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# Up-regulation of [$^3$H]DTG but not [$^3$H](+)-pentazocine labeled $\sigma$ sites in mouse spinal cord by chronic morphine treatment

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**Abstract**

To monitor the possible effect of morphine on $\sigma$ sites, binding characteristics of [$^3$H](+)-pentazocine and [$^3$H]1,3-di-(2-tolyl)guanidine (DTG) to brain and spinal cord membranes of morphine-treated and control mice were compared. For morphine treatment, a single injection (100 mg/kg, s.c.) of morphine was followed 4 h later by pellet implantation (75 mg morphine free base). Animals were sacrificed 24, 72 h or 7 days later. The equilibrium dissociation value ($K_d$) and the density ($B_{max}$) of [$^3$H](+)-pentazocine binding remained unaffected by morphine treatment. Also, no change was found in $K_d$ and $B_{max}$ values of [$^3$H]DTG labeled $\sigma_2$ subtypes after any morphine treatment schedule when measured in the presence of 100 nM (+)-pentazocine. However, the $B_{max}$ of [$^3$H]DTG binding in the spinal cord in the absence of 100 nM (+)-pentazocine, was significantly elevated 72 h after implantation of the morphine pellet and recovered by 7 days, a time when the antinociceptive effect produced by the morphine pellet had dissipated. These data suggest that one population of $\sigma$ sites, that has a high affinity for [$^3$H]DTG, but is not equivalent with the [$^3$H](+)-pentozocine labeled $\sigma_1$ subtype or the [$^3$H]DTG labeled $\sigma_2$ subtype, is upregulated by morphine and, therefore, may play a role in the development of tolerance to or dependence on the effects of morphine. 

*Keywords:* Morphine tolerance; Morphine dependence; G receptor binding; (+)-Pentazocine; DTG (1,3-di-(2-tolyl)guanidine)

## 1. Introduction

The $\sigma$ receptor ligand, (+)-pentazocine, has a high affinity for $\sigma_1$ subtypes (De Costa et al., 1989; DeHaven-Hudkins et al., 1992). (+)-Pentazocine also binds to opioid receptors, but with less affinity than (−)-pentazocine, consistent with the greater selectivity of opioid receptors for (−)- than for (+)-enantiomers (for review, see Walker et al., 1990). (+)-Pentazocine produces some analgesia in humans postsurgically (Sugai et al., 1995), but has a low efficacy and can cause nausea and other problems associated with dysphoria. 1,3-di-(2-tolyl)guanidine (DTG), a $\sigma$ receptor ligand with equally high affinity for $\sigma_1$ and $\sigma_2$ subtypes (Hellewell and Bowen, 1990), produces antinociception in mice at a dose of 10 mg/kg i.p. in both the tail-withdrawal assay and the acute phase of the formalin assay, yet increases pain scores in the tonic phase of the formalin test (Kest et al., 1995a,b). These data suggest a complex modulatory role for $\sigma$ sites in pain transmission. Changes in nociception produced by $\sigma$ receptor ligands may be brought about by the modulatory influence on excitatory amino acid activity (Walker et al., 1990), which is believed to play an important role in nociception.

In addition to their antinociceptive effects, DTG, (+)- and (−)-pentazocine are each equally effective in antagonizing morphine analgesia. The antagonism of morphine by pentazocine was originally thought to be due to its partial agonistic interaction at opioid receptors; it has more recently been found to result from a mechanism that is reversed by haloperidol (Chien and Pasternak, 1993, 1994), which blocks both $\sigma$ sites and dopaminergic receptors, but not (−)-sulpiride, a dopamine receptor antagonist (Chien and Pasternak, 1994). Based on the sensitivity of these effects to haloperidol, antagonism of morphine antinociception by pentazocine and DTG has been proposed to result from an interaction with $\sigma$ sites rather than partial agonism at opioid receptors. Haloperidol alone potentiates the analgesic effects of opioids (Tulunay et al., 1975), suggesting either a tonic level of $\sigma$ activity in the central nervous system (CNS) (Chien and Pasternak, 1995), or indirect activation of $\sigma$ sites by morphine, thereby inhibiting the effect of morphine at opioid sites. The

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potentiative effects of haloperidol are exploited clinically, especially in veterinary medicine, to counteract excitatory effects opioids in some species and to enhance and prolong the sedative and analgesic effects of narcotic analgesics.

In a model of acute morphine dependence in mice, haloperidol decreases the severity (Lal and Numan, 1976) and number of withdrawal behaviors, especially when injected immediately prior to naloxone-induced withdrawal rather than prior to morphine (Kreeger et al., 1994). This underscores the possible involvement of $\sigma$ sites in the expression of symptoms associated with opioid dependence. In contrast, haloperidol prevents, but does not reverse the ability of morphine to potentiate the behavioral response to *N*-methyl-D-aspartate (NMDA) injected intrathecally in mice (Kreeger et al., 1995). Thus, although NMDA activity has been proposed to be critical for the development of opioid tolerance (Marek et al., 1991; Trujillo and Akil, 1991), increased sensitivity to NMDA activity in the spinal cord does not appear to account for the expression of morphine withdrawal behaviors which remain highly sensitive to haloperidol (Kreeger et al., 1995).

There is ample precedent for up- and down-regulation of $\sigma$ binding in response to treatment with a variety of $\sigma$ receptor ligands (reviewed by Matsumoto et al., 1989; Walker et al., 1990). If morphine has the tendency to interact with a $\sigma$ site, either directly or indirectly, one might speculate that sustained exposure to the high concentrations of morphine that are necessary to induce tolerance and dependence may impact on $\sigma$ binding. Because $\sigma$ activity appears to inhibit opioid activity (see above), such changes may account for the decreased analgesic effect of morphine associated with the development of tolerance and/or the production of opioid withdrawal symptoms that characterize dependence.

To determine whether $\sigma$ sites in mouse cortex, cerebellum and spinal cord are affected during the development of opioid tolerance and dependence, we compared the estimated dissociation ($K_d$) and receptor density ($B_{max}$) values measured with [$^3$H](+)-pentazocine and [$^3$H]DTG binding in control mice to that in mice exposed to morphine for 24, 72 h and 7 days. [$^3$H]DTG was used to label high-affinity $\sigma$ sites, binding with high and essentially equal affinity to both $\sigma_1$ and $\sigma_2$ subtypes (Hellewell and Bowen, 1990). [$^3$H](+)-Pentazocine binding was performed to label the $\sigma_1$ subtype (De Costa et al., 1989; DeHaven-Hudkins et al., 1992), and [$^3$H]DTG binding in the presence of 100 nM (+)-pentazocine to mark the $\sigma_2$ subtype. These ligands were selected based on their ability to bind to a unique population of high-affinity $\sigma$ sites in the mouse spinal cord (Kovács and Larson, 1995). Although opiates induce changes throughout the CNS, the mouse spinal cord has been proposed to play an important role in the development of tolerance to morphine (Gutstein and Trujillo, 1993). Thus, changes in the spinal cord were the primary focus, the cortex and cerebellum serving as control tissues.

## 2. Materials and methods

### 2.1. Animals

Male Swiss–Webster mice (20 to 25 g, Sasco, Omaha, NE) were housed 4 per cage under a 12 h light/dark cycle and allowed free access to food and water. Prior to use, acclimatization of 3 days was allowed. Mice were used strictly in accordance with the Guidelines of University of Minnesota Animal Care and Use Committee and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Researches Council (DHEW Publication (NIH) 78–23, revised 1978).

### 2.2. Materials

[$^3$H](+)-Pentazocine (35.3 Ci/mmol) and [$^3$H]DTG (39.4 Ci/mmol) were purchased from Dupont/New England Nuclear (Boston, MA). (+)-Pentazocine was obtained from Research Biochemicals (Natick, MA). Haloperidol was purchased from Sigma (St. Louis, MO). Morphine sulfate was purchased from Mallinkrodt (St. Louis, MO) and morphine base pellets were obtained through the National Institute on Drug Abuse from the Research Triangle Institute (Research Triangle Park, NC).

### 2.3. Treatment of mice with morphine

For chronic morphine treatment, mice were injected s.c. with a single dose of 100 mg/kg of morphine sulfate and 4 h later implanted s.c. with a morphine pellet containing 75 mg morphine-free base. This injection protocol was used as it has been found to attenuate the respiratory depression produced by the implantation of the morphine pellet. Control animals were injected with 0.9% saline and implanted with placebo pellets. Animals were lightly anesthetized with ether during the surgery for pellet implantation. Mice were killed 4 h after the single injection of 100 mg/kg of morphine sulfate and 24, 72 h and 7 days after the injection plus pellet implantation. Brain and spinal cord membranes were used in the radioligand binding assays described below.

### 2.4. Membrane preparation

Crude membranes were prepared for [$^3$H](+)-pentazocine and [$^3$H]DTG binding. Mice were decapitated and brain sections and spinal cords rapidly removed and homogenized in 40 volumes of 10 mM Tris–HCl buffer (pH 7.4) at 4°C with a Brinkmann Polytron (setting 8, for 5 s). The homogenate was centrifuged at 30 000 × $g$ for 20 min at 4°C. The resulting pellet was resuspended in the same amount of buffer and incubated at 37°C for 30 min. The suspension was then centrifuged (30 000 × $g$, 20 min, 4°C) and the final pellet resuspended in 15 volumes (350–400

Table 1
Hot saturation binding parameters for [$^3$H](+)-pentazocine and [$^3$H]DTG measured in the absence or presence of 100 nM (+)-pentazocine in mouse spinal cord membranes

| Time | *n* | Placebo pellet | | Morphine pellet | |
|---|---|---|---|---|---|
| | | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) |
| [$^3$H](+)-Pentazocine hot saturation binding | | | | | |
| 24 h | 3 | 1.26 ± 0.05 | 795 ± 23 | 1.11 ± 0.05 | 801 ± 23 |
| 72 h | 3 | 0.73 ± 0.02 | 767 ± 10 | 0.69 ± 0.05 | 795 ± 20 |
| [$^3$H]DTG hot saturation binding, no (+)-pentazocine added | | | | | |
| 24 h | 3 | 26.0 ± 3.2 | 1504 ± 130 | 25.0 ± 2.5 | 1518 ± 105 |
| 72 h | 6 | 21.0 ± 1.8 | 1386 ± 56 | 22.9 ± 2.2 | 1681 ± 49* |
| 7 days | 3 | 20.7 ± 2.2 | 1149 ± 119 | 20.5 ± 1.0 | 1311 ± 75 |
| [$^3$H]DTG hot saturation binding, +100 nM (+)-pentazocine | | | | | |
| 24 h | 3 | 34.6 ± 7.1 | 1179 ± 185 | 34.0 ± 5.9 | 1065 ± 133 |
| 72 h | 6 | 34.7 ± 1.5 | 1079 ± 53 | 38.1 ± 3.0 | 1031 ± 66 |
| 7 days | 3 | 23.1 ± 2.2 | 938 ± 59 | 23.1 ± 2.1 | 976 ± 105 |

Mice were injected s.c. with a single dose of 100 mg/kg of morphine; 4 h later, they were implanted s.c. with a morphine pellet containing 75 mg morphine free base. Control animals were injected with 0.9% saline and implanted with placebo pellets. Animals were killed 24, 72 h or 7 days later. Hot saturation binding of [$^3$H](+)-pentazocine and [$^3$H]DTG to crude membranes of mouse spinal cord was performed in 50 mM Tris–HCl buffer (pH 7.7) at 37°C for 210 min and 60 min. Experiments were conducted over a concentration range of 0.05–24 nM [$^3$H](+)-pentazocine and 0.75–72 nM [$^3$H]DTG in the absence and presence of 100 nM (+)-pentazocine. Non-specific binding was defined by addition of a final concentration of 10 μM haloperidol. The percentage of non-specific binding for [$^3$H](+)-pentazocine was 2–5%, for [$^3$H]DTG was 20–25%. The values were determined using the iterative curve-fitting program LIGAND and are the mean ± S.E.M. of *n* independent determinations performed in duplicate. *n* represents the number of performed experiments. Asterisk represents a significant difference ($P < 0.05$, ANOVA followed by Scheffe's *F*-test) between placebo and morphine implanted mice.

μg/ml protein) of 50 mM Tris–HCl buffer (pH 7.7) at 37°C. The homogenate was used immediately for binding studies.

### 2.5. Binding assays

Binding of [$^3$H](+)-pentazocine and [$^3$H]DTG to crude membranes of mouse cortex, cerebellum and spinal cord tissues was performed in duplicate in 50 mM Tris–HCl buffer (pH 7.7) at 37°C for 210 min and 60 min respectively, using optimized binding parameters for this tissue that have been previously described (Kovács and Larson, 1995). For determination of equilibrium dissociation values ($K_d$), and the number of binding sites ($B_{max}$), saturation experiments were conducted over a concentration range of 0.05–24 nM [$^3$H](+)-pentazocine and 0.75–72 nM [$^3$H]DTG in the absence and presence of 100 nM (+)-pentazocine. Non-specific binding was defined by addition of a final concentration of 10 μM haloperidol. The assays were terminated by rapid filtration through Whatman GF/C glass fiber filter on a Brandel cell harvester using 3 × 4 ml ice-cold 10 mM Tris–HCl buffer (pH 8.0) at 4°C. Filters were pre-soaked in 0.1% polyethylenimine for 2 h at 4°C prior to use. The filter-bound radioactivity was determined by liquid scintillation spectrometry at a 50% efficiency. Membrane protein concentrations were measured using the method of Lowry et al. (1951) with bovine serum albumin as the standard. Equilibrium-saturation binding data were analyzed with EBDA (Munson and Rodbard, 1980) and LIGAND (McPherson, 1983) computer programs.

### 2.6. Antinociceptive testing

The latency of the tail flick response to a thermal stimulus was determined using the tail immersion, or tail flick assay. Mice were gently restrained and the tail submerged in the water of a bath maintained at 52°C. A cutoff of 12 s was used to avoid tissue damage. Animals that reached this cutoff were assumed to be antinociceptive.

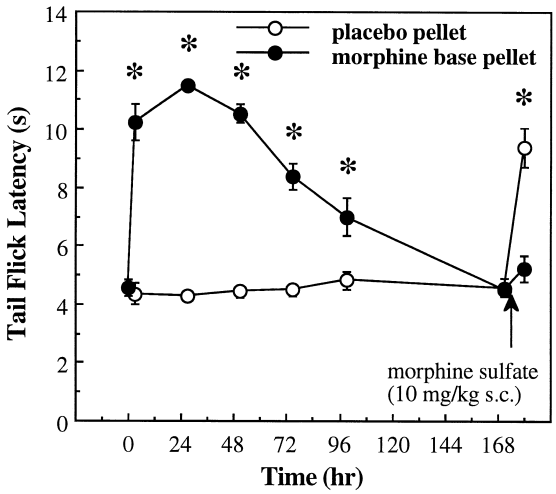


Fig. 1. The degree of antinociception in mice at various times after morphine pellet implantation (75 mg morphine-free base). Complete tolerance to a challenge dose of 10 mg/kg of morphine is observed by day 7 in morphine pellet implanted mice. The tail flick latency values are the mean ± S.E.M. of 3 independent determinations performed using groups of 8 animals in each study. Asterisks represent a significant difference ($P < 0.05$, ANOVA followed by Scheffe's *F*-test) between placebo and morphine pellet implanted mice.

Table 2
Hot saturation binding parameters for [$^3$H]DTG in cortex and cerebellum of morphine treated and control mice 72 h after pellet implantation

| Tissue | $n$ | Placebo pellet | | Morphine pellet | |
|---|---|---|---|---|---|
| | | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) |
| [$^3$H]DTG hot saturation binding, no (+)-pentazocine added | | | | | |
| Cortex | 3 | 20.7 ± 2.0 | 1221 ± 19 | 18.7 ± 2.2 | 1,245 ± 97 |
| Cerebellum | 3 | 21.5 ± 1.8 | 1210 ± 69 | 20.9 ± 1.3 | 1,262 ± 89 |
| [$^3$H]DTG hot saturation binding, +100 nM (+)-pentazocine | | | | | |
| Cortex | 3 | 19.3 ± 0.5 | 811 ± 8 | 16.6 ± 0.7 | 910 ± 10 |
| Cerebellum | 3 | 19.0 ± 0.2 | 806 ± 5 | 16.0 ± 1.1 | 793 ± 26 |

Mice were injected with a single dose (100 mg/kg, s.c.) of morphine; 4 h later they were implanted s.c. with a morphine pellet containing 75 mg morphine free base. Control animals were injected with 0.9% saline and implanted with placebo pellets. Animals were killed 72 h later. Hot saturation binding of [$^3$H]DTG to crude membranes of mouse cortex and cerebellum was performed in 50 mM Tris–HCl buffer (pH 7.7) at 37°C for 60 min. Experiments were conducted over a concentration range of 0.75–72 nM [$^3$H]DTG in the absence and presence of 100 nM (+)-pentazocine. Non-specific binding was defined by addition of a final concentration of 10 $\mu$M haloperidol. The percentage of non-specific binding for [$^3$H]DTG was 8–10%. The values were determined using the iterative curve-fitting program LIGAND and are the mean ± S.E.M. of $n$ independent determinations performed in duplicate. $n$ represents the number of performed experiments.

The mean latency of response for the morphine pellet-implanted group of at least 8 mice was calculated and compared to placebo pellet-implanted control mice tested on the same day. Latencies that were significantly higher than control mice were considered to be antinociceptive.

### 2.7. Statistical analysis

One-way analysis of variance (ANOVA) followed by Scheffe's $F$ test, $P < 0.05$ was used to determine the level of statistical difference between $K_d$ and $B_{max}$ mean values comparing control mice to mice exposed to morphine for various time period. The tail flick latency mean values were statistically analyzed using the same method.

## 3. Results

Computer-assisted Scatchard analysis resulted in the selection of a one-site model as the best fit in every experimental schedule. The values of the Hill coefficients, estimated by the EBDA program, were close to unity. There was no indication of a two-site model.

Our results show that the estimated equilibrium dissociation values ($K_d$) and receptor density ($B_{max}$) values of [$^3$H](+)-pentazocine binding to spinal cord membranes of morphine pellet implanted mice did not differ from control mice sacrificed either 24, 72 h or 7 days later (Table 1). When binding was performed in the presence of 100 nM (+)-pentazocine, to eliminate binding to the $\sigma_1$ subtype, the $K_d$ and $B_{max}$ values of [$^3$H]DTG binding in spinal cord after identical treatment with morphine also remained unchanged compared to that after implantation of a placebo pellet (Table 1). However, measurement of [$^3$H]DTG binding in the absence of (+)-pentazocine, resulted in an moderately elevated binding capacity ($B_{max\ control} = 1386 \pm 56$ fmol/mg protein; $B_{max\ morphine\text{-}treated} = 1681 \pm 49$ fmol/mg protein) 72 h after morphine pellet implantation (Table 1). The increase in $B_{max}$ occurred in the absence of any change in the $K_d$ and recovered by 7 days after implantation of the morphine pellet.

The increase in [$^3$H]DTG binding capacity occurred at a time during the development of tolerance and dependence on morphine when the antinociceptive effect of the morphine pellet implanted mice was beginning to dissipate, as indicated by the gradual return of tail flick latencies to control values (Fig. 1). Typically, morphine pellet-implanted mice did not respond differently than mice implanted with placebo pellets at 7 days after treatment. Complete tolerance to a challenge dose of 10 mg/kg of morphine is observed by day 7 in morphine pellet implanted mice.

Seventy-two hours after morphine pellet implantation, cortex and cerebellum membranes were also used for [$^3$H]DTG binding in the presence and absence of (+)-pentazocine. No significant differences were revealed in either brain section compared the $K_d$ and $B_{max}$ values of placebo and morphine pellet implanted animals (Table 2).

## 4. Discussion

The ability of morphine to enhance [$^3$H]DTG labeled $\sigma$ binding in the mouse spinal cord during the development of morphine tolerance and dependence indicates that morphine treatment does have an impact on $\sigma$ systems. In light of the ability of $\sigma$ receptor ligands to influence morphine activity, such as analgesia (Tulunay et al., 1975; Chien and Pasternak, 1993, 1994), this effect suggests that $\sigma$ activity may play a role in the development of opioid tolerance and/or dependence. However, the identity of the $\sigma$ subtype involved in this effect remains unclear. We have previously shown that the density and distribution of the $\sigma_1$ subtype labeled by [$^3$H]DTG do not parallel those

labeled with [$^3$H](+)-pentazocine (Kovács and Larson, 1995). Based on the present study, 72-h exposure to morphine pellets resulted in an upregulation of [$^3$H]DTG and not [$^3$H](+)-pentazocine labeled $\sigma$ binding, again underscoring the difference between these two sites in the spinal cord area. The increase is presumed to reflect a unique population of high- affinity [$^3$H]DTG binding sites that may be allosterically affected by (+)-pentazocine, but not labeled by [$^3$H](+)-pentazocine. This is supported by the fact that $K_d$ and $B_{max}$ values of the classic, (+)-pentazocine-labeled $\sigma_1$ binding were not altered after the same treatment. However, no increase in [$^3$H]DTG binding was observed when sufficient cold (+)-pentazocine was included to mask $\sigma_1$ binding (Kovács and Larson, 1995), suggesting an allosteric or indirect modulation of morphine-sensitive [$^3$H]DTG binding. Such an interaction is consistent with our previous demonstration of a non-competitive antagonism of [$^3$H]DTG binding by 100 nM (+)-pentazocine, as indicated by its ability to inhibit the $B_{max}$ and not affect the $K_d$. Our previous results also indicate that $\sigma$ sites located in the spinal cord of the mouse are not identical with those in cortex and cerebellum (Kovács and Larson, 1995). Consistent with this, the density of high affinity [$^3H$]DTG labeled binding ($B_{max}$) was elevated 72 h after morphine pellet implantation in the spinal cord but not in the cortex or cerebellum. Thus, the unique binding characteristics of the high-affinity [$^3$H]DTG site may be associated with a unique regulatory system.

Although the results are complicated and the studies have led to conflicting results (for review see Walker et al., 1990; Su, 1993), chronic or repeated treatment with $\sigma$ receptor ligands have been found to alter the estimated $K_d$ and/or $B_{max}$ values of $\sigma$ sites in the CNS. The ability of morphine to up-regulate high affinity [$^3$H]DTG labeled $\sigma$ populations is not likely to occur by a direct interaction at the $\sigma$ site at normal analgesic doses, as morphine has no appreciable affinity ($K_i > 10$ $\mu$M) at $\sigma$ sites in guinea pig brain membranes and PC12 cells labeled with [$^3$H](+)-pentazocine (De Costa et al., 1989; DeHaven-Hudkins et al., 1992), [$^3$H]DTG (Weber et al., 1986; Walker et al., 1992) or [$^3$H](+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ([$^3$H](+)-PPP) (Weber et al., 1986; Hellewell and Bowen, 1990).

Changes in nociception brought about by $\sigma$ ligands may be caused by the modulatory influence of $\sigma$ sites on excitatory amino acid activity (Walker et al., 1990), which are believed to play an important role in nociception. For example, high-affinity $\sigma$ receptor ligands, like DTG, potentiate the excitatory effect of NMDA in the rat hippocampus (Monnet et al., 1990) and in the mouse spinal cord (Hornfeldt et al., 1996), effects that are reversed by haloperidol, but not spiperone or thiothixene, two dopamine receptor antagonists. These data not only support a role for $\sigma$ activity in the NMDA activity, but suggest that DTG-like compounds are $\sigma$ receptor agonists whereas haloperidol-like compounds are $\sigma$ receptor antagonists. Non-NMDA activity is also susceptible to modulation by $\sigma$ receptor ligands as DTG potentiates behaviors induced by the intrathecal injection of kainic acid in mice (Larson and Sun, 1993). An initial and transient increase in kainic acid-induced behaviors occurs within 30 min, which is sensitive to inhibition by haloperidol, while a long-term facilitation of the effect of DTG is observed 24 h after injection of a single dose of DTG. This delayed facilitation is prevented, but not reversed by (+)-5-methyl-10,11-dihydroxy-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine (MK-801), suggesting that sensitization to the effect of DTG occurs via a phencyclidine-sensitive site. One might speculate that a similar MK-801-sensitive pathway may be involved in the generation of morphine tolerance and dependence as (+)-MK-801 (Marek et al., 1991; Trujillo and Akil, 1991) and nitric oxide inhibitors (Cappendijk et al., 1993; Kimes et al., 1993; Kolesnikov et al., 1993; Majeed et al., 1994) attenuate the development of tolerance and dependence to morphine. But as chronic treatment with 1-(1-phenylcyclohexyl)piperidine (PCP) fails to alter $\sigma$ binding (for review see Su, 1993), it is unlikely that inhibition of NMDA activity or an interaction at phencyclidine sites alone accounts for the increased population of [$^3$H]DTG binding sites after morphine treatment. If changes in sigma sites are related to tolerance and/or dependence, one might speculate that calcium flux and/or nitric oxide synthesis, evoked in response to the depolarizing effects of NMDA, may play a role in the up-regulation of $\sigma$ sites observed in the present study.

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