Published in final edited form as:

Horm Behav. 2016 February ; 78: 52–59. doi:10.1016/j.yhbeh.2015.10.008.

Androgen-primed castrate males are sufficient for methamphetamine-facilitated increases in proceptive behavior in female rats

Sarah A. Rudzinkas^a and Jessica A. Mong^{a,b}

^aProgram in Neuroscience, University of Maryland, Baltimore, School of Medicine, Baltimore, Maryland, 21201

^bDepartment of Pharmacology, University of Maryland, Baltimore, School of Medicine, Baltimore, Maryland, 21201

Abstract

Methamphetamine (MA) is a psychomotor stimulant associated with increases in sex drive in both men and women. Women, however, are far more likely to face social disadvantages as a consequence of MA use, and their increased sexual motivation poses additional health concerns such as unplanned pregnancies. To better understand the mechanisms underlying MA-facilitated sexual motivation in females, we previously established a rodent model where a ‘binge’-type administration paradigm of MA to sexually receptive female rats significantly increases proceptive behavior in the presence of a sexually active, gonadally-intact male. Our previous work with this model has led us to consider whether the increases in proceptive behavior are truly indicative of increased sexual motivation, or instead a consequence of heightened motor responsivity. Here, we test whether MA-induced increases in proceptive behaviors are specific to a sexually relevant stimulus. Females’ sexual, social, exploratory behaviors, and interaction times were scored during the exposure to stimulus males, including castrates, and dihydrotestosterone (DHT)-treated castrates. MA-treated females demonstrated significant increases in proceptive behaviors toward DHT-treated castrate males but *not* toward castrate males. While the non-MA-treated females did display proceptive behavior, there was no significant difference between behaviors elicited by DHT-CX males compared to CX males. Our results support the hypothesis that MA facilitates proceptive behavior only in response to specific, androgen mediated sexually-relevant cues.

Keywords

proceptive behavior; methamphetamine; sexual motivation; dihydrotestosterone

Address Correspondences to: Sarah Rudzinkas, 655 W. Baltimore Street, BRB 4-002, Baltimore, MD 21201, Tel: 410-706-5581, Fax: 410-706-0032.

Conflict of Interest: The authors declare no competing financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Methamphetamine (MA) is a highly addictive psychomotor stimulant that has become a widespread drug of abuse and a major health problem, reaching epidemic proportions across the United States and Europe (Gonzales et al., 2010; Rusyniak, 2013). In addition to its potent stimulant effects, MA users report an increase in sex drive and high-risk sexual activity (Rawson et al., 2002; Semple et al., 2004a; Semple et al., 2004b). In women, this increased sexual motivation poses significant health concerns such as high rates of unplanned pregnancies and the spread of sexually transmitted diseases (Corsi and Booth, 2008; Dluzen and Liu, 2008; Mansergh et al., 2006; Semple et al., 2004a). Moreover, MA use is associated with a more pleasurable sexual experience (Lorvick et al., 2012), which may reinforce its addictive qualities (Semple et al., 2004b), thus worsening the health concerns for women abusers. While MA use and its effects are similar between the sexes; among MA abusers, women are more likely to suffer social disadvantages such as increased psychiatric disorders, domestic and sexual abuse, and reproductive health concerns (Du et al., 2013; Lorvick et al., 2012; Polcin et al., 2012; Semple et al., 2004b). Despite these health disparities, there are far fewer studies focusing on women than men.

To understand the mechanisms underlying this phenomenon, our laboratory has established a rodent model in which acute administration of MA increases proceptive, or female-initiated courting, behaviors in sexually receptive female rats over hormones alone (Holder et al., 2010; Holder and Mong, 2010; Holder et al., 2015). These proceptive behaviors are considered to be a relevant gauge of the females' motivation for sexual activity (Hlinak, 1977). Our acute administration paradigm mimics reports of MA binge use in women (Haile et al., 2009) though others demonstrate a similar effect on proceptivity with other administration paradigms (Winland et al., 2011). These findings strongly suggest that MA, working through a release of dopamine (DA), may directly modulate the neural substrates underlying female sexual motivation. Indeed, it has been demonstrated that specific activation of the dopamine receptor type 1 (D₁R) within the medial amygdala (MeA), (Holder et al., 2015) as well as the D₁R/D₂R ratio of activation within other nuclei supporting female sexual behavior such as the medial preoptic area (mPOA) can increase female sexual motivation (Graham and Pfau, 2010, 2012). However, the massive release of neurotransmitter into the synapse, independent of action potential induction, (reviewed in Davidson et al., 2001) may elicit marked changes in general arousal. This raises the possibility that MA-induced arousal may drive females to sexually interact with all other conspecifics, regardless of either their sexual motivation or the conspecific's potential to copulate.

To distinguish between these possibilities, we utilized our established model of MA-induced increases in proceptivity (Holder et al., 2010; Holder and Mong, 2010; Holder et al., 2015), and employed a within-animal design that compared the levels of behavior of sexually receptive, MA or vehicle treated-females during the exposure to stimulus males that were (1) Castrated (CX) and (2) CX-treated with dihydrotestosterone (DHT-CX). Castration of naïve males inhibits normal sex behavior and removes the typical pheromonal profile of the male (Madlafousek et al., 1976). Accordingly, sexually receptive females respond with lower levels of proceptive behavior (Hlinak, 1977). Treatment of CX males with DHT, a

non-aromatizable androgen, restores the normal state of peripheral accessory sex glands, but does not fully restore male sex behavior (Drewett and Spiteri, 1979; Gawienowski et al., 1975; Orsulak and Gawienowski, 1972). Given a choice, receptive females are more likely to approach DHT-CX males than non-treated CX males, but still always prefer intact males (Drewett and Spiteri, 1979).

Given that both CX and DHT-CX have significantly diminished levels of sex behavior, the use of these stimulus males eliminates the influence of male sexual displays seen in gonadally intact males on female proceptive behavior. These males allow us to test the hypothesis that MA-increased proceptive behaviors are specific to 1) the stimulus and/or 2) the sensory cues present during mating. Thus, if MA-facilitated proceptivity is truly reflective of the female's motivation to copulate, we predict that MA-treated females will not exhibit increased proceptivity in the presence of an inappropriate sexual stimulus, such as a CX male.

Materials and Methods

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were approved by and were in accordance with the guidelines of the University of Maryland Institutional Animal Care and Use Committee.

Adult female (225–275g; n=26) and male (325–400g; n=14) Sprague-Dawley rats were purchased from Charles River Laboratories (Kingston, NY) and housed in the animal facility of Health and Science Facility I located at the University of Maryland, Baltimore. Rats had food and water available *ad libitum*, and were maintained under a reversed 12h:12h dark:light cycle, with lights off at 0900 h. All female rats were bilaterally ovariectomized (OVX), and all male rats were castrated (CX) under isoflurane anesthesia. All rats, male and female, were sexually naïve at the time of OVX or CX. Following the surgery, rats were allowed a 10-day recovery period.

Hormones and Methamphetamine Treatment

Stimulus Males—Dihydrotestosterone Benzoate (DHT) was obtained from Sigma-Aldrich (St. Louis, MO) and was dissolved in sesame oil at a concentration of 500ug/100ul for subcutaneous (s.c.) administration. Male rats (n=7) received s.c. injections of DHT (1mg/day) for 5 days, including the morning of sex behavior or social interaction testing. All injections were administered between 0800 and 0830h.

Experimental Females—17- β -Estradiol (E2), Progesterone (P), and MA were obtained from Sigma-Aldrich (St. Louis, MO). The E2 and P were dissolved in sesame oil for s.c. administration. MA was dissolved in sterile saline (0.9%) vehicle to a concentration of 5mg/kg, and administered to rats for intraperitoneal (i.p.) injection at 1ml/kg. All injections were administered between 0830 and 0900h. Rats were treated 52 hours prior to testing with 5ug of E2, and then 24 hours later with 10ug of E2. At least 4 hours before the experiment, females were treated with 500ug of P. This hormonal treatment paradigm mimics the gradual rise of hormones occurring naturally during the estrous cycle (Schwartz and Mong,

2013). At the time of each hormone administration (3 days total), rats were treated with 5mg/kg of MA, or saline vehicle. This treatment paradigm and its corresponding doses of MA and hormones have been previously shown to facilitate proceptive and receptive sex behavior in females and have been validated in previous studies from our lab (Holder et al., 2010; Holder and Mong, 2010).

Sex Behavior Testing

Prior to the first day of drug treatments, each rat (regardless of sex or treatment group) was allowed to acclimate over a 10 minute period to the plexiglass testing chamber (50cm × 38cm × 25cm) which contained shredded cardboard bedding. Females were randomly assigned to one of two groups and treated with either E2+P (non-MA-treated, n=8) or MA + E2+P (MA-treated, n=10). On the third day of treatment, at least 4 hours after the final injection, behavioral studies were conducted under a dim red light.

To test the specificity of MA-facilitation of motivated sex behavior, a novel stimulus male was placed first into the testing chamber. Five minutes later, the experimental female was introduced to the arena for 25 minutes, during which time their behavior was video recorded for later scoring. Immediately after the test, female rats were returned to their home cage until the start of the second experimental test, which would have been approximately 90–120 minutes later. In this within animal design paradigm, the CX-stimulus exposure was conducted prior to the DHT-CX exposure. This experimental order was chosen to avoid potential residual effects from the DHT-CX pheromone profile on female sexual behavior. All males were novel to the test female and were sexually inexperienced at the time of castration.

Quantification of Behavior

An experimenter blinded to treatment groups scored female behavior for both of the 25 minute sessions. Behaviors scored fell under three major categories. 1) Social, *non*-sexual: which included behaviors such as sniffing (a directed nose movement at least briefly having contact with the male's body outside of the anogenital (AG) region), AG investigation, and other social exploration. 2) Social, sexual: which included proceptive behaviors such as hops, darts, ear wiggles, solicitations (a head-wise orientation toward the male followed by a quick runaway into presenting posture), and female on male mounts and 3) Non-social: which included exploratory behaviors such as rearing, corner investigation, digging and grooming. Additionally, an attempt to score receptive behaviors (i.e. lordosis quotation and intensity) was made. However, due to the low sexual competency of the males (both CX and DHT-CX; see below) too few lordotic events were exhibited for a reasonably powered statistical analysis.

Male behavior and interaction times (social vs. non-social) during sex behavior testing were also quantified. This was largely to ensure that any behavioral changes seen in females were due to the female's MA-status and not differential treatment by stimulus males towards any particular group of females. Additionally, to assess the level of male competency (which we anticipated to be low for both stimulus groups), mount latency, mount attempts, and successful mounts were quantified. The latency to mount was scored with a cutoff time of 5

minutes; thus males that never mounted were recorded as having a latency to mount of 5 minutes.

Social Interaction Testing

To test whether MA influenced stimulus male preference when given a choice, a new cohort of females were treated with either E2+P (n=4) or MA + E2+P (n=4). On the day prior to the first day of drug treatments, females were acclimated to the social interaction (SI) arena (90 × 50 × 33 cm) with removable dividers separating the center chamber.

At the start of testing, five hours after the final injection, experimental females were placed into the center of the arena with dividers in place. After 1 minute, the center dividers were removed giving the female access to the entire arena, which she was allowed to freely explore for 5 minutes. Each side of the arena contained either a CX or a gonadally-intact male. These males were confined to a small box with holes within the arena, which allowed social interaction via sniffing but physical interaction with the male was otherwise restricted. Behavior was recorded and scored for interaction time and time spent on each side of the arena. Two hours later, this test was repeated with the same females in the same fashion, but this time with a DHT-CX stimulus on one side and a gonadally-intact male on the other side. For all tests, *all* males were novel to the females, and the sides of the arena containing each possible male stimulus (CX, DHT-CX, or Intact) were balanced and no significant side preference for any treatment group was found.

Statistical Analysis

Results are expressed as mean + SEM. The female within-subject designs were analyzed using a two-way ANOVA where drug treatment (vehicle or MA) was an independent measure, and stimulus male (CX or DHT-CX) was a repeated measure. Stimulus male interactions and female SI tests were analyzed using a two way between-subject ANOVA. Effect size estimates were calculated using eta squared (η^2) for ANOVAs (Levine and Hullett, 2002). Cohen's d values were also calculated to determine effect sizes for the differences in pair-wise comparisons (Cohen, 1988). All two-way ANOVAs were followed by Bonferroni post-hoc analyses. All statistical tests were conducted using GraphPad Prism (San Diego, CA USA).

Results

Sexual Proceptive Behavior

Proceptive behaviors such as hops, darts and ear wiggles solicit the male's attention and are indicators of a female rat's motivational drive to copulate. We used these measures of proceptivity to examine whether the previously observed MA-induced increases in female sexual motivation were directed specifically to sexually relevant conspecifics or resulted from a MA-induced increase in general arousal. Quantification of overall proceptive events (which included hops, darts, solicitations, ear wiggles, and female-male mounts) revealed a significant interaction between stimulus male DHT-treatment (CX, or CX-DHT) and MA-treatment [$F_{(1,16)}=6.04$, $p=0.0258$, $\eta^2=0.17$], as well as a significant main effect of stimulus male DHT-treatment across both groups of females [$F_{(1,16)}=7.49$, $p=0.0146$, $\eta^2=0.21$]. A main

effect of MA-treatment showed a trend toward significance [$F_{(1,16)}=4.20$, $p=0.0571$, $\eta^2=0.17$]. Bonferroni post-hoc analysis revealed that MA-treated females displayed a greater than 5-fold increase in proceptive behaviors in the presence of DHT-CX males compared to CX males ($t_{(16)}=3.9$, $p<0.01$, $d=1.85$; Figure 1A). Moreover, MA-treated females exhibited significantly more proceptive events compared to the non-MA-treated females ($t_{(32)}=3.15$, $p<0.01$, $d=1.49$; Fig 1A) in the presence of a DHT-CX, recapitulating the effect which has been previously demonstrated in our lab with an intact stimulus male. While the non-MA-treated females did display proceptive behavior in response to both stimulus males, there was no significant difference between behaviors elicited by DHT-CX males compared to CX males ($t_{(16)}=0.19$, $p>0.05$, $d=0.09$, ns).

To further understand how MA-treatment was affecting individual components of overall proceptivity, two-way repeated-measures ANOVAs were run independently for specific components of proceptive behavior (Figure 1B–D). For all factors quantified, the MA-treated females exposed to a DHT-CX male stimulus showed a significant increase in the number of proceptive events compared to their CX stimulus response. Total hops and darts showed a significant main effect of stimulus male [$F_{(1,16)}=7.72$, $p=0.0134$, $\eta^2=0.21$], MA treatment [$F_{(1,16)}=5.26$, $p=0.0358$, $\eta^2=0.20$], and a significant interaction between stimulus male and MA treatment [$F_{(1,16)}=5.09$, $p=0.0384$, $\eta^2=0.14$] (Figure 1B). Solicitations were also significantly increased; two-way ANOVA revealed a significant interaction between stimulus male and MA treatment [$F_{(1,16)}=5.833$, $p=0.0281$, $\eta^2=0.20$] but no significant main effects (Figure 1C). Finally, a two-way ANOVA for female-male mounting showed a significant main effect only for MA treatment [$F_{(1,16)}=8.73$, $p=0.0093$, $\eta^2=0.38$], as well as a significant interaction between stimulus male and MA treatment [$F_{(1,16)}=9.06$, $p=0.0083$, $\eta^2=0.22$] (Figure 1D). This was the only factor by which the non-MA-treated group had a higher proceptive response to the CX than the MA-treated females. Still, the presence of a DHT-CX significantly affected the behavior of only the MA-treated group.

Bonferroni post-hoc tests demonstrated the significance of a DHT-CX stimulus on a MA-treated female compared to a non-MA treated female for hops and darts ($t_{(32)}=3.21$, $p<0.01$, $d=1.52$; Figure 1B), as well as solicitations ($t_{(32)}=2.79$, $p<0.05$, $d=1.56$; Figure 1C). However, a DHT-CX stimulus did not significantly change female-male mounts ($t_{(32)}=0.59$, $p>0.05$, $d=0.28$; Figure 1D). Bonferroni post-hoc tests comparing MA-treated females' proceptive responses between a CX vs. DHT-CX stimulus revealed significant increases for hops and darts ($t_{(16)}=3.78$, $p<0.01$, $d=1.79$; Figure 1B), solicitations ($t_{(16)}=3.11$, $p<0.05$, $d=1.48$; Figure 1C), and female-male mounts ($t_{(16)}=2.89$, $p<0.05$, $d=1.37$; Figure 1D) in the presence of a DHT-CX stimulus, with no significant increases between stimuli with non-MA-treated females. Thus, proceptive behavior was significantly increased in only MA-treated female rats and only when a DHT-CX stimulus male was present. Although receptive behavior was quantified when displayed, both CX and CX-DHT males generally mounted very infrequently (Table 1), and thus we did not have sufficient data and statistical power to accurately determine the lordosis intensity and quotient.

Non-Sexual Social Behavior

Sniffing, a specialized behavior used in the acquisition of odors, is a commonly displayed behavior that serves to relay social information about conspecifics (Wesson, 2013). Two-way ANOVA with repeated measures showed that female rats, regardless of MA-treatment, showed significantly fewer instances of sniffing when exposed to a DHT-CX male compared to the CX [$F_{(1,16)}=16.96$, $p=0.0008$, $\eta^2=0.38$], but there was no significant interaction between the factors nor a main effect of female treatment ($F_{(1,16)}=3.15$, $p=0.0948$, $\eta^2=0.26$, ns). Post-hoc analysis revealed a significant decrease in sniffing in MA-treated females after exposure to a DHT-CX stimulus ($t_{(16)}=3.55$, $p<0.05$, $d=1.68$; Figure 2A). There were no differences in female-initiated anogenital investigations [$F_{(1,16)}=0.33$, $p=0.5751$, $\eta^2=0.02$, ns] (Figure 2B).

Non-Social Exploratory Behavior

Two-way ANOVA with repeated measures revealed a significant main effect of male stimulus in decreasing instances of rearing in all females [$F_{(1,16)}=6.37$, $p=0.0226$, $\eta^2=0.22$], but no main effect of female MA-treatment (Figure 2C). There was a significant interaction between the factors [$F_{(1,16)}=5.74$, $p=0.0292$, $\eta^2=0.20$], possibly due to the fact that MA-treated females were spending more time demonstrating proceptive behaviors and thus less time exploring the arena. Bonferroni post-hoc tests were not significant. There were no differences in corner explorations of the arena between any treatment groups at any time during testing (Figure 2D).

Stimulus Male Behavior

Two-way ANOVAs revealed main effects of DHT-treatment on CX males for each behavior scored: instances of AG investigation [$F_{(1,12)}=8.05$, $p=0.015$, $\eta^2=0.39$]; total social investigation [$F_{(1,12)}=10.73$, $p=0.0066$, $\eta^2=0.44$]; and total arena exploration time [$F_{(1,12)}=5.51$, $p=0.037$, $\eta^2=0.30$]. However, there were no significant main effects of female treatment-status, nor any significant interactions (Figures 3A–C). As such, while DHT-treatment did alter the male's social behavior, changes in female proceptivity were clearly due to MA-treatment alone, and not due to differential treatment from the DHT-CX stimulus males toward them in particular.

Total mounts and mount latency were also quantified to understand the mating competency of the males. T-tests revealed that neither overall total mounts ($t_6=0.95$, $p=0.37$, $d=0.68$, ns) nor latency to first mount ($t_6=0.23$, $p=0.82$, $d=0.16$, ns) were significantly different between the CX and DHT-CX stimulus males used (Table 1). Based on a review of the published literature and our own observations, sexually competent males often mount within seconds and rarely take longer than 2 minutes when presented with a sexually receptive female. We found that the mean latency to mount in our CX and DHT males was 4.4 ± 0.56 minutes and 4.25 ± 0.66 minutes, respectively, suggesting a very low level of sexual competency.

Social Interaction Test

To evaluate if, when given a choice between two male rats of varying hormonal status, the MA-treated female rats behaved similarly to non-MA treated rats, a social interaction test was run. First rats were given a choice between a CX male and an intact male. Two-way

ANOVA revealed a main effect of male hormonal status on the time spent exploring each male. [$F_{(1,12)}=30.28$, $P<0.0001$, $\eta^2=0.71$]. However, there were no significant main effects of female treatment-status, nor any significant interactions (Figure 4A). Thus, all females, regardless of MA-treatment, preferred to interact with the intact male. Next, rats were given a choice between a DHT-CX male and an intact male. Two-Way ANOVA again revealed a main effect of male hormonal status on the time spent exploring each male, with all females preferring to spend time interacting with the intact male [$F_{(1,12)}=17.14$, $p=0.0014$, $\eta^2=0.92$]. There were no significant main effects of female treatment-status, nor any significant interactions (Figure 4B). Therefore, changes in female proceptivity toward DHT-CX stimulus males were not indicative of the females' *ability* to distinguish male hormonal status (ie. DHT-CX vs. Intact), since the MA-treated females do this with the same efficacy and prefer the intact male as much non-MA-treated females.

Discussion

MA is a potent psychostimulant known to induce a state of general arousal as defined by enhanced motor activity and intensity of responses to sensory stimuli. Previously, we established that administration of MA to hormonally-primed female rats increases proceptive and receptive behaviors (Holder et al., 2010; Holder and Mong, 2010; Holder et al., 2015). However, these studies did not test whether MA-increased proceptive events were the result of a context-specific arousal or if these behaviors were increased regardless of whether a behaviorally relevant stimulus or sensory cue was present. To distinguish between these possibilities, proceptive behaviors in sexually receptive females treated with MA or vehicle were quantified following exposure to stimulus males of varying levels of sexual relevancy. Our present study demonstrates three novel major findings: (1) MA-induced female sexual motivation is stimulus specific where MA may potentially enhance the salience of the sexual cues; (2) Social interactions are similar in MA- and non-MA-treated females where both groups choose to interact with the most sexually relevant stimulus and (3) Androgen-mediated sexual cues in males are sufficient to elicit increased sexual motivation in MA-treated females.

In a natural or semi-natural environment, female rats initiate the overwhelming majority of sexual interactions with males (Erskine, 1989; Paredes, 1999) with a pattern of approaches and withdrawals from the male that solicits his attention and ultimately results in copulatory behavior. These paracopulatory (proceptive) behaviors have become an accepted measure of female sexual motivation (reviewed in(Agmo, 2011; Kondo and Sakuma, 2005; Paredes, 1999) with the male rat acting as a positive incentive or stimulus (reviewed in(Agmo, 1999, 2011). In the present study, to evaluate the effects of MA on female sexual motivation independent of copulatory behavior, we employed males with significantly diminished sex behavior but partially restored sexually relevant cues (DHT-CX) (Matochik and Barfield, 1991). We found that females treated with MA compared to hormones alone displayed a 5-fold increase in proceptive behaviors toward the DHT-CX males, while the CX-only stimulus elicited equivalent levels of proceptive behaviors with or without MA. These results suggest that MA in hormonally primed females may be acting to enhance the salience of sexual cues from the stimulus males resulting in increased behavioral outputs. In a model for sexual incentive motivation (Agmo, 2011), Agmo and colleagues posit that increased

activation of a central motive state (defined as a set of processes promoting goal-directed behaviors in response to a specific incentive stimulus) results in a more intense response to the stimulus. Thus, if MA were acting to enhance the central motive state we would predict an increased reactivity and awareness in MA-treated females toward only *relevant* sensory cues (e.g., sex pheromones, vocalization, social interactions, etc). Our findings here support this prediction, as MA did not significantly increase proceptivity toward the non-sexually relevant stimuli (i.e. CX male), but did significantly increase proceptivity toward a DHT-CX.

While both CX and DHT-CX males have severely diminished sex behaviors, DHT-CX males have a restored pheromonal profile (Drewett and Spiteri, 1979; Orsulak and Gawienowski, 1972) and differential vocalizations (Cooke et al., 2003). Our findings suggest that the androgen-restored sensory cues were sufficient to elicit increased proceptive responses from MA-treated females but not hormone-only females. Perhaps most importantly, although MA-treated females responded with increased proceptivity to the DHT-treated male, if given the choice, they choose to socially interact with an intact male compared to a DHT-treated male. This distinction indicates that although they are attentive and responsive to sexual cues, MA-treated females are able to discriminate between each stimulus and chose to spend time with the most sexually relevant one, i.e. the male with which they are able to copulate.

These findings are in line with what Pfaff and colleagues have hypothesized about a state of high “generalized arousal” where neurons that serve arousal functions, and in particular sexual arousal, are activated more rapidly (Schober and Pfaff, 2007; Shelley et al., 2006) allowing for a heightened awareness to sensory inputs and quicker context-dependent motivated responses (Lee and Pfaff, 2008; Weil et al., 2010). Pfaff and colleagues have demonstrated a role of gonadal steroids in arousal that suggests steroids, while having modest effects on generalized arousal, have a greater effect on reproductive behaviors/sexual arousal, a component of a general arousal (Chu et al., 2015). In our current model of MA-induced enhancement of proceptive behaviors, MA may prime a specific neural network involved in female sexual behaviors resulting in enhanced activation when EB and P are present.

We have previously shown that the MeA, and particularly the posterior dorsal region (MePD), is necessary for the MA-induced enhancement of proceptive behaviors, but not for the display of baseline sexual behavior (Holder et al., 2015) suggesting that the MePD may be a critical nexus where the combination of ovarian steroids and MA enhance female sexual motivation. The MePD has previously been implicated in the modulation of female sexual behavior (Kondo and Sakuma, 2005; Masco and Carrer, 1980, 1984), and work with male rats also reflects that this nucleus is intricately involved with both sexual behavior and drug induced reward (Frohman et al., 2010a; Frohman et al., 2010b). More recently, Pfaus and colleagues identified a role for the MePD in the “super-solicitalional” behaviors (increased proceptive events and mounting displays) in a naturally occurring variant in Long–Evans rats (Afonso et al., 2009; Afonso and Pfaus, 2006). These super-solicitalional rats have a 2.5-fold increase in cFos-positive cells in the MePD compared to normal rats, and MePD lesions abolish the expression of the mounting displays and proceptive behaviors

(Afonso et al., 2009). In our own work, the combination of MA and ovarian steroids increases the number of cFos-positive cells in the MePD over either one alone (Holder et al., 2010). Similarly, in males, the combination of sex and MA increase the activation of the MePD over either one alone (Frohman et al., 2010b). Together, these functional neuroanatomical studies suggest that specific populations of MePD neurons are involved in heightened proceptive behaviors and that a combination of MA and ovarian steroids may work to activate this subpopulation, allowing for a heightened response to sensory inputs as hypothesized by Pfaff and colleagues (Schober and Pfaff, 2007; Shelley et al., 2006).

The MePD receives direct and indirect neural connections from the main olfactory bulb (MOB) as well as the accessory olfactory bulb (AOB) (Bian et al., 2008; Hosokawa and Chiba, 2007), and itself projects to nuclei known to further modulate female sexual behavior, such as the ventromedial hypothalamus (VMN) and the mPOA (Bian et al., 2008; Martinez and Petrulis, 2013). In particular, it has been demonstrated that the mPOA is an important player in the regulation of DA within the mesolimbic system, where dopaminergic afferents from the VTA activate the mPOA's GABAergic efferents back toward the VTA (Dominguez et al., 2001; Dominguez and Hull, 2001; Tobiansky et al., 2013). In our mechanism, it is possible that heightened activation from both MA and the MePD disrupts the inhibitory feedback of the mPOA on the VTA.

There are also dopaminergic and noradrenergic neuronal groups within the glomerular layer of the olfactory bulb itself, suggesting the possibility that MA may act directly within the bulb to heighten olfactory sensitivity (Corthell et al., 2013; Pignatelli et al., 2005). Thus, whether directly within the bulb and/or indirectly via activation of the MePD, VMN, or mPOA, it is highly likely that inputs from olfactory cues may be enhanced and thus mediate some aspects of the increased proceptive behavior seen in MA-treated females.

As the pheromone producing seminal vesicles, penile papillae, prostate, and coagulating gland in the peripheral nervous system are controlled by androgens, DHT-treated males presumably have a similar pheromonal profile to the intact male without the same exhibition of copulatory behavior (Drewett and Spiteri, 1979; Gawienowski et al., 1975; Orsulak and Gawienowski, 1972). Interestingly, there was a main effect of a DHT-CX stimulus on female sniffing behavior, and post hoc analysis revealed that the MA-treated females paired with the DHT-CX males exhibited a significant reduction in sniffing behavior compared to the MA-treated females paired with CX males. One possible interpretation of this finding is that the MA enhanced the ability for detection olfactory cues, resulting in the females spending less time with social investigation and more time engaging in solicitation behaviors. Nevertheless, when given a choice of social interactions via nose pokes with males restrained in a plexiglass container, both MA- and non-MA treated females spend significantly more time with the intact males versus the DHT-CX males, suggesting other sensory cues or factors are also at play that allow MA-treated females to discriminate the better sexual partner.

One such sensory cue that is altered by CX is ultrasonic vocalizations (USVs). USVs play a large role in rat communication, including sex behavior (Hull and Dominguez, 2007) and it is possible MA-treated females are more attentive to the number or type of calls emitted

from males. Castration significantly reduces male USVs, and a DHT-CX male emits more calls than CX to a female stimulus (Cooke et al., 2003) but not to estrous odors (Matochik and Barfield, 1991). Still, recent work has shown that females did not approach a vocalizing male more than they approached a silent (devocalized) male (Snoeren and Agmo, 2014a, b). Furthermore, the variable in question may not be the number of calls, but the type and frequency (hz) of calls (Snoeren and Agmo, 2014a). Given this mixed data on the importance of USVs in sex behavior, it seems unlikely that USVs alone would be the only factor mediating the behavior change of MA-treated females toward DHT-CX males, but it possibly plays a role.

An additional explanation for the observed increase in MA-induced proceptive behavior is the increased social investigation time exhibited by the DHT-CX males. Perhaps, if the MA-treated females are at a heightened state of arousal, the normally sub threshold levels of sex behavior and/or the slightly increased social investigation exhibited by the DHT-CX males may be sufficient to drive the increase in proceptivity. Our stimulus males showed largely diminished sex behaviors (averaging only 1–5 mount attempts within a 25 minute test), no noted intromissions or ejaculations, and no significant differences between CX and DHT-CX males. Others have shown DHT-CX males do show some increased levels of sex behavior compared to CXs, such as non-contact erections, albeit not to the level of intact males (Drewett and Spiteri, 1979; Hull and Dominguez, 2007; Matochik and Barfield, 1991; Sato et al., 2007).

One possible confound within our study is the testing order, namely that females (regardless of MA-treatment) were always presented with the CX stimulus male first, prior to the DHT-CX male. We specifically chose this experimental order to eliminate the possibility that a pheromonally intact DHT-CX male may induce residual pheromonal and/or olfactory neural activation in the test female, thus influencing potential proceptive responses toward the CX-stimulus. Indeed, even low levels of pheromones can have long lasting effects after they are deposited (He et al., 2010; Wyatt, 2003).

Another consideration related to testing order is whether time after MA-treatment or presentation order contributed significantly to the observed effects. Though possible, our data suggest that time after MA exposure was not a highly significant variable. In our previously published studies, MA-treated females demonstrated increased proceptivity (with no stereotypic effects) up to 9 hours after the final drug/hormone administration. In the present study, testing was conducted well within this period, making it unlikely that the time MA-treated females were tested would fully account for the observed behavioral changes. When the number of proceptive events from MA-treated females was analyzed as a function of time after the last MA-injection, no significant correlations were found and in fact, some of the highest and lowest proceptive responders in the DHT-CX stimulus test occurred within the same hour (data not shown). Finally, if presentation order were a significant factor, we would have predicted an increase in the non-MA-treated females' proceptive behaviors relative to their baseline. To the contrary, *only* the MA primed females exhibited an increase in proceptivity when presented with the DHT-CX male.

Clinically, it is becoming increasingly apparent that the importance of sensory cues and environmental cues in drug seeking behavior cannot be ignored in preventing patient relapse (Hartz et al., 2001; Liu et al., 2013; Liu et al., 2003; Mayo et al., 2013). If particular sensory cues are also important for MA-induced drug seeking and sexual hyperarousal in women, using interventions that reduce cue-reactivity may be an important clinical direction for MA-treatment in humans (Mayo et al., 2013; Voigt and Napier, 2011).

In conclusion, our data demonstrate that MA-increased proceptivity is discriminative and differentially responsive to the stimulus present. It is likely that sensory cues play a large role in mediating MA-increased proceptive responses, and this may be the consequence of MA-induced generalized arousal resulting in heightened awareness to sexually relevant cues. Given the circuitry of the MePD and the fact that a DHT-CX male maintains the pheromonal profile of the male rat, we speculate that olfactory cues play an important role in mediating the MA-induced proceptive behavior. These findings suggest that MA can alter normal social interactions and heighten responses to environmental cues, an implication which may have significant clinical relevance. Ultimately, these data may lend a deeper understanding of underlying neural circuitry mediating sexual motivation and arousal in women.

Acknowledgments

We would like to thank Janell P. Sosa, UMB, for her help in scoring the male behavior. This research was supported by NIH grant DA030517 awarded to Jessica A Mong and an institutional T32 in Membrane Biology, awarded to Sarah A Rudzinkas. The NIH had no further role in study design; in the collection, analysis, and interpretation of the data; in the writing of the report; and in the decision to submit the paper for publication.

References

- Afonso VM, Lehmann H, Tse M, Woehrling A, Pfaus JG. Estrogen and the neural mediation of female-male mounting in the rat. *Behavioral neuroscience*. 2009; 123:369–381. [PubMed: 19331460]
- Afonso VM, Pfaus JG. Hormonal and experiential control of female-male mounting in the female rat. *Hormones and behavior*. 2006; 49:30–37. [PubMed: 15955535]
- Agmo A. Sexual motivation--an inquiry into events determining the occurrence of sexual behavior. *Behav Brain Res*. 1999; 105:129–150. [PubMed: 10553696]
- Agmo A. On the intricate relationship between sexual motivation and arousal. *Hormones and behavior*. 2011; 59:681–688. [PubMed: 20816969]
- Bian X, Yanagawa Y, Chen WR, Luo M. Cortical-like functional organization of the pheromone-processing circuits in the medial amygdala. *Journal of neurophysiology*. 2008; 99:77–86. [PubMed: 17977926]
- Chu X, Gagnidze K, Pfaff D, Agmo A. Estrogens, androgens and generalized behavioral arousal in gonadectomized female and male C57BL/6 mice. *Physiology & behavior*. 2015; 147:255–263. [PubMed: 25936820]
- Cohen, J. *Statistical power analysis for the behavioral sciences*. 2. L. Erlbaum Associates; Hillsdale, N.J: 1988.
- Cooke BM, Breedlove SM, Jordan CL. Both estrogen receptors and androgen receptors contribute to testosterone-induced changes in the morphology of the medial amygdala and sexual arousal in male rats. *Hormones and behavior*. 2003; 43:336–346. [PubMed: 12694644]
- Corsi KF, Booth RE. HIV sex risk behaviors among heterosexual methamphetamine users: literature review from 2000 to present. *Current drug abuse reviews*. 2008; 1:292–296. [PubMed: 19630727]

- Corthell JT, Stathopoulos AM, Watson CC, Bertram R, Trombley PQ. Olfactory bulb monoamine concentrations vary with time of day. *Neuroscience*. 2013; 247:234–241. [PubMed: 23727009]
- Davidson C, Gow AJ, Lee TH, Ellinwood EH. Methamphetamine neurotoxicity: necrotic and apoptotic mechanisms and relevance to human abuse and treatment. *Brain research Brain research reviews*. 2001; 36:1–22. [PubMed: 11516769]
- Dluzen DE, Liu B. Gender differences in methamphetamine use and responses: a review. *Gender medicine*. 2008; 5:24–35. [PubMed: 18420163]
- Dominguez J, Riolo JV, Xu Z, Hull EM. Regulation by the medial amygdala of copulation and medial preoptic dopamine release. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2001; 21:349–355. [PubMed: 11150352]
- Dominguez JM, Hull EM. Stimulation of the medial amygdala enhances medial preoptic dopamine release: implications for male rat sexual behavior. *Brain research*. 2001; 917:225–229. [PubMed: 11640908]
- Drewett RF, Spiteri NJ. The sexual attractiveness of male rats: olfactory and behavioral components. *Physiology & behavior*. 1979; 23:207–209. [PubMed: 515212]
- Du J, Huang D, Zhao M, Hser YI. Drug-abusing offenders with co-morbid mental disorders: gender differences in problem severity, treatment participation, and recidivism. *Biomedical and environmental sciences : BES*. 2013; 26:32–39. [PubMed: 23294613]
- Erskine MS. Solicitation behavior in the estrous female rat: a review. *Hormones and behavior*. 1989; 23:473–502. [PubMed: 2691387]
- Frohman KS, Bateman KL, Lehman MN, Coolen LM. Effects of methamphetamine on sexual performance and compulsive sex behavior in male rats. *Psychopharmacology*. 2010a; 212:93–104. [PubMed: 20623108]
- Frohman KS, Wiskerke J, Wise RA, Lehman MN, Coolen LM. Methamphetamine acts on subpopulations of neurons regulating sexual behavior in male rats. *Neuroscience*. 2010b; 166:771–784. [PubMed: 20045448]
- Gawienowski AM, Orsulak PJ, Stacewicz-Sapuntzakis M, Joseph BM. Presence of sex pheromone in preputial glands of male rats. *The Journal of endocrinology*. 1975; 67:283–288. [PubMed: 1206280]
- Gonzales R, Mooney L, Rawson RA. The methamphetamine problem in the United States. *Annual review of public health*. 2010; 31:385–398.
- Graham MD, Pfau JG. Differential regulation of female sexual behaviour by dopamine agonists in the medial preoptic area. *Pharmacology, biochemistry, and behavior*. 2010; 97:284–292.
- Graham MD, Pfau JG. Differential effects of dopamine antagonists infused to the medial preoptic area on the sexual behavior of female rats primed with estrogen and progesterone. *Pharmacology, biochemistry, and behavior*. 2012; 102:532–539.
- Haile CN, Kosten TR, Kosten TA. Pharmacogenetic treatments for drug addiction: cocaine, amphetamine and methamphetamine. *The American journal of drug and alcohol abuse*. 2009; 35:161–177. [PubMed: 19462300]
- Hartz DT, Frederick-Osborne SL, Galloway GP. Craving predicts use during treatment for methamphetamine dependence: a prospective, repeated-measures, within-subject analysis. *Drug and alcohol dependence*. 2001; 63:269–276. [PubMed: 11418231]
- He J, Ma L, Kim S, Schwartz J, Santilli M, Wood C, Durnin MH, Yu CR. Distinct signals conveyed by pheromone concentrations to the mouse vomeronasal organ. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2010; 30:7473–7483. [PubMed: 20519522]
- Hlinak, JMaZ. Sexual Behaviour of the Female Laboratory Rat: Inventory, Patterning, and Measurement, Behaviour. BRILL. 1977. p. 129-174.
- Holder MK, Hadjimarkou MM, Zup SL, Blustein T, Benham RS, McCarthy MM, Mong JA. Methamphetamine facilitates female sexual behavior and enhances neuronal activation in the medial amygdala and ventromedial nucleus of the hypothalamus. *Psychoneuroendocrinology*. 2010; 35:197–208. [PubMed: 19589643]
- Holder MK, Mong JA. Methamphetamine enhances paced mating behaviors and neuroplasticity in the medial amygdala of female rats. *Hormones and behavior*. 2010; 58:519–525. [PubMed: 20399784]

- Holder MK, Veichweg SS, Mong JA. Methamphetamine-enhanced female sexual motivation is dependent on dopamine and progesterone signaling in the medial amygdala. *Hormones and behavior*. 2015; 67:1–11. [PubMed: 25448531]
- Hosokawa N, Chiba A. Effects of sexual experience on conspecific odor preference and male odor-induced activation of the vomeronasal projection pathway and the nucleus accumbens in female rats. *Brain research*. 2007; 1175:66–75. [PubMed: 17870062]
- Hull EM, Dominguez JM. Sexual behavior in male rodents. *Hormones and behavior*. 2007; 52:45–55. [PubMed: 17499249]
- Kondo Y, Sakuma Y. The medial amygdala controls the coital access of female rats: a possible involvement of emotional responsiveness. *The Japanese journal of physiology*. 2005; 55:345–353. [PubMed: 16409669]
- Lee AW, Pfaff DW. Hormone effects on specific and global brain functions. *The journal of physiological sciences : JPS*. 2008; 58:213–220. [PubMed: 18505601]
- Levine TR, Hullett CR. Eta Squared, Partial Eta Squared, and Misreporting of Effect Size in Communication Research. *Human Communication Research*. 2002; 28:612–625.
- Liu QR, Rubio FJ, Bossert JM, Marchant NJ, Fanous S, Hou X, Shaham Y, Hope BT. Detection of molecular alterations in methamphetamine-activated Fos-expressing neurons from a single rat dorsal striatum using fluorescence-activated cell sorting (FACS). *Journal of neurochemistry*. 2013
- Liu ZJ, Maekawa M, Horii T, Morita M. The multiple promoter methylation profile of PR gene and ER α gene in tumor cell lines. *Life Sciences*. 2003; 73:1963–1972. [PubMed: 12899921]
- Lorvick J, Bourgois P, Wenger LD, Arreola SG, Lutnick A, Wechsberg WM, Kral AH. Sexual pleasure and sexual risk among women who use methamphetamine: a mixed methods study. *The International journal on drug policy*. 2012; 23:385–392. [PubMed: 22954501]
- Madlafousek J, Hlinak Z, Beran J. Decline of sexual behavior in castrated male rats: effects of female precopulatory behavior. *Hormones and behavior*. 1976; 7:245–252. [PubMed: 955584]
- Mansergh G, Purcell DW, Stall R, McFarlane M, Semaan S, Valentine J, Valdiserri R. CDC consultation on methamphetamine use and sexual risk behavior for HIV/STD infection: summary and suggestions. *Public health reports*. 2006; 121:127–132. [PubMed: 16528944]
- Martinez LA, Petrulis A. The medial preoptic area is necessary for sexual odor preference, but not sexual solicitation, in female Syrian hamsters. *Hormones and behavior*. 2013; 63:606–614. [PubMed: 23415835]
- Masco DH, Carrer HF. Sexual receptivity in female rats after lesion or stimulation in different amygdaloid nuclei. *Physiology & behavior*. 1980; 24:1073–1080. [PubMed: 7413786]
- Masco DH, Carrer HF. Pathways conducting amygdaloid influence on feminine sexual behavior in the rat. *Behav Brain Res*. 1984; 11:205–212. [PubMed: 6326783]
- Matochik JA, Barfield RJ. Hormonal control of precopulatory sebaceous scent marking and ultrasonic mating vocalizations in male rats. *Hormones and behavior*. 1991; 25:445–460. [PubMed: 1813373]
- Mayo LM, Fraser D, Childs E, Momenan R, Hommer DW, de Wit H, Heilig M. Conditioned preference to a methamphetamine-associated contextual cue in humans. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2013; 38:921–929. [PubMed: 23416857]
- Orsulak PJ, Gawienowski AM. Olfactory preferences for the rat preputial gland. *Biology of reproduction*. 1972; 6:219–223. [PubMed: 5016868]
- Paredes, RaVB. What do female rats like about sex? Paced mating. *Behavioral Brain Research*. 1999; 105:117–127.
- Pignatelli A, Kobayashi K, Okano H, Belluzzi O. Functional properties of dopaminergic neurones in the mouse olfactory bulb. *The Journal of physiology*. 2005; 564:501–514. [PubMed: 15731185]
- Polcin DL, Buscemi R, Nayak M, Korcha R, Galloway G. Gender Differences in Psychiatric Symptoms among Methamphetamine Dependent Residents in Sober Living Houses. *Addictive disorders & their treatment*. 2012; 11:53–63. [PubMed: 23559891]
- Rawson RA, Washton A, Domier CP, Reiber C. Drugs and sexual effects: role of drug type and gender. *Journal of substance abuse treatment*. 2002; 22:103–108. [PubMed: 11932136]

- Rusyniak DE. Neurologic manifestations of chronic methamphetamine abuse. *The Psychiatric clinics of North America*. 2013; 36:261–275. [PubMed: 23688691]
- Sato SM, Wersinger SR, Hull EM. The effects of nitric oxide-cGMP pathway stimulation on dopamine in the medial preoptic area and copulation in DHT-treated castrated male rats. *Hormones and behavior*. 2007; 52:177–182. [PubMed: 17467707]
- Schober JM, Pfaff D. The neurophysiology of sexual arousal. *Best practice & research Clinical endocrinology & metabolism*. 2007; 21:445–461. [PubMed: 17875491]
- Schwartz MD, Mong JA. Estradiol modulates recovery of REM sleep in a time-of-day-dependent manner. *American journal of physiology Regulatory, integrative and comparative physiology*. 2013; 305:R271–280.
- Sample SJ, Grant I, Patterson TL. Female methamphetamine users: social characteristics and sexual risk behavior. *Women & health*. 2004a; 40:35–50. [PubMed: 15829444]
- Sample SJ, Patterson TL, Grant I. The context of sexual risk behavior among heterosexual methamphetamine users. *Addictive behaviors*. 2004b; 29:807–810. [PubMed: 15135564]
- Shelley DN, Choleris E, Kavaliers M, Pfaff DW. Mechanisms underlying sexual and affiliative behaviors of mice: relation to generalized CNS arousal. *Social cognitive and affective neuroscience*. 2006; 1:260–270. [PubMed: 18985112]
- Snoeren EM, Agmo A. The incentive value of males' 50-kHz ultrasonic vocalizations for female rats (*Rattus norvegicus*). *Journal of comparative psychology*. 2014a; 128:40–55. [PubMed: 24040761]
- Snoeren EM, Agmo A. The role of odors and ultrasonic vocalizations in female rat (*Rattus norvegicus*) partner choice. *Journal of comparative psychology*. 2014b; 128:367–377. [PubMed: 25133463]
- Tobiansky DJ, Roma PG, Hattori T, Will RG, Nutsch VL, Dominguez JM. The medial preoptic area modulates cocaine-induced activity in female rats. *Behavioral neuroscience*. 2013; 127:293–302. [PubMed: 23565937]
- Voigt RM, Napier TC. Context-dependent effects of a single administration of mirtazapine on the expression of methamphetamine-induced conditioned place preference. *Frontiers in behavioral neuroscience*. 2011; 5:92. [PubMed: 22347852]
- Weil ZM, Zhang Q, Hornung A, Blizard D, Pfaff DW. Impact of generalized brain arousal on sexual behavior. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:2265–2270. [PubMed: 20080607]
- Wesson DW. Sniffing behavior communicates social hierarchy. *Current biology : CB*. 2013; 23:575–580. [PubMed: 23477727]
- Winland C, Haycox C, Bolton JL, Jampana S, Oakley BJ, Ford B, Ornelas L, Burbey A, Marquette A, Frohardt RJ, Guarraci FA. Methamphetamine enhances sexual behavior in female rats. *Pharmacology, biochemistry, and behavior*. 2011; 98:575–582.
- Wyatt T. Pheromones and Animal Behaviour: Communication by Smell and Taste (Book). *Choice: Current Reviews for Academic Libraries*. 2003; 41:566.

Highlights

- Androgen-primed castrates are sufficient for increasing MA-facilitated proceptivity
- MA-treatment does not increase proceptivity toward a castrate male
- MA-treated females can discriminate between sexual stimuli
- MA and ovarian hormones may be acting together to enhance the salience of sexual cues

Figure 1. An androgen replaced stimulus male (DHT-CX) increases proceptive behavior in MA-treated females

Compared to their non-MA-treated counterparts, a DHT-CX stimulus significantly increased **A)** Overall Proceptivity (** $p < 0.01$), **B)** Hops and Darts (** $p < 0.01$) and **C)** Solicitations ($p < 0.05$) in MA-treated females. Neither a CX nor DHT-CX stimulus was sufficient to elicit increased proceptivity from a non-MA-treated female. **D)** Compared to non-MA-treated females, a DHT-CX stimulus did not significantly change Female-Male Mounts. However, for MA-treated females, there was a significant increase ($p < 0.05$) compared to their CX response.

Figure 2. An androgen replaced stimulus male decreased sniffing behavior by MA-treated females, but other non-sexual social and exploratory behaviors remained unchanged
A) Sniffing: DHT treatment reduced sniffing behavior in all females, [$F_{(1,16)}=16.96$, $p=0.0008$]. Post-hoc analysis revealed a significant decrease in MA-treated females between a CX vs. DHT-CX stimulus ($*p<0.05$). **B) A/G Investigation:** No significant differences in female-initiated AG investigations. **C) Rears:** There was a significant decrease in rearing in all females, [$F_{(1,16)}=6.368$, $p=0.0226$], and a significant interaction between the factors [$F_{(1,16)}=5.741$, $p=0.0292$], but no main effect of female MA-treatment. **D) Arena Exploration:** There were no significant differences in corner explorations.

Figure 3. Androgen replaced males spend more time socially investigating females, regardless of female MA-treatment

A,B) DHT treatment significantly increased anogenital investigation ♦: [$F_{(1,12)}=8.050$, $p=0.0150$], and total social investigation ♦: [$F_{(1,12)}=10.73$, $p=0.0066$] of all females. **C)** DHT-treatment significantly decreased the amount of time spent exploring the arena ♦: [$F_{(1,12)}=5.507$, $p=0.0369$].

Figure 4. Regardless of MA treatment, female rats spent more time socially interacting with an intact stimulus male over a CX or DHT-CX stimulus male

All females preferred to interact with an intact male over either a CX $***: [F_{(1, 12)} = 30.28, p < 0.0001]$, or DHT-CX $**: [F_{(1, 12)} = 17.14, p = 0.0014]$ male, despite their MA-treatment status.

Table 1

CX males and DHT-CX males displayed very low levels of male typical sex behaviors.

Male Sexual Competency	CX Males	DHT-CX Males	t-test statistics
Average Total Mount Attempts (over 25 minute test)	1.750 ± 1.750 (n=4)	5.675 ± 3.725 (n=4)	$t_6=0.95$, $p=0.37$, $d=0.68$, ns
Average Latency to Mount* (in seconds)	266.5 ± 33.50 (n=4)	254.4 ± 39.78 (n=4)	$t_6=0.23$, $p=0.82$, $d=0.16$, ns

There was no significant difference between the CX and DHT-CX males in total mounts or the latency to mount. Data are represented as mean ± SEM.

* note= latency to mount was examined within 5 minutes (600 seconds) of the test, otherwise the score for that test was recorded as 600 seconds.