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Christian Botz-Zapp April 10, 2018

Effects of Dopamine D3 Receptor Antagonism on Morphine-induced Locomotor Activity

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Abstract

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The misuse of prescription and recreational opioids has led to an alarming increase in the number of overdose deaths nationwide. Recently, the dopamine D3 receptor (D3R) has received attention as a potential site for the development of opioid abuse liability and dependence pharmacotherapies. Interest in the D3R initially peaked due to its primary expression in the ventral striatum, a target region of the mesolimbic dopamine (DA) system that mediates the reinforcing effects of many drugs of abuse. Early studies show that D2-like antagonists can attenuate abuse-related effects of opioids. However, D2-like antagonists non-preferentially bind to D2, D3, and D4 receptors (D2R, D3R, D4R), which makes it difficult to understand the precise role of the D3R in mediating the effects of opioids. Thus, the field has progressed towards the development of D3R-selective agents.

The present study uses the highly selective D3R antagonist PG01037 and the highly selective D2R antagonist L-741,626 to discern the role of the D3R in mediating behavioral effects of morphine. Specifically, we test the effects of PG01037 and L-741,626 pretreatment on morphine-induced locomotion in C57BL/6J mice. Systemic administration of morphine and other opioids stimulates locomotor activity in mice. Given the significant overlap between DA systems that influence motor function and the reinforcing effects of drugs, studying drug-induced locomotor activity is helpful for predicting aspects of abuse liability and addiction. In our study, we found that both PG01037 and L-741,626 significantly attenuate acute morphine-induced locomotion, which implies that DA signaling at the D3R and D2R is important for morphine-induced locomotion. Furthermore, specific time course actions of PG01037 and L-741,626 may reveal that the initial locomotor response to morphine is differentially affected by D3R and D2R antagonism. Next, we tested several morphine-induced locomotor sensitization protocols in hopes of understanding the impact of PG01037 pretreatment in mediating the chronic effects of morphine. After determining a protocol, we found that PG01037 delayed the development of morphine-induced locomotor sensitization. The results presented in this study are helpful for predicting a role of D3R-selective antagonists in the treatment of opioid abuse and addiction.

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(Included with permission from Daniel Manvich and David Weinshenker)

Introduction

The misuse of prescription and recreational opioids has contributed to the growth of a national healthcare crisis that shows no signs of slowing. It is estimated that over 2.5 million Americans misused opioids in 2015, and 33,000 of them died from overdose (drugabuse.gov). The most commonly abused opioids are mu opioid receptor (MOR) agonists, which are widely distributed in the clinic for pain treatment and on the street for recreational use. For example, morphine is a natural MOR agonist used to treat nociception, and heroin is a semisynthetic MOR agonist sold recreationally for its euphoric effects. The typical FDA-approved medications for opioid dependence are methadone and buprenorphine, which act as a full MOR agonist and a partial MOR agonist, respectively, and are effective in reducing symptoms of withdrawal and craving in some people. However, methadone has significant abuse and overdose liability, and buprenorphine may be less effective than methadone even though it is the newer drug (Novick et al., 2015). Given the continued growth of the opioid crisis, research must be devoted to the development of drugs that can more safely and effectively reduce opioid abuse and dependence.

Repeated exposure to drugs of abuse, including opioids, can lead to maladaptive molecular and cellular changes in the brain that result in the development of physical and psychological dependence (Nestler, 2001). Drug-induced plasticity of the mesolimbic dopamine (DA) system is thought to be a driving factor in the establishment of addictive-like behaviors (Kauer and Malenka., 2007). The mesolimbic DA pathway is comprised of dopaminergic cells within the ventral tegmental area (VTA) in the midbrain that project to the ventral striatum (nucleus accumbens; NAc) in the forebrain. While precise actions differ, virtually all drugs of abuse exert their reinforcing effects through increased DA neurotransmission in the NAc (Di Chiara and Imperato, 1988; Pierce and Kumaresan, 2006). For example, MOR agonists such as

morphine increase DA neurotransmission in the NAc through disinhibition of VTA DA cells. GABAerigc cells in the rostral tegmental area (RMTg), the ventral pallidum (VP), and within the VTA provide inhibitory tone on VTA DA cells. Activation of $G\alpha_i$-coupled MORs expressed on the cell bodies, dendrites, and terminals of these GABAergic neurons reduces inhibitory tone, thus increasing activity of VTA DA cells and enhancing DA neurotransmission in the NAc (Fields and Margolis, 2015). It is important to note that the reinforcing properties of MOR agonists are complex and very likely extend beyond the mesolimbic DA system (Merrer et al., 2009). Nevertheless, mesolimbic DA signaling seems to play a role in opioid reinforcement and is the focus of this study.

DA binds to five G protein-coupled receptor subtypes, which are divided into two families. The D1-like family includes the D1 and D5 receptor subtypes (D1R and D5R), which are coupled to $G\alpha_s$ signaling proteins. Conversely, the D2-like family includes the D2, D3, and D4 receptor subtypes (D2R, D3R, D4R), which are coupled to $G\alpha_i$ signaling proteins (Missale et al., 1998). Both D1-like and D2-like receptors are found on the dendrites and cell bodies of neurons that receive dopaminergic input. Additionally, some D2Rs and D3Rs function as autoreceptors and are located on the cell bodies, axons, and terminals of DA-releasing cells. When activated, autoreceptors inhibit neuronal activity and neurotransmitter release (Ford, 2014).

Since its discovery in 1990, the D3R has emerged as an appealing target for the treatment of drug addiction (Sokoloff and Le Foll, 2017). Interest in the D3R initially peaked due to its high expression in target regions of the mesolimbic DA system. In contrast to the D2R, which is expressed widely throughout the brain, the D3R is primarily localized in the ventral striatum (Sokoloff et al., 1990). Postmortem and preclinical studies have revealed that chronic exposure

to cocaine, nicotine, or morphine increases D3R mRNA expression in the substantia nigra (cocaine & morphine), VTA (morphine), NAc (cocaine, morphine, & nicotine), and caudate nucleus/putamen (morphine) (Staley and Mash, 1996; Le Foll and Sokoloff, 2003; Spangler et al., 2003; Liang et al., 2011). Earlier studies indicate that D2-like antagonists can attenuate abuse-related effects of opioids in rodents (Glick and Cox, 1975; Hemby et al., 1996). However, the precise role of the individual DA receptor subtypes is difficult to determine from the results of these studies because the canonical D2-like antagonists non-preferentially bind to all receptors within the D2-like family (Schwartz et al., 1993; Tang et al., 1994). Thus, researchers have worked towards the development of selective D3R agents in hopes of elucidating the role of the D3R in addictive processes.

The purpose of the present study was to compare the impact of pretreatment with highly selective D3R and D2R antagonists on morphine-induced locomotion in mice. Most drugs of abuse, including morphine, induce locomotor hyperactivity when acutely and systemically administered. Although locomotor activity is not considered an addiction-like behavior per se, it is useful in predicting abuse potential because of the overlap between DA systems that influence motor function and the reinforcing effects of drugs (Robinson and Berridge, 2000). It is important to note there is evidence for the involvement of DA-independent mechanisms in opioid-induced locomotion (Vaccarino et al, 1986); however, a substantial amount of research suggests that mesolimbic DA release in the NAc plays a significant role in facilitating the locomotor response to opioids (Broekkamp et al., 1979; Joyce and Iversen, 1979; Kelley et al., 1980; Kalivas et al., 1983). Continuing, repeated exposure to drugs of abuse can lead to the development of locomotor sensitization. Defined as an increase in locomotor response to repeated exposure to the same dose of drug, locomotor sensitization is thought to reflect the

neural plasticity that occurs in addiction. Thus, locomotor activity is helpful in assessing biological and behavioral components of addiction-related processes (Robinson and Berridge, 2000).

This study first establishes dose-response curves to analyze the modulatory effects of selective D3R and D2R antagonists on acute morphine-induced locomotion in mice. We use the D3R antagonist PG01037, which is 133-fold selective for D3Rs over D2Rs, and the D2R antagonist L-741,626, which is 41-fold selective for D2Rs over D3Rs (Newman et al., 2005; Kulagowski et al., 1996). Next, the effects of PG01037 on morphine-induced locomotor sensitization are evaluated. The results from these experiments help to differentiate the functional role of the D3R from that of the D2R in mediating the acute effects of morphine, and suggest how D3R-selective antagonists may affect the neural plasticity that develops as a result of repeated morphine exposure. In sum, this study provides a general basis for evaluating selective D3R antagonists as opioid abuse liability and dependence pharmacotherapies.

Hypothesis

A previous study in our lab found that the D3R-selective antagonist PG01037 significantly enhanced the locomotor stimulating effects of acutely administered cocaine, while conversely the D2R-selective antagonist L-741,626 significantly decreased cocaine-induced locomotion (Sup. Fig 1.; Manvich et al., in preparation). Interestingly, a single-dose pilot study showed that both PG01037 and L-741,626 attenuate morphine-induced locomotion, indicating that the D3R might differentially regulate responses to psychostimulants and opioids. As a result of these preliminary data, we hypothesized that both PG01037 and L-741,626 would shift the morphine dose-response curve to the right, signaling antagonism of morphine-induced

locomotion. Furthermore, we predicted that PG01037 would reduce morphine-induced locomotor sensitization, which is consistent with a study using D3R knockout (KO) mice (Li et al., 2010).

Methods

Subjects

The subjects used in this study were 55 adult male and female C57BL/6J mice (8-12 weeks at start of study) weighing between 19-33 grams. Some mice were bred in our laboratory, and others were ordered from Jackson Laboratory (Bar Harbor, ME). Because we have not observed sex differences in morphine-induced locomotor activity in previous experiments, data from both sexes were combined. Mice were housed in same-sex groups of 3-4 per cage in a climate-controlled vivarium with 12-hour light cycle (lights off 7pm: lights on 7am) and had *ad libitum* access to food and water in the home cage. Procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Emory IACUC.

Drugs

Morphine (NIDA Drug Supply Program) was dissolved in 0.9% bacteriostatic saline. The D3R antagonist PG01037 (generously provided by Dr. Amy Newman of the NIDA Intramural Research Program) was dissolved in filtered distilled water. The D2R antagonist L-741,626 (Tocris Bioscience; Minneapolis MN) was dissolved in vehicle ethanol:Cremophor:saline (5:10:85 v/v). All drugs were injected intraperitoneally (IP) in a volume of 10 ml/kg.

Locomotor Activity Equipment

16 automated locomotor activity chambers (San Diego Instruments; San Diego, California) were used to determine mouse locomotion in response to various drug treatments. Eight y-axis and four x-axis infrared beams detected the ambulatory activity of mice in the test chamber. Ambulatory activity was recorded in 5-min intervals. The test chambers contained a thin layer of mouse bedding and were housed behind a curtain in a well-lit room. All test sessions were conducted between 0900-1400.

Experiment 1: Effects of D3R vs. D2R Antagonism on Acute Morphine-Induced Locomotion

The effects of PG01037 and L-741,626 on acute morphine-induced locomotion were evaluated in eight mice (4M, 4F) using a within-subjects design. Animals were initially placed in the center of the locomotor chambers, and ambulations (defined as consecutive beam breaks) were recorded for 90 min. Next, animals were briefly removed from the chamber and administered either PG01037 (10 mg/kg, i.p.) or its vehicle and placed in the chamber for an additional 30 min for evaluation of locomotor response to antagonist alone. Finally, mice were again removed from the chamber, injected with morphine (10, 18, or 56 mg/kg, i.p.), and placed back in the chamber for 120 min. The 10 mg/kg dose of PG01037 was selected because it produced the most significant enhancement of cocaine-induced locomotion in another study from our lab (Sup. Fig 1.; Manvich et al., in preparation). The 10, 18, and 56 mg/kg morphine doses were selected because they were previously determined to elicit locomotor responses that fell on the ascending limb of the dose-response curve for these eight mice (data not shown). Administration of PG01037 and its vehicle were counterbalanced across animals at each dose of morphine. All eight animals received PG01037 and its vehicle before switching to the next dose

of morphine. The order of morphine dose testing was: 18, 56, 10 mg/kg. Dose-response curves were established by plotting mean ± SEM total ambulations for each drug-drug interaction. Test sessions were separated by at least one week to avoid the development of morphine locomotor sensitization. Once all PG01037/vehicle/morphine combinations were tested, the same experimental design was employed for these eight mice using L-741,626 (10 mg/kg, i.p.) or its vehicle prior to morphine. The 10 mg/kg dose of L-741,626 was selected because it produced the most significant decrease in cocaine-induced locomotion in another study from our lab (Sup. Fig 1.; Manvich et al., in preparation).

Determining a Locomotor Sensitization Protocol for Experiment 2

Experiment 2 aimed to test the impact of PG01037 pretreatment on morphine-induced locomotor sensitization. This was initially evaluated in 16 mice (n=8/group). Days 1-3 were designed to habituate the mice to IP injections. During these three consecutive days, mice were injected with saline and placed in the locomotor chambers for 30 min. Days 4-8 followed a locomotor test session identical to that described for Experiment 1 except that locomotion was recorded for 60 min post morphine (18 mg/kg, i.p.) injection rather than 120 min. The pretreatment groups were as follows: 10 mg/kg PG01037 (n=8) and PG01037's vehicle (n=8). Mice were tested daily for five consecutive days. Mean ± SEM total ambulations from days 4-8 were compared within and across pretreatment groups. This morphine dose and injection schedule did not result in locomotor sensitization in the vehicle pretreatment cohort (see "Results").

Because locomotor sensitization did not occur in the vehicle control group for the experimental design described above, three protocols were tested to determine which morphine

dose and injection schedule resulted in the most robust locomotor sensitization. All new protocols incorporated the aforementioned habituation schedule (3 consecutive days of saline injection) and followed similar test day procedures (90-min habituation, 30-min pretreatment with PG01037's vehicle, and an injection of morphine at 120 min). However, the recording time for locomotor activity post-morphine treatment was extended from 60 min to 180 min, bringing the total time of each test session to 300 min. The original 60-min recording time was followed according to the protocol our lab uses for cocaine-induced locomotor sensitization. However, the locomotor-stimulating effects of morphine last much longer than that of cocaine. Thus, we decided to increase the recording time when we piloted the new protocols. The following dose and injection schedules were implemented:

Protocol 1

Five mice (3M, 2F) were tested once every 48 h and received a dose of 18 mg/kg morphine.

Protocol 2

Five mice (2M, 3F) were tested daily and received a dose of 56 mg/kg morphine.

Protocol 3

Five mice (3M, 2F) were tested once every 48 h and received a dose of 56 mg/kg morphine.

Several studies were used as guidance when deciding morphine doses and intermittency of injections (Li et al., 2010; Koek 2014; Perreau-Lenz et al. 2016). After testing the new

paradigms (see Results section and Fig. 7), we used Protocol 2 as a foundation for the completion of Experiment 2.

Experiment 2: Effects of D3R Antagonism on Morphine-Induced Locomotor Sensitization

The exact procedure from Protocol 2 was employed, except that a PG01037 pretreatment group was included. Thus, one cohort was pretreated with PG01037 (10 mg/kg, i.p.) (n=8) and another with PG01037's vehicle (n=8) before receiving an injection of morphine (56 mg/kg, i.p.) on each test day.

Statistics

For the dose-response curves established in Experiment 1, a two-way ANOVA repeated by both factors (pretreatment and dose of morphine) was used to determine significance. When a main effect was observed, we followed up with a Tukey multiple comparisons test. When ANOVA found a significant interaction, we followed up a Sidak post hoc analysis. When determining a successful sensitization protocol for Experiment 2, a one-way ANOVA was used to assess significance across treatment day in each protocol group. A two-way independent ANOVA repeated by factor of time was used to determine significance of the daily 18 mg/kg morphine and daily 56 mg/kg morphine locomotor sensitization data for Experiment 2. We followed up Experiment 2 with a Dunnett's post hoc test.

Results

Effects of D3R and D2R Antagonism on Acute Morphine-Induced Locomotion

To explore the effects of D3R antagonism on acute morphine-induced locomotion, mice were pretreated with the D3R-selective antagonist PG01037 or its vehicle 30 min prior to receiving an acute injection of morphine (10, 18, or 56 mg/kg, i.p.) (Fig. 1). Locomotor activity, measured as x- and y-axis ambulations, was summed for the 120 min following morphine administration. Two-way repeated measures ANOVA found a main effect of morphine [$F(2,14)=8.211$, $p=0.0044$], and posthoc tests revealed that 56 mg/kg morphine significantly increased locomotor activity compared to 10 mg/kg morphine ($p=0.0094$). There was also a main effect of pretreatment [$F(1,7)=27.47$, $p=0.001$] and a significant interaction between pretreatment and morphine dose [$F(2,14)=3.808$, $p=0.048$]. Relative to vehicle, PG01037 significantly decreased morphine-induced locomotion at the 18 mg/kg ($p=0.002$) and 56 mg/kg ($p=0.0001$) doses. The inhibitory actions of PG01037 on morphine-induced locomotion were evident 10-15 min following morphine administration, and apparent duration of inhibition increased as morphine dose increased (Fig. 2*A*-*C*).

To understand the effects of D2R antagonism on acute morphine-induced locomotion, mice were pretreated with the D2R-selective antagonist L-741,626 or its vehicle 30 min prior to receiving an acute injection of morphine (10, 18, or 56 mg/kg, i.p.) (Fig. 3). Two-way repeated measures ANOVA found a main effect of morphine [$F(2,14)=5.552$, $p=0.0168$], and posthoc tests revealed that 56 mg/kg morphine significantly increased locomotor activity compared to 10 mg/kg morphine ($p=0.0146$). There was also a main effect of pretreatment [$F(1,7)=70.44$, $p<0.0001$]; L-741,626 blocked acute morphine-induced locomotion. ANOVA did not show a significant interaction between morphine dose and pretreatment [$F(2,14)=2.631$, $p=0.1072$). In

contrast to PG01037, the inhibitory effects of L-741,626 were evident immediately after morphine administration and persisted through the entire 120-min window of observation (Fig. 4*A*-*C*).

Due to observed differences in the temporal effects of PG01037 and L-741,626 pretreatment on acute morphine-induced locomotion, we analyzed locomotor activity in the first 10 min following morphine administration to compare the initial consequences of D3R and D2R antagonism (Fig. 5*A*-*B*). Regarding pretreatment with 10 mg/kg PG01037, two-way repeated measures ANOVA revealed a main effect of morphine in the first 10 min following morphine administration [$F(2, 14)=32.38$, $p<0.0001$] but no effect of pretreatment [$F(1, 7)= 0.0049$, $p=0.9461$] (Fig. 5*A*). By contrast, analysis of the same time frame in the L-741,626 locomotor data showed a main effect of morphine [$F(2, 14)=59.11$, $p<0.0001$], pretreatment [$F(1, 7)=29.64$, $p=0.0010$], and a morphine x pretreatment interaction [$F(2, 14)=19.5$, $p<0.0001$], (Fig. 5*B*). Posthoc analysis revealed that 10 mg/kg L-741,626 inhibited the first 10 min of morphine-induced locomotion at 18 mg/kg morphine ($p=0.0068$) and 56 mg/kg morphine ($p<0.0001$). These data suggest that the initial stimulatory effects of morphine may be differentially sensitive to D3R and D2R antagonism.

Daily Exposure to 18 mg/kg morphine does not produce locomotor sensitization

Our initial foray into determining the effects of D3R antagonism on morphine-induced locomotor sensitization was unsuccessful. Mice were pretreated with the D3R-selective antagonist PG01037 (n=8) or its vehicle (n=8) 30 min prior to receiving a daily injection of 18 mg/kg morphine for five days (Fig. 6*B*). Two-way ANOVA revealed that repeated administration of 18 mg/kg morphine did not potentiate morphine-induced locomotion across

daily test sessions [F(4,56=0.3708, p=0.8284]. Mice pretreated with 10 mg/kg PG01037 only trended towards a decrease in morphine-induced locomotion compared to vehicle-pretreated control mice [(F(1,14)=2.446, p=0.1402]. The results from this sensitization trial could not determine the impact of D3R antagonism on morphine-induced locomotor sensitization because sensitization did not occur in the vehicle-pretreated control group.

Three Sensitization Protocols

In order to complete Experiment 2, three protocols were tested to determine which dose of morphine and injection schedule resulted in the most robust morphine-induced locomotor sensitization. The results from Protocols 1, 2, and 3 are shown in Fig. 7. Intermittent injection of 18 mg/kg morphine once every 48 hours did not significantly enhance morphine-induced locomotor activity over the five test days [F(1.635, 6.548)=2.762, p=0.1386] (Fig. 7*A*). However, daily administration of 56 mg/kg morphine produced a gradual increase in morphine-induced locomotion over five test sessions [F(1.789, 7.156)=25.88, p=0.0006; effect of day versus day 1: day 3 (p=0.026), day 4 (p=0.039), day 5 (p=0.016); effect of day versus day 2: day 3 (p=0.046), day 4 (p=0.0181), day 5 (p=0.0097)] (Fig. 7*B*). Similarly, 56 mg/kg morphine injected once every 48 hours also resulted in morphine-induced locomotor sensitization [F(2.468, 9.873)=10.71, p=0.0025; effect of day versus day 1: day 3 (p=0.0399), day 9 (p=0.0337)] (Fig. 7*C*). Enhancement of morphine-induced locomotion in this group plateaued by the third test session [effect of day versus day 5: day 7 (>0.9999), day 9 (0.9833)].

Effects of D3R Antagonism on Morphine-induced Locomotor Sensitization

Using Protocol 2 as a foundation, we retested the impact of PG01037 pretreatment on morphine-induced locomotor sensitization. Mice were pretreated with the D3R-selective antagonist PG01037 (n=8) or its vehicle (n=8) 30 min prior to receiving a daily injection of 56 mg/kg morphine for five consecutive days (Fig. 8*B*). Locomotor activity was recorded for 180 min following morphine injection, and ambulations in the first 120 min of this window were summed for analysis. Two-way ANOVA revealed a main effect of day [$F(4, 56)=17.66$, $p<0.0001$] and pretreatment [$F(1, 14)=4.656$, $p=0.0488$], but no day x pretreatment interaction [$F(4, 56)=0.5055$, $p=0.7318$]. Post hoc analysis showed that the vehicle-pretreated cohort sensitized to the locomotor activating effects of morphine by day 3 (day 1 versus day 3: $p=0.0261$) whereas the PG01037 cohort did not sensitize until day 4 ($p=0.0058$). These data show that daily pretreatment with PG01037 (10 mg/kg, i.p.) can delay the development of locomotor sensitization induced by daily injection of morphine (56 mg/kg, i.p.).

Discussion

It is well-established that DA signaling is important for the locomotor activating effects of opioids (Broekkamp et al., 1979; Joyce and Iversen, 1979; Kelley et al., 1980; Kalivas et al., 1983). However, the precise role of the DA D3 receptor subtype in mediating these effects is much less understood. For example, two studies using D3R KO mice have reported opposite results related to the effect of D3R deletion on the acute morphine locomotor response (Narita et al., 2003; Li et al., 2010). Previous experiments have also tested the impact of D3R antagonism on acute morphine-induced locomotion using nafadotride and U-99194A, both of which display only moderate selectivity for the D3R over the D2R (Audinot et al., 1998; Manzanedo et al.,

1999; Li et al., 2010). Thus, the first aim of the present study was to distinguish the effects of D3R and D2R antagonism on acute morphine-induced locomotion using highly selective D3R and D2R antagonists. The main findings from these experiments were that the D3R-selective antagonist PG01037 significantly attenuated morphine-induced locomotor activity and the D2R-selective antagonist L-741,626 blocked morphine-induced locomotion; each DA receptor antagonist shifted the morphine dose-response function rightward. Our results support the notion that morphine-induced locomotor activity is facilitated by DA signaling. Specifically, both the D3R and D2R are important for acute locomotor responses induced by morphine doses that fall along the ascending limb of the morphine dose-response function.

Differences between the observed time course actions of PG01037 and L-741,626 on morphine-induced locomotion may reveal subtle dissimilarities between the roles of the D3R and D2R. For example, PG01037 did not significantly inhibit morphine-induced locomotion in the first 10 min following morphine administration. Meanwhile, inhibition induced by L-741,626 was evident immediately after morphine injection (Fig. 5*A*-*B*). Using the same pretreatment window of 30 min, PG01037 modulated cocaine-induced locomotion in the first 10 min following cocaine administration (Sup. Fig. 2). Thus, it is unlikely that PG01037's delayed inhibitory action on morphine-induced locomotion was simply due to the antagonist being inactive or absent from the brain at the time morphine was injected. Our data may therefore suggest that the initial locomotor activating effects of morphine are differentially affected by D3R and D2R antagonism. This may also suggest that different mechanisms or circuitries underlie the time course of morphine-induced locomotion. Future locomotor experiments should test several pretreatment times and vary the doses of PG01037 and L-741,626 in order to clarify this phenomenon. In addition to differences in modulating initial morphine response, PG01037-

induced inhibition at all three morphine doses started to gradually subside ~60 min following morphine treatment, while inhibition induced by L-741,626 persisted through the entire 120-min period of observation (Fig. 2, 4). This reduction of inhibition may be attributed to differences in the temporal actions of the D3R on morphine-induced locomotion, or could reflect a difference in drug half-life between PG01037 and L-741,626. Another study found that the bioavailability of PG01037 significantly decreased with intraperitoneal injection compared to intravenous injection in male rats (Mason et al., 2010). Again, varying pretreatment time and the doses of PG01037 and L-741,626 would help to clarify our observations.

Another interesting topic of discussion is the distinct effects of D3R antagonism on morphine- versus cocaine-induced locomotion. Contrary to the inhibition of morphine-induced locomotion we observed in this study, PG01037 *enhanced* cocaine-induced locomotor activity in the same strain of mice (Sup. Fig. 1, Manvich et al, in preparation). The opposite effects are likely due to separate actions of psychostimulants compared to MOR agonists. For example, cocaine stimulates locomotion by blocking reuptake of DA into DA cell terminals and increasing DA neurotransmission in the NAc (Swerdlow et al., 1986). A recent study in our lab determined that potentiation of cocaine-induced locomotion by PG01037 may be due to neuron-specific expression of the D3R in the NAc. Specifically, it was concluded that activation of the D3R inhibits excitatory activity of medium spiny neurons (MSNs) in the NAc that co-express the D1R (Manvich et al., in preparation). DA signaling at the D1R on D1-type MSNs stimulates locomotion through activation of the direct pathway of the basal ganglia (Yager et al., 2015). Antagonizing the D3R on these cells may have caused the observed enhancement of cocaine-induced locomotion. In contrast to cocaine, MOR agonists indirectly enhance DA neurotransmission in the NAc through disinhibition DA cells in the VTA (Fields and Margolis,

2015). Furthermore, MORs expressed in the NAc may directly modulate activity of MSNs. Therefore, D3R antagonism could inhibit morphine-induced locomotion rather than enhance it through complex differences in opioid circuitry and receptor expression. A future step will be to determine the mechanisms underlying the opposing effects of D3R antagonism on cocaine versus morphine responses.

Regarding the potential impact of PG01037 and L-741,626 on morphine abuse liability, more experiments are needed to understand how D3R- and D2R-selective antagonists might alter morphine reward. There is significant overlap of DA systems that modulate the reinforcing effects of drugs and locomotor activity (Robinson and Berridge, 2000), and our study confirms that DA signaling at the D3R and D2R is important for morphine-induced hyperlocomotion. This signaling is likely specific to the mesolimbic DA system as it is heavily implicated in opioid-induced locomotion (Broekkamp et al., 1979; Joyce and Iversen, 1979; Kelley et al., 1980; Kalivas et al., 1983). However, we cannot come to this conclusion because we injected our antagonists systemically. Future locomotor experiments should test site-specific infusions of PG01037 and L-741,626 against morphine-induced locomotion to pinpoint where these drugs exert their main effects. We would first recommend infusions in brain regions associated with the mesolimbic DA system i.e. the VTA and NAc. Regardless, the significant inhibition of morphine-induced locomotion we observed with systemic administration of the antagonists justifies further investigation of the influence of PG01037 and L-741,626 on the rewarding properties of morphine. A future experiment could use a conditioned place preference task to test the effects of PG01037 and L-741,626 on morphine reward.

The second aim of this study was to test the impact of D3R-selective antagonism on morphine-induced locomotor sensitization. Previous studies cite that D1R and D2R antagonism

can reduce the locomotor sensitizing effects of chronic morphine (Kuribara, 1995; Reisi et al., 2014). In regard to the role of the D3R, only a few studies have examined its impact in the development of morphine-induced locomotor sensitization (Li et al., 2010; Liang et al., 2011). In the second portion of our study, we tested the impact of the highly selective D3R antagonist PG01037 on morphine-induced locomotor sensitization. The main findings in this experiment were that PG01037 pretreatment slowed the development of morphine-induced locomotor sensitization compared to vehicle pretreatment.

Our first attempt in addressing Experiment 2 was unsuccessful, as daily exposure to 18 mg/kg morphine failed to sensitize mice in the vehicle pretreatment group (Fig. 6*B*). Without achieving sensitization in this test group, we have no control to compare to the PG01037-pretreated cohort. We have several ideas for why sensitization did not develop in this trial. Firstly, we used a combination of mice bred in our laboratory and from Jackson Laboratory (Bar Harbor, ME). Even though mice were counterbalanced in each pretreatment group by sex, age, and breeding origin, there was a considerable amount of variability within each pretreatment group in response to morphine. This variability may have also been attributed to the dose of morphine itself. Locomotion could vary more greatly between individuals in response to an intermediary morphine dose (18 mg/kg) versus a higher morphine dose (56 mg/kg). This idea is supported in the acute morphine locomotor data where the SEMs were smaller at 56 mg/kg morphine than at 18 mg/kg morphine in the vehicle pretreatment groups (Fig.1, Fig. 3).

After determining a successful sensitization protocol (Fig. 7), we retested Experiment 2 and found that daily pretreatment with PG01037 delayed the development of morphine-induced locomotor sensitization compared to vehicle pretreatment (Fig. 8*B*). A previous study showed that D3R KO mice failed to sensitize to the locomotor activating effects of morphine (Li et al.,

2010). In agreement with this previous study, our results suggest the D3R plays a role in mediating the development of morphine-induced locomotor sensitization. Future morphine sensitization experiments should retest the impact of PG01037 pretreatment to determine if increasing its dose would significantly reduce morphine-induced locomotor sensitization. Another future step will be to test the impact of L-741,626 pretreatment on morphine-induced locomotor sensitization in order to compare the effects of D3R and D2R antagonism.

In summary, the D3R-selective antagonist PG01037 significantly inhibited acute morphine-induced locomotion and the D2R-selective antagonist L-741,626 blocked acute morphine-induced locomotion. We also found that the initial locomotor response to morphine may be differentially affected by D3R and D2R antagonism. Next, PG01037 slowed the development of morphine-induced locomotor sensitization. Given that we only tested locomotor behavior, future experiments should test the effects of PG01037 on morphine reward with a conditioned place preference task; they should also test the effects of PG01037 on more complex models of addiction with an operant self-administration task. To conclude, the results presented in this study are helpful for predicting a role of D3R-selective antagonists in the treatment of opioid abuse and addiction.

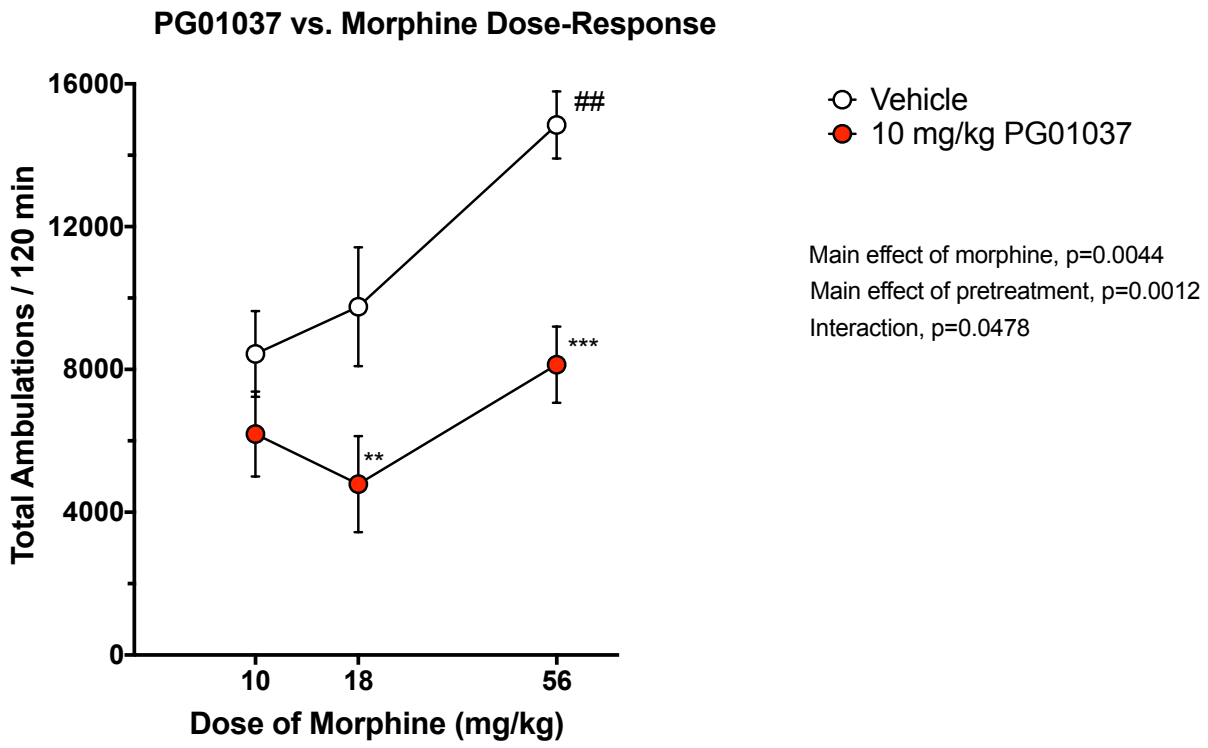


Fig. 1. PG01037 attenuates acute morphine-induced locomotion. Shown are mean ± SEM total ambulations in the 120 min following morphine (10, 18, and 56 mg/kg, i.p.). Red circles denote 30 min pretreatment with PG01037 (10 mg/kg, i.p.), and white circles denote 30 min pretreatment with vehicle. Pretreatment was counterbalanced across test sessions in the same 8 mice (4M, 4F), and the doses of morphine were tested in the following order: 18, 56, 10 mg/kg. Each test session was separated by at least 7 days. ##$p<0.01$, mean total ambulations at 56 mg/kg morphine compared to 10 mg/kg morphine. **$p<0.01$, PG01037 compared to vehicle at 18 mg/kg morphine. ***$p<0.001$, PG01037 compared to vehicle at 56 mg/kg morphine.

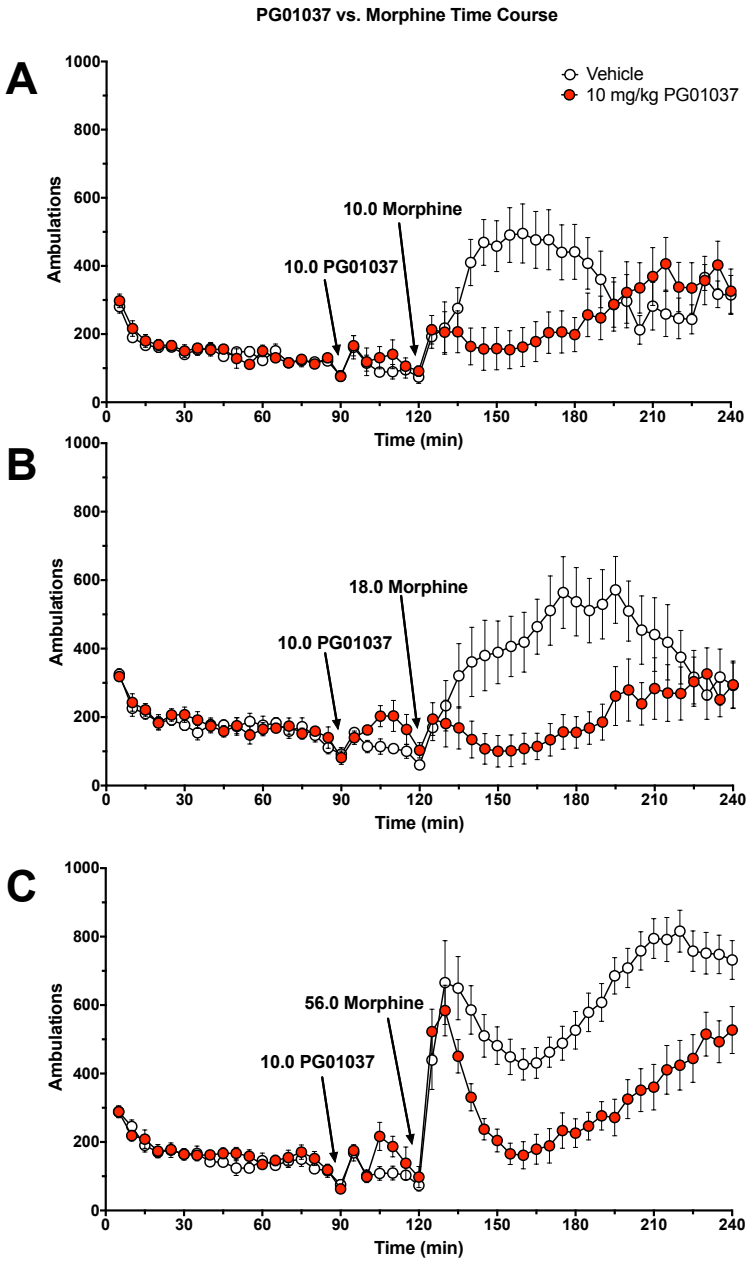


Fig. 2. Time course effects of PG01037 pretreatment on morphine-induced locomotion. Shown is the time course of morphine-induced locomotion following vehicle or PG01037 pretreatment. **(A)** 10, **(B)** 18, and **(C)** 56 mg/kg morphine were tested. Each point is a 5-min bin of total ambulations (mean ± SEM). Red circles denote 30 min pretreatment with PG01037 (10 mg/kg, i.p.). White circles denote pretreatment with vehicle.

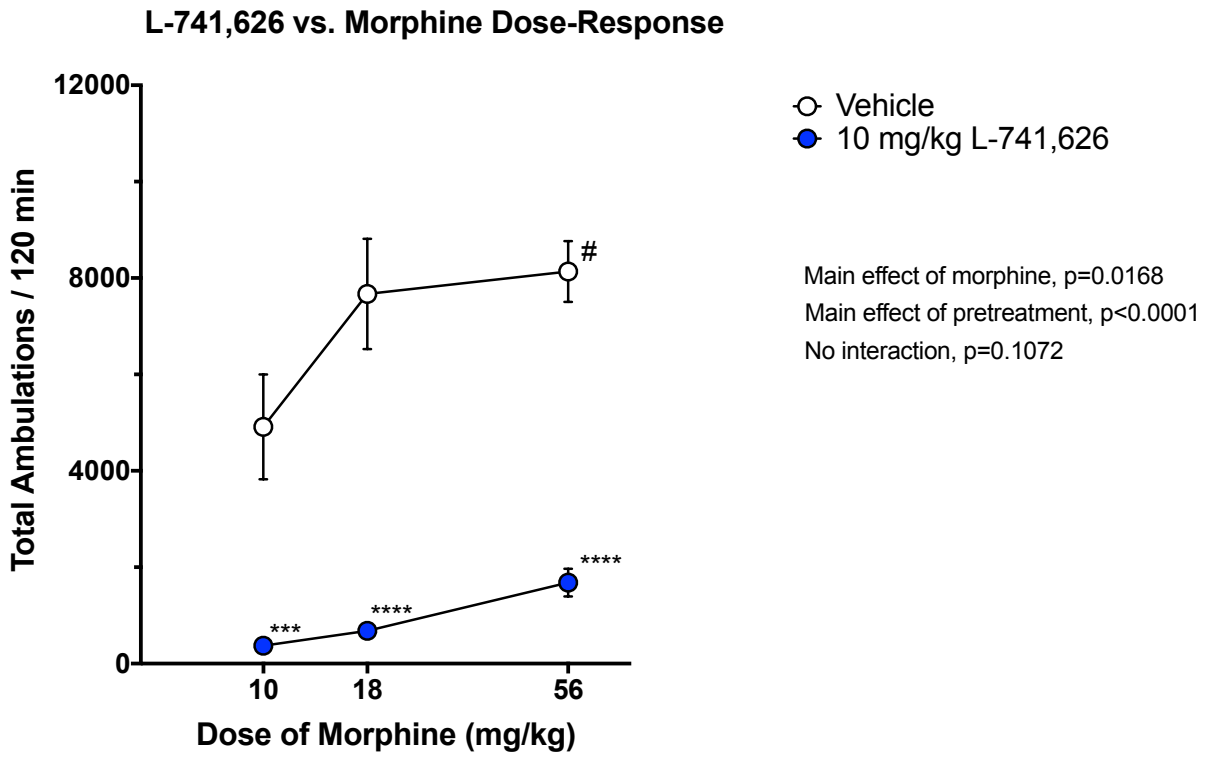


Fig. 3. L-741,626 blocks acute morphine-induced locomotion. Shown are mean ± SEM total ambulations in the 120 min following injection of morphine (10, 18, and 56 mg/kg, i.p.). Blue circles denote 30-min pretreatment with L-741,626 (10 mg/kg, i.p.). White circles denote 30-min pretreatment with vehicle. Pretreatment was counterbalanced across test sessions in the same 8 mice (4M, 4F), and the doses of morphine were tested in the following order: 18, 56, 10 mg/kg. Each test session was separated by at least 7 days. #$p<0.05$ mean total ambulations at 56 mg/kg morphine compared to 10 mg/kg morphine. ***$p<0.001$, L-741,626 compared to vehicle at 18 mg/kg morphine. ****$p<0.0001$, L-741,626 compared to vehicle at 18, 56 mg/kg morphine.

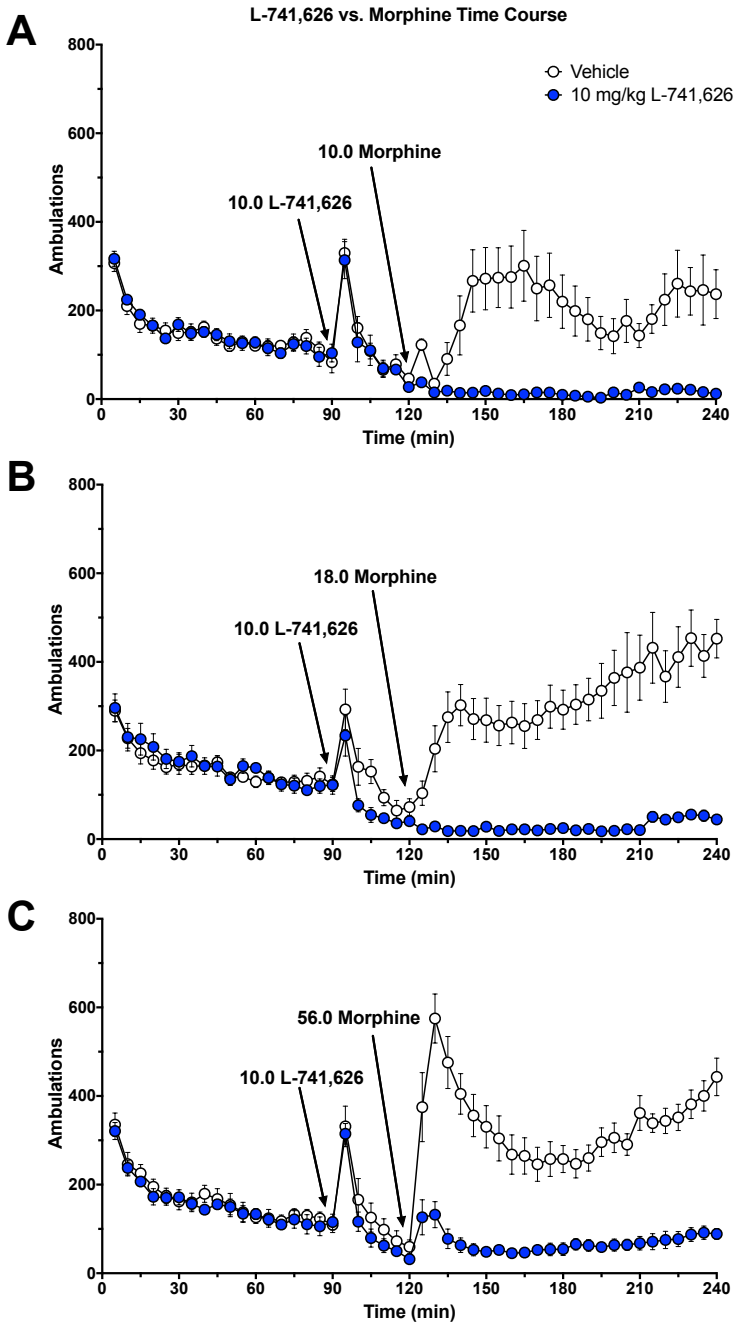


Fig. 4. Time course effects of L-741,626 pretreatment on morphine-induced locomotion. Shown is the time course of morphine-induced locomotion following vehicle or L-741,626 pretreatment. **(A)** 10, **(B)** 18, and **(C)** 56 mg/kg morphine were tested. Each point is a 5-min bin of total ambulations (mean ± SEM). Blue circles denote 30 min pretreatment with L-741,626 (10 mg/kg, i.p.). White circles denote pretreatment with vehicle.

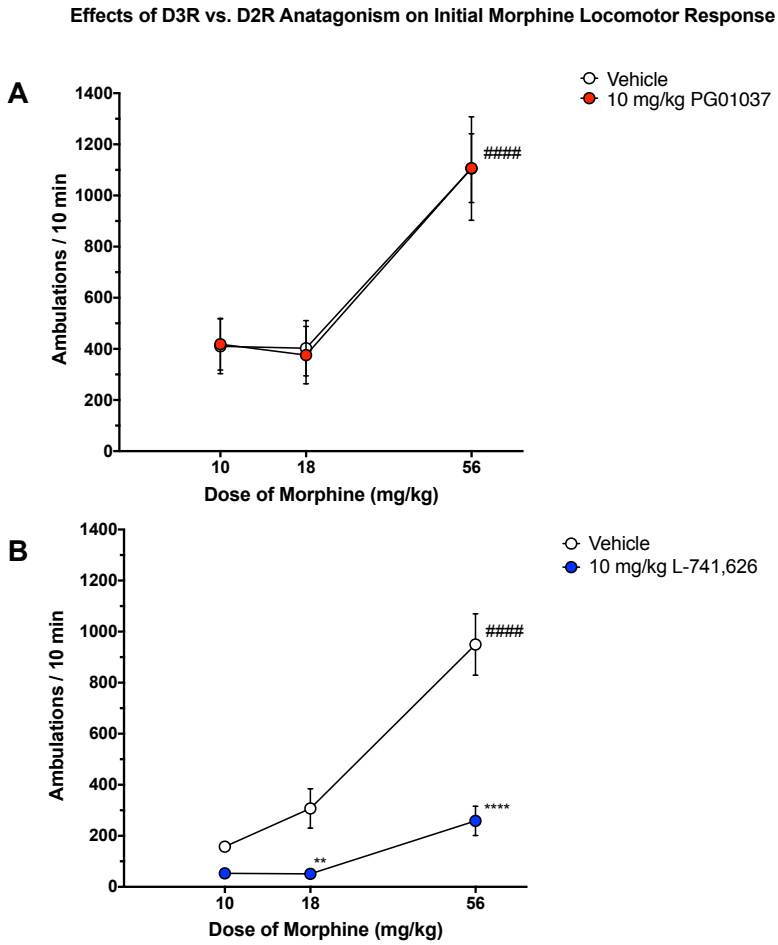


Fig. 5. Initial locomotor response to morphine is sensitive to L-741,626 pretreatment but not PG01037 pretreatment. Shown are mean ± SEM total ambulations in the 10 min following IP injection of morphine (10, 18, and 56 mg/kg, i.p.). **(A)** Red circles denote 30 min pretreatment with PG01037 (10 mg/kg, i.p.). White circles denote 30 min pretreatment with vehicle. ####$p<0.0001$, mean total ambulations at 56 mg/kg morphine compared to 10 mg/kg morphine. **(B)** Blue circles denote 30 min pretreatment with L-741,626 (10 mg/kg, i.p.). White circles denote 30 min pretreatment with vehicle. ####$p<0.0001$, mean total ambulations at 56 mg/kg morphine compared to 10 mg/kg morphine. **$p<0.01$, L-741,626 compared to vehicle at 18 mg/kg morphine. ****$p<0.0001$**,** L-741,626 compared to vehicle at 56 mg/kg morphine. Pretreatment was counterbalanced across test sessions in the same 8 mice (4M, 4F), and the doses of morphine were tested in the following order: 18, 56, 10 mg/kg. Each test session was separated by at least 7 days.

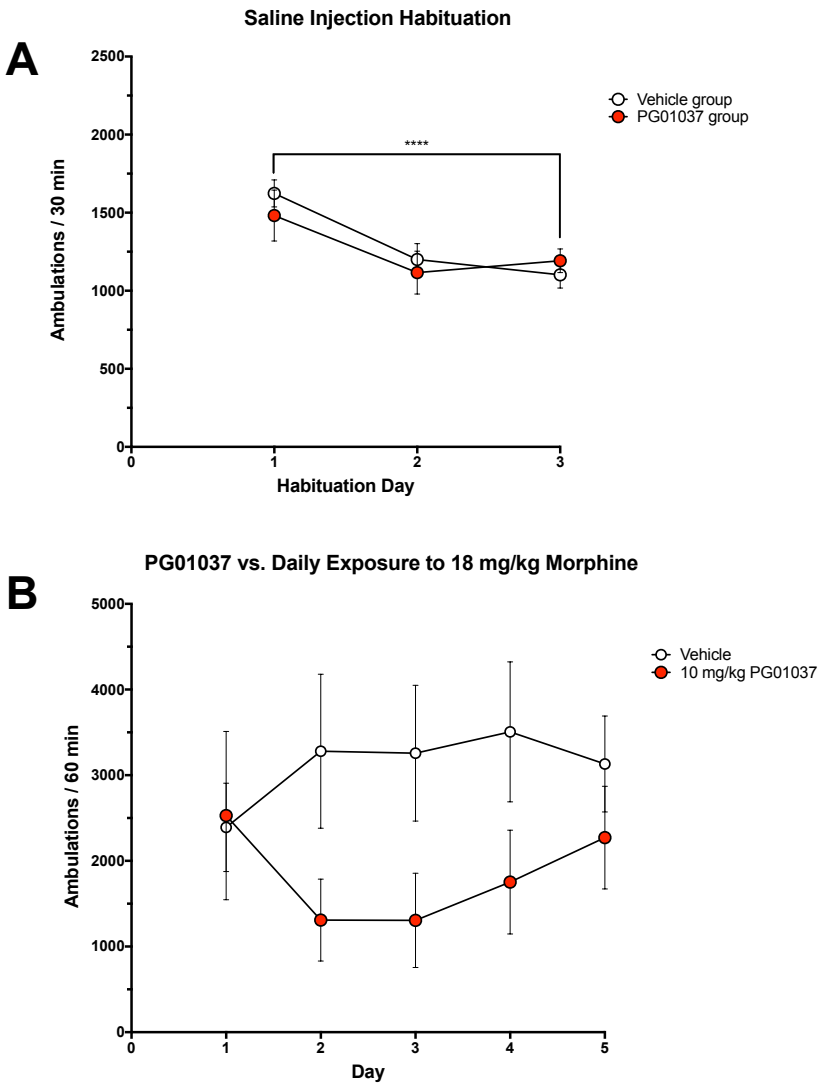


Fig. 6. Daily exposure to 18 mg/kg morphine does not produce locomotor sensitization **(A)** Shown are mean ± SEM total ambulations in the 30 min following daily IP injection of saline. Red circles denote mice that would later receive PG01037 pretreatment during sensitization induction (n=8). White circles denote mice that would later receive vehicle pretreatment during sensitization induction (n=8). ****p<0.0001, compares mean total ambulations between days 1 and 3 of habituation. **(B)** Mean ± SEM total ambulations in the 60 min following daily IP injection of morphine (18 mg/kg, i.p.). Red circles denote mice receiving 30 min pretreatment with PG01037 (10 mg/kg, i.p.) (n=8). White circles denote mice receiving 30 min pretreatment with vehicle (n=8).

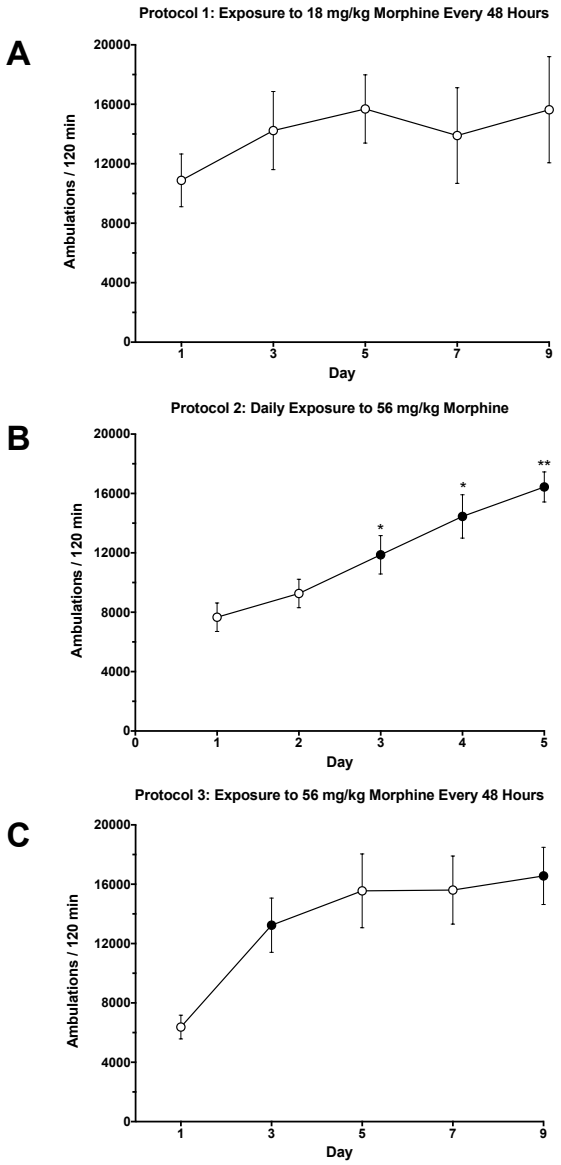


Fig. 7. Protocols 2 and 3 resulted in morphine locomotor sensitization. Shown are mean ± SEM total ambulations in the 120 min following an injection of morphine. All groups received 30-min pretreatment with PG01037's vehicle. **(A)** Mice (n=5) were tested once every 48 h and received a dose of 18mg/kg morphine. **(B)** Mice (n=5) were tested daily and received a dose of 56 mg/kg morphine. Filled circles denote significance compared to day 1 ($p<0.05$), and asterisks denote significance compared to day 2 (*$p<0.05$, **$p<0.01$). **(C)** Mice (n=5) were tested once every 48 h and received a dose of 56 mg/kg morphine. Filled circles denote significance compared to day 1 ($p<0.05$).

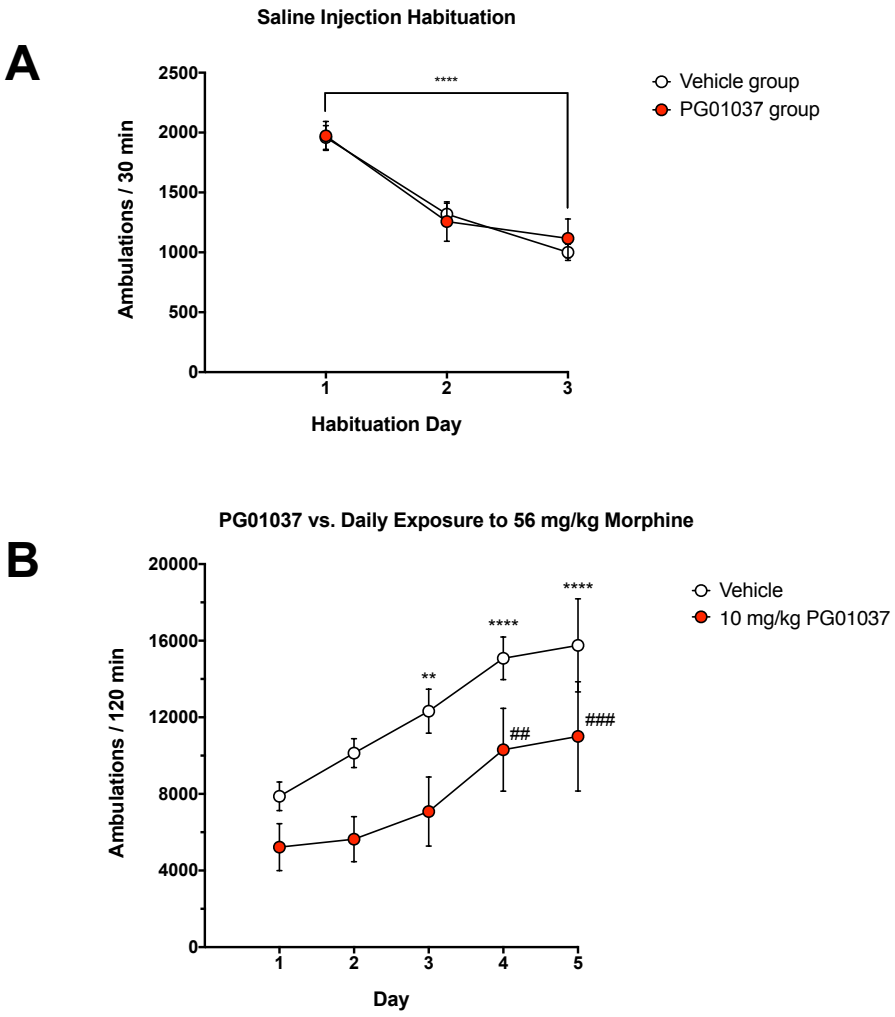
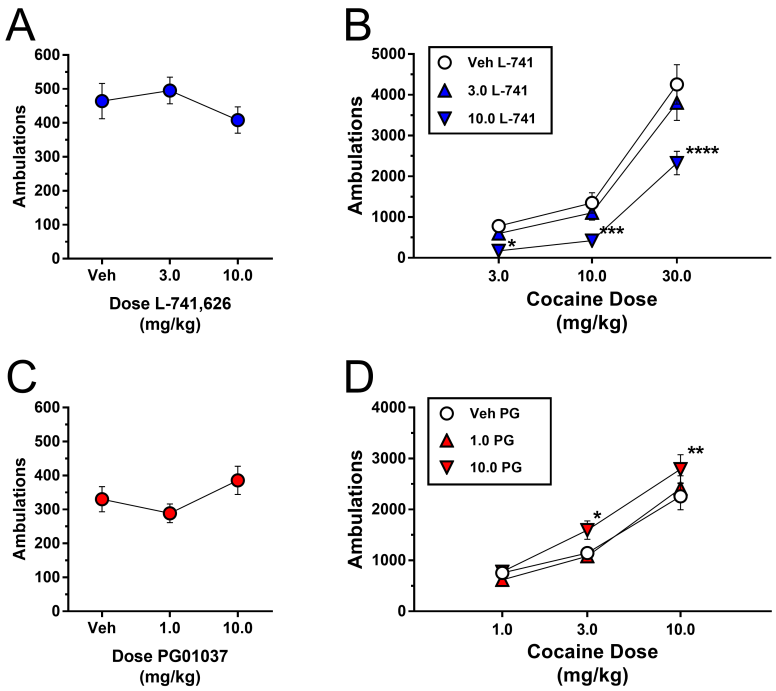


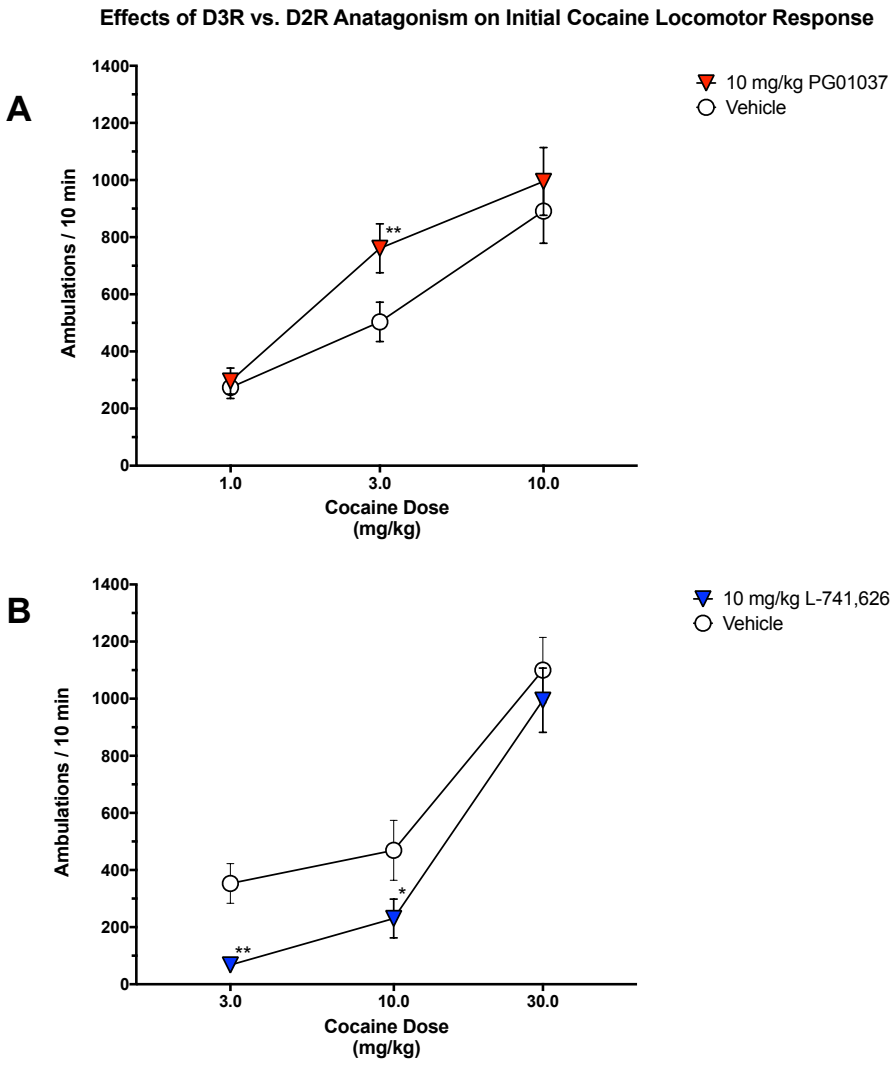
Fig. 8. PG01037 delays the development of morphine locomotor sensitization. (A) Shown are mean ± SEM total ambulations in the 30 min following daily IP injection of saline. Red circles denote mice that would later receive PG01037 pretreatment during sensitization induction (n=8). White circles denote mice that would later receive vehicle pretreatment during sensitization induction (n=8). ****p<0.0001, compares mean total ambulations between days 1 and 3 of habituation. **(B)** Mean ± SEM total ambulations in the 120 min following daily IP injection of morphine (56 mg/kg, i.p.). Red circles denote mice receiving 30 min pretreatment with PG01037 (10 mg/kg, i.p.) (n=8). White circles denote mice receiving 30 min pretreatment with vehicle (n=8). Asterisks denote significance compared to day 1 for vehicle pretreatment (**p<0.01, ****p<0.0001). Pound signs denote significance compared to day 1 for PG01037 pretreatment (##p<0.01, ###p<0.001).

Supplementary Figures from a Weinshenker Lab Study (Manvich et al., in preparation)

(Included with permission from Daniel Manvich and David Weinshenker)



Supplementary Fig. 1. L-741,626 attenuates cocaine-induced locomotion and PG01037 enhances cocaine-induced locomotion. Shown are mean ± SEM total ambulations for **(A)** 30 min pretreatment with L-741,626 (3.0 and 10.0 mg/kg, i.p.) or vehicle and **(B)** the 60 min following cocaine administration (3.0, 10.0, and 30.0 mg/kg, i.p.). Blue triangles denote 30 min pretreatment with L-741,626 (3.0 and 10.0 mg/kg). White circles denote 30 min pretreatment with vehicle. *p<0.05, L-741,626 (10.0 mg/kg) compared to vehicle at 3.0 mg/kg cocaine. ***p<0.001, L-741,626 (10.0 mg/kg) compared to vehicle at 10.0 mg/kg cocaine. ****p<0.0001, L-741,626 (10.0 mg/kg) compared to vehicle at 30.0 mg/kg cocaine. **(C)** Mean ± SEM total ambulations for 30 min pretreatment with PG01037 (1.0 and 10.0 mg/kg, i.p.) or vehicle and **(D)** the 60 min following cocaine administration (1.0, 3.0, and 10.0 mg/kg, i.p.). Red triangles denote 30 min pretreatment with PG01037 (1.0 and 10.0 mg/kg). White circles denote 30 min pretreatment with vehicle. *p<0.05, PG01037 (10.0 mg/kg) compared to vehicle at 3.0 mg/kg cocaine. **p<0.01, PG01037 (10.0 mg/kg) compared to vehicle at 10.0 mg/kg cocaine.



Supplementary Fig. 2. Initial locomotor response to cocaine is sensitive to both L-741,626 and PG01037 pretreatment. (A) Mean ± SEM total ambulations in the 10 min following cocaine administration (1.0, 3.0 and 10.0 mg/kg, i.p.). Red triangles denote 30 min pretreatment with PG01037 (10.0 mg/kg, i.p.). White circles denote 30 min pretreatment with vehicle. **p<0.01, PG01037 (10.0 mg/kg) compared to vehicle at 3.0 mg/kg cocaine. **(B)** Mean ± SEM total ambulations in the 10 min following cocaine administration (3.0, 10.0 and 30.0 mg/kg, i.p.). Blue triangles circles denote 30 min pretreatment with L-741,626 (10.0 mg/kg, i.p.). White circles denote 30 min pretreatment with vehicle. **p<0.01, L-741,626 (10.0 mg/kg) compared to vehicle at 3.0 mg/kg cocaine. *p<0.05, L-741,626 (10.0 mg/kg) compared to vehicle at 10.0 mg/kg cocaine.

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