Morphine tolerance and physical dependence are altered in conditional HIV-1 Tat transgenic mice


SF, DLS, FAK, KLS, RME, PMB, PEK, WLD, KFH, Department of Pharmacology & Toxicology,
PEK, KFH, Department of Anatomy & Neurobiology, Virginia Commonwealth University,
Medical College of Virginia Campus, Richmond, VA 23298, USA
SF, Now at the Department of Psychology & Neuroscience, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
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b) Corresponding Author
Sylvia Fitting, Ph.D. or Kurt F. Hauser, Ph.D.
Dept. Pharmacology & Toxicology
Virginia Commonwealth University
1217 East Marshall Street
Richmond, Virginia 23298-0613, USA
Phone (804) 628-7580 or (804) 628-7579;
FAX (804) 827-9974
Email: sfitting@vcu.edu or kfhauser@vcu.edu

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AIDS – acquired immunodeficiency syndrome
ANOVA – analysis of variance
CNS – central nervous system
DOX – doxycycline
HAND – HIV-1-associated neurocognitive disorders
HIV-1 – human immunodeficiency virus type 1
MPE – maximal possible effect
neuroAIDS – neuro-acquired immunodeficiency syndrome
Tat – transactivator of transcription

e)

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Abstract

Despite considerable evidence that chronic opiate use selectively affects the pathophysiological consequences of HIV-1 infection in the nervous system, few studies have examined whether neuroAIDS might intrinsically alter the pharmacological responses to chronic opiate exposure. This is an important matter, because HIV-1 and opiate abuse are interrelated epidemics and HIV-1-patients are often prescribed opiates as a treatment for HIV-1-related neuropathic pain. Tolerance and physical dependence are inevitable consequences of frequent and repeated administration of morphine. In the present study, mice expressing HIV-1 Tat in a doxycycline (DOX)-inducible manner [Tat(+)], their Tat(−) controls, and control C57BL/6 mice were chronically exposed to placebo or 75-mg morphine pellets to explore the effects of Tat induction on morphine tolerance and dependence. Antinociceptive tolerance and locomotor activity tolerance were assessed using tail-flick and locomotor activity assays, respectively, while physical dependence was measured with the platform jumping assay and recording of other withdrawal signs. We found that Tat(+) mice treated with DOX [Tat(+)/DOX] developed an increased tolerance in the tail-flick assay compared to control Tat(−)/DOX and/or C57/DOX mice. Equivalent tolerance was developed in all mice when assessed by locomotor activity. Further, Tat(+)/DOX mice expressed reduced levels of physical dependence to chronic morphine exposure following a 1 mg/kg naloxone challenge compared to control Tat(−)/DOX and/or C57/DOX mice. Assuming the results seen in Tat transgenic mice can be generalized to neuroAIDS, our findings suggest that HIV-1-infected individuals may display heightened analgesic tolerance to similar doses of opiates compared to uninfected individuals, and show less symptoms of physical
dependence.

**Keywords:** Neuro-acquired immunodeficiency syndrome (neuroAIDS); opioid drug abuse; tolerance; dependence; naloxone; HIV-1 Tat; transgenic mice.
Introduction

Opiates (derivatives of the opium poppy) such as morphine, which is the major bioactive metabolite of heroin in the brain, have considerable abuse liability, but also have great therapeutic value for alleviating moderate to severe chronic pain. Chronic pain, including somatic pain, visceral pain and headache, is often reported by human immunodeficiency virus type 1 (HIV-1)-infected patients (Breitbart and Dibiase, 2002). Opiate drugs can accelerate the central nervous system (CNS) complications of HIV-1 (Bell et al., 1998; Fellin et al., 2006) and can increase the severity of HIV-1-associated neurocognitive disorders (HAND) (Attwell and Laughlin, 2001; Haughey and Mattson, 2002; Yang et al., 2007). In addition, opiates can exacerbate simian immunodeficiency virus progression in experimental models of acquired immune deficiency syndrome (AIDS) (Park et al., 1996; Greenwood et al., 2007; Noel et al., 2008). The increases in HIV-1 pathogenesis caused by opioid abuse have largely been attributed to opioid suppression of immune function (Adler et al., 1993; Carr and Serou, 1995; Peterson et al., 1998), while more recent evidence suggests that opioid drugs additionally interact with neurons and glia directly and thereby worsen the CNS manifestations of HIV-1 (Gurwell et al., 2001; El-Hage et al., 2005; Hu et al., 2005; Turchan-Cholewo et al., 2006).

It has been repeatedly demonstrated that systemically administered morphine produces antinociception via actions at both spinal and supra-spinal sites (Hernandez-Lopez et al., 2000), and that the repeated use of opiates induces tolerance, thus requiring escalating doses to produce pain relief. The neurobiological mechanisms underlying the development of opiate tolerance involve cellular and molecular
adaptations, including the uncoupling of G-proteins from opioid receptors (desensitization), opiate agonist-induced receptor internalization, and/or opioid receptor down-regulation leading to a decrease in the number of functional binding sites (Leshner and Koob, 1999; Hille, 2001; Ellis et al., 2007; Kim et al., 2011). It has been demonstrated that G-protein-coupled opioid and chemokine co-receptors can undergo heterologous, bidirectional cross-desensitization (Rogers et al., 2000; Rogers and Peterson, 2003), including CCR5 and µ-opioid receptors (Rogers and Peterson, 2003; Chen et al., 2004). HIV-1 proteins such as the transactivator or transcription (Tat) have been shown to induce inflammation by elevating the production of CCL5/RANTES and IL-6 (El-Hage et al., 2005; El-Hage et al., 2006; El-Hage et al., 2008). The interaction between Tat and morphine potentiates Tat-induced increases in CCL2/MCP-1 and CCL5/RANTES release in the striata of HIV-1 Tat transgenic mice (Fitting et al., 2010a).

In the present study, we used HIV-1 Tat inducible transgenic mice as a model of HAND to explore the effects of Tat on morphine tolerance and dependence following chronic exposure to an implanted placebo or 75-mg morphine pellet. Tolerance and physical dependence have been studied extensively using subcutaneous implantation of morphine pellets in rodents as a standard technique to produce opioid tolerance and dependence (Maggiolo and Huidobro, 1961; Way et al., 1968; Cicero and Meyer, 1973; Patrick et al., 1975). The results demonstrated that the development of tolerance to chronic morphine exposure is increased by Tat when assessed by tail-flick but not by locomotor activity. In turn, symptoms of physical dependence to chronic morphine exposure are significantly decreased by Tat.
Material and Methods

Animals

Doxycycline (DOX)-inducible, brain-specific HIV-1\text{IIIb} Tat_{1-86} transgenic mice were developed on a C57BL/6J hybrid background as described in detail elsewhere (Bruce-Keller et al., 2008; Hahn et al., 2013). Tat expression, which is under the control of a tetracycline responsive promoter controlled by glial fibrillary acidic protein (GFAP) expression, was induced with a specially formulated chow containing 6 mg/g DOX (Harlan, Indianapolis, IN, product #: TD.09282). Inducible Tat(+) transgenic mice express both GFAP-rtTA and TRE-tat genes, while control Tat(−) transgenic mice express only the GFAP-rtTA genes. All transgenic mice (~4 months of age, ~25 g, males) were genotyped to confirm the presence of tat and/or rtTA transgenes. In addition, non-transgenic C57BL/6 mice (~4 months of age, ~25 g, males) from Harlan Laboratories (Indianapolis, IN, USA) were used to control for the potential effects of the foreign tat and/or rtTA transgenes, as well as any possible effects of the C57BL/6 hybrid background (Bruce-Keller et al., 2008; Hahn et al., 2013). The non-transgenic C57BL/6 mice will be referred to as “C57” throughout the manuscript.

Half of all mice were fed the normal chow and the other half received DOX-supplemented food for 3 weeks before the beginning of the experiment. Mice were housed in groups (2-4 mice per cage) on a 12 h light/dark cycle (lights on at 7:00 AM) and with free access to water and the specified food.

Placebo or Chronic Morphine Administration

Placebo or chronic morphine administration was achieved by the subcutaneous
implantation of a placebo or 75-mg morphine pellet (10.7 mg/day for up to 7 days; NIDA, Rockville, MD) under aseptic conditions and 2.5% isoflurane anesthesia as previously described (Ross et al., 2008). Using morphine pellets is a standard method for continuously administering morphine to prevent cycles of withdrawal in mice, and it produces brain drug levels considered to be similar to blood/tissue levels achieved in humans who are tolerant and dependent on opiates (Ozaita et al., 1998; Ghazi-Khansari et al., 2006), and therapeutic levels seen in patients maintained on chronic opiates/opiate pumps for intractable pain (Balch and Trescot, 2010). Briefly, mice were anesthetized with 2.5% isoflurane before shaving the hair on the back of the neck. Adequate anesthesia was noted by the absence of the righting reflex and a lack of response to a toe pinch, according to Institutional Animal Care and Use Committee guidelines. The skin was cleaned with 10% povidone iodine (General Medical Corp., Prichard, WV) and rinsed with alcohol before making a 1-cm horizontal incision at the base of the skull. By using a sterile glass rod, the underlying subcutaneous space toward the dorsal flanks was opened. Maintenance of a stringent aseptic surgical field minimized any potential contamination of the pellet, incision, and subcutaneous space. A placebo or 75-mg morphine pellet was inserted in the space before closing the site with Clay Adams Brand, MikRon AutoClip 9 mm Wound Clips (BD Biosciences, San Jose, CA) and applying iodine to the skin surface. Mice were allowed to recover in their home cages where they remained throughout the experiment. The sample size of each group was between 6-8 animals [C57/No DOX/Placebo, n = 8; C57/No DOX/Morphine, n = 7; C57/DOX/Placebo, n = 8; C57/DOX/Morphine, n = 8; Tat(−)/No DOX/Placebo, n = 8; Tat(−)/No DOX/Morphine, n = 7; Tat(−)/DOX/Placebo, n = 8; Tat(−)/DOX/Morphine, n
Acute Cumulative Morphine Injections

For acute morphine injections, morphine sulfate was dissolved in pyrogen-free isotonic saline (Hospira, Lake Forest, IL). To test for tolerance, mice received cumulative, subcutaneous morphine injections in the subscapular region following 4 days of chronic exposure of placebo or morphine pellets. Placebo-pelleted mice received cumulative morphine doses of 2, 4, 8, and 16 mg/kg, whereas morphine-pelleted mice received cumulative morphine doses of 8, 16, 32, and 64 mg/kg. Mice were tested before and immediately after the acute cumulative subcutaneous morphine injections.

Testing Procedure
2.1.1. Experimental Design

All animal procedures were approved by the Virginia Commonwealth University of Institutional Animal Care and Use Committee (IACUC) and are in keeping with AAALAC guidelines. The experiments were performed between 9 am and 6 pm (Figure 1). Three weeks before the start of testing, the standard mouse chow was replaced with the specially formulated DOX chow for half of the animals. Body weight was recorded before pellet implants and at the 4th day after pellet implants on the day of testing. Mice chronically received placebo or 75-mg morphine subcutaneously via the time-release pellets for 4 days. To test for morphine tolerance, two different assessments were
conducted; (1) the antinociceptive effects were determined using the warm-water tail-flick test assay, and (2) the increase in locomotor activity using a photocell activity chamber. Baseline response for tail flick and locomotor activity were measured on the 4th day of pellet implants but before animals received acute cumulative subcutaneous morphine injections. Tail-flick response was recorded following a 20 min waiting period after each injection, and after the last injection mice were additionally transferred to an activity chamber to assess locomotor activity.

After assessing tolerance, morphine dependence was examined in response to a naloxone-precipitated withdrawal challenge. Precipitated withdrawal was measured immediately after subcutaneous naloxone (1 mg/kg) injection by using the platform jumping assay, as well as recording other somatic signs of morphine withdrawal, including the number of wet dog shakes, jumps, and forepaw tremors.

Warm-Water Tail-Flick Test

The tail-flick test was performed using a water bath with the temperature maintained at 56 ± 0.1 °C. Each animal was gently wrapped in a cloth by the experimenter. For baseline latency, tail flick was measured before acute cumulative morphine injections. The distal 1/3 of the tail was immersed in a water bath set at 56 °C and mice rapidly remove their tail from the bath at the first sign of discomfort. The duration of time the tail remained in the water bath was counted as the baseline latency. Baseline latency reaction times in untreated mice were 2- to 4-s. Test latency was obtained after each cumulative morphine injection with the latency to remove the tail increasing proportionally to the analgesic potency of the drug. A 10-s maximum cut-off latency
was used to prevent any tissue damage. Antinociception was quantified as the percentage of maximal possible effect (\%MPE), which was calculated as \%MPE = \left[ \frac{(test\ latency - control\ latency)}{(10 - control\ latency)^{-1}} \right] \times 100 \hspace{1em} (Harris\ and\ Pierson,\ 1964). The \%MPE value was calculated for each mouse using 6 to 8 mice per group.

**Locomotor Activity**

Spontaneous motor activity was assessed using activity chambers (Med Associates, Inc, St. Albans, VT). Mice were habituated to the chamber for 10 min prior to drug administration. Ambulatory counts for spontaneous activity were obtained over a 10-min time period. Each individual activity chamber has closeable doors and a ventilation system. The interior of the chamber consists of a 27x27 cm Plexiglas enclosure that is wired with photo-beam cells connected to a computer console that counts the activity of the animal contained within the enclosure. Ambulatory counts were generated and the difference between baseline activity and activity after acute cumulative subcutaneous morphine injections was calculated for each mouse using 6 to 8 mice per group.

**Antagonist-Precipitated Withdrawal Assessment**

The main withdrawal symptom assessed was jumping from an elevated platform at a height of 32 cm and diameter of 17 cm. The number of mice that jumped off their individual platforms was recorded and a 10-min maximal cut-off time was used. This was followed by evaluating additional withdrawal signs during a period of 5 min. Mice were placed in a rectangular clear plastic observation box (16x16x30 cm; max. 3 mice in one box) and observed for 5 min. The number of wet dog shakes, forepaw tremors,
and jumps was counted for each mouse using 6 to 8 mice per group.

Statistical Analysis

All data are presented as mean ± standard error of the mean (S.E.M.). In the behavioral experiments, continuous variables including body weight, warm-water tail flick, and locomotor activity, were subjected to statistical analyses using analysis of variance (ANOVA) followed by Bonferroni’s post-hoc analyses if necessary to determine statistical significance (IBM SPSS Statistics 22, Inc.). The dose-response data for %MPE were additionally analyzed for ED$_{50}$ and potency ratios. ED$_{50}$ values were calculated using sigmoidal curvilinear analysis with a variable slope model fixing bottom and top value constraints of 0 and 100, respectively (Prism 6 for Mac OS X, GraphPad Software, Inc., La Jolla, CA). ED$_{50}$ values were considered significantly different if the upper and lower 95% confidence interval (CI) between the dose-response curves did not overlap. Potency ratio values (specifically, EC$_{50}$ shifts) were calculated using nonlinear regression fixing bottom and top value constraints of 0 and 100, respectively (Prism 6 for Mac OS X, GraphPad Software, Inc., La Jolla, CA). A potency-ratio value of greater than 1.0 with a lower 95% CI of greater than 1.0 was considered a significant difference in potency between two dose-response curves (placebo versus morphine groups for the corresponding mouse group). GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA USA) was used to plot data and regression curves. Jumping off an elevated platform, a nominal scaled measure, was presented as the incidence of jumping (yes/no). Thus, the percentage of mice that jumped off the platform was compared by the z-test of two proportions. Additional non-continuous variables,
including number of wet dog shakes, forepaw tremors, jumps and grooming, are presented as counted observations (ordinal scaled measure) and compared using the Mann-Whitney $U$-test. Differences of $p < 0.05$ were considered significant.
Results

Body Weight

Body weight was unaffected by DOX when comparing the three mouse groups prior to pellet implantation (C57/No DOX: 28.00 ± 0.38 g, C57/DOX: 27.18 ± 0.43 g, Tat(−)/No DOX: 27.20 ± 0.74 g, Tat(−)/DOX: 26.57 ± 0.71 g, Tat(+)/No DOX: 25.44 ± 0.61 g, Tat(+)/DOX: 26.27 ± 0.53 g). The data presented in Figure 2 illustrate the percent change in body weight after pellet implantation represented by the mean ± S.E.M with 100% indicating no change in body weight. An ANOVA indicated a significant effect of DOX \([F(1, 80) = 6.6, p < 0.05]\), with a significant ~3% decrease in body weight for animals receiving the normal chow (97.1 ± 1.02%) compared to DOX animals (100.10 ± 1.00%). Further, a main effect for pellet implant was noted \([F(1, 80) = 13.0, p < 0.001]\), and DOX x pellet implant interaction \([F(1, 80) = 6.4, p < 0.05]\). The DOX x pellet implant interaction indicated that the greatest loss of body weight was shown by the morphine-pelleted group that did not receive DOX food (92.8 ± 0.94 g) compared to the DOX/morphine-pelleted group (99.2 ± 1.87 g), whereas the placebo-pelleted mice that received DOX (101.0 ± 0.90 g) or normal (100.9 ± 1.34 g) chow showed no change. It should be noted that there were no significant effects when conducting post-hoc tests, except for a significant difference between morphine-pelleted Tat(+)/No DOX and Tat(+)/DOX mice \((p < 0.05, \text{Figure 2})\).

Morphine antinociceptive and locomotor activity tolerance in Tat(+) and Tat(−) mice

As depicted in Figure 3, all groups demonstrated antinociceptive tolerance following chronic morphine pellet exposure compared to groups that received placebo pellets.
This is indicated by chronic morphine exposure shifting the morphine dose-response curve to the right for all groups compared to the placebo condition with potency ratio values varying between 3.43 to 11.24-fold (Table 1). Further, the ANOVA results indicate that acute, cumulative morphine injections of 8 and 16 mg/kg significantly increased %MPE in placebo-implanted mice compared to mice receiving chronic morphine via 75-mg pellet implants (p < 0.05). Importantly, in Tat(+)/DOX mice, there was a significant shift (based on non-overlapping 95% CI) in morphine sensitivity revealed by an approximately 11.24-fold increase in morphine ED50 values, from 3.78 (3.28-4.34) mg/kg for placebo-pelleted Tat(+)/DOX to 44.01 (25.87-74.89) mg/kg for morphine-pelleted Tat(+)/DOX mice. This significant shift was absent in C57/DOX and Tat(−)/DOX mice (see Table 1). The ED50 value for morphine-pelleted Tat(+)/DOX mice was significantly different from all other morphine-pelleted groups, except for the Tat(−)/No DOX and C57/No DOX mice groups (based on non-overlapping 95% CI), suggesting Tat induction increased morphine tolerance. Tat(+)/DOX mice displayed a decreased %MPE (31.7 ± 10.47) compared to Tat(−)/DOX mice (61.2 ± 8.81) across all acute morphine injections [F(1, 12) = 4.7, p ≤ 0.05], supporting the observation that Tat increased the tolerance to chronic morphine. Post-hoc tests demonstrated that the Tat-induced increases in tolerance were specifically revealed in response to an acute injection of 32 mg/kg morphine (p < 0.05, Figure 3, bottom right Panel). No other significant effects were noted.

Additionally, tolerance was examined by assessing changes of locomotor activity after acute and cumulative subcutaneous morphine injections (Figure 4). A main effect was noted for mouse group [C57, Tat(−), Tat(+); F(2, 80) = 5.9, p < 0.01], pellet implant...
[placebo, morphine; $F(1, 80) = 177.1, p < 0.001$], mouse group x DOX food interaction
[$F(2, 80) = 8.1, p < 0.01$], and mouse group x pellet implant interaction [$F(2, 80) = 9.6, p
< 0.001$]. Post-hoc tests demonstrated the development of tolerance as indicated by
less stimulatory activity following chronic morphine pellet exposure in all groups, except
C57/No DOX mice treated with placebo pellets ($p < 0.05$). Additionally, for the placebo-
pelleted groups, C57/DOX resulted in significantly less locomotor activity compared to
Tat(−)/DOX ($p < 0.05$) or Tat(+) /DOX ($p < 0.05$) mice. Thus, no significant differences
were noted in the development of tolerance between any of the morphine-pelleted
groups, indicating that Tat did not alter development of tolerance when assessed by
locomotor activity.

Naloxone-Precipitated Withdrawal Assessment to Test for Dependence

Experiments were conducted to determine the development of physical dependence
to morphine in Tat(−) and Tat(+) mice (Figure 5). Physical dependence as quantified by
naloxone-induced jumping was not observed for any of the placebo-pelleted transgenic
mice, while some jumping was observed in the placebo-pelleted C57/No DOX mice
after they received four acute cumulative doses of morphine (2, 4, 8, 16 mg/kg) ($p < 0.05$). In contrast, most of the morphine-pelleted mice jumped off the elevated platform
within the 10 min time period, indicating physical dependence. Chi-Square tests
(corrected for multiple comparisons) indicated significant differences between the
corresponding placebo- vs. morphine-pelleted mice ($p < 0.05$), except for C57/No DOX
and Tat(+)/DOX mice. Whereas the placebo-pelleted C57/No DOX group indicated
dependence, probably due to the acute cumulative subcutaneous morphine injections,
the morphine-pelleted Tat(+)/DOX group indicated significantly less physical dependence to morphine than the C57/DOX or Tat(−)/DOX morphine-pelleted mice ($p < 0.05$). These findings indicate that (1) C57 mice appear to be more sensitive to morphine (Figure 5, top left Panel) and (2) the development of dependence was significantly altered/decreased by Tat induction (Figure 5, bottom right Panel).

Additional somatic signs of withdrawal to morphine were assessed, including number of wet dog shakes, jumps, and forepaw tremors (Figure 6). Incidences of wet dog shakes were higher in chronic morphine exposed mice compared to placebo-pelleted mice ($p < 0.05$), except for Tat(+)/DOX mice, indicating Tat(+)/DOX mice showed no morphine-induced physical dependence (Figure 6A). The alteration of physical dependence by Tat induction is supported by the finding that morphine-pelleted Tat(+)/No DOX mice, showed increased wet dog shakes compared to Tat(+)/DOX mice ($p < 0.05$). The number of jumps indicated the presence of physical dependence in Tat(+)/No DOX ($p < 0.05$), as well as C57/DOX ($p < 0.05$) mice, with Tat(+)/No DOX showing higher numbers of jumps compared to Tat(+)/DOX mice ($p < 0.05$) – again indicating less physical dependence with Tat induction. There were no differences in forepaw tremors, except for the placebo-pelleted Tat(−)/DOX mice, which displayed significantly more forepaw tremors compared to chronic morphine exposed Tat(−)/DOX mice ($p < 0.05$).
Discussion

In this study tolerance and dependence were observed in mice being continuously exposed to morphine via a 75-mg pellet implant for 4 days. Physical dependence on and tolerance to narcotics have been produced in animals by a variety of techniques, including intravenous self-administration, oral self-administration, systemic injection, and intra-ventricular injections. The most widely used method, however, is the implantation of morphine pellets in rodents (Maggiolo and Huidobro, 1961; Way et al., 1968; Cicero and Meyer, 1973). With this procedure the CNS is continuously exposed to morphine (Way et al., 1968) and a marked degree of tolerance and physical dependence can be produced in a very short period of time (Way et al., 1969). The 75-mg morphine pellets result in initial blood levels of 2 µg/mL of morphine and in sustained blood levels (0.6 µg/mL morphine) by 48 h that last 2–5 days (Bryant et al., 1988). Levels of opiates in the blood of addicts who had died of an opiate overdose indicate an average level of opiates in the blood of 0.8 ± 0.1 µg/mL (Ozaita et al., 1998), similar to that seen in 75-mg morphine pelleted mice (Bryant et al., 1988). We realize that the use of a pellet to administer morphine chronically to mice differs from how humans chronically abuse the drug, but in both cases, sufficiently high morphine brain levels are achieved to develop tolerance. Perhaps more importantly, the pellet method has been the method of choice for the chronic administration of morphine to mice for decades and we chose this method to better relate our findings to the decades of work performed using pellets.

Morphine tolerance was assessed by measuring antinociception and locomotor activity in mice chronically exposed to placebo or morphine pellets. Physical
dependence was determined by quantitating different withdrawal signs, elicited by naloxone following chronic administration of the opiate. Importantly, morphine tolerance and physical dependence were differently affected by HIV-1 Tat expression in a transgenic mouse model of neuroAIDS. Tat induction enhanced the development of tolerance, whereas a decrease in physical dependence by Tat was noted after 1 mg/kg naloxone injection.

We used different control conditions to test for morphine, DOX, and Tat effects. First, the induction of tolerance and physical dependence to morphine was clearly shown in the behavioral measures assessed in this study, when comparing placebo groups with their morphine-pelleted counterparts. It should be noted that even though the ED$_{50}$ values for C57/No DOX and Tat(−)/No DOX mice were not significantly different between placebo- and morphine-pelleted conditions, the potency ratios showed significant 5.5- and 5.18- fold increases, respectively. Further, based on the platform-jumping assay after precipitated withdrawal, the placebo-pelleted C57/No DOX mice were more sensitive to morphine as they developed some physical dependence following acute cumulative morphine injections compared to the transgenic mice bred on a C57BL/6J hybrid background. Strain differences in morphine tolerance and dependence are consistent with previous reports (Kest et al., 2002a; Kest et al., 2002b; Liu et al., 2011) who also have reported increased IL-1β expression in C57BL/6 mice after morphine treatment (Liu et al., 2011). However, more detailed experiments with more appropriate controls are necessary to support the notion that C57 mice show higher sensitivity to morphine.

Second, as our conditional HIV-1 Tat transgenic mouse model requires DOX
administration to induce the tat transgene, we also wanted to determine whether chronic DOX exposure might intrinsically affect opiate tolerance or dependence. Although the effect was not significant, it was noticed that there was a tendency for DOX treatment to affect the %MPE response in placebo-pelleted C57 mice receiving an acute 2 mg/kg morphine injection, compared to similarly treated mice that were not administered DOX. Furthermore, a significant DOX effect was noted for platform jumping, with the sensitivity of placebo-pelleted C57 mice to acute morphine being reduced following DOX administration. It is possible that chronic morphine-induced inflammatory effects, such as increases in IL-1β or other cytokines (Liu et al., 2011; Merighi et al., 2013) might be reduced by DOX itself, since DOX is reported to have modest anti-inflammatory effects at high doses (Chen et al., 2009; Chaudhry et al., 2010). Further, DOX increased the ED₅₀ on the %MPE in the tail-flick assay for all morphine-pelleted groups (no increase was noted in placebo-pelleted mice). Importantly however, a statistically significant effect of DOX on the ED₅₀ was noted only in the Tat(+) mice (morphine-pelleted Tat(+)/No DOX versus morphine-pelleted Tat(+)/DOX groups). This indicates that the effect is not due to DOX itself, but rather to the induction of Tat expression.

Third, testing the effects of Tat induction by DOX, Tat(−)/DOX mice are considered the most valid control for their Tat(+) /DOX counterparts, as both mouse groups were developed on the same genetic background and both express the foreign rtTA transgene. The only distinction is that the Tat(−) control mice do not express the tat transgene. The increased tolerance noted in morphine-pelleted Tat(+) /DOX mice on %MPE compared to Tat(−)/DOX mice, indicate that Tat is altering the underlying
mechanism involved in the development of antinociceptive tolerance, which is confirmed by the finding that no differences were noted between Tat(−)/No DOX and Tat(+)/No DOX mice. Cytotoxicity following prolonged morphine or Tat exposure has been previously demonstrated in vitro in glial-restricted precursors isolated from spinal cord (Buch et al., 2007). Interestingly, Tat did not affect tolerance to the effects of morphine in the locomotor activity assay, suggesting that Tat does not interact with morphine’s actions at supraspinal sites governing locomotor activity including the striatum. This was somewhat unexpected since prior studies have shown that Tat and morphine interactions have pronounced neuroinflammatory and neurodegenerative effects on the striatum (Bruce-Keller et al., 2008; Fitting et al., 2010a; Fitting et al., 2010b; Zou et al., 2011; Hauser et al., 2012; Fitting et al., 2014), and prolonged Tat induction was found to disrupt locomotor activity (Hahn et al., 2013). Nevertheless, it has been noted that the effects of Tat on locomotor activity can vary depending on the duration of Tat induction using DOX (Fitting et al., 2012; Hahn et al., 2013). It is clear that depending on the parameter measured, tolerance to morphine, as well as other opiates, develops at different rates and to differing degrees in the same individual. For instance, tolerance develops to respiratory depression and euphoria, but not to constipation, in humans and animals (Freye and Latasch, 2003). Furthermore, the spinal vs. supraspinal mechanisms underlying the development of morphine tolerance differ in regard to which opioid or other receptor types are involved (Porreca et al., 1987; Xu et al., 2014). It has been shown that the mammalian target of rapamycin (mTOR) mediates the induction and maintenance of tolerance to morphine’s antinociceptive effects in the tail flick assay, but mTOR does not affect morphine tolerance as related to locomotor function.
The interaction of mTOR and Tat has been previously reported with mTOR being involved in Tat-induced neurotoxicity (Fields et al., 2015). Assuming that Tat modulates morphine tolerance via mTOR, this might explain why Tat expression affects morphine tolerance in the tail flick assay (spinal level), but not in the locomotor activity test (supraspinal level). Although the reasons for the discrepancy are uncertain, our lab recently reported differences in the onset and in the levels of Tat mRNA expression in the spinal cord and striatum (Fitting et al., 2012). Whether the differential effects of Tat on tolerance are related to regional differences in the effects of Tat within the CNS, e.g., discrepancies in spinal vs. supraspinal actions, or the duration of DOX administration, needs to be further investigated.

Importantly, HIV-1 Tat decreased physical dependence despite increasing tolerance to morphine. It should be noted that a dissociation between tolerance and dependence is not new and has been previously reported when comparing PKC and PKA inhibitors, which reversed tolerance, but failed to block dependence as assessed by naloxone-precipitated withdrawal (Smith et al., 2002; Gabra et al., 2008). PKC and PKA activation has been demonstrated to modulate G protein-coupled receptors and cause heterologous desensitization (Kelly et al., 1999), which is suggested to be one of the molecular adaptations underlying the development of opiate tolerance. The increased tolerance seen with Tat induction has been attributed to the upregulation of heterologous, bidirectional cross-desensitization of opioid and chemokine co-receptors (Rogers et al., 2000; Rogers and Peterson, 2003), as increased CCL2/MCP-1 and CCL5/RANTES levels have been reported in our HIV-1 Tat transgenic mice (Fitting et al., 2010a). In contrast, Tat has been reported to attenuate adenylyl cyclase activity.
(Shpakov et al., 2004), a cellular marker of dependence, thus leading to a decrease in physical dependence with Tat induction. It should be noted that a recent study demonstrated no effects of gp120 on withdrawal-induced weight loss associated with the discontinuation of buprenorphine (Palma et al., 2015). Whether this is specific to buprenorphine as argued by the authors or specific to gp120 needs to be further investigated.

The increase in antinociceptive tolerance and decrease in physical dependence with Tat induction could also be accounted for by the dose of 1 mg/kg naloxone being insufficient to induce precipitated withdrawal symptoms in Tat(+)/DOX. Previous studies from our laboratory have used this dose routinely to induce opioid withdrawal but others have reported the use of 10 mg/kg naloxone to maximize the number and intensity of withdrawal symptoms elicited (Wei, 1981; Smith and Yancey, 2003). The reason for a decrease in naloxone’s effect (efficacy and/or potency) by Tat induction is unclear. One speculation is the reported upregulation of the endogenous opioid transport system by Tat (Hu et al., 2003), suggesting alterations and changes in cellular signaling and expression levels of these opioid receptors.

In conclusion, the present study used a conditional HIV-1 Tat transgenic mouse model to examine the effects of HIV-1 Tat1-86 on morphine tolerance assessed by tail-flick and locomotor activity assays and dependence, as measured by naloxone-precipitated withdrawal. We found that Tat induced an increase in antinociceptive tolerance but decreased physical dependence to chronic morphine exposure. To the extent that Tat expression underlies significant aspects of neuroAIDS in the post-cART era (Olney et al., 1986), these findings in Tat transgenic mice suggest that HIV-infected
individuals may display increased tolerance and decreased symptoms of physical
dependence to opiates compared to uninfected individuals, and that these effects are
mediated by Tat.
Authorship Contributions

Participated in research design: Fitting, Hauser, Knapp, Dewey

Conducted experiments: Fitting, Stevens, Khan, Scoggins.

Performed data analysis: Fitting, Beardsley, Enga.

Wrote or contributed to the writing of the manuscript: Fitting, Hauser, Beardsley.
Reference List


Hahn YK, Podhaizer EM, Farris SP, Miles MF, Hauser KF and Knapp PE (2013) Effects of chronic HIV-1 Tat exposure in the CNS: heightened vulnerability of males...
versus females to changes in cell numbers, synaptic integrity, and behavior.


Harris LS and Pierson AK (1964) Some narcotic antagonists in the benzomorphans.


Footnotes

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Legends for Figures

**Figure 1.** Scheme showing the experimental design of the conducted study on a timeline. After mice received DOX-containing or normal chow for 3 weeks, body weight was taken and a placebo or 75-mg morphine pellet was implanted subcutaneously. On day 4 following pellet implantation, body weight was assessed again and mice were tested for baseline activity in the tail-flick and locomotor activity (10 min) tests. This was followed by four acute, cumulative, subcutaneous morphine injections with a 20 min wait period after each injection before tail-flick responses to warm-water were tested. After the last morphine injection, mice were tested in the tail flick assay and locomotor activity was assessed for 10 min. Lastly, to induce precipitated withdrawal, all mice were given a naloxone (1 mg/kg) injection and were immediately tested for jumping off an elevated platform (10 min). They were observed for other withdrawal signs (5 min), including number of wet dog shakes, forepaw tremor, grooming, and jumping. \( n = 6 \) to 8 mice/group. DOX = doxycycline, TF = tail-flick test.

**Figure 2.** Percent change in body weight following four days of morphine pellet implantation as a function of feeding condition for each test group. No significant change in body weight before or after pellet implantation was noted when conducting post-hoc tests, except for the Tat transgenic mice that expressed the \( tat \) gene. Data are expressed as the mean change in body weight. Capped bars indicate ± S.E.M. \( n = 6 \) to 8 mice/group. Dotted line indicates no change at 100%. DOX = doxycycline. \*\( p < 0.05 \) vs. Tat(+) / No DOX/Morphine.
Figure 3. Percent maximal possible effect in the warm-water, tail-flick assay. Each data point represents the mean of the percent maximal latency of tail withdrawal relative to baseline as a function of morphine dose (mg/kg). Bars through the data points represent ± S.E.M. "PR" = potency ratio, as determined by the EC50 shift between the placebo and morphine-pelleted groups (see text for details). *p < 0.05, indicating a significant difference at a dose comparing placebo- vs. morphine-pelleted group. †p < 0.05, indicating an overall significant difference between Tat(−)/DOX vs. Tat(+) /DOX groups across all morphine injections. §p < 0.05, indicating an overall significant difference between Tat(−)/DOX vs. Tat(+) /DOX groups. Chronic exposure to morphine produced tolerance in all mouse groups (C57, Tat(−), Tat(+) mice in the presence or absence of DOX chow) compared to groups exposed to placebo pellets as indicated by the 3.4-11.2 fold increases in the potency ratios. No significant differences are noted between C57/No DOX or C57/DOX and Tat(−)/No DOX or Tat(−)/DOX mice. Importantly, Tat(+) /DOX mice show increased tolerance when chronically exposed to morphine compared to Tat(−)/DOX mice (†p < 0.05) with an approximately 11.2-fold increase in potency compared to a 4.7-fold increase in potency for Tat(−)/DOX mice (p < 0.05). Bonferroni’s test indicated a significant difference between Tat(−)/DOX and Tat(+) /DOX mice in response to an acute morphine injection of 32 mg/kg (§p < 0.05). n = 6 to 8 mice/group.

Figure 4. Mean changes in locomotor activity counts before and after the four cumulative morphine-dosing regimen. Capped bars indicate ± S.E.M. Changes in locomotor activity after acute, cumulative subcutaneous morphine injections indicate the
development of tolerance in chronic morphine-pelleted mice compared to placebo-
pelleted mice with no effects of Tat on tolerance. *$p < 0.05$ vs. corresponding placebo-
pelleted group. §$p < 0.05$ vs. placebo-pelleted C57/DOX mice. $n = 6$ to 8 mice/group.

**Figure 5.** Tat significantly altered development of physical dependence to chronic
morphine exposure as assessed by platform jumping. Data are expressed as the
percentage of mice jumping off the elevated platform, represented as individual points
as well as the mean ± S.E.M., $n = 6$ to 8 mice/group. DOX = doxycycline. *$p < 0.05$ vs.
corresponding placebo condition, §$p < 0.05$ vs. Tat(−)/No DOX and Tat(+)/No DOX, §$p <
0.05$ vs. Tat(−)/DOX and Tat(+)/DOX. Chronic morphine-exposed mice were physically
dependent as shown by their jumping off the elevated platform in contrast to the
placebo-pelleted groups. Interestingly, morphine-pelleted Tat(+)/DOX mice, in which Tat
has been induced, jump off the elevated platform significantly less compared to their
C57/DOX and Tat(−)/DOX counterparts.

**Figure 6.** Physical signs of withdrawal (wet dog shakes, jumps, and forepaw tremors)
indicate that the sensitivity to morphine-induced physical dependence was reduced by
Tat induction. The incidence of selected somatic signs of withdrawal are presented as
the mean ± S.E.M., $n = 6$ to 8 mice/group. Lines lacking error bars indicate no response.
DOX = doxycycline. *$p < 0.05$ vs. corresponding placebo condition, §$p < 0.05$ vs.
C57/DOX Morphine, §$p < 0.05$ vs. Tat(+)/No DOX Morphine. (A) Wet dog shakes
indicate physical dependence for the chronic morphine exposed groups compared to
the groups that received placebo pellets, except for the Tat(+)/DOX group that did not
show significant increases in wet dog shakes for the chronic morphine-pelleted condition. (B) Incidence of jumps indicates some physical dependence for morphine-pelleted Tat(+)/No DOX and C57/DOX mice compared to placebo-pelleted mice. Additionally, chronic, morphine-exposed Tat(+)/DOX mice showed less jumps compared to C57/DOX mice. None of the placebo-pelleted mice jumped. (C) The placebo-pelleted Tat(−)/DOX group was the only group showing more forepaw tremors compared to chronic morphine exposed Tat(−)/DOX.
Table 1. Morphine analgesic tolerance in the warm-water tail-flick assay (%MPE).

<table>
<thead>
<tr>
<th>Mouse</th>
<th>DOX</th>
<th>Placebo Pellet ( \text{ED}_{50} ) (95% CI)</th>
<th>Morphine Pellet ( \text{ED}_{50} ) (95% CI)</th>
<th>Sig.</th>
<th>Potency Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57</td>
<td>No DOX</td>
<td>2.41 (1.96-2.97)</td>
<td>8.68 (2.05-36.81)</td>
<td>n.s.</td>
<td>5.50 (1.84-9.15)*</td>
</tr>
<tr>
<td></td>
<td>DOX</td>
<td>1.50 (0.73-3.07)</td>
<td>14.89 (10.52-21.07)†</td>
<td>*</td>
<td>10.11 (4.15-16.07)*</td>
</tr>
<tr>
<td>Tat(−)</td>
<td>No DOX</td>
<td>2.83 (2.23-3.59)</td>
<td>8.96 (1.42-56.61)</td>
<td>n.s.</td>
<td>5.18 (1.27-9.10)*</td>
</tr>
<tr>
<td></td>
<td>DOX</td>
<td>3.35 (2.44-4.61)</td>
<td>16.05 (11.51-22.38)†</td>
<td>*</td>
<td>4.68 (2.56-6.80)*†</td>
</tr>
<tr>
<td>Tat(+)</td>
<td>No DOX</td>
<td>3.62 (2.94-4.45)</td>
<td>12.41 (7.46-20.65)†</td>
<td>*</td>
<td>3.43 (1.82-5.03)*†</td>
</tr>
<tr>
<td></td>
<td>DOX</td>
<td>3.78 (3.28-4.34)</td>
<td>44.01 (25.87-74.89)</td>
<td>*</td>
<td>11.24 (7.04-15.43)*</td>
</tr>
</tbody>
</table>

\( \text{ED}_{50} \) values (mg/kg) and potency ratio values are derived from acute cumulative dose-response curves obtained for placebo- and morphine-pelleted mice. n.s., not significant; *\( p < 0.05 \) placebo vs, corresponding morphine group; †Indicates significance from Tat(+) /DOX based on non-overlapping 95% CI. CI = confidence interval.
Figure 1

3 Weeks
DOX or CHOW
Pellet Implant

Day 1
Baseline
Tail Flick
Activity

Day 4
Cumulative s.c. Morphine Injection (mg/kg)
Tail Flick
Tail Flick
Tail Flick
Tail Flick
Tail Flick
Activity

Tolerance Assessment

20 min
20 min
20 min
20 min
20 min
10 min
10 min
5 min

CHOW
Placebo
+ + 2 + 4 + 8 + 16 + + + + + + + + + +

CHOW
Morphine
+ + 8 + 16 + 32 + 64 + + + + + + + + + +

DOX
Placebo
+ + 2 + 4 + 8 + 16 + + + + + + + + + +

DOX
Morphine
+ + 8 + 16 + 32 + 64 + + + + + + + + + +

Naloxone (1 mg/kg)
Platform Jumping
Withdrawal Signs

Body Weight
Figure 4

Change in Ambulatory Count after Acute Morphine Injection

C57/No DOX/Placebo
- C57/No DOX/Morphine

Tat(-)/No DOX/Placebo
- Tat(-)/No DOX/Morphine

Tat(+)/No DOX/Placebo
- Tat(+)/No DOX/Morphine

CHOW

DOX