spinal nerves or streptozotocin-induced diabetic neuropathy. RNase protection assays indicated that nNOS mRNA (1) was upregulated in DRG, but not spinal cord, neurons on the injury side beginning 1 d after nerve ligation, (2) peaked (~10-fold increase) at 2 d, and (3) remained elevated for at least 13 weeks. A corresponding increase in DRG nNOS protein was also observed and localized principally to small and occasionally medium-size sensory neurons. In rats with diabetic neuropathy, there was no significant change in DRG nNOS mRNA. However, similar increases in DRG nNOS mRNA were observed in rats that did not develop allodynia after nerve ligation and in rats fully recovered from allodynia 3 months after the nerve ligation. Systemic treatment with a specific pharmacological inhibitor of nNOS failed to prevent or reverse allodynia in nerve-injured rats. Thus, regulation of nNOS may contribute to the development of neuronal plasticity after specific types of peripheral nerve injury. However, upregulation of nNOS is not responsible for the development and/or maintenance of allodynia after nerve injury.

Key words: neuronal nitric oxide synthase; nerve injury; spinal cord; dorsal root ganglia; sensory neurons; mRNA regulation; diabetic neuropathy; allodynia

Nerve injury of varying etiologies may produce chronic pain states characterized by allodynia, in which innocuous tactile stimuli become frankly aversive. Experimental models of nerve injury–evoked allodynia include traumatic and metabolic etiologies, such as nerve ligation and streptozotocin-induced diabetes. One consequence of such nerve injuries is the appearance of adaptive changes in the expression of a variety of receptors, channels, and enzymes in the dorsal root ganglion (DRG) of the injured nerve and in spinal neurons postsynaptic to the injured afferents.

Changes in spinal nitric oxide (NO) production may contribute to allodynia after nerve injury. Spinal NO release is evoked by NMDA receptor activation (Snyder, 1992; Luo and Vincent, 1994; Montague et al., 1994; Sakai et al., 1998). NO has been shown to enhance the release of excitatory amino acids (Akira et al., 1994; Montague et al., 1994; Mollace et al., 1995; Ohno et al., 1995; Sandor et al., 1995; Ientile et al., 1996; Nei et al., 1996; Bogdanov and Wurtman, 1997). Spinal delivery of NMDA receptor antagonists has been shown to attenuate allodynia (Calcutt and Chaplan, 1997; Chaplan et al., 1997; Siegan et al., 1997). These observations suggest an important role of spinal neuronal nitric oxide synthase (nNOS) in allodynic states observed after nerve injuries. Pharmacological evidence regarding the role of spinal NO in the development of nerve injury–evoked allodynia has been conflicting. Although some investigators observed allodynia inhibition in nerve-injured rats after treatment with L-N^{G}-nitro-arginine methyl ester (L-NAME), a nonspecific NOS inhibitor (Yoon et al., 1998), others have found that L-NAME has no effect on diabetic- or nerve injury–evoked allodynia (Calcutt and Chaplan, 1997) (S. R. Chaplan, unpublished observations).

Immunological and enzymatic studies indicate the presence of nNOS-positive neurons and NOS activity in spinal cord and DRG neurons. Nerve injury after tight spinal nerve ligation has been shown to evoke an increase of NOS expression and activity in DRG but a reduction in spinal cord neurons (Steel et al., 1994; Choi et al., 1996), indicating a NOS-mediated neuronal response to the nerve injury. However, the level of such regulation is not known because nNOS mRNA levels after the nerve injury have not yet been examined. In addition, a direct correlation between nNOS expression and the development and/or maintenance of allodynia has not been established.

This study characterized nNOS expression at the mRNA level and correlated it with the development of tactile allodynia after nerve injury. To do this we have used rats with different genetic backgrounds, either susceptible or resistant to the development of allodynia after tight spinal nerve ligation (Kim and Chung, 1992), nerve-ligated rats with allodynia or fully recovered from allodynia, or diabetic rats with allodynia (Calcutt et al., 1996; Calcutt and Chaplan, 1997). In addition, the effects of 7-nitroindazole (7-NI), a nNOS-specific inhibitor, on the development and/or maintenance of allodynia were evaluated. We established that nNOS expression is upregulated dramatically at the mRNA level after mechanical, but not metabolic, nerve injury but that such

Received Jan. 20, 1999; revised Aug. 9, 1999; accepted Aug. 12, 1999.

This work was supported by National Institutes of Health Grants F32HL09848 (Z.D.L.) and NS01769 (S.R.C.) and an institutional grant from the Howard Hughes Medical Institute, University of California, San Diego (S.R.C. and Z.D.L.).

Correspondence should be addressed to Dr. Z. David Luo, Department of Anesthesiology-0818, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0818. E-mail: zluo@ucsd.edu.

Dr. Cizkova's permanent address: Institute of Neurobiology SAS, Soltesovej 6, 040 01 Kosice, Slovakia.

Copyright © 1999 Society for Neuroscience 0270-6474/99/199201-08$05.00/0

regulation is not responsible for the development and/or maintenance of nerve injury–induced allodynia.

MATERIALS AND METHODS

Materials. Rats were from Harlan Sprague Dawley (Indianapolis, IN), and [^{32}P]UTP (specific activity, 800 Ci/mmol) was from NEN Research Products (Wilmington, DE). Tris-acetate gels (NuPAGE) and buffers were from Novex (San Diego, CA). Monoclonal antibody against rat brain nNOS was from Sigma (St. Louis, MO). Secondary antibody labeled with horseradish peroxidase, its substrate, and enhancer solutions were from Pierce (Rockford, IL). Biotinylated anti-mouse secondary antibody and avidin–biotin complex solution were from Vector Laboratories (Burlingame, CA). 7-Nitroindazole was from Research Biochemicals (Natick, MA). RNases were from Ambion (Austin, TX), and RNA polymerases and restriction enzymes were from Life Technologies (Gaithersburg, MD). Other chemicals were from Sigma.

Animals. Rats (male Harlan or Holtzman Sprague Dawley; 100–150 gm) were housed in separate cages using soft bedding, maintained on a 12:12 hr light/dark cycle, and fed food and water *ad libitum*. Diabetic studies used adult (230–250 gm) female Harlan Sprague Dawley rats that were housed two per cage on wire grates to prevent contact with urine-soiled bedding. All animal care and experiments were performed according to protocols approved by the Institutional Animal Care Committee of the University of California, San Diego.

Neuropathic lesions. The surgical procedure described by Kim and Chung (1992) was used to induce tactile allodynia in rats. Briefly, the left lumbar fifth and sixth spinal nerves (L5/6) of rats anesthetized with halothane were exposed and tightly ligated with 6.0 silk suture distal to their DRG and proximal to their conjunction to form the sciatic nerve. Sham operations were performed in the same way except that spinal nerves were not ligated. Diabetic neuropathy was induced as described (Calcutt and Chaplan, 1997) by a single intraperitoneal injection of 50 mg/kg streptozotocin (freshly dissolved in 0.9% sterile saline) to ablate pancreatic β cells and induce insulin deficiency. Diabetes was confirmed in these rats 2 d later by measuring blood glucose concentrations, using a glucose oxidase–impregnated test strip and reflectance meter (Ames Glucostix and Glucometer II; Miles, Elkhart, IN), in samples obtained by tail prick. Only animals with a blood glucose concentration >15 mmol/l were included as diabetic, and hyperglycemia was reconfirmed at the time of death.

Drug administration. 7-NI was suspended in peanut oil by sonication. For preemptive treatments, a subcutaneous injection of 50 mg/kg in a volume of 1 ml was started 30 min before the surgery and given daily for 6 d. For treatment of tactile allodynia, the same daily dose of 7-NI was administered subcutaneously from day 11 to 13 after the operation when tactile allodynia was fully developed in the nerve-ligated rats.

Behavioral testing. Tactile allodynia was tested as described previously (Chaplan et al., 1994). Briefly, rats were transferred to a clear plastic, wire mesh–bottomed cage and allowed to acclimatize for 15 min. Von Frey filaments (Stoelting, Wood Dale, IL) were used to determine the 50% paw withdrawal threshold using the up–down method of Dixon (1980). A series of filaments, starting with one that had a buckling weight of 2.0 gm, was applied in consecutive sequence to the plantar surface of the left (nerve-ligated) or right (diabetic) hindpaw with a pressure causing the filament to buckle. Lifting of the paw indicated a positive response and prompted the use of the next weaker filament. Absence of a paw withdrawal response after 5 sec prompted the use of the next filament of increasing weight. This paradigm continued until four more measurements had been made after the initial change of the behavioral response or until five consecutive negative (assigned a score of 15 gm) or four consecutive positive (assigned a score of 0.25 gm) responses had occurred. The resulting scores were used to calculate the 50% response threshold by using the formula: 50% gm threshold = $10^{(Xf + \kappa\partial)}/10{,}000$, where Xf = the value (in log units) of the final von Frey filament used, κ = the value [from table in Chaplan et al. (1994)] for the pattern of positive and/or negative responses, and ∂ = the mean difference (in log units) between stimulus strengths. Behavioral tests were performed immediately before or 30 min to 1 hr after drug administrations. Allodynia was considered to be present when paw withdrawal thresholds were <4 gm.

RNA extraction and RNase protection assay. Total RNA was extracted from rat tissues with TRIzol reagent (Life Technologies) and stored at −20°C. nNOS mRNA species were quantified by RNase protection assays as described (Luo et al., 1994, 1996). A partial rat nNOS cDNA subcloned in a Bluescript SK II plasmid (kindly provided by Dr. B. C. Knoe at the University of Texas, Houston Health Science Center, Houston, TX) was linearized with *Eco*RI. After *in vitro* transcription with [^{32}P]UTP, a 623 bp labeled antisense cRNA probe was used for RNase protection. To normalize for sample loading, an antisense probe of rat cyclophilin (gift of Dr. J. N. Wood; University College, London) was included in each RNase protection assay. A *t*RNA lane was included in each RNase protection assay to verify the complete digestion of the free probes. Molecular masses of the protected probes were estimated by electrophoresis on polyacrylamide gels, and protected bands were exposed to BioMax films (Eastman Kodak, Rochester, NY) and quantified by densitometry (UltroScan XL; Pharmacia, Piscataway, NJ).

Western blot. To examine the cellular levels of nNOS, spinal cord and DRG tissues were extracted in 50 mM Tris buffer, pH 8.0, containing 0.5% Triton, 150 mM NaCl, 1 mM EDTA, and protease inhibitors, subjected to NuPAGE Tris-acetate gel electrophoresis, and then transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) electrophoretically. After nonspecific binding sites were blocked with 5% low fat milk in PBS containing 0.1% Tween 20 (PBS-T), monoclonal antibodies against rat brain nNOS (anti-nNOS) were used to blot the membrane in PBS-T for 1 hr at room temperature. After the nitrocellulose membrane was washed twice with the same buffer and once with a buffer containing 150 mM NaCl and 50 mM Tris-Cl, pH 7.5, the antibody–protein complexes were blotted for 1 hr at room temperature with secondary antibodies labeled with horseradish peroxidase. After extensive washing, the protein–antibody complexes were detected with chemiluminescent reagents.

Immunohistochemistry. nNOS immunostaining was performed as described by Dun et al. (1993). At the end of the survival period anesthetized rats were perfused intracardially with heparinized saline followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The spinal cord and DRGs were then removed, post-fixed in the same fixative for 12 hr, and then immersed in PBS containing 15–20% sucrose. Frozen transverse sections (25 μm) were cut 24 hr later. Free-floating tissue sections were then pretreated with 0.3% H_2O_2 in PBS for 30 min, washed and blocked with 5% normal horse serum in PBS, and incubated with the monoclonal anti-nNOS antibody (1:1000) overnight at 4 °C with gentle agitation. After several washes, sections were incubated with biotinylated anti-mouse secondary antibody (1:200) for 2 hr and then with avidin–biotin complex solution (1:50) for 1 hr at 21°C. The protein–antibody complexes were detected by a color reaction with diaminobenzidine-H_2O_2. In some sections, specific nNOS staining was intensified by a 1 min incubation with nickel chloride–enhancing solution. Sections were then washed, mounted, air-dried, dehydrated with alcohol followed by xylene, and coverslipped with Permount. In parallel control sections, the anti-nNOS antibody was omitted from the staining procedures, and no positive staining was observed.

Statistical analyses. Statistical analyses were performed using the unpaired Student's *t* test and the Mann–Whitney test where significance was indicated by a two-tailed p value < 0.05.

RESULTS

Behavioral effects of nerve injury

As shown in Figure 1, tactile allodynia (reduction in the paw withdrawal threshold to mechanical stimulation) developed in nerve-ligated rats by day 4 after the nerve injury. The allodynia peaked at day 6 and lasted for at least 2 weeks. Comparable paw withdrawal thresholds (<4 gm), indicative of tactile allodynia, also developed in diabetic rats (data not shown).

nNOS mRNA

To elucidate the possible regulation of nNOS at the mRNA level in rat models of neuropathic pain, we examined nNOS mRNA levels in the spinal cord and DRGs 2 weeks after tight ligation of the L5/6 spinal nerves or after 8 weeks of diabetes. As indicated in Figure 2*A*, nNOS mRNA levels were higher in dorsal lumbar than in ventral lumbar spinal cord, and L4–6 DRG neurons expressed a low level of nNOS mRNA. L1 DRG neurons expressed a high level of nNOS mRNA, consistent with the high expression level of nNOS protein in L1 DRG neurons (Steel et al., 1994). Interestingly, nNOS mRNA levels were upregulated ~10-fold in L5/6 DRG of the injured side compared with those of the contralateral side, whereas nNOS mRNA levels in L4 DRG

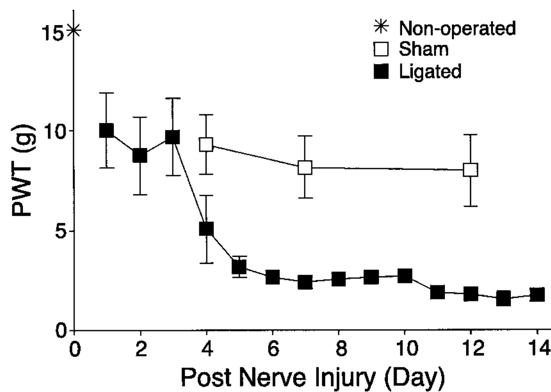

Figure 1. Development of tactile allodynia after L5/6 spinal nerve ligation. Left L5/6 spinal nerve tight ligation was performed in Harlan Sprague Dawley rats, and the paw withdrawal threshold (*PWT*) to mechanical stimulation was tested daily starting 1 d after the surgery. Data are presented as the means ± SEM from 4 (second week) to 10 (first week) nerve-ligated rats and 10 sham rats.

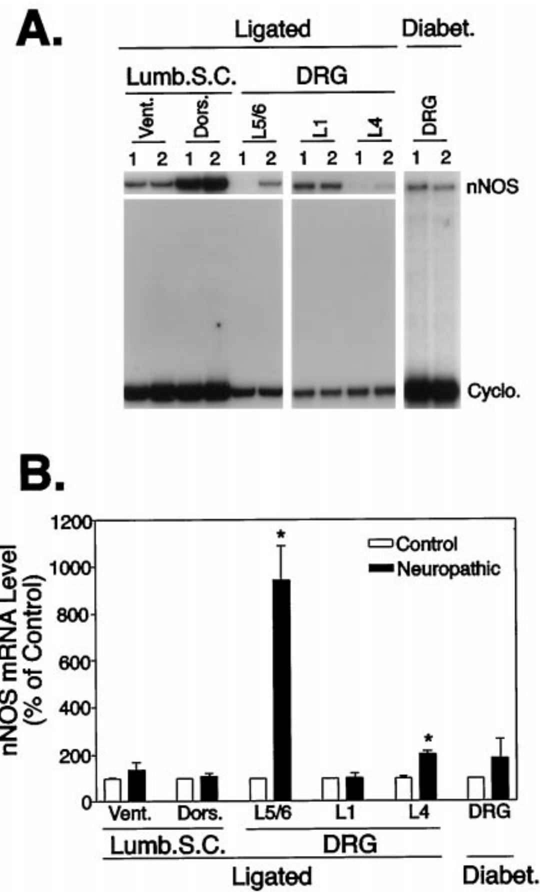

Figure 2. nNOS mRNA level in the spinal cord and DRG of allodynic rats after L5/6 spinal nerve ligation and diabetic neuropathy. Total RNA was extracted from the spinal cord and DRG of Harlan Sprague Dawley rats with allodynia resulting from tight ligation of the left L5/6 spinal nerves (2 weeks) or diabetes (8 weeks). nNOS mRNA was detected by RNase protection assays. *A,* Representative autoradiograms. Twenty micrograms of total RNA from the spinal cord or total RNA from two (L4, L5/6, and diabetic) or three (L1) DRGs were used for each *lane*. nNOS bands had a longer exposure time than did cyclophilin bands because of the low abundance of the nNOS mRNA, except for the separate RNase protection of diabetic samples in which lower specific activity of the cyclophilin probe was used. *Lanes* are labeled *1*, for the contralateral side or nondiabetic rats, and *2*, for the nerve-ligated side or diabetic neuropathy rats. *B,* Percentage change of nNOS mRNA in neuropathic tissues compared with control tissues. Data are presented as the means ± SEM from 6 to 10 rats except for the L5/6 DRG samples that are from 4 rats (*$p < 0.05$ by Student's *t* test or Mann–Whitney test). *Cyclo*., Cyclophilin; *Diabet*., diabetic; *Dors*., dorsal; *Lumb.S.C.*, lumbar spinal cord; *Vent*., ventral.

were slightly upregulated. In contrast, nNOS mRNA levels were not increased in L1 DRG or lumbar spinal cord after nerve ligation, nor was nNOS mRNA upregulated significantly in L4/5 DRG of diabetic rats (Fig. 2).

The relationship between nNOS mRNA upregulation and the development of tactile allodynia was examined by comparing the time courses of injury-induced nNOS mRNA expression in L5/6 DRGs and allodynia onset. Upregulation of nNOS mRNA after the nerve injury was time dependent, starting 1 d but not 8 hr after the surgery, peaked at day 2, and remained elevated for at least 13 weeks (see Figs. 3, 7*B,C*). There was no similar nNOS mRNA increase in DRG neurons from sham-operated rats (Fig. 3*B*). Thus, DRG nNOS mRNA upregulation precedes the tactile allodynia onset (compare Figs. 3*B,* 1).

nNOS protein expression and localization after spinal nerve ligation

The nNOS protein levels after the nerve injury were examined by Western blot analyses 1 week after the nerve ligation. There was a 20-fold increase of nNOS in L5/6 DRG neurons on the injury side compared with that on the contralateral side. No such increase was observed in dorsal lumbar spinal cord (108 ± 12% on the injury side compared with that on the contralateral side; $n = 9$) or in L5/6 DRGs from sham-operated rats (Fig. 4*A*). Data from immunohistochemical experiments indicated that increased nNOS-positive staining is mainly in the small and occasionally medium-size neurons (Fig. 5). Quantitative analyses of the immunohistostaining data revealed that L5/6 DRGs ipsilateral to the nerve injury contain significantly ($p < 0.0001$) more nNOS-positive cells (11.3 ± 1.4%; three sections each from four rats; 2282 cells were counted) than do L5/6 DRGs from sham-operated rats (2.2 ± 0.3%; three sections each from four rats; 2759 cells were counted).

Effect of nerve ligation on nNOS mRNA in a rat strain resistant to allodynia development

To examine the linkage between nNOS mRNA upregulation and allodynia development, we examined nNOS mRNA levels 1 week after the nerve injury in Holtzman rats, a strain that does not develop tactile allodynia (Fig. 6*A*). Data from RNase protection experiments indicated an increase of nNOS mRNA in L5/6 DRGs ipsilateral to the nerve ligation comparable with that seen in the Harlan strain (Fig. 6*B,C*). The similarity of nNOS mRNA upregulation despite genetically based differences in the behavioral response indicates a dissociation between the nNOS mRNA upregulation and the tactile allodynia development after nerve injury.

nNOS mRNA in nerve-ligated rats fully recovered from allodynia

The relationship between nNOS mRNA upregulation and the development and/or maintenance of tactile allodynia was further examined in nerve-ligated rats fully recovered from the neuropathic pain state. As indicated in Figure 7*A*, rats developed tactile allodynia 1 week after the nerve ligation, recovered gradually in 7 weeks, and achieved a full recovery 9 weeks after the surgery.

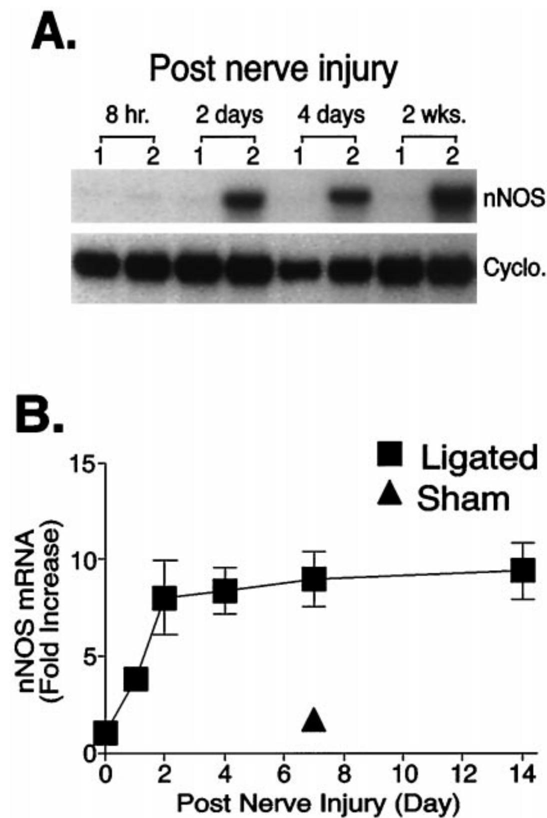

Figure 3. Time-dependent increase of DRG nNOS mRNA in nerve-ligated rats. Total RNA was extracted from two pooled L5/6 DRGs at the designated time after nerve ligation, and nNOS mRNA levels were examined by RNase protection assays as described. *A,* Representative autoradiography showing nNOS and cyclophilin probes protected by their corresponding mRNAs. nNOS bands had a longer exposure time than did cyclophilin bands because of the low abundance of the nNOS mRNA. Each pair of samples was taken from the same animal on the contralateral side (*lane* labeled *1*) or the nerve-ligated side (*lane* labeled *2*). *B,* Summarized time-dependent increase of nNOS mRNA after nerve ligation. The fold increase in nNOS mRNA was defined by comparing nNOS band densities in the injury side with those in the contralateral side after taking the ratio of the nNOS band over the cyclophilin band to correct differences in RNA loading. Data are presented as the means ± SEM from four independent experiments. Values at all time points except for that of the sham control are significantly ($p < 0.05$) different from the value at time 0 as measured by the Student's t test or the Mann–Whitney test.

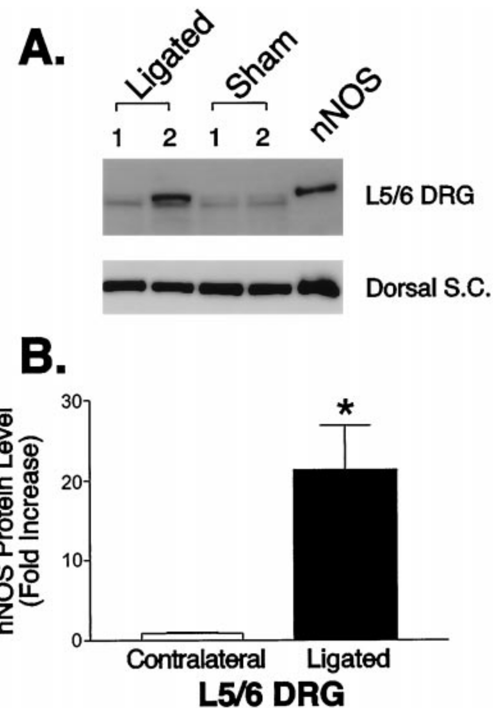

Figure 4. Upregulation of nNOS protein in L5/6 DRGs, but not spinal cord, of nerve-ligated animals. Total protein was extracted from two pooled L5/6 DRGs or the dorsal lumbar spinal cord from each side of the animals, and nNOS protein was identified using monoclonal antibodies against rat brain nNOS. *A,* Representative Western blots. Purified rat brain nNOS was used as a positive control. *B,* Fold increase of nNOS in neuropathic DRGs compared with contralateral DRGs. Data are presented as the means ± SEM from eight independent experiments (*$p < 0.05$ by Student's t test or Mann–Whitney test). *S.C.*, Spinal cord.

However, nNOS mRNA levels in L5/6 DRGs ipsilateral to the nerve ligation remained elevated even when the rats were fully recovered from tactile allodynia (Fig. 7*B,C*), indicating a dissociation between nNOS mRNA upregulation and the maintenance of tactile allodynia after nerve injury.

nNOS inhibition and allodynia

To examine whether nNOS inhibition could alter the expression of nerve injury–evoked allodynia, we treated nerve-ligated rats once daily (50 mg/kg, s.c.) with an nNOS-specific inhibitor, 7-NI, that was started 30 min before the surgery and continued for 6 d. In addition, the role of nNOS inhibition on the maintenance of allodynia was examined by treating allodynic rats with the same treatment for 3 d, starting on day 11 after the operation. At this dose, 7-NI was shown to inhibit effectively rat brain NOS activity and the allodynia-like response induced by spinal cord ischemia *in vivo* (Babbedge et al., 1993; Hao and Xu, 1996). No motor deficit was observed in treated rats. As indicated in Figure 8, neither pre- nor postsurgery treatment of 7-NI affected the development and/or maintenance of tactile allodynia. Intraperitoneal administration of 7-NI (25–50 mg/kg) also failed to reverse allodynia in nerve-injured rats (data not shown).

DISCUSSION

Our study provides the first evidence to indicate that nNOS expression is upregulated at the messenger RNA level in rat DRG, but not spinal cord, neurons after tight spinal nerve ligation, a model widely used to study neuropathic pain after peripheral nerve injury. This finding is similar to the increase of nNOS mRNA in DRG after sciatic nerve transection (Verge et al., 1992). However, the physiological abnormalities resulting from these two nerve injury models are distinct. Although peripheral axotomy results in motor deficit and a complete loss of sensation in the paw ipsilateral to the nerve injury, rats with nerve ligation retain motor function and develop pain syndromes including hyperalgesia, tactile allodynia, and spontaneous pain (Kim and Chung, 1992). Thus, pathways controlling neuropathic pain states are functional and activated in nerve-ligated animals, permitting us to study the coupling of nNOS regulation and the development and/or maintenance of allodynia after nerve injury.

Our study provides genetic, molecular, and pharmacological evidence indicating that nNOS regulation is not directly linked to the development and/or maintenance of tactile allodynia. Even though upregulation of nNOS mRNA precedes the onset of allodynia and persists for the duration of the neuropathic pain state in nerve-ligated animals (Figs. 1, 3), our study indicates a

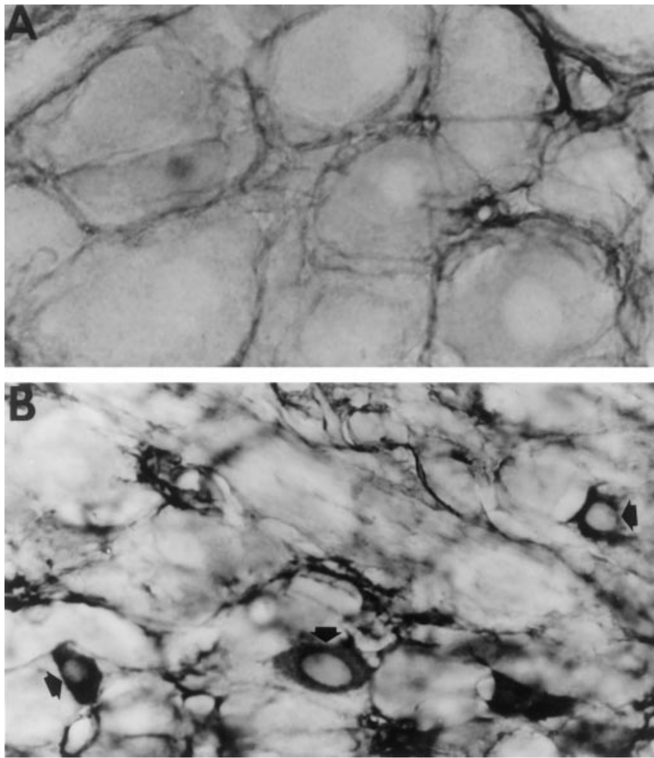

Figure 5. Immunohistological staining of nNOS-positive neurons in L5 DRG. Rat left L5 DRGs from 1 week sham-operated (*A*) or nerve-ligated (*B*) rats were sectioned and stained for nNOS-positive cells as described. Data shown are representative staining from four rats, and photos were taken using a 40× phase-contrast objective. Immunohistostaining of nNOS-positive neurons in L6 DRGs was similar to that in L5 DRG neurons (data not shown). *Arrows* indicate nNOS-positive neurons.

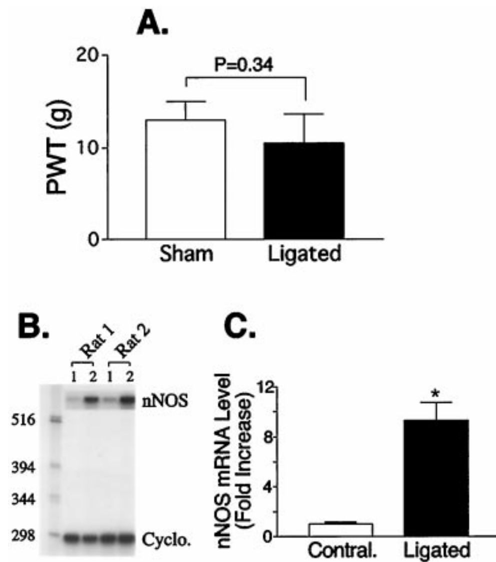

Figure 6. Upregulation of nNOS mRNA in L5/6 DRGs after nerve ligation in Holtzman rats, which do not develop allodynia. Total RNA was extracted from two pooled L5/6 DRGs in Holtzman rats 1 week after the nerve ligation, and nNOS mRNA levels were examined by RNase protection assays. *A,* The paw withdrawal thresholds to mechanical stimulation in the nerve-ligated animals' paws were not significantly different from those in the sham-operated animals' paws (Mann–Whitney test; 4 rats in each group). *B,* Representative autoradiography shows nNOS and cyclophilin probes protected by the corresponding mRNAs from DRGs of two individual rats. *Lanes* are labeled *1,* for the contralateral side, and *2*, for the nerve-ligated side. nNOS bands had a longer exposure time because of the low abundance of the mRNA. *Numbers* on the *left* indicate the positions of DNA markers in base pairs. *C,* Summarized data are from RNase protections shown in *B*. The fold increase of nNOS mRNA was defined by comparing nNOS band densities in the injury side with those in the contralateral side after taking the ratio of nNOS bands over the cyclophilin bands to correct differences in RNA loading. Data are presented as the means ± SEM from four independent experiments (*$p < 0.05$ by Student's *t* test or Mann–Whitney test). *Contral*., Contralateral.

clear separation between nNOS upregulation and the development and maintenance of tactile allodynia after nerve ligation. Nerve-ligated Holtzman rats, which do not develop allodynia, show a remarkable nNOS upregulation in the DRG (Fig. 6), indicating that nNOS upregulation alone is not a determining factor of allodynia susceptibility in this species. Rather, other intrinsic factors may underlie the genetic differences. In addition, Harlan Sprague Dawley rats that are fully recovered from tactile allodynia continue to show levels of expression observed in the earlier period when allodynia was present (Fig. 7). Furthermore, our pharmacological data indicate that inhibition of nNOS does not prevent or block tactile allodynia (Fig. 8). Thus, our conclusions are consistent with the hypothesis that there are NO cGMP-independent pathways that mediate nociception when nNOS is inhibited (Ichinose et al., 1998).

Our findings differ with the conclusion drawn from a recent study showing that treatment with L-NAME partially blocks the development and/or maintenance of allodynia and suggesting that NO may be involved in the neuropathic pain process (Yoon et al., 1998). The lack of specificity of drug action from available pharmacological agents may contribute to the controversial results of NOS inhibition on tactile allodynia. In addition, NOS splice variant expression, as in the case of morphine analgesia and tolerance (Kolesnikov et al., 1997), may contribute to the complexity of NOS inhibition experiments. Finally, it is possible that NO produced by other NOS isoforms such as inducible NOS (iNOS) contributes to the development and/or maintenance of tactile allodynia. This is supported by the findings that iNOS mRNA expression was induced in conditions producing thermal hyperalgesia (Meller et al., 1994; Grzybicki et al., 1996). However, the slow onset of allodynia after nerve injury seems inconsistent with the fast induction of iNOS mRNA that occurs within hours of stimulation (Meller et al., 1994; Grzybicki et al., 1996). In addition, iNOS mRNA was not detected in DRG neurons (data not shown), nor was its protein found in the spinal cord (Goff et al., 1998) after spinal nerve ligation. The role of endothelial NOS in nerve injury–induced allodynia remains to be investigated.

Several pathological conditions occur after nerve ligation that may drive nNOS gene expression in DRG. First, nerve ligation or section leads to an initial barrage of afferent activity in the injured axons, and this is followed over a period of days to weeks by the development of spontaneous afferent traffic arising from the sprouting nerve terminal (neuroma) and from the DRG of the injured axon (Ambron and Walters, 1996). Second, such nerve injuries result in a loss of materials undergoing retrograde axonal transport from the normal target organ. Third, nerve injury leads to the generation of active factors at the injury site that may be retrogradely transported to DRG. These conditions may have either a negative or positive influence on DRG nNOS expression.

Normal afferent activity and retrogradely transported factors may regulate nNOS expression via a negative feedback inhibition mechanism. Nerve ligation may interrupt this feedback regulation

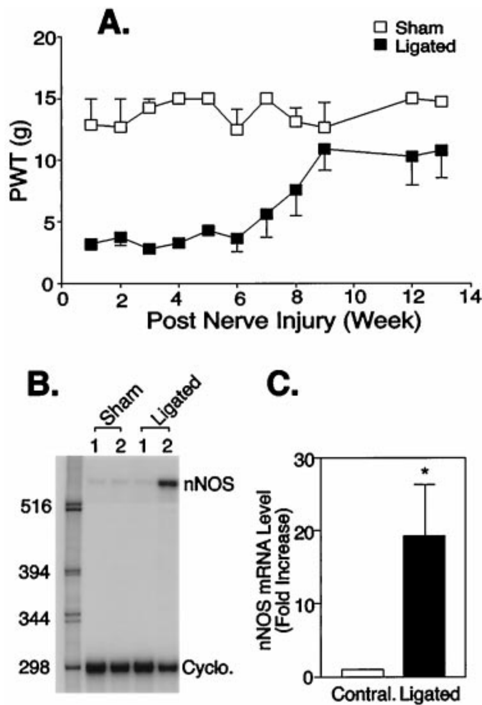

Figure 7. Upregulation of nNOS mRNA in L5/6 DRGs of nerve-ligated rats fully recovered from tactile allodynia. Tight ligation of the left L5/6 spinal nerves was performed in Harlan Sprague Dawley rats, and the paw withdrawal threshold to mechanical stimulation was tested from 1 to 13 weeks after the nerve ligation. Total RNA was extracted from two pooled left L5/6 DRGs from each rat at week 13, and nNOS mRNA levels were examined by RNase protection assays as described. *A,* Full recovery from tactile allodynia in nerve-ligated rats 9 weeks after the spinal nerve ligation. Data presented are the means ± SEM from four sham control rats and six nerve-ligated rats. *B,* Representative autoradiography showing nNOS and cyclophilin probes protected by corresponding mRNAs. nNOS bands had a longer exposure time because of the lower abundance of the nNOS mRNA than that of cyclophilin mRNA. *Numbers* on the *left* indicate the positions of DNA markers in base pairs. *Lanes* are labeled *1*, for the contralateral side, and *2*, for the surgery side. *C,* Summarized data of RNase protections shown in *B*. Data analysis was done as described in the legend for Figure 6*C*. Data are presented as the means ± SEM from four independent experiments ($*p < 0.05$ by Student's *t* test or Mann–Whitney test).

and therefore result in increased nNOS expression. The slow onset of nNOS mRNA upregulation after nerve injury seems inconsistent with the interruption of afferent activity that would occur immediately after the nerve ligation. It is possible that nNOS expression is controlled by factors derived from peripheral nerves or innervated target tissues. This is supported by three observations. (1) The slow onset of nNOS upregulation after nerve injury falls in the time frame of the early phase of nerve regeneration requiring signals conveyed by retrograde transport after axon injury (Ambron et al., 1995; Ambron and Walters, 1996). (2) The upregulation of nNOS mRNA in our model is similar to that induced by sciatic nerve transection (Verge et al., 1992), and retrograde axonal transport is interrupted in both models. (3) nNOS mRNA was mildly, but not significantly, upregulated in DRGs from diabetic rats in which retrograde axonal transport of neurotrophic factors is diminished but not abolished (Fernyhough et al., 1995, 1998). This hypothesis is in accord with the findings that nerve growth factor (NGF) is required by peripheral sympathetic ganglion and sensory neurons to maintain their normal functions and is supplied to ganglion neurons by

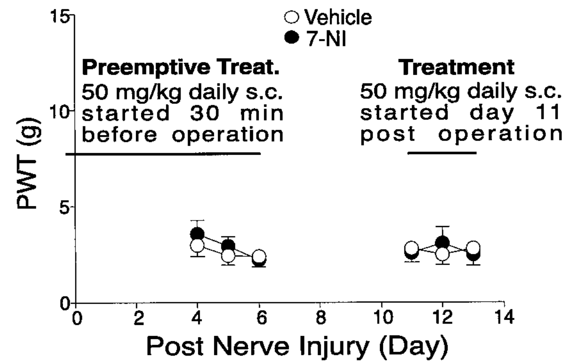

Figure 8. Effects of systemic administration of an nNOS specific inhibitor, 7-NI, on the development and/or maintenance of tactile allodynia in rats after spinal nerve ligation. Preemptive and treatment protocols are indicated, and the paw withdrawal threshold to mechanical stimulation was tested at the designated times. In both cases, control animals were injected with the same volume of peanut oil (vehicle) alone. Similar findings were observed when the drug was administered intraperitoneally. Data presented are the means ± SEM from five (treatment) and six (preemptive treatment) rats.

retrograde axonal transport (Njå and Purves, 1978; Verge et al., 1989, 1990; Anderson et al., 1998). Examples of such feedback regulation include downregulation of the SNS sodium channel subtype in the DRG after axotomy, which is prevented by exogenous NGF administration (Dib-Hajj et al., 1998). Thus, upregulation of nNOS and the subsequent production of NO may serve as a compensatory mechanism for neuronal survival and/or regeneration after DRG neurons sense the change in axonal transport or nerve injury. However, the lack of retrograde control may lead to overexpression of nNOS and subsequent overproduction of NO, which is known to be neurotoxic, and may result in neurodegeneration (Dawson and Dawson, 1996; Dawson et al., 1996).

Alternatively, upregulation of nNOS may result from positive signals derived from increased ongoing afferent activity or active factors generated in the injured terminal as suggested in other studies (Curtis et al., 1993; Yamamoto and Yaksh, 1993; Gunstream et al., 1995; Ambron et al., 1996). The slow onset of nNOS mRNA upregulation after nerve injury suggests that such change is not regulated directly by the initial injury-induced discharges that would reach the cell body immediately after axon injury (Ambron and Walters, 1996). However, it is possible that ongoing injury discharges could indirectly induce nNOS expression requiring *de novo* protein synthesis. This hypothesis is supported by the findings that expression of transcription factors such as Jun, Fos, and Krox was upregulated and colocalized with the increased expression of nitric oxide synthase within spinal cord neurons after noxious stimulation of the rat hindpaw (Herdegen et al., 1994). Similarly, nNOS expression could be regulated by active factors generated at the site of nerve injury. Examples of such retrograde active factors include the ciliary neurotrophic factor (CNTF) that promotes survival of sensory neurons (Barbin et al., 1984; Skaper and Varon, 1986; Thoenen, 1991). This factor is released as a "lesion factor" by damage to Schwann cells in the sciatic nerve (Thoenen, 1991) and induces gene expression (Ip et al., 1992). Most interestingly, retrograde axonal transport of this factor by sensory neurons is enhanced after peripheral nerve injury (Curtis et al., 1993). In fact, nerve CNTF levels are depleted in diabetic rats (Calcutt et al., 1992), perhaps explaining the minimal nNOS induction seen in the present study.

The small but significant increase of nNOS mRNA in L4 DRG neurons after L5/6 spinal nerve ligation is interesting. The L4 spinal nerve is the only remaining afferent connection to the spinal cord after L5/6 spinal nerve ligation. The increase of nNOS mRNA in L4 DRG neurons may reflect adaptive changes, e.g., peripheral neuronal sprouting leading to expended receptive fields. The lack of nNOS mRNA regulation in diabetic rats suggests that DRG neurons respond specifically to certain types of pathological conditions and that the regulatory pathway for nNOS expression may not be markedly altered in diabetes. Taken together, our findings suggest that nNOS regulation in DRG neurons may play an important role in neuroplasticity after nerve injury. However, regulation of nNOS expression is not responsible for the development and/or maintenance of neuropathic allodynia.

REFERENCES

Akira T, Henry D, Baldwin RA, Wasterlain CG (1994) Nitric oxide participates in excitotoxic mechanisms induced by chemical hypoxia. Brain Res 645:285–290.

Ambron RT, Walters ET (1996) Priming events and retrograde injury signals. A new perspective on the cellular and molecular biology of nerve regeneration. Mol Neurobiol 13:61–79.

Ambron RT, Dulin MF, Zhang XP, Schmied R, Walters ET (1995) Axoplasm enriched in a protein mobilized by nerve injury induces memory-like alterations in *Aplysia* neurons. J Neurosci 15:3440–3446.

Ambron RT, Zhang XP, Gunstream JD, Povelones M, Walters ET (1996) Intrinsic injury signals enhance growth, survival, and excitability of *Aplysia* neurons. J Neurosci 16:7469–7477.

Anderson LC, von Bartheld CS, Byers MR (1998) NGF depletion reduces ipsilateral and contralateral trigeminal satellite cell reactions after inferior alveolar nerve injury in adult rats. Exp Neurol 150:312–320.

Babbedge RC, Bland-Ward PA, Hart SL, Moore PK (1993) Inhibition of rat cerebellar nitric oxide synthase by 7-nitro indazole and related substituted indazoles. Br J Pharmacol 110:225–228.

Barbin G, Manthorpe M, Varon S (1984) Purification of the chick eye ciliary neuronotrophic factor. J Neurochem 43:1468–1478.

Bogdanov MB, Wurtman RJ (1997) Possible involvement of nitric oxide in NMDA-induced glutamate release in the rat striatum: an in vivo microdialysis study. Neurosci Lett 221:197–201.

Calcutt NA, Chaplan SR (1997) Spinal pharmacology of tactile allodynia in diabetic rats. Br J Pharmacol 122:1478–1482.

Calcutt NA, Muir D, Powell HC, Mizisin AP (1992) Reduced ciliary neuronotrophic factor-like activity in nerves from diabetic or galactose-fed rats. Brain Res 575:320–324.

Calcutt NA, Jorge MC, Yaksh TL, Chaplan SR (1996) Tactile allodynia and formalin hyperalgesia in streptozotocin-diabetic rats: effects of insulin, aldose reductase inhibition and lidocaine. Pain 68:293–299.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53:55–63.

Chaplan SR, Malmberg AB, Yaksh TL (1997) Efficacy of spinal NMDA receptor antagonism in formalin hyperalgesia and nerve injury evoked allodynia in the rat. J Pharmacol Exp Ther 280:829–838.

Choi Y, Raja SN, Moore LC, Tobin JR (1996) Neuropathic pain in rats is associated with altered nitric oxide synthase activity in neural tissue. J Neurol Sci 138:14–20.

Curtis R, Adryan KM, Zhu Y, Harkness PJ, Lindsay RM, DiStefano PS (1993) Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. Nature 365:253–255.

Dawson VL, Dawson TM (1996) Nitric oxide neurotoxicity. J Chem Neuroanat 10:179–190.

Dawson VL, Kizushi VM, Huang PL, Snyder SH, Dawson TM (1996) Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. J Neurosci 16:2479–2487.

Dib-Hajj SD, Black JA, Cummins TR, Kenney AM, Kocsis JD, Waxman SG (1998) Rescue of alpha-SNS sodium channel expression in small dorsal root ganglion neurons after axotomy by nerve growth factor in vivo. J Neurophysiol 79:2668–2676.

Dixon WJ (1980) Efficient analysis of experimental observations. Annu Rev Pharmacol Toxicol 20:441–462.

Dun NJ, Dun SL, Wu SY, Förstermann U, Schmidt HH, Tseng LF (1993) Nitric oxide synthase immunoreactivity in the rat, mouse, cat and squirrel monkey spinal cord. Neuroscience 54:845–857.

Fernyhough P, Diemel LT, Hardy J, Brewster WJ, Mohiuddin L, Tomlinson DR (1995) Human recombinant nerve growth factor replaces deficient neurotrophic support in the diabetic rat. Eur J Neurosci 7:1107–1110.

Fernyhough P, Diemel LT, Tomlinson DR (1998) Target tissue production and axonal transport of neurotrophin-3 are reduced in streptozotocin-diabetic rats. Diabetologia 41:300–306.

Goff JR, Burkey AR, Goff DJ, Jasmin L (1998) Reorganization of the spinal dorsal horn in models of chronic pain: correlation with behaviour. Neuroscience 82:559–574.

Grzybicki D, Gebhart GF, Murphy S (1996) Expression of nitric oxide synthase type II in the spinal cord under conditions producing thermal hyperalgesia. J Chem Neuroanat 10:221–229.

Gunstream JD, Castro GA, Walters ET (1995) Retrograde transport of plasticity signals in *Aplysia* sensory neurons following axonal injury. J Neurosci 15:439–448.

Hao JX, Xu XJ (1996) Treatment of a chronic allodynia-like response in spinally injured rats: effects of systemically administered nitric oxide synthase inhibitors. Pain 66:313–319.

Herdegen T, Rüdiger S, Mayer B, Bravo R, Zimmermann M (1994) Expression of nitric oxide synthase and colocalisation with Jun, Fos and Krox transcription factors in spinal cord neurons following noxious stimulation of the rat hindpaw. Brain Res Mol Brain Res 22:245–258.

Ichinose F, Mi WD, Miyazaki M, Onouchi T, Goto T, Morita S (1998) Lack of correlation between the reduction of sevoflurane MAC and the cerebellar cyclic GMP concentrations in mice treated with 7-nitroindazole. Anesthesiology 89:143–148.

Ientile R, Picciurro V, Pedale S, Nucci C, Malecka B, Nisticò G, Macaione S (1996) Nitric oxide enhances amino acid release from immature chick embryo retina. Neurosci Lett 219:79–82.

Ip NY, Nye SH, Boulton TG, Davis S, Taga T, Li Y, Birren SJ, Yasukawa K, Kishimoto T, Anderson DJ, et al. (1992) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell 69:1121–1132.

Kim SH, Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50:355–363.

Kolesnikov YA, Pan YX, Babey AM, Jain S, Wilson R, Pasternak GW (1997) Functionally differentiating two neuronal nitric oxide synthase isoforms through antisense mapping: evidence for opposing NO actions on morphine analgesia and tolerance. Proc Natl Acad Sci USA 94:8220–8225.

Luo D, Vincent SR (1994) NMDA-dependent nitric oxide release in the hippocampus in vivo: interactions with noradrenaline. Neuropharmacology 33:1345–1350.

Luo Z, Fuentes ME, Taylor P (1994) Regulation of acetylcholinesterase mRNA stability by calcium during differentiation from myoblasts to myotubes. J Biol Chem 269:27216–27223.

Luo ZD, Pincon-Raymond M, Taylor P (1996) Acetylcholinesterase and nicotinic acetylcholine receptor expression diverge in muscular dysgenic mice lacking the L-type calcium channel. J Neurochem 67:111–118.

Meller ST, Dykstra C, Grzybycki D, Murphy S, Gebhart GF (1994) The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. Neuropharmacology 33:1471–1478.

Mollace V, Colasanti M, Rodino P, Lauro GM, Rotiroti D, Nistico G (1995) NMDA-dependent prostaglandin E2 release by human cultured astroglial cells is driven by nitric oxide. Biochem Biophys Res Commun 215:793–799.

Montague PR, Gancayco CD, Winn MJ, Marchase RB, Friedlander MJ (1994) Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex. Science 263:973–977.

Nei K, Matsuyama S, Shuntoh H, Tanaka C (1996) NMDA receptor activation induces glutamate release through nitric oxide synthesis in guinea pig dentate gyrus. Brain Res 728:105–110.

Njå A, Purves D (1978) The effects of nerve growth factor and its antiserum on synapses in the superior cervical ganglion of the guinea-pig. J Physiol (Lond) 277:55–75.

Ohno M, Arai I, Watanabe S (1995) *N*-Methyl-D-aspartate stimulates dopamine release through nitric oxide formation in the nucleus accumbens of rats. Brain Res 699:332–335.

Sakai M, Minami T, Hara N, Nishihara I, Kitade H, Kamiyama Y, Okuda K, Takahashi H, Mori H, Ito S (1998) Stimulation of nitric oxide release from rat spinal cord by prostaglandin E2. Br J Pharmacol 123:890–894.

Sandor NT, Brassai A, Puskas A, Lendvai B (1995) Role of nitric oxide in modulating neurotransmitter release from rat striatum. Brain Res Bull 36:483–486.

Siegan JB, Hama AT, Sagen J (1997) Suppression of neuropathic pain by a naturally-derived peptide with NMDA antagonist activity. Brain Res 755:331–334.

Skaper SD, Varon S (1986) Age-dependent control of dorsal root ganglion neuron survival by macromolecular and low-molecular-weight trophic agents and substratum-bound laminins. Brain Res 389:39–46.

Snyder SH (1992) Nitric oxide: first in a new class of neurotransmitters. Science 257:494–496.

Steel JH, Terenghi G, Chung JM, Na HS, Carlton SM, Polak JM (1994) Increased nitric oxide synthase immunoreactivity in rat dorsal root ganglia in a neuropathic pain model. Neurosci Lett 169:81–84.

Thoenen H (1991) The changing scene of neurotrophic factors. Trends Neurosci 14:165–170.

Verge VM, Riopelle RJ, Richardson PM (1989) Nerve growth factor receptors on normal and injured sensory neurons. J Neurosci 9:914–922.

Verge VM, Tetzlaff W, Bisby MA, Richardson PM (1990) Influence of nerve growth factor on neurofilament gene expression in mature primary sensory neurons. J Neurosci 10:2018–2025.

Verge VM, Xu Z, Xu XJ, Wiesenfeld-Hallin Z, Hökfelt T (1992) Marked increase in nitric oxide synthase mRNA in rat dorsal root ganglia after peripheral axotomy: in situ hybridization and functional studies. Proc Natl Acad Sci USA 89:11617–11621.

Yamamoto T, Yaksh TL (1993) Effects of colchicine applied to the peripheral nerve on the thermal hyperalgesia evoked with chronic nerve constriction. Pain 55:227–233.

Yoon YW, Sung B, Chung JM (1998) Nitric oxide mediates behavioral signs of neuropathic pain in an experimental rat model. NeuroReport 9:367–372.