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Effect of N$^{\omega}$-nitro-L-arginine methyl ester, a nitric oxide synthesis inhibitor, on stress- and morphine-induced prolactin release in male rats

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1 The effect of the nitric oxide synthesis inhibitor N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) was investigated on stress- and morphine-induced prolactin (PRL) secretion *in vivo* in male rats, by use of a stress-free blood sampling and drug administration method by means of a permanent indwelling catheter in the right jugular vein.

2 Three doses of L-NAME were tested (1, 10 and 30 mg kg^{-1}) and were given intraperitoneally one hour before blood sampling; control rats received saline. After the first blood sample, rats received an initial intravenous injection of morphine (3, 6 or 12 mg kg^{-1}) or were subjected to immobilization stress. In the case of a morphine administration, rats received a second dose of morphine (3, 6 or 6 mg kg^{-1}, respectively) 90 min later, when tolerance had developed, while rats subjected to immobilization stress received 6 mg kg^{-1} morphine 90 min after onset of stress.

3 L-NAME had no effect on basal plasma PRL concentration. However, it potentiated acute morphine-induced PRL secretion and attenuated the subsequent tolerance in a dose-dependent way. Immobilization stress-induced PRL secretion was inhibited dose-dependently by L-NAME, as was its subsequent tolerance to morphine; however, in this case, in a reversed dose-dependent way.

4 When the highest dose of morphine (12 mg kg^{-1}) was combined with the highest dose of L-NAME pretreatment (30 mg kg^{-1}), all rats showed a dramatic potentiation of the morphine-induced PRL rise compared to controls. Moreover, all of these rats died within 90 min after the administration of morphine.

5 These results show that NO plays a role in the acute opioid action on PRL release during stress as well as in the development of tolerance to the opioid effect, and some possible mechanisms are discussed.

Keywords: N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME); morphine; opioid; opiate; stress; prolactin (PRL); nitric oxide (NO)

Introduction

Repeated administration of opiates leads to tolerance for most of the opiate effects, including the prolactin (PRL) rising capacity. Previously we showed that while a first administration of morphine induces a rise of plasma PRL concentration, a second administration was already subject to tolerance (Matton *et al*., 1991). The same accounts for immobilization stress which induces a naloxone-reversible rise in plasma PRL concentration (Bruni *et al*., 1977; Van Vugt *et al*., 1978; Siegel *et al*., 1982; Samson *et al*., 1985) and shows cross-tolerance with μ-opiates, but not with κ-opiates (Matton *et al*., 1991). It is thought that the effects of immobilization stress are mediated by endogenous opioids and more specifically by β-endorphin (Van Vugt *et al*., 1978; Ragavan & Frantz, 1981).

The exact mechanism of opioid action and opioid tolerance is still unclear. Several studies implicate a role for excitatory amino acids (EAA) in opioid action, both after acute immobilization stress (Tocco *et al*., 1991) and in tolerance and withdrawal conditions after morphine administration (Akaoka & Aston-Jones, 1991; Aghajanian *et al*., 1994). More specifically the N-methyl-D-aspartate (NMDA) type of glutamate receptor might be involved, since competitive as well as non-competitive NMDA-antagonists can prevent, attenuate or block the opiate tolerance to morphine analgesia (Marek *et al*., 1991; Trujillo & Akil, 1991; Ben-Eliyahu, 1992; Tiseo & Inturrisi, 1993). Voltammetric studies *in vivo* confirmed the findings of the analgesic studies (Hong *et al*., 1993).

Since NMDA-receptor stimulation by endogenous glutamate can result in the synthesis of nitric oxide (NO), a novel neural messenger molecule (for review see Garthwaite, 1991), inhibitors of this NO synthesis might be able to modulate in their turn the development of opioid tolerance. Indeed, some analgesia studies in rat and mice (Kolesnikov *et al*., 1992; 1993; Thorat *et al*., 1993) confirmed that inhibitors of NO synthesis prevent or attenuate tolerance to morphine-induced analgesia in doses that had no analgesic effect on their own.

The aim of this study was to investigate whether an inhibitor of NO synthesis could also modulate the PRL release inducing effect of opiates. Thus experiments were performed *in vivo* in male rats to see whether the inhibitor of NO synthesis, N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) could influence morphine- or stress-induced PRL secretion in acute as well as tolerance conditions.

Methods

Animals

The experiments were performed on adult male Wistar rats weighing 250 to 300 g (KUL, Leuven, Belgium). The animals were housed individually in wire-bottomed cages and food and water were freely available. Lights were on from 7 h 00 min to 19 h 00 min and the room temperature was constant at 22°C. All blood sampling was performed between 13 h 00 min and 16 h 00 min.

Surgery

For i.v. drug administration and serial blood sampling, a permanent silicone elastomer catheter was implanted in the right jugular vein, after anaesthesia had been induced with sodium pentobarbitone (60 mg kg^{-1}, intraperitoneally), as described previously by Harms & Ojeda (1974).

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Pharmacological agents

L-NAME was purchased from Sigma Chemie (Bornem, Belgium). Morphine-HCl was purchased from Belgopia (Louvain-La-Neuve, Belgium). All solutions were made in physiological saline (0.9% NaCl solution).

Experimental procedures

All experiments were conducted at least 4 days after surgery. On the morning of the experiment a PE60 tubing (Dow Corning, Midland, MI, U.S.A.) was affixed to the free end of the jugular catheter and extended from the animal to the outside of the cage, so that the rat could move freely and was not aware of blood sampling or drug administration, thus avoiding uncontrolled stress. The rats were left undisturbed for about 2 h before the start of the experiment.

Blood samples (0.9 ml) were collected into heparinized tubes by means of a peristaltic pump (Gilson, Villiers-le-Bel, France). The samples were immediately centrifuged and plasma was stored at $-20^{\circ}C$ until assayed for prolactin. Red blood cells were resuspended in saline and reinfused after the following blood sample to minimize the effect of blood loss.

All treated animals received an i.p. injection of L-NAME (1, 10 or 30 mg kg^{-1} body weight) one hour before the first (basal) blood sample. Control rats received an i.p. injection of saline instead of L-NAME.

For the experiments with immobilization stress, each rat was placed in a narrow cylindrical glass cage which allowed almost no freedom of movement. The stress was applied after a first basal blood sampling until the end of the experiment. Morphine was administered i.v. through the catheter, 90 min after the beginning of the stress application.

For the experiments with repeated morphine administration, a first administration of morphine 3, 6 or 12 mg kg^{-1} was given i.v. through the catheter after a first basal blood sampling; the second administration of morphine 3, 6, or 6 mg^{-1} respectively was given 90 min after the first administration.

Hormone assay and data analysis

Plasma samples were assayed for prolactin in duplicate by double-antibody radioimmunoassay. Prolactin for iodination (NIADDK-rPRL I-6) and standard (NIADDK-rPRL-RP3) were kindly supplied by the NIADDK (Torrance, CA, U.S.A.) and the National Hormone and Pituitary Program (Baltimore, MD, U.S.A.). First antibody (rabbit polyclonal rPRL antibody 6-10/90) was used at a final dilution of 1/80 000 and had higher specificity in terms of its low cross-reactivity with anterior pituitary hormones other than PRL, compared to the NIADDK antiserum anti-rPRL-S9 (Bollengier *et al*., 1995). The assays were run according to the NIADDK protocol. The optimal detectability in 100 μl undiluted plasma ranged from 0.25 to 50 ng ml^{-1}. Samples exceeding this upper limit were diluted in assay buffer. Intra- and interassay coefficients of variation were less than 6% and 8%, respectively.

A one-way ANOVA (Analysis of Variance) for repeated measures, followed by an *a posteriori* Scheffé test, was used for analysis of the results in each group. A one-way ANOVA for independent measures, followed by an *a posteriori* Scheffé test, was used for analysis of the results between the different groups. A probability level of $P<0.05$ was considered significant.

Results

Effect of L-NAME pretreatment on basal PRL secretion (Table 1)

One hour before the first blood sample was taken the three groups of rats received pretreatment with 1, 10 or 30 mg kg^{-1} LNAME, respectively; control rats received saline instead. None of the groups showed a significant rise in plasma PRL concentration over a period of 120 min. There were no significant differences between the groups, showing that all plasma PRL concentrations remained at basal values.

Effect of L-NAME pretreatment on stress- and subsequent morphine-induced PRL secretion (Figure 1)

Control rats responded to immobilization stress with a significant rise in plasma PRL concentration. Though stress was

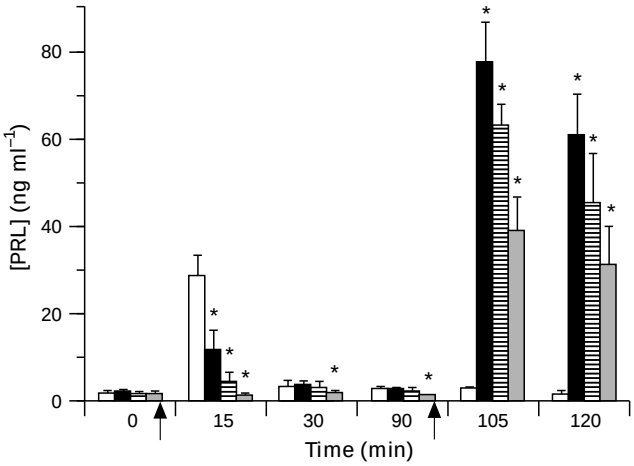

Figure 1 Plasma concentrations of prolactin (PRL; means ± s.e. mean) in rats pretreated with saline (open columns, $n=11$) or L-NAME 1 mg kg^{-1} (solid columns, $n=11$), 10 mg kg^{-1} (hatched columns, $n=12$) or 30 mg kg^{-1} (stippled columns, $n=12$) i.p. one hour before the first blood sampling. Immobilization stress (first arrow) was applied after the first basal blood sample (time = 0). Rats received morphine 6 mg kg^{-1} (second arrow) after blood sampling at time = 90 min. *$P>0.05$ compared to controls at the same time (ANOVA + Scheffé comparisons).

Table 1 Plasma prolactin concentration in rats pretreated with L-NAME (1, 10, or 30 mg kg^{-1}) or saline (controls) one hour before the first blood sampling

	Plasma prolactin (ng ml^{-1})					
Time (min)	*0*	*15*	*30*	*90*	*105*	*120*
Saline ($n=7$)	2.31 ± 0.52	2.33 ± 0.53	2.48 ± 0.40	2.09 ± 0.45	2.03 ± 0.44	2.55 ± 0.60
L-NAME 1 mg kg^{-1} ($n=7$)	2.32 ± 0.48	2.63 ± 0.62	2.66 ± 0.47	2.39 ± 0.45	2.33 ± 0.37	2.59 ± 0.50
L-NAME 10 mg kg^{-1} ($n=7$)	2.20 ± 0.28	2.11 ± 0.27	1.98 ± 0.27	2.02 ± 0.22	2.00 ± 0.31	2.20 ± 0.25
L-NAME 30 mg kg^{-1} ($n=6$)	2.57 ± 0.29	2.69 ± 0.25	2.73 ± 0.21	2.39 ± 0.35	2.42 ± 0.56	2.31 ± 0.34

Values are means ± s.e.mean. There were no significant differences within (ANOVA for repeated measures + Scheffé test, with $P>0.05$) or between the groups (ANOVA for independent measures + Scheffé test, with $P>0.05$).

maintained till the end of the experiment, PRL levels decreased back to basal levels after 30 min. Administration of morphine 6 mg kg^{-1}, 90 min after the start of immobilization stress, did not induce a further PRL rise, suggesting that rats subjected to stress were also tolerant to morphine.

When rats were pretreated with L-NAME (1, 10 or 30 mg kg^{-1}) the stress-induced PRL elevation was attenuated in a significant ($P<0.05$) dose-dependent way. The inhibition of the PRL rise was so strong after 10 and 30 mg kg^{-1} L-NAME, that in this group the plasma PRL concentration was no longer significantly different from basal plasma PRL concentration. Administration of morphine 6 mg kg^{-1}, 90 min after onset of stress, induced a significant rise in plasma PRL concentration in all L-NAME pretreated rats, in contrast to saline treated rats where complete tolerance had developed. In the L-NAME pretreated rats tolerance was attenuated significantly in a reversible dose-dependent way. L-NAME pretreatment had no effect on basal PRL secretion.

Effect of L-NAME on the action of acute and repeated administration of 3 mg kg^{-1} morphine (Figure 2)

Basal PRL secretion was not affected in any of the L-NAME pretreated groups. Figure 2 shows that L-NAME at particular doses had a potentiating effect on the PRL stimulating response to morphine 3 mg kg^{-1}. This potentiation was significantly different compared to controls in the case of 10 and 30 mg kg^{-1} L-NAME. The dose-response relationship seemed dose-related but the difference between the groups given 1 mg kg^{-1} and 10 mg kg^{-1} were not statistically significant, in contrast to the difference between the 1 and 30 mg kg^{-1} L-NAME groups. In all groups the morphine-induced PRL peak was present at 15 min and declined at 30 min.

When morphine 3 mg kg^{-1} was administered a second time, 90 min later, its PRL-rising capacity was already subject to tolerance in the control rats. However, tolerance to the second administration of morphine was attenuated in a significant and dose-dependent way in the L-NAME-pretreated groups.

Effect of L-NAME on the action of acute and repeated administration of 6 mg kg^{-1} morphine (Figure 3)

When morphine 6 mg kg^{-1} was administered for the first time, control rats and L-NAME-pretreated rats showed a significant rise in PRL secretion, with a peak value at 15 min. However, in L-NAME-pretreated rats the morphine-induced rise was potentiated, like in the case of 3 mg kg^{-1} morphine. The potentiation was significant for all doses of L-NAME and appeared to be dose-related, although the difference between the groups given 10 mg kg^{-1} and 30 mg kg^{-1} was not statistically significant, while those of 1 mg kg^{-1} and 30 mg kg^{-1} were significantly different.

When 90 min later morphine 6 mg kg^{-1} was administered for the second time, all control rats showed tolerance. However, rats pretreated with 1, 10 or 30 mg kg^{-1} of L-NAME displayed a significant increase in plasma PRL concentration compared to control rats. Hence tolerance on the second administration of morphine was attenuated in a significant and dose-dependent way in the L-NAME-pretreated groups.

Effect of L-NAME on the action of 12 mg kg^{-1} morphine followed by 6 mg kg^{-1} morphine (Figure 4)

When 12 mg kg^{-1} morphine was administered to the rats, the morphine-induced PRL peaks of rats pretreated with 1 and 10 mg kg^{-1} L-NAME were not significantly different from the control PRL peak at either 15 min, or 30 min. However, when rats were pretreated with 30 mg kg^{-1} L-NAME the PRL-enhancing capacity of morphine was significantly potentiated; in fact these rats showed a dramatic rise in plasma PRL concentration compared to controls. Moreover within 90 min after the morphine administration, all animals in this group died.

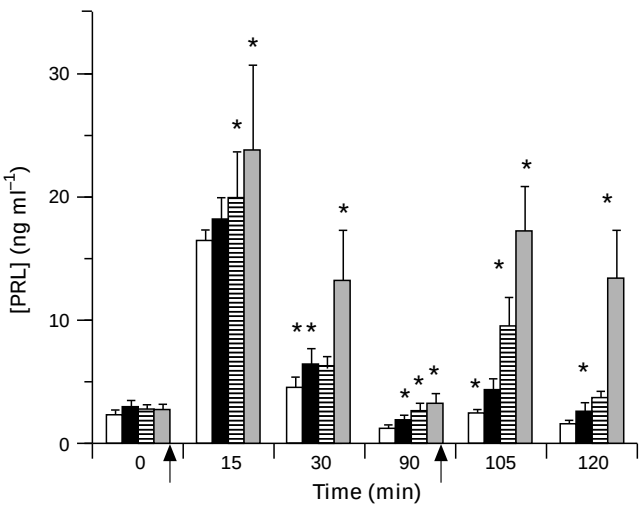

Figure 2 Plasma prolactin levels (PRL; mean ± s.e.mean) in rats injected, after a basal blood sampling, with 3 mg kg^{-1} morphine (first arrow) followed by a second injection of the same dose after blood sampling at time = 90 min (second arrow). One hour before the basal blood sampling rats received an i.p. injection of either saline (open columns, $n=12$) or L-NAME 1 mg kg^{-1} (solid columns, $n=12$), 10 mg kg^{-1} (hatched columns $n=10$) or 30 mg kg^{-1} (stippled columns, $n=11$). *$P<0.05$ compared to controls at the same time (ANOVA + Scheffé comparison).

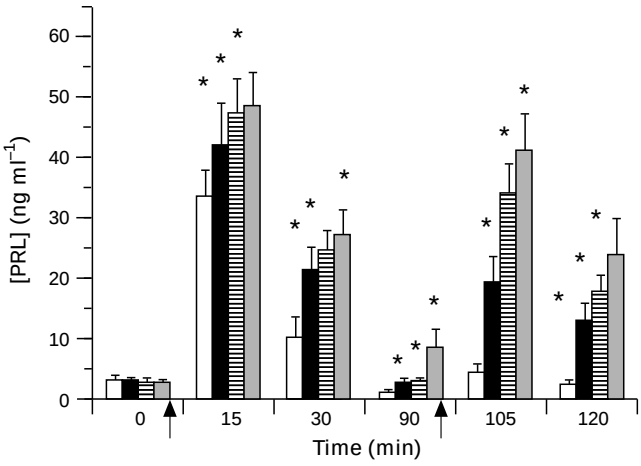

Figure 3 Plasma prolactin levels (PRL; mean ± s.e.mean) in rats injected after a basal blood sample, with 6 mg kg^{-1} morphine (first arrow), followed by a second injection of the same dose after a blood sampling at time = 90 min (second arrow). One hour before the basal blood sampling rats received an i.p. injection of saline (open columns, $n=12$) or L-NAME 1 mg kg^{-1} (solid columns, $n=12$), 10 mg kg^{-1} (hatched columns, $n=10$) or 30 mg kg^{-1} (stippled columns, $n=13$). *$P<0.05$ compared to controls at the same time (ANOVA + Scheffé comparisons).

When a second administration of morphine, in this case 6 mg kg^{-1}, was administered to the remaining groups 90 min later, only rats pretreated with 10 mg kg^{-1} of L-NAME showed a significant attenuation of tolerance.

Discussion

The present studies clearly demonstrate that L-NAME, a nitric oxide synthesis inhibitor, has a modulating effect on the stress- and morphine-induced PRL secretion, while it has no effect on basal PRL secretion. Pretreatment with L-NAME resulted in a significant and dose-dependent attenuation of the stress-in-

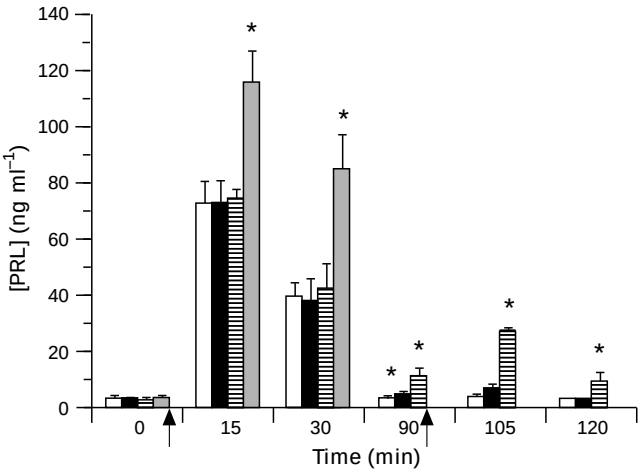

Figure 4 Plasma prolactin levels (PRL; mean ± s.e.mean) in rats injected, after a basal blood sample, with 12 mg kg^{-1} morphine (first arrow), followed by a second injection of 6 mg kg^{-1} morphine after blood sampling at time = 90 min (second arrow). One hour before the basal blood sampling, rats received an i.p. injection of saline (open columns, $n = 10$) or L-NAME 1 mg kg^{-1} (solid columns, $n = 9$), 10 mg kg^{-1} (hatched columns, $n = 10$) or 30 mg kg^{-1} (stippled columns, $n = 8$). *$P < 0.05$ compared to controls at the same time (ANOVA + Scheffé comparisons).

duced elevations of plasma PRL concentration and a significant but reversed dose-dependent inhibition of the subsequent tolerance to morphine. From the experiments with a first administration of 3 or 6 mg kg^{-1} morphine it is clear that L-NAME can potentiate morphine-induced PRL secretion. This is in line with the observation that L-NAME can also potentiate morphine-induced antinociception in rats (Przewlocki *et al*., 1993). When rats received a higher dose of morphine (12 mg kg^{-1}), a significant potentiating effect of L-NAME on the morphine-induced PRL secretion was only apparent with the highest dose of L-NAME (30 mg kg^{-1}). This potentiation was rather dramatic since PRL levels were increased by almost 60% compared to controls. Moreover all rats of this group died within 90 min. It is very unlikely that these deaths were caused by acutely elevated PRL levels; rather a potentiation of other morphine effects, like respiratory depression, seems to be responsible.

When, in our experiments, 3 or 6 mg kg^{-1} morphine was administered repeatedly tolerance developed in all control rats. However, when rats were pretreated with L-NAME, tolerance was overcome in a significant and dose-dependent way. Other studies have obtained similar results with other parameters of opioid action. The blocking effect of NO synthesis inhibitors on opioid tolerance is confirmed by several studies in which analgesia or hypothermia was used as an opioid parameter (Kolesnikov *et al*., 1992; 1993; Thorat *et al*., 1993). In one of these studies, the NO synthesis inhibitor N^G-nitro-L-arginine (L-NOARG), prevented tolerance to the morphine-induced analgesia in mice when coadministered with morphine (Kolesnikov *et al*., 1992), but was not able to potentiate acute morphine analgesia. This discrepancy could be due to the fact that in that study L-NOARG was coadministered subcutaneously with morphine and not given as a pretreatment. This might suggest that an incubation time is necessary or that the drug needs some time to reach the brain. This last hypothesis is strengthened by the fact that potentiation is apparent when the NO synthesis inhibitor is directly injected in the brain, like in the study of Przewlocki *et al*. (1993) where L-NAME was administered intrathecally (i.t.).

Only one other study has examined the effects of a NO synthesis inhibitor on opiate-induced PRL secretion (Rauhala *et al*., 1994). In that study the results contrasted with ours in that L-NAME, in a 5 day pretreatment, attenuated the stimulant action on PRL levels of acute administration of morphine (i.p.), while coadministration of L-NAME with morphine did not attenuate the development of tolerance. These differences could be due to a different experimental setup. Indeed, in that study the assessment of the stimulating effect of morphine in tolerant rats pretreatment with L-NAME was done after a 4-day morphine-free delay. The authors claimed that this was necessary to overcome withdrawal symptoms, but apparently tolerance had already disappeared to a certain extent during the withdrawal period. Also chronic stress associated either with the i.p. L-NAME pretreatment during 5 days or with the i.p. morphine administration itself, could not be ruled out. Hence, it seems that the time of administration of L-NAME is crucial to obtain certain effects on morphine-induced PRL secretion. A recent study by Xu & Tseng (1995) confirmed that a different time course of NO inhibitor pretreatment can give different results on opioid-induced antinociception.

Our results are striking in the fact that stress-induced PRL secretion was inhibited while morphine-induced PRL secretion was potentiated. This might suggest that stress induces PRL secretion through a mechanism different from that of morphine. However, as was pointed out in the Introduction, it is widely accepted that stress induces PRL secretion through an effect on opioid receptors, via the endogenous opioid β-endorphin. Recently, Xu & Tseng (1995) obtained similar opposite effects of a NO inhibitor on morphine and β-endorphin, though using a totally different experimental setup. It was found that antinociception induced by i.c.v. administered morphine was potentiated by i.t. NO inhibitor pretreatment, while antinociception induced by i.c.v. β-endorphin administration was attenuated. To explain the opposite effects of the NO inhibitor on β-endorphin and morphine, it was suggested that the effect on β-endorphin was mediated through another opioid receptor, namely the δ-receptor instead of the μ-receptor like for morphine. However, response induced by a specific δ-receptor agonist was not affected by a NO inhibitor, so this possibility is also excluded. Also, in the present experiments this possibility can be ruled out because δ-receptor agonists do not influence PRL release (Herz, 1984).

The opposite effects of a NO inhibitor on the opioids morphine and β-endorphin could be ascribed to the rather unusual action of NO. NO is a signalling molecule that is not restricted to pass information at discrete loci because it can simply diffuse through cellular membranes and spread rapidly in all directions (Snyder & Bredt, 1991). Gally *et al*. (1990) hypothesized that the effect of NO is activity-dependent, i.e. that NO modifies the response of the target pathways in a manner that depends on their respective activity. This means that NO may have different effects on the same pathway depending on the stimulus that triggered the NO function. Garthwaite & Boulton (1995) have also demonstrated an activity-dependent function of NO so that the net effect of NO on neuronal activity could be either a modulation of the response pattern, or an increase or decrease of the neuronal activity. More studies confirm that the direction of the effect depends on the strength and/or duration of the present stimuli (Zhuo *et al*., 1994; Schuman & Madison, 1994; Garthwaite & Boulton, 1995). From our experiments it is clear that the NO inhibitor L-NAME works in an activity-dependent manner. L-NAME does not affect basal PRL secretion, but it exerts a potentiating or inhibiting effect when PRL secretion is stimulated by morphine or immobilization stress (via β-endorphin), respectively. The different response could be due to a difference in the provoking stimulus by morphine and β-endorphin, in our experiments as well as in those of Xu & Tseng (1995). However, it is not clear which factor is responsible for the different triggering stimulus.

There are many questions regarding the type of 2nd messenger activated by opiods and also the mechanisms of tolerance development. Nestler and coworkers studied these mechanisms extensively, concluding that acute opioid receptor activation inhibits, through the G-protein bound to the re-

ceptor, the enzyme adenylate cyclase thus decreasing adenosine 3′: 5′-cyclic monophosphate (cyclic AMP) levels, while chronic morphine administration leads to an upregulation of this pathway so that cyclic AMP levels rise (Nestler, 1992). However, opioids can affect neural function by influencing ion channels directly via the G-protein without any involvement of cyclic AMP pathway (Childers, 1991; Nestler, 1992), but the significance of these and other mechanisms in relation to the functional effects of opioids is not clear, nor is the point of action for NO. One possibility is the soluble guanylate cyclase, the enzyme responsible for cyclic GMP formation, which is one of the major targets for NO in the central nervous system (Knowles *et al*., 1989). NO can elevate cellular cyclic GMP levels, and hence it can affect cellular cyclic AMP levels or the phosphorylation state of proteins, because cyclic GMP activates several cyclic GMP-dependent protein kinases and phosphodiesterases (Garthwaite & Boulton, 1995). This could then be a possible interaction level of NO and opioid function. Moreover the enzyme producing NO, namely NO synthase, could itself be regulated via phosphorylation by a cyclic AMP-dependent protein kinase. Finally, because of the involvement of NO in a vast array of neuronal pathways, L-NAME could also influence the release rather than the action of endogenous molecules like β-endorphin. This might in part explain the differences between the acute effects of L-NAME on the PRL responses to immobilization and morphine.

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