Nitric oxide-dependent long-term potentiation revealed by real-time imaging of nitric oxide production and neuronal excitation in the dorsal horn of rat spinal cord slices

Nitric Oxide-Dependent Long-Term Potentiation Revealed by Real Time Imaging of Nitric Oxide Production and Neuronal Excitation in the Dorsal Horn of Rat Spinal Cord Slices

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Abstract

Nitric oxide (NO) is thought to be involved in the central mechanism of hyperalgesia

and allodynia at the spinal level. Recently, we reported that NO played an important role in the induction of long-term potentiation (LTP) of synaptic strength in spinal dorsal horn, which is believed to underlie hyperalgesia and allodynia. In this study, to elucidate the relationship of NO to LTP in spinal dorsal horn, we measured the spatiotemporal distribution of NO signal with the NO-sensitive dye, DAR-4M, and neuronal excitation with the voltage-sensitive dye, RH482, in rat spinal cord slices, elicited by dorsal root stimulation. In superficial dorsal horn, neuronal excitation evoked by C fiber-activating dorsal root stimulation was potentiated for more than 2 hours after low-frequency conditioning stimulation (LFS, 240 pulses at 2 Hz for 2 min). In the same slices that exhibited LTP, NO was produced and distributed in the superficial dorsal horn during the delivery of LFS, and the amplitude of LTP and amount of NO production showed close correlation from slice to slice. LTP and production of NO were inhibited in the presence of the NO synthase inhibitors and an inhibitor of heme oxygenase, the synthetic enzyme for carbon monoxide (CO). These results suggest that production and distribution of NO is necessary for the induction of LTP in spinal dorsal horn, and that CO contributes to the LTP inducion and NO production by LFS.

Introduction

Nitric oxide (NO) is recognized as an important intra- and intercellular messenger molecule in synaptic transmission in the peripheral and central nervous system (Garthwaite & Boulton, 1995). In the spinal cord, NO plays an important role in the central sensitization that induces hyperalgesia and allodynia following inflammation or nerve injury. Systemic and intrathecal administration of NO synthase (NOS) inhibitors reduce both central sensitization in the spinal cord and the nociceptive responses to inflammation or nerve injury (Kitto et al., 1992; Haley et al., 1992; Coderre & Yashpal, 1994; Lin et al., 1999). Furthermore, it has been reported that thermal hyperalgesia is facilitated by intrathecal administration of an NO donor (Tao & Johns, 2000). Although there are a number of reports describing the contribution of NO to hyperalgesia and allodynia, the cellular mechanism for inducing these NO-dependent, abnormal pains are still unknown.

NO is also thought to play an important role in inducing long-term potentiation (LTP) of synaptic transmission in the central nervous system (Hawkins et al., 1998; Zhuo et al., 1999), and thus contributes to learning and memory. LTP of synaptic transmission between primary afferents and neurons in the spinal dorsal horn is considered a cellular mechanism for the central sensitization that causes hyperalgesia and allodynia. It has been reported that synaptic transmission between unmyelinated C-afferent fibers and spinal dorsal horn neurons is potentiated by electrical conditioning stimulation or inflammation (Sandkuhler & Liu, 1998; Ikeda et al., 2003). By visualizing optically recorded neuronal excitation at the superficial lamina of the spinal dorsal horn, we recently observed that low-frequency conditioning stimulation (LFS) of the dorsal root induced an NO-dependent presynaptic form of LTP (Ikeda & Murase, 2004). However,

due to technical difficulties, there was no direct evidence to show whether or not NO was produced during the conditioning stimulation. Fluorescent indicators have been developed recently that directly detect NO under physiological conditions (Kojima et al., 1997, 2001; Brown et al., 1999; von Bohlen und Halbach et al., 2002; Takata et al., 2005) making it possible to visualize the spatiotemporal distribution of NO production in real time.

In this study, we performed real time imaging of the spatiotemporal distribution of NO production with the fluorescent NO indicator, DAR-4M, and of neuronal excitation with the voltage-sensitive absorption dye, RH482 (Ikeda & Murase, 2004) following LTP-inducing conditioning stimulation in spinal cord slices.

Materials and methods

All animal studies were undertaken using protocols approved by the university animal ethics committee. The method for slice preparation has been described in detail elsewhere (Ikeda & Murase, 2004). Briefly, transverse slices (500 μm thick) with dorsal root attached were prepared from lumbosacral enlargements of 18- to 25-day-old Wister rat spinal cords in cold Ringer solution. Each slice was first stained in a bath filled with the voltage-sensitive absorption dye RH-482 (0.1 mg/ml; Nippon Kanko Shikiso, Okayama, Japan) and then in a bath filled with the fluorescent NO probe DAR-4M (10 μM; Daiichi Pure Chemicals, Tokyo, Japan). Double stained slices were set in a submersion-type chamber (0.2 ml) on an inverted microscope (IX70; Olympus, Tokyo, Japan) equipped with both a xenon and a halogen lamp. Slices were perfused with Ringer solution containing the following (in mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, 0.2 thiourea, 0.2 ascorbic acid, and 10 glucose

(oxygenated with 95% O_2 and 5% CO_2) at room temperature.

To record neuronal excitation, the light absorption change in RH482 at a wavelength of 700 ± 32 nm was recorded in a 0.83 mm square area in the dorsal horn by an imaging system (Deltalon 1700; Fuji, Tokyo, Japan) with 128×128 pixel photo sensors at a frame rate of 0.6 msec. Eight single pulses were given to the dorsal root at a constant interval of 15 sec, and the resulting images were averaged. The ratio image was calculated by dividing the image data by the initial frame. For test stimulation, 0.5 msec current pulses of 2-2.5mA were delivered via the dorsal root with a glass-suction electrode. To induce LTP, conditioning stimulation of the same strength and duration as the test stimulation was delivered repetitively at 2 Hz for 2 min (LFS).

To record the NO signal during LFS, the change in fluorescence intensity in the dorsal horn was measured using a cooled CCD camera (Coolsnap cf; Photometrics, Tucson, AZ). DAR-4M was excited at 535 ± 15 nm by the xenon lamp, and the resulting emitted fluorescent light was passed through a 580 nm high-pass filter. NO signal was expressed as the ratio of fluorescence intensity of DAR-4M to initial frames.

L-NAME, L-NMMA and D-AP5 were obtained from Sigma (St. Louis, MO), and Zinc protoporphyrin IX from Tocris Cookson (Bristol, UK).

Statistical significance was determined using the Student's t-test.

Results

Fig. 1A, C and D illustrate the spatiotemporal distribution of neuronal excitation recorded by optical imaging with the voltage-sensitive dye, RH482, before and after LFS. As in our previous reports (Ikeda & Murase, 2004), high-intensity single-pulse test stimulation to the dorsal root, which activates A- and C-afferent fibers, evoked an

increase in light absorption, an indication of neuronal excitation, in the spinal dorsal horn. The neuronal excitation was strongest in lamina I-III of dorsal horn, which contains nociceptive neurons.

After LFS, the neuronal excitation was gradually potentiated to as much as $141 \pm 5\%$ of control at 2 hours after LFS (Fig. 1D,E, n = 5). The LTP of neuronal excitation was strongest in the superficial dorsal horn, which includes many projection neurons that convey nociceptive signals to the brain (Fig. 1C).

Fig. 1 near here

Although LFS-induced LTP of optically recorded neuronal excitation in the spinal dorsal horn is strongly inhibited by NOS inhibitors (Ikeda & Murase, 2004), it is not clear if NO is produced as a result of this stimulation. Thus, we measured the spatiotemporal distribution of NO during LFS in the spinal dorsal horn with the fluorescent NO indicator, DAR-4M (Fig. 2A). The NO signal gradually increased during LFS (Fig. 2A,C, $2.4 \pm 0.3\%$, n = 5) especially in lamina I and II (Fig. 2B). The same region as that LTP was induced (Fig. 1C).

Depending on the slice, the degree of LTP varied from 130 % to 160 %. In five slices showing different degrees of LTP, there is close linear correlation between degree of LTP in the neuronal excitation and the intensity of NO signal (Fig. 2D).

Fig.2 near here

We also examined the effect of NOS inhibitors on both neuronal excitation and NO

production. As shown in Fig. 3, 100 μM L-NAME significantly inhibited both the LTP (Fig. 3A, $105 \pm 2\%$, $p < 0.01$ compared with control, n = 5) and NO signals induced by LFS (Fig. 3B, $0.5 \pm 0.3\%$, $p < 0.01$ compared with control, n = 5). L-NMMA also strongly inhibited both LTP (Fig. 3C, 10 μM, $141 \pm 6\%$, n = 5; 50 μM, $117 \pm 5\%$, n = 5; 100 μM, $99 \pm 3\%$, n = 4) and NO signals (10 μM, $2.6 \pm 0.6\%$, n = 5; 50 μM, $1.1 \pm 0.4\%$, n = 5; 100 μM, $0.5 \pm 0.3\%$, n = 4) induced by LFS in a dose-related manner.

It is well known that NO synthase activity is controlled by NMDA receptors (Kawamata & Omote, 1999; Takata et al., 2005). Therefore, we examined contribution of NMDA receptor to LTP and NO signals induced by LFS. A NMDA receptor antagonist D-AP5 (50 μM) did not inhibited neither LTP (Fig. 3C, $166 \pm 8\%$, n = 5) nor NO signals (Fig. 3D, $2.8 \pm 0.5\%$, n = 5).

Since it is reported that carbon monoxide (CO) also acts as a retrograde messenger during LTP (Zhuo et al. 1999), we tested the inhibitor of heme oxygenase, the synthetic enzyme for CO. Both LTP and NO signal induced by LFS were significantly inhibited in the presence of a heme oxygenase inhibitor zinc protoporphyrin IX 10 μM (Fig. 3C,D, LTP: $100 \pm 1\%$, n = 5; NO signal: $0.9 \pm 0.3\%$, n = 5).

Discussion

In this study, we simultaneously visualized the production of NO and neuronal excitation directly in real time during the delivery of a conditioning stimulus in spinal cord slices double-stained with an NO indicator and a voltage-sensitive dye. LFS to the dorsal root induced LTP of neuronal excitation in the superficial dorsal horn, and NO was produced in the same region during the LFS. Furthermore, there was close linear

correlation between the amount of NO produced during LFS and the degree of LTP.

NO imaging with NO-sensitive fluorescent dye has the advantage of measuring the spatio-temporal production and distribution of NO (Kojima et al., 1997, 2001; Brown et al., 1999; von Bohlen und Halbach et al., 2002; Takata et al., 2005). In this study, we employed DAR-4M that developed from DAF-2-type dye, because DAR-4M is pH-independent above 4.0 and is higher excitation wavelength that resulted in smaller background fluorescence comparing to DAF-2-type dye (Kojima et al. 2001). It is reported that the membrane permiable dye DAR-M AM is more suitable for the imaigng than membrane inpermiable DAR-M due to its hydrophobicity (Kojima et al. 2001). In this study, our pupose is to determine the distribution of NO in a whole spinal cord slice but not NO production in individual cells. In preliminary studies, the intensity of NO signals in slices stained with DAR-4M AM was similar to that with DAR-4M. Therefore, we employed DAR-4M, but not DAR-4M AM that primarily responds to intracellular NO.

NO is one of the retrograde messengers involved in the induction of a presynaptic form of synaptic plasticity that plays a role in learning and memory in the hippocampus, cerebellum and other areas of the central nervous system (Hawkins et al., 1998). In some experiments, optical imaging has been used to clarify the involvement of NO in synaptic plasticity. DAR-4M was used in living hippocampal slices, to demonstrate that N-methyl-D-aspartate (NMDA) stimulation or tetanic stimulation produces time-dependent heterogeneous NO (Takata et al., 2005). By measuring NO signals and field potentials, it was shown that the NO signal increases during LTP induction (von Bohlen und Halbach et al., 2002). In the spinal dorsal horn, we recently showed that a presynaptic form of LTP could be induced by LFS by visualizing presynaptic and

postsynaptic neuronal excitation with a voltage-sensitive dye (Ikeda & Murase, 2004). The induction of this LTP was inhibited by the neuronal NOS inhibitor, 7NI, and the inducible NOS inhibitor, AMT. However, it was not clear whether conditioning stimulation produces NO, and the correlation between NO production and LTP induction was not demonstrable because it was technically difficult to directly measure both the NO signal and neuronal excitation in living tissue. In the present study, using the fluorescent NO indicator DAR-4M and voltage-sensitive dye RH-482, we simultaneously visualized the NO signal during conditioning stimulation and the neuronal excitation. By enabling simultaneous measurement of the spatiotemporal distribution of NO production and neuronal excitation, this method furthers our ability to examine the role of NO in the modulation of neuronal activity. With this method, we provide direct evidence that NO contributes to induction of LTP in the spinal dorsal horn.

Ca^{2+} influx through NMDA receptors is a most established pathway for NO synthase activation (Okada et al., 2004; Takata et al., 2005). Since, in this study, both LTP and NO signals induced by LFS were not inhibited in the presence of an NMDA antagonist, other factor(s) may trigger NO production through Ca^{2+}-dependent mechanisms. We have reported that NO-dependent LTP induced by LFS in the spinal dorsal horn is not inhibited by an NMDA receptor antagonist, but inhibited by a metabotropic glutamete receptors (mGluRs) antagonists and that the application of an mGluRs agonist induces LTP without LFS (Ikeda & Murase, 2004). In cerebellum, activation of mGluRs produces acute stimulation of guanylyl cyclase through NO (Okada, 1995; Okada et al., 2004). Although, from these reports, it is likely that NO-dependent LTP in the spinal dorsal horn is triggered by mGluRs activation, further experiments are necessary to

reveal how the NO production is triggered.

CO, that is a gas generated by heme oxygenously, also plays a role as a retrograde messenger for LTP. Zhuo et al. have shown that NO contributes to the induction of LTP produced by one train of tetanus stimulation, and CO is related to the induction of LTP by either two-train or four-train tetanus stimulation. From these results, they suggest that NO synthase and heme oxygenase are not activated in parallel during the induction of LTP, but rather that heme oxygenase plays a more tonic role (Zhou et al., 1999). In this study, a heme oxygenase inhibitor not only blocked LTP, but also reduced NO signals during LFS. Therefore, it is likely that this LTP is induced by a combination or collaboration of NO and CO productions. In ischemic heart, it is shown that an NO-mediated increase of cGMP was reduced by protoporphyrin, and suggested that CO can amplify the NO-stimulated increase of cGMP (Maulik et al. 1996). Further studies are necessary to reveal the role of CO in LFS-induced NO in the spinal dorsal horn.

In this study, LTP was induced mainly in the superficial dorsal horn, which contains many nociceptive neurons (Christensen & Perl, 1970) that receive input from A- and C-fibers (Willis & Coggeshall, 1991). This region includes projection neurons that convey nociceptive information directly to the brainstem or thalamus (Todd et al., 2000; Yu et al., 2005). It has been reported that these projection neurons play an important role in the induction of hyperalgesia and allodynia (Chen & Pan, 2002; Mantyh & Hunt, 2004), and we recently showed that these projection neurons induce LTP (Ikeda et al., 2003; Ikeda & Murase, 2004).

In behavioral studies, hyperalgesia induced by intrathecal injection of NMDA is blocked by administration of the NOS inhibitor, L-NAME (Kitto et al., 1992). The enhancement of formalin-induced paw licking behavior in rats treated with L-glutamate

or substance P is reversed by pretreatment with L-NAME (Coderre & Yashpal, 1994). Topical application of L-NAME onto spinal cord reduces both the first and second peaks of the response of dorsal horn neurons to formalin injection (Haley et al., 1992). The sensitization of spinothalamic neurons induced by intradermal injection of capsaicin is prevented by pretreatment of the dorsal horn with the NOS inhibitor L-NAME or 7-NINA (Lin et al., 1999). The allodynic response, and increased response of dorsal horn neurons to noxious and innoxious stimuli induced by intradermal capsaicin injection are reversed by the administration of the NOS inhibitors 7-NINA or AMT (Wu, 2001). The NO-dependent LTP in the spinal dorsal horn reported in this study indicates a possible cellular mechanism for the induction of these NO-dependent abnormal pains.

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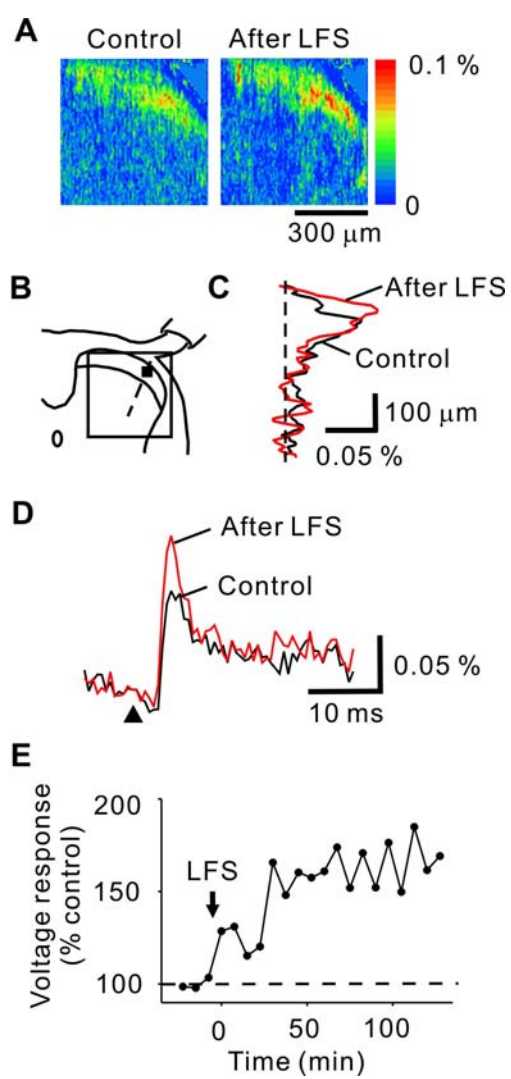
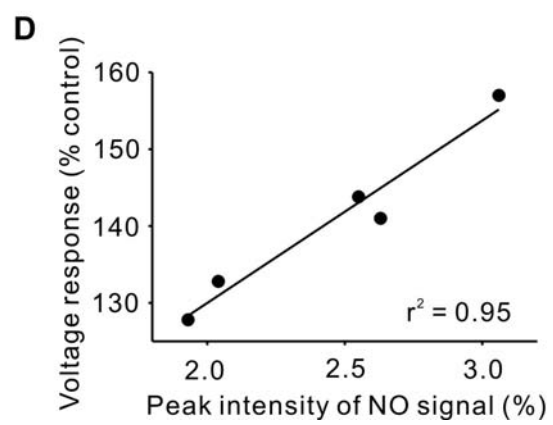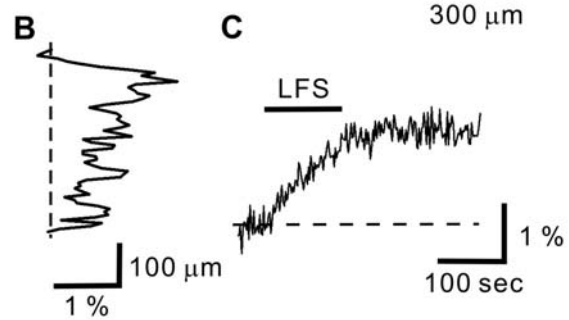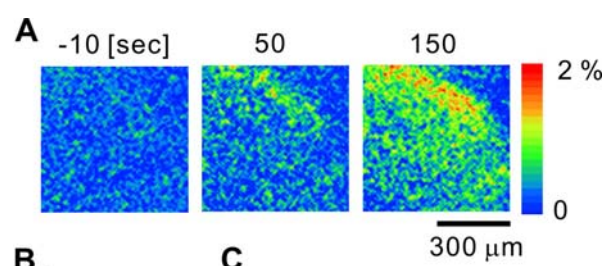layer-specific manner in the rat cerebellar cortex. *Neuroscience,* **125**, 461-472.

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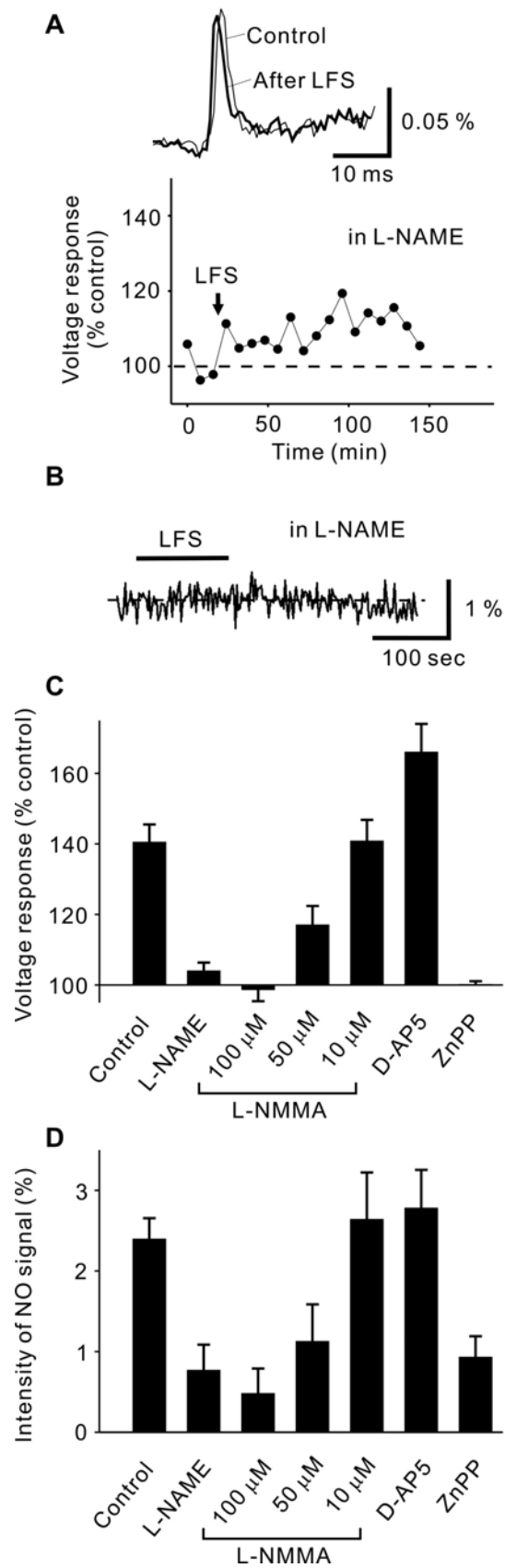of nitric oxide and carbon monoxide in long-term potentiation in the hippocampus.
Learn. Mem., **6**, 63-76.

A Control   After LFS   0.1 %

300 μm   0

B

C   After LFS

Control

100 μm

0.05 %

D   After LFS

Control

0.05 %

10 ms

E

LFS

A −10 [sec]   50   150   2 %

0

300 μm

B   **C**

LFS

1 %

100 μm

100 sec

1 %

D

160

150

140

130

r² = 0.95

2.0   2.5   3.0

Voltage response (% control)

Peak intensity of NO signal (%)

A

Control

After LFS

0.05 %

10 ms

in L-NAME

LFS

B

LFS   in L-NAME

1 %

100 sec

C

D

Figure legends

Fig. 1

An example of LTP of neuronal excitation in the spinal dorsal horn by LFS to the dorsal root. A, Optical responses evoked by single-pulse test stimulation to the dorsal root before (left images) and 120 min after LFS (2 Hz for 2min, right images). The intensity of absorption change is indicated by pseudo-color. B, Images were taken from the area of spinal dorsal horn indicated by a rectangle in the schematic drawing of a transverse spinal cord slice. C, Spatial distribution of changes in the optical responses to test stimuli before (black line) and after LFS (red line) taken along a dorsoventral axis indicated by the dashed line in the schematic drawing. D, Spatial averaged time courses of optical responses before (black line) and after LFS (red line) at the area indicated by the filled square in the schematic drawing in B. Arrowhead indicates the time when test stimulus was given. E, LTP of the optical responses in the superficial layer. Each point represents the magnitude of change in the spatiotemporal average, as a percentage of control, measured during a period of 5 ms after the onset of the response at the area indicated by the filled square in B. Arrow indicates the time when LFS was applied.

Fig. 2

NO signals during LFS to the dorsal root in the same slice shown in Fig.1. A, Pseudo-color images representing intensity of NO were recorded in the same area shown in Fig. 1B. Numbers above the images indicate time after onset of LFS application. B, Spatial distribution of NO signal 2 min after the onset of LFS recorded along the same dorsoventral axis indicated by the dashed line in Fig. 1B. C, Spatial averaged time course of NO signal at the same area indicated by the filled square in the

schematic drawing in Fig. 1B. Horizontal bar indicates the time during which LFS was applied. D, Correlation between degree of potentiation in voltage response 120 min after LFS and peak intensity of NO signal during LFS in five slices. Note that there is a linear correlation between these values.

Fig. 3

LTP of neuronal excitation and NO signals in spinal dorsal horn by LFS under various conditions. A, An example of spatial-averaged time courses of optical responses before (thin line) and after LFS (bold line) in the presence of L-NAME, an NOS inhibitor (upper traces). The percent change in the voltage-response amplitude after LFS in the presence of L-NAME (lower graph). Arrow indicates the time when LFS was applied. B, An example of NO signal recorded during LFS in the presence of L-NAME. Horizontal bar indicates the time during which LFS was applied. C, D, The degree of LTP at 120 min after LFS (C) and the intensity of NO signals at 120 second after onset of LFS (D) (mean ± SE) in control, L-NAME 100 μM, another NOS inhibitor L-NMMA 100 μM, 50 μM and 10 μM, an NMDA receptor antagonist D-AP5 50 μM, and a heme oxygenase inhibitor zinc protoporphyrin IX (ZnPP) 10 μM.