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Neuronal nitric oxide modulates morphine antinociceptive tolerance by enhancing constitutive activity of the μ-opioid receptor

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Abstract

NO is a key mediator of morphine antinociceptive tolerance. This work was conducted to evaluate the specific effects of NO on μ-opioid receptor activity. To investigate the effects of morphine- and L-arginine (the NO precursor)-induced increases in NO, five groups of rats were treated with saline, L-arginine (100-, 300-, or 500-mg/kg/h), or morphine 3-mg/kg/h for 8 h on Day 1; brain tissue was collected on Day 2. To evaluate the effects of additional increases in NO on morphine-induced alterations of the μ-opioid receptor, six groups of rats were treated with 8-h intravenous infusions for two consecutive days as per the following scheme (Day 1:Day 2): saline:saline (control); saline:morphine 3-mg/kg/h (tolerant); L-arginine 500-mg/kg/h:saline (NO control); L-arginine 100-mg/kg/h:morphine 3-mg/kg/h; L-arginine 300-mg/kg/h:morphine 3-mg/kg/h; and L-arginine 500-mg/kg/h:morphine 3-mg/kg/h (supertolerant). Brain tissue was collected at the end of Day 2. The time course of effects on morphine-induced receptor alterations due to increased NO also was evaluated. Brain tissue was analyzed for changes in radioligand (agonist and antagonist) binding and [^{35}S]GTPγS binding (agonist and antagonist). In the absence of agonist exposure, NO produced an alteration in the μ-opioid receptor that increased receptor activity. In the presence of agonist, NO increased constitutive activation of the μ-opioid receptor and reduced the ability of a selective μ-opioid agonist to activate the μ-opioid G-protein-coupled receptor; these molecular effects occurred in a time course consistent with the development of antinociceptive tolerance. This work establishes important NO-induced alterations in μ-opioid receptor functionality, which directly lead to the development of opioid antinociceptive tolerance.

Keywords: Constitutive activity; μ-Opioid receptor; Morphine; Opioid tolerance; Rats

1. Introduction

Opioid analgesics are prescribed widely for management of acute and chronic pain. Morphine, a prototypical opioid, exerts its potent pharmacologic action by binding to the μ-opioid receptor, one of three members of the opioid G-protein-coupled receptor (GPCR) family [1]. Upon morphine-induced activation of the μ-opioid receptor, GDP is released from the $G_i\alpha$ protein subunit and replaced by GTP. This nucleotide exchange facilitates a change in conformation of the $G_i\alpha$ subunit that results in dissociation of the G_i protein heterotrimer into separate $G_i\alpha$ and $G_i\beta\gamma$ subunits. Active GTP-bound $G_i\alpha$ modulates activity of the effector enzyme adenylyl cyclase to decrease intracellular concentration of the second messenger 3′,5′-cyclic monophosphate (cAMP), resulting in activation of inwardly rectifying potassium channels, closing of voltage-gated calcium channels, and subsequently cellular hyperpolarization and reduced pain transmission. While highly effective in the short-term, prolonged opioid use can result in tolerance, or the gradual loss of antinociceptive effect with time. Despite extensive research in the area of opioid tolerance, the exact mechanisms by which this process occurs remain largely unknown.

Extensive in vivo and in vitro investigations have demonstrated that molecular alterations in the μ-opioid

Abbreviations: DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol^5]enkephalin; GDP, guanosine diphosphate; GPCR, G-protein-coupled receptor; GppNHp, 5′guanylylimidodiphosphate; GTPγS, guanosine5′-G-(3-thio)triphosphate; NaCl, sodium chloride; NO, nitric oxide

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receptor and its associated GPCR signaling molecules likely are involved in morphine antinociceptive tolerance. Early investigations identified decreases in μ-opioid receptor number [2–4], attenuated G-protein activation [5–7], and impaired modulation of adenylyl cyclase activity with prolonged morphine administration [8,9]. Furthermore, it has been suggested that morphine tolerance development may be due to enhanced constitutive activity of the μ-opioid receptor [10]. The phenomenon of constitutive activity has been observed for many GPCRs [11], including μ-opioid receptors [10], and is predicted by the extended ternary complex model that assumes GPCRs can exist in two functionally distinct states, inactive (R) and active (R^*) [12]. In the absence of agonist, the level of basal or constitutive activity is determined by the equilibrium between the R and R^* states, while the efficacy of an agonist ligand is determined by the ability of the agonist to alter the equilibrium between the two states. In vitro cellular investigations have demonstrated the presence of an enhanced constitutively active state for the μ-opioid receptor following chronic exposure to μ-opioid agonists. Evidence for this phenomenon includes apparent inverse agonist activity of the μ-opioid antagonists naloxone, naltrexone, and β-chlornaltrexamine [13–15]. Recently, agonist-induced enhancement of μ-opioid receptor constitutive activity also has been demonstrated in vivo [16]. In the presence of enhanced basal stimulation, opioid peptides exhibit reduced agonist-stimulated G protein activation [14].

In addition to specific receptor-based alterations, nitric oxide (NO) appears to be involved in the attenuation of analgesic effect with extended opioid administration [17]. Increased NO production, either prior to or concurrently with opioid administration, results in an enhanced rate and extent of antinociceptive tolerance development [18,19]. Likewise, inhibition of NO production attenuates the tolerance effect with time [20–25]. These effects have been linked to NO production by the neuronal isoform of NO synthase [25–27]. Previous work in this laboratory has established that morphine increases neuronal NO production, and that the changes in brain NO content occur in a close temporal relationship with the loss of antinociceptive effect [28]. Based on these observations, NO appears to be a key modulator of morphine tolerance, and may be involved in molecular receptor-based adaptations observed with chronic μ-opioid receptor agonist administration. This study was conducted to investigate the effects of NO on μ-opioid receptor agonist and antagonist binding and $G_i\alpha$ activation in the presence and absence of morphine. The NO precursor L-arginine was used to increase systematically neuronal NO in vivo in the presence or absence of morphine, according to previously documented dosing regimens [29]. The time course of NO-mediated changes of the μ-opioid receptor and the relationship of these alterations with the development of antinociceptive tolerance also is discussed.

2. Materials and methods

2.1. Chemicals

Morphine sulfate was obtained from Spectrum Chemical. DAMGO, GDP, GppNHp, GTPγS, L-arginine, levallorphan, and naloxone were obtained from Sigma Aldrich. [^{3}H]-Naloxone and [^{3}H]-DAMGO were obtained from American Radiolabeled Chemicals. [^{35}S]GTPγS was obtained from Amersham Biosciences. All other chemicals and reagents used in these experiments were from commercial sources and were of the highest purity available.

2.2. Animals

Adult male Sprague-Dawley rats (320–370 g) were purchased from Charles River Laboratories. Animals were housed in a temperature-controlled facility (72 ± 2 °F) on a 12-h light cycle (7 a.m. to 7 p.m.) and were allowed free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, and were conducted in accordance with accepted standards for laboratory animal care.

Rats were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (10 mg/kg). A silicone rubber cannula (Helix Medical Inc.) was implanted in the right femoral vein and exteriorized at the dorsal neck region of each rat. Patency of the cannulae was maintained with heparinized saline (20 U/mL) during the recovery period. Animals were housed separately following surgery and allowed a minimum of 36 h to recover prior to experimentation.

2.3. Experimental design

To investigate alterations in the μ-opioid receptor system due to elevated NO production per se (in the absence of morphine), four groups of rats (n = 3/group) were treated with saline or L-arginine (100-, 300-, or 500-mg/kg/h, prepared in normal saline; pH 7–8) by intravenous infusion for 8 h starting at 9 a.m. (Day 1). For comparison, fifth group of rats received an 8-h morphine infusion (3-mg/kg/h, administered as morphine sulfate diluted in normal saline). At 9 a.m. the following day (Day 2), the rats were sacrificed by decapitation and brain tissue was collected.

To investigate receptor alterations due to morphine in combination with increased NO production, rats received saline or L-arginine 100-, 300-, or 500-mg/kg/h for 8 h starting at 9 a.m. of Day 1 as in the preceding experiment. On Day 2, rats received an 8-h infusion of saline or morphine at a rate of 3-mg/kg/h (as morphine sulfate). At 5 p.m. on Day 2 (immediately following infusion cessation) the rats were sacrificed by decapitation and brain tissue collected. The following six treatment groups (n = 3/group) were evaluated in this experiment (Day 1:Day 2): (1) saline:saline, (2) saline:morphine 3-mg/kg/h, (3) L-arginine 500-mg/

kg/h:saline, (4) L-arginine 100-mg/kg/h:morphine 3-mg/kg/h, (5) L-arginine 300-mg/kg/h:morphine 3-mg/kg/h, and (6) L-arginine 500-mg/kg/h:morphine 3-mg/kg/h. Throughout this paper, the saline:saline treatment will be referred to as "control," the L-arginine 500-mg/kg/h treatment will be referred to as the "NO control," the saline:morphine treatment will be referred to as "tolerant," and the L-arginine 500-mg/kg/h:morphine treatment will be referred to as "supertolerant." These designations are based on work conducted previously in this laboratory evaluating morphine-induced antinociception in the presence or absence of L-arginine (NO) pretreatment. This work established that an 8-h morphine infusion produced significant tolerance development, and that this effect was amplified substantially (in effect producing a hyperalgesic state) following pretreatment with L-arginine [28].

To investigate the time course of morphine-induced receptor alterations following L-arginine pretreatment, four groups of rats ($n = 3$/group) were treated with L-arginine 500-mg/kg/h for 8 h starting at 9 a.m. on Day 1. On Day 2, morphine 3-mg/kg/h (as morphine sulfate) was infused starting at 9 a.m. Groups of rats were sacrificed and brain tissue collected at 0 h, 2 h, 4 h, and 6 h during the morphine infusion.

All infusions were conducted at a flow rate of 1.2 mL/h. The left and right brain hemispheres (excluding the brain stem and cerebellum) were excised following decapitation and immediately frozen in liquid nitrogen. Brain tissue was stored at −80 °C.

2.4. Preparation of membrane fragments

Membrane fragments were prepared as reported previously with slight modifications [30]. Brain tissue (left hemisphere) was homogenized in 20 volumes of homogenization buffer [50 mM Tris buffer (pH 7.7), 10 μM phenyl-methyl-sulfonyl fluoride, 100 mM NaCl, and 1 mM K^+EDTA] using a Model 100 Ultrasonic Dismembranator (Fisher Scientific) at power setting 1. The mixture was incubated for 15 min at room temperature. The sample then was centrifuged at 48,000 × *g* for 10 min, supernatant was discarded, the pellet was rehomogenized in 20 volumes of homogenization buffer, and centrifugation was repeated. The resultant supernatant again was discarded and the pellet was resuspended in 3 mL of homogenization buffer and frozen at −80 °C in either 1-mL or 200-μL aliquots. Total protein concentration in the membrane fragments was determined, following a 10-fold dilution of the samples, using a BCA (bicinchoninic acid) Protein Assay kit (Pierce Biotechnologies).

2.5. Radioreceptor binding assays

Radioreceptor assays used membrane preparation diluted to 1 mg protein/mL in assay buffer (potassium phosphate 50 mM, magnesium sulfate 5 mM; pH 7.4) and were conducted at 25 °C for 1 h. Nonspecific binding was determined in the presence of 1 μM levallorphan. For saturation isotherms, 500 μL of stock membrane homogenate was incubated with 0.05 to 10 nM [^{3}H]-DAMGO or 1–50 nM [^{3}H]-naloxone. For competition binding assays, 500 μL of the membrane preparation was incubated with [^{3}H]-DAMGO (1.0 nM) or [^{3}H]-naloxone (2.5 nM) and 0.1–10,000 nM test ligand. To investigate differences in ligand functional binding, 25 μM GppNHp (plus 100 mM NaCl) was included in some assays to shift receptor equilibrium to a G protein-uncoupled state; the ternary complex model predicts lower agonist-type ligand affinity under these conditions. Membrane fragments were pooled across treatment groups for determination of μ-opioid receptor density and binding affinity for [^{3}H]-naloxone and [^{3}H]-DAMGO in the presence and absence of GppNHp/NaCl. Binding reactions were terminated by addition of ice-cold buffer followed by rapid filtration through glass fiber filters (GF/C; Brandel) presoaked in 0.5% (w/v) polyethylimine. Filters were washed three times with ice-cold buffer, added to scintillation cocktail (UltimaGold XR; Packard Bioscience), and total radioactivity associated with the filters was determined by liquid scintillation spectroscopy (Packard 1600TR).

2.6. [^{35}S]GTPγS binding assay

Membrane fragments were diluted with reaction buffer [Tris Buffer 50 mM (pH 7.4), EGTA 0.2 mM, NaCl 100 mM, $MgCl_2$ 3 mM] to a total protein concentration of 25 μg/mL. The reaction mixture consisted of GDP (100 μM), [^{35}S]GTPγS (0.05 nM), and varying concentrations of DAMGO or naloxone. GDP was added to the membrane preparation 5 min before reaction initiation. Basal binding was determined in the absence of agonist (presence of GDP and [^{35}S]GTPγS) and nonspecific binding was evaluated with the addition of GTPγS (10 μM) to the agonist-free preparation. Reaction incubations were conducted in a recirculating water bath at 30 °C for 1 h. Reactions were terminated by filtration over glass fiber filters (GF/B; Brandel) presoaked in reaction buffer for 15 s prior to use. Filters then were washed three times with 3 mL of ice cold reaction buffer and radioactivity was determined as described above.

2.7. Morphine analysis in brain tissue

Concentrations of morphine in brain tissue were determined by LC–MS. Morphine was extracted from brain tissue homogenate (90 μL) following the addition of nalorphine (internal standard; 10 μL, 400 ng/mL in DMSO). Sodium bicarbonate [100 μL; 0.1 M (pH 9)] was added to each sample, which was mixed by vortex, and incubated at room temperature for 5 min. An ether:-chloroform (80:20) mixture (2 mL) then was added to each sample and vortexed for 30 s. The sample centrifuged 5000 × *g* for 5 min and the aqueous layer was frozen

immediately by placing the sample tube in an acetone/dry ice bath. The organic layer then was decanted and evaporated to dryness under a stream of nitrogen. The sample was reconstituted in 1 mL of 50:50 methanol:deionized water and injected (5 μL) onto a Phenomenex 2.0 mm × 30 mm 4 μM Synergi Max-RP column (Phenomenex) maintained at room temperature. Chromatographic separation was achieved with a mobile phase gradient of 20 mM ammonium acetate (pH 6; 95–5% linear gradient 0–3 min, 5% for 3–3.5 min, 95% for 3.5–5 min)/methanol. The system was returned to initial conditions at 5 min and allowed to reequilibrate for 1 min. Morphine and nalorphine were quantitated using an 1100 Series Liquid Chromatograph/Mass Selective Detector (LC/MSD; Agilent Technologies) with electrospray-ionization (ESI). Mass detection of morphine (*m/z* 286.1) and nalorphine (*m/z* 312.2) was performed in positive ion monitoring mode. Standard curves were prepared in blank brain tissue homogenate. The lower limit of quantitation was 5 ng/mL. Inter- and intraday coefficients of variation were <15%.

2.8. Data analysis

Nonlinear least-squares regression was used to recover parameter estimates for B_{max} (μ-opioid receptor density), K_{dDAMGO} (DAMGO dissociation constant), and $K_{iMORPHINE}$ (inhibitory dissociation constant for competitive displacement of [^{3}H]-DAMGO by morphine) by simultaneously fitting the following saturation and competitive displacement functions to the binding data:

$$[^3\mathrm{H}] - \mathrm{DAMGO}_{\mathrm{bound}} = \frac{B_{max}S}{K_{dDAMGO} + S} \quad (1)$$

$$[^3\mathrm{H}] - \mathrm{DAMGO}_{\mathrm{bound}} = \frac{B_{max}S}{S + (K_{dDAMGO}(1 + (I/K_{iMORPHINE}))} \quad (2)$$

where S and I represent concentrations of substrate ([^{3}H]-DAMGO) and inhibitor (morphine), respectively.

Membrane fragments were pooled among treatments for determination of B_{max} and K_d of [^{3}H]-naloxone and [^{3}H]DAMGO in the presence and absence of GppNHp/NaCl in the control and supertolerant groups. The K_{iDAMGO} was estimated by fitting the simple saturation curve for [^{3}H]-naloxone with and without GppNHp/NaCl (pooled data) simultaneously with the data defining the competitive displacement of DAMGO displacement of 2.5 nM [^{3}H]-naloxone using Eqs. (1) and (2).

All nonlinear least-squares regression analyses were performed using WinNonlin v3.2 (Pharsight Corporation). Both one- and two-site models were fit to the data collected from both the saturation and competitive binding experiments. However, the two site model was not supported by the data as determined by evaluation of Akaike's Information Criterion between model fits.

A simple E_{max} function was fit to all [^{35}S]GTPγS binding data to recover estimates of E_{max}, $EC_{50,}$ and E_{min} (minimum % stimulation in the presence of agonist).

Analysis of variance (ANOVA) was used to identify statistically significant differences between groups. Tukey post tests were applied when multiple comparisons were made among groups. Where appropriate, a Student's *t*-test was used to compare differences between two groups. In all cases, the criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Effects of L-arginine on agonist binding at the μ-opioid receptor

Parameter estimates for B_{max} and K_D determined from [^{3}H]-DAMGO binding to μ-opioid receptors, and K_i values for competitive inhibition of [^{3}H]-DAMGO binding by morphine are summarized according to the different treatment groups in Tables 1 and 2. Infusion of the NO precursor L-arginine, morphine, or combinations of the two did not alter significantly the B_{max} or K_D for binding of [^{3}H]-DAMGO. Similarly, the experimental treatments did not alter the K_i for morphine.

3.2. Effects of L-arginine on agonist stimulation of [^{35}S]GTPγS binding

Estimates for E_{max}, EC_{50}, and E_{min} for agonist-stimulated [^{35}S]GTPγS binding at the μ-opioid receptor for the different treatment groups are summarized in Tables 3 and 4. Infusion of the NO precursor L-arginine, in the absence

Table 1
Effect of NO and morphine on μ-opioid receptor density and agonist binding affinities

Treatment		[^{3}H]-DAMGO, B_{max} (fmol/mg protein)	[^{3}H]-DAMGO, K_D (nM)	Morphine, K_i (nM)
Day 1	Day 2			
SAL	TC 9 a.m.	36.4 ± 5	0.36 ± 0.2	0.8 ± 0.2
A100	TC 9 a.m.	38.4 ± 10	0.44 ± 0.1	1.2 ± 0.4
A300	TC 9 a.m.	39.9 ± 4	0.44 ± 0.3	0.93 ± 0.4
A500	TC 9 a.m.	38.3 ± 5	0.49 ± 0.4	0.9 ± 0.3
M3	TC 9 a.m.	38.9 ± 9	0.33 ± 0.2	0.51 ± 0.2

Data are presented mean ± S.D. Treatments (infusion regimens over 8 h) are abbreviated as follows: saline (SAL), L-arginine 100-mg/kg/h (ARG 100), L-arginine 300-mg/kg/h (ARG 300), L-arginine 500-mg/kg/h (ARG 500), and morphine 3-mg/kg/h (M3). Time of tissue collection (TC) also is given in the table, no statistically significant changes were observed among treatments ($p > 0.1$).

Table 2
Effect of NO elevations on morphine-induced alterations in μ-opioid receptor density and agonist binding affinities

Treatment		[^{3}H]-DAMGO, B_{max} (fmol/mg protein)	[^{3}H]-DAMGO, K_D (nM)	Morphine, K_i (nM)
Day 1	Day 2			
SAL	SAL, TC 5 p.m.	42.7 ± 10	0.43 ± 0.2	2.0 ± 0.3
SAL	M3, TC 5 p.m.	48.3 ± 10	0.35 ± 0.1	1.7 ± 0.8
A500	M3, TC 5 p.m.	49.4 ± 7	0.47 ± 0.2	1.6 ± 0.6

Data are presented mean ± S.D. Treatments (infusion regimens over 8 h) are abbreviated as follows: saline (SAL), L-arginine 500-mg/kg/h (ARG 500), and morphine 3-mg/kg/h (M3). Time of tissue collection (TC) also is given in the table, no statistical changes were observed between treatments.

of in vivo morphine administration, resulted in a dose-dependent increase in efficacy (E_{max}) of DAMGO to stimulate [^{35}S]GTPγS binding (ANOVA, $p = 0.004$; Table 3, Fig. 1). The potency of DAMGO in stimulating [^{35}S]GTPγS binding also was increased by the highest dose of L-arginine (500-mg/kg/h) as evidenced by a decreased value of EC_{50} when compared to saline treated animals (Table 3, $p = 0.013$). Infusion of morphine alone also increased (~45%) the E_{max} for DAMGO-induced [^{35}S]GTPγS binding when compared to saline (Fig. 1, Table 3, $p = 0.008$), however, no changes in EC_{50} were evident in this treatment group ($p = 0.232$).

Animals pretreated with L-arginine, followed by a prolonged morphine infusion (supertolerant state) evidenced compromised efficacy of DAMGO to stimulate [^{35}S]GTPγS binding when compared to control, NO control, and tolerant groups (Fig. 2A, Table 4, ANOVA, $p = 0.010$). However, decreased efficacy of DAMGO in supertolerant animals also was accompanied by a significant reduction in E_{min} values (Fig. 2A and Table 4). Even after accounting for a reduced E_{min}, the supertolerant animals show a statistically significant reduction in absolute stimulation capacity ($E_{max} - E_{min}$ differential) for DAMGO-induced stimulation of [^{35}S]GTPγS binding compared to the NO control group ($E_{max} - E_{min}$ differential: 37 ± 20 and 130 ± 30 for supertolerant and NO control groups, respectively; ANOVA, $p = 0.007$). There was no statistically significant effect of treatment on $E_{max} - E_{min}$ differentials observed between the tolerant and saline control group, supertolerant and control group, or in the animals infused with intermediate L-arginine infusion rate (100- and 300-mg/kg/h) compared to the control. These observed reductions in efficacy in animals pretreated with L-arginine, followed by morphine, were not associated with alterations in potency of DAMGO-mediated activation of the μ-opioid receptor as evidenced by no detectable changes in EC_{50} across treatment groups (Table 4, ANOVA, $p = 0.508$).

In contrast to a reduced E_{max} for DAMGO stimulation of [^{35}S]GTPγS binding, a significant increase in basal [^{35}S]GTPγS binding (binding in the absence of agonist), a measure of μ-opioid receptor constitutive activity, was observed in animals pretreated with L-arginine followed by morphine (ANOVA, $p = 0.023$, Fig. 2B). While not eliciting statistical changes in μ-opioid receptor constitutive activity, lower L-arginine infusion rates followed by morphine promoted what appeared to be incremental increases in basal [^{35}S]GTPγS binding. In the absence of morphine treatment, however, L-arginine had no effect on μ-opioid receptor constitutive activity (Fig. 2B).

3.3. DAMGO inhibition of [^{3}H]-naloxone binding and naloxone inhibition of [^{35}S]GTPγS binding

Fig. 3 shows results for the four treatment groups regarding competitive displacement of [^{3}H]-naloxone binding by DAMGO in the absence or presence of 25 μM GppNHp plus 100 mM NaCl (a condition that

Table 3
Effect of elevations in NO on DAMGO stimulated [^{35}S]GTPγS binding parameters following L-arginine treatment regimens to increase neuronal NO

Treatment		E_{max} (% basal)	EC_{50} (nM)	E_{min} (% basal)
Day 1	Day 2			
SAL 8 h	TC 9 a.m.	150 ± 5	260 ± 50	100 ± 3
A100 8 h	TC 9 a.m.	150 ± 20	140 ± 80	100 ± 5
A300 8 h	TC 9 a.m.	180 ± 10	260 ± 60	100 ± 3
A500 8 h	TC 9 a.m.	200 ± 20**	57 ± 20*	110 ± 6
M3 8 h	TC 9 a.m.	220 ± 30**	190 ± 70	120 ± 20

Data are presented mean ± S.D. Treatments (infusion regimens over 8 h) are abbreviated as follows: saline (SAL), L-arginine 100-mg/kg/h (ARG 100), L-arginine 300-mg/kg/h (ARG 300), L-arginine 500-mg/kg/h (ARG 500), and morphine 3-mg/kg/h (M3). Time of tissue collection (TC) also is given in the table.
* $p < 0.05$ compared to saline control.
** $p < 0.01$ compared to saline control.

Table 4
Effect of NO on morphine-induced alterations in DAMGO stimulated [^{35}S]GTPγS binding parameters following specified treatments

Treatment		E_{max} (% basal)	EC_{50} (nM)	E_{min} (% basal)
Day 1	Day 2			
SAL 8 h	SAL 8 h, TC 5 p.m.	150 ± 3	110 ± 100	96 ± 2
A500 8 h	SAL 8 h, TC 5 p.m.	220 ± 20	400 ± 300	91 ± 10
SAL 8 h	M3 8 h, TC 5 p.m.	130 ± 50	370 ± 400	65 ± 20
A100 8 h	M3 8 h, TC 5 p.m.	150 ± 80	130 ± 100	80 ± 40
A300 8 h	M3 8 h, TC 5 p.m.	140 ± 50	440 ± 60	70 ± 30
A500 8 h	M3 8 h, TC 5 p.m.	100 ± 20	200 ± 90	60 ± 7

Data are presented mean ± S.D. Treatments (infusion regimens over 8 h) are abbreviated as follows: saline (SAL), L-arginine 100-mg/kg/h (ARG 100), L-arginine 300-mg/kg/h (ARG 300), L-arginine 500-mg/kg/h (ARG 500), and morphine 3-mg/kg/h (M3). Time of tissue collection (TC) also is given in the table, statistical comparisons are presented in Section 3.

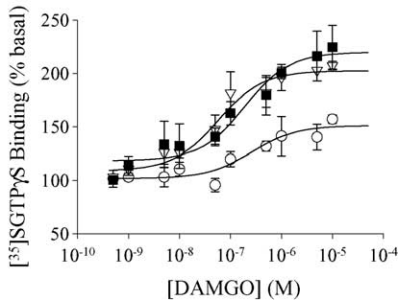

Fig. 1. DAMGO-induced stimulation of [^{35}S]GTPγS binding to membrane fragments prepared from animals infused for 8 h with L-arginine 500-mg/kg/h (▽), morphine 3-mg/kg/h (■), or saline (○). Tissue collections occurred following a washout period. Data are presented mean ± S.E. Intermediate L-arginine treatment groups were omitted for visual clarity (see Table 1).

shifts GPCR equilibrium to a G-protein-uncoupled state). In the supertolerant group, the curve describing the displacement of [^{3}H]-naloxone by DAMGO in the presence of GppNHp and NaCl was shifted upward and to the right when compared to binding to membrane fragments containing receptors in the G-protein-coupled state across all four treatment groups (Fig. 3). K_{iDAMGO} values for the

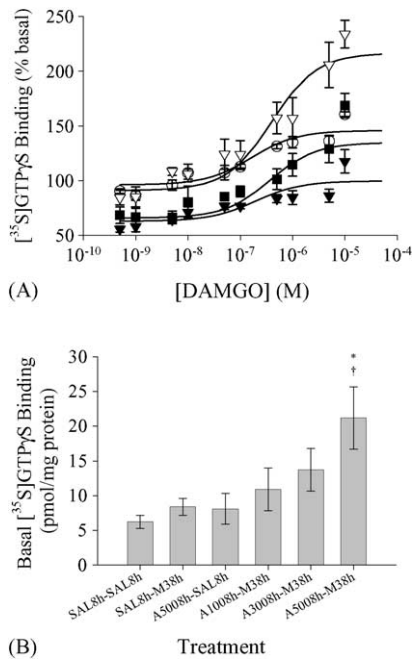

Fig. 2. (A) DAMGO-induced stimulation of [^{35}S]GTPγS binding to membrane fragments prepared from animals infused for 8 h with (Day 1:Day 2): saline:saline (control) (○), saline:morphine 3-mg/kg/h (tolerant) (■), L-arginine 500-mg/kg/h:saline (NO control) (▽), L-arginine 500-mg/kg/h:morphine 3-mg/kg/h (supertolerant) (▼). Intermediate L-arginine treatment groups were omitted for visual clarity (see Table 4). (B) Alterations in basal [^{35}S]GTPγS binding in thoroughly washed membrane fragments from animals infused for 8 h with saline:saline, saline:morphine 3-mg/kg/h, L-arginine 500-mg/kg/h:saline, L-arginine 100-mg/kg/h:morphine 3-mg/kg/h, L-arginine 300-mg/kg/h:morphine 3-mg/kg/h, and L-arginine 500-mg/kg/h:morphine 3-mg/kg/h. Data are presented mean ± S.E. $^{*}p < 0.05$ compared to saline:saline (control), $^{\dagger}p < 0.05$ compared saline:morphine 3-mg/kg/h (tolerant group).

nontolerant control with and without GppNHp/NaCl were 12 ± 10 and 15 ± 5 nM, respectively (*t*-test, $p = 0.444$). However, in the supertolerant animals the K_{iDAMGO} was increased when the receptor was in the uncoupled state (26 ± 5 and 13 ± 4, with and without GppNHp/NaCl, respectively; *t*-test, $p = 0.025$).

Effects of naloxone on [^{35}S]GTPγS binding are depicted in Fig. 4. Naloxone dose-dependently inhibited [^{35}S]GTPγS binding in membranes prepared from supertolerant animals when compared to the saline control (two-way ANOVA, $p < 0.001$), however, this dose-dependent reduction was not detectable in tolerant animals (two-way ANOVA, $p = 0.868$). Naloxone produced neither stimulation nor inhibition of [^{35}S]GTPγS binding in membrane fragments prepared from saline control or NO control groups (data not shown).

3.4. Effects of L-arginine on brain morphine concentrations

Morphine concentrations in tissue prepared from rats comprising the treatment groups saline:morphine 3-mg/kg/h (tolerant), L-arginine 100-mg/kg/h:morphine 3-mg/kg/h, L-arginine 300-mg/kg/h:morphine 3-mg/kg/h, and L-arginine 500-mg/kg/h:morphine 3-mg/kg/h (supertolerant), were 160 ± 60, 110 ± 40, 160 ± 30, and 80 ± 20 ng/g, respectively.

3.5. Time course of NO-induced receptor alterations

A clear time course of receptor activity changes (constitutive activity of the μ-opioid receptor and maximal agonist-stimulated [^{35}S]GTPγS binding) was observed in rats pretreated with an 8 h infusion of L-arginine on Day 1 followed by a morphine 3-mg/kg/h intravenous infusion on Day 2 for increasing durations of time (Fig. 5). Constitutive activity at the μ-opioid receptor increased beginning at 4 h and continued until cessation of the morphine infusion at 8 h; decreases in E_{max} for agonist-stimulated [^{35}S]GTPγS binding exhibited a similar time course over the 8-h time period. In addition, naloxone-induced inhibition of [^{35}S]GTPγS binding occurred in a time-dependent fashion that closely paralleled alterations in constitutive activation of the μ-opioid receptor (data not shown). The NO-induced receptor alterations occurred in a time course corresponding to antinociceptive tolerance development to an intravenous morphine infusion (3-mg/kg/h) (Fig. 5; data adapted from Heinzen et al. [28]).

4. Discussion

This study provides evidence that increased brain neuronal NO production in response to administration of the NO precursor L-arginine modulates μ-opioid receptor activity in rats. Results presented herein indicate that efficacy and

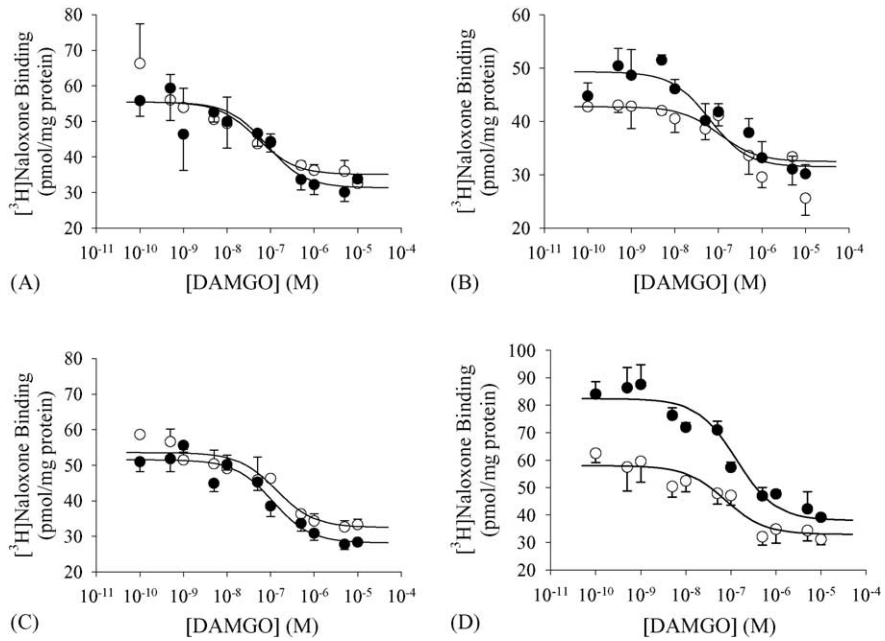

Fig. 3. DAMGO displacement of [^{3}H]naloxone binding in the presence (●) and absence (○) of GppNHp/NaCl in animals treated with a 8 h infusion of (Day 1:Day 2): (A) saline:saline (control), (B) saline:morphine 3-mg/kg/h (tolerant), (C) L-arginine 500-mg/kg/h:saline (NO control), (D) L-arginine 500-mg/kg/h:morphine 3-mg/kg/h (supertolerant). Data are presented mean ± S.E.

potency of the selective μ-opioid receptor agonist DAMGO to activate μ-opioid receptor [^{35}S]GTPγS binding is enhanced after L-arginine infusion (increased E_{max} and decreases EC_{50}; Fig. 1, Table 3). The enhanced efficacy of DAMGO is not due to NO-induced increases in μ-opioid receptor expression, as the B_{max} for [^{3}H]-DAMGO saturation binding was not significantly increased. [^{3}H]-DAMGO μ-opioid receptor affinity also was not changed after L-arginine infusion (Table 1). Although not measured here, it is possible that the increased efficacy in response to L-arginine could be due an up-regulation of $G_i\alpha$ protein and/or enhanced μ-opioid receptor–$G_i\alpha$ coupling.

Results from these experiments also support a second role of NO in modulating μ-opioid receptor activity, one that depends of morphine-induced activation of the receptor. Increased brain neuronal NO production in the presence of morphine produces a supertolerant state that results in compromised efficacy of DAMGO to activate μ-opioid receptor [^{35}S]GTPγS binding (Table 4, Fig. 2A). This effect likely is observed as a consequence of the enhanced μ-opioid receptor constitutive activity that is apparent in the supertolerant state.

The phenomenon wherein GPCRs can couple to and activate G proteins and intracellular signaling cascades independent of agonist (constitutive activity) has been observed for a large variety of GPCRs [31,32]. To account for GPCR constitutive activity, the ternary complex model (ligand, receptor, G protein) has been extended to hypothesize that GPCRs exist in two functionally distinct states, inactive (R) and active (R^*) [12]. In the absence of agonist,

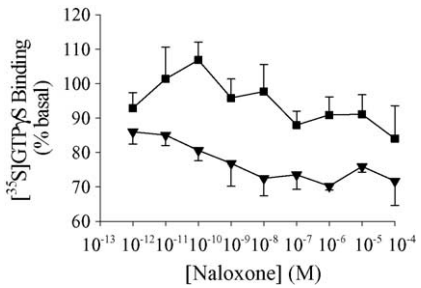

Fig. 4. Naloxone-induced inhibition of [^{35}S]GTPγS binding in membrane fragments prepared from rats treated with an 8 h infusion of (Day1:Day2): saline:morphine 3-mg/kg/h (tolerant rats) (■) or L-arginine 500-mg/kg/h:morphine 3-mg/kg/h (supertolerant rats) (▼). Data are presented mean ± S.E.

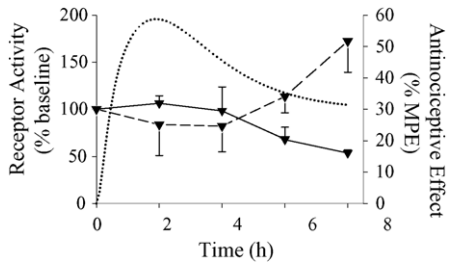

Fig. 5. Receptor activity [constitutive activity (- - - ▼ - - -) or % reduction of maximal DAMGO-induced [^{35}S]GTPγS binding(‾▼‾)] vs. time in rats treated with an 8-h infusion of L-arginine 500 mg/kg/h on Day 1, and a morphine 3-mg/kg/h infusion for 0, 2, 4, 6, and 8 h on Day 2 (primary *y*-axis). Receptor data is reported as % change from data collected at time 0 (% baseline). Dotted line (· · · ·) indicates antinociceptive effect (% MPE) (secondary *y*-axis) following an 8-h morphine infusion 3 mg/kg/h vs. time [data adapted from Heinzen et al. [28]].

the level of basal or constitutive activity is determined by the equilibrium between the R and R^* states, while the efficacy of an agonist is a function of the ability of the agonist to alter the equilibrium between the two states. The isomerization between active and inactive GPCR states is a matter of conformational changes in receptor structure. It has been suggested that stabilizing intramolecular interactions within the α-helical loops of GPCRs serve to constrain the receptor in the inactive conformation, and that these constraints can be released upon agonist binding to the receptor, leading to changes in tertiary structure that facilitate G protein coupling and activation [31,33–35]. Agonist ligands may induce, select, and/or stabilize active conformations of the receptor, while inverse agonists recognize receptors in the inactive state and inhibit release of activating receptor constraints. In theory, a neutral antagonist does not distinguish between active or inactive receptor states [36].

Enhanced μ-opioid receptor constitutive activity following prolonged agonist administration has been well-documented in the literature [10,13–16,37]. The most conclusive evidence for upregulated μ-opioid receptor basal activity in an opioid-tolerant state is the observation that neutral antagonists can evidence inverse agonist functional activity [13,14,16]. Constitutive activity at the μ-opioid receptor, as indicated by apparent conversion of antagonists to inverse agonists, has been well-documented in clonal cell lines transfected with μ-opioid receptor cDNA [10,13,14] and more recently in vivo [16]. Direct evidence for enhanced constitutive activity at the μ-opioid receptor due to prolonged opioid agonist exposure (i.e., evidence for increased basal activity when compared to the untreated state) has been demonstrated in a μ-opioid receptor cell line [14]. Due to analytical sensitivity, in vivo increases in basal μ-opioid receptor activity, which are known to be present due to evaluation of the intrinsic activity of antagonists are not detectable. Consistent with morphine alone eliciting only a slight increase in basal signaling, the experiments conducted herein likewise were unable to document enhanced constitutive activity at the μ-opioid receptor (an increase in [^{35}S]GTPγS binding in the absence of agonist) in animals treated for 8 h with morphine (tolerant group). However, with the addition of NO pretreatment with L-arginine (supertolerant group), an upregulation in basal signaling at the μ-opioid receptor was observed following extended morphine exposure, as evidenced by enhanced [^{35}S]GTPγS binding in the absence of agonist (Fig. 2B). This effect could be attributed to an increased concentration of morphine in the brain tissue of supertolerant animals. However, no changes were observed in brain morphine concentrations between treatment groups suggesting that alterations in constitutive activity observed in these investigations are occurring downstream of agonist presentation at the receptor.

To provide additional validation for the observed increase in μ-opioid receptor constitutive activity in these experiments, naloxone binding and functional analyses also were performed in membrane fragments collected from control rats and animals rendered tolerant and supertolerant. The ability of DAMGO to displace [^{3}H]-naloxone with and without GppNHp/NaCl was unchanged in the control and morphine tolerant animals (Fig. 3A–C). However, the supertolerant group demonstrated approximately a two-fold shift in K_{iDAMGO}. Theoretically, K_{iDAMGO} would reflect the dissociation constant of DAMGO for the μ-opioid receptor. However, supplemental investigations (in membrane fragments pooled within treatment groups) revealed that the addition of GppNHp/NaCl reduced the binding affinity of [^{3}H]-DAMGO consistently across treatment groups, and enhanced affinity of [^{3}H]-naloxone only in the supertolerant group; no changes in B_{max} for [^{3}H]-naloxone or [^{3}H]-DAMGO with the addition of GppNHp/NaCl was observed (data not shown). The decreased affinity of DAMGO with the addition of GppNHp/NaCl was apparently undetectable in the displacement assays, as the control, NO control, and tolerant groups demonstrated no change in the displacement of [^{3}H]-naloxone by DAMGO. This observation suggests that the changes in K_{iDAMGO} in the supertolerant group reflect, at least in part, enhanced binding of naloxone in the uncoupled receptor state, which is consistent with naloxone behaving as an inverse agonist in this preparation. In addition to binding data indicating inverse agonism by naloxone, treatment of membrane fragments with naloxone indicated reduced basal stimulation of the GPCR in the supertolerant state (Fig. 4), consistent with inverse agonist activity of naloxone in the functionally supertolerant state. This conversion was not detectable in the saline treated or tolerant treatment groups. Taken together, these data reveal that constitutive activity at the μ-opioid receptor is enhanced in animals rendered supertolerant by increased NO production in combination with prolonged morphine exposure.

While a decrease in maximum agonist-induced stimulation in [^{35}S]GTPγS binding is consistent with tolerance development [5–7], substantial decreases in [^{35}S]GTPγS binding following the addition of low concentrations of agonist to membranes prepared from supertolerant rats (substantial reductions from basal stimulation with DAMGO treatment, see E_{min} estimates in Table 4) was unexpected. This result could be due to DAMGO binding to the constitutively active receptor state and eliciting an inverse response (conversion of agonist-like intrinsic activity to inverse agonist activity). However, the presence of GppNHp and NaCl reduced the binding affinity of DAMGO across membrane fragments from control, tolerant, and supertolerant rats as compared to membranes evaluated in the absence of receptor uncoupling agents (data not shown). This observation suggests that DAMGO remains an agonist despite alterations in the constitutive activity at the μ-opioid receptor. With the addition of DAMGO, it is possible that the receptor equilibrium

favoring the constitutively active state may be shifted back towards the nontolerant state, thereby reducing basal stimulation. However, in the absence of specific mechanistic detail governing the presence of NO-induced enhanced constitutive activity at the μ-opioid receptor the explanation behind this observation remains unclear and requires further investigation.

The final experiments described herein evaluated the time course of receptor changes that occur during morphine infusion preceded by high-dose L-arginine infusion (supertolerance-inducing treatment). Fig. 5 demonstrates the time-dependent changes in receptor activity over the course of the morphine infusion. It is hypothesized that the receptor changes elucidated in this study are responsible for the development of antinociceptive tolerance development. Establishing a corresponding time course between receptor changes and loss of effect with time is essential in defining the cause-effect relationship. For comparison, data collected from previously conducted experiments shows that tolerance development to the antinociceptive effects of a 3-mg/kg/h infusion begin to occur at approximately 2 h and gradually decline through 8 h [28]. Antinociceptive effect for the comparison shown in Fig. 5 represents animals receiving a tolerance inducing infusion of morphine alone (in the absence of pretreatment with NO), while the time-course of receptor based alterations (reduced maximal agonist-induced [^{35}S]GTPγS binding and enhanced constitutive activity of the μ-opioid receptor) were amplified using L-arginine pretreatment to obtain adequate sensitivity. Changes in receptor activity were detectable beginning at 6 h. By amplifying the receptor based changes using pretreatment with L-arginine, a time-dependent link was established and supports that the observed receptor changes are linked to the development of tolerance development.

The present results support a dual effect on NO on morphine responsivity. One effect occurs in the absence of opioid exposure and increases the ability of agonists to activate G-protein-coupled receptors; the second effect is dependent on increased neuronal NO production in combination with continuous opioid exposure, and results in enhanced constitutive activity at the μ-opioid receptor. It is important to note that upregulation of basal activity at the μ-opioid receptor appears to occur in a time course consistent with the development of antinociceptive tolerance during a continuous morphine infusion.

In conclusion, this work has established that NO, which has been postulated to mediate tolerance development, is involved intimately in stimulating constitutive activity at the μ-opioid receptor, an effect previously linked with tolerance development. While additional mechanistic experimentation is needed to establish the pathways by which NO facilitates the loss of antinociceptive effect, these results provide a vital starting point for deciphering an important role of NO in mediating antinociceptive tolerance development.

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