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Regionally Specific Effects of Oxytocin on Reinstatement of Cocaine Seeking in Male and Female Rats

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Abstract

Background: Oxytocin reduces cued reinstatement of cocaine seeking in male and female rats, but the underlying neurobiology has not been uncovered. The majority of effort on this task has focused on oxytocin and dopamine interactions in the nucleus accumbens core. The nucleus accumbens core is a key neural substrate in relapse, and oxytocin administration in the nucleus accumbens core reduces reinstatement to methamphetamine cues. Further, the nucleus accumbens core has strong glutamatergic innervation from numerous regions including the prefrontal cortex. Thus, we hypothesize that oxytocin regulates presynaptic glutamate terminals in the nucleus accumbens core, thereby affecting reinstatement.
Methods: To begin to evaluate this hypothesis, we examined the effects of intra-nucleus accumbens core oxytocin on extracellular glutamate levels in this region. We next determined if direct infusion of oxytocin into the nucleus accumbens core could attenuate cued reinstatement of cocaine seeking in a manner dependent on metabotropic glutamate 2/3 receptors. Finally, we tested if site-specific application of oxytocin in the prefrontal cortex reduced cued reinstatement of cocaine seeking.
Results: We found an increase in nucleus accumbens core extracellular glutamate for several minutes following reverse dialysis of oxytocin. In male and female rats with a history of cocaine self-administration, site-specific application of oxytocin in the nucleus accumbens core and prefrontal cortex had opposing effects, decreasing and increasing cued reinstatement, respectively. The mGlu2/3 antagonist LY-341495 reversed oxytocin's ability to attenuate cued reinstatement.
Conclusions: While the precise mechanism by which oxytocin increases nucleus accumbens core glutamate is yet to be determined, the present results clearly support oxytocin mediation of glutamate neurotransmission in the nucleus accumbens core that impacts cued cocaine seeking.

Keywords: neuroactive peptides, substance abuse, relapse, reinstatement, glutamate, prefrontal cortex

Introduction

Oxytocin is a well-characterized neuroendocrine hormone produced within the paraventricular nucleus and supraoptic nucleus of the hypothalamus. Oxytocin cells project to a number of areas involved in addiction, including the limbic regions and ventral striatum (Knobloch and Grinevich, 2014). Oxytocin receptors are ubiquitous throughout the brain, are Gq-coupled,

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Significance Statement

In animal models, oxytocin consistently decreases relapse to cues that are associated with the physiological effects of the drug, but very little is known about the neural mechanisms driving this ability. We uncovered bi-directionality of oxytocin's effect on reinstatement of cocaine seeking with increased and decreased responding following site-specific application of the neuropeptide in the PFC or the NAcc, respectively. This study indicates the PFC and NAcc are critical structures involved in the interaction between oxytocin and glutamatergic signaling in males and females and provides critical insight that will aid in the development of oxytocin-based therapies.

and activate transduction pathways which include IP3 receptor activation and the release of intracellular calcium stores (Gimpl and Fahrenholz, 2001). Recently, oxytocin has received increased interest as a treatment for many neuropsychiatric disorders, including addiction.

Addiction remains a persistent problem that affects both men and women and treatments aimed at preventing relapse are needed. Oxytocin shows promise to fill this identified need. In humans, oxytocin administration alleviates stress-induced marijuana craving (McRae-Clark et al., 2013) and reduces some symptoms of alcohol withdrawal (Pedersen et al., 2013). Similarly, in rodents, oxytocin reduces ethanol self-administration and consumption in mice (MacFadyen et al., 2016; King et al., 2017); reduces morphine tolerance and withdrawal effects (Sarnyai and Kovács, 2014), blocks methamphetamine-conditioned behaviors (Qi et al., 2009), and reduces reinstatement of methamphetamine seeking (Carson et al., 2010a; Cox et al., 2013; Baracz and Cornish, 2016). Additionally, oxytocin decreases methamphetamine responding on a progressive ratio schedule of reinforcement in female but not male rats (Cox et al., 2013). In humans, cocaine use decreases plasma oxytocin levels (Light et al., 2004) and our laboratory has shown that systemic oxytocin decreases active lever presses for cocaine, cocaine intake during self-administration, and cue-induced reinstatement of cocaine seeking following extinction in male and female rats (Zhou et al., 2014; Leong et al., 2016, 2017). In spite of this strong evidence indicating the potential of oxytocin as a treatment for cocaine addiction, very little is known about the specific mechanisms behind oxytocin's behavioral effects and whether those mechanisms are sexually dimorphic.

The prefrontal cortex (PFC) exerts top-down control over the nucleus accumbens core (NAcc). A recent report phenotyped oxytocin receptor expressing neurons in mouse PFC and found that 46% and 33% of oxytocin-expressing neurons in the PFC are glutamatergic and GABAergic, respectively. The glutamatergic oxytocin receptor expressing neurons project to the NAcc (Tan et al., 2017). Maladaptive changes in glutamatergic regulation occur in the PFC-NAcc pathway following cocaine exposure (Scofield et al., 2016). Briefly, 2 to 3 weeks following cocaine self-administration, basal extracellular glutamate levels are decreased in the NAcc and the PFC-NAcc glutamate projection is necessary for the reinstatement of cocaine seeking (Baker et al., 2003; McFarland et al., 2003). Compounds such as ceftriaxone and N-acetylcysteine increase NAcc basal extracellular glutamate and attenuate both cocaine- and cue-primed reinstatement of cocaine seeking while attenuating glutamate release in the NAcc (Baker et al., 2003; Knackstedt et al., 2010; Trantham-Davidson et al., 2012). The ability of N-acetylcysteine to attenuate the reinstatement of cocaine seeking is prevented by both systemic and intra-NAcc infusion of the mGlu2/3 antagonist LY341495 (Moran et al., 2005; Moussawi et al., 2011). Conversely, activation of NAcc mGlu2/3 receptors decreases cued cocaine seeking but also reduces consumption of chow (Baptista et al., 2004; Peters et al., 2008). Presynaptic rather than postsynaptic localization of mGlu receptors are believed to drive these effects (Kalivas, 2009; Moussawi et al., 2009; Wang et al., 2013).

Recently, we have shown that site-specific application of oxytocin into the NAcc decreases cued reinstatement of methamphetamine-seeking and, importantly, blockade of mGlu2/3 receptors prevents this effect (Bernheim et al., 2017). This finding is noteworthy, because it suggests that NAcc oxytocin interacts with glutamate via a presynaptic mechanism. In the NAcc, oxytocin receptors have been detected on astrocytes, 5HT-2a-expressing neurons, and on parvalbumin-positive interneurons (Di Scala-Guenot and Strosser, 1992; Di Scala-Guenot et al., 1994; Dölen et al., 2013). Oxytocin receptor mRNA is found in glutamate neurons in the ventral tegmental area (Peris et al., 2017) and PFC (Tan et al., 2017), regions that send glutamate projections to the NAcc.

To begin to understand oxytocin's actions within the PFC-NAcc circuit in a model of cocaine relapse, we first determined if oxytocin had an effect on NAcc glutamate levels and then used the cocaine self-administration, extinction, and reinstatement model to assess reinstated responding to cocaine associated cues using site-specific application of oxytocin into the PFC and NAcc. We also tested whether blockade of mGluR2/3 receptors in the NAcc would block oxytocin's effects and determined whether oxytocin administration increased NAcc glutamate release. Finally, we tested whether systemic administration of oxytocin and mGluR receptor antagonists would also decrease cued reinstatement of cocaine seeking since oxytocin treatment regimens are delivered through systemic circulation in cocaine addicts.

Methods

Subjects

Experiment 1 used 12 male Sprague-Dawley rats (Charles River Laboratories). Experiments 2 to 4 used a total of 45 male and 45 female Sprague-Dawley rats (Harlan). All rats weighed 250 to 300 g (male) and 225 to 250 g (female) at the time of arrival. Rats were single-housed on a reversed 12-hour-light/-dark cycle (lights off at 6:00 AM) in a temperature- and humidity-controlled vivarium. All experimental procedures were conducted during the dark cycle. Rats received water ad libitum and were kept on a stable intake diet 20 to 40 g of standard rat chow daily throughout the study. Procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Rats" (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, 2011) and approved by the IACUC of the University of Florida (Experiment 1) and the Medical University of South Carolina (Experiments 2–4).

Experiment 1: Microdialysis and High Performance Liquid Chromatography (HPLC) for Quantification of Glutamate

Surgery to implant intracranial guide cannulas occurred in a stereotaxic frame (Stoelting). Stainless-steel guide cannulas

(22 gauge; Synaptech) were bilaterally aimed 2 mm above the NAcc according to the following coordinates anterior-posterior (AP) + 1.2 mm, medial-lateral (ML) ± 1.6 mm, dorsal-ventral (DV) −5.5 mm; (Paxinos and Watson, 2007) and were secured to the skull with stainless-steel skull screws and dental acrylic (Co-Oral-Ite Dental MFG. Co.). The analgesic ketorolac (2 mg/kg, i.p.) was administered for 3 days following surgery. One week after surgery, a microdialysis probe was implanted unilaterally. Probes were constructed of a 2-mm cuprophane membrane (20 kDa cut-off weight, outer diameter 0.36 mm; Synaptech). Subjects were placed into a plexiglass box (45 cm × 45 cm) and perfused with artificial cerebral spinal fluid (aCSF: 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl26H2O, 5 mM D-glucose, 1.2 mM CaCl2H20, 0.75 mL 10x phosphate buffered saline). Liquid switches (CMA Harvard Apparatus Company) were utilized to minimize pressure changes when changing perfusion solutions. Following 4 hours of equilibration to aCSF perfusion (2 µL/min), six 10-min baseline samples were collected. Following baseline sample collections, reverse dialysis of oxytocin (36 µM/side in aCSF), with or without the voltage-gated sodium channel blocker tetrodotoxin (TTX; 1 µM) occurred for 5 min, followed by aCSF for 60 minutes. This concentration of oxytocin was chosen to approximate the concentration infused in Experiments 2 to 3, accounting for loss due to dialysis membrane permeability. This concentration of TTX was chosen because it does not reduce basal extracellular glutamate in the NAcc of drug-naïve animals (Baker et al., 2002; Pati et al., 2016) but does prevent synaptic glutamate release in this brain region (McFarland et al., 2003). Furthermore, this concentration suppresses action potentials in the striatum in vitro (Jiang and North, 1991). During oxytocin/TTX perfusion and for the subsequent 60 minutes, dialysate samples were collected once/5 min. The hemisphere of probe insertion was counterbalanced, and probing separated by at least 2 days. Twelve rats were used in this experiment. Five of the 12 rats were probed bilaterally, while 4 were only probed unilaterally, yielding a total of 9 rats (14 hemispheres: oxytocin = 8, oxytocin + TTX = 6) that were used for the data presented in Figure 1. Three rats that received surgeries were eliminated due to illness prior to probing (n = 1), improper cannulae placement (n = 1), and head cap loss (n = 1).

Experiments 2 to 4: Surgery

Rats were anesthetized with i.p. injections of ketamine (66 mg/kg, i.p., Vedco Inc.) mixed with xylazine (1.3 mg/kg, i.p., Lloyd Laboratories), and equithesin (0.5 mL/kg). Ketorolac (2.0 mg/kg, i.p., Sigma) was given before surgery as an analgesic. Surgical procedures were conducted using aseptic techniques. One end of a silastic catheter was implanted into the external right jugular vein. The other end ran subcutaneously, exited from a small incision on the back, and attached to an infusion cannula (PlasticsOne Inc.). Cephazolin (10 mg/0.1 mL) was given post-surgery (0.1 mL i.v.) and during recovery along with 0.05 mL of Taurolidine-Citrate Catheter Solution (TCS; Access Technologies). Experiment 2 and 3 required the use of chronic, indwelling intracranial cannula. In this case, intracranial guide cannulas (Plastics One) directed at the NAcc or PFC, in separate groups of rats, were stereotaxically implanted after the catheter surgery, under the same plane of anesthesia. The rat was placed in the stereotactic apparatus, and the skin overlying the skull was excised. The skull was cleared, and bregma and lambda were positioned at the same DV coordinate. NAcc coordinates were measured from bregma at the skull surface as follows: AP +1.5 mm, ML ± 2.8 mm, DV -7.1mm, at a 10° angle. We targeted the prelimbic (PL) area of PFC using the following coordinates: AP +2.8 mm, ML ± 0.6 mm, and DV -2.7 mm measured from bregma. Jeweler's screws were used to anchor the cannula to the skull and were cemented in place. Dummy wires were inserted through the cannula to prevent obstruction by debris and were the same length as the cannula. Rats recovered 5to 10 days before initiating cocaine self-administration.

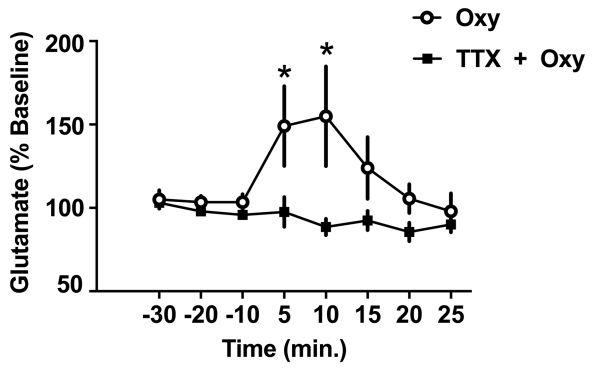

Figure 1. Oxytocin (36 µM/side) increases glutamate levels in the nucleus accumbens core (NAcc) of drug naïve rats when measured using reverse dialysis for up to 20 minutes past application time. The effect was blocked in the presence of tetrodotoxin (TTX). * Significant increase from baseline (-10 relative to 5 and 10 minutes) ($P < .05$).

Experiments 2–4: Self-Administration, Extinction, and Reinstatement

Self-administration procedures (see Figure 2 timeline) were based on recent self-administration studies in males and females (Cox et al., 2013; Leong et al., 2016, 2017). All self-administration experiments were conducted during the rats' dark cycle in standard Plexiglas self-administration chambers (30 x 20 x 20 cm) that were enclosed in sound attenuating cubicles with a ventilation fan (Med Associates) and linked to a computerized data collection program (MED PC, Med Associates). Each chamber was equipped with 2 retractable levers with a white stimulus light above each lever, a house light, and a tone generator. For cocaine self-administration, infusion tubing was enclosed in steel spring leashes (Plastics One Inc) and connected to the infusion cannula and a weighted swivel apparatus (Instech) that was suspended above the box to allow for free movement within the chamber.

Cocaine hydrochloride (provided by the National Institute on Drug Abuse) was dissolved in 0.9% sterile saline and administered at a dose of 0.5 mg/kg/infusion. Self-administration sessions were conducted 6 d/week for 2 h/d. The house light remained on throughout the sessions, and the appropriate number of responses on the active lever resulted in activation of the infusion pump and delivery of a 2-sec i.v. cocaine infusion and a 5-sec presentation of a cue complex (illumination of the white stimulus light over the active lever and activation of tone generator; 78 dB, 4.5 kHz), followed by a 20-second time-out period. During the time-out period, responses on the active and inactive levers were recorded but had no scheduled consequences. Rats initially self-administered cocaine on a fixed ratio 1 (FR1) schedule of reinforcement (1 lever press resulted in a drug infusion) until they reached the criterion of a minimum of 5 days with >10 infusions. Rats then moved to a FR3 schedule for a minimum of 3 days, followed by a FR5 schedule for the remainder of the self-administration sessions. To verify catheter patency when rats were not administering cocaine, a 0.10- to 0.12-mL i.v. infusion of methohexital sodium (Eli Lilly), a short-acting barbiturate

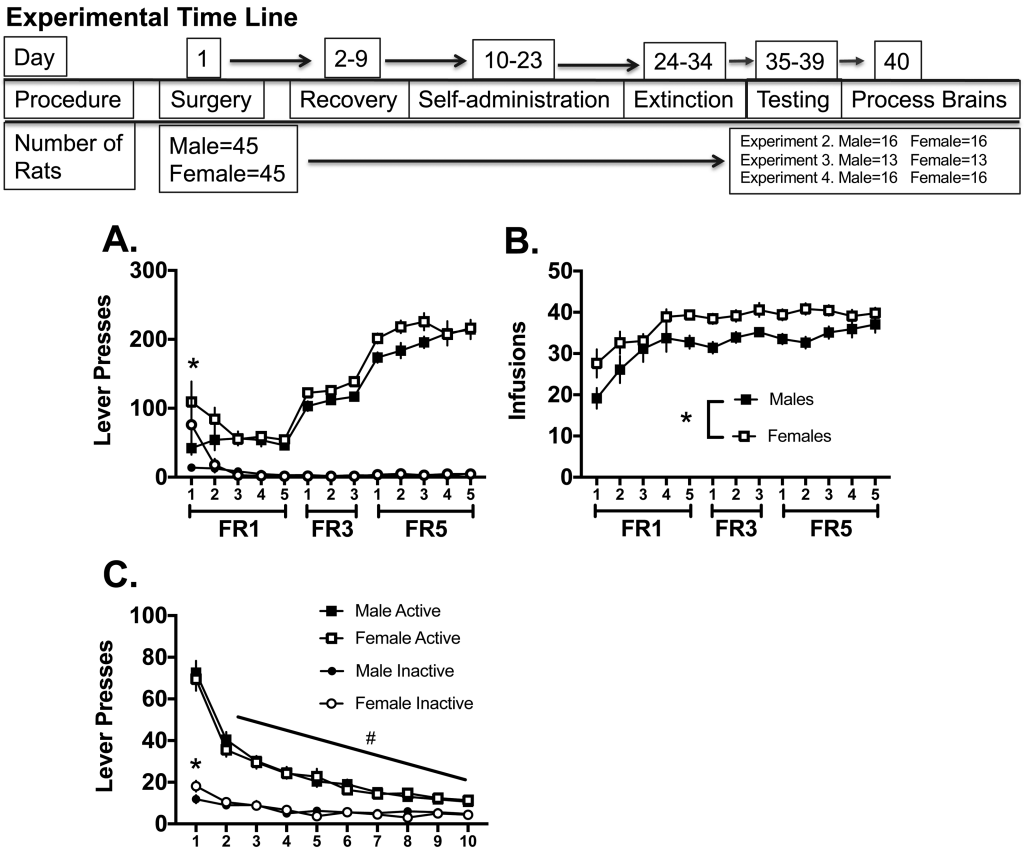

Figure 2. Experimental timeline of self-administration experiments is depicted at the top of the figure. (A) Active lever responding increased as FR values increased and inactive lever responding decreased over time. Females responded more on both levers relative to males on the first day of self-administration. *Significant difference between males and females ($P < .05$). (B) Females received more infusions relative to males ($P < .05$). (C) All animals diminished responding on the active lever over the extinction days. Females responded more than males on the inactive lever on day 1 of extinction. *Significant difference between male and female rats ($P < .05$). # Significant difference from day 1 of extinction ($P < .05$).

that produces a rapid loss of muscle tone when administered i.v. After each self-administration session, rats' catheters were flushed with 0.05 mL TCS.

Following self-administration, rats underwent 2-hour daily extinction sessions for a minimum of 10 days, where responses on both the active and inactive levers were recorded but had no scheduled consequences. Extinction criterion consisted of <25 active lever presses for 3 consecutive days (Leong et al., 2017; Cox et al., 2013; Bernheim et al., 2017). Upon reaching extinction criteria, rats underwent cued reinstatement tests for 2 hours. Responses on the active lever resulted in presentation of only the light + tone stimulus complex (described above) that previously accompanied i.v. cocaine using an FR1 schedule of reinforcement. No cocaine was administered during this test. Responses on the inactive lever were also recorded. Each rat was tested twice with at least 2 extinction sessions (2 h/d) between tests. Test conditions and test order were randomly assigned without replacement until groups were full. Specific experimental methods are detailed below for intracranial microinfusions and drug administration.

Experiments 2 to 3: Intracranial Microinfusions

Animals received a microinfusion of either oxytocin (0.6 nmol/0.25μL/side; Cell Sciences) or saline (0.25 μL/side) in the PFC or NAcc. Some animals received a microinfusion of either LY-341495 (1.3 nmol/0.25 μL/side; Tocris Bioscience) or saline (0.25 μL/side) before oxytocin (0.6 nmol/0.25μL/side) or saline (0.25μL/side) directly into the NAcc. For intracranial administration, oxytocin was infused at a concentration of 0.6 nmol/0.25μL/side (Baracz et al., 2012; Cox et al., 2017; Bernheim et al., 2017). LY341495 was infused at a concentration of 1.3 nmol/0.25μL/side based on a range of 2 efficient doses (Kim et al, 2015; Richard and Berridge, 2011; Bernheim et al., 2017). The microinjector extended 1 mm (PFC) or 1.5 mm (NAcc) beyond the cannula. The PFC infusion took place over 1 minute and the NAcc infusion occurred over 2 minute; injectors were left in place an additional minute to allow diffusion of the drug. Five minutes after the final microinfusion, animals were placed into the chamber for reinstatement testing.

At the end of the experiment, rats were decapitated and brains were collected for histological assessment of cannula placement. In brief, rats were deeply anesthetized with Equithesin and then transcardially perfused with 150 to 200 mL cold 0.9% saline followed by 400 to 500 mL of 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde for 24 hours, submerged in 20% sucrose for 48 hours, and then sectioned on a vibratome. Sections were mounted on microscope slides and cannula placement was verified within the NAcc or PFC.

Experiment 4: Systemic Oxytocin and LY-341495 Administration

Rats received a systemic injection of LY-341495 (1 mg/kg) or vehicle (i.p.) followed by oxytocin (1 mg/kg) or saline (i.p.) 5 minutes later. For systemic injections oxytocin was administered at 1 mg/kg (Cox et al., 2013, Zhou et al., 2015; Leong et al., 2016) and

LY341495 was injected at 1 mg/kg (Scofield et al., 2015; Bernheim et al., 2017). After the second injection, animals waited 30 minutes before being placed into the operant chamber for reinstatement testing to allow the locomotor-suppressing effects of systemic oxytocin to diminish before testing.

Data Analysis

Glutamate levels were normalized to the average baseline glutamate value for each subject and group differences (TTX+oxytocin vs oxytocin) over time were examined using repeated measures (RM) ANOVA with RM conducted on the time variable. The number of lever responses and infusions were the primary dependent variables during drug self-administration and they were analyzed using ANOVA with day (within subjects) and sex (between subjects) as independent variables. For reinstatement testing, lever responses were analyzed using a 2-way ANOVA with sex and treatment as between subject factors. Posthoc comparisons were conducted using a Holm-Sidak's correction for family wise error when appropriate, with the alpha set at 0.05. All analyses were conducted with Prism Software versions 6 and 7. All data are expressed as the mean ± SEM.

Results

Experiment 1: Oxytocin-Induced Glutamate Release in the NAcc Was Blocked by Administration of TTX

Figure 1 illustrates that oxytocin reverse dialyzed in the NAcc increased extracellular glutamate, which was blocked by co-administration of TTX. There was a significant time x treatment interaction, [$F(7,84) = 2.16$, $P < .03$]. Follow-up comparisons show that oxytocin increased glutamate at 5 and 10 min relative to baseline assessed at -10 (Holm-Sidak's multiple comparisons, $P < .05$), while TTX blocked oxytocin induced glutamate release at all time points.

Experiments 2 to 4: Self-Administration and Extinction

Self-administration and extinction data were consolidated between experiments, because they did not differ and were conducted under identical conditions. Figure 2A depicts the active and inactive lever presses throughout the self-administration period for all the male (n = 45) and female (n = 45) rats that were used in the reinstatement testing in Experiments 2 to 4. The timeline in Figure 2 depicts the breakdown of these animals for each experiment. For active lever presses, there was a significant interaction between sex and self-administration day [$F(12,1032) = 1.88$, $P = .03$] with females responding more on day one of self-administration relative to males (Holm-Sidak's, $P < .05$). There was also a main effect of sex [$F(1,86) = 5.63$, $P = .02$] with females pressing the active lever more than males. There was also a significant main effect of day [$F(12,1032) = 91.6$, $P < .0001$] as lever presses increased over time and as the FR increased. On the inactive lever there was also a significant interaction between sex and self-administration day [$F(12,1032) = 2.33$, $P = .0006$] with females responding more on day one of self-administration relative to males (Holm-Sidak's, $P < .05$). The main effect of sex was not significant, but there was a significant main effect of day [$F(12,1032) = 4.3$, $P < .0001$] as inactive lever presses decreased over time.

Figure 2B represents the number of infusions received by males and females during self-administration. Females received a greater number of cocaine infusions than males as indicated by a main effect of sex [$F(12,87) = 10.96$, $P = .0014$]. There was also a significant main effect of day [$F(12,1044) = 13.7$, $P < .0001$] as the number of infusions increased over time. The sex by day interaction was not significant.

Figure 2C depicts active and inactive lever responding during extinction in males and females. On the active lever there a significant main effect of day [$F(9,513) = 125.8$, $P < .0001$] as lever presses decreased over time, specifically day 2 to 10 were significantly lower than extinction day one. The interaction between sex and extinction day and the main effect of sex were not significant. On the inactive lever there was a significant interaction between sex and extinction day [$F(9,513) = 3.71$, $P = .0002$] with females responding more on day one of extinction relative to males (Holm-Sidak's, $P < .05$). There was also a significant main effect of day [$F(9,513) = 24.7$, $P < .0001$] as lever presses decreased over time. The main effect of sex was not significant.

Experiment 2: Intra-Accumbal Oxytocin Reduced Cued Reinstatement of Cocaine Seeking and mGlu2/3 Receptor Antagonism Blocked this Effect in Males and Females

Figure 3A depicts findings for intra-accumbal effects of oxytocin and blockade with LY-341495 in male (n = 16) and female (n = 16) rats. NAcc oxytocin decreased lever responding in response to cues and LY341495 reversed this effect. There was a main effect of drug treatment [$F(3,53) = 8.12$, $P < .002$], but no significant main effect of sex and no significant interaction between sex and drug treatment. Posthoc comparisons on the main effect of drug treatment showed that Veh/Sal, LY/Sal, and LY/Oxy groups had greater lever responding vs the Veh/Oxy group (Holm-Sidak's, $P < .05$). Figure 3B depicts the data for male and female rats separately to visualize the lack of a sex difference on reinstatement testing. Two males and two females were excluded from the study following histological analyses. Figure 3C depicts the terminal point of the injectors used to infuse the test compounds into the NAcc.

Experiment 3: Oxytocin in the PFC Increases Reinstatement to Cocaine-Associated Cues

Figure 3D illustrates that oxytocin microinfusions in the prefrontal cortex of male (n = 13) and female (n = 13) rats increased cued reinstatement of cocaine seeking. There was a main effect of drug treatment [$F(1,24) = 5.69$, $P < .002$], with oxytocin increasing active lever presses. However, the main effect of sex and interaction between sex and drug treatment were not significant. Figure 3E depicts the data for male and female rats separately to visualize the lack of a sex difference on reinstatement testing. Figure 3F depicts the terminal point of the injectors used to infuse the test compounds into the PL area of the PFC.

Experiment 4: Systemic Oxytocin Decreased Cued Reinstatement of Cocaine seeking and mGlu2/3 Receptor Antagonism Blocked this Effect in Males and Females

Figure 4A illustrates that oxytocin reduced cued reinstatement of cocaine seeking and prevention of this effect by LY341395 in males (n = 16) and females (n = 16). There was a

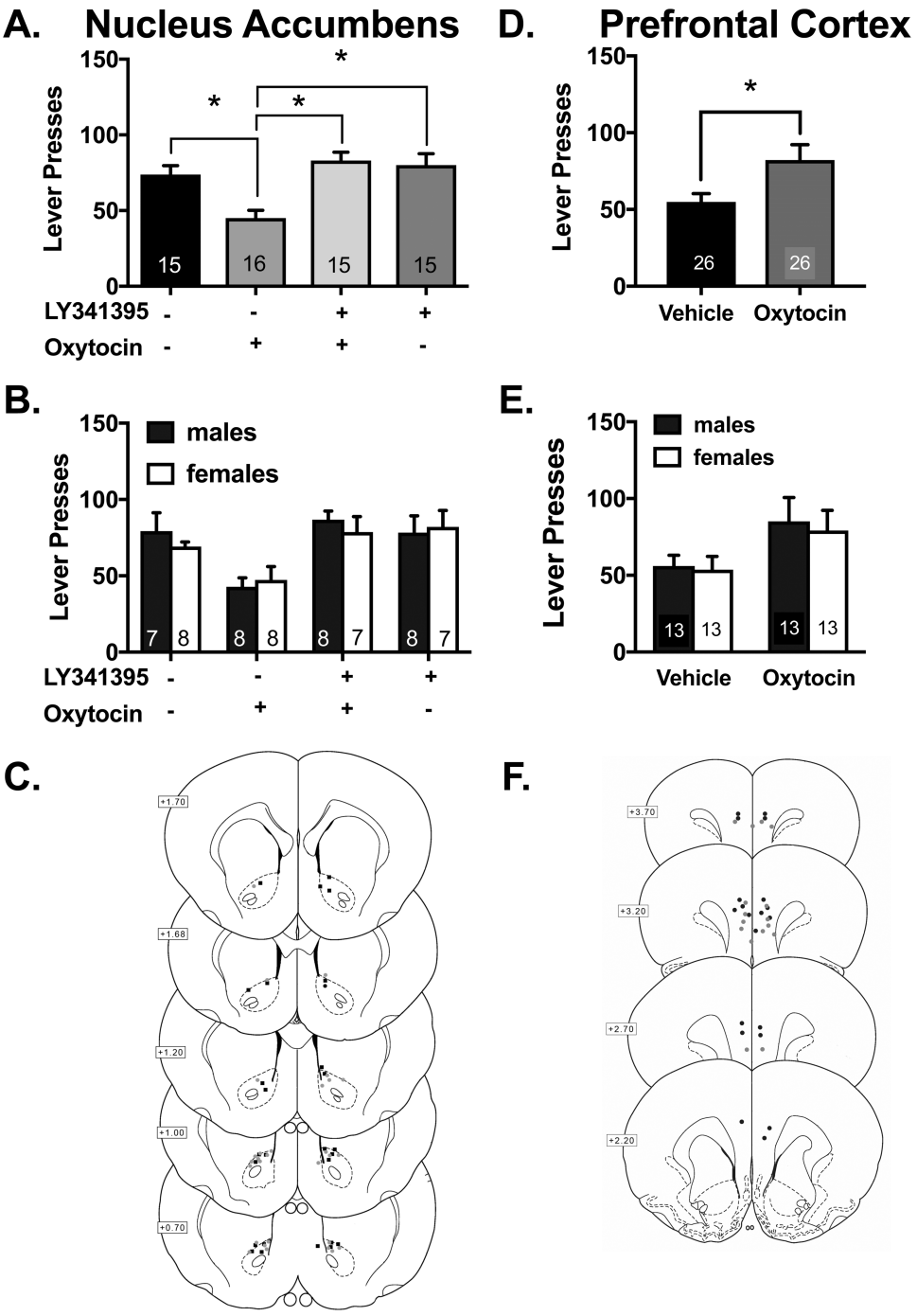

Figure 3. (A-B) Oxytocin (0.6 nmol/0.25 µL/side) infused into the NAcc decreased cued reinstatement of cocaine seeking. LY-341495 (1.3 nmol/0.25 µL/side) blocked oxytocin's effect but was without impact when given alone. *Significant difference from VEH/OXY ($P < .05$). (C) Anatomical depiction of the terminal point of the injectors used to infuse compounds. Numbers depict AP difference from bregma in mm. (D-E) Oxytocin (0.6 nmol/0.25 µL/side) infused into the prefrontal cortex (PFC) increased lever responding during reinstatement of cocaine seeking compared with vehicle in both males and females. *Significant difference from oxytocin ($P < .05$). (F) Depiction of the terminal point of the injectors used to infuse compounds. Inset depicts the number of rats that tested in the specific condition. Black and gray symbols depict males and females, respectively. Numbers depict AP difference from bregma in mm.

main effect of drug treatment [$F(3,53) = 8.12$, $P < .0002$], but no significant main effect of sex or significant interaction between sex and drug treatment. Posthoc comparisons showed that Veh/Sal, LY/Sal, and LY/Oxy groups had greater lever responding than the Veh/Oxy group (Holm-Sidak's, $P < .05$). Figure 4B depicts the data for male and female rats separately to visualize the lack of a sex difference on reinstatement testing.

Discussion

Here we report several potentially important findings regarding regionally specific effects of oxytocin on reinstatement of cocaine seeking. First, we demonstrate that oxytocin applied directly into the NAcc increased extracellular glutamate levels in drug-naïve animals in a TTX-dependent manner. Second, oxytocin delivered directly into the PFC increased reinstated

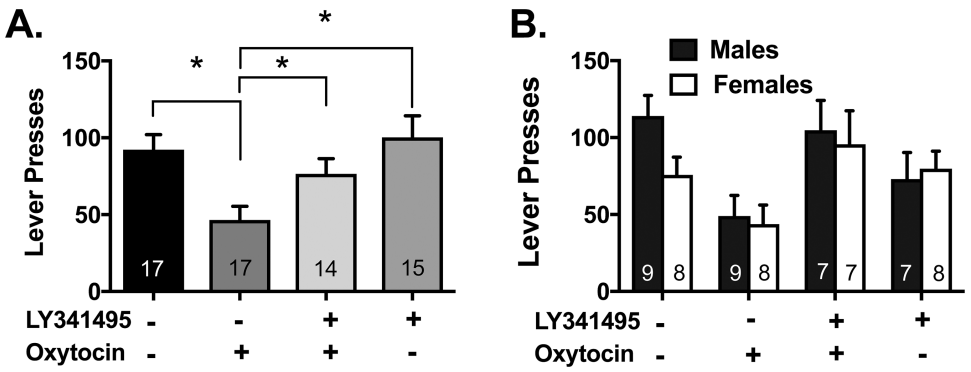

Figure 4. (A-B) Oxytocin (1 mg/kg, i.p.) reduced active presses in both sexes during cued reinstatement. LY-341495 (1 mg/kg, i.p.) blocked oxytocin's effect but was without impact when given alone in males and females. Inset depicts the number of rats that tested in the specific condition. *Significant difference from VEH/OXY ($P < .05$).

responding on the active lever, while oxytocin into the NAcc caused a decrease in cued reinstatement. This opposing pattern of reinstatement is the first to demonstrate regionally specific effects of oxytocin on reinstatement of drug seeking. Third, using an mGlu2/3 receptor antagonist before oxytocin prevented the oxytocin induced decrease in cued reinstatement. These results raise questions regarding oxytocin as a therapeutic option for cocaine addiction and stress the important need to elucidate oxytocin's mechanism.

The NAcc is one of the major neural substrates involved in reward and motivation (Ito et al., 2004; Bjorklund et al., 2008), and glutamate physiology is disrupted in this area following drug self-administration (Cornish and Kalivas, 2000; Kalivas and Volkow, 2011). We show that oxytocin increased extracellular glutamate in the NAcc. Although the source of the extracellular glutamate is beyond this report, the rise in glutamate levels was prevented by co-infusion of TTX. TTX is a sodium channel blocker that inhibits the firing of action potentials in neurons. This finding is critical to oxytocin's action in the NAcc, because TTX blockade of glutamate typically indicates a neuronal source. This finding is surprising, because oxytocin receptors in the NAcc have been detected on astrocytes, parvalbumin positive interneurons, and serotonergic 5HTa receptor-expressing neurons, but not on medium spiny neurons, cholinergic interneurons, or nitric oxide synthase 1 expressing interneurons (Di Scala-Guenot and Strosser, 1992; Di Scala-Guenot et al., 1994; Dolen et al., 2013). As such, the neuronal source of glutamate is unclear, but it is possible that oxytocin receptors may be localized on presynaptic glutamate terminals to modulate glutamate release. Since oxytocin receptors are predominately Gq-coupled, binding by oxytocin would likely stimulate glutamate release. Although this localization has not been demonstrated in the NAcc, oxytocin receptor mRNA is found in glutamate neurons in the ventral tegmental area (Peris et al., 2017) and PFC (Tan et al., 2017) and both of these neuronal populations likely send projections to NAcc.

Surprisingly, oxytocin infused into the PFC increased responding for cocaine cues. The medial (m) PFC glutamatergic innervation of the NAcc is a central pathway involved in relapse (for review, see Koob and Volkow, 2016). Pharmacological inactivation of the PL area of the mPFC or optogenetic inhibition of cortical projection neurons to the NAcc prevents reinstated cocaine seeking (McLaughlin, 2003; Di Pietro et al., 2006; Stefanik et al., 2013). Recently, oxytocin receptors have been identified on glutamate (46%) neurons in the PFC that project to the NAcc (Tan et al., 2017). Importantly, oxytocin also increases expression of GluA1 receptors in the PFC of cocaine experienced male rats (Zhou et al., 2014) and increases the insertion of GluA2-lacking calcium-permeable AMPA receptors (Qi et al., 2012). This neural adaptation is typically found in the NAcc (Conrad et al., 2008), and we recently discovered a similar change in the mPFC after prolonged cocaine abstinence (Pena-Bravo et al., 2017). This interplay between glutamate and oxytocin may explain the potentiated reinstatement we found following oxytocin infusion into the mPFC. Oxytocin-induced excitatory input from the PL-PFC may modulate glutamatergic signaling in downstream pathways such as the NAcc. It should be noted that intra-NAcc and intra-PFC vehicle infusions resulted in different levels of active lever pressing during the test, which may contribute to the interpretations of the ability of intra-PFC oxytocin to increase reinstatement. However, the mean active lever presses fall within the published range of cued reinstatement for animals with cannula in the medial mPFC (James et al., 2018; McLaughlin and See 2003; Fuchs et al., 2004a). Likewise, reinstatement responding following vehicle infusions into the NAcc were within range of previous studies that had cannula or viral infusions into the NAcc (Fuchs et al., 2004b; Scofield et al., 2015; Reissner et al. 2015). We believe that the different values for vehicle-infused rats occurred, because these experiments were conducted separately by different personnel during different time periods.

We have not entirely ruled out astrocyte regulation of glutamate release as a mechanism by which oxytocin decreases cued reinstatement within the NAcc. Wang and colleagues (Wang et al., 2017) recently reported that astrocytes in the supra optic nucleus of the hypothalamus express oxytocin receptors and oxytocin depolarized astrocyte membrane potentials while reducing GFAP expression. Importantly, these effects of oxytocin on GFAP plasticity were modulated by PKA and pERK1/2. In the NAcc, Scofield and colleagues (2015) demonstrated that specific activation of astrocytes (via Gq-coupled DREADDs) decreased cued reinstatement of cocaine seeking through an mGluR2/3-dependent mechanism. Here, we speculate that the localization of astroglial oxytocin receptors in the NAcc reduces relapse via Gq signaling in the cell that can activate transduction pathways including IP3 receptors and the release of intracellular calcium stores resulting in astrocytic glutamate transmitter release (Di Scala-Guenot and Strosser, 1992; Di Scala-Guenot et al., 1994; Hamilton and Attwell, 2010). Extrasynaptic glutamate can thereby activate mGluR2/3 receptors located presynaptically on cortical glutamatergic axons, inhibiting synaptic release of glutamate, thus restoring glutamatergic tone (Moussawi and Kalivas, 2010).

We have recently reported that mGlu2/3 receptor antagonism reversed the ability of oxytocin to reduce reinstatement to

methamphetamine (but not sucrose) cues (Bernheim et al., 2017), and this current report extends this finding to cocaine. Both of these consistent outcomes are in line with Scofield et al., (2015) showing that mGluR2/3 antagonism can block reinstatement to cocaine and methamphetamine (unpublished data) cues initiated by astrocytic GqDREADD stimulation. Other compounds that restore cocaine-induced changes in neuroplasticity also restore tone on mGlu2/3 receptors. For example, the cysteine-glutamate exchanger and glutamate transporters are downregulated following cocaine self-administration (Moran et al., 2005; Kalivas, 2009). Treatments with ceftriaxone and n-acetylcysteine repair these neural adaptations, thereby restoring glutamate tone on mGlu2/3 receptors (Knackstedt and Kalivas, 2009). Whether oxytocin has long-term changes that restore glutamate physiology or whether this neuropeptide is an acute modulator of this system is beyond the scope of this study. We also know that oxytocin acts on local neurons in the NAcc to mediate its effects. Previously, our group has shown that systemic oxytocin reduces motivation and seeking of methamphetamine and that this reduction is blocked by administration of an oxytocin receptor antagonist in the NAcc (Cox et al., 2017). Combined, these data suggest that oxytocin regulates relapse through multiple independent or interdependent mechanisms that are yet to be defined.

We report a sex difference in drug intake with females earning more cocaine infusions relative to males during self-administration. However, this difference did not occur on reinstatement tests in response to cocaine conditioned cues, in agreement with our recent publications (Bechard et al., 2017; Leong et al., 2017). This pattern of findings is consistent with the notion that sex differences in addiction are more robust during drug self-administration, and sex differences are relatively minor once compulsive drug taking has set in (Becker and Koob, 2016). Females have been reported to show greater cocaine-primed reinstatement than males (Lynch and Carroll, 2000; Kippin et al., 2005; Anker and Carroll, 2011), but cue-induced reinstatement is relatively similar between sexes (Fuchs et al., 2005; Feltenstein et al., 2011a; Cox et al., 2017). Previously, we (Bechard et al., 2017; Leong et al., 2017) and others (Feltenstein et al., 2011b) found no sex differences in cocaine-cued reinstatement, as well as similar reinstatement across all estrous cycle phases. This report is consistent with our previous work showing males and females lever press equally to cocaine-associated cues and oxytocin decreases cued reinstatement to cocaine seeking to the same extent in male and female rats (Leong et al., 2016, 2017).

For therapeutic purposes, oxytocin has been administered via intranasal administration in which the peptide enters the blood stream via the nasal passage. In Experiments 2 and 3, we tested site-specific administration of oxytocin, rather than systemic administration. The final experiment demonstrates that a more translationally relevant administration route results in the same pattern of responding as intra-accumbal microinfusions. Oxytocin (i.p.) decreased reinstatement to cocaine seeking, and this was reversed by the mGluR2/3 antagonist similar to what was seen when oxytocin was infused directly into the NAcc. The i.p. delivery of oxytocin results in activation of peripheral oxytocin receptors and can produce a decrease in locomotor activity in male and female rats (Zhou et al., 2014, 2015; Leong et al., 2016). As such, a peripheral mechanism of action for the behavioral effects of oxytocin cannot be ruled out using a systemic delivery route.

Relatively small amounts of oxytocin cross the blood brain barrier after systemic administration (Landgraf et al., 1979; Ermisch et al., 1985). Our laboratory and others (Carson et al., 2010b; Cox et al., 2017; Leong et al., 2017) have offered indirect evidence of oxytocin penetration of the blood brain barrier by showing that systemic oxytocin results in changes in Fos+ oxytocin neurons (Carson et al., 2010b) and normalizes drug and drug-cued increases in Fos expression (Carson et al., 2010b; Cox et al., 2017; Leong et al., 2017). Direct evidence of oxytocin penetrance of the blood brain barrier has also been described in both rodents and primates (Neumann et al., 2013; Freeman et al., 2016). As described above, numerous studies are in agreement that peripherally administered oxytocin is able to decrease intake of addictive substances implying a central mechanism of action (reviewed in Bowen and Neumann, 2017). However, there is no reason to expect that peripherally administered oxytocin, once it crossed the blood brain barrier, would preferentially act in NAcc to inhibit addictive behaviors in contrast to the PFC where oxytocin increased reinstatement to cocaine seeking. Thus, one might expect that peripheral administration of oxytocin would not be as effective in decreasing reinstatement of cocaine seeking due to the additive effects of the opposing actions in NAcc and PFC. There are likely more sites of action of oxytocin in addition to the NAcc and PFC that can impact reward circuitry in a way to decrease drug-seeking. A likely possibility is oxytocin receptor localization on cell bodies in the VTA, which when activated by direct oxytocin administration (Melis et al., 2007; Tang et al., 2014; Xiao et al., 2017), exert primarily excitatory actions on neurotransmission in the NAcc to increase dopamine and/or glutamate release in NAcc, precluding the need for further reward seeking.

In summary, we reveal several important issues regarding oxytocin's mechanism. First, we found that oxytocin increases glutamate in the NAcc. Second, to our knowledge, this is the first study to uncover bi-directionality of oxytocin's effect on reinstatement of cocaine seeking with increased and decreased responding following site specific application of the neuropeptide in the PL-PFC or the NAcc, respectively. Third, we also uncovered that mGluR2/3 receptors can mediate oxytocin-induced attenuation of reinstatement to cocaine seeking. Combined, this study indicates that the PFC and NAcc are critical structures involved in the interaction between oxytocin and glutamatergic signaling in males and females.

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Conflict of Interest

The authors do not have any competing financial interests in relation to this work. The data collected for this manuscript were supported by the National Institute of Health, National Institute of Drug Addiction grants: P50 DA016511 (C.M.R.), R01 DA033049 (C.M.R.), R01 DA033436 (L.K.), and T32 DA728823 (K.C.L. and R.W.). All authors discussed the experimental design, results, and implications at length. R.A.W., K.C.L., and C.L. oversaw and conducted the experiments including surgery, behavior, and microinfusions. C.L. conducted the HPLC study. R.A.W., C.L., and K.C.L. contributed to writing the initial version of the manuscript. C.R.M. conducted the statistical analysis and wrote the final version of the manuscript. L.K. and J.P. edited the final manuscript version.

Statement of Interest

The authors declare there are no conflicts of interest to disclose. All authors contributed in a significant way to the manuscript and all authors have read and approved the final manuscript.

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