Repeated Morphine Produces Sensitization to Reward and Tolerance to Antiallodynia in Male and Female Rats with Chemotherapy-Induced Neuropathy

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ABSTRACT

Paclitaxel is a cancer chemotherapy drug with adverse effects that include chemotherapy-induced neuropathic pain (CINP) as well as depression of behavior and mood. In the clinical setting, opioids are often used concurrently with or after chemotherapy to treat pain related to the cancer or CINP, but repeated opioid exposure can also increase the risk of opioid abuse. In this study, male and female Sprague-Dawley rats were used to test the hypothesis that repeated 3.2-mg/kg doses of morphine would induce tolerance to its antinociceptive effects in a mechanical sensitivity assay and increased expression of its abuse-related rewarding effects in an assay of intracranial self-stimulation (ICSS). Three weeks after four injections of vehicle or 2.0 mg/kg of paclitaxel, the initial morphine dose–effect curves were determined in both assays. Subsequently, rats

were treated with 3.2 mg/kg per day morphine for 6 days. On the final day of testing, morphine dose–effect curves were redetermined in both assays. On initial exposure, morphine produced dose-dependent antiallodynia in the assay of mechanical sensitivity, but it produced little or no rewarding effects in the assay of ICSS. After 6 days of repeated treatment, morphine antiallodynia decreased, and morphine reward increased. Females exhibited greater morphine reward on initial exposure than males, but repeated morphine eliminated this sex difference. These results suggest that repeated morphine may produce tolerance to therapeutically beneficial analgesic effects of morphine but increased sensitivity to abuse-related rewarding effects of morphine in subjects treated with paclitaxel.

Introduction

Opioid abuse has reached epidemic proportions in the United States. Initial opioid exposure can occur during medically supervised treatment of acute or chronic pain, and data published in the 1980s were interpreted to suggest that risk of addiction was low under these conditions (Porter and Jick, 1980; Portenoy and Foley, 1986). This perception likely contributed to the dramatic escalation in clinical opioid use that occurred through the 1990s and up to the present (Sehgal et al., 2012; Wilson-Poe and Morón, 2017). However, more recent evidence suggests that rates of iatrogenic opioid addiction may be high (Boscarino et al., 2010; Manchikanti et al., 2010), and recent data indicate that clinically prescribed opioid exposure for as few as 5 days is associated with increased risk of long-term opioid use (Shah et al., 2017). These findings suggest that opioids retain considerable abuse liability in pain patients, and this concern has triggered the implementation of more restrictive guidelines for opioid prescriptions (Dowell et al., 2016).

Preclinical studies permit controlled research to evaluate the impact of pain on abuse-related opioid effects, and intracranial self-stimulation (ICSS) is one preclinical procedure that can be used to compare abuse-related drug effects in the absence and presence of pain (Wise, 1996; Negus, 2013; Negus and Miller, 2014). In this procedure, subjects are trained to press a lever to receive electrical brain stimulation delivered via a chronically implanted microelectrode targeting a brain reward area, and a drug-induced increase (or “facilitation”) in ICSS responding indicates a rewarding drug effect (Carlezon and Chartoff, 2007; Negus and Miller, 2014).

One advantage of ICSS is that drug effects can be monitored during the earliest stages of drug exposure, and this is especially relevant with opioids because initial exposure produces little or no ICSS facilitation in drug-naïve subjects, but repeated daily exposure for as little as 1 week results in the gradual emergence of rewarding effects (Altarifi and Negus, 2011; Wiebelhaus et al., 2016). Little is known about the degree to which pain states might modify this trajectory of increasing opioid reward during initial opioid exposure; however, we reported previously that repeated exposure to an acute pain stimulus failed to modify this trajectory (Miller et al., 2015), and this agrees with the increased risk of long-term opioid abuse in patients who receive clinically

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ABBREVIATIONS: ANOVA, analysis of variance; CINP, chemotherapy-induced neuropathic pain; ICSS, intracranial self-stimulation; MCR, maximum control rate; MPE, maximum possible effect.

prescribed opioid exposure for as few as 5 days (Shah et al., 2017).

The goal of the present study was to evaluate the impact of a chronic pain state on the emergence of opioid reward that occurs with repeated opioid exposure. Chemotherapy-induced neuropathic pain (CINP) is a common and dose-limiting side effect in the use of chemotherapeutic agents like paclitaxel for cancer treatment (Reeves et al., 2012; Speck et al., 2013; Seretny et al., 2014), and opioid agonists are commonly used to treat CINP (Plante and VanItallie, 2010), despite evidence for marginal therapeutic efficacy (Raja et al., 2002; McNicol et al., 2013; Argyriou et al., 2014). Iatrogenic opioid addiction is well documented in patients with CINP (Ballantyne and LaForge, 2007; Anghelescu et al., 2013; Koyyalagunta et al., 2013; Barclay et al., 2014; Del Fabbro, 2014; Rauenzahn et al., 2017), but it is not clear whether CINP alters opioid abuse liability or merely provides an occasion for opioid exposure.

Accordingly, the present study examined the effects of repeated morphine administration on ICSS in rats treated with paclitaxel or its vehicle. The effects of repeated morphine on ICSS were compared with the effects of the same regimen of repeated morphine treatment on paclitaxel-induced mechanical allodynia (Polomano et al., 2001; Pascual et al., 2010; Boyette-Davis et al., 2011; Hwang et al., 2012; Ko et al., 2014). Studies were conducted in male and female rats because sex differences have been reported previously for some paclitaxel effects (Naji-Esfahani et al., 2016) and for the rewarding and antinociceptive effects of morphine (Cicero et al., 2003; Lynch, 2006; Craft, 2008; Lynch et al., 2013).

Materials and Methods

Subjects

Studies were conducted in adult male and female Sprague-Dawley rats. At the start of the study, males weighed 362–488 g, and females weighed 265–324 g. Rats were housed individually and maintained on a 12-hour light/dark cycle with lights on from 6:00 AM to 6:00 PM in an AAALAC International-accredited housing facility. Food and water were available ad libitum in the home cage. Animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in accordance with the National Academy of Science's Guide for the Care and Use of Laboratory Animals.

Fig. 1. Overview of the experimental timeline for treatment and data collection. Baseline measurements were collected on day 0 (for mechanical sensitivity) and day –2 to day 0 (ICSS). On days 1, 3, 5, and 7, 2.0 mg/kg per day paclitaxel or vehicle was injected (i.p.). The mechanical sensitivity threshold was tested weekly, and ICSS responding was tested daily. Cumulative morphine dose–effect testing was conducted on days 22 and 29, and repeated treatment with either 3.2 mg/kg per day morphine or saline was administered on intervening days 23–28.

Drugs and Experimental Design

Figure 1 shows the overall experimental design. For studies of both mechanical sensitivity and intracranial self-stimulation (ICSS), pre-paclitaxel baseline behavioral measures were determined in each rat before a 29-day protocol of treatment and testing. On days 1, 3, 5, and 7, all rats were treated with either paclitaxel (2.0 mg/kg per day; total paclitaxel dose of 8.0 mg/kg) or vehicle. Paclitaxel was obtained as a clinically available 6.0 mg/ml solution (Cardinal Health, Richmond, VA) and was diluted in vehicle (8.3% ethanol, 8.3% Cremophor EL, and 83.4% saline) to a final concentration of 1.0 mg/ml for intraperitoneal (i.p.) administration in volume of 2.0 ml/kg.

On days 22 and 29, morphine was administered using a cumulative dosing regimen for determination of morphine dose–effect curves. In this regimen, a sequence of five injections was administered at 50-minute intervals, and each successive injection increased the total, cumulative morphine dose by 0.5 log units (0, 0.32, 1.0, 3.2, and 10 mg/kg). On intervening days 23–28, the subjects were treated with a single injection of either 3.2 mg/kg per day morphine or vehicle. The morphine dose for repeated treatment was selected because it was the lowest dose to block paclitaxel-induced mechanical hypersensitivity (see *Results*). Morphine sulfate (National Institute on Drug Abuse Drug Supply Program) was dissolved in sterile water and administered subcutaneously (s.c.) in a volume of 1.0 ml/kg. Morphine doses are expressed in terms of the sulfate salt.

Mechanical Sensitivity Testing with Von Frey Filaments

Testing Procedure. To evaluate mechanical sensitivity, rats were first placed on an elevated mesh galvanized steel platform in individual chambers with a hinged lid and were allowed to acclimate for at least 20 minutes before exposure to the mechanical stimuli. Subsequently, von Frey filaments (ranging from 0.4 to 15.0 g and increasing in ~0.25 log increments; North Coast Medical, Morgan Hill, CA) were applied to the plantar surface of each hindpaw, and the threshold stimulus to elicit paw withdrawal was determined in log grams using the “up-down” method, as previously described elsewhere (Chaplan et al., 1994; Leitel et al., 2014). Filament forces greater than 15.0 g were not used because they physically lifted the paw, and as a result paw movement could not be reliably attributed to a withdrawal response by the subject.

The goal of the study was to establish paclitaxel-induced mechanical hypersensitivity, and then to evaluate the impact of repeated morphine treatment on the dose–effect curve for morphine reversal of hypersensitivity. Baseline mechanical sensitivity thresholds were determined on the day before initiation of paclitaxel treatment.

All rats in this phase of the study then received paclitaxel. A paclitaxel vehicle control was not included because the baseline thresholds in individual rats were generally at the 15.0 g ceiling of the assay, pilot studies indicated no change in baseline after vehicle treatment, and it was not possible to detect morphine-induced increases in thresholds from this high baseline.

After initiation of paclitaxel treatment, thresholds were reassessed weekly on days 8, 15, 22, and (if hypersensitivity criterion was met) 29. Specifically, rats that met a criterion level of paclitaxel-induced mechanical hypersensitivity (mean threshold in log grams <0.90; 15 of 18 males, 17 of 19 females) were subdivided by sex into two cohorts to receive either repeated 3.2 mg/kg per day of morphine ($n = 8$ males, 9 females) or repeated saline ($n = 7$ males, 8 females) on days 23–28. All these rats also received cumulative morphine (0–10 mg/kg) on days 22 and 29 to determine the morphine dose–effect curves before and after the repeated treatment regimen.

Mechanical sensitivity thresholds were determined beginning 30 minutes after each injection. Rats that failed to meet the criterion for paclitaxel-induced mechanical hypersensitivity (3 of 18 males, 2 of 19 females) were removed from the study.

Data Analysis. For each test condition, data were averaged across paws within a rat and then across rats. Changes in thresholds over time were analyzed by one-way analysis of variance (ANOVA), and a

statistically significant ANOVA was followed by Dunnett's post hoc test to compare post-paclitaxel thresholds with the pre-paclitaxel baseline. Additionally, the potential for sex differences in paclitaxel effects was evaluated by two-way ANOVA with day as a within-subjects variable and sex as a between-subjects variable.

Morphine effects were expressed as the percent maximum possible effect (%MPE) using the equation: $\%MPE = [(Test - Daily\ Baseline) \div (Ceiling - Daily\ Baseline)] \times 100$, where "Test" is the threshold determined after a morphine dose, "Daily Baseline" is the threshold determined before any injection on a given test day, and "Ceiling" is the maximum force tested (15 g).

Morphine effects on paclitaxel-induced mechanical hypersensitivity were analyzed by two-way ANOVA with dose and treatment day as the two within-subjects factors. For this and all subsequent two-way ANOVAs, a statistically significant ANOVA was followed by the Holm-Sidak post hoc test.

For all analyses, $P < 0.05$ was considered statistically significant. Additionally, the morphine ED_{50} value was defined as the morphine dose to produce 50% MPE, and morphine ED_{50} values and 95% confidence limits were determined by linear regression of data from the linear portion of each morphine dose-effect curve. ED_{50} values were considered to be significantly different if confidence limits did not overlap.

Intracranial Self-Stimulation (ICSS)

Surgery. Fourteen male and 14 female rats were anesthetized with isoflurane (2.5%–3% in oxygen; Webster Veterinary, Phoenix, AZ) and implanted with electrodes (Plastics One, Roanoke, VA) in the left medial forebrain bundle at the level of the lateral hypothalamus using previously published procedures and coordinates (males: 2.8 mm posterior to bregma, 1.7 mm lateral to the midsagittal suture, 8.8 mm below skull surface; females: 3.8 mm posterior to bregma, 1.6 mm lateral to the midsagittal suture, 8.7 mm below skull surface; Lazenka et al., 2016a,b). The electrode was secured to the skull with orthodontic resin and skull screws. Ketoprofen (5 mg/kg; Spectrum Chemical, New Brunswick, NJ) was administered immediately and 24 hours after surgery as a postoperative analgesic, and the rats recovered for 7 days before initiation of ICSS training.

Apparatus. Studies were conducted in sound-attenuating boxes containing modular acrylic and metal test chambers (29.2 × 30.5 × 24.1 cm; Med Associates, St Albans, VT). Each chamber contained a response lever (4.5 cm wide, 2.0 cm deep, 3.0 cm above the floor), three stimulus lights (red, yellow, and green) centered 7.6 cm above the lever, a 2-W house light, and an ICSS stimulator. Electrodes were connected to the stimulator via bipolar cables routed through a swivel commutator (Model SL2C; Plastics One, Roanoke, VA). Computers and interface equipment operated by custom software controlled all the operant sessions and data collection (Med PC-IV; Med Associates).

Training. Rats were trained to respond for brain stimulation using procedures identical to those previously described elsewhere (Altarifi and Negus, 2011; Negus and Miller, 2014; Miller et al., 2015). Briefly, a white house light was illuminated during behavioral sessions, and responding under a fixed-ratio 1 schedule produced a 500-millisecond train of 0.1-millisecond square-wave cathodal pulses together with 500-millisecond illumination of stimulus lights over the response lever. The terminal schedule consisted of sequential 10-minute components. Each component consisted of 10 1-minute trials, and the available brain-stimulation frequency decreased in 0.05 log Hz increments from one trial to the next (158–56 Hz). Each frequency trial consisted of a 10-second timeout, during which five noncontingent stimulations were delivered at the frequency available during that trial, followed by a 50-second "response" period during which responding resulted in electrical stimulation. Training continued with presentation of three sequential components per day until the following two criteria for stable responding were met for three consecutive days: 1) $\leq 5\%$ variability in the maximum rate of reinforcement in any trial, and 2) $\leq 10\%$ variability in the total number of stimulations per component.

Testing. Three-component ICSS sessions were conducted daily (with occasional exceptions on weekends) throughout the pre-paclitaxel baseline period and subsequent 29-day test period. The final 3 days of training were used to establish pre-paclitaxel baseline data. On days 1, 3, 5, and 7, rats were treated with either 2.0 mg/kg per day paclitaxel ($n = 8$ females and 8 males) or vehicle ($n = 6$ females and 6 males), and three-component ICSS sessions began 2 hours after paclitaxel or vehicle injections. Daily ICSS testing continued on days 8–21 without treatment.

Additionally, mechanical sensitivity was assessed on day 22 using the methods described previously, and all paclitaxel-treated rats met the criterion for paclitaxel-induced mechanical hypersensitivity (mean threshold in log grams < 0.90). Subsequently, all rats received cumulative morphine (0–10 mg/kg) on days 22 and 29 and repeated treatment with 3.2 mg/kg per day morphine on days 23–28.

Control studies were not conducted in rats treated with repeated saline on days 23–28 because we showed in a previous study that repeated saline treatment under these conditions does not alter morphine dose-effect curves on ICSS (Miller et al., 2015). Rather, the goal of this study was to evaluate the hypothesis that repeated morphine would increase expression of morphine-induced ICSS facilitation in rats treated with paclitaxel vehicle but not in rats treated with paclitaxel.

For cumulative-dosing test sessions on days 22 and 29, three daily-baseline components were followed by a series of five consecutive 50-minute morphine test cycles. Treatment injections were administered at the beginning of each test cycle, and two ICSS test components (lasting a total of 20 minutes) began 30 minutes after each injection. For single-dose test sessions conducted on the intervening days 23–28, three daily-baseline components were followed first by administration of 3.2 mg/kg morphine and then 30 minutes later by two ICSS test components.

Data Analysis. Data were analyzed as previously described elsewhere (Altarifi and Negus, 2011; Negus and Miller, 2014; Miller et al., 2015). The primary dependent measure was the total number of reinforcements per component (i.e., the total number of stimulations delivered across all brain-stimulation frequencies during each 10-minute component). All daily sessions consisted of at least three ICSS components. The first component of each daily session was considered to be a warm-up component, and the data were discarded. Data from the remaining pair of components were averaged within each rat and then across rats, and these data provided a measure of "daily baseline" ICSS performance. In addition, behavioral sessions on days 22–29 also included pairs of morphine test components (five pairs on cumulative-dosing days 22 and 29, one pair on single-dose days 23–28). Data from each pair of morphine test components were also averaged first within each rat and then across rats.

Paclitaxel effects on baseline ICSS performance and on morphine-induced changes in ICSS were analyzed separately. First, to compare the effects of vehicle and paclitaxel treatment on daily baseline ICSS, the number of daily baseline stimulations per component on days 1–29 were expressed as a percentage of the pre-paclitaxel baseline using this equation: $\% \text{ Pre-paclitaxel baseline reinforcements per component} = (\text{Daily baseline reinforcements per component on a test day} \div \text{Pre-paclitaxel baseline reinforcements per component}) \times 100$. Data in males and females were analyzed using separate two-way ANOVAs, with treatment day as a within-subjects factor and paclitaxel/vehicle treatment as a between-subjects factor.

Second, to evaluate morphine effects on ICSS in vehicle- and paclitaxel-treated rats, morphine test data on a given day were expressed as a percentage of daily baseline data on that day using this equation: $\% \text{ Daily baseline reinforcements per component} = (\text{Morphine test reinforcements per component} \div \text{Daily baseline reinforcements per component}) \times 100$. The morphine dose-effect data from cumulative dose-effect curves on days 22 and 29 were compared using separate repeated-measures two-way ANOVAs in male and female vehicle- and paclitaxel-treated rats, with morphine dose and treatment day as the two within-subjects factors. The morphine

single-dose test data on days 23–28 were analyzed by separate two-way ANOVAs in males and females, with treatment day as a within-subject factor and treatment as a between-subjects factor.

Finally, the morphine dose–effect data in male and female rats were directly compared to evaluate potential sex differences in the morphine effects on day 22 and day 29. For this comparison, data were collapsed across paclitaxel and paclitaxel vehicle treatments for each day and compared by two-way ANOVA with morphine dose as a within-subjects factor and sex as a between-subjects factor.

A secondary and more granular measure of ICSS performance was the reinforcement rate in stimulations per frequency trial. Raw reinforcement rates for each rat from each trial were converted to percent maximum control rate (%MCR), with MCR defined as the mean of the maximal rates observed at any trial during either the pre-paclitaxel baseline sessions (for analysis of paclitaxel effects) or the daily baseline (for analysis of morphine effects). Thus, %MCR values for each trial were calculated as $[(\text{Reinforcement rate during a frequency trial} \div \text{MCR}) \times 100]$. The %MCR values were then averaged across rats and analyzed by repeated-measures two-way ANOVA, with ICSS frequency and treatment day as the two within-subjects factors.

Results

Repeated Morphine Effects on Paclitaxel-Induced Mechanical Hypersensitivity in Male and Female Rats.

For the 32 rats that completed the mechanical sensitivity studies, baseline mechanical sensitivity thresholds were $1.18 \pm 0.00 \log g$ (males) and $1.12 \pm 0.06 \log g$ (females). Figure 2, A and B, shows that paclitaxel produced significant mechanical hypersensitivity on days 8, 15, 22, and 29 in both males and females. There were no differences between rats treated on days 23–28 with repeated 3.2 mg/kg per day morphine or vehicle in either sex (data not shown), so the data are collapsed to include both subgroups per sex.

Additionally, there was also no sex difference in paclitaxel-induced mechanical hypersensitivity as indicated by two-way ANOVA of the data in Fig. 2, A and B, with sex and time as the two factors (no significant effect of sex or sex $\times$ time interaction). Figure 2, C and D, shows that morphine dose-dependently reversed paclitaxel-induced hypersensitivity in both males and females before (day 22) and after (day 29) repeated vehicle treatment on days 23–28.

Two-way ANOVA indicated no difference between morphine dose-effect curves on day 22 and day 29 in either sex, and Table 1 shows that morphine ED₅₀ values were similar on days 22 and 29 in both sexes. Figure 2, E and F, and Table 1 show that modest but significant tolerance developed to morphine effects in rats treated with repeated 3.2 mg/kg per day morphine on days 23–28. Thus, by day 29 the morphine dose–effect curve had shifted to the right, and the morphine ED₅₀ values were significantly increased in both sexes.

Paclitaxel Effects on ICSS Responding in Male and Female Rats.

For rats used in ICSS studies, pre-paclitaxel/vehicle baseline measures of ICSS performance were 137.9 ± 13.9 (males) and 114.8 ± 12.7 (females) stimulations per component, and MCRs were 57.6 ± 5.7 (males) and 53.1 ± 2.4 (females) stimulations per trial. Paclitaxel treatment had little or no effect on ICSS in either sex. Figure 3, A and B, shows the effects of vehicle and repeated 2.0 mg/kg paclitaxel on the total number of stimulations per component over time in male and female rats. Paclitaxel did not significantly alter ICSS responding in either sex compared with vehicle-treated animals.

Figure 3, C and D, shows the full ICSS frequency-rate curves before and 29 days after initiation of paclitaxel treatment in males and females, respectively. Paclitaxel did not significantly alter ICSS frequency-rate curves in males. In females, there was a significant treatment $\times$ frequency interaction ($P = 0.017$); however, post hoc analysis indicated a significant but small decrease in ICSS responding at only one frequency (89 Hz). Treatment with paclitaxel vehicle did not significantly alter the ICSS frequency-rate curves in males or females (data not shown).

Repeated Morphine Effects on ICSS Responding in Vehicle- versus Paclitaxel-Treated Male and Female Rats.

The effects of repeated morphine treatment on the total-stimulations-per-component measure of ICSS during cumulative-dose testing are shown in Figs. 4 and 5 for male and female rats treated initially with vehicle or paclitaxel, respectively. In general, repeated morphine treatment increased the ICSS-facilitating effects of morphine regardless of sex or vehicle/paclitaxel treatment. Thus, in the vehicle-treated males (Fig. 4A), the initial exposure to morphine on day 22 produced only a dose-dependent decrease in the total-stimulations-per-component measure of ICSS, but on day 29, after 6 days of repeated 3.2 mg/kg per day morphine, ICSS was significantly increased by a dose of 3.2 mg/kg morphine.

Additionally, ICSS rates after administration of 3.2 and 10 mg/kg morphine were significantly higher on day 29 than on day 22. Qualitatively similar effects were observed in vehicle-treated females (Fig. 4B), and most importantly, 3.2 mg/kg morphine significantly facilitated ICSS in females on day 29. Figure 4, C–F highlights effects of 3.2 mg/kg morphine on full frequency-rate curves in vehicle-treated males and females on day 22 (before repeated morphine) and day 29 (after repeated morphine). Relative to saline treatment on the respective test days, 3.2 mg/kg morphine only decreased high rates of ICSS maintained by high brain-stimulation frequencies on day 22 in males (Fig. 4C), but by day 29, tolerance had developed to this rate-decreasing effect, and morphine increased ICSS rates across a broad range of frequencies (63–89 Hz; Fig. 4E). In females, 3.2 mg/kg morphine significantly increased ICSS rates at two frequencies (89–100 Hz) on day 22 (Fig. 4C) and at four frequencies on day 29 (Fig. 4E).

In general, similar effects of repeated morphine were observed in paclitaxel-treated rats. Thus, in the paclitaxel-treated males (Fig. 5A), the initial exposure to morphine on day 22 produced only a dose-dependent decrease in the total-stimulations-per-component measure of ICSS, but on day 29, after 6 days of repeated 3.2 mg/kg per day morphine, ICSS was significantly increased by a dose of 3.2 mg/kg morphine. Similarly, in females, morphine produced only a dose-dependent decrease in this measure of ICSS on day 22, but cumulative doses of 1.0 and 3.2 mg/kg morphine significantly increased ICSS on day 29. In both males and females, ICSS rates were significantly higher after 1.0 and/or 3.2 mg/kg morphine on day 29 than on day 22.

Figure 5, C–F, highlights the effects of 3.2 mg/kg morphine on full frequency-rate curves in paclitaxel-treated males and females on day 22 (before repeated morphine) and day 29 (after repeated morphine). Relative to effects of saline treatment, 3.2 mg/kg morphine only decreased high rates of ICSS maintained by high brain-stimulation frequencies on day 22 in males (Fig. 5C), but by day 29 tolerance had developed

Fig. 2. Effects of morphine on paclitaxel-induced mechanical hypersensitivity in male and female rats. (A and B) The effects of repeated 2.0 mg/kg per day paclitaxel on mechanical sensitivity thresholds in (A) male and (B) female rats. Horizontal axes: Time in days relative to initiation of vehicle/paclitaxel treatment on day 1. Arrows show days of paclitaxel treatment. Vertical axes: Mechanical sensitivity expressed as threshold stimulation to elicit paw withdrawal in log grams. All points show mean $\pm$ S.E.M. (A: $n = 15$; B: $n = 17$), and filled points indicate statistically significant differences from day 0 pre-paclitaxel baseline. (C–F) The effects of morphine on mechanical sensitivity thresholds after paclitaxel treatment. Horizontal axes: Cumulative dose of morphine in milligrams per kilogram. Vertical axes: Percent maximal possible effect (%MPE). All points show mean $\pm$ S.E.M. (C: $n = 7$; D: $n = 8$; E: $n = 8$; F: $n = 9$). Filled points indicate a statistically significant difference from saline (Sal) on a given day, and asterisks denote a statistically significant difference between days at a given morphine dose. Statistical results are as follows. (A) Significant main effect of treatment [$F(3.143, 44) = 62.92$; $P < 0.0001$]. (B) Significant main effect of treatment [$F(3.087, 49.39) = 41.53$; $P < 0.0001$]. (C) Significant main effect of treatment [$F(4, 24) = 51.93$; $P < 0.0001$], no significant main effect of day [$F(1, 6) = 0.5255$; $P = 0.496$], and no significant interaction [$F(4, 24) = 0.46$; $P = 0.765$]. (D) Significant main effect of treatment [$F(4, 28) = 109.2$; $P < 0.0001$], no significant effect of day [$F(1, 7) = 0.77$; $P = 0.789$], and no significant interaction [$F(4, 28) = 0.18$; $P = 0.949$]. (E) Significant main effects of treatment [$F(4, 28) = 65.37$; $P < 0.0001$] and day [$F(1, 7) = 17.27$; $P = 0.004$], and a significant interaction [$F(4, 28) = 5.33$; $P = 0.003$]. (F) Significant main effects of treatment [$F(4, 32) = 135.7$; $P < 0.0001$] and day [$F(1, 8) = 27.00$; $P = 0.001$], and a significant interaction [$F(4, 32) = 4.46$; $P = 0.006$].

to this rate-decreasing effect, and morphine increased ICSS rates at three frequencies (79–100 Hz; Fig. 4E). In females, 3.2 mg/kg morphine significantly increased ICSS rates at three frequencies (71, 89, 100 Hz) on day 22 but also decreased rates at the highest frequency of 158 Hz. On day 29, 3.2 mg/kg

morphine increased ICSS rates at 79–100 Hz with no evidence of rate-decreasing effects at any frequency.

In addition to these data from cumulative-dosing test sessions on days 22 and 29, Fig. 6 shows that data from the single-dose test sessions on days 23–28 also indicated increasing levels of

TABLE 1

Morphine ED₅₀ values in milligrams per kilogram (95% confidence limits) to reverse paclitaxel-induced mechanical hypersensitivity before and after repeated treatment with either saline or 3.2 mg/kg per day morphine on days 23–28

Treatment and Test Day	Males	Females
Repeated saline		
Day 22	1.9 (1.4–2.8)	1.8 (1.3–2.7)
Day 29	2.2 (1.6–3.0)	1.9 (1.4–2.6)
Repeated morphine		
Day 22	1.6 (1.1–2.3)	1.5 (1.2–1.9)
Day 29	3.7 (2.6–5.4) ^a	2.6 (2.1–3.1) ^a

^aStatistically significant difference from day 22 as indicated by nonoverlapping confidence limits.

morphine-induced ICSS facilitation over time. Thus, repeated morphine produced a gradual increase in ICSS facilitation over days, regardless of sex or saline/paclitaxel treatment.

To investigate the role of sex as a determinant for morphine's effects on ICSS, the vehicle- and paclitaxel-treated rats were combined because chemotherapy treatment did not alter the morphine effects. Figure 7A shows the initial effects

of morphine in opioid-naïve rats on day 22. There was a sex difference in effect of 3.2 mg/kg morphine, which produced significant ICSS facilitation in females but not males. A higher dose of 10 mg/kg morphine decreased ICSS in both sexes. Figure 7B shows the effect of morphine on day 29 after the regimen of repeated morphine treatment. There was no longer a sex difference in opioid effects, and morphine-induced ICSS facilitation was enhanced in both males and females. Specifically, morphine produced significant ICSS facilitation at 1 and 3.2 mg/kg in both sexes while still producing ICSS depression at 10 mg/kg.

Discussion

This study compared the effects of repeated morphine treatment on ICSS and mechanical hypersensitivity in male and female rats treated with paclitaxel. There were three main findings. First, paclitaxel produced sustained mechanical hypersensitivity but little change in the baseline ICSS performance in both males and females. Second, initial morphine treatment dose-dependently alleviated paclitaxel-induced mechanical hypersensitivity in both sexes, and repeated morphine

Fig. 3. Effects of treatment with paclitaxel or its vehicle on ICSS in male and female rats. (A and B) Paclitaxel effects on total stimulations per component in (A) males and (B) females. Horizontal axes: Time in days after initiation of treatment. Arrows indicate days for vehicle or paclitaxel administration. Vertical axes: ICSS performance expressed as the % pre-paclitaxel baseline number of reinforcements earned per 10-minute component. All points show mean $\pm$ S.E.M. (for both panels, $n = 6$ vehicle-treated and $n = 8$ paclitaxel-treated rats), and filled points indicate a statistically significant difference from the day 0 pre-paclitaxel baseline. (C and D) Full ICSS frequency-rate curves determined before (Pre-PTX) and 29 days after initiation of paclitaxel treatment. Horizontal axes: Brain stimulation frequency in Hertz (log scale). Vertical axes: ICSS performance expressed as the % maximum control rate (%MCR). All points show mean $\pm$ S.E.M. ($n = 8$ for both panels), and filled points in (D) indicate a statistically significant difference from Pre-PTX at that frequency. Statistical results are as follows. (A) No significant main effect of treatment [$F(1,12) = 1.940$; $P = 0.941$], a significant main effect of time [$F(24,288) = 1.940$; $P = 0.006$], and no significant interaction [$F(24,288) = 0.514$; $P = 0.098$]. (B) No significant main effect of treatment [$F(1,12) = 4.508$; $P = 0.055$], a significant main effect of time [$F(24,288) = 2.128$; $P = 0.002$], and no significant interaction [$F(24,288) = 14.31$; $P = 0.405$]. (C) No significant main effect of treatment [$F(1,6) = 1.898$; $P = 0.218$], a significant main effect of frequency [$F(9,54) = 83.090$; $P < 0.0001$], and no significant interaction [$F(9,54) = 1.883$; $P = 0.0744$]. (D) No significant main effect of treatment [$F(1,7) = 1.660$; $P = 0.239$], a significant effect of frequency [$F(9,63) = 57.45$; $P < 0.0001$], and a significant interaction [$F(9,63) = 2.484$; $P = 0.017$].

Fig. 4. Effects of repeated morphine on ICSS in male and female rats treated with paclitaxel vehicle. Top panels show the effects of cumulative morphine doses on total stimulations per component in (A) males and (B) females. Horizontal axes: Cumulative doses of morphine in milligrams per kilogram. (Sal = saline.) Vertical axes: ICSS performance expressed the % daily baseline number of brain-stimulation reinforcements earned per 10-minute component. Filled symbols indicate a statistically significantly difference from saline within a day, and asterisks indicate a statistically significantly difference between days at a given morphine dose. Bottom panels show the full ICSS frequency-rate curves determined after treatment with saline or cumulative 3.2 mg/kg morphine on day 22 in (C) males and (D) females or on day 29 in (E) males and (F) females. Horizontal axes: Brain stimulation frequency in Hertz (log scale). Vertical axes: ICSS performance expressed as the % maximum control rate (%MCR). Filled symbols indicate a statistically significantly difference from saline at a given frequency. All points show mean $\pm$ S.E.M. in six rats. Statistical results are as follows. (A) Significant main effects of treatment [$F(4,20) = 38.45$; $P < 0.0001$] and day [$F(1,5) = 8.395$; $P = 0.040$], and a significant interaction [$F(4,20) = 7.344$; $P = 0.001$]. (B) Significant main effect of treatment [$F(4,20) = 28.160$; $P < 0.0001$], no significant effect of day [$F(1,5) = 3.280$; $P = 0.130$], and no significant interaction [$F(4,20) = 0.981$; $P = 0.440$]. (C) No significant main effect of treatment [$F(1,5) = 3.999$; $P = 0.102$], a significant main effect of frequency [$F(9,45) = 39.45$; $P < 0.0001$], and a significant interaction [$F(9,45) = 2.551$; $P = 0.018$]. (D) No significant main effect of treatment [$F(1,5) = 5.417$; $P = 0.067$], but a significant main effect of frequency [$F(9,45) = 51.850$; $P < 0.0001$] and a significant interaction [$F(9,45) = 9.864$; $P < 0.001$]. (E) Significant main effects of treatment [$F(1,5) = 9.001$; $P = 0.030$] and frequency [$F(9,45) = 24.74$; $P < 0.0001$], but no significant interaction [$F(9,45) = 2.028$; $P = 0.058$]. (F) Significant main effects of treatment [$F(1,5) = 10.40$; $P = 0.023$] and frequency [$F(9,45) = 73.280$; $P < 0.0001$], and a significant interaction [$F(9,45) = 3.254$; $P = 0.004$].

Fig. 5. Effects of repeated morphine on ICSS in male and female rats treated with 2.0 mg/kg per day paclitaxel. Top panels show the effects of cumulative morphine doses on total stimulations per component in (A) males and (B) females. Horizontal axes: Cumulative doses of morphine in milligrams per kilogram. (Sal = saline.) Vertical axes: ICSS performance expressed as the % daily-baseline number of brain-stimulation reinforcements earned per 10-minute component. Filled symbols indicate a statistically significant difference from saline within a day, and asterisks indicate a statistically significant difference between days at a given morphine dose. Bottom panels show full ICSS frequency-rate curves determined after treatment with saline or cumulative 3.2 mg/kg morphine on day 22 in (C) males and (D) females or on day 29 in (E) males and (F) females. Horizontal axes: Brain stimulation frequency in Hertz (log scale). Vertical axes: ICSS performance expressed as the % maximum control rate (%MCR). Filled symbols indicate a statistically significant difference from saline at a given frequency. All points show mean $\pm$ S.E.M. in eight rats. Statistical results are as follows. (A) Significant main effects of treatment [$F(4,28) = 92.73$; $P < 0.0001$] and day [$F(1,7) = 6.884$; $P = 0.035$], and a significant interaction [$F(4,28) = 7.198$; $P = 0.0004$]. (B) Significant main effects of treatment [$F(4,28) = 59.980$; $P < 0.0001$] and day [$F(1,7) = 6.083$; $P = 0.043$], and a significant interaction [$F(4,28) = 3.16$; $P = 0.029$]. (C) No significant main effect of treatment [$F(1,7) = 1.632$; $P = 0.242$], a significant effect of frequency [$F(9,63) = 61.53$; $P < 0.0001$], and a significant interaction [$F(9,63) = 2.066$; $P = 0.046$]. (D) No significant main effect of treatment [$F(1,7) = 4.19$; $P = 0.080$], but a significant effect of frequency [$F(9,63) = 38.950$; $P < 0.0001$], and a significant interaction [$F(9,63) = 4.594$; $P = 0.0001$]. (E) Significant main effects of treatment [$F(1,7) = 15.85$; $P = 0.005$] and frequency [$F(9,63) = 47.48$; $P < 0.0001$], and a significant interaction [$F(9,63) = 2.281$; $P = 0.028$]. (F) Significant main effects of treatment [$F(1,7) = 10.22$; $P = 0.015$], a significant effect of frequency [$F(9,63) = 33.99$; $P < 0.001$], and a significant interaction [$F(9,63) = 2.662$; $P = 0.011$].

administration produced modest but significant tolerance to this antinociceptive effect. Third, the initial morphine treatment produced greater abuse-related ICSS facilitation in females than males, but repeated morphine treatment enhanced ICSS facilitation in both male and female rats treated with either paclitaxel or its vehicle.

Overall, these results suggest that this model of paclitaxel-induced neuropathy does not alter the trajectory of increasing opioid reward that occurs during initial exposure to repeated morphine administration. More generally, these results suggest that CINP is not protective against opioid reward, and repeated morphine treatment may simultaneously produce both tolerance to

Fig. 6. Effects of repeated 3.2 mg/kg per day morphine on ICSS performance in male and female vehicle- and paclitaxel-treated rats for single-dosing testing on days 23–28. Panels show the effects of daily 3.2 mg/kg morphine on total stimulations per component in (A) males and (B) females. Horizontal axes: Time in days after initiation of paclitaxel treatment. Vertical axes: ICSS performance expressed the % baseline number of brain-stimulation reinforcements earned per 10-minute component. All points show mean $\pm$ S.E.M. for $n = 6$ vehicle-treated rats and $n = 8$ paclitaxel-treated rats in each panel. Line with a superior asterisk denotes days statistically different from day 23. Statistical results are as follows. (A) Significant main effect of day [$F(5,55) = 7.551$; $P < 0.0001$] but not of treatment [$F(1,11) = 0.285$; $P = 0.604$], and no significant interaction [$F(5,55) = 1.596$; $P = 0.177$]. (B) Significant main effect of day [$F(5,60) = 4.704$; $P = 0.001$] but not of treatment [$F(1,12) = 1.525$; $P = 0.241$], and no significant interaction [$F(5,60) = 0.032$; $P = 1.000$].

analgesic effects and increased vulnerability to iatrogenic opioid addiction.

Effects of Paclitaxel on Mechanical Sensitivity and ICSS. The effects of paclitaxel on mechanical sensitivity thresholds reported here agree with previous studies in rodents that examined the time course and extent of mechanical hypersensitivity after paclitaxel treatment (Polomano et al., 2001; Pascual et al., 2010; Boyette-Davis et al., 2011; Hwang et al., 2012; Ko et al., 2014; Toma et al., 2017; Legakis et al., 2018). In addition, although females were found to be more sensitive than males to paclitaxel-induced cold allodynia, the present results agree with previous findings that there were no sex differences in the development of paclitaxel-induced mechanical hypersensitivity (Hwang et al., 2012; Naji-Esfahani et al., 2016; Legakis et al., 2018).

ICSS can be used as one behavioral end point to evaluate the expression and treatment of pain-related behavioral depression and functional impairment (Pereira Do Carmo et al., 2009). ICSS uses positively reinforced operant behavior for electrical stimulation across different frequencies to maintain ranges of low-to-high rates of

reinforcement that are stable over time and can be decreased with noxious stimuli or test treatments. This assay can be used to examine the effects of manipulations that impair reward system function and contribute to affective signs of anhedonia and depression (Carlezon and Chartoff, 2007; Negus and Miller, 2014).

Unlike other noxious stimuli, such as intraperitoneal administration of dilute acid, paw incision, or intraplantar administration of complete Freund's adjuvant or formalin, paclitaxel failed to decrease ICSS rates of reinforcement (Pereira Do Carmo et al., 2009; Ewan and Martin, 2014; Leitel et al., 2014; Brust et al., 2016). This agrees with previous results from our laboratory (Legakis et al., 2018) and suggests that the pain state produced by this paclitaxel treatment regimen in rats is sufficient to produce mechanical hypersensitivity but not pain-related behavioral depression. However, as we discuss later, the lack of paclitaxel effect on baseline ICSS performance did not preclude assessment of paclitaxel effects on the trajectory of increasing opioid reward produced by repeated opioid exposure.

Fig. 7. Effects of morphine on ICSS in male and female rats before and after repeated treatment with 3.2 mg/kg per day morphine. Panels show the effects of cumulative morphine doses on total stimulations per component on (A) day 22 and (B) day 29. Horizontal axes: Cumulative doses of morphine in milligrams per kilogram. (Sal = saline.) Vertical axes: ICSS performance expressed as the % daily baseline number of brain-stimulation reinforcements earned per 10-minute component. All points show mean $\pm$ S.E.M. in 14 rats. Filled points denote a statistically significant difference from saline; asterisk denotes a statistically significant difference between males and females. Statistical results are as follows. (A) Significant main effects of dose [$F(4,104) = 166.700$; $P < 0.0001$] and sex [$F(1,26) = 4.306$; $P = 0.048$], and a significant interaction [$F(4,104) = 3.408$; $P = 0.012$]. (B) Significant main effect of dose [$F(4,104) = 107.600$; $P < 0.0001$] but not of sex [$F(1,26) = 1.727$; $P = 0.200$], and no significant interaction [$F(4,104) = 2.041$; $P = 0.094$].

Effects of Morphine on Paclitaxel-Induced Mechanical Hypersensitivity. Although sex differences in morphine antinociception have been reported in other preclinical injury models in rats (Boyer et al., 1998; Cicero et al., 2002; Craft, 2008), the present results agree with a previous report that failed to observe a sex difference in morphine potency to alleviate paclitaxel-induced mechanical hypersensitivity (Hwang et al., 2012). The present results extend these findings in two ways.

First, to our knowledge, this is the first study to report tolerance to morphine antiallodynia in paclitaxel-treated rats receiving repeated morphine treatment; however, these findings agree with previous reports of tolerance to morphine antiallodynia in rats studied using other nerve-injury models (Bulka et al., 2002; Ledebroer et al., 2006). It is also important to note that, although antiallodynia in preclinical studies is often interpreted as evidence for potential analgesic effects in humans, there are several examples of poor translation between preclinical antiallodynia and clinical analgesia for treatment of CINP (Xiao et al., 2009; Tatsushima et al., 2011; Paton et al., 2017). Thus, the tolerance to morphine antiallodynia shown here is only suggestive of a potential for tolerance to morphine analgesia in human patients with chemotherapy-induced neuropathic pain.

Second, the similarity in morphine potency on days 22 and 29 in rats treated with repeated saline agrees with the similar baseline levels of hypersensitivity on those 2 days to suggest no progression in the underlying neuropathic pain state between these two times. These findings also suggest that the decrease in morphine potency after repeated morphine treatment reflects tolerance and not neuropathy progression.

Effects of Morphine on ICSS. ICSS procedures have a record of predictive validity similar to that of drug self-administration procedures for preclinical abuse-liability assessment, and most drugs that facilitate ICSS also have abuse liability in humans (Wise, 1996; Negus, 2013; Negus and Miller, 2014). We reported previously that repeated morphine treatment produces a progressive increase in the expression of morphine reward in the ICSS procedure, and this trajectory of increasing opioid reward is not altered by a repeated acute-pain stimulus administered in conjunction with morphine (Miller et al., 2015). This preclinical finding agrees with clinical evidence that μ -opioid receptor agonists produced more robust aversive effects and weaker euphoric effects in opioid-naïve than opioid-experienced humans and that repeated opioid exposure in pain patients for as few as 5 days can increase the risk of long-term opioid use (Zacny et al., 1994; Shah et al., 2017).

The present results extend these previous findings in two ways. First, initial morphine exposure produced primarily ICSS depression in both vehicle- and paclitaxel-treated male rats, suggesting that the initial expression of morphine reward was not enhanced by the paclitaxel-induced pain state. It has been argued that chronic pain states can enhance the rewarding effects of analgesic drugs by creating conditions under which those drugs produce negative reward (associated with reversal of an aversive pain state) in addition to whatever positive rewarding effects they may also produce (Navratilova et al., 2015). The present study did not find evidence for this phenomenon with morphine under conditions of a paclitaxel-induced neuropathic pain state in rats.

Second, paclitaxel also failed to alter the trajectory of increasing morphine reward produced by repeated morphine administration. This supports a previous study that found no

effect of paclitaxel on morphine-induced conditioned place preference in rats (Mori et al., 2014) and extends these studies by showing a failure of paclitaxel to alter the changes in morphine reward that occur with initial exposure to a regimen of repeated morphine treatment. It is especially relevant to note that repeated morphine administration produced increasing expression of reward while producing decreased expression of (i.e., tolerance to) antiallodynia. This suggests a risk for iatrogenic addiction during the use of morphine for treatment of CINP, as repeated treatment may produce a vicious cycle of analgesic tolerance and dose escalation that may simultaneously produce increasing sensitivity to morphine reward.

Finally, the present study found that initial morphine exposure produced stronger rewarding effects in vehicle- and paclitaxel-treated female rats than male rats, but repeated morphine exposure eliminated this sex difference and produced the same pattern of increasing reward despite antiallodynic tolerance. These data agree with other rodent studies, which have suggested that females are more sensitive than males to abuse-related effects of μ -opioid agonists (Craft, 2008). In self-administration studies, females acquired operant behavior for heroin and morphine faster than males (Cicero et al., 2003; Lynch, 2006; Lynch et al., 2013); in a conditioned place preference study, females expressed greater place preference for high doses of morphine (Cicero et al., 2000).

Due to the ability of ICSS procedures to detect both the abuse-limiting effects of drugs (e.g., motor depression causing decreases in rate reinforcement) as well as abuse-related facilitation, the lack of expression of abuse-related facilitation in morphine-naïve males may be related to previous observations of increased sensitivity of males to the sedative effects of opioids as opioids are more potent for suppressing locomotion in males compared with females (Holtman et al., 2004; Craft et al., 2006). These preclinical findings in rodents map onto the few studies that have looked at the progression of opioid abuse in humans and are important because paclitaxel is often used to treat cancer in women while morphine is used to treat CINP in both women and men. In particular, women have an earlier age of initiation of opioid substance abuse and a more rapid progression from initial use to dependence (Anglin et al., 1987; Hernandez-Avila et al., 2004) despite no large differences in the overall prevalence of opioid use disorder (Becker et al., 2008; Manubay et al., 2015; Graziani and Nistico, 2016; Serdarevic et al., 2017).

Authorship Contributions

Participated in research design: Legakis, Negus.

Conducted experiments: Legakis.

Performed data analysis: Legakis.

Wrote or contributed to the writing of the experiment: Legakis, Negus.

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