Nitric Oxide in the Rat Spinal Cord in Freund’s Adjuvant-Induced Hyperalgesia

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ABSTRACT—To elucidate the involvement of nitric oxide in spinal nociceptive processing, the correlation of thermal withdrawal latency with nitric oxide synthase-stained neurons in the rat lumbar dorsal horn was analyzed after adjuvant-induced inflammation. From 4 hr through 5 days after subcutaneous injection of complete Freund’s adjuvant into the hind paw, a marked thermal hyperalgesia was observed for heat stimulus applied to the affected region. NADPH-diaphorase and nitric oxide synthase-positive neurons increased significantly in the superficial layers of the dorsal horn ipsilateral to the inflamed hind paw at day 3 of adjuvant-induced inflammation. No change in NADPH-diaphorase-positive neurons was observed at 1 hr and 1 day of adjuvant-induced inflammation. The intravenous administration of N°-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg), an inhibitor of nitric oxide synthase, significantly blocked the adjuvant-induced thermal hyperalgesia at day 3 of inflammation, but not at day 1; and it had no effect in non-inflamed rats. This anti-hyperalgesic effect of L-NAME at day 3 of inflammation was reversed by the prior administration of L-arginine (600 mg/kg, i.p.), a substrate of nitric oxide synthase. These data suggest that nitric oxide producing neurons in the spinal dorsal horn are involved in maintaining and facilitating the hyperalgesia associated with chronic nociception.

Keywords: Nitric oxide synthase, Nicotinamide adenine dinucleotide phosphate-diaphorase, Nociception, Dorsal horn, Arthritis

Nitric oxide (NO) has drawn the interest of numerous investigators since NO was identified as an endothelium-derived relaxing factor (EDRF) (1–5). It has been shown that NO is synthesized from L-arginine by nitric oxide synthase (NOS) (6), which is distributed in a variety of mammalian tissues, and that NO functions as an intracellular messenger or neurotransmitter (7). There are some studies describing that NO inhibits aggregation of platelets (8) and mediates cytotoxicity by macrophages (9, 10). In the brain, NO has been proposed as a mediator in synaptic plasticity, such as long-term potentiation (LTP) (11–13) and long-term depression (14, 15), or as a neurotoxin in situations of excessive production (16–18).

Concerning the physiological roles of NO in the central nervous system, it has been reported that NO is involved in spinal nociceptive processing (19–25). Anatomical studies using immunostaining with specific antibodies for cerebellar constitutive-type NOS (cNOS) or histochemical staining for nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) revealed a dense plexus of positive fibers in lamina I to the outer lamina III (IIIo), except for the outer lamina II (IIo), and positive cells in laminae I through V and X (especially in the superficial layers) of the spinal cord (25–27) and in the dorsal root ganglion (28–30). Hyperalgesia generated by spinal NMDA receptor activation or by protracted afferent input is reversibly blocked by prior administration of N°-nitro-L-arginine methyl ester (L-NAME), an active antagonist of NOS (6, 31, 32), and methylene blue (an inhibitor of soluble guanylate cyclase) without any affect on the baseline nociceptive withdrawal latency (20, 22). Nociceptive and ectopic discharges recorded from peripheral fibers are reduced by NOS inhibitors (19). In addition, with the formalin test, the NOS inhibitor mainly diminishes the second phase (the facilitated processing of afferent activity produced by a continued afferent barrage), but not the first phase of evoked behavior (21).

To elucidate which stage of pain, i.e., manifestation,
Animals

Male Sprague-Dawley rats (180–220 g body weight) were used (Keari Co., Ltd., Osaka). The animals were housed in cages in which the floor was covered with sawdust, and food and water were available ad libitum. Room temperature was maintained at 25°C and 60% humidity. A 12-hr light-dark cycle was used with lights being turned on at 7:00 am. Complete Freund’s adjuvant (CFA, containing Mycobacterium butyricum) was used as the inflammatory substance for the chronic experiment. Rats received a subcutaneous (s.c.) injection of 0.15–0.2 ml (75–100 μg Mycobacterium) CFA into the plantar surface of the right hind paw. All surgical procedures were reviewed and approved by the Osaka University Faculty of Dentistry Intramural Animal Care and Use Committee and conformed to the guidelines of the International Association for the Study of Pain (33).

Behavioral assessments

We determined whether or not the rats were hyperalgesic by using the Plantar Test (Model 7370; Ugo Basile, Varese, Italy). The rats were placed in a small cage on a glass plate. They were not restrained and could move about and explore freely. While the rat stood still with all paws placed on the ground, an infrared (I.R.) generator was positioned directly under a hind paw and switched on to activate both an I.R. generator and a reaction time counter. The intensity of the light beam was controlled to provide a reflex withdrawal latency of approximately 8 sec in the control animals that received saline injection. Latencies less than 7 sec were taken as indicative of hyperalgesia in the inflamed paws. The contralateral paw of the inflamed rats served as an additional control; the withdrawal latency of the contralateral limb did not differ from that of the saline-injected paws.

The change in the relative withdrawal latency (%) was calculated according to the formula of (LCont. – LAdju.) / LCont.) × 100, where LCont. and LAdju. are the withdrawal latency of the hind paw on the control and CFA-injected sides, respectively.

Histochemical staining with NADPH-d

Rats received an intra-plantar injection of CFA were deeply anesthetized with pentobarbital sodium (50 mg/kg) and ethyl carbamate (600 mg/kg) and perfused intracardially with 100 ml saline followed by 500 ml of freshly prepared fixative containing 2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The L5–L6 spinal segments were taken out, postfixed overnight in fresh fixative at 4°C and transferred to a solution of 20% sucrose in 0.1 M PB for cryoprotection. Transverse serial frozen sections were made at 60-μm-thickness and reacted for NADPH-d. The sections were preincubated in 0.1 M PB containing 0.25% Triton X-100 for 5 min and then incubated in a freshly prepared reaction solution containing β-NADPH (0.5 mg/ml) and nitroblue tetrazolium (0.2 mg/ml) for 1–3 hr at 37°C. The sections were rinsed in 0.1 M PB and distilled water and then mounted on gelatin-coated slides, air dried and coverslipped. The number of labeled cells in 10 random sections per animal was counted using a 20 x objective lens.

Immunohistochemistry for cNOS

In other experiments, rats inflamed 3 days after CFA injection into the right hind footpad were anesthetized with pentobarbital sodium (50 mg/kg) and ethyl carbamate (600 mg/kg) and perfused intracardially with 100 ml saline followed by 500 ml of freshly prepared fixative containing 2% paraformaldehyde in 0.1 M PB (pH 7.4). The L5–L6 spinal segments were excised, postfixed in the same fixative for overnight at 4°C and then transferred to a solution of 20% sucrose in 0.1 M PB for cryoprotection. Serial transverse frozen sections, 15-μm-thick, were cut with a cryostat (CM-501; Sakura Coldtome, Tokyo) and thaw-mounted on gelatin-coated slides. The sections were air dried at room temperature for 2 hr. Then the sections were washed in PB for 20 min and blocked with 1% normal goat serum for 20 min and incubated with rat cerebellar NOS antiserum (1:1,000) for 12 hr. Immunostaining was completed according to the protocol for the Vectastain Elite ABC Kit (PK-6101). When double labeling with NADPH-d staining was performed, NADPH-d histochemistry as described above was performed after reaction with biotinylated horseradish peroxidase. To visualize peroxidase activity, the slides were immersed in 0.05% dianimo benzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.2). Control sections were treated in the same way except for the replacement of the antiserum with normal rabbit serum (1:100).

Counts of NADPH-d- or cNOS-positive neurons were made in two regions: laminae I–IIIo and inner lamina III (IIIi)–lamina IV. The ratios of NADPH-d- or cNOS-positive neurons on the inflamed (right) and contralateral (left) sides were determined according to the formula of (NAdju. / NCont.) × 100, where NCont. and NAdju.
indicate the number of labeled neurons on the control and CFA injection sides, respectively.

**Influence of drugs**

L-NAME or its inactive stereoisomer, N\(^\text{°}\)-nitro-d-arginine methyl ester (D-NAME) were given systemically via the tail vein, and then the paw withdrawal latency was calculated at 10 min, 1, 2, 3 and 4 hr post-drug. In some experiments, rats received intravenous (i.v.) injection of L-NAME and were then treated additionally with either L- or D-arginine (i.p.) 15 min prior to the nociceptive test. All drugs used were diluted in physiological saline.

**Agents**

The following compounds were used: CFA (Difco Laboratories, Inc., Detroit, MI, USA); D- and L-NAME (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA); d- and l-arginine (Peptide Institute, Inc., Minoh); nitroblue tetrazolium (Wako Pure Chemical Industries, Inc., Osaka); \(\beta\)-NADPH (Oriental Yeast Co., Ltd., Tokyo); Vectastain Elite ABC Kit (PK-6101) (Vector Laboratories, Inc., Burlingame, CA, USA). A specific antibody against rat cerebellar NOS was obtained by the procedure described previously (34). This type of enzyme was shown to be substantially the same as that of colorectal and another central neurons. The immunohistochemical analysis of rat colorectal tissue indicated that the antibody against the cerebellar NOS stained the ganglion cells and nerve fibers and can cross-react with the colorectal NOS (34).

**Statistical analyses**

Results of in vivo and anatomical experiments are expressed as the mean±S.E. The significance of the difference between the groups was analyzed by Student’s t-test or Dunnett’s non-parametric multiple comparison test (Steel’s test) after ANOVA. P values less than 0.05 were regarded as statistically significant.

**RESULTS**

**Development of hyperalgesia**

Before injection of CFA, the mean of withdrawal latencies for the right and left hind paws were not significantly different (7.9±0.2 sec vs 7.9±0.1 sec, \(n=111\)). The withdrawal latencies of the saline-treated paw and the contralateral paw (untreated) in all groups remained near baseline values throughout the testing sessions.

As shown in Table 1, the s.c. injections of CFA into the plantar surface of the right hind paw rapidly produced hyperalgesia as evaluated by the thermal withdrawal test. A significant decrease in withdrawal latencies was observed during the period of 4 hr to 5 days after induction of inflammation, followed by a gradual return to the control level by 15 days. At 15 days after CFA-induced inflammation, the withdrawal latencies of the CFA-treated paw and the contralateral paw were 7.6±0.4 sec and 7.5±0.2 sec (\(n=38\)), respectively. The histochemical experiments of NADPH-d-stainings were examined at 1 hr, 1 day and 3 days after induction of inflammation to study the difference of NOS activity between early- and late-phase of inflammation. cNOS-stainings were also investigated at 3 days after inflammation.

**Table 1. Time course of shortened withdrawal latencies to noxious heat stimulation after CFA injection**

<p>| Time after | Relative withdrawal | No. of |</p>
<table>
<thead>
<tr>
<th>CFA injection</th>
<th>latencies (%)(^1)</th>
<th>animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>−1.3±1.9</td>
<td>45</td>
</tr>
<tr>
<td>1 hr</td>
<td>10.3±6.0</td>
<td>45</td>
</tr>
<tr>
<td>4 hr</td>
<td>34.7±6.6*</td>
<td>45</td>
</tr>
<tr>
<td>1 day</td>
<td>34.1±3.5*</td>
<td>45</td>
</tr>
<tr>
<td>2 days</td>
<td>31.8±2.0*</td>
<td>45</td>
</tr>
<tr>
<td>3 days</td>
<td>33.6±1.2*</td>
<td>38</td>
</tr>
<tr>
<td>4 days</td>
<td>30.5±2.1*</td>
<td>38</td>
</tr>
<tr>
<td>5 days</td>
<td>18.7±6.0*</td>
<td>38</td>
</tr>
<tr>
<td>8 days</td>
<td>8.3±4.7</td>
<td>38</td>
</tr>
<tr>
<td>10 days</td>
<td>11.7±4.8</td>
<td>38</td>
</tr>
<tr>
<td>15 days</td>
<td>−1.2±5.0</td>
<td>38</td>
</tr>
</tbody>
</table>

\(^1\)Mean±S.E.M. * indicates a significant increase of hyperalgesic behavior at the 0.05 level (Steel’s test), relative to the mean at time zero. Note that the withdrawal latency per animal at respective time points is presented as an average of latencies that are obtained from 4 consecutive stimuli applied at intervals of 5 min and that the value at time zero was obtained 1 hr prior to injection of CFA into the hind paw.
Fig. 1. NADPH-d- and cNOS-positive neurons in the L5 dorsal horn 3 days after CFA injection into the unilateral hind paw. A: NADPH-d staining ipsilateral to the injection. B: NADPH-d staining contralateral to the injection. C: Enlargement of labeled neurons in laminae I through outer III shown in panel A. D: cNOS staining in the L5 dorsal horn in a control animal. Bar=200 μm (A, B). Bar=100 μm (C, D). 1/2=laminae I/II, 3/4=laminae III/IV. Line between 1/2 and 3/4 indicates the border between laminae II and III.

The distribution pattern of neurons stained for cNOS was similar to that of neurons stained for NADPH-d, but they were fewer in number. Like NADPH-d staining, labeled fiber plexuses were observed in lamina I and at the border between laminae II and III, but labeled neurons tended to localize at the border between laminae II and III. Most cNOS-positive neurons were double-labeled with NADPH-d (not shown). In control animals, the total number of stained cells (10 random sections per animal) was much lower in the cNOS-stained sections. The average number of cNOS-positive neurons was 66.0±8.1 (n=6) and 60.5±12.1 (n=6) in the dorsal horns ipsilateral and contralateral to the injection side, respectively. The average number of NADPH-d-positive neurons was 277.0±25.1 (n=6) and 267.2±24.9 (n=6) in the ipsilateral and contralateral dorsal horns, respectively. It should be noted that NADPH-d-positive and cNOS-positive neurons showed a similar distribution pattern in the L5–L6 spinal cord segments (Fig. 1D).

The ratios of NADPH-d neurons in the inflamed vs non-inflamed (contralateral) sides after CFA injection are listed in Table 2. Three days after the CFA injection, the ratio of labeled neurons in laminae I–III increased significantly to 128.0±2.9% (n=6). The ratio in III–IV showed an increased percentage (128.0±14.3%) but the amount was not significant. The ratio in the total (laminae I–IV) was increased significantly to 127.4±3.5%. The morphology of NADPH-d-positive neurons was similar in the contralateral and ipsilateral dorsal horns. No alteration in NADPH-d-positive neurons was observed in the dorsal horn on the inflamed and normal sides at the 1 hr or 1 day time points after CFA-induced inflammation.

The relative change in the number of cNOS-positive
neurons 3 days after the CFA injection was similar to that of NADPH-d-positive neurons. The ratios of cNOS-positive neurons in the inflamed versus non-inflamed sides were $169.3 \pm 15.1\%$ in laminae I–IIIo and $156.5 \pm 6.0\%$ in laminae IIIi–IV (Table 3).

In contrast, the number of NADPH-d and cNOS-positive neurons on the uninjected side remained near the control level (0 day) throughout the experiment sessions.

Table 2. Changes (ratio of treated side/untreated side) in NADPH-d-positive neurons in the L5–L6 dorsal horn after the CFA injection into the right hind paw

<table>
<thead>
<tr>
<th>Time after CFA injection</th>
<th>I–IIlo % of control</th>
<th>III–IV % of control</th>
<th>Total (I–IV) % of control</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>101.4±2.5</td>
<td>105.2±8.9</td>
<td>103.8±1.4</td>
<td>6</td>
</tr>
<tr>
<td>1 hr</td>
<td>96.3±4.7</td>
<td>84.7±5.5</td>
<td>93.8±4.6</td>
<td>6</td>
</tr>
<tr>
<td>1 day</td>
<td>98.5±3.9</td>
<td>102.5±6.6</td>
<td>98.9±3.6</td>
<td>6</td>
</tr>
<tr>
<td>3 days</td>
<td>128.0±2.9*</td>
<td>128.0±14.3</td>
<td>127.4±3.5*</td>
<td>6</td>
</tr>
</tbody>
</table>

All data are presented as means±S.E.M. * indicates a significant increase of labeled neurons at 0.05 level (Steel's test) compared with the value at the 0 time point.

Table 3. Changes (ratio of treated side/untreated side) in cNOS-positive neurons in the L5–L6 dorsal horn after CFA injection into right hind paw

<table>
<thead>
<tr>
<th>Time after CFA injection</th>
<th>I–IIlo % of control</th>
<th>III–IV % of control</th>
<th>Total (I–IV) % of control</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>108.6±11.2</td>
<td>130.2±10.3</td>
<td>113.7±9.5</td>
<td>6</td>
</tr>
<tr>
<td>3 days</td>
<td>169.3±15.1*</td>
<td>156.5±6.0</td>
<td>165.8±11.3*</td>
<td>6</td>
</tr>
</tbody>
</table>

All data are presented as means±S.E.M. * indicates a significant increase at the 0.05 level (Student’s t-test) compared to the value of the 0 time point.

Influence of drugs related to the NO pathway on CFA-induced thermal hyperalgesia

As mentioned above, all animals treated with CFA exhibited a marked hyperalgesia from 4 hr through 5 days (Table 1). In the course of this long-lasting CFA-induced thermal hyperalgesia, increased NOS activity was seen in dorsal horn neurons at 3 days but not at 1 day after inflammation (Table 2). The pharmacological experiments, therefore, were carried out at 1 and 3 days after inflammation.

As shown in Fig. 2, at day 3 of inflammation, the i.v. administration of L-NAME (50 mg/kg) significantly blocked the CFA-induced thermal hyperalgesia, but D-NAME (50 mg/kg) did not. At day 1 of inflammation, however, no change in behavioral hyperalgesia was observed when L-NAME was injected. L-NAME had no effect on the paw withdrawal latency in the absence of intraplantar injection of CFA. The antinociceptive effect of L-NAME at day 3 of inflammation reached a maximum at 1 hr after the injection ($2.3±3.0\%$, $n=12$) and then

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**Fig. 2.** Time course of the effects of L-NAME (50 mg/kg, i.v.) on thermal hyperalgesia one day or 3 days after CFA injection. The data of each group (12 animals) are presented as the means ±S.E.M.; differences between means were analyzed by Steel's test following ANOVA. *Significantly different from the value prior to i.v. administration of L-NAME at 3 days post inflammation (P<0.05). ○: D-NAME, ●: L-NAME, ○: L-NAME (normal rat), ●: L-NAME (1 day after CFA).
gradually returned to the control level. Pretreatment with L-arginine (600 mg/kg, i.p.), a NOS substrate, 5 min prior to administration of L-NAME completely reversed the antinociceptive effect of L-NAME from 2.3 ± 0.070 (n = 12) to 35.1 ± 3.9010 (n = 12) at 1 hr after L-NAME injection. In contrast, D-arginine, the less active enantiomer, at the same dose did not show a significant effect until 1 hr after i.v. injection and then gradually reversed the antinociceptive effect of L-NAME; at 2 hr after injection, the effect of D-arginine finally reached a level similar to that of L-arginine (Fig. 3). When used alone, L or D-arginine failed to exhibit any significant effect on the thermal hyperalgesic response to CFA.

**DISCUSSION**

It has been suggested that the persistence of altered sensations subsequent to tissue injury may result from changes, such as the LTP (23, 27, 35, 36), in intracellular functions in the central nervous system, especially in the spinal cord. An LTP-like process may be one of the factors inducing persistent pain syndromes. Support for this suggestion is that NO relates to nociceptive processing (23, 37) and to the production of LTP (11, 12). In the spinal cord, cNOS- or NADPH-d-positive neurons are located in the superficial layers of the dorsal horn (26, 27, 38). Release of NO changes the excitability of nociceptive neurons which contributes to the persistence of hyperalgesia and allodynia in chronic pain syndromes (22, 39–41). In the present behavioral study, at day 3 of inflammation, the i.v. administration of L-NAME blocked the adjuvant-induced thermal hyperalgesia, but the inactive enantiomer, D-NAME (20, 42), did not. However, no change in behavioral hyperalgesia was observed when L-NAME was injected at day 1 of inflammation. The inhibitory effect of L-NAME at day 3 of inflammation was reversed by the NOS substrate L-arginine. In sham rats, treatment with the same substance did not produce any change in the thermal nociceptive withdrawal latencies. Similar findings have been observed in animals with tonic or persistent pain, a model of neuropathic pain (loose ligation of the sciatic nerve) (24), or with chronic allodynia-like conditions (43). Persistent thermal (24) or mechanical hyperalgesia (43) is reported to be reversed by administration of L-NAME, but not D-NAME, as was observed in the present study. In addition, at day 3 of inflammation, a good correlation between the increase in NO activity and behavioral hyperalgesia was observed. These findings lead to the idea that a sustained production of NO in the spinal cord may be required for, maintenance and facilitation, but not manifestation, of the thermal hyperalgesia induced by prolonged tissue damage.

Most cNOS-positive neurons were stained also for NADPH-d. Both types of neurons were observed at the border between laminae II and III, and some were in laminae I, Ilo and III–IV (see Fig. 1). It has been reported that the distribution pattern of cNOS neurons is nearly identical to that for NADPH-d neurons (25–27). The colocalization of cNOS and NADPH-d in neurons has been confirmed by several investigators (44–46). In the present study, 3 days after the onset of CFA-induced inflammation, the number of NADPH-d and cNOS-positive neurons increased in laminae I–IV, particularly at the border between laminae II and III on the inflamed side. Although the border between laminae II and III is not the region that receives nociceptive primary afferents, the involvement of NADPH-d- or NOS-positive neurons in the maintenance of hyperalgesia might be explained by the action of the diffusible messenger NO or neuronal circuits. The morphology of NADPH-d- and NOS-positive neurons at the border between laminae II and III following inflammation is similar to lamina II-III border cells (27) and islet cells (47, 48); both have been identified as GABAergic (49, 50). These inhibitory interneurons may make axodendritic, dendrodendritic or axoaxonic
synaptic contacts with inhibitory interneurons in lamina IIo, as do IIa islet cells (47, 48). The lamina IIa islet cells make synaptic contacts with stalled cells located at the border between laminae I and IIa (47, 48). The stalled cells send axons into lamina I contact projection cells (47, 48). Therefore, activation of NOS-positive lamina II-III border cells might facilitate lamina I projection cells via disinhibition. An increase of NO activity in lamina II-III border cells may result from an activation of nociceptive primary afferents, because NOS-positive cells have dendrites that extend into laminae I and IIo where nociceptors terminate. In addition, NOS-positive laminae II and III neurons might receive nociceptive input indirectly via interneuronal relays. After peripheral nerve injury, low-threshold mechanoreceptive afferents sprout into laminae IIo and I of the lumbar dorsal horn (51), and after inflammation, low-threshold mechanoreceptive afferent activation produces allodynia. For example, after inflammation, normally non-noxious stimulation of the plantar hind paw during one hr of walking evokes a significant increase of c-fos expression in laminae III, III and IV of the cervical and lumbar spinal cord (52). NOS-positive neurons in laminae III-IV might be involved in this low-threshold mechanoreceptor-driven allodynia.

A significant increase of NADPH-d-stained neurons has been reported in the dorsal horn contralateral to injury (25, 53). In our histochemical study, however, a widespread or bilateral increase in NADPH-d and cNOS-positive neurons after unilateral CFA-induced inflammation was not observed. The reason for the difference between this and previous studies is not clear. Concerning localization of NOS-generating cells in the central nervous system, data from immunocytochemistry, in situ hybridization and NADPH diaphorase histochemistry showed the widespread distribution of this NO-forming enzyme in the basket and granule cells of the cerebellum, striatal neurons, hippocampal neurons and glia. In addition, NO has recently emerged as the major nonadrenergic, noncholinergic transmitters in the peripheral nervous system or as an effector molecule in immunological reactions (54, 55). In this connection, at wounds and other sites of inflammation, activated macrophages and neutrophils generated NO. Taking account of these observations, the possibility that the NO system in other areas except for the spinal cord is also involved in the present phenomena can not be disregarded.

Our findings indicate that there is a delay in the increase of NADPH-d- and cNOS-positive neurons after CFA-induced inflammation. Similar to the present findings, it has been reported that intrathecal injection of L-NAME significantly diminishes the late-phase of formalin-induced electrophysiological responses and prevents the development of thermal hyperalgesia, while having no effect on the early phase (19). These facts suggest that an increase of NOS activity is important for long-lived hyperalgesia, thus, supporting the idea that a substantial accumulation of the expression of immediate-early genes, such as c-jun, may be needed to affect the induction of the cNOS gene (56).

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