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Female rats display higher methamphetamine-primed reinstatement and c-Fos immunoreactivity than male rats

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Abstract

Methamphetamine (meth) dependence is often characterized by persistent and chronic relapse (i.e., return to drug use). Previous work suggests females may be at greater risk to relapse. In this study, we extended this limited evidence and identified sex-dependent neural substrates related to meth-triggered reinstatement. Male and female Sprague-Dawley rats were implanted with indwelling jugular catheters. Half of the rats were then trained to self-administer meth (0.05 mg/kg/inf); the other half self-administered saline during 21 daily sessions (2 h). Rats were then given 12 extinction sessions. Twenty-four hours after the last extinction session, rats received reinstatement testing. Half of the rats received a meth-prime (0.3 mg/kg, IP) injection and the remaining rats received a saline injection. This design resulted in 4 separate groups for each sex, allowing for careful investigation of brain regions related to meth-triggered reinstatement. Brains were harvested following the reinstatement session and c-Fos immunoreactivity was measured in multiple brain regions. Meth triggered reinstatement in both sexes and this effect was more robust in females compared to males. Significant sex differences were detected. Females showed greater c-Fos immunoreactivity in the cingulate cortex area 1, lateral orbitofrontal cortex, prelimbic cortex, caudate-putamen, nucleus accumbens core and shell, and central nucleus of the amygdala following meth-primed reinstatement.

Keywords

Amphetamine; c-Fos; Drug self-administration; Immunohistochemistry; Relapse

1. Introduction

Methamphetamine (meth) use and dependence is a serious public health concern (National Institute on Drug Abuse, 2013). A major impediment to meth dependence treatment is

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relapse, or a return to drug use following a prolonged period of drug abstinence. Relapse can occur months and even years after drug cessation (Baicy and London, 2007; Bamford et al., 2008; Grant et al., 2012). Relapse is often triggered by drug craving, an intense urge or desire to use a drug, induced by drug-associated cues, by stress, or by the drug itself (Blum et al., 2009; Carter and Tiffany, 1999; Chornock et al., 1992; Kaplan et al., 1985; Katz and Higgins, 2003; Paulus and Stewart, 2020; Preston et al., 1992; Self and Nestler, 1998; Stockwell et al., 1982; Walsh et al., 2000).

Drug-associated cues, stress, and drug-primes can also precipitate drug-seeking behavior in animal models (termed reinstatement). Researchers have utilized these pre-clinical reinstatement models to elucidate brain areas associated with drug relapse. Previous studies have implicated the prefrontal cortex (Ciccocioppo et al., 2001; Coordie and McFadden, 2019; Kufahl et al., 2009; Neisewander et al., 2000; Recinto et al., 2012; Thomas and Everitt, 2001; Wexler et al., 2001; Zhou et al., 2014), dorsal and ventral striatum (Di Ciano and Everitt, 2001; Kufahl et al., 2009; Neisewander et al., 2000; Rocha and Kalivas, 2010; Rubio et al., 2015; Zhou et al., 2014; Quinn et al., 2018), hippocampus (Kufahl et al., 2009; Sun and Rebec, 2003; Taepavarapruk and Phillips, 2003; Takashima et al., 2018; Vorel et al., 2001; Zhou et al., 2014), amygdala (Di Ciano and Everitt, 2004; Fuchs et al., 2005; Kufahl et al., 2009; McLaughlin and See, 2003; Rich et al., 2019; See et al., 2001; Whitelaw et al., 1996), hypothalamus (Zhou et al., 2014; Blacktop and Sorg, 2018), ventral tegmental area (VTA: Mahler et al., 2019; McFarland and Kalivas, 2001; Neisewander et al., 2000; Zhou et al., 2014), and substantia nigra (SNR: Bortz and Grace, 2018; Ilango et al., 2014; Kufahl et al., 2009; Neisewander et al., 2000; Rossi et al., 2013) in drug-seeking behavior during reinstatement.

A number of differences between males and females have been documented, including differences in the patterns of meth use and relapse in both clinical and pre-clinical studies. In humans, for example, differences between males and females arise as early as the reported motivation for the initiation of meth use (Simpson et al., 2016). In one study, more women reported using meth for weight control and to increase energy, whereas more men reported being motivated by the desire to work more hours (Brecht et al., 2004). Brecht et al. (2004), as well as several other studies (Dluzen and Liu, 2008; Hser et al., 2005; Lin et al., 2004; Westermeyer and Boedicker, 2000; Wu et al., 2007), found that women initiate use at a younger age than men. Once meth use is initiated, women also tend to transition to regular use more quickly than men (1.6 years for females vs 2.56 years for males; Brecht et al., 2004; Rawson et al., 2005).

Despite evidence that women may be more susceptible to meth dependence, until recently, animal models rarely use female subjects. This leaves a critical need for empirical research on meth-taking/seeking in preclinical models employing females. Some of the limited animal research that has investigated such sex differences in meth effects is consistent with work in humans. In self-administration studies, a greater percentage of female than male rats self-administered a low dose of meth during 6 h sessions, acquired meth self-administration quicker than their male counterparts, and responded more on progressive ratio (PR) schedules (Roth and Carroll, 2004; Reichel et al., 2012). Additionally, females show greater reinstatement induced by a meth-priming injection compared to males (Cox et

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al., 2013; Holtz et al., 2012; Reichel et al., 2012), suggesting that under certain conditions females may be more vulnerable to meth relapse (see Discussion). This literature, along with our preliminary research, led us to examine meth-primed reinstatement in the present study.

In the present report, we are interested in the increased vulnerability to reinstatement found in females and potential neural factors that contribute to this sex difference. To this end, we used the immediate early gene c-Fos as a measure of neural activation (Curran and Morgan, 1985a, 1985b; Kovacs, 1998; Greenberg and Ziff, 1984). This approach will provide critical foundational knowledge on brain regions that may be associated with the behavioral sex differences found during reinstatement. The brain areas examined in this study were the cingulate cortex area 1 and 2 (Cg1; Cg2), prelimbic cortex (PrL), infralimbic cortex (IL), lateral orbital cortex (LO), dorsal medial caudate-putamen (dmCPu), dorsal lateral caudate-putamen (dlCPu), ventral medial caudate-putamen (vmCPu), ventral lateral caudate-putamen, (vlCPu), nucleus accumbens core (NAcC), nucleus accumbens shell (NAcSh), hippocampus proper (CA1; CA2; and CA3) and ventral subiculum (VS), amygdala [central (CEA); basolateral (BLA)], lateral hypothalamus (LH), ventral tegmental area (VTA), and substantia nigra (SNR).

2. Materials and methods

2.1. Subjects

Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN, USA) at approximately 9 weeks of age ($n = 140$). Rats were housed individually in clear polycarbonate cages (35.5 × 32 × 18 cm; length × width × depth) with TEK-Fresh® cellulose bedding. The colony room was temperature- and humidity-controlled and maintained on a 6:00 AM light/6:00 PM dark cycle. Rats were allowed to acclimate to the colony room for 3 days. At that time, 90% free-feeding weights were calculated and maintained for the duration of the experiment. Rats received ad libitum access to water in the home cages. All experimental procedures were conducted during light phase of the cycle. Protocols were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

2.2. Apparatus

Behavioral testing was conducted in chambers purchased from Med Associates (ENV-008CT; Georgia, VT, USA). Each chamber measured 30.5 × 24.1 × 21 cm and was enclosed in a sound-attenuating cubicle. A variable-speed syringe pump (PMH-100VS; Med-Associates) was located outside each cubicle. Tygon® tubing was threaded from the pump syringe, through a leash, into the chamber to be attached to the catheter port that exited below the scapula of the rat. A recessed receptacle (5.2 × 5.2 × 3.8 cm) was centered on one sidewall of each chamber. A dipper arm, when raised, provided access to 0.1 ml of 26% (*w*/*v*) sucrose in this recessed receptacle. A retractable lever was located on each side of the receptacle. A white cue-light (2.54 cm diameter; 28 V, 100-mA) was mounted 7 cm above each lever. A house-light (two white 28 V, 100-mA lamps) was located in the cubicle, 10 cm above the Perspex chamber ceiling.

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2.3. Drugs

(+)-Methamphetamine hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline. Meth was infused intravenous (IV) at a volume of 35.74 μl over 1 s at 0.05 mg/kg/infusion. For reinstatement, 0.3 mg/kg meth was injected intraperitoneal IP at 1 ml/kg. Meth doses are reported in salt form. These meth doses were based on published (Duryee et al., 2009; Reichel et al., 2009) and unpublished research from our lab indicating robust maintenance of self-administration and potential sex differences in reinstatement.

2.4. Preliminary lever training

Following acclimation to the colony room and food restriction to maintain 90% of free-feeding weight, rats were trained to lever press on both levers following previously reported procedures (Charntikov et al., 2015; Charntikov et al., 2013; Pittenger et al., 2016). The start of each session was signaled by illumination of the house light and insertion of a randomly selected lever (right or left). A lever press or a lapse of 15 s resulted in 4-s access to sucrose, retraction of the lever, and commencement of a timeout (average = 60 s; range = 30 to 89 s). Following the timeout, a randomly selected lever was again inserted with the condition that the same lever could not be presented more than twice in a row. This protocol was repeated for 60 sucrose deliveries. Daily sessions range from 65 to 80 min depending on individual performance. Training continued until a lever press was made on at least 80% of the lever insertions for two consecutive days. All rats met criterion between sessions 3 to 5. This autoshaping protocol ensured rats were pressing at robust levels but neither lever was differentially associated with sucrose.

2.5. Catheter surgery and recovery

Indwelling jugular catheters were implanted using previously reported protocols (Charntikov et al., 2015; Charntikov et al., 2013; Pittenger et al., 2016). Rats were anesthetized with a 2:1 ketamine HCl (100 mg/kg; MWI, Boise, ID) plus xylazine HCl (20 mg/kg; MWI, Boise, ID) cocktail (intramuscular; IM). Following surgery, rats were administered buprenorphine (0.1 mg/kg, subcutaneous: SC; MWI, Boise, ID) for pain management and atipamezole (0.5 mg/kg, IM; MWI, Boise, ID) to terminate anesthesia. Buprenorphine was again administered 24-h post-surgery. Rats were allowed to recover for 7 days. During recovery, they remained in their home cages and catheters were flushed daily with a cocktail of 0.2-ml baytril (5.0 mg/ml; MWI, Boise, ID) to prevent infections and heparin (30 Units/ml; MWI, Boise, ID) to minimize blood clotting blocking fluid flow through the catheter. Catheter patency was checked on the last day of recovery by IV infusion of 0.05 ml xylazine (20 mg/ml). Rats that displayed motor ataxia within 20 s were considered patent (cf. Charntikov et al., 2015; Charntikov et al., 2013; Reichel et al., 2008; Pittenger et al., 2016). Patency was again checked upon the completion of the self-administration phase. Twenty eight rats were excluded from the study for failing the patency test.

2.6. Post-surgery lever training

Following recovery, rats were placed on a variable ratio 3 (VR3) schedule of sucrose reinforcement. Under the VR3 schedule, on average every 3rd lever press (range 1 to 5) was followed by 4-s access to sucrose. Levers were again inserted individually with the condition

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that the same lever was not inserted >2 times in a row. These procedures ensured robust responding with both levers having a similar reinforcement history (Charntikov et al., 2015; Charntikov et al., 2013; Pittenger et al., 2016). This training lasted for 3 daily 1-h sessions conducted on consecutive days.

2.7. Self-administration

Following post-surgery lever press training, male and female rats were separated into two self-administration conditions: MethSA or SalineSA. Rats in the MethSA conditions began self-administration of meth during daily 2-h sessions. Rats in the SalineSA condition began daily 2-h sessions identical to those received by the MethSA condition except saline was available in lieu of meth. Drug (meth or saline) was available on a VR3 schedule of reinforcement. During training, two levers were present, active and inactive. Rats were randomly assigned which of the two levers served as the active (meth or saline infusion) vs inactive lever. Before a rat was attached to the leash/tubing at the start of each session, the catheter was flushed with 0.2-ml heparin (30 Units/ml) in sterile saline. The session commenced with insertion of both levers and priming of the catheter with meth or saline [ca. 31 μl (90% of internal catheter volume)]. Requisite VR3 responding on the active lever initiated an infusion of meth or saline, retraction of both levers, and illumination of the house light for a 20-s timeout. Following the timeout, both levers were extended and the house light was terminated. Responding on the inactive lever was recorded but had no programed outcome. After each session, the catheter was flushed with a cocktail of 0.2-ml baytril (5 mg/ml) and heparin (30 Units/ml) in sterile saline. Sessions were conducted 7 days per week for 21 days.

2.8. Extinction

Extinction sessions commenced 24 h after the last self-administration session. Extinction sessions were identical to self-administration sessions except drug was no longer infused. Requisite VR3 responding on the active lever still produced the same cues and the timeout. Responding on the inactive lever was recorded but held no programed consequence. Sessions were 2 h and conducted daily for 12 days.

2.9. Reinstatement

At the end of extinction, the MethSA and SalineSA conditions were split further into 2 different reinstatement-trigger groups: SalineT or MethT. Rats were pseudo-randomly assigned with the caveat that the groups did not differ statistically in responding at the end of extinction. This created 4 groups of males and 4 groups of females: SalineSA/SalineT, SalineSA/MethT, MethSA/SalineT, MethSA/MethT. The first part of each name indicates the drug each group self-administered (Saline or Meth) and the second part of each name indicates the drug that "triggered" reinstatement (Saline or Meth; $n = 14$ per group). Each group provided vital information regarding the activation of brain regions associated with reinstatement. The SalineSA/SalineT group provided baseline c-Fos activation for both sexes in the absence of meth self-administration or an acute meth injection. The MethSA/SalineT groups allowed for examination of c-Fos activation that is related to an extinction (i.e., drug abstinence) period following self-administration of meth. The SalineSA/MethT groups were used to detect neural substrates associated with acute meth injection while controlling

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for exposure to chambers, handling, etc. Finally, the MethSA/MethT groups allowed us to identify neural substrates related with meth-triggered reinstatement following meth self-administration. Rats in the MethT groups were administered a 0.3 mg/kg injection of meth (IP) 15 min before a 70-min reinstatement session. The SalineT groups received a saline injection (IP) 15 min before their 70-min reinstatement session. The reinstatement session was identical to extinction sessions (i.e., no available infusions) except for the truncated time; decreased from 2 h to 70 min. The session length was shortened given preliminary data showing that lever pressing peaked during the first 10 min of reinstatement sessions. c-Fos is primarily expressed approximately 60–90 min after neuronal activation (Kovacs, 1998). Limiting the reinstatement session to 70 min allowed for sufficient time to gather the crucial behavioral data, as well as identify brain regions associated with the meth trigger and the reinstatement behavior.

2.10. Perfusion and brain extraction

Immediately following the reinstatement session, rats were deeply anesthetized by injection with Fatal-Plus® (25 mg/kg; MWI, Boise, ID). Rats were then transcardially perfused with 200 ml of ice cold 0.9% saline followed by 200 ml of ice cold 4% paraformaldehyde. Brains were rapidly removed and post-fixed in 4% paraformaldehyde for 24 h at 4 °C. Brains were then cryoprotected in 30% sucrose for 72 h at 4 °C. Following cryoprotection, brains were frozen on dry ice and stored at −80 °C until sectioning.

2.11. Histology

Brains were sectioned at 40 μm on a freezing microtome. Brain regions were identified according to the atlas of Paxinos and Watson (2007). Coronal sections were collected at 3.24 mm bregma to assess the Cg1, PrL, IL, and LO (see Fig. 1A). Coronal sections at 1.80 mm bregma were used to examine the Cg2, dmCPu, dlCPu, vmCPu, vlCPu, NAcC, and NAcSh (see Fig. 1B). Regions of the hippocampus (CA1; CA2; and CA3), amygdala (CEA; BLA) and LH were identified on sections collected at −2.64 mm bregma (see Fig. 1C). Finally, sections at −5.88 mm bregma contained the VTA, SNR, and VS (see Fig. 1D). Each area of interest was examined in a single hemisphere from 3 separate tissue sections per rat (cf. Zhao and Li, 2010).

2.12. c-Fos immunohistochemistry

Following sectioning, brain sections were stored for no >48 h in a 0.02 M phosphate buffered saline (PBS): 0.1% sodium azide solution (Zhao and Li, 2010). For c-Fos immunohistochemistry, brain sections incubated on ice for 1 h in blocking solution [10% normal goat serum (NGS): 0.3% Triton X-100: 0.02 M PBS]. Sections were then washed 3 times in wash buffer (0.3% Triton X-100: 0.05% NGS: 0.02 M PBS) for 10 min per wash. Washing was proceeded by incubation in 1.5% hydrogen peroxide: 50% methanol for 30 min on ice. This was followed by another round of 3 washes with wash buffer (10 min per wash). Sections were then incubated with c-Fos primary antibody [c-Fos Antibody (4): sc-52 (discontinued), Santa Cruz Biotechnology, Dallas, TX, USA; 1:3000 dilution] in 0.3% Triton X-100:1% NGS: 1% blocking reagent:0.02 M PBS for 48 h at 4 °C. Following incubation with the primary antibody, sections were washed 3 times for 10 min per wash with wash buffer. Then sections were incubated with biotinylated goat anti-rabbit secondary

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antibody (Vector Labs, Burlingame, CA, USA; 1:200 dilution) in 1% NGS:0.02 M PBS for 2 h on ice. This was followed by 3 washes (10 min per wash) with 0.02 M PBS. Sections were then incubated with horseradish peroxide avidin-biotin complex (Vectastain Elite ABC Kit, Vector Labs, Burlingame, CA, USA: 1:200 dilution) in 0.02 M sodium azide- free PBS. This was followed by 3 washes with 0.05 Tris-HCl. Sections were then incubated for 5 min in diaminobenzidine-based peroxide substrate (DAB Substrate Kit, Vector Labs, Burlingame, CA, USA) to aid in protein visualization. Brain sections were then mounted on gelatin-coated slides and allowed to air-dry at room temperature. They were then dehydrated in ascending alcohol concentrations, cleared in xylene and cover slipped with mounting medium (Permount, Fisher Scientific, Suwanee GA, USA).

2.13. c-Fos imaging and quantification

A digital image (20× objective lens magnification; 490 μm^2) was captured from each region of interest from anatomically matched sections (1 image from each tissue section × 3 tissue sections per area for each rat) using a light microscope (Olympus CX41RF, Tokyo, Japan) fitted with a digital camera (Infinity Lite, Ottawa, ON, Canada). For each image, c-Fos immunoreactivity was automatically identified and counted using NIH ImageJ software (Abramoff et al., 2004; Charntikov et al., 2012). Sample photomicrographs of c-Fos expression are provided in Fig. 2. Imaging and immunoreactivity quantification using ImageJ were performed blind to the treatment status of the sections.

2.14. Dependent measures

Lever-pressing was the primary dependent measure during the behavioral phases of the experiment. To show inactive lever responding relative to active lever responding during self-administration, a discrimination index was calculated using the following formula: Discrimination Index = [Active Lever Presses/ (Inactive Lever Presses + Active Lever Presses)]. A Discrimination Index value of 0.5 indicates equal responding on the active and inactive lever (i.e., no discrimination between levers); a value >0.5 indicates more pressing on the active lever. Lever pressing on the inactive lever was near zero following early training and remained there for the rest of the experiment (data not displayed). Positively identified c-Fos cells in each brain region was the primary dependent measure used for neuronal activation. For each rat, the number of positively labeled nuclei was averaged between the 3 tissue sections in each region and used as a unit of measurement (cf. Charntikov et al., 2012; Zhao and Li, 2010; Shram et al., 2007).

2.15. Statistical analyses

Active lever responding in the self-administration and extinction phase were analyzed by separate 3-way mixed measures analysis of variance (ANOVA; Type III Sum of Squares) with *Sex* (Female vs Male) and *Group* (MethSA vs SalineSA) as between-subjects factors and *Session* as a within-subjects factor. This same ANOVA was also utilized to examine the discrimination index. Active lever responding in reinstatement was analyzed by a 3-way ANOVA with *Sex* (Female vs Male) and *Self-Administration Drug* (Meth vs Saline) and *Reinstatement Drug* (Meth vs Saline as between-subjects factors). Three-way ANOVAs with *Sex*, *Self-Administration Drug*, and *Reinstatement Drug* as between-subjects factors were also used to analyze regional c-Fos activation following reinstatement testing. Post-

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hoc analyses were conducted on significant interactions and on planned comparisons in reinstatement behavior and c-Fos activation. The complete list of a priori comparisons can be found in Table 1. To adjust for multiple comparisons, Tukey HSDs were utilized for post-hoc analysis of behavioral data. Statistical significance was declared at $p < 0.05$.

3. Results

3.1. Self-administration

Rats in the meth groups demonstrated robust active lever pressing (Fig. 3). Analysis of active lever pressing revealed significant main effects of Group [F(1, 108) = 240.34; $p < 0.001$], Session [F(20, 2160) = 10.66; p < 0.001], and a Group × Session interaction [F(20, 2160) = 14.47; p < 0.001]. Rats responded significantly more for meth compared to saline in all 21 self-administration sessions. Whereas lever pressing among saline groups sharply decreased from the beginning (sessions 1–9) to the end (sessions 15–21), there was a slight increase in lever pressing among the meth groups (Fig. 3; top versus bottom panel). This finding was not surprising given the preliminary training with food. Females and males responded similarly on the active lever as neither the main effect of Sex (F < 1; $p = 0.982$), the Sex × Group interaction [F(1, 108) = 2.84; $p = 0.095$], the Sex × Session interaction [F(20, 2160) = 1.01; $p = 0.443$], nor the Sex × Group × Session interaction [F(20, 2160) = 1.33; $p = 0.159$] was significant.

Males and females displayed clear discrimination between the active and inactive lever in the meth and the saline conditions (Inset graphs, Fig. 3; Discrimination Index well above 0.5). Analysis of the discrimination index did reveal significant main effects of Group [F(1, 108) = 74.73, $p < 0.001$] and Session [F(20, 2160) = 20.75, p < 0.001], as well as significant Sex × Group [F(1, 108) = 8.71, $p = 0.004$] and Group × Session [F(20, 2160) = 1.76, $p = 0.020$] interactions. Rats displayed better discrimination when receiving meth vs saline infusions. Lever discrimination increased in the meth and saline conditions, however it increased more quickly in the meth condition. Notably, in the saline condition, females showed statistically better discrimination compared to the males. In the meth condition, however, this effect was reversed, with males tending to show better discrimination than their female counterparts, although this effect did not reach significance ($p = 0.074$). The Sex × Group × Session [F(20, 2160) = 1.26, $p = 0.198$] and Sex × Session [F < 1, $p = 0.926$] interactions were not significant.

3.2. Extinction

Active lever pressing in the meth condition was attenuated during the extinction phase of this experiment (Fig. 4). Analysis of active lever pressing during extinction revealed significant main effects of Group [F (1, 108) = 64.89; $p < 0.001$] and Session [F(11, 1188) = 45.43; p < 0.001], as well as a significant Group × Session interaction [F(11, 1188) = 53.32; p < 0.001]. Responding in the meth condition was elevated compared to saline responding for the first 11 sessions, but was reduced to saline levels by session 12 ($p = 0.080$). Responding in the meth condition was higher during initial extinction sessions compared to the later extinction sessions, while responding in the saline condition remained stable throughout extinction. This outcome was expected, given that extinction sessions

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contained the timeout cues that were presumably maintaining a modest level of responding in the saline condition (cf. Pittenger et al., 2017). Responding was again similar between males and females. The main effect of Sex ($F(1,108) = 1.255$ $p = 0.265$), the Sex × Group interaction ($F < 1$; $p = 0.506$), the Sex × Session interaction ($F < 1$; $p = 0.5$), and the Sex × Group × Session interaction ($F < 1$; $p = 0.957$) were not significant.

3.3. Reinstatement

Analysis of active lever pressing during the reinstatement test (Fig. 5) revealed significant main effects of Sex [$F(1, 104) = 12.57$; $p < 0.001$], Self-Administration Drug [$F(1, 104) = 57.93$; $p < 0.001$], and Reinstatement Drug [$F(1, 104) = 59.43$; $p < 0.001$]. The Sex × Reinstatement Drug interaction [$F(1, 104) = 6.67$; $p = 0.012$] and the Self Administration Drug × Reinstatement Drug interaction [$F(1, 104) = 25.297$; $p < 0.001$] were both significant. The overall 3-way Sex × Self-Administration Drug × Reinstatement Drug interaction just missed the cutoff for significance [$F(1, 104) = 3.879$; $p = 0.051$]. The Sex × Self-Administration Drug interaction was not significant [$F(1, 104) = 1.35$; $p = 0.248$].

Post-hoc tests on the planned comparisons revealed several interesting findings. In both sexes, the groups that received meth during self-administration and received meth as a trigger (MethSA/MethT) showed more reinstatement than the groups receiving meth in self-administration and not receiving a drug prime (MethSA/SalineT), receiving a meth trigger without prior meth experience (SalineSA/MethT), or the groups that never had meth at any point in the study (SalineSA/SalineT). The females and males responded similarly in the MethSA/SalineT, SalineSA/MethT, and SalineSA/SalineSA groups. Notably, the only difference between the males and females was in the groups that received meth-primed reinstatement following meth self-administration. Females in this MethSA/MethT group responded significantly more than their male counterparts. This difference in reinstatement behavior between the sexes was not a result of general differences following long-term meth self-administration (MethSA/SalineT), acute meth administration (SalineSA/MethT), nor basal behavioral differences (SalineSA/SalineT). Females only responded more during meth-primed reinstatement of meth-seeking (MethSA/MethT).

3.4. c-Fos immunohistochemistry

Investigation of c-Fos activation in the brain following reinstatement also detected notable findings. Statistical analysis of main effects and interactions for each area are reported in Table 2. Analysis of the planned comparisons (refer to Table 1) revealed in the female rats, the MethSA/MethT group showed more activation than the MethSA/SalineT group in the Cg1, LO, PrL, dlCPu, dmCPu, vmCPu, NacC, NAcSh, and CEA (Fig. 6). The group that received acute meth exposure (SalineSA/MethT) also had higher c-Fos activation levels compared to the long-term self-administration with no prime group in several regions. These regions were the Cg1, vlCPu, NAcC, and CEA (Fig. 6). With the exception of the vlCPu, the activation following an acute meth injection was less robust than the activation in the group that received a meth injection following a self-administration history with meth. This data pattern suggests that the learning history with meth self-administration further potentiated neural activity associated with meth administration, except in the vlCPu which shows more activation following acute meth administration. Differences in the females between

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the baseline group (SalineSA/SalineT) and the reinstatement group (MethSA/MethT) were found in the Cg1, LO, dmCPu, vmCPu, NAcC, NAcS, and CEA (Fig. 6), while differences between the baseline group and acute meth injection group were only detected in the Cg1, vlCPu, NAcC, and CEA (Fig. 6).

Examination of the planned comparisons within the male groups detected substantially fewer differences. In fact, the only significant difference in the males was found in the CEA with the group that received acute meth injection showing higher c-Fos activation than the group that was drug free following long-term self-administration (SalineSA/MethT>MethSA/SalineT; Fig. 7).

There were numerous significant differences detected in the planned comparisons between sexes (Fig. 6 and 7). Females in the reinstatement group (MethSA/MethT) had higher c-Fos activation than their male counterparts in the Cg1, LO, dlCPu, vmCPu, NAcC, and CEA (Fig. 6 and 7). There were also differences between males and females following acute meth administration (SalineSA/MethT). Females had more c-Fos immunoreactivity following acute meth in the LO, vlCPu, vmCPU, NAcC, NAcSh, and CEA (Fig. 6 and 7). No differences were detected between males and females in the baseline group (SalineSA/SalineT) or the meth self-administration group that did not receive a meth-prime (MethSA/SalineT) suggesting that differences between the sexes were not a result of sexually dimorphic basal neuronal activation levels. There were no significant differences in the planned group comparisons in the Cg2, IL, CA1, CA2, CA3, VS, BLA, LH, SNR, or VTA (data not shown).

4. Discussion

Using the standard meth self-administration procedures in our lab, we found that female and male rats readily self-administered meth. Robust meth self-administration is concordant with previous work in our lab (Charntikov et al., 2015; Pittenger et al., 2016; Reichel et al., 2008, 2009) and the findings of other labs (Beardsley et al., 2010; Coordie and McFadden, 2019; Cornish et al., 2012; Cox et al., 2013; Holtz et al., 2012; Hofford et al., 2014; Reichel et al., 2012; Roth and Carroll, 2004; Rubio et al., 2015; Shepard et al., 2004; Sobieraj et al., 2016). In the current study, females and males did not differ in active lever responding in the self-administration phase. This lack of sex difference during self-administration is common (e.g., Pena-Bravo et al., 2019; Pittenger et al., 2017), but not ubiquitous. Differences in sex, with females self-administering more than males, are often established when self-administration sessions are longer in duration than the 2-h protocol used in this study (Roth and Carroll, 2004; Reichel et al., 2012). The lack of difference in our procedures can be viewed as a strength. Given that meth intake was similar during the self-administration phase, we are not concerned with differential intake complicating interpretation of the sex differences found in the later reinstatement phase.

The inclusion of the saline self-administration conditions (i.e., Saline SA/SalineT and SalineSA/MethT) provided the critical controls in which to interpret the outcome of the meth self-administration conditions (i.e., MethSA/SalineT and MethSA/MethT). In fact, their inclusion also served as a methodological strength. Recall that the only difference

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between the meth condition and the saline condition is the type of infusion (meth or saline); pre-training, infusion/timeout cues, progression through the study, handling, transport, etc. were similar. Thus, this saline benchmark allowed for the detection of c-Fos differences specific to drug type in self-administration and for a careful analysis of the behavior controlled by meth compared to that controlled by the weak reinforcing effects of infusion/timeout cues (Caggiula et al., 2009; Chaudhri et al., 2006; Palmatier et al., 2006; Pittenger et al., 2017). Indeed, the groups that received saline during self-administration differentially responded on the active vs inactive lever. However, this responding was significantly lower than responding for meth. Note that our protocol initially trains lever pressing with sucrose. While this approach certainty provided the instrumental response, this training cannot easily explain lever discrimination or persistence of low yet stable active lever presses for 21 sessions. Recall that both levers had a similar history of reinforcement and that the active lever was randomly selected at the start of the meth self-administration phase. Rather, we suggest that this finding supports the notion that the timeout cues (i.e., light illumination and lever extraction for 20 s) have weak reinforcing value that maintains modest levels of responding (Barrett et al., 2020; Caggiula et al., 2009; Chaudhri et al., 2006; Palmatier et al., 2006).

In extinction, responding in the meth groups was attenuated to levels comparable to the saline benchmarks, an expected outcome, as extinction sessions included the timeout cues that were presumably maintaining the low levels of responding in the saline groups. Responding during extinction was similar between females and males. While some work has found females may be more resistant to extinction of meth self-administration (Cox et al., 2013), the current finding matches others that do not report sex differences in extinction rates (e.g., Reichel et al., 2012; Holtz et al., 2012; Pittenger et al., 2017). The cause of this variation remains unknown; however, the lack of sex differences in extinction reported herein allowed the assessment of possible sex differences in subsequent reinstatement without the concern of baseline differences in lever pressing.

Males and females showed significant meth-seeking behavior following a meth-prime injection. Responding was higher in the male and female groups that had a learning history with meth and received a meth trigger (MethSA/MethT) than the group that had a learning history with meth and did not receive a meth prime (MethSA/SalineT), the group that received an acute injection of meth (SalineSA/MethT), and the group that never received meth. Notably, this meth-primed reinstatement effect was potentiated in females compared to males. Responding in the female MethSA/MethT group was significantly higher than responding in the male MethSA/MethT group; this was the only behavioral group that differed between males and females. The difference in these groups alone suggests that differences in reinstatement behavior between the sexes were not a result of general differences following long-term meth self-administration, acute meth administration, nor basal behavioral differences. Females only responded more during meth-primed reinstatement of meth-seeking, which is concordant with previous work that also found amplified reinstatement behavior in females (Cox et al., 2013; Holtz et al., 2012; Reichel et al., 2012).

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There were several notable findings in the examination of c-Fos as a marker of neuronal activation. Generally speaking, c-Fos immunoreactivity showed a similar pattern to the behavioral reinstatement data, particularly in females. c-Fos was higher in females with a learning history with meth and received a meth-prime compared to females that received long-term meth self-administration and no prime in the Cg1, LO, PrL, dlCPu, dmCPu, vmCPu, NacC, NAcSh, and CEA (recall Fig. 6). These differences were a result of both the high activation levels following a prime and lower levels following long-term meth self-administration (i.e., levels in the MethSA/SalineT group were marginally lower than even the baseline SalineSA/SalineT group). These results suggest that hyperactivation was prevalent after a meth prime in females and, notably, there may be hypofunctioning in the female rat brain following long-term meth self-administration and extinction. As extinction and reinstatement in the MethSA/SalineT groups were conducted drug free, this could be conceptualized as a withdrawal period. Accordantly, past work has shown hypofunction in critical areas associated with drug addiction during withdrawal (e.g., Parsegian and See, 2014) and suggests females show heightened sensitivity to withdrawal symptoms (for a review see O'Dell and Torres, 2014).

The increased c-Fos expression in the females in cortical, striatal, and amygdala regions during reinstatement is concordant with several studies investigating c-Fos expression in male rats following reinstatement (Bossert et al., 2012; Ciccocioppo et al., 2001; Cornish et al., 2012; Hamlin et al., 2008a, 2008b; Kufahl et al., 2009; Miller and Marshall, 2005; Recinto et al., 2012; Zavala et al., 2007). These increases in c-Fos during drug-seeking paradigms are not universal (Sobieraj et al., 2016; Zahm et al., 2010; Zhou et al., 2014). In fact, the only other study to examine possible sex dependent neural correlates in reinstatement actually found c-Fos expression in the NAcC and NAcSh lower in reinstating male and female rats compared to control rats that did not receive reinstatement (Zhou et al., 2014). These discrepancies in neuronal activation have been explained by inhibitory GABAergic and dopaminergic neurotransmission in these regions, as well as significant differences between the studies in self-administration drug and reinstatement trigger (Bossert et al., 2012; Ciccocioppo et al., 2001; Cornish et al., 2012; Hamlin et al., 2008a, 2008b; Kufahl et al., 2009; Miller and Marshall, 2005; Neisewander et al., 2012; Recinto et al., 2012; Sobieraj et al., 2016; Zahm et al., 2010; Zhou et al., 2014).

While the research on sex differences and neural processes associated with meth-triggered reinstatement is quite limited, the female-specific increase in c-Fos labeling in the prelimbic cortex for the MethSA/MethT group versus its controls is consistent with the observation that inhibition of the PrL attenuates meth-triggered reinstatement (Coordie and McFadden, 2019). While this finding by Cordie and McFadden was not sex-specific, Pena-Bravo et al. (2019) found that the amplitude of evoked excitatory post-synaptic current in layer V and VI of the PrL was enhanced relative to a saline control only in female rats that had self-administered meth. Male rats, in contrast, did not differ from saline controls in the amplitude of the evoked excitatory post-synaptic current, but its decay was reduced in male rats that had self-administered meth. Pena-Bravo et al. (2019) further reported that this current appears to be mediated in part by *N*-Methyl-*d*-aspartate (NMDA) receptor containing the GluN2B subunit only for male rats. The current study did not utilize double-staining techniques to identify the specificity of neuronal activation. As such, future research

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examining cell-specific neuronal activation in female and male meth reinstatement will be of interest.

Females in the meth-triggered reinstatement group (MethSA/MethT) had higher c-Fos activation than their male counterparts in the Cg1, LO, dlCPu, vmCPu, NAcC, and CEA. There were also differences between males and females following acute meth administration (SalineSA/MethT). Females had greater c-Fos immunoreactivity following acute meth in the LO, vlCPu, vmCPu, NAcC, NAcSh, and CEA. This significant overlap in neural activation when meth was administered, regardless of self-administration drug type, suggests that many of the differences between the sexes may be a result of initial meth administration and not necessarily in differences in meth as a drug trigger for reinstatement of meth-seeking. The two exceptions to this notion were the Cg1 and dlCPu which only showed differences between the sexes in the group that received meth following a learning history with meth (MethSA/MethT). The Cg1 is particularly significant as previous work suggests this region is of particular importance in multiple forms of reinstatement (Ciccocioppo et al., 2001; Neisewander et al., 2000; Thomas and Everitt, 2001; Wexler et al., 2001). In fact, Neisewander et al. (2000) determined that the cingulate cortex was the sole region that was activated by cocaine-primed reinstatement. Recinto et al. (2012) extended this work to a meth-prime model, also determining the cingulate cortex was integral in reinstatement. The work presented here further solidified that notion and places the cingulate cortex as a region associated with behavioral sex differences detected during meth reinstatement.

Rats in the present study were food restricted to 90% of free-feeding weight. Under certain conditions, the extent and pattern of food restriction, can increase self-administration and/or affect reinstatement (e.g., Comer et al., 1995; Glick et al., 1987). Whether food restriction contributed to the pattern of reinstatement, c-Fos activation, or sex-dependent effects will require more research. Along these lines, the experiment reported herein did not examine gonadal hormone levels, however, previous work does show they likely play a role in the amplified vulnerability to drug addiction found in females. In general, estrogen enhances and progesterone inhibits acquisition and escalation of self-administration, resistance to extinction, and reinstatement of drug-seeking [for a review see Carroll and Anker (2010) and Becker and Koob (2016)]. Specific to drug-primed reinstatement, multiple studies have shown that estrogen treatment enhanced cocaine-primed reinstatement in ovariectomized rats (Anker et al., 2007; Larson and Carroll, 2007; Larson et al., 2005). Preclinical work specifically with meth reinstatement is not nearly as extensive as that with cocaine. With meth, studies have not detected differences in reinstatement based on phase of estrous cycle (Ruda-Kucerova et al., 2015; Cox et al., 2013). However, allopregnanolone does reduce meth-primed reinstatement in female, but not male rats, suggesting gonadal hormones may be a factor (Holtz et al., 2012). Future work further elucidating the precise brain areas involved in these hormonal effects on meth-primed reinstatement will be important.

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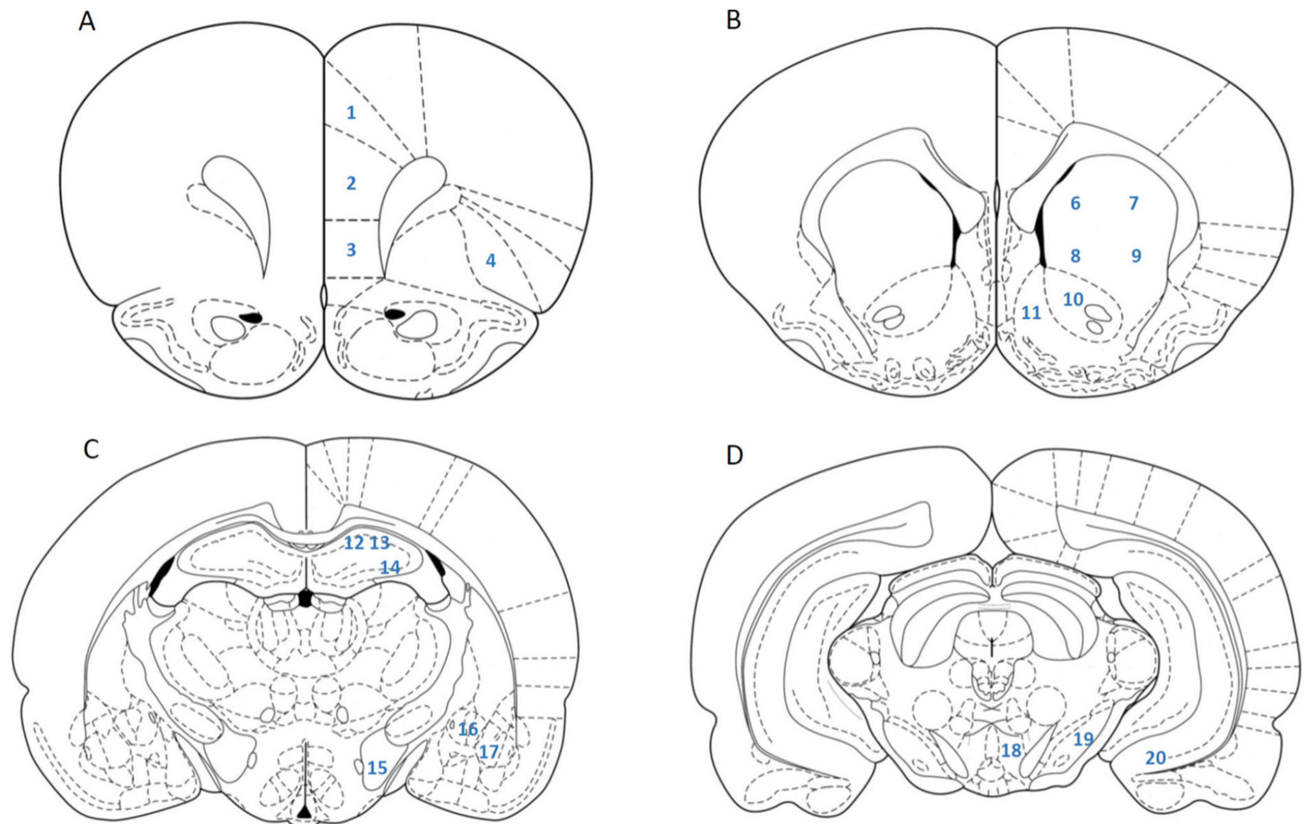

Fig. 1.

Brain regions identified by the atlas of Paxinos and Watson (2007) and examined for c-Fos expression. **A**: Bregma 3.24; **1**-Cg1, **2**-PrL, **3**-IL, **4**-LO. **B**: Bregma 1.80; **5**-Cg2, **6**-dmCPu, **7**-dlCPu, **8**-vmCPu, **9**-vlCPu, **10**-NAcC, **11**-NAcSh. **C**: Bregma −2.64; **12**-CA1, **13**-CA2, **14**-CA3, **15**-LH, **16**-CEA, **17**-BLA. **D**: Bregma −5.88; **18**-VTA, **19**-SNR, **20**-VS.

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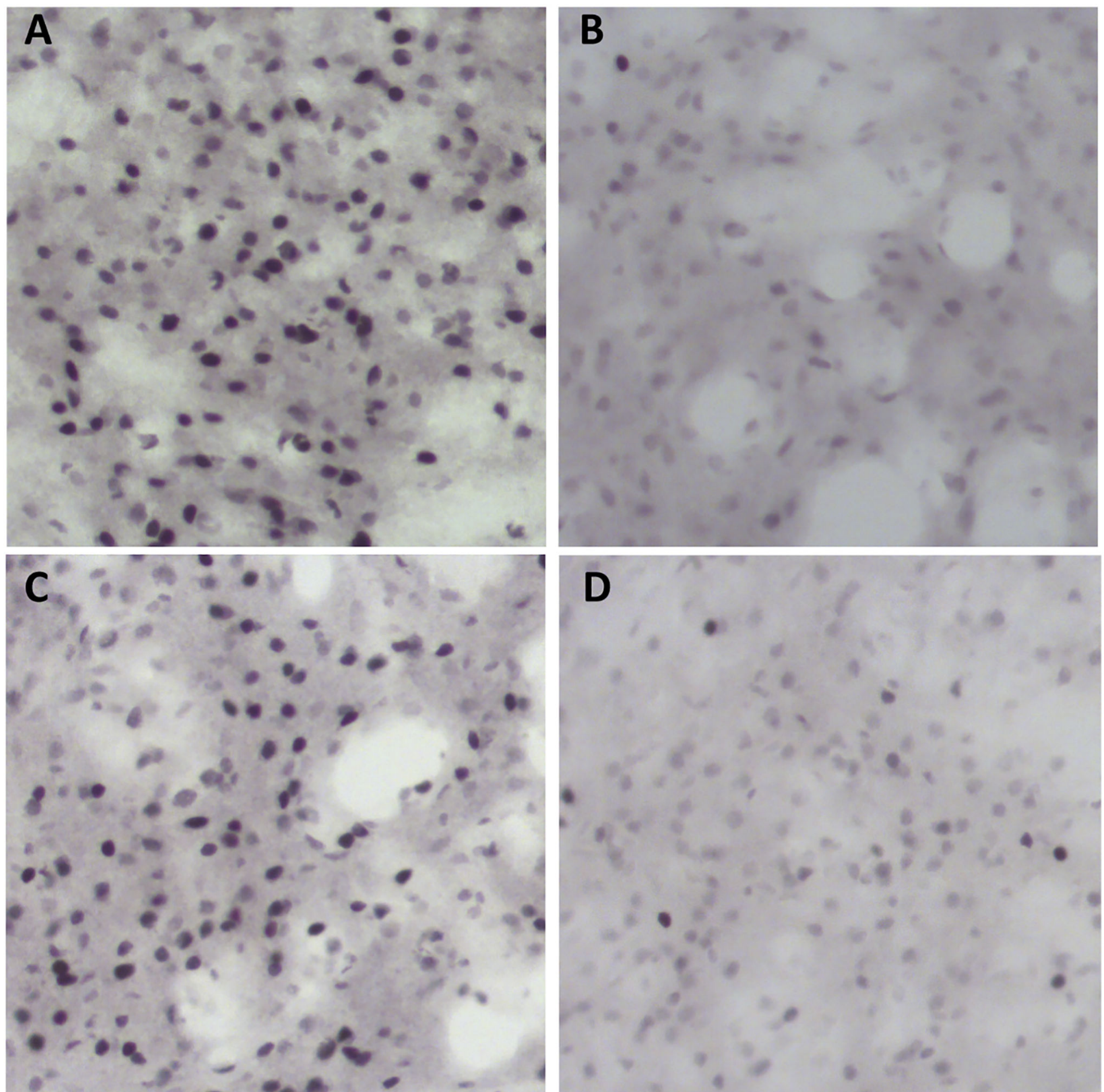

Fig. 2.

Photomicrographs of c-Fos expression in the CEA. **A**: Subject 199; Female MethSA/MethT. **B**: Subject 205; Female MethSA/SalineT. **C**: Subject 184: Female SalineSA/MethT. **D**: Subject 182: Female SalineSA/SalineT.

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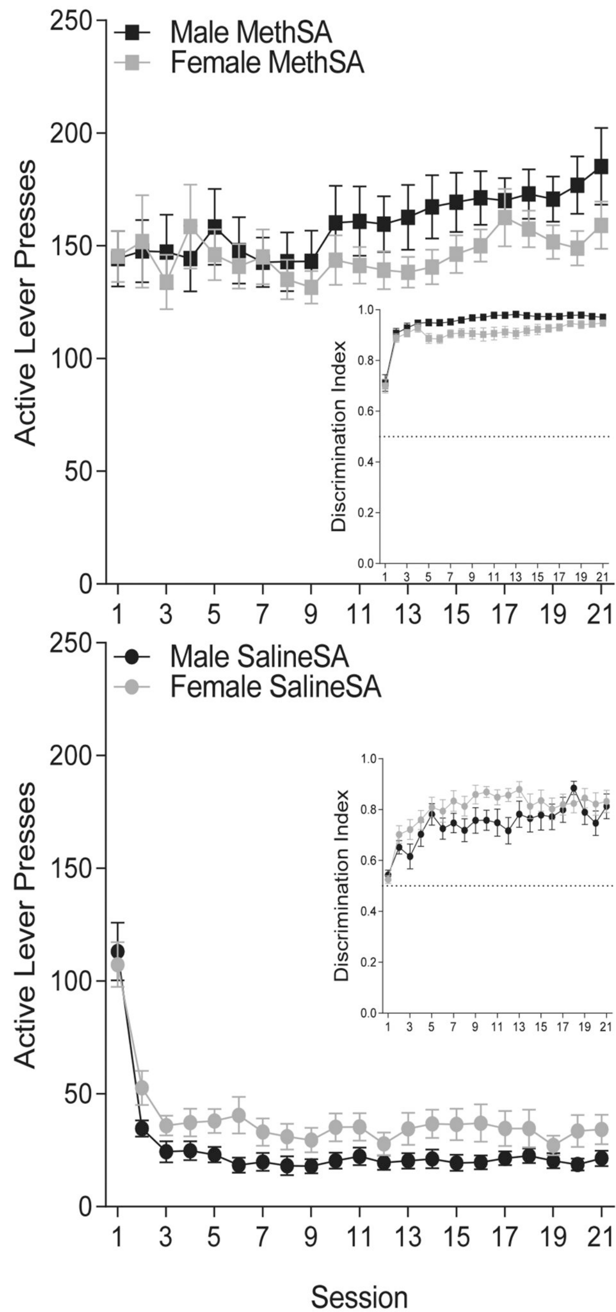

Fig. 3.
Lever pressing (± SEM) during self-administration sessions for males (black) and females (grey) in the meth (square) and saline (circle) conditions. **Inset:** Discrimination Index (± SEM) during self-administration sessions for male and female rats in the meth and saline conditions.

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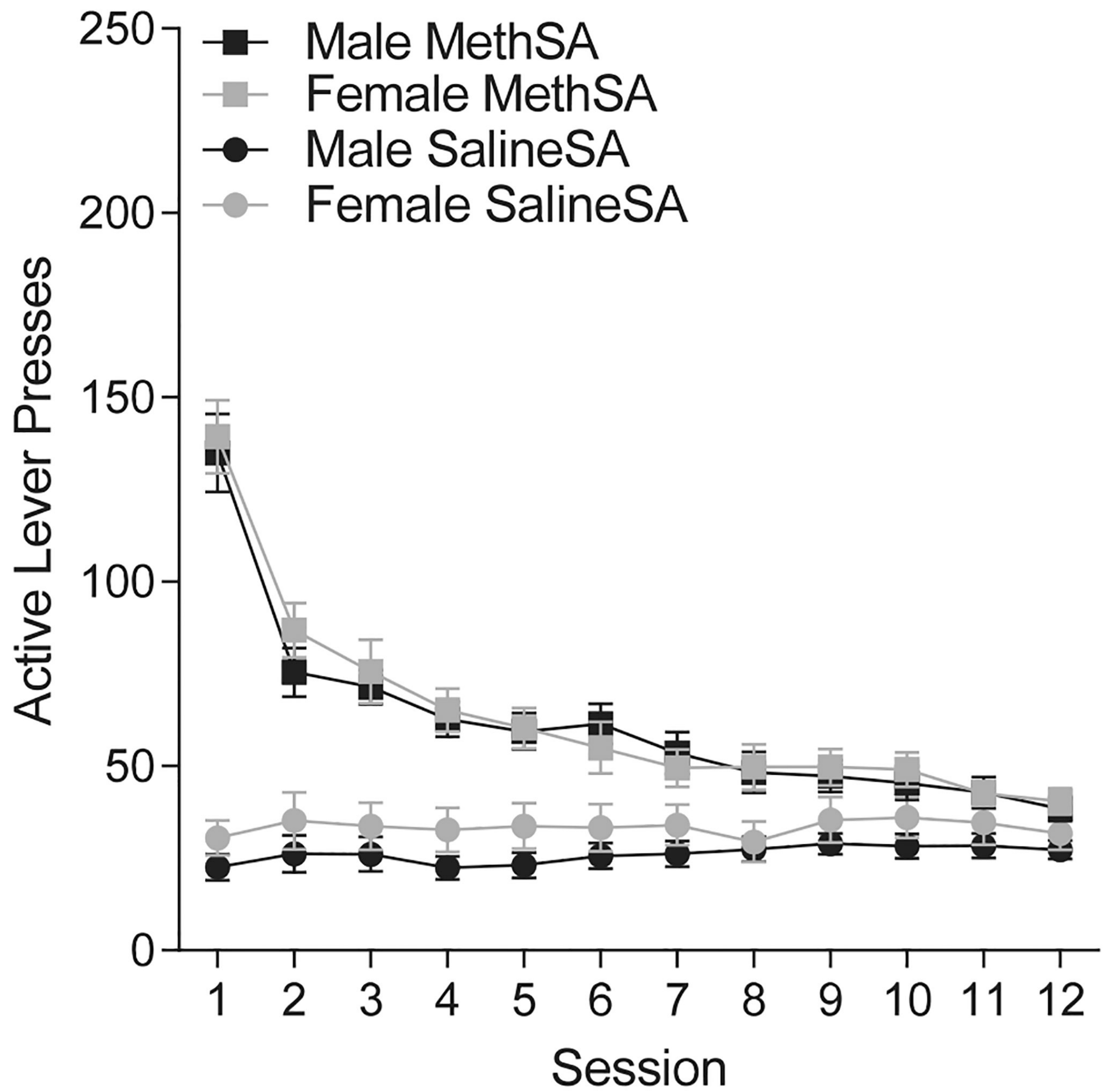

Fig. 4.

Active lever presses (± SEM) in extinction sessions for male (black) and female (grey) rats in the meth (square) and saline (circle) conditions.

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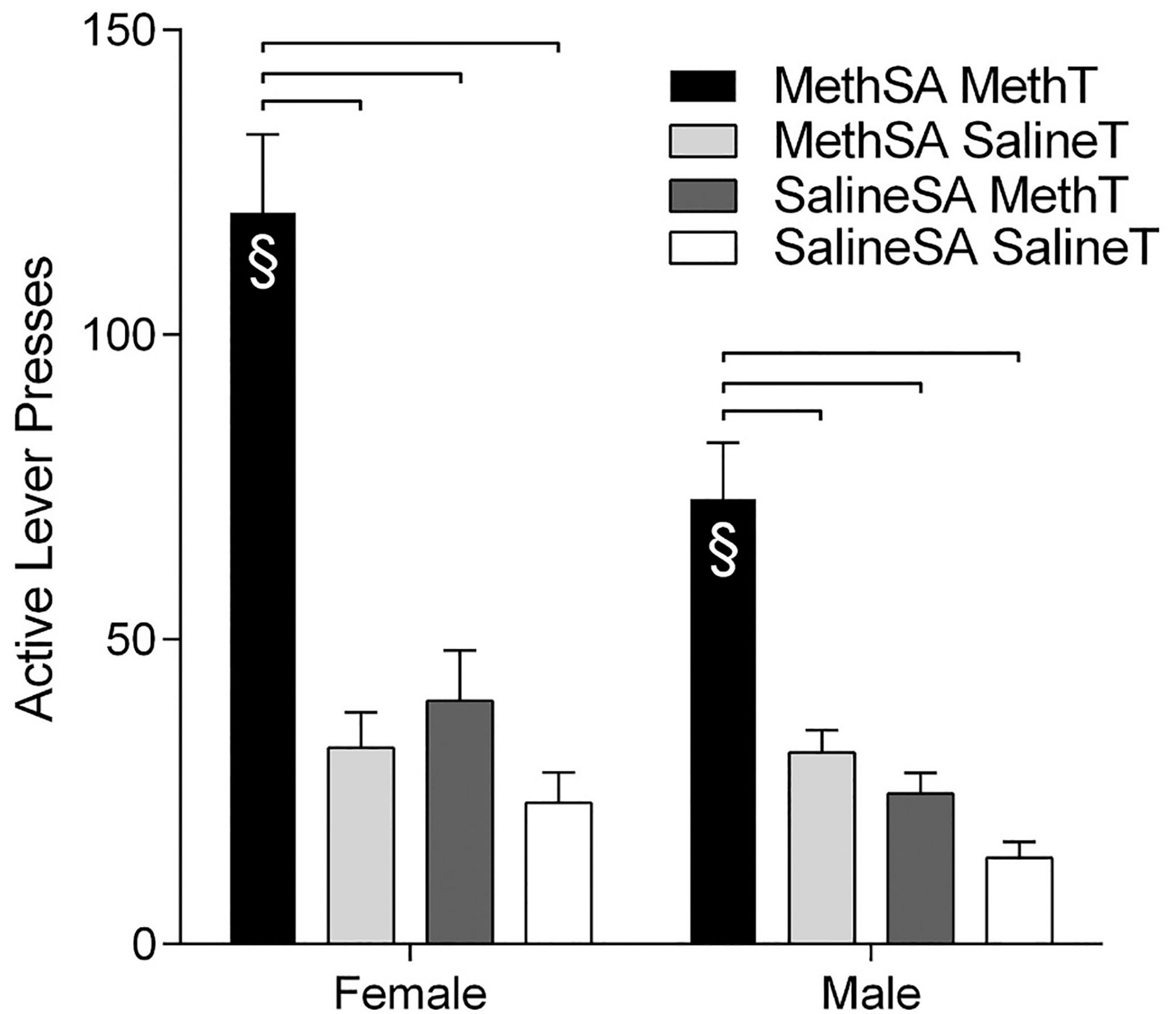

Fig. 5.

Active lever presses (±SEM) for female (left side) and male (right side) rats during the reinstatement session. Groups are delineated by color. Significant differences between groups are marked by hanging bars. Significant sex differences within conditions of Self-Administration Drug and Reinstatement Drug are indicated with §.

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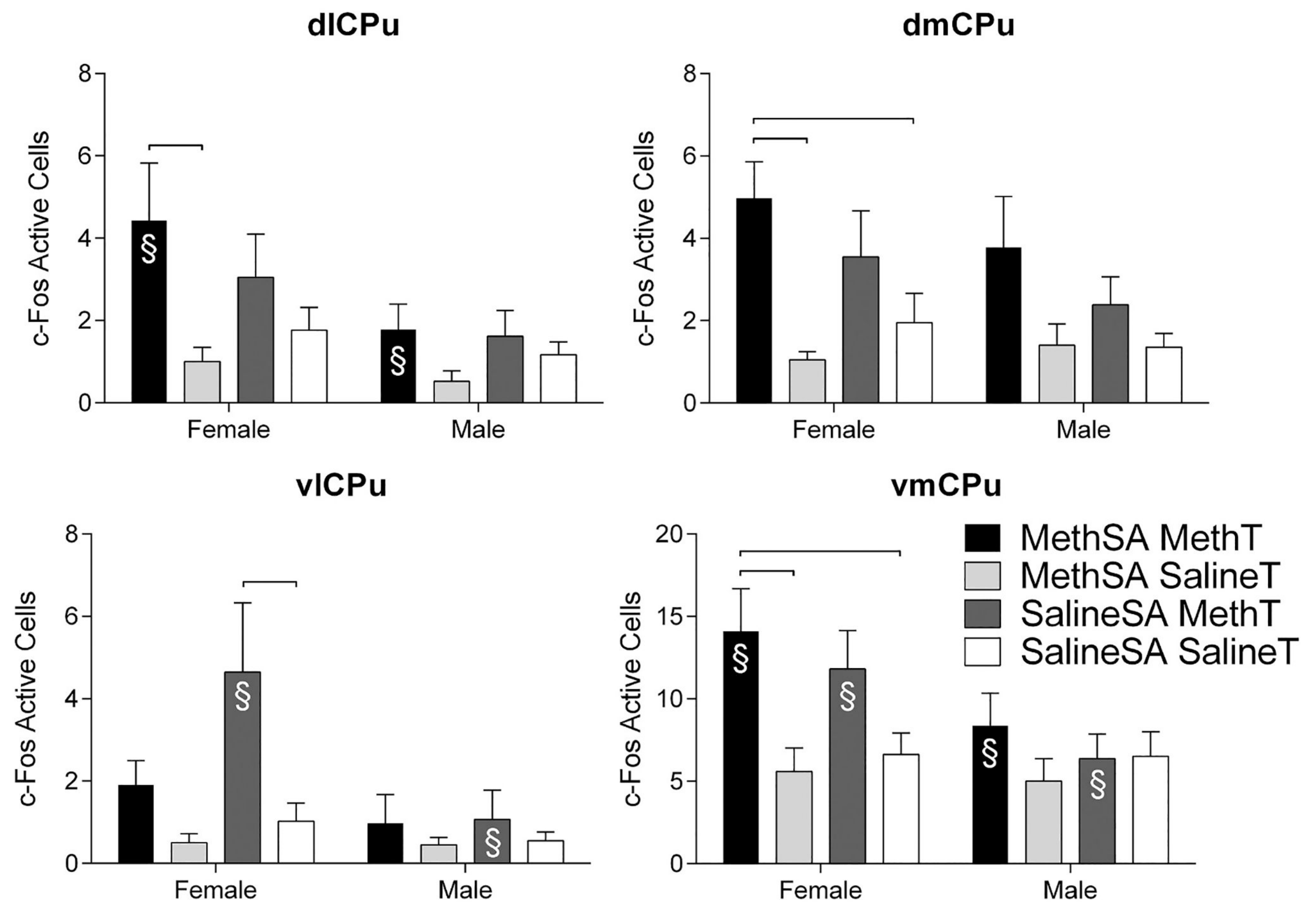

Fig. 6.
Number of c-Fos active cells (± SEM) during reinstatement in females (left) and males (right) in regions of the caudate putamen. Groups are delineated by color. Significant differences between groups within sex are marked by hanging bars. Significant sex differences within conditions of Self-Administration Drug and Reinstatement Drug are indicated with §.

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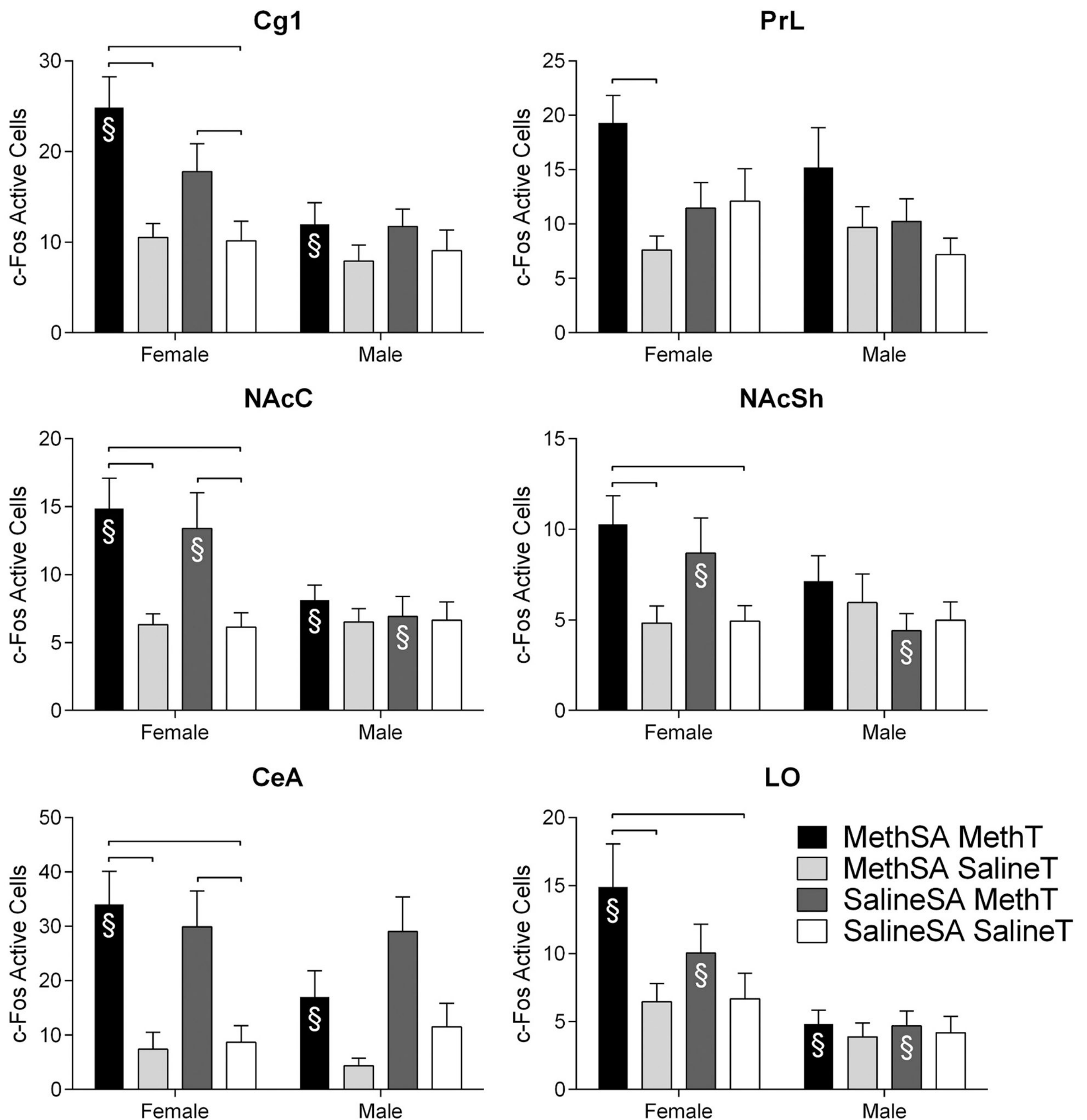

Fig. 7.

Number of c-Fos active cells (± SEM) during reinstatement in females (left) and males (right) in additional regions of interest with significant group differences (regions without group differences are not shown). Groups are delineated by color. Significant sex differences within conditions of Self-Administration Drug and Reinstatement Drug are indicated with §.

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Table 1

List of a priori comparisons examined by post-hoc analysis.

Planned comparisons	
Within females	
1	MethSA/MethT vs MethSA/SalineT
2	MethSA/MethT vs SalineSA/MethT
3	SalineSA/SalineT vs SalineSA/MethT
4	SalineSA/SalineT vs MethSA/SalineT
Within males	
5	MethSA/MethT vs MethSA/SalineT
6	MethSA/MethT vs SalineSA/MethT
7	SalineSA/SalineT vs SalineSA/MethT
8	SalineSA/SalineT vs MethSA/SalineT
Between sex	
9	Female MethSA/MethT vs male MethSA/MethT
10	Female MethSA/SalineT vs male MethSA/SalineT
11	Female SalineSA/MethT vs male SalineSA/MethT
12	Females SalineSA/SalineT vs male SalineSA/SalineT

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Table 2

Statistical analysis of main effects and interactions for each region of interest.

Area	Main effects			Interactions			
	Sex	Reinstatement drug	Self-administration drug	Sex × reinstatement drug	Sex × self-administration	Self-administration drug × reinstatement drug	Sex × Self-Administration Drug × Reinstatement Drug
Cg1	F(1,104)=11.199, p=0.001	F(1,104)=17.923, p<0.001	F<1, p=0.341	F(1,104)=5.061, p=0.027	F(1,104)=1.531, p=0.219	F(1,104)=1.428, p=0.235	F<1, p=0.433
Cg2	F(1,104)=2.230, P=0.138	F(1,104)=3.232, p=0.075	F<1, p=0.618	F(1,104)=1.591, p<0.210	F<1, p=0.521	F<1, p=0.579	F<1, p=0.832
IL	F<1, p=0.423	F(1,104)=2.156, P=0.145	F(1,104), p=0.302	F<1, p=0.962	F<1, p=0.357	F(1,104)=1.613, p=0.207	F<1, p=0.826
LO	F(1,104)=17.256, p=0.001	F(1,104)=7.231, p=0.008	F<1, p=0.361	F(1,104)=4.415, p=0.038	F<1, p=0.337	F(1,104)=1.250, p=0.266	F<1, p=0.352
PrL	F(1,104)=1.456, P=0.230	F(1,104)=8.392, p=0.004	F(1,104)=2.558, P=0.112	F<1, p=0.716	F<1, p=0.543	F(1,104)=4.729, p=0.0319	F(1,104)=2.108, p=0.150
dlCPu	F(104)=6.001, p=0.016	[F(104)=9.377, p=0.002	F<1, p=0.946	F(1,104)=2.042, p=0.156	F<1, p=0.603	F(1,104)=1.978, p=0.163	F<1, p=0.527
draCPu	F(1,104)=1.359, P=0.246	F(1,1o4)=16.082, p<0.001	F<1, p=0.377	F<1, p=0.343	F<1, p=0.677	F(1,104)=2.748	F<l, p=0.662
vlCPu	F(1,104)=5.605, p=0.020	F(1,104)=8.816, p=0.005	F(1,104)=2.647, p=0.107	F(1,104)=3.511, P=0.064	F(1,104)=2.095, p=0.151	F(1,104)=1.088, p=0.299	F(1,104)=1.088, p=0.299
vmCPu	F(1,104)=5.408, p=0.022	F(1,104)=10.978, p=0.001	F<1, p=0.734	F(1,104)=4.185, p=0.043	F<1, p=0.878	F(1,104)=1.764; P=0.187	F<1, p=0.974
NAcC	F(1,104)=7.917, p=0.005	F(1,104)=15.737, p<0.001	F<1, p=0.541	F(1,104)=9.755, p=0.002	F<1, p=0.894	F<1, p=0.562	F<1, p=0.996
NAcSh	F(1,104)=2.752, P=0.100	F(1,104)=6.787, p=0.011	F=1.901, p=0.170	F(1,104)=5.189, p=0.025	F<1, p=0.559	F<1, p=0.362	F<1, p=0.985
CA1	F(1,104)=3.476 p=0.065	F(1, 104)=6.156, p=0.015	F<1, p=0.353	F<1, p=0.355	F<1, p=0.535	F(1,104)=1.537, p=0.217	F<1, p=0.355
CA2	F<1, p=0.450	F(1,104)=1.49, p=0.225	F(1,104)=2.970, p=0.088	F<1, p=0.811	F<1, p=0.460	F(1,104)=4.85, p=0.030	F<1, p=0.811
CA3	F(1,104)=1.932, p=0.168	F(1,104)=2.861, p=0.094	F<1, p=0.883	F(1,104)=1.211, p=0.273	F<1, p=0.902	F(1,104)=1.851, p=0.177	F(1,104)=1.109, p=0.295
VS	F(1,104)=1.492, p=0.224	F<1, p=0.383	F<1, p=0.639	F(1,104)=1.069, p=0.304	F<1, p=0.612	F(1,104)=2.911, p=0.091	F<1, p=0.894
BLA	F<1, p=0.600	F(1,102)=4.478, p=0.036	F(1,102)=2.621, p=0.109	F<1, p=0.814	F(1,102)=1.360, p=0.246	F(1,102)=2.045, P=0.156	F<1, p=0.605

Area	Main effects			Interactions			
	Sex	**Reinstatement drug**	**Self-administration drug**	**Sex × reinstatement drug**	**Sex × self-administration**	**Self-administration drug × reinstatement drug**	**Sex × Self-Administration Drug × Reinstatement Drug**
CEA	F(1,102)=1.751, p=0.189	F(1,102)=32.584, p<0.001	F(1,104)=1.417, P=0.237	F(1,102)=1.689, p=0.197	F(1,102)=2.589, p=0.111	F<1, p=0.975	F<1, p=0.451
LH	F(1,104)=3.417, p=0.067	F(1,104)=6.378, p=0.013	F(1,104)=1.024, P=0.314	F<1, p=0.754	F<1, p=0.570	F<1 p=0.334	F<1, p=0.489
SNR	F(1,103)=2.696, p=0.104	F<1 p=0.648	F<1, p=0.888	F<1, p=0.757	F<1, p=0.727	F(1,103)=1.099, p=0.297	F<1, p=0.998
VTA	F(1,103)=1.890, p=0.172	F<1, p=0.601	F<1, p=0.676	F<1, p=0.780	F<1, p=0.500	F<1, p=0.371	F<1, p=0.732

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