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Effects of nitric oxide availability on responses of spinal wide dynamic range neurons to excitatory amino acids

Dénes Budai [a], George L. Wilcox [b], Alice A. Larson [a,*]

[a] *Department of Veterinary PathoBiology, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, USA*
[b] *Department of Pharmacology, Medical School, University of Minnesota, Minneapolis, MN 55455, USA*

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Abstract

The role of nitric oxide (NO) in responses of spinal dorsal horn neurons to excitatory amino acids and to cutaneous mechanical stimuli was examined. Extracellular recordings were made from wide dynamic range neurons excited with iontophoretically applied excitatory amino acid agonists, *N*-methyl-D-aspartate (NMDA) and (*R*,*S*)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) or kainic acid. Nitric oxide availability was decreased by iontophoretic application of NO synthase inhibitors, N^{ω}-nitro-L-arginine methyl ester (L-NAME) or L-N^5-(1-iminoethyl)ornithine (L-NIO), or elevated by the NO donating compound, *S*-nitroso-*N*-penicillamine (SNAP). When cells were excited with successive application of NMDA and non-NMDA excitatory amino acid receptor agonists, application of NO synthase inhibitors led to a decrease in responses to NMDA in 60% of neurons. In more than a third of the cells tested, inhibition of NO synthase caused reciprocal changes in responses to glutamate receptor agonists: NMDA-evoked responses were significantly decreased whereas responses to the non-NMDA receptor agonists (AMPA or kainic acid) were increased. Application of the NO donating compound, *S*-nitroso-*N*-penicillamine, revealed an opposite tendency, increasing responses to NMDA in more than half of the neurons tested. In approximately 40% of the cells, reciprocal changes in responses to excitatory amino acid receptor agonists of NMDA versus non-NMDA types were observed after application of *S*-nitroso-*N*-penicillamine, such that the increase in NMDA responses was accompanied by decreases in the responses to kainic acid. The inhibitory effect of N^{ω}-nitro-L-arginine methyl ester on the basal firing rate evoked by sustained iontophoretic application of NMDA was specific, dose-dependent and prevented by L-arginine. In the presence of N^{ω}-nitro-L-arginine methyl ester, responses to noxious peripheral stimulation were either unchanged or decreased. Responses to innocuous stimuli, however, were significantly elevated by N^{ω}-nitro-L-arginine methyl ester. These results suggest that availability of NO at the synapses between primary afferent fibers and secondary dorsal horn neurons may contribute to the dominancy of one type of excitatory amino acid receptor class over the other in transmission of somatosensory information.

Keywords: Spinal cord; Nociception; Glutamate receptor; Nitric oxide (NO); N^{ω}-Nitro-L-arginine methyl ester; L-N^5-(1-Iminoethyl)ornithine; *S*-Nitroso-*N*-penicillamine

1. Introduction

Nitric oxide (NO) has been characterized as a novel neuronal messenger involved in a variety of neurotransmitter functions (Bredt and Snyder, 1992; Snyder, 1992; Meller and Gebhart, 1993; Zorumski and Izumi, 1993). The production of NO from the amino acid L-arginine is catalyzed by a Ca^{2+}- and calmodulin-dependent enzyme, NO synthase, which can be selectively inhibited by structural analogs of L-arginine, e.g., N^{ω}-nitro-L-arginine methyl ester. The enzyme NO synthase has been localized within the peripheral and central nervous systems including brain (Garthwaite et al., 1988) and spinal cord (Valtschanoff et al., 1992; Lee et al., 1993; Morris et al., 1994). NO is a small, reactive,

* Corresponding author. Department of Veterinary PathoBiology, University of Minnesota, College of Veterinary Medicine, 295 Animal Science/Veterinary Medicine Building, 1988 Fitch Avenue, St. Paul, MN 55108, USA. Tel. (612) 624 3650, fax (612) 625 0204.

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lipophilic molecule with a half-life of milliseconds to several seconds. It may act in the neuron where it was produced or diffuse through cell membranes to act on adjacent cells as an intercellular messenger (Garthwaite et al., 1988; Schuman and Madison, 1994). The properties of NO may allow it to act as a 'retrograde transmitter', i.e., when produced in a post-synaptic neuron NO might diffuse to pre-synaptic sites, thus altering presynaptic function (Baringa, 1991). This concept is particularly favored in studies of long-term potentiation, where it may account for the use-dependent changes in the efficacy of synaptic transmission.

Considerable evidence suggests that NO plays a role in spinal somatosensory processing. The NMDA receptor subtype, activated by endogenous agonists such as L-glutamate, L-aspartate and L-homocysteate, appears to be involved in nociceptive transmission and plasticity in the spinal cord (for review see Wilcox, 1991; Meller and Gebhart, 1993; Wilcox, 1993). Intrathecal administration of NMDA elicits a transient hyperalgesia which is inhibited by prior treatment with N^{ω}-nitro-L-arginine methyl ester, methylene blue or hemoglobin (Kitto et al., 1992). Intrathecal injection of NO-donating compounds such as L-arginine, sodium nitroprusside or hydroxylamine also results in hyperalgesia (Kitto et al., 1992; Meller et al., 1992a,b; Haley et al., 1992b). In the formalin pain model, inhibition of NO synthase by N^{ω}-nitro-L-arginine methyl ester produces long-lasting antinociception (Moore et al., 1991). Blocking the production of NO resulted in a delay in the onset of thermal hyperalgesia, but did not prevent its development (Meller et al., 1994). Topical application of N^{ω}-nitro-L-arginine methyl ester onto the spinal cord attenuates both the first and second peaks of neuronal responses to formalin (Haley et al., 1992b). L-Arginine, which was surprisingly antinociceptive by itself, reversed the antinociceptive effects of N^{ω}-nitro-L-arginine methyl ester in the formalin-induced paw licking test (Moore et al., 1991) but not the decrease in neuronal firing rate caused by N^{ω}-nitro-L-arginine methyl ester (Haley et al., 1992b). In in vivo electrophysiological studies, inhibition of NO synthase by N^{ω}-nitro-L-arginine methyl ester reduces responses of single dorsal horn neurons to electrical or chemical nociceptive stimulation of peripheral nerves (Haley et al., 1992b) and selectively inhibits the responses of these cells to iontophoretically applied NMDA (Radhakrishnan and Henry, 1993).

Here, we report that responses to NMDA- and non-NMDA-type agonists of excitatory amino acid receptors of spinal wide dynamic range neurons may be differentially affected by changes in NO availability. In addition, neuronal firing evoked by presumably painful peripheral stimuli was decreased, whereas responses to innocuous stimuli were enhanced after inhibiton of NO synthase.

2. Materials and methods

2.1. Surgery

Extracellular single-neuron recordings were made in urethane-anesthetized (1.2 g/kg initial dose i.p.) male Sprague-Dawley rats weighing between 350 and 450 g. A tracheotomy and a cannulation of the external jugular vein were performed after achieving deep anesthesia. Subsequently, smaller doses of urethane were given intravenously to maintain the level of anesthesia. The lumbar enlargement (L3–L5 segments) was exposed by a laminectomy and the spinal cord was covered with a pool of warmed mineral oil to prevent drying. Rectal temperature as well as the temperature of the mineral oil were monitored with temperature probes and kept at 37° C by a warm water-heated blanket beneath the rat and an infrared heat lamp from above. Heart rate and end-tidal CO_2 concentration were monitored and maintained within normal limits. Recordings were only commenced at least 1 h after surgery.

2.2. Extracellular recording

Compound recording/iontophoresis electrodes were constructed from a seven-barreled array of thin-wall borosilicate glass capillary tubings (1.5 mm o.d., 1.12 mm i.d.; Frederick Haer and Co., Brunswick, ME, USA). The recording center barrel contained a low impedance (1–3 MΩ) 7-μm carbon fiber (Thornel T300; Amoco Performance Products, Greenville, SC, USA), and drugs were delivered from the surrounding outer barrels. Single unit extracellular recordings were made from selected dorsal horn neurons responding to both innocuous (brush, pressure) and noxious (pinch, squeeze that was felt as painful by the experimenter) stimuli applied to the excitatory receptive fields of the hind paw. Manually applied innocuous stimuli (light touch or pressure) were used as searching stimulus which was followed by characterization of neuronal responses to trials of mechanical stimuli. Mechanical stimuli of increasing strength were delivered by a small brush and three serrated clamps with a graduated force. The experimenter released clamps so that force was standard for each mechanical stimulus delivered over a period of 3 s, and 5–10 min were allowed to pass between trials. Action potentials were displayed on an oscilloscope and detected with a window discriminator (Frederick Haer and Co., Brunswick, MA, USA). The number of action potentials per second (Hz) was counted by the computer and the resulting peristimulus time histograms were displayed. Iontophoretic drug delivery and collection of experimental data were performed by a multifunction instrument control and data acquisition board (NB-MIO-16, National Instruments, Austin, TX, USA) placed in a Macintosh IIvx com-

puter programmed in LabVIEW 3. Detailed description of data acquisition hardware and software is given elsewhere (Budai, 1994).

2.3. Drugs

Microiontophoresis was performed using an E 104 B/5 controller with automatic current balancing (Fintronics, Orange, CT, USA). Drug barrels of the combined recording/iontophoresis electrode contained one of the following freshly made solutions: 100 mM *N*-methyl-D-aspartate Na (NMDA) in 100 mM NaCl (pH 8.0), 10 mM (*R,S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid HBr (AMPA) in 190 mM NaCl (pH 8.0), 20 mM kainic acid in 180 mM NaCl (pH 8.0), 20 mM L-arginine HCl in 180 mM NaCl (pH 7.2), 50 mM N^{ω}-nitro-L-arginine methyl ester (L-NAME) in 150 mM NaCl (pH 6.5), 50 mM N^{ω}-nitro-D-arginine methyl ester (D-NAME) in 150 mM NaCl (pH 6.5), 10 mM L-N^5-(1-iminoethyl)ornithine (L-NIO) in 150 mM NaCl (pH 6.5) or 10 mM *S*-nitroso-*N*-penicillamine (SNAP) in 150 mM NaCl containing 5% dimethyl sulfoxide. Excitatory amino acid agonists (NMDA, AMPA and kainic acid) were delivered by negative currents and N^{ω}-nitro-L-arginine methyl ester, N^{ω}-nitro-D-arginine methyl ester or L-N^5-(1-iminoethyl)ornithine by positive currents. *S*-Nitroso-*N*-penicillamine was ejected by pressure because preliminary experiments indicated that this compound could not reliably be ejected iontophoretically. NMDA, kainic acid, D-NAME, N^{ω}-nitro-L-arginine methyl ester, and L-arginine were purchased from Sigma (St. Louis, MO, USA). AMPA, L-N^5-(1-iminoethyl)ornithine and *S*-nitroso-*N*-penicillamine were obtained from RBI (Natick, MA, USA).

2.4. Data analysis

Digital records of unit activity were retrieved and analyzed off-line. Statistical evaluations were made using the mean total number of spikes per second or the total number of spikes evoked during each epoch of excitation by mechanical stimuli or iontophoretic application of an excitatory agent. The background neuronal discharges were calculated by averaging 15 s of activity preceding each epoch of excitation and subtracted from all drug-evoked and mechanically evoked responses. Differences between responses for individual iontophoretic pulses of a single cell were confirmed by one-factor analysis of variance (with Student-Newman-Keuls test for post-hoc analysis) by comparing the mean total number of spikes per second. A P value of < 0.05 was considered significant in all cases. The changes induced by applications of various drugs were evaluated at their maxima or, when no maximum was found (e.g. in case of an irreversible inhibitor or antagonist), at the end of the recording. The total number of spikes evoked in response to mechanical stimulation of the cutaneous receptive field by brush, pressure, pinch and squeeze was quantified as described above. Differences between the responses before and after administration of L-NAME were determined using a Wilcoxon rank test for pair comparison, and changes with a P value < 0.05 were considered to be significant.

3. Results

3.1. Effects of NO synthase inhibitors

Recordings were made in 38 rats from a total of 74 spinal dorsal horn neurons responding to both innocu-

Table 1
Effects of changes in NO availability on responses of spinal wide dynamic range neurons to iontophoretically applied *N*-methyl-D-aspartate (NMDA) and kainic acid (KA)

	L-NAME			L-NIO			SNAP		
	n	Control	+L-NAME	*n*	Control	+L-NIO	*n*	Control	+SNAP
NMDA									
Decrease	24	23.6 ± 6.1	11.5 ± 5.7	7	31.6 ± 6.6	14.5 ± 9.9	6	32.3 ± 10.0	16.9 ± 8.9
Unchanged	7	27.6 ± 5.8	24.8 ± 3.8	2	26.5 ± 8.3	22.1 ± 5.0	2	25.8 ± 9.9	30.7 ± 8.2
Increase	11	24.1 ± 9.3	40.9 ± 8.5	2	30.1 ± 5.9	41.8 ± 8.3	13	30.3 ± 8.7	64.2 ± 10.8
KA									
Decrease	13	30.8 ± 5.3	9.8 ± 10.0	2	23.0 ± 4.9	13.1 ± 5.5	10	28.0 ± 6.9	10.5 ± 9.7
Unchanged	7	33.5 ± 6.0	31.3 ± 8.4	4	29.5 ± 6.5	32.1 ± 7.9	6	31.1 ± 9.1	33.5 ± 8.9
Increase	22	28.1 ± 7.1	59.8 ± 9.3	5	31.1 ± 7.1	44.5 ± 9.8	5	26.0 ± 7.2	50.9 ± 10.7

All values are the group combined mean total spikes/s ± S.E. for control responses to iontophoretic pulses prior to coapplication of N^{ω}-nitro-L-arginine methyl ester (L-NAME), L-N^5-(1-iminoethyl)ornithine (L-NIO) or *S*-nitroso-*N*-penicillamine (SNAP), and for responses that showed maximum effect after changing the NO availability by one of these test compounds. Neurons are grouped according to the direction of change in their individual responses to the excitatory amino acids produced by co-release of one of the test compounds. 'Decrease' or 'Increase' indicates significant ($P < 0.05$, by Student-Neuman-Keuls test) changes in responses to NMDA or KA after application of L-NAME, L-NIO or SNAP.

ous and noxious stimuli delivered to the cutaneous receptive field. All cells had very little or no spontaneous activity and were located between 100 and 600 μm from the surface of the dorsal horn, as estimated by microdrive readings. Ejection currents for NMDA and kainic acid or AMPA were selected to produce a maximal peak height of 40–60 spikes/s per stimulation epoch.

Neurons were excited with trials of brief iontophoretic application of NMDA and kainic acid and/or AMPA as shown in Fig. 1. Co-release of N^{ω}-nitro-L-arginine methyl ester or L-N^5-(1-iminoethyl)ornithine, compounds known to inhibit NO synthase, resulted in significant changes ($P < 0.05$, by Student-Neuman-Keuls test) in responses to glutamate analogs in 70–80% of the 53 neurons tested under these conditions (Table 1). Responses to NMDA were decreased by inhibition of NO synthase in approximately 60% of the cells. Responses to kainic acid were increased in half of the cells whereas 20–30% of the cells showed less sensitivity to kainic acid after the inhibition of NO synthase. In 40% of the cells, application of N^{ω}-nitro-L-arginine methyl ester or L-N^5-(1-iminoethyl)ornithine was followed by changes in the opposite directions for responses to NMDA versus non-NMDA excitations. In these cells inhibition of NO synthase significantly decreased responses to NMDA application while increasing responses to kainic acid and/or AMPA (Figs. 1 and 2). In three cells, by contrast, NMDA responses were increased and non-NMDA responses were decreased by N^{ω}-nitro-L-arginine methyl ester or changes in the same direction for both NMDA and non-NMDA responses were also observed.

In contrast, with N^{ω}-nitro-L-arginine methyl ester the effect of L-N^5-(1-iminoethyl)ornithine was usually

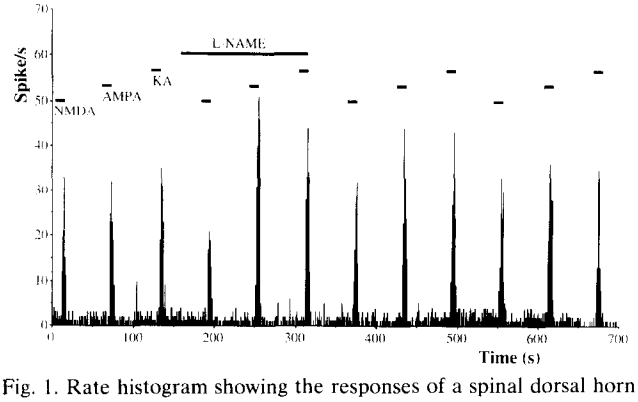

Fig. 1. Rate histogram showing the responses of a spinal dorsal horn neuron to iontophoretic applications of NMDA (40 nA), AMPA (15 nA) and kainic acid (KA; 10 nA) for 5 s in every minute as indicated by the horizontal bars. N^{ω}-Nitro-L-arginine methyl ester (L-NAME) was ejected as shown with 50 nA for 15 s from a barrel containing 50 mM solution. Note the decrease in the responses to NMDA we observed most frequently, despite an increase in responses to the non-NMDA type of excitatory amino acid receptor agonists.

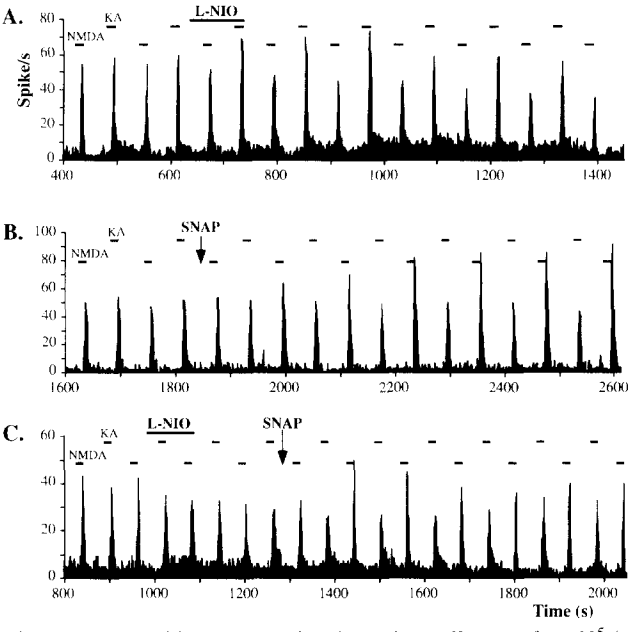

Fig. 2. Rate histograms showing the effects of L-N^5-(1-iminoethyl)ornithine (L-NIO, ejected by 80 nA) and *S*-nitroso-*N*-penicillamine (SNAP, ejected by pressure) on the responses of spinal wide dynamic range neurons to iontophoretic application of NMDA and kainic acid (KA). NMDA and kainic acid were ejected using iontophoretic currents of 23 and 8 nA, respectively.

slow in onset and long in duration. In cells where a decrease by L-N^5-(1-iminoethyl)ornithine of NMDA responses was accompanied by an increase in kainic acid-induced responses, the maximum effects of this NO synthase inhibitor on NMDA and kainic acid responses were observed 16 ± 12 min ($n = 9$) and 6 ± 6 min ($n = 7$, mean ± S.E.) after its application, respectively (Fig. 2, panel A). There was no significant difference in the latency to peak effects for NMDA and kainic acid responses. In most experiments, excitatory amino acid responses that were significantly altered by L-N^5-(1-iminoethyl)ornithine did not return to the control level but could be overcome by the NO releasing compound, *S*-nitroso-*N*-penicillamine (Fig. 2, panel C).

3.2. Effects of increased NO availability

In a separate set of 21 cells, effects of the NO-donating compound, *S*-nitroso-*N*-penicillamine, were tested (Table 1, Fig. 2). Application of *S*-nitroso-*N*-penicillamine increased neuronal sensitivity to NMDA and decreased responses to kainic acid. In doing so, NMDA responses were significantly increased ($P < 0.05$, by Student-Neuman-Keuls test) in 13 cells and kainic acid responses were significantly decreased ($P < 0.05$) in 10 cells. *S*-Nitroso-*N*-penicillamine had no effects on responses to NMDA in 2 cells or responses to kainic acid in 6 cells. In 8 cells, an increase in NMDA responses was accompanied with a decrease in

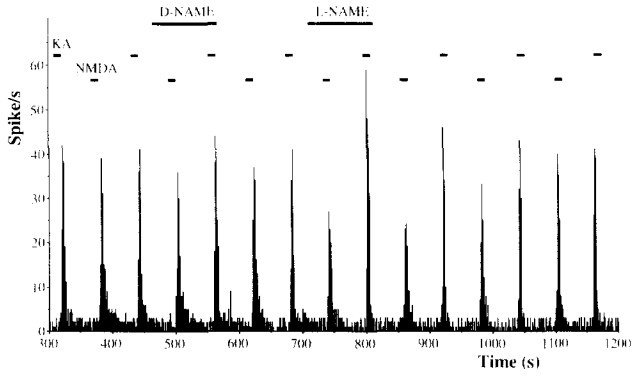

Fig. 3. Specificity of N^{ω}-nitro-L-arginine methyl ester (L-NAME) effects on the excitatory amino acid receptor agonist-evoked neuronal firing. Iontophoretic application of N^{ω}-nitro-L-arginine methyl ester but not N^{ω}-nitro-D-arginine methyl ester (D-NAME), both ejected as shown with 50 nA from barrels containing 50 mM solutions, caused reciprocal changes in responses to NMDA versus non-NMDA agonists. NMDA and kainic acid (KA) were ejected for 5 s as shown using 25 nA and 12 nA, respectively.

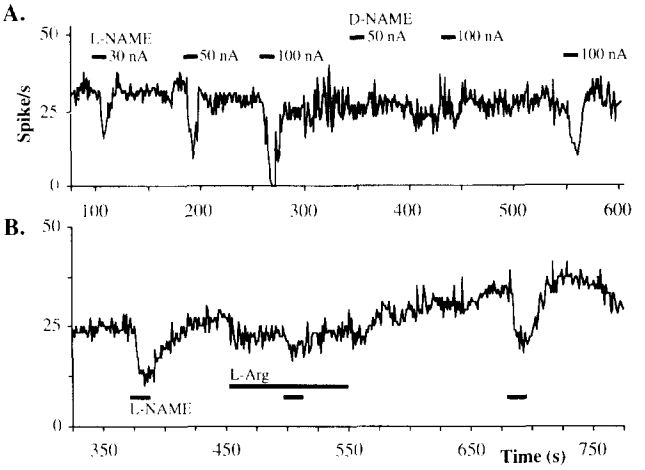

Fig. 4. Specific effects of N^{ω}-nitro-L-arginine methyl ester L-NAME on the steady basal firing rate maintained by continuous iontophoresis of NMDA (10 nA). (A) Dose-dependent inhibition of the NMDA-evoked basal firing rate by N^{ω}-nitro-L-arginine methyl ester but not N^{ω}-nitro-D-arginine methyl ester (D-NAME). (B) Reversal of N^{ω}-nitro-L-arginine methyl ester inhibition of NMDA-evoked neuronal firing by L-arginine. N^{ω}-Nitro-L-arginine methyl ester and L-arginine were ejected with 50 and 100 nA, respectively. Experiments shown in panels A and B were carried out on two separate neurons.

responses to kainic acid after *S*-nitroso-*N*-penicillamine application. A representative ratemeter recording of this reciprocal changes is shown in Fig. 2, panel B. Effects of L-N^5-(1-iminoethyl)ornithine on the NMDA- and kainic acid-evoked responses were reversed by application of *S*-nitroso-*N*-penicillamine (Fig. 2, panel C). The effect of *S*-nitroso-*N*-penicillamine on the excitatory amino acid responses was slow in onset and long in duration, requiring 10 ± 8 min (mean ± S.E., $n = 19$) for NMDA and 19 ± 14 min (mean ± S.E., $n = 15$) for kainic acid to achieve its full effect (Fig. 2, panels B

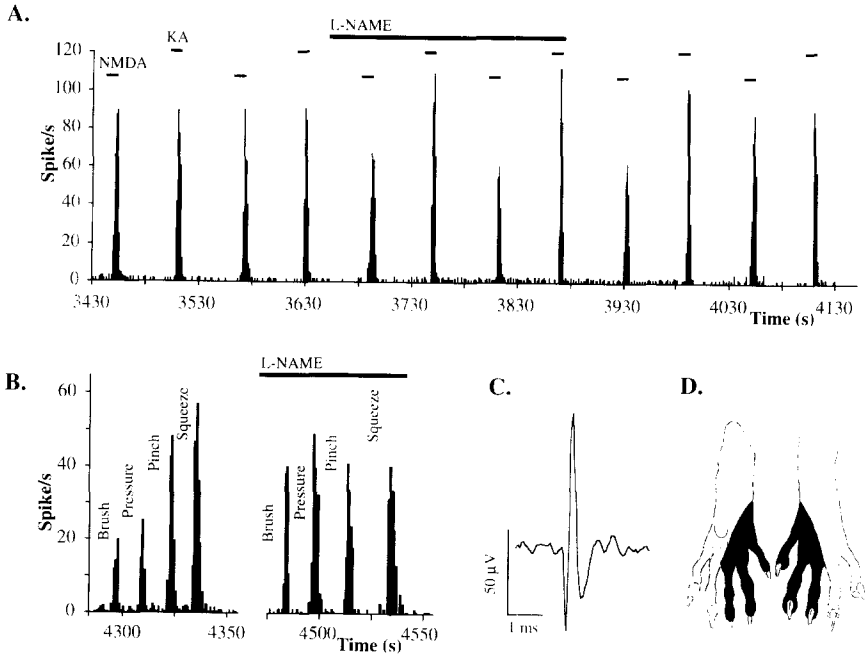

Fig. 5. Rate histograms showing the effects of N^{ω}-nitro-L-arginine methyl ester on the responses of a representative wide dynamic range neuron to chemical excitation by NMDA or kainic acid and mechanical stimulation of the cutaneous receptive field. (A) Reciprocal changes in responses to NMDA (ejected with 38 nA) and kainic acid (KA) (17 nA) were evoked by N^{ω}-nitro-L-arginine methyl ester (L-NAME). (B) Characterization of the same neuron by innocuous and noxious mechanical stimuli delivered to the receptive field before and in the presence of N^{ω}-nitro-L-arginine methyl ester. Note the increased and decreased responses to innocuous and noxious stimulation, respectively. (C) An average of ten action potential traces recorded from the neuron. (D) Drawing of the left hind paw showing the cutaneous receptive field.

and C). There was no statistically significant difference in these values.

3.3. Specificity of L-NAME effects

The specificity of N^{ω}-nitro-L-arginine methyl ester-induced changes in responses to excitatory amino acid agonists in 7 spinal wide dynamic range neurones was tested utilizing N^{ω}-nitro-D-arginine methyl ester, an isomer of N^{ω}-nitro-L-arginine methyl ester inactive in inhibiting nitric oxide synthase. N^{ω}-Nitro-D-arginine methyl ester was applied prior to the N^{ω}-nitro-L-arginine methyl ester application. Neither NMDA nor non-NMDA responses of these cells were significantly altered (one-factor analysis of variance with Student-Neuman-Keuls test for post-hoc, $P < 0.05$ for significance) by iontophoretic application of N^{ω}-nitro-D-arginine methyl ester (Fig. 3), whereas N^{ω}-nitro-L-arginine methyl ester, ejected with the same iontophoretic parameters, elicited significant changes in the same cells as described above and illustrated in Fig. 3.

Specificity of N^{ω}-nitro-L-arginine methyl ester was further tested in 5 wide dynamic range neurones in which a steady basal firing rate was maintained by continuous application of NMDA. Iontophoretic co-application of N^{ω}-nitro-L-arginine methyl ester, but not N^{ω}-nitro-D-arginine methyl ester, both ejected with the same iontophoretic parameters, reduced cell firing in a dose (current)-dependent manner (Fig. 4, panel A). The inhibition of NMDA-maintained cell firing by N^{ω}-nitro-L-arginine methyl ester was prevented by iontophoretic application of L-arginine. Occasionally, application of L-arginine led to an elevation in the NMDA-evoked neuronal firing rate (Fig. 4, panel B).

3.4. Effect of L-NAME on responses to innocuous and noxious cutaneous stimuli

Fig. 5 exemplifies the effects of N^{ω}-nitro-L-arginine methyl ester on the responses of wide dynamic range neurons to NMDA and non-NMDA excitations as well as to natural stimulation of its cutaneous receptive field. After having observed reciprocal changes in NMDA- versus kainic acid-evoked responses (panel A), innocuous (brush, pressure) and noxious stimuli (pinch, squeeze) were applied to the receptive field (panel D) in the absence and presence of N^{ω}-nitro-L-arginine methyl ester (panel B). In summary, responses of wide dynamic range neurons to innocuous and noxious stimuli were altered by N^{ω}-nitro-L-arginine methyl ester showing two types of response patterns: (a) inhibition of responses to all stimulation intensities observed (4/11 neurons, not shown), and (b) enhancement of responses to innocuous and no change or reduction in responses to noxious stimuli (7/11 neurons, shown in Figs. 5 and 6). Fig. 6 shows the effects of L-NAME on the averaged responses of the latter 7 neurons to mechanical stimulation of their cutaneous receptive fields. Statistical analysis by the Wilcoxon rank test for paired comparisons of the total number of spikes evoked above baseline revealed significant increases ($P < 0.01$) in responses to brush or pressure stimuli after L-NAME application. In contrast, responses to noxious stimuli remained statistically unchanged.

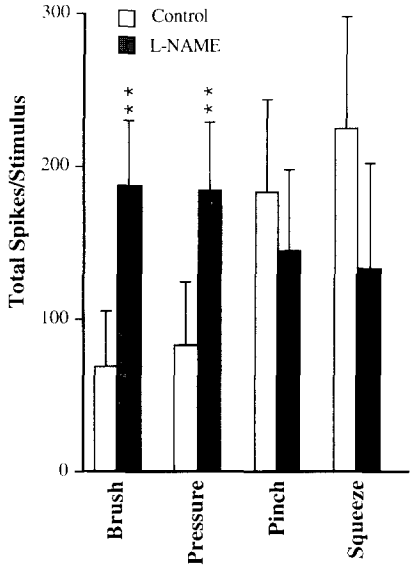

Fig. 6. Summary of the responses of 7 wide dynamic range neurons which showed an increase in responses to innocuous mechanical stimulation of the cutaneous receptive field after application of L-NAME. Bars indicate the mean total spikes per stimulation epochs ± S.E before and after L-NAME application and grouped by individual stimulus types. Significant changes were found by Wilcoxon rank test for pair comparison (* * $P < 0.01$).

4. Discussion

This study demonstrates that alterations in NO availability can significantly change responses of spinal wide dynamic range neurons to iontophoretic application of NMDA and non-NMDA receptor agonists as well as responses to mechanical stimulation of their cutaneous receptive fields. Application of selective NO synthase inhibitors decreased neuronal responsiveness to NMDA in more than half of the cells tested; this was often accompanied by an increase in the non-NMDA responses. Our experiments also revealed notable differences in the time course of effects of N^{ω}-nitro-L-arginine methyl ester versus L-N^{5}-(1-iminoethyl)ornithine application on the neuronal responsiveness to excitatory amino acids. N^{ω}-Nitro-L-arginine methyl ester exerted relatively brief (few minutes-long) effects which were fast in onset and showed uniform

time course for both NMDA- and non-NMDA-evoked responses. In contrast, effects of L-N^5-(1-iminoethyl)ornithine on the responses evoked by excitatory amino acids were slow in onset and usually very long in duration (up to more than an hour). Although L-N^5-(1-iminoethyl)ornithine has been characterized as the most potent NO synthase inhibitor (Radomski et al., 1990; Rees et al., 1990), its slower diffusion from the ejecting pipette to the cell and different kinetics with which it penetrates membranes of intact neurons may account for its slower onset found under our experimental conditions. Finally, the long-lasting effects of L-N^5-(1-iminoethyl)ornithine in our study seem to confirm its characterization as an irreversible inhibitor of NO synthase in phagocytic cells (McCall et al., 1991). The long-lasting effects of the NO donating compound, *S*-nitroso-*N*-penicillamine, which were also slow in onset, were probably due to slow rate of NO release from this compound after its application. This time course is similar to effects that previously have been seen in behavioral studies (Kitto et al., 1992). Moreover, different time course for the effects of changing NO concentrations by application of L-N^5-(1-iminoethyl)ornithine or *S*-nitroso-*N*-penicillamine on the neural responsiveness to NMDA and kainic acid may be explained by differences in the rate, direction or magnitude of NO effects on the NMDA versus non-NMDA classes of excitatory amino acid receptors (Manzoni et al., 1992; Manzoni and Bockaert, 1993; Dev and Morris, 1994).

In approximately one-third of the tested neurons, changes in responses to glutamate receptor agonists were characterized by a decrease in responses to one class of excitatory amino acid receptor agonist (mainly NMDA), and an increase in responses to the other type of excitatory amino acid receptor agonist (mainly non-NMDA). Similar effects have in fact been observed in response to a variety of drugs under a variety of experimental conditions including dissociated spinal cord neurons (Vlachova et al., 1987), cultured retinal horizontal cells (Ishida and Neyton, 1985), hippocampal slices (Palmer et al., 1989) as well as in vivo spinal cord preparations (Budai et al., 1992; Dougherty et al., 1992). These observations were reviewed and the term 'reciprocal changes' was coined by Dougherty et al. (1993). Reciprocal changes in excitatory amino acid responses may represent a switching of the dominant input to a particular cell from one type of glutamate receptor to another. It has been proposed that non-NMDA (AMPA/kainic acid) receptors mediate rapid monosynaptic excitatory neurotransmission whereas NMDA receptors have a special role in use-dependent synaptic plasticity mediating responses of dorsal horn neurons to polysynaptic inputs (Davies, 1988). It is tempting to speculate that NO acts as a retrograde transmitter in spinal dorsal horn neurons such that changes in the post-synaptic production of NO lead to differential alterations in the efficacy of synaptic transmission by changes in the release of excitatory amino acids from primary afferent fibers or in the activities of NMDA and non-NMDA glutamate receptors. Such a hypothesis is consistent with our data in which neuronal excitation evoked by innocuous mechanical stimulation was enhanced while responses to noxious pinch or squeeze were unchanged or significantly reduced in the presence of N^{ω}-nitro-L-arginine methyl ester. It has been shown that NO can, in fact, increase the release of excitatory amino acids from presynaptic terminals (Lawrence and Jarrott, 1993; Guevara-Guzman et al., 1994), inhibit NMDA receptor activity (Manzoni et al., 1992) and increase specific binding at AMPA/kainate receptors (Dev and Morris, 1994).

The link between excitatory amino acid receptor activation and NO-related physiological changes has recently become the subject of intensive research. Activation of excitatory amino acid receptors can lead to the Ca^{2+}-dependent formation of NO which, in turn, can stimulate the enzyme guanylate cyclase and thus trigger cGMP synthesis (Southam et al., 1991; Morris et al., 1994). Differences in rate and magnitude of NO production activated by NMDA- versus non-NMDA-type excitatory amino acid receptor agonists have also been reported. The non-NMDA agonists, AMPA and kainic acid, caused a prompt rise in cGMP in cerebellar slices, whereas an equimolar concentration (100 μM) of NMDA evoked a much slower and smaller increase. In addition, the inhibitory effects of L-arginine analogues on NO production evoked by individual excitatory amino acid agonists may differ greatly. In adult cerebellar slices, L-N^{ω}-monomethyl-L-arginine had an IC_{50} value of about 6 μM against the AMPA-evoked rise in cGMP level; by contrast L-N^{ω}-nitroarginine was more potent ($IC_{50} = 0.6$ μM) against the NMDA-induced cGMP formation (Southam et al., 1991). In primary cultures of mesencephalic neurons, NMDA (EC_{50} 50 μM), kainic acid (EC_{50} 28 μM) or AMPA treatments led to a NO synthase-dependent increase in cGMP accumulation of 164, 202 or 115% of the basal values, respectively (Ambrosini and Racagni, 1993).

Recent studies have shown that NO can have feedback inhibitory effects on its synthesizing enzyme (Assreuy et al., 1993) further contributing to the complexity of NO effects on the efficiency of neurotransmission. A decrease in NO availability following inhibition of NO synthase might lead to a rebound increase in either NO synthase activity or in NMDA receptor-mediated physiological events. A number of reports are consistent with these possibilities. Competitive NO synthase inhibitors inhibit the induction of long-term potentiation, whereas NO donating compounds produce long-term potentiation in the CA1 region of hippocampus (Haley et al., 1992a). Although NO may contribute to long-term potentiation only under certain experimental

circumstances (Bliss and Collingridge, 1993), NO has been found to produce long-lasting increases (long-term potentiation) or decreases (long-term depression) in synaptic transmission depending on the timing and degree of post-synaptic depolarization (for review, see Zorumski and Izumi, 1993). Nitroso compounds that generate NO have been found to inhibit NMDA receptor-mediated responses in rat cortical neurons (Lei et al., 1992). Development of amygdala kindling and behavioral seizures is facilitated by inhibition of NO synthase (Rondouin et al., 1992, 1993; Buisson et al., 1993). By contrast, the facilitatory effects of L-arginine on electrocortical profiles or mean seizure scores following excitatory amino acid microinjection are prevented by inhibition of NO synthase using N^{ω}-nitro-L-arginine methyl ester (Mollace et al., 1991; De Sarro et al., 1993). Although NMDA neurotoxicity was reduced by inhibiting NO synthase (Dawson et al., 1991; Kollegger et al., 1993), this finding was opposed or not confirmed in other studies (Demerle-Pallardy et al., 1991; Lerner-Natoli et al., 1992; Regan et al., 1993). To resolve the apparent paradox, a novel regulatory mechanism of NO on NMDA receptor activity has been proposed: the redox versatility of NO generated upon activation of NMDA receptors allows for its interconversion from neurotoxic to neuroprotective species by a change in the redox milieu (Lipton et al., 1993).

In our present extracellular electrophysiological study, reduction of NMDA-evoked responses after inhibition of NO synthase is largely in agreement with data reported by Radhakrishnan and Henry (1993). Our data also indicate that an increase or decrease in the concentrations of NO can exert reciprocal effects on NMDA versus non-NMDA receptor-mediated physiological events.

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References

Ambrosini, A. and G. Racagni, 1993, Glutamate receptor-induced cyclic GMP formation in primary cultures of mesencephalic neurons, Biochem. Biophys. Res. Commun. 193, 1098.

Assreuy, J., F.Q. Cunha, F.Y. Liew and S. Moncada, 1993, Feedback inhibition of nitric oxide synthase activity by nitric oxide, Br. J. Pharmacol. 108, 833.

Baringa, M., 1991, Is nitric oxide the 'retrograde messenger'?, Science 254, 1296.

Bliss, T.V.P. and G.L. Collingridge, 1993, A synaptic model of memory: long-term potentiation in the hippocampus, Nature 361, 31.

Bredt, D.S. and S.H. Snyder, 1992, Nitric oxide, a novel neuronal messenger, Neuron 8, 3.

Budai, D., 1994, A computer-controlled system for post-stimulus time histogram and wind-up studies, J. Neurosci. Methods 51, 205.

Budai, D., G.L. Wilcox and A.A. Larson, 1992, Modulation of *N*-methyl-D-aspartate and (*R*,*S*)-alpha-amino-3-hydroxy-5- methylisoxazole-4-propionate (AMPA) responses of spinal nociceptive neurons by a N-terminal fragment of substance P, Eur. J. Pharmacol. 216, 441.

Buisson, A., N. Lakhmeche, C. Verrecchia, M. Plotkine and R.G. Boulu, 1993, Nitric oxide: an endogenous anticonvulsant substance, NeuroReport 4, 444.

Davies, J., 1988, A reappraisal of the role of NMDA and non-NMDA receptors in neurotransmission in the cat dorsal horn, in: Frontiers in Excitatory Amino Acid Research, eds. E.A. Cavalhiero, J. Lehman and L. Turski (Alan R. Liss, New York) p. 355.

Dawson, V.L., T.M. Dawson, E.D. London, D.S. Bredt and S.H. Snyder, 1991, Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures, Proc. Natl. Acad. Sci. USA 88, 6368.

Demerle-Pallardy, C., M.O. Lonchampt, P.E. Chabrier and P. Braquet, 1991, Absence of implication of L-arginine/nitric oxide pathway on neuronal cell injury induced by L-glutamate or hypoxia, Biochem. Biophys. Res. Commun. 181, 456.

De Sarro, G., E.D. Di Paola, A. De Sarro and M.J. Vidal, 1993, L-Arginine potentiates excitatory amino acid-induced seizures elicited in the deep prepiriform cortex, Eur. J. Pharmacol. 230, 151.

Dev, K.K. and B.J. Morris, 1994, Modulation of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) binding sites by nitric oxide, J. Neurochem. 63, 946.

Dougherty, P.M., K.A. Sluka, L.S. Sorkin, K.N. Westlund and J.D. Willis, 1992, Enhanced responses of spinothalamic tract neurons to excitatory amino acids parallel the generation of acute arthritis in monkey, Brain Res. Rev. 17, 1.

Dougherty, P.M., J. Palacek, S. Zorn and J.D. Willis, 1993, Combined application of excitatory amino acids and substance P produces long-lasting changes in responses of primate spinothalamic tract neurons, Brain Res. Rev. 18, 227.

Garthwaite, J., S.L. Charles and R. Chess-Williams, 1988, Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain, Nature 336, 385.

Guevara-Guzman, R., P.C. Emson and K.M. Kendrick, 1994, Modulation of in vivo striatal transmitter release by nitric oxide and cyclic GMP, J. Neurochem. 62, 807.

Haley, J.E., G.L. Wilcox and P.F. Chapman, 1992a, The role of nitric oxide in hippocampal long-term potentiation, Neuron 8, 211.

Haley, J.E., A.H. Dickenson and M. Schachter, 1992b, Electrophysiological evidence for a role of nitric oxide in prolonged chemical nociception in the rat, Neuropharmacology 31, 251.

Ishida, A.T. and J. Neyton, 1985, Quisqualare and L-glutamate inhibit retinal horizontal cell responses to kainate, Proc. Natl. Acad. Sci. USA 82, 1837.

Kitto, K.F., J.E. Haley and G.L. Wilcox, 1992, Involvement of nitric oxide in spinally mediated hyperalgesia in the mouse, Neurosci. Lett. 148, 1.

Kollegger, H., G.J. McBean and K.F. Tipton, 1993, Reduction of striatal *N*-methyl-D-aspartate toxicity by inhibition of nitric oxide synthase, Biochem. Pharmacol. 45, 260.

Lawrence, A.J. and B. Jarrott, 1993, Nitric oxide increases interstitial excitatory amino acid release in the rat dorsomedial medulla oblongata, Neurosci. Lett. 151, 126.

Lee, J.H., R.H. Price, F.G. Williams, B. Mayer and A.J. Beitz, 1993, Nitric oxide synthase is found in some spinothalamic neurons and in neuronal processes that appose spinal neurons that express Fos induced by noxious stimulation, Brain Res. 608, 324.

Lei, S.Z., Z.-H. Pan, S.K. Aggarwal, H.-S.V. Chen, J. Hartman, N.J. Sucher and S.A. Lipton, 1992, Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex, Neuron 8, 1087.

Lerner-Natoli, M., G. Rondouin, B.F. De and J. Bockaert, 1992, Chronic NO synthase inhibition fails to protect hippocampal neurones against NMDA toxicity, NeuroReport 3, 1109.

Lipton, S.A., Y.-B. Choi, Z.-H. Pan, S.Z. Lei, H.-S.V. Chen, N.J. Sucher, J. Loscalzo, D.J. Singel and J.S. Stamler, 1993, A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso compounds, Nature 364, 626.

Manzoni, O. and J. Bockaert, 1993, Nitric oxide synthase activity endogenously modulates NMDA receptors, J. Neurochem. 61, 368.

Manzoni, O., L. Prezeau, P. Marin, S. Deshager, J. Bockaert and L. Fagni, 1992, Nitric oxide-induced blockade of NMDA receptors, Neuron 8, 653.

McCall, T.B., M. Feelisch, R.M. Palmer and S. Moncada, 1991, Identification of *N*-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells, Br. J. Pharmacol. 102, 234.

Meller, S.T. and G.F. Gebhart, 1993, Nitric oxide (NO) and nociceptive processing in the spinal cord, Pain 52, 127.

Meller, S.T., C. Dykstra and G.F. Gebhart, 1992a, Production of endogenous nitric oxide and activation of soluble guanylate cyclase are required for *N*-methyl-D-aspartate-produced facilitation of the nociceptive tail-flick reflex, Eur. J. Pharmacol. 214, 93.

Meller, S.T., P.S. Pechman, G.F. Gebhart and T.J. Maves, 1992b, Nitric oxide mediates the thermal hyperalgasia produced in a model of neurpathic pain in the rat, Neuroscience 50, 7.

Meller, S.T., C.P. Cummings, R.J. Traub and G.F. Gebhart, 1994, The role of nitric oxide in the development and maintenance of the hyperalgesia produced by intraplantar injection of carrageenan in the rat, Neuroscience 60, 367.

Mollace, V., G. Bagetta and G. Nistico, 1991, Evidence that L-arginine possesses proconvulsant effects mediated through nitric oxide, NeuroReport 2, 269.

Moore, P.K., A.O. Oluyomi, R.C. Babbedge, P. Wallace and S.L. Hart, 1991, L-N^G-Nitro arginine methyl ester exhibits antinociceptive activity in the mouse, Br. J. Pharmacol. 102, 198.

Morris, R., E. Southam, S.R. Gittins, J. Devente and J. Garthwaite, 1994, The NO-cGMP pathway in neonatal rat dorsal horn, Eur. J. Neurosci. 6, 876.

Palmer, E., D.T. Monaghan and C.W. Cotman, 1989, Glutamate receptors and phosphoinositide metabolism: stimulation via quisqualate receptors is inhibited by *N*-methyl-D-aspartate receptor activation, Mol. Brain Res. 4, 161.

Radhakrishnan, V. and J.L. Henry, 1993, L-NAME blocks responses to NMDA, Substance-P and noxious cutaneous stimuli in cat dorsal horn, NeuroReport 4, 323.

Radomski, M.W., R.M. Palmer and S. Moncada, 1990, Characterization of the L-arginine:nitric oxide pathway in human platelets, Br. J. Pharmacol. 101, 325.

Rees, D.D., R.M. Palmer, R. Schulz, H.F. Hodson and S. Moncada, 1990, Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo, Br. J. Pharmacol. 101, 746.

Regan, R.F., K.E. Renn and S.C. Panter, 1993, NMDA neurotoxicity in murine cortical cell cultures is not attenuated by hemoglobin or inhibition of nitric oxide synthesis, Neurosci. Lett. 153, 53.

Rondouin, G., M. Lerner-Natoli, O. Manzoni, M. Lafon-Cazal and J. Bockaert, 1992, A nitric oxide (NO) synthase inhibitor accelerates amygdala kindling, NeuroReport 3, 805.

Rondouin, G., J. Bockaert and M. Lerner-Natoli, 1993, L-Nitroarginine, an inhibitor of NO synthase, dramatically worsens limbic epilepsy in rats, NeuroReport 4, 1187.

Schuman, E.M. and D.V. Madison, 1994, Locally distributed synaptic potentiation in the hippocampus, Science 263, 532.

Snyder, S.H., 1992, Nitric oxide: first in a new class of neurotransmitters?, Science 257, 494.

Southam, E., S.J. East and J. Garthwaite, 1991, Excitatory amino acid receptors coupled to the nitric oxide/cyclic GMP pathway in rat cerebellum during development, J. Neurochem. 56, 2072.

Valtschanoff, J.G., R.J. Weinberg, A. Rustioni and H.H. Schmidt, 1992, Nitric oxide synthase and GABA colocalize in lamina II of rat spinal cord, Neurosci. Lett. 148, 6.

Vlachova, V., L. Vyklicky, L. Vyklicky, Jr. and F. Vyskocil, 1987, The action of excitatory amino acids on chick spinal cord neurones in culture, J. Physiol. (London) 386, 425.

Wilcox, G.L., 1991, Excitatory neurotransmitters and pain, in: Proceedings of the VIth World Congress on Pain, eds. M.R. Bond, J.E. Charlton and C.J. Woolf (Elsevier Science Publishers, New York) p. 97.

Wilcox, G.L., 1993, Spinal mediators of nociceptive neurotransmission and hyperalgesia. Relationships among synaptic plasticity, analgesic tolerance, and blood flow, APS J. 2, 265.

Zorumski, C.F. and Y. Izumi, 1993, Nitric oxide and hippocampal synaptic plasticity, Biochem. Pharmacol. 46, 777.