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Supplemental Online Material

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 Results with statistical reporting
Table S1 (statistical reporting)

Supplemental Results

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. 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. The mixed ANOVA for pellets earned, which included the between-subjects factors of BU08028 Dose (0, 0.05, 0.1 mg/kg/d) and the within-subjects factor of Session (1-7 post minipump surgery), showed a significant effect of BU08028 dose x Session ($F(5.5, 46.8)=7.2, p<0.001$). The mixed ANCOVA for active lever presses (covariate, inactive lever presses) with the same factors also showed a significant effect of BU08028 dose x Session ($F(6.2, 49.5)=4.0, p<0.001$).

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. One female rat from the 0.1 mg/kg/d BU08028 group died four days after minipump surgery.

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. The ANCOVA of number of active lever presses on Abstinence Day 8, which included the between-subjects factors of BU08028 Dose and Sex (covariates Abstinence day 1 active lever and Abstinence day 8 inactive lever), showed a significant effect of BU08028 Dose ($F(2,52)=7.6, p=0.01$) but no significant effects of Sex or interaction (p values > 0.1). Subsequent ANCOVAs within each sex showed significant effects of BU08028 Dose for both males ($F(2,24)=6.2, p=0.008$) and females ($F(2,34)=5.1, p=0.012$).

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. The ANCOVA of number of active lever presses, which included the between-subjects factors of BU08028 dose and Sex and the within-subjects factor of

Extinction Session, showed significant effects of Extinction session ($F(1.9, 101.5)=21.0, p<0.001$) and approaching significant effect of BU08028 dose x Extinction session ($F(3.8, 101.5)=2.4, p=0.06$). However, visual inspection of the data in Fig. 3B suggested that BU08028 decreased extinction responding in males but not females. This impression was statistically supported by subsequent exploratory post-hoc ANCOVAs within each sex that showed significant effects of BU08028 Dose ($F(2,21)=3.9, p=0.037$) and BU08028 Dose x Extinction Session ($F(3.5, 36.9)=3.9, p=0.012$) for males but not females (p values > 0.1). The significant effect of BU08028 dose on extinction responding in male rats should be interpreted with caution, because it is based on post-hoc analyses within each sex in the absence of a significant BU08028 dose x Sex interaction in the initial ANCOVA.

Context-induced reinstatement (Fig. 3C)

BU08028 had no effect on context-induced reinstatement of heroin seeking in either sex. The ANCOVA of number of active lever presses, which included the between-subjects factors of BU08028 Dose and Sex and the within-subjects factor of Context (A, B), showed a significant effect of Context ($F(1,52)=43.1, p>0.001$) but no other main or interaction effects (p values > 0.1). The data of one female rat were excluded from only this test because SPSS identified the data as being a statistical outlier.

Reacquisition (Fig. 3D)

BU08028 *increased* reacquisition of heroin self-administration in female but not male rats. The ANCOVA of number of infusions, which included the between-subjects factors of BU08028 Dose and Sex and the within-subjects factor Session hour (1,6) (covariate, mean number of infusions on the last 3 self-administration training days), showed significant effects of BU08028 Dose ($F(2,50)=7.6, p=0.001$), Sex ($F(1,50)=10.6, p=0.002$), Sex x Session Hour ($F(5,250)=2.3, p=0.049$), and BU08028 Dose x Sex x Session Hour ($F(10,250)=2.2, p=0.016$). Subsequent analyses within each sex showed significant effects of BU08028 Dose ($F(2,28)=4.5, p=0.019$) and BU08028 Dose x Session Hour ($F(10,140)=2.2, p=0.022$) for female but not male rats (p values >0.1). The significant interaction with session hour is due to a greater effect of BU08028 earlier in the reacquisition session.

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. The analysis, which included the within-subjects factors of BU08028 Dose (0, 0.03, 0.1 mg/kg/day) and Heroin Dose (0, 0.032, 0.01, 0.032, 0.1 mg/kg/infusion), showed a significant effect of Heroin dose ($F(2.1, 33.1)=30.1, p<0.001$), but no effect of BU08028 dose or an interaction. 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 Experiment 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. The analysis showed a significant main effect of Heroin dose ($F(1.7,27.4)=57.0, p<0.001$), but no effect of BU08028 Dose or an interaction. 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.

Table S1. Statistical analysis for Experiments 1-3. For Experiments 1-2, analyses were performed with SPSS GLM repeated-measures module, unless indicated otherwise. For Experiment 3, analyses were performed with SAS repeated measures Multivariate Analysis of Variance module within JMP Pro 15.1. Partial Eta² = proportion of explained variance. NP, not possible to calculate partial Eta². For ANCOVAs, the covariate is inactive lever presses, unless indicated otherwise. Sphericity correction for degrees of freedom and p values was performed using Greenhouse-Geisser test. RM, repeated measures

Exp. 1: Effect of BU08028 on food self-administration: male and female rats

Figure number	Factor name	F-value	p-value	Partial Eta ²
Figure 1B. Heroin self-administration training <u>Infusions</u> RM-ANOVA	Sex (M, F) between-subjects Session (1-14) within-subjects Sex x Session <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Session 2-3,5-14 p>0.05 Session 1,4, p=0.008, 0.001	F(1, 18)=2.5 F(4.1, 73.7)=20.2 F(4.1, 73.7)=0.6	0.134 <0.001* 0.661	0.12 0.53 0.03
Figure 1B. Heroin self-administration training <u>Lever presses</u> RM-ANCOVA	Sex (M, F) between-subjects Session (1-14) within-subjects Sex x Session <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Session 2-3,5-14 p>0.05 Session 1,4, p=0.017, 0.029	F(1, 17)=2.9 F(1.5, 25.4)=4.1 F(13, 221)=1.1	0.107 0.039* 0.325	0.15 0.19 0.06
Figure 1C. Food self-administration <u>Pellets</u> RM-ANOVA	BU08028 Dose between-subjects Session (1-7) within-subjects BU08028 Dose x Session <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Sessions 1-6, p>0.05 Session 7, p=0.029	F(2, 17)=7.5 F(2.7, 46.8)=26.1 F(5.5, 46.8)=7.2	0.005* <0.001* <0.001*	0.47 0.61 0.46
Figure 1C. Food self-administration <u>Lever presses</u> RM-ANCOVA	BU08028 Dose between-subjects Session (1-7) within-subjects BU08028 Dose x Session <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Sessions 1, 6-7, p>0.05 Sessions 2-5, p=0.036, 0.037, 0.025, 0.024	F(2, 16)=3.0 F(3.1, 49.5)=2.9 F(6.2, 49.5)=4.0	0.079 0.023* 0.002*	0.27 0.15 0.33

Exp. 2: Effect of BU08028 on extinction, context-induced reinstatement, and reacquisition: male and female rats

Figure number	Factor name	F-value	p-value	Partial Eta2
Figure 2B. Heroin self-administration training <u>Infusions</u> RM-ANOVA	Sex (M, F) between-subjects Session (1-14) within-subjects Sex x Session <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Sessions 1,3,5-14 p>0.05 Sessions 2,4, p=0.024, 0.039	F(1, 58)=0.3 F(2.8, 164.7)=80.7 F(2.8, 164.7)=0.7	0.566 <0.001* 0.538	0.07 0.58 0.01
Figure 2B. Heroin self-administration training <u>Lever presses</u> RM-ANCOVA	Sex (M, F) between-subjects Session (1-14) within-subjects Sex x Session <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Sessions 1-14 p>0.05	F(1, 53)=0.9 F(3.7, 194.7)=16.3 F(3.7, 194.7)=1.0	0.360 <0.001* 0.404	0.02 0.24 0.02
Figure 2C. Incubation of heroin seeking <u>Lever presses</u> ANCOVA (GLM univariate) (covariates: active lever abstinence day 1 [pre-minipump], inactive lever day 8)	BU08028 Dose between-subjects Sex (M, F) between-subjects BU08028 Dose x Sex <u>Mauchly Sphericity test:</u> not provided <u>Levene homogeneity of variance test:</u> Abstinence day 8, p>0.05	F(2, 52)=7.8 F(1, 52)=0.4 F(2, 52)=0.2	0.01* 0.84 0.86	0.23 0.01 0.06
Figure 3B. Extinction <u>Lever presses</u> RM-ANCOVA	BU08028 Dose between-subjects Sex (M, F) between-subjects Session (1-7) within-subjects BU08028 Dose x Sex BU08028 Dose x Session Sex x Session BU08028 Dose x Sex x Session <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Sessions 1-2 & 4-7, p>0.05 Session 3, p=0.02	F(2, 53)=1.5 F(1, 53)=1.2 F(1.9, 101.5)=21.0 F(2, 53)=1.3 F(3.8, 101.5)=2.4 F(1.9, 101.5)=1.9 F(3.8, 101.5)=1.1	0.240 0.282 <0.001* 0.279 0.06 0.16 0.366	0.05 0.02 0.28 0.05 0.08 0.03 0.04
Figure 3C. Context-induced reinstatement <u>Lever presses</u> RM-ANCOVA	BU08028 Dose between-subjects Context (B, A) within-subjects Sex (M, F) between-subjects BU08028 Dose x Sex BU08028 Dose x Context Context x Sex BU08028 Dose x Context x Sex <u>Mauchly Sphericity test:</u> not provided <u>Levene homogeneity of variance test:</u> Context A & B, p>0.05	F(2, 52)=0.4 F(1, 52)=43.1 F(1, 52)=0.2 F(2, 52)=1.6 F(2, 52)=0.2 F(1, 52)=0.1 F(2, 52)=0.1	0.667 <0.001* 0.644 0.205 0.839 0.737 0.911	0.01 0.45 0.004 0.16 0.007 0.002 0.004

Figure 3D. Reacquisition <u>Infusions</u> RM-ANCOVA (covariate, mean number of infusions during the last 3 training days)	BU08028 Dose between-subjects	F(2, 50)=7.6	0.001*	0.23
	Sex (M, F) between-subjects	F(1, 50)=10.6	0.002*	0.17
	Hour (1-6) within-subjects	F(5, 250)=0.9	0.491	0.02
	BU08028 Dose x Sex	F(2, 50)=0.2	0.814	0.008
	BU08028 Dose x Session Hour	F(10, 250)=1.1	0.383	0.04
	Sex x Session Hour	F(5, 250)=2.3	0.049*	0.04
	BU08028 Dose x Sex x Session Hour	F(10, 250)=2.2	0.016*	0.08
	<u>Mauchly Sphericity test:</u> p>0.05			
	<u>Levene homogeneity of variance test:</u> Hours 2-6, p>0.05			
	Hour 1, p=0.036			

Exp. 2: Effect of BU08028 on extinction, context-induced reinstatement, and reacquisition: male rats

Figure number	Factor name	F-value	p-value	Partial Eta2
Figure 2B. Heroin self-administration training <u>Infusions</u> RM-ANOVA	Session (1-14) within-subjects <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Not applicable	F(2.5, 60.3)=35.4	<0.001*	0.60
Figure 2B. Heroin self-administration training <u>Lever presses</u> RM-ANCOVA	Session (1-14) within-subjects <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Not applicable	F(2.8, 64.4)=5.4	0.003*	0.19
Figure 2C. Incubation of heroin seeking <u>Lever presses</u> ANCOVA (GLM univariate) (covariates: active lever abstinence day 1 [pre-minipump], inactive lever day 8)	BU08028 Dose between-subjects <u>Mauchly Sphericity test:</u> not provided <u>Levene homogeneity of variance test:</u> Abstinence day 8, p>0.05	F(2, 24)=6.2	0.008*	0.38
Figure 3B. Extinction <u>Lever presses</u> RM-ANCOVA	BU08028 Dose between subjects Session (1-7) within-subjects BU08028 Dose x Session <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Sessions 1-3, 5-7 p>0.05 Session 4, p=0.041	F(2, 21)=3.9 F(1.8, 36.9)=19.0 F(3.5, 36.9)=3.9	0.037* <0.001* 0.012*	0.27 0.48 0.27
Figure 3C. Context-induced reinstatement <u>Lever presses</u> RM-ANCOVA	BU08028 Dose between-subjects Context (B, A) within-subjects BU08028 Dose x Context <u>Mauchly Sphericity test:</u> p>0.05 <u>Levene homogeneity of variance test:</u> Context A & B, p>0.05	F(2, 21)=1.5 F(1, 21)=16.8 F(2, 21)=0.2	0.251 0.001* 0.788	0.12 0.44 0.02
Figure 3D. Reacquisition <u>Infusions</u> RM-ANCOVA	BU08028 Dose between-subjects Hour (1-6) within-subjects BU08028 Dose x Session Hour <u>Mauchly Sphericity test:</u> p>0.05 <u>Levene homogeneity of variance test:</u>	F(2, 21)=3.0 F(5, 105)=1.1 F(10, 105)=1.2	0.072 0.380 0.309	0.22 0.05 0.10

RM-ANCOVA (covariate, mean number of infusions during the last 3 training days)	Hours 1-6, p>0.05			
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Exp. 2: Effect of BU08028 on extinction, context-induced reinstatement, and reacquisition: female rats

Figure number	Factor name	F-value	p-value	Partial Eta2
Figure 2B. Heroin self-administration training <u>Infusions</u> RM-ANOVA	Session (1-14) within-subjects <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Not applicable	F(2.7, 94.0)=46.7	<0.001*	0.579
Figure 2B. Heroin self-administration training <u>Lever presses</u> RM-ANCOVA	Session (1-14) within-subjects <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Not applicable	F(3.1, 104.6)=30.2	<0.001*	0.471
Figure 2C. Incubation of heroin seeking <u>Lever presses</u> ANCOVA (GLM univariate) (covariates: active lever abstinence day 1 [pre-minipump], inactive lever day 8)	BU08028 Dose between-subjects <u>Mauchly Sphericity test:</u> not provided <u>Levene homogeneity of variance test:</u> Abstinence day 8, p>0.05	F(2, 34)=5.1	0.012*	0.25
Figure 3B. Extinction <u>Lever presses</u> RM-ANCOVA	BU08028 Dose between subjects Session (1-7) within-subjects BU08028 Dose x Session <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Sessions 1-6, p>0.05 Session 7, p=0.02	F(2, 31)=0.6 F(1.9, 57.8)=10.2 F(3.7, 57.8)=1.4	0.567 <0.001* 0.240	0.04 0.25 0.08
Figure 3C. Context-induced reinstatement <u>Lever presses</u> RM-ANCOVA	BU08028 Dose between-subjects Context (B, A) within-subjects BU08028 Dose x Context <u>Mauchly Sphericity test:</u> p>0.05 <u>Levene homogeneity of variance test:</u> Context A & B, p>0.05	F(2, 30)=0.2 F(1, 30)=24.5 F(2, 30)=0.01	0.802 <0.001* 0.985	0.02 0.45 0.001
Figure 3D. Reacquisition <u>Infusions</u> RM-ANCOVA RM-ANCOVA (covariate, mean number of infusions during the last 3 training days)	BU08028 Dose between-subjects Session Hour (1-6) within-subjects BU08028 Dose x Session Hour <u>Mauchly Sphericity test:</u> p>0.05 <u>Levene homogeneity of variance test:</u> Hours 1-4, p>0.05 Hour 5-6, p=0.025, .010	F(2, 28)=4.5 F(5, 140)=0.7 F(10, 140)=2.2	0.019* 0.598 0.022*	0.25 0.03 0.14

Exp. 3: Effect of BU08028 on heroin choice: male and female rats

Figure number	Factor name	F-value	p-value	Partial Eta2
Figure 4B. Heroin Choice <u>Percent heroin choice</u> RM-ANOVA	Heroin unit dose within-subjects BU08028 dose within-subjects Interaction <u>Mauchly Sphericity test:</u> p<0.001 <u>Levene homogeneity of variance test:</u> Not applicable	F(2.1, 33.1)=30.1 F(2, 16)=0.92 F(4.1, 33.1)=1.3	<0.001* 0.42 0.29	NP NP NP
Figure 4C. Choice <u>Reinforcement rates</u> RM-ANOVA	Heroin unit dose within-subjects BU08028 dose within-subjects interaction <u>Mauchly Sphericity test:</u> not provided <u>Levene homogeneity of variance test:</u> Not applicable	F(1.7, 27.4)=57 F(2, 16)=0.64 F(3.4, 27.4)=2.0	<0.001* 0.54 0.13	NP NP NP