Morphine Tolerance and Physical Dependence Are Altered in Conditional HIV-1 Tat Transgenic Mice

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ABSTRACT

Despite considerable evidence that chronic opiate use selectively affects the pathophysiologic consequences of human immunodeficiency virus type 1 (HIV-1) infection in the nervous system, few studies have examined whether neuro-acquired immune deficiency syndrome (neuroAIDS) might intrinsically alter the pharmacologic 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 of 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, and 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 with 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 after a 1-mg/kg naloxone challenge compared with 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 with uninfected individuals and show fewer symptoms of physical dependence.

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 patients infected with human immunodeficiency virus type 1 (HIV-1) (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 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), but 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 supraspinal 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

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ABBREVIATIONS: ANOVA, analysis of variance; CCR, C-C chemokine receptor; CNS, central nervous system; DOX, doxycycline; GFAP, glial fibrillary acidic protein; HIV-1, human immunodeficiency virus type 1; IL, interleukin; MPE, maximal possible effect; mTOR, mammalian target of rapamycin; neuroAIDS, neuro-acquired immune deficiency syndrome; RANTES, regulated on activation, normal T expressed and secreted; Tat, transactivator of transcription.

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 coreceptors can undergo heterologous, bidirectional cross-desensitization (Rogers et al., 2000; Rogers and Peterson, 2003), including C-C chemokine receptor type 5 (CCR5) and μ -opioid receptors (Rogers and Peterson, 2003; Chen et al., 2004). HIV-1 proteins such as the transactivator of transcription (Tat) have been shown to induce inflammation by elevating the production of CCL5/regulated on activation, normal T expressed and secreted (RANTES) and interleukin-6 (El-Hage et al., 2005, 2006, 2008). The interaction between Tat and morphine potentiates Tat-induced increases in CCL2/monocyte chemoattractant protein-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 HIV-1-associated neurocognitive disorders (HAND) to explore the effects of Tat on morphine tolerance and dependence after 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_{IIIB} Tat₁₋₈₆ transgenic mice were developed on a C57BL/6J hybrid background as described in detail elsewhere (Bruce-Keller et al., 2008; Hahn et al., 2015). 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 (product TD.09282; Harlan, Indianapolis, IN). 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, nontransgenic C57BL/6 mice (~4 months of age, ~25 g, males) from Harlan Laboratories (Indianapolis, IN) 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., 2015). The nontransgenic C57BL/6 mice will be referred to as C57 mice throughout this report.

Half of all mice were fed 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 to 4 mice per cage) on a 12-hour 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 (National Institute on Drug Abuse, Rockville, MD) under aseptic conditions and 2.5% isoflurane anesthesia as previously described

elsewhere (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, or 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, $n = 8$; Tat(+)/no DOX/placebo, $n = 8$; Tat(+)/no DOX/morphine, $n = 8$; Tat(+)/DOX/placebo, $n = 8$; and Tat(+)/DOX/morphine, $n = 6$].

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 after 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

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 (Fig. 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 was determined 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. The tail-flick response was recorded after a 20-minute 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.

Fig. 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 measured, and a placebo or 75-mg morphine pellet was implanted subcutaneously. On day 4 after pellet implantation, body weight was assessed again, and the mice were tested for baseline activity in the tail-flick and locomotor activity (10-minute) tests. This was followed by four acute, cumulative subcutaneous morphine injections with a 20-minute 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 minutes. Finally, 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 minutes). They were observed for other withdrawal signs (5 minutes), including the number of wet-dog shakes, forepaw tremor, grooming, and jumping. Per group, $n = 6$ to 8 mice.

Warm-Water Tail-Flick Test. The tail-flick test was performed using a water bath with the temperature maintained at $56 \pm 0.1^\circ\text{C}$. 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 one-third of the tail was immersed in a water bath set at 56°C , and mice rapidly removed 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 seconds. 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-second maximum cutoff latency was used to prevent any tissue damage. Antinociception was quantified as the percentage of maximal possible effect (%MPE), which was calculated as $\%MPE = [(\text{Test latency} - \text{Control latency}) / (10 - \text{Control latency})] \times 100$ (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, St. Albans, VT). Mice were habituated to the chamber for 10 minutes before drug administration. Ambulatory counts for spontaneous activity were obtained over a 10-minute time period. Each individual activity chamber has closeable doors and a ventilation system. The interior of the chamber consists of a 27×27 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-minute maximal cutoff time was used. This was followed by evaluating additional withdrawal signs during a period of 5 minutes. Mice were placed in a rectangular clear plastic observation box ($16 \times 16 \times 30$ cm; maximum of three mice in one box) and observed for 5 minutes. 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 $\pm$ 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 (SPSS Statistics 22; IBM, Chicago, IL). 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, La Jolla, CA). The 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). A potency-ratio value of greater than 1.0 with a lower 95% CI > 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) 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 noncontinuous variables, including number of wet-dog shakes, forepaw tremors, and 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 statistically significant.

Results

Body Weight. Body weight was unaffected by DOX when comparing the three mouse groups before 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 Fig. 2 illustrate the percentage change in body weight after pellet implantation represented by the mean $\pm$ S.E.M. with 100% indicating no change in body weight. An ANOVA indicated a statistically significant effect of DOX [$F(1, 80) = 6.6$, $P < 0.05$], with a significant $\sim 3\%$ decrease in body weight for animals receiving the normal chow ($97.1 \pm 1.02\%$) compared with DOX animals ($100.10 \pm 1.00\%$).

Further, a main effect for pellet implant was noted [$F(1, 80) = 13.0$, $P < 0.001$], and DOX $\times$ pellet implant interaction [$F(1, 80) = 6.4$, $P < 0.05$]. The DOX $\times$ pellet implant

Fig. 2. Percentage of change in body weight after 4 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 $\pm$ S.E.M. Per group, $n = 6$ to 8 mice. Dotted line indicates no change at 100%. [†] $P < 0.05$ versus Tat(+)/no DOX/morphine.

interaction indicated that the greatest loss of body weight was shown by the morphine-pelleted group that did not receive DOX food ($92.8 \pm 0.94\%$) compared with the DOX/morphine-pelleted group ($99.2 \pm 1.87\%$), whereas the placebo-pelleted mice that received DOX ($101.0 \pm 0.90\%$) or normal ($100.9 \pm 1.34\%$) chow showed no change. It should be noted that there were no statistically significant effects when conducting post hoc tests, except for a statistically significant difference between morphine-pelleted Tat(+)/no DOX and Tat(+)/DOX mice ($P < 0.05$, Fig. 2).

Morphine Antinociceptive and Locomotor Activity Tolerance in Tat(+) and Tat(-) Mice. As depicted in Fig. 3, all groups demonstrated antinociceptive tolerance after chronic morphine pellet exposure compared with 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 with the placebo condition with potency ratio values varying between 3.43- and 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 with mice receiving chronic morphine via 75-mg pellet implants ($P < 0.05$).

Importantly, in Tat(+)/DOX mice, there was a significant shift (based on nonoverlapping 95% CI) in morphine sensitivity revealed by an approximately 11.24-fold increase in morphine ED_{50} 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. The significant 11.24-fold shift for Tat(+)/DOX mice was markedly higher compared to the significant 4.68-fold shift observed for Tat(-)/DOX mice (see Table 1). The ED_{50} 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 mouse groups (based on nonoverlapping 95% CI), suggesting Tat induction increased morphine tolerance.

Tat(+)/DOX mice displayed a decreased %MPE ($31.7 \pm 10.47\%$) compared with Tat(-)/DOX mice ($61.2 \pm 8.81\%$) across

all acute morphine injections [$F(1, 12) = 4.7, P \leq 0.05$], 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 ($P < 0.05$, Fig. 3, bottom right panel). No other statistically significant effects were noted.

Additionally, tolerance was examined by assessing changes of locomotor activity after acute and cumulative subcutaneous morphine injections (Fig. 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 $\times$ DOX food interaction [$F(2, 80) = 8.1, P < 0.01$], and mouse group $\times$ 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 after chronic morphine pellet exposure compared to placebo pellet exposure in all groups, except for the C57/no DOX mice. ($P < 0.05$). Additionally, for the placebo-pelleted groups, C57/DOX resulted in significantly less locomotor activity compared with 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 (Fig. 5). Physical dependence as quantified by naloxone-induced jumping was not observed for any of the placebo-pelleted transgenic mice, although some jumping was observed in the placebo-pelleted C57/no DOX mice after they received four acute cumulative doses of morphine (2, 4, 8, and 16 mg/kg) ($P < 0.05$). In contrast, most of the morphine-pelleted mice jumped off the elevated platform within the 10-minute time period, indicating physical dependence.

Chi-square tests (corrected for multiple comparisons) indicated statistically significant differences between the

Fig. 3. Percentage of maximal possible effect in the warm-water, tail-flick assay. Each data point represents the mean of the percentage of maximal latency of tail withdrawal relative to baseline as a function of morphine dose (mg/kg). Bars through the data points represent $\pm$ S.E.M. PR is the potency ratio, as determined by the EC_{50} shift between the placebo and morphine-pelleted groups (see the text for details). * $P < 0.05$, indicating a statistically significant difference at a dose comparing placebo- versus morphine-pelleted groups. $^{\dagger}P < 0.05$, indicating an overall statistically significant difference between Tat(-)/DOX versus Tat(+)/DOX groups across all morphine injections. $^{\S}P < 0.05$, indicating an overall statistically significant difference between Tat(-)/DOX versus Tat(+)/DOX groups. Chronic exposure to morphine produced tolerance in all mouse groups (C57, Tat(-), and Tat(+)) mice in the presence or absence of DOX chow compared with groups exposed to placebo pellets as indicated by the 3.4- to 11.2-fold increases in the potency ratios. No statistically 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 with Tat(-)/DOX mice ($^{\dagger}P < 0.05$, with an approximately 11.2-fold increase in potency compared with a 4.7-fold increase in potency for Tat(-)/DOX mice ($P < 0.05$). Bonferroni's test indicated a statistically significant difference between Tat(-)/DOX and Tat(+)/DOX mice in response to an acute morphine injection of 32 mg/kg ($^{\S}P < 0.05$). Per group, $n = 6$ to 8 mice MPE, maximal possible effect.

corresponding placebo- versus 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 (Fig. 5, top left panel); and 2) the development of dependence was significantly altered/decreased by Tat induction (Fig. 5, bottom right panel).

Additional somatic signs of withdrawal to morphine were assessed, including number of wet-dog shakes, jumps, and

TABLE 1

Morphine analgesic tolerance in the warm-water tail-flick assay (%MPE)

ED_{50} values (mg/kg) and potency ratio values are derived from acute cumulative dose-response curves obtained for placebo- and morphine-pelleted mice.

Mouse DOX	Pellet ED_{50} (95% CI)		Significance	Potency Ratio (95% CI)
	Placebo	Morphine		
C57				
No DOX	2.41 (1.96–2.97)	8.68 (2.05–36.81)	NS	5.50 (1.84–9.15) ^a
DOX	1.50 (0.73–3.07)	14.89 (10.52–21.07) ^b	^a	10.11 (4.15–16.07) ^a
Tat(-)				
No DOX	2.83 (2.23–3.59)	8.96 (1.42–56.61)	NS	5.18 (1.27–9.10) ^a
DOX	3.35 (2.44–4.61)	16.05 (11.51–22.38) ^b	^a	4.68 (2.56–6.80) ^{a,b}
Tat(+)				
No DOX	3.62 (2.94–4.45)	12.41 (7.46–20.65) ^b	^a	3.43 (1.82–5.03) ^{a,b}
DOX	3.78 (3.28–4.34)	44.01 (25.87–74.89)	^a	11.24 (7.04–15.43) ^a

NS, not statistically significant.

^a $P < 0.05$ placebo versus corresponding morphine group.

^b Indicates significance from Tat(+)/DOX based on nonoverlapping 95% CI.

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

forepaw tremors (Fig. 6). Incidences of wet-dog shakes were higher in chronic morphine-exposed mice compared with placebo-pelleted mice ($P < 0.05$), except for Tat(+)/DOX mice, indicating Tat(+)/DOX mice showed no morphine-induced physical dependence (Fig. 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 with morphine-pelleted Tat(+)/DOX mice ($P < 0.05$). The number of jumps indicated the presence of physical dependence in morphine-pelleted Tat(+)/no DOX ($P < 0.05$) as well as C57/DOX ($P < 0.05$) mice compared to placebo-pelleted mice, again indicating less physical dependence with Tat induction. There were no differences in forepaw tremors

except for in the placebo-pelleted Tat(-)/DOX mice, which displayed statistically significant more forepaw tremors compared with 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 intraventricular injections. The most widely used method, however, is the

Fig. 5. Tat significantly altered the 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 $\pm$ S.E.M. Per group, $n = 6$ to 8 mice. * $P < 0.05$ versus corresponding placebo condition; § $P < 0.05$ versus Tat(-)/no DOX and Tat(+)/no DOX; § $P < 0.05$ versus Tat(-)/DOX and Tat(+)/DOX. Chronic morphine-exposed mice were physically dependent, as shown by their jumping off the elevated platform in contrast with the placebo-pelleted groups. Interestingly, morphine-pelleted Tat(+)/DOX mice, in which Tat has been induced, jump off the elevated platform significantly less compared with their C57/DOX and Tat(-)/DOX counterparts.

Fig. 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 $\pm$ S.E.M., $n = 6$ to 8 mice/group. Lines lacking error bars indicate no response. * $P < 0.05$ versus corresponding placebo condition; † $P < 0.05$ versus C57/DOX morphine; § $P < 0.05$ versus Tat(+)/no DOX morphine. (A) Wet-dog shakes indicate physical dependence for the chronic morphine exposed groups compared with the groups that received placebo pellets, except for the Tat(+)/DOX group which 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 with placebo-pelleted mice. Additionally, chronic, morphine-exposed Tat(+)/DOX mice showed fewer jumps compared with

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 $\mu\text{g/ml}$ of morphine and in sustained blood levels (0.6 $\mu\text{g/ml}$ morphine) by 48 hours that last 2–5 days (Bryant et al., 1988). The 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 $0.8 \pm 0.1 \mu\text{g/ml}$ (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 after 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 neuro-acquired immune deficiency syndrome (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 after acute cumulative morphine injections compared with 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,b; Liu et al., 2011), which also have reported increased IL-1 β 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

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 with chronic morphine-exposed Tat(-)/DOX.

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 with 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 after DOX administration. It is possible that chronic morphine-induced inflammatory effects, such as increases in IL-1 β or other cytokines (Liu et al., 2011; Merighi et al., 2013) might be reduced by DOX itself because 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 with 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 after 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 because 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,b, 2014; Zou et al., 2011; Hauser et al., 2012), and prolonged Tat induction was found to disrupt locomotor activity (Hahn et al., 2015). 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., 2015).

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 versus 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 (Xu et al., 2014). 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 laboratory 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, such as discrepancies in spinal versus 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 protein kinase C and protein kinase A inhibitors, which reversed tolerance but failed to block dependence as assessed by naloxone-precipitated withdrawal (Smith et al., 2002; Gabra et al., 2008). Protein kinase C and protein kinase A 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 might be attributable to the up-regulation of heterologous, bidirectional cross-desensitization of opioid and chemokine coreceptors (Rogers et al., 2000; Rogers and Peterson, 2003), as increased CCL2/monocyte chemoattractant protein-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 up-regulation of the endogenous opioid peptide transport system by Tat (Hu et al., 2003), may differentially alter and change the cellular signaling and expression levels of individual opioid receptor types.

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 by 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 with 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, Dewey, Knapp, Beardsley.

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