Title

Effects of Selective Dopamine D_2 or D_3 Receptor Antagonism on Morphine-Induced Locomotion in Mice

Authors

Christian A. Botz-Zapp[1], Stephanie L. Foster[1,2], Amy H. Newman[3], David Weinshenker[1], and Daniel F. Manvich[1,4]

Affiliations

[1]Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322

[2]Graduate Program in Neuroscience, Emory University, Atlanta, GA 30322

[3]Medicinal Chemistry Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse-Intramural Research Program, National Institutes of Health, Baltimore, MD 21224

[4]Current Address: Department of Cell Biology and Neuroscience, Rowan University School of Osteopathic Medicine, Stratford, NJ 08084

Corresponding Author

Daniel F. Manvich Ph.D., Department of Cell Biology and Neuroscience, Rowan University School of Osteopathic Medicine

Address: 2 Medical Center Drive, Room A204, Stratford, NJ 08084

Phone: (856) 566-6424

Fax: (856) 566-2881

Email: manvich@rowan.edu

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Abstract (250 words or less)

Rationale: The dopamine D_3 receptor (D_3R) has garnered interest as a pharmacotherapeutic target for the treatment of opioid use disorder (OUD). Recent evidence suggests that D_2R and D_3R antagonism oppositely affect the locomotor-activating effects of cocaine, but whether this pattern extends to opioid-induced hyperactivity remains unresolved.

Objective: This study compared the impact of selective D_2R vs. D_3R antagonists on the locomotor-activating effects of acute and repeated morphine administration in mice. Catalepsy following D_2R vs. D_3R antagonism alone or in combination with morphine was also assessed.

Methods: C57Bl/6J mice were pretreated with either the highly-selective D_3R antagonist PG01037 (vehicle, 10.0 mg/kg) or the selective D_2R antagonist L-741,626 (vehicle, 10.0 mg/kg) and tested for 1) locomotor activity induced by acute morphine administration (10.0 – 56.0 mg/kg), 2) locomotor sensitization following repeated morphine administration (56.0 mg/kg), or 3) catalepsy after administration of either antagonist alone or in combination with morphine (10.0 – 56.0 mg/kg).

Results: In locomotion studies, both PG01037 and L-741,626 shifted the acute morphine dose-response function rightward/downward, although the inhibitory effect of L-741,626 pretreatment was more robust. Likewise, PG01037 pretreatment partially attenuated, while L-741,626 pretreatment fully abolished, morphine-induced locomotor sensitization. L-741,626 produced

catalepsy that was blunted by morphine, whereas PG01037 did not induce catalepsy under any conditions.

Conclusions: D_2R or D_3R antagonism attenuates morphine-induced locomotor activity and sensitization. D_2R antagonism produces a stronger suppression of these effects, but also induced modest cataleptic effects which were not observed following D_3R antagonism. The results lend additional support to the investigation of selective D_3R antagonists as treatments for OUD.

Keywords: Dopamine, D_3 receptor, D_2 receptor, morphine, PG01037, L-741,626, locomotion, sensitization, opioid abuse, catalepsy

Introduction

The abuse of prescription and recreational opioids has culminated in a growing national healthcare crisis, with substantial efforts being devoted to the development of novel pharmacotherapeutics that can safely and more effectively reduce opioid misuse and dependence as compared to currently-available medications (Blanco and Volkow 2019; Kreek et al. 2019; Volkow et al. 2019). It is well-established that the abuse-related effects of opioids are mediated largely via their capacity to increase dopamine (DA) neurotransmission within the mesolimbic reward system (for review, see Di Chiara and North 1992; McBride et al. 1999; Pierce and Kumaresan 2006; Reiner et al. 2019; Wise 1989), a projection that arises from DAergic cells located in the ventral tegmental area (VTA) and terminates in the nucleus accumbens (NAc) (Bjorklund and Dunnett 2007; Moore and Bloom 1978). Opioids administered either systemically (Chefer et al. 2003; Gysling and Wang 1983) or directly into the VTA (Devine et al. 1993; Gysling and Wang 1983; Leone et al. 1991; Spanagel et al. 1992) produce increases in NAc DA levels by disinhibiting VTA DA neurons via activation of G_i-coupled mu opioid receptors located on the cell bodies and terminals of GABAergic neurons that normally provide inhibitory tone (Johnson and North 1992; Matsui and Williams 2011). Accordingly, the locomotor-activating, reinforcing, and reinstatement-inducing effects of systemic or intra-VTA opioid administration may be dampened following perturbation of NAc DA neurotransmission (Hand and Franklin 1985; Kelley et al. 1980; Phillips et al. 1983; Shippenberg et al. 1993; Smith et al. 1985; Spyraki et al. 1983; Stinus et al. 1980; Wang et al. 2003). These results implicate indirect activation of NAc DA transmission as a significant contributor to the abuse-related effects of opioids.

DA binds to five G protein-coupled receptor subtypes which are divided into two families. The D1-like receptor family includes the G_s-coupled D_1 and D_5 receptor subtypes (D_1R and D_5R) while the D2-like receptor family includes the G_i-coupled D_2, D_3, and D_4 receptor subtypes (D_2R, D_3R, D_4R) (Beaulieu and Gainetdinov 2011). Preclinical evidence indicates that antagonism of either D1-like receptors or D2-like receptors reduces opioid-induced locomotor activation, opioid self-administration, and opioid seeking (for review, see Di Chiara and North 1992; Pierce and Kumaresan 2006; Reiner et al. 2019; Wise 1989). However, adverse side effect profiles associated with these subtype-nonselective compounds have hampered their potential clinical utility to treat substance use disorder (Cho et al. 2010; Haney et al. 2001; Kishi et al. 2013; Millan et al. 1995). Thus, attention has shifted towards pharmacological interrogation of the individual subtypes within these receptor families in an effort to maintain or perhaps improve pharmacotherapeutic efficacy for treatment of opioid use disorder (OUD) while reducing undesirable side effects.

In recent years, the D_3R has emerged as an appealing target in this regard for several reasons (Heidbreder and Newman 2010; Sokoloff and Le Foll 2017). First, the D_3R exhibits a restricted pattern of expression in the brain, being primarily localized to the NAc and other associated limbic regions (Bouthenet et al. 1991; Heidbreder and Newman 2010; Sokoloff et al. 1990). Second, postmortem studies in humans and animals have revealed that chronic exposure to drugs of abuse including opioids increases D_3R mRNA expression within components of the mesolimbic DA system including the VTA and NAc (Le Foll et al. 2003; Liang et al. 2011; Spangler et al. 2003; Staley and Mash 1996). Finally, accumulating preclinical evidence suggests that pretreatment with selective D_3R antagonists attenuates ongoing opioid self-administration under various schedules of reinforcement as well as opioid-seeking behaviors in the general

absence of adverse side effects associated with nonselective D2-like receptor antagonists (Boateng et al. 2015; Galaj et al. 2015; Hu et al. 2013; Jordan et al. 2019; Lv et al. 2019; You et al. 2019). Despite these promising findings, the underlying neuropharmacological mechanisms by which D_3R antagonism alters the neurochemical and/or behavioral effects of opioids remain largely unresolved.

Stimulation of locomotor activity in rodents is a useful behavioral measure with which to selectively probe NAc DA neurotransmission following systemic administration of drugs of abuse, including opioids (Broekkamp et al. 1979; Delfs et al. 1990; Kalivas et al. 1983; Kelly and Iversen 1976; Kelly et al. 1975). Using locomotor activation as a behavioral proxy for NAc DA signaling, we recently reported that selective D_3R antagonism enhances, while selective D_2R antagonism attenuates, cocaine-induced locomotor activity and locomotor sensitization, and these opposite effects on behavior are associated with the unique ability of D_3R blockade to augment DA-induced excitation of D_1 medium spiny neurons (Manvich et al. 2019). However, whether this pattern of effects extends to other classes of drugs of abuse has not been systematically evaluated. The present study therefore aimed to resolve the impact of D_3R and D_2R subtype-selective antagonism upon opioid-induced modulation of locomotor activity. We directly compared the effects of pretreatment with the highly-selective D_3R antagonist PG01037 (133-fold selectivity for the D_3R over D_2R (Grundt et al. 2005)) or the selective D_2R antagonist L-741,626 (41-fold selectivity for the D_2R over D_3R (Kulagowski et al. 1996)) on acute morphine-induced hyperactivity, as well as the induction of sensitization to morphine-induced hyperactivity, in mice. To determine whether nonspecific disruptions of motor function may contribute to any observed reductions of morphine-induced hyperactivity in the present study, we

also assessed whether administration of PG01037 or L-741,626 induces catalepsy when administered either alone or in combination with morphine.

Materials and methods

Subjects

Subjects used in this study were 56 adult male and female C57BL/6J mice (28/sex, 8-12 weeks old at the start of study) weighing between 19-33 g. 48 mice were ordered from Jackson Laboratory (Bar Harbor, ME) and 8 mice were bred at Emory University. Mice were housed in same-sex groups of 3-5 per cage in a climate-controlled vivarium with a 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 were approved by the Emory University Institutional Animal Care and Use Committee.

Locomotor Activity Apparatus

Locomotor activity was assessed in transparent polycarbonate cages (22 x 43 x 22 cm) that allowed passage of 8 infrared beams through the long wall and 4 infrared beams through the short wall of the enclosure at 4.9 cm intervals (San Diego Instruments; San Diego, California). Horizontal ambulations, defined as the sequential disruption of two adjacent infrared beams, were recorded in 5-min bins. The test chambers contained a thin layer of clean bedding and were located behind a closed hospital curtain within the animals' housing room. All test sessions were conducted during the light cycle between 0900-1400. Prior to the onset of experiments, mice were injected intraperitoneally (i.p.) with saline and placed in the test chambers for 30 min for 3 consecutive days in order to habituate the mice to injections and the test apparatus.

Effects of Selective D_3R or D_2R Antagonism on Acute Morphine-Induced Locomotion

The effects of PG01037 and L-741,626 on acute morphine-induced locomotion were evaluated in separate groups of 8 mice (4 males, 4 females) using a within-subjects design. Animals were initially placed in the center of the locomotor chamber and ambulations were recorded for 90 min. Next, animals were briefly removed from the chamber and administered either PG01037 (vehicle or 10 mg/kg i.p.) or L-741,626 (vehicle or 10 mg/kg i.p.), and returned to the locomotor chamber for an additional 30 min for evaluation of the locomotor response to antagonist administration 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 doses of PG01037 and L-741,626 were carefully selected for use based on our previous work showing that these doses do not appreciably disrupt basal locomotion and are the lowest effective doses capable of significantly modulating the locomotor-activating effects of cocaine in C57BL/6J mice (Manvich et al. 2019). Therefore, these doses were expected *a priori* to demonstrate behavioral effects in the present study while maintaining optimal selectivity for their respective D2-like receptor subtypes. The 10 – 56 mg/kg dose range of morphine was selected based on the results of pilot studies in our laboratory showing that they encompass the ascending limb of the morphine dose-response curve (data not shown). Interaction studies with PG01037 and morphine were completed first in all mice, followed by interaction studies with L-741,626 and morphine. For each interaction study, the order of morphine dose testing was: 18, 56, 10 mg/kg. Administration of the active dose of the antagonist pretreatment or its vehicle were counterbalanced across animals within each dose of morphine. Test sessions were separated by at least 1 week to prevent the development of locomotor sensitization to morphine. All mice received all treatments.

Effects of Selective D_3R or D_2R Antagonism on Morphine-Induced Locomotor Sensitization

Sensitization induction took place over 5 consecutive days and was performed in 4 separate groups of mice (n = 8/group, 4 male and 4 female). Mice were initially placed in the center of the locomotor chamber, and locomotor activity was recorded for 90 min. They were then briefly removed from the chamber and administered either PG01037 (vehicle or 10 mg/kg i.p.) or L-741,626 (vehicle or 10 mg/kg i.p.) and returned to the locomotor chamber for 30 min, then were again removed and injected with morphine (56 mg/kg i.p.) and placed back in the chamber for 120 min. Mice received the same pretreatment for each of the 5 test days. Seven days following the last induction session, locomotor activity was again assessed as described above with the exception that all mice received a vehicle injection as the pretreatment 30 min prior to challenge with 56 mg/kg morphine.

Effects of Selective D_3R or D_2R Antagonism on Catalepsy

The effects of PG01037 and L-741,626 on catalepsy were studied in two separate groups of mice (n = 8/group, 4 male and 4 female). Mice received a pretreatment of either PG01037 (vehicle, 10 mg/kg i.p.) or L-741,626 (vehicle, 10 mg/kg i.p.) followed 30 min later by administration of 0, 10, or 56 mg/kg morphine. Catalepsy was evaluated using the bar test (Sanberg et al. 1988), during which a thin bar was secured horizontally 1.75” above a flat surface. Each test was conducted by lifting the mouse by the tail and allowing it to grab the bar with its front paws, then releasing the tail so that the mouse was positioned sitting upright on its hind legs. Upon assuming this position, the latency to remove at least one paw from the bar was recorded. The test was stopped if the subject failed to withdraw one paw within 60 s. Mice that could not be placed in the testing position after 3 attempts received a latency score of 0 s. In each

test, catalepsy was measured 0, 15, 30, 60, and 120 min following administration of morphine. The order of dose-combinations was randomized across mice in each group. Mice were tested once per week until each mouse received all treatment combinations. After testing was complete, all mice received a final catalepsy test in which they were administered 3 mg/kg risperidone i.p. followed by saline i.p. 30 min later. Catalepsy in these tests was measured up to 60 min following saline injection. The3 mg/kg risperidone test was chosen as a positive control because it induces prominent catalepsy in mice (Fink-Jensen et al. 2011).

Drugs

Morphine sulfate (National Institute on Drug Abuse Drug Supply Program, Bethesda, MD) was dissolved in sterile saline. PG01037 was synthesized by Ms. J Cao in the Medicinal Chemistry Section, National Institute on Drug Abuse Intramural Research Program as described previously (Grundt et al. 2005) and dissolved in sterile water. L-741,626 (Tocris Bioscience; Minneapolis, MN) and risperidone (Sigma-Aldrich; St. Louis, MO) were dissolved in vehicle containing ethanol:CremophorEL (Sigma-Aldrich):saline (5:10:85 v/v). All drugs were administered i.p. at a volume of 10 ml/kg.

Statistics

For acute morphine-induced locomotion studies, total ambulations during the 2 h following morphine administration were analyzed via two-way ANOVA with repeated measures on both factors (pretreatment dose x morphine dose) followed by post hoc Holm-Sidak multiple comparisons tests. To assess the effects of either PG01037 or L-741,626 alone on basal locomotor activity over the 30-min period prior to morphine administration, total ambulations in

the 30-min pretreatment window were summed for each mouse, averaged across replicate tests, and analyzed using paired two-tailed t-test. For the induction phase of sensitization studies (i.e. days 1-5), total ambulations during the 2 h following morphine administration were analyzed via mixed two-way ANOVA with repeated measures on one factor (day) and independent measures on the other factor (treatment condition). Dunnett's multiple comparisons tests were used to compare morphine-induced locomotor activity on each of induction days 2-5 vs. morphine-induced locomotor activity on induction day 1. For the challenge day of sensitization studies (day 12), total ambulations during the 2 h following morphine administration were analyzed via independent two-tailed t-test. Latency scores in catalepsy experiments were analyzed using two-way ANOVA with repeated measures on both factors (treatment condition x time), followed by post hoc Bonferroni's test to compare the effects of treatment condition within each time point. The effects of 3 mg/kg risperidone + saline were excluded from statistical analyses since risperidone was included for study only as a positive control to validate the catalepsy detection procedure.

Results

Effects of D_3R or D_2R Antagonism on Acute Morphine-Induced Locomotion

To examine the effects of D_3R antagonism on acute morphine-induced locomotion, mice were pretreated with the highly-selective D_3R antagonist PG01037 (vehicle or 10 mg/kg i.p.) 30 min prior to acute administration of morphine (10, 18, or 56 mg/kg i.p.). In the 30 min following PG01037 administration (i.e. prior to morphine administration), paired t-test indicated that 10 mg/kg PG01037 produced a small albeit significant increase in basal locomotor activity as compared to vehicle ($t_{(7)} = 3.51$, $P = 0.016$) (Fig. 1a). The slight increase in locomotion returned to baseline prior to morphine administration at the 120-min time point in each condition (Fig. 2a-c). Morphine produced a robust increase in locomotor activity with dose-dependent maximal magnitudes and durations of action that was attenuated by PG01037 (Fig. 1b, 2a-c). Two-way repeated measures ANOVA indicated significant main effects of morphine dose ($F_{(2,14)} = 8.21$, $P = 0.004$) and PG01037 dose ($F_{(1,7)} = 27.47$, $P = 0.001$) and a significant morphine x PG01037 interaction ($F_{(2,14)} = 3.81$, $P = 0.048$). Post hoc comparisons revealed pretreatment with PG01037 significantly attenuated the locomotor-activating effects of 18 mg/kg by ~ 51% and the locomotor-activating effects of 56 mg/kg morphine by ~ 45% ($P < 0.05$) (Fig. 1b). The inhibitory actions of PG01037 on morphine-induced locomotion emerged within 10-15 min following morphine administration and persisted until either the stimulated locomotor activity ceased (Fig. 2a-b) or until the 2-h observation period elapsed (Fig. 2c).

To determine the effects of D_2R antagonism on acute morphine-induced locomotion, mice were pretreated with the selective D_2R antagonist L-741,626 (vehicle or 10 mg/kg i.p.) 30 min prior to acute administration of morphine (10, 18, or 56 mg/kg i.p.). In the 30 min following L-741,626 administration (i.e. prior to morphine administration), 10 mg/kg L-741,626 produced

a small decrease in locomotor activity, but paired t-test indicated that it was not significantly different from vehicle ($t_{(7)} = 2.31$, $P > 0.05$) (Fig. 3a). Morphine administration produced a dose-dependent increase in locomotor activity that was almost completely abolished by L-741,626 (Fig. 3b, Fig. 4a-c). Two-way repeated measures ANOVA indicated significant main effects of morphine dose ($F_{(2,14)} = 5.55$, $P = 0.017$) and L-741,626 dose ($F_{(1,7)} = 70.44$, $P < 0.0001$) but not a significant morphine x L-741,626 interaction ($F_{(2,14)} = 2.63$, $P = 0.107$). Post hoc comparisons revealed that pretreatment with L-741,626 reduced morphine-elicited ambulations between 78-92% as compared to vehicle pretreatment ($P < 0.0001$) (Fig. 3b). In contrast to the effects of PG01037, the inhibitory actions of L-741,626 on morphine-induced locomotion were evident immediately upon morphine administration. Similar to PG01037 however, the effects of L-741,626 persisted throughout the duration of morphine's effects (Fig. 4a-c).

Effects of D_3R or D_2R Antagonism on Morphine-Induced Locomotor Sensitization

To test the impact of selective D_3R antagonism on the development of morphine-induced locomotor sensitization, mice were pretreated daily for 5 consecutive days with vehicle or PG01037 (10 mg/kg i.p.) 30 min prior to morphine (56 mg/kg, i.p.). Morphine administration produced a sensitized response, as revealed by an increase in locomotion over days, that was attenuated by PG01037 (Fig. 5, 6a-e). Two-way mixed factors ANOVA revealed significant main effects of induction day ($F_{(4, 56)} = 17.66$, $P < 0.0001$) and PG01037 dose ($F_{(1, 14)} = 4.67$, $P = 0.049$), but not a significant day x PG01037 interaction ($F_{(4, 56)} = 0.51$, $P = 0.73$). Post hoc analyses indicated that, collapsed across vehicle and 10 mg/kg PG01037 pretreatments, mice exhibited a sensitized locomotor response to morphine by day 3 of the induction phase (Fig. 5; time course, Fig. 6a-e). Furthermore, the significant main effect of PG01037 treatment without a

significant day x PG01037 interaction suggests that selective D_3R antagonism attenuated this effect equally across all 5 days, effectively reducing morphine-induced locomotion on average by ~ 36.5% (Fig. 5). One week after the final induction session, all mice received vehicle pretreatment followed by a morphine challenge (56 mg/kg i.p.). Independent t-test showed no difference in locomotor activity between mice that had previously been pretreated with 10 mg/kg PG01037 during induction as compared to mice pretreated with vehicle during induction ($t_{(14)}$ = 0.67, P = 0.52) (Fig. 5; time course, Fig. 6f).

To assess the impact of selective D_2R antagonism on morphine-induced locomotor sensitization, mice were pretreated daily for 5 consecutive days with vehicle or L-741,626 (10 mg/kg i.p.) 30 min prior to morphine (56 mg/kg, i.p.). Morphine administration produced a sensitized response, as revealed by an increase in locomotion over days, that was completely abolished by L-741,626 pretreatment (Fig. 7, 8a-e). Two-way mixed factors ANOVA revealed significant main effects of induction day ($F_{(4, 56)}$ = 9.87, P < 0.0001) and L-741,626 dose ($F_{(1, 14)}$ = 35.57, P < 0.0001) and a significant day x L-741,626 interaction ($F_{(4, 56)}$ = 10.53, P < 0.0001). Post hoc analyses indicated that mice pretreated with vehicle prior to morphine exhibited a sensitized locomotor response by day 3 of the induction phase (Fig 7; time course, Fig. 8a-e). However, morphine-induced locomotor sensitization was reduced by > 90% across the entire induction phase in mice pretreated with 10 mg/kg L-741,626 (Fig. 7). One week after the final induction session, all mice received vehicle pretreatment followed by a morphine challenge (56 mg/kg i.p.). Mice that had previously been administered 10 mg/kg L-741,626 daily during the induction phase showed an attenuated locomotor response to morphine challenge as compared to mice pretreated with vehicle during induction sessions ($t_{(14)}$ = 3.48, P = 0.004). Notably, the

locomotor response on challenge day in L-741,626-treated mice was similar in magnitude (Fig. 7) and time course (Fig. 8f) to vehicle-treated mice on day 1 of the induction phase (Fig. 8a).

Effects of D_3R or D_2R Antagonism Alone or in Combination with Morphine on Catalepsy

To test whether selective D_3R antagonism induced catalepsy, mice were administered vehicle or PG01037 (10 mg/kg i.p.) 30 min prior to morphine (0, 10, 56 mg/kg, i.p.). Neither administration of PG01037 alone, morphine alone, nor their combination resulted in appreciable catalepsy (Fig. 9a). Analysis of these treatment conditions using two-way ANOVA showed no significant main effects of treatment condition ($F_{(4,28)} = 1.00$, $P = 0.424$), time ($F_{(4,28)} = 1.00$, $P = 0.424$), or the treatment condition x time interaction ($F_{(16,112)} = 1.00$, $P = 0.462$). For all mice tested, the latency to withdraw a forepaw in any of the aforementioned conditions did not exceed 1 s. By contrast, 3 mg/kg risperidone produced a robust increase in catalepsy across the 60 min test period, ranging on average from ~ 48 s up to the procedural maximum of 60 s (Fig. 9a).

To test whether selective D_2R antagonism induced catalepsy, mice were administered vehicle or L-741,626 (10 mg/kg i.p.) 30 min prior to morphine (0, 10, 56 mg/kg, i.p.). As was observed in the PG01037 cohort (Fig. 9a), administration of either 10 or 56 mg/kg morphine alone (i.e. 30 min following vehicle of L-741,626) resulted in forepaw withdrawal latency of < 1 s in all mice tested (Fig 9b). However, administration of 10 mg/kg L-741,626 alone or combined with morphine increased latency scores. Two-way ANOVA revealed significant main effects of treatment condition ($F_{(4,28)} = 10.13$, $P < 0.0001$), time ($F_{(4,28)} = 7.58$, $P = 0.0003$), and a significant treatment condition x time interaction ($F_{(16,112)} = 5.12$, $P < 0.0001$). Post hoc analyses indicated that administration of 10 mg/kg L-741,626 alone (i.e. 30 min prior to saline administration) increased catalepsy as compared to the vehicle + 10 mg/kg morphine or vehicle

+ 56 mg/kg morphine treatment conditions at 30, 60, and 120 min post-morphine injection. The increase in catalepsy produced by L-741,626 was significantly attenuated at the 120 min time point by treatment with either 10 or 56 mg/kg morphine (Fig. 9b). Notably, 3 mg/kg risperidone produced a more robust increase in catalepsy across the 60 min observation period, at levels similar to those observed in the PG01037 cohort (Fig. 9a-b).

Discussion

The primary objective of the present work was to compare the effects of selective dopamine D_2R antagonism and selective D_3R antagonism on the locomotor-activating effects of acutely-administered morphine, as well as on the development of sensitization following repeated morphine administration. Our results indicate that antagonism of either dopamine receptor subtype attenuates acute morphine-induced hyperactivity, although differences were observed with respect to the magnitude of this attenuation. Specifically, pretreatment with the selective D_2R antagonist L-741,626 fully abolished the locomotor-activating effect of morphine and prevented the development of sensitization, while pretreatment with the selective D_3R antagonist PG01037 resulted in a significant but partial reduction of morphine-induced hyperlocomotion as compared to that produced by L-741,626 pretreatment. Furthermore, daily pretreatment with PG01037 attenuated, but did not abolish, the locomotor-activating effects of each of five repeated morphine administrations and ultimately did not disrupt the induction of locomotor sensitization.

The present observation that D_2R antagonism more effectively suppresses the behavioral-stimulant effects of morphine as compared to D_3R antagonism is in agreement with a few previous studies that have explored this question using pharmacological tools with poorer subtype selectivity. For example, pretreatment with the D2-like receptor antagonist eticlopride, which exhibits ~15-fold selectivity for D_2R vs. D_3R (Levant 1997), fully abolished morphine-induced hyperlocomotion in mice (Cook and Beardsley 2003), similar to what we observed with the selective D_2R antagonist L-741,626. By contrast, pretreatment with nafadotride, which exhibits ~10-fold selectivity for D_3R vs. D_2R (Audinot et al. 1998), produced a significant but less effective attenuation of morphine-induced hyperlocomotion in the same study (Cook and

Beardsley 2003). A similar pattern of results has also been reported in a comparison between the nonselective D2-like receptor antagonist haloperidol and the modestly-selective D_3R antagonist, U99194A (Manzanedo et al. 1999). To our knowledge, the present study is the first to assess the impact of pretreatment with the selective D_2R antagonist L-741,626 on the locomotor-activating effects of morphine, which is notable given that L-741,626 is presently considered to be among the most selective D_2R antagonists available for *in vivo* preclinical investigation. Our results suggest that the robust attenuation of morphine-induced locomotion previously observed with nonselective D2-like receptor antagonism is primarily attributable to blockade of the D_2R subtype. Moreover, our finding that pretreatment with the selective D_3R antagonist PG01037 produces a significant, albeit weaker, attenuation of morphine-induced locomotion is in accord with a number of recent studies that also demonstrate modest reductions of the locomotor-activating effects of opioids following pretreatment with other highly-selective D_3R antagonists (Kumar et al. 2016; Lv et al. 2019; You et al. 2017). Based on these prior findings and our present results with PG01037 and L-741,626, we conclude that D_3R antagonism and D_2R antagonism exert qualitatively similar reductions of morphine-induced hyperlocomotion, however a pharmacological shift away from D_3R selectivity towards either nonselective antagonism or selective D_2R antagonism renders this modulation far more robust.

It could be argued that the attenuation of morphine-induced hyperactivity following PG01037 pretreatment may have been more effective had higher doses of PG01037 been tested. Although we cannot presently rule out this consideration entirely, this seems unlikely for several reasons. First, the dose of 10 mg/kg PG01037 used in these studies was carefully selected based on our previous finding that 10 mg/kg is the lowest dose that produces significant modulation of cocaine-induced locomotion in C57Bl/6J (Manvich et al. 2019). Second, we tested higher doses

of PG01037 (up to 30 mg/kg) and did not find them to be more efficacious than 10 mg/kg (unpublished observations). Finally, testing higher doses of PG01037 runs the risk of exerting larger effects on basal locomotion and/or limiting D_3R vs. D_2R selectivity *in vivo*, each of which would undoubtedly confound the interpretations of the data. Based on these considerations, our strategy in the present experiments was to assess the effects of the lowest behaviorally-relevant dose of PG01037 (10 mg/kg) in order to maintain maximal D_3R vs. D_2R selectivity. A similar strategy was employed in selecting the dose of L-741,626, inasmuch as 10 mg/kg is the lowest pretreatment dose to significantly alter cocaine-induced locomotion in C57BL/6J mice (Manvich et al. 2019). It could also be suggested that testing lower doses of L-741,626 may have resulted in a partial attenuation of morphine-induced hyperactivity akin to what was observed following PG01037 pretreatment, however this too seems unlikely given that reducing the dose of L-741,626 one-half log step to 3 mg/kg renders this compound ineffective in modulating cocaine-induced locomotor activity (Manvich et al. 2019). Our studies were therefore designed to investigate optimal doses of both PG01037 and L-741,626 that achieve the goal of assessing selective D_3R vs. selective D_2R antagonism.

Not only did D_2R antagonism abolish morphine-induced locomotion, but it also prevented the development of locomotor sensitization. By contrast, D_3R antagonism attenuated acute morphine-induced locomotion but evidently did not prevent the induction of sensitization, since mice appeared sensitized when challenged with morphine alone 1 week after the last drug combination was administered. The reasons for these discrepant effects remain unclear. One possible explanation is that systemic D_2R blockade disrupts the neurobiological adaptations underlying sensitization to opioid-induced locomotion, while systemic D_3R blockade does not. It is generally accepted that changes in mesolimbic DA neurotransmission mediate opioid-induced

locomotor sensitization, and that the primary site at which these neuroadaptations take place is the VTA (Kalivas and Duffy 1987; Vanderschuren and Kalivas 2000; Vezina et al. 1987). Although both D_2Rs and D_3Rs are expressed as autoreceptors within the VTA (Beaulieu and Gainetdinov 2011; Diaz et al. 2000; Lejeune and Millan 1995; Mercuri et al. 1997; Missale et al. 1998), it is possible that they differentially modulate the development of opioid-induced sensitization via distinct neuropharmacological effects that are not yet fully understood. While the NAc does not play a prominent role in the induction of opioid-induced locomotor sensitization (Vanderschuren and Kalivas 2000; Vezina et al. 1987), it is known to play a role in the locomotor-activating effects of acute systemic morphine administration (Schildein et al. 1998; Teitelbaum et al. 1979; Vaccarino and Corrigall 1987; Vezina et al. 1987), and intra-NAc administration of the highly-selective D_3R antagonist SB-277011A has been reported to attenuate the *expression* of morphine-induced locomotor sensitization (Liang et al. 2011). Therefore, our finding that systemic D_3R antagonism attenuates the acute locomotor-activating effects of morphine but does not alter induction of sensitization to this phenomenon could be due to differential modulatory impacts on neural activity within the NAc and VTA, respectively. Consequently, D_3R antagonism may "mask" the overt appearance of sensitization during the induction phase due to its capacity to attenuate morphine-induced hyperactivity, but the development of the underlying sensitization can be "unmasked" and observed when morphine is tested in the absence of D_3R antagonism. It is noteworthy that this hypothesis is in conflict with a recent report that pretreatment with the highly-selective D_3R antagonist VK4-116 attenuated the induction of oxycodone-induced locomotor sensitization (Kumar et al. 2016) as assessed by a challenge test with oxycodone alone. However, some key procedural differences may underlie these discrepant findings including the use of different opioids (morphine vs. oxycodone), use of

different D_3R antagonists (PG01037 vs. VK4-116), and imposition of 7 days vs. 2 days between the final induction session and the expression test.

A clear distinction can be drawn from our results between the impact of D_3R antagonism and D_2R antagonism on morphine-induced hyperactivity, as the combination of the D_2R antagonist L-741,626 with morphine resulted in a dramatic and sustained loss of locomotion that was not evident following the combination of the D_3R antagonist PG01037 with morphine. We suspected that the pronounced reduction in locomotion following combined administration of L-741,626 with morphine may be due in part to the emergence of catalepsy, based on the findings that combined administration of nonselective D2-like receptor antagonists with opioids induces catalepsy (Kiritsy-Roy et al. 1989; Rodriguez-Arias et al. 2000) and administration of L-741,626 alone has been found to induce catalepsy in squirrel monkeys (Achat-Mendes et al. 2010), rats (Millan et al. 2000), and mice (Hattori et al. 2006). However, to our knowledge, the cataleptic effects of combined administration of a selective D_2R antagonist such as L-741,626 with morphine had not been investigated prior to the present work. Our results replicate the previous finding that L-741,626 pretreatment alone is sufficient to induce a modest cataleptic effect 1 h after its administration in mice (Hattori et al. 2006). Interestingly however, we found that co-administration of moderate doses of morphine that fail to induce catalepsy alone (but which promote hyperactivity) attenuated, rather than potentiated, the cataleptic effects of L-741,626 120 min after morphine administration. Moreover, we note that the locomotor-activating effects of morphine are disrupted as early as 30 min after L-741,626 administration, a time point at which its cataleptic effects are nonsignificant. Given these pieces of evidence, we conclude that the emergence of a cataleptic state does not underlie the robust suppression of morphine-induced hyperactivity following L-741,626 pretreatment. Nevertheless, the cataleptic effects exhibited by

the selective D_2R antagonist L-741,626 suggest that catalepsy (and possibly other adverse motoric/extrapyramidal effects) induced by nonselective D2-like receptor antagonists are mediated by blockade of the D_2R subtype. By contrast, PG01037 showed no cataleptic effects in this study at a dose which significantly modulated morphine-induced hyperactivity. This finding is in agreement with previous reports that catalepsy is not observed in rodents following pretreatment with other selective D_3R antagonists, S33084 (Millan et al. 2000) and SB-277011A (Reavill et al. 2000). However, it should be pointed out that ours is the first study to assess catalepsy following combined administration of a highly-selective D_3R antagonist and an opioid. Given that D_3R antagonists are being considered as potential pharmacotherapeutics for OUD, the lack of measurable catalepsy following D_3R antagonism alone or in combination with morphine adds to accumulating evidence that D_3R antagonists exhibit a desirable safety profile as compared to nonselective D2-like receptor antagonists or D_2R-selective antagonists, even when opioids are concurrently administered (Jordan et al 2019).

One key question remaining is how pretreatment with a D_3R antagonist attenuates opioid-induced hyperactivity (present results) but potentiates cocaine-induced hyperactivity (Manvich et al. 2019). This remains an elusive question that will require further investigation to clarify. The enhancement of cocaine-induced locomotion by D_3R antagonism occurs coincidentally with an increase in DA-induced excitability and activity of DA D_1 receptor-expressing medium spiny neurons within the NAc (Manvich et al. 2019). However, this mechanism is unlikely to explain the attenuation of morphine-induced locomotion by D_3R antagonism because i.c.v infusion of the D1-like receptor agonist SKF38393 enhances, rather than attenuates, morphine-induced hyperactivity in mice (Funada et al. 1994). There are some notable disparities between hyperactivity produced by opioids vs. psychostimulants that may partially or wholly explain the

bidirectional effect of D_3R antagonism. For example, cocaine increases locomotion in a DA-dependent manner via blockade of the dopamine reuptake transporter (DAT) and a consequent rise in extracellular DA levels within the NAc (Giros et al. 1996; Ritz et al. 1987; Yamamoto et al. 2013). By contrast, opioid receptor agonists like morphine are capable of increasing locomotion via both DA-dependent and DA-independent processes. More specifically, intra-VTA opioid receptor agonist administration induces locomotor activity via disinhibition of DAergic neurons, and as such requires an intact mesolimbic DA projection (Devine et al. 1993; Gysling and Wang 1983; Johnson and North 1992; Matsui and Williams 2011; Spanagel et al. 1992). By contrast, intra-NAc opioid receptor agonists induce locomotion via direct actions on NAc medium spiny neurons, an effect that does not require intact DA neurotransmission (Churchill and Kalivas 1992; Kalivas et al. 1983; Pert and Sivit 1977; Stinus et al. 1985). Collectively, our prior and current findings may therefore indicate that D_3R antagonism potentiates drug-induced hyperactivity that is DA-dependent, but attenuates drug-induced hyperactivity that is DA-independent. This hypothesis is further supported by the findings that intra-NAc administration of a selective D_3R antagonist potentiates the locomotor-activating effects of cocaine (Manvich et al. 2019), but attenuates the locomotor-activating effects of morphine (Liang et al. 2011). Alternatively, the temporal patterns of cocaine-induced vs. morphine-induced increases in NAc DA are different (Di Chiara and Imperato 1988; Pontieri et al. 1995; Rouge-Pont et al. 2002; Sorge and Stewart 2006; Zocchi et al. 2003), likely owing to their dependence upon DAT inhibition vs. VTA DA neuron disinhibition respectively, and it is plausible that these temporally-distinct influences on DA signaling may be differentially modulated by D_3R antagonism. Still another potential explanation for the opposing influence of D_3R antagonism on stimulant- vs. opioid-induced hyperactivity may be that the reliance of

cocaine and related psychostimulants on the DAT to induce increases in locomotion renders them potentially susceptible to neuropharmacological modulation by recently-described direct interactions between DAT and the D_3R (Castro-Hernandez et al. 2015; McGinnis et al. 2016; Zapata et al. 2007). By contrast, DAT/D_3R interactions would not be predicted to play a role in the DA-independent drivers of opioid-induced locomotion since they do not require functional DAergic terminals to exert their behavioral effects (Churchill and Kalivas 1992; Kalivas et al. 1983; Stinus et al. 1985). At present, such hypotheses remain speculative and additional studies will be required to elucidate the specific mechanisms and neuroanatomical substrates underlying the ability of D_3R antagonism to oppositely modulate the locomotor-activating effects of cocaine and morphine, as well as determine whether these observed differences extend to other drugs within the classes of psychostimulants and opioids.

In summary, we report here that pretreatment with either the highly-selective D_3R antagonist PG01037 or the selective D_2R antagonist L-741,626 attenuates morphine-induced increases in locomotor activity in mice. D_2R antagonism resulted in a far greater suppression of morphine-induced locomotion as compared to D_3R antagonism and fully prevented the induction of sensitization to morphine's locomotor-activating effects, but also exhibited undesirable cataleptic effects that were not produced by doses of PG01037 which significantly affected morphine-induced hyperactivity. Taken together, these findings lend further support to the potential use and safety of D_3R antagonists in the treatment of OUD.

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Figure Legends

Fig. 1 Effects of the highly-selective D_3R antagonist PG01037 on acute morphine-induced locomotor activity. **a** Total ambulations in the 30-min period immediately following administration of 10.0 mg/kg PG01037 (filled symbol) or its vehicle (open symbol). * $P < 0.05$, 10.0 mg/kg PG01037 compared to vehicle. **b** Total ambulations in the 2-hr period immediately following administration of morphine (10 – 56 mg/kg). 30 min prior to morphine injection, animals had been pretreated with either 10.0 mg/kg PG01037 (filled symbols) or its vehicle (open symbols). ** $P < 0.01$, *** $P < 0.001$, 10.0 mg/kg PG01037 compared to vehicle within each dose of morphine. Each data point represents mean ± SEM total ambulations. For both panels **a** and **b**, all subjects received all treatments (n = 8)

Fig. 2 Time course of locomotor activity following pretreatment with the highly-selective D_3R antagonist PG01037 and subsequent administration of morphine. 10.0 mg/kg PG01037 (filled symbols) or its vehicle (open symbols) were administered 30 min prior to **a** 10 mg/kg morphine, **b** 18 mg/kg morphine, or **c** 56 mg/kg morphine. Each data point represents mean ± SEM ambulations recorded in 5-min bins. Arrows indicate time of pretreatment injection ("Veh/PG", i.e. vehicle or 10.0 mg/kg PG01037 respectively) or morphine. All mice received all treatments (n = 8)

Fig. 3 Effects of the selective D_2R antagonist L-741,626 on acute morphine-induced locomotor activity. **a** Total ambulations in the 30-min period immediately following administration of 10.0 mg/kg L-741,626 (shaded symbol) or its vehicle (open symbol). **b** Total ambulations in the 2-hr period immediately following administration of morphine (10 – 56 mg/kg). 30 min prior to

morphine injection, animals had been pretreated with either 10.0 mg/kg L-741,626 (shaded symbols) or its vehicle (open symbols). **** $P < 0.0001$, main effect of 10.0 mg/kg L-741,626 compared to vehicle (collapsed across morphine doses). Each data point represents mean ± SEM total ambulations. For both panels **a** and **b**, all subjects received all treatments (n = 8)

Fig. 4 Time course of locomotor activity following pretreatment with the selective D_2R antagonist L-741,626 and subsequent administration of morphine. 10.0 mg/kg L-741,626 (shaded symbols) or its vehicle (open symbols) were administered 30 min prior to **a** 10 mg/kg morphine, **b** 18 mg/kg morphine, or **c** 56 mg/kg morphine. Each data point represents mean ± SEM ambulations recorded in 5-min bins. Arrows indicate time of pretreatment injection ("Veh/L7", i.e. vehicle or 10.0 mg/kg L-741,626 respectively) or morphine. All mice received all treatments (n = 8)

Fig. 5 Effects of the highly-selective D_3R antagonist PG01037 on morphine-induced locomotor sensitization. Mice received the combination of either 10.0 mg/kg PG01037 + 56.0 mg/kg morphine or vehicle + 56.0 mg/kg morphine daily for 5 days. One week later (day 12), all mice received vehicle pretreatment 30 min prior to a challenge with 56.0 mg/kg morphine. Shown for days 1-5 are the total number of ambulations in the 2-hr period following injection of 56.0 mg/kg morphine, which was administered 30 min after pretreatment with either 10.0 mg/kg PG01037 (filled symbols) or its vehicle (open symbols). Shown on day 12 are total ambulations in the 2-hr period following administration of 56.0 mg/kg morphine in mice pretreated with 10.0 mg/kg PG01037 during induction (filled symbols) or mice pretreated with vehicle during induction (open symbols). ## $P < 0.01$, #### $P < 0.0001$, significant difference compared to Day

1 (collapsed across pretreatment doses). * $P < 0.05$, main effect of pretreatment dose (collapsed across days 1-5). N.S., not significant

Fig. 6 Time course of locomotor activity following pretreatment with the selective D_3R antagonist PG01037 and subsequent administration of 56.0 mg/kg morphine during induction days 1-5 of sensitization, and challenge test with morphine alone one week later. 10.0 mg/kg PG01037 (filled symbols) or its vehicle (open symbols) were administered 30 min prior to 56.0 mg/kg morphine over 5 consecutive days. Shown are mean ± SEM ambulations recorded in 5-min bins on **a** day 1, **b** day 2, **c** day 3, **d** day 4, and **e** day 5 of sensitization induction, or **f** challenge day one week later in which 56 mg/kg morphine was administered to all mice after vehicle pretreatment. Arrows indicate time of pretreatment injection ("Veh/PG" i.e. vehicle or 10.0 mg/kg PG01037 respectively) or 56.0 mg/kg morphine. n = 8 per group

Fig. 7 Effects of the selective D_2R antagonist L-741,626 on morphine-induced locomotor sensitization. Mice received the combination of either 10.0 mg/kg L-741,626 + 56.0 mg/kg morphine or vehicle + 56.0 mg/kg morphine daily for 5 days. One week later (day 12), all mice received vehicle pretreatment 30 min prior to a challenge with 56.0 mg/kg morphine. Shown for days 1-5 are the total number of ambulations in the 2-hr period following injection of 56.0 mg/kg morphine, which was administered 30 min after pretreatment with either 10.0 mg/kg L-741,626 (shaded symbols) or its vehicle (open symbols). Shown on day 12 are total ambulations in the 2-hr period following administration of 56.0 mg/kg morphine in mice pretreated with 10.0 mg/kg L-741,626 during induction (filled symbols) or mice pretreated with vehicle during induction (open symbols). #### $P < 0.0001$, compared to Day 1 in vehicle-treated

mice. **** $P < 0.0001$, main effect of pretreatment dose (collapsed across days 1-5). Ψ $P < 0.05$, vehicle vs. 10.0 mg/kg L-741,626 on challenge day

Fig. 8 Time course of locomotor activity following pretreatment with the selective D_2R antagonist L-741,626 and subsequent administration of 56.0 mg/kg morphine during induction days 1-5 of sensitization, and challenge test with morphine alone one week later. 10.0 mg/kg L-741,626 (shaded symbols) or its vehicle (open symbols) were administered 30 min prior to 56.0 mg/kg morphine over 5 consecutive days. Shown are mean ± SEM ambulations recorded in 5-min bins on **a** day 1, **b** day 2, **c** day 3, **d** day 4, and **e** day 5 of sensitization induction, or **f** challenge day one week later in which 56 mg/kg morphine was administered to all mice after vehicle pretreatment. Arrows indicate time of pretreatment injection ("Veh/L7" i.e. vehicle or 10.0 mg/kg L-741,626 respectively) or 56.0 mg/kg morphine. n = 8 per group

Fig. 9 Catalepsy following administration of the highly-selective D_3R antagonist PG01037 or the selective D_2R antagonist L-741,626 alone or in combination with morphine. **a** Mice were pretreated with PG01037 (vehicle or 10 mg/kg) followed 30 min later by administration of 1 of 3 doses of morphine (0, 10, or 56 mg/kg), or 3 mg/kg risperidone followed 30 min later by administration of saline. **b** Mice were pretreated with L-741,626 (vehicle or 10 mg/kg) followed 30 min later by administration of 1 of 3 doses of morphine (0, 10, or 56 mg/kg), or 3 mg/kg risperidone followed 30 min later by administration of saline. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, compared to both "Veh + 10 Morph" and "Veh + 56 Morph" within the same time point. ## $P < 0.01$, ### $P < 0.001$, compared to "10 L7 + 0 Morph" within the same time point. Each data point represents mean ± SEM latency in seconds to withdraw paw in the bar test.

Latencies were measured at 0, 15, 30, 60, and 120 min relative to the second injection. For both panels **a** and **b**, all subjects received all treatments (n = 8/group).

Fig. 1

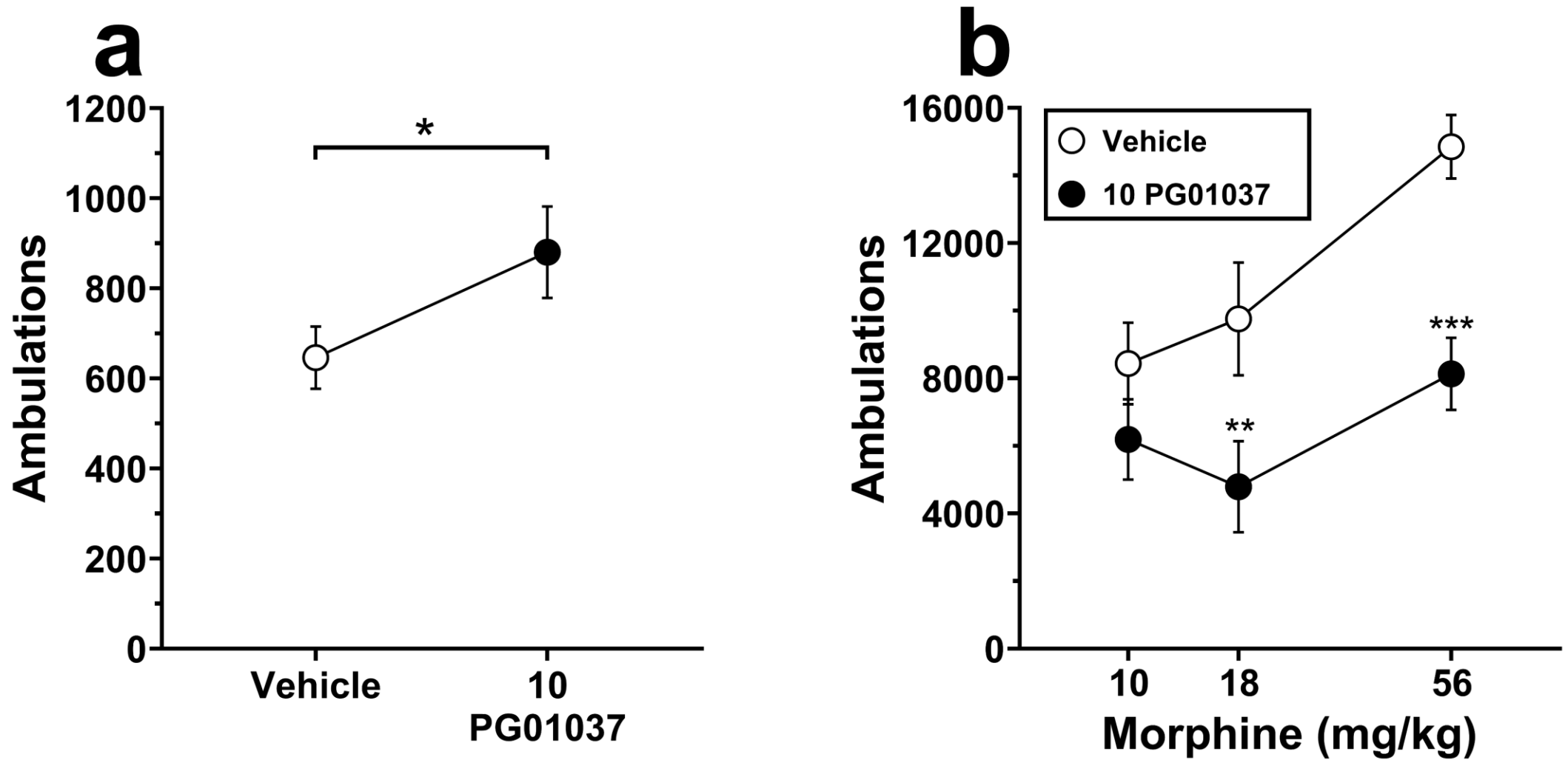

a
Ambulations
1200
1000
800
600
400
200
0
*
Vehicle
10
PG01037
b
Ambulations
16000
12000
8000
4000
0
Vehicle
10 PG01037
**
***
10
18
56
Morphine (mg/kg)

Fig. 2

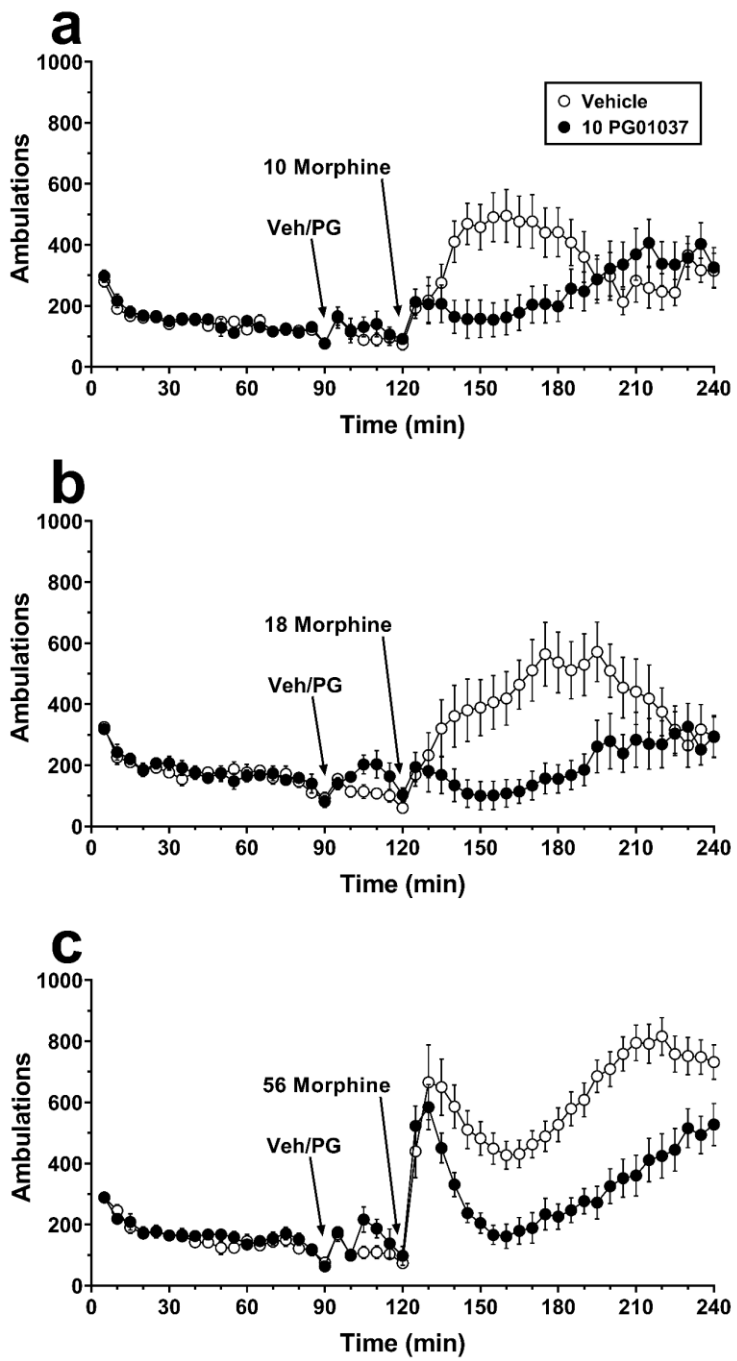

a
Vehicle
10 PG01037
10 Morphine
Veh/PG
Ambulations
Time (min)
b
18 Morphine
Veh/PG
Ambulations
Time (min)
c
56 Morphine
Veh/PG
Ambulations
Time (min)

Fig. 3

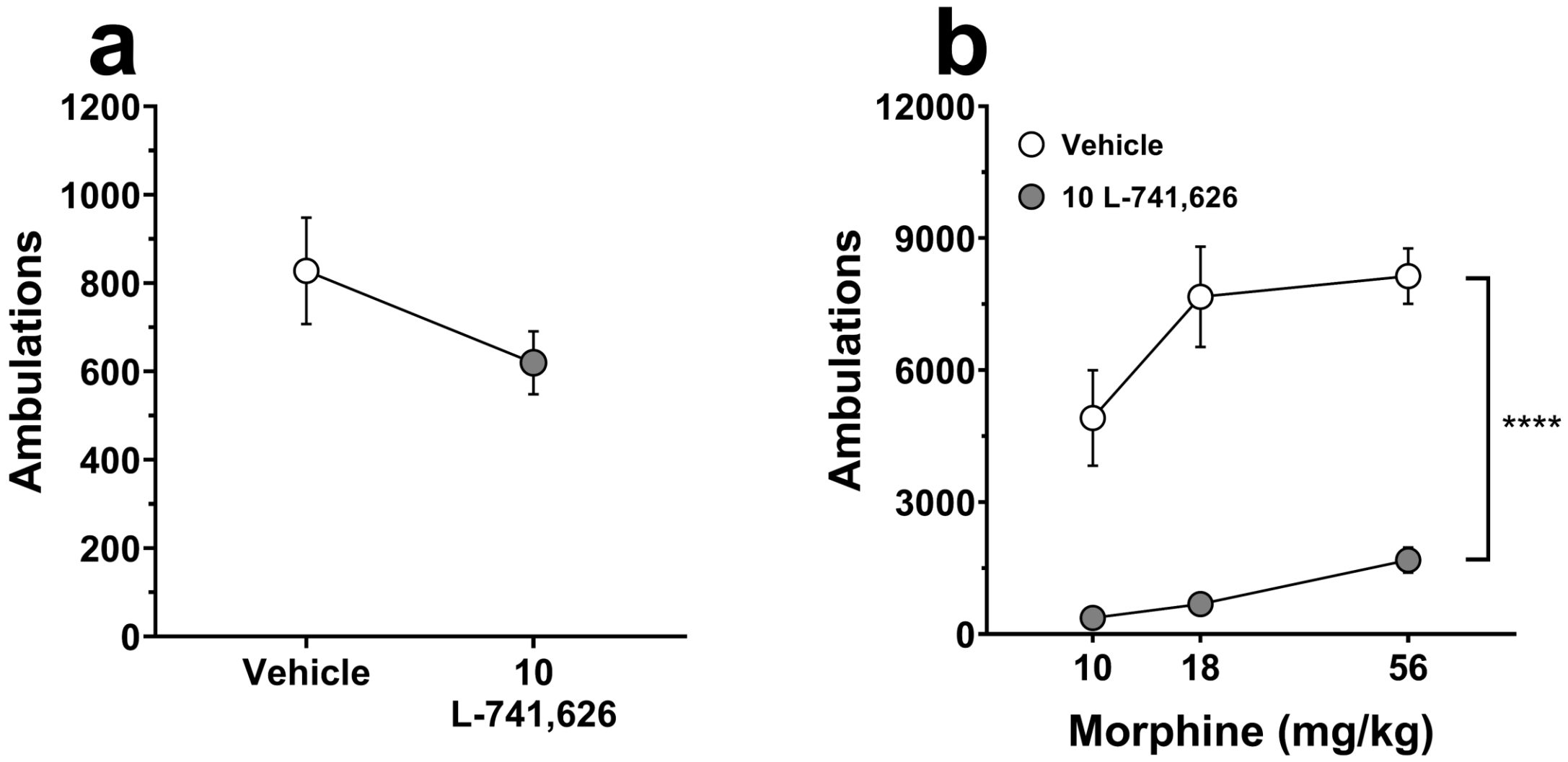

a
Ambulations
1200
1000
800
600
400
200
0
Vehicle
10
L-741,626
b
Ambulations
12000
9000
6000
3000
0
Vehicle
10 L-741,626
****
10
18
56
Morphine (mg/kg)

Fig. 4

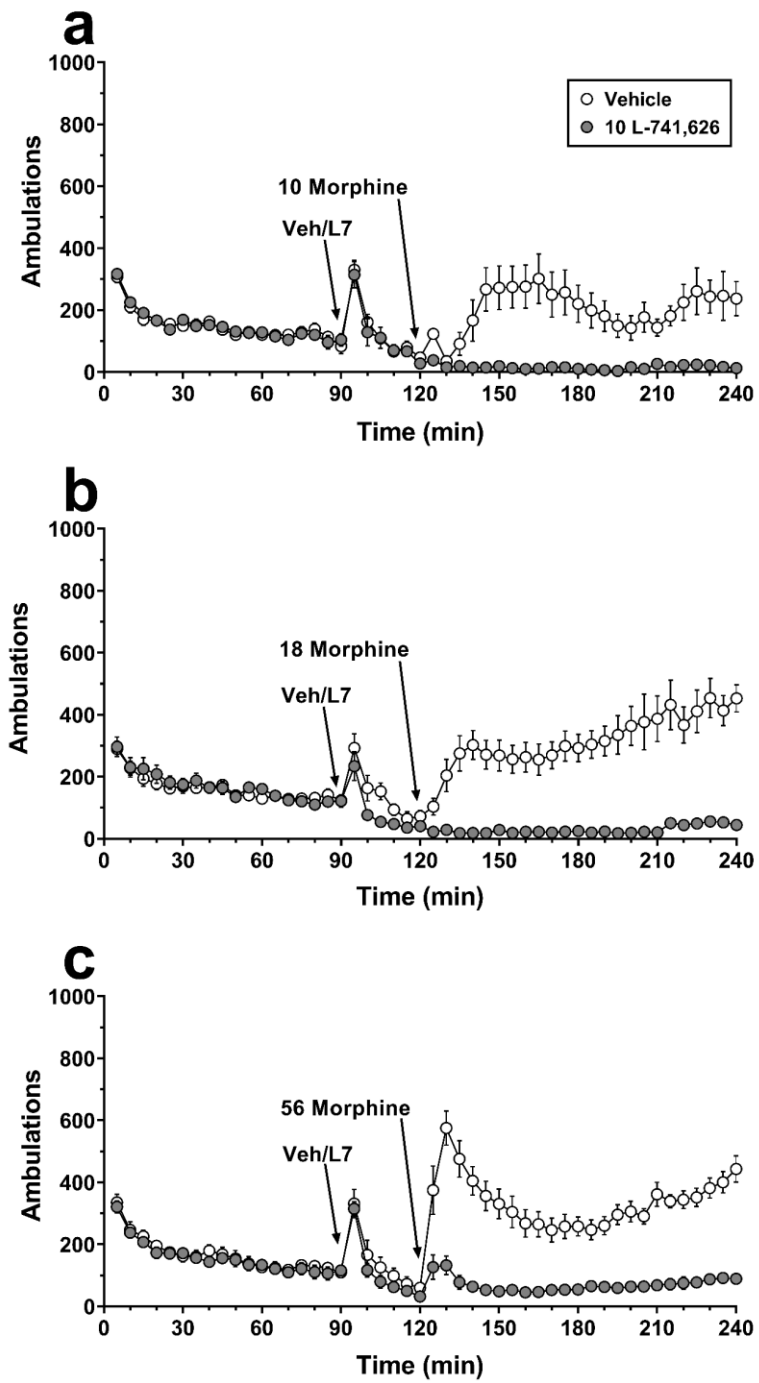

a
Vehicle
10 L-741,626
10 Morphine
Veh/L7
Ambulations
Time (min)
b
18 Morphine
Veh/L7
Ambulations
Time (min)
c
56 Morphine
Veh/L7
Ambulations
Time (min)

Fig. 5

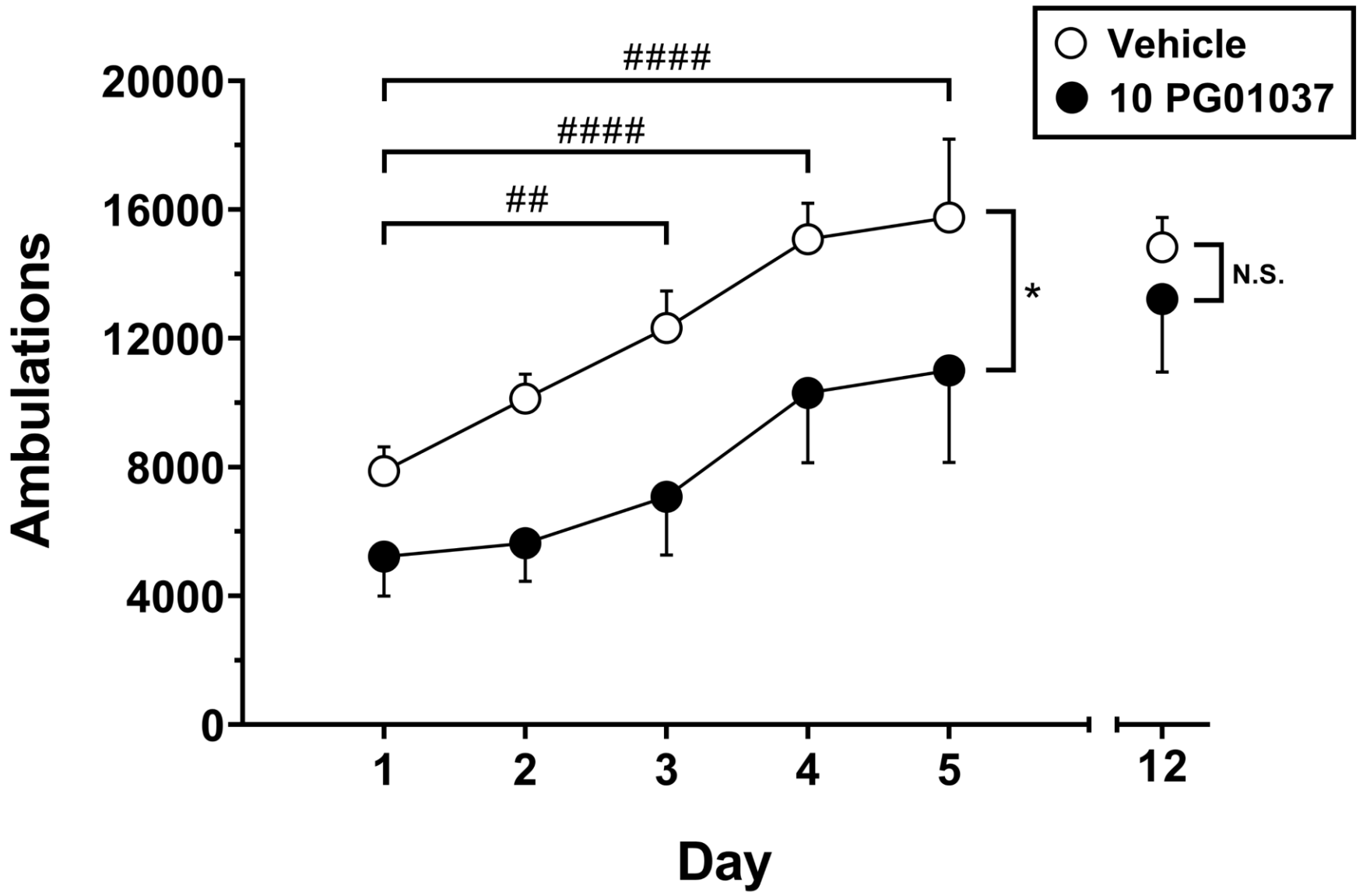

Vehicle
10 PG01037
####
####
##
*
N.S.
Ambulations
20000
16000
12000
8000
4000
0
1
2
3
4
5
12
Day

Fig. 6

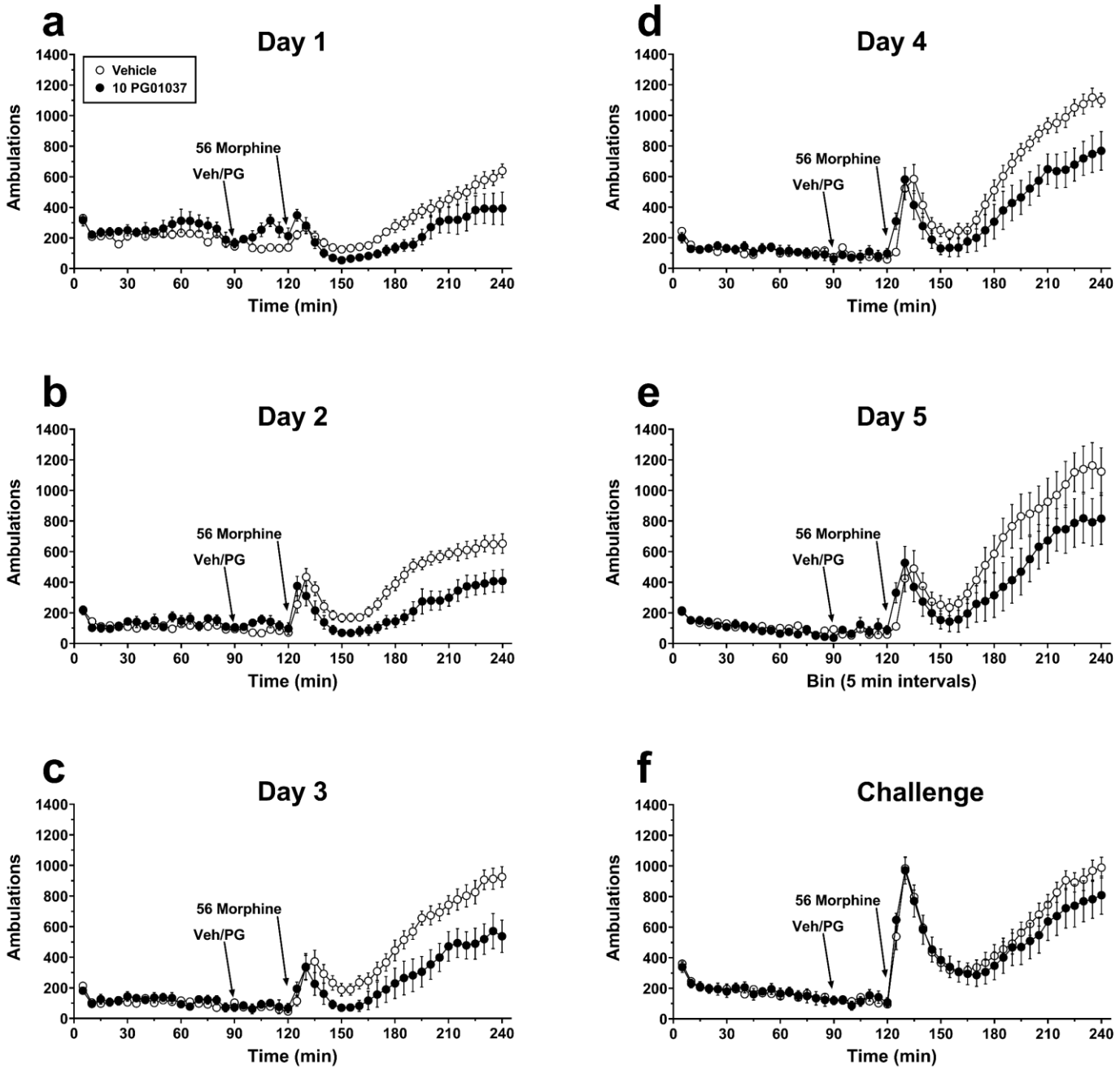

a
Day 1
Vehicle
10 PG01037
56 Morphine
Veh/PG
Ambulations
Time (min)
b
Day 2
56 Morphine
Veh/PG
Ambulations
Time (min)
c
Day 3
56 Morphine
Veh/PG
Ambulations
Time (min)
d
Day 4
56 Morphine
Veh/PG
Ambulations
Time (min)
e
Day 5
56 Morphine
Veh/PG
Ambulations
Bin (5 min intervals)
f
Challenge
56 Morphine
Veh/PG
Ambulations
Time (min)

Fig. 7

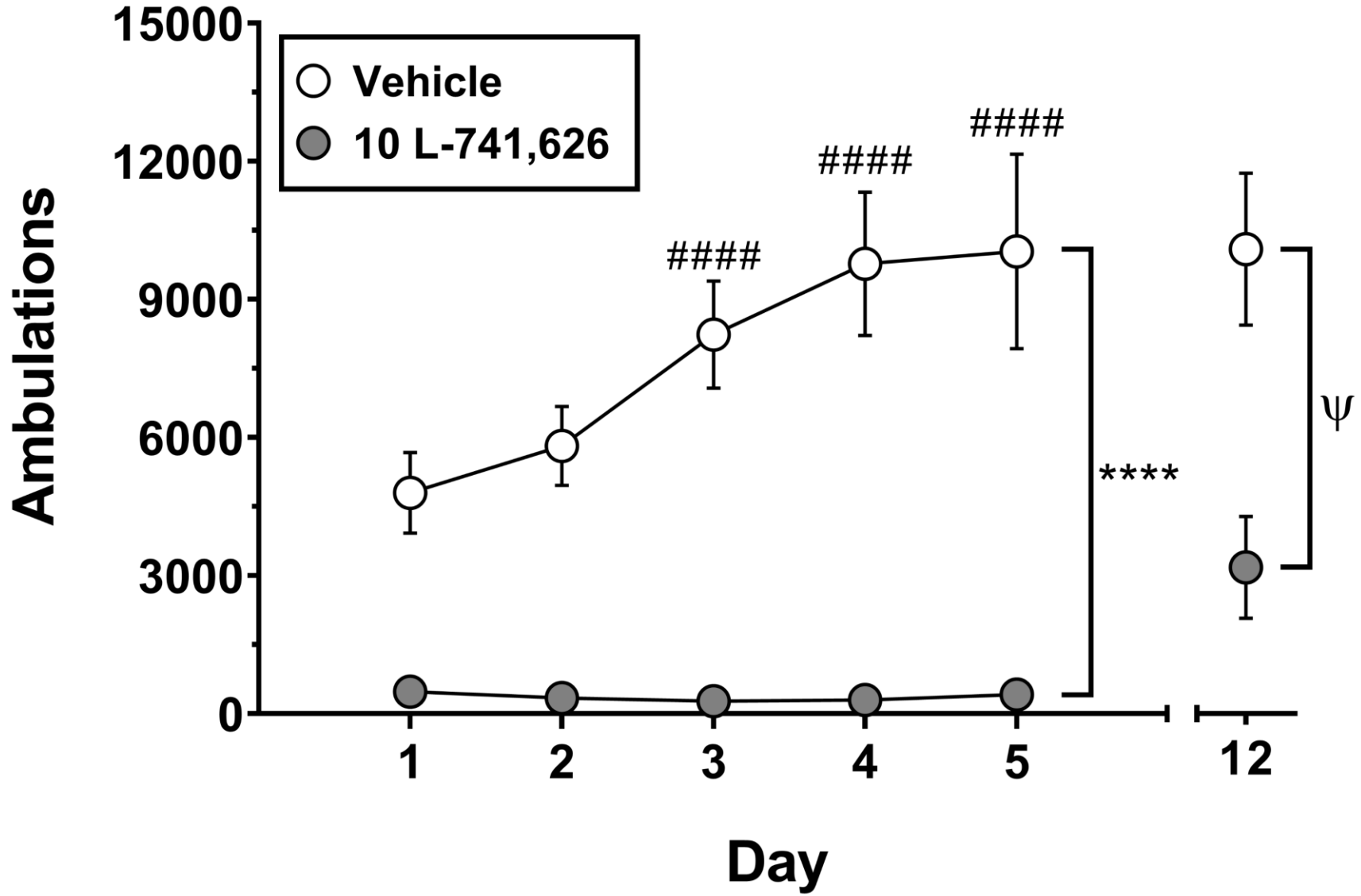

15000
12000
9000
6000
3000
0
Ambulations
Vehicle
10 L-741,626
####
####
####
****
ψ
1
2
3
4
5
12
Day

Fig. 8

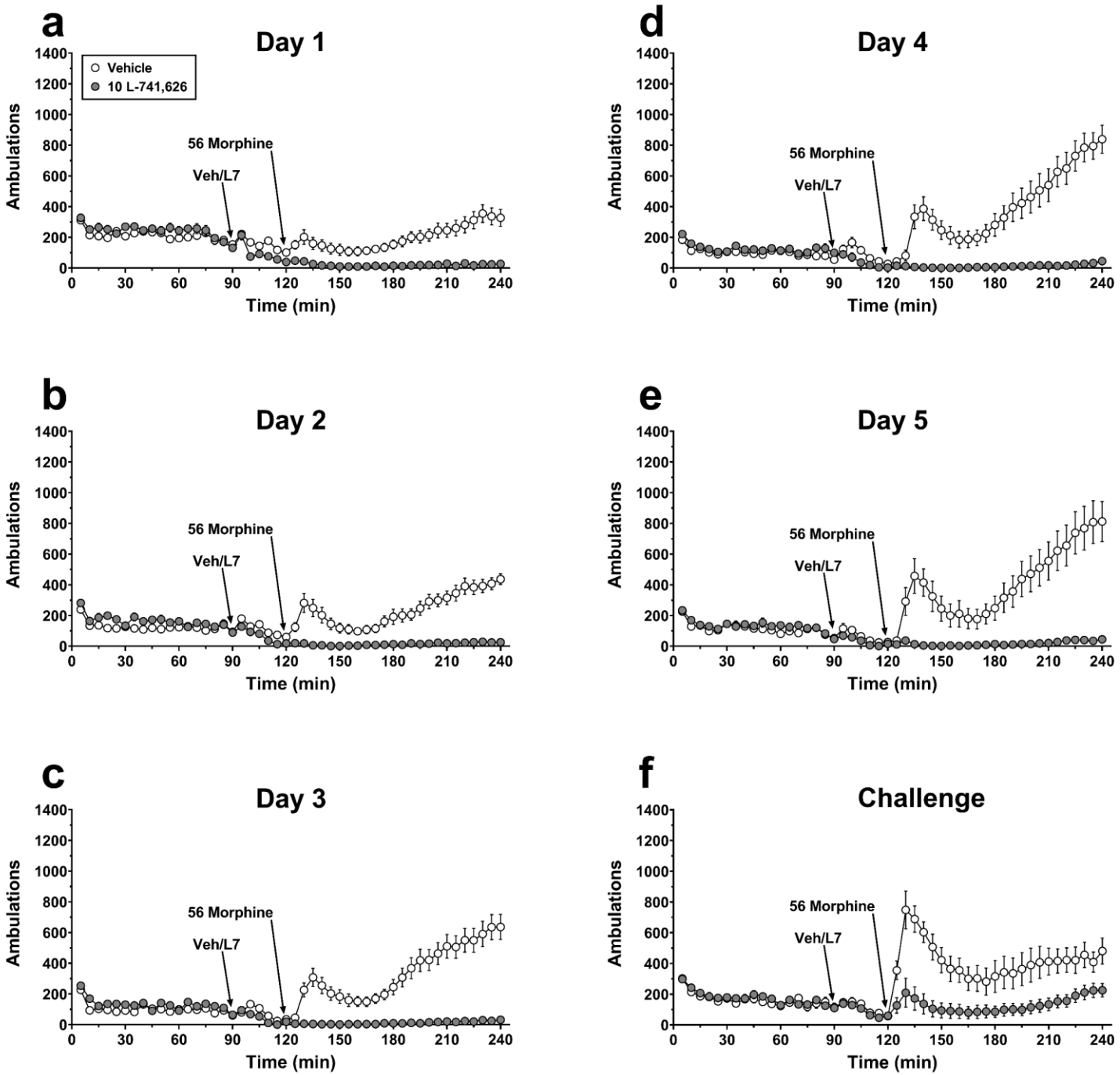
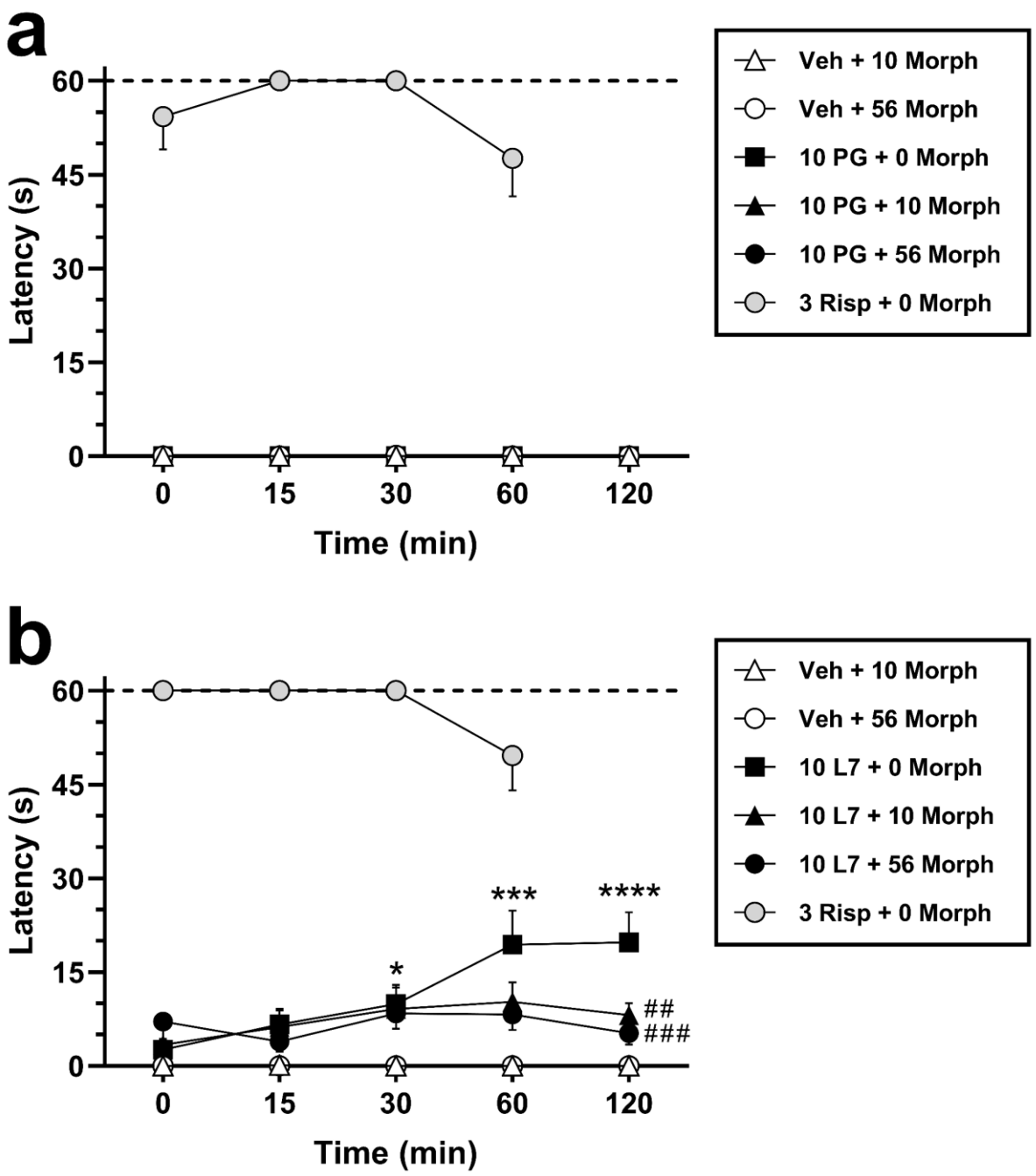

a
Day 1
Vehicle
10 L-741,626
56 Morphine
Veh/L7
Ambulations
Time (min)
b
Day 2
c
Day 3
d
Day 4
e
Day 5
f
Challenge

Fig. 9


a
Latency (s)
60
45
30
15
0
0
15
30
60
120
Time (min)
Veh + 10 Morph
Veh + 56 Morph
10 PG + 0 Morph
10 PG + 10 Morph
10 PG + 56 Morph
3 Risp + 0 Morph
b
Latency (s)
60
45
30
15
0
0
15
30
60
120
Time (min)
*
***
****
##
###
Veh + 10 Morph
Veh + 56 Morph
10 L7 + 0 Morph
10 L7 + 10 Morph
10 L7 + 56 Morph
3 Risp + 0 Morph