

SUPPLEMENTARY INFORMATION

NOP Receptor Antagonism Attenuates Reinstatement of Alcohol Seeking through Modulation of the Mesolimbic Circuitry in Male and Female Alcohol- Preferring Rats.

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MATERIALS AND METHODS

Animals

Male ($n = 101$) and female ($n = 102$) genetically selected alcohol-preferring mSP rats were used. Animals were bred at the University of Camerino (Italy) and were 9-10 weeks old at the beginning of the experiments. The rats were randomly assigned to treatment groups and housed in standard ventilated clear plastic cages (2 per cage) with conventional wood chips bedding in the animal facility of the University of Camerino. They were maintained in a pathogen free-environment on 12h light/dark cycle (lights off at 8:30 AM and on at 8:30 PM) at controlled temperature (20-22°C) and humidity (45-55%). Food (4RF18, Mucedola, Settimo Milanese, Italy) and water were available *ad libitum*. To familiarize animals to the procedures, they were handled for 5 days before behavioral testing. Experiments were conducted during the dark phase of the light/dark cycle and all efforts were made to minimize the number of animals and their discomfort. All animal care and experimental procedures were performed in accordance with the guidelines of the European Community Council Directive for Care and Use of Laboratory Animals and European legislation (2010/63/EU). Formal approval to conduct the experiments was obtained from the Italian Ministry of Health and the Organism Responsible for Animal Welfare of the University of Camerino (protocol no. 1D580.1).

Drugs

The alcohol (10% v/v) drinking solution was prepared by diluting 95% alcohol (FL Carsetti SNC, Camerino, Italy) in tap water. The saccharin (Sigma-Aldrich, Milan, Italy) was dissolved in tap water to obtain a 0.2% (w/v) solution. The alpha-2 adrenoreceptor antagonist yohimbine (Sigma-Aldrich, Milan, Italy), at the dose of 1.25 mg/kg, was dissolved in distilled water and administered intraperitoneally (i.p.) 30 minutes prior to the reinstatement tests. The NOP receptor antagonist LY2817412 was synthesized and kindly provided by Eli Lilly (Indianapolis,

IN, USA). For systemic administration, LY2817412 (3.0, 10.0, and 30.0 mg/kg) was dissolved in a vehicle that consisted of a 1:1 mixture of distilled water and 1 M H₃PO₄ (Sigma-Aldrich, Milan, Italy) and administered in a volume of 1 ml/kg given by gavage (p.o.) 1 hour before the beginning of reinstatement tests. For intracerebral microinjections, LY2817412 (1.0, 3.0 and 6.0 µg/µl/rat) was suspended in 10% dimethylsulfoxide (DMSO; Sigma-Aldrich, Milan, Italy), 3% Tween-80, and 87% deionized water and administered bilaterally in a volume of 0.5 µl/rat, approximately 15 min before the beginning of reinstatement tests. Solutions were freshly prepared before the tests. All pharmacological treatments were carried out according to a *Latin square* within-subjects counterbalanced design. Drug treatments were separated by at least 3 days.

Intracranial Surgery and Infusion Procedure

Animals were anesthetized by intramuscular injection of a solution (100-150 µl) containing tiletamine (58.17 mg/ml) and zolazepam (7.5 mg/ml). Bilateral guide cannulas (0.65 mm outside diameter) aimed at the VTA, CeA and NAc, were implanted and cemented on the skull. We used the following stereotaxic coordinates (relative to bregma) according to the rat brain atlas [3] and adjusted for the animals' body weight: male: [VTA: anterior/posterior (AP): -5.7 mm; medial/lateral (ML): ±2.2 mm; dorsal/ventral (DV): -7.4 mm, 12° angle; CeA: AP: -2.3 mm, ML: ±4.2 mm, DV: -6.5 mm; NAc: AP: +1.5 mm; ML: ±1.1 mm, DV: -5.5 mm] and female: [VTA: AP: -5.6 mm, ML: ±2.0 mm, DV: -7.2 mm, 10° angle; CeA: AP: -1.8 mm, ML: ±4.0 mm, DV: -6.4 mm; NAc: AP: +1.40 mm, ML: ±1.0 mm, DV: -5.2 mm].

Following surgery, animals received a single subcutaneous injection of ketoprofen (2.5 mg/kg; s.c.) and were allowed to recover for 1 week in their home cages. During this period rats were handled daily. Before starting the tests, animals were habituated to the microinjection procedure, consisting of insertion of the injector into the guide cannulas. Approximately 15

min before the reinstatement tests, rats were bilaterally injected with either vehicle or LY2817412 over a period of 60 s, using a stainless-steel injector, 1.5 mm longer than the guide cannula. The injector was left in place for few seconds after injections to allow diffusion of the solution. At the completion of experiments, animals were deeply anesthetized with isoflurane and injected with a black India ink (0.3 μ l per site) into the brain areas. Rats were then immediately euthanized and brains were collected for histological analysis of cannula placement (see **Supplementary Information**).

Alcohol and Saccharin Self-Administration Training

Operant training and testing were performed in standard self-administration operant chambers (Med Associate, Inc.) located in sound-attenuating rooms. Each chamber was equipped with a drinking reservoir (volume capacity: 0.3 ml) and two retractable levers. An infusion pump was activated by responses on the right active lever, while responses on the left inactive lever, were recorded but did not result in activation of the pump. Auditory and visual stimuli were presented via a speaker and a light located on the front panel. A microcomputer controlled the delivery of fluids, presentation of auditory and visual stimuli, and recording of the behavioral data. Male and female msP rats were trained to self-administer 10% (v/v) alcohol or 0.2% (w/v) saccharin for five days a week, in 30-min daily sessions under a fixed-ratio one (FR1) schedule of reinforcement in which each active lever response resulted in delivery of 0.1 ml fluid, as previously described [1]. During each session, alcohol or saccharin delivery was associated with 5 seconds time-out (TO) signaled by illumination of a white house light. Before the beginning of the operant alcohol training phase, rats were subjected to an overnight session (12 hours) in order to accustom them to the chambers and lever pressing. Alcohol and saccharin self-administration training was continued until animals reached a stable

baseline of responding ($\pm 10\%$ variability in number of reinforcements earned for at least three consecutive self-administration sessions).

After the acquisition phase for alcohol rats have been subjected to an extinction procedure followed by the relapse tests, whereas after saccharin training animals underwent directly to the testing phase.

EXPERIMENTAL PROCEDURES

Effect of Systemic Administration of LY2817412 on Yohimbine-Induced Reinstatement of Alcohol Seeking in Male and Female msP Rats

The experimental procedure consisted of three phases: operant training, extinction and reinstatement (for details, see the experimental timeline in **Fig. 1A**). A group of male ($n = 10$) and female ($n = 10$) msP rats was trained to self-administer 10% (w/v) alcohol for five days a week, in 30-min daily sessions as previously described. Training phase (total number of daily sessions: 20) continued until animals reached a stable baseline of responding ($\pm 10\%$ variability in number of reinforcements earned for at least three consecutive self-administration sessions). The mean of g/kg/30 min of alcohol consumed in the last 3 self-administration days was 1.30 for female rats and 0.86 for males. Following this training phase, rats were subjected to a total of 15 daily 30-min extinction sessions, in which responses at the active lever triggered the delivery mechanism but did not result in the delivery of alcohol. Extinction training was maintained until the number of presses of the previously active lever dropped below 20 presses (average of the last three sessions). The last 3 extinction days, animals were habituated to the LY2817412 and the yohimbine injection procedures by administering their respective vehicles. On the test days, animals were injected with either vehicle or LY2817412 (3.0 and 30.0 mg/kg; p.o.) 30 min prior to yohimbine (1.25 mg/kg; i.p.). Reinstatement sessions started 30 min after

yohimbine administration. Experiments were carried out in a *Latin square* within-subjects counterbalanced design. A 3-day interval between drug tests, during which animals underwent to extinction sessions, was employed. Responding at the inactive lever was recorded to monitor possible non-specific behavioral effects. The self-administration training and extinction data are reported as **Supplementary Fig. 1**.

Effect of Systemic Administration of LY2817412 on Cue-Induced Reinstatement of Alcohol-Seeking in Male and Female msP Rats

The experimental procedure consisted of four phases: operant training, conditioning, extinction and reinstatement (for details, see the experimental timeline in **Fig. 2B**). Once a stable baseline ($\pm 10\%$ variability in number of reinforcements earned for at least three consecutive self-administration sessions) of responding for alcohol was reached (total number of daily sessions: 20; mean alcohol (g/kg/30min) consumed in the last 3 alcohol self-administration was 1.44 for females and 1.01 for males), male ($n = 8$) and female ($n = 10$) msP rats were trained to discriminate between 10% (v/v) alcohol and water in 30-min daily sessions, as previously described [2]. During the first three days of the conditioning phase the rats were given alcohol sessions only. Subsequently, alcohol and water sessions were conducted in random order across training days, with the constraint that all rats received a total of 10 alcohol and 10 water sessions. Discriminative stimuli (SD) predictive of alcohol (CS⁺, odor of an orange extract) versus water availability (CS⁻, odor of an anise extract) were presented during alcohol and water self-administration sessions, respectively. The olfactory stimuli were generated by depositing six to eight drops of the respective extract into the bedding of the operant chamber one minute before the beginning of the self-administration sessions and remained present throughout the 30-min sessions. In addition, each lever press resulting in the delivery of alcohol was followed by a 5-second time-out period contingently paired with the

illumination of the chamber's house light, while lever presses resulting in water delivery were accompanied by a 5-second time-out period contingently paired with a 70 dB tone. The bedding of the chamber was changed and bedding trays were cleaned between sessions.

After completion of the conditioning phase, rats were subjected to a total of 15 daily 30-min extinction sessions. During this phase, each trial began with the extension of the levers without presentation of the olfactory discriminative stimuli. Responses at the lever activated the delivery mechanism but resulted neither in the delivery of liquids nor in the trigger of response-contingent cues. Extinction training was maintained until the number of presses of the previously active lever dropped below 20 presses (average of the last three sessions).

Following completion of the extinction phase, rats were tested for 30-min reinstatement tests to evaluate the effect of the NOP antagonist LY2817412 on cue-induced reinstatement of alcohol seeking. Reinstatement sessions were conducted under initial S⁻ condition on day 1 and under the S⁺ condition on day 2, with the exception that alcohol and water were not made available. The reinstatement test under S⁺ condition was repeated every 3 days to test the effect of LY2817412 (0.0, 3.0, 10.0, and 30.0 mg/kg; p.o.) or its vehicle in a *Latin square* within-subjects counterbalanced design. The drug was given 1-hour prior to the beginning of the sessions. Between reinstatement tests, animals remained confined in their home cages. Responding at the inactive lever was recorded to monitor possible non-specific behavioral effects. Training, conditioning phase and extinction data are reported as **Supplementary Fig. 2**.

Effect of Intracranial Administration of LY2817412 on Yohimbine- and Cue-Induced Reinstatement of Alcohol-Seeking in Male and Female msP Rats

To investigate whether NOP receptor blockade in the VTA, CeA and NAc prevents yohimbine- or cue-induced reinstatement of alcohol seeking, LY2817412 (1.0, 3.0, 6.0

$\mu\text{g}/0.5\mu\text{l}/\text{rat}$) or its vehicle was bilaterally microinjected into these regions. Male and female msP rats were used [yohimbine: (male: $n = 12$ for VTA, $n = 9$ for CeA, $n = 8$ for NAc; female: $n = 9$ for VTA, $n = 9$ for CeA, $n = 9$ for NAc); cue: (male: $n = 10$ for VTA, $n = 10$ for CeA, $n = 9$ for NAc; female: $n = 10$ for VTA, $n = 10$ for CeA, $n = 10$ for NAc); saccharin: (male: $n = 13$ for VTA, $n = 12$ for CeA; female: $n = 12$ for VTA, $n = 13$ for CeA)]. The experimental procedures were identical to those described for systemic administration of LY2817412 with the exception that the NOP receptor antagonist was administered into the brain region of interest approximately 15 min prior to the reinstatement tests. In the yohimbine-induced reinstatement experiment, animals were subjected to the stereotaxic surgery for the bilateral implantation of cannulas after the completion of alcohol self-administration training (for details, see experimental timeline in **Fig. 3A**). For cue-induced reinstatement, the stereotaxic surgery occurred after the conditioning phase (for details, see experimental timeline in **Fig 4A**). Experiments were carried out in a *Latin square* within-subjects counterbalanced design. During the last 3 self-administration training days the mean values of alcohol intake ($\text{g}/\text{kg}/30\text{min}$) were: yohimbine experiment, male: 0.92 for VTA, 0.96 for CeA, 1.24 for NAc; female: 1.06 for VTA, 1.15 for CeA, 1.31 for NAc; cue experiment, male: 0.92 for VTA, 0.93 for CeA, 0.95 for NAc; female: 1.58 for VTA, 1.53 for CeA, 1.44 for NAc. For all experiments extinction data are reported as **Supplementary Fig. 3 and Fig. 4**.

Effect of Intracranial Administration of LY2817412 on Saccharin Self-Administration in Male and Female msP Rats

To investigate the effect of NOP receptor blockade on 0.2% (w/v) saccharin self-administration, LY2817412 (1.0, 3.0, 6.0 $\mu\text{g}/0.5\mu\text{l}/\text{rat}$) or its vehicle were bilaterally microinjected into the VTA and CeA. Male ($n = 13$ for VTA, $n = 12$ for CeA) and female ($n = 12$ for VTA, $n = 13$ for CeA) msP rats were used. After acquisition of saccharin self-

administration (number of sessions: 10), animals underwent to the stereotaxic surgery for the implantation of the cannulas into the VTA or CeA. After recovery from surgeries, animals were subjected to other 10 sessions of saccharin self-administration to re-establish a stable baseline of responding ($\pm 10\%$ variability in number of reinforcements earned for 3 consecutive self-administration sessions). LY2817412 or its vehicle were administered into the brain region of interest 15 min prior to the beginning of the self-administration session. Experiments were carried out in a *Latin square* within-subjects counterbalanced design. A 3-day interval between drug tests was employed. Responding at the inactive lever was recorded to monitor possible non-specific behavioral effects. Training data are reported as **Supplementary Fig. 5**.

Effect of Systemic Administration of LY2817412 on the First Extinction Day in Male and Female msP Rats

To rule out a specific effect of LY2817412 on reinstatement behavior, we evaluated its effect during the first day of extinction in rats previously trained to alcohol self-administration. Male ($n = 8$) and female ($n = 7$) genetically selected alcohol-preferring msP rats were used. Once a stable alcohol self-administration baseline was established rats were subjected to a 30-min extinction session under conditions identical to those of the alcohol self-administration phase, except that alcohol was not made available. On the test days, animals were injected with either vehicle or LY2817412 (10.0 mg/kg, p.o.) 1 hour before the extinction session. Experiments were carried out in a *Latin square* within-subjects counterbalanced design. At least 3-days intervene between drug tests. The day after the test rats remained in their home cage whereas in the second and third days a baseline of alcohol self-administration was re-established. Active and inactive lever responses were recorded throughout the experiment. Data are reported as **Supplementary Fig. 6**.

Histological Verification of Correct Cannula Placement

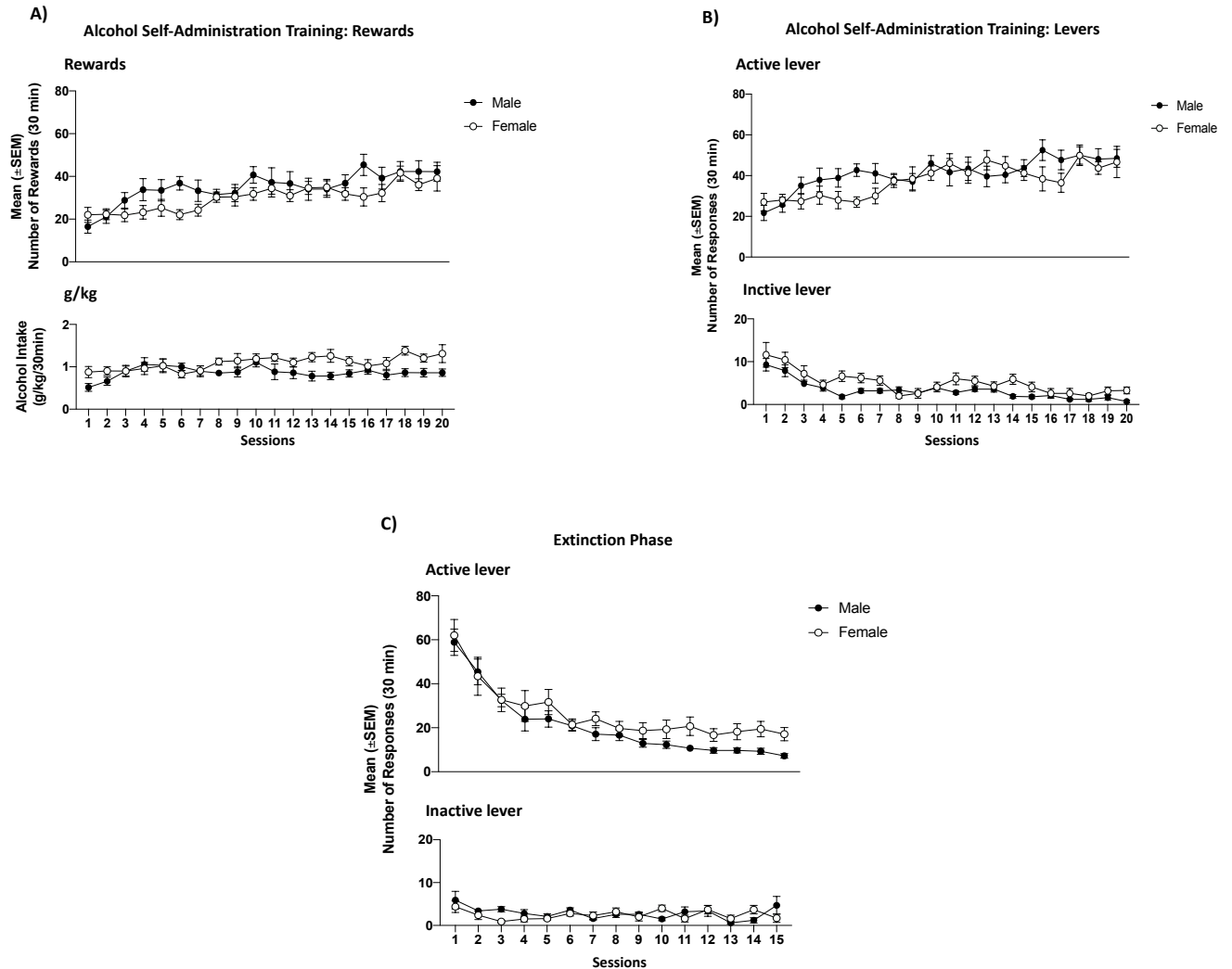
Upon completion of the experiments, the rats were anesthetized with isoflurane, and black India ink (0.3 μ l per site) was injected into the studied brain areas. The rats were then immediately euthanized to remove the brain and histologically analyse the cannula placements (see **Supplementary Information**). Only data from rats with correct cannula placements were included in the statistical analysis [yohimbine: (male: $n = 9$ for VTA, $n = 7$ for CeA, $n = 7$ for NAc; female: $n = 7$ for VTA, $n = 7$ for CeA, $n = 8$ for NAc); cue: (male: $n = 7$ for VTA, $n = 7$ for CeA, $n = 8$ for NAc); saccharin: (male: $n = 10$ for VTA; $n = 10$ for CeA; female: $n = 9$ for VTA; $n = 9$ for CeA)].

Statistical Analysis

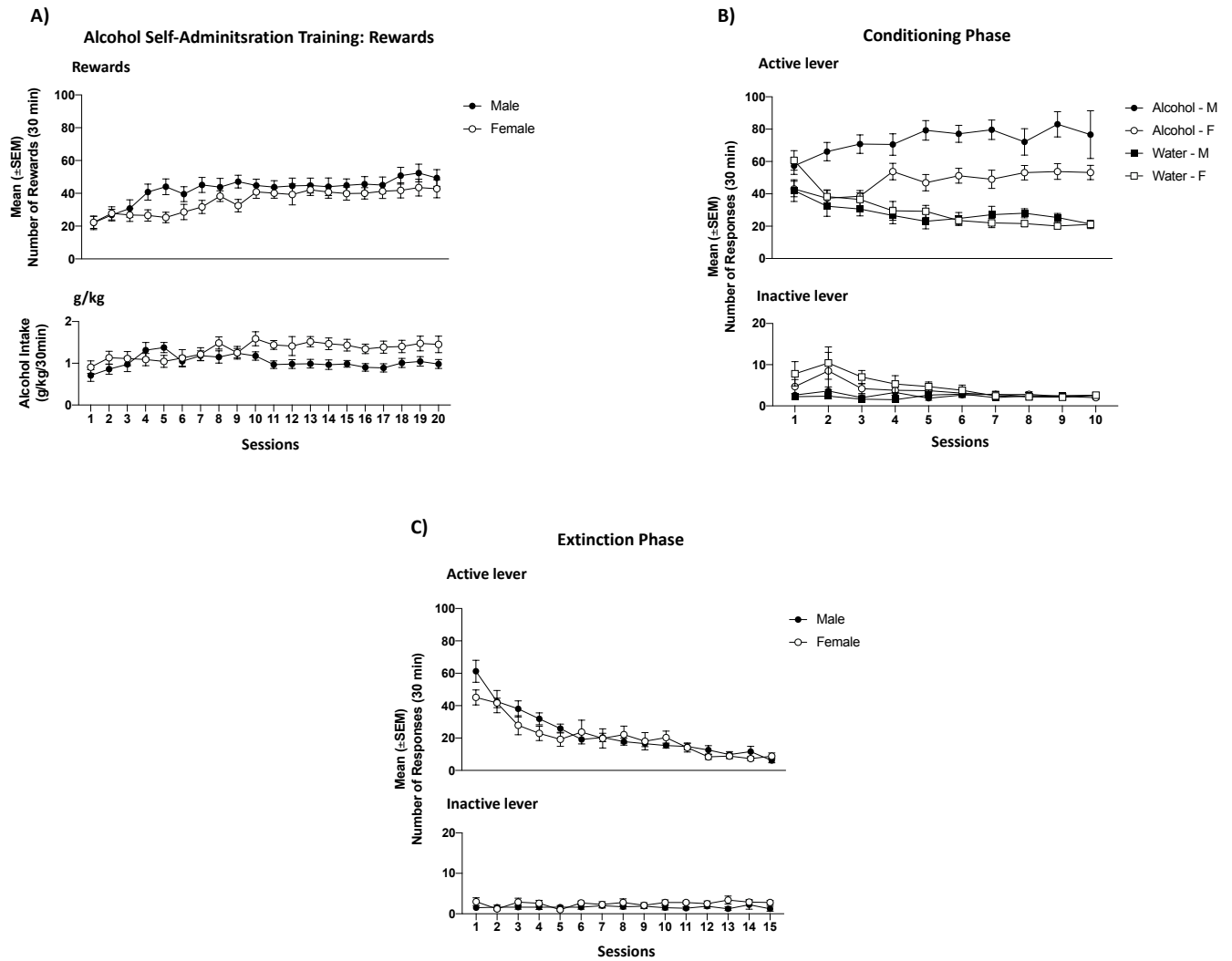
For alcohol and saccharin self-administration active or inactive lever were analyzed using a two-way ANOVA with ‘sex’ as a between-subjects factor and ‘time’ as a within-subject factor. During the conditioning phase for cue-induced reinstatement, lever responding for alcohol and water on the last 3 days was assessed by using a three-way ANOVA with ‘sex’ (male vs. female) as a between-subjects factor, ‘time (days)’ and ‘cues condition’ (S^+/CS^+ vs. S^-/CS^-) as within-subject factors. Differences between responding during the extinction (EXT) and reinstatement (VEH) sessions in the vehicle-treated group and the effect of systemic and intracranial administration of LY2817412 on yohimbine- and cue-induced reinstatement were analysed separately as two different phases using a two-way ANOVA with ‘sex’ as a between-subjects factor and ‘reinstatement’ or ‘treatment’ as a within-subject factor. All the analysis described were conducted both for the active and inactive lever. Data represent the mean \pm SEM. The level of significance was set at $p < 0.05$. Newman-Keuls *post hoc* test was used

where appropriate. The statistical analyses were performed using Prism 7 software (GraphPad Prism, La Jolla, CA, USA).

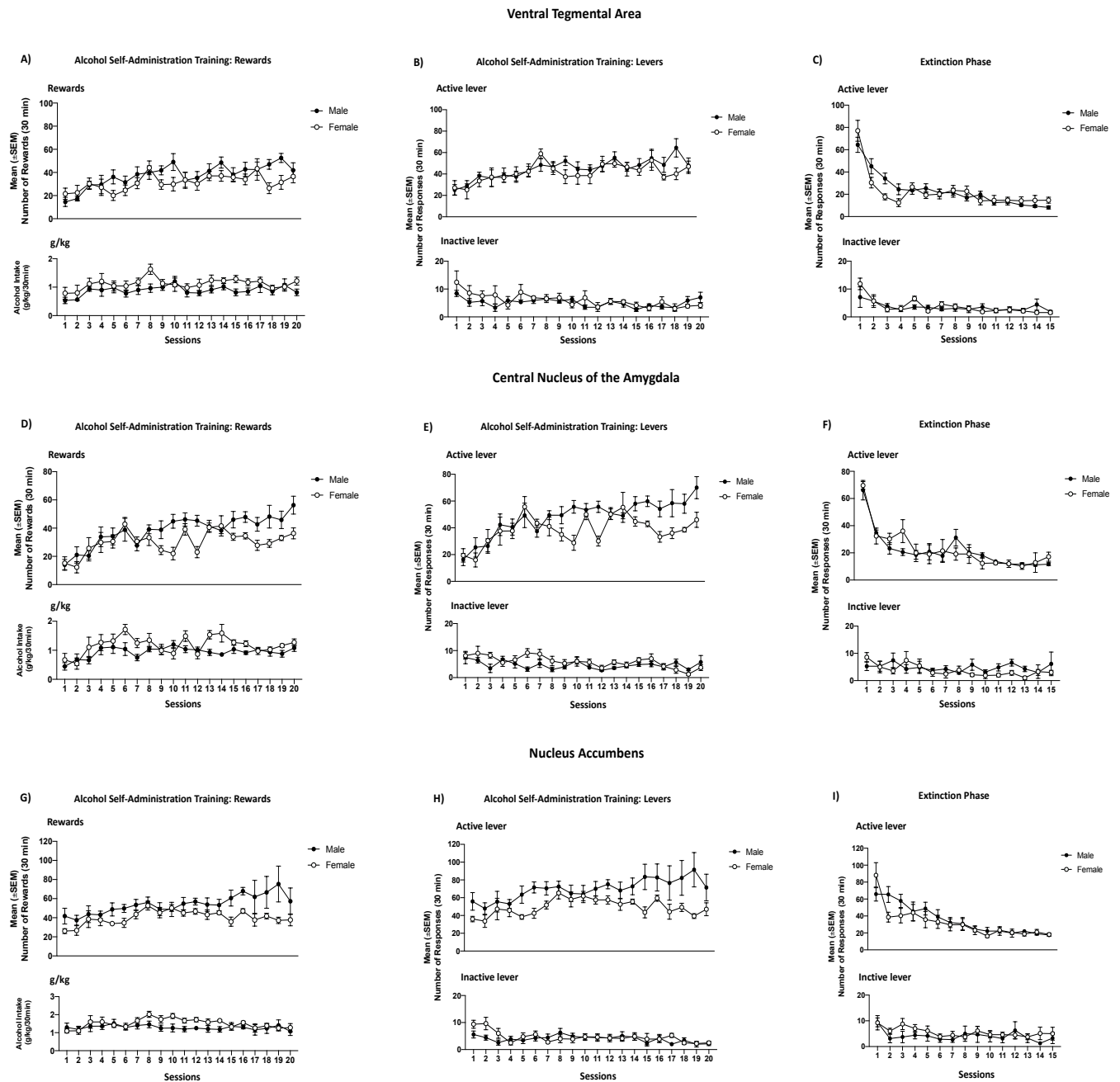
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Male ($n = 10$) and female ($n = 10$) msP rats received 20-daily alcohol self-administration sessions and 15-daily extinction sessions before testing systemic LY2817412 (0.0, 3.0 and 30.0 mg/kg, p.o.) on reinstatement induced by 1.25 mg/kg of yohimbine (**Experiment 1**). **A.** Number of rewards (upper panel) and g/kg of alcohol intake (lower panel) in 30-min daily session during alcohol self-administration training. **B.** Number of active (upper panel) and inactive (lower panel) lever presses in 30-min daily session during alcohol self-administration training. **C.** Number of active (upper panel) and inactive (lower panel) lever presses in 30-min session during extinction phase. Values represent the mean (\pm SEM). Statistical analyses are reported in **Table 1**.

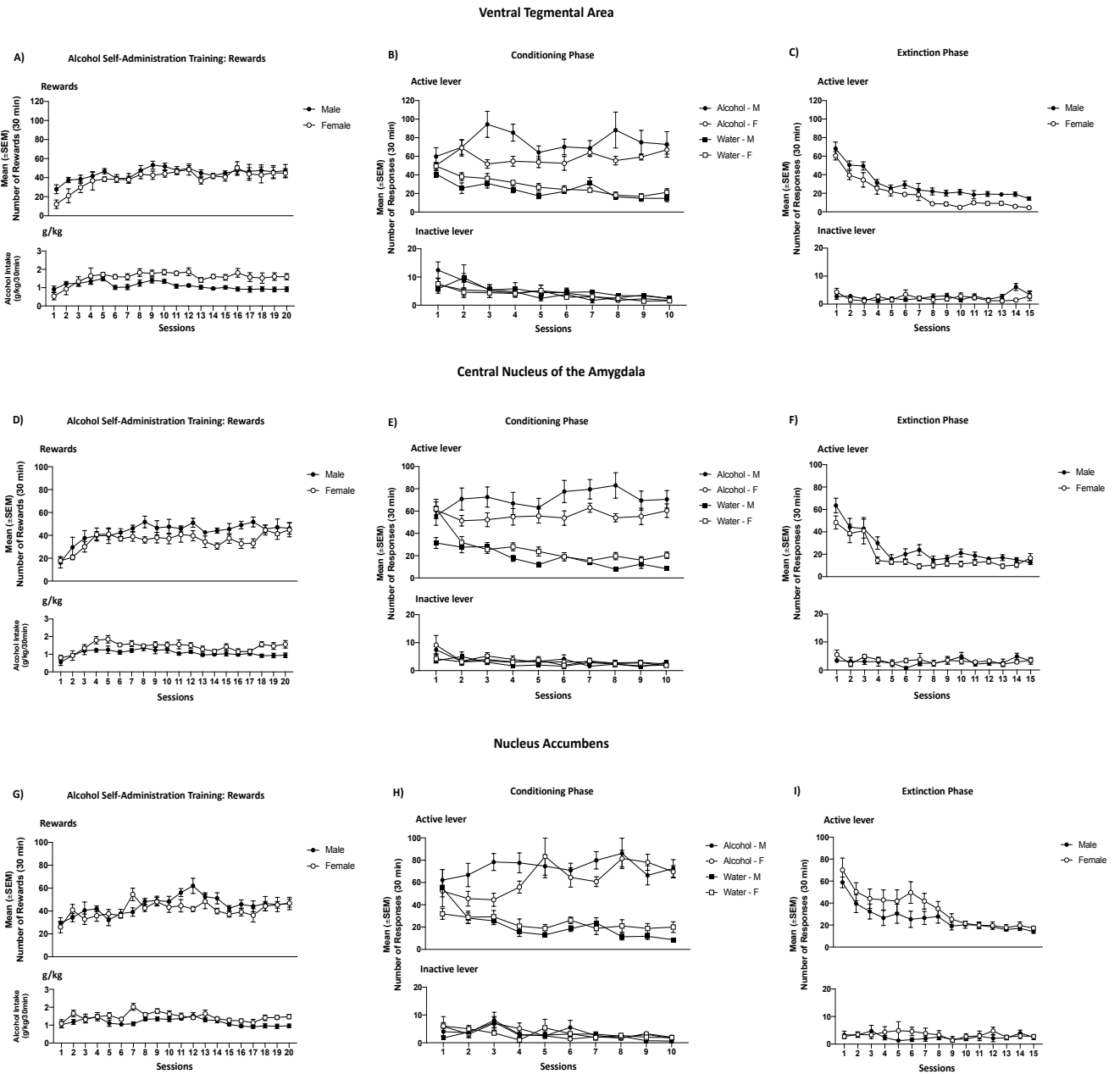


Supplementary Figure 2. Male ($n = 8$) and female ($n = 10$) msP rats received 20-daily alcohol self-administration (A), 20-daily conditioning sessions (10 alcohol sessions and 10 water sessions) (B) and 15-daily extinction sessions (C) before testing systemic LY2817412 (0.0, 3.0, 10.0 and 30.0 mg/kg, p.o.) effects on cue-induced reinstatement (Experiment 2). A. Number of rewards (upper panel) and g/kg of alcohol intake (lower panel) in 30-min session during alcohol self-administration training. B. Number of active (upper panel) and inactive (lower panel) lever presses in 30-min session during the conditioning phase. C. Number of active (upper panel) and inactive (lower panel) lever presses in 30-min session during extinction phase. Values represent the mean (\pm SEM). Statistical analyses are reported in Table 2.



Supplementary Figure 3. Male and female msP rats were implanted with bilateral cannulas aimed at the VTA (A-C), CeA (D-F) and NAc (G-I). All experimental groups received 20-daily alcohol self-administration sessions and 15-daily extinction sessions before receiving LY2817412 (1.0, 3.0, 6.0 $\mu\text{g}/0.5\mu\text{l}/\text{rat}$) or its vehicle in the brain regions of interest on reinstatement induced by 1.25 mg/kg of yohimbine (Experiment 3). A, D, G. Number of rewards (upper panel) and g/kg of alcohol intake (lower panel) in 30-min daily alcohol self-administration training sessions. B, E, H. Number of active (upper panel) and inactive (lower panel) lever presses in 30-min session during alcohol self-administration training. C, F, I.

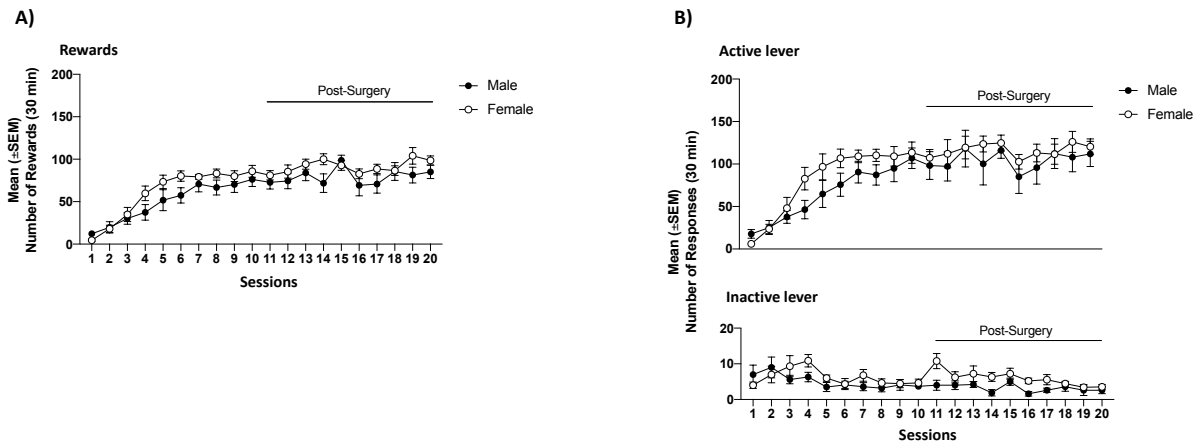
Number of active (upper panel) and inactive (lower panel) lever presses in 30-min session during extinction phase. Male: $n = 9$ for VTA, $n = 7$ for CeA, $n = 7$ for NAc; female: $n = 7$ for VTA, $n = 7$ for CeA, $n = 8$ for NAc. Values represent the mean (\pm SEM). Statistical analyses are reported in **Table 3**.



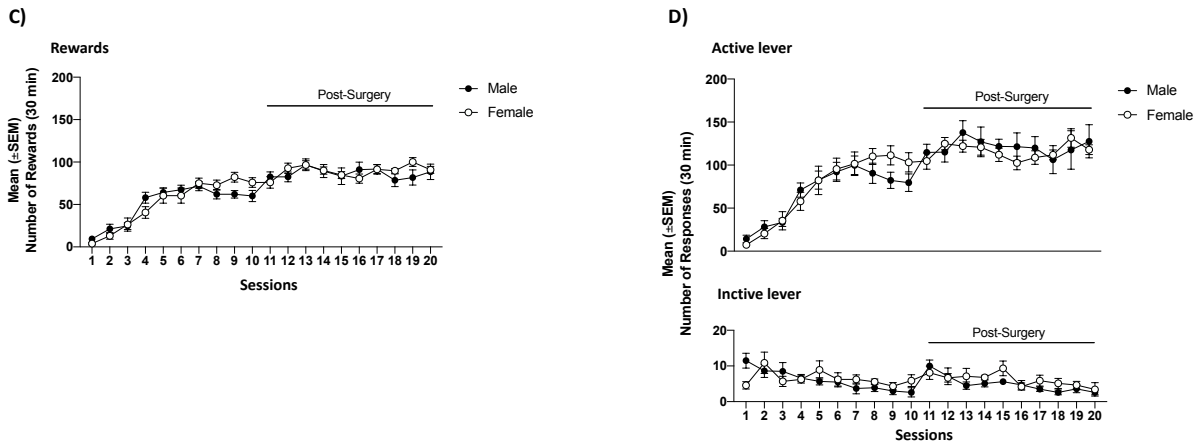
Supplementary Figure 4. Male and female msP rats were implanted with bilateral cannulas aimed at the VTA (A-C), CeA (D-F) and NAc (G-I). All experimental groups received 20-daily alcohol self-administration (A, D, G), 20-daily discrimination sessions (10 alcohol sessions and 10 water sessions; B, E, H) followed by 15-daily extinction sessions (C, F, I) before testing LY2817412 (1.0, 3.0, 6.0 $\mu\text{g}/0.5\mu\text{l}/\text{rat}$) in the brain regions of interest on cue-induced reinstatement (**Experiment 3**). A, D, G. Number of rewards (upper panel) and g/kg of alcohol intake (lower panel) in 30-min session during alcohol self-administration training. B, E, H. Number of active (upper panel) and inactive (lower panel) lever presses in 30-min session

during the conditioning phase. **C, F, I.** Number of active (upper panel) and inactive (lower panel) lever presses in 30-min session during extinction phase. Male: $n = 7$ for VTA, $n = 7$ for CeA, $n = 8$ for NAc; female: $n = 7$ for VTA, $n = 8$ for CeA, $n = 8$ for NAc. Values represent the mean (\pm SEM). Statistical analyses are reported in **Table 4**.

Ventral Tegmental Area

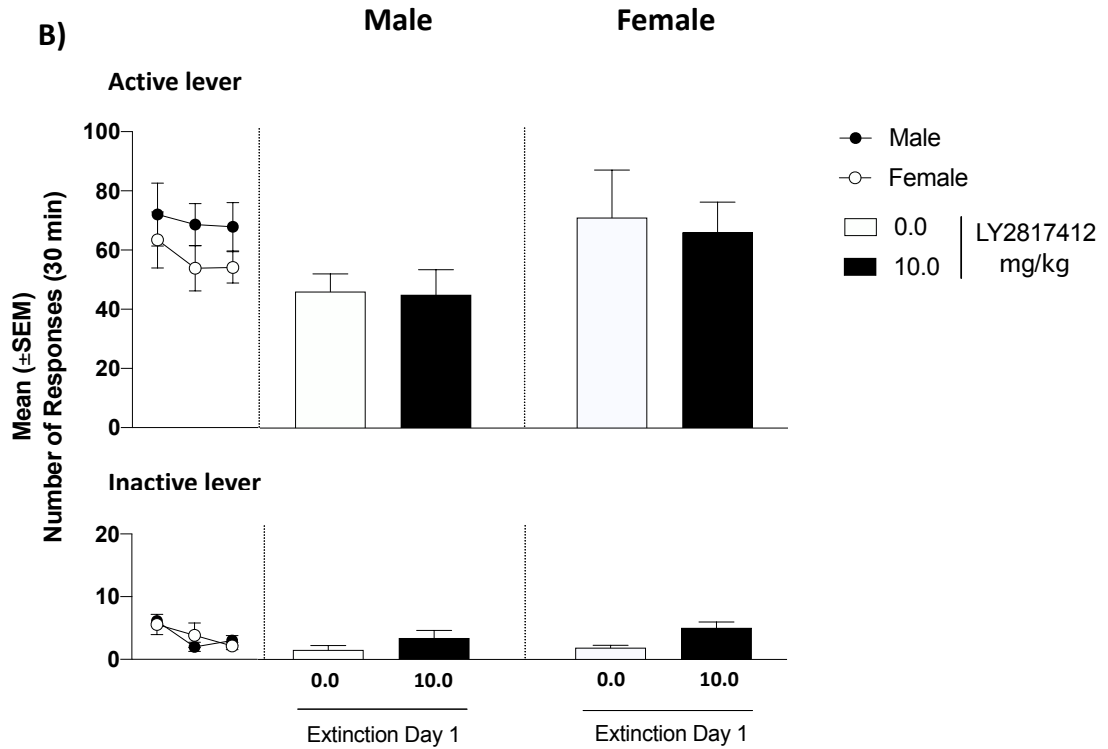
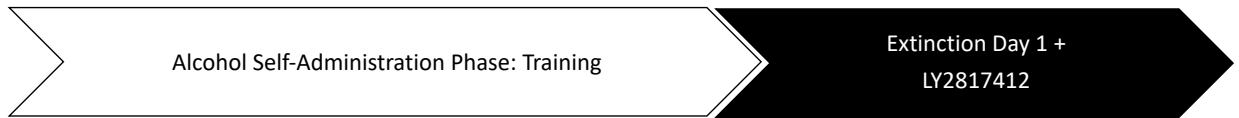


Central Nucleus of the Amygdala



Supplementary Figure 5. Male and female msP rats were implanted with bilateral cannulas aimed at the VTA (A-B) and the CeA (C-D). All experimental groups received 20-daily sessions of saccharin self-administration before testing LY2817412 (1.0, 3.0, 6.0 μ g/0.5 μ l/rat) in the brain regions of interest on saccharin self-administration (**Experiment 5**). **A, C.** Number of rewards in 30-min daily session during saccharin self-administration training. **B, D.** Number of active (upper panel) and inactive (lower panel) lever presses in 30-min session during saccharin self-administration training. Male: $n = 10$ for VTA; $n = 10$ for CeA; female: $n = 9$ for VTA; $n = 9$ for CeA. Values represent the mean (\pm SEM). Statistical analyses are reported in **Table 5**.

A)



Supplementary Figure 6. Effect of Systemic Administration of LY2817412 on the First Extinction Day in Male and Female msP Rats.

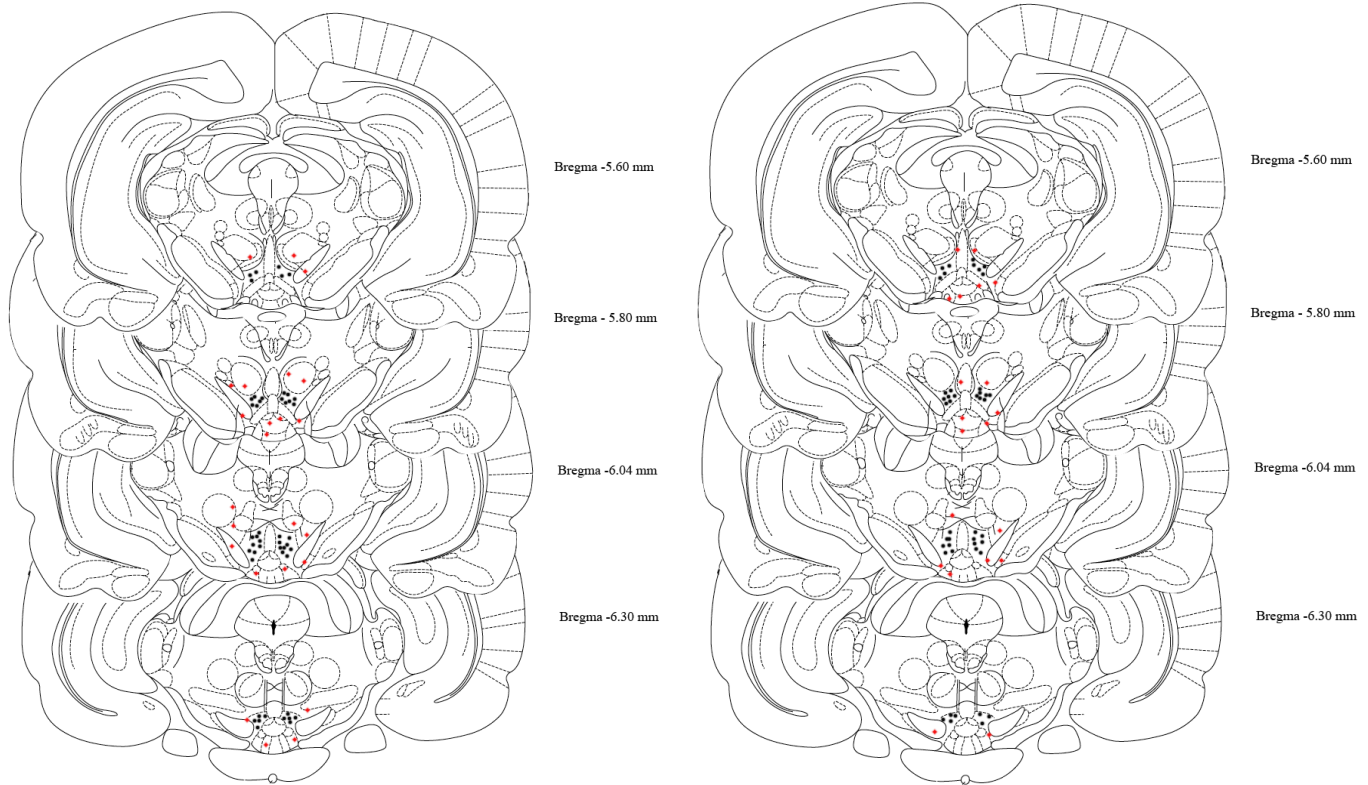
A. Schematic representation of the experimental timeline: male ($n = 8$) and female ($n = 7$) msP rats were trained to self-administer alcohol and then tested to evaluate the effect of LY2817412 (0.0 and 10.0 mg/kg, p.o.) on the first extinction day. Tests were carried out in a *Latin square* within-subjects counterbalanced design. **B.** Self-administration: black circle (male) and white circle (female) represent the mean number of the responses during the last 3 days of alcohol self-administration. No differences were denoted in the number of active or inactive lever presses during this phase. LY2817412 pretreatment (10.0 mg/kg, p.o.) on the first day of extinction did not modify active or inactive lever presses both in male and female rats. Active and inactive lever presses are presented in the upper and lower panels, respectively. Values represent the mean (\pm SEM).

Ventral Tegmental Area

Yohimbine-induced reinstatement

Male msP rats

Female msP rats

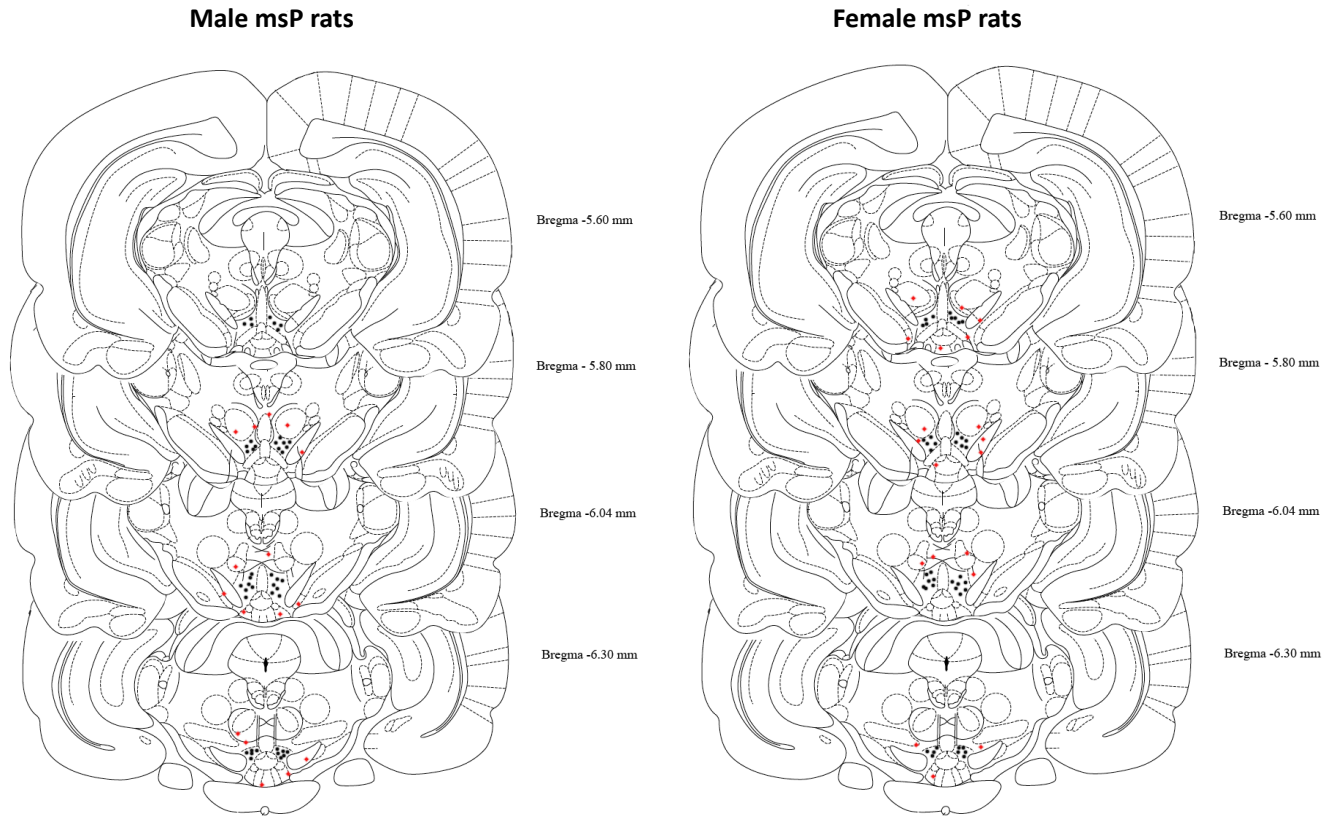


Supplementary Figure 7. Schematic representation of intra-VTA sites of injection in the yohimbine-induced reinstatement test assessed by histological analysis.

Male ($n = 12$) and female ($n = 9$) msP rats were implanted with bilateral cannulas aimed at the VTA and then subjected to yohimbine-induced reinstatement test. Black dots represent the correct cannula placement ($n = 9$ male; $n = 7$ female). Red dots indicate animals excluded for the incorrect cannula placement. Only data resulting from correct cannula placement were included in the statistical analysis.

Ventral Tegmental Area

Cue-induced reinstatement

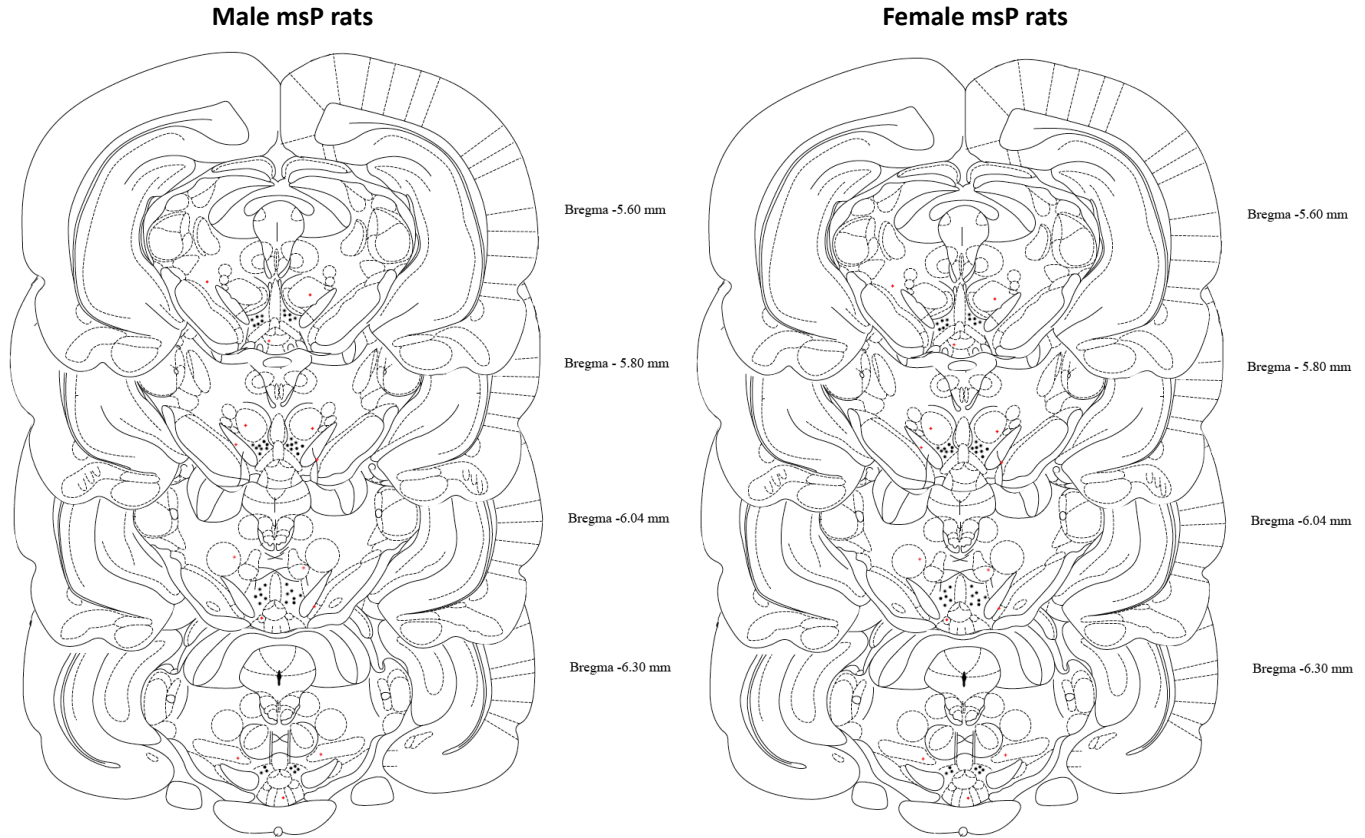


Supplementary Figure 8. Schematic representation of intra-VTA sites of injection in the cue-induced reinstatement test assessed by histological analysis.

Male ($n = 10$) and female ($n = 10$) msP rats were implanted with bilateral cannulas aimed at the VTA and then subjected to yohimbine-induced reinstatement test. Black dots represent the correct cannula placement ($n = 7$ male; $n = 7$ female). Red dots indicate animals excluded for the incorrect cannula placement. Only data resulting from correct cannula placement were included in the statistical analysis.

Ventral Tegmental Area

Saccharin Self-Administration



Supplementary Figure 9. Schematic representation of intra-VTA sites of injection in the saccharin self-administration test assessed by histological analysis.

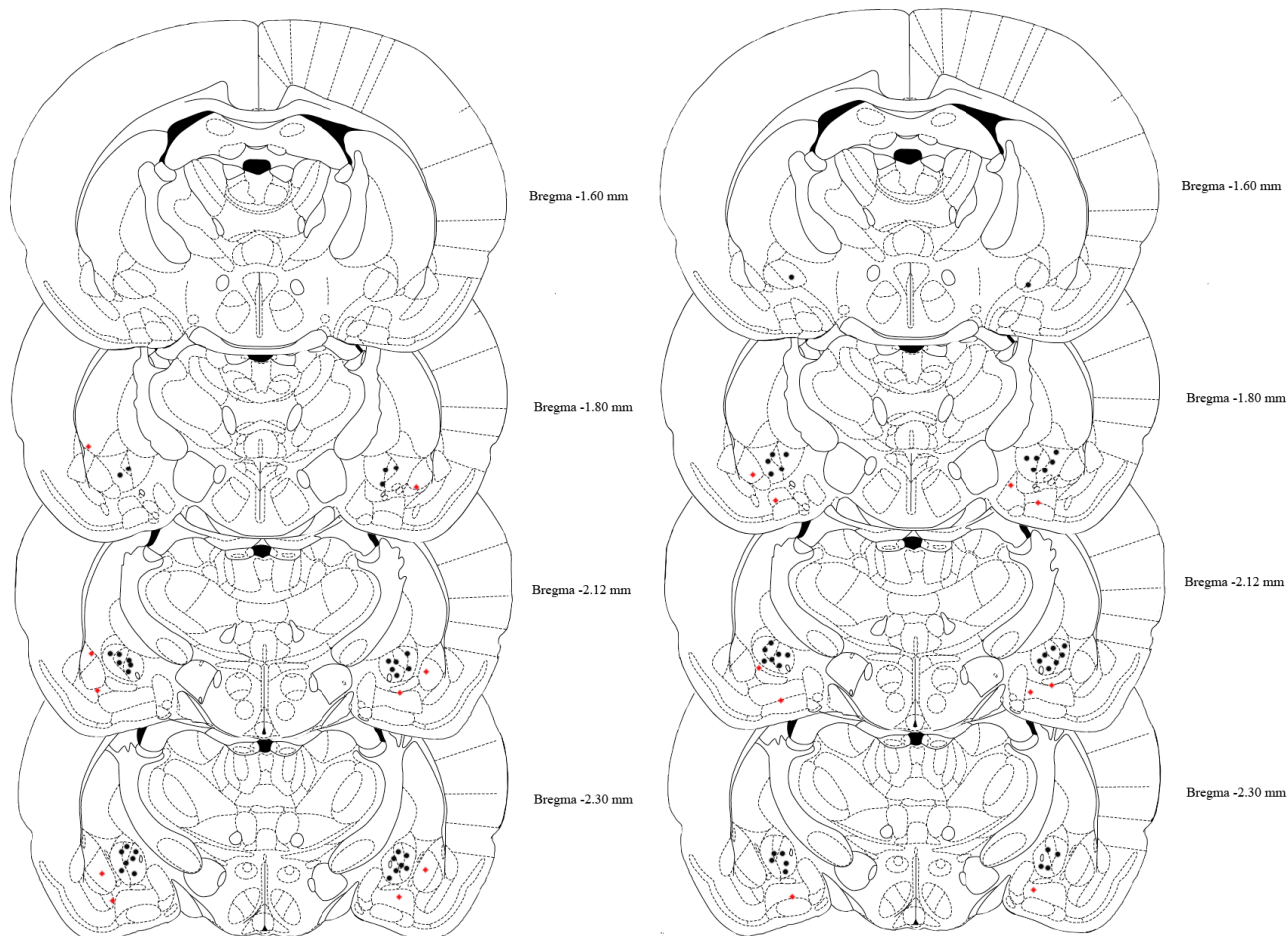
Male ($n = 13$) and female ($n = 12$) msP rats were implanted with bilateral cannulas aimed at the VTA and then subjected to yohimbine-induced reinstatement test. Black dots represent the correct cannula placement ($n = 10$ male; $n = 9$ female). Red dots indicate animals excluded for the incorrect cannula placement. Only data resulting from correct cannula placement were included in the statistical analysis.

Central Nucleus of the Amygdala

Yohimbine-induced reinstatement

Male msP rats

Female msP rats

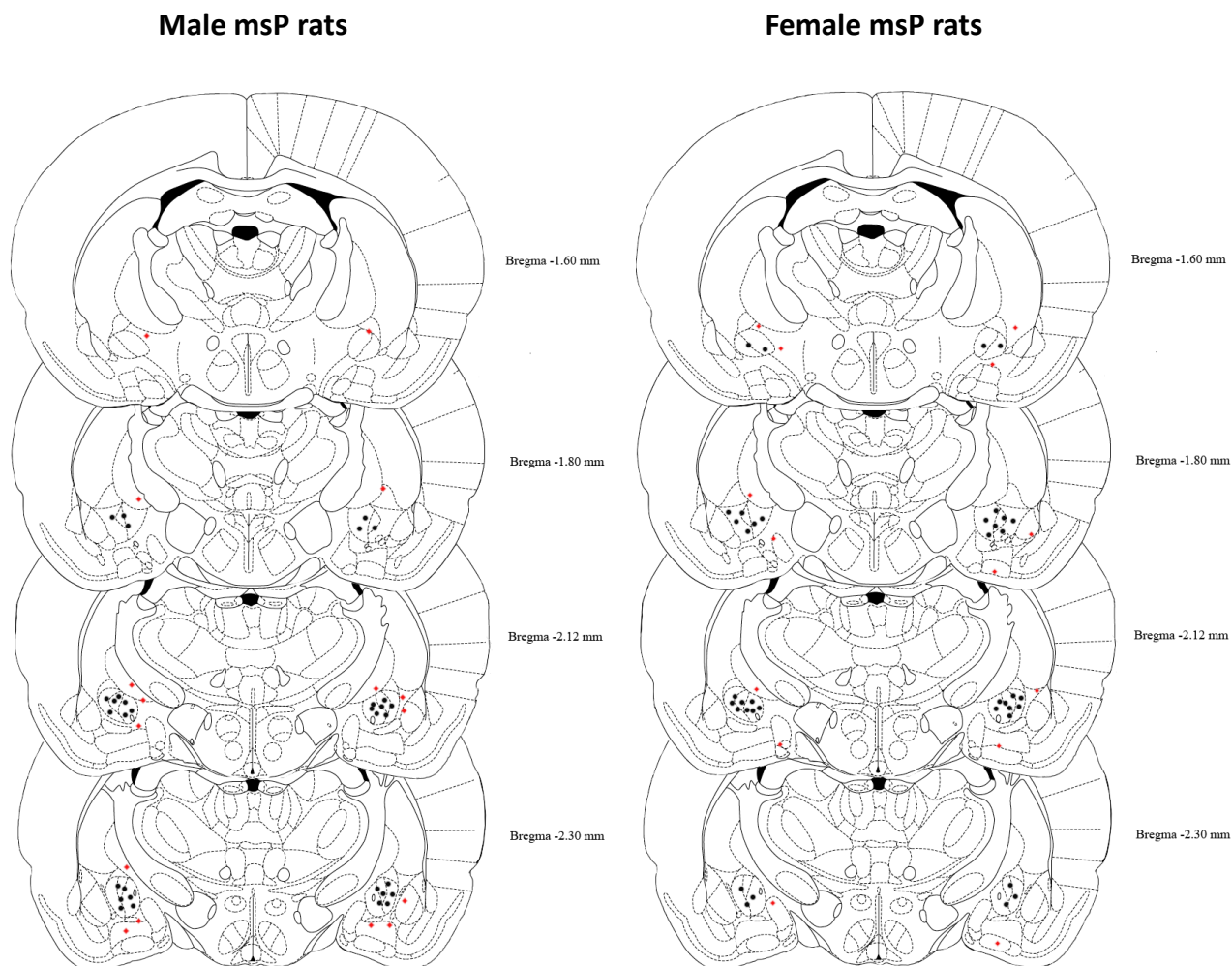


Supplementary Figure 10. Schematic representation of intra-CeA sites of injection in the yohimbine-induced reinstatement test assessed by histological analysis.

Male ($n = 9$) and female ($n = 9$) msP rats were implanted with bilateral cannulas aimed at the CeA and then subjected to yohimbine-induced reinstatement test. Black dots represent the correct cannula placement ($n = 7$ male; $n = 7$ female). Red dots indicate animals excluded for the incorrect cannula placement. Only data resulting from correct cannula placement were included in the statistical analysis.

Central Nucleus of the Amygdala

Cue-induced reinstatement

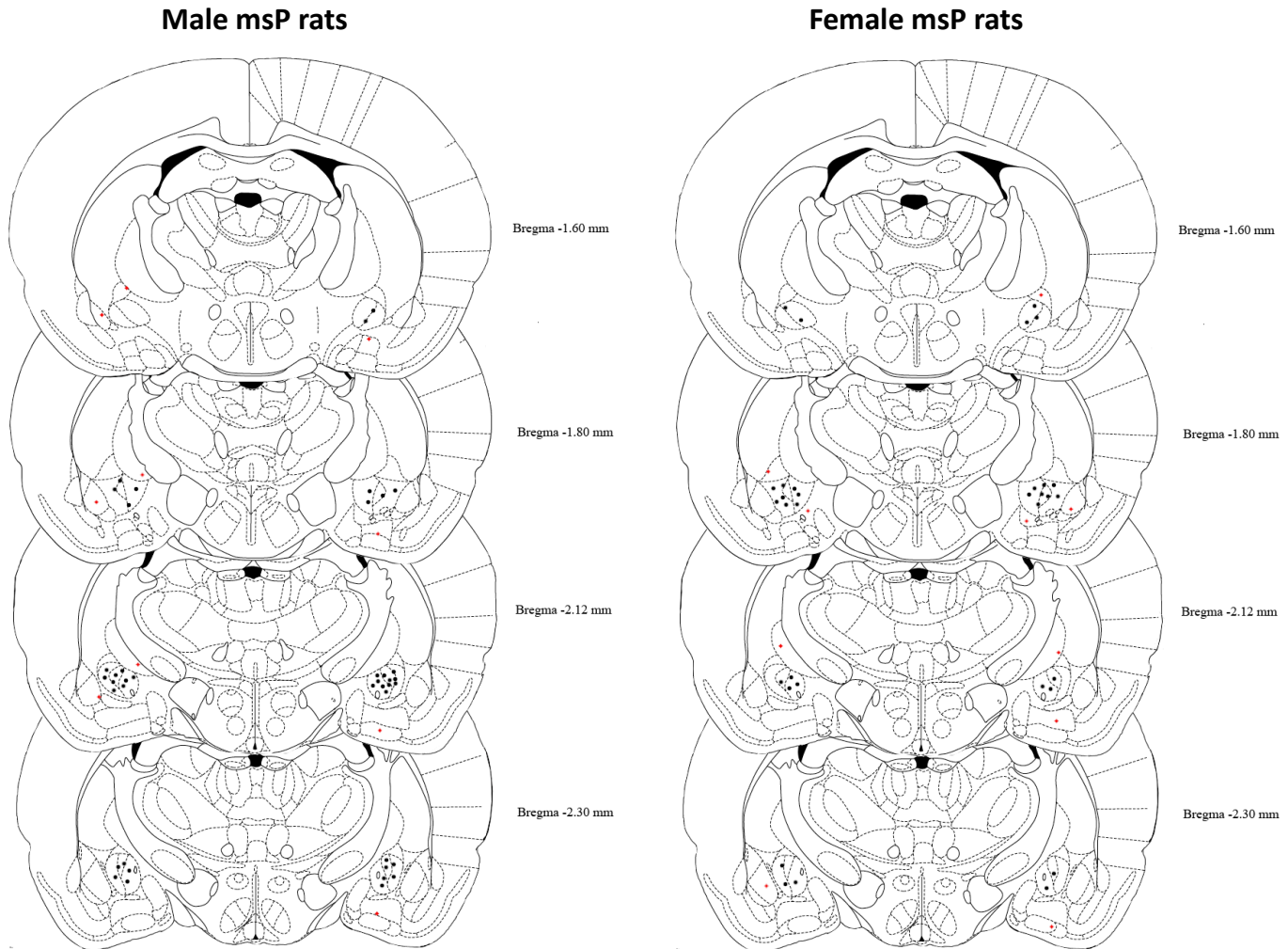


Supplementary Figure 11. Schematic representation of intra-CeA sites of injection in the cue-induced reinstatement test assessed by histological analysis.

Male ($n = 10$) and female ($n = 10$) msP rats were implanted with bilateral cannulas aimed at the CeA and then subjected to yohimbine-induced reinstatement test. Black dots represent the correct cannula placement ($n = 7$ male; $n = 8$ female). Red dots indicate animals excluded for the incorrect cannula placement. Only data resulting from correct cannula placement were included in the statistical analysis.

Central Nucleus of the Amygdala

Saccharin Self-Administration

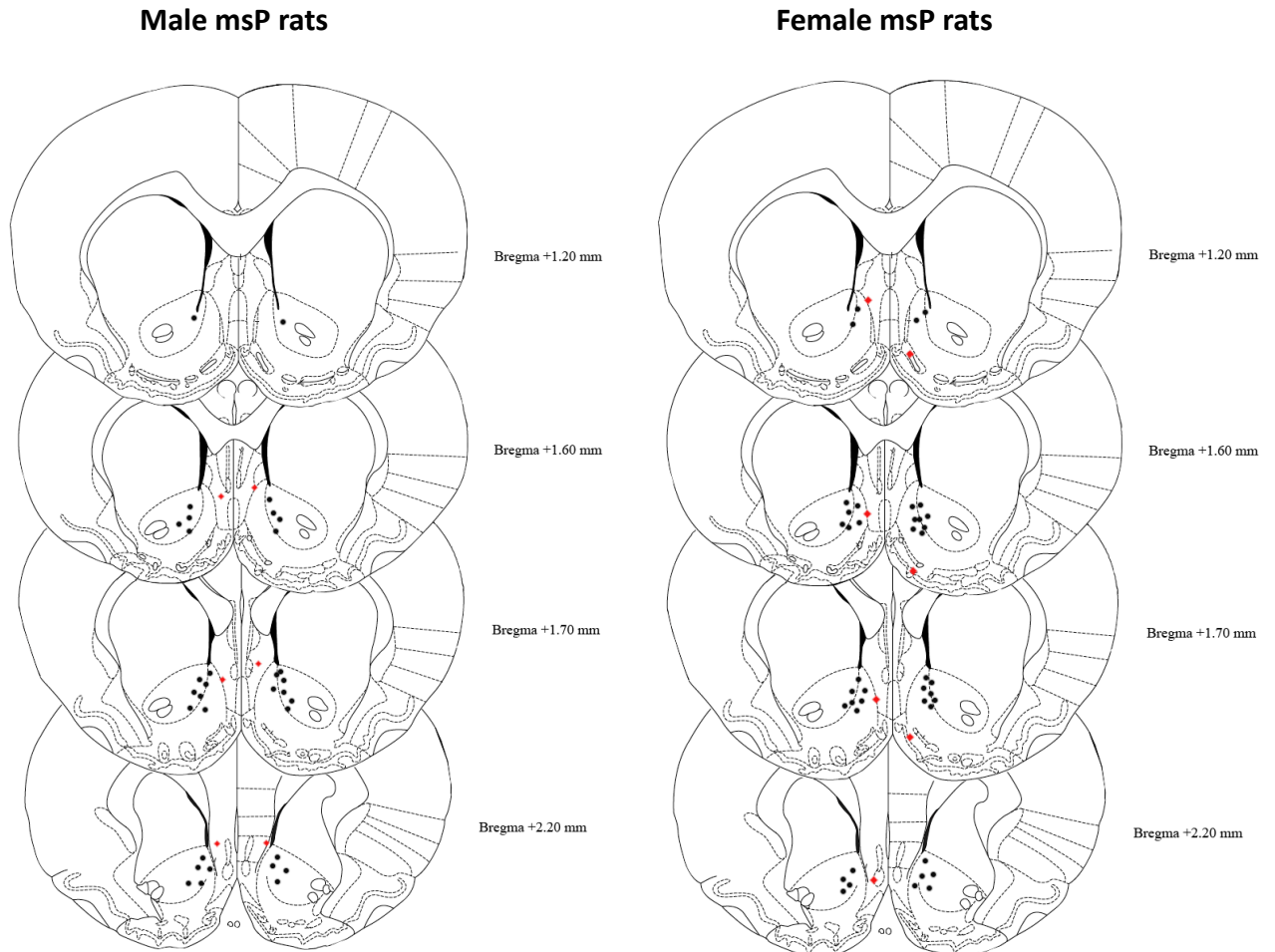


Supplementary Figure 12. Schematic representation of intra-CeA sites of injection in the saccharin self-administration test assessed by histological analysis.

Male ($n = 12$) and female ($n = 13$) msP rats were implanted with bilateral cannulas aimed at the CeA and then subjected to saccharin self-administration. Black dots represent the correct cannula placement ($n = 10$ male; $n = 9$ female). Red dots indicate animals excluded for the incorrect cannula placement. Only data resulting from correct cannula placement were included in the statistical analysis.

Nucleus Accumbens

Yohimbine-induced reinstatement

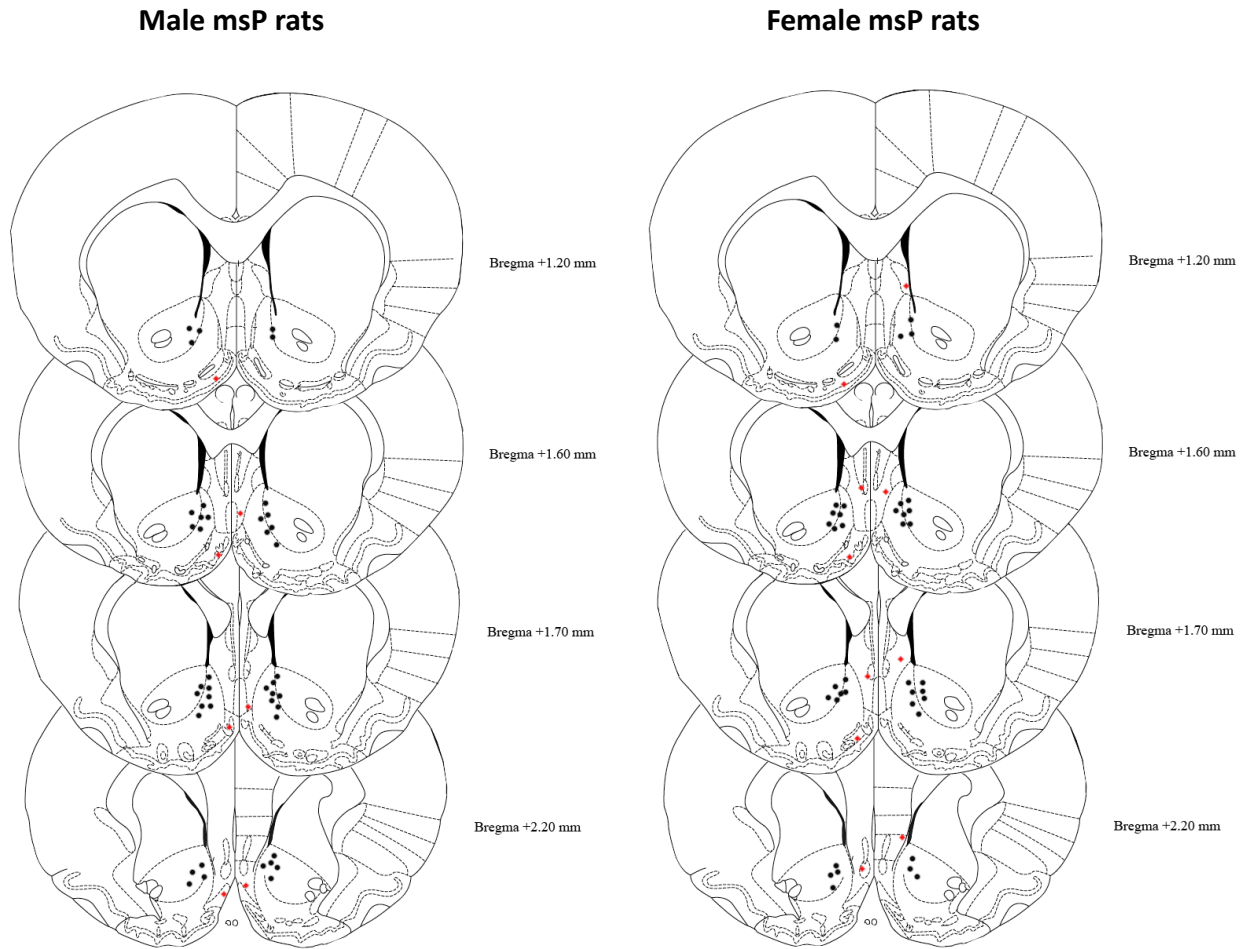


Supplementary Figure 13. Schematic representation of intra-NAc sites of injection in the yohimbine-induced reinstatement test assessed by histological analysis.

Male ($n = 8$) and female ($n = 9$) msP rats were implanted with bilateral cannulas aimed at the NAc and then subjected to yohimbine-induced reinstatement test. Black dots represent the correct cannula placement ($n = 7$ male; $n = 8$ female). Red dots indicate animals excluded for the incorrect cannula placement. Only data resulting from correct cannula placement were included in the statistical analysis.

Nucleus Accumbens

Cue-induced reinstatement



Supplementary Figure 14. Schematic representation of intra-NAc sites of injection in the cue-induced reinstatement test assessed by histological analysis.

Male ($n = 9$) and female ($n = 10$) msP rats were implanted with bilateral cannulas aimed at the NAc and then subjected to yohimbine-induced reinstatement test. Black dots represent the correct cannula placement ($n = 8$ male; $n = 8$ female). Red dots indicate animals excluded for the incorrect cannula placement. Only data resulting from correct cannula placement were included in the statistical analysis.

Table 1. Statistical analysis of alcohol self-administration training (rewards, g/kg, active/inactive lever) and extinction phase (active/inactive lever) for the systemic effect of LY2817412 on yohimbine-induced reinstatement (**Experiment 1**). Two-way ANOVA with ‘sex’ as a between-subjects factor and ‘time’ as a within-subject factor for all the phases.

Number of Figure	Behavior	Statistic
Figure S1A	Rewards	Sex: $F_{(1, 18)} = 3.82, p > 0.05$ Time: $F_{(19, 342)} = 5.26, p < 0.001$ Sex x Time: $F_{(19, 342)} = 1.06, p > 0.05$
Figure S1A	g/kg	Sex: $F_{(1, 18)} = 6.53, p < 0.05$ Time: $F_{(19, 342)} = 1.91, p < 0.05$ Sex x Time: $F_{(19, 342)} = 1.68, p < 0.05^{\$}$
Figure S1B	Active lever	Sex: $F_{(1, 18)} = 1.23, p > 0.05$ Time: $F_{(19, 342)} = 5.63, p < 0.001$ Sex x Time: $F_{(19, 342)} = 1.38, p > 0.05$
Figure S1B	Inactive lever	Sex: $F_{(1, 18)} = 16.29, p < 0.001$ Time: $F_{(19, 342)} = 10.02, p < 0.001$ Sex x Time: $F_{(19, 342)} = 1.05, p > 0.05$
Figure S1C	Extinction Active Lever	Sex: $F_{(1, 18)} = 2.90, p > 0.05$ Time: $F_{(14, 252)} = 29.28, p < 0.001$ Sex x Time: $F_{(14, 252)} = 0.58, p > 0.05$
Figure S1C	Extinction Inactive Lever	Sex: $F_{(1, 18)} = 0.50, p > 0.05$ Time: $F_{(14, 252)} = 2.18, p < 0.01$ Sex x Time: $F_{(14, 252)} = 1.77, p < 0.05^{\$}$

$\$$: no relevant differences in post-hoc analysis.

Table 2. Statistical analysis of alcohol self-administration training (rewards, g/kg), conditioning phase (active/inactive lever) and extinction phase (active/inactive lever) for the systemic effect of LY2817412 on cue-induced reinstatement (**Experiment 2**). Two-way ANOVA with ‘sex’ as a between-subjects factor and ‘time’ as a within-subject factor for rewards, g/kg and, extinction phases; three-way ANOVA with sex as a between-subjects factor, time and cues condition (associated with drugs availability: alcohol vs. water) as within-subject factors.

Number of Figure	Behavior	Statistic
Figure S2A	Rewards	Sex: $F_{(1, 16)} = 15.19, p < 0.05$ Time: $F_{(19, 304)} = 4.97, p < 0.001$ Sex x Time: $F_{(19, 304)} = 0.72, p > 0.05$
Figure S2A	g/kg	Sex: $F_{(1, 16)} = 11.12, p < 0.01$ Time: $F_{(19, 304)} = 1.54, p > 0.05$ Sex x Time: $F_{(19, 304)} = 1.59, p > 0.05$
Figure S2B	Conditioning Phase Active Lever	Sex: $F_{(1, 16)} = 23.71, p < 0.001$ Time: $F_{(9, 144)} = 39.21, p < 0.001$ Sex x Time: $F_{(9, 144)} = 7.07, p < 0.001$ Drugs: $F_{(1, 16)} = 0.02, p > 0.05$ Drugs x Sex: $F_{(1, 16)} = 0.024, p > 0.05$ Time x Drugs: $F_{(9, 144)} = 1.97, p < 0.05$ Time x Drugs x Sex $F_{(9, 144)} = 1.39, p > 0.05$
Figure S2B	Conditioning Phase Inactive Lever	Sex: $F_{(1, 16)} = 2.72, p > 0.05$ Time: $F_{(9, 144)} = 1.31, p > 0.05$ Sex x Time: $F_{(9, 144)} = 1.88, p > 0.05$ Drugs: $F_{(1, 16)} = 0.03, p > 0.05$ Drugs x Sex: $F_{(1, 16)} = 1.61, p > 0.05$ Time x Drugs: $F_{(9, 144)} = 0.29, p > 0.05$ Time x Drugs x Sex $F_{(9, 144)} = 0.23, p > 0.05$
Figure S2C	Extinction Active Lever	Sex: $F_{(1, 16)} = 0.63, p > 0.05$ Time: $F_{(14, 224)} = 24.64, p < 0.001$ Sex x Time: $F_{(14, 224)} = 1.39, p > 0.05$
Figure S2C	Extinction Inactive Lever	Sex: $F_{(1, 16)} = 13.15, p < 0.01$ Time: $F_{(14, 224)} = 0.50, p > 0.05$ Sex x Time: $F_{(14, 224)} = 0.67, p > 0.05$

Table 3. Statistical analysis of alcohol self-administration training (rewards, g/kg, active/inactive lever) and extinction phase (active/inactive lever) for the intra-VTA, intra-CeA and intra-NAc LY2817412 infusions on yohimbine-induced reinstatement (**Experiment 3**). Two-way ANOVA with ‘sex’ as a between-subjects factor and ‘time’ as a within-subject factor for all the phases.

Number of Figure	Behavior	Statistic
Figure S3A	Rewards	Sex: $F_{(1, 14)} = 2.40, p > 0.05$ Time: $F_{(19, 266)} = 3.59, p < 0.001$ Sex x Time: $F_{(19, 266)} = 0.87, p > 0.05$
Figure S3A	g/kg	Sex: $F_{(1, 14)} = 5.22, p < 0.05$ Time: $F_{(19, 266)} = 2.00, p < 0.01$ Sex x Time: $F_{(19, 266)} = 0.70, p > 0.05$
Figure S3B	Active lever	Sex: $F_{(1, 14)} = 0.75, p > 0.05$ Time: $F_{(19, 266)} = 3.74, p < 0.001$ Sex x Time: $F_{(19, 266)} = 0.81, p > 0.05$
Figure S3B	Inactive lever	Sex: $F_{(1, 14)} = 1.14, p > 0.05$ Time: $F_{(19, 266)} = 3.17, p < 0.001$ Sex x Time: $F_{(19, 266)} = 1.29, p > 0.05$
Figure S3C	Extinction Active Lever	Sex: $F_{(1, 14)} = 0.12, p > 0.05$ Time: $F_{(14, 196)} = 29.23, p < 0.001$ Sex x Time: $F_{(14, 252)} = 1.30, p > 0.05$
Figure S3C	Extinction Inactive Lever	Sex: $F_{(1, 14)} = 0.08, p > 0.05$ Time: $F_{(14, 196)} = 4.66, p < 0.001$ Sex x Time: $F_{(14, 196)} = 1.10, p > 0.05$
Figure S3D	Rewards	Sex: $F_{(1, 12)} = 7.77, p < 0.05$ Time: $F_{(19, 228)} = 6.66, p < 0.001$ Sex x Time: $F_{(19, 228)} = 1.99, p < 0.01^s$
Figure S3D	g/kg	Sex: $F_{(1, 12)} = 3.35, p > 0.05$ Time: $F_{(19, 228)} = 3.97, p < 0.001$ Sex x Time: $F_{(19, 228)} = 1.88, p < 0.05^s$
Figure S3E	Active Lever	Sex: $F_{(1, 12)} = 7.03, p < 0.05$ Time: $F_{(19, 228)} = 7.09, p < 0.001$ Sex x Time: $F_{(19, 228)} = 2.14, p < 0.01^s$
Figure S3E	Inactive Lever	Sex: $F_{(1, 12)} = 2.80, p > 0.05$ Time: $F_{(19, 228)} = 2.61, p < 0.001$ Sex x Time: $F_{(19, 228)} = 1.63, p > 0.05$
Figure S3F	Extinction Active Lever	Sex: $F_{(1, 12)} = 0.03, p > 0.05$ Time: $F_{(14, 168)} = 33.77, p < 0.001$ Sex x Time: $F_{(14, 168)} = 1.52, p > 0.05$
Figure S3F	Extinction Inactive Lever	Sex: $F_{(1, 12)} = 0.97, p > 0.05$ Time: $F_{(14, 168)} = 1.25, p > 0.05$ Sex x Time: $F_{(14, 168)} = 1.08, p > 0.05$
Figure S3G	Rewards	Sex: $F_{(1, 13)} = 14.22, p < 0.01$ Time: $F_{(19, 247)} = 2.02, p < 0.01$

		Sex x Time: $F_{(19, 247)} = 0.97, p > 0.05$
Figure S3G	g/kg	Sex: $F_{(1, 13)} = 4.18, p > 0.05$ Time: $F_{(19, 247)} = 1.34, p > 0.05$ Sex x Time: $F_{(19, 247)} = 0.97, p > 0.05$
Figure S3H	Active Lever	Sex: $F_{(1, 13)} = 21.37, p < 0.001$ Time: $F_{(19, 247)} = 1.68, p < 0.05$ Sex x Time: $F_{(19, 247)} = 1.03, p > 0.05$
Figure S3H	Inactive Lever	Sex: $F_{(1, 13)} = 1.12, p > 0.05$ Time: $F_{(19, 247)} = 2.41, p < 0.01$ Sex x Time: $F_{(19, 247)} = 1.36, p > 0.05$
Figure S3I	Extinction Active Lever	Sex: $F_{(1, 13)} = 0.32, p > 0.05$ Time: $F_{(14, 182)} = 18.63, p < 0.001$ Sex x Time: $F_{(14, 182)} = 1.91, p > 0.05$
Figure S3I	Extinction Inactive Lever	Sex: $F_{(1, 13)} = 0.66, p > 0.05$ Time: $F_{(14, 182)} = 2.24, p < 0.01$ Sex x Time: $F_{(14, 182)} = 0.73, p > 0.05$

§: no relevant differences in post-hoc analysis.

Table 4. Statistical analysis of alcohol self-administration training (rewards, g/kg), conditioning phase (active/inactive lever) and extinction phase (active/inactive lever) for the intra-VTA, intra-CeA and intra-NAc LY2817412 infusions on cue-induced reinstatement (**Experiment 4**). Two-way ANOVA with ‘sex’ as a between-subjects factor and ‘time’ as a within-subject factor for rewards, g/kg and, extinction phases; three-way ANOVA with sex as a between-subjects factor, time and cues condition (associated with drugs availability: alcohol vs. water) as within-subject factors.

Number of Figure	Behavior	Statistic
Figure S4A	Rewards	Sex: $F_{(1, 12)} = 0.55, p > 0.05$ Time: $F_{(19, 228)} = 5.09, p < 0.001$ Sex x Time: $F_{(19, 228)} = 1.68, p < 0.05^s$
Figure S4A	g/kg	Sex: $F_{(1, 12)} = 14.68, p < 0.01$ Time: $F_{(19, 228)} = 3.33, p < 0.001$ Sex x Time: $F_{(19, 228)} = 2.13, p < 0.05^s$
Figure S4B	Conditioning Phase Active Lever	Sex: $F_{(1, 12)} = 2.12, p > 0.05$ Time: $F_{(9, 108)} = 33.18, p < 0.001$ Sex x Time: $F_{(9, 108)} = 3.55, p < 0.001$ Drugs: $F_{(1, 12)} = 0.02, p > 0.05$ Drugs x Sex: $F_{(1, 12)} = 0.03, p > 0.05$ Time x Drugs: $F_{(9, 108)} = 2.63, p < 0.01$ Time x Drugs x Sex: $F_{(9, 108)} = 1.55, p > 0.05$
Figure S4B	Conditioning Phase Inactive Lever	Sex: $F_{(1, 12)} = 0.45, p > 0.05$ Time: $F_{(9, 108)} = 9.55, p < 0.001$ Sex x Time: $F_{(9, 108)} = 1.48, p > 0.05$ Drugs: $F_{(1, 12)} = 1.75, p > 0.05$ Drugs x Sex: $F_{(1, 12)} = 1.04, p > 0.05$ Time x Drugs: $F_{(9, 108)} = 0.35, p > 0.05$ Time x Drugs x Sex: $F_{(9, 108)} = 0.76, p > 0.05$
Figure S4C	Extinction Active Lever	Sex: $F_{(1, 12)} = 31.76, p < 0.001$ Time: $F_{(14, 168)} = 31.50, p < 0.001$ Sex x Time: $F_{(14, 168)} = 0.42, p > 0.05$
Figure S4C	Extinction Inactive Lever	Sex: $F_{(1, 12)} = 0.33, p > 0.05$ Time: $F_{(14, 168)} = 1.50, p > 0.05$ Sex x Time: $F_{(14, 166)} = 1.36, p > 0.05$
Figure S4D	Rewards	Sex: $F_{(1, 13)} = 2.85, p > 0.05$ Time: $F_{(19, 247)} = 5.13, p < 0.001$ Sex x Time: $F_{(19, 247)} = 0.92, p > 0.05$
Figure S4D	g/kg	Sex: $F_{(1, 13)} = 10.46, p < 0.01$ Time: $F_{(19, 247)} = 4.04, p < 0.001$ Sex x Time: $F_{(19, 247)} = 0.88, p > 0.05$
		Sex: $F_{(1, 13)} = 0.74, p > 0.05$

Figure S4E	Conditioning Phase Active Lever	Time: $F_{(9, 117)} = 55.09, p < 0.001$ Sex x Time: $F_{(9, 117)} = 5.58, p < 0.001$ Drugs: $F_{(1, 13)} = 0.19, p > 0.05$ Drugs x Sex: $F_{(1, 13)} = 2.64, p > 0.05$ Time x Drugs: $F_{(9, 117)} = 2.06, p < 0.05$ Time x Drugs x Sex: $F_{(9, 117)} = 1.67, p > 0.05$
Figure S4E	Conditioning Phase Inactive Lever	Sex: $F_{(1, 13)} = 0.74, p > 0.05$ Time: $F_{(9, 117)} = 4.80, p < 0.001$ Sex x Time: $F_{(9, 117)} = 0.40, p > 0.05$ Drugs: $F_{(1, 13)} = 7.15, p < 0.05$ Drugs x Sex: $F_{(1, 13)} = 3.25, p > 0.05$ Time x Drugs: $F_{(9, 117)} = 2.04, p < 0.05$ Time x Drugs x Sex: $F_{(9, 117)} = 0.19, p > 0.05$
Figure S4F	Extinction Active Lever	Sex: $F_{(1, 16)} = 9.27, p < 0.01$ Time: $F_{(14, 182)} = 17.59, p < 0.001$ Sex x Time: $F_{(14, 182)} = 0.70, p > 0.05$
Figure S4F	Extinction Inactive Lever	Sex: $F_{(1, 13)} = 0.27, p > 0.05$ Time: $F_{(14, 182)} = 1.12, p > 0.05$ Sex x Time: $F_{(14, 182)} = 0.97, p > 0.05$
Figure S4G	Rewards	Sex: $F_{(1, 14)} = 2.37, p > 0.05$ Time: $F_{(19, 266)} = 4.43, p < 0.001$ Sex x Time: $F_{(19, 266)} = 1.65, p < 0.05^{\$}$
Figure S4G	g/kg	Sex: $F_{(1, 14)} = 10.09, p < 0.01$ Time: $F_{(19, 266)} = 3.38, p < 0.001$ Sex x Time: $F_{(19, 266)} = q.68, p < 0.05^{\$}$
Figure S4H	Conditioning Phase Active Lever	Sex: $F_{(1, 14)} = 1.23, p > 0.05$ Time: $F_{(9, 126)} = 43.76, p < 0.001$ Sex x Time: $F_{(9, 126)} = 2.43, p < 0.05$ Drugs: $F_{(1, 14)} = 0.50, p > 0.05$ Drugs x Sex: $F_{(1, 14)} = 0.37, p > 0.05$ Time x Drugs: $F_{(9, 126)} = 2.00, p < 0.05$ Time x Drugs x Sex: $F_{(9, 126)} = 1.20, p > 0.05$
Figure S4H	Conditioning Phase Active Lever	Sex: $F_{(1, 14)} = 0.02, p > 0.05$ Time: $F_{(9, 126)} = 3.78, p < 0.001$ Sex x Time: $F_{(9, 126)} = 1.50, p > 0.05$ Drugs: $F_{(1, 14)} = 4.92, p > 0.05$ Drugs x Sex: $F_{(1, 14)} = 0.25, p > 0.05$ Time x Drugs: $F_{(9, 126)} = 1.54, p > 0.05$ Time x Drugs x Sex: $F_{(9, 126)} = 0.97, p > 0.05$
Figure S4I	Extinction Active Lever	Sex: $F_{(1, 14)} = 3.17, p > 0.05$ Time: $F_{(14, 196)} = 12.37, p < 0.001$ Sex x Time: $F_{(14, 196)} = 0.84, p > 0.05$
Figure S4I	Extinction Inactive Lever	Sex: $F_{(1, 14)} = 0.53, p > 0.05$ Time: $F_{(14, 196)} = 0.62, p > 0.05$ Sex x Time: $F_{(14, 196)} = 0.99, p > 0.05$

§: no relevant differences in post-hoc analysis.

Table 5. Statistical analysis of saccharin self-administration training (rewards and active/inactive levers) for the intra-VTA and intra-CeA LY2817412 infusions on saccharin self-administration (**Experiment 5**). Two-way ANOVA with ‘sex’ as a between-subjects factor and ‘time’ as a within-subject factor for rewards and active/inactive lever presses.

Number of Figure	Behavior	Statistic
Figure S5A	Rewards	Sex: $F_{(1, 17)} = 1.86, p > 0.05$ Time: $F_{(19, 323)} = 37.83, p < 0.001$ Sex x Time: $F_{(19, 323)} = 1.56, p > 0.05$
Figure S5B	Active Lever	Sex: $F_{(1, 17)} = 0.92, p > 0.05$ Time: $F_{(19, 323)} = 23.04, p < 0.001$ Sex x Time: $F_{(19, 323)} = 0.85, p > 0.05$
Figure S5B	Inactive lever	Sex: $F_{(1, 17)} = 5.57, p < 0.05$ Time: $F_{(19, 323)} = 2.72, p < 0.001$ Sex x Time: $F_{(19, 323)} = 1.34, p > 0.05$
Figure S5C	Rewards	Sex: $F_{(1, 17)} = 0.00, p > 0.05$ Time: $F_{(19, 323)} = 46.20, p < 0.001$ Sex x Time: $F_{(19, 323)} = 1.585, p > 0.05$
Figure S5D	Active Lever	Sex: $F_{(1, 17)} = 0.00, p > 0.05$ Time: $F_{(19, 323)} = 30.74, p < 0.001$ Sex x Time: $F_{(19, 323)} = 1.17, p > 0.05$
Figure S5D	Inactive Lever	Sex: $F_{(1, 17)} = 1.18, p > 0.05$ Time: $F_{(19, 323)} = 3.55, p < 0.001$ Sex x Time: $F_{(19, 323)} = 1.68, p > 0.05$

Table 6. Statistical analysis of the last 3 days of alcohol self-administration training (active/inactive levers) and the systemic effect of LY2817412 (10 mg/kg; p.o.) on the first extinction day in male and female msP rats (**Experiment 6**). Two-way ANOVA with ‘sex’ as a between-subjects factor and ‘time’ as a within-subject factor for active/inactive lever presses; two-way ANOVA with ‘sex’ as a between-subjects factor and ‘treatment’ as a within-subject factor for LY2817412’s effect on active/inactive lever presses.

Number of Figure	Behavior	Statistic
Figure S6A	Active Lever	Sex: $F_{(1, 13)} = 1.52, p > 0.05$ Time: $F_{(2, 26)} = 1.10, p > 0.05$ Sex x Time: $F_{(2, 26)} = 0.19, p > 0.05$
Figure S6A	Inactive Lever	Sex: $F_{(1, 13)} = 0.01, p > 0.05$ Time: $F_{(2, 26)} = 6.55, p < 0.01$ Sex x Time: $F_{(2, 26)} = 1.12, p > 0.05$
Figure S6B	Active Lever	Sex: $F_{(1, 13)} = 3.27, p > 0.05$ Treatment: $F_{(1, 13)} = 0.16, p > 0.05$ Sex x Treatment: $F_{(1, 13)} = 0.06, p > 0.05$
Figure S6B	Inactive Lever	Sex: $F_{(1, 13)} = 1.38, p > 0.05$ Treatment: $F_{(1, 13)} = 6.95, p < 0.05$ Sex x Treatment: $F_{(1, 13)} = 0.44, p > 0.05$

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