

Effect of Chronic Delivery of the Toll-like Receptor 4 Antagonist (+)-Naltrexone on Incubation of Heroin Craving

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Background: Recent evidence implicates toll-like receptor 4 (TLR4) in opioid analgesia, tolerance, conditioned place preference, and self-administration. Here, we determined the effect of the TLR4 antagonist (+)-naltrexone (a μ -opioid receptor inactive isomer) on the time-dependent increases in cue-induced heroin seeking after withdrawal (incubation of heroin craving).

Methods: In an initial experiment, we trained rats for 9 hours per day to self-administer heroin (.1 mg/kg/infusion) for 9 days; lever presses were paired with a 5-second tone-light cue. We then assessed cue-induced heroin seeking in 30-minute extinction sessions on withdrawal day 1; immediately after testing, we surgically implanted rats with Alzet minipumps delivering (+)-naltrexone (0, 7.5, 15, 30 mg/kg/day, subcutaneous) for 14 days. We then tested the rats for incubated cue-induced heroin seeking in 3-hour extinction tests on withdrawal day 13.

Results: We found that chronic delivery of (+)-naltrexone via minipumps during the withdrawal phase decreased incubated cue-induced heroin seeking. In follow-up experiments, we found that acute injections of (+)-naltrexone immediately before withdrawal day 13 extinction tests had no effect on incubated cue-induced heroin seeking. Furthermore, chronic delivery of (+)-naltrexone (15 or 30 mg/kg/day) or acute systemic injections (15 or 30 mg/kg) had no effect on ongoing extended access heroin self-administration. Finally, in rats trained to self-administer methamphetamine (.1 mg/kg/infusion, 9 hours/day, 9 days), chronic delivery of (+)-naltrexone (30 mg/kg/day) during the withdrawal phase had no effect on incubated cue-induced methamphetamine seeking.

Conclusions: The present results suggest a critical role of TLR4 in the development of incubation of heroin, but not methamphetamine, craving.

Key Words: Craving, extinction, glia, heroin self-administration, opioid drugs, reinstatement, relapse, TLR4

A high rate of relapse to drug use is a main feature of heroin addiction (1,2). One factor thought to contribute to heroin relapse and craving in humans, even after prolonged abstinence, is exposure to environmental cues previously associated with drug use (3). In rat models of drug relapse and craving (4), response to cues previously associated with self-administration of heroin (5,6) and other abused drugs (7–11) progressively increases after withdrawal. We have termed this phenomenon incubation of drug craving (7,12). Over the last decade, we and others have identified several critical mechanisms of incubation of cocaine craving (13,14). In contrast, mechanisms underlying incubation of craving for heroin and other drugs are largely unknown (13). Here, we assessed the role of toll-like receptor 4 (TLR4) in incubation of heroin craving.

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Emerging evidence indicates that exposure to opioids and other abused drugs activates nonneuronal (glia, microglia, astrocytes) cells of the central immune system and that this activation plays a role in the behavioral effects of opioids and possibly other drugs (15–18). TLR4 is an innate immune system pattern recognition receptor and a member of the TLR family; this family includes 13 innate immune system receptors traditionally thought to primarily respond to pathogen-derived (pathogen associated molecular patterns) and tissue damage-related (damage associated molecular patterns) ligands (19,20). TLR4, the first discovered mammalian TLR, was initially found to recognize and to be activated by bacterial lipopolysaccharide (21). Subsequent studies have demonstrated that TLR4 is also activated by other foreign substances, such as small molecule xenobiotics (xenobiotic associated molecular patterns) (22) and several abused drugs (15,16).

TLR4 activation within the central nervous system causes the release of proinflammatory and neuroexcitatory cytokines, such as tumor necrosis factor- α and interleukin-1 β (20,23). TLR4 and other TLRs are widely distributed in the brain, where they form an essential link between the innate immune system and the central nervous system (20,24). These innate immune receptors are expressed in different immunocompetent cells (20,24), including microglia (25), astrocytes (26), and oligodendrocytes (27). There is also evidence that TLR4 is expressed in cortical central nervous system neurons (28).

Recent studies indicate that morphine and other μ -opioid receptor (MOR) agonists, which stereoselectively activate MOR (29), induce nonstereoselective activation of TLR4 by binding to an accessory protein of TLR4, myeloid differentiation protein 2. Activation of TLR4 triggers oligomerization and subsequent

glia-mediated proinflammatory responses (22,30). Conversely, the preferential MOR antagonists (–)-naloxone and (–)-naltrexone nonstereoselectively inhibit TLR4 activation by opioid agonists and other stimuli (e.g., stressors, pain manipulations) (16,24). Results from *in vivo*, *in vitro*, and *in silico* studies demonstrate that (+)-naloxone and (+)-naltrexone, the MOR inactive isomers of (–)-naloxone and (–)-naltrexone, are selective TLR4 antagonists (30–32). Importantly, blockade of TLR4 with (+)-naloxone or (+)-naltrexone attenuates neuropathic pain, morphine analgesic tolerance, and opioid withdrawal symptoms (16,32). Most recently, Hutchinson *et al.* (30) reported that blockade of TLR4 with (+)-naloxone decreased morphine conditioned place preference (CPP) and remifentanil (a short-acting MOR agonist) self-administration in rats.

The studies described above implicate TLR4 in the acute rewarding effects of opioid drugs, as assessed in CPP (33) and drug self-administration (34) procedures. The role of TLR4 in relapse to opioid seeking is unknown; additionally, mechanisms of drug reward, as assessed in these procedures, are often dissociable from those mediating relapse to drug seeking in rat models (35,36). Therefore, in the present study, we explored the role of TLR4 in relapse to heroin seeking using an incubation of heroin-craving procedure in which the response to heroin cues in extinction tests progressively increases after withdrawal from the drug (5,6). In the experiments described below, we used (+)-naltrexone as a long-acting TLR4 antagonist. After assessing its receptor selectivity, we determined the effect of acute and chronic (+)-naltrexone exposure on incubation of heroin craving. We also studied the effect of chronic delivery and acute injections of (+)-naltrexone on ongoing heroin self-administration and incubation of methamphetamine craving. To the degree that (+)-naltrexone is a selective TLR4 antagonist, our results demonstrate a novel role of TLR4 in the development of incubation of heroin but not methamphetamine craving.

Methods and Materials

Overview of the Behavioral Experiments

Using procedures similar to the ones described in the Supplemental Online Methods section in *Supplement 1*, we found that acute injections of the short-acting TLR4 antagonist (+)-naloxone (10 or 30 mg/kg, subcutaneous [SC]) had an inconsistent effect on cue-induced heroin seeking in extinction tests (3 hours) on withdrawal days 1 and 15 (F.R. Theberge, unpublished data). We also found in these pilot studies that twice daily repeated injections of (+)-naloxone (30 mg/kg) during the withdrawal period had no effect on incubated cue-induced heroin seeking on day 15.

Thus, in experiment 1 reported here, we employed an extended access heroin self-administration training procedure (9 hours of heroin access per day over 9 days) and used Alzet minipumps (Durect Corporation, Cupertino, California; 14-day delivery) to chronically deliver the long-acting TLR4 antagonist (+)-naltrexone during the 2 weeks of withdrawal from heroin. We tested the rats for incubated cue-induced heroin seeking in 3-hour extinction tests on withdrawal day 13. Before minipump implantation, we gave rats a 30-minute extinction session on day 1. This was done to verify that incubation of craving is reliably observed in each experiment in the minipump-vehicle condition and to allow us to match the different groups for baseline early withdrawal extinction responding.

In experiment 2, we determined whether the effect of chronic delivery of (+)-naltrexone on incubated cue-induced heroin seeking is mimicked by acute pretest injections of the TLR4 antagonist. We also used 12 rats that previously participated in experiment 2 to assess the effect of chronic delivery of (+)-naltrexone on operant responding maintained by palatable food pellets (37). In experiment 3, we surgically implanted rats with the minipumps containing (+)-naltrexone 2 days before the training phase to determine whether chronic delivery of the TLR4 antagonist would decrease ongoing extended-access heroin self-administration. We also assessed the effect of acute systemic injections of both (+)-naltrexone (both SC and intraperitoneal [IP]) and for comparison purposes (+)-naloxone (used in Hutchinson *et al.* [30] study) on ongoing extended-access heroin self-administration. Finally, in experiment 4, we used the same experimental conditions used in experiment 1, with the exception that lever presses during the training phase led to methamphetamine infusions, to determine whether chronic delivery of (+)-naltrexone would also decrease incubated cue-induced methamphetamine seeking. The details of the experimental procedures for these experiments are provided in the Supplemental Online Methods section in *Supplement 1*, which also provides a description of the initial *in vitro* experiments to assess potential non-TLR4 receptor binding sites or enzymatic activity of (+)-naltrexone.

Results

In Vitro Assays

Results from the target screening performed by Caliper Life Sciences showed that (+)-naltrexone displayed no significant activity at the 64 biological targets examined. The summary data in *Table S1 in Supplement 1* show that (+)-naltrexone had greater than 10 μ mol/L affinity for the receptors and ion channels tested and failed to exhibit significant inhibition of the enzymes tested. *Figure 1* depicts the dose-response curves for (+) and (–) isomers of naltrexone in the binding assay for human MOR. K_i for (+)- and (–)-naltrexone were 1634 ± 146 nmol/L and $.68 \pm .04$ nmol/L, respectively; the K_i for DAMGO was $11.1 \pm .08$ nmol/L. The binding data indicate that (+)-naltrexone is at least 2400-fold less potent than (–)-naltrexone in its binding affinity at MOR.

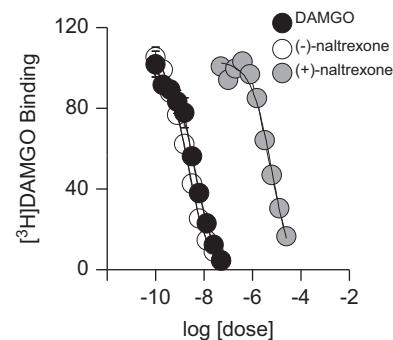
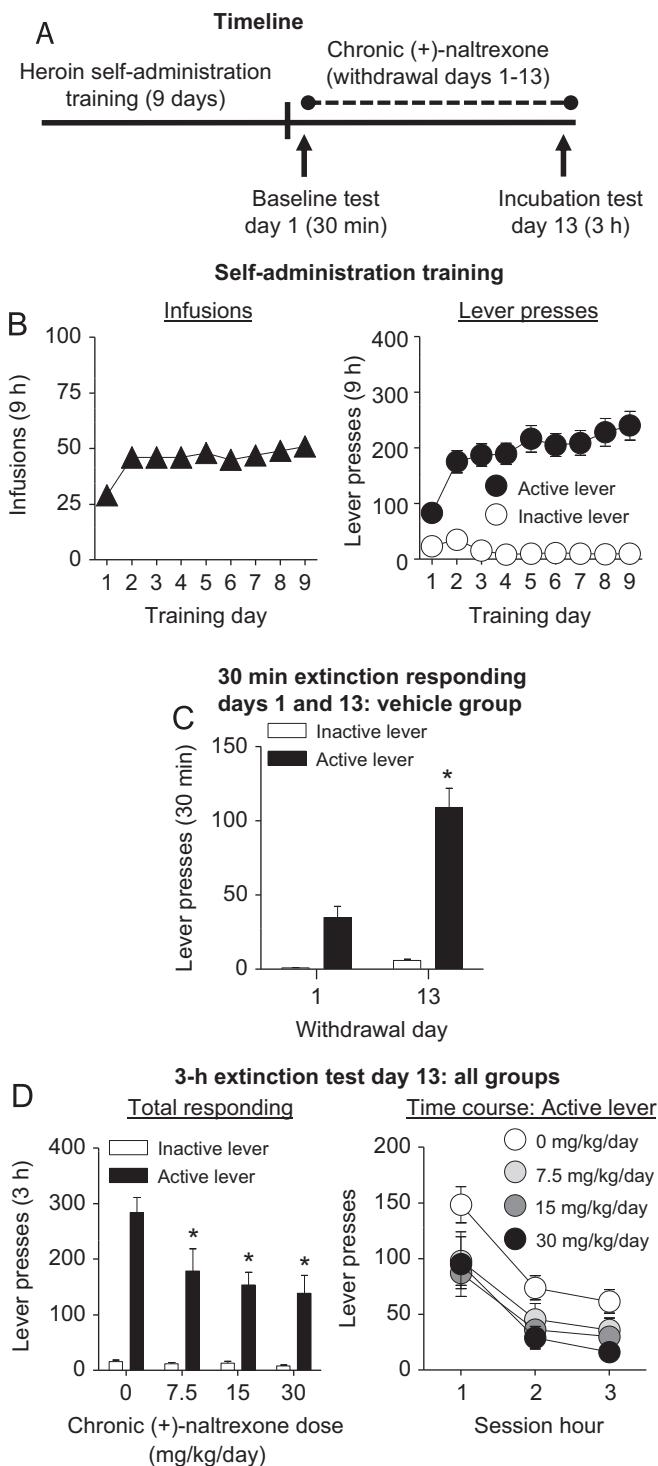


Figure 1. Dose-response curves for inhibition of $[^3\text{H}]$ DAMGO binding for isomers of naltrexone: (–)-naltrexone and (+)-naltrexone. Membranes from Chinese hamster ovary cells expressing human μ opioid receptors were prepared as described in Methods and Materials. Ten concentrations of each test drug were incubated in the presence of 3 nmol/L $[^3\text{H}]$ DAMGO to generate curves. Data are expressed as mean \pm SD for three separate runs performed in triplicate.

Experiment 1: Effect of Chronic Delivery of (+)-Naltrexone During the Withdrawal Phase on Incubated Cue-Induced Heroin Seeking. *Self-Administration Training:* The rats increased their number of heroin infusions over days ($F_{8,424} = 12.0, p < .001$; Figure 2B). Additionally, active but not inactive lever presses increased over days (lever \times day interaction [$F_{8,424} = 10.9, p < .001$]; Figure 2B). *Extinction Tests:* The rats in the chronic vehicle group demonstrated time-dependent increases in cue-induced heroin seeking in the extinction tests (incubation of

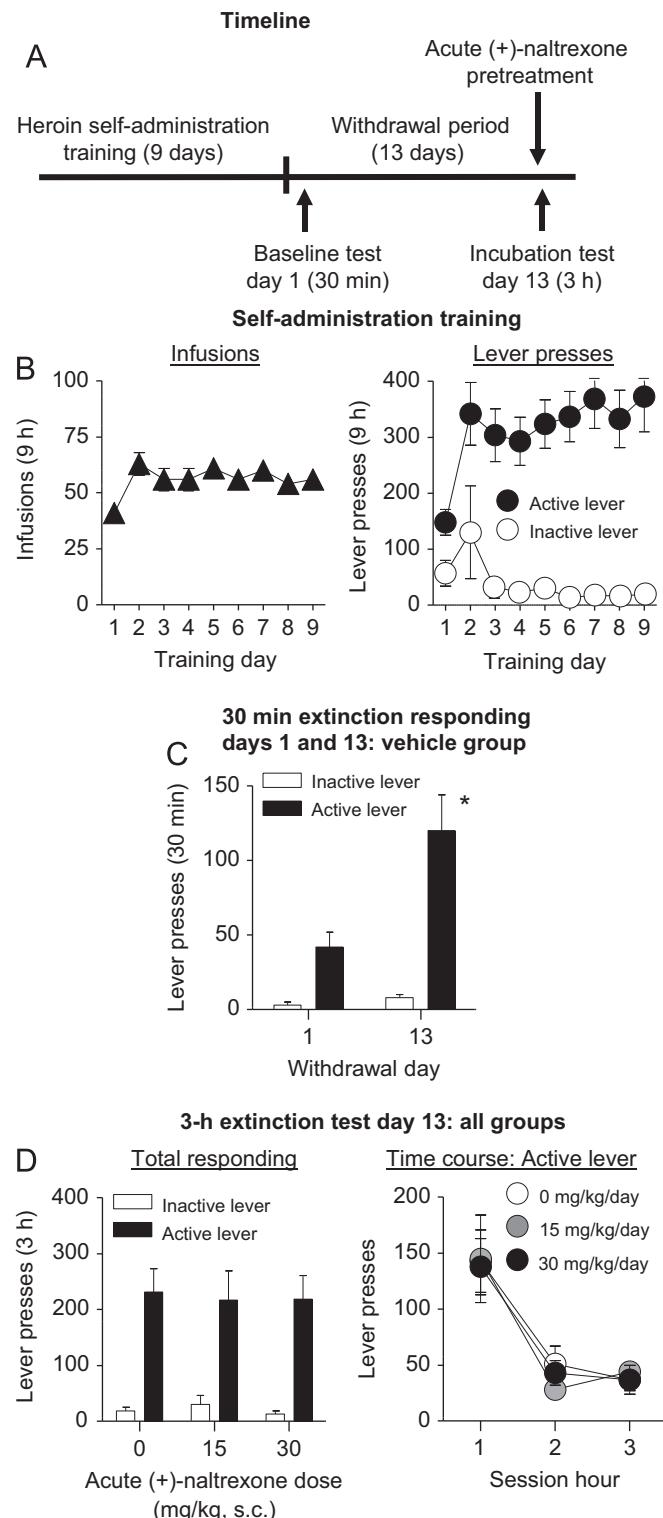


heroin craving, Figure 2C). The statistical analysis, which included the within-subjects factors of withdrawal day and lever, demonstrated a significant interaction of withdrawal day \times lever ($F_{1,27} = 27.6, p < .001$). Chronic delivery of (+)-naltrexone decreased incubated cue-induced heroin seeking on withdrawal day 13 (Figure 2D). The analysis of total active and inactive lever presses, which included the between-subjects factor of (+)-naltrexone dose and the within-subjects factor of lever, demonstrated a significant interaction of (+)-naltrexone dose \times lever ($F_{3,53} = 4.9, p = .005$). Subsequent one-way analysis of covariance of active lever presses on withdrawal day 13, using day 1 active lever presses (30 minutes) as a covariate, demonstrated a main effect of (+)-naltrexone dose ($F_{3,52} = 5.02, p = .004$); post hoc tests (false discovery rate corrected) demonstrated that the 7.5, 15, and 30 mg/kg/day (+)-naltrexone groups were significantly different from the vehicle group ($p < .05$). Analysis of time course of active lever presses, which included the between-subjects factor of (+)-naltrexone dose and session time (hour 1, 2, 3), demonstrated significant effects of (+)-naltrexone dose ($F_{3,53} = 5.1, p = .004$) and session time ($F_{2,106} = 27.9, p < .001$) but no interaction between the two factors (Figure 2D).

Experiment 2: Effect of Acute Injection of (+)-Naltrexone on Incubated Cue-Induced Heroin Seeking. *Self-Administration Training:* The rats increased their number of heroin infusions over days ($F_{8,216} = 6.1, p = .001$; Figure 3B). Additionally, active but not inactive lever presses increased over days (lever \times day [$F_{8,216} = 3.9, p < 0.001$]; Figure 3B). *Extinction Tests:* The rats in the acute vehicle group demonstrated time-dependent increases in cue-induced heroin seeking in these tests (withdrawal day \times lever [$F_{1,9} = 13.1, p = .006$]; Figure 3C). Acute subcutaneous injections of (+)-naltrexone before the extinction test on withdrawal day 13 had no effect on cue-induced heroin seeking on that day (a significant effect of lever [$F_{1,27} = 72.8, p < .001$] but no effects of (+)-naltrexone dose or (+)-naltrexone dose \times lever [p values $> .05$]; Figure 3D). Analysis of the time course of active lever presses demonstrated a significant effect of session time ($F_{2,54} = 37.2, p < .001$) but no effects of (+)-naltrexone dose or interaction between the two factors (p values $> .05$; Figure 3D). *Food-Reinforced Responding:* Seven days after withdrawal day 13 testing, 12 rats received surgically implanted minipumps containing sterile water (vehicle, $n = 6$) or (+)-naltrexone (30 mg/kg/day, $n = 6$) to determine the effect of (+)-naltrexone on operant responding for food pellets (fixed ratio-1, 20-sec time-out

Figure 2. Chronic delivery of (+)-naltrexone during the withdrawal phase decreased incubated cue-induced heroin seeking. (A) Timeline of the experiment. (B) Heroin self-administration training. Data are mean \pm SEM number of heroin infusions (.1 mg/kg/infusion) and active and inactive lever presses during the nine 9-hour daily self-administration sessions (total $n = 57$). During training, active lever presses were reinforced on an fixed ratio-1 20-second time-out reinforcement schedule and heroin infusions were paired with a 5-second tone-light cue. (C) Extinction test withdrawal days 1 and 13 (vehicle group). Data are mean \pm SEM of responses on the previously active lever and on the inactive lever in the vehicle-treated rats ($n = 28$) during the 30-minute extinction test on withdrawal day 1 and the first 30 minutes of the 3-hour extinction test on withdrawal day 13. *Different from withdrawal day 1, $p < .05$. (D) Extinction test withdrawal day 13. Data are mean \pm SEM of responses on the active and inactive levers during the 3-hour extinction test. During testing, lever presses led to contingent presentations of the tone-light cue previously paired with heroin infusions during training but not heroin. The rats were tested on withdrawal day 13 with Alzet osmotic minipumps that were implanted subcutaneous on withdrawal day 1 with either vehicle (sterile water, $n = 28$) or (+)-naltrexone: 7.5, 15, or 30 mg/kg/day ($n = 9$ –10 per dose). *Different from vehicle, $p < .05$.

reinforcement schedule). (+)-Naltrexone had no effect on food-reinforced responding (Figure S1 in Supplement 1). The analysis of total pellets earned, which included (+)-naltrexone dose as the between-subjects factor and training day as the within-subjects factor, did not show significant effects of (+)-naltrexone dose or interaction between the two factors (p values $> .05$). The analysis of total active and inactive lever presses demonstrated a



significant effect of lever ($F_{1,10} = 37.5$, $p < .001$) but no effects of (+)-naltrexone dose or interaction between the two factors (p values $> .05$) (Figure S1 in Supplement 1).

Experiment 3: Effect of Chronic Delivery or Acute Injections of (+)-Naltrexone on Heroin Self-Administration. *Experiment 3a: Chronic Minipump Delivery:* Chronic delivery of (+)-naltrexone during the training phase had no effect on acquisition and maintenance of heroin self-administration. The analysis of the number of heroin infusions, which included the between-subjects factor of (+)-naltrexone dose and the within-subjects factor of training day, demonstrated a significant effect of day ($F_{8,200} = 9.1$, $p < .001$) but no significant effects of (+)-naltrexone dose or interaction between the two factors (p values $> .05$). The analysis of the number of active and inactive lever presses demonstrated significant effects of lever ($F_{1,25} = 68.1$, $p < .001$) and lever \times training day ($F_{8,200} = 10.5$, $p < .001$) but no significant effects of (+)-naltrexone dose or interactions between this factor and lever or training day (p values $> .05$). *Experiment 3b: Acute Injections:* We trained rats ($n = 11$) to self-administer heroin for 8 days (3×3 -hour session days separated by 1 hour) and then tested them repeatedly (counterbalanced order) for the effects of acute systemic injections of (+)-naltrexone and (+)-naloxone before the first 3-hour session of the 9-hour daily sessions on heroin self-administration. Acute IP or SC systemic injections of (+)-naltrexone had no effect on heroin self-administration (Figure 4B). Data (infusions/3 hours) were analyzed using the within-subjects factors of (+)-naltrexone dose and session time (first, second, and third 3-hour session). For SC injections, there were no effects of (+)-naltrexone dose, session time, or interaction between the two factors (p values $> .05$). For IP injections of (+)-naltrexone, there was a significant (+)-naltrexone dose \times session time interaction ($F_{4,40} = 2.9$, $p = .032$) but no effects of (+)-naltrexone dose or session time (p values $> .05$); this interaction is due to the somewhat higher and lower heroin intake in the 30 mg/kg dose condition in the first and third sessions, respectively. There were no statistically significant effects of acute IP or SC (+)-naloxone injections on heroin self-administration (p values $> .05$; Figure S2 in Supplement 1).

Experiment 4: Effect of Chronic Delivery of (+)-Naltrexone During the Withdrawal Phase on Incubated Cue-Induced Methamphetamine Seeking. *Self-Administration Training:* The rats increased their number of methamphetamine infusions over days ($F_{8,192} = 48.3$, $p < 0.001$; Figure 5B). Analysis of active and inactive lever presses demonstrated significant effects of lever ($F_{1,192} = 4.4$, $p = .047$) and training day ($F_{8,192} = 2.15$, $p = .033$; Figure 5B) but not lever \times training day ($p > .1$). The lack of significant interaction is likely due to the fact that 5 of the 26 rats developed stereotypic responding on the inactive lever on some of the training sessions, resulting in a high rate of responding

Figure 3. Acute injection of (+)-naltrexone had no effect on incubated cue-induced heroin seeking on withdrawal day 13. **(A)** Timeline of the experiment. **(B)** Heroin self-administration training. Data are mean \pm SEM number of heroin infusions (.1 mg/kg/infusion) and active and inactive lever presses during the nine 9-hour daily self-administration sessions (total $n = 30$). **(C)** Extinction test withdrawal days 1 and 13 (vehicle group). Data are mean \pm SEM of responses on the active and inactive levers in the vehicle-treated group ($n = 10$) during the 30-minute extinction test on withdrawal day 1 and the first 30 minutes of the 3-hour extinction test on withdrawal day 13. *Different from withdrawal day 1, $p < .05$. **(D)** Extinction test withdrawal day 13. Data are mean \pm SEM responses on the active and inactive levers during the 3-hour extinction test. On withdrawal day 13, rats were injected acutely with either vehicle (sterile water, $n = 10$) or (+)-naltrexone (15 or 30 mg/kg, subcutaneous [s.c.], $n = 10$ per dose) 10 to 15 minutes before the extinction test.

on this lever (over 300 per day). This stereotyped responding occurred on 13 daily sessions across these 5 rats; these outlier values (>3 standard deviations from the sample mean) were included in the statistical analysis but were excluded from the data present in Figure 5B, which includes 221 individual data points out of the 234 possible data points from the 26 rats across the 9 training days. *Extinction Tests:* The rats in the vehicle group demonstrated time-dependent increases in cue-induced methamphetamine seeking in the extinction tests (incubation

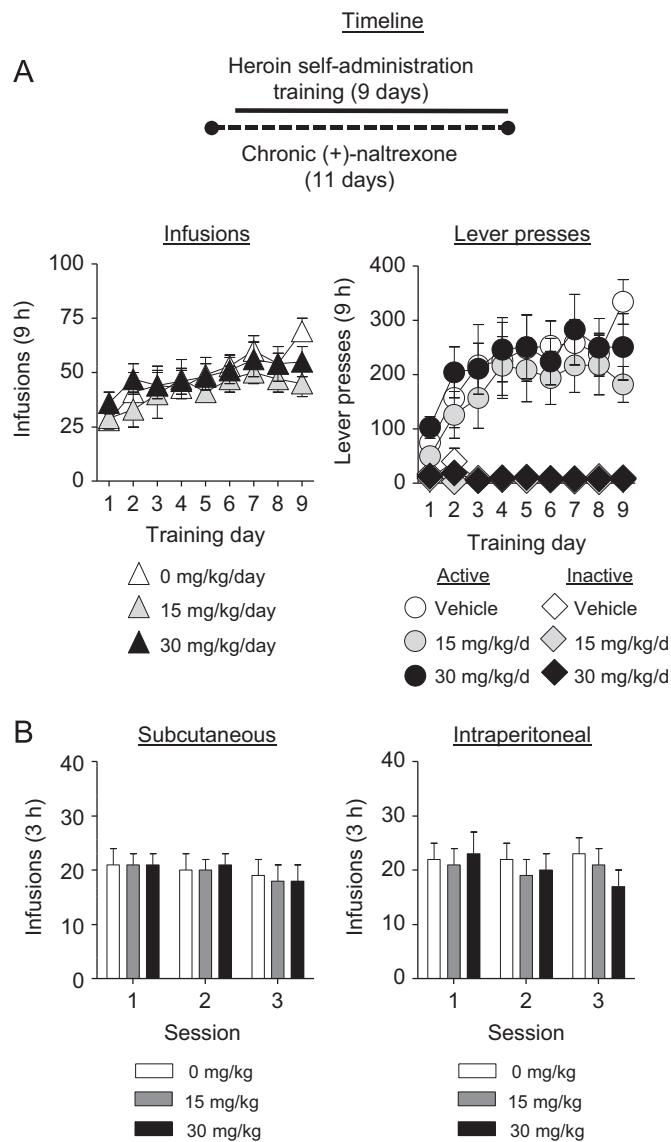


Figure 4. Chronic (minipump) delivery or acute systemic injections of (+)-naltrexone had no effect on ongoing heroin self-administration. **(A)** Chronic delivery. Data are total mean \pm SEM of heroin infusions (.1 mg/kg/infusion) and active and inactive lever presses during heroin self-administration training (three 3-hour sessions separated by 1 hour). Two days before training, rats were implanted with subcutaneous Alzet osmotic minipumps that delivered either vehicle (sterile water, $n = 10$) or (+)-naltrexone (15 or 30 mg/kg/day, $n = 8-10$ per dose) during the training period. **(B)** Acute injections. Data are mean \pm SEM of heroin infusions (.1 mg/kg/infusion) during the first, second, and third daily sessions. Systemic injections (subcutaneous or intraperitoneal) of (+)-naltrexone (0, 15, and 30 mg/kg; $n = 10-11$) were given 10 to 15 minutes before the start of the first 3-hour daily session.

of methamphetamine craving, Figure 5C). The analysis demonstrated a significant interaction of withdrawal day \times lever ($F_{1,12} = 39.8$, $p < .001$).

Chronic delivery of (+)-naltrexone had no effect on incubated cue-induced methamphetamine seeking on withdrawal day 13 (Figure 5D). The analysis demonstrated a significant effect of lever ($F_{1,24} = 101.2$, $p < .001$) but no effect of (+)-naltrexone dose or (+)-naltrexone dose \times lever (p values $> .1$). Analysis of the time course of active lever presses demonstrated a significant effect of session time ($F_{2,48} = 40.6$, $p < .001$) but no significant effect of (+)-naltrexone dose or interaction between the two factors (p values $> .05$; Figure 5D).

Discussion

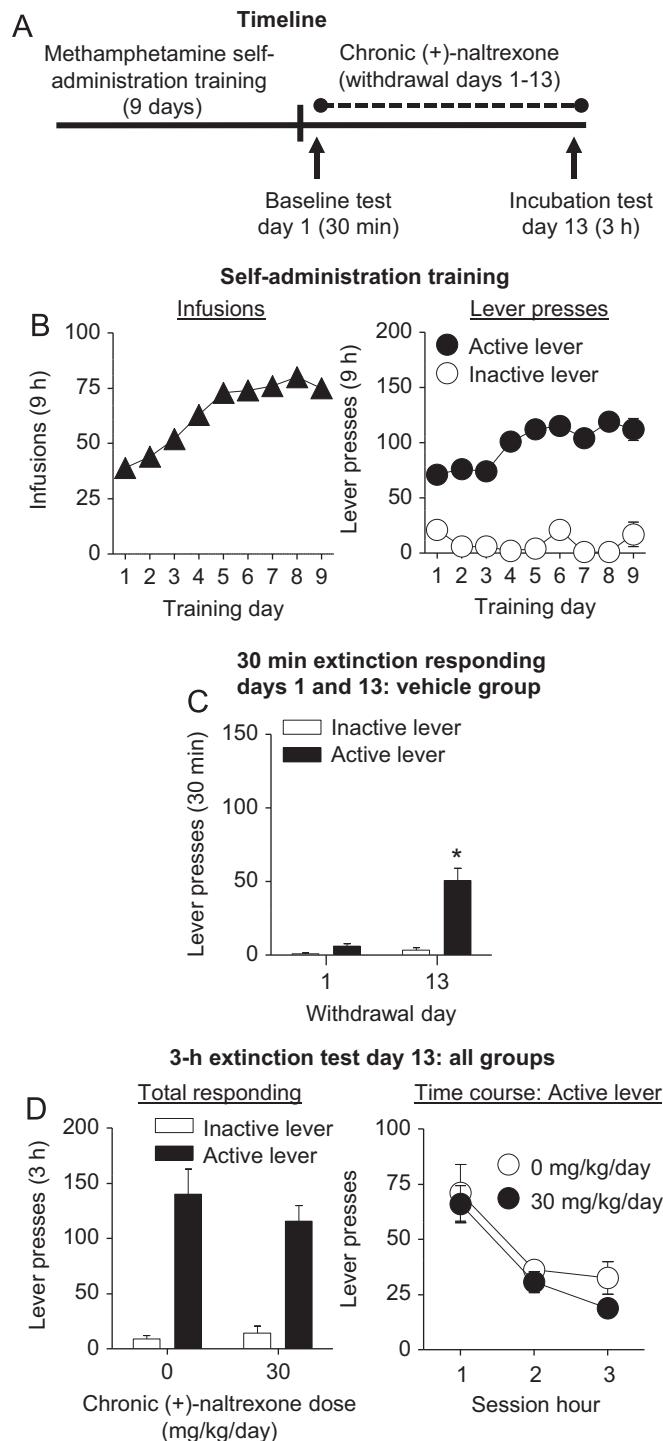
We used (+)-naltrexone to study the role of TLR4 in incubation of heroin craving, operationally defined as time-dependent increases in cue-induced heroin seeking in extinction tests after withdrawal from self-administered heroin. We first performed in vitro binding experiments to determine the possibility of non-TLR4 effects of (+)-naltrexone and found that (+)-naltrexone had minimal activity at a number of biologically relevant targets, as well as low binding affinity to MOR. In the in vivo experiments, we found that chronic delivery of (+)-naltrexone during the withdrawal phase attenuated incubated cue-induced heroin seeking in extinction tests performed on withdrawal day 13. This effect was not statistically dose-dependent due to large individual variability in nonreinforced lever presses during testing, a common observation in extinction reinstatement (38,39) and incubation (40) studies. In contrast, acute (+)-naltrexone injections immediately before withdrawal day 13 extinction tests were ineffective. Chronic delivery of (+)-naltrexone or acute pretest injections of (+)-naltrexone (or [+]-naloxone) had no effect on ongoing extended access heroin self-administration; additionally, chronic delivery of (+)-naltrexone had no effect on high-rate food-reinforced responding. Finally, we assessed the generality of our findings to incubation of psychostimulant craving and found that chronic delivery of (+)-naltrexone during the withdrawal phase had no effect on incubated cue-induced methamphetamine seeking. Our data indicate a role of TLR4 in the development of incubation of heroin, but not methamphetamine, craving. The present findings provide additional evidence for the important role of nonneuronal glia-related mechanisms in the behavioral effects of opioid drugs (17,18,30).

Methodological Considerations

Several methodological issues should be considered in the interpretation of our data. One issue is the behavioral specificity of chronic (+)-naltrexone's effect for incubated cue-induced heroin seeking. Decreased active lever responding after chronic delivery of (+)-naltrexone may be caused by motor deficits or other nonspecific performance deficits. However, this is unlikely because chronic delivery of (+)-naltrexone had minimal effects on heroin self-administration, lever responding for palatable food, or cue-induced methamphetamine seeking. It is also unlikely that a short extinction session on withdrawal day 1 confounds data interpretation. In the present and previous studies, we observed reliable incubation of craving for both heroin (5,41) and cocaine (42,43) in rats repeatedly tested during early and late withdrawal. Additionally, it is unlikely that a short extinction session on withdrawal day 1 promotes long-term extinction learning and consequently decreased cue responding

on day 13, because it takes several weeks to extinguish heroin self-administration behavior in rats (44,45).

Another issue is the pharmacologic specificity of (+)-naltrexone to TLR4. We found that *in vitro* (+)-naltrexone had no significant activity at a number of potential non-TLR4 sites, including MOR. A MOR-mediated effect is also unlikely, because we recently found that acute injections of the preferential MOR antagonist, (–)-naloxone (1 mg/kg), decreased incubated cue-induced heroin seeking on withdrawal day 15 (41). In contrast, acute injections of higher (+)-naltrexone doses (15–30 mg/kg)



before withdrawal day 13 testing were ineffective. A MOR-mediated effect of (+)-naltrexone, or potentially its metabolites, is also unlikely, because with this scenario, (+)-naltrexone would have also decreased heroin self-administration, a MOR-dependent behavior (46,47). Finally, other non-TLR4 targets of (+)-naloxone (and by extension [+]-naltrexone) were recently reported, including filamin A (48) and nicotinamide adenine dinucleotide phosphate oxidase (49). However, it is unlikely that these targets mediated (+)-naltrexone's effect on incubation of heroin craving, because the effects of (+)-naloxone or (+)-naltrexone on behavioral effects of opioid drugs (e.g., tolerance, dependence, CPP) are not observed in the TLR4 knockout mice (16).

Role of TLR4 in Opioid Reward

Hutchinson *et al.* (30) recently reported that acute injections of the TLR4 antagonist (+)-naloxone decreased morphine-induced CPP and remifentanil self-administration in rats. They also reported that TLR4 or MyD88 (a TLR4 accessory signaling protein) knockout mice do not develop CPP for the opioid agonist oxycodone. In contrast, we found that chronic delivery of (+)-naltrexone or acute injections of (+)-naltrexone or (+)-naloxone had no effect on heroin self-administration. What might account for these different results beyond differences in the opioid agonist (remifentanil or oxycodone versus heroin)?

It is perhaps not surprising that TLR4 antagonism prevented CPP for response-independent morphine injections but had no effect on response-contingent operant heroin self-administration. While both CPP and drug self-administration procedures have been used to measure opioid reward (50–52), previous studies demonstrated dissociable neurobiological mechanisms for opioid CPP versus self-administration. For example, mesoaccumbens dopamine plays a critical role in morphine and heroin CPP (53,54) but not heroin self-administration (46,55,56).

It is somewhat more difficult to reconcile our negative findings for chronic (+)-naltrexone or acute injections of (+)-naltrexone or (+)-naloxone effects on heroin self-administration with those reported by Hutchinson *et al.* (30) who found that acute (+)-naloxone decreased remifentanil self-administration. These differences might be due to two main factors. The first is that Hutchinson *et al.* (30) trained rats for cocaine self-administration and then assessed the effect of (+)-naloxone on remifentanil self-administration during substitution sessions in which cocaine was intermittently replaced with remifentanil, an opioid agonist with a half-life that is significantly shorter than heroin (1). Another potential factor is that the rats in Hutchinson *et al.* (30) were trained under a limited-access drug self-

Figure 5. Chronic delivery of (+)-naltrexone during the withdrawal phase had no effect on incubated cue-induced methamphetamine seeking. **(A)** Timeline of the experiment. **(B)** Methamphetamine self-administration training. Data are mean \pm SEM number of methamphetamine infusions (.1 mg/kg/infusion) and active and inactive lever presses during the nine 9-hour daily self-administration sessions (total $n = 26$). **(C)** Extinction test withdrawal days 1 and 13 (vehicle group). Data are mean \pm SEM of responses on the active and inactive levers in the vehicle-treated group ($n = 13$) during the 30-minute extinction test on withdrawal day 1 and the first 30 minutes of the 3-hour extinction test on withdrawal day 13. *Different from withdrawal day 1, $p < .05$. **(D)** Extinction test withdrawal day 13. Data are mean \pm SEM responses on the active and inactive levers during the 3-hour extinction test. During testing, lever presses led to contingent presentations of the tone-light cue previously paired with methamphetamine infusions during training but not methamphetamine. The rats were tested on withdrawal day 13 with Alzet osmotic minipumps that were implanted subcutaneous on withdrawal day 1 with either vehicle (sterile water, $n = 13$) or (+)-naltrexone, 30 mg/kg/day ($n = 13$).

administration condition (2 hours/day) for cocaine, while our rats were trained under an extended-access condition (9 hours/day) for heroin. Even within a given drug class, these different access conditions lead to different patterns of drug self-administration (57,58), brain neuroadaptations (59–62), and differential responses to pharmacologic manipulations (63–66).

Mechanisms of TLR4 Role in Incubation of Heroin Craving

Our pharmacologic finding with (+)-naltrexone suggests a role of TLR4 in incubation of heroin craving. As in other systemic pharmacology studies, our positive findings inspire follow-up questions on downstream molecular mechanisms. Below, we briefly speculate on potential mechanisms within a conceptual framework of two distinct molecular mechanisms of incubation of drug craving (13). The first involves the acute expression of incubation of drug craving or the acute incubated response to drug cues after prolonged withdrawal that occurs on a time scale of minutes. The second involves the development of incubation of drug craving or the time-dependent drug-induced neuroadaptations that take weeks to develop after withdrawal but are not directly involved in the acute incubated response to drug cues during testing (13).

Regarding the first mechanism, one possibility is that acute conditioned TLR4 activation by heroin cues in brain areas critical for cue-induced heroin seeking (e.g., nucleus accumbens [45]) directly mediates the incubated response on withdrawal day 13. Since the seminal work of Ader and Cohen (67), many studies have shown that conditioned cues can activate (or inhibit) the immune system (68), including cues associated with opioid-induced immune activation/suppression (69). There is also evidence for modulation of conditioned responses to opioids by central glia immune-related mechanisms (15,17), including TLR4-related mechanisms (30). However, it is unlikely that direct heroin cue-induced TLR4 activation contributes to the acute expression of incubation of heroin craving. This is because acute injections of high (+)-naltrexone doses before the extinction tests on withdrawal day 13 had no effect on incubated cue-induced heroin seeking.

The finding that chronic but not acute (+)-naltrexone delivery decreased incubated cue-induced heroin seeking suggests that TLR4 plays a unique role in the development of incubation of heroin craving. The causes of these TLR4-related neuroadaptations, induced by heroin self-administration and subsequent withdrawal, are unknown. One potential downstream mechanism is TLR4-mediated activation of nuclear factor kappa-B (NFκB) (19), which is activated by opioid agonists (70) and recently implicated in the maintenance of memories for morphine-associated cues (71) and opioid withdrawal symptoms (72), as well as other behavioral effects of drugs (73). We assessed the potential role of this downstream mechanism by determining the effect of chronic (minipump) delivery of the NFκB antagonist sc-514 (74) into the lateral ventricles during the withdrawal period using experimental conditions similar to those used in experiment 1 (see legend of Figure S3 in Supplement 1). We found that this manipulation had no effect on incubated cue-induced heroin seeking on withdrawal day 13 (Figure S3 in Supplement 1). These data may suggest that NFκB is not a downstream mechanism for the TLR4-mediated effect of chronic delivery of (+)-naltrexone on development of incubation of craving. However, an alternative interpretation of these negative data with the NFκB antagonist is that ventricular delivery of sc-514 (a compound that is very difficult to dissolve, even in 50% dimethyl sulfoxide) either did not reach critical brain areas involved in incubation of heroin craving or that the drug

did not remain in solution in the minipump for the duration of the experiment. Thus, whether or not NFκB is a potential downstream mechanism for the putative TLR4-mediated effect of chronic delivery of (+)-naltrexone on incubation of heroin craving is a subject for future research.

Another downstream mechanism of TLR4 activation that may contribute to the development of incubation of heroin craving is activation of tumor necrosis factor-alpha (TNF- α) and subsequent regulation of synaptic strength at glutamate synapses (75). In hippocampal cultured neurons and slices, TNF- α promotes the insertion of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors into plasma membranes (75) and the formation of GluA2-lacking AMPA receptors (76). Time-dependent accumulation of GluA2-lacking AMPA receptors in nucleus accumbens after withdrawal is critical for incubation of cocaine craving (14,77). However, whether this speculative mechanism contributes to incubation of heroin craving is a subject for future research, because it has not been established that withdrawal from heroin self-administration induces the accumulation of GluA2-lacking AMPA receptors in nucleus accumbens or that TNF- α modulates glutamatergic synapse strength in this brain area.

Concluding Remarks

Our results suggest a novel role of TLR4 in incubation of heroin, but not methamphetamine, craving. This selective role of TLR4 in incubation of heroin craving is in agreement with results from our recent studies suggesting different mechanisms for incubation of opioid versus psychostimulant craving (5,13,41). These previous and present results also extend previous reports demonstrating that mechanisms of opioid- and psychostimulant-taking behaviors are often dissociable (47,78,79). Finally, a question for future research will be to identify brain sites and downstream cellular mechanisms that contribute to incubation of heroin craving whose function is altered by chronic delivery of (+)-naltrexone during the withdrawal phase.

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Supplementary material cited in this article is available online.

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