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Main text

Sex differences in the effect of chronic delivery of the buprenorphine analog BU08028 on heroin relapse and choice in a rat model of opioid maintenance

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Supplemental Online Material
Figure S1
Table S1 (statistical reporting)

Abstract

Background and Purpose: Maintenance treatment with opioid agonists (buprenorphine, methadone) decreases opioid use and relapse. We recently modeled maintenance treatment in rats and found that chronic delivery of buprenorphine or the mu opioid receptor (MOR) partial agonist TRV130 decreases relapse to oxycodone seeking and taking. Here, we tested the effect of the buprenorphine analog BU08028 on different heroin relapse-related measures and heroin vs. food choice.

Experimental Approach: For relapse assessment, we trained male and female rats to self-administer heroin (6-h/d, 14-d) in context A and then implanted osmotic minipumps containing BU08028 (0, 0.03, or 0.1 mg/kg/d). We then tested the effect of chronic BU08028 delivery on (1) incubation of heroin seeking in a non-drug context B, (2) extinction responding reinforced by heroin-associated discrete cues in context B, (3) reinstatement of heroin seeking induced by reexposure to context A, and (4) reacquisition of heroin self-administration in context A. For choice assessment, we tested the effect of chronic BU08028 delivery on heroin vs. food choice.

Results: Chronic BU08028 delivery decreased incubation of heroin seeking. Unexpectedly, BU08028 *increased* reacquisition of heroin self-administration selectively in females. Chronic BU08028 had minimal effects on context-induced reinstatement and heroin vs. food choice in both sexes. Finally, exploratory post-hoc analyses suggest that BU08028 decreased extinction responding selectively in males.

Conclusions and Implications: Chronic BU08028 delivery had both beneficial and detrimental sex-dependent effects on different triggers of heroin relapse and minimal effects on heroin choice in both sexes. Results suggest that BU08028 will not be an effective opioid maintenance treatment in humans.

Key words: opioid maintenance, incubation of craving, extinction, context-induced reinstatement, heroin self-administration, heroin choice, reacquisition

1. Introduction

Over the past decade, there has been a large increase in opioid-related deaths (Hedegaard et al., 2017; Rudd et al., 2016). This 'opioid crisis' persists despite the availability of effective opioid agonist maintenance treatments (methadone and buprenorphine) (Epstein et al., 2018; Skolnick, 2018). Therefore, there is an unmet need to advance novel medications for the treatment of opioid use and relapse. In this regard, one class of drugs that has recently received considerable attention are those that act as partial agonists at both the mu opioid receptor (MOR) and the nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor (Ding & Ko, 2021; Zaveri, 2016).

One such compound is the buprenorphine analog BU08028 (Ding et al., 2016). Like buprenorphine, BU08028 has high affinity to MOR and it acts as a partial agonist at this receptor; however, unlike buprenorphine which has low affinity and efficacy at the NOP receptor, BU08028 has high affinity to NOP and acts as a partial agonist at this receptor (Ding et al., 2016). In rodent models, acute injections of BU08028 produce antinociception that is longer-lasting than that of buprenorphine (Khroyan et al., 2011; Sukhtankar et al., 2013). In non-human primates, acute injections of BU08028 also produce antinociception. However, unlike classical full MOR agonists (e.g., heroin, oxycodone, fentanyl) or partial agonists (e.g., buprenorphine), BU08028 is not self-administered by rhesus monkeys (Ding et al., 2016). A more recent study also showed that buprenorphine and BU08028 both acutely and chronically decrease alcohol drinking in rhesus monkeys, with BU08028 showing higher potency than buprenorphine (Flynn et al., 2019). Together, these findings suggest that BU08028 can mimic the therapeutic effects of buprenorphine while carrying lower risk for abuse liability.

We recently combined a rat model of opioid maintenance (Shaham et al., 1996; Sorge et al., 2005) with a modified version of our ABA renewal model of context-induced reinstatement (Bossert et al., 2019; Crombag & Shaham, 2002) to determine the effect of chronic delivery (maintenance treatment) of the novel partial MOR agonist TRV130 (DeWire et al., 2013) on relapse in male and female rats trained to self-administer oxycodone (Bossert et al., 2020). We first established the predictive validity of our rat model using chronically delivered buprenorphine via Alzet osmotic minipumps and then compared the efficacy of buprenorphine to that of TRV130 (DeWire et al., 2013). We found that in oxycodone-trained male and female rats, chronic buprenorphine delivery decreased extinction responding, context-induced

reinstatement, and reacquisition of oxycodone self-administration (Bossert et al., 2020). Chronic TRV130 delivery mimicked buprenorphine's effect in all three drug-seeking and drug-taking measures in male rats, but only decreased extinction responding in in female rats.

In the present study, we used our rat model of opioid maintenance to test the efficacy of chronic delivery of BU08028 on extinction responding, context-induced reinstatement, and reacquisition in male and female rats trained to self-administer heroin. We used these three measures because we wanted to directly compare the effects of BU08028 to buprenorphine (Bossert et al., 2020). Additionally, these behavioral measures are commonly employed in animal models of relapse (Fuchs et al., 1998; McNally, 2014; Reiner et al., 2019; Venniro et al., 2016). We also used both male and female rats because we observed sex differences in our relapse measures with TRV130 (Bossert et al., 2020) and there is evidence for sex differences in the behavioral effects of opioids in rats (Craft, 2008). We also added to our experimental procedure an assessment of BU08028's effect on incubation of heroin craving (Shalev et al., 2001; Theberge et al., 2013). Incubation of drug craving refers to time-dependent increases in drug seeking during abstinence (Fredriksson et al., 2021; Grimm et al., 2001). Finally, we assessed BU08028's effect on heroin vs. food choice in a rat model in which the choice for intravenous opioid agonists progressively increases with increasing the drugs' unit dose (Townsend et al., 2019b; Townsend et al., 2021). As discussed elsewhere, drug choice procedures uniquely predict medications' efficacy (or their lack of) in humans who use drugs (Banks & Negus, 2017; Venniro et al., 2020).

2. Methods

2.1. Subjects

For Experiments 1-2 (conducted at IRP/NIDA/NIH, Baltimore, MD), we used male (n=34) and female (n=50) Sprague-Dawley rats (Charles River) weighing 250-350 g (males) or 175-225 g (females) before surgery. We used more females than males because in our experience, female rats' behavior in response to pharmacological manipulations is more variable in the relapse model used in the present study (Bossert et al., 2020). We maintained the rats under a reverse 12:12 h light/dark cycle (lights off at 8:00 A.M) with food and water freely available. We housed two rats/cage prior to surgery and individually after

surgery. We excluded 3 rats due to health problems during the experiment (n=2) or failure to acquire heroin self-administration during training (n=1), and death after the minipump surgery (n=1).

For Experiment 3 (conducted at Virginia Commonwealth University), 3 male and 3 female Sprague-Dawley rats (body weight at the time of arrival: 290-310 g for males, 240-260 g for females, Envigo) completed the experiment (we excluded one female rat due to health problems during the experiment). Following i.v. surgery, we housed the rats individually and maintained them on a 12-h light/dark cycle (lights off at 6:00 PM) with food (Teklad Rat Diet, Envigo) and water freely available in the homecage.

We performed Experiments 1-3 in accordance with the NIH Guide for the Care and Use of Laboratory Animals (8th edition), under protocols approved by the Animal Care and Use Committee of the NIDA IRP or Virginia Commonwealth University.

2.2. Drugs

For Experiments 1 & 2, we received heroin hydrochloride (HCl) from the NIDA pharmacy and dissolved it in sterile saline. We chose a unit dose of 0.1 and 0.05 mg/kg/infusion for self-administration training (Exp. 1 & 2) based on our previous work (Bossert et al., 2004; Bossert et al., 2016). BU08028 (2S)-2-[(5R,6R,7R,14S)-N-cyclopropylmethyl-4,5-epoxy-6,14-ethano-3-hydroxy-6-methoxymorphinan-7-yl]-3,3-dimethylpentan-2-ol hydrochloride) was synthesized in six steps from thebaine, using a previously reported process for a large-scale production of buprenorphine applying tert-pentylmagnesium chloride as a Grignard reagent (Allen et al., 2010; Bentley & Hardy, 1967; Cami-Kobeci et al., 2011; Greedy et al., 2013). BU08028 free base was converted to a hydrochloride salt and the formulation was confirmed by elemental analysis. We dissolved BU08028 in a solution of 30% dimethyl sulfoxide (DMSO), 15% ethanol (EtOH), and sterile water at concentrations that yielded chronic delivery of doses of 0.03, 0.05, or 0.1 mg/kg/day. The vehicle chosen for BU08028 was based on our buprenorphine vehicle (Bossert et al., 2020). BU08028 doses are based on pilot minipump implantation studies and on the time course of recovery of food-reinforced responding after BU08028 implantation (Exp. 1). For Experiment 3, we obtained heroin HCl from NIDA Drug Supply Program (Bethesda, MD) and dissolved it in sterile saline to yield doses of 0.0032, 0.01, 0.032, & 0.1 mg/kg/infusion. The investigators were not blind to the minipump dose conditions.

2.3. Surgery

Intravenous surgery: For Experiments 1-2, we anesthetized the rats with isoflurane (5% induction; 2-3% maintenance, Covetrus). We attached silastic catheters to a modified 22-gauge cannula cemented to polypropylene mesh (Amazon or Industrial Netting), inserted the catheter into the jugular vein, and fixed the mesh to the mid-scapular region of the rat (Caprioli et al., 2015; Fredriksson et al., 2020). We injected the rats with ketoprofen (2.5 mg/kg, s.c., Covetrus) during surgery and on the following day to relieve pain and decrease inflammation. Rats recovered for 6-8 days before heroin self-administration training. During all experimental phases, we flushed the catheters daily with gentamicin in sterile saline (4.25 mg/mL, Fresenius Kabi).

For Experiment 3, we anesthetized rats with 2-3% isoflurane in oxygen and implanted them with polyurethane catheters into the right jugular vein using methods previously described (Townsend et al., 2021). We injected the rats with ketoprofen (5 mg/kg, s.c.) immediately after the surgery and then 24-h later. We allowed the rats to recover for 5 days prior to the start of the experiment. After each behavioral session, we flushed catheters with 0.1 mL gentamicin (4 mg/ml; Aspen Veterinary Resources), followed by 0.1 mL of heparinized saline (10 U/mL).

Minipump surgery: Chronic delivery of BU08028 was achieved by implanting osmotic minipumps subcutaneously (Alzet model 2ML2, 5 µl/h for 14-16 days, Durect Corporation) (Bossert et al., 2020). For Experiments 1-2, we anesthetized the rats with isoflurane as described above and made a small incision on the right side of the intravenous backmount. We used a hemostat to spread apart the subcutaneous connective tissue to make a small pocket for the pump. We placed the osmotic pumps into the pocket with the flow moderator directed away from the incision. We then closed the incisions with sterile surgical suture or wound clips. For Experiment 3, we aseptically implanted an osmotic pump (Alzet Model 2001) in the mid-scapular subcutaneous region under isoflurane anesthesia on a Friday afternoon after the heroin vs. food choice session.

2.4. Apparatus

For Experiments 1-2, we trained and tested the rats in standard Med Associates self-administration chambers. Each chamber had two levers located 7.5-8 cm above the grid floor on opposing walls. Lever presses on the active, retractable lever activated the infusion pump, whereas lever presses on the inactive, non-retractable lever had no programmed consequences. As in our previous studies (Bossert et

al., 2016; Bossert et al., 2019; Bossert et al., 2020), the active lever was inserted into the chamber at the start of the self-administration and test sessions and retracted at the end of these sessions, while the inactive lever was constantly present in the chamber. For Exp. 2, the two contexts differed in their auditory, visual, and tactile cues, as in our previous studies (Adhikary et al., 2017; Bossert et al., 2019). We refer to the contexts as A and B, where A is the context for self-administration training and reacquisition, and B is the context for extinction. We counterbalanced the physical environments of contexts A and B.

For Experiment 3, we used modular operant chambers located in sound-attenuating cubicles (Med Associates) equipped with two retractable levers, a set of three LED lights (red, yellow, green) mounted above each lever, and a retractable "dipper" cup (0.1 mL) located between the levers for presenting diluted Ensure® (32% v/v vanilla flavor Ensure® in tap water; Abbott Laboratories). Activation of a syringe pump delivered heroin solutions intravenously as previously described (Townsend et al., 2021).

2.5. Heroin self-administration (Exp. 1-2)

Training in context A (14 days): We trained the rats to self-administer heroin HCl in context A for 6 h/day (six 1-h sessions separated by 10 min) for 14 days. Each session began with the illumination of a houselight that remained on for the entire session; the active lever was inserted into the chamber 10 s after the houselight was illuminated. During training, the rats earned heroin infusions by pressing on the active lever; infusions were paired with a compound tone–light cue for 3.5 s under a fixed-interval 20s (FI20) reinforcement schedule in which the first response after 20s has elapsed results in a heroin infusion followed by a 20s timeout. Heroin was infused at a volume of 100 µl over 3.5 s at a dose of 0.10 mg/kg/infusion (the first 7 sessions) and then 0.05 mg/kg/infusion (the last 7 sessions). Responses on the active lever during the timeout period were recorded but did not result in heroin infusions. Presses on the inactive lever were recorded but had no programmed consequences. At the end of each session, the houselight was turned off and the active lever was retracted. If we suspected catheter failure during training, we tested patency with Diprivan (propofol, NIDA pharmacy, 10 mg/mL, 0.1-0.2 mL injection volume, i.v.). If the catheter was not patent, we catheterized the left jugular vein. We implanted the minipumps 7 days (Exp. 1) or 2 days (Exp. 2) after the last heroin self-administration training session (see below for details).

2.6. Specific experiments

The number of subjects in Experiments 1-3 are based on previous studies in which we obtained reliable effects of minipump-implanted drugs on the different relapse- and choice-related measures used in Exp. 1-3 (Bossert et al., 2020; Townsend et al., 2021) and many other studies in which we and others determined the effects of other pharmacological and brain manipulations on incubation of drug craving, extinction responding, context-induced reinstatement, and choice (Bossert et al., 2013; Pickens et al., 2011; Townsend et al., 2019a; Townsend et al., 2019b; Venniro et al., 2016). Because of this large body of historical data, we did not perform a formal power analysis to determine the sample size in the study.

Experiment 1: Effect of chronic BU08028 on food self-administration

The timeline for Exp. 1 is shown in Figure 1A. The goal of Experiment 1 was to rule out sedative or other non-specific effects of BU08028 as explanations for the relapse-related effects to be determined in Exp. 2. For this purpose, we tested the effect of 0.05 and 0.1 mg/kg/d BU08028 on high-rate food-maintained operant responding in heroin-experienced male and female rats.

The experiment consisted of four phases: heroin self-administration (14 days, 6 h/d as described above), food self-administration training (6 days, 1 h/d), minipump-implantation surgery, and food self-administration (7 days, 1 h/d). Heroin self-administration training is described above. We began food self-administration 24 h after the last day of heroin self-administration. Each session began with the illumination of a houselight that remained on for the entire session; the active lever was inserted into the chamber 10 s after the houselight was illuminated (Note: the same active lever was used for both heroin and food training). On the first day, we gave the rats a 1-h magazine-training session during which 1 pellet was delivered noncontingently every 2 min; during this session, the active lever was not inserted into the chamber. During the food self-administration sessions, lever presses under the FI20 reinforcement schedule led to the delivery of one 45-mg pellet of palatable food (TestDiet, Cat # 1811155, 12.7% fat, 66.7% carbohydrate, and 20.6% protein) (Calu et al., 2014); pellet deliveries were paired with a 20-s white-light cue. At the end of the session, the houselight was turned off and the active lever was retracted. We gave the rats 6 consecutive days of food self-administration training prior to the minipump-implantation surgery.

We then implanted the rats (8 males, 12 females) with minipumps containing vehicle (30% DMSO, 15% EtOH, and sterile water), or 0.05 or 0.1 mg/kg/d BU08028 (n=6-7 per group; 2-3 males and 4 females per group) and ran the rats for 7 consecutive days thereafter. We matched the rats in the different dose groups for total heroin infusions during heroin self-administration training and for total pellets and active lever presses during food self-administration training.

Experiment 2: Effect of chronic BU08028 on incubation of heroin seeking, extinction responding, context-induced reinstatement, and reacquisition

The timeline for Exp. 2 is shown in Figures 2A & 3A. The goal of experiment 2 was to test the effect of chronic delivery of BU08028 on our relapse-related measures. Prior to the relapse tests, we trained the rats to self-administer heroin in context A for 6 h per day for 14 days as described above. Next, after a short 30-min extinction session on abstinence day 1 (see below), we implanted the rats on abstinence day 2 with minipumps containing vehicle (12 females, 9 males), or 0.03 mg/kg/d (11 females, 8 males), or 0.1 mg/kg/d (14 females, 8 males) BU08028. During recovery from surgery, we observed signs of emesis and pica (side effects of high doses of MOR agonists) (Aung et al., 2004) with the higher BU08028 dose. Thus, we kept all of the rats (vehicle and both doses of BU08028 rats) in the operant chambers for 48 h to prevent them from swallowing the homecage bedding. [Note: in previous studies, we found reliable incubation of drug seeking after forced abstinence in either the homecage or the self-administration chambers with the active lever retracted (Grimm et al., 2001; Lu et al., 2004)]. We then moved the rats to their homecage for 4 more days before resuming testing 7 days after minipump implantation. We chose 0.03 mg/kg/d as the lower dose in Exp. 2 (vs. 0.05 mg/kg/d in Exp.1) because 0.05 and 0.1 mg/kg/d dose produced similar behavioral effects on food self-administration in Exp. 1. We matched the rats in the different dose groups for total heroin infusions during heroin self-administration training and for extinction responding in context B on abstinence day 1.

Incubation of heroin seeking in context B (abstinence days 1 and 8)

We tested rats in a brief (30 min) extinction session in context B one day after the last day of heroin self-administration training (abstinence day 1). During the test session, responses on the previously active lever led to presentations of the tone-light cue but not heroin infusions. We used the first 30 min of the 6-h extinction session on abstinence day 8 to evaluate whether chronic BU08028 delivery would

decrease incubation of heroin seeking. In previous studies, we observed reliable incubation of heroin seeking in context A and incubation of methamphetamine seeking in context B after one week of abstinence (Adhikary et al., 2017; Shalev et al., 2001).

Extinction responding in context B (abstinence days 8-14):

We ran the rats under extinction conditions in context B for 6 h per day (six 1-h sessions separated by 10 min) for 7 days. During this phase, responses on the previously active lever led to presentation of the discrete tone-light cue but not heroin infusions.

Context-induced reinstatement in contexts A and B (abstinence days 15-16): We tested the rats under extinction conditions (see above) for 6 h per day for 2 days in context A and context B in a counterbalanced order.

Reacquisition of heroin self-administration in context A (abstinence day 17): We tested reacquisition of heroin self-administration during one 6-h session in context A. During testing, lever presses were reinforced by heroin (0.05 mg/kg/infusion, FI20 reinforcement schedule) and the discrete tone-light cue. After the 6-h session, we tested catheter patency with propofol (NIDA pharmacy, 10 mg/mL, 0.1-0.2 mL injection volume, i.v.). We excluded 4 rats from the reacquisition test because they did not demonstrate an immediate anesthetic response after propofol injections.

Experiment 3: Effect of chronic BU08028 on heroin choice

The timeline for Exp. 3 is shown in Figure 4A. We trained the rats in a heroin vs. food choice procedure as described previously (Townsend et al., 2021). Briefly, we first trained the rats to respond on the right lever for heroin (0.032 mg/kg/infusion) beginning under an FR1 reinforcement schedule and progressing to an FR5 reinforcement schedule (every 5th lever press was reinforced and the next available heroin infusion could be earned 20 s after the last heroin infusion). Illumination of a green stimulus light signaled heroin availability. Next, we trained the rats to respond on the left lever for a 5-s presentation of 32% Ensure® beginning under an FR1 reinforcement schedule and progressing to an FR5 reinforcement schedule. Illumination of a red stimulus light signaled Ensure® availability. Once the rats responded for heroin and 32% Ensure® in isolation, we made both reinforcers available under a concurrent FR5 reinforcement schedule.

The behavioral session consisted of five 10-min response components, each preceded by a 4-min "sample" component. Each sample component started with either no infusion (first component only) or a non-contingent infusion of the unit heroin dose available during the subsequent response component, followed by a 2-min timeout, and subsequently a 5-s presentation of liquid food, followed by a 2-min timeout. The response component began after this second timeout. During each response component, both levers extended, a red stimulus light above the left lever was illuminated to signal liquid food availability, and a green stimulus light above the right lever was illuminated to signal heroin availability. Response requirement (FR5) completion on the left lever resulted in a 5-s presentation of liquid food whereas response requirement (FR5) completion on the right lever resulted in the delivery of the unit heroin dose available for that component. Responding on one lever reset the ratio requirement for the other lever.

We held the Ensure® concentration constant throughout the session, but varied the heroin dose during each of the five successive response components (0, 0.0032, 0.01, 0.32, and 0.1 mg/kg/infusion during components 1–5, respectively) by changing the infusion duration (e.g., for a 315-g rat: 0, 0.5, 1.56, 5, and 15.6 s during components 1–5, respectively). To indicate a new heroin unit dose, the green light above the heroin lever flashed on and off in 3-s cycles (i.e., longer flashes corresponded with larger heroin doses). During each response component, the rats could complete up to 10 total ratio requirements between the food- and heroin-associated levers. Each ratio requirement completion initiated a 20-s timeout, the retraction of both levers, and turning off the red and green stimulus lights. If a rat completed all 10 ratio requirements before 10-min had elapsed, then both levers retracted, and the stimulus lights were turned off for the remainder of that component. We considered choice behavior stable when the smallest heroin dose that maintained at least 80% of completed ratio requirements on the heroin-associated lever was within a 0.5 log unit of the running mean for three consecutive days with no trends. We conducted heroin vs. food choice sessions five days per week from approximately 2:00 PM – 3:10 PM unless otherwise noted.

Once heroin vs. food choice was stable, we determined the effects of 7-day chronic BU08028 treatment on heroin vs. food choice (see figure Fig. 4A for experimental timeline). We counterbalanced the order of vehicle and BU08028 doses (0.032 and 0.1 mg/kg/day) between rats. Following pump

implantation on a Friday after a week of baseline heroin vs food choice, heroin vs food choice resumed on Monday to Friday. After the Friday heroin choice session, the rats were again anesthetized with isoflurane and the pump was removed. Once heroin vs. food choice returned to pre-test levels after at least one week of baseline heroin choice, another osmotic pump was aseptically implanted. Counterbalanced treatment conditions were generally, but not always, separated by one week of baseline heroin vs. food choice. We based this experimental design on our published methods for evaluating candidate medications on opioid vs. food choice in rats (Townsend et al., 2019a; Townsend et al., 2021)

Statistical analysis

The statistical analysis was undertaken only for studies where each group size was at least n=5. The group sizes are the number of independent observations and the statistical analyses were performed using these independent observations. We analyzed the data with repeated-measures or univariate ANOVA and ANCOVA using SPSS (Version 27, GLM procedure) or SAS (Version 8.4.3). The covariates we used are described in the Results section and the Supplemental Online Section. We tested the data for sphericity and homogeneity of variance when appropriate (see Table S1). When the sphericity assumption was not met, we adjusted the degrees of freedom using the Greenhouse-Geisser correction. Because our ANOVAs yielded multiple main and interaction effects, we report only effects that are critical for data interpretation. For complete statistical results, see Supplemental Results section and Supplemental Table 1 (Table S1).

3. Results

We report the results of the statistical analyses in the Supplementary Online Material.

3.1. Heroin self-administration (Fig. 1B & 2B)

In Exp. 1-2, we trained the rats to self-administer heroin at 0.1 mg/kg/infusion for the first 7 days, followed by 0.05 mg/kg/infusion for the next 7 days. The rats of both sexes demonstrated reliable heroin self-administration as indicated by an increase in the number of heroin infusions and active lever presses over days, and a compensatory increase in the number of infusions earned when we halved the dose (Fig. 1-2 and Table S1 for statistics). There were no significant sex differences in heroin self-administration (Table S1).

3.2. Experiment 1: Effect of BU08028 on food self-administration

Food training (Fig. 1C)

Chronic delivery of BU08028 decreased food-reinforced responding for up to 3 days after the minipump surgery; responding recovered to the level of the vehicle group after that.

3.3. Experiment 2: Effect of BU08028 on incubation of heroin seeking, extinction responding, context-induced reinstatement, and reacquisition

In Exp. 2, we trained male and female rats to self-administer heroin and tested them for 30 min under extinction conditions on abstinence day 1. On abstinence day 2, we implanted vehicle- or BU08028-containing Alzet minipumps and starting on abstinence day 8 performed the drug-seeking and drug-taking tests described below.

Incubation of heroin seeking in context B (Fig. 2C)

Active lever presses in the vehicle condition were higher on abstinence day 8 than on day 1 (incubation of heroin seeking) and this incubation effect was decreased by BU08028 in both sexes.

Extinction responding in context B (Fig. 3B)

BU08028 decreased active lever presses reinforced by the discrete tone+light cue in context B (extinction responding) in male but not female rats. However, the significant effect of BU08028 dose on extinction responding in male rats should be interpreted with caution, because it is based on exploratory post-hoc analyses within each sex without a significant BU08028 dose x Sex interaction in the initial ANCOVA (see Supplemental Online Material).

Context-induced reinstatement (Fig. 3C)

BU08028 had no effect on context-induced reinstatement of heroin seeking in either sex

Reacquisition (Fig. 3D)

BU08028 *increased* reacquisition of heroin self-administration in female but not male rats.

The results of Exp. 2 suggest that chronic BU08028 resulted in sex-dependent different effects on the relapse-related measures. BU08028 decreased incubation of heroin seeking in both sexes, selectively decreased extinction responding in male rats, had no effect on context-induced reinstatement in either sex, and unexpectedly selectively *increased* reacquisition of heroin self-administration in female rats.

3.4. Experiment 3: Effect of chronic BU08028 on heroin vs. food choice

In Experiment 3, we trained male and female rats to self-administer heroin during daily 2-h heroin vs. food choice sessions. Once heroin choice behavior was stable, we implanted vehicle- or BU08028-containing Alzet pumps and tested the effect of chronic BU08028 delivery on heroin choice over five consecutive days.

Heroin choice (Fig. 4B)

BU08028 had no effect on heroin vs. food choice. Under the vehicle and the two BU08028 dose conditions, increasing the heroin unit dose caused a dose-dependent increase in heroin choice. Neither 0.03 nor 0.1 mg/kg/day BU08028 significantly altered heroin choice. We did not include Sex as a between-subjects factor in the statistical analysis, because Exp. 3 was not adequately powered to investigate potential sex differences in BU08028 effects on heroin choice.

Reinforcement rates (Fig. 4C)

BU08028 did not significantly alter rates of operant responding at the doses tested during the heroin choice session. Because these BU08028 doses did not significantly alter either heroin choice or reinforcement rates, we piloted a higher BU08028 dose (0.32 mg/kg/day) in several rats. This dose produced toxic effects such as severe lethargy for many hours that precluded further investigation.

4. Discussion

We used our rat model of opioid maintenance to test the effects of chronic delivery of the mixed NOP/MOR agonist BU08028 on four relapse-related measures of heroin seeking and taking and on heroin vs. food choice. We report four main findings: First, chronic BU08028 delivery decreased incubation of heroin seeking in both male and female rats. Second, chronic BU08028 delivery decreased extinction responding in male rats only. Third, chronic BU08028 had minimal effects on context-induced reinstatement of heroin seeking and heroin vs. food choice in either sex. Fourth, unexpectedly, chronic BU08028 delivery *increased* reacquisition of heroin self-administration selectively in female rats. Together, chronic BU08028 delivery had sex-dependent beneficial and detrimental effects on some measures of heroin relapse, and sex-independent null effects on other relapse measures and heroin choice. In the sections below, we first discuss the “behavioral measures-dependent” effects of BU08028, and then discuss sex differences in BU08028 effects.

Dissociable effects of BU08028 on different relapse-related measures and drug choice

A main finding in our study was that in male and female rats, chronic delivery of BU08028 had different effects on the relapse-related measures. In males, BU08028 decreased incubation of heroin seeking and extinction responding but had no significant effects on context-induced reinstatement or reacquisition. In females, BU08028 decreased incubation of heroin seeking, had no significant effects on extinction responding and context-induced reinstatement, and increased reacquisition. This pattern of results is different from what we previously reported with the parent compound, buprenorphine, which decreased extinction responding, context-induced reinstatement, and reacquisition in both sexes (Bossert et al., 2020). Buprenorphine is a MOR partial agonist and kappa opioid receptor (KOR) antagonist (Jaffe, 1990; Khroyan et al., 2011); however, the results of our previous study indicate that its suppressing effect on relapse is due to its action at MOR. We found that chronic blockade of KOR with the selective long-lasting antagonist nor-BNI (Portoghese et al., 1987) has no effect on the three relapse-related measures (Bossert et al., 2020).

What might account to the different results of buprenorphine and BU08028 on relapse in our model? Unlike buprenorphine, a partial MOR agonist/KOR antagonist with low affinity and efficacy at NOP receptors, BU08028 binds at high affinity to NOP receptors and is a partial agonist at both MOR and NOP receptors (note that both buprenorphine and BU08028 bind at high affinity to the delta opioid receptor (DOR) but do not activate this receptor) (Khroyan et al., 2011). We suspect that the most likely pharmacological account of the different effects of buprenorphine and BU08028 on the different relapse measures is BU08028's partial agonist action on the NOP receptor. In a previous acute pharmacological study with oxycodone as the self-administered drug, we found a role of MOR but not KOR or delta opioid receptor (DOR) in both context-induced reinstatement and oxycodone self-administration (Bossert et al., 2019). More recently, using the same experimental procedure we used in the present study, we found that chronic delivery of AT-201, a mixed partial MOR/NOP receptor agonist (Kallupi et al., 2018), mimicked the potentiation effect of BU08028 on reacquisition of heroin self-administration in females (Bossert et al. unpublished data).

Another main finding in our study is that BU08028 had no effect on heroin choice using an experimental procedure in which buprenorphine strongly decreases heroin choice and promotes

behavioral reallocation to an alternative nondrug reinforcer (Townsend et al., 2021). We suspect that like the heroin relapse measures, the different effects of BU08028 and buprenorphine on heroin choice are due to BU08028's actions on NOP receptors that counteract the beneficial effects of partial MOR activation on choice.

One potential reason for the different effect of BU08028 on the relapse-related measures and choice is that its effect on operant responding is dependent upon the baseline response rates in the different tasks, which are not the same for the different relapse and choice measures. In this regard, many studies have shown that the effect of drugs on operant responding are dependent on baseline response rates (e.g., psychostimulant drugs increase low rate operant responding but decrease high rate responding) (Sanger & Blackman, 1976). However, it is unlikely that a rate-dependent effect can account for our data, primarily because this putative mechanism cannot explain the sex-specific effects of BU08028 on extinction responding and reacquisition of heroin self-administration in which responding under the vehicle condition was similar in males and females. However, as choice measures are less sensitive to rate-dependent drug effects (Woolverton & Johanson, 1984), we cannot rule out a rate-dependent account for the different effects of BU08028 on some of the relapse measures versus the drug's lack of effect on heroin vs. food choice. We also cannot rule out a rate-dependent account for some of the different effects of BU08028 in female rats: decreased incubation of heroin seeking (high response rate) vs. increased reacquisition of heroin self-administration (low response rate).

More generally, the different effects of BU08028 on the different relapse-related measures agree with results from many studies indicating that different neurobiological mechanisms control incubation of drug seeking vs. reinstatement after extinction, as well as reinstatement after extinction vs. reacquisition after extinction (Bossert et al., 2013; Khoo et al., 2017; Prasad et al., 2020; Reiner et al., 2019). For example, reversible inactivation of ventral medial prefrontal cortex (mPFC) decreases incubation of cocaine seeking but induces reinstatement after extinction (Koya et al., 2009; Peters et al., 2009), while inactivation of dorsal mPFC decreases context-induced reinstatement of alcohol seeking after extinction but increases reacquisition (Willcocks & McNally, 2013). Additionally, the data on the different effects of BU08028 on heroin choice vs. some of the relapse-related measures agree with results from many studies showing that the neuropharmacological mechanisms of drug choice are different and distinct from

those controlling drug self-administration and relapse as assessed in rodent and monkey models (Banks & Negus, 2017; Venniro et al., 2020).

A methodological issue to consider is that unlike other studies on incubation of drug craving after homecage forced abstinence where the incubation tests were performed in the drug self-administration context (Pickens et al., 2011; Venniro et al., 2016; Wolf, 2016), we measured incubation of heroin seeking in a non-drug context (context B). However, it is unlikely that this design change can confound the interpretation of the data on the inhibitory effect of BU08028 on this incubation. This is because in previous studies using the ABA procedure, we observed reliable incubation in context B after drug self-administration in context A and homecage forced abstinence (Adhikary et al., 2017; Bossert et al., 2019). Indeed, an unexpected conclusion from the parametric study of Adhikary et al. (2017) was that incubation of drug craving is primarily driven by time-dependent increases in operant responding, and that this effect is context independent.

Finally, the complicated set of results from our studies suggesting both beneficial and detrimental effects of BU08028 on heroin seeking, taking, and choice do not agree with previous studies in non-human primates suggesting beneficial effects on opioid-taking behavior of BU08028 and another mixed partial agonist at MOR and NOP receptors (AT-121) (Ding et al., 2016; Ding et al., 2018). The reasons for different results between rodents and monkey models of drug self-administration and relapse are complex and beyond the scope of our paper. We suspect, however, that the main reason for the different results is that we used chronic steady-state delivery of BU08028, while in the previous monkey studies, the beneficial effects of BU08028 and AT-121 were either inferred from the observation that the drugs are not self-administered, or in the case of AT-121, from its acute effect on oxycodone self-administration (Ding et al., 2016; Ding et al., 2018). Another potential reason for the different results is that we used different relapse-related and choice measure, while the monkey studies only assessed ongoing drug self-administration. As discussed elsewhere and above, results from many studies showed that the neuronal mechanisms of ongoing drug self-administration are different from those controlling relapse/reinstatement or choice in animal models (Kalivas & McFarland, 2003; Shalev et al., 2002; Venniro et al., 2020).

Sex differences in the effect of BU08028 on relapse-related behaviors

An unexpected finding in our study was the sex-dependent effects of chronic BU08028 on the relapse-related measures. BU08028 decreased incubation of heroin seeking in both sexes, selectively decreased extinction responding in males, and selectively increased reacquisition in females. This pattern of results is different from our previous results with buprenorphine: decreased extinction responding, context-induced reinstatement, and reacquisition of oxycodone self-administration in both sexes (Bossert et al., 2020). Similarly, Bakhti-Suroosh et al. (2021) reported that chronic buprenorphine decreases extinction responding and discrete cue-induced reinstatement of fentanyl seeking in both sexes. However, the sex-specific effect of BU08028 in the current study extend results from our previous study with TRV130, a selective partial agonist at MORs (Gillis et al., 2020), which decreases extinction responding in both sexes, but selectively decreases context-induced reinstatement and reacquisition of oxycodone self-administration in males (Bossert et al., 2020). Based on these results and evidence for sex differences in both MOR and NOP receptors expression and function (Becker & Chartoff, 2019; Craft, 2008; Zhang et al., 2012; Zhang et al., 2018), we speculate that the sex-specific effects of BU08028 are due to its sex-specific effects on both MOR and NOP receptors.

Another finding in our study was that in the vehicle condition, we found no evidence for sex differences in the four relapse-related measures. These results agree with those from our previous study with oxycodone in which we did not observe sex differences in extinction responding, context-induced reinstatement, and reacquisition (Bossert et al., 2020), and other studies using the extinction-reinstatement procedure showing similar magnitude of reinstatement of opioid seeking in males and female rats (Nicolas et al., 2021). Our negative sex differences data also agree with those from our recent studies on relapse to opioid seeking after voluntary abstinence induced by either providing rats with alternative rewards (palatable food or social interaction) in a choice procedure or exposing them to an electric barrier near the drug-paired lever (Fredriksson et al., 2020; Fredriksson et al., 2021; Reiner et al., 2020; Venniro et al., 2019; Venniro et al., 2017). However, in a recent study, Bakhti-Suroosh et al. (2021) reported higher extinction responding but not discrete cue-induced reinstatement in female rats trained to self-administer fentanyl using the intermittent access self-administration procedure.

Together, the results from the present study and our previous studies with oxycodone (Bossert et al., 2020; Fredriksson et al., 2020) highlight the importance of including both sexes in studies aimed to

identify novel medications for relapse prevention in animal models because even under conditions where the relapse-related behavior is similar in males and females, the response to the target medications can be sex-specific (Fredriksson et al., 2021).

Concluding remarks

We used a rat model of opioid maintenance to test the effect of chronic BU08028 delivery on relapse-related measures and heroin choice. BU08028 had sex-dependent beneficial and detrimental effects on some measures of heroin relapse, and sex-independent null effects on other relapse measures and heroin choice. To the degree that our rat models of relapse and choice predict medication efficacy in humans (Banks & Negus, 2017; Epstein et al., 2006; Venniro et al., 2020), our results do not support the notion, derived from non-human primate studies (Ding & Ko, 2021), that mixed NOP/MOR partial agonists should be considered for the treatment of opioid addiction in humans.

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Author's contributions

JMB, SL, LA, IF, and AS carried out the experiments; JMB, SL, LA, MB, and YS performed data analysis. JMB, SL, MB and YS designed the study and wrote the manuscript. SH, AS, and KCR synthesized BU08028. All authors critically reviewed the content and approved the final version before submission.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigor

The authors acknowledge that the paper adheres to the principles for transparent reporting and scientific rigor of preclinical research as stated in the BJP guidelines and as recommended by NIH.

Data availability

A summary Excel file of the raw data used for the statistical analyses and shown in the figures is available from the authors upon request. The manuscript complies with the British Journal Pharmacology's recommendations and requirements on experimental design and analysis (Curtis et al., 2018).

Figure legends

Figure 1. *Effect of BU08028 on food self-administration in heroin-experienced male and female rats.* **(A)** Timeline of Exp. 1. **(B)** Heroin self-administration training: Mean±sem number of heroin infusions (Left panel) and inactive and active lever presses (Right panel) during heroin self-administration training for male (n=8) and female (n=12) rats. **(C)** Food self-administration after minipump surgery: Mean±sem number of pellets consumed (Left panel) and active lever presses (Right panel) after the rats were implanted with Alzet osmotic minipumps containing vehicle (3 males, 4 females), 0.05 mg/kg/day (3 males, 4 females), and 0.1 mg/kg/day (2 males, 4 females). The data were combined for the males and females. * Significant BU08028 Dose x Session interaction, $p<0.05$.

Figure 2. *Effect of chronic delivery of BU08028 on incubation of heroin seeking in context B.* **(A)** Timeline of Exp. 2. **(B)** Heroin self-administration training in context A: Mean±sem number of heroin infusions (Left panel) and inactive and active lever presses (Right panel) during heroin self-administration training for male and female rats (n =25 males and n=35 females). **(C)** Incubation of heroin seeking in context B: Mean±sem number of active (Left panel) and inactive (Right panel) lever presses on abstinence day 1 (before minipump implantation) and on abstinence day 8. Also shown are individual data for each rat. During testing, active lever presses led to contingent presentations of the tone-light cue previously paired with heroin infusions, but not heroin. Vehicle (9 males, 12 females), 0.05 mg/kg/d (8 males, 10 females), and 0.1 mg/kg/day (8 males, 13 females). See text for details of the experimental procedure. * Significant main effect of BU08028 Dose, $p<0.05$.

Figure 3. *Effect of chronic delivery of BU08028 on extinction responding in context B, context A–induced reinstatement, and reacquisition in context A.* **(A)** Timeline of Exp. 2. **(B)** Extinction in context B: *Left panel:* Mean±sem number of active and inactive lever presses during the seven 6-h extinction sessions in male and female rats implanted with Alzet osmotic minipumps containing vehicle (9 males, 12 females), 0.05 mg/kg/d (8 males, 10 females), and 0.1 mg/kg/day (8 males, 12 females). Also shown are individual data for each rat. *Right panel:* Mean±sem number of active lever presses at each extinction session. Active lever presses led to contingent presentations of the tone-light cue previously paired with heroin infusions, but not heroin. **(C)** Context A–induced reinstatement: *Left panel:* Mean±sem number of

active lever presses during the 6-h reinstatement tests in context B and context A. Active lever presses led to contingent presentations of the tone-light cue, but not heroin; the number of subjects for the vehicle and BU08028 groups are the same as that for extinction in context B. *Right panel:* Mean±sem number of active lever presses in context A at each hour of testing. **(D)** Reacquisition in context A: *Left panel:* Mean±sem number of heroin infusions (0.05 mg/kg/infusion) during reacquisition. Active lever presses led to the delivery of heroin infusions and the tone-light cue. vehicle (9 males, 11 females), 0.05 mg/kg/d (8 males, 9 females), and 0.1 mg/kg/day (8 males, 12 females). *Right panel:* Mean±sem number of infusions at each hour of testing. * Significant BU08028 Dose, BU08028 Dose x Session interaction, or BU08028 Dose x Sex x Session interaction $p<0.05$.

Figure 4. *Effect of chronic delivery of BU08028 on heroin-vs-food choice.* **(A)** Timeline of Exp. 3. **(B)** Heroin choice: Mean ± sem percent heroin choice across within-session heroin unit doses in rats implanted with Alzet osmotic minipumps containing vehicle or a BU08028 dose (0.03, 0.1 mg/kg/day) (n=6, 3 males, 3 females). **(C)** Reinforcement rates: Mean ± sem total choices completed per component across the within-session reinforcement-rate dose-response function. Some symbols may obscure visualization of other symbols on the graph due to overlapping data points between BU08028 treatment conditions (i.e., zero-heroin dose condition in Panel B).

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