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Delayed stress-induced antinociceptive effect of nitric oxide synthase inhibition in the dentate gyrus of rats

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Abstract

Stimulation of the hippocampal formation can modulate nociceptive mechanisms, whereas painful stimuli can activate this structure. Stress exposure can produce plastic changes in the hippocampus. Nitric oxide (NO) is an important neuroregulatory agent present in the hippocampus. The objective of the present study was to investigate the effects of intrahippocampal administration of N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), an inhibitor of NO synthase (NOS), on nociceptive processes in stressed and nonstressed rats. Male Wistar rats ($n = 6–11$/group) received unilateral microinjection of L-NAME (50–300 nmol/0.2 μl) into the dentate gyrus (DG) of the dorsal hippocampus. Immediately after the injection tail-flick reflex latency was measured. Stressed animals were submitted to 2 h of restraint and tested immediately or 1, 2, 5 or 10 days later. L-NAME failed to modify nociception in nonstressed rats. However, 5 days after, restraint L-NAME, at all doses tested, produced an antinociceptive effect (ANOVA, $P < .05$). The dose–response curve had an inverted U shape. L-NAME antinociceptive effect was antagonized by previous treatment with L-arginine (150 nmol/0.2 μl, $P < .05$). The results suggest that the modulation of nociceptive processes by NO in the dorsal hippocampus is dependent on previous stress exposure and on poststress interval.

Keywords: Antinociception; Hippocampus; Tail-flick; Restraint stress; Analgesia; Nitric oxide; Stress-induced analgesia

1. Introduction

Spinal pathways to limbic and cortico-limbic structures provide direct inputs to brain areas involved in affective aspects of pain and integrate nociceptive input with contextual information and memory (Price, 2000). Physiological adaptive mechanisms in limbic regions are probably involved in the decrease of pain responsiveness following exposure to noxious and non-noxious stressors, usually referred to as stress-induced analgesia (SIA, Madden et al., 1977; Bodnar et al., 1980; Amit and Galina, 1988; Jorum, 1988; Bechara et al., 1997; Ploghaus et al., 2000).

The hippocampus, although one of the main structure of the limbic system, is usually not recognized as an important region in pain perception (Melzack and Casey, 1968). However, electrical stimulation of guinea pig dorsal hippocampus elicited painful expression (Lico et al., 1974) and evokes painful sensations in humans (Delgado, 1955; Halgren et al., 1978). Partial hippocampectomy can be used for chronic pain treatment (Gol and Faibish, 1967). Moreover, antinociceptive effects were found after lidocaine microinjection (McKenna and Melzack, 1992), or electrical (Prado and Roberts, 1985) or chemical (Klamt and Prado, 1991) stimulation of the hippocampal formation. Finally, nociceptive stimuli modify the electrical activity of the hippocampus (Khanna and Sinclair, 1989, 1992; Khanna and Zheng, 1999) and are able to induce c-Fos expression in this structure (Ceccarelli et al., 1999).

The original finding that severe stress can produce plastic and functional changes of the hippocampus was reported by

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Foy et al. (1987) and confirmed by several studies (McEwen, 1999; Sapolsky, 2000; Xu et al., 1997; Shors and Dryver, 1994). Glutamate-mediated neurotransmission may play an important role on both pain perception and hippocampal stress-induced changes. For example, NMDA receptor antagonist prevents the restraint-induced increase of immediate early genes expression in this region (Titze-de-Almeida et al., 1994; Lino de Oliveira et al., 1997) and, when injected into the dentate gyrus (DG), show antinociceptive effects (McKenna and Melzack, 2001).

Production of nitric oxide (NO) is one of the transduction mechanism of NMDA receptor-mediated neurotransmission (Garthwaite, 1991). NO is a short-lived, highly reactive molecule (Garthwaite, 1991; Moncada et al., 1994), synthesized from L-arginine by the enzymes nitric oxide synthases (NOS; Bredt and Snyder, 1990). The constitutive, cytosolic, Ca^{2+}/calmodulin-dependent neuronal enzyme occurs only in neuronal cell bodies, dendrites and axons (Bredt and Snyder, 1990), with a discrete localization in several brain structures, including the hippocampus (Barjavel and Bhargava, 1995; Valtschanoff et al., 1993; Wendland et al., 1994).

NO has been implicated in many physiological and pathological brain processes including hippocampal responses to stress (Reagan et al., 1999; Radomski et al., 1990; Weber et al., 1994; Haulica et al., 1995), SIA (Spinella and Bodnar, 1994) and nociception (Haley et al., 1992; Kolesnikov et al., 1993; Babbedge et al., 1993a,b). Concerning the latter, however, there are controversial reports on its role. Although antinociceptive effects of intracerebroventricular injection of NOS inhibitors have been shown by several groups (Moore et al., 1991; Shibuta et al., 1995; Babbedge et al., 1993a,b) both antinociceptive and nociceptive effects of L-arginine have been described (Kawabata et al., 1993, 1994; Zhuo et al., 1993; Kumar et al., 1993; Iwamoto and Marion, 1994; Haulica et al., 1995). Moreover, the possible brain sites of the modulatory effect of NO on nociception are largely unknown.

The objective of the present study was to investigate if the NO system in the hippocampal formation could interfere with nociceptive processes. In addition, considering the influence of stressful stimuli on both hippocampal function and nociception, a possible influence of restraint stress exposure on this interference was also investigated.

2. Materials and methods

2.1. Animals

Male Wistar rats weighting 200–220 g were kept in a temperature controlled room (23 °C) with a 12-h light/dark cycle (lights on at 7:00 a.m.) and with free access to water and food. The experiments were carried out according to the Brazilian Society of Neuroscience and Behavior guidelines that are in compliance with National Institutes of Health Guide for care and use of laboratory animals. All efforts were made to minimize animal suffering. Experiments were performed between 8:00 and 12:00 a.m. A white noise generator provided a constant background noise and the apparatus was cleaned and dried before each session with 70% ethanol to minimize olfactory cues.

2.2. Drugs

N^G-nitro-L-arginine methylester (L-NAME, Sigma, 50–300 nmol) and L-arginine (Sigma, 150 nmol) were diluted in sterile saline. The doses were selected based on a previous study showing anxiolytic effects of intracerebral administration of L-NAME into the dorsal periaquedutal grey matter (Guimarães et al., 1994).

2.3. Stereotaxic surgery

Animals were anaesthetized with 2.5% 2,2,2-tribromoethanol (10 ml/kg ip) and fixed on a stereotaxic frame (David-Kopf) with the incisor bar 5 mm above the interaural line. A stainless steel guide cannula (O.D. 0.7 mm) was implanted unilaterally into the DG of the hippocampal formation (coordinates: A: −4.0 mm posterior to bregma, L: 2.8 mm, D: 2.1 mm; Paxinos and Watson, 1986). The cannula tip was 1.5 mm above the injection site and was attached to the bone with stainless steel screws and acrylic cement. To control for the possibility that the drug may have seeped into the ventricles and produced its effects at distal sites four animals had their cannulas implanted into the lateral ventricle.

2.4. Microinjection

Microinjections were performed with a Hamilton microsyringe (US) in a volume of 0.2 μl. Rats are allowed to freely move within a $20 \times 24 \times 45$ cm box during the microinjection. The solutions were injected slowly (over 1 min) and the cannulas were left in place for an additional 120 s to prevent reflux. The movement of an air bubble inside the PE-10 polyethylene tubing connecting the microsyringe with the dental needle confirmed drug flow. The microinjections were carried out in the same room of the behavioral tests.

2.5. Tail-flick test

Antinociception was assessed using the tail-flick test (TFT). Each rat was placed in a ventilated glass tube with the tail laid across a nickel-chrome wire coil maintained at room temperature (23 ± 2 °C). The coil temperature was then raised at the rate of 9 °C/s by the passage of electric current, which was adjusted to ensure a tail withdrawal reflex within 2.5–3.5 s. A cut-off time of 6 s was established to minimize the probability of skin damage. Animals were tested every 5 min for 15 min until stable baseline tail-flick latency (BTFL) was obtained over three

consecutive trials. Antinociception measurements were taken before and after drug treatment. The drug effect was assessed immediately after the injection and every 5 min since for 30 min. Tail flick latencies (TFL) were also measured before and after stress in animals submitted to restraint. Each TFL was normalized by an Index of Antinociception (IA) using the formula: IA=(TFL − average BTFL)/[6 − (average BTFL)].

2.6. Restraint stress procedure

Rats were submitted to 2 h of forced restraint in a semicylindrical wire cage (25 × 7 × 5 cm) with an adjustable roof and perforations to allow ventilation.

2.7. Experimental procedure

The animals were handled daily before the experimental procedures. Seven to ten days after surgery, they were randomly assigned to experimental groups. Three independent experiments were carried out in a sound attenuated, temperature-controlled room, illuminated with two 40-W fluorescent lights placed 1.3 m away from the animal.

2.7.1. Experiment 1

Animals ($n = 6–9$/group) were submitted to the 2-h restraint session or remained undisturbed in their home cage for the same period. They were tested immediately or 1, 2, 5 or 10 days after restraint. Independent groups were used at each assessment point. BTFL were measured before the injection of saline (0.2 μl) or L-NAME (150 nmol/0.2 μl) and TFL measures were immediately repeated.

2.7.2. Experiment 2

The procedure was similar to that described in the previous experiment. All animals ($n = 7–11$/group), however, were submitted to restraint stress and tested 5 days later. Three doses (50, 150 or 300 nmol) of L-NAME were tested.

2.7.3. Experiment 3

The procedure was similar to that described in Experiment 1. Animals ($n = 8–9$/group) were submitted to restraint stress and tested 5 days later. However, 30 min before the administration into the DG of saline or L-NAME (150 nmol), they were pretreated with saline (0.2 μl) or L-arginine (150 nmol). The TFT was performed immediately after the last DG microinjection. In all experiments, control and treated animals were evaluated in parallel.

2.8. Histology

After the experimental procedures, animals were deeply anesthetized with urethane 25% (0.4 ml/0.1 kg) and received a 0.2-μl microinjection of Evans Blue into the DG. The brains were removed and stored in 10% formalin solution during 4 days. Cannula placement was verified in 40-μm coronal sections obtained in a cryostat (Leika CM 1850). The slides were stained with Nissl and histological localization was performed blindly to the treatment condition. Data were included in the statistical analysis only if the dye was visible in the target area, there was no evidence of drug leakage to other brain regions and there was no significant necrosis along the cannula track.

3. Statistical analysis

The IA data were analyzed by a repeated measures multivariate analysis of variance (MANOVA), factors being treatment, time and session (in case of measures taken to verify the effects of restraint). In case of significant interactions, one-way ANOVA was performed at each time. The Duncan test was used for multiple comparisons. A repeated measure MANOVA was also employed to compare non-normalized TFL obtained at baseline measurements. The significant level was set at $P < .05$.

4. Results

Fifty-three subject's injection sites were localized in areas outside of the DG. These included the cortex ($n = 26$), CA1 ($n = 4$), CA2 ($n = 11$), CA3 ($n = 12$) and thalamus ($n = 2$). Results from these animals, as well as from those that received the drug into the lateral ventricle ($n = 4$), did not show any difference between groups (data not shown). These results were excluded from subsequent analysis. A representative photomicrograph of an injection site is shown in Fig. 1.

4.1. Experiment 1

The restraint stress procedure induced a significant increase in TFL [$F(1,79) = 5.84$, $P = .018$; Fig. 2]. No difference in baseline measurements was found between treatment groups [$F(3,138) = 1.75$, $P = .16$]. L-NAME produced a significant increase in IA 5 days after restraint [$F(3,27) = 13.39$, $P = .039$; Duncan, $P < .05$; Fig. 3]. No significant drug effect was found at any other poststress interval [$F(3,28)$ ranging from 0.29 to 0.90, $P > .05$]. A significant overall time effect was found in the measurements taken 1, 2, 5 or 10 days after restraint [$F(6,28)$ ranging from 2.5 to 4.16, $P < .05$], indicating that the IA decreased along the session.

4.2. Experiment 2

A significant increase in IA was produced by all doses of L-NAME [$F(3,30) = 14.05$, $P < .001$; Duncan test, $P < .05$; Fig. 4]. The dose–response curve, however, had an inverted U-shape, with both the lower and higher doses producing

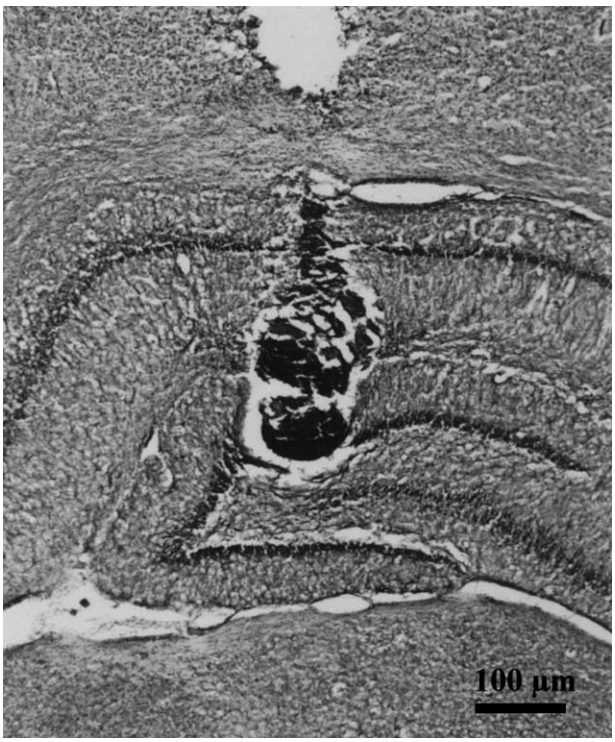

Fig. 1. Photomicrography of an injection site into the dentate gyrus of the dorsal hippocampus.

significant smaller effects than the 150-nmol dose (Duncan test, $P<.05$). No significant time effect [$F(6,25)=1.4$] or drug vs. time interaction [$F(18,71)=0.84$] was found.

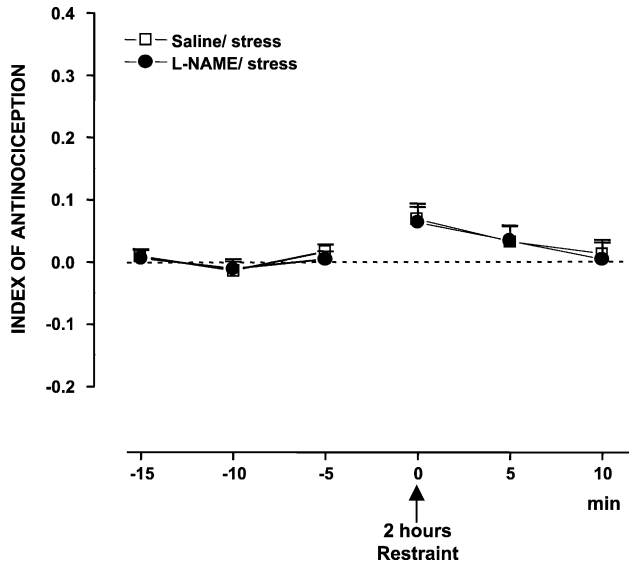

Fig. 2. Effect of restraint stress on TFL. Seven days after surgery, animals ($n=40-41$/group) with cannulas aimed at the dentate gyrus were submitted to 2 h of forced restraint. Pre- and postrestraint TFL were evaluated at 5-min intervals. Results are expressed as index of antinociception [IA= (TFL − average baseline TFL)/6 − average TFL]. Points and bars represent the means ± S.E.M. The IA results after restraint were significant higher than before (ANOVA, $P<.05$).

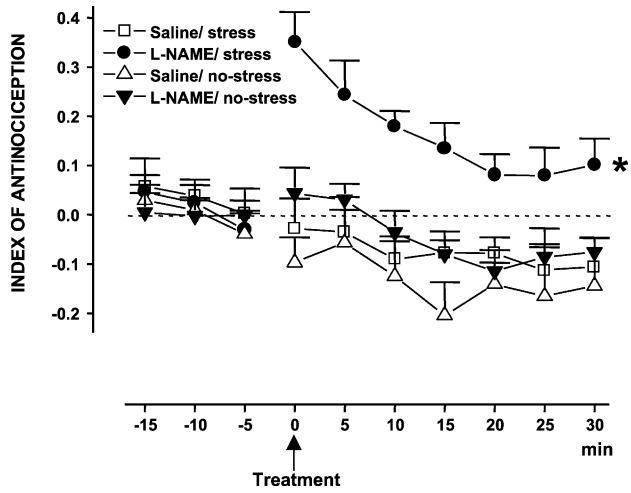

Fig. 3. Effects of L-NAME on TFL after restraint stress. Animals ($n=6-9$/group) with cannulas aimed at the dentate gyrus were submitted to 2 h of forced restraint or left undisturbed in their home cage (no-stress) and tested 5 days later. After pretreatment (baseline) measurements, they received intrahippocampal injection of saline (0.2 µl) or L-NAME (150 nmol) and TFL was evaluated immediately after at 5-min intervals. * Indicates an overall significant difference from all the other groups (Duncan test, $P<.05$). Further specifications as in Fig. 2.

4.3. Experiment 3

The significant increase in IA induced by L-NAME [150 nmol, $F(3,29)=35.31$, $P<.001$] was prevented by pretreatment with L-arginine (Duncan test, $P>.05$; Fig. 5). No

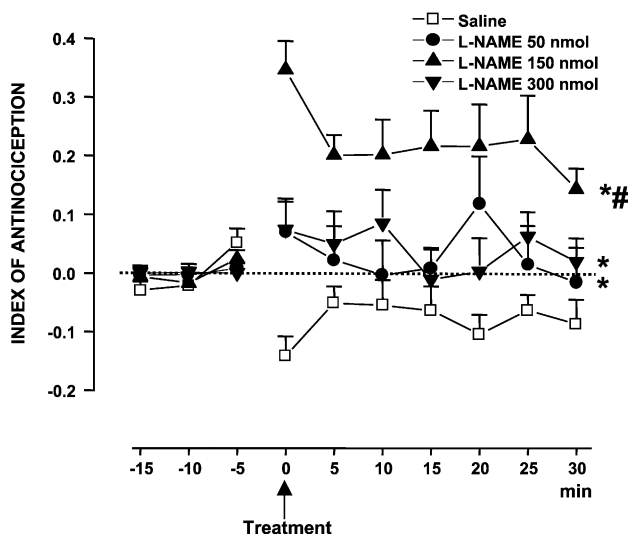

Fig. 4. Effects of different doses of L-NAME on TFL after restraint stress. Animals ($n=7-11$/group) with cannulas aimed at the dentate gyrus were submitted to 2 h of forced restraint and tested 5 days later. After pretreatment (baseline) measurements, they received intrahippocampal injection of saline (0.2 µl) or L-NAME (50–300 nmol) and TFL was evaluated immediately after at 5-min interval. #Significant overall difference from all the other groups (Duncan test, $P<.05$). * Significant difference from saline (Duncan test, $P<.05$). Further specifications as in Fig. 2.

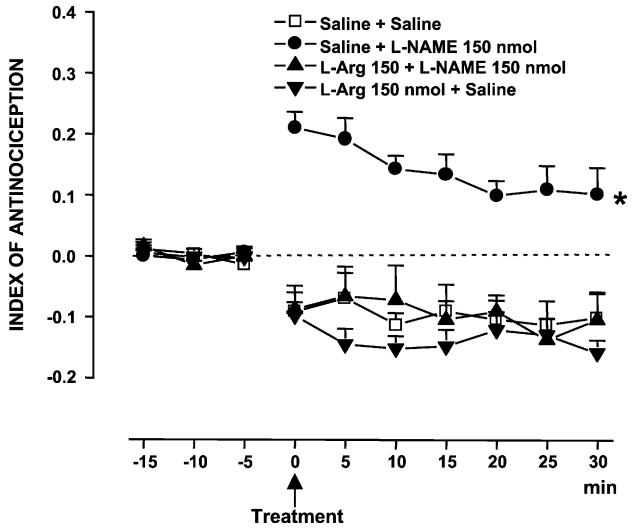

Fig. 5. Effects of L-arginine (L-Arg) (150 nmol) on TFL changes induced by L-NAME after restraint stress. Animals ($n = 8-9$/group) with cannulas aimed at the dentate gyrus were submitted to 2 h of forced restraint and tested 5 days later. After pretreatment (baseline) measurements, they received intrahippocampal injection of saline (0.2 μl) or L-arginine (150 nmol) followed, 30 min later, by a second microinjection of saline or L-NAME (150 nmol). TFL was evaluated immediately after the last injection at 5-min interval. Further specifications as in Fig. 2. * Indicates an overall significant difference from all the other groups (Duncan test, $P < .05$).

significant difference between any other groups was found (Duncan test, $P > .05$). There was no significant time [$F(6,24) = 1.2$] or drug vs. time [$F(18,68) = 0.9$] effects.

5. Discussion

In the present study, a small but significant antinociceptive effect was induced by restraint exposure. Similar results have been shown by several studies, but the magnitude of the SIA varies greatly among them (Madden et al., 1977; Maier, 1986; Calgnetti and Holzman, 1990). This may depend on different stressor procedures used. For example, repeated exposure to cold water swims has been shown to interfere with SIA (Bodnar and Komisaruk, 1984). A factor that could help to explain the small antinociceptive effect of restraint observed in our study is stress duration (Grau et al., 1981). Corroborating this possibility Calgnetti and Holzman (1990) found that increasing the immobilization period from 1 to 6 h produced tolerance to the potentiation of morphine analgesia induced by restraint stress. Depletion of neurotransmitters release by stress (Calgnetti and Holzman, 1990) or interaction between opioid and non-opioid analgesic systems (Bodnar, 1986) could help to explain this finding.

Intrahippocampal administration of L-NAME in non-stressed animals failed to produce any antinociceptive effect in the TFT. Moreover, even considering the small antinociceptive effect of restraint in our study no interaction between L-NAME and restraint was found immediately or up to 2 days after stress. A clear antinociceptive drug effect was obtained 5 days after restraint. This result was replicated in three independent experiments. The effect was probably not due to L-NAME diffusion to other parts of the hippocampal formation, since injections into the CA region did not have any effect. The same applies for the overlying cortex or some more distal sites. These results do not preclude, however, the possibility that L-NAME applied over a larger extension of the CA region, the cerebral cortex or the thalamus could modulate pain behavior. The regional specificity of L-NAME effect in the DG may be explained by the funneling of cortical signals trough this region (McKenna and Melzack, 2001), which would allow a discrete drug injection to interfere with a relatively larger portion of the hippocampal formation.

The results show, therefore, that stress exposure, in addition to causing analgesia by itself, can also interact with antinociceptive drug effects. Similar results have been shown in other studies. For example, restraint stress can potentiate, both immediately or 1 week later, morphine analgesia (Calgnetti and Holzman, 1990) whereas NOS inhibition can potentiate swim stress antinociception (Spinella and Bodnar, 1994). As a main component of the "limbic system" the hippocampus is proposed to play an important role on both the affective component of pain perception (McKenna and Melzack, 2001) and on the behavioral responses to stress (McEwen, 1999).

Initial NOS inhibition by L-NAME is competitive with L-arginine and the extend of inhibition is diminished by this amino acid (Griffith and Stuehr, 1995; Dawson et al., 1991). Therefore, our results showing that L-NAME effect is prevented by L-arginine pretreatment suggest that the drug is probably acting by inhibition of NO formation (Moncada et al., 1991; Griffith and Stuehr, 1995).

Although antinociceptive effects of L-NAME were observed in all doses tested (50–300 nmol), the dose–response curve had an inverted U-shape. Similar bell shaped curves have been described with NOS inhibitors in several studies, involving either systemic (Harkin et al., 1999; Volke et al., 1995) or intracerebral injections (Guimarães et al., 1994). The reason for such effect is not known. The effective doses of L-NAME used in this study were compatible with NOS inhibitory potency (Ayers et al., 1997; Salter et al., 1995) and were similar to those used in other studies with intracerebral injection (Guimarães et al., 1994). NO effects may depend on several factors such as the functional state of the target neurons and the instant composition of the extracellular fluid. In addition, due to its very high liposolubility NO can modify the function of neurons located up to hundreds of microns from its origin. Small changes in local NO concentration, therefore, could be a key factor in determining its biological effect (Contestabile, 2000).

In Experiment 1, there was a significant general time effect, indicating a decrease in TFL along the session. Paradoxical decrease in response latencies with repeated stress exposition (Madden et al., 1977) or holding stress (Vidal and Jacob, 1986) has been previously described. The latter effect was attributed to anxiety occurring in the absence of knowledge regarding forthcoming events (Vidal and Jacob, 1986). Similar mechanisms could be related to our findings since exposure to restraint stress has been shown to increase anxiety in rats tested 24 or 48 h later (Padovan and Guimarães, 2000).

One can only speculate on possible mechanisms for the delayed stress-potentiation effect of L-NAME in the hippocampus. The intensity and temporal pattern of the stressor are proposed to be critical factors determining the nature of SIA (Vidal and Jacob, 1982a,b) and delayed stress influence on nociception has already been reported (Caggiula et al., 1989; Calgnetti and Holzman, 1990). Exposure to severe stressors can result on long-term structural and functional modifications of the brain, the hippocampal formation being especially sensitive to such effects (McEwen, 1999). In this structure, restraint stress has been found to produce ultrastructural and molecular changes, long-lasting increases in synaptic efficacy (McEwen and Magarinos, 1997; Shorts et al., 1997; Xu et al., 1997) and modification of corticotrophin-releasing hormone (Givalois et al., 2000) and glucocorticoid receptor mRNAs expression (Paskitti et al., 2000). Stress-induced plastic changes of neuronal NOS have also been described. A significant up-regulation of nNOS mRNA was described after restraint stress in the paraventricular nucleus of the hypothalamus, medial amygdala and dorsal periaqueductal gray (de Oliveira et al., 2000). Although no hippocampal nNOS expression change was found, in this study the animals were sacrificed only 24 h after restraint. Longer intervals might be needed to detect changes in this region. This possibility remains to be tested.

In summary, the present data suggest that NO-sensitive mechanisms in the hippocampal formation may modulate noxious sensory processing depending on previous stress stimulation and on poststress interval. The mechanisms underlying the present findings are not clear but may represent a physiological adaptive mechanism in a brain structure subserving affective motivational response to pain.

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