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Nitroglycerin inhibits the development of morphine tolerance and dependence in rats

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Abstract

The development of tolerance to and physical dependence on opioids remains a significant barrier to their clinical use. *N*-Methyl-D-aspartate (NMDA) receptor antagonists inhibit tolerance and dependence. However, many NMDA antagonists have undesirable side effects. It has been shown that nitroglycerin (NTG) can antagonize NMDA receptor activity. This study was designed to determine whether NTG could inhibit the development of morphine tolerance and dependence. Rats were anesthetized and implanted with either morphine or placebo pellets, and pumps infusing vehicle or NTG (doses from 0.1 μg/kg/day to 10 mg/kg/day). Tolerance development was assessed by tail-flick latency (TFL). After 6 days, withdrawal was precipitated by subcutaneous injection of 2 mg/kg naloxone. Withdrawal signs were observed for 15 min. Placebo-pelleted rats showed no changes in TFL over the course of the study and no withdrawal signs. Morphine-pelleted rats developed tolerance. The 0.1 mg/kg/day NTG dose significantly attenuated tolerance development, while the other doses had no significant effect. The 0.1 mg/kg/day dose also attenuated some withdrawal signs. Higher or lower doses were not effective, possibly because of competing biochemical effects.

Keywords: Narcotic; Modulation; Naloxone; NMDA receptor; Opioids; Withdrawal

1. Introduction

The development of tolerance to opioid analgesia and physical dependence upon opioids remain significant impediments to their effective use in chronic pain management. Tolerance is defined as a reduction in effect with continued exposure to a drug, while dependence has been defined as the physiological alterations that lead to unpleasant (and, with some drugs, life-threatening) signs and symptoms upon withdrawal of the drug (Trujillo and Akil, 1991c, 1995). Also, a desire to avoid withdrawal symptoms can be a powerful impetus for continued drug usage. It has been shown that antagonists of the NMDA subtype of glutamate receptors can inhibit the development of tolerance to and dependence on opioids (Marek et al., 1991; Trujillo and Akil, 1991a). NMDA antagonists can inhibit tolerance development at both supraspinal and spinal levels (Gutstein and Trujillo, 1993), suggesting that the pharmacological mechanisms underlying opioid tolerance are operative at all levels of the central nervous system, not just in higher cognitive centers.

While these drugs hold great promise for the treatment of opioid tolerance and other conditions such as neuropathic pain and stroke, wide clinical usage of many of these compounds has been limited because of undesirable side effects of the NMDA antagonists themselves (Olney et al., 1991). These compounds can cause a wide array of neuropsychiatric side effects. In addition, they could potentially decrease the therapeutic index (LD_{50}–ED_{50}) of opioids (Trujillo and Akil, 1991b), requiring stricter attention to dosing and opioid side effects such as somnolence and respiratory depression. Several strategies have been proposed for minimizing these drawbacks (Gutstein, 1996). While the NMDA antagonist dextromethorphan inhibits opioid tolerance in rodents and has been shown to have efficacy as an analgesic in the clinical treatment of diabetic neuropathy (Elliott et al., 1994; Sindrup and Jensen, 1999), more drugs of this type are needed.

The NMDA receptor has several potential modulatory sites that could be useful for therapeutic interventions (Fig. 1). Competitive antagonists at the glutamate site have been

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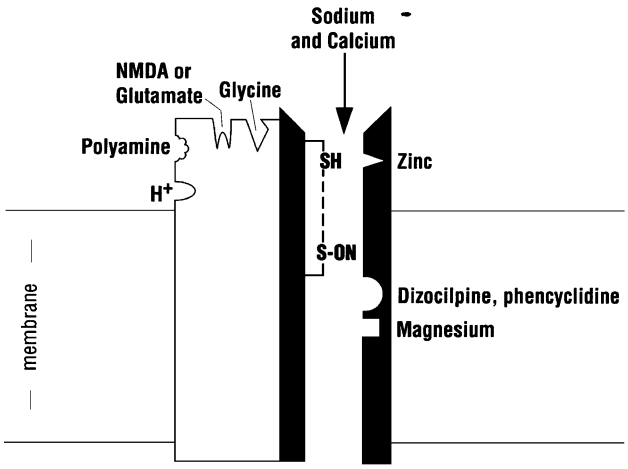

Fig. 1. Schematic representation of the NMDA receptor. Cellular depolarization or receptor activation by agonist causes release of the magnesium block of the ion channel and influx of calcium or sodium ions. Receptor activation can be modified competitively by antagonists at the glutamate-binding site, or noncompetitively by drugs binding to the glycine, polyamine and H^+ sites. Channel blocking compounds inhibit channel function in an uncompetitive manner (see text). Compounds acting at the redox site modify channel function by altering the oxidation state of the sulfhydryl groups. Channel activity is increased if the sulfhydryl groups are reduced, and is decreased if the groups are oxidized and a disulfide bridge is formed.

shown to inhibit (and, under some circumstances, reverse) opioid tolerance and dependence (Tiseo et al., 1993; Tiseo and Inturrisi, 1993). A potential drawback to the use of competitive antagonists (and drugs that act at the glycine co-agonist site) is that competitive antagonists could interfere with normal neural functions mediated by excitatory amino acids (such as learning and memory) before blocking effects induced by higher glutamate levels or increased glutamate release (Lipton, 1993; Lipton and Rosenberg, 1994). An attractive option that has been pursued in the past is the use of uncompetitive inhibitors. These are drugs that require receptor activation (in the case of the NMDA receptor, that the channel be opened) before inhibition can occur (Chen et al., 1992; Lipton, 1993). In this situation, a greater degree of receptor activation would cause a proportionately greater degree of inhibition, leading to a relatively constant low level of neurotransmission. Many of the NMDA antagonists that have been shown to inhibit opioid tolerance and dependence, such as MK-801, ketamine and dextromethorphan, are drugs of this type (also known as "open-channel blockers" because of their requirement for receptor activation and channel opening to be effective). Unfortunately, most of these drugs have proven neurotoxic as well because once bound in the channel they are released very slowly, leading to a cumulative increase in receptor blockade (Chen et al., 1992; Lipton, 1993; Lipton and Rosenberg, 1994).

Another mechanism for altering NMDA receptor function would be to use noncompetitive inhibitors of NMDA receptor modulatory sites that do not completely block NMDA receptor activation. These compounds could decrease activation without eliminating basal transmission, functioning like a "gain control" in the presence of higher agonist concentrations. A great deal of attention has focused on the redox modulatory site in altering NMDA receptor function (Lei et al., 1992; Lipton et al., 1993). This site consists of two closely approximated sulfur atoms that can be reduced and form a disulfide bridge, or oxidized by other agents with loss of the disulfide bond (Fig. 1). Oxidizing agents lead to a decrease, but not complete blockade, of NMDA receptor function (Lipton, 1993). It has been shown that the commonly used compound nitroglycerin (NTG) is an effective oxidizing agent at the NMDA receptor (Lei et al., 1992; Lipton, 1993). Therefore, this study was to determine whether NTG could inhibit the development of morphine tolerance and physical dependence in rats.

2. Methods and materials

After approval of the Committee for the Use and Care of Animals, 72 adult male Sprague–Dawley rats were used as subjects for the study. Animals were housed six to a cage in a 12-h light/dark cycle with unlimited access to food and water. Rats were allowed to habituate in the colony room for 1 week before experimental manipulations were undertaken. Morphine pellets (75 mg) were from NIDA, naloxone was from Sigma (St. Louis, MO) and NTG was from Abbott (North Chicago, IL).

Prior to experimental manipulations, animals underwent tail-flick latency (TFL) testing. Animals were loosely restrained in a towel and a hot light was shined on the tail. Withdrawal times were measured using a photocell. Three measurements were obtained and the average used in all calculations. A cutoff latency of 10 s was used to avoid damage to the tail. Morphine tolerance was induced using a paradigm that has previously been well characterized in our laboratory (Gutstein and Trujillo, 1993; Gutstein et al., 1995). After baseline TFL testing, rats were anesthetized with methoxyflurane and implanted between the scapulae with either a morphine or placebo pellet, and osmotic minipumps (Alza, Foster City, CA) infusing either NTG at 0.1, 1 or 10 mg/kg/day or 5% dextrose in water (D5W) vehicle ($n=6$ per group). An additional experiment was performed to assess the effects of lower NTG doses on morphine tolerance and physical dependence. In this experiment, animals were implanted with morphine pellets as described above, and implanted with minipumps infusing either NTG at 0.1, 1 or 10 μg/kg/day or D5W ($n=6$ per group). A continuous infusion paradigm was utilized in both experiments to minimize any potential hemodynamic side effects from bolus NTG administration, and to provide a constant level of NTG in combination with the sustained continuous morphine release provided by the pellets (Cerletti et al., 1976; Yoburn et al., 1985). Animals then underwent TFL testing 4, 24, 48 and 72 h after implantation. At 72 h, animals were implanted with three more pellets of

the same type under anesthesia. TFLs were obtained 4 h after the second implantation and daily for the next 3 days (until 144 h after the initial implantation). After the final TFL test, animals were weighed and withdrawal was precipitated by the injection of 2 mg/kg sc naloxone. Animals were then placed in clear Plexiglas withdrawal cages $25 \times 25 \times 100$ cm high and observed for 15 min. The occurrence of withdrawal signs (escape jumps, wet dog shakes, teeth chattering, lacrimation, diarrhea, ptosis, belly lying) was recorded. The counted withdrawal signs (escape jumps, wet dog shakes and teeth chattering) were also grouped to determine a "withdrawal score" using a modification of the primary "graded" signs of the Gellert and Holtzman scale (Gellert and Holtzman, 1978). Instead of counting abdominal constrictions, a withdrawal behavior we have not observed, quantitative differences in teeth chattering were included in the grouping. Animals were weighed again 2 h after naloxone injection and weight changes determined.

2.1. Statistical analysis

TFL data from the initial experiment were analyzed using a mixed-model three-factor ANOVA with one repeated measure (time) and two crossed factors (pellet type and NTG dose). A series of one-factor ANOVAs were then computed because significant interactions were found between time and the crossed factors. Post-hoc comparisons were computed for each pellet type at each time using the Dunn test. TFL data from the second experiment were analyzed using a mixed-model two-factor repeated measure ANOVA. Withdrawal signs that were ordinal variables, as well as the grouped withdrawal signs, were analyzed using two-factor ANOVAs for each pellet type, and post-hoc comparisons were computed using the one-tailed Tukey's test, since we did not predict an increase in withdrawal symptoms. Nominal variables were analyzed using the chi-square test. $P < .05$ was required for significance.

3. Results

Baseline TFLs were not significantly different between groups in both studies, ranging between 3.1 and 3.3 s. Placebo-pelleted animals in the initial experiment did not show any significant effect of NTG or vehicle infusion on TFL during the study (Fig. 2). Therefore, to minimize animal usage, placebo-pelleted animals were not included in the subsequent experiment to investigate lower NTG doses. Morphine-pelleted animals in both experiments became analgesic and developed tolerance to the analgesic effect over time (Figs. 3 and 4). Three-factor mixed-model ANOVA analysis of the initial experiment (Fig. 3) demonstrated that all the statistically considered factors [NTG dose ($F = 13.74$), pellet type ($F = 2245.69$) and time ($F = 93.94$)], two-factor [Dose × Pellet ($F = 15.68$), Dose × Time ($F = 2.80$), Pellet × Time ($F = 95.81$)] and three-factor [Dose × Pellet × Time ($F = 2.76$)] interactions were statistically significant. The two-factor interaction terms indicate that tolerance developed differently in the groups when the interactions between NTG dose and pellet type, NTG dose over time and pellet type over time were considered individually. The three-factor interaction indicates that all of the factors taken together impacted on each of the groups differentially. After the first pelleting, the 0.1 mg/kg/day group demonstrated statistically significant differences from controls at 48 and 72 h after pelleting (Fig. 3). After the second pelleting, the 0.1 mg/kg/day group showed significant inhibition of tolerance compared with both control and other NTG-treated groups at 120 and 144 h (Fig. 3). Three of the six rats in the

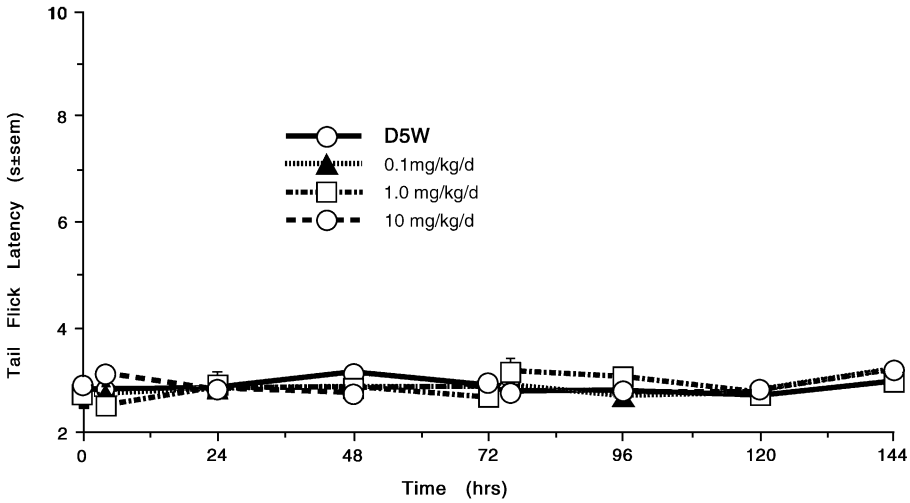

Fig. 2. Tail flick latency responses of placebo-pelleted animals. Pellets and pumps were implanted as described in the text and were tested under an 80-V lamp while loosely restrained in towels. Three days after initial implantation, three additional placebo pellets were placed. A 10-s cutoff was employed to avoid tissue damage. D5W-5% dextrose in water vehicle control. Values reported are mean TFLs ± S.E.M.

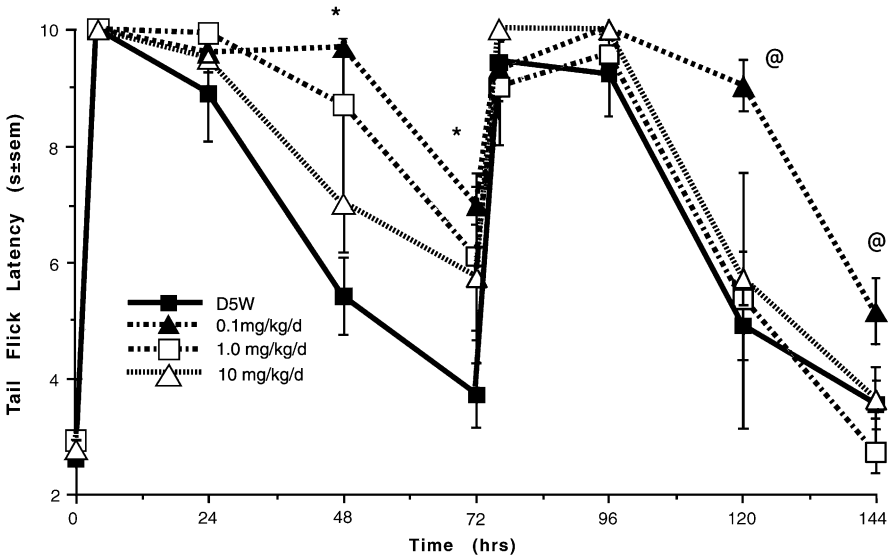

Fig. 3. Tail flick latency responses of morphine-pelleted animals receiving "high-dose" (0.1, 1 and 10 mg/kg/day) NTG treatment. Pellets and pumps were implanted as described in the text, and animals were tested under an 80-V lamp while loosely restrained in towels. Three days after initial implantation, three additional morphine pellets were placed. A 10-s cutoff was employed to avoid tissue damage. Values reported are mean TFLs ± S.E.M. * 0.1 mg/kg/day group significantly different from saline infusion group, $P < .05$. @ 0.1 mg/kg/day group significantly different from all other treatment groups, $P < .05$.

group receiving 10 mg/kg/day NTG and morphine died after the second pelleting. The reason for this mortality was not clear.

Two-factor mixed-model ANOVA analysis of the subsequent experiment utilizing lower NTG doses (0.1, 1 and 10 μg/kg/day; Fig. 4) revealed that the two-factor interaction between NTG Dose × Time was not statistically significant ($F = 0.26$) and that NTG dose alone was also not statistically significant ($F = 0.32$). As expected, TFL responses varied significantly over time ($F = 109.82$). However, the lack of effect of dose or interaction indicates that all groups were affected equally over time, demonstrating that lower

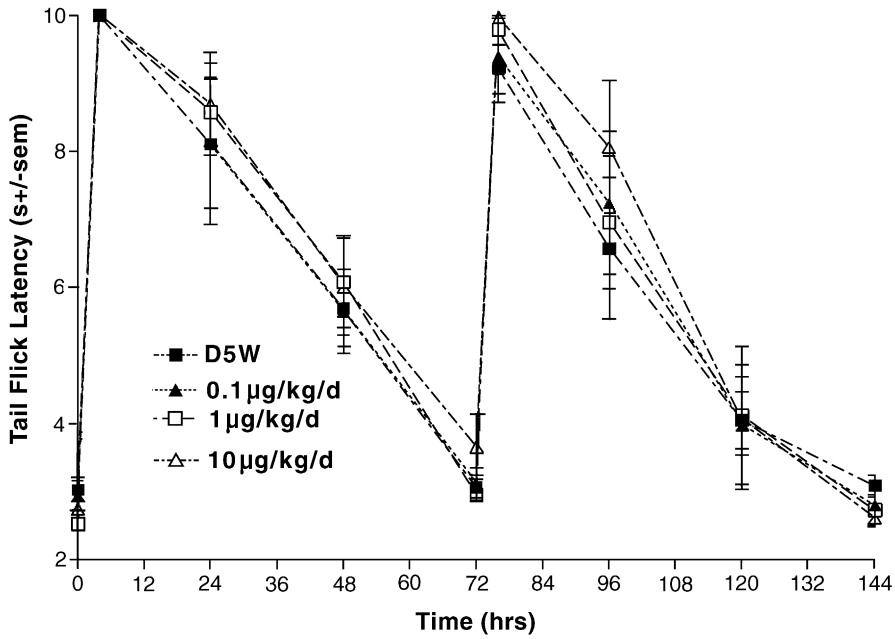

Fig. 4. Tail flick latency responses of morphine-pelleted animals receiving "low-dose" (0.1, 1 and 10 μg/kg/day) NTG treatment. Pellets and pumps were implanted as described in the text, and animals were tested under an 80-V lamp while loosely restrained in towels. Three days after initial implantation, three additional morphine pellets were placed. A 10-s cutoff was employed to avoid tissue damage. Values reported are mean TFLs ± S.E.M. No significant differences were observed between the groups at any time point.

NTG doses had no effect on the development of tolerance after morphine pelleting (Fig. 4).

In the initial "high dose" experiment, after naloxone injection, two-factor (NTG dose and pellet type) ANOVA revealed that percent weight change was a significant variable (NTG dose, $P<.01$; pellet type, $P<.0001$; NTG Dose × Pellet interaction, $P<.01$). As expected, placebo-pelleted animals did not show a significant weight change, while morphine-pelleted animals lost weight. Animals receiving NTG lost between 5% and 7% of their body weight in 2 h, while control animals lost over 10% (Fig. 5). Differences between NTG doses were not significant. In the "low dose" experiment, a similar analysis revealed no significant difference in weight loss between control and NTG-treated groups.

Escape jumps, teeth chattering, and wet dog shakes were observed counted withdrawal signs. In the high dose experiment, the only significant effects for individual signs were found for pellet type [i.e., all of these effects occurred in the morphine-pelleted groups ($P<.005$ for pellet type for each of the three symptoms)], not NTG dose. While there was a trend toward decreased number of symptoms in the 0.1 mg/kg/day NTG group (Table 1), a significant effect of NTG dose and a Dose × Pellet interaction was not observed for any of the symptoms individually. This was probably due to the fact that individual animals showed a wide variation in the expression of these withdrawal signs. For instance, some animals exhibited primarily escape jumping and very little teeth chattering, while the main sign expressed by other animals was teeth chattering. This variability is reflected in the large standard deviations observed. However, when escape jumping, wet dog shakes and teeth chattering were summed and expressed as grouped withdrawal signs (Table 1), the number of grouped signs observed was more consistent between animals (reflected in lower relative standard deviations) and significant effects of NTG dose and pellet type were observed (NTG dose, $F=3.32$, $P<.03$; pellet type, $F=87.66$, $P<.0001$). Post-hoc tests of the morphine-pelleted animal data using the one-tailed Tukey's test revealed a significant decrease in the number of grouped withdrawal signs in the 0.1 mg/kg/day NTG dose group when compared with all other treatment groups (Table 1).

Lacrimation, diarrhea, belly lying and ptosis were analyzed using the chi-square test. No attenuation by any NTG dose was noted for these withdrawal signs.

Table 1
Counted withdrawal signs in morphine-pelleted animals

Treatment group	Escape jumps	Teeth chattering	Wet dog shakes	Grouped W/D signs
D5W	8.2 ± 7.8	6.7 ± 4.3	2.5 ± 3.3	17.2 ± 7.2
NTG 0.1 mg/kg/day	4.2 ± 4.2	2.3 ± 2.1	2.0 ± 3.1	8.5 ± 4.3 *
NTG 1.0 mg/kg/day	6.2 ± 5.3	6.7 ± 3.3	4.0 ± 1.8	17.0 ± 6.8
NTG 10 mg/kg/day	6.6 ± 6.4	7.3 ± 4.5	3.7 ± 0.6	17.7 ± 2.5

Grouped withdrawal signs are the sum of escape jumps, teeth chattering and wet dog shakes for each treatment group (values expressed as mean ± S.D.).
* Significantly different from all other treatment groups, $P<.05$.

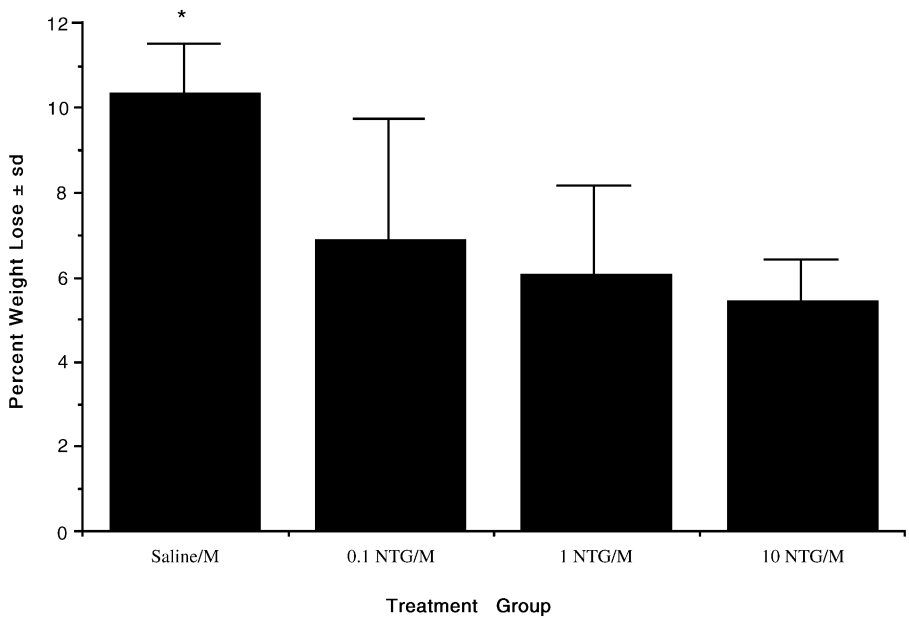

Fig. 5. Weight loss after naloxone-precipitated withdrawal in morphine-pelleted animals receiving "high-dose" (0.1, 1 and 10 mg/kg/day) NTG treatment. After 6 days of treatment, animals were weighed and withdrawal was precipitated with 2 mg/kg sc naloxone. Two hours after injection, animals were weighed again and percent body weight loss calculated. Values are mean percentage weight change ± S.D. * Significantly different from all "high-dose" NTG treatment groups, $P<.05$. * Significantly different from all other treatment groups, $P<.05$.

4. Discussion

The main finding of this study was that continuous infusion of NTG could inhibit the development of morphine tolerance and some signs of physical dependence in rats. The effect was observed only at the 0.1 mg/kg/day dose, not at higher or lower infusion rates. This response was unexpected, but not unprecedented. Amphetamines can exhibit similar dose–response properties, and a lack of therapeutic effect at higher doses is being recognized more frequently in pharmacological studies (Calabrese and Baldwin, 2001). While the effect was not as robust as for tolerance, some signs of physical dependence were also inhibited by the 0.1 mg/kg/day NTG dose. As noted above, a wide variation in the expression of individual counted withdrawal signs was observed between animals in the same treatment groups. This finding is consistent with the classic observations of many investigators summarized by Blasig and Herz (1977). They suggested that behavioral signs of withdrawal could be divided into two broad classes: ''recessive'' signs that decrease with the degree of dependence and ''dominant'' signs that increase with the degree of dependence. Thus, the variability in expression of different withdrawal signs could be due to differential expression of ''dominant'' and ''recessive'' signs in individual animals in our paradigm. The degree of weight loss induced by precipitated withdrawal was reduced to a similar extent by the higher doses of NTG studied, suggesting that higher doses of NTG could possibly be reducing the intensity of some aspects of withdrawal syndrome. However, some withdrawal signs (ptosis, diarrhea and belly lying) were seen regardless of the NTG dose employed.

It is also important to note that the 0.1 mg/kg/day dose attenuated morphine tolerance throughout the 6-day course of this study. In contrast, it has been known for almost 100 years that tolerance to the vasodilatory effect of NTG occurs, in some cases, quite rapidly (Elkayam, 1991; Shlevin, 1982). While physical dependence to NTG in the sense of markedly disordered functioning of the organism in the absence of drug does not occur, a rebound vasoconstriction can be noted after prolonged NTG therapy (Munzel et al., 1995). However, the effective NTG dose for attenuating morphine tolerance is substantially (100–300-fold) lower than doses used in previous studies to induce tolerance to the vascular effects of NTG (Needleman, 1970). The mechanism underlying vascular tolerance is still not entirely known (Munzel et al., 1995; Needleman, 1970; Parker, 1989), but these characteristics and other findings suggest that it is different than the mechanism we are postulating for morphine tolerance inhibition.

While the mechanism by which NTG inhibited morphine tolerance and dependence was not determined in this study, it is intriguing to speculate that this effect could be to NMDA receptor inhibitory effects of NTG. The redox site of the NMDA receptor is a site in the ion channel where two sulfur atoms in close proximity can be induced to form a disulfide bond by reducing agents and, alternatively, the bond can be broken by oxidizing agents (Fig. 1). Nitroso compounds such as NTG act as oxidizing agents at this site via formation of a RS-ON (*S*-nitrosoprotein) complex with these sulfur atoms, leading to a decrease in NMDA receptor function (Lei et al., 1992; Lipton et al., 1993). This requires that the NO moiety be present as a nitrosonium ion in the NO^+ oxidation state (nitric oxide is electrically neutral, containing one more electron than the nitrosonium ion) (Stamler et al., 1992). However, nitrosonium can be converted to nitric oxide (Lipton et al., 1993), which could actually accentuate opioid tolerance development (Mao et al., 1995). However, the reaction of NTG to produce nitric oxide via nitrosonium proceeds very slowly (Lipton et al., 1993), so the nitrosonium form should predominate. Perhaps increased nitric oxide production by higher doses of NTG could account for the decreased effectiveness of these doses in blocking the development of narcotic tolerance and dependence (Mao et al., 1995). Alternatively, other pharmacological or physiological effects of NTG could interfere with tolerance inhibition at higher doses (Calabrese and Baldwin, 2001). Further investigation of these possibilities seems warranted.

In sum, we have shown that continuous infusion of NTG can inhibit the development of morphine tolerance and attenuate some signs of physical dependence in rats. Additional studies are needed to further characterize this effect.

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