SEXUAL BEHAVIOR AS A MODEL FOR THE STUDY OF MOTIVATIONAL DRIVE AND RELATED BEHAVIORS

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EDITORIAL
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Editorial: Sexual Behavior as a Model for the Study of Motivational Drive and Related Behaviors

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Keywords: sexual behavior, sexual motivation, mesocorticolimbic system, dopamine, sex hormones, motivational drive, animal models, individual differences

Editorial on the Research Topic

Sexual Behavior as a Model for the Study of Motivational Drive and Related Behaviors

Sex is a strong, primary natural reinforcer with a pervasive role in the life of animals and human beings. However, sexual motivation differs from other classes of motivation, i.e., thirst and hunger, being necessary for the preservation of species but not for the survival of individuals. The study of the neurobiological underpinnings of sexual behavior may provide useful information about the mechanisms underlying its motivational determinants and, in more general terms, about the neural correlates of motivation, whose processes are dysfunctional in many psychopathological conditions, including major depression and addiction. Sexual behavior and its motivational determinants could therefore serve as a general model for investigating physiologically and pathologically motivated behaviors with the ultimate goal of identifying new therapeutic approaches for the treatment of psychogenic sexual dysfunctions and psychopathological conditions characterized by altered motivation and related dysfunctional behaviors.

Sexual behavior, and specifically sexual motivation, has traditionally been "hard matter" for the scientist who attempts to investigate it. Difficulties are immediately present in its definition, methodological approach and data interpretation. This Research Topic brings together 18 contributions (11 Original researches, four Reviews, two Mini-reviews, and one Brief Research Report) where comprehensive overviews of current knowledge on the neurobiology of sexual behavior and original findings on innovative approaches and models for studying sexual motivation in males and females are provided by leading experts in the field.

Firstly, a key point in the comparative research on sexual behavior and motivation, i.e., the generalization from animals to humans, is discussed by Le Moëne and Ågmo. Authors report that while huge behavioral differences exist between humans and rodents in terms of consummatory aspects, analogies can be observed, to some extent, in the motivational determinants that allow sexual interaction and copulation as well as in the underlying hormonal and neurochemical correlates. However, unlike in animals, the determinants of sexuality in humans are strongly influenced by social factors. The use of animal models of sexual behavior to investigate the features of human pathological conditions is elegantly discussed by Bialy et al.. Based on the analysis of the parameters that describe the complex structure of sexual behavior in laboratory rodents, the authors propose an interesting approach for delineating the distinct mechanisms affecting sexual motivation and performance in several (psycho)pathological conditions and assessing the efficacy of therapeutic approaches in preclinical investigations. To better characterize the multiple facets and complexities of sexuality, Portillo and Paredes discuss the broad spectrum of reproductive strategies in mammals in which biological variability points to the importance of understanding

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their neurobiological bases in different species and provide a description of asexuality and monogamous bonds in mammals—two conditions that can model and help us to understand some important aspects of human sexual motivation.

Employing selected rat lines is a relatively new strategy for the study of sexual behavior that allows investigation of the genetic and neurobiological underpinnings of individual differences. Esquivel-Franco et al. investigate the role of 5-HT1A auto- and hetero-receptors in sexual behavior in wildtype and knockout rats lacking the serotonin transporter (SERT), which model the alterations in ejaculatory function observed under conditions of chronically elevated levels of serotonin, e.g., during SSRI therapy. A transgenic animal model is also used by Sanna et al. who demonstrate altered motivational and performance aspects of sexual behavior in rats partially or totally lacking the dopamine transporter (DAT). Behavioral alterations were accompanied by an imbalanced dopamine/glutamic acid interaction and an altered expression of neural activation and plasticity markers in mesocorticolimbic areas, suggesting a possible use of these animals as a model of hypersexuality due to chronic hyperdopaminergia. The respective roles of genetic background and maternal care in affecting sexual responses are investigated by Dorantes-Nieto et al. in rat lines selected for differences in their yawning response. Authors observe that while some behavioral features/responses are resilient to environmental factors, thus revealing the strong influence of the genetic background, others can be modified by selective fostering, showing that maternal care is capable of changing innate behavioral responses, including yawning, penile erection and grooming.

Several contributions identify a key component of the neurobiological core of sexual motivation and drive in the dopamine mesocorticolimbic system. However, beyond the well-established involvement of this system in motivational processes, what emerges is the need for broader research to better understand the complex neurochemical interactions between dopamine and other neurotransmitters in modulating its activity. Moore et al. employ biosensors and DREADD technique to investigate fronto-accumbal glutamatergic activity during sexual behavior in the female hamster. What emerges from this study is the importance of the fronto-cortical glutamatergic input to mesolimbic dopaminergic areas in fine modulation of the behavioral output, mainly regarding its motivational aspects, which provides new insight into the neurobiology of the motivational control of female sexual behavior. Canseco-Alba and Rodríguez-Manzo examine the interaction between mesolimbic dopamine and endocannabinoids in regulating sexual motivation and satiation in the male rat. They show that endocannabinoid activity reverses sexual satiety by modulating dopaminergic transmission, presumably at the mesolimbic system, with anandamide and 2-arachidonoylglycerol, displaying different actions on D1- and D2-like receptors.

Sexual dysfunction in women is poorly understood, perhaps due to its subtle expression; this can include loss of motivation and loss of pleasure during sex, which can affect intimate relationships, self-esteem and, ultimately, psychological well-being and quality of life. Animal models of female sexual behavior have provided insight on how the interaction of neurotransmitters and steroid hormones are required to modulate the central motivation state and thus affect sexual motivation. Some of the contributions to this Research Topic focus on the neurobiological mechanisms involved in female sexual motivation at both preclinical and clinical levels. Guarraci and Frohardt review the current knowledge on models of sexual motivation in female rats and discuss the main patterns of behavior that reflect either increases or decreases in motivation to advance our understanding of female sexual behavior with particular attention to partner choice and preference models. The role of sex hormones in modulating female sexual motivation when multiple rewards (e.g., food and sex) are available is examined by Yoest et al.. They provide a neurobiological framework for understanding how ovarian hormones, released over the course of the estrous cycle, modulate adaptive behavioral choices in females when multiple rewards are available.

The role of sex hormones in modulating pathologically motivated behavior is investigated by Bakhti-Suroosh et al. who examine how estradiol affects different aspects of psychostimulant addiction. Notably, a dual role for this steroid hormone in drug addiction was revealed. Estradiol was found to both enhance and reduce vulnerability by amplifying drug reward and facilitating new learning during the extinction process, respectively. The interactions between psychostimulants, ovarian hormones and sexual motivation are reviewed by Rudzinskas et al. who discuss a model of increased sexually-motivated behaviors induced by administration of the psychostimulant methamphetamine in females. They suggest that the combination of ovarian hormones, olfactory information and methamphetamines could produce enhanced sexual motivation by inducing activation and neural plasticity within a key integration site for sexually relevant sensory information, i.e., the posterodorsal medial nucleus of the amygdala.

Besides sex hormones, the neurosteroid allopregnanolone is now attracting the attention of researchers for its ability to modulate specific aspects of female sexuality. In their first contribution, Frye et al. show that the effects of intra-VTA allopregnanolone on female sexual behavior involve NMDA receptors and are likely mediated through GABA-A receptors. A second contribution of Frye and Chittur investigates the neuroplastic modifications induced by mating in the mesocorticolimbic system of female rats and shows that mating significantly enhances midbrain mRNA expression of genes involved in hormonal and trophic actions, revealing a complex fine-tuning of mating-induced neuroplastic processes.

Preclinical research on sexual behavior has almost exclusively been conducted in mammals, mainly rats. Here, Sato et al. review the role of Fru proteins in the sexual behavior of *Drosophila melanogaster* and discuss how the selective expression of some forms of these proteins might confer male-specific roles by interacting at transcriptional level with partner proteins, thus contributing to the regulation of male sexual behavior with particular reference to courtship and mating.

Three clinical studies conclude the Research Topic. In the first study, Regier et al. investigate possible differences in the activity of the mesolimbic system in women with

different propensities of engaging in unsafe sexual intercourse. Authors report that women who have protected sex may view sexually related stimuli more positively than young women at increased risk of sexually transmitted diseases (STIs)/HIV, in which they also evidenced lower mesolimbic responses to sexual cues. The study thus enriches the list of prevention factors and may help to identify young women at greatest risk of contracting STIs and/or HIV. The large multicenter study by Zamboni et al. focuses on sexual functioning in opioid-addicted women under opioid maintenance treatment and reveals that the majority of them have sexual dysfunction, regardless of the treatment protocol (buprenorphine vs. methadone), and report a poor quality in intimate relationships and mental health. These results suggest that female sexual well-being should also be taken into account during treatment detoxification given that it may impact adherence to therapy and, thus, interfere with its beneficial outcomes. Finally, Soares et al. investigate the association between infatuation/passionate love and impulsivity in adolescents with Attention Deficit and Hyperactivity/impulsivity Disorder (ADHD). Intriguingly, although an association between infatuation intensity, behavioral urgency, and sensation-seeking was observed, this association does not change in the presence of ADHD, pointing to the need for further studies to clarify an increased risk for negative social outcomes due to sexually related risky behaviors in some population groups.

In conclusion, the classical Beach's distinction between appetitive and consummatory aspects of sexual behavior seems to be, in its core aspects, still valid and constitutes a conceptual framework for most of the recent research in the field. However, current challenges lie in depicting the extremely complex intricacies between these two main aspects of sexual behavior, accounting for its complexity at both behavioral and neurobiological levels. The disclosure of the neurobiological underpinnings of sexual behavior at molecular, neuronal, and system levels is dramatically improving our knowledge of this complex matter. The studies on the specific roles of different neurosteroids and their interactions with brain neurotransmitters as well as on the neuroplastic changes induced by sexual activity will be highly informative in accounting for such a complexity. Genetic and (bio)behavioral selection have allowed for a more accurate investigation of the determinants and neurobiological correlates of individual differences related to sexual motivation and behavior. Significant strides have been made in the study of difference between the sexes, and the increasing number of studies on female sexuality is important to note, as research on female sexual behavior has often been "neglected."

Overall, we feel that the present Research Topic provides an interesting and valuable picture of the current field and contributes to our understanding of the mechanisms that control sexual motivation and reward.

AUTHOR CONTRIBUTIONS

FS, PP, and LF equally contributed to this Editorial for the Research Topic entitled "Sexual Behavior as a Model for the Study of Motivational Drive and Related Behaviors." All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Sexual Functioning and Opioid Maintenance Treatment in Women. Results From a Large Multicentre Study

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Opioid maintenance treatment (OMT) is the most widespread therapy for both females and males opioid addicts. While many studies have evaluated the OMT impact on men's sexuality, the data collected about the change in women's sexual functioning is still limited despite the fact that it is now well-known that opioids - both endogenous and exogenous - affect the endocrine system and play an important role in sexual functioning. The present study aims to determine how OMT with buprenorphine (BUP) or methadone (MTD) affects sexual health in women; examining also any possible emerging correlation between sexual dysfunction (SD), type of opioid and patients' mental health. This multi-center study case recruited 258 female volunteers attending Italian public Addiction Outpatients Centers that were stabilized with OMT for at least 3 months. SD was assessed with the Arizona Sexual Experience Scale. The twelve-item General Health Questionnaire was used to assess participants' mental health conditions. The results show that 56.6% of women receiving OMT for at least 3 months presented SD without significant differences between MTD e BUP groups. The majority of the subjects with SD have a poorer quality of intimate relationships and worse mental health than the average. To the best of our knowledge, the present study is the largest report on the presence of SDs in women as a side effects of MTD and BUP used in OMT. Since SDs cause difficulties in intimate relationships, lower patients' quality of life and interfere with OMT beneficial outcomes, we recommend that women undertaking an opioid therapy have routine screening for SD and we highlight the importance to better examine opioid-endocrine interactions in future studies in order to provide alternative potential treatments such as the choice of opioid, opioid dose reduction and hormone supplementation.

Keywords: methadone, addiction, quality of life, women sexuality, buprenorphine

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INTRODUCTION

The World Health Organization (WHO) describes sexual health as a state of physical, emotional, mental and social well-being in relation to sexuality itself and recognizes it as a right to every human being (World Health Organization [WHO], 2006). It is commonly accepted that a healthy sexuality is fundamental to one's sense of self-worth (Kaplan, 1974); it represents the integration of the biological, emotional, social, and spiritual aspects of who one is and how one relates to others (Covington, 1997).

Despite its proven capital importance for human self-esteem, sexuality has been a neglected topic in scientific research and in treatment of women with Substance Use Disorders (SUD) to this day. Reproduction intended as a combination of contraception, pregnancy, parenthood and risky sexual behavior (i.e., sexually transmitted diseases and prostitution), is the aspect drawing most of the attention in terms of research and special health services for women. Although these aspects deserve priority in regards to feminine sexuality, they are not fully exhaustive on the matter.

The lack of professional study cases on the relationship between SUD and sexual functioning appears more evident considering sexual health in opioid dependent women under Opioid Maintenance Treatment (OMT) on buprenorphine (BUP) and methadone (MTD). To this day we have a serious lack of data in regards to this topic despite the fact that opioids -both endogenous and exogenous- evidently affect the endocrine system (Katz and Mazer, 2009; Rhodin et al., 2010; Voung et al., 2010) and play an important role in sexual functioning (Palha and Esteves, 2008).

Sexual dysfunctions (SDs) are a frequent adverse effect during opioid treatment for both men and women however, most of the studies had been conducted basing the researches on male candidates receiving OMT (Brown and Zueldorff, 2007; Lugoboni et al., 2017).

To date most of the studies that have analyzed the impact of opioid treatment on female sexuality had been conducted on women treated with opioid analgesics aimed to cure non-malignant chronic pain. These studies demonstrated that opioids inhibit the production of multiple hypothalamic, pituitary, ovarian and adrenal hormones, causing opioid-induced hypogonadotropic hypogonadism that can determine amenorrhea or hypomenorrhea, SDs, fatigue and depression in female patients (Daniell, 2008; Rhodin et al., 2010).

Opioid maintenance treatment combined with psychosocial interventions is the most widespread treatment for opioid dependence. In Europe MTD is currently the most prescribed medicine against heroine dependency: 69% of opioid dependent patients are undergoing this treatment, meanwhile the 28% of the subjects is assuming BUP. Among 700.000 European patients in OMT, 20% is represented by women (EMCDDA, 2015).

Given the fact that one of the main goals of the OMT is the amelioration of the patients' quality of life and their reintegration in a gratifying social life, it is clear how assessing and curing eventual SDs in both female and male OMT patients is of primary importance. It is also demonstrated that iatrogenic sexual disorders can act against treatment retention and achievement of a good quality of life for the patients (Xia et al., 2013).

Taking into account the limited number of monitored and rigorous studies regarding OMT and SDs as a side effect of this therapy in women, the present study puts its focus on the presence of SDs in Italian female patients treated for opioid dependence with MTD or BUP in specialized outpatient centers. The authors hypothesize that OMT impacts the sexual health of opioid addicted women, as it is already ascertained in men, and they aim to examine if there is any correlation between possible SD, type of opioid, daily dose administration and patients' mental health. Due to the factors explained above, the results of this study could help to find evidence-based models that would allow assisting clinicians to address and treat sexual issues and related concerns with aimed therapy.

MATERIALS AND METHODS

The study was conducted in 20 Addiction Treatment for Outpatients Centers of the Italian public health system. The philosophy of intervention, policies and procedures applied were similar in each Center and the accessibility threshold was the same across all structures.

Italian Addiction Treatment Services provide outpatient treatment programs with a variety of therapeutic and rehabilitative strategies: MTD, BUP, and naltrexone are administered in association with possible psychosocial interventions, such as psychotherapy, family therapy, group therapy, social support and medications for psychiatric co-morbidity.

The selected centers did not differ in the psychosocial treatment protocols associated with MTD and BUP, or in the admission criteria. In the Italian Addiction Services the majority of patients are heroin addicts. There are no exclusion criteria regarding the access to the public health system. Patients who fail to respond to interventions such as OMT and continue to inject heroin are not dismissed by these centers.

Between 1st July and 31st December, 2015, a cross-sectional survey was administered to a large sample of patients receiving MTD or BUP maintenance treatment for heroin dependence. The sample included 258 women between the age of 18 and 61 (mean age: 37) enrolled in a drug recovery program in treatment centers for clinically diagnosed heroin dependence (American Psychiatric Association, 2000) DSM IV TR. Patients were receiving either MTD (N 198, 76.7%; mean daily dose 60.5 mg) or BUP (N 56, 23.3%; mean daily dose 10.8 mg) maintenance in combination with psychosocial treatment. At the time of the study they had been stabilized with an OMT for at least 3 months.

Underage patients, subjects following a drug-free treatment or an opioid substitution therapy for less than 3 months (and/or other than MTD/BUP) were excluded from the study case and also those who presented difficulty of language comprehension.

The questionnaires and data sheets were delivered by a nurse to the participating patients. This was done to optimize patients' privacy and to minimally affect responses, as nurses are less

involved in therapy compared to doctors and psychologists. Patients filled the questionnaires at the facility or at home and handed the documents anonymously. As indicated by a previous focus group among surveyed patients the collection of the questionnaires was carried out using an urn and not by direct delivery to the staff. Patients gave a written informed consent in order to take part to this survey, which was approved by the Public Health System ethical committee of Verona University Hospital in Verona, Italy. All participants were volunteers and were not paid for their participation. The patients could stop the survey's compilation at any time. Study procedures did not interfere with the daily protocols of the centers.

Measures

Sexual dysfunction was assessed by the Arizona Sexual Experience Scale (ASEX) (McGahuey et al., 2000). It is composed by five items rated on a 6-point Likert-type scale, with higher scores reflecting greater or lower dysfunction level. Each item quantifies a major domain of sexual function, sexual drive, psychological arousal, physiologic arousal (vaginal lubrication for women), ability to reach orgasm, and orgasm satisfaction (e.g., "How easily are you sexually aroused?"). Cases of SD were established according to three criteria: (a) a total score $\geq$19; (b) any item with an individual score $\geq$5; and (c) any three or more items scoring $\geq$4 were considered as SD (McGahuey et al., 2000). These three criteria showed optimal sensitivity and specificity for SD. The main dependent variable for all the analyses will use cases applying all those criteria (0, "not SD," 1 "SD"), and analyses will be repeated for each specific criterion in order to test possible differences with more restrictive definitions. In the present study, the internal reliability was 0.86.

The twelve-item General Health Questionnaire (GHQ-12) was used to assess the mental state of the participants. This tool is intended to screen for general (non-psychotic) psychiatric morbidity (Goldberg and Williams, 1988; e.g., "Have you recently felt you couldn't overcome your difficulties?"). It has been widely used and translated into many languages and extensively validated in general and clinical populations worldwide (Werneke et al., 2000). Items were answered on a 4-point scale from 0 (not at all) to 3 (much more than usual).

Higher scores indicate poorer mental health. In the present study, the internal reliability was 0.89.

The questionnaire also included demographic and drug related variables such as age, marital status, education level, and use and dosage of MTD and BUP. The respondents completed anonymously all questionnaires.

Statistical Analysis

At first, differences in proportions or means between patients with or without SD were compared with chi-square tests (categorical variables) and *t*-tests for unrelated samples (continuous).

In order to assess the strength of the associations with categorical variables the Cramer's V coefficient of association (range from 0 for no association to 1 as perfect association) was calculated, whereas the effect size (Hedge's g) was measured on associations with continuous variables.

Hedge's g provides values that are very similar to Cohen's d [d = g/sqrt (N/df)] for which the following arbitrary rules of thumb are often used: 0.2–0.3, small effect; 0.5, moderate effect; and 0.8, large effect (Cohen, 1988).

A confirmatory factor analyses (CFA) was carried out for examining the distinctiveness of the scales used in this study. More specifically, we compared a full measurement model to a one-factor structure (where items were set to load into a common factor). The model ft was tested considering the Comparative Fit Index (CFI), the Incremental Fit Index (IFI), and the Root-Mean-Square Error of Approximation (RMSEA). According to Kline (2005) and Byrne (2016), the CFI and IFI values should have a cutoff value of $\geq$0.90, and RMSEA a value of $\leq$0.08 to indicate a good ft of the model. Reliability analysis was performed using Cronbach's α measure.

Finally, to examine whether demographics (age, marital status, and education level), use of MTD and BUP, and overall psychological well-being were predictive of sex dysfunction, a stepwise multiple regression analysis was carried out. A *P*-value $<$0.05 was considered statistically significant.

Statistical analyses were carried out using PASW Statistics 18·0 and AMOS 16·0 (Chicago, IL, United States, Arbuckle, 2007).

RESULTS

Factorial Validity of the Scales

Results from CFA showed that the hypothesized two-factor model ($\chi 2$ = 297.25 *df* = 113, $P < 0.01$, RMSEA = 0.079, CFI = 0.92, IFI = 0.92) fits the data significantly better than the one general factor model ($\chi 2$ = 606.26, *df* = 114, $P < 0.01$, RMSEA = 0.130, CFI = 0.78, IFI = 0.78) providing evidence of discriminable different factors.

Descriptive Statistics

A total of 56.6% (*SE* = 3.1; *n* = 146) of the sampled patients manifested SD considering the three criteria explained above. 18.6% (*SE* = 2.4; *n* = 48) fulfilled criterion a, 43.0% criterion b (*SE* = 3.1; *n* = 111), and 43.4% criterion c (*SE* = 3.1; *n* = 112). In the total sample the mean age was 37.7 years (*SD* = 10.6; range: 18–61); 61.3% of the subjects were single, 15.2% married, 18.4% divorced or separated and 5.1% widowed; 69.0% presented a secondary education level while the 26.4% had a higher education, and 4.7% an elementary level of education. 76.4% (*n* = 197) were taking MTD with an average of 60.6 mg (*SD* = 73.5) and 20.6% (*n* = 53) were taking BUP with an average of 10.5 mg (*SD* = 7.6). The mean score in the GHQ for the total sample was 14.6 (*SD* = 7.0).

Comparisons between those with and without SDs indicated that women with SDs were older, were more often separated or divorced, had lower levels of education, assumed higher doses of MTD (among those consuming the drug), and presented a poorer mental health as measured by the GHQ12. No differences among groups were found in regards to the percentage of patients taking MTD or BUP or the doses of this last drug. All these results are summarized in **Tables 1**, **2**.

TABLE 1 | Characteristics of the sample according to the presence of sexual dysfunction.

Variables	Sexual dysfunction *N* = 146	Not sexual dysfunction *N* = 112	*p*	*g (95% CI) or V*
Age	39.1 (11.1)	35.5 (9.9)	**0.006**	**0.34 (0.09,0.59)**
Marital status (%)				
Married	13.2 (2.8)*	17.9 (3.6)*	**0.048**	**0.176**
Widowed	6.9 (2.1)*	2.7 (1.5)*		
Divorced/ separated	22.9 (3.5)*	12.5 (3.1)*		
Single	56.9 (4.1)*	67.0 (4.5)*		
Education level (%)			**0.004**	**0.209**
Elementary	6.9 (2.1)*	1.8 (1.3)*		
Secondary	74.0 (3.6)*	62.5 (4.6)*		
Higher	19.2 (3.3)*	35.7 (4.5)*		
Methadone (% yes)	80.1 (3.3)*	71.4 (4.3)*	0.103	0.102
Methadone, mg	69.4 (91.1)	47.6 (30.3)	**0.041**	**0.30 (0.01,0.58)**
Buprenorphine (% yes)	17.2 (3.1)*	25.0 (4.1)*	0.127	0.095
Buprenorphine, mg	8.9 (8.2)*	12.0 (6.7)*	0.132	0.41 (−0.13,0.95)
GHQ	16.0 (7.1)	12.8 (6.4)	**<0.001**	**0.47 (0.22,0.42)**

*Values between brackets are standard deviations unless indicated: *, Standard errors. Comparisons: t-tests for continuous variables, chi-square tests for categorical variables g, Hedge's effect size, V, Cramer's V. Values in bold are statistically significant (p < 0.05).*

TABLE 2 | Results of logistic regression analyses, persons without sexual dysfunction (reference category) vs. persons with sexual dysfunction, unadjusted and controlling for age (except for the age effect).

Variables	OR, unadjusted (95% CI)	OR, adjusted (95% CI)
Age	1.03 (1.01,1.06)	
Marital status (ref.: single)		
Married	0.87 (0.43,1.75)	0.73 (0.35,1.51)
Widowed	3.05 (0.81,11.50)	2.15 (0.54,8.53)
Divorced/separated	**2.16 (1.07,4.34)**	1.56 (0.72,3.36)
Education level (ref.: elementary)		
Secondary	0.31 (0.07,1.45)	0.33 (0.07,1.58)
Higher	**0.14 (0.03,0.69)**	**0.16 (0.03, 0.80)**
Methadone (ref.: No)	1.61 (0.91,2.87)	1.70 (0.95,3.07)
Methadone, mg	**1.01 (1.001,1.02)**	**1.01 (1.0001,1.02)**
Buprenorphine (ref.: No)	0.62 (0.34,1.15)	0.58 (0.31,1.07)
Buprenorphine, mg	0.94 (0.87,1.02)	0.94 (0.87,1.02)
GHQ	**1.07 (1.03,1.12)**	**1.09 (1.04,1.13)**

Numbers in bold indicate statistically significant effects (p < 0.05).

Interestingly, when performing the same analyses using more restrictive definitions of SD, results did not change for criteria b (any one item with a score ≥5) and c (any three or more items with scores ≥4), while in regards to criteria a (cut-off score in the total scale ≥19) there was no difference shown in the severity of mental health symptoms.

As shown in **Table 3**, only one significant effect emerged for item # 2 (easiness for sexual activation) when analyzing distribution of scores for specific ASEX items in women taking or not taking MTD. Subjects taking MTD reported higher difficulty for getting aroused, with an average effect size (Cramer's $V = 0.286$). The total ASEX score, however, did not significantly differ [$t(256) = 0.15$; $p = 0.882$] between those taking MTD (mean = 14.77, $SD = 4.58$) or BUP (mean = 14.67, $SD = 4.51$).

Hierarchical Regression Analyses

Stepwise multiple regression analysis was conducted with individual characteristics as shown in **Table 4**, including age, marital status, and education level, use of MTD and BUP, and overall psychological well-being as predictor variables and sex dysfunction as criterion (dependent) variables. **Table 4** shows that the model accounted for 6.5% of the criterion variance.

Overall psychological well-being was the only significant predictor ($\beta = 0.27$, $p < 0.001$).

DISCUSSION

The scientific literature that treats gender differences in SUD is rather recent and it highlights substantial differences between men and women. It is demonstrated by these studies that gender influences the prevalence, the origin, the progression and the outcome of these disorders. Women showed a quicker transition from use to dependence (Becker and Hu, 2008), worse clinical conditions at the time of admission, more frequent comorbidity for depression and anxiety, increased suicide risk, and worse physical health compared to men presenting opioid use disorder. Psychiatric comorbidity often precedes and favor onset of SUD in women, such as post-traumatic stress disorder which is found related to physical and sexual abuse in all ages and worst socioeconomic conditions (Cotto et al., 2010; Eiroá-Orosa et al., 2010; Back et al., 2011). Women indulge in sexual risky behavior more than men by avoiding condom use, choosing a greater number of sexual partners and using sex in exchange of money and/or drugs more frequently; women also tend to choose stable partners with SUD (Quaglio et al., 2004, 2006). They often accept unprotected sex in order to grant the continuity of the relationship (Sheeran et al., 1999). Numerous studies verified that intimate partner violence and childhood sexual abuse in general population are strongly related to risky sexual behaviors and to the occurrence of sexually transmitted diseases (Urada et al., 2013). These dynamics facilitate the manifestation of unbalanced love or sexual relationships that favor the masculine partner's power. Together with SUD these situations jeopardize women's determination to look for and find a healthy sexual life (Engstrom et al., 2012; Gilbert et al., 2015).

To this day gender studies have largely neglected the sexual aspects of opioid dependent women and to the best of our knowledge, the present study is the largest report on women

TABLE 3 | Association between responses to specific items from the ASEX and the use of MTD (% of persons using MTD responding to each category).

Items	1	2	3	4	5	6	*p*	*V*
How strong is your sex drive?	1.52	12.69	22.34	31.47	21.83	10.15	0.210	0.167
How easily are you sexually aroused (turned on)?	**1.53**	**10.71**	**20.92**	**39.80**	**21.94**	**5.10**	**0.001**	**0.286**
How easily does your vagina becomes moist or wet during sex?	6.12	22.45	25.51	28.57	12.76	4.59	0.265	0.159
How easily can you reach an orgasm?	1.80	15.32	27.03	33.33	16.22	6.31	0.146	0.146
Are your orgasms satisfying?	9.91	31.53	29.73	16.22	7.21	5.41	0.071	0.259

From 1 "extremely strong/ easily/ satisfying" to 6 "no sex drive/ never/ can't reach orgasm." Higher scores means more sexual dysfunction. Numbers in bold indicate statistically significant effects ($p < 0.05$); tests: chi-square (df = 5).

with SD on MTD or BUP maintenance treatment. It focuses on the sexual health of 258 women in OMT using consistent and validated measures of SD and also evaluating other factors (i.e., demographic data, mental health, and opioid dose), that could contribute to SD. The results show that 56.6% of women receiving BUP or MTD for at least 3 months show SD without significant differences between MTD e BUP groups.

These results differ from the ones reported by Moreira et al. (2008), in a large community survey that showed how 30.1% of adult women in Southern Europe (Italy, Spain, France) suffer from lack of sexual interest, while 22.7% experience lack of sexual pleasure and 24.8% incur inability to reach orgasm. These percentages indicate that female patients in OMT have a higher rate of SDs in comparison to the general population.

In the present study the MTD group shows a significantly higher excitation disturbance compared to the BUP group while considering specific ASEX issues. These results are consistent with those of the study conducted by Giacomuzzi et al. (2009), which demonstrated how, in a small sample of 30 women in OMT, it is harder to reach orgasms while taking MTD instead of BUP.

Furthermore, demographic variables emerged from this study, BUP and\or MTD intake are not significant predictors of SDs, and the majority of subjects with SD have a quality of intimate relationship and mental health poorer than the average. The results from the stepwise regression show how women's overall psychological well-being is positively linked to SD. These findings are consistent with those of other studies reporting SD, anxiety and depression in women treated with opioid in chronic pain (Daniell, 2008; Katz and Mazer, 2009). The relationship between mood disorders and SD is actually still unsettled in women in OMT, but in many cases it could be directly associated with opioid-induced hypogonadotropic hypogonadism, especially for impaired androgen production. The testosterone opioid-induced suppression can have important consequences other than SD, such as potential anxiety, depression, fatigue and a generally reduced quality of life. These symptoms were reported to have improved with androgen supplementation in women undergoing long-term opioid treatment (Brown and Zueldorff, 2007; Katz and Mazer, 2009). As a matter of fact, the presence of depression, anxiety and a generally reduced quality of life are common in women in OMT and could be due to associated conditions and co-morbidities (i.e., other medications, primary psychiatric disorders, other medical conditions, use of other substances low socioeconomic status), regardless of the opioid treatment. In case of co-presence of these symptoms and SDs, female patients in OMT should be assessed for opioid-induced hypogonadism by laboratory endocrine evaluation to investigate if alterated gonadal hormon levels play any role in SDs and in mood and/or anxiety disorders.

Furthermore, demographic variables taking BUP and\or MTD were not significant predictors of SD.

It is important to mention the correlation between MTD dose and SD emerged by this study, dynamic which is not present in BUP groups. Other studies have shown a dose-response effect in patients undergoing MTD treatment due to boosting testosterone suppression by increasing the dose of MTD. This result is clearer in men than in women, due to limited scientific information on testosterone levels in female patient undergoing MTD treatment (Bawor et al., 2014). Our outcomes are in line with the previous study carried out by Parvaresh et al. (2015) that used ASEX and focused on MTD dose-related effect in sexual functioning in adult women. Conversely there is no evidence in literature of a link between SD and BUP dosage in women in OMT treatment or about testosterone level in these subjects. If further studies on women will confirm the correlation between SDs and MTD dosages and on the contrary no correlation with BUP dose, this issue should be taken into account at the moment of choice of opioid medication, especially because there are findings that women need higher MTD doses compared to man in order to avoid quitting the treatment (Vigna-Taglianti et al., 2016). The reason behind this last result is still unclear, hypothetically it could be partially associated to the evidence that higher MTD dosages are requested in patients diagnosed with post traumatic stress disorder or depression (Trafton et al., 2006). These illnesses are more frequent in women than in men as explained above.

TABLE 4 | Stepwise linear regression analysis of predictors of sexual dysfunction (*N* = 258).

Variables	B	SE	ß	*P*-value
Age	0,008	0,03	0,019	0,784
Marital status	0,33	0,271	0,085	0,224
Education level	0,109	0,3	0,023	0,716
MET	−1,566	1,605	−0,147	0,33
BUP	−1,743	1,684	−0,155	0,302
Overall psychological well-being	0,176	0,041	0,271	0,000

Predictors of sexual dysfunction final model produced at p = 0.05, F = 3,85, P < 0.01, R^2 = 0.063.

Moreover it should be noted that, despite the lack of evidence in literature of the correlation between the severity of SUD and MTD or BUP dosages needed in OMT, higher MTD doses are predictive of major reduction in illicit opioid consuming in both men and women (Fareed et al., 2009).

CONCLUSION

Sexual dysfunctions may cause difficulties in intimate relationships, lower patients' quality of life, can favor and maintain the SUD, interfere with OMT beneficial outcomes and influence adherence to treatment (Brown and Zueldorff, 2007; Xia et al., 2013; Bawor et al., 2014). It is important to explore the cause of SDs through a multidimensional evaluation. It is very important to inform patients on the possible side effects of opioid therapy on their sexuality and when present, to their treatment also. Spreading the information can avoid the arousal of negative thoughts about themselves and their sexual self-efficacy.

This study shows how OMT can determine sexual side effects in women despite being an essential and effective treatment in opioid addicted patients. Unfortunately, the lack of evidence about SD in women in OMT implicates absence of intervention models in case of sexual disturbances. This can be an obstacle to clinicians to carefully enquire about sexual health in these subjects. Women on opioid therapy should have routine screening for SD longitudinally, and should be treated with appropriate measures.

In the light of the above-mentioned considerations, we now understand the necessity of continuing the studies in order to overcome the existing limited literature about opioid induced SD in women and therefore better examine hypogonadism in women in OMT. The aim is to provide female patients the chance to eventually apply potential treatments like the choice of opioid, opioid dose reduction and androgen supplementation.

GICS MEMBERS

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Limitations of the Study

Whereas the strength of the present study is its larger sample size compared to previous researches, the limitations concern the questionnaire as it is self-reported and the definition of SD as it is subjective. A lack of sexual activity, for example, is not always perceived as SD; personal views (i.e., cultural, religious, or other) often bias interpretation. Furthermore this research lacks a longitudinal perspective. As this research was cross-sectional, we were unable to analyze causal influence and changes in the studied variables across time.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

FL was responsible for the study concept and design. GICS contributed to the data acquisition. LZ assisted with the data analysis and interpretation of findings. AF and LM drafted the manuscript. All authors critically reviewed the content and approved the final version of the manuscript for publication.

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frontiers in Behavioral Neuroscience

BRIEF RESEARCH REPORT
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Investigation on the Attention Deficit Hyperactivity Disorder Effect on Infatuation and Impulsivity in Adolescents

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Introduction: In this study, we proposed to investigate the association between infatuation/passionate love and impulsivity in a context of potential high impulsivity: adolescents with attention deficit and hyperactivity/impulsivity (ADHD) diagnosis compared with typically developing adolescents.

Methods: Impulsivity was understood as an exploratory and a sensation seeking behavior, a trend to engage in novel and exciting activities, and was evaluated using the UPPS Impulsive Behavior Scale. Eighty-one adolescents from 13-to-18 years old with and without ADHD diagnosis were compared regarding infatuation intensity, behavioral impulsivity, and social and educational profiles.

Results: After correlation analysis, we found association between higher scores on the infatuation intensity with fewer years of formal education, heightened urgency and sensation seeking. On the other hand, using the generalized equation model, we showed that the association of passionate love with behavioral urgency and sensation seeking did not change in the presence of the ADHD diagnosis.

Conclusion: The understanding of the relationship of impulsivity with infatuation might help to clarify why some population groups show an increased risk for many negative social outcomes.

Keywords: attention deficit/hyperactivity disorder, ADHD, impulsivity, passionate love, infatuation

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INTRODUCTION

Human pair-bonding and reproduction are complex cross-cultural phenomena involving physiological, cognitive, and emotional changes with high impact on behavior (Hatfield and Rapson, 1993; Fisher et al., 2016). Specifically, the passionate love strategy may have increased human offspring survivability as partners focusing time and energy on one another would probably rear a child as a team. Infatuation, also known as passionate love, is the falling in love or simply

an intense amorous feeling for one individual. Passionate love is the first phase of a romantic relationship, a phase of positive emotions, but also a negative and stressful one if marked by break-ups and life dissatisfaction. Passionate love has been argued as a "natural addiction," including aspects such as obsessive and intrusive thinking, euphoria, and craving (Fisher et al., 2016). Furthermore, the process of falling in love engages brain reward system areas involved in chemical or behavioral addiction, specifically dopamine pathways, with the ventral tegmental area (VTA) consistently associated with intense infatuation (Fisher et al., 2016).

Attention-deficit/hyperactivity disorder (ADHD) is the most prevalent neurodevelopmental disorder in childhood and adolescence, affecting as much as 5% of young people around the world (Barkley et al., 2006; Polanczyk et al., 2007). ADHD is highly complex regarding its etiology but has a strong genetic base with evidence of reward processing alteration and dopaminergic dysfunction (Dalley and Roiser, 2012; Beauchaine et al., 2017). Impulsivity is a core deficit and an important long-term symptom of ADHD. Children with high levels of hyperactive-impulsive symptoms have higher rates of academic drop out and fewer years of education (Fredriksen et al., 2014). Adolescents with ADHD have greater rates of car accidents and delinquency, poorer performance in educational and employment settings, lower occupational status, and social problems with family members and peers (Polanczyk et al., 2007; Bussing et al., 2010). Additionally, ADHD negatively impacts romantic relationships, which is evidenced by lower marital satisfaction, higher rates of divorce (Biederman et al., 1993; Murphy and Barkley, 1996), an aggressive attitude toward others (Wymbs et al., 2012), and poorer conflict resolution (Canu and Carlson, 2007). Antisocial and impulsivity-driven behaviors may partly explain many social and academic outcomes (Fredriksen et al., 2014).

Impulsivity can be considered action with no conscious thinking, a behavior delivered without enough self-control, and a tendency to respond without planning (Moeller et al., 2001). Different approaches have been used to evaluate several aspects of impulsivity, ranging from self-report questionnaires to cognitive measures (Salgado et al., 2009). One of such aspects related to impulsivity is associated with an exploratory behavior, a trend to engage in novel and exciting activities, and a sensation-seeking behavior. Sensation seeking may serve an adaptive purpose, increasing the chances of reproductive success and food obtention (Irwin and Millstein, 1986; Spear, 2000), but also can lead to negative outcomes (Whiteside and Lynam, 2001). Relative to human development, sensation seeking likely peaks in adolescence (Zuckerman, 1974; Roth et al., 2005). In adolescence, youngsters start to engage in new and exciting experiences and begin to expand and have more complex social interactions and relationships. Adolescence is a vulnerable phase for risk-taking. Statistics point to a peak in dangerous activities, such as car accidents, auto and hetero-aggression, drug and alcohol abuse, unprotected sex, and a rise in psychopathologies' rate (Steinberg and Monahan, 2007; Casey et al., 2008). The high sensitivity to incentives and contexts related to impulsivity in adolescence was associated with an imbalance in the self-control circuitry modulated by dopamine (Casey, 2015).

Based on that, this study was conceived to better understand the relationship between impulsivity and the intensity of romantic love in conditions of high impulsivity (ADHD and adolescence). Specifically, this study aimed to investigate a putative association between ADHD, infatuation intensity, and behavioral impulsivity, since they appear to share some neural substrates. Then, we sought to evaluate a possible moderation effect of ADHD on the relationship between infatuation and impulsivity in adolescence.

METHODS

Participants and Study Design

This was a transversal study of adolescents (n = 81) aged 13-to-18 years old, being 55 (67,9%) girls and 26 (32,1%) boys, with 51 (63%) of them presenting a typical development and 30 (37%) with ADHDs. Of these, 18 (60%) were only inattentive, 5 (16.7%) were only hyperactive/impulsive and 7 (23.3%) had a combined profile. Typically-developing participants were from two local urban schools or from a collaborative school from a nearby city, and adolescents with ADHD were recruited in an impulsivity and attention research center. The ADHD screening was performed with the MTA-SNAP-IV (Mattos et al., 2006), which shows good accuracy in detecting this condition among Brazilian children and adolescents (Costa et al., 2018). Adolescents with typical development were classified based on parents reporting a lack of history of psychiatric or neurological disease and may not have borderline or clinical scores in the internalizing and externalizing scales of the Child Behavior Checklist (parent form) (Achenbach et al., 2011). The study was approved by the local ethics board. All the volunteers gave written informed consent and their main caregiver consented for participation. The study is in accordance with the Declaration of Helsinki.

Instruments

Juvenile Love Scale (JLS)

To measure infatuation/passionate love intensity, we used the Brazilian version of the Juvenile Love Scale (JLS) (Hatfield and Young, 1998). The JLS is composed of 30 items rated on a nine points-Likert scale with higher scores indicating a higher passionate love intensity. The JLS access cognitive, emotional, and behavioral features of passionate love such as intrusive thinking and idealization of the other, attraction toward the partner (especially sexual), positive and negative feelings, physiological arousal, physical proximity, and be available to the other (Hatfield and Sprecher, 1986; Cacioppo et al., 2012). We used the Brazilian version short version of JLS, containing 15 items, which was previously validated to our culture (Soares et al., 2017). In this sample, JLS internal consistency was higher than 0.90 suggesting good reliability.

Juvenile Love Scale scores, obtained by summing all items, provides a global measure on how much infatuated the respondent is. In the original study, Hatfield and Young (1998) described five different categories based on percentile data,

ranging from "the thrill is gone," the lower level of passionate love, and "wildly, even recklessly, in love," the higher one. However, since our sample is relatively small, we divided our participants in only two groups (more infatuated and less infatuated groups), based on the sample median JLS score.

UPPS Impulsive Behavior Scale (UPPS)

We administered the UPPS Impulsive Behavior Scale as a behavioral measure of impulsivity (Whiteside and Lynam, 2001). The scale was adapted and validated to the Brazilian context (Sediyama et al., 2017). The UPPS is a self-report scale with 45 items addressing four behavioral dimensions of impulsivity: (1) urgency, a tendency to act precipitously under distress or extreme negative emotions; (2) (lack of) premeditation or acting without thinking; (3) (lack of) persistence, related to the ability of remain focused on a task; and (4) sensation seeking, a tendency to engage in novel and exciting experiences (Whiteside and Lynam, 2001; Cyders et al., 2007). The scale is presented in a Likert-type format ranging from 1 to 4: (1) strongly agree, (2) partially agree, (3) partially disagree, and (4) strongly disagree. Higher scores are suggestive of higher impulsivity. UPPS internal consistency in this sample was higher than 0.90.

TABLE 1 | Participants' characteristics and group comparisons.

	Control		ADHD		Comparison		
	M	*SD*	*M*	*SD*	*U*	*Z*	Parameter
Age	16.0	1.3	15.3	1.4	546.5*	−2.2	–
Socioeconomic status	1	15.4	39.6	19.3	603.5	−1.4	–
Education	10.0	1.0	10.0	1.2	405.5**	−3.3	–
Inattention (SNAP-IV)	10.5	10.4	26.0	9.8	1430.0**	−4.9	>15[1]
Hyperactivity/impulsivity (SNAP-IV)	4.1	3.7	12.2	6.1	1513.0**	−3.7	>10[1]
ODD symptoms (SNAP-IV)	4.0	4.4	10.1	6.1	1609.0**	−2.4	>7[1]
Urgency (UPPS)	28.6	6.8	30.9	8.0	654.5	−1.1	29.0 (6.0)[2]
Premeditation (UPPS)	24.5	8.0	25.5	7.2	688.0	−0.7	24.0 (5.0)[2]
Perseverance score (UPPS)	23.1	6.3	24.5	6.8	688.0	−0.7	21.0 (5.0)[2]
Sensation seeking (UPPS)	31.7	7.2	34.9	6.8	583.0	−1.8	34.0 (7.0)[2]
Juvenile Love Scale (15 items)	76.3	31.6	77.7	35.2	738.5	−0.3	.

*SE, standard error; U, Mann–Whitney U-test; Z, difference between standard scores. *p = 0.05; **p < 0.01. [1]Cutoff for ADHD according to Costa et al. (2018), [2]We found no Brazilian normative values for adolescents in UPPS, so we added parameters – Mean (SD) based in d'Acremont and Van der Linden (2005) community study. This second parameter should be interpreted carefully, since the referred study was conducted with French Adolescents, a different culture from our sample.*

TABLE 2 | Participants' correlations among Passionate Love and Impulsivity measures on adolescence.

Variable(s)	1	2	3	4	5	6	7	8
(1) Passionate Love (JLS)	–							
(2) Age (years)	−0.03	–						
(3) Education	−0.21	0.71**	–					
(4) Socioeconomic level (CCEB)	0.02	0.03	0.06	–				
(5) Urgency (UPPS)	0.28*	0.13	−0.10	−0.20	–			
(6) Premeditation (lack of) (UPPS)	−0.01	−0.01	−0.02	−0.07	0.37**	–		
(7) Perseverance (lack of) (UPPS)	−0.17	0.08	−0.01	−0.10	0.37**	0.60**	–	
(8) Sensation seeking (UPPS)	0.26*	−0.17	−0.19	−0.08	0.29**	0.09	−0.11	–

*JLS, Juvenile Love Scale; CCEB, Brazilian Economic Classification Criteria (higher scores suggest higher socioeconomic situation); UPPS, UPPS Impulsive Behavior Scale. *p < 0.05; **p < 0.01.*

TABLE 3 | Effect of Passionate Love on adolescents' Impulsivity measures depending on ADHD status.

Outcome	Predictor	*F*	*df*	*p*-value	β_p^2
Urgency (UPPS)	Passionate Love (JLS)	4.14*	1	0.045	0.051
	Group	0.34	1	0.561	0.004
	Group*Passionate Love (JLS)	1.63	1	0.130	0.021
Sensation seeking (UPPS)	Passionate Love (JLS)	4.41*	1	0.039	0.054
	Group	0.76	1	0.386	0.010
	Group*Passionate Love (JLS)	0.16	1	0.205	0.021

*JLS, Juvenile Love; UPPS, UPPS Impulsive Behavior Scale; df, degrees of freedom. *p < 0.05.*

TABLE 4 | Description of participants' scores on the UPPS scale stratified by JLS scores.

	Less infatuated group		More infatuated group	
Variable(s)	Control Mean (SD)	ADHD Mean (SD)	Control Mean (SD)	ADHD Mean (SD)
Urgency	30,0 (8,7)	33,1 (± 7,3)	28,7 (± 6,1)	27,5 (± 6,8)
Premeditation	24,8 (± 9,2)	25,1 (± 8,3)	24,4 (± 7,1)	25,4 (± 6,5)
Perseverance	20,5 (± 6,1)	24,4 (± 7,2)	25,0 (± 6,1)	24,2 (± 6,2)
Sensation seeking	35,4 (± 6,4)	34,9 (± 6,1)	30,0 (± 7,3)	33,4 (± 7,3)

SD, standard deviation.

Sociodemographic Characteristics

Information about adolescents' age, sex, and education was given by parents. Participants' socioeconomic status (SES) was characterized by the *Brazilian Economic Classification Criteria (CCEB)* which provides evidence about purchasing power and general situation of the households through questions about possession of durable goods and educational level of the head of the household. Scores can vary from 0 to 100 and fall in one of six socioeconomic strata: A (monthly household income estimation of U$ 6464.42), B1 (monthly household income estimation of U$ 2863.93), B2 (monthly household income estimation of U$ 1501.60), C1 (monthly household income estimation of U$ 837.14), C2 (monthly household income estimation of U$ 502.90), and DE (monthly household income estimation of U$ 237.68) (ABEP, 2008). Higher scores suggest better SES. In this sample, participants had the following economic classifications: 4 (9%) A, 7 (16%) B1, 15 (35%) B2, 9 (21%) C1, 7 (16%) C2, 1 (2%) DE (ABEP, 2008).

Statistical Procedures

All analyses were performed with SPSS 22.0. We conducted descriptive statistics and correlational analysis to investigate variables distribution and their associations. Then, we tested whether significant associations between passionate love and impulsivity measures would change depending on group diagnosis. General Linear Models were built independently for each significant impulsivity measure associated with passionate love (dependent variables). Main effects and group-by-passionate love interaction were computed controlling for significant demographic differences between typically developing and ADHD adolescents.

RESULTS

Table 1 shows descriptive information regarding adolescents' age, education, and socioeconomic situation. Additionally, descriptive information was given for passionate love intensity through the JLS and UPPS factors (i.e., urgency, lack of premeditation, lack of perseverance, and sensation seeking). Typically developing adolescents were mostly girls (n = 43) and ADHD participants mostly boys (n = 17). We found no group differences regarding sex in any measure of ADHD, impulsivity or in passionate love (all $p > 0.05$).

We found an association between higher scores on the JLS, heightened urgency and sensation seeking (**Table 2**). We didn't find any association between participants' sex, education, and socioeconomic level and love intensity. The general linear model (see **Table 3**) showed that the association of passionate love with behavioral urgency and sensation seeking did not change depending on ADHD diagnosis. Additionally, we tested if any of these associations were moderated by the age of the participant, stratified by the samples median (16 years). We found no interaction between age, diagnosis, passionate love on any impulsivity measure.

Table 4 shows the participants' scores on all four impulsivity domains, divided into infatuated (above average and below average on JLS scores), and then categorized in ADHD group (6 or more hyperactivity and/or inattentive symptoms on SNAP-IV) and control group (5 or less hyperactivity and/or inattentive symptoms on SNAP-IV).

DISCUSSION

To the best of our knowledge, this is the first study investigating the possible effects of romantic love on impulsivity in adolescents and its relationship with ADHD symptoms. Our results showed that love affects sensation seeking and urgency, suggesting that passionate love intensity may exert some influence on these aspects of impulsivity. There is also a correlation between love and urgency, as well as there is between love and ADHD symptoms. These results point toward a positive direction relationship, which means that the more passionate, more sensation seeking behaviors are exhibited, and more reckless they tend to act when under negative emotions.

Self-control appears to be an important factor for maintaining a long-term relationship. According to early studies, cognitive control would predict behaviors which could contribute to a lasting relationship, including staying faithful, resist flirting and being forgiving (Finkel and Campbell, 2001; Ritter et al., 2010). In the early stages of love, however, the impulsivity related to sensation-seeking could be beneficial (Van Steenbergen et al., 2014). Sensation seeking refers to individual high motivation for novelty and intense and unusual sensory experiences (Norbury and Husain, 2015). Our results of higher rates of sensation seeking behaviors among the more infatuated subjects could indicate an openness for new habits and experiences,

which would allow incorporating the other in one's life (Aron and Aron, 1986). Unbalanced top-down cognitive control might enhance impulsive activity (Hofmann et al., 2009), just as observed in addiction (Dalley et al., 2011). Some authors have suggested that passionate love is a "natural addiction" since they share these brain processes drive, such as impulsivity (Fisher et al., 2016). Indeed, the striatal dopamine release on the in-love brain is similar to the process involved in addiction, with an enhancement of reward regulation networks, and is also related to sensation seeking (Fisher et al., 2005; Frascella et al., 2010; Xu et al., 2011). This dopaminergic modulation could change the balance between striatal and prefrontal connections, favoring an increase of impulsivity in the early stages of passionate love (Cools, 2008; Van Steenbergen et al., 2014). Additionally, the hypothalamic pituitary adrenal (HPA) axis increases its activity during the early stages of passionate love, indicating a high-stress level (Marazziti and Canale, 2004). Stress can be seen as an altered mood state and insecurity (Stárka, 2007; Berscheid, 2010), too typical of the early stages of passionate love, contributing to non-rational behavior in a love situation. According to our results, more infatuated individuals may tend to act rashly (urgency), especially in contexts associated with the loved one.

We found no moderating effect of ADHD diagnosis over love and impulsivity interaction, indicating that passionate love intensity seems to affect ADHD and typically-developing adolescents in the same way. Despite the changes in cortico-striatal pathways associated with diverse forms of impulsivity in ADHD, there was no additive role of the disorder in adolescents' passionate love influence on urgency or sensation seeking tendencies.

Our study has limitations that must be addressed. One of the study's limitations was the sample size, which was insufficient to detect small effect sizes. In this sense, more discrete associations may not be perceived in our data. The control group was not interviewed by a child psychiatrist due to the context of their data collection (schools). To minimize this bias, we used the CBCL scale as a screening tool, an instrument that shows high concordance with a clinical interview when responded by the participants' parents (Brasil and Bordin, 2010). We also did not have detailed data on participants' relationship status, even though they could just think about someone when informing scores in the JLS. We had no information about how long possible couples were together, partners' age and SES, neither on participants' pubertal development. Of the ADHD sample, 60% were only inattentive, 16.7% were only hyperactive/impulsive, and 23.3% had a combined presentation profile. The inattentive predominance might partially explain why there was no difference in UPPS impulsive scores between adolescents with and without ADHD in our sample. As it is inherent to any correlation approach, we cannot determine whether passionate love increases urgency and sensation seeking behaviors when one is in love, or if adolescents for whom extreme emotions act like a boost to impulsiveness, or that are inclined to live exciting new experiences, get to experience more intense infatuation.

Intense passionate/romantic love is a near-universal human phenomenon. The turmoil that accompanies adolescence may serve as a motivation enhancer to courtship attraction and the pursuing of a mating partner, which can lead to a love that is returned or rejected. Anyhow, passionate love likely increases activity related to impulsivity for better or for worse. The understanding of the relationship of impulsivity with infatuation might help to clarify why some population groups show an increased risk for many negative social outcomes.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the local ethical board (UFMG/Plataforma Brasil) with written informed consent from all subjects. All subjects and primary caregivers gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the UFMG Ethics committee.

AUTHOR CONTRIBUTIONS

LS conceptualized and designed the study, contributed to data collection, conducted the initial analyses, drafted the initial manuscript, and reviewed and revised the manuscript. DC and JdP conceptualized and designed the study, conducted additional analyses, contributed to interpretation of data analysis, drafted the initial manuscript, and reviewed and revised the manuscript. LM-D, MR-S, and DdM obtained funding, conceptualized and designed the study, contributed to interpretation of data analysis, and reviewed and revised the manuscript. DdM also drafted the manuscript. All authors approved the final manuscript as submitted. All authors agreed to be accountable for all aspects of the work, including the accuracy and integrity of this study.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Endocannabinoids Interact With the Dopaminergic System to Increase Sexual Motivation: Lessons From the Sexual Satiety Phenomenon

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In male rats, copulation to satiety induces a long-lasting sexual inhibitory state, considered to rely on a decreased sexual motivation. Dopaminergic transmission at the mesolimbic system plays a central role in the regulation of male sexual motivation. Endocannabinoids (eCBs) modulate the activity of the mesolimbic system and both dopamine (DA) and cannabinoid receptor activation reverses the sexual inhibition that characterizes sexually satiated rats. The eCB anandamide reverses sexual satiety when systemically administered or infused into the ventral tegmental area (VTA), the region where the activity of mesolimbic dopaminergic neurons is regulated. Thus, it could be thought that sexual motivation is diminished during the long-lasting sexual inhibition of sexually satiated rats and that eCBs reverse that inhibition through the modulation of the dopaminergic system. To test this hypothesis, we assessed the motivational state of sexually satiated male rats and determined if 2-arachidonoylglycerol (2-AG), the most abundant eCB and a full cannabinoid receptor agonist, also reversed the sexual inhibitory state. To establish the possible interaction between 2-AG and anandamide with the dopaminergic system for the reversal of sexual satiety, we analyzed the effects of the co-administration of each eCB and DA receptor agonists or antagonists. Results showed that 24-h after copulation to satiety, when the sexual inhibition is well established, the males' sexual motivation is diminished as measured in the sexual incentive motivation test. 2-AG, similarly to anandamide, reverses sexual satiety through the activation of CB1 receptors and both eCBs interact with the dopaminergic system to reverse the sexual inhibitory state. 2-AG effects are mediated by the modulation of the D2-like DA receptor family, whereas anandamide's effects are clearly mediated by the modulation of the D1-like DA receptor family and the activation of D2-like DA receptors. Present results evidence that a reduced sexual motivation underlies the sexual inhibitory state of sexually satiated rats and support the notion that eCBs reverse sexual satiety by modulating dopaminergic transmission, presumably at the mesolimbic system. Anandamide and 2-AG have a different interaction with D1-like and D2-like DA receptor families. Altogether present data endorse the association of the eCB system with the regulation of the motivational tone at the mesolimbic system.

Keywords: endocannabinoids (eCBs), CB1 receptors, D1-like/D2-like DA receptors, sexual motivation, sexual satiety, natural reward, mesolimbic circuit

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INTRODUCTION

Sexually experienced male rats allowed to copulate without restriction with a single female will ejaculate repeatedly until becoming sexually exhausted (Beach and Jordan, 1956; Rodríguez-Manzo and Fernández-Guasti, 1994). Copulation to satiety has as its main outcome the installation of a long- lasting sexual behavior inhibition (up to 72 h) that gradually fades away, requiring a 15-day period of sexual rest for exhausted males to completely recover their initial ejaculatory capacity (Rodríguez-Manzo et al., 2011). Twenty-four hours after copulation to satiety, when exposed to a new sexually receptive female, the majority of these animals (two-thirds of the population) does not show any sexual activity and the remaining third displays a single ejaculatory series after which males will not resume copulation (Rodríguez-Manzo and Fernández-Guasti, 1994).

Copulation is a highly rewarding behavior and the mesolimbic dopaminergic (MSL) system is involved in the control of its motivational component and reinforcing properties (Kelley and Berridge, 2002). The dopamine (DA) neurons of the MSL system, originating in the ventral tegmental area (VTA) of the midbrain, project to the nucleus accumbens (NAcc; Swanson, 1982; Ikemoto and Panksepp, 1999). DA has been suggested to be important for the assignment of the motivational value to rewarding behaviors (Berridge and Kringelbach, 2011) and motivation plays a central role in the maintenance of rewarding behaviors that are triggered by salient environmental stimuli, such as sexual behavior (Everitt, 1990). Copulation activates the MSL system increasing DA release at the NAcc (Mas et al., 1990; Pfaus et al., 1990; Wenkstern et al., 1993) and augmenting c-Fos protein expression in the DA neurons of the VTA (Balfour et al., 2004). During repeated copulation, DA levels at the NAcc remain elevated, indicating a continued activation of the MSL system (Fiorino et al., 1997).

The long-lasting sexual behavior inhibition that characterizes sexually exhausted male rats is considered to rely on a decreased sexual motivation (Guadarrama-Bazante and Rodríguez-Manzo, 2019), as their performance in a sexual motivation paradigm, immediately after reaching sexual satiety, is diminished (Ågmo et al., 2004). Besides, it has also been shown that interfering with the sexual motivation decline that follows copulation to exhaustion, by means of the Coolidge effect (renewal of sexual activity in satiated rats induced by changing the female partner), hinder the establishment of the long-lasting sexual behavior inhibition 24 h after copulation to satiety (Rodríguez-Manzo, 1999a). These data suggest that changes in the motivational component of copulatory behavior might play an important role in the sexual satiety phenomenon.

Interestingly, 24 h after copulation to exhaustion, once the sexual inhibitory state is established, changing the female partner has no effect on the sexual responsiveness of the satiated rats (Rodríguez-Manzo, 1999a). Though, the established sexual inhibition can be reversed by a number of pharmacological agents (a 5-HT1A receptor agonist, an α2-adrenoceptor antagonist, μ and δ opioid antagonists, among others), acting at different neurotransmitter systems, which seem to directly or indirectly interact with the dopaminergic system (Rodríguez-Manzo and Fernández-Guasti, 1995; Rodríguez-Manzo, 1999b). In addition, DA receptor agonists, systemically administered or infused into the NAcc, also reverse the sexual inhibition of satiated rats (Guadarrama-Bazante et al., 2014; Guadarrama-Bazante and Rodríguez-Manzo, 2019). Together, these data suggest that DA transmission plays a central role in the reversal of sexual satiety.

Endocannabinoids (eCBs) are retrograde transmitters, of which anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized (Di Marzo et al., 1998). Unlike classical neurotransmitters, eCBs are synthesized and released on demand, during periods of high neural activity (Freund et al., 2003). At the MSL system, eCBs are released from the DA cell bodies in the VTA and from the medium spiny neurons in the NAcc (Lupica and Riegel, 2005). Once in the synaptic cleft, they retrogradely activate CB1 cannabinoid receptors, located on GABAergic and glutamatergic axon terminals in each of these brain regions, thereby inhibiting neurotransmitter release (Alger, 2002; Wilson and Nicoll, 2002). Through the modulation of MSL system's activity, eCBs regulate rewarding behaviors (Lupica et al., 2004; Gardner, 2005). Sexual behavior is rewarding and eCBs are involved in its control (Gorzalka et al., 2008), playing a complex role in its expression (for review, see Rodríguez-Manzo and Canseco-Alba, 2015).

In sexually satiated male rats, low doses of AEA reverse the sexual inhibition that characterizes sexual satiety (Canseco-Alba and Rodríguez-Manzo, 2014), an effect mimicked by its direct infusion into the VTA (Canseco-Alba and Rodríguez-Manzo, 2016).

Based on these data, it could be thought that sexual motivation is diminished during the long-lasting sexual inhibitory period that characterizes sexually exhausted rats and that eCBs reverse that inhibition through the modulation of the dopaminergic system. To test this hypothesis, in this work we first assessed the motivational state of sexually exhausted male rats 24 h after copulation to satiety, by means of a sexual incentive motivation test. We then determined if 2-AG, the most abundant eCB in the brain, also reversed sexual satiety through the activation of CB1 receptors. Finally, we analyzed the possibility of an interaction between AEA or 2-AG and the dopaminergic system for the reversal of sexual satiety, determining the possible participation of each of the two DA receptor families in this effect.

MATERIALS AND METHODS

Animals

Sexually experienced adult male Wistar rats (250–300 g b. wt.) were used in this study. Animals were housed, eight per cage, under inverted light/dark cycle conditions (12 h light: 12 h dark, lights on at 22:00 h), at 22°C, and with free access to food and water. For the selection of sexually experienced males, rats were subjected to five independent sexual behavior tests, and those males showing ejaculation latencies (EL) shorter than 15 min, in at least three of these tests, were considered

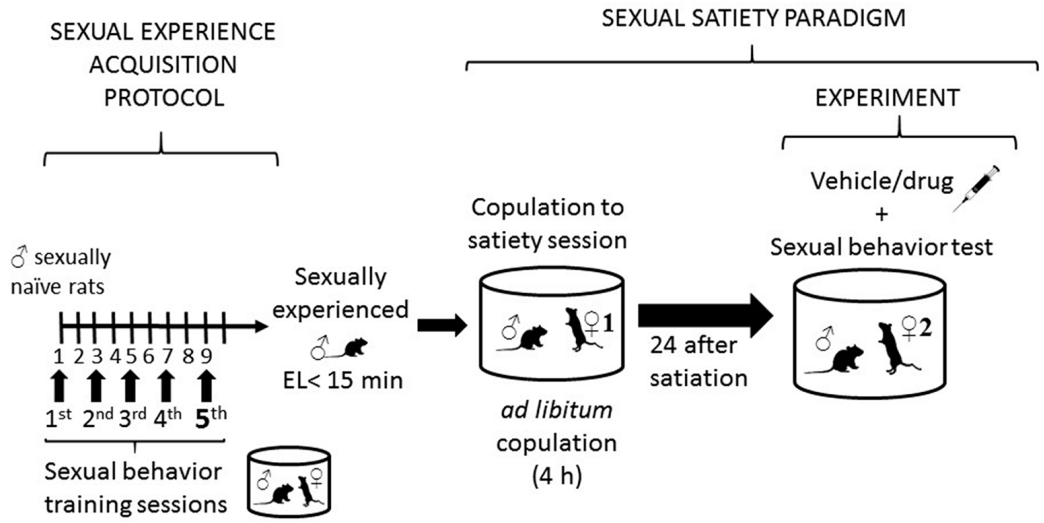

FIGURE 1 | Description of sexual behavior protocols. The left side of the figure describes the protocol followed to render sexually experienced male rats, consisting in subjecting sexually naïve rats to five independent sexual behavior sessions, run every other day. At the end of this process males showing ejaculation latencies (EL) shorter than 15 min are considered sexually experienced and selected for the study. The right side of the figure describes the sexual satiety paradigm, involving sexually experienced male rats that copulate *ad libitum* with a single sexually receptive female (♀1) until reaching the sexual satiety criterion (around 4 h). Twenty-four hours later these males receive the pharmacological treatment or its vehicle and are subjected to a sexual behavior test with a new sexually receptive female (♀2).

sexually experienced (see **Figure 1**). Receptive female Wistar rats served as sexual stimuli. Sexual receptivity was induced in intact females by the sequential s.c. injection of estradiol benzoate (12 μg/rat) followed 24 h later by progesterone (6.0 mg/rat). Our institutional Internal Committee for the Care and Use of Laboratory Animals (Comité Institucional para el Cuidado y Uso de Animales de Laboratorio, CICUAL) approved all experimental procedures (Protocol 0230-16), which followed the regulations established in the Mexican Official Norm for the use and care of laboratory animals NOM-062-ZOO-1999.

Sexual Exhaustion Paradigm

Sexual behavior observations were conducted in a room under dim red light, during the dark phase of the cycle. Male rats were introduced into polycarbonate cylindrical arenas (62 cm diameter, 52 cm height), with the floor covered with fine sawdust, and a 5-min adaptation period was allowed to the males before introducing a receptive female. The males copulated with a single receptive female during 4 h, without restriction. Previous data from our laboratory have shown that this period is sufficient for all animals to reach the sexual exhaustion criterion, i.e., 90 min from the last ejaculation without attaining another ejaculation. At the end of the sexual exhaustion session, the animals were returned to their home cages. Twenty-four hours later, the same animals were subjected to a sexual behavior test with a new sexually receptive female, after receiving the pharmacological treatments or the vehicle (see **Figure 1**).

In this last test, we recorded the percentage of males displaying sexual behavior, i.e., mount, intromission, ejaculation and copulation resumption after ejaculation. Since these animals are sexually inhibited, the display of each of these sexual responses indicates a facilitation of sexual behavior expression. When the proportion of satiated animals capable of resuming copulation after a first ejaculation, during the 24 h test, is significantly increased in response to a pharmacological treatment, it is considered that sexual satiety was reversed. In those animals ejaculating, we recorded the following specific sexual parameters: intromission latency (IL, time from the introduction of the female to the appearance of the first intromission); mount and intromission number displayed prior to ejaculation (M and I); EL (time from the first intromission until ejaculation) and post-ejaculatory interval (PEI, time from ejaculation to the first intromission of the next copulatory series). These specific parameters are regularly used to evaluate the sexual performance of sexually experienced male rats.

Locomotor Activity

In order to discard non-specific effects of the drug treatments that could have interfered with sexual behavior execution, the animals' spontaneous locomotor activity was recorded immediately after the sexual behavior tests that followed drug treatments. To this purpose, male rats were placed into an acrylic box (33 × 44 × 20 cm), with the floor divided into 12 squares (11 × 11 cm for each quadrant), and the number of crossings from one quadrant to another during a 5-min period was recorded. The cage was carefully cleaned between tests.

Sexual Incentive Motivation Test

The sexual incentive motivation test was conducted in a room under dim red light following the method described by Ågmo

(2003). This is a non-conditioned test measuring the sexual incentive motivation induced in male rats by a sexually receptive female as opposed to the social incentive motivation induced by another male rat. The apparatus consists of a solid plastic elliptic open field arena (85 × 50 × 40 cm) that has two diagonally opposed windows (one in each long wall extreme), separated from the central arena by wire mesh. Each of these windows communicates with a removable incentive animal cage (20 × 10 × 15 cm), separated from the arena by the wire mesh in which the incentive animals, i.e., a sexually receptive female or a sexually experienced male are placed. In front of each window, a rectangular zone in front of each incentive animal cage (measuring 30 × 20 cm) is designated as the incentive zone. Between tests, the female and male cages are semi-randomly changed from one position to another and the apparatus cleaned to eliminate odor traces from other animals.

Prior to the experimental session, the male subjects are habituated to the arena for three consecutive days in the absence of incentive animals and allowed to freely explore it for 10 min. On the test day, the incentive animals are introduced into their cages, the experimental male is then placed into the center of the arena where it can hear, see and smell the inaccessible incentive animals and its behavior is videotaped during 10 min, in the absence of the experimenter. An observer, blind to the experimental groups, analyzed video recordings. The cumulative time spent by the experimental subjects in the respective incentive zones is considered as indicative of the incentive motivation generated by each animal (male or female).

Drugs

All drugs were purchased from Sigma-Aldrich Chem. Company (St. Louis, MO, USA). Arachidonoylethanolamide (anandamide, AEA) and 2-AG were dissolved in a vehicle composed by a mixture of ethanol (2%), Tween80 (2%) and saline solution (96%). AM251 was dissolved in a vehicle composed by a mixture of DMSO (1 drop), Tween (2%) and saline solution (98%). Haloperidol was dissolved in distilled water adding three drops of ascorbic acid (0.01%). Apomorphine, quinpirole, SKF38399, SCH23390 and raclopride were dissolved in saline solution. All drugs were i.p. injected in a volume of 1 ml/kg. All the CB1 ligands (AEA, 2-AG and AM251) were administered 5 min before subjecting the animals to the sexual behavior tests. The DA receptor ligands had different latencies, which are specified for each drug in the experimental design. Estradiol benzoate and progesterone were dissolved in sesame oil and s.c. injected to the females as described above, under the animals' heading.

Statistical Analyses

Comparison of the proportions of sexually exhausted rats exhibiting the different sexual behavior responses, i.e., mount, intromission, ejaculation and copulation resumption after ejaculation, was conducted by means of the Fisher *F*-test. The distinct sexual behavior parameters of sexually experienced males in the dose-response curves, as well as the locomotor activity data were compared by means of the Kruskal–Wallis ANOVA followed by Dunn's test when pertinent. The differences in the time spent by male rats in the different incentive zones were established by means of the Mann-Whitney *U* test. All statistical analyses were performed with the Sigma Plot program (version 12.0).

Experimental Design

Experiment 1: Incentive Sexual Motivation of Sexually Exhausted Male Rats

Two independent groups of sexually experienced male rats (n = 12 each) were used. One group was directly tested for incentive motivation and served as the control group. The experimental group was first subjected to the sexual exhaustion paradigm and 24 h after copulation to satiety, tested for incentive motivation.

Experiment 2: Effects of 2-AG on Sexual Behavior Expression of Sexually Experienced and Sexually Exhausted Male Rats

A dose-response (D-R) curve of the effects of 2-AG (0.03–3.0 mg/kg) in sexually experienced rats was run to establish the effects of this eCB on copulation of sexually active animals. To establish the effects of 2-AG in sexually satiated rats, six independent groups of sexually experienced males (n = 8 each) were subjected to the sexual exhaustion paradigm and 24 h later, injected with different doses of 2-AG (0.03–3.0 mg/kg) or its vehicle and their sexual activity recorded. An additional group of sexually exhausted rats was employed to establish if 2-AG effects were mediated by CB1 receptors. In this case, the CB1 receptor antagonist AM251 (0.1 mg/kg) was injected to the satiated male rats immediately before the administration of an effective 2-AG dose (0.3 mg/kg) and after 5 min the sexual behavior test was run. The AM251 dose was chosen from a previously reported D-R curve (Canseco-Alba and Rodríguez-Manzo, 2014).

Experiment 3: Interaction of the eCBs AEA and 2-AG With the Dopaminergic System in Sexually Exhausted Male Rats

Four independent groups of sexually exhausted males (n = 8 each) were used to establish the effects of the unspecific DA receptor antagonist haloperidol (125 μg/kg, −30 min) on the reversal of sexual exhaustion induced by an effective dose of AEA (0.3 mg/kg) or 2-AG (0.3 mg/kg). The AEA dose was chosen from the D-R curve of AEA effects on sexually satiated males previously reported (Canseco-Alba and Rodríguez-Manzo, 2014). The haloperidol dose was chosen from a published D-R curve run in sexually satiated animals (Rodríguez-Manzo, 1999b) and was injected 30 min prior to either eCB; the control group received the combination of vehicles.

The possible interaction of the unspecific DA receptor agonist, apomorphine with the eCBs AEA and 2-AG was determined by the co-administration of the DA agonist and each of the eCBs, at doses that were subthreshold for reversing sexual satiety. To this aim, four additional independent groups of satiated males (n = 8 each) were used; one receiving the combination of vehicles, another receiving the

TABLE 1 | Specific sexual behavior parameters of the first copulatory series of sexually experienced male rats treated with specific doses of dopamine (DA) receptor agonists.

Treatment	*n*	IL	M	I	EL	PEI
Vehicle (saline)	8	0.91 ± 0.17	3	8	8.07 ± 0.76	5.68 ± 0.34
Apomorphine 10 μg/kg	8	0.68 ± 0.14	2	6.5	6.04 ± 0.79	5.78 ± 0.54
SKF-38399 0.1 mg/kg	8	0.80 ± 0.18	5	10	9.77 ± 1.0	6.03 ± 0.47

IL, intromission latency; M, number of mounts; I, number of intromissions; EL, ejaculation latency; PEI, postejaculatory interval. Temporal measures are expressed in minutes (min) as mean ± SEM and M&I as median number.

sub-effective dose of apomorphine (10 μg/kg, −15 min) and two for the combinations of apomorphine with either AEA or 2-AG, at sub-effective doses (0.03 mg/kg each). Apomorphine's ineffective dose was chosen from a pilot study with sexually experienced male rats. The data of this pilot study are shown in **Table 1**.

Experiment 4: Participation of D1-Like DA Receptors in the eCB-Induced Reversal of Sexual Satiety

Two independent groups of sexually satiated rats (n = 8 each) were used to establish the effects of the combined treatment of an effective dose of AEA or 2-AG (0.3 mg/kg each) with a dose of the D1-like receptor antagonist SCH23390 that lacked effects *per se* (0.1 mg/kg, −30 min). A dose-response curve of SCH23390 was run in sexually experienced male rats to identify the dose not modifying sexual behavior *per se* (**Figure 6**).

Two additional groups of sexually exhausted males (n = 8 each) were employed to establish the effects of the combination of previously determined suboptimal doses of AEA or 2-AG (0.03 mg/kg each) with a sub-effective dose of the D1-like receptor agonist SKF38399 (0.1 mg/kg, −30 min), which was determined in a pilot study with sexually experienced male rats. The data of this pilot study are included in **Table 1**.

Experiment 5: Participation of D2-Like DA Receptors in the eCB-Induced Reversal of Sexual Satiety

Two independent groups of sexually satiated rats (n = 8 each) were used to establish the effects of the combined treatment of an effective dose of AEA or 2-AG (0.3 mg/kg each) with a dose of the D2-like receptor antagonist raclopride that lacked effects *per se* (0.03 mg/kg, −20 min). A dose–response curve of raclopride was run in sexually experienced male rats to identify the dose not modifying sexual behavior (**Figure 8**).

Finally, another two groups of sexually exhausted males (n = 8 each) were employed to establish the effects of the combination of suboptimal doses of AEA or 2-AG (0.03 mg/kg each) with a suboptimal dose of the D2-like receptor agonist quinpirole (0.03 mg/kg, −15 min) chosen from a previously reported D-R curve in sexually satiated rats (Guadarrama-Bazante et al., 2014).

RESULTS

Sexual Incentive Motivation of Sexually Exhausted Male Rats

In the sexual incentive motivation test it was found that sexually experienced male rats spent significantly more time in the incentive zone of the sexually receptive female as compared to the time spent in the male's incentive zone (Mann-Whitney U test, $U = 2$, $P < 0.001$). In contrast, in the sexually exhausted males, tested 24 h after copulation to satiety, there was no difference between the time spent by the males in the incentive zones of the sexually receptive female and the sexually active male (Mann-Whitney U test, $U = 82.5$, $P = 0.93$; **Figure 2**).

Effects of 2-AG on Sexual Behavior of Sexually Experienced and Sexually Exhausted Male Rats

Figure 3 depicts the dose-response curve of the effects of different doses of 2-AG on the sexual behavior of sexually experienced male rats. It can be observed that none of the sexual parameters were statistically significantly modified by any 2-AG dose. The percentage of sexually exhausted male rats showing mounts and intromissions (M&I), ejaculating (E) and resuming copulation after ejaculation (CR), 24 h after copulation to satiety, in response to different doses of the eCB 2-AG, are shown in **Figure 4**. As it can be seen in panel **A**, the majority of the tested doses (0.1–3.0 mg/kg) significantly increased the proportion of satiated rats attaining ejaculation [seven out of eight (87.5%; Fisher F test, $P = 0.01$) for 0.1 mg/kg; eight out of eight (100%; Fisher F test, $P = 0.001$) for 0.3 mg/kg and six out of eight (75%; Fisher F test, $P < 0.05$) for both the 1.0 and 3.0 mg/kg doses] and resuming copulation thereafter [five out of eight (62.5%; Fisher F test, $P < 0.05$) for 0.1 mg/kg; six out of eight (75%; Fisher F test, $P < 0.01$) for 0.3 mg/kg; and five out of eight (62.5%; Fisher F test, $P < 0.05$) for the 1.0 and 3.0 mg/kg doses], while the lowest dose tested (0.03 mg/kg) failed to increase these proportions. Thus, 2-AG doses between 0.1 and 3.0 mg/kg reversed sexual satiety.

Figure 4B depicts the action of the CB1 receptor antagonist, AM251, at a dose that lacks effects *per se* (0.1 mg/kg), on the increase in the percentages of sexually exhausted rats ejaculating and resuming copulation after ejaculation induced by 0.3 mg/kg 2-AG. It can be observed that the 2-AG-induced reversal of sexual satiety was canceled, indicating that this effect is mediated by CB1 receptors.

Interaction of the eCBs AEA and 2-AG With the Dopaminergic System in Sexually Exhausted Male Rats

Figure 5 shows the effects of the combined injection of the unspecific DA receptor antagonist haloperidol with AEA or 2-AG (panels **A** and **B**, respectively) and those of the

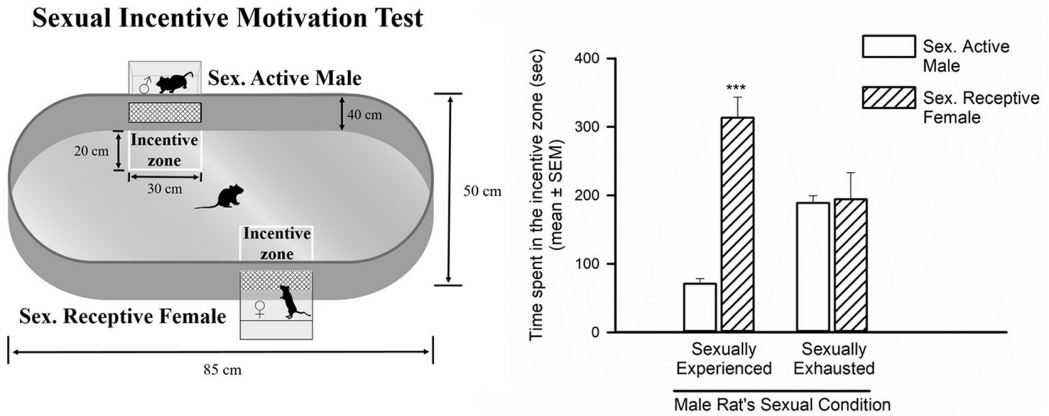

FIGURE 2 | Sexual incentive motivation is measured as the time spent by the male rat in the incentive zones of the sexually receptive female (dashed bars) or the male (empty bars), in control sexually experienced (n = 12) and in sexually satiated male rats (n = 12). Data in the graph are expressed in seconds (s) as mean ± SEM. A statistically significant difference was found in the sexually experienced males in the time spent in each of the incentive zones. Mann-Whitney U test, ***$P < 0.001$. The features of the apparatus used to evaluate sexual incentive motivation are depicted on the left side of the figure.

combined injection of apomorphine, a non-specific DA receptor agonist, with AEA or 2-AG (panels **C** and **D**, respectively) in sexually exhausted male rats. Haloperidol injection (125 μg/kg) *per se* did not induce mating behavior in sexually satiated rats, while a dose of 0.3 mg/kg AEA (panel **A**) or 2-AG (panel **B**), statistically significantly increased the proportions of satiated rats ejaculating (Fisher F test, $P < 0.01$ for AEA; $P < 0.001$ for 2-AG) and resuming copulation after ejaculation (Fisher F test, $P < 0.05$ for both eCBs), as compared to vehicle-treated satiated males. Pre-treatment with haloperidol canceled both, AEA- and 2-AG-induced increases in these percentages.

Apomorphine, at the dose of 10 μg/kg, lacked effects on copulation of satiated rats, as did AEA (panel **C**) and 2-AG (panel **D**) at the dose of 0.03 mg/kg. However, the combined administration of AEA with apomorphine increased the proportion of sexually exhausted males copulating. These increases were statistically significant for M (Fisher F test, $P < 0.05$), for I (Fisher F test, $P < 0.01$) and for CR (Fisher F test, $P < 0.05$). The combined treatment of apomorphine with 2-AG statistically significantly increased the proportion of satiated rats showing I (Fisher F test, $P < 0.05$), E (Fisher F test, $P < 0.05$) and CR (Fisher F test, $P = 0.01$). Thus, sub-effective doses of apomorphine and each of the eCBs synergized to reverse sexual satiety.

Participation of D1-Like DA Receptors in the eCB-Induced Reversal of Sexual Satiety

A dose-response curve of the effects of the D1-like receptor antagonist SCH23390 on the sexual behavior of sexually experienced male rats is shown in **Figure 6**. SCH23390 significantly increased the temporal parameters in these animals at the 0.3 and 1.0 mg/kg doses (Kruskal–Wallis ANOVA $H_{(3)} = 24.66$, $P < 0.001$; Dunn's Test, $P < 0.05$ for IL), (Kruskal–Wallis ANOVA $H_{(3)} = 16.36$, $P < 0.001$; Dunn's Test, $P < 0.05$ for EL; Kruskal–Wallis ANOVA $H_{(3)} = 20.33$, $P < 0.001$; Dunn's Test, $P < 0.05$ for PEI). The lowest SCH23390 dose (0.1 mg/kg) reduced the I number (Kruskal–Wallis ANOVA $H_{(3)} = 8.18$, $P < 0.042$; Dunn's Test, $P < 0.05$), considered a sexual facilitative outcome, and lacked effects on any other parameter; therefore, this dose was selected for the combined treatments.

Figure 7 depicts the effects of the combined injection of the D1-like receptor antagonist SCH23390 with effective doses of AEA (panel **A**) or 2-AG (panel **B**), as well as the effects of the combination of sub-effective doses of the D1-like receptor agonist SKF-38399 with sub-effective doses of AEA (panel **C**) or 2-AG (panel **D**), on sexual behavior expression of sexually satiated rats. It can be seen that the sole administration of 0.1 mg/kg SCH23390 lacked effects in sexually satiated rats, however, it canceled the increase in the percentage of satiated rats showing sexual behavior induced by an effective dose of AEA (panel **A**). By contrast, this same SCH23390 dose did not block the actions of the 2-AG effective dose on copulation of sexually satiated rats (panel **B**). This figure also shows that combination of sub-effective doses of the D1-like receptor agonist with AEA synergized to significantly increase the proportion of satiated rats showing each of the sexual behavior responses (Fisher F test, $P < 0.01$ for M; $P < 0.05$ for I, E and CR; panel **C**), whereas its combination with a sub-effective dose of 2-AG failed to significantly increase these proportions (panel **D**).

Participation of D2-Like DA Receptors in the eCB-Induced Reversal of Sexual Satiety

Figure 8 shows a dose-response curve of the effects of the D2-like receptor antagonist raclopride on the sexual behavior of sexually experienced male rats. Raclopride had sexual effects at doses from 0.1 to 1.0 mg/kg, increasing the EL (Kruskal–Wallis ANOVA,

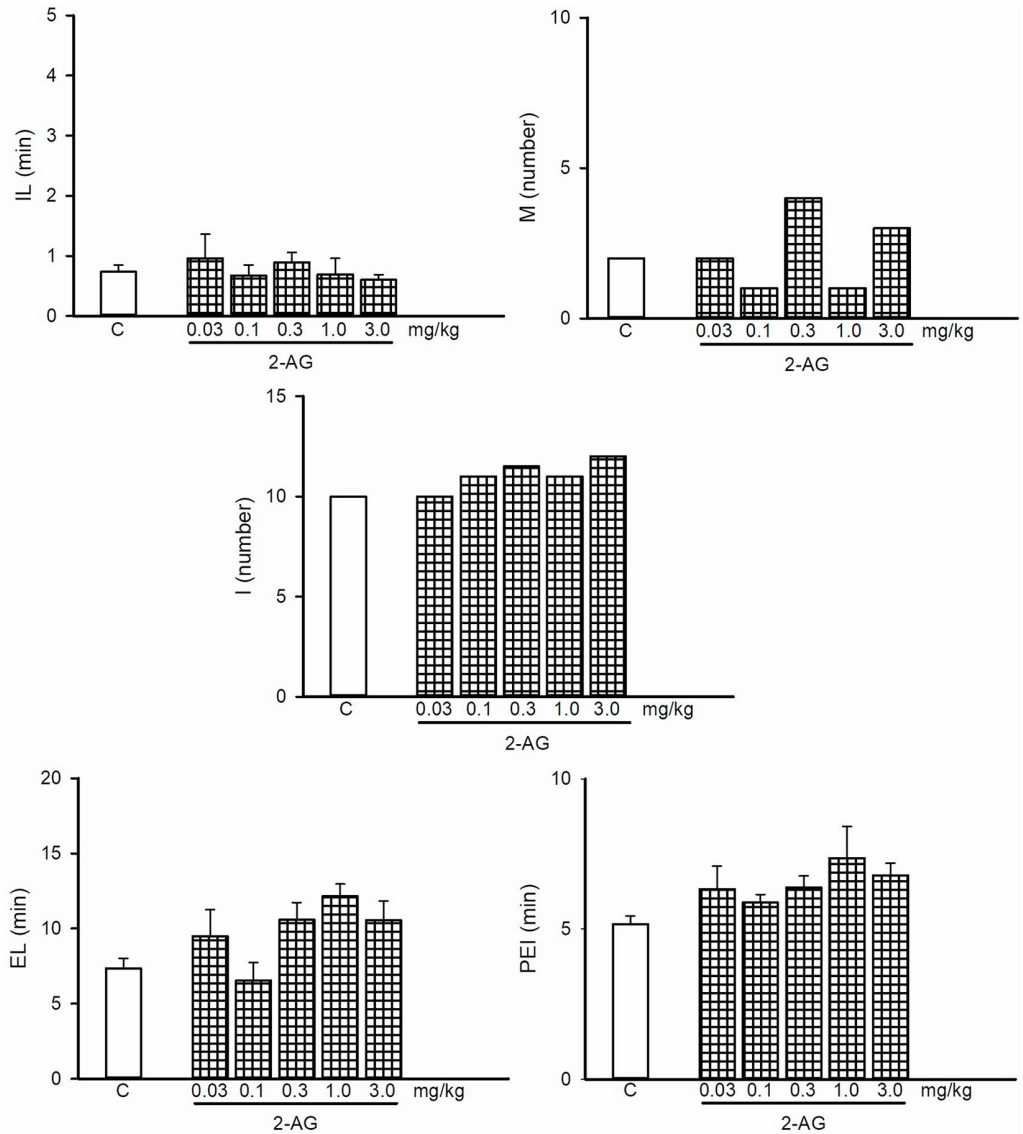

FIGURE 3 | Dose-response curve of the effects of different doses of 2-arachidonoylglycerol (2-AG; 0.03–3.0 mg/kg, $n = 8$ each) or vehicle (C) on the specific sexual behavior parameters of sexually experienced male rats. IL, intromission latency; M, number of mounts; I, number of intromissions; EL, ejaculation latency; PEI, postejaculatory interval. Latencies are expressed in minutes, as mean ± SEM, and numbers as medians.

$H_{(3)} = 26.36$, $P < 0.001$; Dunn's test, $P < 0.05$) at the two higher doses and the PEI at the lower dose (Kruskal–Wallis ANOVA, $H_{(3)} = 20.33$, $P < 0.001$; Dunn's test, $P < 0.05$). We selected the lowest raclopride dose tested (0.03 mg/kg) for the combined treatments, as it lacked sexual effects.

The effects of the combined injection of raclopride with effective doses of AEA or 2-AG on sexual behavior expression of sexually exhausted male rats are shown in **Figure 9** (panels **A** and **B**, respectively), as well as the effects of the combination of sub-effective doses of the D2-like receptor agonist quinpirole with sub-effective doses of AEA (panel **C**) or 2-AG (panel **D**). It can be observed that 0.03 mg/kg raclopride, *per se*, lacked effects on copulation of satiated rats. When combined with an effective dose of AEA, raclopride did not block the increases induced by this eCB (panel **A**), however, it canceled the 2-AG-induced increases in the proportion of satiated animals capable of displaying the different sexual behavior responses (panel **B**). Combination of sub-effective doses of the D2-like receptor agonist quinpirole and the eCBs, AEA or 2-AG, synergized to significantly increase the proportion of satiated rats showing each of the sexual behavior responses. The combination of quinpirole with AEA promoted M, I and E display in all animals (Fisher F test, $P = 0.02$ for M; $P = 0.007$ for I and E) and seven out of eight animals resumed copulation thereafter (Fisher F test, $P = 0.01$). Combined treatment of quinpirole with 2-AG induced M

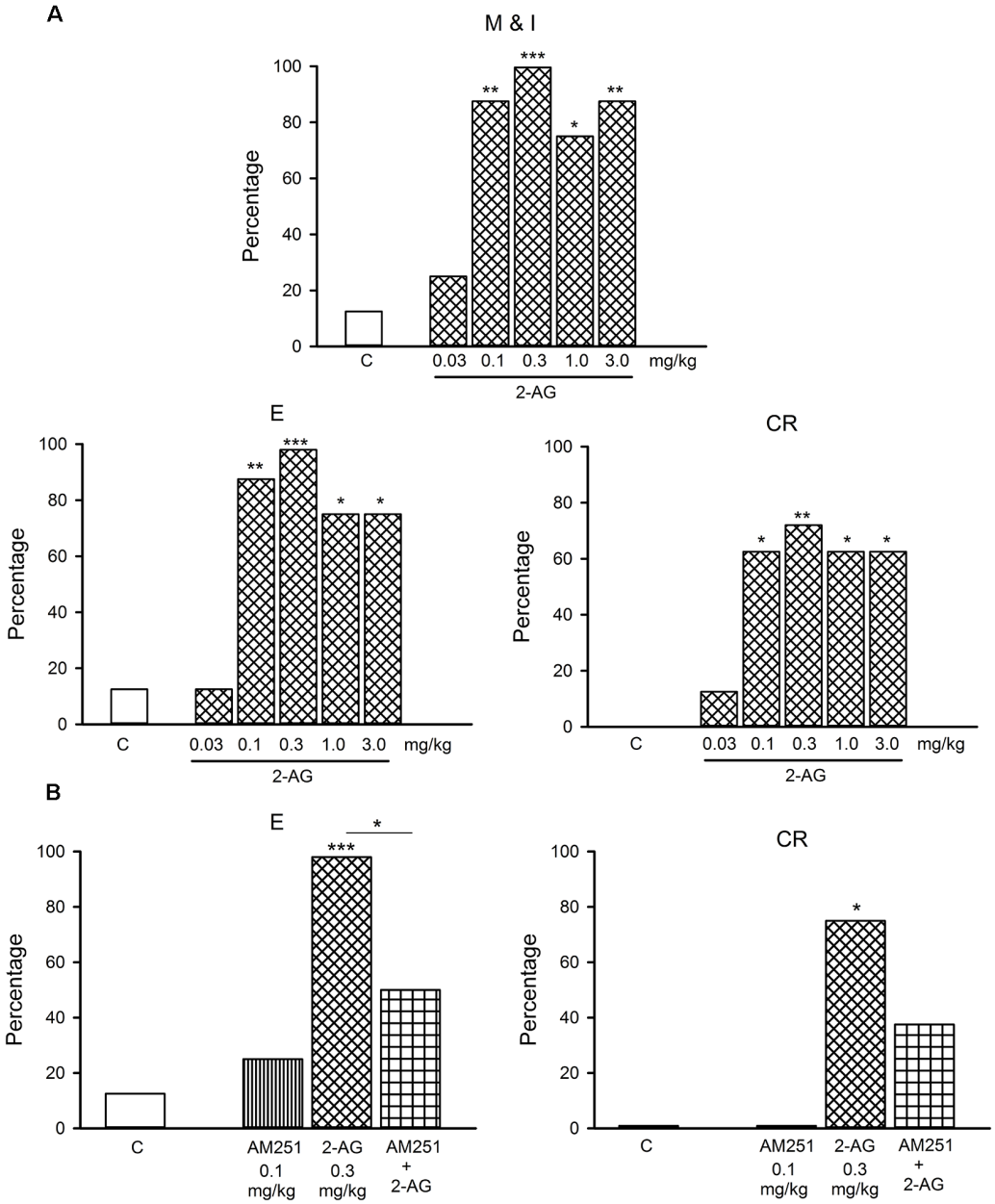

FIGURE 4 | (A) Dose-response curve of the effects of different doses of 2-AG (0.03–3.0 mg/kg) or vehicle (C) on the percentage of sexually satiated rats that exhibited the different sexual behavior responses: mount and intromission (M and I), ejaculation (E) and copulation resumption after ejaculation (CR). Fisher *F* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. C. Panel **(B)** shows that the CB1 receptor antagonist, AM251 (0.1 mg/kg), blocks the reversal of sexual exhaustion induced by 0.3 mg/kg 2-AG, i.e., the increase in the percentage of satiated rats ejaculating (E) and resuming copulation after ejaculation (CR). Fisher *F*-test $^{*}P < 0.05$; $^{***}P < 0.001$; $n = 8$ for each group. Asterisks over bars indicate statistical significance vs. the control group; other comparisons are indicated.

and I behavior in all animals (Fisher *F* test, $P = 0.026$ for M and $P = 0.007$ for I), while seven out of eight animals ejaculated (Fisher *F* test, $P = 0.041$) and resumed copulation after ejaculation (Fisher *F* test, $P = 0.01$; panels **C** and **D**, respectively).

The specific sexual behavior parameters of the satiated animals in which treatments reversed satiety are shown in **Table 2**.

None of the pharmacological treatments significantly affected the spontaneous ambulatory behavior of sexually satiated male rats. These data are presented in **Table 3**. **Table 1** includes the data of the pilot studies showing the doses of apomorphine and SKF-38399 that lacked effects *per se* on the sexual behavior of sexually experienced male rats which were selected for experiments involving combined treatments of sub-effective drug doses.

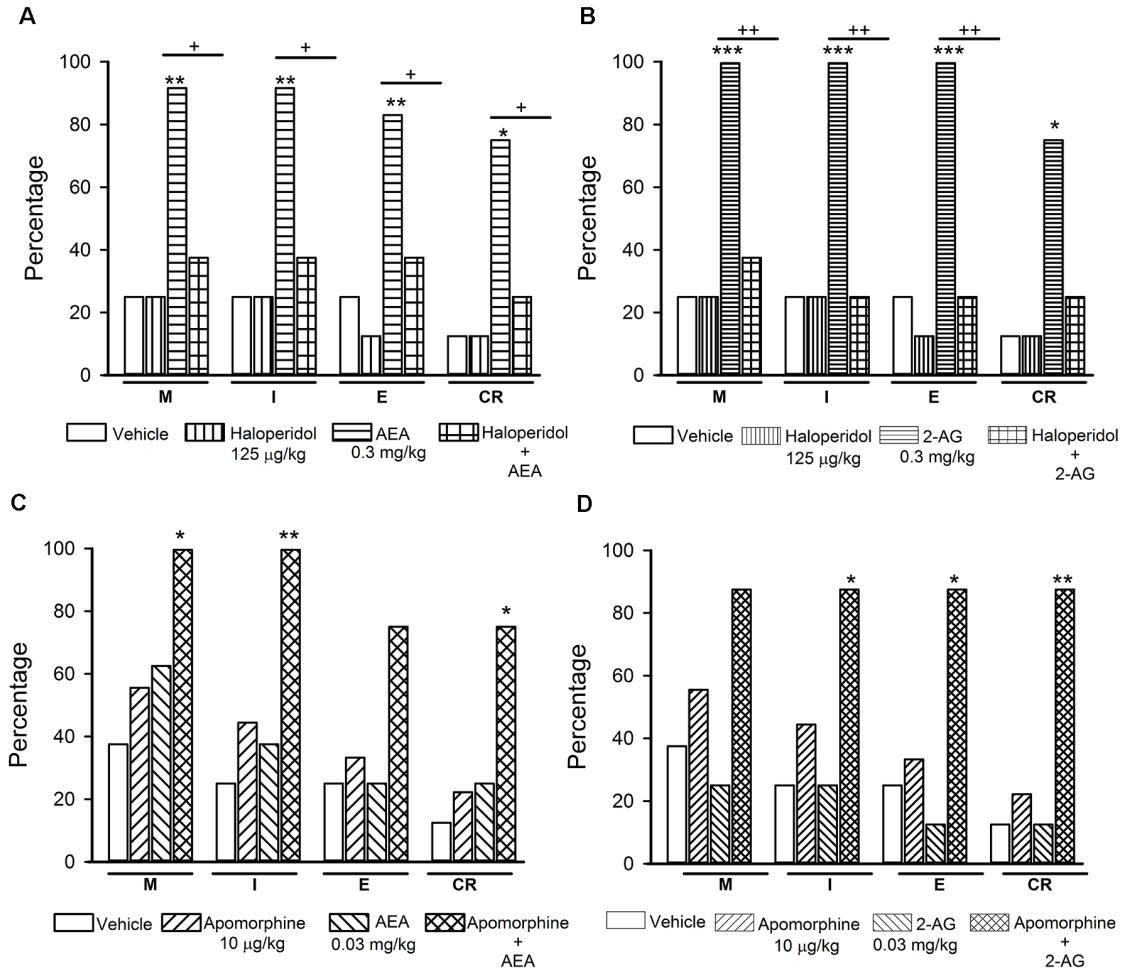

FIGURE 5 | Interaction of the endocannabinoids (eCBs) AEA and 2-AG with dopamine (DA) transmission in the reversal of sexual satiety. The upper graphs show the percentages of sexually exhausted rats that are able to mount (M), intromit (I), ejaculate (E) and resume copulation after ejaculation (CR) following vehicle (1 ml/kg), Haloperidol (125 μg/kg), AEA (0.3 mg/kg) or 2-AG (0.3 mg/kg) and the combined treatment of Haloperidol with AEA (panel **A**) or 2-AG (panel **B**). The lower graphs depict the effects of vehicle (1 ml/kg), Apomorphine (10 μg/kg) and their combined treatment with AEA (0.03 mg/kg; panel **C**) or 2-AG (0.03 mg/kg; panel **D**) on the percentages of satiated rats showing M, I, E and CR. Fisher *F*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{+}P < 0.05$, $^{++}P < 0.01$; $n = 8$ for each group. Asterisks over bars indicate statistical significance vs. the control group; crosses show significance for other indicated comparisons.

TABLE 2 | Specific sexual behavior parameters of those sexually satiated male rats achieving ejaculation in response to drug treatment and of a group of sexually experienced male rats as a reference.

Treatment	*n*	IL	M	I	EL	PEI
2-AG 0.3 mg/kg	6/8	13.55 ± 2.45	6	9	9.29 ± 2.93	15.67 ± 2.92
Apomorphine 10 μg/kg + AEA 0.03 mg/kg	7/8	3.91 ± 0.68	3	7	5.62 ± 1.05	16.02 ± 2.70
Apomorphine 10 μg/kg + 2-AG 0.03 mg/kg	7/8	3.01 ± 1.24	6	10	10.07 ± 3.02	16.41 ± 1.58
SKF-38399 0.1 mg/kg + AEA 0.03 mg/kg	6/8	3.62 ± 0.56	3	8	8.57 ± 1.80	20.32 ± 3.03
Quinpirole 0.03 mg/kg + AEA 0.03 mg/kg	8/8	4.22 ± 2.43	1	3	9.17 ± 2.43	23.92 ± 3.18
Quinpirole 0.03 mg/kg + 2-AG 0.03 mg/kg	7/8	13.18 ± 6.32	1	7	6.37 ± 0.84	38.05 ± 8.23
Sexually experienced	8/8	2.22 ± 0.28	0.5	6.5	5.97 ± 0.64	5.50 ± 0.61

IL, intromission latency; M, number of mounts; I, number of intromissions; EL, ejaculation latency; PEI, postejaculatory interval. Temporal measures are expressed in minutes (min) as mean ± SEM and M and I as median number.

DISCUSSION

The main findings of the present series of experiments can be summarized as follows: (a) sexually exhausted male rats exhibit a reduced sexual motivation 24 h after copulation to satiety, when the sexual inhibitory period is well established; (b) low doses of the eCB 2-AG reverse sexual satiety through a CB1 receptor-dependent mechanism, but do not modify copulatory behavior

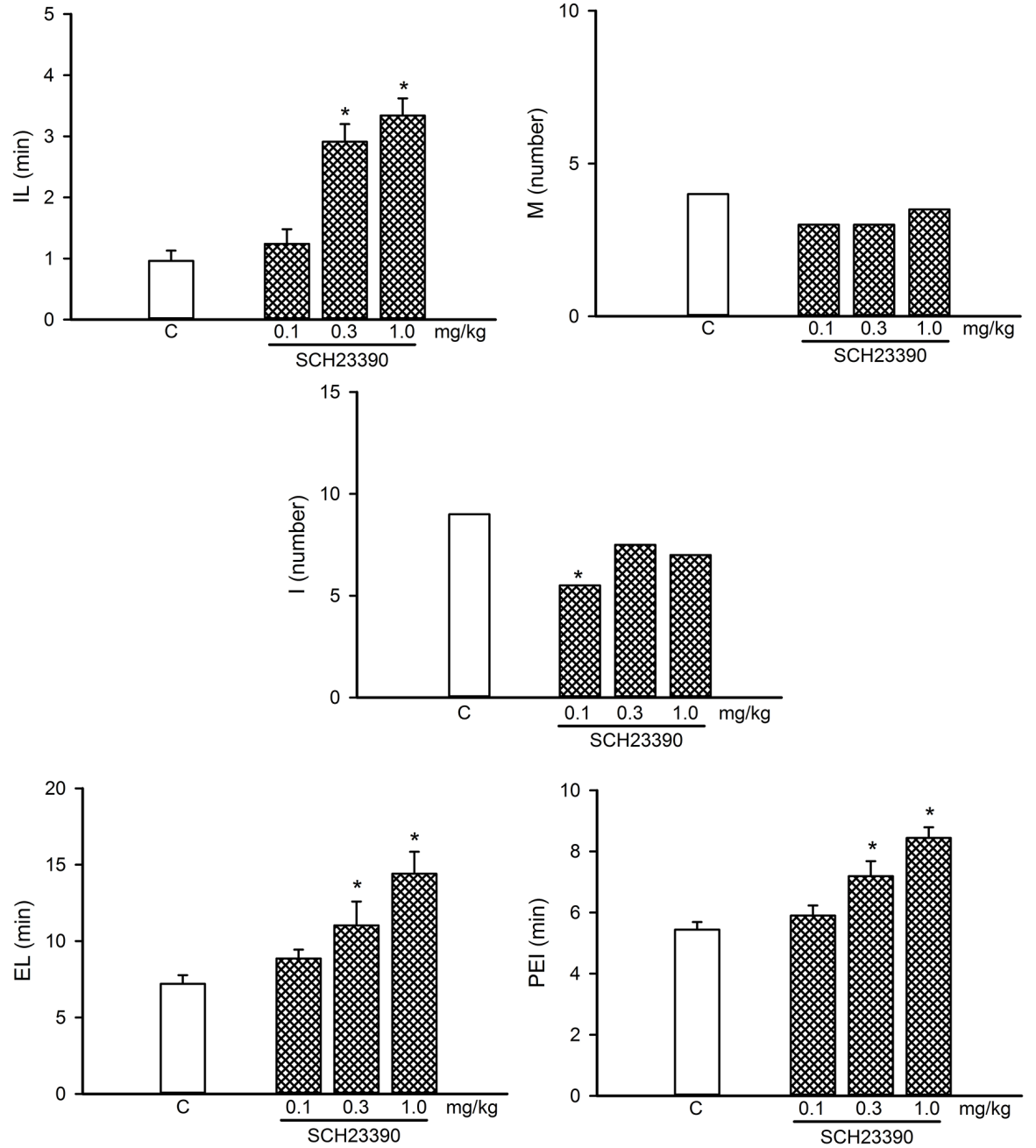

FIGURE 6 | Dose-response curve of the effects of different doses of the D1-like receptor antagonist, SCH23390 (0.1–1.0 mg/kg, $n = 8$ each), on sexual behavior of sexually experienced male rats. IL, intromission latency; M, number of mounts; I, number of intromissions; EL, ejaculation latency; PEI, postejaculatory interval; C, control. Latencies are expressed in minutes, as mean ± SEM, and numbers as medians. Kruskal–Wallis ANOVA followed by Dunn's test, $^*P < 0.05$ vs. control.

of sexually experienced male rats; (c) the eCBs AEA and 2-AG interact with the dopaminergic system to induce sexual behavior expression in sexually exhausted male rats; (d) D2-like, but not D1-like DA receptors, participate in the 2-AG-induced sexual satiety reversal; and (e) AEA-induced satiety reversal is mediated by D1-like DA receptors. Notwithstanding, D2-like receptor agonists also synergize with AEA to induce sexual activity in sexually exhausted males.

The sexual incentive motivation test revealed that sexually exhausted male rats do not show the preference for a sexually receptive female exhibited by sexually experienced animals, thereby confirming that during the sexual inhibitory period that characterizes sexual satiety, male rats have a reduced sexual motivation. As mentioned in the introduction section, previous data implied that sexual motivation might play a role in the sexual satiety phenomenon (Rodríguez-Manzo, 1999a;

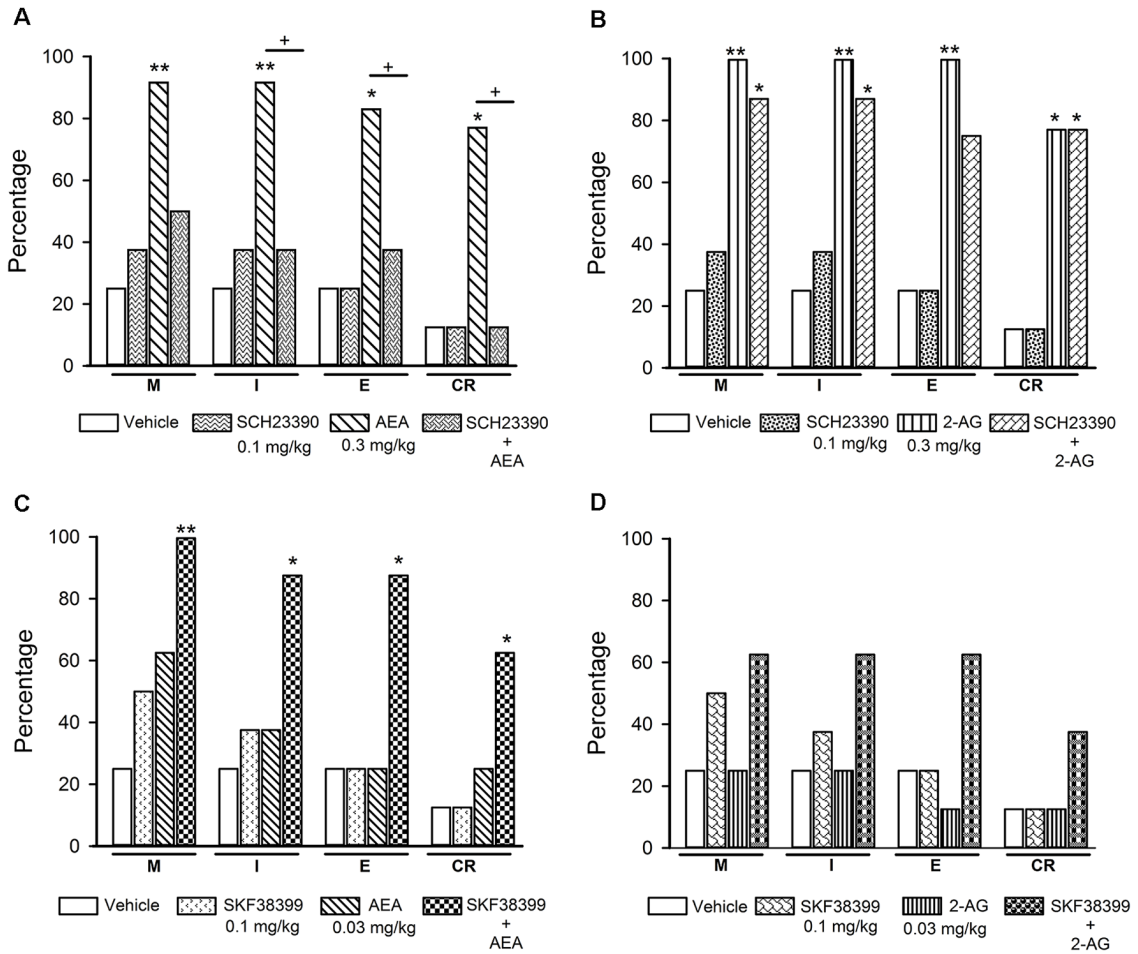

FIGURE 7 | Effects of the combined treatment of eCBs and D1-like receptor ligands on sexual behavior of sexually exhausted male rats. The upper graphs show the percentages of sexually satiated rats that are able to mount (M), intromit (I), ejaculate (E) and resume copulation after ejaculation (CR) following vehicle (1 ml/kg), the D1-like receptor antagonist SCH23390 (0.1 mg/kg), AEA (0.3 mg/kg) or 2-AG (0.3 mg/kg) and the combined treatment of SCH23390 with AEA (panel **A**) or with 2-AG (panel **B**). The lower graphs depict the effects of vehicle (1 ml/kg), the D1-like receptor agonist SKF38399 (0.1 mg/kg) and their combined treatment with AEA (0.03 mg/kg; panel **C**) or 2-AG (0.03 mg/kg; panel **D**) on the percentages of satiated rats showing M, I, E and CR. Fisher *F* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{+}P < 0.05$; $n = 8$ for each group. Asterisks over bars indicate statistical significance vs. the control group; crosses show significance for other indicated comparisons.

Guadarrama-Bazante and Rodríguez-Manzo, 2019), including a report on a diminished sexual motivation measured in male rats immediately after reaching sexual satiety (Ågmo et al., 2004). However, the sexual motivational state of the satiated animals during the long-lasting sexual inhibitory period (i.e., 24–72 h later) had not been directly assessed. This assessment is important, because 24 h after copulation to satiety, not only the characteristic sexual inhibitory state is well established, but also other possible confounding factors are absent. For instance, the fatigue due to intense copulation is no longer present, since the animals rested overnight. Males had free access to water and food when returning to their home-cages after copulation to satiety, eliminating hunger and thirst as factors playing a role in their lack of interaction with the receptive female rat. Finally, during the sexual incentive motivation test, the sexually satiated rats are exposed to a new sexually receptive female, eliminating the possible habituation to the sexual partner as another factor involved in the absence of preference. Under these conditions, we believe that the lack of interest for the sexually receptive female exhibited by sexually satiated males 24 h after copulation to satiety reflects an actual decrease in sexual motivation, thus validating the notion that this factor is involved in the long-lasting sexual inhibitory state that characterizes the sexual satiety phenomenon.

eCB signaling in the brain has been found to regulate the motivation for natural rewards (Parsons and Hurd, 2015). Reinforcing this notion, previous data from our group showed that the eCB AEA reversed sexual satiety after its direct infusion into the VTA (Canseco-Alba and Rodríguez-Manzo, 2016). This result supports the idea that eCBs' actions at the MSL system might modify the sexual motivational tone of sexually satiated rats. AEA and 2-AG are the best-characterized eCBs and both activate CB1 receptors; however, AEA binds with moderate affinity and is a partial agonist at CB1 receptors,

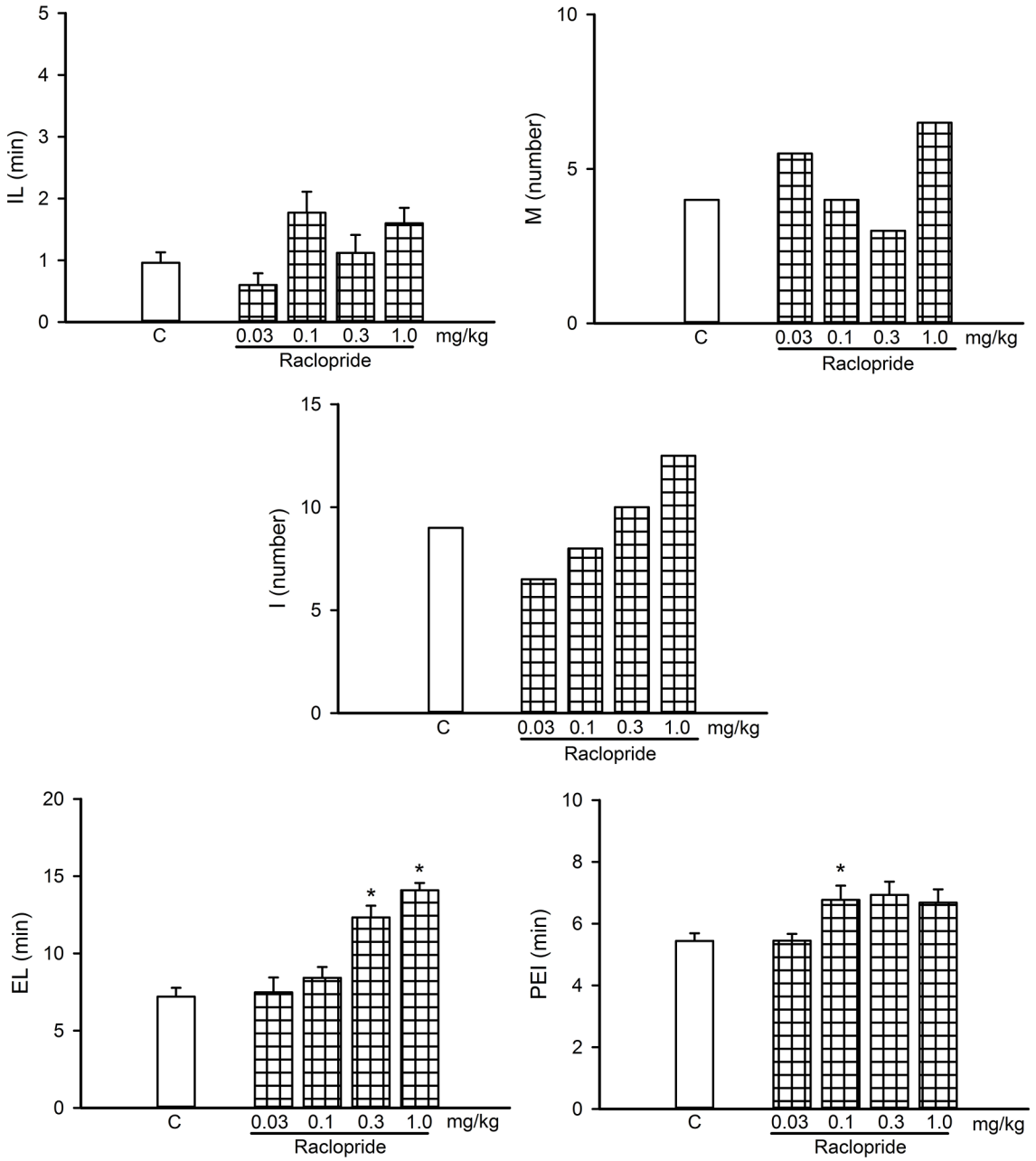

FIGURE 8 | Dose-response curve of the effects of different doses of the D2-like receptor antagonist Raclopride (0.03–1.0 mg/kg, $n = 8$ each) on sexual behavior of sexually experienced male rats. IL, intromission latency; M, number of mounts; I, number of intromissions; EL, ejaculation latency; PEI, postejaculatory interval; C, control. Latencies are expressed in minutes as mean ± SEM and numbers as medians. Kruskal–Wallis ANOVA followed by Dunn's test, $^*P < 0.05$ vs. control.

whereas 2-AG binds with low affinity but exhibits full efficacy at these receptors (Hillard, 2000). Besides, 2-AG is more abundant than AEA in the brain (Stella et al., 1997; Nomura et al., 2008) and is considered as the key eCB released on demand by VTA DA neurons to modulate its own activity (Melis et al., 2004; Tanimura et al., 2010). Therefore, it was crucial to determine if 2-AG was also capable of reversing the sexual inhibitory state of sexually exhausted rats. Results showed that similar to AEA's effects (Canseco-Alba and Rodríguez-Manzo, 2014), low doses of 2-AG reversed sexual satiety through the activation of CB1 receptors. However, 2-AG was capable of inducing copulation in satiated rats within a broader dose range than AEA (0.1–3.0 mg/kg vs. 0.1–0.3 mg/kg, respectively), which is compatible with the higher efficacy of the former at CB1 receptors. Interestingly, 2-AG induced the display of sexual behavior in sexually satiated rats but did not modify the sexual performance of sexually experienced animals. A possible explanation for this differential action could be based on the

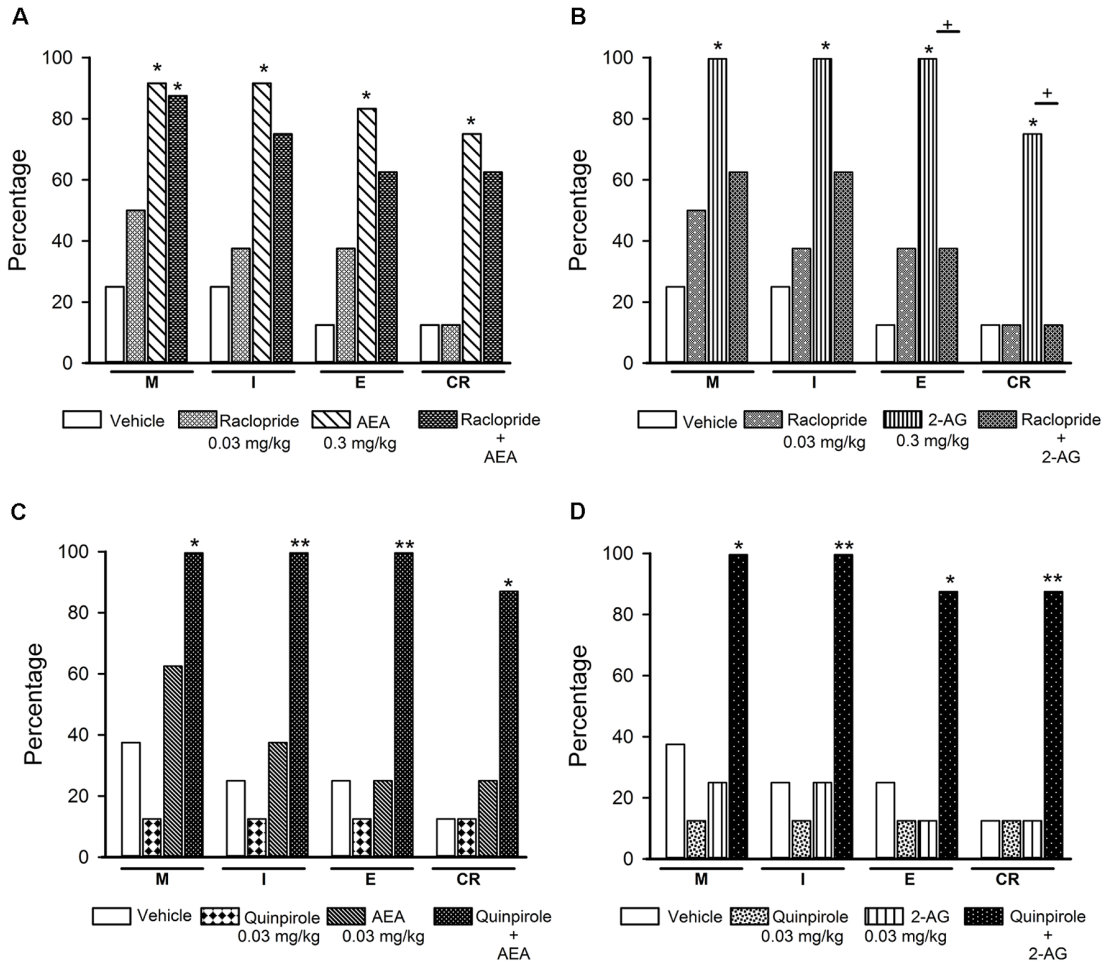

FIGURE 9 | Effects of the combined treatment of eCBs and D2-like receptor ligands on sexual behavior of sexually exhausted male rats. The upper graphs show the percentages of sexually satiated rats that are able to mount (M), intromit (I), ejaculate (E) and resume copulation after ejaculation (CR) following vehicle (1 ml/kg), the D2-like receptor antagonist Raclopride (0.03 mg/kg), AEA (0.3 mg/kg) or 2-AG (0.3 mg/kg) and the combined treatment of Raclopride with AEA (panel **A**) or 2-AG (panel **B**). The lower graphs depict the effects of vehicle (1 ml/kg), the D2-like receptor agonist Quinpirole (0.03 mg/kg) and their combined treatment with AEA (0.03 mg/kg; panel **C**) or 2-AG (0.03 mg/kg; panel **D**) on the percentages of satiated rats showing M, I, E and CR. Fisher *F* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{+}P < 0.05$; $n = 8$ for each group. Asterisks over bars indicate statistical significance vs. the control group; crosses show significance for other indicated comparisons.

proposal that the net effect of CB1 receptor-mediated actions at the MSL system depend on the level of baseline activity of midbrain DA neurons, such that enhancing DA neuronal firing may have a larger effect when baseline frequency is low compared to when neurons are burst firing (Covey et al., 2017). Following this idea, when sexually experienced rats are exposed to sexually receptive females as well as during copulation, DA neurons fire in the bursting mode rendering a phasic DA release in the NAcc (Robinson et al., 2002). Although, eCBs are capable of removing the tonic inhibition exerted by GABAergic inputs onto midbrain dopaminergic neurons to promote the phasic mode of DA release (Oleson et al., 2012), in the sexually experienced males this effect is also elicited by the rewarding stimulus (Grace, 1991; Grace et al., 2007), i.e., the presence and interaction with the sexually receptive female, which could account for the lack of 2-AG sexual facilitative effects in these animals. By contrast, in sexually satiated rats the inhibition of their sexual responsiveness suggests that DA neuron baseline activity is low, explaining the ability of 2-AG to produce sexual behavior facilitative effects in animals with this sexual condition.

A central finding of the present work is that both AEA and 2-AG interact with the dopaminergic system to reverse sexual satiety. Sub-optimal doses of each of these eCBs synergized with a dose of apomorphine that was subthreshold for reversing sexual satiety, whereas the reversal of the sexual inhibitory state, induced by effective doses of AEA or 2-AG, was canceled by the DA receptor antagonist haloperidol. These results contribute to strengthening the notion of a relationship between eCB and DA signaling in the facilitation of reward-motivated behaviors (Oleson and Cheer, 2012; Wenzel and Cheer, 2018). They also suggest that the deficient motivational tone of sexually exhausted rats might be increased by eCBs' actions, through the modulation of DA activity, enabling sexually satiated males to respond with

TABLE 3 | Effect of the different drug treatments on spontaneous locomotor activity of sexually exhausted male rats.

Drug	Dose	Number of counts/ 5 min mean ± SEM
2-AG Veh	0	44.25 ± 3.41
2-AG	0.03 mg/kg	47.50 ± 2.86
	0.1 mg/kg	47.25 ± 6.05
	0.3 mg/kg	51.75 ± 6.18
	1.0 mg/kg	46.75 ± 5.76
	3.0 mg/kg	48.88 ± 6.08
AM251 Veh + 2AG Veh	0	42.00 ± 3.13
AM251+2-AG	0.1 mg/kg + 0.1 mg/kg	49.50 ± 1.60
Haloperidol	125 μg/kg	46.71 ± 2.19
Hal Veh + AEA/2-AG Veh	0	41.10 ± 2.31
Haloperidol + AEA	125 μg/kg + 0.3 mg/kg	38.12 ± 1.97
Haloperidol + 2-AG	125 μg/kg + 0.3 mg/kg	41.03 ± 2.47
saline + AEA/2-AG Veh	0	35.87 ± 2.09
Apomorphine	10 μg/kg	36.25 ± 1.81
Apomorphine+ AEA	10 μg/kg + 0.03 mg/kg	38.25 ± 1.08
Apomorphine+ 2-AG	10 μg/kg + 0.03 mg/kg	35.75 ± 3.29
SCH23390	0.1 mg/kg	37.00 ± 2.61
SCH23390 + AEA	0.1 mg/kg +0.3 mg/kg	32.62 ± 1.48
SCH23390 + 2-AG	0.1 mg/kg + 0.3 mg/kg	50.38 ± 3.77
Raclopride	0.03 mg/kg	30.37 ± 1.58
Raclopride + AEA	0.03 mg/kg + 0.3 mg/kg	32.87 ± 2.60
Raclopride + 2-AG	0.03 mg/kg + 0.3 mg/kg	47.01 ± 3.20
SKF38399	0.1 mg/kg	38.28 ± 3.47
SKF38399 + AEA	0.1 mg/kg + 0.03 mg/kg	33.14 ± 2.25
SKF38399+ 2-AG	0.1 mg/kg + 0.03 mg/kg	44.57 ± 4.65
Quinpirole	0.03 mg/kg	56.11 ± 7.85
Quinpirole + AEA	0.03 mg/kg + 0.03 mg/kg	47.86 ± 1.90
Quinpirole + 2-AG	0.03 mg/kg + 0.03 mg/kg	50.25 ± 3.83

Kruskal–Wallis ANOVAs, non-significant.

sexual activity to the rewarding stimulus, represented by the receptive female.

It has been proposed that the dopaminergic system might be the common final pathway for the pharmacological reversal of sexual satiety. This idea emerged from the fact that several drugs, acting at different neurotransmitter systems, are capable of reversing sexual satiety by interacting, directly or indirectly, with the dopaminergic system (Rodríguez-Manzo and Fernández-Guasti, 1995; Rodríguez-Manzo, 1999b; Hull and Rodríguez-Manzo, 2017). Present results are in line with this proposal ascribing to the MSL system a central position in the dopaminergic-mediated regulation of male rat sexual behavior expression.

Midbrain DA neurons are involved in the signaling of reward-related stimuli by changing their firing pattern (Grace, 1991; Grace et al., 2007). The basal activity of these neurons involves low-frequency firing resulting in a dopaminergic tone capable of activating high-affinity D2-like DA receptors in the NAcc. Upon the presentation of a rewarding stimulus, this firing pattern changes to a high-frequency burst firing that is accompanied by an increase in NAcc DA release, which activates low-affinity D1-like DA receptors (Grace et al., 2007; Dreyer et al., 2010). On these bases, establishing the DA receptor family/families involved in the 2-AG/DA and AEA/DA interactions to induce sexual behavior display in sexually satiated rats appeared relevant. Interestingly, our data showed that there was a differential interaction between each of these eCBs and the two DA receptor families. 2-AG synergized with D2-like, but not with D1-like DA receptor agonists, to induce sexual satiety reversal; an effect that was completely prevented by the D2-like receptor antagonist raclopride. In contrast, AEA synergized with both D1- and D2-like DA receptor agonists to reverse the sexual inhibition, but only the D1-like DA receptor antagonist SCH23390 was able to block the AEA-induced sexual satiety reversal.

It has been documented that 2-AG and AEA, in spite of activating the same cannabinoid receptors and signal transduction pathways (Janero et al., 2009), do not always play the same physiological role, acting sometimes in concert and sometimes not (Di Marzo and Cristino, 2008; Luchicchi and Pistis, 2012). It is important to recall that in the present work eCBs were exogenously administered and, therefore, could activate CB1 receptors in different brain regions. Within the MSL system, CB1 receptors are expressed both in the NAcc (Pickel et al., 2006) and in the VTA (Herkenham et al., 1991); therefore, systemically administered eCBs might have reversed satiety by acting at each of these brain regions, where they behave as retrograde messengers suppressing presynaptic glutamate and GABA release (Lupica and Riegel, 2005). However, substantial evidence indicates that exogenously administered cannabinoids increase DA release in rat NAcc (Chen et al., 1990; Gardner and Vorel, 1998; Gessa et al., 1998) and excite midbrain DA neurons in the VTA (French et al., 1997). These data suggest that the actions of exogenously administered eCBs in the present work would be exerted at the VTA; a proposal supported by the finding that intra-VTA infusion of AEA reverses sexual satiety (Canseco-Alba and Rodríguez-Manzo, 2016).

In relation to DA receptors, D1-like and D2-like receptors within the MSL system are mainly expressed in the NAcc, segregated in different populations of medium spiny neurons, which constitute 95% of the cells in this brain region (Yang et al., 2018). According to present data, the 2-AG/DA interaction for the reversal of sexual satiety clearly involves only the activation of D2-like receptors, probably from the NAcc, since in the VTA these receptors are somatodendritic autoreceptors that regulate the firing rate of DA neurons and DA release in terminal fields. D2 autoreceptor activation reduces DA release at the NAcc and also limit somatodendritic DA release in the VTA (Rice and Patel, 2015). In support of the notion that the 2-AG/DA interaction takes place in the NAcc is the finding that direct infusion of the selective D2-like DA receptor agonist, quinpirole, into this brain region reverses the sexual inhibition of satiated rats (Guadarrama-Bazante and Rodríguez-Manzo, 2019).

Results of this work also suggest that AEA's interaction with DA transmission in sexually satiated rats is essentially, although not exclusively, mediated by D1-like DA receptor activation. This conclusion derives from the fact that D1-like DA receptor blockade with the antagonist SCH23390 canceled AEA-induced reversal of sexual satiety and a sub-effective dose of the D1-like receptor agonist SKF33939 synergized with a dose of AEA, that was subthreshold for reversing sexual satiety, to promote sexual behavior display. In line with this finding, our group observed that systemically administered DA receptor agonists reverse sexual satiety through the activation of D1-like DA receptors (Guadarrama-Bazante et al., 2014).

AEA also synergized with D2-like DA receptor activation to reverse sexual satiety, but reversal of sexual satiety induced by an effective dose of AEA, was not canceled by the D2-like receptor antagonist raclopride. These results evidence that AEA effects are mediated by the modulation of D1-like DA receptors, though the independent activation of D2-like DA receptors with quinpirole could synergize with the AEA-mediated activation of D1-like DA receptors to reverse satiety. Interestingly, activation of D2-like DA receptors in the NAcc increases the extracellular levels of AEA in this brain region (Giuffrida et al., 1999). In fact, DA exerts modulatory effects on both AEA and 2-AG content in the NAcc (Patel et al., 2003), where medium spiny neurons synthesize and release these eCBs in response to DA stimulation. Remarkably, the NAcc's content of AEA and 2-AG is differentially modulated by the activation of D1-like and D2-like DA receptors, respectively (Patel et al., 2003). These data further support the relationships described in the present work, between AEA and D1-like DA receptors and between 2-AG and D2-like DA receptors. Thus, a possible contribution to the reversal of sexual satiety of changes in NAcc's content of these two eCBs, resulting from the activation of the two DA receptor families, cannot be discarded.

In spite of the differential interaction found for each eCB with the DA receptor families, it has been considered that the simultaneous activation of D1-like and D2-like DA receptors in the NAcc is required for the processing of reward relevant information (Ikemoto et al., 1997). Moreover, it has been reported that only the combination of D1-like and D2-like receptor agonists is able to enhance NAcc core cell firing *in vitro*—an effect in which the participation of eCBs is required—, while the independent activation of each of these receptor families does not reproduce this result (Seif et al., 2011). Remarkably, we found that the direct infusion of the non-selective DA receptor agonist, apomorphine, into the NAcc induced a full sexual satiety reversal, not obtained with any other pharmacological treatment so far tested in sexually satiated rats. In this case, the most effective apomorphine dose induced sexual behavior to ejaculation and copulation resumption after ejaculation in every sexually satiated animal. Besides, the copulatory performance of these males was as efficient as that of sexually experienced males, an outcome not commonly seen with other treatments reversing satiety (Guadarrama-Bazante and Rodríguez-Manzo, 2019). This result reinforces the notion that the regulation of the rewarding properties and motivational tone at the NAcc is mediated by the cooperative actions of the two DA receptor families.

Based on the data here presented, it could be proposed that repeated activation of the MSL system by intense copulation during sexual satiety development, induces a diminished sexual motivational tone, responsible for their characteristic long-lasting sexual behavior inhibition. AEA and 2-AG seem to reverse this sexual inhibitory state by modulating dopaminergic transmission, presumably at the MSL system. Specific experiments further analyzing the role played by eCBs in the regulation of DA-mediated motivation for natural rewards at the MSL system are warranted.

The motivational component of male rat sexual behavior and the initiation of the copulatory behavioral pattern (Blackburn et al., 1992) have not only been linked to mesolimbic DA, but also to DA transmission at the hypothalamic medial preoptic area (mPOA; Pfaus and Phillips, 1991; Hull et al., 1999). Indeed, DA levels increase simultaneously in the NAcc and mPOA in response to the presence of an inaccessible sexually receptive female (Blackburn et al., 1992; Hull et al., 1999). However, present and previous data from our lab do not suggest a participation of this brain region in the eCB/DA-mediated increase in sexual motivation that leads to the reversal of sexual satiety. To the extent of our knowledge, eCBs have not been associated to the control of mPOA DA neuron activity and, infusion of DA receptor agonists into this brain region does not reverse sexual satiety, except for a specific low dose of the D2-like receptor agonist quinpirole (Guadarrama-Bazante and Rodríguez-Manzo, 2019). However, the systemic administration of several quinpirole doses failed to reverse the sexual inhibition of satiated rats (Guadarrama-Bazante et al., 2014). Notwithstanding, the indirect participation of the mPOA, a brain region that is crucial for male sexual behavior expression in all vertebrate species (Hull et al., 1999), through its efferent projections, which have been considered essential for the initiation of the copulatory pattern (Everitt, 1990), cannot be discarded. Actually, some of these projections target specifically the VTA (Simerly and Swanson, 1988; Stolzenberg and Numan, 2011; Zahm et al., 2011) and might influence the activity of mesolimbic DA neurons favoring an increase in sexual motivation of sexually satiated rats.

The present work uses sexual satiety as an animal model to provide new evidence showing that eCBs modulate the MSL system with an impact on sexual motivation. This model offers the opportunity to explore the nature of adaptive inhibitory mechanisms (Bancroft, 1999) controlling the expression of an innate behavior normally triggered by specific incentive stimuli, i.e., copulation in the presence of an accessible sexually receptive female, which appears to involve changes in the male's motivational tone. This research also suggests that CB1 receptor activation might have therapeutic potential to positively influence sexual arousal and desire, which deficiency underlies several human sexual disorders, the hypoactive sexual desire disorder (American Psychiatric Association, 2013) among them; this possibility should be explored.

Like all studies, the present work has its limitations. Since all drug treatments in this study were systemically administered, the involvement of brain structures outside the mesolimbic system as well as peripheral effects of eCBs cannot be discarded. The participation of the MSL system in the eCB-mediated actions here described has to be confirmed, however, this study is a good starting point motivating more specific experiments directed to identify the brain loci within the mesolimbic system where the described eCB/DA interactions occur.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Animal Subjects

The animal study was reviewed and approved by Cinvestav Internal Committee for the Care and Use of Laboratory Animals (Comité Institucional para el Cuidado y Uso de Animales de Laboratorio, CICUAL) following the regulations established in the Mexican Official Norm for the use and care of laboratory animals NOM-062-ZOO-1999; Protocol 0230-16.

AUTHOR CONTRIBUTIONS

AC-A and GR-M designed the experiments, performed statistics, interpreted data, drafted the manuscript, wrote the final manuscript and analyzed behavioral data. AC-A performed some behavioral experiments. GR-M conceptualized the study. Both authors contributed to the scientific discussions and approved the final manuscript.

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REVIEW
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Modeling Human Sexual Motivation in Rodents: Some Caveats

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Sexual behavior is activated by motivation. An overwhelming majority of experimental studies of the intricacies of sexual motivation has been performed in rodents, most of them in rats. Sometimes it is desirable to generalize results obtained in this species to other species, particularly the human. It is hoped that studies of the neurobiology of rodent sexual behavior may shed light on the central nervous mechanisms operating in the human, and the search for efficient pharmacological treatments of human sexual dysfunctions relies partly on studies performed in rodents. Then the issue of generalizability of the rodent data to the human becomes crucial. We emphasize the importance of distinguishing between copulatory acts, behavior involving the genitals, and the preceding event, the establishment of physical contact with a potential mate. Comparisons between the structure of copulatory behavior in rats and humans show abysmal differences, but there may be some similarity in the underlying mechanisms. The endocrine control of sex behavior is shortly mentioned, and we also compare the effects of the few drugs known to affect both rodent and human copulatory behavior. The stimuli activating sexual motivation, often called desire in the human literature, are examined, and the sexual approach behaviors in rats and humans are compared. There is a striking similarity between these species in how these behaviors respond to drugs. It is then shown that the intensity of sexual approach is unrelated to the intensity of copulatory behavior. Even though the approach is a requisite for copulation, an activity that requires at least two individuals in close physical contact, these two aspects of sexuality do not covary. This is similar to the role of the testosterone in men and male rats: although the hormone is needed for sex behavior, there is no correlation between serum testosterone concentration and the intensity of copulation. It is also pointed out that human sexual behavior is mostly determined by social conventions, whereas this is not the case in rats and other rodents. It is concluded that some observations in rats can be generalized to the human, but extreme caution must be exercised.

Keywords: sexual motivation, sexual behavior, orgasm, ejaculation, paracopulatory behavior, lordosis

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INTRODUCTION

The typical textbook definition states that motivation is a concept referring to the mechanisms responsible for the activation, direction and persistence of behavior. According to this definition, the organism would be completely inactive in the absence of motivation. Once the organism has been activated, motivational systems determine which of all possible

behaviors should be performed, and for how long the organism should persist with that behavior. Thus, motivation is underlying all activity and the choice of the specific activities to be performed at any moment. It is difficult to imagine a more fundamental concept in the science of behavior. These basic notions have been extensively discussed elsewhere (Hernández-González et al., 2008; Ågmo, 2011).

The early search for understanding motivational processes concentrated on rather basic behaviors, such as drinking, eating, and sex. It was believed that the motivational control of these basic behaviors was similar in all animal species. Consequently, the choice of species as an experimental subject was often based on convenience. However, already in 1949, at a meeting with the American Psychological Association, Frank Beach expressed concern about the overly frequent use of rats, hamsters and guinea-pigs in behavioral research (Beach, 1950). He feared that the concentration on a few, similar species, was incompatible with a real comparative psychology, and would make it impossible to determine if and how behavioral principles established in one species were at work in other species. The question of the generalizability of observations in one species to another is still unresolved.

In the present review article, we will discuss the generalizability of observations made on rat sexual behavior to the human. In other words, we will ask the question of whether we can use rat sex as a model of human sex. Some general notions about rat models and their potential utility have been outlined elsewhere (Ågmo et al., 2004; Ågmo, 2014), and they will not be repeated here. Instead, we will provide an in-depth analysis of the usefulness of observations of copulatory behavior on one hand and of sexual approach behaviors on the other, in rats and humans. Long ago, it was pointed out that the validity of generalization between species is strictly dependent on the quality of the description and understanding of the behavior in each of the species we want to generalize between (Beach, 1976). Therefore, we will include an analysis of the characteristics of rat and human sexual behavior. We will also discuss similarities and differences in the endocrine control of sexual behavior, and of the effects of drugs on these behaviors. In the end, we will ascertain that we are not now in possession of sufficient data of sufficient quality for any firm conclusion. Before turning to these issues, however, we will define the essential concepts employed here. This should reduce the possibility of misunderstanding and enhance clarity of all subsequent arguments.

DEFINITIONS

Sexual motivation, often called sexual desire in the human literature, is an abstract concept referring to the *probability of displaying copulatory behavior when a mate is available or the intensity of that behavior when displayed*. It can also refer to the *intensity of approach to a potential sexual partner*. Since sexual activities (except masturbation) require at least two individuals in close physical proximity, any sexual encounter is preceded by approach behaviors.

The intensity of copulatory behavior can be quantified in many ways in male rats. We consider short latencies to mount, intromission or ejaculation as well as large number of mounts and intromissions as indicators of high intensity, whereas long latencies and low numbers indicate low intensity. High copulatory rate (number of sexual acts per unit time) and short interintromission intervals can likewise be considered indicators of high intensity, and low rate and long intervals constitute evidence for low intensity. In female rats, the indicators range from lordosis quotient and number of paracopulatory behaviors in the standard observation cage and these plus the temporal aspects of interaction with the male in the divided cage and seminatural environment (see "Rodents" section for explication of the terms used). In humans, the intensity of copulatory behavior is rarely defined or quantified. It appears that simple self-report of the number of copulatory encounters per unit time is used as indicator of the intensity of that behavior. Throughout this article, we refer to one or several of the abovementioned criteria whenever we mention the intensity of copulation. The intensity of sexual approach behaviors in rats and humans will be operationally defined in the "Sexual Approach Behaviors" section.

Copulatory behavior *is any action leading to sexual reward*. Sexual reward is a state of positive affect activated by physical stimulation of the genitalia or mental representations of such stimulation (Ågmo, 2007, p. 3). Evidence for the capacity of mental representations to cause sexual reward indistinguishable from that obtained by genital stimulation is limited to the human female. Fantasies alone can lead to the subjective experience of orgasm and the physiological manifestations of that state identical to those observed after orgasm caused by clitoral stimulation (Whipple et al., 1992). Likewise, imaging studies have revealed that the brain areas activated by fantasies or clitoral stimulation are similar (Wise et al., 2016). In men as well as in males and females of non-human species, physical stimulation of the genitals seems to be required for the obtainment of sexual reward.

It may appear inadequate to consider fantasizing leading to orgasm as a copulatory behavior. However, the fantasies are often about genital interaction with a mate (Seehuus et al., 2019), i.e., about copulatory behavior in a strict sense. The fact that the mate is imaginary rather than real is not crucial, according to our judgment. It may also be argued that humans may engage in sexual activities without obtaining or expecting to obtain sexual reward. In those cases a different reward, for example money, improved relationship or favors of all kinds, operates. Thus, motor patterns similar to those constituting copulatory behavior become instrumental for obtaining non-sexual reward. We do not consider such behavior as sexual. It may also be noted that these behaviors probably are determined by motives other than sexual. In fact, Meston and Buss (2007) have listed more than 200 possible motives, most of them unrelated to sexuality, for engaging in motor patterns similar to copulation.

We prefer the term "copulatory behavior" rather than "sexual behavior", since the former more explicitly refers to genital activities. Another reason for avoiding "sexual behavior" is to clearly distinguish between non-genital sexual approach behaviors and acts involving the genitals. Thus, from here on

we stick to "copulatory" instead of "sexual" when referring to behaviors involving the genitals.

A model of the relationship between external stimuli, central nervous processes and somatic as well as visceral responses to these stimuli is presented in **Figure 1**. Much of the ensuing discussion is based on this model.

ANIMAL SEX IS NOT ALWAYS A MODEL

For a long time, copulatory behavior in rats, hamsters, guinea-pigs, rabbits and many more exotic species was studied without any explicit intention of generalizing to other species. The basic purpose of these studies was simply to describe the nature of copulatory behavior and the internal and external stimuli controlling it in a particular species and even in particular strains of some species. Species and strain comparisons were frequent, but generalizations from one species or strain to another were made only with great caution (for example see Beach, 1976) or not at all. The influential normative descriptions of male rat sexual behavior (e.g., Beach and Jordan, 1956; Larsson, 1956) were never intended to be generalized to other species. The detailed analysis of the circuits and hormones controlling female rat lordosis is perhaps a still better example of this. The sensory pathways transmitting the stimulus required for activating the behavior, from the cutaneous receptors to the diencephalon, as well as the descending output to *musculus longissimus lateralis* and *musculus transversospinalis*, both responsible for the lordosis posture, have been painstakingly described (Pfaff, 1980). This is also the case for the action of the ovarian hormones in hypothalamic structures, down to the molecular level (reviewed in Pfaff, 2017; see also Micevych and Sinchak, 2018). The applicability of these findings to humans was not of any major

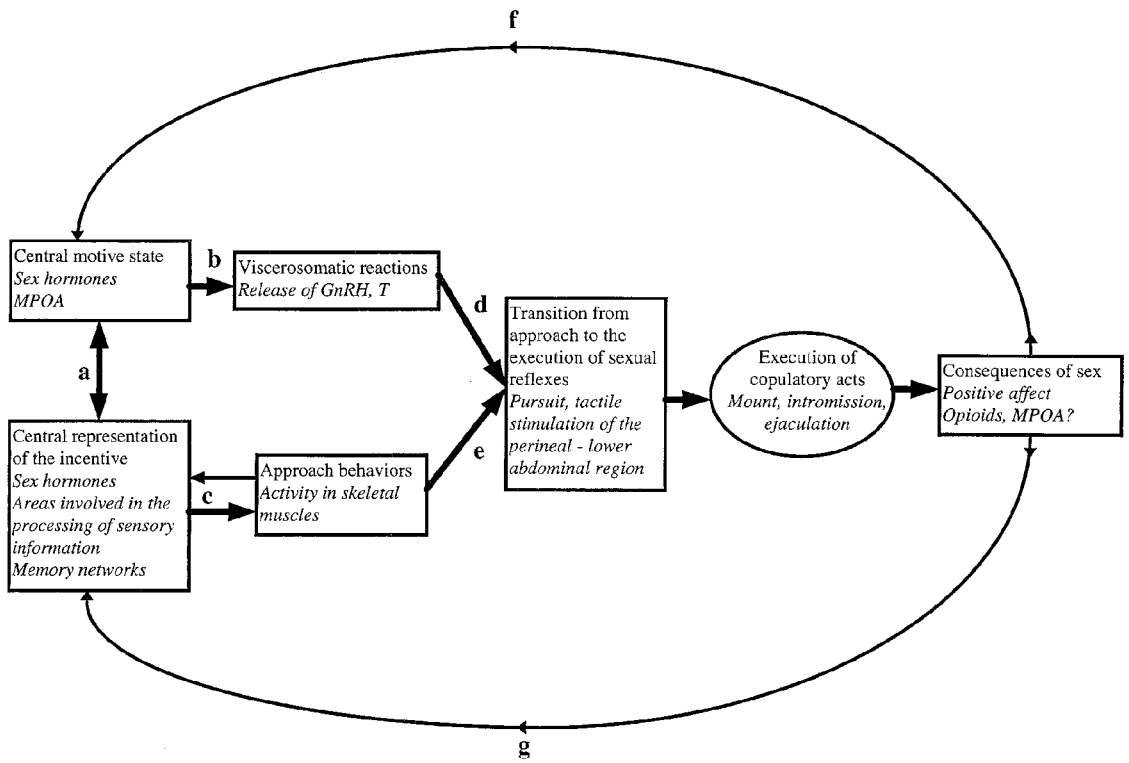

FIGURE 1 | A model for sexual incentive motivation. The text in italics represents the example of the male rat. **(a)** A reciprocal excitatory relationship functioning in such a way that the central motive state enhances the sensory system's sensibility to stimuli with sexual significance. When such stimuli are perceived, the sensory system excites the central motive state which in turn further sensibilizes the sensory system, i.e., the relationship is one of reciprocal positive feedback. **(b)** At a certain threshold level of activity, the central motive state engages a series of viscerosomatic activities preparing the subject for sexual interaction. **(c)** The appropriate environmental stimuli activate motor patterns that bring the subject in contact with the source of stimulation. During approach, additional incentive stimuli may be encountered. These will be centrally represented and enhance the central motive state through **(a)**. **(d,e)** Provided that approach behaviors have been successful and that appropriate viscerosomatic reactions are being accomplished, the subject's behavior may change from unconditioned or conditioned instrumental responses to the execution of sexual reflexes. These are activated by tactile stimulation of the perineal or lower abdominal region. If the subject is sexually inexperienced such stimulation is obtained accidentally. If the subject already has acquired sexual experience, then conditioned instrumental responses may facilitate the attainment of tactile stimulation necessary for activation of sexual reflexes. At the point of transition from approach to execution of copulatory reflexes, the behavioral sequence is aborted in the absence of tactile stimulation. In case that sexual reflexes indeed are activated, sex behavior will normally continue until ejaculation. **(f)** The positive affect induced by ejaculation will feed back to the central motive state where a short-lasting inhibitory system is activated. **(g)** At the same time, the positive affect and associated processes of reinforcement will strengthen the learning of associations between itself and environmental cues. These cues will acquire incentive properties in relation to the intensity of the positive affect that is experienced. For further details, see Ågmo (1999, 2011) and Paredes and Ågmo (2004). Reprinted from Ågmo (1999). Copyright (1999), with permission from Elsevier.

concern to the brilliant scientists behind these discoveries. The rat was not used as a model for something; it was studied in its own right. Whether the knowledge about the molecular actions of steroid hormones are applicable to other animals, including humans, is a completely different and perhaps irrelevant question in this context.

WHY DO WE NEED MODELS FOR STUDYING HUMAN SEXUAL BEHAVIOR AND MOTIVATION?

Satisfaction of scientific curiosity, for example understanding the intricacies of rat copulatory behavior and its hormonal control, is not of basic importance for organizations financing research or for scientists with utilitarian inclinations. To both of them, the use of non-human animals is a means of enhancing human well-being. Then, the discoveries made in non-human animals are of interest only if applicable to humans. Moreover, the problems addressed should preferably be related to important public health issues. Since sexual dysfunction neither is a cause of death nor of great expenses to society, research on such dysfunctions is not necessarily of high priority. Nevertheless, sexual activities have been reported to positively contribute to human well-being, as assessed by different types of questionnaires (Blanchflower and Oswald, 2004; Cheng and Smyth, 2015; Kashdan et al., 2018). Disorders of sexual function can lead to reduced quality of life (Hisasue et al., 2005; Rosen and Bachmann, 2008; Rosen et al., 2009). Thus, even though these disorders are not life-threatening, they may disrupt the life of the affected individuals.

The most common of the sexual disorders in women is sexual interest/arousal disorder (West et al., 2008; Burri and Spector, 2011). Before the DSM-5 (American Psychiatric Association, 2013) this condition was known as female hypoactive sexual desire disorder. We will use this old name. In men, the prevalence of hypoactive sexual desire disorder is somewhat below that of erectile dysfunctions and premature ejaculation (Beutel et al., 2006; McCabe and Connaughton, 2014). The opposite condition, hyperactive sexual desire, was rejected for inclusion in the DSM-5, but is nevertheless of some clinical concern (Kafka, 2014). It is often assumed that the paraphilias are associated with hyperactive desire, and treatments reducing desire may be viable therapeutic approaches to this kind of disorder (Kafka, 2003). The high prevalence of the reduced desire disorders and the social apprehension caused by the paraphilias, notably pedophilia, have prompted a search for efficient pharmacological treatment. This search was also inspired by the commercial success of treatments for erectile dysfunction. Regardless of the reasons behind the pursuit of drugs able to stimulate low sexual desire and to inhibit excessive desire, the need for preclinical models with acceptable predictive validity became apparent.

Other human sexual dysfunctions that have been modeled in non-human animals include premature ejaculation, a condition common in young men. Even though the role of sexual motivation in the etiology of premature ejaculation is unclear, this is another example of the search for a treatment of a sexual disorder using rodent models.

An entirely different condition, persistent lack of sexual attraction or asexuality, has attracted some attention during the last few decades. It has been estimated that between 0.4% and 3.3% of the adult population consider themselves as asexual (Aicken et al., 2013; Höglund et al., 2014). The condition is not included in diagnostic manuals like the ICD-11 or DSM-5 and is often regarded as a sexual orientation or identity (e.g., Hinderliter, 2013; Bogaert, 2015). There are, nevertheless, reports showing that some male rats and mice also may display a persistent lack of sexual attraction (Portillo and Paredes, 2003; Portillo et al., 2013). However, asexuality is not a clinical condition and consequently there is no interest in developing treatments. This means that there is no need for rodent models. The condition will not be further discussed.

HUMAN COPULATORY BEHAVIOR

Generalities

Even though Moll (1897) and Ellis (1933) had analyzed human copulatory behavior in elegant ways, the groundbreaking work of Kinsey et al. (1948, 1953) can be considered the origin of modern enquiries into human sexuality. Since the times of Kinsey, scientists have reported quantitative data concerning most aspects of human sexual behavior. The overwhelming majority of these data stems from self-reports of sexual activities. The Kinsey group obtained their data through highly structured interviews performed by well-trained interviewers whereas most of the subsequent work has been based on the use of questionnaires. Answers have been provided in either written form (e.g., Alexander and Sherwin, 1993; Merghati Khoei et al., 2018) or as responses to questions made over the telephone (e.g., Lewin et al., 1998). More recently, internet-based questionnaires have become widespread (e.g., Ritter et al., 2018). Regardless of the way in which the self-reports are obtained, they are notoriously unreliable. The most eloquent example of this is that men systematically report a considerably higher number of heterosexual partners than women. However, when a man has sex with a new woman, there is always a woman having sex with a new man. Thus, in societies where the proportion of men in the population is approximately equal to that of women, which is the case in most societies, the number of partners must be close to equal for the two sexes. This has been pointed out many times (e.g., Smith, 1992; Wiederman, 1997). Possible causes for the discrepancy between men and women in reporting the number of partners may be different accounting strategies (women counting, men estimating) and misreporting due to social norms, among others (Mitchell et al., 2019). In any case, the questionnaire-based notion that men are more promiscuous than women survives facts showing that it is impossible.

Since most of the knowledge about human copulatory behavior is based on questionnaires, it must be considered as approximate, in the best of cases. There are, however, notable exceptions. Masters and Johnson (1966) made careful observations of humans during actual copulation, and their work is still unsurpassed. Others have studied genital arousal (erection and vaginal lubrication) under various conditions, thereby obtaining objective data on sexual responses. Still,

others have analyzed cerebral blood flow or oxygenation when humans are exposed to sexually relevant stimuli (e.g., Mouras et al., 2003), or during masturbation (e.g., Stoléru et al., 2012) while in a magnetic resonance scanner. Due to the constraints of the scanner tube, brain imagery during actual copulation has not been performed. Nevertheless, the imaging studies have given rise to sophisticated models of the cerebral control of human sexual behavior (e.g., Georgiadis and Kringelbach, 2012). However, the fact that a brain area is activated or inhibited during sexual activities does not constitute evidence for that area actually being important for these activities. Lesions in some of the areas showing intense fos activation during female rat sexual behavior can leave the behavior unaffected (Guarraci et al., 2004). This can be an example of the typical redundancy of brain systems mediating basic behaviors. The functions of one area can be fulfilled by other areas when needed.

Despite the fact that a large quantity of descriptive and a limited amount of experimental data concerning human copulatory behavior are available, we are seriously lacking knowledge about many basic aspects of that behavior. This becomes particularly evident as soon as we are interested in the mechanisms activating the behavior. Neither the central nervous mechanisms underlying human sexual motivation nor the stimuli that render a human attractive to other humans are more than vaguely understood. Since sexual motivation is activated by stimuli emitted from other individuals, knowledge of these stimuli and how they affect the receiving individual are essential. It must be observed that even if humans sometimes replace the external stimuli from another individual with mental representations of such stimuli, the mechanisms activating sexual motivation are probably similar.

Description

In his classical description of human copulatory behavior (van de Velde, 1926), it was assumed that this behavior was a continuous activity, starting with sexual arousal (erection and vaginal lubrication) followed by vaginal penetration and male thrusting until ejaculation in the male and orgasm in the female. van de Velde's (1926) graphical illustration of human sexual intercourse is shown in **Figure 2**. The continuous nature of human copulation was later confirmed by direct observations (Masters and Johnson, 1966). In fact, these scientists adapted van de Velde's (1926) scheme of copulation in their famous three-phase model (excitement, plateau, orgasm). A more recent account, based on clinical experience, added desire as an event preceding excitement (Kaplan, 1979), but the notion of a continuous process has not been challenged. The continuous flow of sexual behavior patterns in human encounters have been brilliantly described (Schick et al., 2016), although the descriptions are based on self-reports rather than on direct observation.

Classical accounts of human sexual activities only considered heterosexual encounters in pairs. Sex among groups of humans as well as copulation in same-sex pairs has not been studied and analyzed with the same care. As far as we know, however, the continuous nature of the interaction is still present, probably even in groups where the members may change partner in the midst of copulation (Tewksbury, 2002; Friedman et al., 2008; Meunier, 2014). Nevertheless, it can be maintained that the vast majority of human sexual activities occurs in heterosexual pairs, and that vaginal—penile intercourse is the most common of these activities (Laumann et al., 1994; Lewin et al., 1998). In fact, 95% of adult men and women reported to have engaged in penile–vaginal intercourse during the last 3 months regardless of whether the survey was made in Germany or Australia (Rissel et al., 2014; Goethe et al., 2018). None of the studies mentioned inquired about continuous or interrupted sexual encounters, probably because it is *a priori* assumed that human sexual encounters indeed are continuous.

Endocrine Control

In men, there is clear-cut evidence for the crucial importance of androgens, acting on the androgen receptor, for the activation and maintenance of adequate sexual functioning (Bagatell et al., 1994; Schmidt et al., 2009). Estrogens are not required (Sartorius et al., 2014). In women, it is not clear if estrogens coming from the ovaries and from aromatization of androgens in other tissues, acting on estrogen receptors, or androgens, mainly coming from the adrenal cortex, and acting on androgen receptors, are needed. There are strong proponents for both opinions (Waxenberg et al., 1959; Tuiten et al., 2000; Cappelletti and Wallen, 2016). However, recent data showing the efficiency of testosterone therapy for treating low sexual motivation in women may settle the issue in favor of actions at the androgen receptor (Traish et al., 2009; Guay and Traish, 2010; Khera, 2015).

Drugs and Sex

There is no lack of anecdotal evidence for the most spectacular drug effects in humans, but there are very few controlled studies. Still worse, the results of these studies are often contradictory. In fact, there are very few drugs for which there is solid evidence for some effect on human copulatory behavior. We will now examine these drugs. We do not consider the drugs improving erection as drugs modifying copulatory behavior, even though they make that behavior possible.

The time from vaginal penetration until ejaculation is called the intravaginal ejaculation latency. Some men ejaculate with a very short latency. Even though this is an expression of normal interindividual variation, it is considered a pathology with the label premature (early) ejaculation. A drug, dapoxetine, prolongs this latency in men diagnosed with premature ejaculation (Yue et al., 2015; Russo et al., 2016). The drug is, in fact, the only pharmacological treatment for premature ejaculation approved in Europe and in many other countries (excepting the United States).

Dapoxetine is a fast-acting specific serotonin reuptake inhibitor (SSRI). Not surprisingly, some of the SSRIs used for the treatment of depression have also been employed for the treatment of premature ejaculation, with results equally good or better than those reported for dapoxetine (reviewed in Waldinger, 2007). It is noteworthy that the ejaculation-delaying effect is desirable in men suffering from premature

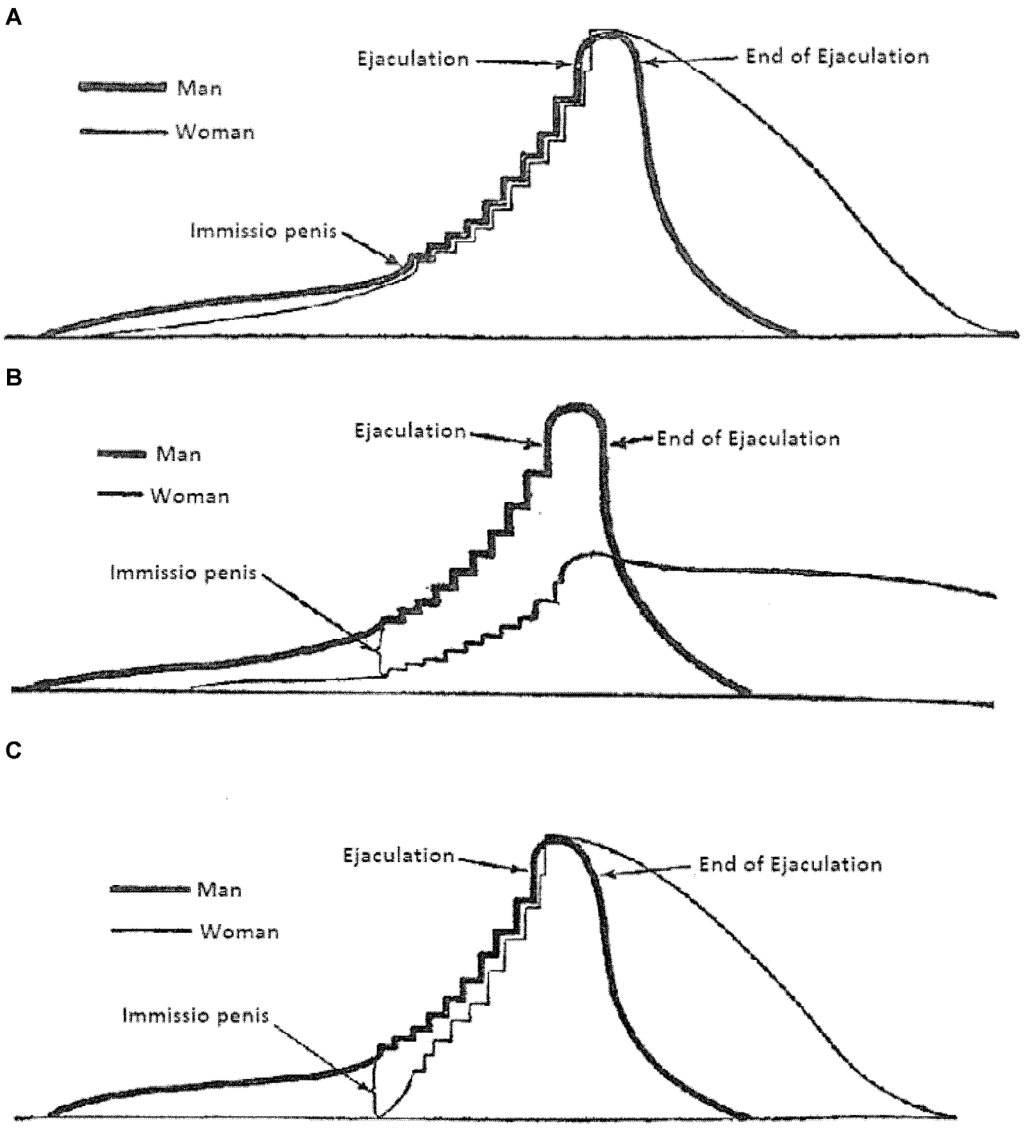

FIGURE 2 | van de Velde's (1926) illustration of human coital interactions. **(A)** The changes in sexual excitation during an ideal copulatory encounter. With ideal it is understood that the man and the woman reache orgasm at the same time. Excitation is defined as the summation of sexual desire and pleasure, bodily and psychic. **(B)** Similar to panel **(A)**, but here the sexual interaction occurs with an inexperienced woman without adequate coital stimulation. **(C)** Similar to panel **(A)**, but now the woman is sexually experienced. The prelude was omitted, but the woman's low initial excitation was compensated for by her experience. From van de Velde (1926).

ejaculation, while it is regarded as an unpleasant side effect in men taking SSRIs for the treatment of depression. In fact, iatrogenic (caused by a presumably therapeutic treatment) sexual dysfunction is considered a serious problem with the SSRIs, and it is sometimes supposed to be the most frequent cause for abandoning treatment (e.g., Kennedy and Rizvi, 2009). This assertion, however, has no support in clinical data. Non-sexual side effects or lack of antidepressant effect are the main causes for discontinuation of treatment with the SSRIs (Bull et al., 2002). Nevertheless, deleterious effects of these drugs on sexual functions are not uncommon. All facets of sexuality, from desire through arousal to orgasm, have been reported to be affected by the SSRIs, in both men and women (reviewed in Rosen et al., 1999; La Torre et al., 2013). Delayed ejaculation in men and anorgasmia in women might be the most common adverse effects, but the poor quality of the clinical data precludes any firm conclusion (Kronstein et al., 2015). Indeed, in a carefully conducted, double-blind study on healthy, young men fluoxetine had no significant effect on any parameter of sexual function (Madeo et al., 2008). It appears that the effects of SSRIs on human sexual performance are inconsistent.

Multiple Orgasms and Ejaculations

We have not been able to find any experimental data concerning the latency to orgasm from the moment of penile penetration into the vagina until orgasm in women. However, orgasm induced by masturbation (clitoral stimulation) has been carefully studied. The mean orgasm latency is usually around 7 min, and the duration of orgasm is about 20–30 s when objective measurements (change in vaginal blood flow or vaginal and anal contractions) are used (see Levin and Wagner, 1985, and references therein). Interestingly, self-reports of orgasm duration did not correlate with the physiological measurements, prompting Levin and Wagner (1985; p. 439) to remind us of the fact that "data obtained . . .. . . from questionnaires or interviews have suspect validity."

There are many reports of women experiencing multiple orgasms in the course of a single sexual encounter (see Darling et al., 1991). Estimates of the proportion of multiorgasmic women range from 42.7% in the Darling et al.'s (1991) study to 14% in Kinsey et al.'s (1953) classical study. The interval between successive orgasms varies between a few seconds and a few minutes, and the number of sequential orgasms varies between 2 and 20 (Kinsey et al., 1953; Darling et al., 1991). The duration of a sexual encounter, from vaginal penetration until the last orgasm, is not known.

In healthy, young men the mean intravaginal ejaculation latency has been found to be 3.01 min (Kreutzer et al., 2001) in one study and 5.4 min in another (Waldinger et al., 2005). It appears that most sexual encounters end after the first ejaculation, although there are scant data supporting this assertion. In any case, detumescence follows ejaculation, and there is a period of time, called the post-ejaculatory refractory period, during which another erection is impossible.

The fact that there are almost no studies of the "refractory period" in men, has not impeded scientists from publishing reviews of the subject with irregular intervals (e.g., Levin, 2009; Seizert, 2018). One of the few published experimental studies used young men as subjects. They were asked to watch a pornographic video while erection (tumescence and rigidity) was monitored (Ekmekçioğlu et al., 2005). When erection was complete, the men applied mechanical stimulation to the penis until ejaculation. The mean ejaculation latency (time from the start of stimulation until ejaculation) was 2.2 min, not dramatically different from that measured in copula. The sexually relevant stimulation (pornographic video) continued after ejaculation. About 80% of the men showed complete detumescence after ejaculation, whereas the remaining proportion showed only partial detumescence. However, 68.2% of the men showed a second erection, indistinguishable from the first. The mean interval between ejaculation and the following erection was 19 min. Other studies employing a similar procedure have reported mean post-ejaculatory refractory periods of 11 (Aversa et al., 2000) and 13.8 (Mondaini et al., 2003) min. However, in these studies, the subsequent erection was not detected by objective procedures. The subjects themselves judged when it occurred.

Considering that a majority of men are able to have a new erection a couple of minutes after ejaculation, and that women may experience many orgasms in rapid succession, we need to explain why sexual encounters usually terminate after the man's first ejaculation. Many explanations have been launched, but none is beyond the stage of speculation (for a good example, see Turley and Rowland, 2013). It must also be mentioned that human copulatory behavior in informal settings, such as sex clubs, have been reported to consist of a series of ejaculations with different partners in men, and sequential orgasms with different partners in women. Also in the latter cases, the cause for ending copulatory activity remains unknown.

We propose that a very simple mechanism, negative alliesthesia, can offer a conceptual, but not neurobiological, explanation. Briefly, alliesthesia refers to the frequently observed fact that exposure to a reward momentarily reduces the value of that reward. For example, rats and humans like sweet solutions, and avidly consumes such solutions when made available. If they are pre-exposed to a small amount of the solution, they will consume far less than when non-exposed (Cabanac and Duclaux, 1973). Although negative alliesthesia first was reported for tastants, it also operates for other kinds of stimuli (Brondel and Cabanac, 2007). In the context of sex, having achieved one ejaculation or orgasm may reduce the reward value of sexual activity, and consequently the incentive value of sexually relevant stimuli. Thus, sexual activity ceases. Some humans may require more prolonged sexual activity before the negative alliesthesia has built up to the level required for ceasing sexual activity, and therefore continue copulating beyond the first ejaculation. The mechanisms underlying sexual alliesthesia are unknown, but the present notion provides at least a conceptual framework for the pursuit of these mechanisms.

Negative alliesthesia should not be confounded with habituation. The latter phenomenon requires repeated exposure to a constant stimulus, whereas negative alliesthesia may occur after a single exposure, as in humans ceasing to copulate after one orgasm. Furthermore, habituation is a case of non-associative learning, whereas alliesthesia refers to change in the reward value of a stimulus. However, in multiple ejaculators, like male rats, habituation to a female probably contributes to the end of sexual activity. In humans, this is probably not the case.

RODENTS

Copulatory behavior in rodents consists of a series of stereotyped motor patterns performed in an ordered sequence. Since the rat is the most studied and still most used species, we will limit the following description to male and female rats. Sexual behavior in mice, hamsters, and guinea pigs are somewhat different, but in all these species it is still a series of stereotyped motor patterns, and the central nervous control of this behavior is quite similar.

Description: The Female Rat

The basic element of female rat sexual behavior is the lordosis posture, a concave arching of the back, stretched hind-legs, and the tail moved to one side (Pfaff et al., 1973). This posture exposes the vaginal opening, making it possible for the male to achieve vaginal penetration, in the rat literature called intromission. We will consistently use the term intromission when talking

about copulatory behavior in rodents. In addition to lordosis, the female will often rapidly shake her head up and down and sideways, giving the impression that she wiggles the ears. Lordosis and ear-wiggling are activated by tactile stimulation from the male. Although stimulation of the flanks and rump is most efficient for activating lordosis, stimulation of any part of the body can be enough. There may also be some ear-wiggling without direct physical contact with the male. Finally, the female can approach the male, and then suddenly run away with darting or hopping movements. This behavior is called solicitation. The exact stimulus responsible for activating solicitation is unknown. Ear-wiggling and solicitation are frequently grouped together under the label paracopulatory or proceptive behavior (Erskine, 1989) Sexual encounters between a male and a female rat can be arranged in many ways. The most common is to put the animals together in a small cage and observe what they are doing. A variant is to divide the cage in halves with a wall having one or several holes. The size of the holes can be adjusted so that the slim female can move between halves whereas the fat male remains confined to one half. The female can thus escape from the male to her own half whenever she finds it convenient. An entirely different procedure is to create an environment somewhat similar to rats' natural habitat. This can be done by combining a large open space with an artificial burrow, and allow a mixed sex group to live in the environment for some time. Such seminatural environments have been used only in a handful of studies of sexual behavior (reviewed in Chu and Ågmo, 2016b).

In the small cage, the members of the pair have no escape from each other. In the divided cage, the female has the privilege to escape from the male. It is often maintained that the female controls sexual interaction in this situation. In seminatural environments, both the female and the male can escape whenever they want, simply because of the size of the environment and the availability of easily defended nest boxes. In the latter situation, both males and females can and do control sexual interactions. If we want to study the ordered sequence of events constituting copulatory behavior, and obtain meaningful results, the small cage must be avoided. Since it fails to give the rats an opportunity to escape, and since escape is a fundamental part of sexual interactions among wild rats observed in their natural habitat (Calhoun, 1962; Robitaille and Bouvet, 1976), the small cage lacks external validity in the brunswikian sense. According to Brunswik (1955), an externally valid design should either be a random sample of experimental procedures in which the target event may occur or the test procedure should be as similar as possible to the subjects' natural habitat (see also Petrinovich, 1980). Studies failing to incorporate at least one of these criteria lack external validity, and results cannot, therefore, be generalized beyond the specific procedure used. The divided cage and the seminatural environment offers surprisingly similar descriptions of the structure of female sexual behavior, and can probably be considered as externally valid.

In the divided cage, the female will sooner or later enter the male's half, and the male will sooner or later mount the female. The mount may or may not be transformed into an intromission. If it is, the female will usually return to her half of the cage. If the mount ends without intromission, the likelihood for the female to escape to her own half is not above random (Ellingsen and Ågmo, 2004). In case the male ejaculates, the likelihood for the female escaping to her half of the cage is higher than it is after an intromission. Furthermore, the time she will remain in her half of the cage is longer than after an intromission. Thus, the likelihood of escape from the male and the time the female remains inaccessible are directly proportional to the intensity of sexual stimulation received (Erskine, 1989). **Figure 3** illustrates the typical temporal sequence of female rat sexual behavior. The important thing to observe here is that female rat sexual behavior is a series of approach–avoidances.

Approach is activated by attractive stimuli whereas avoidance is a response to aversive stimuli. Therefore, during sexual interaction, the male is transformed from an attractive to an aversive stimulus by intromission and ejaculation. At the same time, intromission and ejaculation cause positive affect (see "Multiple Ejaculations and Orgasm" section).

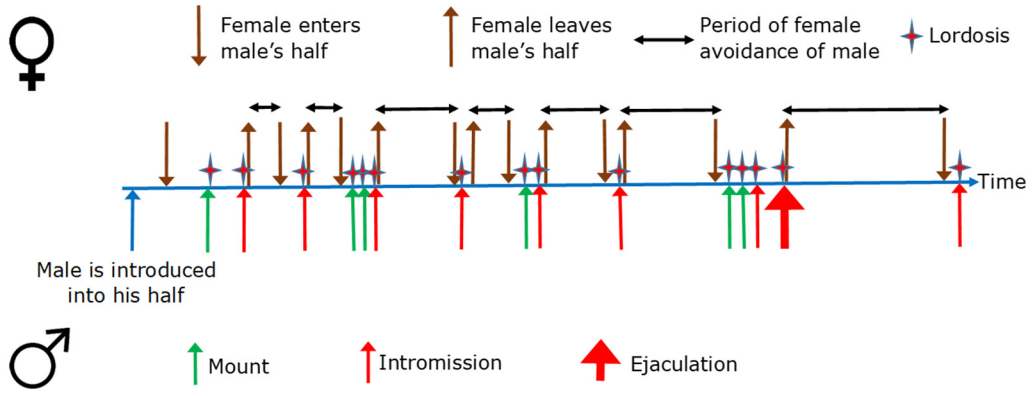

FIGURE 3 | Sexual behavior in a cage in which the female can escape from the male copulation partner. The female is present already at the beginning of the timeline. The horizontal, double-ended black arrows represent the time the female has escaped from the male into her own half. The illustration ends after the first intromission following the first ejaculation. Copulation may have continued for several ejaculations, however.

The mechanisms behind the contradictory reactions of the female are not entirely known, but some informed speculations have been made (Komisaruk and Whipple, 2000). One possible explanation is that mechanical stimulation of the vaginal wall momentarily reduces sexual motivation and causes short-lived pain.

In females in the seminatural environment, the interval to the next sexual event is less than 3 min after having received a mount. After an intromission it is about 5 min, and after an ejaculation it is about 13 min (Chu and Ågmo, 2014). During these intervals, the females are engaged in non-sexual activities or resting. These data show that sexual interactions in a seminatural environment have consequences similar to what was described for the divided observation cage. Despite the fact that three rather than one male were able to copulate with the female in the seminatural environment, the relationship between the amount of sexual stimulation received by the female and the interval to the next sexual event remained similar to that observed in the divided cage. Thus, female sexual behavior is a sequence of approach–avoidance also in a seminatural environment (Chu and Ågmo, 2014).

The female rat copulatory behavior pattern, lordosis, has a duration of 1–2 s (e.g., Ellingsen and Ågmo, 2004). In a seminatural environment, intact females display a total of about 200 lordosis during the period of behavioral estrus. This period has a mean duration of 7.3 h (Chu and Ågmo, 2014). For about 400 s of this time, the female is engaged in actual copulatory behavior, i.e., 0.015% of the time. The overwhelming majority of time was spent in other activities, unrelated to sex. These other activities were now and then interrupted by sexual acts. Data from a seminatural environment confirm that copulation in the female rat consists of a series of intermittent, short interactions with males.

Description: The Male Rat

Turning to the male, we find the same sequence of approach–avoidance as in the female. In fact, it will soon become evident that there is a surprising similitude between male and female rat sexual behavior. Whereas the basic female sexual behavior pattern is the lordosis, the mount is the basic male behavior pattern. When mounting, the male stands on his hind legs with his forepaws placed on another rat's rump from behind while performing a series of antero-posterior pelvic movements, thrusting. Accelerometric studies of the movements during copulation have shown that the mount is extremely stereotyped (reviewed in Moralí and Beyer, 1992) with a mean duration of about 400 ms and a thrusting frequency of about 18 Hz. During some mounts, the erect penis will make contact with the vaginal orifice. The male will then make a strong forward thrust leading to intromission. The duration of the intromission is about 400 ms. The male will thereafter dismount with a vigorous backward thrust. After a couple of intromissions, ejaculation will occur. Penile insertion lasts longer (1–2 s) and is accompanied by intravaginal thrusting and the expulsion of semen. The male dismounts slowly, without any backward thrust.

A mount not ending in intromission may be succeeded by another mount within a few seconds. An intromission will be followed by a short period of inactivity or non-sexual activities. In our laboratory, sexual quiescence after a mount bout with or without intromission lasts 42 ± 13.6 s (median ± semi-interquartile range), based on data from 143 rats tested in heterosexual pairs in a small cage. In these same males, quiescence following ejaculation lasted 301 ± 40.3 s. The conclusion to be drawn from this is that the period of sexual inactivity following a sexual interaction depends on the intensity of that interaction in males as well as in females.

In a seminatural environment, male sexual behavior is also a sequence of discrete events followed by long periods of non-sexual activities or complete inactivity. In fact, during periods of sexual activity the males spend 77% ± 4% of the time resting and grooming, while 8% ± 2% was spent on pursuing the female. Only 0.3% of the time was used for the execution of copulatory acts, i.e., mount, intromission and ejaculation (Chu and Ågmo, 2015b). Periods of sexual activity were defined as the time between the first mount or intromission recorded until the last copulatory event before a period of inactivity exceeding 60 min. An example of male sexual behavior in a seminatural environment can be found in **Figure 4**.

Endocrine Control

It is established beyond doubt that gonadal hormones are required for the display of copulatory behaviors in male and female rats (for recent reviews, see González-Flores et al., 2017; Hull and Rodríguez-Manzo, 2017). In many strains of rats, simultaneous activation of both androgen and estrogen receptors are needed for male sexual behavior to occur. In female rats, androgen receptors do not contribute to sexual behaviors.

Drugs and Rat Sex

Pharmacological studies of rat sex behavior were once upon the time very popular. We will make no intent to review the voluminous literature. This declining field has been reviewed many times before (e.g., Bitran and Hull, 1987; Paredes and Ågmo, 2004; Snoeren, 2015; Uphouse, 2014). Instead, we will focus on the few kinds of drugs having known effects on human copulatory behavior. These are, as mentioned, limited to dapoxetine and other SSRIs. Although the effect of flibanserin is questionable in humans, we will also mention the few studies performed in rats.

The SSRIs fluoxetine and paroxetine have been shown to enhance ejaculation latencies in rats (e.g., Vega Matuszczyk et al., 1998; Waldinger et al., 2002). However, we were not able to see any effect of fluoxetine (**Figure 5**). This negative finding is in agreement with other studies (e.g., Frank et al., 2000). The only possible conclusion is that the effects of SSRIs in male rats are inconsistent. This is not surprising since only about 20%–30% of men treated with SSRIs for depression report sexual side effects and only part of those report delayed ejaculation. It is unlikely that prolonged ejaculation latency in such a small proportion of the experimental subjects could render the effect statistically significant.

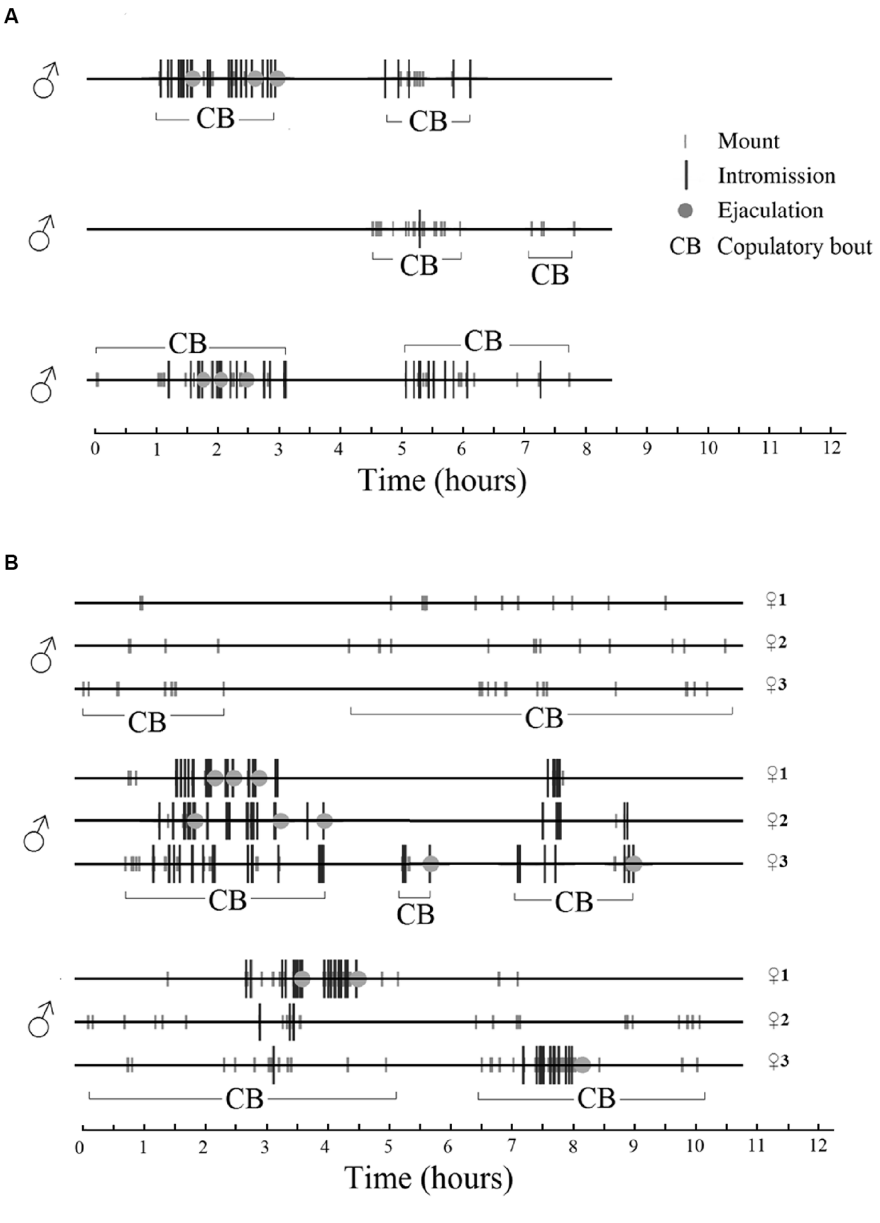

FIGURE 4 | Sexual behavior displayed by male rats in a seminatural environment during females' natural estrus. There were three males in the environment. Time 0 represents the beginning of estrus, i.e., when the female or females presented their first lordosis. A copulatory bout (CB) is a period of continuous (adjacent copulatory events are separated by less than 60 min) male sexual activity. **(A)** One single female is in estrus. All males copulate with the female during overlapping periods. **(B)** Three females are in estrus simultaneously. Each male copulates with the three females, and each female copulates with all the males in overlapping periods. Rat copulatory behavior seems to be entirely promiscuous, perhaps similar to what is observed in sex clubs frequented by humans (see "Multiple Orgasms and Ejaculations" section). For further details, see Chu and Ågmo (2015b). Reprinted with permission from the American Psychological Association.

Dapoxetine enhances the ejaculation latency in male rats as it does in men, but only in rats selected because of their initially short latency (Clement et al., 2012; Olayo-Lortia et al., 2015). For obvious reasons, there has been no interest in studying the effects of dapoxetine on female rat copulatory behavior.

There are several reports of reduced lordosis and paracopulatory behavior in female rats treated with fluoxetine (e.g., Matuszczyk et al., 1998; Maswood et al., 2008; Ventura-Aquino and Fernández-Guasti, 2013b). However, another SSRI, paroxetine, does not alter female rat copulatory behavior in any way (Kaspersen and Ågmo, 2012), not even after a very long treatment period (Snoeren et al., 2011). As was the case for the male rat, SSRIs have inconsistent effects on female copulatory behavior.

Flibanserin, an agonist at 5-HT_{1A} receptors and a weak antagonist at 5-HT_{2A} receptors, has been tested in rats.

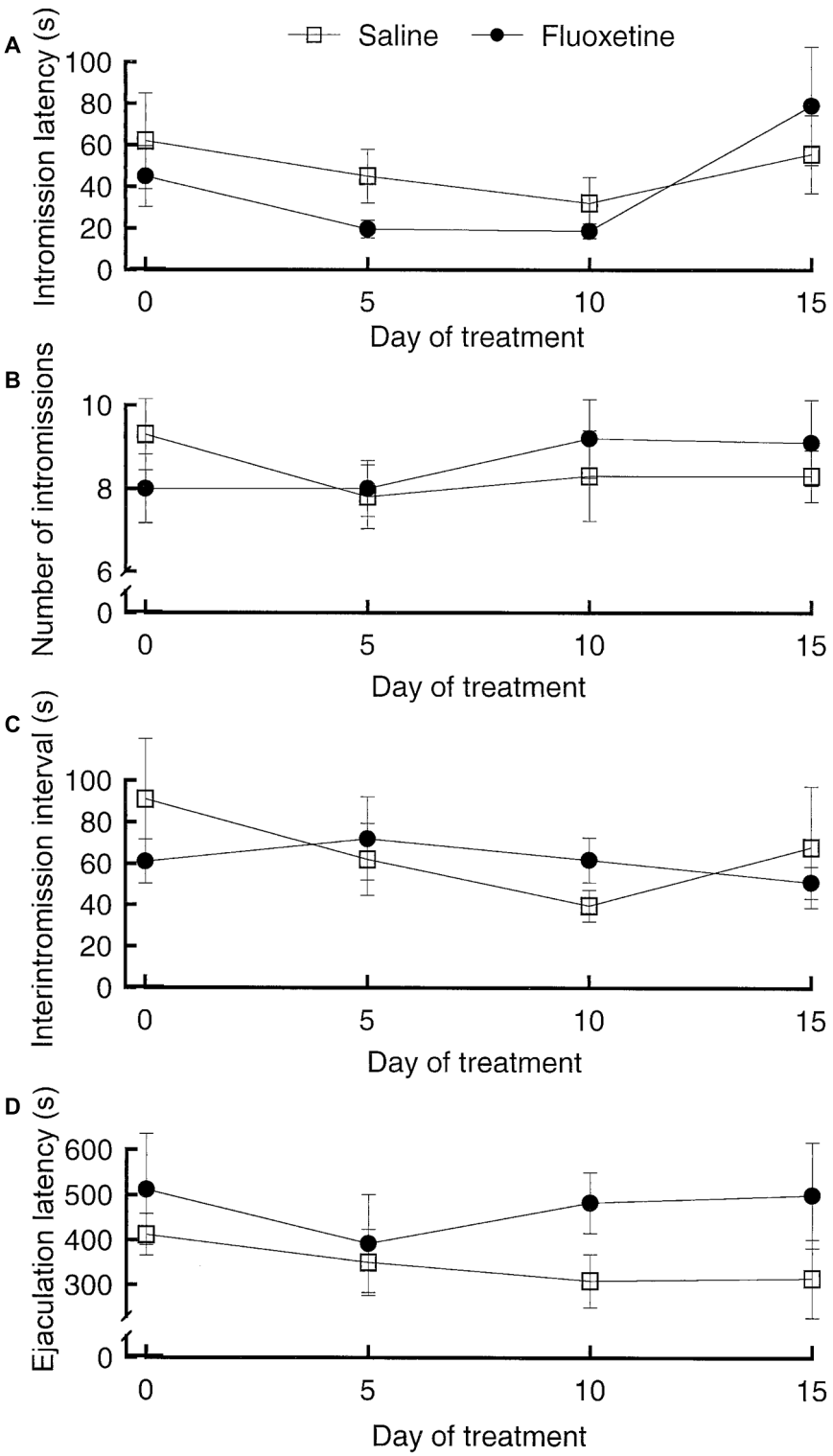

FIGURE 5 | Parameters of copulatory behavior in male rats treated with fluoxetine, 10 mg/kg per day orally, or saline for 15 days. The test on Day 0 was performed 1 h after the first fluoxetine administration. **(A)** Intromission latency. Analysis of variance (ANOVA) for repeated measures on the factor Day of treatment and independent measures on the factor Treatment failed to reveal any statistically significant effect of Treatment, of Day of treatment and of the interaction Day × treatment (all *p*s > 0.07). **(B)** Number of intromissions. Also here, ANOVA failed to detect any significant effect (all *p*s > 0.51). **(C)** Interintromission interval. No effect (all *p*s > 0.39). **(D)** Ejaculation latency. No effect (all *p*s > 0.13). Data are mean ± standard error of the mean (SEM). Data are from an unpublished experiment, performed by one of the present authors (AÅ) together with Juoni Sirviö, Gro Sandberg and Live Sørensen.

The drug enhanced the number of solicitations displayed by ovariectomized females after 2 weeks of treatment and on. Females treated with estradiol benzoate (EB) only or with EB combined with progesterone (P) responded in the same way (Gelez et al., 2013).

Multiple Ejaculations and Orgasm

It is not known whether rats experience something similar to the human orgasm. Some argue that they do (Pfaus et al., 2016), whereas others consider it unnecessary to employ anthropomorphisms to explain rat behavior (e.g., Ågmo, 2007). Nevertheless, there are much data showing that sexual activity leads to positive affect in both male and female rats. Events or activities causing positive affect are considered to be rewarding. Sexual reward has been extensively studied with the conditioned place preference procedure, a procedure often used to evaluate positive affect induced by natural rewards as well as by drugs like morphine, cocaine and amphetamine. Ejaculation produces a robust place preference (e.g., Ågmo and Berenfeld, 1990). Several intromissions without ejaculation are also able to produce place preference, although ejaculation seems to be more efficient (Camacho et al., 2009; Tenk et al., 2009). Mounts without intromission are not enough. In the female, the receipt of several intromissions causes place preference, regardless of whether she copulates in the divided observation cage (Paredes and Alonso, 1997; Paredes and Vazquez, 1999) or in a small cage (Meerts and Clark, 2007, 2009b). Even artificial stimulation of the cervix works well in this procedure (Meerts and Clark, 2009a). It may be interesting to note that even prolonged copulation, leading to the receipt of several ejaculations, is just as rewarding as copulation limited to one ejaculation or 15 intromissions (Arzate et al., 2011). Regardless of whether the sexual reward experienced by rats have anything in common with the human experience of orgasm or not, we can conclude that sexual activities are rewarding for both rats and humans.

The female rat will display lordosis to every male mount during the entire period of behavioral estrus, and show undiminished amounts of paracopulatory behaviors until the abrupt end of estrus when observed in a seminatural environment (Chu and Ågmo, 2014, 2015a). In the divided cage, there is no reduction in lordosis responses after prolonged copulation, but the rate of paracopulatory behaviors is reduced when the female has received several ejaculations (Ventura-Aquino and Fernández-Guasti, 2013a). We have confirmed that observation in females having received five ejaculations during a prolonged test in a divided cage. However, although the rate of paracopulatory behaviors was lower in the 5th ejaculatory series than in the first, the number of these behaviors remained constant (**Figure 6**). Since the intensity of male behavior was much reduced in the 5th series, the interval between copulatory interactions increased, and the duration of the series was far longer than for the first series. Thus, even though a constant number of behaviors were displayed, the rate was inevitably reduced.

In conclusion, male and female rats continue to copulate for extended periods. The mechanisms underlying the end of a

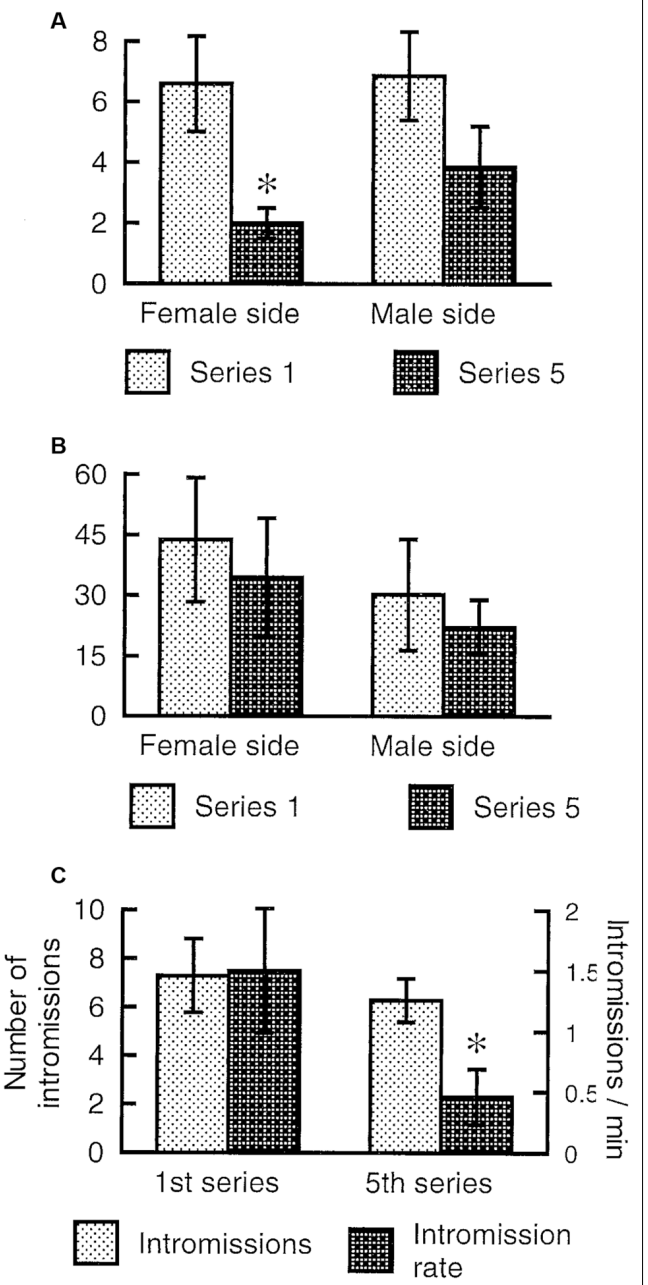

FIGURE 6 | Paracopulatory behaviors displayed by female rats tested in a divided cage until the male had achieved five ejaculations. We determined the number of those behaviors displayed in the male's half as well as the number displayed in the female's half during the 1st and 5th ejaculatory series. **(A)** The rate expressed as number per minute of paracopulatory behavior. Two-factor ANOVA with repeated measures on one factor (Series) revealed a main effect of Series ($F_{(1,6)} = 20.269$, $p = 0.004$) but not of Side (female, male; $F_{(1,6)} = 0.778$, $p = 0.412$). The interaction Series × Side was also non-significant ($F_{(1,6)} = 1.326$, $p = 0.293$). **(B)** Number of behaviors. ANOVAs found no effect (all $ps > 0.24$). **(C)** Male copulatory behavior. The number of intromissions performed during the 1st and 5th series was similar ($t_{(6)} = 0.786$, $p = 0.462$, paired t-test). The rate of intromission, calculated as number per minute, was lower in the 5th series than in the 1st ($t_{(6)} = 2.691$, $p = 0.018$). Data (mean ± SEM) are from an experiment performed by Ellinor Ellingsen and Ana Lene Turi. *Different from 1st series ($p < 0.05$).

period of sexual activity remain obscure. In the male, the last event of a bout of copulatory activity is equally often a mount, an intromission or an ejaculation (Chu and Ågmo, 2015b). In the female, it seems that the end of sexual activity is associated with a sudden loss of attractivity to the males, at least in a seminatural environment (Chu and Ågmo, 2015a). As was the case with humans, the neurobiological mechanisms behind the end of copulatory activity in rats are unknown. Again, we propose that negative alliesthesia can be used as a conceptual basis for future work.

COPULATORY BEHAVIOR IN RATS AND HUMANS: SOMETHING IN COMMON?

Concerning the structure of copulatory behavior, the differences between rats and humans are abysmal. Whereas a sexual encounter in rats consists of a series of very short periods of genital contact interrupted by long periods of non-sexual activities, in humans a sexual encounter is continuous, normally without any intrusion of non-sexual activities. The apparent dissimilarity in copulatory behavior may not apply to the underlying mechanisms controlling basic processes, though. In the human, ejaculation is triggered by continuous mechanical stimulation of the glans penis. In rats, the mechanical stimulation is intermittent. However, each intromission leads to a gradually increasing excitation, continuing to increase for several minutes post-intromission. The excitation is reinforced by the following intromissions until an ejaculatory threshold is reached (Larsson, 1960; extensively discussed in Ågmo, 2011). Thus, the difference between rats and humans is that in rats, a gradually increasing excitatory state is produced by intermittent mechanical stimulation whereas humans require continuous mechanical stimulation. In both species, ejaculation occurs when the excitation surpasses a threshold. Since the ejaculation latency is somewhat shorter in men than in rats, it appears that continuous stimulation causes a more rapid increase in excitation than intermittent stimulation does.

Male rats ejaculate many times before reaching sexual exhaustion, whereas most humans end a sexual encounter after the man's first ejaculation. Whether this is a result of social learning or of the inherent nature of human copulatory behavior is not known, since the mechanisms causing cessation of copulatory activity are unknown. Likewise, it is not known if female rats experience something similar to orgasm in women. Consequently, we cannot know if rats, like women, may have multiple orgasms during a single sexual encounter.

Copulatory behavior is dependent on gonadal hormones in rats and humans, even though the crucial hormones may be different. In male rats, the simultaneous action of androgens and estrogens is necessary, whereas only androgens may be sufficient in men, as already mentioned. In female rats, estrogen and progesterone synergize to induce sexual behavior, whereas the role of these ovarian hormones is unclear in women, as pointed out above. Androgens do not contribute to female rat sexual behavior, but they may be important in women. There are also similarities in drug actions in rats and humans, despite the large differences in copulatory behavior. The most important similarities and differences in copulatory behavior between rats and humans are summarized in **Table 1**.

TABLE 1 | Comparison of some basic characteristics of copulatory behavior in rats and humans and the responses to drugs used clinically for the treatment of sexual dysfunctions.

	Rats	Humans
General		
Copulation is continuous	No	Yes
Copulatory motor patterns	Highly stereotyped	Extremely variable
Multiple ejaculations	The rule	Occasionally
Ejaculation latency[a]	∼7 min	3–5 min
Post-ejaculatory period[b]	∼5	∼19 min
Multiple female orgasms	?	Yes
Latency to orgasm	?	∼7 min[c]
Depends on gonadal hormones[d]	Yes	Yes
Drug effects		
SSRI	May enhance ejaculation latency	Inhibition in some individuals
Dapoxetine	Enhances ejaculation latency	Enhances ejaculation latency
Flibanserin	Stimulates paracopulatory behaviors	Stimulates desire in women?

?Unknown or uncertain effect; [a]time from the first vaginal penetration until ejaculation in rats, time from penile insertion until ejaculation in men; [b]time from ejaculation until the following vaginal penetration in rats, time from ejaculation until next erection in men; [c]time from the start of clitoral stimulation until orgasm. Latency in copula is unknown. [d]Males and females collapsed.

SEXUAL APPROACH BEHAVIORS

We have already mentioned that copulatory behavior requires physical proximity of at least two individuals, and that copulation, therefore, must be preceded by approach behaviors. In fact, van de Velde (1926) described the first phase of a sexual encounter, the prelude, in the following words: "As soon as the first stirrings of the impulse of approach are perceptible, the prelude to sexual union begins" (from a reprint of the English translation, van de Velde, 1926, p. 102). This idea is not much different from Kaplan's (1995) notion of the desire phase. For these and other reasons, we have suggested that the intensity of sexual approach behaviors is an exquisite indication of the intensity of sexual motivation (Ågmo et al., 2004; Spiteri and Ågmo, 2006; Ågmo, 2014). It has even been argued that the intensity of copulatory behavior is not an indicator of sexual motivation. In fact, Meyerson and Lindström (1973, p. 1) wrote: "However, the intensity of the copulatory act or the readiness to respond to mating attempts of another individual cannot be taken as a measure of sexual motivation. It is the eagerness to seek sexual contact, not the consummatory act which interests us." This is certainly an exaggeration, but there is no doubt that studies of sexual approach are most informative in rodent studies (for a discussion, see Ågmo, 2014).

The approach behaviors are as variable in rats as they are in humans. A rat can walk, run, jump, swim or dig in order to approach a potential mate, and a human can engage in all kinds of activities with the purpose of establishing contact with and approach to a desired individual. This

means that we cannot describe sexual approach behaviors in terms of particular motor patterns. Consequently, it seems reasonable to consider all actions leading to reduced distance to a potential mate as sexual approach behavior. It is, however, most important to distinguish sexual approach from non-sexual or social approach. van de Velde (1926) simplified the issue by making the rather grotesque assumption that any "stirring of the impulse of approach" is a manifestation of the desire to establish a sexual encounter. However, both humans and rats are social animals, and most approaches to other individuals are made because of purely social motivation. In rats, experimental setups can be arranged so that sexual approach can be clearly distinguished from social approach. To the contrary, in humans this distinction can rarely, if ever, be made. It could be assumed that a purely heterosexual woman approaches other women for uniquely social reasons. However, if she would approach a man, it could be either because of social motivation, sexual motivation, or a combination of both. In fact, the quantification of the intensity of human sexual approach behavior, uncontaminated by social approach, is extremely difficult or perhaps impossible. As we soon will see, this conundrum has been solved by replacing studies of human approach behaviors with studies of genital responses. Such responses cannot be regarded as manifestations of social motivation. To the contrary, it is generally accepted that they represent sexual motivation and nothing else.

Sexual Approach in Men and Women

There are, for the reasons mentioned in the preceding section, no experimental studies of the behavior patterns employed by humans when sexually approaching other individuals. There are many literary or anecdotal descriptions, but the scientific value of these anecdotes is most doubtful. Likewise, the many manuals explaining how to successfully approach and seduce men or women are of little help for scientists. Nevertheless, there are some possibilities to objectively evaluate something that might approximate human sexual approach behavior.

As was outlined in the model of sexual motivation illustrated in **Figure 1**, a sexual incentive will activate approach behavior and visceral responses, provided the stimulus is presented in an adequate context. Among the most reliable visceral responses to sexual incentives is enhanced genital blood flow, manifested as erection in men and vaginal lubrication in women. Thus, the magnitude of the genital response can be used as a proxy for the impact of sexually relevant stimuli on sexual motivation. The latter is the factor causing the individual to engage in approach behaviors. It must be mentioned that in the human literature, the genital response to sexual incentives is called "sexual arousal."

The complex relationship between the genital responses and the subjective experience of these responses, as reported on a questionnaire, is beyond the scope of the present discussion. It has been brilliantly reviewed elsewhere (Meston and Stanton, 2019). In our opinion, the notion of subjective sexual arousal does not contribute to our understanding of the mysteries of sexual motivation (Ågmo, 2008). It cannot be used for non-human animals, for example. In the following, we will use genital blood flow as the sole reliable indicator of sexual motivation or desire in the human.

External Stimuli and the Activation of Sexual Motivation in Men and Women

We have already mentioned that humans may use mental representations (fantasies) of sexually relevant stimuli instead of external stimuli for the activation of sexual responses, including orgasm in women. There are also observations showing that fantasies make an important contribution to sexual desire (Birnbaum et al., 2019). Unfortunately, these private events are difficult to investigate with experimental methods, and are beyond the scope of the present communication. We will, therefore, only discuss external stimuli. The initial activation of sexual motivation, and consequently of sexual approach behaviors and genital responses, must be achieved by distant stimuli, i.e., olfactory, auditory, or visual. Once approach has been successful, tactile stimuli will become crucial for the further enhancement of sexual motivation and the eventual initiation of copulatory activities.

To our knowledge, there is only one study in which the stimuli important for sexual approach in the human has been described in a non-laboratory setting. The probability for a woman to be approached by a man in a nightclub depended on the amount of naked flesh exposed and the amount of sexually suggestive dance movements made (Hendrie et al., 2009). None of the other stimuli emitted by a woman, for example facial expressions or verbal activities, had any effect. This study seems to be unique in the way that direct approach rather than genital responses to sexually relevant stimuli was observed. However, the nightclub setting imposes many limitations, and experimental studies of actual approach behavior are desperately needed before any conclusion can be presented as to the exact stimuli causing this approach. In the meantime, we need to base our knowledge of the stimuli activating sexual motivation on studies of genital responses.

In both men and women, visual and auditory stimuli with sexual content are efficient for activating genital responses, and the combination of these modalities is still more efficient (McConaghy, 1974; Gaither and Plaud, 1997). In fact, moving pictures of diverse sexual activities, in heterosexual or homosexual pairs, and sometimes in groups, are routinely used in laboratory studies of genital responses. In vernacular language, this kind of movies are called pornographic. In the scientific literature, the euphemism erotic is often used, for some unknown reason. There is an extensive literature on the importance of the content in written descriptions of sexual activities or in pornographic movies, in relation to the sex of those depicted as well as of those observing, and of preferences for the own or the opposite sex (reviewed in Rupp and Wallen, 2008). We will ignore this literature, and simply conclude that the modalities of vision and audition are crucial in human sexual approach. There is no evidence for any role of olfactory stimuli, despite the widespread belief to the contrary (for discussion and references, see Ågmo, 2007; Le Moëne and Ågmo, 2017). We regret that this might be inconvenient for the perfume industry and for the sociobiologists.

It must be mentioned that neutral stimuli may acquire the capacity to activate genital responses through learning. In a most elegant study, the presentation of a neutral picture was associated with clitoral stimulation in women. Clitoral stimulation is an unconditioned stimulus causing enhanced vaginal blood flow. After a few pairings, the picture enhanced this blood flow by itself, i.e., it worked as a conditioned stimulus (Both et al., 2008, 2011). There are several other studies showing that classical conditioning can transform any stimulus into a sexually relevant stimulus in men and women (reviewed in Hoffmann, 2017). It is generally believed that this kind of learning is the basis of fetishism (Köksal et al., 2004). In any case, the fact that learning can make any stimulus capable of activating sexual motivation in humans should not be ignored.

Drugs and the Activation of Sexual Motivation (Desire)

Women

The vaginal response to pornographic movies is not altered by menopause (Laan and van Lunsen, 1997; Suh et al., 2004), despite the strong reduction in circulating estrogens associated with that state. This observation can suggest that estrogens are not important for responses to sexually relevant stimuli, or that even in menopause they are above the level required for maximal responding.

One single drug (flibanserin, Addyi®) has been approved by the Federal Drug Administration for the treatment of female hypoactive sexual desire disorder. Initially, the drug was developed as an antidepressant, but it failed both some preclinical tests and a phase II study (Gellad et al., 2015). Since some of the participants in the clinical study reported heightened sexual desire, it was decided to develop the drug for treatment of low sexual desire rather than for depression. Although the phase III studies indeed suggested some effect on sexual desire (DeRogatis et al., 2012; Thorp et al., 2012), it is questionable whether this drug is superior to placebo (Saadat et al., 2017; Anderson and Moffatt, 2018). Perhaps this is related to the fact that flibanserin was approved because of political pressure rather than because of proven efficiency (Woloshin and Schwartz, 2016). Interestingly, the effects of flibanserin on vaginal responses to sexual stimuli has not been evaluated and, as mentioned, its effect on subjective measures, mainly self-reports, of sexual desire is questionable.

Another kind of drugs that might affect sexual motivation in women is the SSRI. As was the case with flibanserin, the effect of SSRIs on vaginal responses to sexually relevant stimuli has not been studied. Thus, we do not know if these drugs are affecting anything else than performance on questionnaires. It is amazing that the rather simple procedures needed for objectively assessing vaginal responses are so rarely used, whereas the notoriously unreliable questionnaires are omnipresent. Most unfortunately, we are constrained to conclude that the effects of clinically used drugs on vaginal responses to sexual stimuli are entirely unknown.

Even though adrenergic compounds are not used for the clinical treatment of sexual desire disorders, a nonspecific adrenergic α and β agonist, ephedrine, has been tested for effects on female sexual functions. The drug increases the vaginal response to pornographic movies (Meston and Heiman, 1998). To the contrary, an α_2 antagonist, yohimbine, has no effect (Meston and Worcel, 2002). This is cumbersome, since blocking the α_2 receptor generally enhances the release of noradrenaline (Gobert et al., 2004). Consequently, yohimbine and ephedrine should have similar effects. To further complicate things, it has been reported that the α_2 agonist clonidine reduces the vaginal response to a pornographic movie (Meston et al., 1997). It seems that the role of the adrenergic receptors in sexual responses in women needs to be further evaluated before any conclusion can be reached.

Men

Seventy-five percentage of castrated men show a drastically diminished penile response to a pornographic movie segment (Greenstein et al., 1995). This is also the case in men suffering from severe hypogonadism after treatment with leuprolide (Schober et al., 2005), a compound inhibiting gonadotropin release from the pituitary. These observations suggest that androgens are needed for the activation of genital responses to sexually relevant stimuli, hence for the activation of sexual motivation.

Some SSRIs are used for treating paraphilia because it has been suggested that this condition may be related to unusually high levels of sexual motivation (e.g., Kafka, 2003). Although there are some data suggesting good effects (Briken and Kafka, 2007), there is no consensus regarding the long-term usefulness of SSRIs (Holoyda and Kellaher, 2016). This may be related to the observation that SSRIs like fluoxetine and citalopram do not alter the penile response to a pornographic movie in healthy young men, not even after 4 weeks of treatment (Haensel et al., 1998; Madeo et al., 2008). This interesting observation suggests that SSRIs do not reduce sexual desire, at difference to widely held beliefs. In fact, in the Madeo et al. (2008) study, the SSRIs not only failed to affect objectively measured sexual arousal but also sexual desire as evaluated by a questionnaire.

A review of sexual functions in people using drugs for recreational purposes, including tobacco and alcohol, concluded that not even these commonly used and socially acceptable drugs have been adequately studied and that no firm conclusion as to their sexual effects could be presented (Zaazaa et al., 2013). Therefore, we will end the account of drug effects here.

We cannot leave this section without addressing the limitations of using genital responses as a proxy for sexual approach behaviors. Even though we maintain that both genital blood flow and sexual approach are manifestations of sexual motivation, we must accept that these expressions of motivation do not always coincide. An eloquent example is the reliable, stimulating effect of sildenafil on penile responses to sexually relevant stimuli, such as pornographic movie segments, in men with (e.g., Gingell et al., 2004) and without erectile dysfunction (e.g., Kolla et al., 2010). However, there is no evidence showing that sildenafil enhances any other aspect of sexual function than erection (Jones et al., 2008). This means that we cannot automatically infer effects on sexual approach behaviors from effects on genital responses. Additional data are always required.

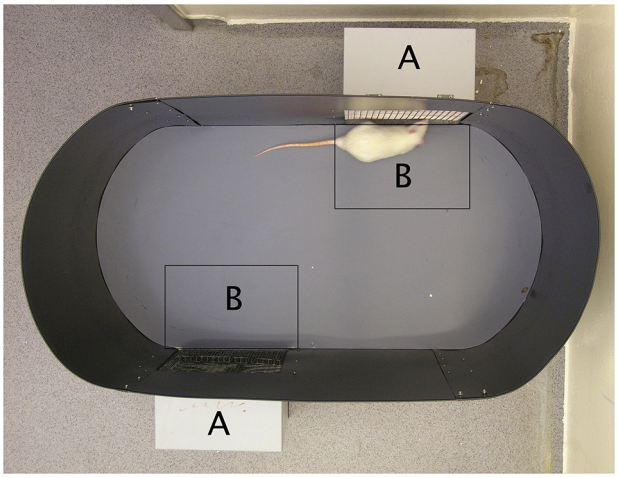

FIGURE 7 | A photograph of the sexual incentive motivation test arena. The incentive animal cages (marked with an A on the photograph) are located on the outside of the oval arena (100 × 50 cm). They are detachable from the outside of the wall so that the position of the incentive animals can be changed randomly. The side facing the arena is made of a wire mesh that allows the experimental subject to see, smell and hear the incentives. A virtual zone of 21 × 29 cm (marked with a B on the photograph) is defined outside each incentive animal cage. A computerized videotrack system determines the experimental subject's position, the time spent in the incentive zones, the number of visits to them, the distance moved during the test, the mean speed of movement while moving, and the immobility time. Reproduced from Spiteri and Ågmo (2006). Copyright 2006, republished with permission from Elsevier.

Unfortunately, in the absence of experimental studies, these additional data have to come from self-reports or questionnaires of some kind.

Sexual Approach in Rodents

The direct observation of sexual approach behavior in rodents does not pose the slightest problem, and there are many established procedures available (reviewed in Ventura-Aquino and Paredes, 2017). We will briefly describe the one that we have used for many years (Ågmo, 2003; Ågmo et al., 2004). The setup is illustrated in **Figure 7**. It has been validated in several ways, and it allows for a clear distinction between approach behavior to a potential mate (sexual approach) on one hand and to a social stimulus (social approach) on the other. In order to determine whether the approach to the sexual incentive really represents sexual motivation, we performed a series of experiments. First, we replaced the sexual incentive with a second social incentive, so that the experimental male or female rat only had social incentives to approach. There was no systematic difference between these two incentives regardless of which specific incentive was used. In fact, all social incentives were about equally attractive. With the regular setup, with a sexual and a social incentive, we then tested male and female subjects that should have no sexual motivation. Castrated males did not distinguish between incentives, and when they were treated with testosterone, they enhanced the approach to the sexual incentive without altering approach to the social incentive (Ågmo, 2003; Attila et al., 2010). In females, there was no variation in approach to the social incentive during the estrus cycle, whereas approach to the sexual incentive peaked in proestrus. Ovariectomized females approached equally the sexual and the social incentive, whereas females given EB alone or EB + P approached the sexual incentive far more than the social incentive (Spiteri and Ågmo, 2006). Finally, we tested males that should have reduced sexual motivation because of immediately preceding sexual activity. Actually, the test was performed after that the males had had continuous access to a receptive female for 4 h. The males did not distinguish between the social and the sexual (a different female) incentive. Likewise, females tested immediately after having received three ejaculations did not approach the sexual incentive more than the social (Ågmo et al., 2004). All these observations made us conclude that the procedure indeed can be used for quantifying sexual motivation expressed as approach behavior.

The Stimulus Control of Rodent Sexual Approach

Exactly as is the case in humans, sexual approach in rats must be activated by a distant stimulus. There is no reason to believe that rats produce mental representations of sexually relevant stimuli, meaning that any manifestation of sexual motivation must have its origin in an external stimulus. We have carefully determined the role of olfactory, auditory and visual stimuli (Ågmo and Snoeren, 2017) in the procedure described in the preceding section. The results of the corresponding experiment are shown in **Figure 8**. It turned out that olfactory stimuli are necessary but not sufficient. The odor of a sexual incentive is not superior to a social incentive. The odor employed was produced by a sexually receptive female left in the incentive cage for 6 h and withdrawn just before the test. Urine, feces and body odors left on the walls and floor were the odor sources. To become superior to a social incentive, odor must be combined with another stimulus, either auditory or visual. The exact auditory and visual stimuli required could not be identified, but we excluded the ultrasonic vocalizations that rats emit in the presence of conspecifics, particularly conspecifics of the opposite sex. Devocalized sexual incentives were not less approached than vocalizing incentives. The lack of importance of ultrasonic vocalizations had already been established in a series of studies in this same procedure (Snoeren and Ågmo, 2013, 2014a,b) and in a seminatural environment (Chu et al., 2017). It must be added that, as is the case in the human, any stimulus may acquire sexual significance through conditioning (see Kvitvik et al., 2010; Chu and Ågmo, 2012; and references therein). For a much more extensive analysis of the stimulus control of sexual approach behaviors, the reader is referred to Le Moëne and Ågmo (2017).

Even though not systematically evaluated in other rodent species, we assume that olfaction is of prime importance. The role of additional sensory modalities is not known.

Drugs and Sexual Approach in Female Rats

Because of scientists' fascination for copulation, studies of sexual approach behavior in rodents are not often performed. For example, the only drug in clinical use for the explicit treatment of sexual motivation, flibanserin, has not been studied with regard to its effects on sexual approach. To the contrary, there are some data concerning the SSRIs. We have reported that 20 days of treatment with paroxetine

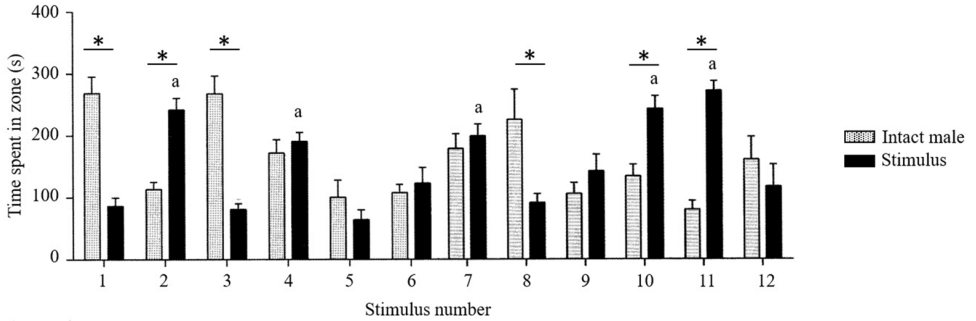

FIGURE 8 | Sexual approach behavior in male rats in response to different kinds of stimuli (black bars). As control for social approach, an intact male was used (gray bars). The experimental subject could choose between spending its time close to the male stimulus or close to the alternative stimulus. The ordinate shows time spent in proximity to the different stimuli. The stimuli are indicated on the abscissa. 1. An intact male and an empty, clean cage, *N* = 11. The time spent close to the male was far superior to the time spent close to the empty cage. This illustrates nicely the male's social attraction. 2. An intact male and a sexually receptive female, *N* = 10. The difference between the time spent with the female and the time spent with the male is the intensity of sexual attraction [(sexual + social approach) – social approach] = sexual approach. 3. Intact male and playback of female ultrasonic vocalizations, *N* = 10. As can be seen, the vocalizations were not more attractive than a silent, empty cage. 4. Intact male and the odor of a sexually receptive female, *N* = 11. The female had spent 6 h in the cage before being removed just before the test. She left behind urine, feces and other body odors that may stick to the floor and walls of the small cage. The odor was not more attractive than the male. Thus, odor by itself has no sexual attractant properties according to our definition (see Stimulus 2). 5. As always, an intact male. The other stimulus was here an anesthetized female and the experimental subject was anosmic. Thus, the only stimulus modality available to the male was vision, *N* = 9. Neither of the stimuli was more attractive than an empty cage. Thus, olfaction is necessary for social as well as for sexual approach. Visual stimuli have no impact. 6. Intact male and a devocalized female were the available stimuli, and the experimental subject was anosmic. The test was performed in complete darkness. Thus, neither visual nor olfactory stimuli were available, and no stimuli from the female's vocal cords, *N* = 10. None of the stimuli was attractive to the experimental male. 7. Intact male and the odor of a female + playback of female ultrasonic vocalizations were the alternatives, *N* = 10. Both were equally attractive, showing that odor + vocalizations have no sexual attractant properties. 8. Intact male and anesthetized female + playback of vocalizations. The experimental subject was anosmic, *N* = 9. There was no difference between these latter stimuli and an empty cage, showing that the sight and vocalizations from a female does not attract a male at all. 9. Intact male and sexually receptive female. The experimental subject was anosmic, and the test was performed in complete darkness. The only stimulus available to the male was auditory, *N* = 10. They did not produce sexual attraction. 10. Intact male and anesthetized female, providing olfactory and visual stimulation but no sounds, *N* = 11. She was as attractive as an active female. 11. Intact male and devocalized female, test performed in complete darkness, *N* = 9. This female was as attractive as an intact female. 12. Intact male and devocalized female. The subject was anosmic, *N* = 10. No social or sexual attractivity was observed. *Different from the social incentive (male rat), $p < 0.05$. [a]Different from the empty cage (stimulus 1), $p < 0.05$. The conclusion from this experiment was that olfactory stimuli need to be combined with some other stimulus in order to activate sexual attraction. The other stimulus cannot be produced by the female's vocal cords. Further details can be found in Ågmo and Snoeren (2017).

reduces sexual approach in female rats (Kaspersen and Ågmo, 2012). Similar data have been reported after treatment with fluoxetine (Adams et al., 2012). This drug also affects female rat behavior in an operant procedure. The authors interpreted the effects as signs of reduced sexual motivation (Uphouse et al., 2015). Contradictory data have also been reported. In a study employing a procedure almost identical to ours, Matuszczyk et al. (1998) failed to detect any effect of fluoxetine on sexual approach, even though the drug reduced lordosis. Nevertheless, we conclude that the majority of data suggests that the SSRIs reduces sexual approach in the female rat.

Drugs and Sexual Approach in Male Rats

As was the case with females, sexual approach behaviors have rarely been evaluated in drug studies in males. This makes it easy to summarize the literature, particularly since we will limit ourselves to clinically used drugs with established or presumed effects on sexual motivation in men. The only candidate drugs for inclusion in this group are the SSRIs, as mentioned. It has been reported that treatment of male rats with fluoxetine for 14 days reduces their approach to a sexually receptive female (Vega Matuszczyk et al., 1998). We have replicated this finding. As can be seen in **Figure 9**, fluoxetine treatment reduced sexual approach, particularly during the tests performed after 10 and 15 days of treatment. It seems that fluoxetine consistently reduces sexual approach in males.

Sexual Approach in Rats and Humans: Any Similarities?

Table 2 summarizes the main characteristics of sexual approach behaviors in rats and humans. As can be seen, there are striking similarities. The main difference is with regard to the stimulus modalities involved in the activation of sexual motivation, hence approach. Furthermore, it is likely that mental representations of sexually relevant stimuli as sources of sexual motivation are exclusive to humans.

Except for the two differences mentioned in the preceding paragraph, it seems that the mechanisms of sexual approach are most similar in these species. Unfortunately, the scarcity of drugs with established effect on human sexual approach make comparisons of drug effects extremely limited. Moreover, the complete absence of experimental evaluation of human sexual approach forces us to use genital responses to sexual stimuli as a proxy for actual approach as soon as we search for objective data. Nevertheless, the conclusion that sexual approach is controlled

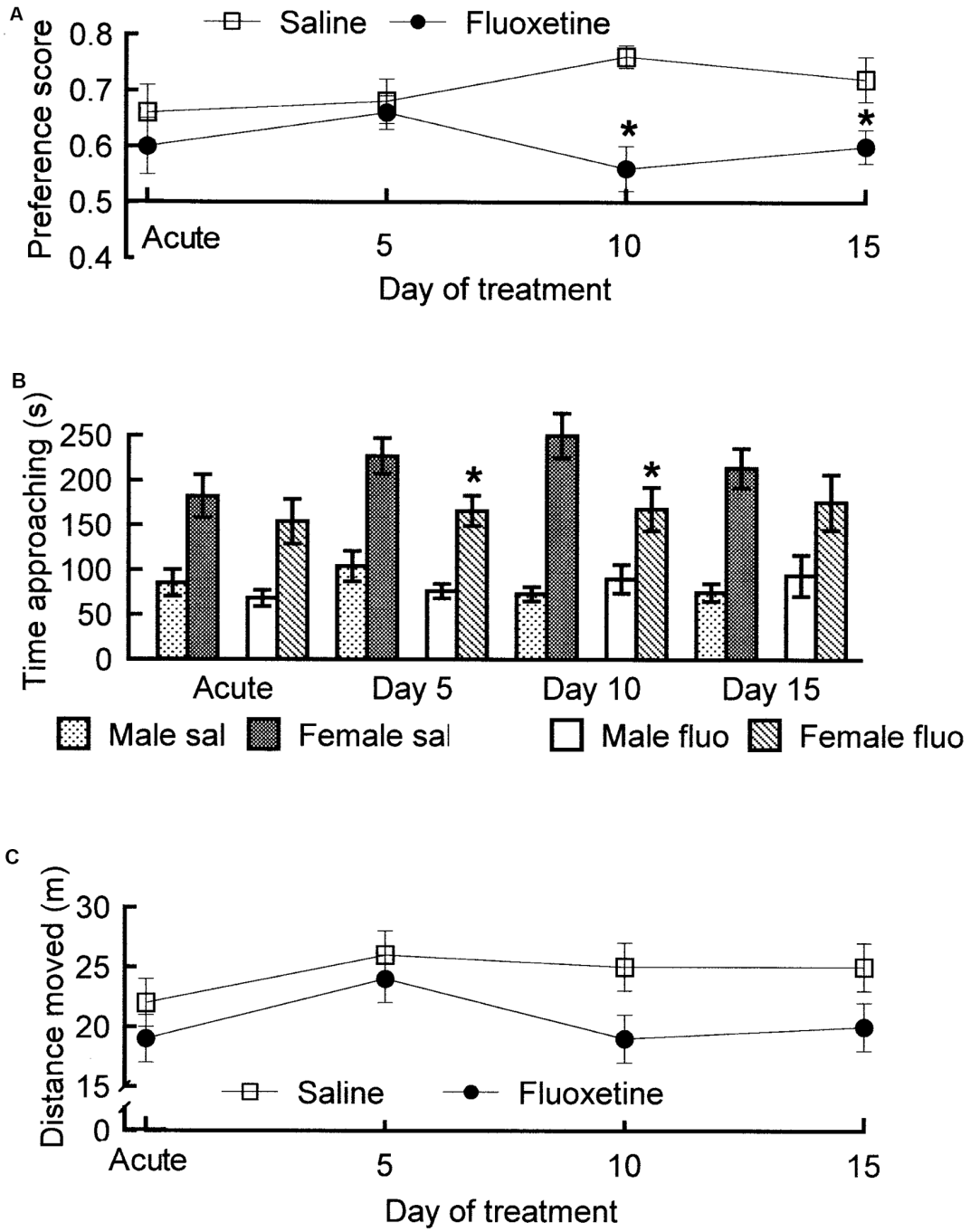

FIGURE 9 | The effects of fluoxetine, 10 mg/kg and day given orally, on sexual approach behaviors in male rats. The procedure described in "Sexual Approach in Rodents" section was used. Test duration was 10 min. The acute test was performed 60 min after drug administration. **(A)** Preference score. Mixed two-factor ANOVA with Treatment and Day as factors revealed a significant Treatment effect ($F_{(1,34)} = 7.528$, $p = 0.010$), but there was no effect of Day ($F_{(3,102)} = 0.192$, $p = 0.902$) and no interaction Treatment × Day ($F_{(3,102)} = 1.239$, $p = 0.300$). **(B)** Time spent in the vicinity of the social incentive (another male) and in the vicinity of the sexual incentive (receptive female rat). Three-factor ANOVA with the factors Incentive, Treatment and Day found an effect of Incentive ($F_{(1,34)} = 86.072$, $p < 0.001$) and of Treatment ($F_{(1,34)} = 7.134$, $p = 0.012$) but not of Day ($F_{(3,102)} = 1.789$, $p = 0.154$). The time spent in the vicinity of the female was superior to that spent in the vicinity of the social incentive at all tests according to the Tukey HSD test. There was also an interaction between Incentive and Treatment ($F_{(1,34)} = 7.993$, $p = 0.008$). Fluoxetine did not alter the time spent in the vicinity of the male incentive whereas the time spent in the vicinity of the sexual incentive was reduced. There were no other interactions ($ps > 0.300$). Thus, fluoxetine did not modify social approach but reduced sexual approach. Since there was no interaction Treatment × Day, this effect was present already at the acute test. **(C)** The distance moved during the test, a measure of ambulatory activity, was not altered by treatment and did not vary between days ($ps > 0.102$). Thus, the inhibition of sexual approach cannot be explained as a secondary effect of reduced activity. Data are mean ± SEM. *Different from saline, $p < 0.05$. From an unpublished experiment performed by AÅ, Juoni Sirviö, Gro Sandberg and Live Sørensen.

TABLE 2 | Comparison of some basic characteristics of sexual approach behaviors in rodent and humans.

	Rats	Humans
Efficient stimulus	Olfactory + any other modality	Visual, auditory (fantasies)
Neutral stimuli may become sexual incentives through learning	Yes	Yes
Behavioral responses	Context dependent	Context dependent
Depends on gonadal hormones	Yes	Yes
Effects of SSRIs	May inhibit	May inhibit

by similar behavioral and neural mechanisms in rats and humans seem warranted.

Relationship Between Sexual Approach Behavior and Copulatory Behavior

In "Sexual Approach in Rodents" section, we have discussed sexual motivation, expressed as the intensity of genital responses in humans and approach to a potential mate in rodents. We also have mentioned that sexual motivation is a determinant of the intensity of copulatory behavior. A fundamental issue is whether sexual motivation is a unitary concept or not. If it is, then the intensity of genital responses and approach should always covary with the intensity of copulatory behavior. In humans, there does not seem to exist any systematic study of the relationship between genital responses to sexually relevant stimuli and the intensity of copulatory behavior. The typical setup for evaluating genital responses in men and women is such that no copulatory activity can occur in the testing situation. The only way to determine any possible relationship between the magnitude of the individual's genital response and the intensity of copulatory behavior displayed by the same individual would be to enquire about the person's sexual activity outside the laboratory. Under the conditions that magnitude of the genital response is stable between the laboratory and bedroom contexts and that the individual correctly reports his or her sexual activity, this might be an acceptable approximation. Unfortunately, this kind of study has not been performed, at least not published. Instead, much effort has been invested in finding out how genital responses relate to subjective sexual arousal, as discussed earlier. The issue of whether subjective arousal has any relationship to actual copulatory activities or if it is a useless concept has not been of much concern.

In rodents, there is direct experimental evidence showing that the intensity of copulatory behavior can be experimentally manipulated independently of the intensity of sexual approach behaviors (Ågmo, 2002). After repeatedly pairing ejaculation with a female smelling of fish oil with an injection of LiCl, a compound producing stomach ache and diarrhea, both approach to and copulation with such females were suppressed. However, the experimental males approached non-scented females with undiminished intensity, but they did not copulate with them when given the opportunity. Thus, the conditioned inhibition of approach was specific to an olfactory stimulus (fish oil) whereas inhibition of copulation generalized to any female. Pharmacological studies have also revealed that approach and copulation can be modified in opposite directions by drugs. The adrenergic α_2 antagonist RX 821002 enhances approach behavior whereas copulation is reduced, for example (Chu and Ågmo, 2016a).

We have further evaluated the notion that approach can vary independently of copulation by calculating the correlation between sexual approach and copulatory behavior in a large number of rats. As shown in **Table 3**, there was no significant correlation between the intensity of approach and the intensity of copulatory behavior. Thus, at the level of the individual, there is no relationship between approach and copulation. However, if we instead look at the group level, for example comparing the mean intensity of sexual approach in a group of intact rats with that in a group of castrated males, we find a highly significant difference. The preference score was 0.70 ± 0.04 [mean $\pm$ standard error of the mean (SEM)] in the intact group vs. 0.46 ± 0.03 in the castrated group ($p < 0.001$). This is also the case with every aspect of copulatory behavior. In fact, the castrated males did not display a single mount, and obviously no intromission or ejaculation. Thus, the intact group shows a higher level of approach than the castrated group, and also a far more intense copulatory behavior.

Turning to females, we again find that there is no correlation between the intensity of approach and copulatory behavior in the divided cage, a procedure in which the female can pace sexual interaction (**Table 4**). As was the case with males, however, we find a clear relation between approach and copulation at the group level. When comparing data from ovariectomized females given either oil or EB + P, we find clear-cut differences both in approach and copulatory behavior. The mean $\pm$ SEM preference score was 0.72 ± 0.03 in hormone-treated females whereas it was 0.55 ± 0.06 in oil-treated females ($t_{(16)} = 2.980$, $p = 0.009$). The

TABLE 3 | Pearson correlations between sexual approach behavior (quantified as a preference score[a] obtained in the procedure described in Ågmo, 2003) and copulatory behavior in intact male rats having displayed at least one mount or one intromission in a test for copulatory behavior performed immediately after the test for approach behavior.

Copulatory behavior parameter	Correlation	*N*
Mount latency	0.016	195
Number of mounts	−0.009	195
Intromission latency	−0.067	173
Number of intromissions	0.064	173
Ejaculation latency	−0.008	154
Post-ejaculatory interval	−0.153	154

[a] *Preference score = [time spent approaching a sexually receptive female/(that time + the time spent approaching another male)]. Not all animals displaying mounts achieved intromission, and not all animals displaying intromission ejaculated during the test. The test duration was determined by the following criteria: end of the first post-ejaculatory interval, no intromission within 15 min of introduction of the female, no ejaculation within 30 min of the first intromission. Data were pooled from several experiments performed in the laboratory.*

TABLE 4 | Pearson correlations between sexual approach behavior and copulatory behavior in ovariectomized female rats given EB, 25 μg and P, 1 mg, 48 and 4 h before being subjected to a test for sexual approach behavior immediately followed by a test for copulatory behavior in the divided cage.

Copulatory behavior parameter	Correlation	*N*
Latency to enter male's half	−0.156	30
Exit after male mount	0.010	29
Return latency after mount	−0.063	29
Exit after intromission	−0.300	25
Return latency after intromission	0.201	25
Return latency after ejaculation	0.056	24
Number of paracopulatory behaviors	−0.122	30

Data are pooled from three separate experiments performed in the laboratory by Chiara Pozzato and AÅ. Approach behavior was quantified as a preference score established in the sexual incentive motivation test (Ågmo, 2003). Latency to enter male's half, time between introduction of the female into her half and entry into the male half with all four paws; exit after mount, proportion of mounts followed by escape to the female's half within 10 s; return latency after mount, time between the female's entry into her half and her return, with all four paws, to the male's half after having received a mount. Exit and return latency after intromission are self-explanatory. All females escaped after ejaculation. Thus, the proportion of exits is a constant and cannot be used for calculating a correlation.

former displayed intense copulatory behavior whereas the latter showed none. It is evident that sexual approach at the individual level is unrelated to copulatory behavior, exactly as it is in males, but that there is a relationship at group level.

The contradictory observations between the lack of relationship between approach and copulation within the individual and the clear relationship at the group level is similar to the lack of relationship between serum testosterone concentration and the intensity of copulatory behavior at the individual level (e.g., Damassa et al., 1977, in rats and Brown et al., 1978, in men) even though testosterone is necessary for that behavior. This fact is normally explained by posing that above a minimum serum concentration of testosterone, further increases in concentration has no consequence. We propose that a similar principle also holds for sexual approach behavior. Although approach is a requisite for copulation, once the intensity of approach surpasses a certain level, further increase has no consequence.

THE CAVEATS

Female rat copulatory behavior is relatively straightforward, and relevant behavior patterns are limited to lordosis and paracopulatory behaviors. Other behaviors displayed by females, such as sniffing or anogenital sniffing of the male, have no relationship to copulatory behavior (Chu and Ågmo, 2014; Le Moëne and Ågmo, 2018). It can obviously be argued that rat lordosis has no equivalent in women, and that treatment effects on that behavior cannot be generalized to women. Nevertheless, the ease by which lordosis is displayed is determined by motivation, and since lordosis is a sexual response, that motivation is sexual. This same argument could be used for the paracopulatory behaviors. Most women are not ear-wiggling during copulation, yet rat ear-wiggling is, like any other behavior, controlled by motivation. Since this behavior is a response to a sexually relevant stimulus [a sexually active male induces far more ear-wiggling than a castrated male (Vreeburg and Ooms, 1985)], it can be supposed to be controlled by sexual motivation. Thus, any treatment effects on ear-wiggling represent effects on motivation. Even though the behavioral manifestations of sexual motivation are drastically different in rats and women there may well be similar underlying mechanisms in operation.

Likewise, in the male rat there is a series of behavioral parameters that have no equivalence in men. Rat measures such as the interval between intromissions or the proportion of mounts ending in vaginal penetration are probably meaningless. It is also uncertain whether these parameters represent motivation of any kind. The ease with which intromission is achieved depends on vascular erection as well as the activity in the penile striated muscles (Sachs, 1982; Giuliano et al., 1994). Any alteration in the coordinated activity of these processes can modify copulatory behavior, even though they are unrelated to sexual motivation. A complete description of male copulatory behavior might make it possible to distinguish effects on peripheral mechanisms from effects on motivation. However, any speculation about motivation based on copulatory behavior suffers from a considerable degree of uncertainty. It appears far more difficult and risky to infer changes in sexual motivation from changes in copulatory behavior in males than in females.

Sexual approach behaviors have sometimes been considered as the only acceptable indicator of sexual motivation. The data showing that the intensity of approach is unrelated to the intensity of copulatory behavior at the individual level makes this assertion somewhat exaggerated. If the proposal made above, that variations in the intensity of approach behaviors have no consequence for the intensity of copulatory behavior when the former are above some minimum level, is true it appears of limited interest to pursue treatments that might lead to enhancement beyond the minimum. To the contrary, in the case of search for treatments of hypoactive sexual desire disorder, it can be assumed that sexual approach behaviors in those affected are below the minimum, and any increase would consequently be beneficial. Thus, rodent models of human conditions involving reduced desire should evaluate approach rather than copulation.

The uncertainties regarding the correspondence between copulatory and sexual approach behaviors in rodents and humans are considerable, as mentioned. Nevertheless, there is no doubt that in both species, these behaviors are determined by one or another aspect of sexual motivation. We will now turn to an additional complication, jeopardizing any generalization from rodent to human. Whereas rodent sexual approach and copulation are mainly determined by preprogrammed mechanisms in the central nervous system, human sexual relationships are basically determined by social conventions. Anthropologists and sociologists have elegantly shown that human sexual behaviors, in the widest sense, are social constructions (Ford and Beach, 1951; Marshall and Suggs, 1971; Gagnon and Simon, 2002). Conventions determine to whom, when and where we can manifest sexual approach, how this approach should be manifested, and how to proceed in order to initiate copulatory activity. The nature of that activity, i.e., the motor patterns employed, are also largely determined by conventions, acquired through social learning. Even the impact of sexually relevant stimuli are made context-dependent because

of social learning. A naked human body will not function as a sexual incentive on a nudist beach, whereas it usually is most efficient in the intimacy of a bedroom.

The fundamental role of social determinants in human sexual activities has obviously no equivalence in rodents. Insofar as those determinants influence the activation and behavioral manifestations of sexual motivation, rodent models are of limited help. However, even if social factors are crucial for human sexuality, there are basic neurobiological and behavioral mechanisms on which these factors act. According to the incentive motivation model presented in **Figure 1**, the central motive state prepares the ground for the actions of sexual incentives and for the responses to those. The incentives and the responses may well be heavily dependent on social learning, but without an appropriate central motive state no stimulus would act as an incentive and no response would be performed. There is no reason to believe that the nervous basis for the central motive state is drastically different in rodents and humans. This means that we should pursue means to discover the workings and manifestations of the activity of the central motive state underlying sexual motivation.

CONCLUSION

Like any other behavior, sexual approach and copulation are determined by motivation. We assume that a particular motivational state makes organisms sensitive to sexually relevant stimuli which in turn activate responses, normally approach and copulation, eventually leading to sexual reward. This motivational state is called sexual motivation. It appears that there are some differences between the motivational state leading to the establishment of physical contact with a potential mate, approach, and the motivational state leading to the execution of copulatory acts. Since "motivational state" is an abstract concept, it must be anchored in reality through its behavioral manifestations. These manifestations can be rather different in rodents and humans, but the neurological underpinnings of the motivational state behind behavior are probably very similar. It should always be borne in mind that human sexual activities are a result of social learning and that we must go around the confound caused by this fact if we are to understand their motivational basis. This is often forgotten.

AUTHOR CONTRIBUTIONS

OLM and AÅ contributed equally to this manuscript.

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REVIEW
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Sex, Drugs, and the Medial Amygdala: A Model of Enhanced Sexual Motivation in the Female Rat

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Methamphetamine (METH) is a psychomotor stimulant that is reported to enhance sexual desire and behavior in both men and women, leading to increases in unplanned pregnancies, sexually-transmitted infections, and even comorbid psychiatric conditions. Here, we discuss our rodent model of increased sexually-motivated behaviors in which the co-administration of METH and the ovarian hormones, estradiol and progesterone, intensify the incentive properties of a sexual stimulus and increases measures of sexually-motivated behavior in the presence of an androgen-specific cue. We then present the neurobiological mechanisms by which this heightened motivational salience is mediated by the actions of METH and ovarian hormones, particularly progestins, in the posterodorsal medial nucleus of the amygdala (MePD), a key integration site for sexually-relevant sensory information with generalized arousal. We finally demonstrate the cellular and molecular mechanisms underlying this facilitation of sexual motivation by METH, including the upregulation, increased phosphorylation, and activation of progestin receptors (PRs) in the MePD by METH in the presence of ovarian hormones. Taken together, this work extends our understanding of the neurobiology of female sexual motivation.

Keywords: methamphetamine, dopamine, proceptive behavior, progesterone, sexual motivation, medial amygdala

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INTRODUCTION

Sexual behaviors are a complex, coordinated suite of actions that arise from the integration of psychological and physiological processes with external elements. One key component of sexual behaviors is that of sexual motivation, a hypothetical, internal willingness to engage in sexual behaviors (Holder and Mong, 2017). Although research into female sexual motivation is an active and growing field, relatively little is understood about the neurobiological origins of sexual motivation in women. Many of these mechanistic questions cannot be currently answered in women, so rat models are most frequently used to study sexual motivation and behavior (Pfaus et al., 2003; Blaustein, 2008).

In this review article, we discuss the modulators of female sexual motivation, using the concept of incentive motivation as a foundational working model. Next, we summarize what is known in regard to the neurobiology of female sexual motivation in rats. We then describe our methamphetamine (METH) model of increased sexually-motivated behaviors in female rats. We finally detail insights into the neurobiology and mechanisms of enhanced female sexual motivation gained using this model.

RODENT SEXUAL BEHAVIORS

The female rats show a wide range of specific sexual behaviors that are displayed in the presence of a male rat. Following anogenital investigations, the female will typically engage in approach and solicitation behaviors, which serve to initiate sexual contact with a male (McClintock and Adler, 1978; Erskine, 1989; Pfaus et al., 2003). The female approaches the male with a head-wise orientation then quickly runs away (Pfaus et al., 2003). This runaway takes the form of proceptive behaviors such as hopping and darting, with and without ear wiggling, in a traditional behavioral arena (Madlafousek and Hlinák, 1983). Hopping is distinct from general locomotion as it is a rapid, stiff-legged upward jump, followed by a bow-shaped return to the floor, and ends in a crouch. A hop covers the distance of approximately one extended body length (Madlafousek and Hlinák, 1977). Darting is a specialized form of a runaway from the male in which the female accelerates swiftly, using rapid low steps with the body held near the floor (Hemmingsen, 1933; Beach, 1942). The series of hopping-and-darting typically ends with a presentation behavior, or a pre-lordotic crouch (Madlafousek and Hlinák, 1977). This crouch serves to help support the male's mounting behaviors. Upon a successful mount by the male, the female rat displays a behavioral reflex known as lordosis, in which the female arches her back, elevates her head and rump, and deflects her tail to one side (reviewed in Erskine, 1989). Proceptive behaviors typically precede the first lordosis during the period of sexual receptivity, and the numbers of proceptive events increase in the minute preceding lordosis (Chu and Ågmo, 2015). Indeed, females that display more proceptive behaviors are pursued more frequently by males (Chu and Ågmo, 2014). In addition, the female's display of proceptive behaviors precedes nearly all male sexual behaviors (Bergheim et al., 2015).

In arenas that allow for separation between the male and female rat, such as a paced mating arena with escape chamber(s) or bilevel chambers, the female rat controls the tempo and occurrence of the sexual behaviors (McClintock and Adler, 1978; Erskine and Baum, 1982; Erskine, 1985; Pfaus et al., 2003). If female rats are given the opportunity to choose between two males, they display a consistent partner preference, as indicated by increased time with a preferred male and by returning to him more rapidly in a paced-mating environment even across multiple encounters (Lovell et al., 2007).

MODULATORS OF SEXUAL MOTIVATION

Central Motive State

There are two necessary components of any motivated behavior: (i) the incentive properties of an external stimulus; and (ii) a central motive state (Bindra, 1974; Ågmo, 1999). The external stimulus has incentive or aversive qualities that serve to influence the hedonic, or pleasurable values. Incentive stimuli create a tendency for an individual to approach the object; whereas, aversive stimuli create a tendency for avoidance behaviors (Bindra, 1974). The central motive state is the integration of the physiological processes, such as hormones, with the neural processes that direct the motivational behaviors (Bindra, 1974; Ågmo, 1999). It is the interplay between the incentive qualities of the stimulus and the central motive state that ultimately determine the likelihood of a particular behavioral response, whether it be approach or avoidance behaviors (**Figure 1**).

One major assumption of the central motive hypothesis is that of the hedonic value of the external stimulus. In order to apply this hypothesis to the study of female sexual motivation, it must, therefore, be established that females engage in sexual behavior to experience sexual pleasure (Pfaus et al., 2003; Ågmo, 2007). Sexually-motivated female rats lever press (Bermant, 1961; French et al., 1972), cross an electrified grid (Meyerson and Lindström, 1973), and nose-poke (Matthews et al., 1997; Cummings and Becker, 2012) to gain access to a sexually-active male. When female rats can pace sexual behavior, as in bilevel arenas or those with escape chambers, they show conditioned place preference in that they spend more time in the portion of an arena in which a sexual encounter occurred (Paredes and Alonso, 1997; Meerts and Clark, 2007). Subsequent studies indicate that female rats will only develop this conditioned place preference when copulation is at their preferred pacing interval (Jenkins and Becker, 2003b). Finally, female rats show evidence of orgasm-like behavior as indicated by contractions of the pelvic-floor muscles and short-term changes associated with reward state such as ultrasonic vocalizations (USVs; Pfaus et al., 2016). Taken together, these studies indicate that sexual behavior may, in itself, be rewarding to the female rat, at least under certain conditions.

The central motive state determines the external stimulus's incentive value through modulation of the hedonic value of that stimulus (Berridge, 2004). It would then follow that changes to the central motive state could increase the attractivity to a sexually-relevant stimulus (Pfaus et al., 2003; Ågmo, 2007). That is, an increased activation of the central motive state would enhance the strength of behavioral responses toward sexually-salient cues. This may take the form of more olfactory investigations and displays of the solicitation, proceptive, and pacing behaviors in the presence of a male rat. In addition, it is possible that this enhanced activation of the central motive would also lead to the abolition of mate preferences, as the same sensory cues may increase in incentive qualities.

We can conceive of the central motive state as arising from two components: (i) a generalized state common to all forms of motivated behavior; and (ii) a specific drive that depends on physiological needs (Pfaff, 1999). The first component is generalized arousal which energizes all motivated behaviors (Pfaff et al., 2008). The specific neurobiological signals known to mediate the sexual motivation and behavior in the female rat are the ovarian hormones estradiol and progesterone (Cummings and Becker, 2012; Uphouse et al., 2015). Although these are not the only neurobiological factors that could alter the central motive state to lead differences in sexual motivated behaviors, we will focus discussion on the factors of generalized arousal and ovarian hormones in this review article.

Generalized Arousal

Generalized arousal is a hypothetical construct that energizes all behavioral processes by promoting wake, alertness, and

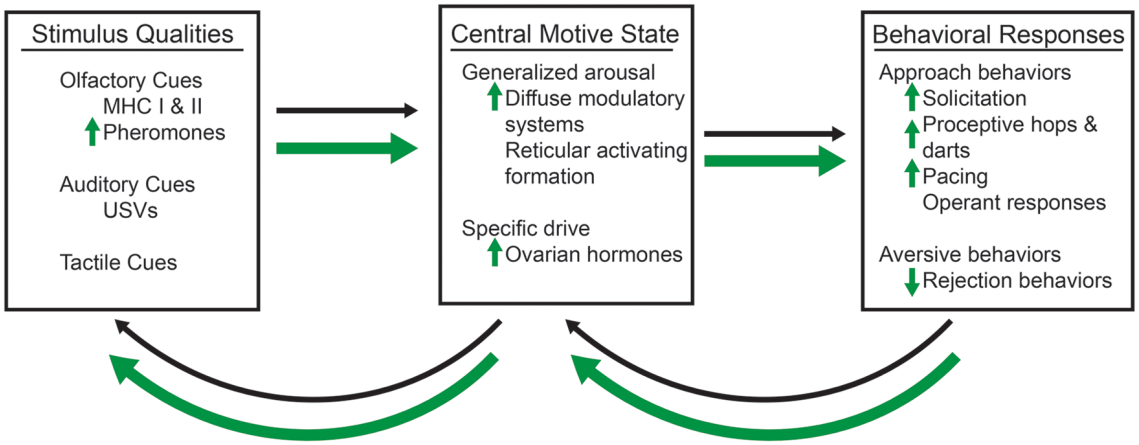

FIGURE 1 | A conceptual model of sexual motivation, which are represented by thin, black arrows and enhancements by methamphetamine (METH), which are represented by thick, green arrows. The olfactory, such as the major histocompatibility complex I and II (MHC I and MHC II), which contribute to pheromones, auditory, such as ultrasonic vocalizations (USVs), and tactile, such as the flank stimulation, qualities of a sexual stimulus interact with the central motive state, which itself is modulated by the generalized arousal state and the activation of a specific drive (e.g., the presence of ovarian hormones and activation of their cognate receptors). METH requires the activation of dopaminergic and progestin receptors (PRs) to enhance the activation of the central motive state and female sexual motivation. The presence of a sexual stimulus and activation of the central motive state then influences the likelihood of sexual behavioral responses, which then have the ability to feedback and alter the central motive state itself. Changes to the central motive state then feedback to alter the salience of particular qualities of the sexual stimulus. METH increases the salience of androgen-specific cues of a sexual stimulus, which in term lead to increases in display of solicitations, proceptive hops and darts, and paced mating behaviors. There is also a decrease in the number of rejection behaviors.

responses to and interaction with the environment (Pfaff et al., 2008). Generalized arousal has been demonstrated by: (i) a responsiveness to sensory stimuli across multiple modalities; (ii) motor activity; and (iii) emotional or affective reactivity (Pfaff et al., 2008). The ascending, diffuse neuromodulatory systems that form the reticular activating formation contribute to generalized arousal. Noradrenergic projections to the cerebral cortex modulate the sensory responsiveness, whereas the nigrostriatal dopaminergic projections mediate the motor activity directed towards salient stimuli (Pfaff et al., 2008). Mesolimbic dopaminergic projections, which comprise part of the natural reward circuit, facilitate the incentive salience, or the "wanting" of some stimulus (reviewed in Berridge, 2007, 2019). As such, certain types of sexual behaviors (e.g., female-paced sexual behavior) will result in a release of dopamine in the nucleus accumbens (Jenkins and Becker, 2003a). Further, the administration of agonists of noradrenergic or dopaminergic receptors will enhance, and antagonists will reduce, measures of female sexual behavior (Foreman and Moss, 1979; Fernández-Guasti et al., 1985a,b, 1987; Grierson et al., 1988; Petitti and Etgen, 1990; Chu and Etgen, 1999; Chu et al., 1999). Thus, both neurotransmitters appear to work in conjunction to modulate general arousal and prime a female towards sexual behavior.

Ovarian Hormones

The period of sexual receptivity in rats is limited to a few hours prior to the onset of ovulation (Nequin et al., 1979; Freeman, 1994). Several classic studies have demonstrated the role of both estradiol and progesterone in triggering both proceptive and receptive sexual behaviors in the rat (Beach, 1976). High levels of estradiol are sufficient and activation of the estrogen receptors (ERs) is necessary to induce lordosis behaviors; however, the intensities of lordosis, based on the degree of spinal curvature, is highly variable with frequent displays of rejection behaviors (Boling and Blandau, 1939; Beach et al., 1942; Whalen, 1974; Spiteri et al., 2010). Progesterone increases the efficacy of estradiol in the induction of lordosis. In addition, progesterone and the activation of the PRs is necessary for the occurrence of the solicitation, proceptive, and paced mating behaviors (Boling and Blandau, 1939; Beach et al., 1942; Beach, 1976; Whalen, 1974; Fadem et al., 1979; Tennent et al., 1980; Edwards and Pfeifle, 1983; Olster and Blaustein, 1988; Blaustein, 2008). These hormones strongly affect the responses to olfactory and tactile stimuli, with modest effects on generalized arousal (Chu et al., 2015), providing evidence that the ovarian hormones contribute to the central motive state to modulate the incentive qualities of the male rat.

NEUROBIOLOGY OF SEXUAL MOTIVATION

The historical focus of the neurobiology of female sexual behavior has been focused on the neurocircuit that controls lordosis. As lordosis is a behavioral reflex, the neural mechanisms of it are more readily elucidated than the neural mechanisms of sexual motivations. The lordosis circuit has been exquisitely detailed using multilateral approaches including electric stimulation and lesions of each of the nuclei in the circuit (Mathews and Edwards, 1977; Davis et al., 1979; Pfaff and Sakuma, 1979; Sakuma and Pfaff, 1979; Brink and Pfaff, 1980; Schwartz-Giblin and Pfaff, 1980; Femano et al., 1984a,b), patterns of neuronal activation (Flanagan et al., 1993; Tetel et al.,

1993; Flanagan-Cato and McEwen, 1995; Polston and Erskine, 1995; Pfaus et al., 1996; Pfaus and Heeb, 1997), and viral tract tracing studies to map the anatomical connections (Daniels et al., 1999). Of primary importance for lordosis is the ventrolateral portion of the ventromedial nucleus of hypothalamus (VMN; reviewed in Pfaff et al., 1994). The ovarian hormones serve to activate the neurons of the VMN, which then overcomes the tonic inhibition on lordosis (Powers and Valenstein, 1972; Moss et al., 1974; Pfaff and Sakuma, 1979; Kow et al., 1985; Fahrbach et al., 1989).

The mechanisms and the neural circuitry controlling female sexual motivation have not been as well elucidated. Furthermore, if motivated behavior arises from both the incentive properties of a sensory stimulus and mediators of the central motive state, it is likely that the neural circuitry that processes these sensory cues also contribute to sexual motivation. The work of ourselves and others indicates that sexual motivation arises from an interplay of activation of the natural reward circuity and the processing of olfactory cues in the limbic/hypothalamic social behavior circuitries.

The posterodorsal nucleus of the medial amygdala (MePD) is a good candidate region for the regulation of sexual motivation and the modulation of the output sexual behavior (Mascó and Carrer, 1980, 1984; Erskine, 1989; Kondo and Sakuma, 2005; Afonso et al., 2009). Changes to generalized arousal would influence the activation of the MePD as it receives both noradrenergic and dopaminergic input (Gray, 1999; Pitkänen, 2000). The MePD contains both ERs and PRs (Pfaff and Keiner, 1973; Simerly et al., 1990), making it sensitive to the specific drivers of sexual motivation. The MePD also receives chemosensory signals of pheromones from the accessory olfactory bulb (Keller et al., 2009), so it would be activated by sexually relevant olfactory cues. The projections of the MePD target and can activate several key output nuclei involved in social and sexual behaviors including the VMN (Kevetter and Winans, 1981; Simerly, 2002; Keller et al., 2009). Finally, lesions of the MePD lead to fewer lordosis responses (Mascó and Carrer, 1984), proceptive behaviors (Mascó and Carrer, 1980; Afonso et al., 2009), and a reduction in conditioned place preference (García-Horsman et al., 2008) and sensitivity to sexual stimulation (Guarraci, 2010).

METHAMPHETAMINE INCREASED MEASURES OF SEXUAL MOTIVATION TOWARDS AN INCENTIVE STIMULUS

To better explore the neurobiology of sexual motivation in females, we created a model of enhanced motivation by administering METH. METH is a drug of abuse that intensifies sexual drives, desires, and sexual activities in women (Rawson et al., 2002; Semple et al., 2004a). In addition, METH use is also associated with a more pleasurable sexual experience (Lorvick et al., 2012). These anecdotal and clinical self-reports are supported by the increased rates of sexually-transmitted infections and of unplanned pregnancies (Semple et al., 2004b; Mansergh et al., 2006). As users of METH tend to administer it several times over the course of a few days (Haile et al., 2009), we administer METH (5 mg/kg/day) once a day for 3 days to ovariectomized female rats at the same time as the ovarian hormones estradiol benzoate and progesterone (Holder et al., 2010). The optimal time to test for female sexual behavior is 4–6 h following the administration of progesterone (Nequin et al., 1979; Freeman, 1994). Importantly, neither stereotyped behavior nor hyper-locomotor behavior are present 4–6 h after METH administration, suggesting that any increase in sexual behavior due to METH reflects heightened sexual motivation, not motor responses (Holder et al., 2010).

The acute administration of METH enhances measures of sexual motivation in hormonally-primed female rats (Holder and Mong, 2010; Holder et al., 2010; Winland et al., 2011). METH treatment increases the lordosis response in addition to doubling the frequency of proceptive behavior of hops, darts, and ear-wiggles (**Figure 2A**; Holder et al., 2010). When tested in a paced-mating arena, female rats treated with METH are less likely to leave the male rat following sexual stimulation, and if they leave, they return to him more rapidly compared to saline-treated, hormonally-primed females (Holder et al., 2010; Winland et al., 2011). In addition, these METH-treated female rats displayed more solicitation and proceptive behaviors, especially during the post-ejaculatory interval (Holder and Mong, 2010). The possibility remains that METH may alter the timing and displays of sexual behavior instead of sexual motivation *per se*; however, there is growing evidence that motivation and timing of behaviors are not independent processes such that changes to the hedonic value lead to alterations in interval duration, indicating that the changes in timings of a behavior are produced by changes in motivational state (reviewed in Galtress et al., 2012). While the decreased latency to return to the male is suggestive of an increased tempo for sexual behavior, the timing aspects should be further explored using more direct measures of sexual motivation in female rats (e.g., operant responding).

METH may also alter the preferences of specific sexual partners based upon relevant sensory cues. For example, METH-treated, hormonally-primed female rats make more approaches and spend more time with a potential sexual partner (e.g., a male or a castrated male treated with dihydrotestosterone) compared to a non-sexual partner (e.g., a female or a castrated male; Winland et al., 2011; Rudzinskas and Mong, 2016). Dihydrotestosterone provides the necessary androgen-mediated cues, such as pheromones (Orsulak and Gawienowski, 1972; Drewett and Spiteri, 1979), sufficient to elicit solicitation, hops, and darts, with METH treatment increasing the number of proceptive behaviors (**Figure 2B**; Rudzinskas and Mong, 2016). These pheromonal cues are olfactory in nature, and while there are no differences in anogenital investigations induced by METH, there are significantly fewer sniffing behaviors. Consistent with an increase in generalized arousal as part of the central motive state, this work suggests that METH may enhance the detection of olfactory cues. Future work is necessary to explore the potential effects of METH on olfaction. Taken together, these data suggest that METH does not alter the ability of females to discriminate between stimuli, but rather enhances

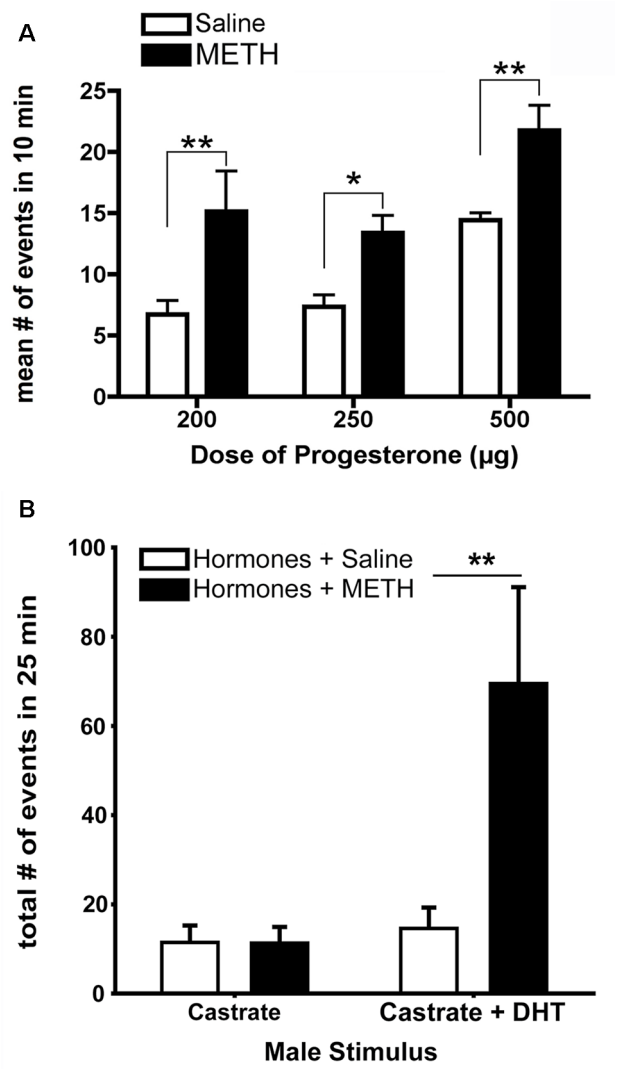

FIGURE 2 | The enhancement of sexually-motivated behaviors by METH. **(A)** METH treatment doubles the number of proceptive events displayed in 10 min regardless of progesterone (P) dose, compared to the respective saline-treated females. **(B)** Replacement of androgen-specific cues in castrated males induces more markedly increased proceptive behaviors in hormonally-primed female rats treated with METH. Data represents means ± standard error of the mean (SEM); $*p < 0.05$, $**p < 0.01$. Reprinted with permission from Elsevier, Inc., Amsterdam, Netherlands.

central motive state arousal to increase sexual motivation in a context-specific manner by potentiating the behavioral responses towards an incentive stimulus.

A LOCUS FOR ENHANCED SEXUAL MOTIVATION

The combination of METH and ovarian hormones enhances the measures of sexual motivation; therefore, we hypothesized that METH would converge with ovarian hormone actions to increase neuronal activity and induce neuroplasticity of the neurocircuitry that underlies sexual motivation and behavior. There is an additive effect of METH and ovarian hormones on the expression of cFos, an immediate early gene that is used as a marker of neuronal activation, in both the MePD and VMN (**Figure 3A**; Holder et al., 2010; Williams and Mong, 2017). The MePD projects to and can activate the VMN (Kevetter and Winans, 1981; Simerly, 2002; Keller et al., 2009). Therefore, it is likely that the increase in cFos in the VMN follows the increase in neuronal activation of the MePD. In further support, spinophilin, a cytoskeleton-associated protein found in dendrite spines, has a 60% increase in the MePD, but not the VMN, following the administration of METH and ovarian hormones (**Figure 3B**; Holder and Mong, 2010). This increase in spinophilin suggests that METH and ovarian hormones synergize to increase the density of dendritic spines and, thus, synaptic connectivity in the MePD. Taken together, the increase in neuronal activation and spinophilin in the MePD suggest that the METH-induced enhancement of female sexual motivation and behavior arise from converging actions of the ovarian hormone in the MePD.

It has been previously reported that lesions of the MePD do not abolish the expression of female sexual behavior, but rather, reduces the expression of sexually-motivated behaviors (Mascó and Carrer, 1980, 1984; Afonso et al., 2009). Lesions of the MePD also prevent the METH-induced increase in proceptive behaviors (**Figure 3C**; Holder et al., 2015). The Daun02 inactivation techniques allow for a more precise investigation of the cells activated in the MePD and the interactions of METH and ovarian hormone signaling on sexually motivated behaviors. Briefly, neuronal activation induces both cFos and β-galactosidase expression in Sprague–Dawley cFos-lacZ trans-genetic rats (Koya et al., 2016). The β-galactosidase both serves as another method of visualizing activated neurons, but it also converts the prodrug Daun02 into daunorubincin, which then triggers apoptosis of the activated cell populations (Santone et al., 1986; Farquhar et al., 2002; Pfarr et al., 2015). Therefore, this Daun02 inactivation technique produces selective lesions of the cells that are activated by METH and/or ovarian hormones. As with the cFos expression, the combination of METH and ovarian hormones produces an increase of β-galactosidase over that of ovarian hormones in a discrete population of cells within the MePD (**Figures 3D,E**; Williams and Mong, 2017). In addition, successful Daun02 lesions of this neuronal ensemble reduce the proceptive behaviors to baseline levels, further supporting the notion that the MePD utilizes signals from METH on hormonally responsive neurons to augment the behavioral response (**Figure 3F**; Williams and Mong, 2017). Furthermore, the Daun02 inactivation does not alter receptive/reflexive sexual behaviors, supporting the MePD's role as an integration center specifically for sexual motivation. Taken together, these results indicate a synergy of intracellular signaling cascades induced by METH and ovarian hormones within MePD cells. This will be further explored in subsequent sections.

One way in which this synergy of intracellular signaling cascades could result in an increase in sexual motivation is *via* changes in epigenetic modifications, which can then lead to marked changes in gene expression. These epigenetic changes could occur on the DNA directly, leading to localized regulation of gene transcription, or by modification of the histones, an

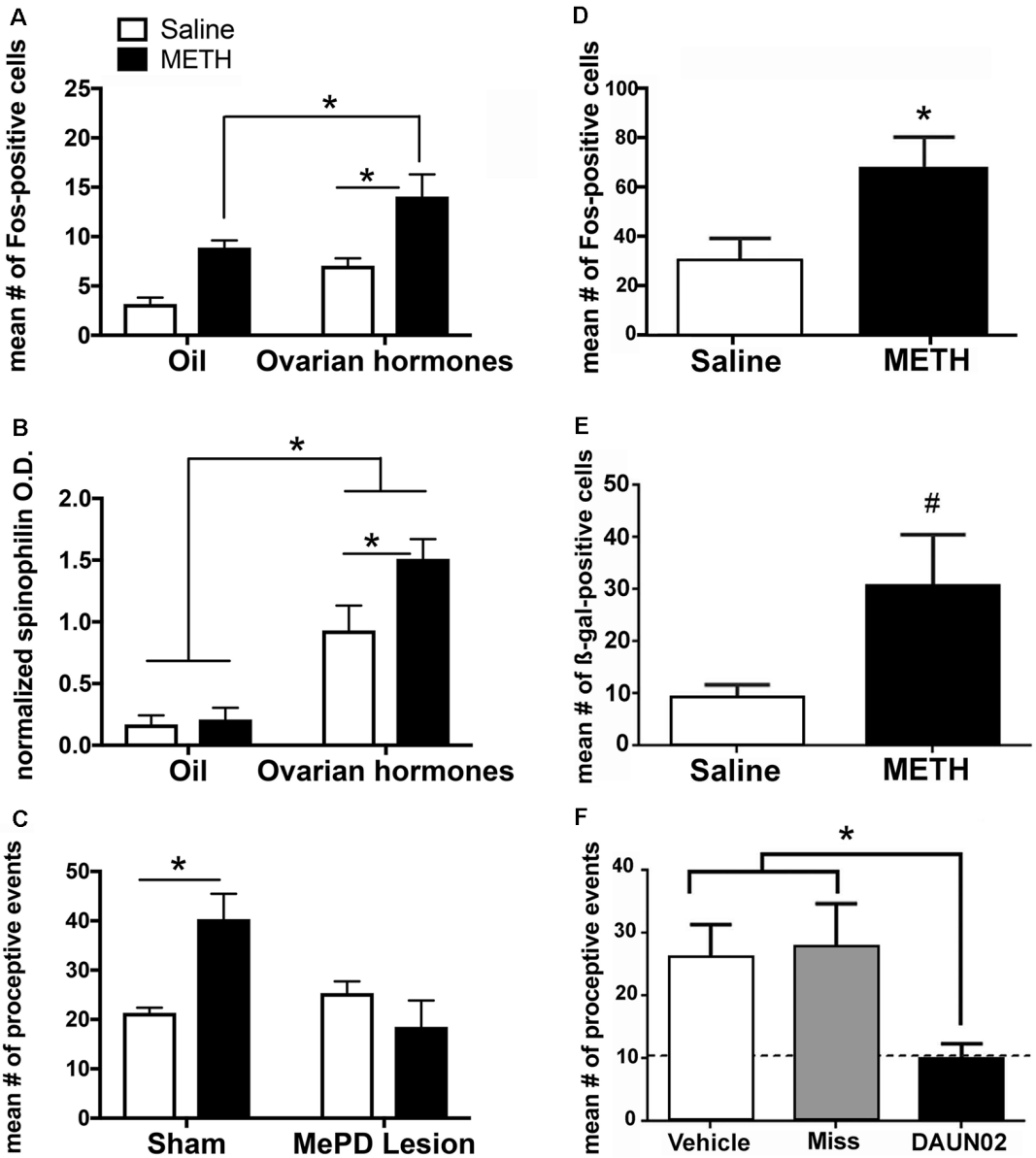

FIGURE 3 | The posterodorsal medial amygdala (MePD) is a locus for the enhancement of sexual motivation by METH. **(A)** The combination of METH and the ovarian hormones estradiol and progesterone increases Fos-immunoreactivity in the MePD, compared to either METH-oil controls and saline-hormone controls. **(B)** METH treatment significantly increases spinophilin protein levels, compared to saline-hormone controls. **(C)** There was a significant interaction of METH and the MePD lesion, such that the lesion of the MePD blocks the METH-induced increases in proceptive behaviors. **(D)** METH increases Fos-immunoreactivity in the presence of ovarian hormones in cFos-lacZ transgenic rats. **(E)** There is a strong trend towards an increase in β-galactocidase (β-gal)-immunoreactivity in the presence of both ovarian hormones and METH in cFos-lacZ transgenic rats. **(F)** DAUN02 inactivation of ovarian hormone- and METH-responsive cells in the MePD prevents the METH-induced increase in proceptive behavior, compared to vehicle-controls and animals in which DAUN02 is infused into areas other than the MePD (Miss). The dashed line represents the baseline levels of proceptive behavior induced by ovarian hormones. Data represents means ± SEM; $^{*}p < 0.05$, $^{\#}p = 0.05$. Reprinted with permission from Elsevier, Inc., Amsterdam, Netherlands; **(A–C)** and under the use of the Creative Commons license **(D–F)**.

integral part of the chromatin around which the DNA spools, which lead to more global alteration of gene transcription (reviewed in Robison and Nestler, 2011). DNA methylation, in which methyl groups are added to DNA molecules by DNA methyltransferase (DNMT), results in repression of gene transcription. Both METH and ovarian hormones reduce the enzymatic activity of DNMT in the MePD (Rudzinskas and Mong, 2018). Acetylation of the histones enables gene transcription by allowing chromatin expansion, and histone deacetylases (HDAC) are enzymes that remove the acetyl groups,

leading to more tightly coiled DNA and a reduction of gene transcription. The combination of both METH and ovarian hormones reduces the enzymatic activity of HDAC in the MePD (Rudzinskas and Mong, 2018). Reduced activity of both HDAC and DNMT should allow for enhanced gene transcription in cells of the MePD. Moreover, these data further support the notion that the MePD is a locus for this enhanced sexual motivation, as no significant changes in HDAC or DNMT activity occur in the VMN. In addition, these changes in enzymatic activity are not the result of changes in the total protein levels of the enzymes (Rudzinskas and Mong, 2018). Taken together, these data suggest that dynamic epigenetic changes may play some role in the genetic mechanisms which underlie METH-enhanced proceptivity. As such, these changes should be investigated further on a gene-by-gene basis, particularly in relationship to the genes explored in the next section of this review article.

MECHANISMS OF ENHANCED SEXUAL MOTIVATION

Dopamine Receptors

While cells within the MePD mediate the METH-facilitated increase in proceptive behavior, it remains unclear how METH and ovarian steroids specifically activate this cellular population to increase neural activation. One likely source of neural activation is dopamine, as one of the primary responses following METH administration is the release of a bolus of dopamine into the synapse (Sulzer et al., 2005; Fleckenstein et al., 2007). The MePD receives both direct and indirect inputs from the ventral tegmental area, a major source of dopaminergic synthesis in the mesolimbic, natural reward pathway (reviewed in Ikemoto, 2007). Thus, it is likely that dopamine receptor (DR) activation in the MePD mediates the enhanced sexually motivated behaviors by METH.

Activation of the excitatory D1-type DRs (D1Rs), which comprise both D_1R and D_5R, in the MePD in the absence of METH increases the number of proceptive events above levels induced by ovarian hormones alone (**Figure 4A**). In addition, administration of an antagonist to these D1Rs in the MePD prevents the METH-induced increase in proceptive behaviors (**Figure 4B**; Holder et al., 2015). In contrast, administration of agonists or antagonists to the D2-type DRs, which comprise D_2R, D_3R and D_4R, in the MePD has no effect on the number of proceptive behaviors displayed. Interestingly, the overall quantity of D1Rs in the MePD remained unchanged between treatment groups (Rudzinskas, 2017). These experiments suggest that METH, through a release of dopamine, may work through the activation of a stable population of D1Rs in the MePD to directly modify the expression of genes underlying female sexual motivation.

The DR agonists and antagonists were administered once a day, for 3 days; therefore, it is probable that the genes affected would be both those necessary for the immediate display of sexual motivation and behavior and those involved in more long-lasting changes to the sexual motivation circuit. One such gene whose expression could be modified *via* changes in D1R activation is the PR (Olesen et al., 2005, 2007). Indeed, METH alone, in the absence of estradiol, increases PRs in the MePD (Holder et al., 2015). However, in a follow-up study, a D1R antagonist infused into the MePD at the same time of METH administration failed

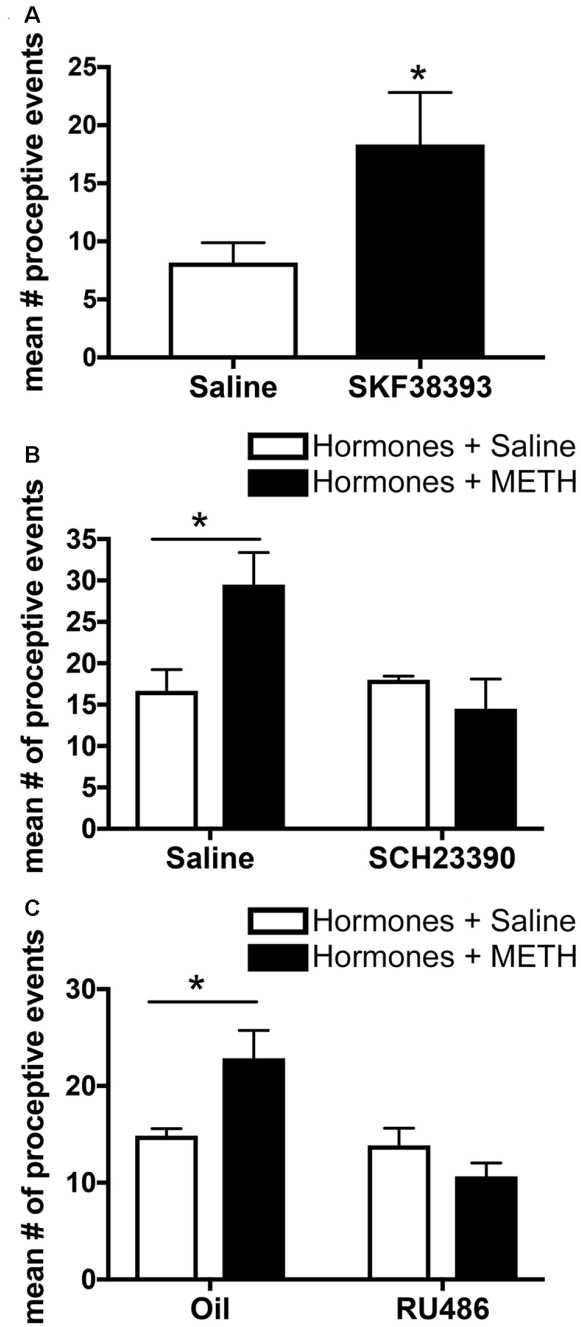

FIGURE 4 | Effects of microinfusions into the posterodorsal medial amygdala (MePD) on sexually-motivated behaviors. **(A)** Infusion of the dopamine type-1 receptor (D1R) agonist SKF38393 increases the number of proceptive events displayed in 10 min. **(B)** Infusion of the D1R antagonist SCH23390 prevents the METH-induced increase in proceptive behaviors. **(C)** Infusion of the progestin receptor (PR) antagonist RU486 prevents the increase in proceptive behavior induced by METH treatment. Data represents means ± SEM; $^*p < 0.05$. Reprinted with permission from Elsevier, Inc., Amsterdam, Netherlands.

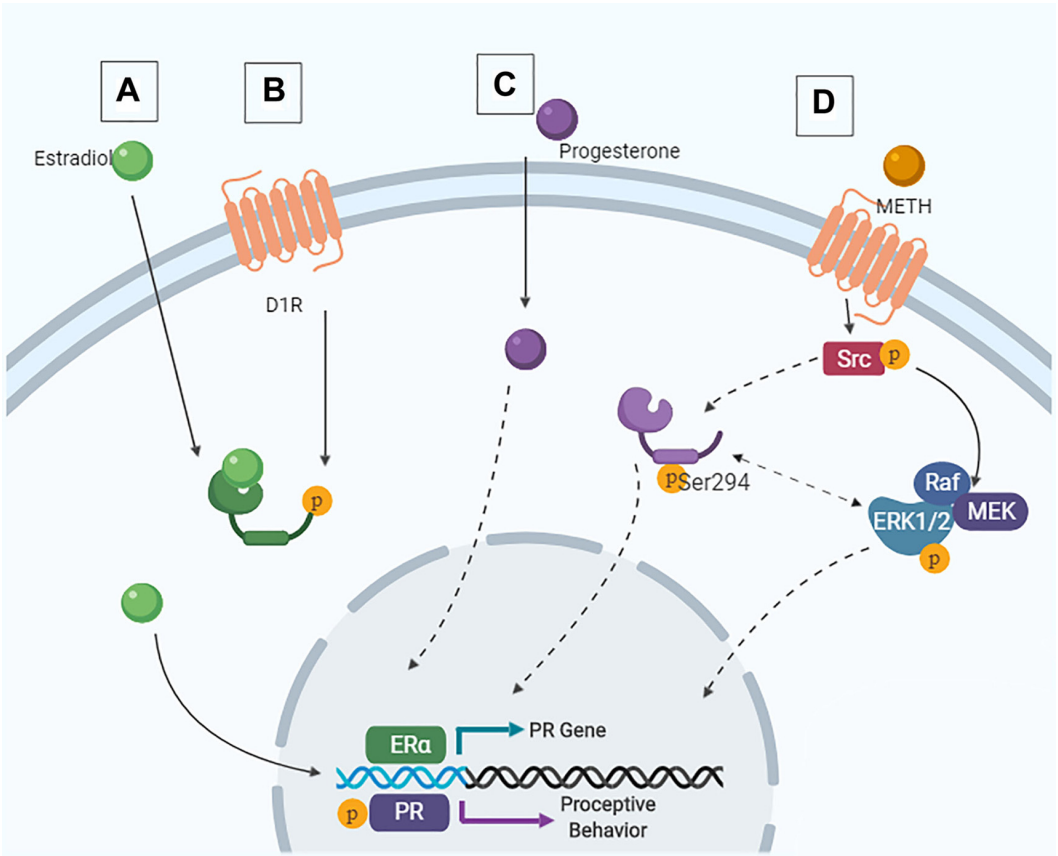

FIGURE 5 | Intracellular signaling cascades in the MePD that contribute to female sexual motivation. **(A)** Estradiol enters the cell and binds to estrogen receptor (ER), leading to ligand-dependent gene transcription. **(B)** Following METH-induced dopamine release, signaling *via* the D1R also leads to ER translocation to the nucleus and ligand-independent transcription of the PR. These are the priming stages. **(C)** Similarly, progesterone can enter the cell and lead to ligand-dependent signaling. **(D)** Following activation of the D1R, Src interacts with the PR, leading to phosphorylation of Src and PR at Ser294. This results in downstream ERK1/2 phosphorylation and increases proceptive behavior.

to change PR levels in the MePD (Williams et al., 2018). It may be microinfusions of the antagonist at a longer time course prior to METH administration may have prevented the METH-induced increase in PRs.

Progesterone Receptors

Progesterone and activation of the PRs are necessary for the display of proceptive behavior, and there is functional specificity of the two isoforms of the nuclear receptor. PR_A activation contributes primarily to the display of lordosis, whereas PR_B activation seems to contribute primarily to proceptivity (Mani et al., 2006). PR activation, primarily through PR_B, in the MePD may facilitate increases in proceptive and other sexually-motivated behavior. While the contributions of the PR isoforms in the MePD to enhancement of sexual motivation by METH has not been determined, it has been demonstrated that the microinfusion of RU486, a PR antagonist, into the MePD decreases the METH-facilitated proceptive behaviors (**Figure 4C**; Holder et al., 2015). Finally, recent work demonstrates that increasing PR protein expression with a lentiviral overexpression vector injected into the MePD in the absence of METH increases proceptive behaviors and lordosis intensity, with no other noted effects on social, exploratory, or rejection behaviors (Williams et al., 2018). Taken together, it is clear that PRs in the MePD have functional relevance toward the induction of female sexual motivation.

Intracellular Mechanisms

In addition to activated D1R increasing the number of PRs in the absence of estradiol, D1R activation can also activate PRs in the absence of progesterone (Auger, 2001); therefore, METH-facilitated activation of D1R could work *via* other, intracellular mechanisms in conjunction with the increased PRs to enhance sexual motivation. The ligand-bound PR is necessary to modulate proceptive behaviors; however, METH can facilitate these proceptive behaviors even in the presence of subthreshold doses of progesterone (**Figure 2A**; Holder et al., 2010). Taken together, this evidence suggests that METH administration enhances PR sensitivity to ligand in the MePD (Weigel et al., 1995).

The changes to PR sensitivity and/or functionality may arise from post-translational modifications, which include

phosphorylation, acetylation, sumoylation, and ubiquitination (Hagan et al., 2012). Of these, the phosphorylation is thought to be the primary regulator of PR actions, such that phosphorylation of specific sites on the PR enhances transcriptional activity (Denner et al., 1990; Bai et al., 1997; Weigel and Moore, 2007). In fact, activation of D1Rs leads to a sequence of kinase phosphorylation events, which could then modulate the activational state of the PRs (Auger, 2001). The PR is highly promiscuous, as it is able to dock onto activated mitogen-activated protein kinases, such as the extracellular signal-regulated kinases (ERK1/2), Src kinases, and the ERs (Lu and Xu, 2006; Dressing et al., 2009). ERK1/2 has been reported to directly phosphorylate the progesterone receptor, while both ERK1/2 and Src kinase have been reported to act in a complex with both ERs and PRs (Migliaccio et al., 1998; Boonyaratanakornkit et al., 2001). The activation of these kinase cascades leads to enhancements of both receptive and proceptive behaviors (González-Flores et al., 2009, 2010; Lima-Hernández et al., 2012).

As the activation of both D1Rs and PRs in the MePD are necessary for the METH-induced enhancement of proceptive behaviors, it is likely that the behaviorally-relevant neurons contain both D1Rs and PRs and that the METH-induced enhancement of sexual motivation arises due to the activity of kinases. In support, METH administration leads to phosphorylation of the ubiquitous kinases ERK1/2 and cSrc in hormonally intact or primed rats (Hebert and O'Callaghan, 2000; Choe et al., 2002; Zhang et al., 2004; Pascoli et al., 2005; Williams et al., 2018). The cytosolic-dependent kinase pathways that could be induced by D1R activation converge with the hormone-dependent kinase pathways at two serine sites in the PRs, serine 294 and 345, suggesting a molecular mechanism through which METH may modulate PR activity (**Figure 5**).

The combined actions of METH and the ovarian hormones increased the phosphorylation of PR serine 294, but not serine 345, in the MePD. Moreover, the administration of a D1R antagonist prevented this increase in phosphorylation of the PR_B at serine 294 (Williams et al., 2018), further supporting the role of the PR_B isoform in the mediation of proceptive behaviors. The activity of the Src kinase to phosphorylate serine 294 of the PR is required for the enhancements of sexual motivation of METH; however, blocking the activation of the ERK1/2 also prevents the METH-induced increases of proceptive behaviors without affecting the serine 294 phosphorylation. Taken together, these data provide evidence of a direct molecular interaction of D1R and PR actions such that the intracellular signaling cascades initiated by D1R activation phosphorylate a site on the PRs in order to modulate the activational states of the PRs. Further studies are necessary to elucidate the role of serine 294 in the MePD and in the relative contributions of the different kinase activation pathways in the MePD on the enhancement of sexual motivation in the female rat. The utilization of modern tools such as CRISPR/Cas-9 may provide further insights into the specificity of this signaling cascade as it relates to female sexual motivation. Ultimately, though, the activation of phosphorylation kinases and enhanced activation of PR would lead to an increase in the transcription of PR-dependent genes. The gene targets of the activated PRs in the MePD that enhance female sexual motivation and influence the central motive state have yet to be determined.

CONCLUSION

One of the key components for sexual behavior is that of sexual motivation. We have presented one model system in which we can further study the motivational aspects of sexual behavior. The data presented in this review article indicates that sexual motivation arises from interactions of neurotransmitters and steroid hormones to change the central motive state. In addition, these interactions can be influenced by pharmacological agents, such as METH, to further increase the central motive state and drive the response to sexually-relevant stimuli. With the advent of technologies that enable us to examine and determine the nature of these interactions on epigenetic and molecular levels, we approach answers to such fundamental questions as the origins of sexual motivation. The use of the METH-model of enhanced sexually-motivated behaviors has already revealed complexities to an admittedly intricate and multifaceted system; however, this model also presents new avenues for research that may ultimately reveal the origins of sexual desire.

AUTHOR CONTRIBUTIONS

SR, KW, JM, and MH designed the studies referenced. SR, KW, and MH performed the experiments and wrote sections of the manuscript. MH wrote the outline of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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"What a Girl Wants": What Can We Learn From Animal Models of Female Sexual Motivation?

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Sexual motivation is notably different than other motivations such as hunger and thirst, because it lacks homeostatic drive. Sexual motivation poses no threat to physical well-being; individual survival is not at stake. Nevertheless, sexual motivation is a powerful drive and is critical for species survival. Understanding the complexity of sexual motivation has the potential to advance our understanding of other motivations, even pathological motivations, such as those associated with substance abuse. The study of motivation that is unique to females has often been neglected. A number of paradigms have been developed to investigate female sexual motivation beyond measuring only the lordosis reflex. Lordosis is a reflexive posture displayed by female mammals in response to male sexual stimulation to facilitate intromission. The lordosis reflex is essential, but studying the drive to mate is compromised in the absence of robust lordosis. Therefore, appetitive measures of sexual behavior (e.g., preferences, solicitation behaviors) are more specific and more sensitive indicators of sexual motivation than lordosis alone. Paradigms designed to study female sexual motivation often provide a female subject with the choice to interact with a sexually vigorous male or either a non-sexual partner (i.e., female, castrated male) or to remain alone. The study of appetitive measures of sexual motivation has elucidated the role of hormones in female sexual motivation, as well as the underlying neural pathways. The present review describes methods for studying female rats to advance our understanding of sexual motivation and sexual dysfunction.

Keywords: paced-mating behavior, partner-preference test, mate choice, solicitation behavior, rats

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FEMALE SEXUAL MOTIVATION MODELED IN RATS

Motivation for sex is unlike many other drives, in that sex lacks a homeostatic drive for balance. Early theories of motivation relied on the assumption that an organism is motivated by an experience of deprivation that creates a need, subsequently activating drives, and then behaviors, which are directed toward a beneficial goal, relieving deprivation (Hull, 1943). Because there is no necessary "deprivation state", "set point" or "optimal" amount of sex, it is difficult to account for the motivation resulting in sexual behavior with a concept that starts with deprivation. However, in most females – across species – sexual motivation can only be observed when fertilization is possible; if the female is not approaching ovulation, no sexual behavior is displayed and sexual motivation is low. A female rat will avoid a male rat during all phases of her estrous

cycle (metestrus, diestrus), except for behavioral estrus (i.e., proestrus). The day of proestrus is characterized by a rise in gonadal hormones (e.g., estrogen followed by progesterone) in anticipation of ovulation. This period of behavioral estrus lasts approximately 24 h. It starts abruptly and ends abruptly (Chu and Agmo, 2015a,b). Therefore, sexual motivation in most mammalian females can only be measured during a limited period of time. During this time, females will display the lordosis reflex. The lordosis reflex is defined as the dorsal flexion of the female rat's back in response to physical contact (e.g., mounting) from a male rat (Beach, 1976). The lordosis posture facilitates penile penetration and reflects a female's willingness to receive sexual stimulation from the male (i.e., sexual receptivity). However, because lordosis is a reflex in response to physical contact from the male, it lacks elements of what many consider the basic element of motivation – drive. In many species, including humans, the time-sensitive willingness to engage in sex contributes to the observation that in most species, males have a higher drive for sex than females. Approach behavior is often used as a measure of, and surrogate for, drive. If organisms are motivated to acquire a goal (e.g., food, water, drugs), they will actively seek out and approach the goal. Initially, goal-directed sexual motivation was studied using instrumental conditioning (Everitt and Stacey, 1987; Everitt et al., 1987; Everitt and Wolf, 2002), much like early studies of drug reward. However, these experiments required extensive training and pairing of sexual stimuli with instrumental responses. The present review attempts to identify more parsimonious measures of female sexual motivation. Over the last 40 years, a number of paradigms have been developed to specifically measure female sexual behavior and quantify sexual motivation. The study of female sexual motivation has turned out to be a complicated and nuanced endeavor.

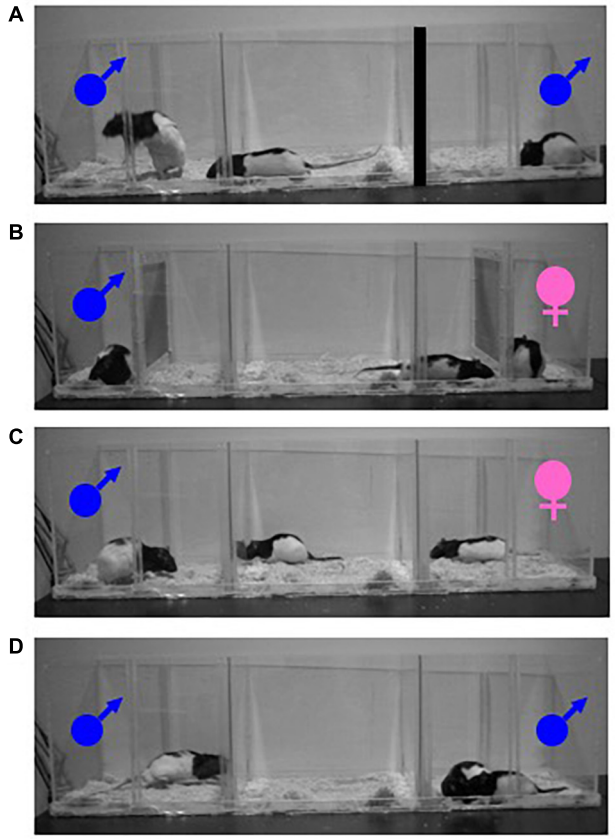

FIGURE 1 | Photograph of a typical paced-mating behavior test where a female rat can mate with one male rat **(A)**. Photograph of a partner-preference test where physical contact is restricted between a female rat (center compartment) and a male stimulus (left compartment) or a female stimulus (right compartment). Both stimulus animals behind wire mesh **(B)**. Photograph of a partner-preference test where physical contact is not restricted between a female rat (center compartment) and a male stimulus (left compartment) or a female stimulus (right compartment) **(C)**. Photograph of a mate choice test where a female rat could interact freely with either of two male stimulus animals **(D)**.

LABORATORY PARADIGMS THAT MEASURE FEMALE SEXUAL BEHAVIOR AND MOTIVATION

One of the first advances in the study of female sexual motivation involved studying wild and domesticated rats in a semi-natural environment (McClintock and Adler, 1977; McClintock and Anisko, 1982; McClintock et al., 1982). The environment was developed such that female rats could control the rate, or pace, of sexual contact. In the *paced-mating behavior* paradigm, a sexually receptive female is given the opportunity to approach and withdraw from a sexually vigorous male, thereby controlling the timing of mounts, intromissions, and ejaculations (i.e., sexual stimulations). Female rats will pace the receipt of sexual stimulation in semi-naturalistic conditions, as well as in more minimal laboratory settings. This paradigm has been used extensively to model naturalistic aspects of female sexual behavior and quantify female responses (Erskine, 1989; Blaustein and Erskine, 2002). Typically, in this paradigm a female rat is given the opportunity to enter through holes in a divider that separate the subject from a male rat. In **Figure 1A**, a female rat is depicted leaving the center compartment and approaching the male rat on the left. Access to another male on the right is prevented by blocking the holes in the divider. Furthermore, when female rats can control the rate at which they receive sexual stimulation from one or more males simultaneously, we can assess how the specific measures of paced-mating behavior (e.g., percentage of exits: likelihood of leaving the male after sexual stimulation; contact-return latency: latency to return to the male after sexual stimulation) reflect female sexual motivation. For example, changes in the latency to return to the male after receiving sexual stimulation reflect changes in motivation, with faster return latency indicating an increase in motivation to mate. Differences in percentage of exits are also sensitive to motivational state. For instance, more intense genital stimulation (mount < intromission (mount + penetration) < ejaculation) increases the likelihood of the female's withdrawal and leads to longer periods away from the male (Erskine, 1989). Allowing

the female to pace sexual contact with one or more males is similar to the mating conditions of rats in their natural habitat (Calhoun, 1962). Paced-mating behavior is associated with larger litters (Coopersmith and Erskine, 1994) and is more rewarding (Paredes and Vazquez, 1999; Martinez and Paredes, 2001) for the female, when compared to non-paced conditions. The observation of paced-mating behavior in females, makes understanding female sexual motivation more complicated than male sexual motivation, given that there are aspects of a sexual encounter that seem to drive females *away* from males in the middle of a sexual encounter. The complexity of female sexual behavior is even more problematic when viewing this behavior through a lens common to the study of motivation; more approach = more motivation. Female sexual behavior is not endless approach behavior, suggesting that not all aspects of sexual contact are equally motivating. Therefore, sexual behavior in the female rat becomes a delicate balance between approaching the male and avoiding the male (Paredes and Vazquez, 1999). Somatosensory stimulation received from the male, and female motivation, act in concert to affect female behavior and likely contribute to the avoidance of the male during mating (Erskine et al., 2004; Clark et al., 2011). Because receipt of sexual stimulation triggers withdrawal, the amount of time spent with the male is reduced when mating is possible, relative to when the female can only exchange olfactory, visual, and auditory stimuli, but not mate with the male (Clark et al., 2004). The control of the timing of sexual contact is not only rewarding for females (Paredes and Vazquez, 1999; Martinez and Paredes, 2001), but also increases fertility. Therefore, the somatosensory stimulation experienced during intromission and insemination (Komisaruk and Wallman, 1977) may have been essential for the development of paced mating in the species and contributes to the rewarding qualities of vaginocervical stimulation.

The *partner-preference test* is a paradigm commonly used to evaluate approach and the appetitive aspects of sexual behavior (Paredes and Alonso, 1997; Avitsur and Yirmiya, 1999; Paredes and Vazquez, 1999; Bakker, 2003). During a partner-preference test, a sexually receptive female is given the choice to spend time in the vicinity of either a sexual partner (e.g., sexually vigorous male) or a non-sexual partner (e.g., same-sex conspecific, castrated male). A sexually receptive female rat will spend more time with the sexual partner when the sexual partner is placed behind a wire mesh thereby restricting physical contact (**Figure 1B**), than when physical contact is not restricted and mating is possible (**Figure 1C**). The difference between preferences observed when physical contact is restricted vs. when physical contact is unrestricted indicates that the distal cues (i.e., auditory, visual and olfactory) of a sexual partner are not only sufficient for approach behavior but these cues elicit a more robust preference in female rats (Clark et al., 2004). Because female rats spend less time with a sexual partner under conditions that also allow them to engage in paced-mating behavior, it is possible that some aspects of sexual stimulation received during paced mating may be aversive to female rats. Alternatively, the difference between the two conditions of the partner-preference test could also be a function of the very nature of paced-mating behavior. Specifically, leaving the male after the receipt of sexual stimulation followed by periods of time remaining away from the male could artificially reduce the time that a female rat can spend with a sexual partner.

The *conditioned-place preference (CPP)* paradigm has also been used to assess the rewarding aspects of sex. Although the CPP paradigm has been useful in assessing the rewarding properties of drugs that are commonly abused, such as opiates and psychomotor stimulants (Carlezon, 2003), it has also been used to identify which aspects of sex and under which conditions do female rats find sex rewarding. In the CPP paradigm, aspects of a sexual encounter (e.g., conditions for mating, types of mating stimulation) are repeatedly paired with spending time in one distinct context (e.g., white walls, gravel floor), whereas another distinct context (e.g., black walls, grate floor) is paired with a control condition (e.g., no mating). If aspects of a sexual encounter were sufficiently rewarding, an association between the context and sexual encounter will develop. Evidence of this reward state will be expressed by subjects as a preference to spend time in that conditioned context when given the opportunity to spend time in either context. Initial studies found that paced-mating behavior could be conditioned, therefore female control over the timing of mating is rewarding (Paredes and Alonso, 1997). Furthermore, pre-treatment with naloxone (i.e., opiate antagonist) blocks the formation of a CPP associated with female paced sexual stimulation, indicating that the rewarding properties of paced-mating behavior depend on opioid receptors (Paredes and Martinez, 2001). However, a number of studies have since suggested that what is rewarding is not necessarily control *per se*, but allowing the female to take a break between sexual stimulation. For example, Becker and colleagues reported increases in mesencephalic (i.e., striatum, nucleus accumbens) dopamine release in response to copulation if the female experiences her "preferred pacing interval" between sexual stimulations, even when the female had no active control of this interval (Jenkins and Becker, 2001, 2003a,b). Meerts and Clark (2007, 2009) have also found that vaginocervical stimulation (VCS) is rewarding when measured using the CPP paradigm, independent of active control (i.e., artificial VCS or non-paced mating conditions), as long as females are given a brief period of time without any sexual stimulation following ejaculations (the most intense sexual contact), suggesting that the reprieve from sexual stimulation is critical for the reward state.

The *mate choice* paradigm is another methodology that has been used to advance our understanding of the rewarding properties of sex in female rats. Although rats are promiscuous, preference for one mate over another has been observed. In this paradigm, female rats are given the choice to mate with multiple male rats simultaneously (**Figure 1D**). Choice of one mate over another can be determined by which mate the female spends more time with and/or which mate is visited first. Results from our lab have consistently found that a female rat will spend more than twice as much time with one mate (i.e., her preferred mate) than another (i.e., her non-preferred mate), as well as return faster to her preferred mate than to her non-preferred mate following sexual stimulation. In addition, female rats receive more sexual stimulations from their preferred mate than their non-preferred mate. Female rats will visit and display solicitation

behaviors more frequently with their preferred mate than their non-preferred mate (Ferreira-Nuño et al., 2005; Lovell et al., 2007; Zewail-Foote et al., 2009). The pattern of behavior displayed with a preferred mate further supports the conclusion that measures of paced-mating behavior reflect sexual motivation. Specifically, females are less likely to leave their preferred mate than their non-preferred mate after receiving sexual stimulation, but if they do leave, they return to their preferred mate faster than their non-preferred mate. In addition to describing the patterns of mate choice in female rats, we have also investigated the effects of mate choice on reproductive success (Lovell et al., 2007; Zewail-Foote et al., 2009). From these studies, we have found that female rats consistently prefer the same mate across multiple tests, as well as between different females. However, using the mate choice paradigm or olfactory preferences for particular mates, we have been able to determine that it is unlikely that preference for a particular male rat is related to urinary testosterone levels, body weight, or testes weight (Winland et al., 2012) but instead female rats are attracted to males with high levels of major urinary proteins, which could communicate health, nutritional status, and social rank (Kumar et al., 2014). Surprisingly, mate choice does not seem to provide any reproductive advantage (Winland et al., 2012; Chu et al., 2015).

During any mating encounter, in any paradigm, female rats also display species-specific, sex-specific behaviors, such as hopping, darting, ear wiggling, and presenting (Erskine, 1989). These additional behaviors displayed by sexually receptive female rats seem to attract or "solicit" the attention of potential mates, hence the common use of the term "solicitation behaviors" to describe this cluster of behaviors. Although the underlying neural mechanism of these behaviors is not well known, solicitation behaviors often precede the receipt of sexual stimulation from a male, suggesting that there is a functional purpose for these behaviors. Not all female rats display solicitation behaviors consistently throughout a mating encounter, nevertheless, interest in sexual contact has been inferred from the rate at which females attract a male rat's attention with the display of hops, darts, ear wiggles, and presentations. New qualitative analyses of complex sequences of behavior may be useful in furthering our understanding of the role solicitation behaviors play in a sexual interaction.

INDICATIONS OF MOTIVATION

A consistent pattern of behavior has been identified in the many recent experiments investigating female sexual behavior using the aforementioned paradigms. For example, following the administration of a number of different psychomotor stimulants that are known to enhance, or cross-sensitize with, other reinforcing drugs, female rats have been shown to spend more time with a sexual partner, leave a sexual partner less frequently, and display more solicitation behaviors (Guarraci, 2010; Guarraci and Bolton, 2014). This pattern of behavior likely reflects an increase in a female rat's motivation to spend time interacting with a male rat. Alterations in motivation do not always follow the above pattern perfectly; rarely do we observe females spending more time, leaving less often, coming back faster, and displaying more solicitations as the result of a drug treatment or other experimental manipulation. More often than not, we see the pattern with two or three of these behaviors affected. For instance, ketamine, at doses comparable to what is being used off-label to treat depression, increased time spent with a male rat during a partner-preference test and decreased the likelihood of leaving the male after sexual stimulation (Guarraci et al., 2018). In addition to pharmacological studies, we have also observed this pattern of enhanced motivation to mate under other conditions. The pattern is observed while a female is mating with her preferred mate (as mentioned above). This pattern is also observed after repeated mating encounters, as female rats transition from virgins to experienced breeders. With regular repeated sexual experience, female rats spend more time with a male, are less likely to leave after sexual stimulation, return to the male faster, and display more solicitation behaviors, when compared to virgin females during their first sexual encounter (Meerts et al., 2014, 2016; Guarraci and Meerts, 2017; Arnold et al., 2019; Piergies et al., 2019).

In contrast, a variety of conditions result in a consistent pattern indicating a disruption of sexual motivation beyond the lordosis reflex. This pattern is characterized by female rats spending less time with a sexual partner, leaving the male more frequently, taking longer to return after receiving sexual stimulation, and displaying fewer solicitation behaviors. We have observed this disruptive pattern when female rats are exposed to drugs that block estrogen receptors or drugs that inhibit PDE-5 (Clark et al., 2003, 2009). Lesions of the medial preoptic area of the hypothalamus also decrease time spent with a sexual partner, increase the likelihood of leaving, and delay returning to the male after receiving sexual stimulation (Yang and Clemens, 2000; Guarraci et al., 2004; Guarraci and Clark, 2006) when subjects are tested for partner preference and during paced-mating behavior. Interestingly, this pattern was observed even though the lordosis reflex remained intact; motivation of the female rat to actively pursue sexual contact was diminished by the lesions despite robust lordosis.

An important consideration must be made when a manipulation or treatment changes levels of general locomotor behavior. For example, psychomotor stimulants increase locomotion. Such increases in locomotion can artifactually affect measures we record during mating tests, such as visits to the stimulus animals. In contrast, opiates and aging have been shown to decrease general locomotor behavior. In lieu of these changes in locomotion, we have had to rely on discrimination between the male and the female stimulus. Specifically, even when locomotor behavior is increased following administration with caffeine, we have noted that visits to the male stimulus outpace visits to the female, indicating discriminating motivation (Guarraci and Benson, 2005). Similarly, we have noted increases to the male stimulus compared to the female stimulus despite overall decreases in visits to both stimulus animals in middle-aged female rats (under review).

Taken together, the growing literature investigating female sexual motivation indicates that sexual behavior in the female rat a complex balance between approach and withdrawal that

can be measured with a number of paradigms. Studies of sexual motivation in female rats can be used to advance our understanding of the underlying neural pathways of healthy motivation, as well as dysfunctional motivation.

AUTHOR CONTRIBUTIONS

Both authors worked equally on the development of the ideas and the writing.

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REVIEW
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Motivational Drive in Non-copulating and Socially Monogamous Mammals

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Motivational drives guide behaviors in animals of different species, including humans. Some of these motivations, like looking for food and water, are crucial for the survival of the individual and hence for the preservation of the species. But there is at least another motivation that is also important for the survival of the species but not for the survival of the individual. Undoubtedly, sexual motivation is important for individuals to find a mate and reproduce, thus ensuring the survival of the species. In species with sexual reproduction, when males find a female in the appropriate hormonal conditions, they will display sexual behavior. However, some healthy males do not mate when they have access to a sexually receptive female, even though they are repeatedly tested. These non-copulating (NC) individuals have been reported in murine, cricetid and ungulates. In humans this sexual orientation is denominated asexuality. Asexual individuals are physically and emotionally healthy men and women without desire for sexual intercourse. Different species have developed a variety of strategies to find a mate and reproduce. Most species of mammals are polygamous; they mate with one or several partners at the same time, as occur in rats, or they can reproduce with different conspecifics throughout their life span. There are also monogamous species that only mate with one partner. One of the most studied socially monogamous species is the Prairie vole. In this species mating or cohabitation for long periods induces the formation of a long-lasting pair bond. Both males and females share the nest, show a preference for their sexual partner, display aggression to other males and females and display parental behavior towards their pups. This broad spectrum of reproductive strategies demonstrates the biological variability of sexual motivation and points out the importance of understanding the neurobiological basis of sexual motivational drives in different species.

Keywords: sexual motivation, polygamy, monogamy, wanderer, non-copulating males, asexuality

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INTRODUCTION

Mammals display several reproductive strategies that can be influenced by population density, group size, distribution, home range size, abundance of food and resources. In mammals, the most common mating strategy is polygamy with the polygyny (one male more than one female) and polyandry (one female, more than one male, rare or inexistent in no human species) as subtypes. In polygamy, there is no sexual exclusivity and reproductive success is maximized through multiple mating partners (Kleiman, 1977). Social monogamy is a reproductive strategy in species in which resources are evenly distributed but sparse, females can disperse and have large home

ranges, and males are not able to defend the access to more than one female. Also, a low density of females and food can favor monogamy. Monogamy is also present when successful rearing of offspring requires paternal and maternal care. Males help carry the litter, provide food for them and the mother when this resource is energetically costly to obtain, and the litter size is larger (Clutton-Brock and Harvey, 1978). Socially monogamous males and females after mating establish a pair bond that can last more than one reproductive cycle. However, in monogamous species some males and females do not form this pair bond and only mate opportunistically.

Interestingly, there are males and females in polygamous and socially monogamous species that do not mate even if they have the opportunity. In humans, around 1% of healthy men and women are not interested in engaging in sexual activity and are denominated as asexual. However, asexual individuals are interested in other motivational aspects of sexuality such as romantic relationships (Bogaert, 2004; Prause and Graham, 2007; Brotto and Yule, 2017; Jones et al., 2017). The biological bases of asexuality in humans are not well understood due to their complexity and ethical issues. However, the physiological bases of asexuality have been studied in murine, cricetid and ungulates, where some males do not mate even if they are tested with several sexually receptive females. In this manuscript, we will briefly outline different motivational strategies associated with reproduction in mammals and then we will describe in more detail the possible neurobiological factors associated with non-copulating (NC) males and the socially monogamous prairie vole.

In most mammals, sexual behavior consists of stereotyped movements usually organized in predictable patterns that are similar between individuals, but which vary between species. The specific patterns displayed by males and females reflect the motivational or consummatory aspects of sexual behavior. The comparative analysis between species showing different mating strategies including monogamy, polygamy and the case of asexuality could help us understand the biological variability of sexual motivational drives in mammals.

MOTIVATIONAL DRIVE IN RODENTS

Under the appropriate hormonal conditions, females in estrus will display a series of stereotyped behaviors to attract a male. Originally described by Beach (1976), proceptive behaviors are displayed to attract the male and they include approach, orientation, and runaway. After a receptive female approaches the male, she positions herself placing her anogential region in contact with his face. After that, she may display hopping and darting as if running away and ear wiggling. In some rodents, these proceptive behaviors can be accompanied by scent marking and/or ultrasonic vocalizations (Gonzalez-Flores et al., 2017). After these behaviors are displayed by the female, the male will usually follow her and display mounts and intromissions. In the case of a sexually experienced male rat, he will display around 15 intromissions before ejaculating. If the female is receptive, she will arch her back, elevate the pelvis and deviate the tail. This lordosis reflex facilitates intromissions and ejaculations (Hardy and DeBold, 1972). It has been suggested that the male rat is an unconditional incentive stimulus for the female which she will approach without a previous learning or rewarding experience (for a discussion see Ågmo, 2003). Consistent with this hypothesis studies in seminatural and natural conditions have demonstrated that the female rat has a very active role in mating, controlling and spacing the stimulation she receives during a sexual interaction (McClintock and Adler, 1978). Classical studies have shown that under laboratory conditions females can also control (pace) the sexual interaction (Erskine, 1989). Many studies indicate that when subjects (males or females) pace the sexual interaction a reward state is induced that ensures that the behavior will be repeated in the future (reviewed in Paredes, 2014). Moreover, mating under pacing conditions induces the formation of new cells and neurons in the olfactory bulbs (OBs) and dentate gyrus of the hippocampus indicative of permanent plastic changes after mating (for a review see Bedos et al., 2018; Portillo et al., 2019).

Another important characteristic that is observed in natural and/or seminatural conditions is that rats are promiscuous. Usually, several females will be in estrus at the same time and they will mate with one or several partners repeatedly changing partners in the middle of copulation (McClintock and Anisko, 1982). In this way, a female could receive as first stimulation an ejaculation from a male that had been mating with another female and a male could mate with a female that has received several intromissions or ejaculations. Other studies in which subjects can choose between different mating partners indicate that the females spend more time with a male, but the preferred male is different across the estrous cycle (Ferreira-Nuño et al., 2005). It has also been shown that rats can develop conditioned mate preference for a partner that has been associated with sexual reward cues (Pfaus et al., 2001). More recent studies in seminatural observations indicate that females have a preferred male with whom they copulate more but receive intromissions and ejaculations from both the preferred and non-preferred males (Chu and Ågmo, 2014). One important characteristic of group mating is that males and females eventually receive the same amount of stimulation with both sexes controlling sexual interaction. It thus appears that sexual behavior in rats has evolved to ensure that sexual interaction will be rewarding for both sexes and hence increase the probability that the behavior will be repeated (for a discussion see Paredes, 2014).

Non-copulating (NC) Males

Under appropriate conditions and when the female is in estrous most males will mate with her. However, it is well documented that some males will not mate even though they area repeatedly tested with receptive females. The existence of NC animals in different species confirms the biological variability in sexual motivational drives and allows the opportunity to study and understand the biological bases of asexuality (see below). NC males have been identified in sheep, guinea pigs, gerbils, hamsters, rats and mice (Whalen et al., 1961; Harding and Feder, 1976; Paredes et al., 1990; Alexander et al., 1999; Clark and Galef, 2000; Portillo et al., 2006, 2010, 2013; De Gasperín-Estrada et al., 2008; Borja and Fabre-Nys, 2012;

Canseco-Alba and Rodríguez-Manzo, 2013; Mirto et al., 2017; Ventura-Aquino and Paredes, 2017). They represent between 1% and 5% of murine (Portillo et al., 2006, 2013) and 16%–20% of ungulates (Alexander et al., 1999). To our knowledge, no research group has evaluated whether there are asexual females in different mammalian species. Therefore, this is a field of great scientific potential and interest. Some studies have evaluated females that display low levels of sexual behavior. For example, Snoeren and coworkers evaluated the sexual motivation of a female rat to approach a male using an arena with two compartments. One of the compartments was empty and the other contained a sexually active male, only females were able to move from one compartment to the other. Females were classified into three groups: those that avoid the male, females that approach the male and a middle group. The females that avoid the males show low preceptive behaviors. The authors suggest that the females that avoid the males represent an animal model to evaluate hypoactive sexual desire disorder (Snoeren et al., 2011). In the following section, we will describe studies of NC males in murine and ungulates, which are the most studied species.

Several studies have suggested that NC males can have alterations in brain regions that control sexual behavior. In mammal's the medial preoptic area (MPOA) regulates different motivated behaviors such as aggression, parental and sexual behavior (Pfaff and Baum, 2018; Yoshihara et al., 2018; Tsuneoka, 2019). With respect to sexual behavior, the MPOA modulates the appetitive (motivational) and consummatory (execution, mount, intromission and ejaculation) aspects of male sexual behavior (Paredes, 2003; Pfaff and Baum, 2018). Bilateral lesions of the MPOA eliminate consummatory components of sexual behavior in several species including fish, lizard, snake, quail, rat, guinea pig, marmoset, chicken, frog, mouse, hamster, ferret, goat, cat, dog and rhesus monkeys. On the other hand, stimulation of the MPOA induces penile erections in squirrel monkeys. In rats stimulation of this brain region increases mating; review in Paredes (2003) and references therein. The lack of sexual behavior in NC males is not associated with a decrease in plasmatic testosterone levels or a reduction of testis and seminal vesicle weight (Stefanick and Davidson, 1987). Also, males with lesions in the MPOA do not present alterations in penile erection or seminal emission (Larsson and Heimer, 1964; Lisk, 1968; Stefanick and Davidson, 1987; Liu et al., 1997). As already mentioned, the MPOA also plays a fundamental role in the appetitive components of male sexual behavior. Male rats with MPOA lesions show a decrease in the time they pursue the female. Partner preference tests have also demonstrated the importance of the MPOA in the motivational components of male sexual behavior. When given the choice to interact with a sexually receptive female or a male, both male rats and ferrets show a clear preference for the sexually receptive female. However, after bilateral lesions of the MPOA the males do not mate with the females and they show a preference for the male in both ferrets (Cherry and Baum, 1990) and rats (Paredes et al., 1998). Male rats also show a clear preference for odors from estrous females as opposed to odors from anestrous females or clean odors. Again, rats with MPOA lesions lose this preference and equally prefer estrus and anestrus female odors. This change in olfactory preference was not associated with alterations in the neuronal processing of sexually relevant odors in the accessory olfactory system (Hurtazo and Paredes, 2005).

Much like males with MPOA lesions, NC male rats, do not have genital dysfunction as they show penile reflexes and spontaneous seminal emission similar to copulating males (Stefanick and Davidson, 1987). Also, NC rats and mice do not have alterations in plasmatic testosterone or estradiol levels that could explain the lack of sexual interest and systemic hormone replacement fails to induce sexual activity (Whalen et al., 1961; Stefanick and Davidson, 1987; Portillo and Paredes, 2003; Portillo et al., 2006, 2013). Although there are no differences in their plasmatic hormonal levels, NC rats have alterations in their steroid receptors. Androgen receptors (ARs) are higher and estrogen receptors alpha are lower in the MPOA of NC males and the activity of the aromatase enzyme (enzyme that converts testosterone to estradiol) is reduced in the MPOA of NC males (Portillo et al., 2006, 2007). Interestingly, our research group has demonstrated, that testosterone or estradiol implants in the MPOA induces mating in previously NC male rats (**Figure 1**). These effects are specific to the MPOA since estradiol or testosterone implants outside this area fail to induce sexual behavior (Antonio-Cabrera and Paredes, 2014). Similarly, NC or sexually sluggish rams do not have alterations in testosterone or luteinizing plasmatic levels. However, when copulating rams cohabit with sexually receptive females their plasmatic levels of luteinizing hormone (LH) increase. This physiological response is not observed in NC rams or in males that do not mount receptive females but display the behavior with other males (male-oriented males; Alexander et al., 1999). NC rams also have alterations in their hormone receptors. NC rams have a reduced number of estrogen receptors in the MPOA and higher number in the anterior adenohypophyses in comparison to sexually active males (Alexander et al., 1993). Moreover, studies in rats and rams have shown that the MPOA of NC or sexually sluggish males, those that do not mate consistently or take a long time to ejaculate, is smaller than that of copulating males and similar to the MPOA of females (Rhees et al., 1999; Alexander et al., 2001) suggesting that these males show neuroanatomical feminization of the MPOA.

NC male rats also have alterations in different aspects of sexual motivation. NC rats show less social behavior such as autogenital grooming and display reduced grooming partner and vocalizations than copulating males (Pottier and Baran, 1973). Our research group has shown that NC males show a reduced preference for odors or for the presence of sexually receptive females. Whereas copulating male rats and mice show a clear preference for a receptive female with whom they can mate (sexual preference) or for one they can only see, hear and smell (sexual incentive motivation) as opposed to a male or a non-receptive female, NC mice and rats do not show any preference (Portillo and Paredes, 2003, 2004; Portillo et al., 2013). In rodents, the sense of smell is very important to identify conspecifics and their pheromones. Copulating males show a strong preference for bedding exposed to secretions of receptive females as opposed to anestrous, male or clean bedding.

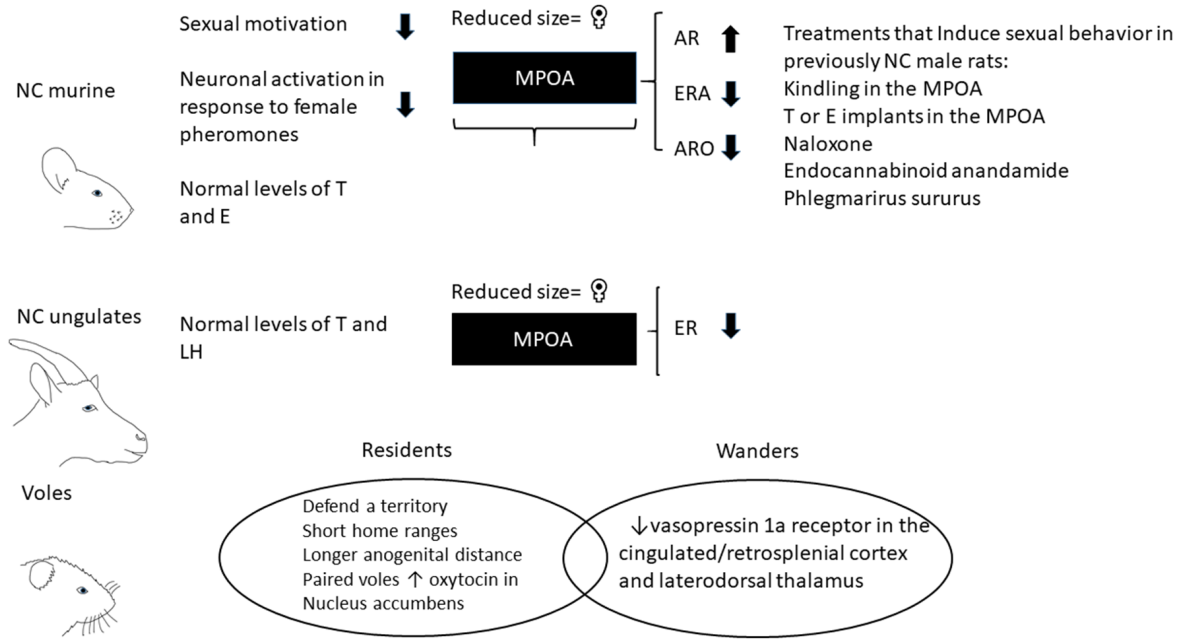

FIGURE 1 | Characteristics of non-copulating males (NC) and voles with resident and wandering mating strategies. NC rats have normal plasmatic levels of testosterone (T) and estradiol (E) and ungulates have normal plasmatic levels of T and luteinizing hormone (LH). The medial preoptic area (MPOA) is involved in sexual motivation and shows a reduced size in NC rats and rams in comparison to copulating males. The MPOA in NC males has increase androgen receptors (ARs), reduce estrogen receptor alpha (ERA) and low activity of aromatase enzyme (ARO). Treatments that induce sexual activity in previously NC male rats: MPOA kindling stimulation; T or E implants in the MPOA; systemic administration of naloxone, endocannabinoid anandamide and *Phlegmarirus sururus* extract. Prairie voles are socially monogamous species. However, around 30% of the males show a promiscuous mating strategy (wanderers) which have shorter anogenital distances, decrease in oxytocin receptors in the nucleus accumbens (NAc) and vasopressin 1a receptors in the cingulated/retrospenial cortex and thalamus in comparison to males that form pair bonds (residents).

Although NC males also choose the estrous odors this preference is significantly reduced compared to copulating males (Portillo and Paredes, 2003, 2004; Portillo et al., 2013). NC mice can discriminate volatile urine odors from males and females, but they spend less time smelling them compared with copulating males (Portillo et al., 2013). Taken together, these results suggest that NC males are not sexually motivated by the receptive females or their odors (**Figure 2**).

The lower preference for estrous female odors in NC males may be due to deficits in the neuronal processing of sexually relevant odors. For example, when copulating males detect odors from estrus females, the MPOA, and other neuronal regions in the vomeronasal projection pathway increase their neuronal activity, evaluated by the expression of the protein of the early gen c-Fos. On the contrary, the MPOA and central structures of the vomeronasal projection pathway in NC males do not increase their neuronal activity (Portillo and Paredes, 2004; Portillo et al., 2013). Thus, NC males have an alteration in the neuronal processing of sexually relevant cues. This reduction in neural activity could simply reflect the reduce motivation that these males have for sexually receptive females or their odors (**Figure 2**).

Sexual behavior can be induced in NC males using different experimental strategies. Systemic injection of the opioid receptor antagonist naloxone can induce mating behavior in formerly NC rats (Gessa et al., 1979; Canseco-Alba and Rodríguez-Manzo, 2019). Administration of the endocannabinoid anandamide induces sexual activity in 50% of previously NC male rats (Canseco-Alba and Rodríguez-Manzo, 2013). These males were able to mate 14 days after the drug treatment without needing another administration of the compound. Endocannabinoid anandamide induces sexual behavior in previously NC male rats through the activation of the CB1 cannabinoid receptor (Canseco-Alba and Rodríguez-Manzo, 2019). Endocannabinoids modulate presynaptic neurotransmitter release. Rodriguez-Manzo group reports a high proportion of NC male rats in their experiments, around 20% of their male Wistar rats were classified as NC. In our studies, using the same rat strain, we found that only around 1%–3% of the males can be classified as NC. The high frequency of NC males reported in other research groups could be due to different housing and or breeding conditions.

Another compound that induces sexual behavior in previously NC male rats is the aphrodisiac *Phlegmarirus saururus*. This compound is rich in alkaloids, principally sauroine, sauroxine and 6-hydroxylycopodine and when administered to NC males induces sexual behavior (Birri et al., 2017).

Kindling is a model of epilepsy in which an initially subconvulsive electrical stimulation of a specific region of the brain eventually develops a generalized seizure. Kindling

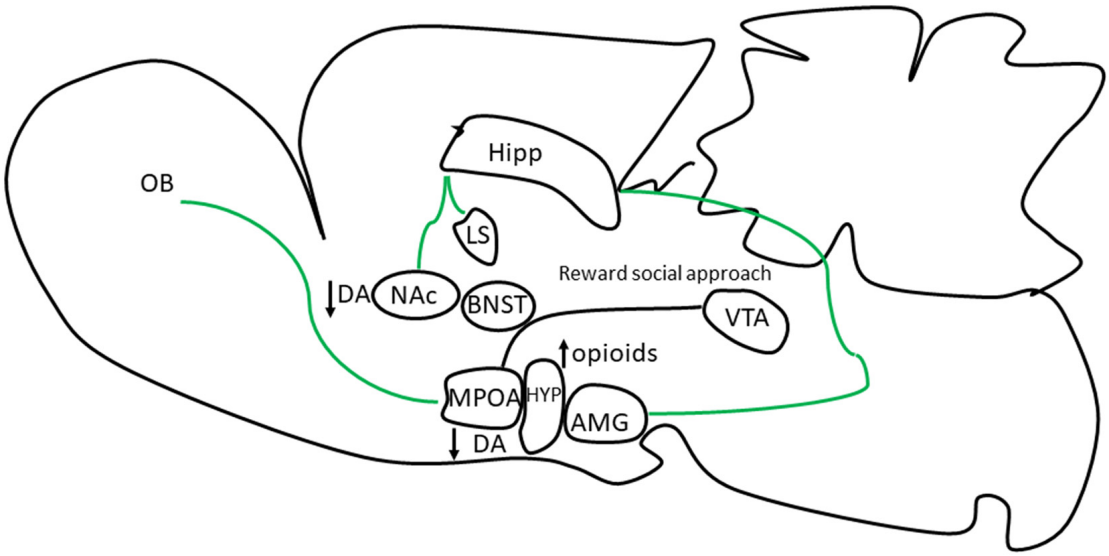

FIGURE 2 | Neuronal brain regions and neuromodulators involved in sexual motivation in NC rats. Copulating male rats and mice show an increase in neuronal activity in response to odors from sexually receptive females in the olfactory bulbs (OBs) a region involved in conspecific recognition and in neuronal areas involved in the social behavior network (SBN; bed nucleus of the stria terminalis, BNST, amygdala, AMG) and MPOA, and mesolimbic reward system (MRS; NAc and ventral tegmental, VTA). However, in NC male rats and mice no increase in neuronal activity is observed in some of these neuronal regions. In NC male rats DA is not increase when males are exposed to estrous females indicative of a lack of interest reducing approach behavior to the incentive. The MPOA is an important interface with the VTA to establish a reward state that assures that the behavior will be repeated (McHenry et al., 2017).

can induce several plastic changes in the brain such as modulation of neurotransmitters (GABA), monoamines, several opioid peptides, long term potentiation (LTP) and changes in cellular protein synthesis (Gorter et al., 2016). Even though kindling did not modify sexual behavior in copulating rats when induced in the MPOA, the development of MPOA kindling in previously NC male rats induced sexual activity in seven out of nine animals. The sexual behavior displayed by previously NC male rats with MPOA kindling was very similar to that observed in copulating males. This effect of kindling over sexual behavior was specific to the stimulated area, because kindling in the AMG in NC males did not induce sexual behavior (Paredes et al., 1990). The induction of sexual activity in previously NC male rats with MPOA kindling is long lasting since males displayed sexual behavior even 8 months after kindling stimulation had ceased (Portillo et al., 2003). The induction of sexual activity in previously NC rats could be associated with changes in neuromodulatory systems, protein synthesis or LTP.

NC males are poorly studied in other species. In male Mongolian gerbils (which are socially monogamous; Scheibler et al., 2004), hormone exposure during fetal development modifies their sexual behavior when adults. Males that develop between two females have lower levels of circulating testosterone and deficits in the development of genital musculature in comparison to males gestated between two males. Around 22% of males located between two females when reach adulthood did not mount the females and when they cohabited with them, they failed to induce pregnancy. These NC gerbils show high levels of alloparental behavior, they spend 30%–50% more time caring for pups than males that developed between two males (Clark et al., 1992; Clark and Galef, 2000). Clark and Galef propose that although NC gerbils are unable to have descendants, they can increase their fitness by contributing to rear collateral kin.

From the above-described studies, it is evident that asexuality or the lack of copulation in different species has an important biological component that can modify the structure of the central nervous system and consequently its function reducing sexual motivation. The MPOA is a brain region where these changes might occur as part of the circuits controlling sexual behavior. NC males are a valuable animal model to study the factors that modulate motivational sex drive and hence sexual behavior.

ASEXUALITY

The NC males that have been identified in several species could be equivalent to asexual individuals in humans. However, it is necessary to recognize the limitations of these comparisons since the psychological (fantasies) and romantic aspects of human sexuality cannot be studied in animals. In general, asexual individuals are healthy men and women without physical or emotional disorders, who report low or absent sexual desire and/or attraction (erotic and sensual allure). That is, they do not feel sexual attraction to any congener (Bogaert, 2004; Prause and Graham, 2007; Brotto et al., 2010). Asexual individuals have more negative explicit and implicit attitudes toward sex as well as explicit negative attitudes toward romance (feeling of infatuation or emotional attachment) than individuals who engage in sex. Thus, asexual people have a neutral or negative view of sex,

low passion but can have romantic attraction (Bogaert, 2012; Bulmer and Izuma, 2018; Zheng and Su, 2018). However, this sexual orientation does not prevent them from engaging in emotional relationships, and some of them have relationships with other asexual individuals (Bogaert, 2004; Prause and Graham, 2007; Brotto et al., 2010; Brotto and Yule, 2017; Jones et al., 2017).

Bogaert in 2004 reported that approximately 1% of the population of Britain and the United States identify themselves as asexual (Bogaert, 2004, 2015). In New Zealand students, asexual individuals represent about 2% of the population (Lucassen et al., 2011), and in Finland 3.3% and 1.5% of women and men, respectively (Höglund et al., 2014). Asexual individuals are more likely to be women (70%; Bogaert, 2012, 2015). Bogaert reported that asexual individuals, in general, experience their first sexual interaction at an older age than sexual persons and throughout their lives they have fewer sexual partners. Asexual and sexual women differ in parameters such as age, socioeconomic status, education, race, weight, age of menarche and religiosity (Bogaert, 2004). In contrast, asexual and sexual men differ in socio-economic status, education, weight and religiosity; review in Prause and Graham (2007). However, recent studies did not find significant differences between sexual and asexual individuals regarding education level and physical health (Greaves et al., 2017; Yule et al., 2017; Zheng and Su, 2018). Asexual people report more frequent anxiety disorders such as somatization, depression, more interpersonal problems and suicidal and psychotic symptoms than sexual participants (Yule et al., 2013).

Both asexual men and women report falling curiosity about sexual relationships during adolescence, but they report having less frequent sexual intercourse experience because it is unpleasant. In fact, a low percentage of asexual individuals reported to be in a relationship. Moreover, some asexual individuals who are married engage in sexual activity only to please their partners. That is, they have unwanted but consensual sex (Carrigan, 2011; Van Houdenhove et al., 2014, 2015a; Zheng and Su, 2018). Asexuality is not due to physical alterations, because asexual men do not have erection deficiencies and sometimes masturbation is pleasurable, but not sexual contact with a partner (Brotto et al., 2010). An early study found no significant differences in masturbation frequency between asexual and sexual men. However, while sexual men masturbate for reasons associated with sexual needs; their partners are not interested in sex, are unavailable, or they simply want sexual satisfaction, asexual men masturbate because they report to be bored, or because it helps them relax and/or fall sleep (Bogaert, 2012).

Asexual as well as sexual women participants respond to audiovisual erotic stimuli with an increase in genital congestion, which is an indication that they experience normal levels of genital arousal. Even though masturbation is usually enjoyable, asexual women masturbate less frequently than sexual women (Brotto and Yule, 2011, 2017; Zheng and Su, 2018). Similar to asexual men, asexual women masturbate to relax and release stress or tension and they feel that this activity is not sexual because it does not involve sexual thoughts or sexual emotions (Van Houdenhove et al., 2015a). In a recent study (Yule et al., 2017), asexual women and men reported to be less likely to masturbate for sexual pleasure or fun. Around 40% of asexual individuals reported that they had never had a sexual fantasy in comparison with sexual participants of both genders (8%). Sexual fantasies are less exciting in asexual than in sexual participants. Asexual people (12% men and 14% women) that have sexual fantasies, do not see themselves in the fantasies. Their fantasies are about other people, voyeurism and fictional human characters. Asexual men or woman reported to have fantasies that do not include sexual or romantic content, for example cuddling (Yule et al., 2017).

Although asexual individuals are not interested in the physical part of a relationship, they experience the need and desire to develop emotional bonds, and they look for the romantic side of relationships and a stable emotional partner. Some asexual men and women reported that they like kissing and cuddling but without a sexual connotation (Scherrer, 2008). Asexual individuals can self-categorize into aromantic with no romantic feelings and romantic. The ideal relationship of aromantic asexual men and women is a friendship-like interaction. On the other hand, romantic asexual people which represent the majority (79%–72%) have the same romantic desires and needs as sexual individuals. Romantic asexual men and women can be homo-romantics (14%), hetero-romantics (32%) and bi-romantics (26%). Other asexual individuals identify themselves as gender-neutral (not referring to either sex), genderqueer (individuals who see their gender as fluid or hybrid), or reject the binary between male and females (Scherrer, 2008; Brotto et al., 2010; MacNeela and Murphy, 2015; Van Houdenhove et al., 2015b; Zheng and Su, 2018).

Asexual people describe different benefits to their orientation. Among those are that they keep away from the common problems of intimate relationships, which include high risk of acquiring a sexually transmitted infection, unwanted pregnancies and finding a partner. Among the main disadvantages of asexuality are that asexual men and women are seen as less human than people with other sexual orientations, difficulties in establishing intimate non-sexual relationships and the positive effects of sex are missing. Some asexual individuals worry that there is something wrong with them and wonder if they are the only ones with this sexual orientation (MacInnis and Hodson, 2012). This could increase because there is a lack of awareness and disbelief that asexuality exists in the general population. Thus, the asexual community lacks visibility and credibility in social media and communications (MacNeela and Murphy, 2015; Robbins et al., 2016). In an attempt to reduce the lack of awareness and increase visibility asexual societies have been created. The Asexual Visibility and Education Network (AVEN) founded in 2005 stands out among them. AVEN is a social network that focuses on informing about asexuality. This network links members with scientific studies related to this orientation and makes information available to contact other asexual members with the possibility of finding an emotional partner. There is a clear need to understand the biological bases of asexuality. Due to ethical limitations, studies in humans have mainly concentrated on questionnaires

and clinical descriptions. However, studies in NC animals suggest that they are present in different species representing a biological variability in which sexual motivation is reduced. More research is needed in this area, not to cure asexuality, but to understand and give support to those that could need it.

Monogamous Prairie Vole

As already described, there are different reproductive strategies in mammals that are influenced by external and internal factors in trying to assure the survival of the species. While in mammals the most common mating strategy is polygamy, there are species that have developed a socially monogamous reproductive strategy (around 3%–9% of mammals) demonstrating the biological variability in sexual motivation. *Microtus ochrogaster* is a socially monogamous species (Lukas and Clutton-Brock, 2013). Sexually naïve females and males form long-lasting pair bonds after mating or cohabitation for at least 6 h, sharing a nest and home range, showing a preference for their sexual partner, displaying selective aggression to other males and females, defending a territory and displaying parental behavior to their pups. When the sexual partner dies, the survivor usually does not form a new pair (Getz and McGuire, 1993; Gobrogge, 2014; Walum and Young, 2018).

However, not all voles pair bond (residents), in natural and laboratory conditions some males have home ranges that overlap with territories of other males and females. These voles mate when they find an available receptive female but do not form a pair bond or defend the territory (Getz and McGuire, 1993; Carter et al., 1995; Getz and Carter, 1996; Ophir et al., 2008). These males represent around 30% of the population and have been denominated as wanderers. Females can also be wanderers but less than 15% have been found to adopt this reproductive strategy (Ophir et al., 2008). This behavioral pattern is not fixed since some wanderers had been residents or become residents during the same season. Studies have evaluated the socially monogamous or wandering reproductive strategies in voles. Resident male voles defend their territory and have shorter home ranges than wanderers (Solomon and Jacquot, 2002). Residents have more possibility to sire a litter probably by mate guardian. Resident males with litters had fewer home range overlaps than reproductively successful wanderers. As expected wandering males that sired a litter had a higher home range overlap than that of unsuccessful wanderers (Ophir et al., 2008, 2012).

In semi-natural conditions, resident male voles have longer anogenital distances than wanderers (Ophir and Delbarco-Trillo, 2007). Studies in rodents indicate that the anogenital distances depend on prenatal levels of testosterone; pre and neonatal treatment with an AR blocker (flutamide) decrease anogenital distance in male rats and impairs sexual behavior (Domínguez-Salazar et al., 2002). Male gerbils with longer anogenital distances have higher testosterone levels, higher testes weight, scent mark more frequently and display sexual behavior more than males with shorter anogenital distance (Clark et al., 1990). These results suggest that changes in testosterone levels could be associated with resident and wanderer mating strategies.

Female voles show a clear sexual preference for males with longer anogenital distances and larger testes. Male voles with longer anogenital distances had higher levels of seminal fluid and sperm than males with short anogenital distances (Ophir and Delbarco-Trillo, 2007). Thus, resident pair voles are more masculinized and fertile than wanderers. Females can identify these characteristic to choose a mate and eventually form a pair bond (Ophir and Delbarco-Trillo, 2007). These reproductive strategies have no impact on the general health of the voles since there are no significant differences in their body mass and survival (Solomon and Jacquot, 2002). Differences in neurotransmitters have been reported between residents and wanderers. In male prairie voles, vasopressin facilitates pair bonding. Moreover, vasopressin receptor 1a (V1aR) is higher in the ventral pallidum (VP) of prairie voles in comparison to polygamous *Microtus montanus* (montane voles) and *Microtus pennsylvanicus* (meadow vole; Nair and Young, 2006). Resident male voles that have extra-pair copulation (sexual infidelity) and wandering males in a seminatural enclosure show low levels of vasopressin 1a receptor V1aR expression in neuronal regions involved in spatial memory such as the posterior cingulate/retrosplenial cortex and laterodorsal thalamus. However sexual fidelity is not associated with vasopressin 1a receptor in the VP or lateral septum (LS) areas involved in pair bonding formation (Ophir et al., 2008).

Another neurotransmitter involved in pair-bonding is oxytocin. Prairie voles have a higher density of oxytocin receptors in the nucleus accumbens (NAc) medial prefrontal cortex (mPFC) and AMG compared to the closely related non-socially monogamous montane and meadow voles (Insel and Shapiro, 1992). Interestingly, the density of oxytocin receptors in the NAc and caudate putamen is highly variable in prairie voles (Ophir et al., 2012). Ophir and coworkers showed that sexual exclusivity is not related to oxytocin receptor density. They demonstrated that males that sired offspring only with their sexual partners did not differ in oxytocin receptors in the forebrain in comparison with males that sired offspring with a female that was not their partner. However, paired male voles had more oxytocin receptors in the NAc than wandering males (Ophir et al., 2012).

As already described, pair-bonding can be induced by mating or cohabitation for 6 h (Williams et al., 1992; Carter et al., 1995; Wang et al., 1997). We evaluated if mating and pair-bonding endure because they induce a positive affective state. In male voles, the pair bond resulting from mating until one ejaculation or copulation for 6 h induces a positive affective state evaluated by the conditioned place preference (CPP) test. This positive state is not induced if males are exposed to auditory, olfactory and visual stimulation with a receptive female, but without physical contact for 6 h. This rewarding state induced by mating is opioid-dependent because the administration of the opioid antagonist naloxone to males that ejaculate once or mate for 6 h blocked the induction of a reward state (Ulloa et al., 2018). Female voles that were exposed to a sexually active male without mating or that mated for 6 h or mated until one ejaculation did not develop a reward state. The failure to develop CPP and hence a reward state in female voles after mating could be due to the fact that

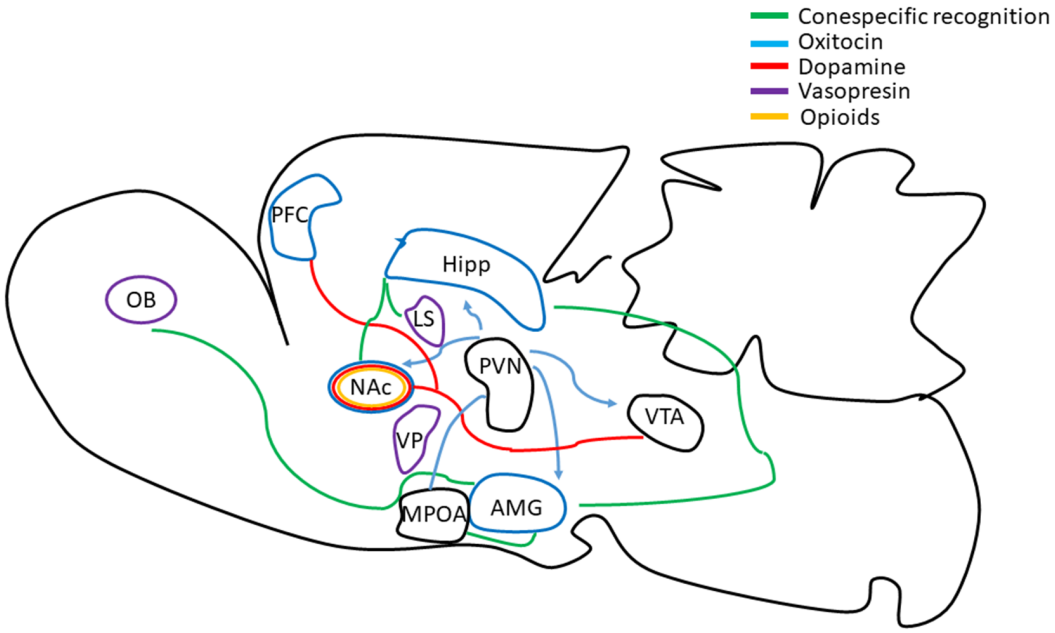

FIGURE 3 | Schematic representation of emotional, reward and sensory brain circuits involved in pair-bonding formation. Recognition and memory formation of the sexual partner cues are encoded by the OB, the AMG, the MPOA, the Hipp, the NAc and the lateral septum (LS; green lines). The prefrontal cortex (PFC) and NAc modulate affiliative behavior. The VTA is known to modulate motivational, reward and emotional salient stimuli. The VP is related to the hedonic or motivational stimuli of the partner, and the paraventricular nucleus is involved in social recognition and bond separation. Oxytocin (blue lines), dopamine (red lines) and vasopressin (purple lines) play a fundamental role in pair bonding. Oxytocin is involved in individual discrimination and partner preference. Dopamine induces approach behavior facilitating partner preference without mating and is involved in pair bond maintenance. Vasopressin is relevant in social recognition, territory marking and aggressive behavior. Opioids (yellow lines) are involved in sexual and partner associated reward that contributes to the establishment of long term pair bond (review in Lieberwirth and Wang, 2016; Walum and Young, 2018).

females were not allowed to control, pace, the sexual interaction. As described above in order for sexual behavior to be rewarding in female rats, they need to pace the sexual interaction (Martinez and Paredes, 2001; Arzate et al., 2011). When females receive at least 10 intromission, the sexual stimulation is rewarding. Similarly, sexual behavior is rewarding only in those males that mate pacing the sexual interaction (Ågmo and Berenfeld, 1990; Paredes and Alonso, 1997; Martinez and Paredes, 2001; Parada et al., 2010; Pfaus et al., 2012). Further studies in female voles are needed to determine if sexual stimulation in pacing conditions induces a reward state.

A recent study demonstrated that female voles that mate, but not those exposed to a peer formed a place preference for cues associated with their mates (Goodwin et al., 2019). The differences between our study and that of Goodwin et al. (2019) is that we allowed the females to mate with the male for 6 h in each of the three reinforcing conditioning days. After mating females were returned to their home cage without the male partner. In Goodwin's study females cohabited with the male for 12 h in the reinforcing conditioning days. Our females were sexually naïve and in order to avoid pregnancy females were ovariectomized and treated with intraperitoneal (i.p.) administration of estradiol benzoate. In the study of Goodwin et al. (2019), females were sexually experienced and had previously produced litters. Futures studies need to address possible rewarding differences between residents and wanderers.

Neural Control of Sexual Motivation

In a recent review, we described in detail the possible neural circuits that control sexual motivation (Ventura-Aquino et al., 2018). Briefly, there are two brain circuits that have homologies in different vertebrate lineages which integrates internal and external stimuli. Both are part of the social decision-making network facilitating adaptation and survival of the individual. The first circuit is the social behavior network (SBN) important for the control of sexual behavior that includes brain regions such as the MPOA, the AMG, the anterior hypothalamus and the ventro medial hypothalamus. The second network, the mesolimbic reward system (MRS), includes the ventral tegmental area (VTA) and the NAc and is important for reward, including the reward associated with sexual incentives. When a potential mate is present and mating occurs, under appropriate conditions (pacing for the female, for example) a reward state will be induced that will favor the repetition of the behavior (Paredes, 2014).

As described above the MPOA is a key brain area regulating sexual motivation (for a review see Paredes, 2003) and the release of opioids in this brain region is important for sex to be rewarding in both males and females (Paredes, 2014). The NAc is also important for sexual motivation and dopamine (DA) is released in anticipation and prediction of reward (Berridge et al., 2009; Berridge and Robinson, 2016). Different lines of evidence indicate that a variety of events enhance DA release in the NAc, including eating, drinking, as well

as aversive stimuli such as tail pinch, restraint stress, foot-shock, social defeat, and aggressive encounters (for a review see Paredes and Ågmo, 2004). Taken together these results suggest that DA is involved in the wanting response for different motivated behaviors. In rats, DA participates in the consummatory aspects of mating, whereas opioids are involved in the reward state associated with mating. In voles, mating induces oxytocin and DA release facilitating the association of sexually relevant cues of the partner with mating inducing pair bonding (Lieberwirth and Wang, 2016; Walum and Young, 2018; **Figure 3**).

CONCLUSION

The motivational drives that control and influence sexual behavior produce great biological variability between species that induce different behavioral patterns. These behavioral patterns under the appropriate conditions allow males and females to reproduce and ensure the survival of the species. The promiscuous, monogamous and NC (asexual in humans) patterns represent different motivational drives that need to be studied to understand the neurobiology of sexual behavior.

AUTHOR CONTRIBUTIONS

WP and RP contributed equally to this manuscript.

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Glutamate Afferents From the Medial Prefrontal Cortex Mediate Nucleus Accumbens Activation by Female Sexual Behavior

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Low levels of desire and arousal are the primary sexual dysfunctions in women, necessitating neurobiological studies of sexual motivation in female animal models. As the mesocorticolimbic system is a primary neural circuit underlying sexual motivation, the goal of this study was to test the hypothesis that medial prefrontal cortex (mPFC) glutamate mediates sexual behavior activation of the nucleus accumbens. Glutamatergic neurons in the mPFC were activated by sex behavior, and these sex-activated cells shown to project to the nucleus accumbens. During sexual interactions with the male, glutamate transients recorded in the nucleus accumbens of female hamsters were specifically associated with the receipt of intromissions from the male. Further, inhibition of the mPFC during sex significantly decreased nucleus accumbens activation. Glutamatergic medial prefrontal cortical input to the nucleus accumbens mediates the activity in the nucleus accumbens during female sexual behavior. These results offer novel insights into the neurobiology of the motivational control of female sexual behavior and provide attractive avenues for pursuing target-specific and clinically-relevant therapies for sexual dysfunction in women.

Keywords: sexual behavior, reward, glutamate, medial prefrontal cortex, nucleus accumbens, DREADD

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INTRODUCTION

Unlike in men where sexual dysfunction is primarily reflected in performance issues (i.e., erectile failure), sexual dysfunction in women is often more subtle, characterized by a loss of motivation to initiate sex and a loss of pleasure during sex (McCabe et al., 2016). This lack of interest in sex, especially among women in intimate relationships, carries with it serious psychological consequences that include relationship problems, low self-esteem, and importantly, decrements in health-related quality of life (Biddle et al., 2009). Unfortunately, there are few therapeutic options for women with low levels of sexual motivation, and available options are relatively ineffective (Jaspers et al., 2016; Goldstein et al., 2017). A reason so few therapeutic options exist comes from the erroneous belief that animal models cannot capture the essential components of sexual motivation in women. As a result, very little is known about neural mechanisms underlying female sexual desire in women.

In contrast to the view that female sexual desire cannot be modeled in animals, we and others have developed behavioral analyses that measure the incentive value and pleasurable consequences of female sexual behavior in rodents, including the female's willingness to engage in copulatory activity with a male (Mendelson and Pfaus, 1989; Paredes and Vazquez, 1999; Meisel and Mullins, 2006; Cummings and Becker, 2012; Georgiadis et al., 2012). An important component of these behavioral analyses is the separation of the motivational components of female sexual behavior from the overt expression of female sexual behavior, i.e., the lordosis posture (Pfaus et al., 1990; Georgiadis et al., 2012). Hypothalamic circuits have long been known to mediate female sexual behavior in rodent models (e.g., Pfaff, 1980). More recent studies have pointed to the mesocorticolimbic circuits controlling female sexual motivation (Meisel and Mullins, 2006; Micevych and Meisel, 2017), consistent with the role of this system in motivational control in general (e.g., Salamone and Correa, 2012).

The nucleus accumbens has a key role in the incentive motivational processes (Bindra, 1969; Berridge, 2007) of both "wanting" and "liking" female sexual behavior (Micevych and Meisel, 2017). Damage to the nucleus accumbens of rats dramatically reduces the female's willingness to engage in sexual interactions with a mounting male, i.e., wanting sex (Dohanich and McEwen, 1986; Rivas and Mir, 1991; Guarraci et al., 2002), though the lesions do not reduce the incidence of lordosis if the male is able to successfully mount the female. Separate studies link the nucleus accumbens to the rewarding consequences of sexual behavior for female rodents (Hedges et al., 2009; Been et al., 2013), indicating the role of this region in the liking of sexual behavior.

We have spent several decades studying sexual motivation in a female Syrian hamster model (reviewed in Meisel and Mullins, 2006). Syrian hamsters offer a distinct advantage over the more commonly used rat model. Early studies of the mesocorticolimbic circuits linked this system to locomotor activity (Mogenson et al., 1980). The primary limitation of using female rat sexual behavior to understand its control by this system is that female rats have a high level of locomotor activity during sexual interactions (e.g., Guarraci et al., 2002). This characteristic of female rat sexual behavior makes it very difficult to separate the locomotor activation of the mesocorticolimbic system from the activation resulting from sexual behavior. We explicitly chose female Syrian hamsters for our studies as they remain relatively immobile during sex, maintaining the lordosis posture for upwards of 9 min of a 10 min test (Meisel et al., 1988). As a result, it is much easier to associate mesocorticolimbic activation with components of sexual behavior in female Syrian hamsters than in female rats.

The neurobiological underpinnings of our model of the motivational control of female sexual behavior have focused on the mesocorticolimbic dopamine system and its innervation of the nucleus accumbens. We know that the pleasurable aspects of sexual behavior in female rodents and the willingness of these females to regulate sexual contacts with a male both correlate with nucleus accumbens dopamine release and are modulated by manipulations of dopamine innervation of the nucleus accumbens (Meisel and Mullins, 2006). At the same time, that dopamine is an important regulator of the nucleus accumbens with respect to female sexual behavior, concordant stimulation by both dopamine and glutamate is key to nucleus accumbens activity (Carlezon and Thomas, 2009). Research on male rats highlights the key role of mesocorticolimbic glutamate regulating copulation (Hernández-González et al., 2008; Beloate and Coolen, 2017), and in this light it is surprising that so little attention has been paid to the effects of glutamate in this system on female sexual behavior. In a similar vein, the medial prefrontal cortex (mPFC) and its glutamatergic inputs to the nucleus accumbens are at the core of this circuit, though very little is known about medial prefrontal cortical control of the nucleus accumbens during female sexual behavior.

In this report, we provide a converging set of experiments that identify for the first time the importance of mPFC glutamate innervation on the activation of the nucleus accumbens during female sexual behavior. We first analyzed c-Fos staining to evaluate neuronal activation in the mPFC and nucleus accumbens during sexual behavior in female Syrian hamsters. We next demonstrated that efferents from the mPFC to the nucleus accumbens core are activated during female sexual behavior. Further, c-Fos activation in the mPFC during sexual behavior was localized to glutamatergic neurons. *In vivo* recordings of extracellular glutamate in the nucleus accumbens were associated with the female's receipt of intromission from the mounting male. Finally, we used viral expression of inhibitory DREADDs in the mPFC to demonstrate that silencing the mPFC during sexual behavior prevented the increase in nucleus accumbens c-Fos expression by female sexual behavior.

MATERIALS AND METHODS

Animals

Adult (about 55 days old at arrival) female hamsters (Charles River Laboratories, Wilmington, MA, USA) were used as experimental subjects, whereas similar-aged adult male hamsters were used as stimulus animals for the sexual behavior tests. Females were housed individually and males housed in pairs in polycarbonate cages (females: 51 × 41 × 20 cm; males: 43 × 23 × 20 cm). The colony room was maintained on a reversed 14 h light/10 h dark photoperiod with lights off between 13:00 and 23:00. Behavioral testing was performed during the nocturnal animals' dark phase. The animal room was maintained at 22°C, with food and water available for the animals *ad libitum* except during periods of behavioral testing. All procedures in these experiments were approved by the University of Minnesota IACUC and are in accordance with The Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 2011).

Surgeries

One week after arrival to the laboratory, female hamsters were bilaterally ovariectomized under sodium pentobarbital anesthesia (Nembutal, 8.5 mg/100 g body weight, i.p., Abbott Laboratories, Abbott Park, IL, USA). Stereotaxic surgery was performed directly following ovariectomy. Depending on the

experiment, one of two stereotaxic approaches was taken. For the neural tracing study, unilateral intracranial injections were made by lowering a microinjection syringe (Model #701, Hamilton Company, Hamilton, Reno, NV, USA) under stereotaxic control (Microinjection Unit, Model 5002, David Kopf Instruments, Tujunga, CA, USA) into the NAc core and injecting a volume of 50 nL cholera toxin subunit β (CTB; Product #104, List Biological Laboratories, Campbell, CA, USA) over the course of 30 s. For viral vector delivery of an inhibitory DREADD, bilateral injections of 1.0 μL pAAV5-CaMKIIα-hM4D(Gi)-mCherry (Addgene, Cambridge, MA, USA) were infused over the course of 10 min. To minimize the flow of infused solution up the needle tract, the syringe was left in place for 10 min after each injection.

Female hamsters in the biosensor study were stereotaxically implanted with a unilateral BASi guide cannula (0.7 mm diameter; Bioanalaytical Systems, West Lafayette, IN, USA). The guide cannula was fixed to the skull using dental acrylic (Patterson Dental, St. Paul, MN, USA) extending to three stainless steel screws secured to the skull (Pinnacle Technology, Lawrence, KS, USA), and a stainless steel post was inserted into the cannula shaft to prevent occlusion.

Post-surgical analgesic (Butorphanol, 10 mg/kg, s.c., Fort Dodge Animal Health, Fort Dodge, IA, USA or meloxicam, 2 mg/kg, s.c., Norbrook, Overland Park, KS, USA) and antibiotic (0.1 mL Baytril, 2.27% solution s.c., Bayer Animal Health, Monheim, DE, USA) were provided on the day of surgery and for each of the next three postsurgical days for all animals.

Sexual Behavior Testing

One or 3 weeks (viral vector studies) following surgery, female hamsters were hormone-primed for sexual behavior testing *via* subcutaneous injections of 10 μg of estradiol benzoate (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mL of cottonseed oil (Sigma-Aldrich) at approximately 48 and 24 h prior to the sexual behavior test, followed by a subcutaneous injection of progesterone (500 μg in 0.1 mL of cottonseed oil, Sigma-Aldrich) 4 h prior to the testing. Females were paired with a male hamster in either the biosensor testing chamber or in the female's home cage for a 10 min session. Copulatory parameters of the females (lordosis latency and total lordosis duration) and males (mounts, intromissions, ejaculations) were obtained to ensure that the females received comparable levels of sexual stimuli. For c-Fos experiments, control females were not given a sexual behavior test following hormonal priming; instead their cage was placed in the same behavioral testing room with the male hamsters present for 10 min. In the DREADD experiment, female hamsters were given either 5 mg/kg CNO in 0.9% saline (Enzo Life Sciences, Farmingdale, NY, USA) or an equivalent volume of saline (0.1 mL/100 g body weight) 30 min prior to behavioral testing.

Perfusion and Tissue Sectioning

Sixty minutes after sexual behavior testing or soon after biosensor recordings, female hamsters were deeply anesthetized with the euthanizing agent Buthanasia-D (0.2 mL i.p., Merck Animal Health, Summit, NJ, USA) and transcardially perfused with 25 mM phosphate buffer (∼50 mL) containing 0.9% saline (PBS, pH = 7.6) followed by 4% paraformaldehyde in PBS (∼500 mL). The brains were removed and post-fixed for either 2 h or overnight (c-Fos/CTB experiment only) in 4% paraformaldehyde and then placed in a 10% sucrose solution in PBS overnight. Serial coronal sections (40 μm) of frozen brain tissue were sectioned on a microtome (American Optical, model 860) and every fourth section was processed for immunohistochemical localization of the proteins of interest.

Immunohistochemistry

c-Fos Staining

Free-floating sections were rinsed in PBS with 0.1% IgG-free bovine serum albumin (Jackson ImmunoResearch, West Grove, PA, USA) wash buffer and then incubated in a polyclonal antibody to c-Fos primary (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat# sc-52) in wash buffer with 0.3% Triton-X100 (Sigma-Aldrich) at room temperature for 48 h at 4°C. After rinsing in wash buffer, sections were incubated for 60 min at room temperature in biotinylated anti-rabbit IgG secondary antibody (1:600; Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA), rinsed in wash buffer, and then incubated in an avidin-biotin horseradish peroxidase complex (1:200, Vectastain Elite ABC Kit) for 60 min at room temperature. The sections were then rinsed in wash buffer and reacted in a 0.1 M Tris buffer (pH = 7.6) with 0.56 mM 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) solution, 0.003% hydrogen peroxide and 63 mM nickel ammonium sulfate (Sigma-Aldrich). After 5 min, sections were rinsed in Tris buffer to stop the chromagen reaction. Immunostained sections were mounted onto glass slides (Adhesion Superfrost Plus Microscope Slides, Brain Research Laboratories, Newton, MA, USA), cover slipped with DPX mounting media (Sigma-Aldrich), and imaged using bright field microscopy.

Dual Labeling for c-Fos With GAD or CaMKIIα

Free-floating sections were treated as described for c-Fos staining. After the DAB reaction and appropriate rinses, sections were incubated for 72 h at 4°C in a primary antibody against GAD67 (1:3,000, EMD Millipore, Burlington, MA, USA, Cat#MAB5406) or for 48 h at 4°C in a primary antibody against CaMKIIα (1:1,000, Abcam, Cambridge, UK, Cat# ab111890), and rinsed as described. The sections were then incubated in secondary antibody for 1 h followed by avidin-biotinylated HRP complex for 1 h with appropriate rinses. Finally, sections were incubated a second time in DAB, though without the nickel ammonium sulfate, washed, mounted on slides, and coverslipped.

Dual Labeling for c-Fos With CTB

Free-floating sections were treated as described for c-Fos staining. After the DAB reaction and appropriate rinses, sections were incubated for 48 h at 4°C in a primary antibody against CTB (1:70,000, List Biological Laboratories, Campbell, CA, USA, Cat# 703) and rinsed. The sections were then incubated in secondary antibody for 1 h followed by avidin-biotinylated HRP complex for 1 h with appropriate rinses. Then sections were incubated for 5 min in DAB in 0.175 M sodium acetate buffer

without nickel ammonium sulfate, washed, mounted on slides, and coverslipped.

Viral Vector Visualization and Fluorescent Histochemistry

Sixty minutes following the sexual experience, subjects were injected with an overdose of Buthanasia-D, intracardially perfused and the brains sectioned as described in "Perfusion and Tissue Sectioning" section. Free-floating sections were rinsed in PBS wash buffer and then incubated in anti-c-Fos (1:10,000) in wash buffer with 0.3% Triton-X100 (Sigma-Aldrich) at 4°C for 24 h. After rinsing in wash buffer, sections were incubated in a biotinylated secondary antibody (1:600, Vector Laboratories) for 60 min at room temperature, rinsed in wash buffer, and then incubated in streptavidin DyLight® 488 (1:200, SA-5488, Vector Laboratories) for 60 min at room temperature. Sections were then rinsed in wash buffer before being mounted onto glass slides, cover slipped with VectaShield® HardSet™ mounting medium (H-1500, Vector Laboratories) and examined under a confocal microscope (Leica SPE personal confocal, Wetzlar, Germany) for c-Fos localization as well as the rostral-caudal spread of AAV injection, visualized directly with the AAV-expressed mCherry.

Image Analysis

Brightfield microscopic analyses of regions of interest in the mPFC and the NAc were obtained with a Leica microscope (Leica DN4000 B) equipped with a digital camera connected to a computer running Leica software. Our approach to counting fields of stained neurons was modeled after Bradley and Meisel (2001). Digital images were obtained and adjusted to match brightness and contrast as seen through the microscope. The same digital settings were used to capture all images for an individual experiment. The digital images were then opened in Adobe Photoshop (Adobe, San Jose, CA, USA) to place boxes identifying the regions of interest to analyze.

Analyses for the caudal NAc were based on Bradley and Meisel's (2001) findings. Within rostral to caudal coronal sections containing the NAc, the anterior commissure is monotonically shifted in a medial direction. We can take advantage of this to precisely identify a rostral-caudal level to analyze that is matched across all brains. For the NAc, we took a single section in which the distance from the ventral tip of the lateral ventricle was 300 μm from the medial edge of the anterior commissure, and from this section took cell counts from the right hemisphere in each animal. For the mPFC a single section was also measured to be consistent with the approach for the NAc. In this case, the section to be counted represented the mid rostral-caudal level of the mPFC and was matched to a template histological section based on the position and shape of the corpus collosum to ensure that the region of interest was sampled from the same location among all brains analyzed. A rectangular box was placed within the region of interest to ensure a consistent area in which cell counts were obtained. ImageJ Fiji software (Schindelin et al., 2012) was used to count labeled cells within these regions.

Fluorescent images were obtained with a Leica SPE personal confocal microscope for c-Fos localization as well as for parameters of the AAV injection. For all cell counts, digital images of the regions of interest were transferred to Photoshop to superimpose the rectangle outlining the counting region and the labeled cells were counted manually using the ImageJ cell-counter plugin.

Biosensor Testing

Glutamate oxidase-based sensors (Pinnacle Technology, Lawrence, KS, USA) were used to detect glutamate release in the female hamsters during sexual behavior (Moore et al., 2017). First, sensors were calibrated before use. Animals were then lightly anesthetized using a subthreshold dose of sodium pentobarbital (Nembutal, 3 mg/100 g body weight, i.p.) and the calibrated sensor was inserted through the guide cannula. Each animal was then placed in the testing chamber consisting of a 10-gallon glass aquarium with pine bedding taken from the animal's home cage, after which the sensor was connected to a potentiostat *via* an electrically shielded cable and electrical swivel (Pinnacle Technology). After the animal was injected with progesterone, the sensor was allowed to equilibrate for the next 4 h. A male hamster was then placed in the testing chamber for approximately 10 min while the amperometric signal and time-locked video data were simultaneously recorded. During sexual interactions with a male, female hamsters maintain a tonic lordosis posture for minutes on end while the male intermittently mounts the female (e.g., Meisel et al., 1988). Consequently, we were able to measure glutamate transients in the female's brain in response to discrete mating stimulation provided by the male hamster and in the absence of non-specific movement artifacts.

After recordings were completed, females were perfused as described, the brains frozen sectioned at 40 μm, and the free floating sections stained conventionally with cresyl violet acetate. The stained sections were mounted on slides and coverslipped. Brightfield images of the location of the biosensor were obtained with a Leica microscope (Leica DN4000 B) and the digital images used to plot the location of the biosensor.

Biosensor Analyses

Finding Peaks

After importing the raw amperometric data and behavioral annotations acquired during experimental recording, the raw current vs. time was plotted. A moving average taken over 20 data points (one point collected each second) was then used to create a smoothed data set from the raw voltage; this represented more tonic changes occurring throughout the experimental recording. Then, to obtain a normalized signal producing a flat basal response, the smoothed dataset was subtracted from the raw dataset. Using the standard "findpeaks" MATLAB function, we located peaks in the data using a threshold value determined by calculating half of the root-mean-square (RMS) of the normalized signal.

Peak Characterization

Peak characterization was performed by examining both the peak prominence in units of amplitude (nanoamps) and the

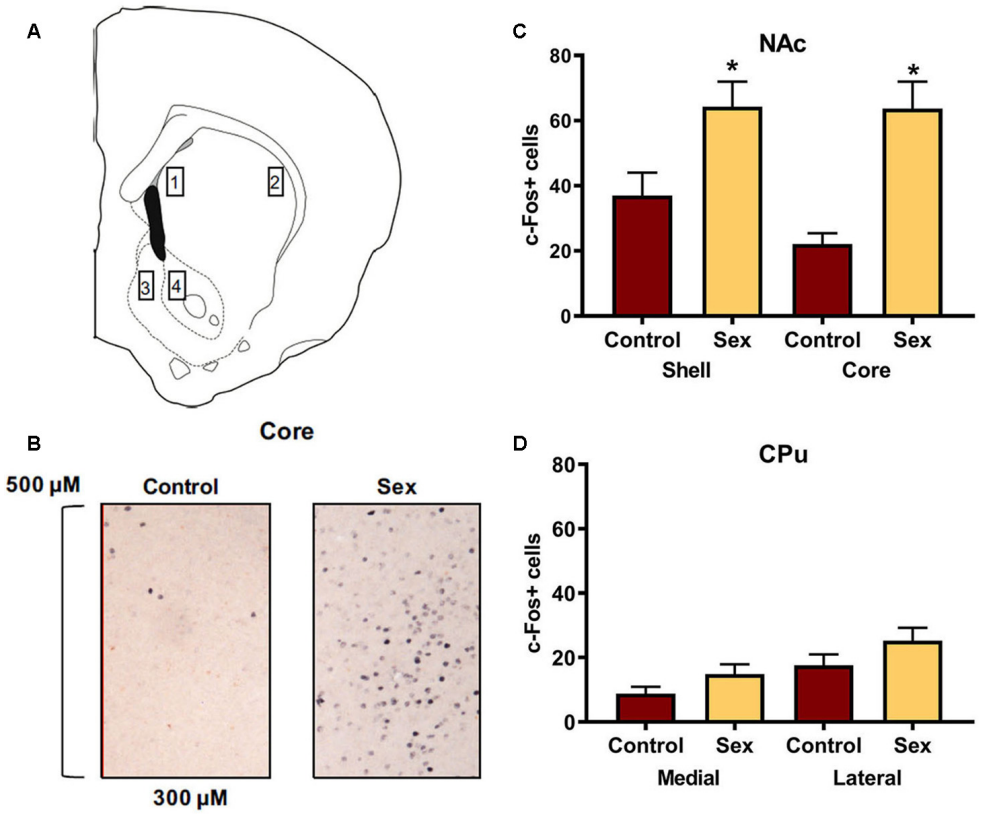

FIGURE 1 | Female sexual behavior activates the nucleus accumbens (NAc) shell and core, but not the medial or lateral caudate-putamen (CPu). **(A)** Hamster atlas plate (+2.1 mm from Bregma) indicating counting domains for 1-medial CPu, 2-lateral CPu, 3-NAc shell, and 4-NAc core. **(B)** Representative c-Fos counting boxes for the NAc core. **(C)** There was no significant difference between animals that received sex experience and controls in either the medial or lateral CPu. **(D)** There were significantly more c-Fos positive cells in animals that received sex experience than for their control counterparts in both the shell and core of the NAc (*indicates $p < 0.05$).

width of the peak in units of time (seconds). Both the average peak prominence and width were compared across each subsequent mating bout using a repeated measures analysis of variance (ANOVA) to determine if tonic changes occurred during the course of experimental testing. A peri-peak analysis was also performed using combined data from all mating bouts using frequency histograms of the number of peaks that occurred within a 5 s window of each behavioral annotation (e.g., mount or mount with subsequent intromission). Peri-event time analyses between mounts with and without intromission were also performed that included the first 5 min after the start of the first mating bout.

Statistics

Parametric statistical tests were based on the demonstration of homogeneity of variance among treatment groups. For the immunohistological experiments, unpaired *t*-tests were used to evaluate possible differences between animals receiving sexual behavior testing and untested controls. Proportions of mounts with and without intromission associated with glutamate peaks for individual animals were compared by Chi-squared tests using the online Graph Pad 2 × 2 contingency table calculator[1]. One-way ANOVAs were used to analyze the DREADD experiment, with Tukey multiple comparison *post hoc* tests probing significant ANOVAs. All significant differences were based on $p < 0.05$.

RESULTS

Female Sexual Behavior Activates the NAc and mPFC

Figure 1A identifies the locations for the regions of interest in which c-Fos cells were counted in the dorsal and ventral striatum. An example of c-Fos staining is illustrated in **Figure 1B**. Confirming previous findings, female hamsters ($n = 14$) who received a 10 min sexual behavior test had significantly more c-Fos positive cells in the NAc core compared with control female hamsters ($n = 12$) who remained in their cage in the presence of male hamsters ($t_{(24)} = 4.41$, $p < 0.0002$, **Figure 1D**). A similar increase in c-Fos labeling was observed in the NAc shell of these animals ($t_{(24)} = 2.59$, $p < 0.02$, **Figure 1D**). We used the caudate

[1]https://www.graphpad.com/quickcalcs/contingency2/

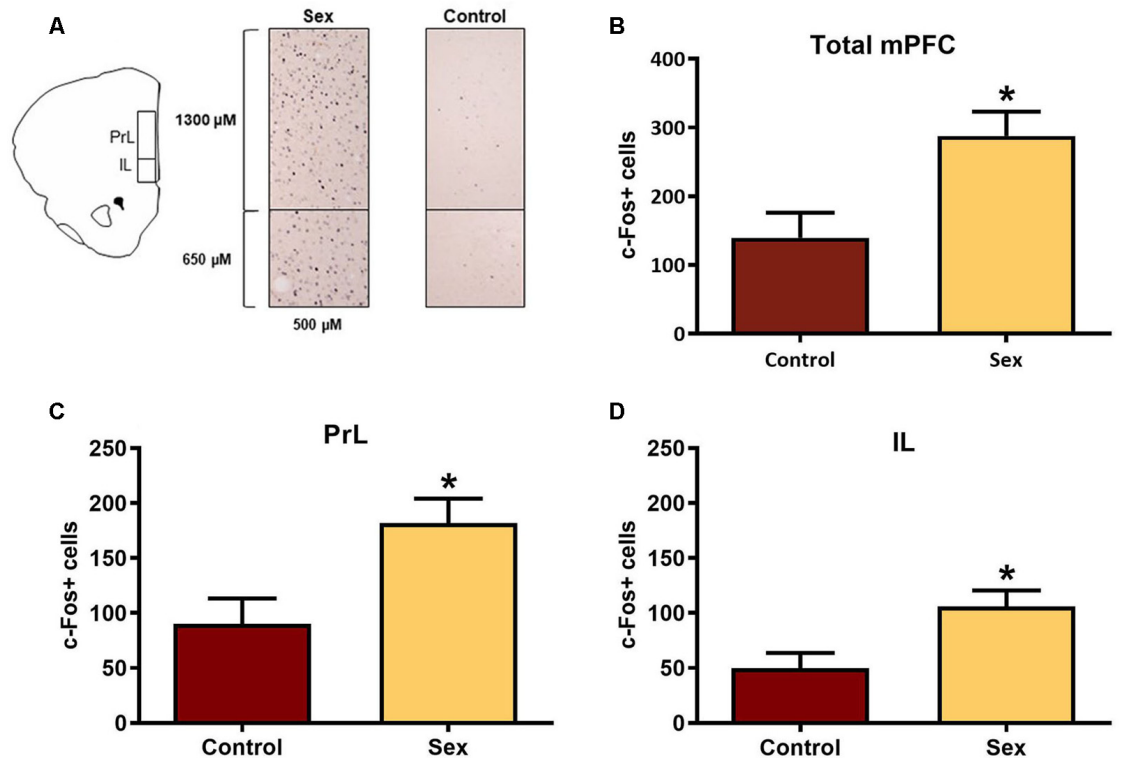

FIGURE 2 | Female sexual behavior activates the medial prefrontal cortex (mPFC). **(A)** Hamster atlas plate (+3.2 mm from Bregma) counting domains for the prelimbic (PrL) and infralimbic (IL) regions of the mPFC. **(B)** There was a significant increase in c-Fos labeled cells after sex behavior in the combined regions of the mPFC (Total mPFC), which was represented individually in both the **(C)** PrL and **(D)** IL subregions (*$p < 0.01$).

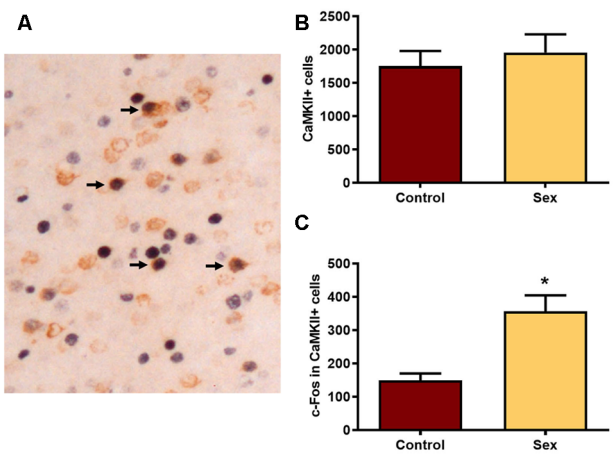

FIGURE 3 | Female sexual behavior increases the number of c-Fos neurons in CaMKIIα neurons in the mPFC. **(A)** A histological image illustrating the double labeling for c-Fos and CaMKIIα. Arrows point to several double labeled cells in which nuclear staining for c-Fos is a black reaction product and cytoplasmic CaMKIIα is brown. **(B)** There were no differences in the total numbers of CaMKIIα labeled neurons in the total mPFC following sex behavior. **(C)** Sex behavior increased the number of c-Fos cells within CaMKIIα neurons for the total mPFC, demonstrating increased activation of PFC glutamatergic neurons following a single sexual experience (*indicates $p < 0.01$).

as an anatomical control and did not find any change in c-Fos labeling in the medial or lateral caudate as a function of sex behavior testing (**Figure 1C**).

The mPFC is part of the mesocorticolimbic circuit that includes the nucleus accumbens, yet this region had not been examined previously with respect to activation by female sexual behavior. **Figure 2A** illustrates the regions of interest for the c-Fos analyses in the mPFC. Females receiving a sex behavior test ($n = 15$) had significantly more total c-Fos labeled cells ($t_{(26)} = 2.90$, $p < 0.01$, **Figure 2B**) and more c-Fos labeled cells in both the infralimbic ($t_{(26)} = 2.73$, $p < 0.01$, **Figure 2C**) and prelimbic ($t_{(26)} = 2.86$, $p < 0.01$, **Figure 2D**) subdivisions of the mPFC when compared with female hamsters who remained in their cage in the presence of male hamsters ($n = 13$).

Sex-Activated mPFC Neurons Are Glutamatergic

Given our finding of activation of the mPFC following female sexual behavior, we wished to determine the neuronal phenotype of these sex-activated cells. We used double label immunohistochemistry to identify sex-activated GABAergic and glutamatergic cells in this region. After confirming equal numbers of GAD labeled neurons in female hamsters tested for sexual behavior ($n = 6$) or control females ($n = 7$), we found no

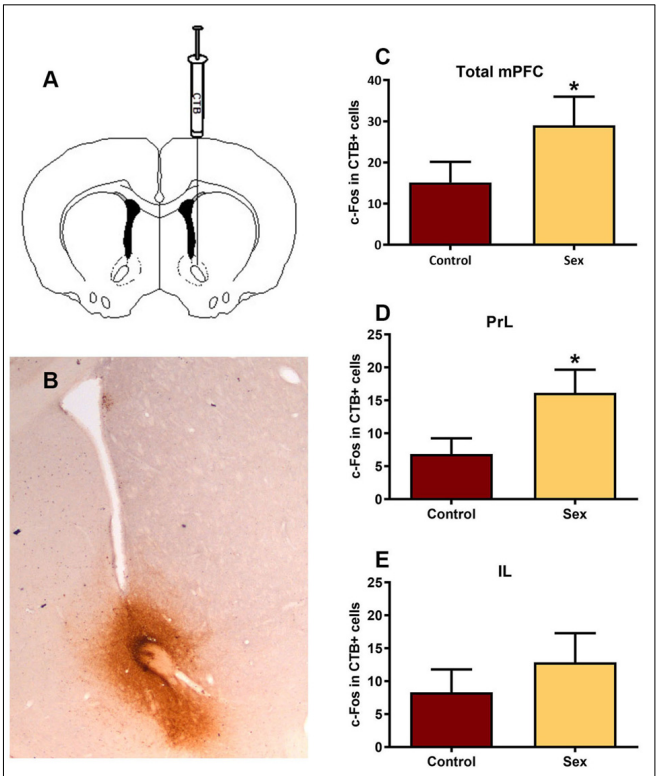

FIGURE 4 | Double immunolabeling revealed a significant increase in activated inputs to the NAc core from the prelimbic region of the mPFC. **(A)** Illustration of the unilateral infusion of cholera toxin B (CTB) targeted to the core of the NAc. **(B)** Immunohistochemical labeling of the retrograde tracer CTB confirmed injection sites were biased to the core of the NAc. **(C)** There was no significant change in c-Fos labeling in CTB cells within the total mPFC, though **(D)** there was a significant increase in c-Fos labeling within CTB cells in the prelimbic (PrL) subregion following sex behavior. **(E)** The infralimbic (IL) region of the mPFC did not exhibit a change in c-Fos within CTB labeled cells following sex (*$p < 0.05$).

difference in the number of c-Fos positive cells between these groups (data not shown).

We next used CaMKIIα labeled cells (**Figure 3A**) in the mPFC as a marker for glutamatergic neurons. There were no group differences in the numbers of CaMKIIα labeled cells (**Figure 3B**) between females receiving sexual behavior testing ($n = 6$) or control females ($n = 7$). Across all levels of the mPFC female sexual behavior increased the number of c-Fos labeled CaMKIIα positive cells compared to controls ($t_{(11)} = 4.20$, $p < 0.002$; **Figure 3C**). These results indicate that female sexual behavior activates mPFC glutamatergic neurons.

Characterization of Sex-Activated Afferents to the NAc Core

After identifying the neurotransmitter phenotype of these sexual behavior-activated mPFC neurons, we directly mapped the underlying circuitry using dual labeling of the retrograde tracer cholera toxin B (CTB, **Figure 4**) and c-Fos to determine active afferents to the NAc during sex. Immunohistochemical analysis revealed that all of the CTB tracer injections were core biased in the NAc (**Figure 4A**). mPFC neurons providing afferents to the NAc core had increased numbers of c-Fos labeled cells (**Figure 4B**) in females tested for sexual behavior ($n = 15$) compared with control females ($n = 13$), with an increase in the prelimbic ($t_{(26)} = 2.11$, $p < 0.05$, **Figure 4C**), but not infralimbic (**Figure 4D**) portions of the mPFC.

Characterization of NAc Glutamate Release During Sexual Behavior

Given that sexual behavior activated glutamatergic neurons in the mPFC that project to the NAc, we used glutamate biosensor recordings to test whether there were elevations in glutamate in the female hamster NAc during sexual interactions with a male. Our hypothesis was that glutamate would be elevated during sex, with the specific expectation that there would be glutamate transients associated with the receipt of copulatory stimulation from the mounting male hamster. Hence for this experiment, the focus of our analyses was on individual biosensor cases rather than on the grouped data.

NAc Core

During sexual interactions with the male hamster, glutamate transients were regularly recorded in NAc core biosensor placements ($n = 4$). **Figure 5A** demonstrates one representative case (see **Supplementary Figures S1–S3** for additional core cases). The glutamate transients were differentially associated with the receipt of intromission by the mounting male hamster (**Figure 5B**). In this female hamster (and in two of the three cases reported in the **Supplemental Results**) there were significantly more peaks within 5 s of the start of a mount that resulted in intromission than for mounts without a subsequent intromission (see **Table 1**). The temporal patterning of these peaks was consistent among all animals tested, as illustrated in **Figure 5C** with all peaks occurring within 3 s of the start of a mount with intromission. Very few glutamate peaks were measured in conjunction with mounts without subsequent intromission, and for these peaks there was no concordance in the timing of the fluctuations in the glutamate signal (**Figure 5C**).

NAc Shell

As in the NAc core, glutamate transients were found in NAc shell recordings ($n = 4$). **Figures 6A,B** demonstrates one representative case (see **Supplementary Figures S4–S6** for additional shell cases 2–4). In each case, there were significantly more peaks within 5 s of the start of a mount that resulted in intromission than for mounts without a subsequent intromission (see **Table 2**). In contrast to glutamate activity in the NAc core during sexual interactions, in the shell there was no concordance in the timing of the glutamate peaks relative the start of mounts with intromission (**Figure 6C**).

Medial Caudate

There were no peaks identified in any of the recordings in association with behavioral events for any of the four hamsters with probes in the medial caudate used as anatomical controls (**Figure 7**).

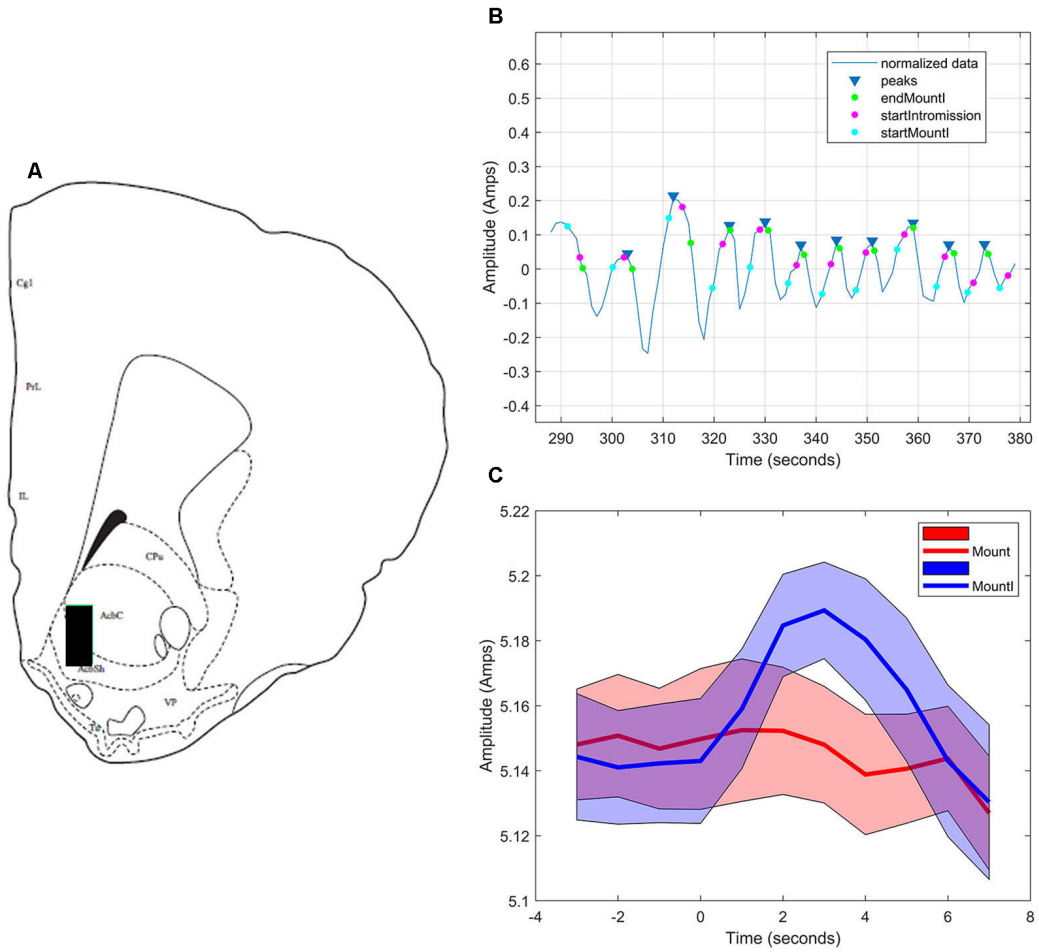

FIGURE 5 | Glutamate biosensor recording in NAc core, Subject #1. **(A)** This probe was in the center of the rostral NAc core (+2.6 mm from Bregma). **(B)** MATLAB annotated signal and peak analysis for the first mating bout. Blue rectangles indicate peaks determined by a threshold value of half of the root-mean-square (RMS) of the normalized signal. Blue circles (startMountI) indicate the start of a mount that results in an intromission. Magenta circles (startIntromission) indicate the start of a penile intromission from a male. Green circles (endMountI) indicate the end of a mount that resulted in an intromission. **(C)** Mounts with subsequent intromissions were collapsed across the first 5 min of the sex test. These mounts with intromission had a coincident increase in glutamate (blue signal) that peaked 3 s after the start of the mount. No coincident signal was seen in glutamate among mounts without intromission (red signal).

TABLE 1 | Proportion of mounts with and without subsequent intromission associated within 5 s of a glutamate peak in NAc core.

Subject	Mounts w/Intromission	Mounts alone	Chi Square analysis
NAcC-1	49/53 (93%)	16/52 (31%)	$\chi^2 = 42.3, p < 0.001$
NAcC-2	29/45 (64%)	0/5 (0%)	$\chi^2 = 7.7, p < 0.01$
NAcC-3	23/27 (85%)	12/21 (57%)	$\chi^2 = 4.7, p < 0.05$
NAcC-4	10/14 (71%)	1/4 (25%)	$\chi^2 = 2.8$, NS

Inhibitory DREADD Silencing of mPFC During Sexual Behavior

In this study we used viral expression of DREADDs to silence glutamatergic mPFC neurons in female hamsters during sexual behavior to test whether c-Fos activation in the NAc core was driven by mPFC glutamatergic afferents. Because the sex-activated neurons in the mPFC were CaMKIIα-expressing neurons (**Figure 3D**), we inhibited the mPFC using an inhibitory DREADD (Roth, 2016) with a CaMKIIα promoter. Viral injection sites were verified as being localized to the mPFC using fluorescent microscopy for the mCherry reporter. Only AAV-injections that spanned the prelimbic and infralimbic subdivisions of the mPFC were included in the analyses (**Figure 8A**). Control females not receiving sexual behavior received either a saline injection or CNO. There were no differences between saline and CNO animals in this condition (data not shown), so these sex behavior controls (n = 6) were combined into a single treatment group. Analyses of the mPFC confirmed that the CNO decreased c-Fos labeling following sexual behavior. There was a significant treatment effect across groups ($F_{(2,15)}$ = 16.64, p < 0.001; **Figure 8B**), with *post hoc* Tukey's multiple comparisons tests confirming that saline-treated females tested for sexual behavior (n = 6) had more c-Fos

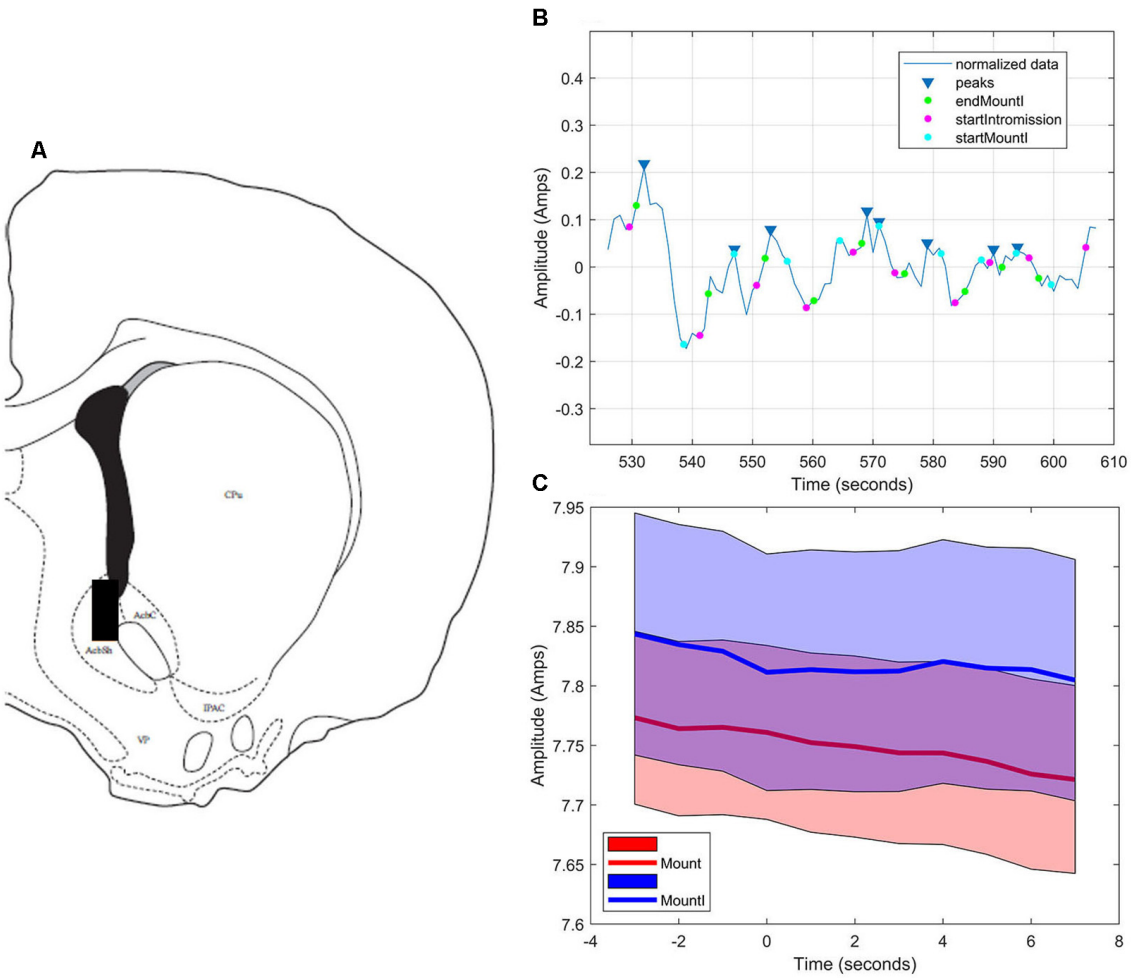

FIGURE 6 | Glutamate biosensor recording in NAc shell, Subject #1. **(A)** This probe was in the caudal NAc shell (+1.5 mm from Bregma). **(B)** MATLAB annotated signal and peak analysis for the first mating bout. Blue rectangles indicate peaks determined by a threshold value of half of the RMS of the normalized signal. Blue circles (startMountI) indicate the start of a mount that results in an intromission. Magenta circles (startIntromission) indicate the start of a penile intromission from a male. Green circles (endMountI) indicate the end of a mount that resulted in an intromission. **(C)** Although there are significantly more glutamate peaks associated with mounts that resulted in intromission, there was no coincident timing of those peaks (blue signal), a finding similar to that of mounts without intromission (red signal).

TABLE 2 | Proportion of mounts with and without subsequent intromission associated within 5 s of a glutamate peak in the NAc shell.

Subject	Mounts w/Intromission	Mounts alone	Chi Square analysis
NAcSh-1	52/58 (90%)	5/31(16%)	$\chi^2 = 47.4, p < 0.001$
NAcSh-2	38/40 (95%)	3/48 (6%)	$\chi^2 = 69.1, p < 0.001$
NAcSh-3	35/41(85%)	6/45 (13%)	$\chi^2 = 44.6, p < 0.001$
NAcSh-4	45/45 (100%)	6/30 (20%)	$\chi^2 = 52.9, p < 0.001$

labeling in the mPFC than did control females not tested for sexual behavior ($p < 0.01$). Females tested for sexual behavior and receiving CNO ($n = 6$) had fewer c-Fos labeled cells than did the saline-treated females receiving sex ($p < 0.01$). There was no significant difference between the no sex controls and sex tested females receiving CNO.

Key to our overarching hypothesis, this inhibition of the mPFC resulted in significantly attenuated c-Fos labeling in the NAc core following female sexual behavior ($F_{(2,15)} = 60.76$, $p < 0.0001$; **Figure 8C**) with *post hoc* Tukey's multiple comparisons tests confirming that saline-treated females tested for sexual behavior had more c-Fos labeling in the NAc core than did control females not tested for sexual behavior ($p < 0.01$). Females tested for sexual behavior and receiving CNO had fewer c-Fos labeled cells than did the saline-treated females receiving sex ($p < 0.01$), but were not reduced to control levels, with these groups demonstrating a significant difference from each other ($p < 0.01$). The CNO injections did not affect the levels of lordosis shown by the hamsters (data not shown), so the effects of the CNO on c-Fos in the NAc were independent of the levels of sexual behavior displayed by the female hamsters. These results suggest that glutamate neurons in the medial PFC activated during sex are at

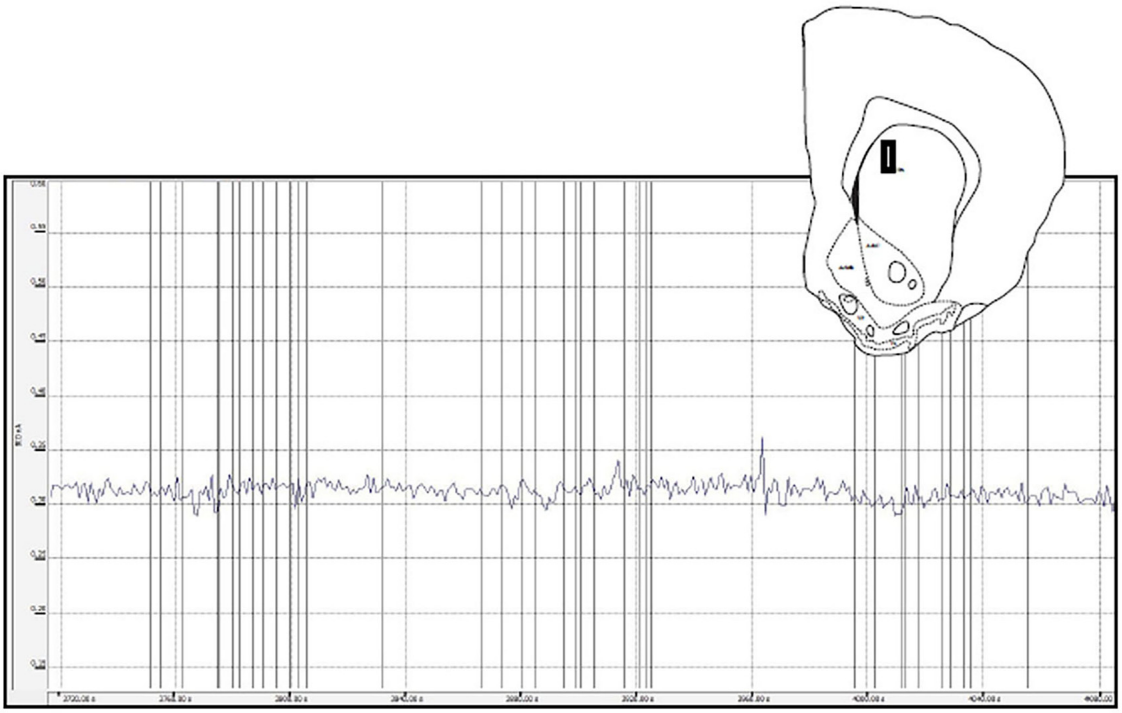

FIGURE 7 | This figure depicts a glutamate biosensor placement within the medial caudate (insert) as well as the raw trace for the glutamate signal during a sex behavior test. For this and the other caudate animals there were few peaks recorded and no systematic glutamate changes associated with behavioral events (vertical lines).

least partially responsible for driving neuronal excitability in the NAc.

DISCUSSION

Despite a large literature detailing dopaminergic neurotransmission in mesolimbic circuitry during female sexual behavior (Meisel et al., 1993; Mitchell and Gratton, 1994; Mermelstein and Becker, 1995; Kohlert et al., 1997; Kohlert and Meisel, 1999; Becker et al., 2001; Jenkins and Becker, 2003), the role of glutamate has been disproportionately understudied. The overarching goal of this study was to help close this gap in knowledge, adding to our understanding of the complex underpinnings of female sexual reward and motivation.

Our discovery that the mPFC (see Wise, 2008 for a discussion of the prefrontal cortex in rodents) is activated by sexual behavior led us to consider the functional relationship between the mPFC and the nucleus accumbens with respect to the control of female sexual behavior. The ventral tegmental area (VTA) sends distinct dopaminergic projections to both the mPFC and the NAc, rather than collateral inputs to each region (Swanson, 1982; Lammel et al., 2008; Yetnikoff et al., 2014). Based on this pattern of innervation, we explored the serial connection between the mPFC and NAc in conjunction with activation by female sexual behavior. Our results demonstrate that sexual behavior had no effect on the numbers of glutamate neurons in the mPFC (as measured by CaMKIIα staining). Instead, the existing glutamate neurons within the mPFC were activated by female sexual behavior, and these activated neurons project to the NAc. Finally, DREADD-mediated inhibition of glutamatergic mPFC neurons prevented increased activity in the NAc following sex, providing converging evidence of the importance of mPFC glutamatergic projections driving activity in the NAc. What is missing from this work, and is a focus of ongoing research, is what behavioral functions are affected by inhibition of PFC glutamatergic inputs to the NAc. We found that the expression of lordosis is not affected by direct inhibition of the PFC (and in turn indirect inhibition of the NAc). Our next step is to test different motivational endpoints to see whether these behaviors are mediated by PFC glutamatergic activation of the NAc.

In addition to anatomically demonstrating the involvement of prefrontal glutamatergic neurotransmission in female sexual behavior, we sought to characterize glutamate release in the NAc during sexual behavior. Using enzymatic biosensors, we discovered that the core of the NAc releases glutamate with a short latency preceding individual intromissions from the male. These glutamate peaks had a coincident timing with respect to the onset of intromission. These results are consistent with the view that one function of the nucleus accumbens core is to coordinate sensory cues (in this case intromission by the mounting male) with an appropriate action, a process not affected by repeated presentation of the stimulus (e.g., Brown

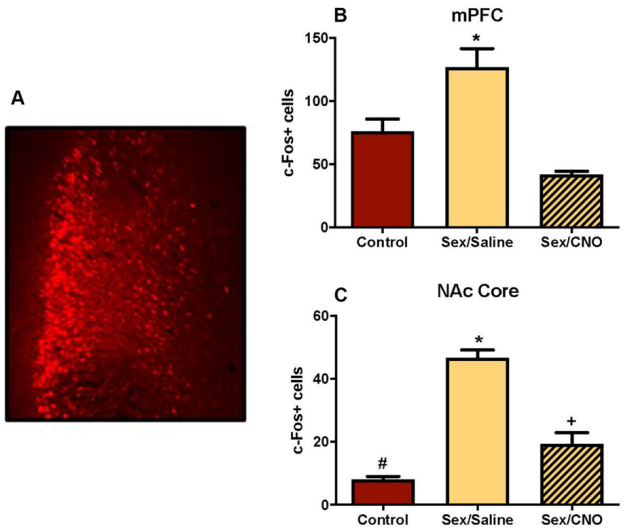

FIGURE 8 | Silencing glutamatergic mPFC projections decreases NAc c-Fos in response to sex. **(A)** An example of a mPFC injection sites identified based on the location of the mCherry reporter. **(B)** Female sexual behavior significantly increased c-Fos stained cells within the mPFC, an effect that was blocked by pretreatment with CNO (*indicates $p < 0.05$). **(C)** Female sexual behavior increased c-Fos labeling in the core of the NAc, an effect that was similarly blocked by DREADD inhibition of the mPFC. Each group is significantly different from each other as indicated by distinct symbols ($p < 0.05$).

et al., 2011). Intromission also elicited glutamate transients in the NAc shell, though these peaks were not coordinated in time. We hypothesize that the shell is responding to the rewarding consequences of intromission, a process that is more abstract and altered by repeated exposure to the stimulus, and therefore is more variable in its timing (e.g., Brown et al., 2011; Sackett et al., 2017).

The timing of the onset of glutamate release was an interesting finding that came from the characterization of glutamate release in the NAc during sexual behavior. Although the peak of the transient occurred after the onset of a mount culminating in intromission, the initial rise in glutamate was found to actually precede the actual receipt of intromission. We and others have demonstrated that female hamsters can control whether the mounting male hamster can achieve intromission (Noble, 1979, 1980; Bradley et al., 2005; Parada et al., 2014). These data suggest that glutamate in the nucleus accumbens is signaling this anticipatory response. A higher proportion of glutamate peaks occur in response to mounts with subsequent intromission as opposed to mounts alone, though a smaller proportion of mounts that do not result in intromission are also associated with glutamate transients. This small proportion of mounts without subsequent intromission that are still associated with glutamate release may indicate prediction error by the female (Hart et al., 2014; Saddoris et al., 2015; Gmaz et al., 2018).

In these experiments, we focused on the role of glutamate innervation mediating the effects of sex-induced NAc activation; however, we know that the NAc receives both dopamine and glutamate functional inputs (Zahm and Brog, 1992; Brog et al., 1993; O'Donnell and Grace, 1995; Kelley, 2004; Britt et al., 2012). Dopamine functions as a neuromodulator within the NAc coordinating with glutamatergic afferents to regulate the excitability of NAc neurons (O'Donnell et al., 1999; Nicola et al., 2000). Dopamine can affect intracellular signaling to modulate the functional responsiveness of both metabotropic and ionotropic glutamate receptors (Chen and Roche, 2007; Cahill et al., 2014). Understanding the coordinated actions of both dopamine and glutamate on nucleus accumbens neurons in regulating female sexual behavior is a goal of our current research.

Consistent with the literature from rodent models, mesocorticolimbic circuitry mediates the rewarding consequences of sexual behavior in people (Stahl, 2010; Georgiadis and Kringelbach, 2012; Oei et al., 2012; Kingsberg et al., 2015). Increased PFC and nucleus accumbens activation has been demonstrated in response to different components of sexual arousal in human subjects (Bocher et al., 2001; Sabatinelli et al., 2007; Voon et al., 2014; Wehrum-Osinsky et al., 2014; Lee et al., 2015; Ruesink and Georgiadis, 2017). Within this circuitry, dopamine and glutamate are the predominant signaling molecules, though how dopamine or glutamate release may be associated with sexual function or dysfunction in women is unknown. It is abundantly clear that animal models of sexual reward and motivation provide a logical preclinical avenue to a mechanistic understanding for the development of targeted therapeutics (Kingsberg et al., 2015; Jaspers et al., 2016), despite obvious differences in behavior patterns between animals and people. In this context, we have developed behavioral tests to measure both the pleasurable consequences of sexual interactions in female hamsters as well as the motivational control (Meisel and Mullins, 2006). The emphasis for future studies will be to evaluate neuronal mechanisms underlying sexual motivation and pleasure in our Syrian hamster model to provide viable directions for the development of therapeutics for low sexual desire in women.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Minnesota Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LB, RM and KM: project conception and experimental design. LB, MG and KM: execution of experiments. LB and KM: data analyses. MJ and WO: Matlab analyses of biosensor data. LB, RM, KM and WO: manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00227/full#supplementary-material

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REVIEW
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Sex Mysteries of the Fly Courtship Master Regulator Fruitless

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The *fruitless* (*fru*) gene of *Drosophila melanogaster* generates two groups of protein products, the male-specific FruM proteins and non-sex-specific FruCOM proteins. The FruM proteins have a 101 amino acids (a.a.)-long extension at the N-terminus which is absent from FruCOM. We suggest that this N-terminal extension might confer male-specific roles on FruM interaction partner proteins such as Lola, which otherwise operates as a transcription factor common to both sexes. FruM-expressing neurons are known to connect with other neurons to form a sexually dimorphic circuit for male mating behavior. We propose that FruM proteins expressed in two synaptic partners specify, at the transcriptional level, signaling pathways through which select pre- and post-synaptic partners communicate, and thereby pleiotropic ligand-receptor pairs for cell-cell interactions acquire the high specificity for mutual connections between two FruM-positive cells. We further discuss the possibility that synaptic connections made by FruM-positive neurons are regulated by neural activities, which in turn upregulate Fru expression in active cells, resulting in feedforward enhancement of courtship activities of the male fly.

Keywords: ***Drosophila*, sexually dimorphic circuit, social effect, mating behavior, transcription factors**

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PREFACE

fruitless (*fru*) mutant males in *Drosophila* are known to exhibit strong male-to-male courtship activities with reduced or no female-directed courtship (Hall, 1978; Villella et al., 1997; Yamamoto and Koganezawa, 2013). The gene responsible for *fru* mutant phenotypes encodes, when wild type, a group of transcriptional regulators with a masculinizer function FruM (Ito et al., 1996; Ryner et al., 1996), which organize, together with the other sex-determinant protein Doublesex (Dsx), a subset of neurons into the sexually dimorphic neural circuitry for mating behavior (Kimura et al., 2005, 2008; Cachero et al., 2010; Rideout et al., 2010; Robinett et al., 2010; Ruta et al., 2010; Yu et al., 2010; Kohl et al., 2013; Tanaka et al., 2017). However, there remain uncertainties regarding the mechanisms of action of the *fru* gene in achieving this organizer role in the sexual dimorphism formation of the brain. This article discusses three major questions. Do non-sex-specific products (FruCOM) of the *fru* gene have nothing to do with sex-type specification? Is the neural masculinizing action of FruM ascribable entirely to its cell autonomous function? Does the *fru* gene affect adult behavior exclusively through its developmental functions before adult emergence? In this article, we discuss the importance of the finding that nearly all neuroblasts in both FruM-positive and FruM-negative lineages express FruCOM, the finding that postsynaptic tissues form through interactions with a *fru*-positive presynaptic neuron (non-cell autonomy), and the finding that the *fru*-positive circuit appears to accommodate itself to ambient conditions to best tune the male's behavior from time to time.

MULTIFACETED FRU PROTEIN ACTIVITIES RELY ON COMPLEX SPLICING

The *fru* gene spans over 150 kb of the genome, and harbors at least four promoters, *P1–P4* (Ryner et al., 1996; Usui-Aoki et al., 2000; **Figure 1A**). The distally located *P1* promoter is dedicated to sex-specific functions of the *fru* gene, whereas the *P2–P4* promoters contribute to the production of FruCOM proteins, which are shared by both sexes (Ryner et al., 1996; Anand et al., 2001; Song et al., 2002; **Figures 1B,C**). Structurally, FruM proteins have a unique N-terminal extension composed of 101 amino acids (a.a.), followed by the main body of the protein, which is composed of a sequence identical to full-length FruCOM (except for small variations; Ryner et al., 1996; Song et al., 2002; **Figure 1D**). Thus, although the C-termini are common to FruM and FruCOM, there are five types of C-terminal splice variants called types A to E (**Figures 1A,B**). For example, the FruM isoform with the C-terminus of type B is referred to as FruBM. Types A, B and E in our terminology (Usui-Aoki et al., 2000) correspond to types A, C and B in the terminology adopted by the Barry Dickson (Demir and Dickson, 2005; Stockinger et al., 2005) and Stephen Goodwin groups (Song et al., 2002). Thus far, the type A, B and E isoforms (following the terminology of Usui-Aoki et al., 2000, which is adopted throughout this article) have been studied in some detail, and so we will focus on these three isoforms in the following discussion. The 101 a.a. extension unique to FruM proteins has no known motif, whereas the main body of the protein has a BTB domain near the N-terminus and two zinc finger motifs at the C-terminus (Ito et al., 1996; Ryner et al., 1996; **Figure 1D**). The BTB-Zn finger proteins are dominated by transcriptional regulators, and indeed, this proved to be true for FruM as well; FruBM binds to the DNA region named FROS to repress transcription of a target gene (e.g., *robo1*, Ito et al., 2016) that forms a complex with other transcription regulators, including HDAC1, HP1a, Bonus, TRF2 and Lola (Ito et al., 2012; Chowdhury et al., 2017; Sato et al., 2019), some of which are well known for their involvement in chromatin modifications. Although C-terminal variations likely contribute to target specificities (Neville et al., 2014; von Philipsborn et al., 2014), the absence of the male-specific N-terminal extension probably does not narrow the range of target choice, because major portions of the behavioral and cellular phenotypes of FruM-null mutants are rescuable by artificial expression of FruCOM instead of FruM (Ferri et al., 2008). This observation, however, does not exclude the possibility that FruCOM might have additional transcriptional targets to which FruM proteins are unable to bind for transcriptional regulation.

Whereas FruCOM functions as well as FruM in terms of masculinizing neural and behavioral traits, FruCOM and FruM have different endogenous tissue distributions (Lee et al., 2000). The *P1* promoter seems to be active only in neurons, as FruM expression is strictly confined to neurons (Sato et al., 2019). *P1*-derived *fru* mRNAs are transcribed in both females and males (Usui-Aoki et al., 2000), but the FruM protein is male-specific and absent from females (Lee et al., 2000; Usui-Aoki et al., 2000). The male-specific FruM expression is a result of sex-specific splicing of the *fru* primary transcript (**Figures 1B,C**), which yields *fru* mRNA encoding a full-length ORF in males and an ORF prematurely interrupted by a stop codon (and thus non-coding) in females (Heinrichs et al., 1998). Thus, the presence or absence of FruM (FruCOM is not expressed in adult neurons of either sex) is decisive in directing the sexual fate of a neuron to the male fate or female fate.

The sex-determination in *Drosophila* is achieved on a cell-by-cell basis, i.e., each cell composing the entire organism establishes its sexual identity according to the genetic code without any involvement of sex hormone signaling. When the ratio (X/A) of the number of X-chromosomes over the number of autosome pairs (typically "2") is 1.0 (such as when somatic cells in an individual carry two X chromosomes) or larger, the cell adopts the female fate, whereas, when the X/A value is 0.5 (in an individual carrying a single X chromosome) or smaller, the cell adopts the male fate. Counting of the relative numbers of X-chromosomes is performed by a transcriptional two-directional switch at the *Sex-lethal* (*Sxl*) gene, which is transcribed only when X/A exceeds 1.0. Thus the *Sxl* gene typically produces the Sxl protein only in XX individuals. The female-specific Sxl protein functions as a splicing regulator that induces female-specific splicing of its target, the *transformer* (*tra*) gene primary transcript. Only a transcript spliced in the female pattern can encode a functional Tra protein, which in turn induces female-specific splicing distinct from a default splicing that occurs in males in its targets, e.g., the primary transcript from the *P1* promoter of the *fru* gene (*fru-P1*). Upon binding to the Tra target motif in the *fru-P1* primary transcript (Ito et al., 1996; Ryner et al., 1996; Heinrichs et al., 1998), the Tra protein induces splicing of the *fru-P1* primary transcript at the site 3′ to the binding site in females, leading to the production of an mRNA whose ORF is interrupted by a termination signal (Ito et al., 1996; Ryner et al., 1996; Heinrichs et al., 1998; **Figure 1C**). In males, default splicing in the absence of Tra takes place at a more 5′ site, which excludes the termination signal from the mature *fru* mRNA (Ito et al., 1996; Ryner et al., 1996; Heinrichs et al., 1998). *fru* is therefore considered to be an effector transcription factor gene in the sex determination cascade, together with the other Tra target, *dsx*.

DOES MALE-SPECIFIC FRUM SIGNALING INTERSECT NON-SEX-SPECIFIC FRUCOM SIGNALING?

No Tra-binding motif has been identified in primary transcripts from *P2–P4* promoters. The *P1* promoter dedicated to sex-related functions is active only in neurons, while the *P2–P4* promoters are active in a variety of tissues. The apparent absence of FruCOM (*P2–P4* products) in neurons and neuron-restricted FruM (*P1* products) expression do not necessarily mean that FruCOM is "non-neural." Lee et al. (2000) observed a large number of cells labeled by the anti-FruCOM but not anti-FruM antibodies in the brain and ventral nerve cord of third instar female and male larvae. Our recent analysis with wandering stage larval brains convincingly showed that nearly all neuroblasts transiently express FruCOM proteins, which rapidly fade out and

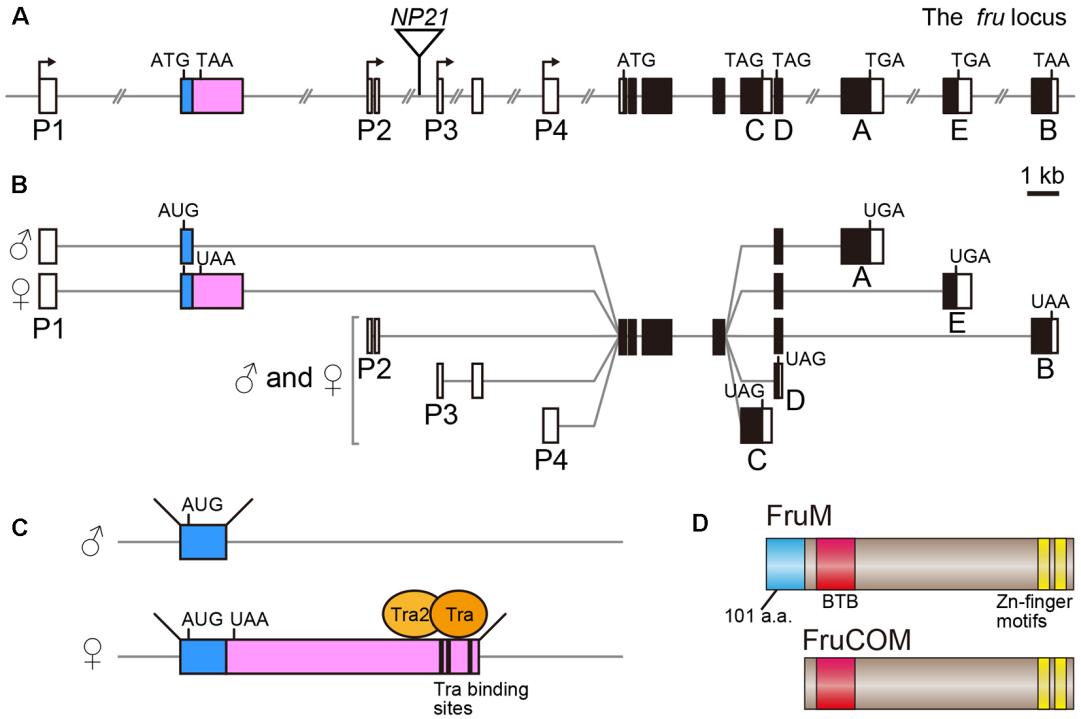

FIGURE 1 | Schematic representation of the *fruitless (fru)* gene structure. **(A)** Locations of four promoters (P1–P4), the exon-intron organization and the *fru*^{NP21} *P*-element insertion point (an inverted triangle) are shown. Filled and open boxes indicate coding and non-coding exons, respectively. The second exon subjected to sex-specific splicing is highlighted in color. A-E denote isoform-specific exons for types A-E. The start and termination codons are also shown. **(B)** Splicing variations and the resulting protein isoform variants are illustrated. **(C)** The Tra-binding sites and sexually dimorphic splicing mechanism are depicted. **(D)** Schematic representation of the FruM and FruCOM protein structures.

disappear in the daughter cells (i.e., ganglion mother cells and neurons; Sato et al., 2019). This raises the intriguing possibility that FruCOM proteins have a hitherto uncharacterized function in proliferating neuroblasts, such as specifying the types of neurons the neuroblast should produce. A comprehensive analysis of clonal cell lineages unraveled that *P1*-dependent *fru*-positive neurons (hereinafter *fru*[+]-neurons) that are sexually dimorphic derive from multiple neuroblasts rather than a few dedicated neuroblasts: in fact, all type II neuroblast lineages bring about sexually dimorphic *fru*[+]-neurons (Ren et al., 2016). It remains to be determined whether larval expression of FruCOM could have any sustained effect on the transcriptional state of Fru-responsive genomic elements—such as, for example, to sensitize them for subsequent exposure to FruM.

DO FruM PROTEINS SHAPE ONLY NEURONS IN WHICH THEY ARE EXPRESSED?

With a few exceptions, transcription factors act within cells in which they are expressed. Indeed, the FruM proteins, in their capacity as transcription factors, specify the structure of a *fru*[+]-neuron by their cell autonomous functions. The best characterized *fru*[+] neurons are those that compose the mAL cluster in the brain. mAL neurons are sexually dimorphic in three respects (Kimura et al., 2005). First, the number of neurons that comprise the cluster is five in females and 30 in males. Second, the ipsilateral neurite forms only in males. Third, the posteriorly extending contralateral neurite bifurcates near its tip only in females. These three sex-specific characteristics are determined by the presence or absence of FruM. Reducing functional FruM levels in males (e.g., in *fru* hypomorphic mutant males) leads to an increase in the proportion of female-typical neurons at the expense of the male-type neurons in the mAL cluster (Ito et al., 2012). In principle, the neurons with intersexual structures are not produced; every neuron in the mAL cluster is either a perfect female-type or male-type neuron under *fru* loss-of-function conditions (Ito et al., 2012). By contrast, manipulations of a *fru* downstream element or some *fru*-interacting partners result in malformation of one or more sexually dimorphic characteristics of mAL neurons (Goto et al., 2011; Ito et al., 2016; Chowdhury et al., 2017; Sato et al., 2019). These observations suggest that FruM proteins operate as two-directional switches between the female-type and male-type developmental pathways in mAL neurons, whereas the specification of each sex-specific neural structure is achieved by pathway-specific molecules downstream of FruM. FruM and the FruM-downstream components function in the cell that produces these molecules, i.e., they function cell autonomously in conferring the sex-specific characteristics onto mAL neurons.

Sex differences in neurons other than mAL are also produced by a similar cell autonomous mechanism, and sexually dimorphic neurons thus specified on a cell-by-cell basis may form synapses to establish a sex-specific circuitry. On the other hand, synaptogenesis inevitably involves coordinated tuning of pre- and postsynaptic elements. Thus, it is conceivable that cell-to-cell interactions during synaptogenesis would also contribute to sexually dimorphic refinement of dendritic arbors and axonal terminals. There is a precedent case in which FruM expression in a cell was shown to be pivotal for normal development of another cell; that is, the male-specific adult muscle called the muscle of Lawrence (MOL) was shown to form only when innervated by a male motoneuron named the Mind (MOL-inducing) neuron (Nojima et al., 2010), irrespective of whether the muscle on its own is composed of female cells or male cells (Lawrence and Johnston, 1984, 1986; Taylor, 1992). Muscle cells do not express FruM and the MOL is not an exception to this rule. Exploring how the MOL induction is achieved by the Mind neuron will provide insights into the molecular mechanism whereby FruM in a neuron exerts non-cell autonomous effects on its synaptic partners for the formation of a sexually dimorphic circuit.

DO FruM FUNCTION ONLY IN DEVELOPMENT OR DO FruM ALSO FUNCTION IN A BEHAVING ADULT FLY?

The nervous system of holometabolous insects such as *Drosophila* is largely reorganized during the pupal stage when sexually dimorphic circuitry is newly established under the control of FruM and Dsx. Consistent with this fact, FruM expression commences at the wandering third instar larval stage, peaks at the pupal stage, and thereafter declines but does not disappear after the adult emergence (Lee et al., 2000). The functions of FruM in the adult stage have been ill-defined. However, clues to the roles of FruM in adults were obtained by Hueston et al. (2016). They found that *fru-GAL4* expression in the *Or47b*-expressing olfactory neurons is sustained through the adult stage only when these cells are functionally active: *fru-GAL4* expression is activity-dependent in *Or47b* neurons (Hueston et al., 2016). Or47b is activated by the fatty acid ligand methyl laurate, which is an endogenous aphrodisiac for both sexes and is contained in the adult cuticle of both sexes (Dweck et al., 2015). The major sex pheromones in *Drosophila* are several hydrocarbon compounds in the body surface cuticle (Jallon, 1984). Notably, genetic deprivation of all hydrocarbons from wild-type male flies makes them extremely attractive for other males and results in male-male courtship, which is rarely seen under normal conditions (Billeter et al., 2009). These unusual homosexual activities among males are likely evoked by the fatty acid attractants remaining in the cuticle, from which hydrocarbon pheromones, both excitatory and inhibitory ones, have been deprived. Notably, male-male courtship is a hallmark of *fru* mutants that lack *fru* expression (Hall, 1978; Villella et al., 1997). Recent studies have demonstrated that male-male courtship in *fru* mutants is enhanced by rearing these flies in a group and suppressed by social isolation (Pan and Baker, 2014; Kohatsu and Yamamoto, 2015). Olfactory experience appears important for the development of this trait because genetic deprivation of olfaction abrogated the induction of male-male courtship in grouped *fru* mutant males (Pan and Baker, 2014). These observations tempt us to postulate that activity-dependent *fru* expression might play a role in experience-dependent changes in behavior after adult emergence. Another study showed that juvenile hormone (JH; known to stimulate reproductive maturation in the adult) acts on *Or47b* olfactory neurons in mature adult males to boost their ligand sensitivity, making these elder males more successful in copulation than younger males (Lin et al., 2016). This finding invites speculation that JH might act through FruM to elevate Or47b sensitivity. Remarkably, Wu et al. (2018) suggested that some of the JH actions are mediated by a FruM-dependent mechanism: they showed that a sex difference in sleep patterns disappears and FruM expression in the brain declines in male flies when JH signaling is inhibited. Of note, sleep activities and sexual activities are reciprocally regulated by a group of *fru*[+] neurons called P1 neurons (Chen et al., 2017), which were originally identified to be the primary decision-making cells for the initiation of male courtship (Kimura et al., 2008). It would be of interest to examine whether the mechanism by which JH elevates male mating success by acting on Or47b is dependent on functional FruM in these neurons. A recent study revealed that *IR52a*-expressing *fru*[+]-chemosensory neurons on the wing margin mediate input to stimulate male-male courtship (He et al., 2019). It remains to be examined whether *fru* expression in the *IR52a*-sensory neurons as positive regulators for male-male courtship is also modulated by neural activities during the adult stage.

PERSPECTIVES

The *fru* gene produces two major protein groups: FruM and FruCOM. The FruM proteins have an N-terminal extension that FruCOM proteins lack, but we do not know how important this structural difference is in terms of the protein functions. The expressions of the FruM and FruCOM proteins are mutually exclusive both spatially and temporally (e.g., neuroblasts vs. neurons in the postembryonic nervous system; Sato et al., 2019), implying that each protein group acts in a different developmental context, possibly through partially redundant signaling mechanisms.

Molecular studies on the actions of FruBM protein have revealed that this protein forms a transcriptional complex with an isoform of Lola, a pleiotropic transcription factor, to transcriptionally repress the *robo1* gene, a direct target of FruM (Ito et al., 2016). In male flies, FruM protects Lola from truncation upon binding to Lola through each-others' BTB domains; the N-terminal portion of Lola is otherwise truncated by ubiquitin proteasome digestion (Sato et al., 2019). Robo1 functions to inhibit the extension of the male-specific neurite of mAL neurons, thereby contributing to the formation of sexual dimorphism in these neurons (Ito et al., 2016). Full-length Lola represses *robo1* in males, whereas truncated

Lola inhibits full-length Lola's action to repress *robo1*, with the result that the ipsilateral neurite forms in males but not females (Sato et al., 2019). Lola is known to drive neuroblasts to exit the stem cell state and enter the differentiation pathway (Southall et al., 2014). An intriguing possibility is that FruCOM contributes to this process together with Lola in both sexes by playing a transcriptional role similar to that of FruM in sexual-type specification in males, and yet its target specificity or its preference for interaction partners differs from that of FruM. Notably, fasciculation and path-finding of pioneering axons in the embryo were disrupted by *fru* mutations that lost FruCOM while retaining FruM proteins (Song et al., 2002). In the embryonic nervous system, FruCOM but not FruM proteins are expressed in neuroblasts, ganglion mother cells (GMCs) and some neurons and glial cells (Song et al., 2002). Remarkably, axon guidance and fasciculation defects were rescued by the type A or type B isoform of FruCOM (but not by any of the FruM isoforms) when these proteins were overexpressed in neuroblasts and GMCs (but not neurons), suggesting that FruCOM functions are required in cells before neural differentiation for normal axonogenesis that occurs after differentiation (Song et al., 2002). Intriguingly, FruM overexpression even exaggerated the axonal defects in *fru* mutants (Song et al., 2002). These observations imply that FruCOM proteins with no N-terminal extension have biological activities distinct from those of FruM with the N-terminal extension. One may envisage, for example, that the male-specific N-terminal extension of FruM affects the stability of the FruM-containing transcriptional complex by modulating the proteasomal degradation of FruM-interaction partners within the complex. We presume that FruM is evolutionarily a derivative of FruCOM that was co-opted for sex-specific functions in neurons, whereas FruCOM expression was eliminated through negative selection in evolution.

The *robo1* gene is the sole established target of FruM (more specifically, FruBM; Ito et al., 2016), although the total number of FruBM targets is expected to exceed 100 based on immunolabeling of FruBM that bound to the target sites on polytene chromosomes (Ito et al., 2012). Robo1 is a transmembrane protein that functions as a receptor for Slit proteins, membrane-anchored ligands that mediate cell-to-cell interactions (Kidd et al., 1998, 1999). Robo proteins of vertebrates and invertebrates exert pleiotropy, working in neural midline crossing/turning/stopping, angiogenesis, kidney development, heart development, mammary gland morphogenesis and other developmental processes, and this pleiotropy partly depends on the pleiotropic processing of Robo and Slit upon their binding to each other (Blockus and Chédotal, 2016), which occurs in two facing cells that interact with each other. This leads to an important question. How do FruM-expressing neurons recognize each other and specifically make connections with an appropriate partner? An intriguing possibility is that FruM proteins determine, at the transcriptional level, the manner of processing of Robo and Slit upon ligand-receptor interactions. One can anticipate that only FruM-expressing cells display coherent processing patterns in both pre- and postsynaptic membranes, allowing stable connections to be made and inductive interactions to occur between them.

The loss of FruM expression by the olfactory receptor mutations observed in adult pheromone neurons (Hueston et al., 2016) might suggest that functional synaptic connections are maintained by FruM, whose expression is maintained in a use-dependent manner: a feedforward loop between the neural activity and FruM expression could operate to enhance courtship activities for improved fitness of elder males.

These considerations prompt us to speculate that the *fru* gene became potentiated to achieve a specialist role—i.e., a neural masculinizer role—by creating structurally distinct FruM proteins in addition to FruCOM proteins. We assume that FruM proteins specify coherent signaling pathways in the pre- and postsynaptic neuron pair to form a Fru-labeled neural circuit. This circuit is probably consolidated by the fly's experience *via* use-dependent synaptic enhancement. However, this model describing how the actions of *fru* could induce adaptive changes in the nervous system of a fly during its individual lifetime remains to be tested in future experiments.

AUTHOR CONTRIBUTIONS

DY: conceptualization, review and editing. KS and DY: funding acquisition and writing the original draft. JG: experimental work. KS: result analysis and visualization.

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Tamoxifen Blocks the Development of Motivational Features of an Addiction-Like Phenotype in Female Rats

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Women become addicted sooner after initiating cocaine use as compared to men. Preclinical studies reveal a similar vulnerability in females, with findings from ovariectomized rats suggesting that estradiol mediates the enhanced vulnerability. However, since ovariectomy depletes not only estradiol, but all ovarian hormones, its role in a physiological context is not clear. Thus, the goal of this study was to determine the role of estradiol in the development of an addiction-like phenotype in ovary-intact females treated chronically with the selective estrogen receptor (ER) modulator tamoxifen. We hypothesized that tamoxifen, by antagonizing ERs, would block the development of an addiction-like phenotype as defined by an enhanced motivation for cocaine (assessed under a progressive-ratio schedule), and a heightened vulnerability to relapse (assessed under an extinction/cue-induced reinstatement procedure). Effects were examined following extended access cocaine self-administration (24-h/day; 4-discrete trials/h; 1.5 mg/kg/infusion) and 14-days of abstinence, conditions optimized for inducing an addiction-like phenotype. As predicted, motivation for cocaine was increased following extended-access self-administration and protracted abstinence in the vehicle (sesame oil) and no-injection control groups, but not in the tamoxifen group indicating that ER signaling is critical for the development of this feature of an addiction-like phenotype. Surprisingly, the increase in motivation for cocaine following abstinence was also attenuated in the vehicle group as compared to no-injection controls suggesting that oil/injections also affected its development. Contrary to our hypothesis, tamoxifen did not decrease vulnerability to relapse as this group responded at similar levels during initial extinction sessions and cue-induced reinstatement testing as compared to controls. Tamoxifen did, however, impair extinction learning as this group took longer to extinguish as compared to controls. Taken together, these findings indicate that estradiol is critical for the extinction of drug-associated cues and the development of motivational features of addiction.

Keywords: cocaine, estradiol, extended access, self-administration, sex differences

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INTRODUCTION

Cocaine use is a leading cause of overdose deaths in the U.S., second only to opioids (Hedegaard et al., 2018). Among African-Americans, it's the number one cause (Shiels et al., 2018), with rates of overdose deaths from cocaine on par with, or exceeding, overdose deaths from opioids in white Americans (Shiels et al., 2018). Furthermore, cocaine use is on the rise with 2.2 million Americans reporting current use in 2017 vs. 1.9 million in 2016 (Center for Behavioral Health Statistics and Quality, 2015; Substance Abuse and Mental Health Services Administration, 2017)—a trend predicted to continue as levels of coca cultivation and potential cocaine production in Colombia rise (United States Drug Enforcement Administration, 2016). Women users are particularly concerning as they become addicted to the drug more quickly and display more serious drug-related medical and psychological complications (Center for Substance Abuse Treatment, 2009; Greenfield et al., 2010; Becker and Koob, 2016). Once addicted, women also have longer periods of use after relapse, relapse for different reasons, and have a greater risk of admittance to treatment facilities as compared to men (White et al., 1996; Luchansky et al., 2000; Gallop et al., 2007; Potenza et al., 2012).

Preclinical studies also reveal a faster time-course for the development of features of an addiction-like phenotype in females vs. males (Lynch and Carroll, 2000; Lynch and Taylor, 2004; Kawa and Robinson, 2019; Nicolas et al., 2019). For example, female rats given extended access (ExA) to cocaine (6–24 h/day) take more cocaine and display a greater disruption of diurnal control over intake as compared to males (Lynch and Taylor, 2004, 2005; Roth and Carroll, 2004; Kawa and Robinson, 2019; Nicolas et al., 2019). Furthermore, females show an enhanced motivation for cocaine following 7 days of ExA self-administration and 10 days of abstinence—conditions that do not impact motivation for cocaine in males (Lynch and Taylor, 2004). However, when conditions are optimized (i.e., 10 days of ExA self-administration and 14 days of abstinence), both males and females show an enhanced motivation for cocaine (Ramôa et al., 2013). These findings are consistent with humans and indicate that the enhanced time-course for the development of cocaine addiction in females is biologically-based.

The biological mechanisms underlying these sex differences are unknown but likely involve the ovarian hormone estradiol (Segarra et al., 2010; Quinones-Jenab and Jenab, 2012; Ramôa et al., 2013). Women report the greatest sensitivity to the euphoric effects of cocaine and other stimulants during the follicular phase when estradiol levels are high and progesterone levels low (Justice and de Wit, 1999; Evans et al., 2002). Similarly, in female rats, motivation for cocaine and cocaine-seeking vary across the estrous cycle with the highest levels observed during estrus, when the ratio of estradiol to progesterone is relatively high (Lacy et al., 2019; Nicolas et al., 2019). Additionally, ovariectomy (OVX) has been reported to decrease cocaine self-administration under ExA conditions (Larson et al., 2007; Martinez et al., 2016), and to prevent the development of an enhanced motivation for cocaine, even when assessed under optimized conditions (Ramôa et al., 2013). Notably, estradiol replacement restores both levels of self-administration and the development of an enhanced motivation for cocaine (Ramôa et al., 2013), indicating that estradiol may be necessary for the development of an addiction-like phenotype in females.

One important caveat, however, is that OVX depletes all ovarian hormones, not just estradiol. OVX also results in the cessation of rhythmic fluctuations in levels of hormones, which may be critical for effects on addiction (Di Paolo, 1994; Bossé et al., 1997; Zhang et al., 2008; Segarra et al., 2014). Thus, the role of estradiol in a physiological context is not clear. This is especially important considering that findings obtained in ovary-intact females are somewhat contradictory to those observed in OVX females in that levels of drug intake, motivation, and seeking are highest during estrus, and not proestrus when levels of estradiol peak (Kippin et al., 2005; Feltenstein and See, 2007). Furthermore, the activity of dopaminergic neurons in the ventral tegmental area is lowest in intact females during proestrus and highest during estrus (Zhang et al., 2008). While the ratio of estradiol to progesterone has been suggested as a means to reconcile the differential results observed in intact vs. OVX rats, neurochemical data in OVX rats showing that both estradiol and progesterone similarly increase dopamine release in the nucleus accumbens (NAc) contradict this idea (Zhang et al., 2008). These disparities indicate a need to explore the role of estradiol in the development of an addiction-like phenotype in ovary-intact females.

Thus, in this study, we examined the effects of chronic treatment with tamoxifen, a selective estrogen receptor (ER) modulator, on the development of an addiction-like phenotype in ovary-intact females. Tamoxifen has been used extensively as an ER antagonist both clinically, primarily as a treatment for breast cancer (Huang et al., 2015), as well as preclinically, in intact and estradiol-treated OVX female rats and mice since it readily crosses the blood-brain barrier, inhibits estradiol-dependent behaviors (i.e., lordosis), and antagonizes both alpha and beta ERs (Halbreich and Kahn, 2000; Wilson et al., 2003; Smith and O'Malley, 2004; Flynn et al., 2017; Sá et al., 2018). Tamoxifen has also been reported to block estradiol-induced increases in striatal dopaminergic signaling in OVX females (Ferretti et al., 1988; McDermott et al., 1998, 1999; Dluzen et al., 2001; Landry et al., 2002), and to prevent the acquisition of cocaine self-administration (Lynch et al., 2001), the development of tolerance to opioids (Chiang et al., 2017; Withey et al., 2017), and the expression of a morphine-induced conditioned place preference in gonad-intact rats and mice (Esmaeili et al., 2009). It also attenuates the acquisition of an estradiol-induced conditioned place preference and estradiol-induced anxiolytic effects in OVX and ovary-intact females (Walf and Frye, 2005; Walf et al., 2007; Azizi-Malekabadi et al., 2015).

As in our previous work (Ramôa et al., 2013), the development of an addiction-like phenotype was assessed by comparing motivation for cocaine prior to and following ExA self-administration and 14 days of abstinence. We also examined the effect of tamoxifen on the development of another key feature of addiction, relapse vulnerability, as measured following protracted abstinence using an extinction/cue-induced

reinstatement procedure. Tamoxifen's effects were compared to effects observed in vehicle-treated rats. We also included additional groups of non-treated rats as a control for the effects of daily vehicle treatment (sesame oil). We hypothesized that chronic tamoxifen treatment in ovary-intact females would prevent the development of an addiction-like phenotype, including an enhanced motivation for the drug and heightened vulnerability to relapse.

MATERIALS AND METHODS

Animals

Subjects were sexually mature intact female (N = 62) Sprague-Dawley rats (Charles River), weighing 235–300 g at the start of the study. Upon arrival, rats were individually housed in operant testing chambers (Med Associates, St. Albans, VT, USA) and randomly assigned to one of two groups: vehicle-treated (VEH, n = 24) or tamoxifen-treated (TAM, n = 20). We also included additional non-treated, no-injection (NO INJ, n = 18) controls since initial results indicated that the development of an enhanced motivation for cocaine was attenuated by VEH treatment/injections (Lynch and Taylor, 2004; Ramôa et al., 2013). This group was run contemporaneously with the TAM and VEH groups as a control for vehicle injections. Throughout the study, rats were maintained on a 12-h light/dark cycle (house and room lights on at 7.00 AM), with *ad libitum* access to food and water (except as noted below for some animals during cocaine self-administration training). Following a 2-day habituation period, in order to encourage rapid subsequent acquisition of cocaine self-administration, rats were pre-trained to lever press for sucrose pellets (45 mg) using methods previously described (fixed-ratio 1; 24-h/day sessions; $\geq$50 sucrose pellets/session for 2 days; Lynch, 2008). Body weights were recorded three times/week and health was examined daily. The University of Virginia Animal Care and Use Committee approved all animal protocols, which adhered to the guidelines set by the National Institute of Health.

Tamoxifen Treatment and Vaginal Cytology

Rats were given subcutaneous injections of tamoxifen (1.0 mg/kg) or an equal volume of sesame oil (∼0.3 ml) between 8:30 and 11:30 AM 5 days/week beginning 1 day after arrival and continuing throughout the duration of the study, with the dose adjusted three times/week based on changes in body weight. Based on findings showing that a 5-day treatment regimen with estradiol prevents changes in dopamine receptor sensitivity that occur following daily administration, tamoxifen and vehicle treatments were administered 5 days/week (Di Paolo et al., 1981). Rats in the NO INJ group were handled similarly but did not receive injections. In order to verify the effectiveness of the tamoxifen treatment and to determine estrous cycle phase, vaginal samples were collected daily during the first week of treatment, and thereafter, weekly. Swabs were examined under light microscopy and the phase of the estrous cycle was determined using methods previously described (Lynch et al., 2001; Lynch and Taylor, 2005). Vaginal smears obtained from rats in the TAM group contained predominantly necrotic epithelia and leukocytes, which are indicative of metestrus and diestrus.

Surgery and Catheter Maintenance

Following lever pre-training, rats were anesthetized with ketamine/dexdomitor in order to implant a chronic, indwelling catheter (Silastic tubing; 0.51 and 0.94 mm o.d.; Dow Corning, Midland, MI, USA) into the right jugular vein, using methods previously described (Lynch, 2008). Catheter patency was tested 3 days/week by flushing with heparinized saline (∼0.5 ml), and by periodically administering methohexital (1.5 mg/kg). During the 14-day abstinence period, rats were given daily infusions of cefazolin (17 mg/kg) to help maintain patency. If a catheter was leaking, pressure prevented flushing, or the animal did not lose muscle tone immediately following the infusion of methohexital, the catheter was considered no longer patent and data collected between this assessment and the last patency check were discarded. If patency was lost, a new catheter was implanted into the left jugular vein. Cocaine self-administration resumed after 1–2 days of recovery.

Experimental Procedures

Cocaine Self-administration Training

Rats were initially trained to self-administer cocaine (1.5 mg/kg/infusion) under a fixed-ratio 1 schedule with a maximum of 20 infusions/day, using methods previously described (Lynch et al., 2010). Acquisition was defined as two consecutive days wherein all 20 infusions were obtained. A relatively high dose of cocaine was used to encourage rapid rates of acquisition and moderate food restriction (20 g/day) was used briefly (2–3 days) when necessary. All groups acquired rapidly under these high dose conditions and rates of acquisition did not differ between groups. Responses on the right (non-active) lever were counted during self-administration sessions as a measure of general activity, but they did not have any programmed consequence.

Motivation for Cocaine

Following acquisition, motivation for cocaine was assessed using a progressive-ratio (PR) schedule wherein the response requirement to obtain a cocaine infusion increased progressively throughout the session in the following steps: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, etc. PR sessions were conducted as previously described (Ramôa et al., 2013), and were run for 22-h each day (responding typically ceased within 2–4 h) until a stable baseline was achieved (defined as no increasing or decreasing trend in the number of infusions obtained over three consecutive sessions; typically achieved within 3–4 sessions). The moderate dose of cocaine tested (0.5 mg/kg/infusion) has been shown to reveal motivational differences between OVX females with and without estradiol replacement following ExA self-administration and abstinence, while producing comparable levels of responding at baseline (Ramôa et al., 2013).

ExA Cocaine Self-administration

After achieving a stable PR baseline, rats were given ExA (24-h/day) to cocaine under a discrete trial procedure using methods

previously described (1.5 mg/kg/infusion, 4-discrete trials/h, 10 days; Ramôa et al., 2013, 2014). Briefly, 10-min trials began every 15 min (96 infusions/day) with the extension of the active-lever into the chamber; after either 10 min or a response on the active-lever the trial was terminated and the lever retracted. These conditions have been shown to induce high levels of cocaine intake and dysregulated patterns of use (Ramôa et al., 2013). After the last ExA session, responding was again assessed under a fixed-ratio 1 schedule with a maximum of 20 infusions in order to equate levels of cocaine intake between groups before abstinence. A 14-day abstinence period began following the second fixed-ratio 1 session, during which animals remained in their test chambers.

Enhanced Motivation for Cocaine

In order to determine the impact of tamoxifen on the development of an addiction-like phenotype, motivation for cocaine was assessed following ExA self-administration and abstinence. This was conducted in a subset of rats (VEH, $n = 16$; TAM, $n = 13$; NO INJ, $n = 10$) using a PR schedule as described above.

Enhanced Relapse Vulnerability

The impact of tamoxifen on vulnerability to relapse was assessed in a subset of rats (VEH, $n = 8$; TAM, $n = 7$; NO INJ, $n = 8$) following ExA cocaine self-administration and 14 days of abstinence using a within-session extinction/cue-induced reinstatement procedure and methods previously described (Lynch et al., 2010). Briefly, extinction responding was examined in a minimum of 6, 1-h extinction sessions, and once responding extinguished (<15 responses) or a maximum of nine extinction sessions, reinstatement responding elicited by the cues formerly associated with cocaine (light above the lever and sound of the pump) was assessed in a 1-h session.

Hormone Measurements

Serum concentrations of estradiol and progesterone were assessed in a subset of rats using methods previously described (Lynch, 2008). Trunk blood collection occurred between 10 AM and 12 PM following the completion of the last PR session (VEH, $n = 6$; TAM, $n = 7$; NO INJ, $n = 7$) or the extinction/reinstatement test (VEH, $n = 6$; TAM, $n = 4$; NO INJ, $n = 7$). Rats in the PR experiment also underwent pharmacological testing prior to serum collection; however, this testing did not appear to impact hormone levels as no differences were observed for either estradiol or progesterone levels in serum collected following PR testing vs. extinction/reinstatement testing. Radioimmunoassays were conducted at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

Drugs

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse and prepared in sterile saline (7 mg/ml). The mg/kg dose was adjusted for changes in body weight three times a week by adjusting the infusion duration. Tamoxifen and sesame oil (vehicle) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Data Analysis

Intake during the ExA component of the study was compared between groups using a linear mixed-effects model with group, session, and their interaction as fixed factors and subject as a random effect. A paired-samples *t*-test was used to compare average intake between sessions 1–2 vs. 3–10. The primary measure for the development of an addiction-like phenotype was an enhanced motivation for cocaine which was determined following ExA self-administration and abstinence and, based on our previous findings in intact males and females (Lynch and Taylor, 2004; Ramôa et al., 2013, 2014; Doyle et al., 2014), was defined as a 15% or greater increase in PR responding during retest as compared to baseline (averaged across the three sessions within each phase). A linear mixed-effects model was also used to examine group differences in PR responding for cocaine (number of infusions obtained during the three baseline and retest sessions) with phase (baseline vs. retest) as an additional fixed factor. *Post hoc* comparisons within each group were made using Bonferroni-corrected paired *t*-tests and within phase *post hoc* comparisons to control (VEH group) were performed using Dunnett's *t*-tests. In order to control for group differences in PR responding at baseline, data were also examined as percent change from baseline to retest using similar statistical procedures (i.e., a linear mixed-effects model, Dunnett's *t*-test for *post hoc* between-group comparisons and Bonferroni-corrected one-sample *t*-tests for within-group comparisons).

Similar procedures were used to examine group differences during extinction (active-lever responses during sessions 1–9), with a paired-samples *t*-test used to compare responding during session 1 vs. later sessions (2–9) and univariate analysis of variance (ANOVA) used to examine differences within each session. Since not all rats required greater than 6 sessions to extinguish responding, zero responses were used in the analyses for rats who had already reached the extinction criteria. We also verified that the effects were similar when analyzed during the first six extinction sessions only. Univariate ANOVAs were used to analyze group differences in total responses on the inactive-lever during extinction and reinstatement, with *post hoc* comparisons made using Dunnett's *t*-test. A Kruskal-Wallis test was used to compare the number of sessions required to meet the extinction criteria (i.e., 6, 7, 8, or 9). A linear mixed-effects model was used to compare the number of active-lever responses during the last extinction session vs. the reinstatement session. Serum levels of estradiol, progesterone, and the ratio of estradiol to progesterone were collapsed across experiments (PR and reinstatement) and analyzed using univariate ANOVAs with *post hoc* comparisons performed using Dunnett's *t*-tests. Changes in body weight across 10 time-points from arrival to PR/relapse testing following abstinence (i.e., at arrival and during cocaine self-administration training, PR baseline, early, mid, and late ExA self-administration, early, mid, and late abstinence, and at test) were examined using a linear mixed-effects model with Dunnett's *t*-tests used for *post hoc* comparisons. One-tailed *t*-tests were used for predicted differences in motivation for cocaine, and two-tailed *t*-tests were used for all other comparisons. Statistical analyses were performed using

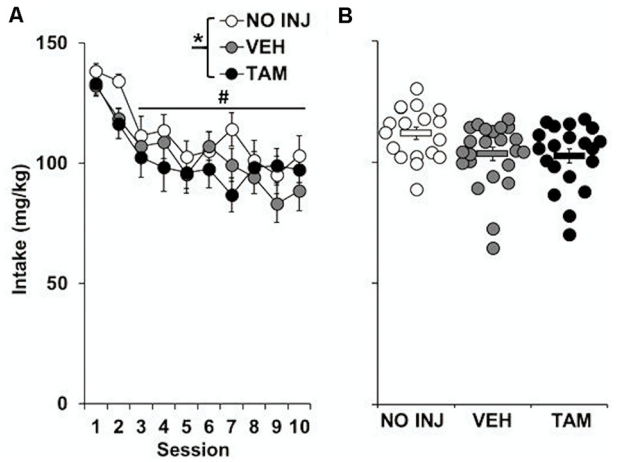

FIGURE 1 | Effect of tamoxifen on ExA cocaine self-administration. **(A)** Mean (±SEM) cocaine intake (mg/kg) for the no-injection (NO INJ; $n = 18$), vehicle (VEH; $n = 24$) and tamoxifen (TAM; $n = 20$) groups for each of the 10 ExA cocaine self-administration sessions. *Significant group effect ($P = 0.048$); #Significant effect of session (1–2 vs. 3–10, $P < 0.0001$) **(B)** Scatterplot of each individual rat's mean cocaine intake across the 10 days of ExA cocaine self-administration. Solid bars represent the average values within each group (±SEM).

SPSS (V26). Alpha was set at 0.05. Data are presented as the mean ± SEM.

RESULTS

Effect of Tamoxifen on ExA Cocaine Self-administration

Although a significant effect of group was observed for cocaine intake ($F_{(2,59)} = 3.190$, $P = 0.048$; **Figure 1**), this effect appears to be due to a non-significant trend for higher intake in the NO INJ group as compared to VEH ($P = 0.07$); no differences were observed between the VEH and TAM groups. All groups self-administered cocaine in a similar pattern (group-by-session, $P = 0.801$), with the highest levels of intake occurring during the first two sessions (session, $F_{(9,531)} = 11.608$, $P < 0.0001$; session 1–2 vs. 3–10, $t = 12.189$, $df = 61$, $P < 0.0001$).

Effect of Tamoxifen on the Development of an Enhanced Motivation for Cocaine

As predicted, following ExA self-administration and 14 days of abstinence, PR responding for cocaine increased from baseline for the VEH and NO INJ groups, but not for the TAM group (**Figure 2A**), with results revealing significant group-by-phase ($F_{(2,180)} = 22.406$, $P < 0.0001$) and phase effects ($F_{(1,180)} = 35.253$, $P < 0.0001$), a trend for a session effect ($F_{(2,180)} = 2.963$, $P = 0.054$), but a non-significant group effect ($P = 0.452$). Subsequent analysis within baseline (**Figure 2A**, Pre) revealed a trend for a session effect ($F_{(2,72)} = 3.026$, $P = 0.055$), but non-significant group ($P = 0.785$) and group-by-session effects ($P = 0.176$). In contrast, analysis within retest (**Figure 2A**, Post) revealed a significant group effect ($F_{(2,36)} = 4.268$, $P = 0.022$), with *post hoc* comparisons revealing a trend for higher PR responding in the VEH vs. the TAM group ($P = 0.054$), and no difference between the VEH and NO INJ groups. Subsequent analysis within each group showed that the number of infusions obtained significantly increased from baseline to retest for the NO INJ and VEH groups ($t = 7.156$, $df = 9$, $P < 0.001$; $t = 2.773$, $df = 15$, $P = 0.042$, respectively), but not for the TAM group ($P = 0.286$).

Similar differences in motivation for cocaine were observed in the analysis of percent change from baseline to retest (**Figure 2B**), with results revealing a significant group effect ($F_{(2,36)} = 10.088$, $P < 0.0001$), but non-significant effects of session ($P = 0.508$) and group-by-session ($P = 0.784$). *Post hoc* comparisons to VEH revealed a significant difference for both the TAM ($P = 0.019$) and NO INJ groups ($P = 0.044$). Analysis within each of the groups revealed that motivation for cocaine increased by 15% or more in all 10 NO INJ rats, 7 of the 16 VEH rats, and 2 of the 13 TAM rats (**Figure 2C**), with the average percent change found to be significantly increased from baseline (0) for the NO INJ ($t = 4.496$, $df = 9$, $P = 0.003$) and VEH groups ($t = 2.857$, $df = 15$, $P = 0.036$), but not for the TAM group ($P = 0.405$). Together, these findings confirm the development of an addiction-like phenotype in the NO INJ and VEH groups, and show that it is blocked by tamoxifen treatment, and surprisingly, attenuated by vehicle (oil) injections.

Effect of Tamoxifen on Relapse Vulnerability

Analysis of responding on the formerly-active lever over the nine extinction sessions revealed significant effects of group ($F_{(2,20)} = 7.812$, $P = 0.003$; **Figure 3A**), session ($F_{(8,160)} = 25.759$, $P < 0.0001$; session 1 vs. 2–9, $t = 5.974$, $df = 22$, $P < 0.0001$), and group-by-session ($F_{(16,160)} = 1.743$, $P = 0.044$), with *post hoc* comparison to VEH revealing significantly higher responding in the TAM group ($P = 0.004$). Similar effects were also observed in the analysis of the first six extinction sessions, which all rats completed (group, $F_{(2,20)} = 4.323$, $P = 0.028$; session, $F_{(5,100)} = 28.428$, $P < 0.0001$; group-by-session, $F_{(10,100)} = 1.930$, $P = 0.05$). Analysis within each session revealed non-significant effects of group within each of the first four sessions; however, a significant group effect was observed for each of the subsequent sessions (session 5, $F_{(2,20)} = 5.410$, $P = 0.026$; session 6, $F_{(2,20)} = 5.470$, $P = 0.026$; session 7, $F_{(2,20)} = 3.570$, $P = 0.047$; session 8, $F_{(2,20)} = 6.935$, $P = 0.005$; session 9, $F_{(2,20)} = 5.147$, $P = 0.016$). *Post hoc* comparisons to VEH within each session revealed significantly higher responding in the TAM group (session 5, $P = 0.017$; session 6, $P = 0.029$; session 7, $P = 0.035$; session 8, $P = 0.007$; session 9, $P = 0.02$). A significant group difference was also found for inactive-lever responses during extinction (group, $F_{(2,20)} = 3.521$, $P = 0.049$), with *post hoc* comparisons to VEH revealing decreased responding in the NO INJ group ($P = 0.028$; data not shown). The number of sessions required to extinguish responding also differed between groups ($P = 0.003$; **Figure 3B**), as seven out of eight rats in both the NO INJ and VEH groups extinguished responding within six sessions, while all TAM rats, except one, required more than six sessions. Comparison of responses on the formerly-active lever during the last extinction session vs. the reinstatement session revealed a significant effect of phase ($F_{(1,40)} = 19.603$,

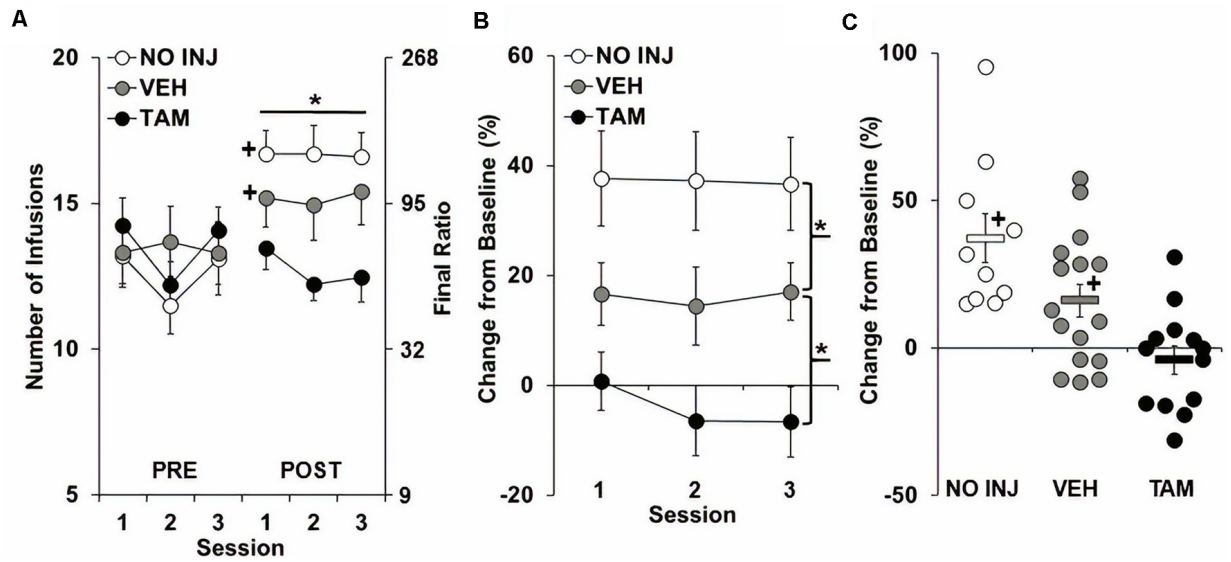

FIGURE 2 | Tamoxifen blocked the development of an enhanced motivation for cocaine. **(A)** Mean (±SEM) number of cocaine infusions obtained under the progressive-ratio (PR) schedule during each of the three sessions at baseline (PRE) and retest following ExA self-administration and abstinence (POST) for the no-injection (NO INJ; $n = 10$), vehicle (VEH; $n = 16$) and tamoxifen (TAM; $n = 13$) groups. *Significant group effect ($P = 0.022$); $^{+}$Significant increase from baseline within the VEH ($P = 0.042$) and NO INJ groups ($P < 0.001$). **(B)** Percent change (±SEM) in the number of infusions obtained at retest following ExA self-administration relative to baseline (PRE) averaged across the three sessions. *Significant difference between the NO INJ and VEH groups ($P = 0.044$) and the VEH and TAM groups ($P = 0.019$). **(C)** Scatterplot of each individual rats' data for percent change in the number of infusions from baseline to retest. Solid bars represent the average values within each group (±SEM). $^{+}$Significant increase from zero within each group (NO INJ, $P = 0.003$; VEH, $P = 0.036$).

$P < 0.0001$; **Figure 3C**) but non-significant effects of group and group-by-phase indicating that, while responding was reinstated by the cues formerly associated with cocaine, this occurred similarly between the groups. Thus, while tamoxifen impaired the extinction process, it did not affect initial responding during extinction or responding during reinstatement.

Effect of Tamoxifen on Serum Hormone Levels

Each of the groups had similar serum levels of estradiol (group effect, $P = 0.212$; **Figure 4A**). While a significant group effect was observed for progesterone ($F_{(2,34)} = 5.948$, $P = 0.006$; **Figure 4B**), *post hoc* comparisons to the VEH group were not significant due to a high level of variability in this group (P's > 0.10). The ratio of estradiol to progesterone differed between groups ($F_{(2,34)} = 16.102$, $P < 0.0001$; **Figure 4C**), with a higher ratio observed in the TAM group vs. the VEH group ($P < 0.0001$). Thus, while tamoxifen did not significantly affect either estradiol or progesterone, it increased the ratio of estradiol to progesterone.

Effect of Tamoxifen on Body Weight

Body weights were markedly reduced in the TAM group compared to the NO INJ and VEH groups (**Figure 5**), as analysis across the 10 phases of the study revealed significant effects of group ($F_{(2,59)} = 24.195$, $P < 0.0001$), phase ($F_{(9,531)} = 130.605$, $P < 0.0001$) and group-by-phase ($F_{(18,531)} = 8.604$, $P < 0.0001$) with *post hoc* comparisons to the VEH group revealing significantly lower weight in the TAM group ($P < 0.0001$). Subsequent analyses within each phase revealed significant effects of group (P's < 0.0001) as well as significantly lower weight in the TAM vs. VEH group at every phase except arrival (P's < 0.0001).

DISCUSSION

The purpose of this study was to determine the role of estradiol in the development of an addiction-like phenotype in ovary-intact females treated chronically with tamoxifen. As predicted, tamoxifen prevented the increase in motivation for cocaine following ExA self-administration and protracted abstinence, suggesting that ER signaling is critical for the development of this feature of an addiction-like phenotype. Surprisingly, the increase in motivation for cocaine following abstinence was also attenuated in the vehicle group as compared to no-injection controls, suggesting that oil/injections also affected the development of this feature of an addiction-like phenotype. Contrary to our hypothesis, tamoxifen did not decrease vulnerability to relapse as this group responded at similar levels during initial extinction sessions (1–4) and cue-induced reinstatement testing as compared to controls. Tamoxifen did, however, impair extinction learning as this group continued to respond at high levels during later extinction sessions and took longer to extinguish as compared to controls. Taken together, these findings indicate that estradiol is critical for the extinction of drug-associated cues and the development of motivational features of addiction.

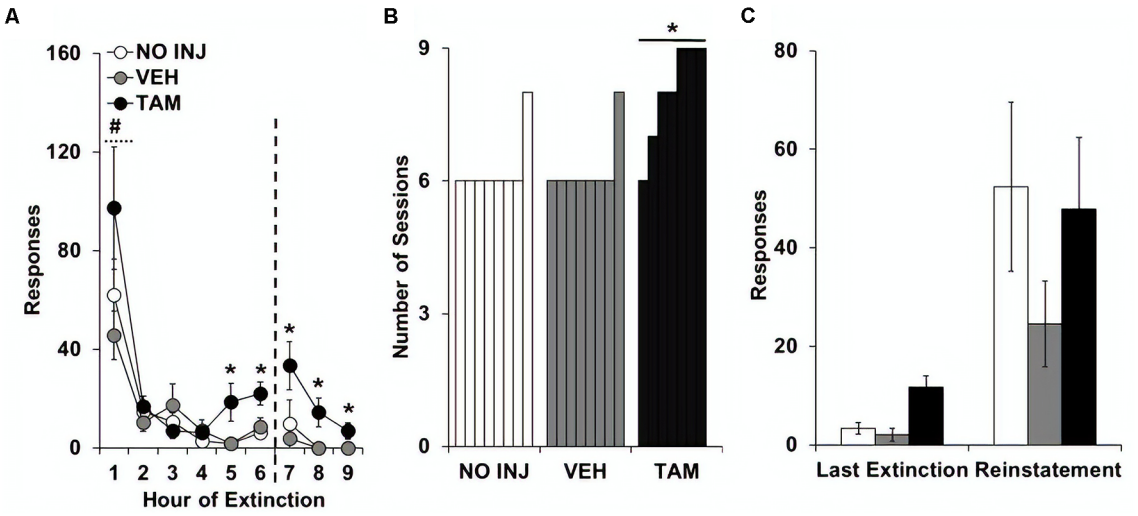

FIGURE 3 | Tamoxifen impaired extinction learning. **(A)** Mean (±SEM) active-lever responses made during the nine extinction sessions for the no-injection (NO INJ; $n = 8$), vehicle (VEH; $n = 8$) and tamoxifen (TAM; $n = 7$) groups. The dotted line indicates that additional extinction sessions were run for rats that did not meet the extinction criterion within the first six sessions (≤15 responses; one of the eight rats in the NO INJ and VEH groups and six of the seven rats in the TAM group). *Significant difference between the VEH and TAM groups (session 5, $P = 0.017$; session 6, $P = 0.029$; session 7, $P = 0.035$; session 8, $P = 0.007$; session 9, $P = 0.02$); #Significant effect of session (1 vs. 2–9, $P < 0.0001$). **(B)** Number of sessions required to extinguish responding (≤15 responses). *Unequal distribution across groups. **(C)** Mean (±SEM) active-lever responses made during the last extinction session and reinstatement test session.

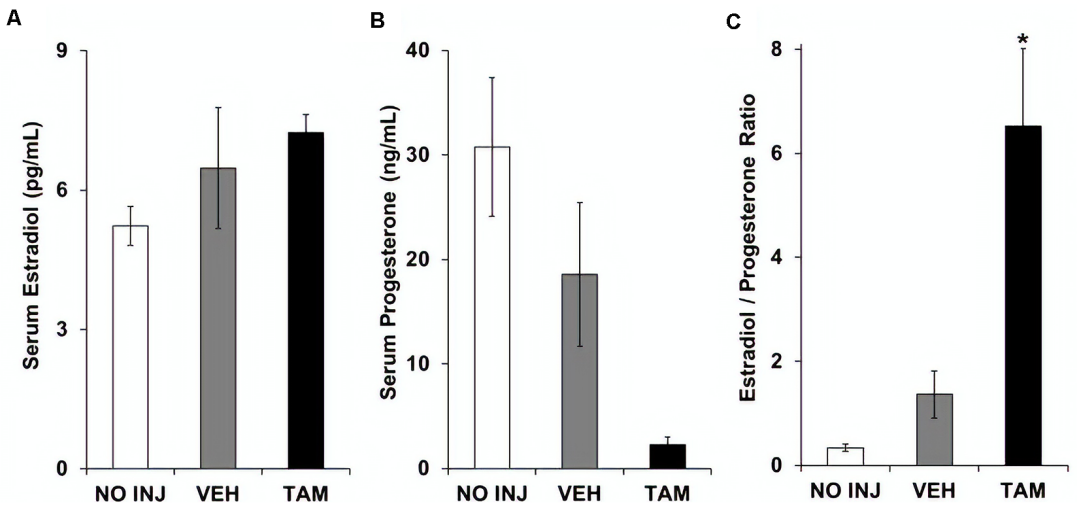

FIGURE 4 | Tamoxifen was associated with an increased ratio of serum estradiol to serum progesterone. **(A)** Mean (±SEM) serum estradiol concentration in the no-injection (NO INJ, $n = 14$), vehicle (VEH, $n = 12$), and tamoxifen (TAM, $n = 11$) groups. **(B)** Mean (±SEM) serum progesterone concentration. Despite a significant overall effect of group ($P = 0.006$), Dunnett-corrected pair-wise comparisons to the VEH group were not significant due to a high level of variability in this group. **(C)** Mean (±SEM) ratio of estradiol to progesterone. *Significant difference between the VEH and TAM groups ($P < 0.0001$).

As predicted, motivation for cocaine was increased following ExA self-administration and protracted abstinence in the NO INJ and VEH groups (37.2% and 16%, respectively), but not in the TAM group (−4.1%). These findings are consistent with previous work showing that OVX prevents the development of an enhanced motivation for cocaine following ExA self-administration and protracted abstinence, as well as the development of a preference for cocaine over food, while estradiol replacement restores these phenotypes (Kerstetter et al., 2012; Ramôa et al., 2013, 2014). That similar findings were observed here in ovary-intact females as compared to previous work in OVX females indicates that the effects of estradiol are reliable and robust. Taken together, these findings strongly support the hypothesis that

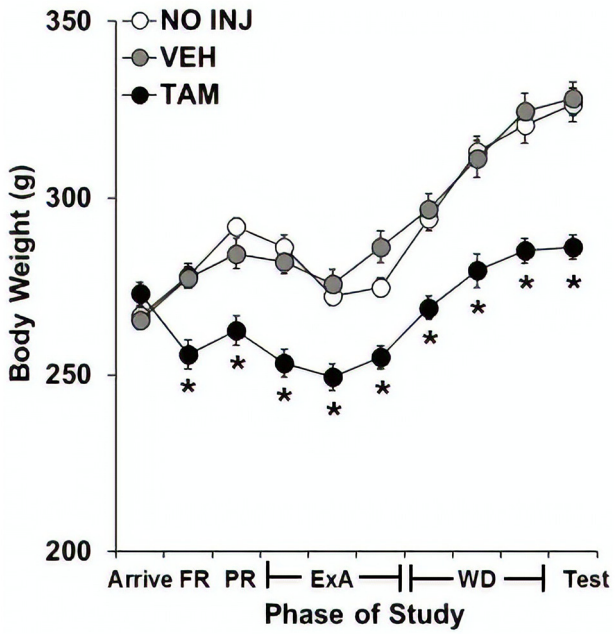

FIGURE 5 | Tamoxifen decreased body weight. Mean (±SEM) body weight across 10 phases of study for the no-injection (NO INJ, $n = 18$), vehicle (VEH, $n = 24$), and tamoxifen (TAM, $n = 20$) groups. *Significant difference between the VEH and TAM groups (P's < 0.0001). FR, fixed ratio; PR, progressive-ratio; ExA, extended-access; WD, withdrawal/abstinence.

estradiol is critical for the development of motivational features of an addiction-like phenotype, and likely underlies the accelerated time-course for the development of addiction in women and an addiction-like phenotype in female laboratory animals (Lynch and Carroll, 2000; Lynch and Taylor, 2004; Center for Substance Abuse Treatment, 2009; Greenfield et al., 2010; Becker and Koob, 2016; Kawa and Robinson, 2019; Nicolas et al., 2019).

While the mechanisms underlying these effects are not yet clear, they likely involve estradiol-dopamine interactions in the reward pathway. Numerous studies have shown that estradiol enhances drug-induced increases in dopaminergic signaling in the ventral tegmental area and striatum (Becker, 1990a,b; Becker and Rudick, 1999; Russo et al., 2003; Song et al., 2019). Results also show that antagonizing ER signaling, either with tamoxifen, ICI 182, 780, or ER knockdown, offsets estradiol-induced increases in striatal dopamine release and decreases the rewarding effects of drugs of abuse (Walf et al., 2007; Satta et al., 2018; Song et al., 2019). There is also evidence indicating that the development of an enhanced motivation for cocaine in females may depend on estradiol's ability to potentiate dopaminergic signaling during initial exposure. Specifically, Calipari et al. (2017) showed that females conditioned during proestrus/estrus, when levels of estradiol are relatively high, had a heightened behavioral and neurochemical response to cocaine as compared to females conditioned during diestrus. In addition, Johnson et al. (2019) further demonstrated that only cues that had initially acquired their value during estrus led to a subsequent increase in motivation for cocaine when compared to males or females initially trained during diestrus. Thus, it is possible that ER-induced amplification of dopaminergic signaling in the ventral tegmental area and NAc, possibly during initial drug exposure, underlies the accelerated time-course observed in females with estradiol. It is also possible that the effects of estradiol/ER signaling are mediated *via* other signaling pathways. For example, we previously showed that the development of an addiction-like phenotype is accompanied by a shift in the mechanism motivating cocaine self-administration, from NAc dopamine to glutamate (Doyle et al., 2014; Ramôa et al., 2014). We further showed that OVX prevented not only the behavioral phenotype, but also the diminished role for dopaminergic signaling in the NAc (Ramôa et al., 2014). Thus, an alternative, non-mutually exclusive possibility is that estradiol is necessary for the shift from NAc dopamine to glutamate. Future research is necessary to investigate these possibilities. Additionally, since tamoxifen was administered throughout the study, future research is needed to determine which time-points during the development of an addiction-like phenotype that estradiol is critical (i.e., during initial exposure, ExA self-administration, or abstinence). Such studies are also necessary to address the possibility that tamoxifen prevents the expression rather than the development of an addiction-like phenotype.

Interestingly, unlike OVX (Lynch and Taylor, 2005; Ramôa et al., 2013, 2014; Martinez et al., 2016), tamoxifen treatment did not significantly decrease cocaine intake during ExA self-administration, yet both OVX and tamoxifen prevent the subsequent increase in motivation for cocaine (Ramôa et al., 2013, 2014). These discrepant results could indicate a less robust effect of estradiol on intake vs. motivational features of an addiction-like phenotype; however, future research will be necessary to resolve this inconsistency given that the effects reported previously with OVX appear to be robust and reliable for both intake and motivational features of an addiction-like phenotype (Lynch and Taylor, 2005; Ramôa et al., 2013, 2014; Martinez et al., 2016). The effects of tamoxifen treatment in intact females are also likely very different than the effects of OVX and estradiol replacement. The fact that intake did not differ in the current study is nonetheless a strength of the tamoxifen ovary-intact model, considering that reduced intake was a confounding factor in previous studies investigating the role of estradiol in OVX rats.

Contrary to our hypothesis, tamoxifen did not decrease relapse vulnerability as responding during both the initial extinction sessions and reinstatement testing were similar between the groups. These findings are consistent with results in both women and female rats showing that levels of progesterone, but not estradiol, are predictive of cue-induced craving/seeking (Feltenstein and See, 2007; Sinha et al., 2007). However, they are in contrast to results from studies examining drug-primed reinstatement, which show that the reinstatement of drug-seeking is decreased by OVX and restored by estradiol replacement (Larson et al., 2005; Anker et al., 2007; Larson and Carroll, 2007). Thus, while these findings indicate that the role of ER signaling is different for relapse vs. motivational features

of addiction, further research is necessary to determine its role under other relapse testing conditions, particularly in response to drug primes.

We also observed a paradoxical increase in extinction responding as a consequence of tamoxifen treatment. This effect appears to be due to impairment of extinction learning as the tamoxifen group continued to respond at high levels, even after responding had extinguished in controls. This interpretation is consistent with recent results in intact females showing that estradiol, through its learning enhancing functions, can be used to facilitate the extinction of cocaine-seeking following cocaine self-administration (Yousuf et al., 2019). Similar findings have also been observed in OVX female rats where estradiol markedly accelerated the extinction of a cocaine-induced place preference leading to extinguished expression in 8 days vs. over a month in vehicle-treated controls (Twining et al., 2013). Indeed, learning and memory varies across the menstrual/estrous cycle (Frick and Berger-Sweeney, 2001; Frye et al., 2007; Paris and Frye, 2008; Pompili et al., 2010; Luine and Frankfurt, 2013; Frick et al., 2015; Kromrey et al., 2015), is impaired by both tamoxifen treatment and OVX (Chen et al., 2002; Rissman et al., 2002; Heikkinen et al., 2004; Sarkaki et al., 2008; Esmaeili et al., 2009; Su et al., 2012; Twining et al., 2013; Lichtenfels et al., 2017; Djiogue et al., 2018), and can be restored in OVX rats by estradiol replacement (Luine et al., 1998; Gibbs, 2000; Frye and Rhodes, 2002; Rhodes and Frye, 2004; Frye et al., 2005; Gresack and Frick, 2006; Jasnow et al., 2006). These learning enhancing effects of estradiol may serve to both heighten vulnerability to addiction by enhancing drug-associated learning, and paradoxically reduce vulnerability by facilitating the extinction of drug-associated learning.

We also observed modest, but surprising, protective effects of vehicle treatment on the development of an enhanced motivation for cocaine as this group showed less of an increase in motivation for cocaine as compared to the non-treated controls. Additionally, while 100% of non-treated controls showed a 15% or more increase in motivation for cocaine following protracted abstinence, only 44% of the vehicle-treated group displayed this phenotype. We selected sesame oil for the vehicle in this study as it is commonly used to dissolve fat-soluble hormones in not only addiction studies (Perrotti et al., 2000; Roth-Deri et al., 2006; Silverman and Koenig, 2007; Russo et al., 2008; Mello et al., 2011; Van Swearingen et al., 2013; Ghazvini et al., 2016; Rauhut and Curran-Rauhut, 2018), but also in general biomedical studies (Dubal et al., 2001; Babaei et al., 2010; Asarian et al., 2012; McClure et al., 2013; Hiroi et al., 2016; González-Garcia et al., 2018; Khariv et al., 2018; Matsumoto et al., 2018). Given its widespread use, we were reluctant to attribute effects in the vehicle group to sesame oil. However, it is a strong possibility given findings from two recent studies with fish oil. Specifically, these studies showed that chronic treatment with fish oil, which like sesame oil is rich in essential polyunsaturated fatty acids (Sowmya et al., 2009), prevented the reinstatement of an amphetamine or morphine-induced CPP and associated molecular changes (Metz et al., 2019; Milanesi et al., 2019). Sesame oil is also rich in linoleic acid, which has been reported to have antagonistic effects on ER signaling (Durgam and Fernandes, 1997; Kenny et al., 2000; Tanmahasamut et al., 2004; Liu and Sidell, 2005). In fact, one study found that while estradiol dissolved in propylene glycol produced a conditioned place preference in OVX female rats, when dissolved in sesame oil, this same dose of estradiol failed to induce a conditioned place preference (Frye and Rhodes, 2006). The other possibility is that effects are due to stress from chronic subcutaneous injections, an alternative that will be addressed in future studies by measuring corticosterone levels. However, this possibility seems less likely considering that the stress associated with chronic injections should enhance, rather than reduce, vulnerability (Goeders and Guerin, 1994; Piazza and Le Moal, 1998). Further research is needed to address the potential mechanism for effects observed following vehicle treatment.

There are two potential confounds in this study. First, we observed significantly diminished weight gain with tamoxifen treatment. This is a seemingly unavoidable confound that occurs as a consequence of chronic estradiol manipulation. Indeed, OVX also dramatically impacts body weight and is thus plagued with the same confound (Roesch, 2006; Wegorzewska et al., 2008). While direct manipulation of ERs in the brain through site-specific infusion would likely minimize effects on body weight (Wade and Heller, 1993; Wade et al., 1993; Sibonga et al., 1998), such techniques also limit translational value. Second, although we did not observe a significant impact of tamoxifen treatment on serum levels of estradiol or progesterone, likely due to the high variability in the vehicle group, tamoxifen treatment did produce markedly higher ratios of estradiol to progesterone, as would be expected given previous findings (Sibonga et al., 1998; Wilson et al., 2003; Messinis, 2006). However, it is important to note that given the complex nature of the positive and negative feedback mechanisms regulating estradiol, progesterone, FSH, and LH release (Kubota et al., 2016), all hormone manipulation models are confounded by unintended effects on hormone levels and hormone-dependent behaviors (i.e., anxiety and depression; Azizi-Malekabadi et al., 2015).

In summary, tamoxifen prevented the development of an enhanced motivation for cocaine following ExA self-administration and abstinence indicating that ER signaling is critical for the development of motivational features of addiction and likely contributes to the accelerated time-course observed in females for the development of addiction. Contrary to our hypothesis, however, tamoxifen did not decrease vulnerability in response to cocaine-associated cues indicating that the role of ER signaling in relapse may differ from its role in motivating cocaine use. However, future research is necessary to examine its role in other forms of relapse (e.g., drug-primed). Tamoxifen also impaired the extinction of cocaine-seeking indicating that ER signaling may be critical for not only establishing and maintaining drug self-administration but also for facilitating new drug-associated learning during extinction training. Future research is needed to determine the mechanisms that underlie estradiol's differential effects on relapse, extinction learning, and motivational features of addiction.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All animal protocols were reviewed and approved by The University of Virginia Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AB-S and WL designed the study, performed the statistical analysis, and wrote the manuscript. AB-S and TN collected the data. All authors contributed to manuscript revision, read and approved the submitted version.

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Ovarian Hormones Mediate Changes in Adaptive Choice and Motivation in Female Rats

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In female rodents, sexual receptivity is coordinated with cyclic changes in the release of gonadal hormones. Increases in estradiol (E) and progesterone (P) during proestrus and estrus not only induce ovulation but also modulate behaviors that increase the likelihood that the female will find a mate and reproduce. This includes changes in receptive behaviors, such as lordosis, as well as changes in appetitive or proceptive behaviors, including motivation. Interestingly, the direction of these changes in motivation is dependent on the type of reward that is being pursued. While induction of sexual receptivity by E and P increases motivation for access to a male, motivation for a palatable food reward is decreased. These concurrent changes may facilitate adaptive choice across the estrous cycle; females bias their choice for sex when fertilization is most likely to occur, but for food when copulation is unlikely to result in impregnation. In order to test this hypothesis, we developed a novel paradigm to measure the motivated choice between a palatable food reward and access to a male conspecific. Ovariectomized, hormone primed females were trained to operantly respond for both food and sex on a fixed interval (FI) schedule. After training, unprimed and primed females were tested in a chamber that allows them to choose between food and sex while still requiring responding on the FI schedule for reach reward. From this we can not only determine the impact of hormone priming on female choice for food or sex, but also how this is reflected by changes in motivation for each specific reward, as measured by the average number of responses made during each fixed interval. Induction of sexual receptivity by hormone priming biases choice toward sex over food and this change is accompanied by an increase in motivation for sex but a decrease in motivation for food. This work provides evidence in support of a novel framework for understanding how the release of ovarian hormones over the course of the estrous cycle modulates adaptive behavioral choice in females by directly assessing motivation *via* operant responding when multiple rewards are available.

Keywords: sexual behavior, feeding, motivation, ovarian hormones, females

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INTRODUCTION

All behaving organisms are continually faced with alternative and competing demands from which they must direct behavior in order to enhance their adaptive success. Motivation is a key regulator of these goal-directed behaviors and has been proposed to modulate not only decision-making processes but also the vigor with which these behaviors are executed (Niv et al., 2006). In order for behavior to be appropriately selected based on physiological needs, internal signals for hunger, thirst, and reproductive status have evolved the ability to direct motivation for specific stimuli based on the organism's internal state (Zardetto-Smith et al., 1993; Balleine, 1994; Dickinson and Balleine, 1994; Salamone et al., 2003; Robinson and Berridge, 2013; Cone et al., 2014; Aitken et al., 2016).

Discrete components of motivated behaviors have classically been categorized as being either appetitive or consummatory (Craig, 1917; Ball and Balthazart, 2008). Importantly, appetitive and consummatory aspects of a behavior can be dissociated, and often are regulated by discrete neural systems (Everitt, 1990; Baldo and Kelley, 2007). Behaviors involved with the consumption of a reward or the act of copulation, in the case of sexual behavior, are considered consummatory behaviors. On the other hand, appetitive behaviors serve to prepare the animal to engage in consummatory behaviors and are inherently more variable as they depend on the circumstances in which the animal engages in the behavior. Appetitive behaviors include behaviors that the animal engages in to locate and obtain rewards.

One example of a motivated behavior, for which the neural circuitry underlying both appetitive and consummatory components have been extensively investigated, is sexual behavior in male rats. When presented with a sexually receptive female, male rats will approach and investigate the female, after which they will attempt to mount and intromit (Hull and Dominguez, 2007). Following repeated intromissions and ejaculation, the male then enters the refractory period, during which locomotor activity and interest in the female are suppressed (Beach and Jordan, 1956). The neural circuitry underlying the consummatory aspects of male sexual behavior (e.g., mounting, intromissions, and ejaculations) and the ability of male gonadal hormones (e.g., testosterone) to modulate this circuitry has been well studied. For example, lesions to the medial preoptic area (MPOA) result in significant impairments in consummatory behaviors. Furthermore, testosterone replacement to the MPOA of castrated male rats, who do not normally exhibit sexual behavior, can reinstate mounting, intromissions, and ejaculation (Christensen and Clemens, 1974; Everitt and Stacey, 1987).

However, examination into the appetitive aspects of male reproductive behavior have yielded different results. Training animals to exhibit an operant response to receive access to a reward, like pressing a lever or poking their nose in a hole, is a paradigm that has been used extensively in a variety of fields when it is important to directly evaluate the level of motivation an animal. Importantly, lesions to the MPOA that abolish consummatory sexual behavior in males have no effect on operant responding for access to a sexually receptive female, indicating that the MPOA does not play a role in male sexual motivation (Everitt, 1990). Conversely, lesions to the basolateral amygdala abolish motivated responding for access to a female without altering the consummatory aspects of sexual behavior (Everitt, 1990).

Applying these same principles to the study of female sex behavior has proved to be more challenging. One reason for this disparity in our understanding of male vs. female sexual behavior is that there are several important differences in the expression and regulation of male and female reproductive behaviors. Male sexual behavior is dependent on the presence of the gonadal hormone testosterone, but adult male rats with intact testes are continuously capable of engaging in sexual behavior (Hull and Dominguez, 2007). Males also find sexual activity most rewarding when the male has free access to the female so that he regulates the mating encounter (Martínez and Paredes, 2001). Females, however, require the coordinated sequential release of estradiol and progesterone in order to induce sexual receptivity (Beach et al., 1942) and motivation (Cummings and Becker, 2012), limiting the time during which she will engage in sexual behavior to the time around ovulation. In addition, females only find a sexual encounter rewarding when the rate of mounts, intromissions, and ejaculations are regulated by the female, a pattern of sexual activity known as pacing behavior (Adler and McClintock, 1978; Jenkins and Becker, 2003b).

Substantial research has shown that when given the opportunity, females will actively pace a sexual encounter by running away from the male following a mount, intromission, or ejaculation (Erskine, 1989). Pacing behavior reflects the sensitivity of the female rat to the intensity of cervical stimulation received during an encounter with the male and modulates the female's response to that stimulation. Female paced mating also allows for the activation of a neuroendocrine reflex that increases the probability of conception (Erskine et al., 1989). During pacing the female will engage in a complex pattern of behaviors that includes ear wiggling, hops and darts, and other general approach behaviors, that serve not only to attract attention from conspecifics but also to hold the male's attention between intromissions (Adler and McClintock, 1978; Erskine, 1989). Female sexual behaviors are thus crucial for the full display of reproductive behavior, particularly in the context of ethological relevant mating paradigms, and demonstrate that females play an active role in mating (McClintock and Anisko, 1982; McClintock et al., 1982; McClintock, 1984).

The motivational circuitry important for appetitive behaviors in the female may be anatomically dissociable from the neural circuitry that mediates consummatory aspects of sexual behavior, as was demonstrated in the male rat (Everitt, 1990). However, the majority of research on female sexual behavior has used reflexive behaviors such as lordosis quotient, ear wiggling, and the number of hops and darts as indices of female sexual arousal and motivation (Pfaus et al., 1999; Mazzucco et al., 2008). That these behaviors can be elicited by the experimenter rubbing the rump of the female rat when she is hormone primed calls into question their validity as a measure of sexual motivation. This necessitates that more direct measures of motivation be utilized. In support of this idea, SSRI-induced sexual dysfunction,

which primarily affects precopulatory and appetitive sexual behaviors in women, is detectable using operant tasks that measure sexual motivation, but not using classic tests of female proceptive behaviors (Uphouse et al., 2015). This highlights the importance of using behavioral paradigms that specifically measure sexual motivation, without the confound of potentially reflexive components of sexual behavior.

Operant paradigms have been applied to the study of female sexual behavior as early as 1961 (Bermant, 1961; Bermant and Westbrook, 1966). In these studies, females were trained to make an operant response for the presentation of a sexually experienced male rat. The experimenters then measured how the magnitude of stimulation received by the female altered the latency to initiate an operant response and found that the response latencies were positively correlated with the magnitude of stimulation. This is consistent with findings that the latency for females to return to a male when pacing sexual behavior varies with the intensity of stimulation (Erskine, 1989). Operant tasks have also been used to measure female preference for a specific mate (French et al., 1972). Using an FR1 schedule of reinforcement, researchers found that females make more responses during estrus compared to diestrus, and that preferences for one mate over another are only apparent when females are sexually receptive. However, while these early studies provide the first evidence that female rats will work for access to a mate, the use of an FR1 schedule does not allow the response rate to be compared without also comparing the consumption of the primary reward.

By allowing the female to control the rate at which the male was introduced, experimenters were allowing the female to pace the mating encounter, even though the phenomenon of paced mating had not yet been formally described (Adler and McClintock, 1978). Additional research has confirmed and extended these findings. Paced mating is rewarding for receptive female rats: they will develop a conditioned place preference following paced mating and will readily work for access to a mate when they are able to pace the rate of copulation (Paredes and Alonso, 1997; Jenkins and Becker, 2003b; Cummings and Becker, 2012). This demonstrates that sexual behavior is both motivating and rewarding for female rats when it occurs under the right conditions, and thus is likely mediated, at least in part, by the neural circuitry underlying motivation for other rewards.

The major brain system underlying motivated behaviors includes the dopaminergic projections from the substantia nigra (SN) and ventral tegmental area (VTA) to the dorsal striatum (DS) and nucleus accumbens (NAc), respectively (Yoest et al., 2014; DiFeliceantonio and Berridge, 2016). Dopamine (DA) cell bodies in the SN and VTA show altered firing in response to salient environmental events and the cues that predict them, including exposure to a novel environment, delivery of unexpected rewards or cues that predict reward, and even aversive stimuli such as aggressive encounters (Horvitz, 2000). These changes in DA cell firing induce changes in DA signaling and downstream activity that correlates with motivation (Bromberg-Martin et al., 2010). Importantly, DA signaling is responsive to homeostatic changes in the animal's internal state, both through direct effects of peripheral signaling molecules on mesolimbic DA circuitry, as well as through projections from extra-striatal areas involved in maintaining homeostasis (Jerlhag et al., 2006; Cone et al., 2014; Nieh et al., 2016; Woods et al., 2016; Baimel et al., 2017; McHenry et al., 2017). This has been hypothesized to facilitate changes in motivation for specific rewards in accordance with the organism's physiological or adaptive need.

Ovarian hormones may regulate changes in motivated behavior through effects on DA responsivity. Stimulated DA release in both the DS and NAc is enhanced during proestrus and estrus, when levels of circulating hormones are at their highest (Xiao and Becker, 1994; Thompson and Moss, 1997; Calipari et al., 2017). A large body of work has demonstrated that the ovarian hormone estradiol rapidly enhances stimulated DA release in females but not in males (Yoest et al., 2018). But while the majority of research has focused on the effect of estradiol on the DA system, the induction of many motivated behaviors associated with the ovulatory cycle requires the release of both estradiol and progesterone (Tennent et al., 1980). Thus, treatment with estradiol alone potentiates stimulated DA release, and treatment of estradiol-primed animals with progesterone further enhances stimulated striatal DA release, above the effects of estradiol alone (Dluzen and Ramirez, 1984; Becker and Rudick, 1999). Interestingly, the effect of progesterone on DA release is biphasic, 30 min–4 h following progesterone treatment DA release is enhanced, but DA release is attenuated 24 h after progesterone administration (Dluzen and Ramirez, 1984). This time course coincides with the maximal induction of sexual receptivity following hormone priming, as well as an increase in female sexual motivation (Cummings and Becker, 2012). This suggests that modulation of striatal DA release around the time of ovulation is important for specific components of sexual behavior, but the role of hormone-mediated changes in DA release during periods of sexual receptivity has not yet been examined.

Research in male rats has implicated DA in copulatory ability as well as appetitive components of sexual behavior. DA levels in both the NAc and striatum is increased in response to a sexually receptive female and subsequent copulation, and this rise in DA is seen regardless of sexual experience, indicating that it is not a consequence of learning (Wenkstern et al., 1993; Pfaus et al., 1995; Robinson et al., 2001). Additionally, pharmacological inactivation of DA receptors in male rats increases, while administration of the DA agonist amphetamine decreases, operant responding for access to a female latency to mount and intromit in male rats (Everitt, 1990). Importantly, extracellular DA levels in striatum and NAc in the female increase during sexual behavior only when copulation occurs at the female's preferred interval (Mermelstein and Becker, 1995; Pfaus et al., 1995; Becker et al., 2001; Jenkins and Becker, 2003a). The greatest increase in DA release is seen prior to the male's intromission, and is not due to sensory stimulation or non-copulatory social interaction, indicating that DA is involved in anticipation of sexual behavior, rather than the sensorimotor aspects of sexual behavior (Jenkins and Becker, 2003a).

The conditioned place preference induced by mating may also be DA dependent. In female hamsters, administration of

a D2 DA receptor antagonist prevents the formation of a conditioned place preference (Meisel et al., 1996). However, other groups have found that DA antagonists do not block place preferences induced by female paced mating, but instead that the opioid system regulates mating induced conditioned place preference in female rats (Paredes and Martínez, 2001; García Horsman and Paredes, 2004). While this may be due to species-specific regulation of sexual reward, this discrepancy may also be explained by methodological differences. When copulation occurs in the conditioning chamber, the place preference is sensitive to DA receptor manipulations (Meisel et al., 1996). Alternatively, when the female is placed into the conditioning chamber immediately following copulation, the formation of the place preference is dependent on opioid transmission (Paredes and Martínez, 2001; García Horsman and Paredes, 2004). Taken together with findings that DA dynamically increases during female paced mating, this may indicate that DA mediates active, motivated components of female sexual behavior, while the opioid system is involved in post-copulatory components of the rewarding aspects of sexual behavior.

Increases in hormones during proestrus and estrus that induce ovulation and enhance striatal DA release also modulate behaviors that increase the likelihood that the female will find a mate and reproduce (Fessler, 2003). Changes in receptive behaviors (e.g., lordosis), proceptive behaviors (e.g., ear wiggling), and other aspects of appetitive behavior (including motivation) are modulated by ovarian hormones. Importantly, while estradiol and progesterone appear to non-specifically enhance stimulated DA release, the effect of these hormones on motivation is dependent on the type of reward that is being pursued. Increases in estradiol and progesterone lead to enhanced motivation for access to a mate but decrease motivation for food (Cummings and Becker, 2012; Richard et al., 2017).

The adaptive benefit of coordinating sexual behaviors with ovulation is quite clear. Copulatory behaviors are accompanied by necessary danger: risk of predation during mate-seeking, injury due to the copulatory act itself, or infectious disease (Daly, 1978). Therefore, females that are not in estrus are not motivated to find a mate and will actively reject male advances (Hardy, 1972; Cummings and Becker, 2012). However, estrogens, and to a lesser extent, progesterone, have also been implicated in the regulation of feeding behavior and motivation for food. Food intake and body weight are both decreased around the time of ovulation (Blaustein and Wade, 1976). Removal of ovarian hormones through ovariectomy increases body weight by increasing food intake, and cyclic estradiol replacement in ovariectomized females restores normal feeding patterns and body weight (Wade, 1972; Asarian and Geary, 2002). The effects of estradiol on the neural circuitry regulating food intake are diffuse; estradiol has been shown to interact with both orexigenic and anorexigenic peptides in a number of different brain areas (Eckel et al., 2002; Clegg et al., 2006; Brown and Clegg, 2010; Santollo et al., 2011; Mela et al., 2016). While estradiol has been shown to be sufficient for the regulation of consummatory feeding behavior in females, progesterone may also modulate feeding. Progesterone is positively correlated with feeding in women, and others have speculated that progesterone may inhibit the anorexigenic effects of estradiol on feeding (Wade, 1972; Yu et al., 2011; Roney and Simmons, 2017).

These changes in feeding behavior around the time of ovulation are less obviously adaptive than changes in sexual behavior. Copulation and reproduction are energetically costly, and would be expected to require greater food intake, but females show decreased food intake and motivation for food around the time of ovulation (Asarian and Geary, 2006; Richard et al., 2017). In order to explain this paradoxical change in feeding behavior, researchers have speculated that changes in feeding behavior serve to reduce the amount of time and energy that animals dedicate toward obtaining and consuming food, thus increasing the amount of time available for mate-seeking and reproductive activities (Fessler, 2003; Schneider et al., 2013). Although this hypothesis has powerful explanatory potential as an ultimate explanation of animal behavior, it remains untested within experimental settings.

Similarly to sexual behavior, research on hormonal regulation of feeding behavior in female rats has focused on changes in consummatory aspects of food intake (Rivera and Stincic, 2018). Few studies have evaluated the direct effect of estradiol on motivation for food, even though many of the signaling molecules regulated by estradiol have been shown to regulate motivated feeding (Cone et al., 2014; Olarte-Sánchez et al., 2015; Stouffer et al., 2015; van der Plasse et al., 2015; Hayes and Schmidt, 2016). The limited work that has been done has shown that motivation for palatable food reward is reduced during proestrus and estrus, an effect that is mediated by estradiol acting directly on the VTA (Richard et al., 2017). Further, repeated treatment with estradiol potentiates the ability of GLP-1 to attenuate motivation for food *via* ERα (Richard et al., 2016).

Even less work has been done to investigate the role of progesterone in feeding behavior. Progesterone is positively correlated with feeding in women, and others have speculated that progesterone may inhibit the anorexigenic effects of estradiol on feeding (Wade, 1972; Yu et al., 2011; Roney and Simmons, 2017). The effect of progesterone on motivation for food is unknown.

In addition to the paucity of work investigating the effect of ovarian hormones on motivated behaviors, what work that has been done has evaluated motivation when only one reward is available. This context strongly contradicts the natural environment, where organisms are continuously faced with opportunities for alternate rewards. In the natural environment, decisions must be made based on the value of the reward, likelihood of receiving the reward, as well as the animal's internal state (Carr, 1996; Niv et al., 2006; Porter-Stransky et al., 2013; Aitken et al., 2016; Bach and Dayan, 2017). Often, pursuit of one reward precludes the ability to earn other available rewards, requiring the animal to not only decide which reward they should pursue but also which reward they must forgo.

To this end, our lab has developed an operant paradigm that quantitatively measures the female's motivation to obtain access to a sexually active male, while allowing the female to actively pace the sexual encounter (Cummings and Becker, 2012). In this paradigm, females respond on a fixed interval schedule

for access to a sexually experienced male conspecific that is tethered in an adjacent chamber. Importantly, the use of the fixed interval schedule allows us to dissociate the effects of ovarian hormones on consummatory aspects of sexual behavior from effects on motivation by quantifying the number of responses the female makes to gain access to the male within a fixed period of time. We have adapted this paradigm to measure concurrent changes in motivation for both food and access to a mate when multiple rewards are available. Therefore, the current experiment aimed to evaluate whether concurrent changes in motivation for food vs. a mate are able to facilitate motivated choice during periods of sexual receptivity. Ovariectomized (OVX) female rats were trained to respond to both food and a mate simultaneously. During testing, animals were able to choose between the two rewards, after which the number of responses animals made for each reward was used as an indicator of their motivation. Thus, the present study demonstrates that increases in ovarian hormones associated with induction of sexual receptivity directs both choice of and motivation for food and a mate in female rats.

MATERIALS AND METHODS

Animals

Ten female Long Evans rats 50–55 days of age and 15 proven breeder stimulus males (Charles River Breeding Laboratory; Portage, MI, USA) were maintained on a 14:10 L:D cycle (lights of at 13:00 h) and housed in same-sex pairs in large laboratory cages (Allentown NextGen 1800; Allentown, NJ, USA) with ad libitum access to water and phytoestrogen free rat chow (2017 Teklad Global, 14% protein rodent maintenance diet, Harlen rat chow; Harlan Teklad, Madison, WI, USA). All procedures were carried out in accordance with the National institutes of Health Guidelines on laboratory animal use and care, using a protocol approved by the University of Michigan Institutional Animal Care and Use Committee. Experimental animals were OVX as previously described (Cummings et al., 2014). Vaginal lavage samples were collected daily starting 10 days after surgery in order to verify absence of estrous cycle.

Drug Preparation and Hormone Priming

Estradiol benzoate (EB; Sigma Aldrich, MO, USA; 5 μg/0.1 ml) and P (Sigma Aldrich, MO, USA; 500 μg/0.1 ml) were administered subcutaneously in order to induce sexual receptivity in OVX females. Both hormones were emulsified in peanut oil and stored at room temperature for the duration of use. EB was administered at 13:00 h for 2 days, followed by P at 1,000 h on the third day. Animals were considered fully hormone primed and sexually receptive 4–6 h after P administration.

Operant Task

All training and testing took place in custom-built operant pacing chambers (**Figure 1**). Control of the apparatus and video recording and analysis was performed using AnyMaze (Stoelting, Wood Dale, IL, USA). Two compartments within the chamber were separated by a horizontal sliding door. The larger compartment contained a tethered stimulus male (Male Side).

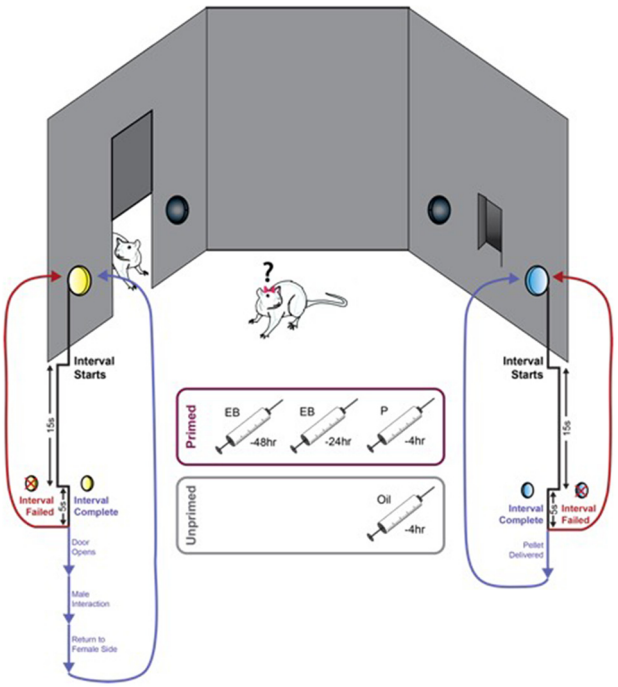

FIGURE 1 | An operant paradigm for concurrent measurement of motivation for food and sex. Female rats were trained to respond for both food and sex on a 15 s fixed-interval (FI15) schedule. Activation of either active port initiated an FI15 for that specific reward (food or sex), and animals could only respond for access to the initially selected reward for the duration of the trial. Completion of the FI15 on the active port located adjacent to the food trough resulted in the delivery of a palatable food pellet (45 mg banana flavor, BioServ, Flemington, NJ, USA) accompanied by presentation of a discrete light cue for 1 s. Completion of the FI15 on the active port located adjacent to the door leading to the other chamber resulted in the door opening and presentation of a different light cue for 1 s. The door remained open until the female crossed into the male side to interact with the male and then closed upon her return to the female side. Failure to complete the FI15 did not result in reward delivery, and the next response made on either port would initiate a new trial.

The smaller of the two compartments (Female Side) was outfitted with four nose poke ports. Two ports were located on the wall adjacent to the sliding door and served as response elements (active and inactive) to open the door. The additional two ports were located on the wall opposite the door spaced around the food tray in which a palatable food pellet (45 mg banana flavor, BioServ, Flemington, NJ, USA) would be delivered. Responding on the active port for each reward resulted in activation of a discrete light cue located directly above the port and initiation of the FI15. During this interval, all responses were counted and resulted in presentation of the cue light but had no other consequence. This second-order schedule, in which responding during the fixed interval is maintained by presentation of the reward-paired light cue, allows for a distinction to be made between the primary reinforcement value of the reward and the incentive motivational value of the cue. This is particularly important when using the number of responses during a fixed interval schedule as the index of motivation for sex as the primary reward (Everitt, 1990). The first response made within

5 s of the conclusion of this 15 s interval resulted in either delivery of a single food pellet or activation of the sliding door to allow access to the second compartment. If the female did not make a response within this 5 s window, the interval was failed, and no rewards were delivered until the animal initiated and completed a new trial. Importantly, although both rewards were concurrently available, initiation of the FI15 for one reward precluded responding to earn the other reward until the initial FI15 was either completed or failed. Any responses made for the other reward during this window were counted but did not result in activation of the cue light and could not initiate a new trial or earn the reward. Thus, animals must first choose between the two available rewards, and then sustain responding for this choice until the end of the 15 s interval in order to be rewarded.

Training Paradigm and Schedule

Animals were initially trained to respond for each reward separately. All training sessions lasted 30 min and animals had no more than one training session per day. Animals started training on a fixed-ratio (FR) 1 schedule, during which every response on the active port resulted in delivery of the respective reward. Once animals made at least 10 active responses during training for sex, and 20 active responses during training for food, the FR requirement was increased to five. The FI schedule was introduced after animals mastered the FR5 (same criterion as FR1). Animals continued training on the FI15 for each reward separately for 1 week, at which point they started training on the concurrent FI15 schedule. At this point, animals were trained twice a week, once when unprimed and once when primed. Training on the concurrent FI15 continued for 3 weeks. Eight out of 10 animals reached stable levels of responding after this point. The two animals that failed to successfully learn the task were excluded from subsequent analyses.

Testing Schedule

During the week of testing, animals were tested once when primed and once when unprimed. The order of testing was counterbalanced across animals and animals were always tested with a novel stimulus male. Four hours prior to testing (10:00 h), animals were given a single subcutaneous injection of either P, if they had been primed with EB, or oil, if they were unprimed. At this time, food hoppers were removed from the home cage and animals were lavaged to verify hormonal status. Although we have previously found that female rats will perform an operant task for food reward even when fed ad libitum, the removal of food from the home cage and the decision to test females at the beginning of the dark phase when food intake is at its highest were both intended to increase the likelihood that females would work for the palatable food reward (Rosenwasser et al., 1981; Perry et al., 2013). At 02:00 h, animals were transported to the testing room. Testing sessions lasted 30 min, after which animals were returned to their home cage.

Video Scoring

Behavioral video was scored offline by an observer blind to the animal treatment group. Videos were analyzed to verify the amount of time animals spent in each chamber during testing, as well as to determine which components of the apparatus animals engaged with. All durations were normalized to the total amount of time in the chamber prior to analysis. Finally, sexual behavior was scored in order to account for the effect of the male's behavior on female sexual motivation.

Statistical Analysis

Group comparisons were performed using GraphPad Prism v7.0a (GraphPad, San Diego, CA, USA). Shapiro–Wilk normality tests were used to test for normal distributions. The effect of hormone priming on discrete variables was analyzed using paired *t*-tests or Wilcoxon matched-pairs signed-rank tests when data violated the assumption of normality. Interactions between hormone priming and other variables, e.g., trial type, were analyzed using two-way repeated-measures ANOVA with Holm–Sidak *post hoc* tests (**Supplementary Table S1**). The effect of ejaculation on motivation for sex in hormone primed animals was analyzed using one-way ANOVA. Changes in responding for food pellets were analyzed within treatment groups by linear regression to determine if the slope of the line was significantly different from zero. Data are presented as mean ± SEM except where stated otherwise.

RESULTS

Induction of Sexual Receptivity Alters Preference for Food vs. Sex

The number of trials that a female initiated in pursuit of each reward was used as an indicator of the primary reinforcement value of each reward. There was a significant main effect of hormone priming on the number of trials that animals initiated ($F_{(1,7)} = 19.27$, $p < 0.01$), that differed between food and mate trials ($F_{(1,7)} = 7.30$, $p < 0.05$). As shown in **Figure 2B**, hormone primed animals initiated fewer food trials than unprimed animals ($p < 0.05$), but a similar number of mate trials overall ($p = 0.96$). However, unprimed animals also initiated a greater number of trials overall ($t_{(7)} = 4.53$, $p < 0.01$; **Figure 2A**). Therefore, in order to account for differences in the total number of trials that animals initiated, we normalized the number of mate vs. pellet trials to the total number of trials initiated during each session. This normalized value reflects the preference for one reward over the other. After normalizing, there was still a significant effect of hormone priming on the number of mate vs. food trials that animals initiated relative to the total number of trials initiated that differed between food and mate trials ($F_{(1,14)} = 22.20$, $p < 0.001$; **Figure 2C**). Hormone primed animals initiated a smaller proportion of food trials ($p < 0.01$) but a greater proportion of mate trials ($p < 0.01$) than unprimed animals, indicating that hormone priming does indeed bias choice toward a sexual partner and away from a palatable food reward.

Hormone priming specifically increased the number of completed food ($p < 0.001$) or mate ($p < 0.001$) trials, without altering the number of failed trials for either reward (food: $p = 0.94$, mate: $p = 0.88$; **Figure 2D**). After further controlling for the total number of trials initiated in pursuit of each reward, we found that hormone priming significantly

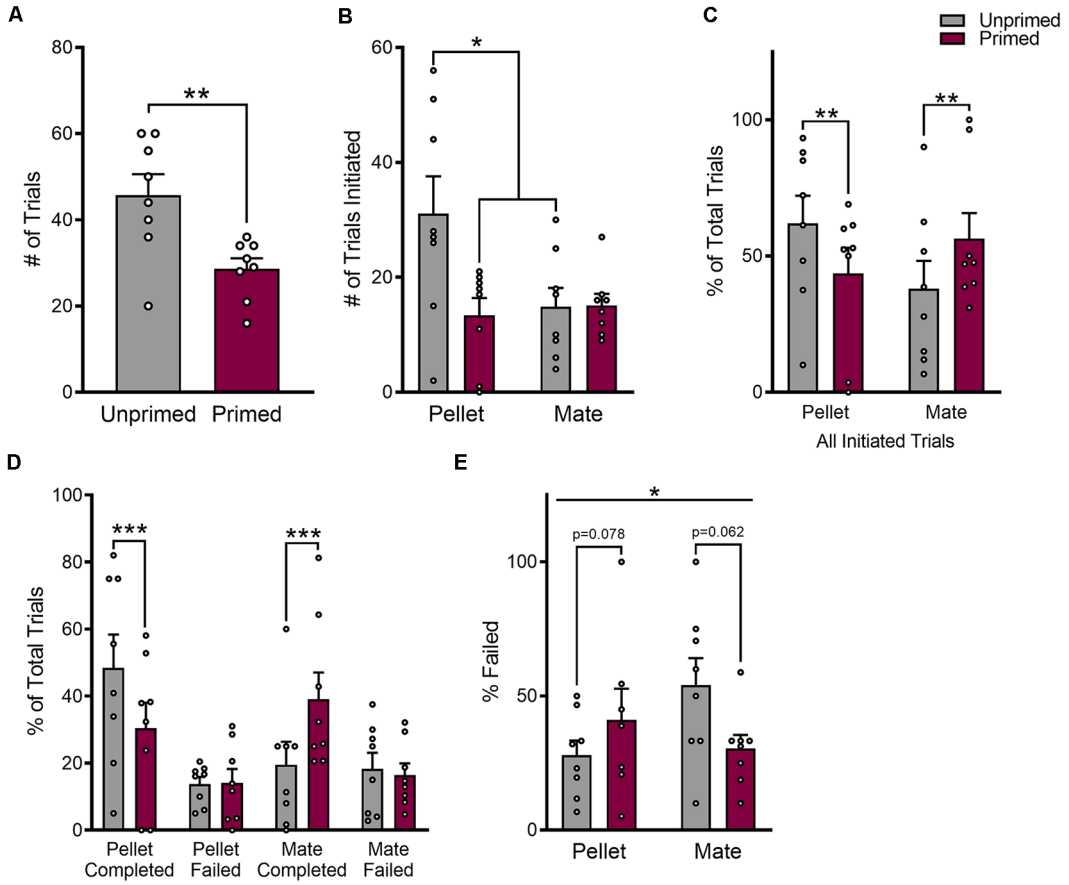

FIGURE 2 | Reproductive status biases choice for food vs. sex. Hormone treatment reduced the total number of trials that animals initiated **(A)**, specifically by reducing the number of pellet trials **(B)**. The proportion of mate or pellet trials that animals initiated was altered following hormone treatment **(C)**. Animals initiated more mate trials when hormone primed than when unprimed, and more pellet trials when unprimed than when hormone primed. Changes in the proportion of pellet or mate trials that animals initiated were driven by increases in completed trials, without altering the total number of trials that animals failed **(D)**. Although the total number of failed trials remained unchanged, changes in the corresponding number of completed trials resulted in a significant interaction between hormone treatment and trial type on the proportion of trials that animals failed **(E)**. Data are shown as mean ± SEM. Data points represent within session means for individual animals, $n = 8$, within subject design. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

altered the proportion of failed trials, but this effect was again dependent on the reward that was being pursued ($F_{(1,27)} = 4.88$, $p < 0.05$; **Figure 2E**).

Pre-treatment with ovarian hormones also altered the amount of time that animals spent engaging in food or mate-seeking. There was a significant effect of hormone priming on the average duration of food and pellet trials ($F_{(1,7)} = 11.43$, $p < 0.05$) where all trials were longer when animals were hormone primed (**Figure 3A**). In addition, mate trials were longer than pellet trials overall ($F_{(1,7)} = 7.11$, $p < 0.05$) but this effect did not differ by hormone treatment ($F_{(1,7)} = 0.76$, $p = 0.41$; **Figure 3A**).

When looking at total duration of time animals spent engaging in either food-seeking or mate-seeking, a measure that accounts for both differences in the number of trials animals initiated as well as the duration of each trial, there was a significant interaction between trial type and hormone treatment ($F_{(1,7)} = 25.16$, $p < 0.01$). As shown in **Figure 3B**, unprimed animals spent a similar amount of time engaged in mate and pellet trials ($p = 0.40$), while in primed animals the total amount of time spent in mate trials was significantly greater than the amount of time spent in pellet trials ($p < 0.001$).

Hormone Priming Alters Motivation for Food vs. Sex

The number of responses that females made during each fixed interval trial were used as a quantitative measure of incentive motivation for each reward. In addition to altering which reward females chose more frequently, hormone priming increased motivation for sex ($p < 0.05$), while simultaneously reducing motivated responding for pellets ($p < 0.05$; **Figure 4A**). This effect of hormone priming on responding was dependent on whether or not the trial was rewarded (Pellet trials: $F_{(2,14)} = 8.10$, $p < 0.01$; Mate trials: $F_{(2,14)} = 19.18$, $p < 0.0001$). Hormone priming only reduced motivated responding for sex when the trial resulted in the delivery of reward ($p < 0.001$), and not during failed trials ($p = 0.91$; **Figure 4C**). The same was true during pellet

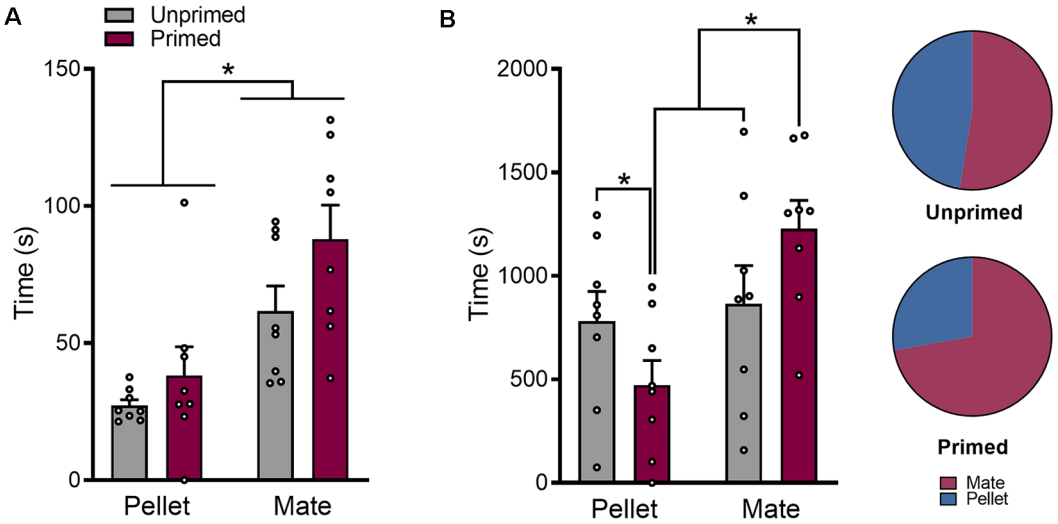

FIGURE 3 | Hormone treatment alters the amount of time animals spend in pursuit of each reward. **(A)** Mate trials are on average longer than pellet trials, and trial length for both rewards is increased in after hormone priming. **(B)** The total amount of time that animals spent engaged in mate trials was increased following hormone priming, which was accompanied by a decrease in the amount of time spent engaged in pellet trials. Data are shown as mean ± SEM. Data points represent within-session means for individual animals, $n = 8$, within-subject design. $^*p < 0.05$.

trials (**Figure 4D**); where primed animals made fewer responses than unprimed animals during completed trials ($p < 0.01$), but not during failed trials ($p = 0.07$).

Although hormone treatment did not alter motivated responding for the initially chosen reward during failed trials, there were important differences in responding for the alternate reward during failed trials (Pellet trials: $F_{(2,14)} = 4.40$, $p < 0.05$; Mate trials: $F_{(2,14)} = 4.67$, $p < 0.05$). Animals made significantly more mate responses during failed pellet trials than completed pellet trials ($p < 0.05$), but only when they were hormone primed (**Figure 4F**). Interestingly, hormone primed animals also made more pellet responses during failed mate responses ($p < 0.05$; **Figure 4E**).

Motivation for Access to a Mate Is Reduced Following Ejaculation

Animals were only sexually receptive following hormone treatment (**Figure 5B**). Hormone treated animals received significantly more mounts ($t_{(7)} = 4.02$, $p < 0.01$), intromissions ($t_{(7)} = 4.12$, $p < 0.01$), and ejaculations ($t_{(7)} = 3.97$, $p < 0.01$). In order to determine the effect of male ejaculation on sexual motivation, we compared the average number of mate responses during the trials leading up to and following ejaculation. As shown in **Figure 5A**, ejaculation significantly reduced motivation for access to a mate ($F_{(5,57)} = 3.487$, $p < 0.01$). Animals decreased responding during the three trials following ejaculation (Trial 1: $p < 0.01$; Trial 2: $p < 0.05$; Trial 3: $p < 0.05$).

Motivation for Food Increases Over the Test Session

In order to determine if animals altered their motivation for food over the course of the session, we plotted the number of responses females made during pellet trials as a function of trial number. We found that overall the number of responses animals made during the session increased over time (slope compared to zero: $F_{(1,54)} = 15.91$, $p < 0.001$), indicating that satiety is not influencing motivation for food during the test session. Interestingly, when animals were grouped by hormone treatment, there was a significant increase in the number of active pellet responses unprimed animals (**Figure 6A**) made during pellet trials (slope compared to zero: $F_{(1,54)} = 17.96$, $p < 0.0001$), but no change in responding over time in hormone-treated animals (slope compared to zero: $F_{(1,21)} = 0.41$, $p = 0.53$; **Figure 6B**). This indicates ovariectomized females increase their motivation for food over the course of the session, but not after hormone priming.

Hormone Priming Biases Where Animals Are Located in the Chamber

Females were willing to work for access to a mate regardless of hormone treatment. However, receptive and non-receptive animals differed in their behavior once they gained access to the male chamber (**Figure 7B**). Receptive females spent a greater proportion of time in the male side ($t_{(7)} = 2.37$, $p < 0.05$). Alternatively, when animals were not sexually receptive, they spent more time in the door to the chamber, where they could see the male but he could not physically interact with them ($W_8 = -34$, $p < 0.05$). Although females spent a comparable amount of time on the female side regardless of hormonal status ($t_{(7)} = 1.357$, $p = 0.22$; **Figure 7A**), there were important differences in how they directed their focus within the operant chamber (**Figure 7C**).

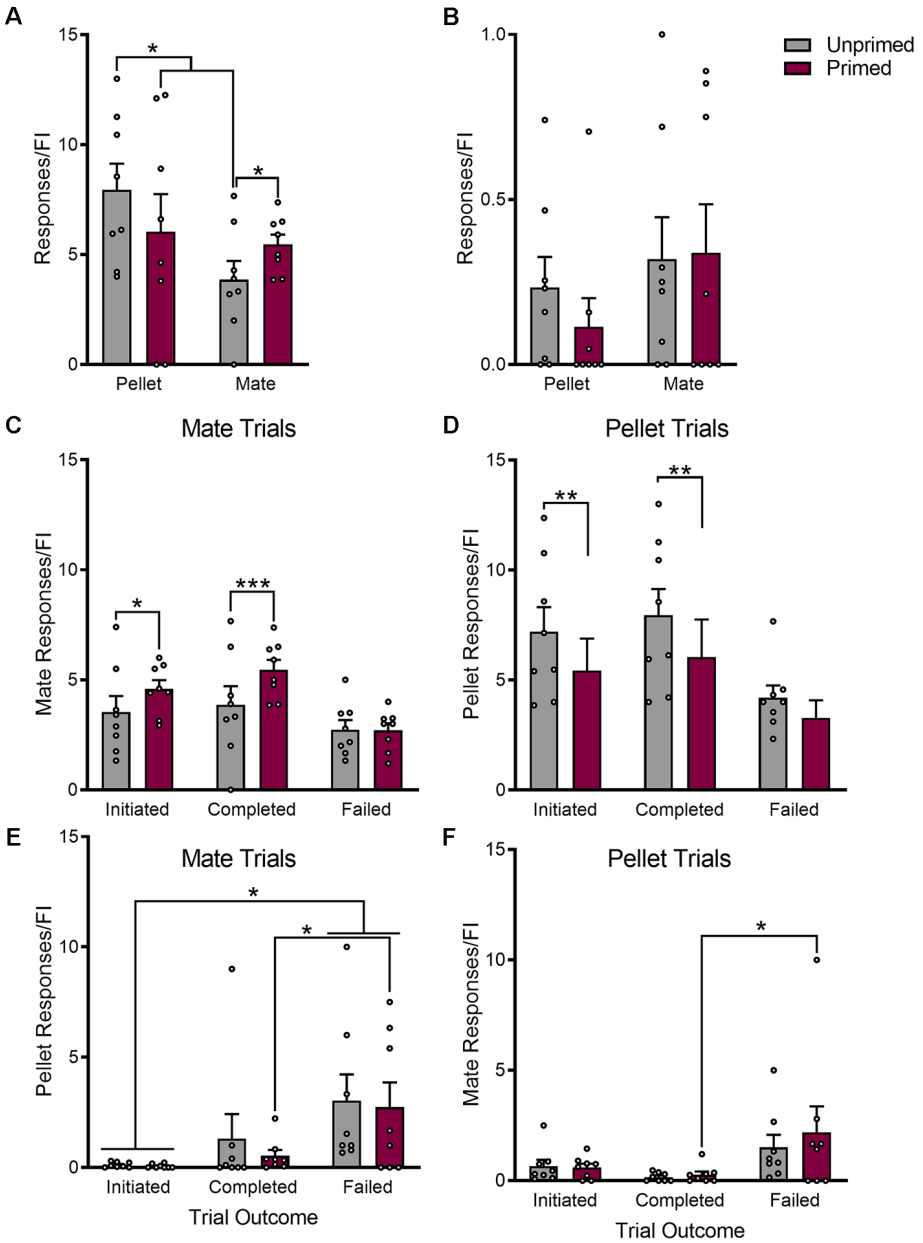

FIGURE 4 | Hormone priming increases motivation for sex while simultaneously decreasing motivation for palatable food reward. The effect of hormone priming on motivated responding is dependent on the reward being pursued **(A)**. Unprimed animals show greater motivation for food than for access to a mate. Induction of sexual receptivity by hormone priming reduces motivation for food, but increases motivation for access to a mate, resulting in similar levels of motivated responding for both rewards. The effect of hormone priming on motivated responding is not mediated by changes in overall locomotor behavior, as there was no effect of hormone priming on the number of responses made on the inactive ports **(B)**. Hormone priming specifically increased responding during completed mate trials, without changing the number of active mate responses during failed trials **(C)**. Similarly, hormone priming only reduced responding for pellet during completed pellet trials, but not during failed pellet trials **(D)**. Although responding for the active reward was not altered during failed trials, hormone primed animals made more responses for the inactive reward during failed trials than completed trials during both mate **(E)** and pellet **(F)** trials. Both primed and unprimed animals made more responses for the alternate reward during failed trials when compared to all trials that were initiated. Data are shown as mean ± SEM. Data points represent within-session means for individual animals, $n = 8$, within-subject design. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Non-primed females spent more time oriented toward and engaging with the pellet nose poke hole, cue, and food tray than primed females ($t_{(7)} = 5.42$, $p = 0.001$; **Figure 7D**, right panel). This was also true when we measured attention toward the food associated cues ($t_{(7)} = 4.86$, $p < 0.01$) or food tray alone ($t_{(7)} = 4.77$, $p < 0.01$; **Figure 7F**). However, there was no effect of hormone treatment on the amount of time that animals spent engaging with the mate nose poke hole,

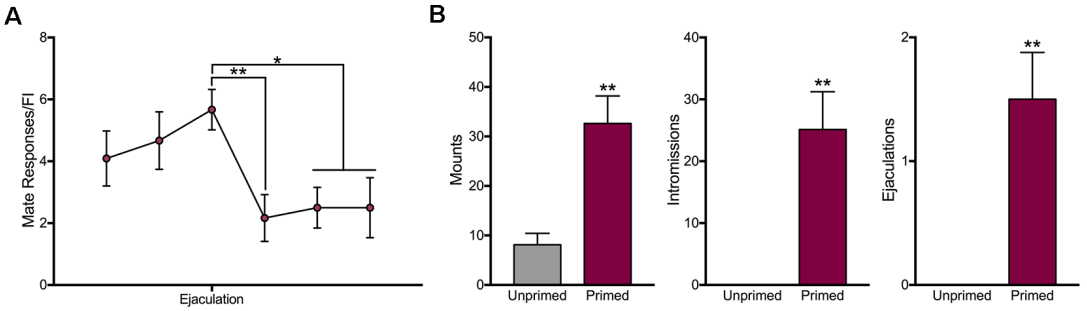

FIGURE 5 | Motivation for access to a mate decreases following ejaculation in sexually receptive female rats. **(A)** Motivation for access to a mate was attenuated during three trials following ejaculation. **(B)** As expected, only sexually receptive animals engaged in sexual behavior. Data are shown as mean ± SEM. For panel **(A)**, data are shown as mean $n = 12$ across eight animals. For panel **(B)**, data shown are mean ± SEM, $n = 8$, within-subject design. $^{*}p < 0.05$, $^{**}p < 0.01$.

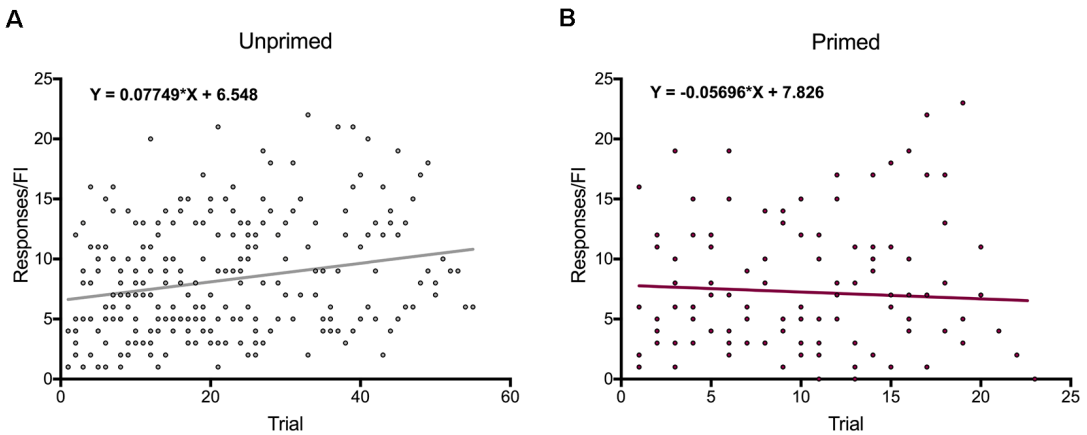

FIGURE 6 | Changes in motivation for food over time. **(A)** Motivation for food increases significantly in over the course of each session in ovariectomized female rats. **(B)** Primed females do not increase their motivation for food from the beginning to the end of each session. Data represent the number of responses during individual trials, $n = 56$ across eight animals for unprimed trials and $n = 23$ across eight animals for primed trials.

cue, or door ($t_{(7)} = 1.00$, $p = 0.35$; **Figure 7D**, left panel, **Figure 7E**). This indicates that hormone primed females, when they are not actively attending to the task, are engaging in some third behavior, presumably waiting for the desired time period between intromissions.

DISCUSSION

Scholars have long speculated that seemingly paradoxical reductions in food intake and body weight during periods where energetic demand is increased and food remains freely available serve to decrease the likelihood that feeding behaviors will disrupt other, more important, activities (Mrosovsky and Sherry, 1980). One such example that has been the subject of much research is the peri-ovulatory decrease in food consumption seen in most female mammals (Tarttelin and Gorski, 1971; Wade, 1972, 1975; Fessler, 2003; Asarian and Geary, 2006). However, while the proximate mechanisms underlying the effects of the ovulatory cycle on food intake are well understood, enquiry into the ultimate or adaptive purpose of these changes remains mostly speculative. Here, we describe experimental evidence that administration of EB + P to induce sexual receptivity in female rats simultaneously biases both choice and motivation for sex over food and propose an adaptive framework for the interpretation of these behavioral changes.

Induction of Sexual Receptivity Biases Choice Between Sex and Food

OVX female rats trained to respond on a concurrent FI operant paradigm for both food and sex show a bias toward choice of food reward over access to a sexually experienced male conspecific. This is indicated by both the number of trials that animals initiate in pursuit of the palatable food reward, as well as a shift in the proportion of pellet vs. mate trials. Hormone priming that induces sexual receptivity reduces the number of pellet trials that animals initiate, therefore shifting the proportion of pellet vs. mate trials toward a preference for pursuit of access to the male, but does not increase the total number of mate trials that animals initiate. This may be due to differences in the average length of each trial, and particularly in the amount of

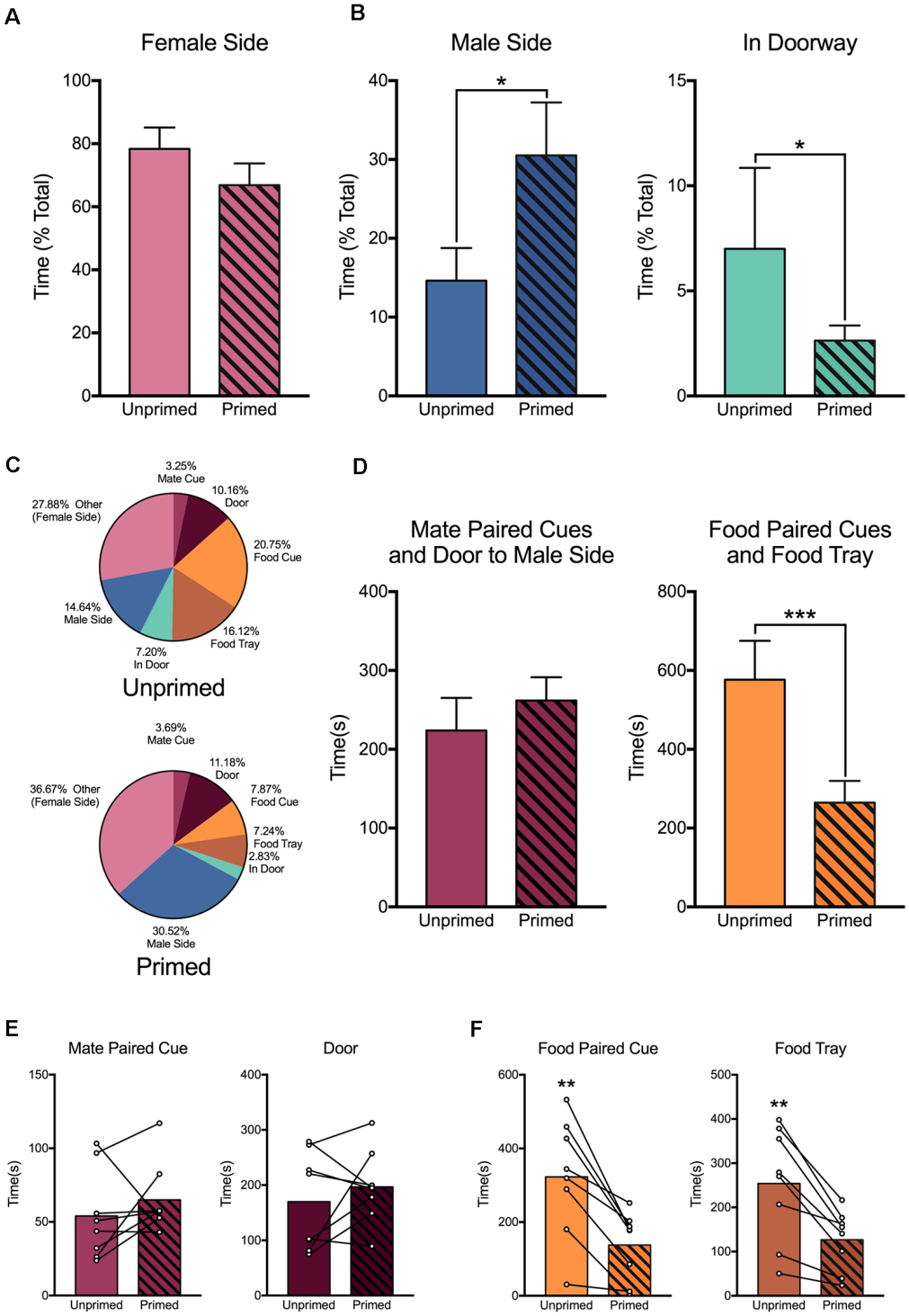

FIGURE 7 | Hormone priming alters the behavior of female rats within the chamber. **(A)** Females spent a similar amount of time in the female (instrumental) compartment regardless of hormone treatment. **(B)** However, when females were given access to the male chamber, hormone primed animals spent more time with the male, while unprimed animals spent more time in the doorway, out of reach of the male. **(C)** Hormone priming altered the distribution of time that animals spent engaging in various aspects of the task. **(D)** Hormone primed animals spent a similar amount of time engaging with the mate paired cue, active mate port, and door, but reduced the amount of time they spent engaging with the food paired cue, active food port, and food trough. This was true when considering the amount of time animals engaged with each aspect of the apparatus individually for both mate **(E)** and food **(F)** paired cues. For panels **(A,B,D)**, data are shown as mean ± SEM, $n = 8$, within-subject design. Mean values are used in panel **(C)**. In panels **(E,F)**, bars represent mean values and data points for individual values, connected to indicate within-subject changes. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

time that animals spend with the male after gaining access to the male compartment. Indeed, hormone-treated animals spent more time engaged in mate-seeking and copulation compared to food-seeking, as well as when compared to the amount of time unprimed animals spent engaged in mate-seeking behaviors. Taken together, this suggests that measurement of the raw number of times the female will attempt to gain access to the male is a poor indicator of her sexual motivation and that other measures must also be considered.

Parsing Sexual vs. Social Motivation

Rats are social animals and will work to gain access to a same-sex conspecific even over drug reward (Venniro et al., 2018). Thus, it is not surprising that non-receptive females will still engage with the operant task in pursuit of a social reward. However, when non-receptive females do gain access to the male, their behavior differs in several important ways. When hormone treated, females engage in sexual behavior (**Figure 4B**) and spend more time with the male and less time in the doorway between the two sides of the cage (**Figure 5B**). Unprimed females spend comparatively less time with the male and instead will remain in the doorway where they can see the male but are out his reach and cannot physically interact with him. This further indicates when unprimed females respond for access to the male, they are not doing so in pursuit of sexual reward, but instead for social reward or general novelty seeking. In addition, motivated responding on the fixed interval schedule was sensitive to trial-by-trial changes in sexual motivation. Motivation for access to a mate was attenuated during the male's post-ejaculatory refractory period. This is consistent with previous work showing that the latency for females to respond for access to a male is increased following ejaculation compared to following a mount or intromission (Bermant, 1961). If operant responding for access to the male was driven by social motivation, we would not expect response rates to be sensitive to changes in sexual satiety.

We used a second-order schedule, in which the reward contingent light cue gains incentive motivational properties, in order to maintain responding during the fixed interval leading up to the presentation of the primary reinforcer. It is possible that the light cue was able to drive responding for access to the male independently of changes in motivation for the actual sexual reward. However, previous work has demonstrated that rats will alter their cue-motivated operant responding in response to changes in homeostatic state (Robinson and Berridge, 2013). This suggests that cue-driven responding is not independent of motivation for the primary reward, but instead reflect dynamic changes in motivation.

Induction of Sexual Receptivity Has Reward-Specific Effects on Motivation

The number of responses that a female made on the FI schedule can be used as an indicator of her motivation for each reward without being confounded by changes in the number of rewards received. As such, females were more motivated for a palatable food pellet when unprimed, but more motivated for access to a mate when sexual receptivity was induced. Interestingly, this shift in motivation for sex vs. food appears to equalize motivation for the two rewards during periods of sexual receptivity. When females are not sexually receptive, they show greater motivation for food, as opposed to the access to the male. After hormone priming, motivation for food decreases, while motivation for sex increases, leading to a comparable level of responding for both rewards in hormone-treated animals.

This is somewhat surprising, as one would expect that sexually receptive animals would show greater operant responding for sex compared to food. There are a number of potential explanations for this difference. One explanation is that motivation for food remains high because it is still adaptive for females to be motivated for food, even when sexually receptive. Even sated animals will respond to palatable food reward, a strategy that is clearly beneficial in unpredictable environments where food availability is sporadic. Indeed, estradiol alters food intake by decreasing meal size without altering the meal frequency by enhancing the effects of satiety hormones (Blaustein and Wade, 1976; Eckel et al., 2002; Santollo et al., 2007; Brown and Clegg, 2010; Maske et al., 2017). This makes sense within the adaptive explanation that has been proposed—specifically enhancing satiety mechanisms ensures that females will not overlook opportunities to eat but instead spend less time eating during each bout in order to return to the important business of mate-seeking and reproduction. In support of this, we found that females generally increased their motivation for food over time, but this did not happen when they were hormone primed. This does not indicate satiety specifically, as primed females do not show decreased motivation for food over time, but does suggest that there is an effect of hormone treatment on how motivation for food changes over the course of the session, where normal increases in motivation for palatable food reward are blunted in primed females. Alternatively, although animals in the current experiment were not food deprived at any point, we did mildly restrict access to food by removing their food from the home cage 4 h prior to testing, and 3 h prior to the start of the dark cycle. Ad lib fed female hamsters show a strong bias toward sex when both food and males are freely available, which is reversed following food deprivation (Schneider et al., 2007). It is possible that even the marginal food restriction used in the current experiment prevents any further decrease in motivation for food in hormone-treated female rats.

Effects of Hormone Priming on Task Performance

The effect of hormone treatment on choice between food and sex was specifically driven by an increase in the number of trials that were rewarded, without altering the number of trials that animals failed. This resulted in an overall shift in the proportion failed trials that was specific to which reward was being pursued. Although the number of trials that animals failed was unchanged after hormone treatment, the animal's behavior during failed vs. completed trials did differ based on reproductive status.

Increased motivation for each reward was driven by an increase in responding during completed trials. During failed

trials, animals show no changes in the number of responses for the active reward as a consequence of hormone treatment. However, during both pellet and mate trials, hormone primed, but not unprimed, failed trials were characterized by an increase in the number of responses that animals made for the alternate reward.

Hormone Priming Biases Attention for Reward Paired Cues

In addition to changes in instrumental responding for each reward, the shift in preference for food vs. sex was apparent in what elements of the apparatus animals attended to during the task. There was no effect of hormone priming on the amount of time animals spent interacting with elements of the apparatus associated with access to the mate, including the mate paired light cue and door. However, females spent significantly less time interacting with the food tray and the food paired cue after hormone treatment. Within the female side of the chamber, where all of the response elements are located, animals have limited options for what to direct their attention toward. The decrease in time spent focused on the food associated elements, without a concurrent increase in time spent focused on the mate paired elements, indicates that hormone primed females are instead increasing the amount of time they spend engaging in some third behavior. One possibility is that the animals are waiting for the desired time period between intromissions to elapse before returning the male side. As mentioned previously, females will actively pace the rate of copulation when given the opportunity. The length of the interval between intromissions is dependent on the intensity of stimulation and is necessary for the induction of the progestational reflex required for successful implantation of a fertilized embryo as well as sexual reward (Erskine et al., 1989, 2004; Jenkins and Becker, 2003b). DA release during female sexual behavior rises during the time leading up to, but not during intromissions (Jenkins and Becker, 2003a). This may indicate that this waiting period, rather than being a passive phase in between bouts of sexual behavior, is instead an active behavior that is important for the rewarding aspects of the female sexual experience.

Operant responding on the fixed interval was maintained by the contingent presentation of a reward paired light cue. During reward learning, increases in DA release shifts from the presentation of the primary reinforcer to the predictive cue (Day et al., 2007). In addition to facilitating learning, this shift in DA release has been proposed to mediate the incentive salience of reward paired cues (Berridge, 2007). In the current paradigm, which allows us to distinguish between changes in consummatory behaviors and changes in appetitive reward-seeking, changes in operant responding during each trial can be interpreted as changes in the incentive value of the reward-paired cue. This is supported by findings that response rates are altered during trial by trial changes in motivational state (e.g., following ejaculation). Within this model, the ability of ovarian hormones to increase responding for one reward, while simultaneously reducing responding for another, suggests that induction of sexual receptivity can selectively alter the incentive salience of reward paired cues, thereby directing motivated behaviors in pursuit of specific rewards.

CONCLUSION

Understanding how organisms balance motivations for competing rewards is key to understanding how motivation influences decision making. The majority of research evaluates motivation for a reward when only one reward is available, and while this approach can be helpful in understanding the neural circuitry underlying motivation, it does not help us to understand how these processes are integrated during adaptive decision making. In our paradigm, female rats first choose between two available rewards, then make a variable number of responses to indicate how motivated they are for each reward. This allows us, for the first time, to measure how concurrent changes in feeding and sexual behavior, during periods of sexual receptivity are reflected by changes in their motivation for both rewards simultaneously. These findings demonstrate experimentally that changes in motivation for food after hormone treatment act to enhance motivation for sex. When given the opportunity to choose between sex and food, OVX female rats show a preference for food reward that is reversed following administration of EB and P. This shift in the choice between food and sex is reflected by concurrent changes in their motivation for each reward, as measured by operant responding on a FI schedule. We propose that these findings provide experimental evidence for the ultimate or adaptive purpose of periovulatory changes in feeding behavior. While scholars have speculated that seemingly paradoxical reductions in food intake and body weight during ovulation reflect a shift in the female's behavioral priorities, this hypothesis remained untested. The paradigm described herein allows us to disentangle the effects of ovarian hormones on motivation from their effects on consummatory aspects of feeding and reproductive behavior. The results presented here demonstrate the adaptive value of periovulatory changes in feeding behavior.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Michigan Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

KY collected and analyzed the data. KY, JC, and JB designed the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00250/full#supplementary-material.

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The Sexual Motivation of Male Rats as a Tool in Animal Models of Human Health Disorders

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Normal or dysfunctional sexual behavior seems to be an important indicator of health or disease. Many health disorders in male patients affect sexual activity by directly causing erectile dysfunction, affecting sexual motivation, or both. Clinical evidence indicates that many diseases strongly disrupt sexual motivation and sexual performance in patients with depression, addiction, diabetes mellitus and other metabolic disturbances with obesity and diet-related factors, kidney and liver failure, circadian rhythm disorders, sleep disturbances including obstructive sleep apnea syndrome, developmental and hormonal disorders, brain damages, cardiovascular diseases, and peripheral neuropathies. Preclinical studies of these conditions often require appropriate experimental paradigms, including animal models. Male sexual behavior and motivation have been intensively investigated over the last 80 years in animal rat model. Sexual motivation can be examined using such parameters as: anticipatory behavior and 50-kHz ultrasonic vocalizations reflecting the emotional state of rats, initiation of copulation, efficiency of copulation, or techniques of classical (pavlovian) and instrumental conditioning. In this review article, we analyze the behavioral parameters that describe the sexual motivation and sexual performance of male rats in the context of animal experimental models of human health disorders. Based on analysis of the parameters describing the heterogeneous and complex structure of sexual behavior in laboratory rodents, we propose an approach that is useful for delineating distinct mechanisms affecting sexual motivation and sexual performance in selected disease states and the efficacy of therapy in preclinical investigations.

Keywords: sexual motivation, general arousal, sexual arousal, ultrasonic vocalizations, depression, anxiety, metabolic disorders, male behavior

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INTRODUCTION

Sexual interaction has been one of the most intensively studied appetitive behaviors over the last 80 years. Copulation differs between species, but detailed investigation of the mechanisms regulating the behavior of one species seems to be important from the perspective of comparative physiological research. Furthermore, effective sexual interactions involve activation of a sequence of behavioral patterns that depend on distinct brain structures, neural networks,

and neurotransmitters. The amygdala (A), bed nucleus of stria terminalis (BNST), medial preoptic area (MPOA), and central tegmental field/subparafascicular nucleus of the thalamus constitute the core central structures. They connect with the dopaminergic mesolimbical, mesocortical, and nigrostriatal tracts, lateral and ventromedial hypothalamus, paraventricular nucleus of the hypothalamus, ventral premammilary nucleus, midbrain periaqueductal gray, nucleus paragigantocellularis of the medulla, and autonomic regions of the spinal cord and regulate sexual motivation, arousal, and copulatory performance. Detailed analysis of neural networks and neurotransmitters in the context of sexual behavior is outlined in several recent reviews (Hull and Rodríguez-Manzo, 2017; Hill and Elias, 2018; Seizert, 2018; Le Moëne and Ågmo, 2019).

With this background, analysis of the sexual activity of laboratory rodents provides a powerful experimental tool for studying the inheritable traits, endocrine factors, neurotransmitter systems, and neural networks involved in evolutionarily preserved as well as experience-dependent aspects of behavior.

In this review article, we analyze the behavioral parameters describing the sexual motivation and sexual performance of male rats in the context of health disorders in humans. Based on analysis of parameters describing the heterogeneous and complex structure of sexual behavior in laboratory rodents, we propose an approach that is useful for delineating distinct mechanisms affecting sexual motivation and sexual performance in disease states and the efficacy of therapy in preclinical investigations. Furthermore, we argue that this approach could be applied for more precise determination of specific mechanisms involved in abnormal or disturbed sexual behavior in rats that are translationally related to human health disorders. In particular, translational research in rodent models of sexual behavior has provided important insights into the pathomechanisms and pharmacotherapy of clinical conditions that are described in the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM 5) and in the International Statistical Classification of Diseases and Related Health Problems 10th revision (ICD-10), including premature ejaculation, paraphilias, mood and anxiety disorders as well as neurological and metabolic diseases (McVary et al., 1997; Grønli et al., 2005; Giuliano and Clément, 2006; Hawley et al., 2013; Pfaus et al., 2013; Kang et al., 2014; Olayo-Lortia et al., 2014; Sanna et al., 2014; Faulkner et al., 2015; Babaei-Balderlou and Khazali, 2016; Oosting et al., 2016; Ramírez-Rodríguez et al., 2017; Hernández and Fernández-Guasti, 2018; Novati et al., 2018). In this light, we propose that in various rodent models of human disease states, sexual motivation and performance may be differently affected, which is reflected in distinct changes of specific components of male rat sexual behavior. However, this translational potential of animal models of sexual behavior for investigating human disorders should be exploited cautiously, as not all aspects of sexual behavior and health disorders are identical in rats and humans (Le Moëne and Ågmo, 2019). Here, we present an outline of male rat sexual behavior in the context of rodent models of human diseases, which should be helpful in finding appropriate experimental models for evaluation of pathomechanisms, therapeutic interventions, and alternatives to the current therapies in preclinical studies. Owing to the specificities and differences of male and female sexual motivation and behavior under physiological conditions and in health disorders (Pfaff, 2017; Hill and Elias, 2018), we did not analyze female sexual behavior in the review.

SEXUAL BEHAVIOR AS AN EXPERIMENTAL MODEL

The sexual behavior of male rats consists of the anticipatory stage during which a male searches for a receptive female, followed by an initiation stage during which a male and a female show mutual investigation. At the end of the initiatory stage, rats begin to copulate. Female behaviors, including sex-soliciting behavior, receptivity, and occurrence of the lordosis reflex (measured as % displaying lordosis), influence the initiation stage and copulatory performance, as lordosis allows for intromission. Copulation comprises specific highly stereotypical motor patterns, including mounting, intromission, and ejaculation. Mounting is a pattern when a male lifts his forebody over the female hindquarters, clasping her flanks with his forepaws, and begins a series of rapid shallow movements of the pelvis. When the glans penis detects the vagina, a male can perform rapid erection, with a deeper intravaginal thrust, which is followed by immediate dismounting. This mounting-intromission-dismounting pattern is repeated until ejaculation is achieved. Ejaculation is marked by a long-lasting intromission (about 1–2 s), which a male rat usually achieves after a few to a dozen intromissions. Rats usually copulate for up to eight ejaculations until copulatory satiation (Larsson, 1956; Sachs and Barfield, 1976). During all these stages, rats produce a complex series of ultrasound vocalizations of various frequencies and temporal patterns (Barfield et al., 1979). Furthermore, fully expressed sexual behavior requires both sexual motivation and sexual arousal, which should be treated as two distinct phenomena (Sachs, 2000). Sexual arousal depends on the activation of brain networks within the brainstem that simultaneously control behavioral and autonomic nervous system responses during sexual interaction and is mainly manifested by penile erection, whereas sexual motivation drives and maintains subsequent stages of sexual behavior (Schober et al., 2011; Ågmo, 2011). Differences between sexual motivation and sexual arousal, or more precisely the fact that these processes are not interchangeable or equivalent, can be explained by the analysis of non-copulating male rats or asexual orientation in humans. In healthy non-copulating rats, noncontact erections are present during exposure to the receptive female in spite of the absence of an attempt to copulate, which is especially visible after medial preoptic lesions (Stefanick and Davidson, 1987; Liu et al., 1997; Portillo and Paredes, 2019). Similar dissociation between sexual arousal and sexual motivation can be seen in asexually oriented men. The level of masturbation in healthy asexual men is similar to that in heterosexual counterparts, but with no motivation for either

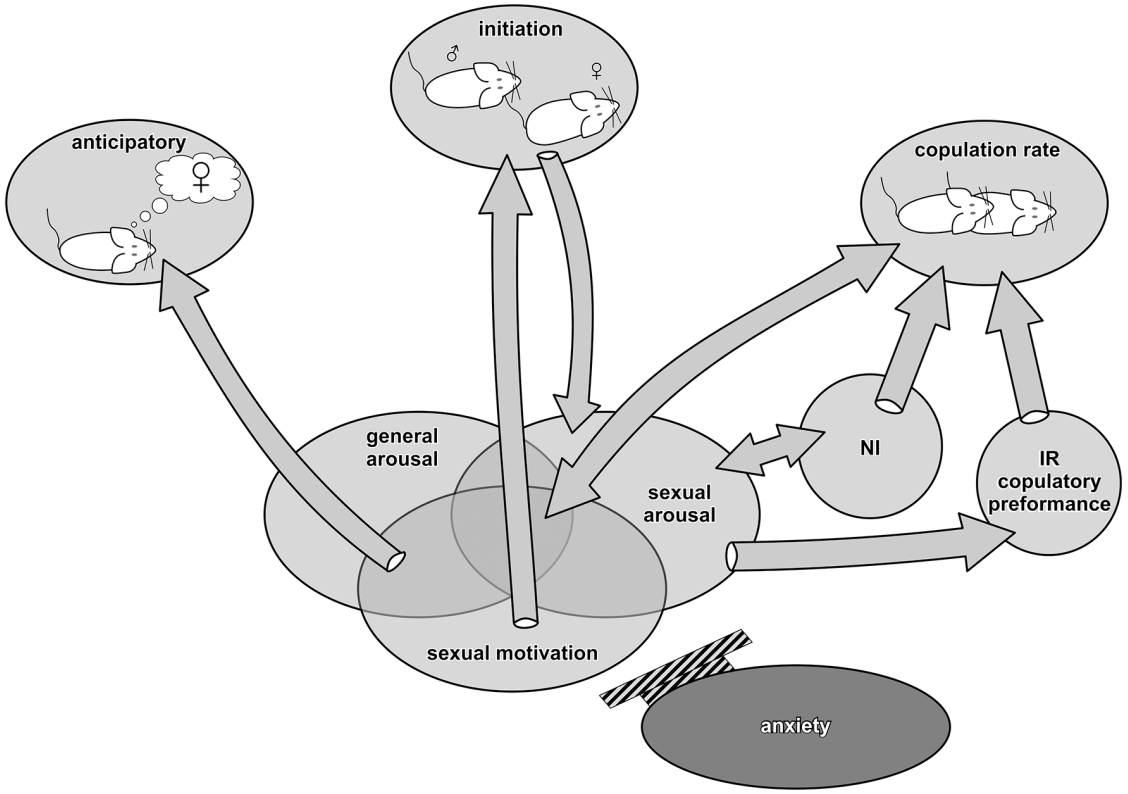

FIGURE 1 | Components—factors of male sexual behavior in relation to sexual motivation, general arousal, and sexual arousal. Anticipatory behavior is related to sexual motivation and general arousal. The initiation phase is powered by sexual motivation and leads to the enhancement of sexual arousal necessary to evoke penile erection. Anxiety inhibits this phase. Copulation rate is related to the reciprocally augmented level of sexual motivation and sexual arousal as well as to general arousal. NI, number of intromissions, is reciprocally related to sexual arousal accumulation and affects the copulatory rate. IR, intromission ratio, is dependent on erectile function/dysfunction and strongly affects copulatory efficiency.

hetero or homosexual interactions (Brotto et al., 2010; Portillo and Paredes, 2019).

The sexual behavior of a male rat contains both inheritable arousal-activated neuronal networks and networks that are experience-dependent and modified by classical and instrumental conditioning (Pfaus et al., 2001; Hull and Rodríguez-Manzo, 2017). Based on statistical factor analysis of sexual behavior in copulatory experienced rats, five independent components of sexual behavior have been distinguished: anticipatory, initiation, rate of copulation, number of intromissions, and intromission ratio (IR; Sachs, 1978; Pfaus et al., 1990). All five components of sexual interactions are summarized in **Figure 1** and discussed below from the perspectives of sexual motivation, general arousal, and sexual arousal.

Anticipatory Behavior

In standard laboratory procedure, a male rat is usually placed in the experimental chamber for 5 min before introduction of a receptive female (Larsson, 1956). During this time, anticipatory behavior is measured by intensiveness of chamber exploration, number of rearings, or changes of levels in special bi-level apparatus (Mendelson and Gorzalka, 1987; Mendelson and Pfaus, 1989). In sexually experienced males, intensiveness of exploration with looking for cues from a female co-occurs with intensive ultrasonic vocalizations in the 50-kHz band, termed precontact vocalizations (PVs; Bialy et al., 2000). Ultrasounds emitted by rodents, in addition to olfactory cues, are a signal for identification of individuals (Holy and Guo, 2005; Asaba et al., 2014). Ultrasonic vocalizations in the 50-kHz band reflect the emotional state of rodents and are related to the activation of the nucleus accumbens (Hamed et al., 2016; Mulvihill and Brudzynski, 2018). Ultrasounds also cause rats to react by approaching a sound source (Wöhr and Schwarting, 2007; Pultorak et al., 2016). The number of PVs during the acquisition of sexual experience is related to the level of sexual experience and conditioning to odor cues and conditioning stimuli (CS) from the experimental chamber, and it depends on the rewarding value of sexual contacts (Bialy et al., 2000).

PVs and other elements of anticipatory behavior can be completely suppressed by repeated dopamine D1 receptor activation in the nucleus accumbens without significant effects on subsequent copulatory behavior (Bialy et al., 2010). Furthermore, increase in the number of PVs during the acquisition of sexual experience is inhibited by NMDA antagonists but is not related to the initiation of copulation

measured by mount latency (ML; see below; Bialy et al., 2000). These observations indicate that anticipatory behavior depends on different neural networks than initiation and copulatory behaviors.

In the sexual context, penile erection is treated as an indicator of an elevated level of sexual arousal (Sachs, 2000). However, our studies indicate that penile erection is not observed during anticipatory behavior (Bialy et al., 2000, 2010). This implies that the anticipatory behavior depends on stimulation of the general arousal system and motivation to look for cues related to sexual activity rather than on the sexual arousal itself.

Initiation Behavior

The time between exposure of a male rat to a receptive female and the first mounting determines the length of the initiation stage and is described as ML. When a female is introduced, both male and female show mutual investigation and mutually emit ultrasonic vocalizations. Odor, visual, auditory, and tactile cues enhance both the level of sexual motivation, leading to copulatory behavior, and the level of sexual arousal, making it sufficient to evoke erection and effective intromission (Hull and Rodríguez-Manzo, 2017). ML depends on sexual motivation enhanced by mutual male-female investigation. In addition to the motivational aspect measured by ML, the latency between the introduction of a female to the first intromission, termed intromission latency (IL), indicates the time required to reach a sufficiently high level of sexual arousal to induce erection (Hull and Rodríguez-Manzo, 2017). ML is prolonged in sexually naïve males and is significantly shorter in sexually experienced rats. Sexual experience and conditioning to cues from a female or experimental chamber significantly reduce both ML and IL (Larsson, 1959; Dewsbury, 1969; Bialy et al., 2000).

The initiation of copulation is strongly related to sexual motivation, and it is inhibited by an enhanced level of anxiety (Pfaus and Wilkins, 1995; Miwa et al., 2011). Activation of cAMP-response element-binding protein (CREB) in the nucleus accumbens reduces anxiety level and ML, but it has no effect on copulatory efficiency as measured by ejaculation latency (EL; Barrot et al., 2005). Similarly, acute administration of D1 receptor agonist into the nucleus accumbens significantly increases the percentage of sexually naïve males that display mounting, but without an increase in PVs or shortening of EL (Bialy et al., 2010). ML is also dramatically prolonged by lesion of the anterior cingulate cortex (Ågmo et al., 1995), suggestive of a critical role for the nucleus accumbens–anterior cingulate cortex/medial prefrontal cortex network in sexual motivation and initiation of a new behavior (Bialy et al., 2010; Sanna et al., 2017). Sexual motivation can also be described by approach behavior in the sexual incentive motivation test arena (Le Moëne and Ågmo, 2019).

Copulatory Efficiency (Copulatory Rate Factor)

Sexual motivation during copulation controls the behavior directed towards the satisfaction of sexual drive by achieving intromissions and ejaculations. Copulatory efficiency describes the ability to satisfy the sexual drive and depends on sexual experience (Larsson, 1959; Dewsbury, 1969; Bialy et al., 2000). Simultaneously with sexual motivation, adequate sexual arousal has to be achieved and accumulated to elicit penile erection and ejaculation, which are mediated by activation of the autonomic nervous system (Giuliano and Rampin, 2004).

The most important measure of copulatory efficiency is EL, which describes the time from first intromission to ejaculation. Additionally, copulatory efficiency can be measured by inter-intromission interval (III), which provides the mean time between intromissions before each ejaculation (Sachs and Barfield, 1976; Sachs, 1978).

Acquisition of sexual experience has been shown to involve different neuronal networks in copulatory efficiency and in the initiation of copulation (Bialy et al., 2000, 2010). The key neural network that regulates copulatory rate involves connections between the amygdala, BNST, central tegmental field, and MPOA (Hull and Rodríguez-Manzo, 2017). MPOA is one of the brain structures in which acquisition of sexual experience leads to an increase in Fos expression, and higher levels of neurotransmitters, receptors, or enzymes and hormones important for regulation of sexual activity (Hull and Rodríguez-Manzo, 2017). Specifically, it was shown that acquisition of sexual experience is associated with an increase in nitric oxide with higher levels of nitric oxide synthase (Dominguez et al., 2006), glutamate and dopamine (Will et al., 2014), D1 receptor signaling (McHenry et al., 2012) and D2 receptor signaling (Nutsch et al., 2016), and oxytocin receptors (Gil et al., 2013), and an increased number of neurons containing androgen receptors (Swaney et al., 2012). Moreover, acquisition of sexual experience and improvement in copulatory efficiency was shown to involve neuronal plasticity as measured by *c-fos* expression in the parieto-occipital cortex (Bialy et al., 1992; Bialy and Kaczmarek, 1996). Additionally, dopamine and noradrenaline levels in the medial prefrontal cortex correlate with sexual experience (Sanna et al., 2017).

Number of Intromissions

The number of intromissions indicates the level of genital stimulation required to induce ejaculation and describes the accumulation of sexual arousal. The cortico-medial part of the amygdala accumulates arousal in rats (de Jonge et al., 1992), as lesions in this region lead to a dramatic increase in number of intromissions (Harris and Sachs, 1975). Similar effects were observed after BNST lesion (Valcourt and Sachs, 1979). Strong reductions in intromission number were observed after serotonin 5HT1A receptor agonist (Snoeren et al., 2014) and D2 agonist and, less effectively, D1 agonist, but not D4 agonists (Cagiano et al., 1989; Beck et al., 2002; Sanna et al., 2015).

Intromission Ratio

Males display intromissions and/or mounts without intromission from the initiation of copulation to ejaculation. The IR describes the proportion of intromissions to the sum of mounts and intromissions. A low value of this parameter is strongly related to erectile dysfunction, which may be due to a low level of NO synthesis (neuronal and epithelial source),

TABLE 1 | Particular components of male sexual behavior, parameters that describe them and their relation to abnormal sexual motivation, arousal and performance due to psychiatric, cardiovascular, neurologic, endocrine and metabolic health disorder, and additional tests to confirm causes of changes in parameters in the rat models.

Parameter	→ Possible Cause	→ Preclinical Models	Additional Tests / Parameters
Anticipatory Behavior – Emotional Value, Reward Value of Previous Contacts			
PVs (No.) **Rearing** (No.) **Bi-Level** (No. of changes) **Exploration** (Distance in meters)	**Lower vs Control**		
	↓ Reward	**Dysregulation of Rewarding System/Drug Withdrawal/ Anhedonic States/Depressive Like-Behavior [1]** **Emotional Memory Dysfunctions [2]** **Sexual Fetishism** (Absence of Fetish) **[3]**	• CPP • Instrumental Reaction • Glucose Preference Test • Forced Swim Test • Open Field (General Activity)
	Higher vs Control		
	↑ Reward	**Drug-Induced Behavior [4]**	• CPP • Open Field (General Activity)
Initiation Behavior – Motivation Value			
ML (Mount Latency in sec.)	**Shorter vs Control**		
	↑ Sexual Motivation	**Neurodegenerative Hypersexualism [5]** **Sexual Fetishism (Presence of Fetish) [3]**	• Open Field (General activity, Time Spent in Center)
	↓ Level of Anxiety	**Low Anxiety Level [6]**	• Open Field (Time Spent in Center) • CRH/ACTH/ Corticosterone Levels
	Longer vs Control		
	↓ Sexual Motivation	**Anhedonic States [7]** **Insulin-Resistance** (mice) **[8]** **HPG Axis Disorders [9]** **Depressive-Like Behavior [10]** **Alcoholism [11]** **Diabetes Related Neurological Impairment [12]**	• Medical Examination • Additional Laboratory Tests for Metabolic Disorders • Glucose Preference Test • Forced Swim Test
	↑ Level of Anxiety	**Anxiety Disorders [13]**	• Open Field (Time Spent in Center) • CRH/ACTH/ Corticosterone Levels
	↓ Preference to Female	**Same-Sex Preference/Sex Identity Formation Disruptions (Gender Dysphoria) [14]**	• Sexual/Copulatory Preference Test
Copulatory Rate – Efficiency (Motivation) to Achieve Ejaculation(s)			
EL (Ejaculation Latency in sec.)	**Shorter vs Control**		
	↑ Sexual Experience	**Acquisition of Sexual Experience [2]** **Sexual Fetishism (Presence of Fetish) [3]**	• Normal or Lower NI
	↓ Ejaculatory Threshold	**Premature Ejaculation [15]**	• Lower NI • Higher EF
	Longer vs Control		
	↓ Sexual Motivation	**Stress Induced Behavior/Depressive Like-Behavior [16]** **Alcoholism [11]** **HPG Axis Disorders [9]** **Diabetes Related Neurological Impairment [12]**	• Longer Time Between Intromissions (III)
	↑ Ejaculatory Threshold	**Delayed ejaculation, SSRI Induced Ejaculatory Dysfunction [17]**	• Normal III • Ex copula mechanically evoked pattern of ejaculation
Number of Intromissions – Accumulation of Sexual Arousal			
NI (No. intromissions)	**Lower vs Control**		
	↓ Ejaculatory Threshold	**Premature Ejaculation [18]** **Sexual Fetishism (Presence of Fetish) [3]**	• Short EL
	Higher vs Control		
	↓ Arousal Accumulation	**Neurodegenerative Diseases [19]**	• Normal or Shorter III • Higher EL
Intromission Ratio – Erectile Function			
IR (No. intromissions / No. mountings + intromissions)	**Lower vs Control**		
	Erectile Dysfunction	**Neuropathy [12]** **NO synthesis inhibition [20]**	• ML • Ex copula test
	Higher vs Control		
	Improved Erection	**PDE5 Inhibition [21]**	• ML • Ex copula test

Since sexual behavior depends on specific strain of rats, laboratory environment, nutrition and housing (Hansen et al., 1978; Bialy et al., 2014; Molenda-Figueira et al., 2017; Sanna et al., 2017), changes in specific parameters describing male sexual behavior should be evaluated against values obtained in control groups in a given experimental paradigm. Abbreviations: ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; EF, ejaculation frequency; HPG, hypothalamic-pituitary-gonadal axis; III, inter-intromission interval; PDE5, phosphodiesterase 5; PVs, precontact vocalizations in the 50-kHz band; SSRI, selective serotonin reuptake inhibitor. References in the table: [1] (Pfaus and Phillips, 1991, Van Furth et al., 1994, Barr et al., 1999); [2] (Bialy et al., 2000); [3] (Pfaus et al., 2013); [4] (Fiorino and Phillips, 1999); [5] (Novati et al., 2018); [6] (Barrot et al., 2005, Miwa et al., 2011); [7] (Pfaus and Phillips, 1991); [8] (Faulkner et al., 2015); [9] (Babaei-Balderlou and Khazali, 2016); [10] (Bialy et al., 2014); [11] (Sadeghzadeh et al., 2018); [12] (McVary et al., 1997); [13] (Hawley et al., 2013, Sanna et al., 2014); [14] (Ramírez-Rodríguez et al., 2017, Hernández and Fernández-Guasti, 2018); [15] (Coolen et al., 1997, Pattij et al., 2005a, Clément et al., 2007, Kang et al., 2013, Olayo-Lortia et al., 2014); [16] (Grønli et al., 2005); [17] (de Jong et al., 2005, Hueletl-Soto et al., 2012); [18] (Coolen et al., 1997, Beck and Bialy, 2000); [19] (Harris and Sachs, 1975, Valcourt and Sachs, 1979, Novati et al., 2018); [20] (Hull et al., 1994, Bialy et al., 1996); [21] (Ferraz et al., 2016).

peripheral neuropathy, or vascular pathology (Hull et al., 1994; Bialy et al., 1996).

Evaluation of copulatory efficiency is critically important in rat models of premature ejaculation. Short ejaculation latencies with a very low number of intromissions (1 or 2 in a copulatory series) were observed in rats treated with 5HT-1A agonist, which can be considered a model of premature ejaculation (Coolen et al., 1997). Another model of premature ejaculation is based on the fact that in sexually experienced rats, there are two extreme endophenotypes. One represents premature ejaculation, with male rats achieving rapid and frequent ejaculations, up to five ejaculations during a short 30-min session of sexual interactions. The other phenotype represents the animal model of retarded ejaculation, with sexually experienced rats achieving only intromissions without any ejaculations during such a session (Pattij et al., 2005a; Waldinger and Olivier, 2005). Such model can be useful for understanding the mechanisms and pharmacological background of premature ejaculation and the role of serotonin receptors, selective serotonin reuptake inhibitors (SSRI), and oxytocin receptors (Giuliano and Clément, 2006; Clément et al., 2007, 2012, 2013; Kang et al., 2013, 2014; Oosting et al., 2016) but only in the case when less genital stimulation is required to achieve ejaculation (fewer intromissions). However, findings from our laboratory indicated that the majority of normal male rats were capable of achieving extravaginal ejaculations when mounting a female with a closed vaginal orifice, provided the male rats received sufficient genital stimulation during at least two intromissions preceding the extravaginal ejaculation. Furthermore, this phenomenon was independent of the number of mountings and was present without any pharmacological intervention (Beck and Bialy, 2000).

In addition to the retarded ejaculation model described above, copulatory efficiency and sexual motivation are strongly affected by metabolic disorders, especially type 2 diabetes mellitus (McVary et al., 1997; Faulkner et al., 2015), depressive-like/anhedonic states (Pfaus and Phillips, 1991; Van Furth et al., 1994; Bialy et al., 2014), or high anxiety levels (Hawley et al., 2013; Sanna et al., 2014).

Postejaculatory Behavior

The mechanisms behind the postejaculatory period are relatively poorly understood and involve numerous spinal and supraspinal structures of the central nervous system (Seizert, 2018; Le Moëne and Ågmo, 2019). In the postejaculatory period, all three processes: general arousal, sexual motivation, and sexual arousal, which control the male's behavior are reflected in different parameters. After ejaculation, a male usually moves to one of the corners of a chamber and starts to emit a vocalization in the 22-kHz band. Most of the time, a male does not move when vocalizing. About 2 min after the first ejaculation (and significantly later after the second one), a male starts to explore the experimental chamber, even before termination of the postejaculatory vocalizations (Sachs and Bialy, 2000). This exploratory behavior reflects increasing general arousal but not sexual motivation or sexual arousal. Operant behavior shows that, at this time, male rats very often perform instrumental reactions—bar-pressing or run in a runway—but that after arriving in the compartment with a female, they evidently escape any socio-sexual contact and show a departure reaction (Beck, 1986; Beck et al., 2002). On the other hand, sexual arousal measured by penile erection occurs later than first exploratory behavior, at least during the first postejaculatory period. Such erection is visible even before the termination of postejaculatory 22-kHz vocalizations, suggesting that sexual arousal increases before a male starts to show interest in a female due to enhanced sexual motivation (Sachs and Bialy, 2000). Sexual motivation and interest in a receptive female appear after the termination of vocalizations. Furthermore, weak painful stimuli that increase sexual motivation in a non-specific way enhance a male's interest in a female and mating, but this is present only after the end of postejaculatory vocalizations (Sachs and Barfield, 1974, 1976). These findings suggest that sexual motivation during postejaculatory ultrasonic vocalizations remains at a very low level. Therefore, after ejaculation, three parameters, latency to the first exploration, latency to the first noncontact erection, and latency to approaching a female, can be treated as measures of general arousal, sexual arousal, and sexual motivation, respectively. The initiation of enhanced sexual motivation later than of enhanced sexual arousal indicates that the postejaculatory interval is not simply the mirror state of the anticipatory and initiation phases of sexual behavior. Ultrasonic postejaculatory vocalizations, on the other hand, reflect, in our opinion, a relaxation state after ejaculation (Bialy et al., 2016). An enhanced level of general arousal and sexual arousal before the termination of postejaculatory vocalizations can be distinguished by spectral analysis of postejaculatory calls. Before the termination of vocalizations, at the time as exploration or noncontact erection take place, some frequency modulations or a shift from about the 45-kHz to the 28–23-kHz band are more often detected, and these differ from the very flat 22-kHz frequency ultrasonic vocalizations at the beginning and middle of the postejaculatory period (Bialy et al., 2019).

Rewarding Value of Sexual Interactions

The rewarding properties of mountings, intromissions, and ejaculations can be evaluated by conditioning procedures. In fact, the process of conditioning in appetitive behavior usually requires several sessions before there are visible effects. Ultrasound vocalizations in the 50-kHz band seem to be the most robust parameter reflecting positive emotional states (Brudzynski, 2007). In this line, high numbers of PVs convey the rewarding value of previous sexual contacts (Bialy et al., 2000). Additionally, conditioning during a second-order procedure (Everitt et al., 1989), conditioned place preference procedure (Camacho et al., 2009; Tenk et al., 2009), and instrumental conditioned reflexes during copulation (Beck, 1971; Beck et al., 2002) are useful in the evaluation of the rewarding value of subsequent events during sexual interactions. In addition, postejaculatory vocalizations—the most of time, extremely flat long-lasting vocalizations in the 22-kHz frequency band—probably reflect abrupt decreases in sexual arousal and a relaxation state following ejaculation. Thus, in this sense, these

22-kHz vocalizations can be used as an additional measure of reduction in sexual arousal and motivation related to the preceding ejaculation (Bialy et al., 2016). Postejaculatory vocalizations usually co-occur with a male's inactivity or grooming. In addition, we found that males vocalize for significantly longer when a female is present in the copulatory chamber after ejaculation (Sachs and Bialy, 2000). Furthermore, such vocalizations are present only in a familiar environment, and cues that increase anxiety level (odor cues from unfamiliar males) significantly reduce such postejaculatory vocalization (Bialy et al., 2016). Moreover, the postejaculatory vocalizations are distinct from shorter low-frequency vocalizations that are produced by a male rat expressing a sexually related frustration state (Bialy et al., 2019).

RAT SEXUAL BEHAVIOR AND HUMAN DISEASES ASSOCIATED WITH SEXUAL DYSFUNCTION

Table 1 summarizes the key components of rat sexual behavior with relevant parameters that describe specific aspects of the behavior (first column). These parameters may be used to quantify disturbances of sexual motivation and performance (second column) that are observed in various rodent models of human diseases (third column). Moreover, additional parameters and tests can be used to further delineate and confirm the underlying causes of abnormal sexual behavior (fourth column), for example, level of anxiety or anhedonia. Furthermore, analysis of these parameters may be useful for evaluation of the efficacy of therapeutic interventions in preclinical investigations.

The translational application of animal models should be exploited cautiously, as not all aspects of sexual behavior and health disorders are identical in rats and humans (Le Moëne and Ågmo, 2019). Additionally, rodent models usually comprise only selected aspects of the complex pathogenesis of neurological, cardiovascular, and metabolic diseases in humans (Zaragoza et al., 2011; Dawson et al., 2018; Lutz, 2018). Even though the sexual behavior of a male rat is not identical to that seen in humans, neurotransmitters, brain structures, and neuronal networks and the motivational, and consummatory aspects of the sexual behavior seem to be fundamentally similar (Larsson and Ahlenius, 1999; Pattij et al., 2005b; Chan et al., 2008; Georgiadis et al., 2012). Since the sexual behavior of a male rat is well defined in terms behavioral, anatomical, and neurochemical characteristics, investigation of sexual behavior in various rodent models of human diseases provides a translational framework for better recognition of the underlying mechanisms of the sexual dysfunction seen in numerous human health disorders and their potential treatment.

AUTHOR CONTRIBUTIONS

MB conceived the study, analyzed the literature, prepared the figure, wrote and revised the manuscript, and secured funding. WB-R analyzed the literature, prepared the table, and wrote the manuscript. JP analyzed the literature, reviewed the manuscript, and secured funding. TZ analyzed the literature, prepared the table, and wrote and revised the manuscript.

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Women at Greater Sexual Risk for STIs/HIV Have a Lower Mesolimbic and Affective Bias Response to Sexual Stimuli

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Young adult women in the United States have high rates of sexually transmitted infections, increasing the risk of human immunodeficiency virus (HIV). The underlying neurobiology of behaviors that increase the probability of contracting sexually-transmitted diseases (STIs) and HIV is just beginning to be explored. The current study assessed the link between sexual risk and the brain and behavioral response to sexual cues in emerging adult women. Our hypothesis was that women with more activity in reward/motivational circuitry would report higher sexual risk behaviors and would evidence higher positive affective bias to visual sexual stimuli. Women (n = 52; age = 18–24 years) who had protected sex 100% of the time (n = 17) vs. those who did not (n = 35), in the past 3 months, were compared on their brain response to 500 ms evocative (sex, aversive, food) vs. neutral cues in a blood-oxygen-level-dependent (BOLD) functional magnetic resonance imaging (fMRI) fast event-related design. Based on existing literature, an *a priori* anatomical "cue-reactive" mask was used to constrain the analyses. Self-reported sexual activity and the affective bias scores to sexual cues were examined as correlates with the brain response to cues. In contrast to our initial hypothesis, the higher sexual risk (Unprotected) group had significantly *less* activation in mesolimbic brain regions and lower (less positive) affective bias scores to sexual cues compared to the lower risk (Protected) group. As predicted, the brain response was positively correlated with sexual bias. Follow-up analyses showed an effect of partner "risk" (e.g., more vs. less knowledge of partner's STIs/HIV status). This evidence suggests that women who have protected sex may view sexual-related stimuli more positively, reflected by a neural response in reward/motivational regions and more positive sexual bias scores. In contrast, young women at increased risk for STIs/HIV may feel more negatively about sexual-related stimuli, evidenced by a lower mesolimbic response and a less positive affective bias to sexual cues. These data may help identify young women who are at greatest risk for acquiring STIs and/or HIV, which carries added importance with the availability of new medications that can prevent HIV.

Keywords: risk-taking, women, fMRI, STIs, HIV, condoms

INTRODUCTION

Rates of sexually-transmitted infections (STIs) have been on the rise since the early 2010s. In 2017, the Center for Disease Control reported a 22% increase in chlamydia infections, a 67% increase in gonorrhea, and a 76% increase in syphilis (Centers for Disease Control and Prevention, 2017). Individuals in late adolescence and emerging adulthood, whose regulatory brain regions are still in development, are particularly at risk, with 50% of STIs occurring in these age groups (Centers for Disease Control and Prevention, 2017). Women aged 20–24 years had the highest rate of reported chlamydia cases compared with any other age group, and rates of gonorrhea among women aged 15–24 years was higher than in men of the same age group (Centers for Disease Control and Prevention, 2017). In addition to adverse health outcomes (such as pelvic inflammatory disease and ectopic pregnancy), women with STIs are also at increased risk of contracting human immunodeficiency virus (HIV; Centers for Disease Control and Prevention, 2018a). Although the overall rate of new HIV infections has decreased in the United States over the past decade, the epidemic persists.

For women, who made up 19% of new HIV diagnoses in 2017, 87% of which were due to heterosexual contact (Centers for Disease Control and Prevention, 2018b), condoms can effectively prevent new infections. However, the use of a condom requires participation from a male partner, and this process of negotiation (Pulerwitz et al., 2002) may be especially challenging during the period of adolescence and emerging adulthood (Teitelman et al., 2011) when the brain is still developing (Sowell et al., 2004; Casey et al., 2008). For example, motivational circuits that encode reward may receive considerably less oversight from still-developing inhibitory brain regions (Ernst et al., 2005; Steinberg, 2005; Eshel et al., 2007; Casey et al., 2008; van Duijvenvoorde et al., 2010). Thus, investigating the motivational circuits that underlie behaviors that increase risk of STIs/HIV may help to identify vulnerable phenotypes and lead to interventions.

Research has begun to reveal neural correlates associated with behavior that increases the risk of STIs, much of which has focused on adolescents and the role of regulatory circuits. These circuits allow an individual to evaluate choices and future consequences associated with a particular behavior (e.g., whether to have sex or not) and enable inhibition of behavior associated with risks (e.g., sex without a condom, Miller, 2000; Bechara and Van Der Linden, 2005; Ghazizadeh et al., 2012). Studies have shown, for example, that activation in the dorsolateral prefrontal cortex and other regulatory regions during inhibition of perseverative responses and cognitive interference is correlated with more sexual risk behaviors (Feldstein Ewing et al., 2015; Barkley-Levenson et al., 2018; Hansen et al., 2018; but see Goldenberg et al., 2013), with researchers suggesting a greater potential compensatory regulatory action to inhibit prepotent responses (Hansen et al., 2018), presumably driven by hyperactive reward and emotional brain regions.

Emerging research is investigating reward-processing motivational circuits as neural correlates of sexual risk behaviors. Stimuli associated with reward act as powerful incentives for individuals to make decisions that lead to rewarding goals and previous literature has suggested that sexual cues presented in a laboratory setting can act as rewards (Gola et al., 2016). Thus, probing motivational and reward circuits with evocative stimuli, such as sexual images, may reveal differences in brain response associated with sexual risk. Prior studies suggest a heightened response in striatal and other mesolimbic regions to sexual stimuli is associated with greater sexual risk (Seok and Sohn, 2015) and compulsive sexual (Voon et al., 2014) behaviors in males. However, to our knowledge, very few previous studies have investigated the brain response to sexual stimuli as it relates to sexual risk behaviors in females. One study in females found that a heightened reward response to sexual images was associated with future sexual desire (Demos et al., 2012), though sexual risk behaviors *per se* were not investigated. Based on previous findings showing a relationship of increased mesolimbic response to greater sexual risk in males, in addition to studies generally suggesting sensitivity to cues is associated with higher risk behavior (Flagel et al., 2009; Morrow et al., 2011), we hypothesized that activation of mesolimbic regions to sexual cues would be associated with higher sexual risk in emerging adult women. Worth noting, though the direction of effects in women may differ from men, they would be important to characterize.

Passive viewing of explicit sexual stimuli can elicit feelings of embarrassment and/or shame, potentially complicating interpretation of the neural response. However, *implicit* measures of affective bias can provide a greater understanding of the brain's response to evocative visual stimuli, without the confounds of embarrassment and/or shame. Previous research has shown that affective bias can aid in understanding more automatic decision-making (e.g., classical conditioning), such as approach or avoidance behaviors triggered with little or no conscious thought (Olson and Fazio, 2001). Affective bias allows one to measure "positive" and "negative" emotional valences paired with specific stimuli; these are likely to map on to approach and avoidance behaviors, respectively (Berridge and Robinson, 1998). In the present study, we hypothesized that the affective bias toward sexual cues and the brain response to these cues would be positively correlated (i.e., a stronger brain response to sexual cues would correspond to a more positive bias towards sexual stimuli).

MATERIALS AND METHODS

Participants

Participants (n = 60) were recruited from a federally-supported Title X (serving low-income individuals) family planning clinic and from a nearby university; both recruitment sites were located in a large urban area in the mid-Atlantic region of the United States. Flyers were posted and handed to participants by study team recruiters in the clinic waiting room and posted in the surrounding university campus. Potential participants expressed interest in the study by calling the phone number on the flyer or talking with recruiters in person. Eligibility screening was performed in a private location in the clinic or over the phone. The eligibility criteria included: women of ages 18–24 years who had vaginal sex (defined as penis in vagina) in the past

3 months, who were able to speak and read English at a 6th-grade level or above, and who were able to independently provide written informed consent. Exclusion criteria beyond standard fMRI contraindications (e.g., claustrophobia; metal in the body) included: pregnancy or plans to become pregnant in the next year or having given birth in the last 3 months, use of a copper IUD for birth control, being HIV-infected, having serious medical abnormalities (e.g., cardiovascular, neurological, endocrine, etc.) or untreated diabetes or hypertension, history of head trauma, history of seizure disorder, or currently under the influence of drugs or alcohol. Participation in any other studies was assessed and if these involved medications that might interfere with the fMRI, participants were excluded. Substance use was assessed by urine screens, recent alcohol use was assessed by breathalyzer, and pregnancy was assessed by a urine test prior to the fMRI session. Eligibility screening was supervised by an individual with a master of social work degree. After the fMRI session, the participant received compensation of $110 for the two-session visit. This study had Institutional Review Board approval and complied with the Declaration of Helsinki.

Data Collection

Subjects participated in two sessions, typically scheduled on consecutive days. In the first session, participants completed informed consent, surveys, and an interview about sexual behaviors in the past 3 months using the Timeline Follow-Back (TLFB) method (Copersino et al., 2010). Information on other sensitive topics (e.g., intimate partner violence) was gathered using Audio Computer-Assisted Self-Interviewing (ACASI) that increases the accuracy of self-reported data (Newman et al., 2002). During the ACASI portion, participants wore headphones and listened as questions were read to them while also viewing the written questionnaire on a computer screen and entered responses on the computer. Participants were asked to provide demographic and health information by completing a paper survey.

Behavioral and Environmental Variables

Questions assessed for age, education (participant and mother's), race, ethnicity, substance use, and sexual behavior history. Validated scales were used to measure impulsivity (Stanford et al., 2009), sensation seeking (Stephenson et al., 2007), risk-taking (Lejuez et al., 2002), anxious attachment (Kershaw et al., 2007), depression (Radloff, 1977), maltreatment (Bernstein et al., 2003), and intimate partner violence (IPV) (Garcia-Moreno et al., 2005).

STI/HIV Sexual Risk Behavior Measure

The primary measure for sexual risk was assessed by condom use during sex in the past 3 months. Participants who had sex with a condom 100% of the time in the past 3 months were considered the "Protected" group, and participants who had sex with a condom, less than 100% of the time in the past 3 months were considered the "Unprotected" group. To further define the Protected and Unprotected groups, a follow-up analysis incorporated the STIs/HIV risk of the participants' partner. Participants who reported (or did not know) their partner had HIV, multiple partners, or an STI were considered "Risky Partners" (RP).

Other Sex-Related Behaviors

In addition to condom use, data were collected on other types of sex-related behaviors. These included: number of lifetime sexual partners, number of sexual partners in the past 3 months, anal sex since the age of 15, drug and alcohol use prior to sex, frequency of vaginal sex, and knowledge of partner's STI and/or HIV status as well as partner's sexual behaviors outside of their relationship (Centers for Disease Control and Prevention, 2018c).

fMRI Data Collection

In the second session, participants completed a blood-oxygen-level-dependent (BOLD) fMRI scan. The imaging center contains a Siemens 3 Tesla (Trio) research-dedicated magnet, an 8-channel head-coil, an LCD projector for stimulus presentation, air-conducting earphones, and a fiber optic response pad. Mirrors, attached to the head coil, are adjusted so that participants can focus attention on projected stimuli and instructions. Prior to the functional scans, a 3 min localizer scan and a T1-weighted high-resolution resting scan (5 min) were acquired. For functional scans: T2*-weighted BOLD images were obtained with a single-shot gradient echo-planar imaging sequence (field of view = 192 mm, matrix 64 × 64, TR = 2 s, TE = 30 ms, flip angle = 80°).

Fifty-four participants (of the 60 enrolled) completed the fMRI scanning session. Six subjects were unable to proceed with the fMRI visit [claustrophobia (n = 4), heart murmur (n = 1), dental retainer (n = 1)]. As in previous studies from our lab (Childress et al., 2008; Wetherill et al., 2014), the session included a "fast" event-related fMRI task with 24 novel 500 ms target cues in four categories [food (n = 24), sexual (n = 24), aversive (n = 24), and neutral (n = 24); **Figure 1**], from which usable data was gathered from 52 patients. More than half of the sexual cues and all of the aversive cues were selected from the top quartile (e.g., "most unpleasant" and "most pleasant," respectively) of the International Affective Picture System (Lang et al., 1999). The remainder of the sexual cues were specifically generated to reflect the diversity of our sample. Target stimuli were interspersed with gray screens with a single crosshair presented at a random duration between 1,000 ms and 2,000 ms, an average of approximately 1,500 ms (**Figure 1**).

Affective Bias

After the fMRI scan, participants completed an off-scanner affective priming task, that determined the hedonic valence of visual sexual and condom cues by measuring the ability of these cues to influence (i.e., prime) the identification of nouns as positive (e.g., joy, paradise) or negative (e.g., murder, vomit). Images with positive valence (e.g., sexual cues) have been shown to facilitate the speed and accuracy for identifying positively-valenced nouns, and, conversely, to slow the reaction time of negatively-valenced nouns. Images with a negative valence (e.g., aversive) have the opposite effect (Olson and Fazio, 2001; Childress et al., 2008). A total of 12 sexual cues were chosen from a subset of those used on the scanner task (see below), and

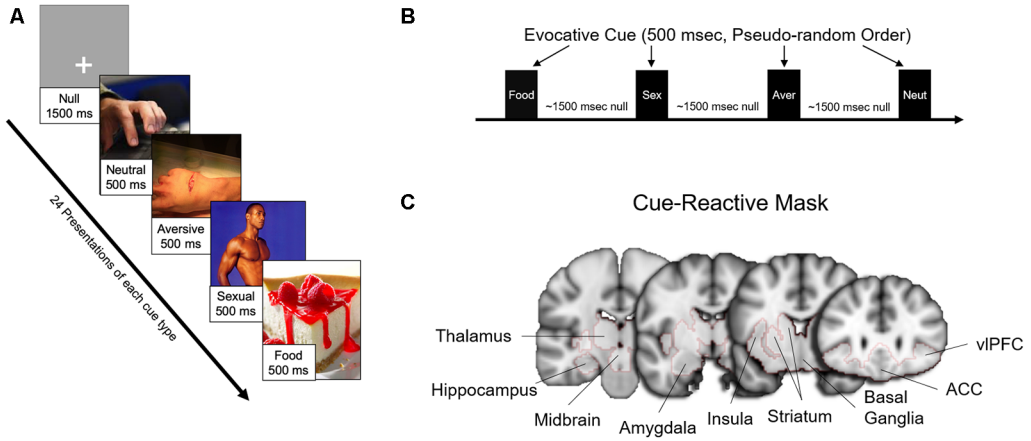

FIGURE 1 | Task design, evocative cues, and cue-reactive mask. **(A)** Examples of evocative (sexual, aversive, food) and neutral cues. **(B)** Each cue was presented 24 times each for 500 ms in pseudorandom order interspersed with approximately an average of 1,500 ms (random duration between 1,000 and 2,000 ms) of a gray screen with crosshair (null). **(C)** Brain activity for the main findings was examined with a mesolimbic "cue-reactive" mask, with regions of interests based on previous studies of sexual stimuli and other evocative cues.

another 12 images of condoms were free-to-use images chosen from internet sites.

Data Analysis

Demographic Health and Behavioral Data

Survey data were analyzed descriptively for frequency, mean, median and range. Demographic and sexual risk behavioral data were compared between sexual risk subgroups (Protected vs. Unprotected), using Chi-square and Fisher's exact test for categorical variables, and *t*-tests for continuous variables. Health and behavioral data were analyzed with SPSS (IBM, 2016) and MATLAB (The MathWorks, 2019).

Imaging Data

Data processing was carried out in SPM12[1] run under MATLAB R2019a. Each participants' images were slice-timing corrected, realigned, co-registered to high-resolution to structural images, and subsequently normalized to MNI standard space and smoothed with the FWHM kernel of 9 mm. The motion statistics for each subject were examined to ensure that motion did not exceed 2 mm in any plane. For the first-level analysis, a canonical hemodynamic response function with time and dispersive derivatives was fitted to the onset of each event. The following contrasts were defined to assess the cue effect: sexual vs. neutral, aversive vs. neutral and food vs. neutral.

Mesolimbic "Cue-Reactive" Mask

For each contrast, independent *t*-tests were conducted between sexual risk groups (Protected vs. Unprotected). Primary analyses were limited to subcortical regions [e.g., caudate, putamen, insula, amygdala, hippocampus, caudal orbitofrontal cortex (e.g., ventrolateral prefrontal cortex, or vlPFC), thalamus] associated with neural responses to sexual stimuli (Mitricheva et al., 2019) and other evocative cues (Childress et al., 1999; Franklin et al., 2007; Noori et al., 2016; Regier et al., 2017). These regions were combined into a mesolimbic "cue-reactive" mask (**Figure 1**) using the Harvard-Oxford probabilistic anatomical atlas included with FMRIB Software Library (FSL). Clusters were considered significant at $p < 0.005$, cluster-corrected ($k > 130$) with Monte-Carlo simulations, using 3dClustsim included in the most recent AFNI software (Cox et al., 2017). Images were displayed with Mango (Multi-Image Analysis GUI) software[2]. Results are displayed both at cluster-corrected $p < 0.01$ and $p < 0.005$ to illustrate the spread of activation surrounding the peaks. Parameter estimates were extracted from nodes to explore differences between sexual risk subgroups—Protected (with and without RP), Unprotected (with and without RP)—and to examine relationships with sexual risk variables and bias scores.

Affective Bias

An affective bias score was calculated for those who correctly completed the task (at least 70% of the nouns correctly identified as positive or negative). For each image category, mean reaction time for positive word trials was subtracted from the mean reaction time for negative word trials to obtain the mean affective bias score. Thus, positive reaction time scores reflected a more positive affective bias, and negative reaction time scores reflected a more negative affective bias. Bias scores were compared between Protected vs. Unprotected groups (and RP subgroups) with *t*-tests. Bias scores were also used to examine the relationship with the brain response to sexual (-neutral) cues within the mesolimbic mask. As described above, clusters were considered significant at $p < 0.005$, cluster-corrected ($k > 130$) with Monte-Carlo simulations, and images were displayed with at both at cluster-corrected $p < 0.01$ and $p < 0.005$ to illustrate the spread of activation surrounding the peaks.

[1]http://www.fil.ion.ucl.ac.uk/spm

[2]http://ric.uthscsa.edu/mango/mango.html

RESULTS

Demographic and Health Variables

The average age of participants was 21. The population had a diverse racial/ethnic profile; participants self-identified as African American (67%), Caucasian (24%), Asian (7%), Hispanic/Latino (6%), and American Indian/Taino (4%). The majority of participants were students (59%) with an average of 12.4 highest grade completed. In the past 30 days, 67% used alcohol, 31% used marijuana, and 9% used cigarettes.

Thirty-three percent (n = 18) of the emerging adult women participants used condoms 100% of the time in the past 3 months (Protected) and 67% (n = 36) used condoms less than 100% of the time, 26 of whom (72%) did not use condoms at all, in the past 3 months (Unprotected). For the 52 participants that completed the 500 ms brain imaging task, there were no differences of demographic variables between the Protected (n = 17) and Unprotected (n = 35) groups (**Table 1**), and the Protected and Unprotected groups did not differ on impulsivity, sensation seeking, BART scores, or anxious attachment. Significantly more of the Unprotected group had been victims of intimate partner violence (80% vs. 47%, χ^2 = 5.73, $p < 0.05$; **Table 1**), but they did not differ from the Protected group on childhood maltreatment scores.

Except for alcohol use before sex and partner status, measures related to sexual risk were generally higher in the Unprotected group (**Table 1**). Significant differences (FDR-corrected) were found for anal sex since the age of 15″ (51% vs. 12%, χ^2 = 7.78, $p < 0.01$), total amount of lifetime sexual partners (7.8 vs. 4.4, $t_{(52)}$ = 2.27, $p < 0.05$), and frequency of vaginal sex in the past 3 months (16.8 vs. 5.2, $t_{(52)}$ = 2.79, p = < 0.01; **Table 1**). In contrast, significantly fewer women in the Unprotected group had "Risky Partners" (RP) in the past 3 months (43% vs. 82%, $p < 0.01$).

Imaging

Brain Response to Evocative Cues

Compared to the Unprotected group, the brain response to sexual (-neutral) cues (controlling for IPV) in the Protected group was higher in the cue-reactive mask, with nodes in the dorsal striatum (caudate and putamen), anterior insula, and vlPFC [voxel-level threshold: $p < 0.005$, cluster-corrected ($k > 130$); **Figure 2**]. No significant results were found within the cue-reactive mask when comparing Protected vs. Unprotected on brain response to aversive or food cues. Whole-brain results are presented in **Table 2** and displayed in the **Supplementary Figure S1**.

Sexual Cues Response by Sexual Risk Subgroups

Because the brain response to sexual cues may have differed due to differences in partner status [see "Materials and Methods" and "Results" section above; the majority of the Protected group (15/18) had a "risky partner" (RP), while less than half of the Unprotected group (16/36) had an RP], we investigated groups by sexual risk subgroups (Protected-RP, n = 15; Unprotected-NoRP, n = 20; and Unprotected-RP, n = 16; only three of the women in the Protected group did not have RP and thus were excluded from the analysis, due to the small number). Extracted parameter estimates from significant clusters (caudate, putamen, and vlPFC) were compared between three subgroups.

TABLE 1 | Demographics and health variables.

	Protected (n = 17)	Unprotected (n = 35)	P-value
Age	20.8	21.4	[1]p = 0.78
Mom finished high school	88%	77%	[2]p = 0.34
Recruitment site	47%	69%	[2]p = 0.13
Race:			
African American	41%	69%	[3]p = 0.09
Caucasian	24%	20%	
Asian/American Indian/Other	35%	11%	
Hispanic	24%	11%	[2]p = 0.26
Smokes cigarettes	0%	14%	[2]p = 0.11
Alcohol use	69%	66%	[2]p = 0.83
Cannabis use	38%	29%	[2]p = 0.52
Intimate Partner Violence	47%	80%	[2]p = *0.02*
Impulsivity	60.5	60.5	[1]p = 0.99
Anxious attachment	7.6	7.9	[1]p = 0.74
Sensation seeking	24.8	24.3	[1]p = 0.75
Depression	20.2	15.9	[1]p = 0.21
BART	28.6	29.4	[1]p = 0.60
Sexual risk (and other sex-related) variables			
Number of lifetime partners	4.2	7.8	[1]p = *0.02*
Multiple partners (past 3 months)	18%	23%	[2]p = 0.67
Anal sex since age 15	12%	51%	[2]p = *0.006*
Alcohol use before sex	59%	37%	[2]p = 0.14
Drug use before sex	18%	6%	[2]p = 0.17
Frequency of sex (past 3 months)	5.3	17.3	[1]p = *0.01*
Risky Partner	82%	43%	[2]p = *0.007*
History of STI	24%	46%	[2]p = 0.12

[1]*Independent t-test;* [2]*Chi-Squared test (2 × 2);* [3]*Chi-Squared test (2 × 3).*

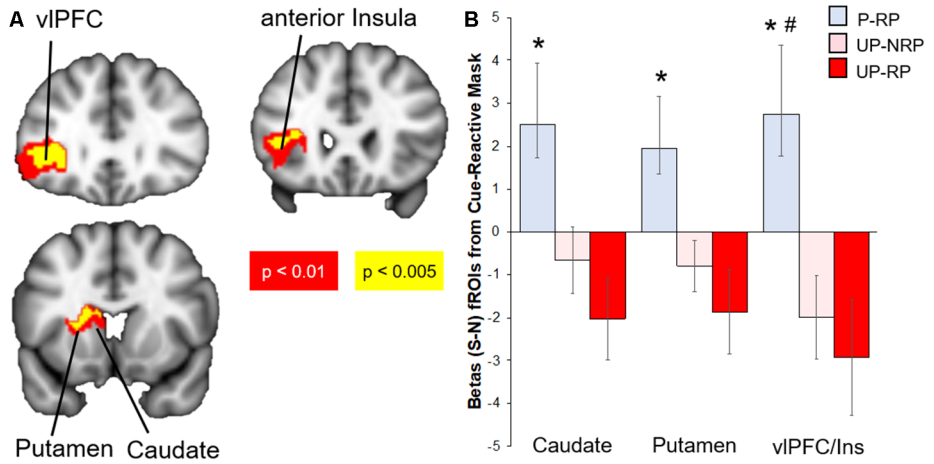

FIGURE 2 | Differential brain response to sexual (S)–neutral (N) cues among sexual risk groups. **(A)** Protected > Unprotected groups: activity in the mesolimbic mask, with nodes in the putamen, caudate, anterior insula, and lateral OFC, was higher in the Protected vs. Unprotected group. **(B)** The Protected (P, blue) with risky partners (RP) group had the highest brain mesolimbic response that significantly differed from both the Unprotected-RP (UP-RP, orange) and Unprotected-NoRP (UP-NRP, red) groups in the vlPFC but only the Unprotected-RP group in the caudate and putamen. *Significant difference vs. Unprotected-RP group. #Significant difference vs. Unprotected-NoRP group.

TABLE 2 | Whole-brain results.

Region	Coordinates x, y, z	Peak *t*-Value	Number of Voxels
	Protected > Unprotected: Sexual Cues		
vlPFC	−30, 34, −6	3.12	328
Fusiform gyrus	52, −38, −18	3.69	563
	Protected > Unprotected: Aversive Cues		
Sup. front lobe	−20, 20, 60	4.7	920
Mid. front lobe	18, 26, 58	3.44	296
	Protected > Unprotected: Food Cues		
Cerebellum	32, −58, −36	3.66	435
	Sexual Cues with Sexual Bias		
dlPFC (left)	−40, 38, 24	4.07	437
dlPFC (right)	38, 34, 30	3.78	508
Putamen (right)	26, 16, 8	3.68	261
Inf. Par. Lobe	58, −30, 54	3.53	268

Results at $p < 0.005$, cluster corrected ($k > 256$). vlPFC, ventrolateral prefrontal cortex; MTL, middle temporal lobe; sup front lobe, superior frontal lobe; mid front lobe, middle frontal lobe; dlPFC, dorsolateral prefrontal cortex; inf par lobe, inferior parietal lobe.

The results (FDR-corrected) showed that the Protected-RP group had greater brain response to sexual cues in the vlPFC compared to both the Unprotected-NoRP ($t_{(32)} = 2.67$, $p < 0.05$) and Unprotected-RP groups ($t_{(27)} = 2.71$, $p < 0.05$). In addition, the results show that the Protected-RP group had greater brain response to sexual cues in the caudate ($t_{(27)} = 2.67$) and putamen ($p < 0.05$; $t_{(27)} = 2.48$, $p < 0.05$) compared to the Unprotected-RP group, while the difference of brain response between the Protected-RP and Unprotected-*NoRP* group trended towards significance in the caudate ($p = 0.067$) and putamen ($p = 0.058$). The brain response between the Unprotected (NoRP vs. RP) groups did not differ (**Figure 2**).

Sexual Cue Response: Correlation With Sex Frequency

To further explore the reduction of brain response to sexual cues observed in the Unprotected (vs. Protected) group, extracted parameter estimates were correlated with the frequency of sex in the past 3 months. The distribution of the sex frequency variable was not gaussian but instead positively skewed, thus sex frequency was log-transformed prior to analyses and plotting (Manikandan, 2010). There was a significant inverse correlation (FDR-corrected) of frequency of sex in past 3 months and the brain response to sexual (-neutral) cues in the caudate ($r = -0.47$, $p < 0.01$), putamen ($r = -0.49$, $p < 0.01$), and vlPFC ($r = -0.47$, $p < 0.01$; **Figure 3**).

Affective Bias

Sexual Cue Response: Correlation With Sexual Bias

For the subset of participants ($n = 39$) who correctly completed the affective bias test (achieved at least 70% accuracy), extracted parameter estimates from the difference between Protected > Unprotected groups brain response to sexual (-neutral) cues (see "Results" section above) were used to test the relationship with implicit affective bias to sexual cues. Results showed a significant positive correlation (FDR-corrected) with parameter estimates from the putamen ($r = 0.38$, $p < 0.05$; **Figure 3**) but only a trend was found with dorsal caudate parameter estimates ($r = 0.28$, $p = 0.09$), and no significant relationship was found with vlPFC parameter estimates.

Affective Bias Scores

The Protected group had a positive bias to both sexual and condom cues, while the Unprotected group had significantly lower bias (vs. the Protected group) scores to sexual ($t_{(37)} = 3.71$, $p < 0.05$) and condom cues ($t_{(37)}$, $p < 0.01$). To check whether bias scores differed by the risk of the sexual partner (RP variable), we examined bias scores in subgroups of Protected and Unprotected groups (see "Materials and Methods" and "Results" section; **Figure 4**). The Protected-RP group had higher affective bias scores to sexual cues compared to the Unprotected-RP

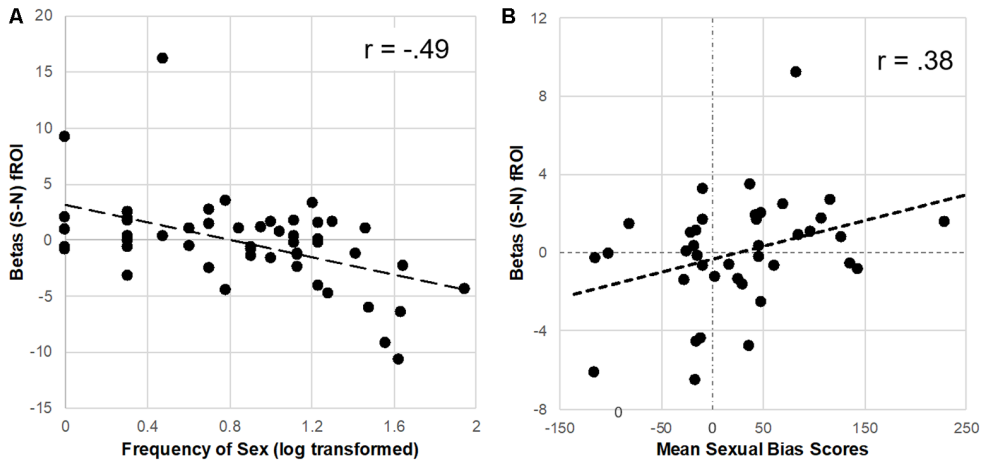

FIGURE 3 | Parameter estimates (betas) from the Protected > Unprotected [sexual (S)–neutral (N) cues] differential brain response correlated with the frequency of sex and affective sexual bias scores. **(A)** Neural responses in the dorsal striatum (fROI) to sex (-neutral) cues were inversely correlated with the frequency of sex in the past 3 months. **(B)** In contrast, neural responses in the putamen to sexual (-neutral) cues were positively correlated with affective sexual bias scores.

group ($t_{(20)}$ = 3.54, $p < 0.01$) but not the Unprotected-*NoRP* group ($t_{(24)}$ = 1.82, p = 0.11). There were no differences between Unprotected (NoRP vs. RP) groups. The Protected-RP group had higher (FDR-corrected) condom bias scores compared to both the Unprotected-RP group ($t_{(20)}$ = 2.52, $p < 0.05$) and the Unprotected-NoRP group ($t_{(24)}$ = 4.21, $p < 0.01$). Again, there were no differences between Unprotected (NoRP vs. RP) groups.

Exploratory: Sexual Bias and Brain Response to Sexual Cues

For the subset of 39 participants who successfully completed the affective bias task and the 500 ms brief cue fMRI task, the brain response to sexual (-neutral) cues were correlated with sexual bias scores. Results showed a significant positive relationship within the cue-reactive mask, with nodes centered in the bilateral putamen (**Figure 4**). Whole-brain results are presented in **Table 2** and displayed in the **Supplementary Figure S2**.

DISCUSSION

In this study, emerging adult women (ages 18–24) who had sex in the past 3 months were divided into two groups: individuals who used a condom 100% of the time (Protected group) and individuals who did not use a condom 100% of the time (Unprotected group). These two groups were compared on their brain response to evocative [sexual, food, aversive (vs. neutral) cues] in several mesolimbic regions (**Figure 1**). Based on prior studies, primarily in males, it was expected that heightened mesolimbic response to sexual cues would correspond with higher sexual risk (i.e., unprotected sex). However, in the present study, it was instead women in the "Protected" (vs. Unprotected) group who exhibited a heightened mesolimbic brain response to sexual cues (**Figure 2**). Because the Protected group had more "risky" partners (RP, i.e., reported or did not know that their partner had HIV, STIs, and/or other sexual partners), follow-up analyses were conducted to account for partner status. Compared to both the Unprotected (NoRP and RP) groups, generally, the Protected-RP group had more activation in nodes within the cue-reactive mask (**Figure 2**). Even though there was a tendency of the Protected-RP group to have the highest response and Unprotected-RP to have the lowest response, there were no differences between the Unprotected (NoRP vs. RP) groups. Interestingly, activation nodes within the mesolimbic mask were inversely correlated with the frequency of sex in the past 3 months (**Figure 3**). In other words, the more sex individuals reported from the past 3 months, the lower the mesolimbic response to sexual cues. Finally, in a subset of participants who successfully completed an affective bias task, the Protected (vs. Unprotected) group showed a higher positive affective bias for sexual and condom cues. In line with our secondary hypothesis, sexual bias scores were positively correlated with the mesolimbic response to sexual cues, with nodes in the bilateral putamen (**Figures 3**, **4**). In other words, it was expected that sexual bias scores would have a positive relationship with the mesolimbic response to sexual cues, however, it was unexpected that higher sexual bias scores and correlation with an increased brain response to sexual cues was higher in the group at *lower* risk for STIs/HIV.

Our hypotheses were based on prior literature generally reporting greater reward circuit activation for those with higher sexual risk behaviors; however, most of the emerging studies on the relationship of brain response to sexual cues and sexual risk behaviors have thus far been in males (Voon et al., 2014; Seok and Sohn, 2015). Previous literature indicates that there is a difference between male and female attitudes about condom use. For example, males have reported that condom use is associated with a lack of pleasure, whereas females have reported that condom use by their partner is associated with protection from negative consequences (Martinez-Donate et al., 2004; Hill et al., 2011; Calsyn et al., 2013). Our findings suggest that sexual associations at the level of the brain and behavior (affective

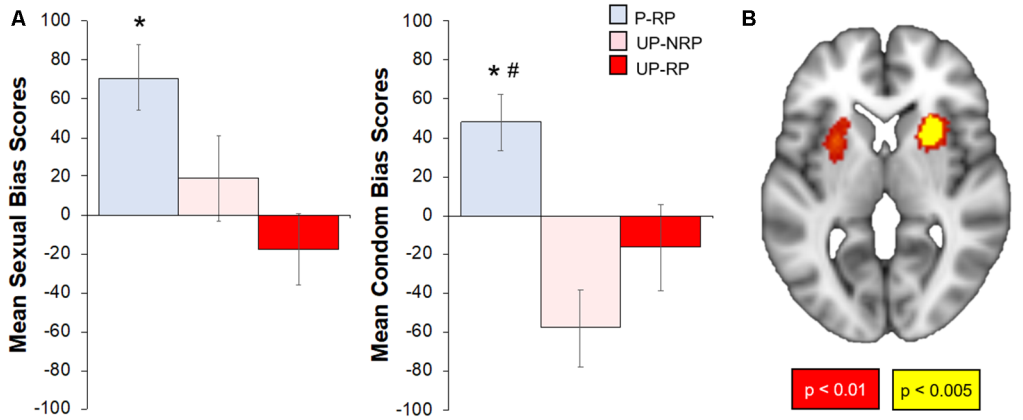

FIGURE 4 | Differences in affective sexual bias scores by sexual risk group; correlation of affective sexual bias scores with the brain response to sexual (-neutral) cues. **(A)** Overall, the Protected group had higher affective bias scores to sexual and condom cues. When accounting for RP, the Protected RP (P-RP, blue) group had significantly higher sexual bias scores than the Unprotected RP (UP-RP, red) group but not the Unprotected-NoRP (UP-RP, orange) group; the P-RP group had higher condom bias scores than both Unprotected (RP and NoRP) groups, but bias (sexual or condom) scores did not differ between UP groups. **(B)** Sexual bias scores positively correlated with regions within the cue-reactive mask, including nodes in the bilateral putamen ($p < 0.01$–0.005, cluster corrected). *Significant difference vs. Unprotected-RP group. #Significant difference vs. Unprotected-NoRP group.

biases) are more favorable for women whose partners use condoms. While some studies have reported male vs. female differences of neurobiological and behavioral responses to sexual stimuli (Rupp and Wallen, 2008; Hill et al., 2011), a recent meta-analysis found that females and males generally activate the same brain regions in response to sexual cues (Mitricheva et al., 2019). In addition, while a recent study found that differential patterns in the subcortical response to *non-sexual* cues between males vs. females were predictive of sexual risk behaviors (Victor et al., 2015), it is unclear whether there would be differences in the mesolimbic response to *sexual* cues between males vs. females with varying degrees of STIs/HIV sexual risk behaviors.

Given the previous literature and present study, one interpretation of our results might involve a level of safety, in that women may feel more protected, worry less about the negative consequences, and may, therefore, enjoy sex more when their partners use condoms. Though not mutually exclusive, another interpretation may be that the Protected group represents the standard response (heightened reward activity and positive bias to sexual stimuli), while the Unprotected group, particularly the Unprotected-RP subgroup, might represent an atypical response (diminished reward activity and sexual bias to sexual stimuli). Given that the Unprotected-RP subgroup reported significantly more negative experiences with sexual partners, such as a history of STIs and physical IPV, compared to the other subgroups (uncorrected; Protected-RP: $p = 0.04$, $p = 0.01$, respectively; Unprotected-NoRP: $p = 0.03$, $p = 0.03$, respectively), that may partially explain the decreased reward response to sexual stimuli, potentially exacerbated by more sexual encounters (i.e., frequency of sex). Though our numbers were too few to examine the interaction of all these variables, future studies would provide further elucidation.

Significant nodes within the cue-reactive mask (e.g., dorsal striatum, insula) that differed between the Protected and Unprotected groups have been shown to be involved in the processing of visual sexual stimuli (Mitricheva et al., 2019). The caudate and putamen process both positive and negative stimuli (Lammel et al., 2014), driving reward-seeking behaviors and motivational states (Wise, 2004), such as pleasurable eating (e.g., Small et al., 2003), drug craving (e.g., Breiter et al., 1997; Wong et al., 2006; Volkow et al., 2006), sexual-related activities (see review, Gola and Draps, 2018), and the pursuit and loss of monetary value (e.g., Knutson et al., 2000). However, they have been found to differ in other processes, such as those associated with deliberative (caudate) and habit-based (putamen) behaviors (e.g., Yin and Knowlton, 2006; Graybiel, 2008; Regier et al., 2015). In the present study, positive sexual bias associated with activation to sexual cues may indicate an increased reward response in the Protected group, whereas the decreased response in the Unprotected group may indicate an attenuated reward response to sexual stimuli. This attenuated reward response may be particularly relevant to the Unprotected-RP group, which on average had negative sexual bias scores and lower striatal responses to sexual cues. Other brain responses that differed between groups included nodes primarily in the vlPFC but that also overlapped with the anterior insula. The vlPFC receives projections from dopaminergic regions and has been implicated in reward-related decisions (Sakagami and Pan, 2007; Treadway et al., 2012) and interoceptive signals related to the processing of evocative stimuli (Seo et al., 2014). The anterior insula has also been shown to process visceral experiences (e.g., increased heart rate, nervous stomach) associated with evocative stimuli (Craig, 2009), and has recently been posited as a hub of appetitive motivational systems in risky reward-seeking behaviors (Naqvi and Bechara, 2009). Therefore, abnormally low activity in the insula and vlPFC, as observed in the Unprotected group, may be associated with more risky behavior.

The challenges of the current study may be used to stimulate and guide future research. For example, although differences between groups were found for affective bias scores of condoms,

condom images were not included in the fMRI imaging design. Results from the present study may imply sex differences of the brain response to sexual cues between females and males at higher and lower risk for STIs/HIV; however, a future study explicitly testing these apparent differences, within male and female cohorts tested in similar paradigms, would be highly informative. It is notable that there were potential differences in condom use practices by race/ethnicity with proportionally more Hispanic and Asian/Pacific Islander and fewer African-American women in the protected group. Given the multiple factors underlying race/ethnicity categories, this is an interesting result that merits further considering in future research, employing larger samples allowing for disaggregation of these categories. Though the current study featured passive exposure to brief evocative cues, the results encourage future studies with tasks that can explicitly probe decision-making systems (e.g., reinforcement learning) utilizing these evocative cues. Additional tasks, parameters, or even different analyses might reveal brain structures (e.g., ventral striatum, amygdala, hippocampus) undetected by the current study. Finally, although the current study has an adequate sample size (n = 52) for examining the brain response to sexual stimuli associated with sexual risk behaviors in the overall group (even) larger future sample sizes would enable examination of other heterogeneities, as mentioned above, as well as others (e.g., mood and anxiety disorders) that may both impact the brain response to evocative cues and sexual risk behaviors.

CONCLUSIONS

Individuals at higher risk for STIs/HIV had lower activation in subcortical areas in response to sexual cues; they had a less positive affective bias to sexual cues and condoms compared to individuals at lower risk for STIs/HIV, and the bias to sexual cues was positively correlated with the subcortical brain response to sexual cues. Together, these results indicate that women whose partners use condoms may have a higher reward response to sexual cues, or that the women whose partners did not use condoms may have an attenuated reward response to sexual cues. Understanding the relationship of brain response to appetitive cues associated with greater sexual risk can help to inform treatment interventions that target these brain responses with behavioral therapy, medication, or both. In addition, the availability of medications for pre-exposure prophylaxis (PrEP) to prevent HIV infection (Flash et al., 2014) has energized research efforts toward identifying individuals at increased STI/HIV risk.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AT, AC, and PR contributed to the conception and design of the study. ZM acquired fMRI data. KJ, ZM, and CM performed initial analyses. PR performed all other statistical analyses and wrote first draft of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00279/full#supplementary-material.

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Central Actions of 3α,5α-THP Involving NMDA and $GABA_A$ Receptors Regulate Affective and Sexual Behavior of Female Rats

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The neurosteroid, 5α-pregnan-3α-ol-20-one (known as "allopregnanolone" or 3α,5α-THP), is produced in the midbrain ventral tegmental area (VTA), independent of peripheral sources of progestogens, where it has potential actions at N-methyl-D-aspartate (NMDA) and $GABA_A$ receptors to facilitate rodent sexual behavior. Progestogens can also have anti-anxiety effects, but whether these involve actions of centrally-derived 3α,5α-THP or these receptors to support reproductively-relevant behavior is not well understood. We investigated the extent to which 3α,5α-THP's actions *via* NMDA and/or $GABA_A$ receptors in the midbrain VTA influence reproductive behaviors. Estradiol-primed, ovariectomized/adrenalectomized (OVX/ADX) rats received midbrain VTA infusions of vehicle, an NMDA receptor blocker (MK-801; 200 ng), or a $GABA_A$ receptor blocker (bicuculline; 100 ng) followed by a second infusion of vehicle or 3α,5α-THP (100 ng). Reproductively-relevant behaviors were assessed: sexual (paced mating), anxiety-like (elevated plus maze), and social (partner preference, social interaction) behavior. Compared to vehicle, intra-VTA infusions of MK-801 exerted anxiolytic-like effects on elevated plus maze behavior and enhanced lordosis. Unlike prior observations in gonadally-intact rats, intra-VTA bicuculline had no effect on the behavior of OVX/ADX rats (likely due to a floor effect). Subsequent infusions of 3α,5α-THP reversed effects on lordosis and infusions of bicuculline inhibited 3α,5α-THP-facilitated lordosis. Thus, NMDA and $GABA_A$ receptors may act as mediators for reproductive behavioral effects of 3α,5α-THP in the midbrain VTA.

Keywords: allopregnanolone, anxiety, bicuculline, dizocilpine, lordosis

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INTRODUCTION

Progesterone (P_4) plays a key role in the regulation of reproductive behavior in female rodents. In the brain, P_4 can exerts its effects either *via* "genomic" or "non-genomic" action. In the hypothalamus, P_4 facilitates lordosis, the reflexive posture that allows copulation. In this brain region, P_4 actions are mediated by intracellular cognate progestin receptors, which act as nuclear transcription factors to alter RNA transcription and protein synthesis (Meisel and Pfaff, 1985). However, In the midbrain ventral tegmental area (VTA), P_4 can exert actions to mediate both

"consummatory" reproductive behavior (e.g., lordosis) and "appetitive" reproductive behaviors (e.g., proceptivity indicators such as ear wiggling, hopping, darting, and inhibition of normative anxiety-like behavior) which are etiologically important for successful reproduction. Given that the VTA is largely devoid of progestin receptors, P_4 actions in this brain region are mediated by conversion of P_4 to its 3α-hydroxy, 5α-reduced metabolite, 5α-pregnan-3α-ol-20-one (known as "allopregnanolone" or 3α,5α-THP). Unlike P_4, 3α,5α-THP lacks affinity for progestin receptors and instead acts at neurotransmitter receptors. We have found that engaging in a pseudo-naturalistic mating paradigm (termed "paced mating") enhances steroidogenesis of 3α,5α-THP in the midbrain and additional brain regions (hippocampus, prefrontal cortex, and diencephalon; Frye et al., 2007). Blocking 3α,5α-THP formation in the VTA increases anxiety-like behavior and attenuates lordosis of female rats or hamsters (Frye and Vongher, 2001; Petralia et al., 2005; Frye et al., 2008a,b, 2009a,b; Frye and Paris, 2011). Thus, 3α,5α-THP actions in the VTA are necessary for the full expression of consummatory and appetitive reproductive behavior. However, the mechanisms of 3α,5α-THP action in this brain region are not well-understood.

One mechanism by which 3α,5α-THP may alter reproductive behavior occurs *via* actions at inhibitory and excitatory neurotransmitter receptors. A well-characterized, non-genomic signaling pathway by which 3α,5α-THP acts is *via* modulation of inhibitory γ-aminobutyric acid type A ($GABA_A$) receptor complexes. 3α,5α-THP is among the most potent, positive allosteric modulators of $GABA_A$ Cl^- channels and a direct agonist in high concentrations (Majewska et al., 1986; Lambert et al., 1987; Morrow et al., 1987; Paul and Purdy, 1992; Gunn et al., 2011). Pharmacologically-enhancing or -attenuating actions at $GABA_A$ receptors in the VTA has commensurate effects to enhance or attenuate P_4-facilitated lordosis (DeBold and Frye, 1994). Less well understood are 3α,5α-THP's actions that may involve excitatory N-methyl-D-aspartate (NMDA) receptors. While free 3α,5α-THP has little affinity for NMDA receptors (Maurice et al., 2006), when sulfated it acts as a negative allosteric modulator of NMDA Ca^{2+} channels, binding the NR2B subunit (Johansson and Le Grevès, 2005). Blocking NMDA receptors in the VTA also enhances progestogen-facilitated lordosis in gonadally-intact, or ovariectomized, rats (Petralia et al., 2007; Frye and Paris, 2011), but it is not known if these effects involve upstream actions of 3α,5α-THP.

Beyond lordosis, progestogens' actions at $GABA_A$ and NMDA receptors in the midbrain VTA may also be important for the expression of additional reproductively-relevant behaviors such as anxiety. Indeed, 3α,5α-THP exerts robust anti-anxiety effects in the VTA (Frye et al., 2006a) and $GABA_A$ receptor agonists in the VTA can underlie positive changes in mood and affect (Gifkins et al., 2002; Genud et al., 2009). Less is known about the role that NMDA receptors may play in the VTA; albeit, NMDA receptor antagonism facilitates lordosis and may underlie aspects of anxiolysis, in part, *via* actions of peripheral glands (adrenals and/or ovaries) and/or neurosteroidogenesis (Frye and Paris, 2011). Notably, the NMDA receptor antagonist, MK-801 (a.k.a. dizocilpine), blocks 3α,5α-THP's anti-depressant-like effects in the amygdala of rats (Shirayama et al., 2011) supporting a modulatory role for affective behavior. We have previously utilized ovariectomized (OVX) and/or adrenalectomized (ADX) rats to reveal that central neurosteroid enhancement in the VTA is important for the expression of consummatory (i.e., lordosis) and appetitive (i.e., social and anti-anxiety-like) behavior. Antagonizing $GABA_A$ and NMDA receptors within the VTA alters the anxiety-like and lordosis response to pharmacologically-promoted increases in steroidogenesis (Frye and Paris, 2011), but the identity of the important steroids that underlie actions at these sites are not known. The present work aimed to assess the importance of $GABA_A$ and NMDA receptor targets in the mediation of 3α,5α-THP's central effects to influence reproductively-relevant behavior. We hypothesized that female OVX/ADX, estradiol (E_2)-primed rats administered the NMDA receptor blocker, MK-801, would demonstrate reduced anxiety-like behavior (general anxiety assessed *via* the elevated plus maze and social anxiety assessed *via* a social interaction test) and enhanced sexual receptivity (assessed *via* a propinquity test and a paced mating test), while those administered the $GABA_A$ receptor blocker, bicuculline, to the VTA, would demonstrate opposite effects on these behaviors. We further hypothesized that subsequent 3α,5α-THP infusions to the VTA would reverse the effects of blockers.

MATERIALS AND METHODS

These methods were approved by the Institutional Animal Care and Use Committee at The University at Albany-SUNY and were conducted in accordance with ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85-23).

Animals

Adult (50–60 days old), Long-Evans female rats (N = 100) were bred in the Life Sciences Laboratory Animal Care Facility at The University at Albany-SUNY (original stock obtained from Charles River, Raleigh, NC, USA). Rats were housed in polycarbonate cages with woodchip bedding (45 × 24 × 21 cm) in a temperature-controlled room (21 ± 1°C) and were maintained on a 12:12 h reversed light cycle (lights off at 08:00 h) with continuous access to Purina Rat Chow and tap water in their home cages.

Surgical Protocol

Rats were stereotaxically-implanted with bilateral guide cannulae aimed over the medial aspect of the VTA (from bregma: AP = −5.3, ML = ± 0.4, DV = −7.0) under xylazine (12 mg/kg) and ketamine (70 mg/kg) anesthesia. Immediately following stereotaxic surgery, rats were OVX/ADX as previously described (Frye and Paris, 2011). Following surgery and prior to testing, animals were monitored for loss of weight, righting response, flank stimulation response, and/or muscle tone (Marshall and Teitelbaum, 1974). One rat failed these assessments and was immediately euthanized. All rats were screened for complete-ADX *via post hoc* assessment of corticosterone in plasma (methods below). All the rats included in analyses had circulating concentrations of corticosterone that were below

baseline levels (<1 μg/dl). Sixteen rats were excluded due to circulating corticosterone >1 μg/dl, which prior work suggests is indicative of incomplete adrenalectomy and can alter behavioral and endocrine measures (Frye and Paris, 2011). Remaining rats all had circulating corticosterone levels <1 μg/dl.

Preparation of Pharmacological Blockers

The NMDA receptor blocker, MK-801 hydrogen maleate (Sigma Chemical Co., St. Louis, MO, USA) was diluted to a concentration of 200 ng/μl in sterile saline as elucidated in prior investigations (Frye and Paris, 2011). MK-801 is a long-lasting non-competitive antagonist that acts in the receptor channel pore, where it blocks opening (Dravid et al., 2007). While, MK-801 and 3α,5α-THP have not previously been co-infused in a mating model, this dose has previously been demonstrated to facilitate reproductive behaviors and antagonize the effects of a general neurosteroidogenesis enhancer (Petralia et al., 2007; Frye and Paris, 2011).

The GABA$_A$ blocker, bicuculline (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in sterile saline to a concentration of 100 ng/μl as previously demonstrated (Frye and Paris, 2011). Bicuculline is an antagonist that inhibits the GABA$_A$ ion channel by competing for the GABA binding site on GABA$_A$ receptors but does not compete with the allosteric steroid-binding site (Ueno et al., 1997). This dose has previously been demonstrated to reduce anxiety-like behavior and lordosis of sexually-receptive rodents (Frye et al., 2006b; Frye and Paris, 2009).

Study Procedure

Seven days after surgery, OVX/ADX rats were primed with E_2 (10 μg, SC) and tested 44 h later to evaluate sexual behavior (**Figure 1**). On the day of testing, E_2-primed rats were randomly assigned to receive a bilateral infusion (1 μl) of saline vehicle, bicuculline (100 ng/μl), or MK-801 (200 ng/μl) to the midbrain VTA. Thirty min later, rats received a subsequent infusion of 25% β-cyclodextrin vehicle or 3α,5α-THP (100 ng/μl). Rats were tested 10 min later in all tasks described below (**Figure 1**). We have previously systematically assessed the effects of exposure to the tasks utilized in this behavioral battery (Frye et al., 2007). We find that performance in one task does not significantly influence subsequent behavioral performance or central/circulating steroid levels, with the exception of an engagement in paced mating which promotes central steroidogenesis of 3α,5α-THP (Frye et al., 2007). As such, the paced mating task is always performed last (**Figure 1**). There were six experimental conditions based on intra-VTA infusions: vehicle/vehicle (n = 13), vehicle/3α,5α-THP (n = 17), MK-801/vehicle (n = 15), MK-801/3α,5α-THP (n = 12), bicuculline/vehicle (n = 15), and bicuculline/3α,5α-THP (n = 11). The numbers of rats per group were reduced when those with infusions to sites other than the VTA were taken into account.

Behavioral Outcome Measures

Behavioral data were collected using ANY-maze animal tracking software (Stoelting Co., Wood Dale, IL, USA). All rats were placed in an open field apparatus (76 × 57 × 35 cm) consisting of a 48-square grid floor (6 × 8 squares, 9.5 cm/side) for a 5 min habituation to the apparatus and testing room. The frequency of crossings into each of the 48 squares on the floor was recorded as an assessment of general motor behavior. While entries into the central 24 squares of the open field is a common measurement of anti-anxiety behavior (Frye and Rhodes, 2006a), we observed no differences in the number of central squares entered among experimental groups in this study, or prior studies utilizing this OVX/ADX model (Frye and Paris, 2011).

Elevated Plus Maze

The elevated plus maze was conducted per previous methods (File, 1990). Briefly, the maze has four opaque arms (49 cm long, 10 cm wide) elevated off the ground (50 cm high). Two arms (east and west) are enclosed by walls (30 cm high), while the other two arms (north and south) are exposed. The amount of time spent on, and the number of entries into, open or closed arms were recorded during the 5 min task. Open arm time is an index of exploratory and anti-anxiety behavior, while total arm entries are an index of motor behavior.

Partner Preference

Partner preference was conducted as previously described (Frye and Rhodes, 2006a). Briefly, stimulus rats (one diestrous female and one male) are confined to opposite corners of an open field *via* Plexiglass compartments that are permeated with small holes for olfactory exchange. Sexually-receptive rats will seek an opposite-sex partner for the purposes of copulation (Nofrey et al., 2008). The amount of time that an experimental rat spends in proximity (one body length or less) to either stimulus rat is recorded during a 5 min test. In the lab setting, preference for a male vs. female stimulus rat is considered a measure of sexual receptivity.

Social Interaction

Social interaction was assessed in the open field apparatus per previous methods (File, 1990). Briefly, an experimental female was placed in one corner, while a diestrous stimulus female was placed in the opposite corner of the apparatus. The amount of time that the experimental rat spent interacting (sniffing, crawling over or under, following with contact, tumbling, boxing, or grooming) with the stimulus rats was recorded in a 5 min test. Total time spent in social interaction is a measure of social anxiety-like behavior.

Paced Mating

Paced mating was conducted per previous methods (Erskine, 1985). In brief, the paced mating apparatus (37.5 × 75 × 30 cm) was equally divided by a Plexiglas partition, which contained a small (5 cm in diameter) hole in the bottom center, allowing the female (but not the stimulus male) free access to both sides of the apparatus. Frequency of mounts + intromissions + ejaculations was recorded and a lordosis quotient was calculated [(frequency of female dorsiflexion during a sexual contact/total sexual contacts by a male) * 100] during a 15 min test. As well, the percentage of aggressive behaviors (vocalizing, attack) and the percentage of times the experimental female left the chamber containing the male (% exits) following sexual contacts were recorded.

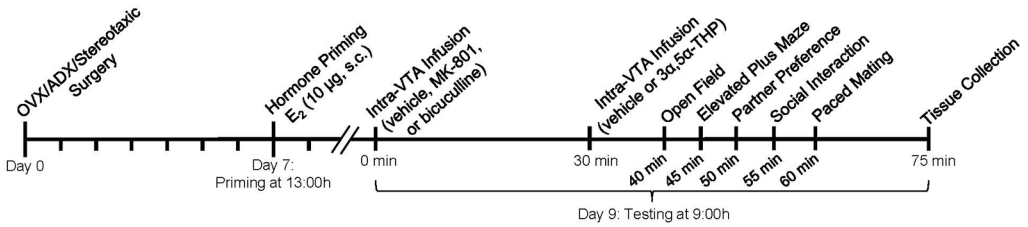

FIGURE 1 | Time-course of the experimental procedure.

Cannulae Placement and Neuroendocrine Assessment

Immediately after testing, rats were decapitated and trunk blood and whole brains were collected and stored as described (Frye and Rhodes, 2006a). Infusion site analyses were conducted on fresh tissue, as described (Frye and Paris, 2011). Of the rats that had not been excluded for receiving a partial-ADX, 22 had infusions to sites other than the VTA; however, all experimental groups were not represented, which precluded factorial analyses of behavioral measures based on-site placement. As such, rats with cannulae placement incongruous with a hit to the VTA were excluded from all analyses. Thus, the experimental groups with complete-ADX and verified cannulae placement to the VTA yielded: vehicle/vehicle (n = 9), vehicle/3α,5α-THP (n = 11), MK-801/vehicle (n = 9), MK-801/3α,5α-THP (n = 12), bicuculline/vehicle (n = 11), and bicuculline/3α,5α-THP (n = 9). Plasma was extracted for radioimmunoassay of corticosterone.

Steroid Extraction

Corticosterone was extracted from serum *via* incubation with ether and 800 cpm of [^{3}H] corticosterone (Frye and Bayon, 1999). Ether-incubated steroids were snap-frozen twice and supernatant was evaporated in a speed drier. Samples were reconstituted with phosphate assay buffer to the original serum volume (Frye et al., 2008a,b).

Radioimmunoassay

Levels of corticosterone were measured by radioimmunoassay, per previously reported methods (Frye et al., 1998). Concentrations of [^{3}H] corticosterone were assessed *via* the logit-log method of Rodbard and Hutt (1974) with "AssayZap" interpolation software published by Biosoft (1994). Inter- and intra-assay reliability coefficients were 0.04 and 0.07, respectively.

Statistical Analyses

Data were analyzed using StatView (SAS Institute Inc.). Group differences were assessed *via* two-way analyses of variance (ANOVAs) with central blocker condition (vehicle, MK-801, bicuculline) or central 3α,5α-THP condition (vehicle, 3α,5α-THP) as factors. Significant main effects were followed by Fisher's protected least significant differences *post hoc* tests to determine group differences. Significant interactions were delineated *via* follow-up one-way ANOVA with alpha corrected for all possible comparisons. The alpha-level for statistical significance was $p < 0.05$.

RESULTS

MK-801 and 3α,5α-THP Infusions to the VTA Altered Anti-anxiety Behavior

Two-way ANOVAs revealed that intra-VTA infusion of blockers and 3α,5α-THP significantly interacted to influence open arm time in the elevated plus-maze ($F_{(2,55)} = 3.05$, $p = 0.05$; **Figure 2**). Contrasts revealed that OVX/ADX rats infused with MK-801/vehicle spent a significantly increased amount of time on the open arms of the elevated plus-maze, compared to rats receiving infusions of bicuculline/vehicle ($p = 0.04$) or control infusions of vehicle/vehicle ($p = 0.046$). Rats infused with subsequent 3α,5α-THP did not significantly differ from vehicle-infused controls or any other group. Notably, central manipulations did not significantly alter motor behavior in the open field (indicated by the number of total squares entered) or in the elevated plus-maze (indicated by the number of arms entered; **Table 1**).

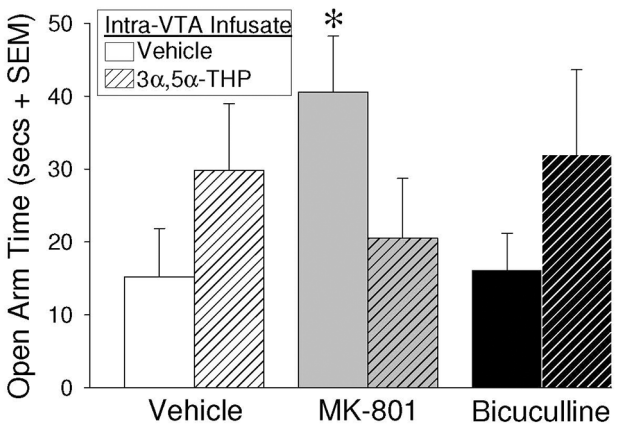

FIGURE 2 | Depicts time spent on the open arms of an elevated plus-maze among estradiol-primed (10 µg, SC), ovariectomized/adrenalectomized (OVX/ADX) female rats infused with the vehicle, MK-801, or bicuculline, followed by subsequent infusions of vehicle or 3α,5α-THP, to the ventral tegmental area (VTA) of the midbrain. *Indicates significantly different from vehicle/vehicle- or bicuculline/vehicle-infused rats, $p < 0.05$.

TABLE 1 | Motor and social behavior measures of female ovariectomized/adrenalectomized rats infused with the vehicle, 3α,5α-THP, bicuculline, and/or MK-801 to the ventral tegmental area of the midbrain (mean ± SEM).

Infusate #1	**Vehicle**		**MK-801**		**Bicuculline**	
Infusate #2	**Vehicle (n = 9)**	**3α,5α-THP (n = 11)**	**Vehicle (n = 9)**	**3α,5α-THP (n = 12)**	**Vehicle (n = 11)**	**3α,5α-THP (n = 9)**
Open field						
Number of total entries	265 ± 20	273 ± 18	259 ± 22	270 ± 21	274 ± 21	254 ± 25
Elevated plus maze						
Number of total arm entries	8 ± 1	11 ± 2	12 ± 2	10 ± 2	9 ± 2	11 ± 3
Partner preference						
Time with male (s)	159 ± 14	152 ± 18	133 ± 33	138 ± 27	120 ± 25	171 ± 26
Time with female (s)	90 ± 12	96 ± 19	107 ± 30	91 ± 28	95 ± 21	72 ± 16
Social interaction						
Interaction time (s)	84 ± 15	87 ± 10*	132 ± 12	84 ± 16*	99 ± 10	74 ± 14*
Paced mating						
Pacing exits (%)	19 ± 6	15 ± 5	11 ± 5	12 ± 6	11 ± 5	6 ± 4

**Indicates significant main effect for the performance of rats receiving subsequent infusions of 3α,5α-THP to differ from those that received subsequent infusions vehicle, irrespective of additional MK-801 or bicuculline treatment, $p < 0.05$.*

3α,5α-THP Influenced Non-sexual Social Behavior

Central manipulations also altered non-sexual social behavior. There was a main effect for subsequent 3α,5α-THP infusions to decrease the duration of time spent in social interaction with a conspecific ($F_{(1,55)} = 4.65$, $p < 0.05$); this was observed irrespective of whether vehicle, MK-801, or bicuculline was first infused (**Table 1**). While, it was apparent that this effect was observed only in MK-801 and bicuculline-infused groups when 3α,5α-THP was co-administered, these factors did not significantly interact.

In the partner preference task, neither infusions of central pharmacological blockers (MK-801, bicuculline) nor infusions of subsequent 3α,5α-THP, significantly influenced the number of time rats spent in proximity to a male (**Table 1**).

MK-801 and 3α,5α-THP Facilitated Lordosis and Defensive Aggression Behavior in Response to Mounting

Intra-VTA infusions of blockers and 3α,5α-THP significantly interacted to alter lordosis ($F_{(2,55)} = 6.54$, $p < 0.05$; **Figure 3**, top). Infusions of vehicle/3α,5α-THP significantly enhanced lordosis compared to vehicle/vehicle-infused controls ($p = 0.03$) or bicuculline/vehicle-infused rats ($p = 0.002$). MK-801/vehicle also significantly enhanced lordosis compared to vehicle/vehicle controls ($p = 0.004$), or rats infused with MK801/3α,5α-THP ($p = 0.01$), bicuculline/vehicle ($p = 0.0002$), or bicuculline/3α,5α-THP ($p = 0.02$). Thus, infusions 3α,5α-THP or MK-801 with vehicle significantly enhanced lordosis, but 3α,5α-THP attenuated MK-801's effects.

Defensive aggression in response to mounting was also significantly altered by intra-VTA infusions (**Figure 3**, bottom). Blockers and 3α,5α-THP interacted ($F_{(2,55)} = 5.22$, $p < 0.05$), such that MK-801/3α,5α-THP infusions attenuated defensive aggression compared to MK-801/vehicle infusions ($p = 0.02$), or infusions of any other compound co-administered with 3α,5α-THP (vehicle/3α,5α-THP, $p = 0.006$; bicuculline/3α,5α-THP, $p = 0.008$). The pacing of mating contacts, following mounting by males, was reduced when blockers were infused to the VTA compared to vehicle infusions; however, this was not a statistically significant difference (**Table 1**). Thus, the co-infusion of MK-801 and 3α,5α-

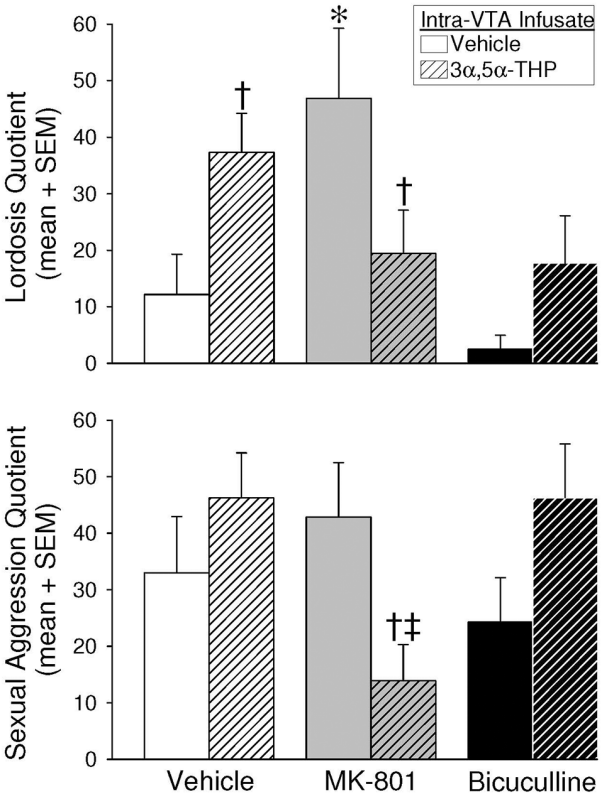

FIGURE 3 | Depicts lordosis (top) and defensive sexual aggression (bottom) quotients in the paced mating paradigm among estradiol-primed (10 μg, SC), OVX/ADX female rats infused with vehicle, MK-801, or bicuculline, followed by subsequent infusions of vehicle or 3α,5α-THP, to the VTA of the midbrain. *Indicates significantly different from vehicle/vehicle-infused controls. †Indicates a significant difference between rats receiving subsequent 3α,5α-THP infusions compared to their respective vehicle/vehicle or MK-801/vehicle infused controls. ‡Indicates significantly different from vehicle/3α,5α-THP or bicuculline/3α,5α-THP-infused rats, $p < 0.05$.

THP to the VTA reduced defensive aggression compared to other manipulations.

DISCUSSION

The hypothesis that intra-VTA infusions of MK-801 would enhance sexual and reproductively-relevant (exploratory, affective, social) behaviors, and bicuculline would reduce these behaviors, was partly upheld. E_2-primed, OVX/ADX rats that were infused with MK-801 demonstrated significantly enhanced lordosis in the paced mating task, and significant anxiolysis as assessed *via* the elevated plus-maze, compared to bicuculline- or vehicle -infused controls. Similarly, investigations in mouse models have revealed that systemic administration of MK-801 has commensurate anxiolytic effects to those of 3α,5α-THP (Reddy and Kulkarni, 1997) and genetically perturbing global NMDA receptor expression yields an aberrant sexual, social, and anxiety-like phenotype (Mohn et al., 1999). However, MK-801 infusions in the present study did not significantly alter non-sexual social behavior (partner preference or free social interaction). Alternatively, infusions of bicuculline significantly blocked the effects of 3α,5α-THP to enhance lordosis, but did not significantly reduce sexual, social or affective behaviors on their own. These findings are commensurate with those of past observations wherein sexually-receptive rodents that were gonadally-intact or OVX (and/or ADX and hormone primed with E_2 and P_4) demonstrated enhanced lordosis when NMDA receptors were blocked in the VTA (Petralia et al., 2007; Frye et al., 2008a,b; Frye and Paris, 2011) and reduced lordosis when bicuculline was infused to the midbrain VTA or central gray (McCarthy et al., 1991; Frye and Paris, 2009, 2011). These effects are dampened when peripheral steroid glands (ovaries and adrenals) are completely extirpated such that sexual and anxiety-like behavior of rats is greater in gonadally-intact > OVX > OVX/ADX rats (Fernández-Guasti et al., 1991; Gorzalka and Moe, 1994; Frye and Paris, 2009, 2011). E_2-priming alone may not sufficiently reinstate anti-anxiety and lordosis in the OVX/ADX model to a level that intra-VTA bicuculline can efficaciously be observed to attenuate these behaviors (Frye and Paris, 2011). Indeed, circulatory P_4 is a critical factor for several aspects of paced mating including its reinforcing properties (Paredes and Alonso, 1997; Paredes and Vazquez, 1999; González-Flores et al., 2004). For these reasons, we may have observed a "floor effect" on anxiety-like responding with exogenous 3α,5α-THP non-significantly increasing anti-anxiety-like behavior when administered alone. Thus, NMDA and/or GABA$_A$ receptors in the midbrain VTA regulate lordosis and anti-anxiety-like behavior of rats; but, appetitive sexual behaviors (e.g., ear wiggling, pacing) and social interactions may require circulatory progestogens for assessment of full expression.

The second hypothesis, that subsequent 3α,5α-THP infusions to the VTA would reverse effects of pharmacological blockers, was partly supported. Enhancements of anxiolysis and lordosis that were promoted by MK-801 infusions were not observed when 3α,5α-THP was co-administered. Co-infusion of MK-801 and 3α,5α-THP also reduced defensive aggression, but this was not observed when either compound was infused with the vehicle. These data support the notion that 3α,5α-THP may play an important reproductive regulatory role *via* intra-VTA NMDA receptors. Similarly, others have seen intracerebroventricular infusions of an NMDA receptor antagonist to block P_4-enhancement of lordosis among OVX, E_2-primed rats (Gargiulo et al., 1992; Gargiulo and Donoso, 1995). We also observed 3α,5α-THP-mediated lordosis to be significantly attenuated when bicuculline was co-infused, supporting a regulatory role for 3α,5α-THP at intra-VTA GABA$_A$ receptors. Indeed, 3α,5α-THP has been observed to regulate GABA$_A$ subunit expression *in vitro* and *ex vivo* (Shen et al., 2005; Zhou and Smith, 2007) and orally-active micronized P_4, which can metabolize to 3α,5α-THP, is seen to enhance the positive and negative effects of benzodiazepines in premenopausal women (Babalonis et al., 2011a,b). In the present animal model, it is known that neither gonadal nor adrenal, P_4 are necessary for the expression of lordosis (Foreman and Moss, 1977; Auger et al., 1997); rather, central neurosteroidogenesis in the VTA is critical for expression and maintenance of this behavior (Frye and Paris, 2011). The present investigation extends these findings to reveal 3α,5α-THP as the important neurosteroid product acting in the VTA to mediate reproductively-relevant anxiety and sexual behavior of female rats.

We have previously observed 3α,5α-THP infusions to the VTA to be associated with enhanced 3α,5α-THP production in other brain regions (hippocampus, prefrontal cortex, diencephalon; Frye and Rhodes, 2006a). These effects are not thought to be due to infusate diffusion given that we have previously observed central infusate to spread ∼1 mm and for intra-VTA infusions not to diffuse beyond the midbrain (Frye and Rhodes, 2008). Moreover, 3α,5α-THP infusions targeted to sites in proximity to the VTA (substantia nigra or central gray) are not observed to promote 3α,5α-THP formation in midbrain, hippocampus, or striatum (Frye and Rhodes, 2006b, 2008; Frye et al., 2008a,b). Rather, steroids can be synthesized in neural cells, independent of peripheral sources (King, 2008). Notably, women administered P_4 exhibited shifts in metabolite:prohormone ratio that was indicative of depression status, supporting the importance of steroid metabolites (Girdler et al., 2012). Others find that exogenous progestins and oral contraceptives that do not metabolize 3α,5α-THP increase anxiety-like behavior of rodents (Porcu et al., 2012). Formation of 3α,5α-THP may play an important role in the benefits of pregnane steroids.

The behavioral effects of inhibitors observed herein likely involve modulation of VTA efferents to limbic and extralimbic brain regions. In the present report, we observed either 3α,5α-THP or MK-801 actions in the VTA to facilitate lordosis. The midbrain VTA consists of a mixture of dopaminergic (∼65%) and non-dopaminergic neurons, the latter of which are largely GABAergic (∼30%) or glutaminergic (∼5%; Zessen et al., 2012). Activation of dopaminergic efferents from the VTA to forebrain structures (particularly, within the striatum) are generally observed to inhibit lordosis and lesioning or quiescing these neurons facilitates lordosis (Caggiula et al.,

1979; Sirinathsinghji et al., 1986; Pednekar and Mascarenhas, 1993; Frye et al., 2010). By virtue of 3α,5α-THP's potent affinity for $GABA_A$ receptors, it rapidly promotes Cl^- influx into neurons, reducing excitability (Majewska et al., 1986; Lambert et al., 1987). When acting in the VTA, 3α,5α-THP may dampen the activity of dopaminergic efferents, thereby promoting lordosis. As well, intra-VTA NMDA receptors are important for dopamine neurotransmission (Gu and Lu, 2018) and infusion of MK-801 decreases Ca^{2+} influx into neurons, similarly attenuating excitation and promoting lordosis. Despite achieving their endpoints by different mechanisms, actions of 3α,5α-THP and MK-801 to inhibit dopaminergic projection neurons to other brain regions, particularly the hippocampus, mPFC, and striatum (caudate/putamen and nucleus accumbens), may underlie their effects to facilitate reproductive behavior. It is of interest that the subsequent addition of 3α,5α-THP reversed MK-801's effects on lordosis. The capacity for 3α,5α-THP to restore behavioral homeostasis may be conferred by its capacity to potently activate $GABA_A$ Cl^- channels, restoring ion homeostasis. Following inhibition of Ca^{2+} channels *via* MK-801, cells may become hyperpolarized; under these circumstances, subsequent activation of $GABA_A$ channels *via* 3α,5α-THP may efflux, rather than influx, Cl^-, thus, restoring the excitatory/inhibitory ion balance within the VTA.

The present study reveals the capacity for modulation of intra-VTA NMDA receptors to influence appetitive and consummatory reproductive behaviors in female rats. Subsequent administration of 3α,5α-THP restored behavioral homeostasis, presumably *via* actions at $GABA_A$ receptors to re-establish ion homeostasis within the VTA. These data further reveal the importance of excitatory/inhibitory substrates within the VTA for reproductively-relevant behaviors. Formation of 3α,5α-THP may act to balance ion homeostasis within the VTA, thereby influencing efferents to regions involved in the processing of natural reward.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at The University at Albany-SUNY and were conducted in accordance with ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85-23).

AUTHOR CONTRIBUTIONS

CF participated in experimental design. DL and JP acquired data. CF, DL, and JP performed data analyses. CF, AQ, DL, and JP wrote and contributed to the writing of the manuscript. All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Yawning and Penile Erection Frequencies Are Resilient to Maternal Care Manipulation in the High-Yawning Subline of Sprague–Dawley Rats

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Yawning is a stereotyped behavioral pattern characterized by wide opening of the mouth associated with deep inspiration followed by short expiration. All vertebrate species yawn, but with low frequencies. We obtained two sublines of Sprague–Dawley (SD) rats by a strict inbreeding process: one with a high-yawning frequency (HY) of 20 yawns/h, which is one order of magnitude higher with respect to the low-yawning frequency (LY) subline, with 2 yawns/h. Outbred SD rats had a yawning frequency of 1 yawn/h. HY dams had a different organization of maternal care with respect to that displayed by LY and SD dams because HY dams constructed lower quality nests and had more re-retrieving and atypical retrieving. The aim of this study was to analyze the changes in maternal care using in- and cross-fostering between the sublines and SD dams and to measure spontaneous and dopaminergic-induced yawning, penile erections, grooming and scratching bouts. We also measured the expression of dopamine D_2 receptors in the striatum using Western blot analysis. Our results showed that HY male rats reared by SD or LY dams did not significantly differ in yawning frequencies with respect to HY male rats reared by mothers of their own phenotype. Maternal care did not differ between sublines and SD dams independent of the litter they reared. However, LY rats reared by HY dams showed a significant increase in the number of spontaneous penile erections. Importantly, in-fostered HY male rats had the highest number of yawns induced by systemic administration of (−)-quinpirole supporting that higher maternal care display can influence the frequency of dopaminergic-induced yawning. In fact HY male rats in all conditions yawned more than did LY and SD male rats independent of the dam that raised them supporting a strong influence of genetic background. However SD male rats raised by LY dams showed significantly increased the dopamine D_2 receptor expression.

In conclusion, maternal care and the environmental nest conditions during the lactation period did not change the phenotypic characteristics of the yawning sublines supporting that their genetic background is fundamental for the expression of spontaneous or dopaminergic-induced yawning.

Keywords: anxiety, sexual behavior, maternal care, grooming, epigenetic, cross-fostering, depression, scratching

INTRODUCTION

Yawning is a stereotyped behavioral pattern that is characterized by deep inspiration followed by short expiration across all vertebrate species (Barbizet, 1958; Argiolas and Melis, 1998; Collins and Eguibar, 2010). In mammals, including rats, yawning frequency is very low, approximately 1 yawn/h (Baenninger, 1997).

We selectively inbred for more than 85 generations two sublines of Sprague–Dawley (SD) rats: a high-yawning (HY) subline, with a mean of 20 yawns/h, which is one order of magnitude higher than the low-yawning (LY) subline, with a mean of 2 yawns/h (Urbá-Holmgren et al., 1990; Eguibar et al., 2015). We used as a control group and outbred SD rats that have a mean of 1 yawn/h. HY rats allow us to analyze the environmental influences on yawning behavior. Yawning frequency has a circadian rhythm with a peak before dusk (Anías et al., 1984). The yawning circadian rhythm is not an endogenous mechanism because it is not free running, and it can be synchronized by a restricted feeding period, with food availability being a stronger zeitgeber than a light–dark cycle (Holmgren et al., 1991). These sublines also differ in their responses to stress, as seen in the open-field arena, where HY rats are more active than LY rats, indicating that HY rats are less emotionally reactive than the latter (Moyaho et al., 1995).

The physiological role of yawning has not been clearly established until now, and there are several hypotheses about the role of this innate motor pattern in respiratory and circulatory roles. Several studies have demonstrated that yawning can change the respiratory rhythm, increasing heart frequency and blood oxygenation, as well as vasodilatation, but these variables are not triggering yawning. In conclusion, there is not a clear physiological association between respiration or circulation and yawning (Guggisberg et al., 2010). Another hypothesis about the physiological role of yawning is the role of drowsiness as an inducer of yawning and a concomitant increase in arousal levels as a method of global activation of brain activity from brain stem to cortical areas, but there is no convincing evidence of such an association (Guggisberg et al., 2010). A third hypothesis is the role of yawning in cooling down the brain temperature, the so-called thermoregulatory hypothesis (Gallup and Gallup, 2008). Based on the high yawning frequency of HY male rats, we can demonstrate that when a yawn happens, there is a decrease in the cornea and ear concha temperatures, two hairless facial structures, using thermographic analysis. In fact, 10 s after a yawn happens, there is a reduction in both facial areas that correlates with the reduction in the cortical temperature measured with implanted thermocouple probes in non-HY rats that then returns to basal levels within a short time period (Shoup-Knox et al., 2010; Eguibar et al., 2017b). In another study, we demonstrated in budgerigars that the beak temperature decreased when a yawn happened (Gallup et al., 2017). These experimental data indirectly support the thermoregulatory role of yawning, but it is necessary to obtain more empirical data to support the hypothesis. Finally, the blood gas (CO_2/O_2) hypothesis was rejected because breathing neither pure oxygen nor high CO_2 had a significant effect on yawning frequency, although both increased the breathing rate (Provine et al., 1987).

On the other hand, HY male rats allow us to show that this innate behavioral pattern is strongly correlated with spontaneous penile erections in a very short time window of only 3 min; in fact, 50% of yawns and penile erections happen together (Holmgren et al., 1985). Yawning frequency also correlated with the number of penile erections after systemic administration of D_2-like dopaminergic agonists such as apomorphine in low doses, bromocriptine, or (−)-quinpirole (Urbá-Holmgren et al., 1993; Eguibar et al., 2003); this behavioral correlation is present even in a myelin mutant rat with progressive demyelination (Eguibar et al., 2012).

Yawning behavior is regulated by several neurotransmitters, including cholinergic, muscarinic, or D_2-like dopaminergic agonists; it is inhibited by opioids and GABAergic mechanisms (for review, see Argiolas and Melis, 1998; Collins and Eguibar, 2010); and it is increased by the central administration of adrenocorticotrophic, α-melanocyte-stimulating hormone (MSH), oxytocin, and prolactin peptides and inhibited by bombesin (Argiolas and Melis, 1998; Díaz-Romero et al., 2002; Collins and Eguibar, 2010). Among all of these neurotransmitters and neuromodulators, the dopaminergic system, through D_2-like receptors, is the most potent inducer of yawning and penile erections, acting in the paraventricular nucleus (PVN) of the hypothalamus (Sanna et al., 2012), and the motor output is regulated by the striatum (Dourish and Cooper, 1990).

On the other hand, yawning is part of a behavioral syndrome induced by exposure to a stressor, with a strict temporal organization and an initial increase in alertness, grooming, yawning, and finally somnolence or even sleep (Delius, 1988). Therefore, grooming and yawning are two behaviors that reduce stress responses and have adaptive properties (Fentress, 1988). In the case of HY rats, they groomed more when exposed to a novel environment (Eguibar and Moyaho, 1997) or after wetting the fur (Moyaho et al., 1995), indicating that they had different coping strategies to confront a stressor. This approach is supported when HY rats are exposed to an open-field arena because they ambulate more and have a lower number of fecal boluses; therefore, they are less emotionally reactive (Moyaho et al., 1995). This finding

is corroborated by a preliminary study in which HY rats explore more the open arms in the elevated-plus maze, supporting that they are less anxious (Eguibar et al., 2017a).

It is well established that maternal care plays a substantial role in physiological and behavioral stress responses in adulthood (Caldji et al., 2000). In this context, HY dams spent less time in the nest, retrieved their pups faster, and showed a longer latency to licking and mouthing the pups than LY dams. The HY dams also had atypical retrieving, and they built nests of less quality, supporting that they are motivated to take care of their pups, but the "fine tuning" of maternal care is different (Ugarte et al., 2011). In a previous study, we analyzed yawning frequencies in male and female rats after cross-fostering, but all subjects were raised in individual cages to increase their stress responses. Our results showed that sex ratio and littermate size influenced yawning frequency in adulthood (Moyaho et al., 2009), but no further analysis was performed until now.

Based on these data, the aim of this study was to first analyze the role of maternal care in spontaneous yawning and penile erections in male rats raised in conditions of in- and cross-fostering between the sublines or with SD dams. Second, we analyzed the maternal behavior during the initial phase of the lactation period in conditions of in- or cross- fostering between the sublines or with SD dams. Third, in another group of rats, fostered by different types of dams, we determined yawning, penile erection, grooming, and scratching frequencies after systemic administration of a specific D_2-like receptor agonist (−)-quinpirole, and finally in another group of rats, we measured the relative expression of D_2 receptors in the striatum using Western blot analysis.

MATERIALS AND METHODS

Animals

We used a total of 198 HY, LY, and SD male rats, with 63 being dams. For the (−)-quinpirole experiments, we evaluated 99 rats: 33 HY, 33 LY, and 33 SD. Additionally, we used 36 rats for Western blot experiments: 12 HY, 12 LY, and 12 SD. All subjects (Ss) were bred in our animal room facilities at the Institute of Physiology of the Benemérita Universidad Autonóma de Puebla. After weaning (28 days), the rats were maintained at 3–4 rats per transparent acrylic cage (46 × 32 × 20 cm), with the floor covered with wood shavings (Beta Chip, Warrensburg, NY, USA). The Ss were maintained in a room with controlled temperature (22°C ± 2°C) and relative humidity between 30% and 45%. Ss were maintained under a 12:12 h light–dark schedule (lights on at 07:00), with free access to balanced rodent pellets (Purina Mills 5001, Richmond, IN, USA) and purified water (Ciel, FEMSA, Estado de México, México), and they weighed between 280 and 310 g when the experiment began. All trials were conducted in the light phase between 10:00 and 13:00.

All procedures described have been performed in compliance with the Laws and Codes approved in the seventh title of the Regulations of the General Law of Health regarding Health Research of the Mexican Government (NOM-062-ZOO-1999) and following the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition, 2011). All experiments procedures were approved by Benemérita Universidad Autónoma de Puebla Animal Care and Use Committee, no. EGC SAL-G-2019.

In- or Cross-fostering Procedures and Measuring Maternal Behavior

Pregnant dams were housed individually in transparent acrylic cages (46 × 32 × 20 cm; **Figure 1**). As soon as parturition happened, the pups were weighed and sexed, and the litter was curled to four females and four males and swapped between cross- and in-fostered dams. Therefore, HY, LY, or SD litters were fostered to SD dam mothers of the same subline or SD (in-fostered) or to mothers of the opposite subline or SD (cross-fostered). Then, the pups were returned to the dams until weaning at 28 days of age and housed four males/collective acrylic cage (46 × 32 × 20 cm). Spontaneous yawning and penile erections were determined at 60 days of age following the procedure described by Holmgren et al. (1985), and dopaminergic-induced yawning was determined at 90 days of age in a novel cage environment following previous procedures (see Experimental design in **Figure 1**; Eguibar et al., 2015).

For maternal behavior analysis, all dams were tested inside the maternal cage without disturbing them between 10:00 and 12:00 to minimize stress responses. Maternal behavior was observed for 20 min and videotaped using a Sony HD camera (model HDR-PJ260V, Tokyo, Japan) at postpartum days 3, 5, 7, and 9. All videos were stored on an internal HD of a PC computer under Windows 10 software for ulterior analysis. The behavioral analyses were performed using Observer XT software v. 11.0 (Noldus Information Technology, Wageningen, The Netherlands). All measurements were performed offline by a trainer observer who was blinded to dam characteristics and evaluated the percentage of time that the dam spent in different maternal behaviors, such as arched-back nursing, blanket-nursing, and side-nursing postures. We also evaluated licking and grooming of the young, self-grooming inside the nest, food intake, and time spent outside the nest area. In the video digital records, we measured the total amount of time spent in the following behaviors: nursing, body licking, genital licking, the time that the dams spent outside the nest, and nest building.

Evaluation of Spontaneous Yawning and Penile Erections in Young Adult Male Rats

First, in all Ss, we determined their spontaneous yawning frequency at 2 months of age in a transparent glass cylinder (diameter 190 mm, height 100 mm) in which the floor was covered with a sheet of clean filter paper and the top was covered with a Plexiglas plate, leaving a 1-cm-wide gap for ventilation. The standard period of observation was 1 h, starting at 09:00, and the number of yawns, penile erections, and grooming and scratching bouts were recorded. Yawning was characterized as a prolonged (∼1 s) wide opening of the mouth accompanied by deep inspiration and sometimes associated with tongue protrusion (Ushijima et al., 1984). The yawn ended when rats closed their mouths and returned to normal breathing (Holmgren et al., 1985; Eguibar et al., 2012, 2015). Penile erection

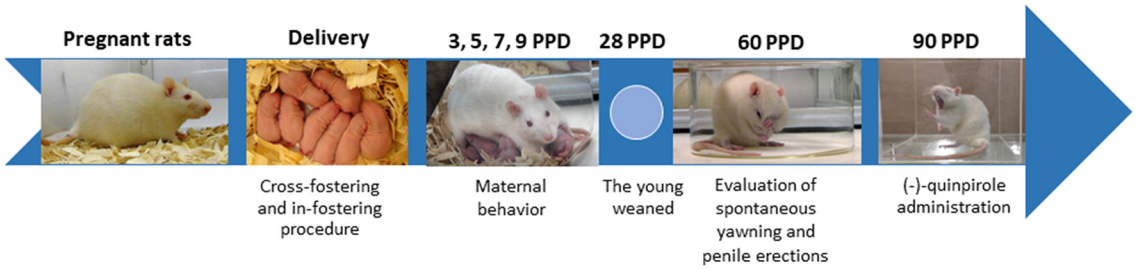

FIGURE 1 | Experimental design. Pregnant female rats from the high-yawning (HY), low-yawning (LY), and Sprague–Dawley (SD) lines were maintained in individual acrylic cages until they delivered the pups. In the first 8 h, the pups were culled to eight pups (four males and four females), and they were weaned at 28 days of age. At 60 days of age, we evaluated spontaneous yawning and penile erections in a glass cylinder, and later, the dose-response to systemic (−)-quinpirole administration was evaluated in a novel environment. PPD, post-partum days.

consisted of pelvic thrusts immediately followed by the rat sitting in an upright position, an engorged penis, and subsequent licking of the perigenital area and even ingesting the ejaculate (Bagdy and Makara, 1995). A mirror was placed behind two stacked glass cylinders to allow the simultaneous observation of four rats by a trained observer following previous criteria (Eguibar et al., 2015; **Figure 1**).

Dose-Response Analysis of Systemic (−)-Quinpirole, A Specific D_2-Dopaminergic Agonist That Produces Yawning, Penile Erections, Grooming, and Scratching

One month after the spontaneous yawning frequencies evaluation, all Ss were distributed randomly from the different fostered groups. For the determination of the dose–response curve to the intraperitoneal (i.p.) injection of (−)-quinpirole hydrochloride (RBI, Inc., Natick, MA, USA; hereafter quinpirole), the drug was dissolved in sterile water with a constant volume of 1 mL/kg of body weight. We administered an increasing dose–response scheme of quinpirole hydrochloride administered every half hour in the order 0, 25, 50 and 100 μg/kg following the procedure used by two other groups and it is possible to use a cumulative dose scheme with (−)-quinpirole D_2 agonist (Baladi and France, 2009; Serafine et al., 2015). In brief all observations started at 09:00 in an observation area that was 1 m from the housing place. The Ss were placed inside of a transparent acrylic cage 23 × 20 × 20 (cm), and the upper part of the cage was covered with a lid of the same material to allow ventilation. Under these circumstances, stress responses were reduced to a minimum. We injected i.p. each rat, and then behavior was continuously observed over a period lasting 30 min, and then the second i.p. dose was administered. We repeated the procedure until the four doses had been administered to the same animal, with a total experiment duration of 2 h 15 min. All sessions were also recorded with a Sony HD camera model HDR-PJ260 V, Tokyo, Japan and stored on the HD of a personal computer for analysis offline by a trained and blinded observer using Observer XT software v. 11.0; the number of yawns and penile erections, as well as the number of grooming and scratching bouts, were recorded.

A grooming episode was scored, as previously described (Eguibar et al., 2004), when any of the following components occurred: face washing, which consisted of vibrating movements of the forepaws in front of the snout, licking of the same paws followed by strokes along the snout, and semicircular movements over the top of the head; and body grooming, which consisted of the licking of body fur, genital grooming when licking the genital area, and paw licking of the forepaws and hindpaws. We also analyzed the scratching of the neck and thoracic body areas with the alternating pattern of hind limbs and licking the toes. Interruptions greater than 5 s determined separate grooming bouts.

Relative Expression of the D_2 Receptor in the Striatum of HY, LY, and SD Rats

We evaluated 12 rats from cross-fostered groups of male rats from each subline as well as SD rats. The rats were rapidly decapitated with a guillotine; the brains were obtained in cold conditions and kept on ice, and the striatum was dissected, placed in an Eppendorf tube, and stored at −80°C until measurements were performed. Total protein extracts were prepared from the striata by disruption in lysis buffer (20 mM Tris-HCl pH 7.4, 100 mM glycine, 100 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM DL-dithiothreitol), added with a protease inhibitor cocktail, with an electronic homogenizer (TissueTearor; BioSpec Products, Inc. IL, USA). Equal amounts of protein (70 μg by group) were denatured in Laemmli's sample buffer, separated through 10% sodium dodecyl sulfate–polyacrylamide gels and electroblotted to nitrocellulose membranes (Bio-Rad Laboratories Headquarters, Mexico City, México). Blots were stained with Ponceau red (Amresco; 0.3% in acetic acid 1%) to confirm that the protein content was equal in all lines. Membranes were soaked in phosphate-buffered saline (PBS; containing 0.2% Tween-20) and incubated in 5% dry milk diluted in PBS for 1 h to block nonspecific protein-binding sites. Membranes were incubated overnight at 4°C with the primary antibody (rabbit polyclonal anti–rat dopamine receptor D_2L, 1:200; cat no. AB1792P; Thermo

Fisher Scientific Inc., Mexico City, México) diluted with milk 1% in PBS followed by secondary antibody (goat anti–rabbit immunoglobulin–horseradish peroxidase, 1:2,000; cat no. SC 2357; Industrias Bioselect, Mexico City) for 2 h. Immunoreactive polypeptides were detected using a chemiluminescence kit (West Pico Signal; Thermo Fisher Scientific, Monterrey, México) and were exposed to a Carestream Medical X-ray film. The relative expression of the D_2 receptor was measured by densitometry and normalized against the signal obtained from Ponceau red staining used as loaded control (Romero-Calvo et al., 2010); for this, ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used. Data are presented as the percentage of change against the control group. Measurements were done in two independent experiments and averaged, afterward.

Statistical Analysis

All statistical analyses were performed using the R statistical environment (R Core Team, 2019). $P \leq 0.05$ was accepted as indicative of a significant difference. All data are presented as the mean ± SEM unless otherwise stated.

Experiment 1. Basal Spontaneous Yawning Frequency, Penile Erection and Grooming of In-fostered and Cross-fostered Male Rats Raised by Different Dams

For each of the behaviors, three different effects were tested independently using the Kruskal–Wallis test: the effect of pup type (HY, LY, or SD), the effect of dam subline (HY, LY, or SD), and the effect of the cross-in-fostering manipulation (manipulated or control). When a test detected a significant effect, *post hoc* group comparisons were performed using Dunn test with Holm–Šidák correction.

Experiment 2. Maternal Care Organization in the In- or Cross-fostering Conditions Between the Sublines or With the SD Dams

Maternal components were modeled as proportions in the (0.1) interval by dividing the time each dam spent in each component over the total observation time. The proportions were then used as the outcome variable in hierarchical regression using a generalized linear model (GLM) of the beta distribution family. Dam subline, pup strain, and in- or cross-fostering manipulation were used as predictive factors.

Experiment 3. Systemic Administration of Quinpirole Differentially Increased Yawning and Penile Erection and Decreased Grooming and Scratching Frequencies in Both Sublines and SD Rats

All statistical analyses were performed to test for two predictive factors: pup strain (i.e., HY, LY, or SD) and the strain of the foster mother (i.e., HY, LY, or SD). All behavioral data were analyzed using a GLM using Poisson, beta, or binomial families depending on the empirical distribution of the data (Quinn and Keough, 2003). The number of yawns, penile erections, and grooming or scratching bouts were each used as a dependent variable, and the strain of the foster mother or the type of male rats were the predictors. All second-order interactions were tested for inclusion in the models.

The effective dose (ED_{50}) was defined as the estimated dose within the model expected to produce 50% of the maximum behavioral response measured in each of the dependent variables. The only exception was penile erections, which were modeled as a binary response, and the ED_{50} in this case represented the dose at which the probability of occurrence was estimated at 50%.

Some coefficients are presented as a change in log-odds, which represents the change in probability after undergoing a logit transformation, which consists of transforming probability into odds (event probability/1 − event probability) and then odds into log-odds (logarithm base 10 of the odds).

Experiment 4. Dopamine D_2 Receptor Expression in the Striatum

The levels of D_2 receptor expression in the striatum were analyzed using Kruskal–Wallis analysis of variance (ANOVA) followed by Dunn multiple-comparisons test using Prism software v. 7 for Windows 10, San Diego, CA, USA.

RESULTS

Experiment 1. Basal Spontaneous Yawning Frequency, Penile Erection, and the Grooming of In-fostered and Cross-fostered Male Rats Raised by Different Dams

The aim of this experiment was to determine which factor has a stronger impact on yawning and penile erection frequencies, the genetic background (sublines or SD male rats), or the raising conditions during the lactation period. **Figure 2** shows that spontaneous yawning frequencies did not differ among in-fostered or cross-fostered sublines or SD male rats raised by different dams (see **Figures 2A,C**). However, HY male rats had a significantly greater number of yawns with respect to their LY and SD counterparts ($\chi^2_{(2)} = 44.7$, $P < 0.001$). No significant effects were found for the dam subline or SD strain ($\chi^2_{(2)} = 3.47$, $P < 0.17$) or for the in- or cross-fostering manipulations ($\chi^2_{(1)} = 1.91$, $P = 0.17$). However, Dunn comparison tests used in the *post hoc* analysis showed a significant difference between HY and LY ($P < 0.001$, Holm–Šidák correction) and between HY and SD ($P < 0.001$) but not between LY and SD male rats ($P = 0.12$; see **Figures 2A,C**).

Figure 3 shows that spontaneous penile erection frequencies significantly differ between HY and LY or SD male rats ($\chi^2_{(2)} = 6.90$, $P < 0.05$). No significant effects were found with respect to dam subline or SD strain ($\chi^2_{(2)} = 0.35$, $P = 0.83$) or for in- or cross-fostering manipulations ($\chi^2_{(2)} = 0.18$, $P = 0.66$; **Figures 3A–C**). A *post hoc* Dunn test showed significant differences between HY and SD rats ($P < 0.05$, Holm–Šidák correction; **Figure 3C**), but no significant differences were found

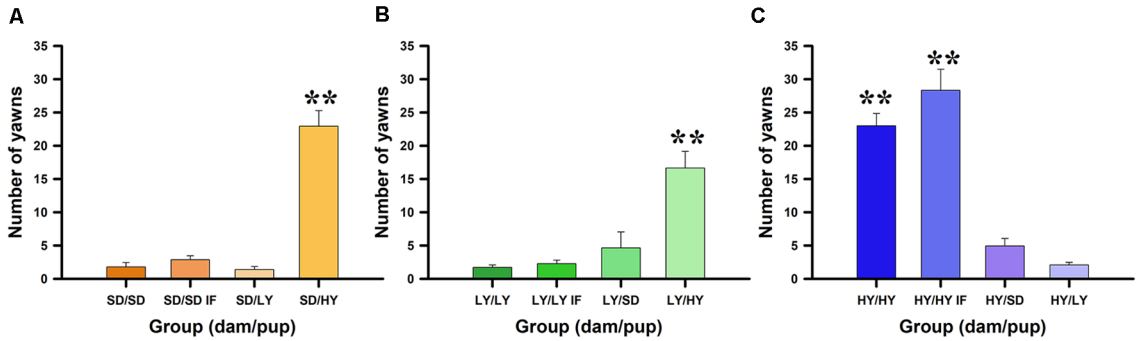

FIGURE 2 | Spontaneous yawning frequency dependents on the dam that take care of male rats. (**A**) Sprague-Dawley (SD) dams do not change the behavioral trend, being the high yawners male rats belonging from HY subline independently of the dam that raised them, see pairs dam/male rats SD/HY (panel **A**), LY/HY (panel **B**), and HY/HY or HY/HY IF (panel **C**); $^{**}P < 0.01$. Note that LY dams can reduce yawning frequencies in all pairs LY/LY, LY/LY IF or LY /SD (panel **B**). Contrary, HY dams do not increase yawning frequencies in SD or LY male rats (see HY/SD and HY/LY; panel **C**).

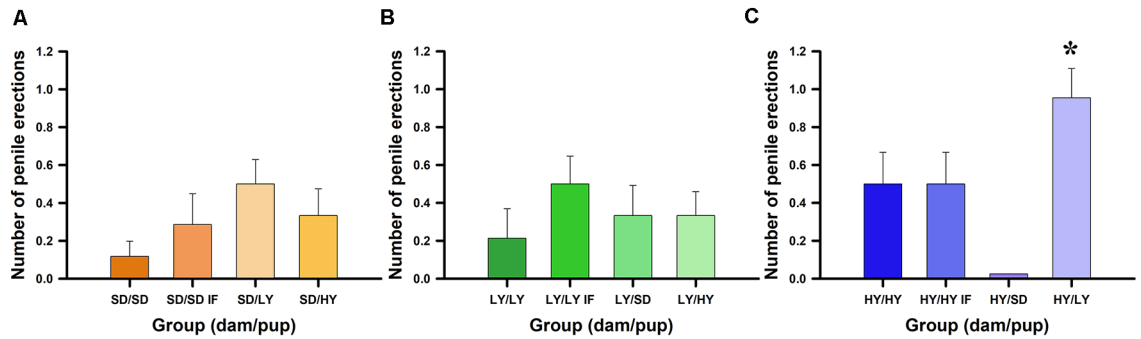

FIGURE 3 | Spontaneous penile erections dependent on the dams that take care of male rats. **(A)** Sprague-Dawley (SD) had similar impact across groups, rearing male rats with lower frequencies of penile erections. **(B)** Low-yawning (LY) dams do not change the penile erection frequencies in the different groups of male rats. **(C)** However, High-yawning (HY) dams are capable to increase the number of spontaneous penile erections on LY male rats (see HY/LY, $^{*}P < 0.05$).

between HY and LY ($P = 0.30$) or between LY and SD male rats ($P = 0.22$). Only HY dams were able to significantly increase the number of spontaneous penile erections in male rats ($P < 0.05$, Holm–Šidák correction).

The spontaneous number of grooming bouts showed a significant effect among the different groups of rats tested ($\chi^2_{(2)} = 22.40$, $P < 0.001$). No significant effects were found for the dams between sublines or with respect to SD dams ($\chi^2_{(2)} = 2.17$, $P = 0.33$); this was also the case for in- or cross-fostering manipulations ($\chi^2_{(1)}$, $P = 0.97$). A *post hoc* Dunn test showed that HY rats had significantly more grooming bouts with respect to LY and SD rats ($P < 0.001$). No significant differences were found among the male rats tested ($P = 0.08$, data not shown).

Experiment 2. Maternal Care Organization in the Cross-fostered Male Rats Raised by HY, LY, or SD Dams

The aim of this experiment was to determine the changes in the behavioral components of maternal care due to cross-fostering among the sublines and SD rats. A beta family GLM was used to analyze the proportion of time for each of the maternal components displayed in the early lactation period and showed that arched-back and blanket-nursing postures differed among the sublines and SD rats ($\chi^2_{(2)} = 22.33$, $P < 0.001$ and $\chi^2_{(2)} = 26.78$, $P < 0.001$, respectively) as illustrated in **Figure 4**, which shows the percentage of time SD dams (**Figures 4A,B**, orange pie charts) spent in each maternal behavioral component on cross-fostered male rats from both sublines. **Figure 4** shows the maternal behavioral components displayed by LY (**Figures 4C,D**, green pie charts) and HY (**Figures 4E,F**, blue pie charts) dams. The dams differed in the time spent in self-grooming in the nest ($\chi^2_{(2)} = 14.31$, $P < 0.001$), as well as in the time spent outside the nest ($\chi^2_{(2)} = 45.90$, $P < 0.001$), but not in the other maternal behaviors measured. In general, both sublines showed a higher proportion of blanket-nursing posture and time spent outside the nest area with respect to that displayed by SD dams ($P < 0.05$). The latency to approach the litter after cross-fostering is higher in HY dams than in SD dams ($P < 0.05$).

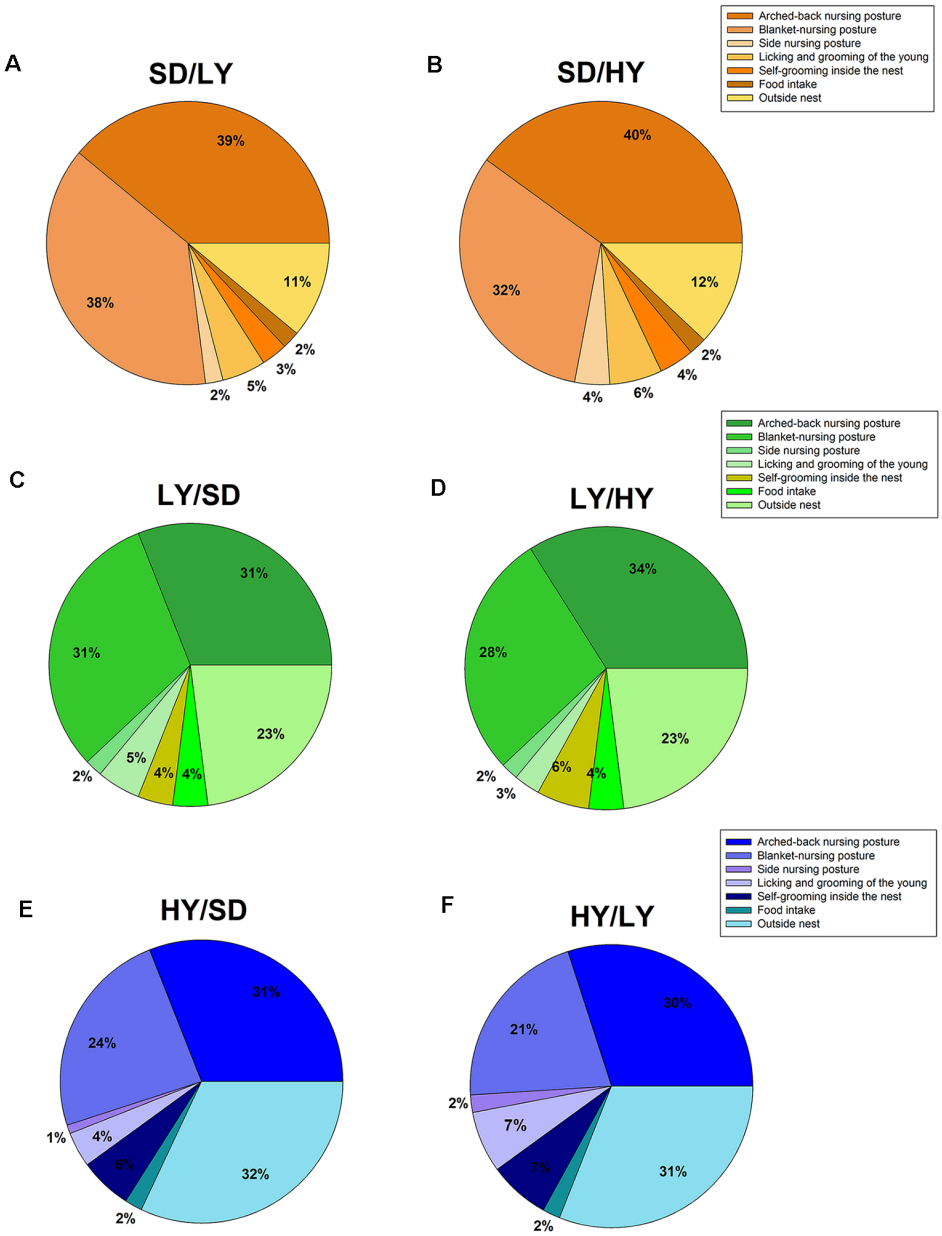

FIGURE 4 | Maternal care in cross-fostered male rats raised by HY or LY sublines or by SD dams. **(A)** SD dams that raised LY male rats (SD/LY) did not differ in their maternal behavior components. **(B)** SD dams showed an increase time spend outside the nest, but the difference was not significant (SD/HY). **(C,D)** LY dams showed a decreased frequency of the arched-back nursing posture compared to SD dams (SD/LY) or with respect to LY dams that raised HY male rats (LY/HY). **(E,F)** HY dams showed a lower proportion of blanket-nursing posture with respect to SD (HY/SD) or LY rats (HY/LY).

Note that HY, LY, and SD dams showed similar values to those obtained in a previous study, but in this study, the maternal components were determined without disturbing the mother and their pups; that is, we measured the ongoing maternal behavior without disturbing them (**Figure 5**). Note that HY and LY dams showed fewer arched-back nursing postures than SD dams ($P < 0.05$). Dams from the HY subline spent more time in self-grooming ($P < 0.05$; **Figure 5E**) and outside the nest ($P < 0.05$; **Figure 5G**) than LY and SD dams.

Experiment 3. Systemic Administration of (−)-Quinpirole Differentially Increased Yawning and Penile Erection and Decreased Grooming and Scratching Frequencies in Both Sublines and SD Rats

The aim of this experiment was to analyze whether the cross-fostering manipulation changed the sensitivity to the D_2 dopaminergic agonist (−)-quinpirole. A beta family GLM was

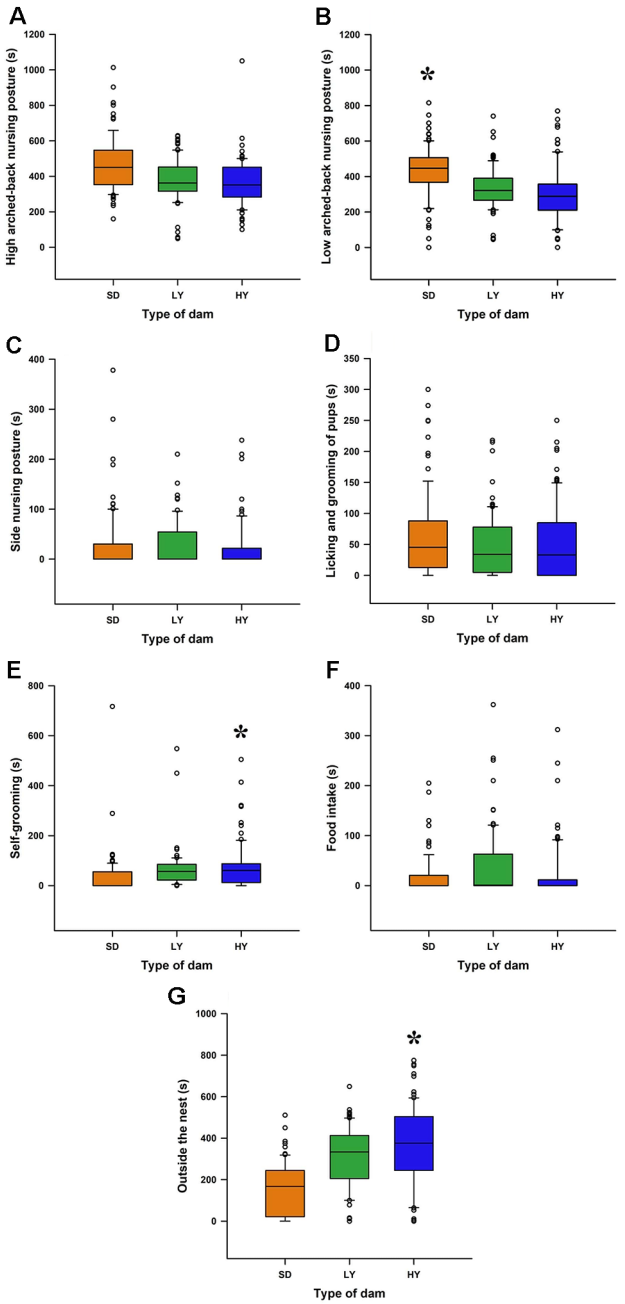

FIGURE 5 | Spontaneous maternal care display in the early lactation period in HY and LY dams or in SD dams. **(A)** High arched-back nursing posture did not differ between sublines or with respect to SD dams. **(B)** SD dams had a higher time spent in the low arched-back nursing position with respect to HY and LY dams (*$P < 0.05$). **(C)** The time spent in the side-nursing posture was similar between the sublines or in SD dams. **(D)** The time spent in licking or grooming the pups was similar among HY, LY, or SD dams. **(E)** The time spent in self-grooming is higher in HY dams with respect to LY or SD dams (*$P < 0.05$). **(F)** The time the dams spent on eating pellets was similar in the different group of rats. **(G)** HY dams spent more time inside the nest with respect to LY or SD dams (*$P < 0.05$).

fitted using the yawning frequency as the dependent variable. Systemic injections of different doses of (−)-quinpirole had a significant effect on yawning frequencies ($\chi^2_{(2)} = 9.66$, $P < 0.01$; **Figure 6**). There was also evidence for a significant effect of the subline or SD strain of the male rats ($\chi^2_{(2)} = 96.20$, $P < 0.001$), with the HY rats being significantly more responsive to this agonist than LY or SD rats. At larger dosages, the yawning frequency decreased, which significantly contributed to the linear regression model ($\chi^2_{(2)} = 165.99$, $P < 0.001$). Finally, a significant interaction was found between the sublines or SD male rats and the (−)-quinpirole dose ($\chi^2_{(2)} = 17.62$, $P < 0.001$; **Figure 6**). The estimated ED_{50} was 4.27 μg/kg for HY rats; in the case of SD rats, the estimated ED_{50} for male rats reached 5.96 μg/kg, which is 1.39 times higher than that of the HY subline, and the estimated ED_{50} for LY rats reached 7.86 μg/kg, which is 1.84 times higher than that of the HY subline. We did not find evidence against the null hypothesis for the dam subline, either for in- or cross-fostered manipulations or in the latency to approach their litter ($\chi^2_{(2)} = <1$, $P > 0.05$).

A binomial family GLM was fitted using the occurrence of penile erections as the dependent variable. Systemic administration of (−)-quinpirole had a significant effect on the log-odds of HY penile erections ($\chi^2_{(2)} = 10.53$, $P < 0.01$; **Figure 7**). Additionally, there was evidence for an effect of the different groups of rats on the logarithm of the probability of penile erections ($\chi^2_{(2)} = 14.51$, $P < 0.001$), as well as an interaction between the type of rats and the dosage of (−)-quinpirole ($\chi^2_{(2)} = 11.823$, $P < 0.01$; **Figure 7**). The estimated ED_{50} for HY rats was 32.23 μg/kg, and for SD rats, the estimated dose was 144.2 μg/kg, which is 4.47 higher than that for HY male rats. In the case of the LY subline, the ED_{50} could not be computed because the drug decreased the odds of penile erections in the case of this subline instead of increasing them. We did not find evidence against the null hypothesis for the dams, either in- or cross-fostered rats or for the latency to approach the litter by the dams ($\chi^2_{(2)} = <1$, $P > 0.05$).

A Poisson family GLM was fitted using the frequency of grooming episodes as the dependent variable. The dose of (−)-quinpirole was found to have a significant inhibitory effect on grooming bouts ($\chi^2_{(2)} = 159.78$, $P < 0.001$). A significant effect was also found for the group of rats ($\chi^2_{(2)} = 63.29$, $P < 0.001$) and the interaction of the group of rats and dosage ($\chi^2_{(2)} = 54.42$, $P < 0.001$).

The ED_{50} was estimated as the middle point between grooming bouts at baseline and total inhibition, with zero mean grooming bouts expected (see **Supplementary Table S1**). Thus, the estimated ED_{50} for HY rats was 42.76 μg/kg, for SD rats was 29.72 μg/kg, and for LY rats was 140.90 μg/kg, which was 3.29 times higher than that of HY rats. We did not find a significant effect of the dams that took care of the different male rats or for in- or cross-fostered manipulations ($\chi^2_{(2)} = <1$, $P > 0.05$, **Supplementary Table S1**).

A binomial family GLM (logistic regression) was fitted using the occurrence of scratching as the dependent variable. There was a significant inhibitory effect of (−)-quinpirole on the odds of scratching ($\chi^2_{(2)} = 37.019$, $P < 0.001$). We did not find an effect of the pup's subline or SD, the dam's subline or SD, or the in-fostering or cross-fostering manipulation. As no subline

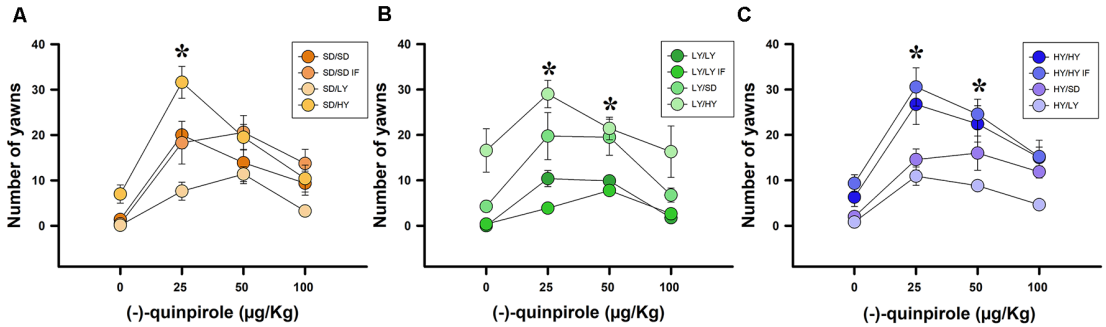

FIGURE 6 | Systemic administration of (−)-quinpirole increased yawning frequencies, which was dependent on the phenotype of male rats. **(A)** SD dams that raised HY male rats yawned significant higher with 25 mg/kg of quinpirole with respect to the rest of pairs (SD/HY, $^*P < 0.05$). **(B)** LY male rats had the lowest yawning frequencies when raised by a dam of their own phenotype, with the highest frequency of yawning occurring when LY dams raised HY male rats (LY/HY; $^*P < 0.05$). **(C)** HY male rats have the greatest yawning frequencies independent of the phenotype of the dams that raised them. Note that HY/HY IF and HY/HY had significantly higher yawning frequencies with respect to HY/SD and HY/LY ($^*P < 0.05$).

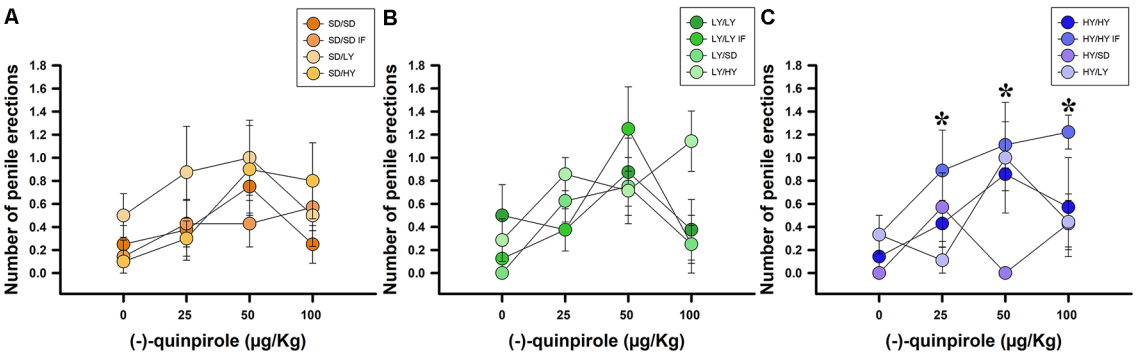

FIGURE 7 | Systemic administration of (−)-quinpirole increased penile erection frequencies in all groups of rats. **(A)** SD dams that raised HY male rats (SD/HY) showed higher penile erection frequencies with respect to the other pairs of dams/male rats. **(B)** LY male rats do not differ in penile erection frequencies with respect to the phenotype of the dam that raised them. **(C)** HY male rats in-fostered with another HY dam (HY/HY IF) had the highest penile erection frequencies with respect to the other pairs of HY male rats ($^*P < 0.05$).

effects were detected, a single ED_{50} was estimated at 53.31 µg/kg (**Supplementary Table S2**).

Experiment 4. Dopamine D_2 Receptor Expression in the Striatum

The aim of this experiment was to determine the D_2 receptor relative expression in the striata of HY, LY, and SD of male rats. Two main immunoreactive bands were observed around 50 kDa. We measured these both as coincided with the technical specifications for the antibody indicating additional bands could be observed in a range from 60 to 100 kDa.

The levels of the striatal D_2 receptor expression in both sublines or in SD male rats did not significantly differ in the different groups of rats raised by SD dams (**Figures 8A,D**). Furthermore, HY dams did not affect the expression of D_2 dopamine receptors in SD, LY, or HY male rats (**Figures 8B,E**). However, SD male rats reared by LY dams showed a significant increase in the striatal dopamine D_2 receptor expression but not in HY male rats (**Figures 8C,F**, respectively; Kruskal–Wallis ANOVA, $H = 7.4$, $P < 0.02$; followed by Dunn test, $P < 0.02$).

DISCUSSION

Our results showed that the higher number of spontaneous and (−)-quinpirole-induced yawning bouts and penile erections in HY male rats is independent of the type of dam that raises them, supporting that the yawning frequency is specific to each subline. Therefore, genetic backgrounds are the main component due to strong inbreeding, and nurturing conditions during the lactation period did not play a significant role in determining yawning frequencies (**Figure 2**). A similar trend was obtained with LY and SD male rats, which had lower yawning frequencies independent of the type of dam that raised them, supporting that the genetic background is the main variable that determines yawning frequency.

In the case of the spontaneous number of penile erections, we clearly demonstrated that HY dams were able to significantly increase the number of penile erections in LY male rats. It is

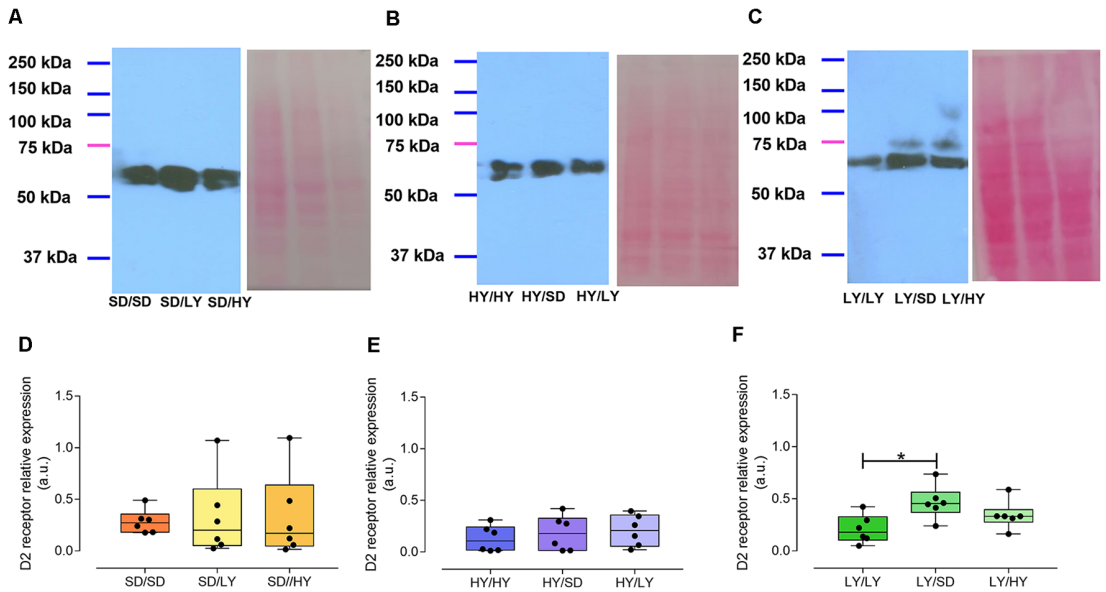

FIGURE 8 | Relative expression of the D_2 receptor (DR2) in the striatum of rats of both sublines and of SD male rats raised by different dams. Representative immunoblots from the striatal D_2 receptor expression and matched Ponceau's red staining in **(A)** SD male rats raised by SD dams (SD/SD), or by LY (SD/LY), or HY (SD/HY) male rats; **(B)** HY, SD, or LY male rats raised by HY dams (HY/HY, HY/LY, and HY/SD); **(C)** LY, SD, or HY male rats raised by LY dams (LY/LY, LY/SD, and LY/HY, respectively). The D_2 receptor relative expression was normalized against the matching Ponceau's red stained lane and expressed as arbitrary units. **(D–F)** Data are shown as the median and interquartile ranges. Only SD male rats raised by LY dams significantly differ from LY/LY group [Kruskal–Wallis analysis of variance (ANOVA), $H = 7.4$, $P < 0.02$, followed by Dunn multiple-comparisons test, $^*P < 0.02$].

relevant that male rats subjected to in-fostering generally had almost double the number of penile erections with respect to their nonfostered counterparts (**Figure 3**), but these differences did not reach significant levels due to, at least in part, the higher variability and lower incidence of noninduced penile erections. It has been demonstrated that the amount of prepubescent self–anogenital grooming is greater in male rats than in their female counterparts, and this grooming component is androgen-dependent because castration reduced it in male rats and androgenization in female rats increased it (Moore, 1986a). The amount of anogenital grooming is also context dependent because living in isolation conditions significantly increased genital grooming (Moore, 1986b). In our conditions, all groups of rats lived in groups, with 3–4 Ss in acrylic cages, and tests were performed in transparent glass cylinders. Therefore, considering that all the conditions are equal, it was outstanding and unexpected that HY dams could increase the number of penile erections in the LY male rats (**Figure 3C**), supporting a strong maternal component in the expression of penile erections out of a sexual context.

It has been demonstrated that cross-fostering is a postnatal environmental manipulation that is capable of inducing behavioral and physiological changes in mice and rats (Bartolomucci et al., 2004). In fact, mother–pup interaction in rats occurs within the nest and consists of approaching the litter, licking and grooming the pups, and nursing them in different positions (Fleming et al., 1999). To control the effects of manipulation due to the fostering of the pups, we also used an in-fostering group of rats as an additional control of pup manipulation, but we obtained an increase in yawning frequencies and in penile erections in in-fostered male rats, supporting that dams are able to discern that they are different pups and change in some way the strategy in which they care for them, which is not reflected in the percentage of time spent in the different behavioral measurements. However, these changes are due to neonatal handling, as already demonstrated in the Roman lines (Fernández-Teruel et al., 1997). It is important to mention that Roman lines share various behavioral characteristics with our yawning sublines (Melis et al., 2019).

Our results also showed that arched-back and blanket-nursing postures differed between both sublines, as well as the time spent in self-grooming and outside the nest or even the time that HY dams approach the litter, supporting a different organization of maternal care in the HY subline with respect to the LY and SD dams, as previously demonstrated (Ugarte et al., 2011). Importantly, the general trend is maintained within each group of dams instead of the characteristic organization of maternal care in the different fostered groups evaluated in these experiments as HY dams that had a different organization of maternal care (Ugarte et al., 2011). These results clearly support that the genetic background between the sublines is quite stable because, as already mentioned, HY and LY rats were maintained by a strict inbreeding process for more than 85 generations (∼35 years), which implies that the presence of different pups with different genetic pools is not able to change maternal care. A similar trend has been obtained with the naturally occurring variations in maternal licking/grooming of pups that were significantly correlated when the mother nursed in the

arched-back position and persisted in the first and even second litters, supporting a possible epigenetic mechanism (Caldji et al., 2000). In future experiments, we could analyze the relationship of the amount of anogenital grooming displayed by the mother and the frequency of spontaneous and drug-induced penile erections in both sublines in the first and second generations to evaluate persistent effects due to maternal care.

In this context, it is relevant that Fleming et al. (1999) showed that there is an interaction between the newborn and the mother that is capable of altering the basic mechanisms of behavioral expression in both, including in neuroanatomical, neurochemical, and behavioral aspects (Fleming et al., 1999). If we consider the degree of interaction of the dams with their offspring and the ways that individuals respond later in life and then changing the response of hypothalamic–hypophysis–adrenal gland axis regulation and affiliative and social behaviors (Fleming et al., 1999), in which yawning and grooming play a central role (Collins and Eguibar, 2010; Melis et al., 2019). So, nurture had a strong influence on behavior, but it is not the case with yawning and penile erections in our experimental conditions. Because yawning is an emphatic behavior that is also related to communication, it is highly remarkable that maternal care did not influence the spontaneous expression of yawning, and in the case of HY male rats, they were noted as the rats that yawned more with respect to LY and SD rats independent of the subline or SD dams that raised them. Another possibility is that social interaction between the dams and their littermates can reverse the effects due to the quality and quantity of maternal care.

A similar trend was obtained with spontaneous penile erections, with HY rats showing more of this spinal reflex without any sexual context, neither motivational nor somesthetic. In future experiments, we will analyze the penile erections induced by an inaccessible estrous female, the so-called noncontact penile erections, or in the case of the retraction of the preputial skin as a mechanism to increase the frequency of penile erections in both sublines (Sachs et al., 1988; Sachs, 2000). As we already demonstrated in sexually experienced male HY rats, they showed a different copulatory pattern because they had more sexual bouts, with longer interintromission intervals that delayed ejaculation (Eguibar et al., 2016). It is quite relevant that HY dams are able to significantly increase the number of penile erections in the LY male rats, and this effect is probable due to an increase in the genital grooming given by HY dams because the number of penile erections in adulthood is strongly dependent on the genital grooming displayed in the first week of life (Moore, 1986a,b). This is the first report that shows an increase in the number of penile erections after a cross-fostering technique, which implies that early-life experiences have an important impact on the central and/or peripheral mechanisms that participate in penile erections. Another possible explanation is that maternal care is capable of changing oxytocinergic and dopaminergic neurotransmission and perhaps nitric oxide levels in key integrative areas in the hypothalamus, such as in the PVN of the hypothalamus, which are capable of increasing penile erections (Argiolas, 1994; Giuliano and Rampin, 2000). In fact, the local administration of oxytocin, dopaminergic agonist, or even nitric oxide increased the number of penile erections, supporting a central role of the PVN (Argiolas, 1994).

In a previous study, we showed that HY male rats have a higher percentage of noncopulators with respect to SD rats (Portillo et al., 2010). In Roman high-avoidance and in the novel exploration higher responder sublines, all of them showed deficits in sexual behavior that are proposed to be due to changes in the dopaminergic tone (Melis et al., 2019). Another group of rats selected on the basis of susceptibility to cholinergic agonists was the sensible and resistant Flinders sublines, FSL and FSR, respectively. These two groups of rats also differed in their sexual performance because both had a marked decrease in the ejaculatory frequencies and reached exhaustion sooner with respect to SD controls (Ferreira-Nuño et al., 2002). Importantly, the FSL rats had higher levels of anxiety and depressive-like behavior that were like those obtained in the LY subline of rats (Overstreet, 1993; Overstreet et al., 2005; Eguibar et al., 2017b). When cross-fostered with SD dams, FSL rats did not show a change in depressive-like symptoms in the forced swing test, and in adulthood, an SD liter showed clearly deteriorated depressive-like behavior when raised by FSL dams (Malkesman et al., 2008). Similar results were obtained when cross-fostering was used between stress-vulnerable and stress-resilient rats (Uchida et al., 2010).

These results are relevant, considering that HY male rats being less emotionally reactive (Moyaho et al., 1995) and having an increased frequency of grooming bouts when exposed to a novel environment (Eguibar and Moyaho, 1997), supporting this hypothesis. A recent experiment showed that HY rats explore and enter more the open arms of an elevated plus maze, supporting that they are resilient to stress manipulations (Eguibar et al., unpublished results). Cross-fostering between Lewis rats, a group of rats with higher anxiety rates, and Fischer 344 rats, which are resilient to stress, is relevant. When cross-fostering was performed, dams induced a nonsignificant increase in the offspring's time spent in the open arms in the elevated plus-maze, but they were able to induce an increase in the offspring's exploration of the center in an open-field arena, supporting that maternal care was able to change the anxiety responses (McCarty, 2017; Siviy et al., 2017).

Another relevant aspect is that maternal care can impact spontaneous penile erections, but this is not the case when we determined D_2-like dopaminergic agonist-induced yawning and penile erections, in which the HY phenotype had higher frequencies independent of the dams that raised them. It was unexpected that LY dams were capable of increasing dopamine D_2 receptor expression in the striatum in SD male rats, supporting that, during neurodevelopment, maternal care and the presence of their siblings and nest environment are capable of changing dopaminergic function independent of the genetic background of the male rats, but not in the (−)-quinpirole-induced yawning and penile erections. We use a cumulative dosing because it allows us to quickly evaluate behavioral responses to this dopaminergic agonist in a valuable group of rats as they are the in- and cross-fostered which require shorter time to obtain a dose–response curve with similar distribution

to that obtained with other dose schemes. Previously this type of approach has been used in rats under food restriction that decreases or with high-fat diet that enhances sensitivity to the actions of quinpirole (Baladi and France, 2009). It is also the case with streptozotocin-treated rats that had a reduced response to quinpirole-induced yawning (Sevak et al., 2007).

Additionally, (−)-quinpirole decreased grooming bouts and abolished scratching; in the case of grooming, this effect was mainly due to the stimulation of D_1 receptors (Berridge and Aldridge, 2000a,b; Eguibar et al., 2003). Our results showed a clear inhibitory effect in all groups of rats, with the HY rats being more sensitive than the LY and SD rats, supporting the participation of D_2-like dopamine receptors in the modulation of this behavior. Dopamine had a modulatory role in the striatum because it is a key component for the serial order of the sequence of grooming components, including the transitions in the face components through the lateral parts of the body (Aldridge and Berridge, 1998). It is relevant that in mice with a knockdown mutation in the dopamine transporter gene, extracellular dopamine levels in the striatum increase by 170% with respect to wild-type counterparts, and they show an increased grooming stereotypy that emulates obsessive–compulsive disorder (Berridge et al., 2005). In animal models of obsessive–compulsive symptoms, there were excessive dopamine levels, and it is relevant that subchronic treatment of (−)-quinpirole produced enhanced checking behavior in the open-field arena (Szechtman et al., 1998). Our results of decreased grooming support a role for dopamine D_2 receptors in this behavior, which can be a display of obsessive–compulsive disorder, as previously reported (Robbins et al., 2019), but further experimental data are necessary to support the role of each type of dopamine D_2-like receptor. HY male rats had higher D_1 binding in the striatum with respect to LY rats, which supports their higher grooming bout frequency (Eguibar and Moyaho, 1997; Díaz-Romero et al., 2005).

Scratching behavior depends directly on a spinal command and can be modulated by higher brain centers that are capable of exciting or inhibiting the activity of the scratch generator (Gelfand et al., 1988). On the other hand, central administration of gastric releasing hormones and related peptides, such as bombesin, induced scratching, and this spinal reflex can be inhibited by the administration of D_1 antagonists, such as SCH 23390 (Van Wimersma Greidanus and Maigret, 1991), supporting again a role of dopamine neurotransmission in scratching. In HY rats, intracerebroventricular administration of bombesin significantly reduced yawning frequency and increased scratching independent of grooming bouts (Díaz-Romero et al., 2002).

CONCLUSION

Our results showed that yawning is a stereotyped and genetically determined behavior with little influence of initial stages of development, including maternal care and nesting conditions and sibling interactions during the lactation period. In fact, the behavioral responses after the administration of dopaminergic D_2-like agonist (−)-quinpirole clearly showed higher responses in the HY male rats, supporting a strong role of the genetic background.

In future experiments, we will analyze the specific role of dopaminergic neurotransmission in the PVN of the hypothalamus or in the striatum by applying dopaminergic agonists directly to each area. The increase in spontaneous penile erections in LY male rats raised by HY dams and the increase in D_2 receptor expression levels induced by LY dams In SD male rats require further experimental data, but these results support a specific role of maternal care in the expression of yawning and penile erections changing some biochemical characteristics in the central nervous system taking into account due to the advantage of high spontaneous expression of yawning and penile erections of HY subline with respect to SD and even Wistar rats.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article and are available by request.

ETHICS STATEMENT

The animal study was reviewed and approved by Benemérita Universidad Autónoma de Puebla Animal Care and Use Committee No. EGC SAL-G-2019.

AUTHOR CONTRIBUTIONS

Tasks of individual authors: JE and CC: conceptualization. ÁD-N, ÁC, CC, AU, AT, and JE: data curation and investigation. ÁD-N, ÁC, HC-F, and JE: formal analysis. CC and JE: funding acquisitions and resources. ÁD-N, CC, ÁC, and JE: methodology. ÁD-N, CC, ÁT, and JE: writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00020/full#supplementary-material.

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Pharmacological Studies on the Role of 5-HT_{1A} Receptors in Male Sexual Behavior of Wildtype and Serotonin Transporter Knockout Rats

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Brain serotonin (5-HT) neurotransmission plays an important role in male sexual behavior and it is well established that activating 5-HT_{1A} receptors in rats facilitate ejaculatory behavior. However, the relative contribution of 5-HT_{1A} somatodendritic autoreceptors and heteroreceptors in this pro-sexual behavior is unclear. Moreover, it is unclear whether the contribution of somatodendritic 5-HT_{1A} autoreceptors and postsynaptic 5-HT_{1A} heteroreceptors alter when extracellular 5-HT levels are chronically increased. Serotonin transporter knockout ($SERT^{-/-}$) rats exhibit enhanced extracellular 5-HT levels and desensitized 5-HT_{1A} receptors. These rats model neurochemical changes underlying chronic SSRI-induced sexual dysfunction. We want to determine the role of presynaptic versus postsynaptic 5-HT_{1A} receptors in the pro-sexual effects of 5-HT_{1A} receptor agonists in $SERT^{+/+}$ and in $SERT^{-/-}$ rats. Therefore, acute effects of the biased 5-HT_{1A} receptor agonists F-13714, a preferential 5-HT_{1A} autoreceptor agonist, or F-15599, a preferential 5-HT_{1A} heteroreceptor agonist, and S15535 a mixed 5-HT_{1A} autoreceptor agonist/heteroreceptor antagonist, on male sexual behavior were assessed. A clear and stable genotype effect was found after training where $SERT^{+/+}$ performed sexual behavior at a higher level than $SERT^{-/-}$ rats. Both F-15599 and F-13714 induced pro-sexual activity in $SERT^{+/+}$ and $SERT^{-/-}$ animals. Compared to $SERT^{+/+}$, the F13714-dose-response curve in $SERT^{-/-}$ rats was shifted to the right. $SERT^{+/+}$ and $SERT^{-/-}$ rats responded similar to F15599. Within both $SERT^{+/+}$ and $SERT^{-/-}$ rats the potency of F-13714 was much stronger compared to F-15599. S15535 had no effect on sexual behavior in either genotype. In $SERT^{+/+}$ and $SERT^{-/-}$ rats that were selected on comparable low sexual activity ($SERT^{+/+}$ 3 or less ejaculations and $SERT^{-/-}$ 5 or less ejaculations in 10 weeks) S15535 also did not influence sexual behavior. The two biased compounds with differential effects on 5-HT_{1A} auto- and hetero-receptors, exerted pro-sexual activity in both $SERT^{+/+}$ and

SERT$^{-/-}$ rats. Applying these specific pharmacological tools has not solved whether pre- or post-synaptic 5-HT$_{1A}$ receptors are involved in pro-sexual activity. Moreover, the inactivity of S15535 in male sexual behavior in either genotype was unexpected. The question is whether the *in vivo* pharmacological profile of the different 5-HT$_{1A}$ receptor ligands used, is sufficient to differentiate pre- and/or post-synaptic 5-HT$_{1A}$ receptor contributions in male rat sexual behavior.

Keywords: serotonin, male sexual behavior, rat, 5-HT$_{1A}$ receptor, serotonin transporter, 5-HT$_{1A}$ autoreceptors, 5-HT$_{1A}$ heteroreceptors

INTRODUCTION

The serotonergic system plays an important modulatory role in sexual behavior (Uphouse and Guptarak, 2010; Olivier et al., 2019). This is, for example, illustrated by the effects of chronic SSRI treatment in depressed patients that results in enhanced 5-HT levels often causing sexual dysfunctions like in men delayed ejaculation and libido problems (Segraves and Balon, 2014). Early studies in male rats identified 5-HT$_{1A}$ receptor (R) agonists like 8-OH-DPAT, the azapirones (e.g., buspirone, ipsapirone, and gepirone) and others (e.g., flesinoxan) as pro-sexual drugs (Ahlenius et al., 1981; Ahlenius and Larsson, 1985; reviewed in: Snoeren et al., 2014). The prototypal 5-HT$_{1A}$ receptor agonists (±) and (+) – 8-OH-DPAT, potently stimulate male rat sexual behavior; in a certain time frame (e.g., 30 min), the number of ejaculations increases associated with shortened ejaculation latencies and fewer intromissions to reach ejaculation (Hillegaart and Ahlenius, 1998; Uphouse and Guptarak, 2010; Chan et al., 2011). Although (±) 8-OH-DPAT has also 5-HT$_7$ R agonistic effects (Thomas et al., 1999), this mechanism cannot explain the pro-sexual effects because other 5-HT$_{1A}$ receptor agonists without 5-HT$_7$ R agonistic activity, also display pro-sexual effects (Snoeren et al., 2014). 5-HT$_{1A}$ receptors are present as presynaptic inhibitory autoreceptors on soma and dendrites of raphé serotonergic neurons projecting to many forebrain areas (Fernandez-Guasti et al., 1992; Le Poul et al., 1995; Marek, 2010; Altieri et al., 2013). Moreover, 5-HT$_{1A}$ receptors are also present as post-synaptic heteroreceptors in various brain areas, mainly in the forebrain (Frink et al., 1996; Garcia-Garcia et al., 2017). Systemic acute administration of non-selective 5-HT$_{1A}$ receptor agonists like (±)-8-OH-DPAT and flesinoxan (activation of pre- and post-synaptic receptors) leads to decreased serotonergic release, but at the same time to activation of post-synaptic 5-HT$_{1A}$ heteroreceptors (Müller et al., 2007; Lladó-Pelfort et al., 2012). These non-selective 5-HT$_{1A}$ receptor agonists often display biphasic dose-response curves, and it is suggested that low doses of 8-OH-DPAT preferentially activate autoreceptors, whereas higher doses of 8-OH-DPAT preferentially activate post-synaptic heteroreceptors (De Vry et al., 2004; Depoortère et al., 2019). However, in male sexual behavior 8-OH-DPAT exerts a linear dose-dependent increase in sexual activities, without any evidence for differential effects on 5-HT$_{1A}$ auto- or heteroreceptors (Mos et al., 1991). The pro-sexual effects of these non-selective 5-HT$_{1A}$ receptor agonists are therefore not yet explained in terms of pre- or post-synaptic mechanisms. To further explore the role of pre- and post-synaptic 5-HT$_{1A}$ receptors in male sexual behavior, more recently developed selective and high-affinity 5-HT$_{1A}$ receptor agonists are useful. These so-called "biased" or "functionally selective" agonists (Newman-Tancredi, 2011; Garcia-Garcia et al., 2014) display selectivity for either pre- or post-synaptic 5-HT$_{1A}$ receptors. F15599 is a high-affinity, selective 5-HT$_{1A}$ receptor agonist (Ki = 3.4 nM) for post-synaptic 5-HT$_{1A}$ heteroreceptors, whereas F13714 (Ki = 0.1nM) is a preferential 5-HT$_{1A}$ autoreceptor agonist (Koek et al., 2001; de Boer and Newman-Tancredi, 2016; Hazari et al., 2017). In contrast to 8-OH-DPAT, both F15599 (Newman-Tancredi et al., 2009) and F15714 (Assié et al., 2006) are devoid of 5-HT$_7$ receptor activity. We studied both compounds in a dose-response study in male rat sexual behavior. Another high-affinity (Ki = 1.8 nM) 5-HT$_{1A}$ receptor ligand, S-15535 acts *in vivo* as a preferential agonist at presynaptic autoreceptors and as antagonist at post-synaptic 5-HT$_{1A}$ heteroreceptors (Millan et al., 1993; Carli et al., 1999).This compound is an interesting tool to study in male sexual behavior as it may shed further light on the complex role of 5-HT$_{1A}$ receptors in male rat sexual behavior.

As mentioned before, chronic SSRI treatment results in enhanced 5-HT levels often causing sexual dysfunctions (Segraves and Balon, 2014). The exact mechanisms for these dysfunctions remain unclear, but are high likely due to alterations in the 5-HT$_{1A}$ receptor. Male rats lacking the serotonin transporter (SERT$^{-/-}$) display a robust genotype that has a lower basal ejaculatory performance than wildtype rats (SERT$^{+/+}$) or heterozygous serotonin transporter knockout (SERT$^{+/-}$) rats (Chan et al., 2011; Esquivel-Franco et al., 2018). More specific, due to the lack of the serotonin transporter SERT$^{-/-}$ rats have a nine-fold increase in extracellular 5-HT levels (Homberg et al., 2007), decreased number of ejaculations and an increased ejaculation latency (Chan et al., 2011) compared to SERT$^{+/+}$ rats. This genetic animal model has therefore been proposed and used as an animal model of spontaneous or SSRI-induced delayed ejaculation in humans. Chronic SSRI use in men may result in several side-effects including increased ejaculation threshold, resulting in a delayed ejaculation latency or sometimes even absent ejaculation, associated with a reduction in sexual desire (Waldinger et al., 1998; Hirschfeld, 2003; Balon, 2006; Rubio-Casillas et al., 2015). This is believed to be caused by the combination of enhanced 5-HT levels and diminished 5-HT$_{1A}$ receptor functioning (both pre- and post-synaptic) similar to chronic SSRI-treatment in normal animals (Chan et al., 2011),

or short acting SSRIs like dapoxetine in fast ejaculating rats (Clément et al., 2012). Although conflicting findings on the effects of acute and chronic SSRI treatment have been reported, Clément et al. (2012) mention this is explained by distinct pharmacokinetics rather than pharmacodynamic properties as dapoxetine has rapid peak plasma concentrations which delays ejaculation frequencies in men with premature ejaculation. In rats the dapoxetine profile is less clear, although it is suggested by the authors that in faster ejaculating rats dapoxetine seems to delay the ejaculation latency (Clément et al., 2012). In particular this 5-HT$_{1A}$ receptor desensitization phenomenon is relevant here to further provide more clarity as to the potency of the biased agonists to stimulate sexual behavior. SERT$^{-/-}$ rats have higher extracellular serotonin levels than SERT$^{+/+}$ animals which is comparable to levels after chronic SSRI administration (Homberg et al., 2007). Pharmacological experiments in these rats indicated that rats lacking the SERT have altered 5-HT$_{1A}$ receptor reactivity; the altered 5-HT$_{1A}$ receptor functioning is probably not a global phenomenon, but might be limited to some specific subpopulations of 5-HT$_{1A}$ receptors (not necessarily pre- or post-synaptic), as indicated by changed autonomic responses like core body temperature in SERT$^{+/+}$ and SERT$^{-/-}$ animals. The 5-HT$_{1A}$ receptor population involved with hypothermia was not sensitive, while the 5-HT$_{1A}$ receptor population involved with hyperthermia was still sensitive (Homberg et al., 2008; Olivier et al., 2008). Experiments performed in male sexual behavior (Chan et al., 2011) also indicated that likely at least two populations of 5-HT$_{1A}$ receptors are involved in its expression. However, it is worthwhile to mention that 5-HT$_{1A}$ receptors can co-localize with 5-HT$_7$ receptors in the cell-membrane (Renner et al., 2012). It has been postulated that heterodimerization of these receptors may influence the desensitization of 5-HT$_{1A}$ autoreceptors caused by SSRIs (Naumenko et al., 2014). In addition, 5-HT$_{1A}$ receptors are G-protein coupled receptors activating different intracellular signaling pathways, which are brain region specific. Activation of different G-protein cascades may therefore play a role in the activation of 5-HT$_{1A}$ receptors in specific cellular environments, while having no effect on other subpopulations of the same receptor (Newman-Tancredi, 2011). For performing sexual behavior, activation of one population of 5-HT$_{1A}$ receptors is needed and we postulated that this pool is desensitized in SERT$^{-/-}$ rats. The pro-sexual effects of 8-OH-DPAT are probably mediated via 5-HT$_{1A}$ receptors, which are not changed or somewhat less sensitive in SERT$^{-/-}$ rats. This difference makes the SERT$^{-/-}$ rat a further attractive model to test the different 5-HT$_{1A}$ receptor-modulating drugs, F15599, F13714 and S-15355, as it may provide information on the adaptation of pre- and post-synaptic 5-HT$_{1A}$ receptors due to chronic high 5-HT levels, which may aid in the treatment of sexual dysfunction caused by SSRI treatment.

Finally, we selected male rats that, after extensive training (Pattij et al., 2005), display a low level of sexual behavior, i.e., low number of ejaculations. Because 5-HT$_{1A}$ receptor agonists facilitate ejaculation, a too high initial level of the number of ejaculations would probably interact with the pro-sexual effects of these drugs. The purpose of this study was to use functionally selective agonists for either pre- or post-synaptic 5-HT$_{1A}$ receptors to identify the role of somatodendritic (auto) receptors and post (hetero) receptors in sexual behavior. A second goal was to use SERT$^{-/-}$ rats because they model SSRI-induced delayed ejaculation in humans, and hence may provide insight in the adaptation of specific 5-HT$_{1A}$ hetero- or auto receptors due to chronic increased extracellular 5-HT levels. Thus, we investigated whether chronic exposure to high 5-HT levels affected the pro-sexual effects of 5-HT$_{1A}$ agonists, and whether pre- or post-synaptic receptors were differently affected in SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats. We used F13714, F15599, and S15535 in normal (SERT$^{+/+}$) and SERT$^{-/-}$ rats, and hypothesized that these drugs would have differential effects on sexual behavior and that SERT$^{-/-}$ rats would display desensitized response to these drugs.

MATERIALS AND METHODS

Animals

Wistar rats were bred in our animal facility (University of Groningen, GELIFES) using serotonin transporter (SERT) heterozygous males and females, resulting in male and female SERT wild type (SERT$^{+/+}$), heterozygous (SERT$^{\pm}$) and homozygous or knock out (SERT$^{-/-}$) rats. On postnatal day 21 pups were weaned and ears were punched for individual recognition and genotyped as reported previously (El Aidy et al., 2017). We used two groups of animals, the first one (normal ejaculating rats) consisting of sixty-three male SERT (SERT$^{+/+}$, n = 32), and (SERT$^{-/-}$, n = 31) rats and the second one (slow ejaculating rats) of 32 male (16 SERT$^{+/+}$ and 16 SERT$^{-/-}$) rats, all of them of at least 12 weeks old when used for sexual behavior experiments.

Female SERT$^{\pm}$ and SERT$^{+/+}$ were used as sexual stimulus females (n = 120) as SERT$^{+/+}$ and SERT$^{\pm}$ rats do not differ in basal sexual activity (Snoeren et al., 2010). Rats were housed under reversed dark-light conditions (12 h light:12 h dark, lights off from 8:00 AM to 8 PM). After 6-weekly training tests (30 min/test), male rats were considered sexually trained and classified based on ejaculation frequencies per test. Male rats display, after extensive training, a rather stable sexual phenotype (Pattij et al., 2005; Olivier et al., 2006; Chan et al., 2008). In these experiments, for the normal ejaculating 24 rats were selected (from 14 different dams, a maximum of 3 SERT$^{+/+}$ and/or 3 SERT$^{-/-}$ rats were used from the same litter) that showed a normal ejaculatory phenotype (between 1 and 2 ejaculations per test after training, for the last three sessions) and for the slow ejaculating rats 20 (from 8 dams, a maximum of 5 SERT$^{+/+}$ and/or 2 SERT$^{-/-}$ rats were used from the same litter) that showed a rather low sexual phenotype (between 0 and 1 ejaculation per test after training, for the last three sessions) were selected. We summed all ejaculations per rat for all training weeks in **Supplementary Figure 1** (group 1) and 2 (group 2). The most left tail-side of the distribution was selected. Animals were socially housed (2–5 per cage, maximum 4 for males). Cages were enriched with wooden gnawing blocks and nesting material (Envirodri).

Thus, to select normal ejaculating rats 32 SERT$^{+/+}$ and 31 SERT$^{-/-}$ rats were sexually trained for 6 weeks and a total

of 12 SERT$^{+/+}$ and 12 SERT$^{-/-}$ rats were selected with a normal average number of ejaculations. For selection of the slow ejaculating rats 16 SERT$^{+/+}$ and 16 SERT$^{-/-}$ rats were sexually trained for 6 weeks and a total of 10 SERT$^{+/+}$ and 10 SERT$^{-/-}$ rats were selected with a normal and low average number of ejaculations (because this enhances the sensitivity of the anticipated improvement in sexual behavior by the 5-HT$_{1A}$ compounds and to match the control group as much as possible to the knock-out animals). Experiments in the normal ejaculating rats lasted 13 weeks in total (after training), and 4 weeks in total (after training) for sow ejaculating rats. Animals were used only once a week to guarantee sufficient drug washout time. Rats had *ad libitum* access to food and water. This study was carried out in accordance with the principles of the EU Directive 2010/63/EU. All efforts were made to minimize the number of animals and their suffering.

Female Rats

Female stimulus rats were tubal ligated in order to prevent pregnancies. To perform tubal ligation surgery, females were anesthetized (Isoflurane) and given pain relief (Fynadine, 0.1 mg/100 g) before surgery, and 24 and 48 h after surgery. Females were at least 12 weeks old when surgery was performed, and 2 weeks of recovery were given before they were made intentionally receptive with estradiol (50 μg in 0.1 ml oil, S.C., 36–48 h before the test) before the sexual behavior training tests and experiments. Females were used not more than once in 2 weeks and not more than two times per experimental day.

Drug Treatment and Behavioral Experiments

For the first experiment in normal ejaculating rats, animals received all dosages of F13714 and F15599 in a crossover-randomized design in order to prevent order effects; after this experiment, S15535 was administered in a randomized design. For the second experiment in slow ejaculating rats, animals were only administered S15535 in a randomized design similar to the first set of animals. As described previously in Olivier et al. (2017), when pharmacological tests are performed, male rats are given a 30-min habituation time in the test boxes right after drug administration via IP injection, before the female rat is introduced. All behavior during the 30-min test is video-recorded after introduction of the female and were also live-scored; the following parameters of the ejaculation series were deduced (Chan et al., 2011): number of ejaculations/test (E), number of mounts (M), number of intromissions (I), latency (s) to first mount (ML), latency (s) to first intromission (IL) and latency (s) to the first ejaculation (EL). After ejaculation, the post ejaculatory latency (PEL(s)) was calculated, using the time from the first ejaculation and the time of the first mount/intromission (whatever occurred first) of the second ejaculation series. Intromission Ratio (IR) was calculated as: IR = (#I/(#I + #M)) × 100%. EL was calculated using the time of the EL from the first ejaculation series minus the intromission latency of the first ejaculation series (EL = EL – IL). These parameters were used to run the statistical analysis.

Because it is important to have comparable pharmacodynamics and kinetics in pharmacological studies, a test of fixed duration has been chosen: 30 min (1800 s). In the cases where drug-treatment had no "effect" on ejaculation and sexual behavior, or few or no animal achieved a first ejaculation it was not possible to perform statistical analyses and for those cases we assigned values of 1800 s (i.e., the maximum test duration) for some latencies (ejaculation, mount and intromission latency), although this is undoubtedly a matter of discussion as we have discussed before (Chan et al., 2011; Olivier et al., 2017). All tables and figures show the results for the first Ejaculation Series only.

Drugs

F15599 and F13714 (Pierre Fabre Pharmaceuticals, France; Lot # SBR1401003 and # JLM3001201, resp.) and S-15535 (Servier Pharmaceuticals, France; Lot B01JLP061A) were dissolved in NaCl 0.9% (saline) and each solution was freshly prepared on each testing day. All drugs were administered via intraperitoneal (IP) injection 30 min before the test.

Training (Table 1)

For the normal ejaculating group, rats were sexually trained for 6 times (30 min, once a week). For the slow ejaculating group, rats were sexually trained 10 times (30 min, once a week). The latter animals received extra training due to the extreme low sexual performance to assess and stabilize their basal sexual activity. Rats habituated for 10 min to the testing box right before the training session. After the habituation period a receptive female was introduced in the box and sexual behavior was assessed for 30 min. Non-receptive females were switched for a different receptive female. The training and testing occurred in wooden rectangular (57 cm × 82 cm × 39 cm; glass wall) testing boxes filled with regular bedding material. To stimulate sexual behavior, bedding material was not changed during the training and testing to preserve pheromones of previous rounds and to create a more competitive sexual environment.

Only males showing stable normal (1–2 ejaculations, for experiments 1 and 2 and low (0–1 ejaculations for experiment 3) ejaculation levels in the last three tests were used in the

TABLE 1 | Overview of training of the various genotypes and pharmacological experiments in selected male rats.

Selection	# Rats trained	# Rats in pharmacological experiments	Drug (doses) tested
Normal ejaculating rats	32 SERT$^{+/+}$	Exp. 1: 12 SERT$^{+/+}$	F15599 (0.01,0.04, 0.16,
	31 SERT$^{-/-}$	12 SERT$^{-/-}$	and 0.64-mg/kg, IP) F13714 (0, 0.0025, 0.01, 0.04, and 0.16-mg/kg, IP)
		Exp. 2: 12 SERT$^{+/+}$ 12 SERT$^{-/-}$	S15535 (0, 0.25, 1, and 4-mg/kg, IP)
Slow ejaculating rats	16 SERT$^{+/+}$	Exp. 3: 10 SERT$^{+/+}$	S15535 (0, 0.25, 1, and 4-mg/kg, IP)
	16 SERT$^{-/-}$	10 SERT$^{-/-}$	

pharmacological experiments. For Experiments 1 and 2 (normal ejaculating rats) 24 rats were selected (N = 12 per genotype). In Experiment 3 (slow ejaculating rats) 20 animals were selected (10 per genotype). All training sessions and experiments were performed under red light conditions between 10:00 AM and 17:00 PM.

Pharmacological Experiments (Table 1)

Experiment 1 (Normal Ejaculating Rats): F15599 and F13714 Dose Response

Twenty-four normal ejaculating male rats were selected (N = 12 per SERT genotype) and were tested in a crossover design. Rats received vehicle (saline), 0.01, 0.04, 0.16, and 0.64-mg/kg F15599 and 0.0025, 0.01, 0.04, and 0.16-mg/kg F13714 via intraperitoneal (IP) administration. Experiments were performed once per week on the same testing day, over 9 weeks and animals and treatment were randomized over the 9 weeks. Although the experiments with these two drugs were performed together, we performed the statistical analysis separately for each compound.

Experiment 2 (Normal Ejaculating Rats): S15535 Dose Response

The same 24 animals from experiment one received vehicle (saline), 0.25, 1 and 4-mg/kg S15535, IP in a randomized design. Testing was performed over 4 weeks and always on the same day per week.

Experiment 3 (Slow Ejaculating Rats): S15535 Dose Response

10 SERT$^{+/+}$ and 10 SERT$^{-/-}$ rats were selected for low numbers of ejaculation. Rats received vehicle (saline), 0.25, 1, and 4-mg/kg S15535, via IP administration in a randomized design. Testing was performed over 4 weeks and always on the same day per week.

Statistical Analyses

Differences in baseline ejaculation numbers during the training between genotypes were analyzed using two-way ANOVA for repeated measures, with genotype as between- and time (weeks) as within-subjects factors. Where appropriate, an independent t-test was performed. For the F19955, F13714, and S15535 dose-response experiments, a two-way ANOVA for repeated measures was performed with dose as within-subject factor (5 levels) and genotype as between-subject factor (2 levels). Where appropriate one way-ANOVA with LSD *post hoc* was performed. All statistical analyses were performed using the Statistical Package for Social Sciences for Windows version 25 (LEAD technologies, Chicago, United States). Level of significance was set at $p < 0.05$.

RESULTS

Sexual Stability

The sexual performance of the selected experimental animal groups that exhibited a normal (1–2 ejaculations) and a low basal ejaculation frequency (0–1 ejaculation) during the six training days was registered and from the 63 male rats sexually trained from the first group and 32 from the second group, only 24 and 20 animals (respectively) that showed stable normal and low sexual performance and ejaculations respectively, were selected to run the pharmacological studies (see **Supplementary Figures 1**, **2**). For the first group (selection for normal ejaculation rats), there was a significant week (time) effect $F_{(7,154)} = 13.86$, $p < 0.001$, a significant week × genotype effect $F_{(7,154)} = 3.40$, $p < 0.01$ and a significant genotype effect ($F_{(1,22)} = 23.81$, $p < 0.001$). In SERT$^{+/+}$ rats from week 3 onward they performed significant more ejaculations (all p-values are < 0.05) compared to the first 2 weeks (**Figure 1** and **Table 2**). In SERT$^{-/-}$ rats only week 16–20 significantly differed (all p-values are < 0.05) from all other weeks (**Figure 1** and **Table 2**). SERT$^{-/-}$ rats ejaculated significantly less compared to SERT$^{+/+}$ rats in week 3 ($p < 0.05$), week 4 ($p < 0.05$), week 5 ($p < 0.05$), week 6 ($p < 0.05$), weeks 7–14 (0.05), and weeks 16–20 ($p < 0.01$).

For the second group of animals trained (for selection of slow ejaculating rats), there was a significant difference in weeks of training ($F_{(10,180)} = 3.453$, $p < 0.001$). In week 11–14, SERT$^{+/+}$ and SERT$^{-/-}$ rats had significant more ejaculations compared to all other weeks (all p-values < 0.01). No significant differences in time × genotype, and genotype effects were found during the training weeks (**Figure 2** and **Table 3**).

We included in **Figure 1** the saline data gathered in the pharmacological experiments performed on animals in group one (selection for normal ejaculating rats). The saline data obtained for all animals in weeks 7–15 (Exp. 1) were comparable to the last training data, but the saline data from the last (S15535) experiment (Exp. 2) (during week 16–20) showed significantly higher values. This "enhanced" baseline level of sexual behavior made us decide (because of possible ceiling effects) to repeat the S15535 experiment in rats with very low levels of sexual ejaculation activity (group two, Exp. 3: data shown in **Table 3**). In

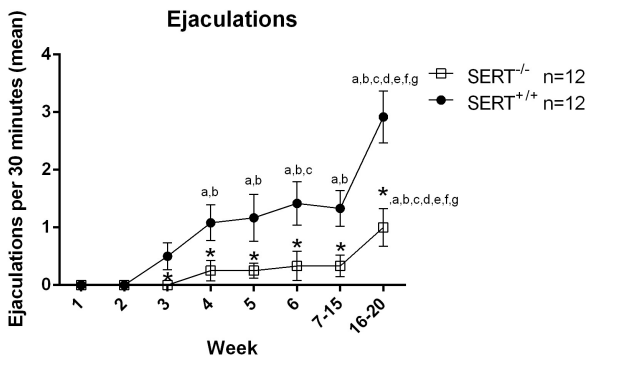

FIGURE 1 | Mean ejaculation frequencies (±SEM) over 6 weeks of training of male Wistar rats of group one (selection for normal ejaculating rats). Added are also the mean ± SEM of the saline data from experiment one (F13714 and F15599) and two (S15535) of group one. a: significantly different ($p < 0.05$) from week 1; b: significantly different ($p < 0.05$) from week 2;c: significantly different ($p < 0.05$) from week 3; d: significantly different ($p < 0.05$) from week 4; e: significantly different ($p < 0.05$) from week 5; f: significantly different ($p < 0.05$) from week 6; g: significantly different ($p < 0.05$) from week 7 to 15; *significantly different ($p < 0.05$) from SERT$^{+/+}$. Detailed statistical analyses are provided in **Table 2**.

TABLE 2 | Sexual Behavior performance during training weeks of male SERT$^{+/+}$ and SERT$^{-/-}$ Wistar rats from group 1 (selection of normal ejaculating rats).

SERT	Week								
	1	2	3	4	5	6	7–15	16–20	One way ANOVA time effect
	Mean ± SEM A	Mean ± SEM B	Mean ± SEM C	Mean ± SEM D	Mean ± SEM E	Mean ± SEM F	Mean ± SEM G	Mean ± SEM H	
+/+	0.0 ± 0.0	0.0 ± 0.0	0.50 ± 0.23	1.08 ± 0.31	1.16 ± 0.40	1.41 ± 0.37	1.33 ± 0.30	2.91 ± 0.45	$F_{(7.88)} = 9.37$; $p < 0.001$
				A,B	A,B	A,B,C	A,B	A,B,C,D,E,F,G	
−/−	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.25 ± 0.17	0.25 ± 0.13	0.33 ± 0.25	0.33 ± 0.18	1.00 ± 0.32	$F_{(7,88)} = 3.25$; $p < 0.01$
								A,B,C,D,E,F,G	
t-test genotype per week	ns	ns	*	*	*	*	*	*	
			$T_{(1,22)} = 2.171$, $p < 0.05$	$T_{(1,22)} = 2.311$, $p < 0.05$	$T_{(1,22)} = 2.154$, $p < 0.05$	$T_{(1,22)} = 2.370$, $p < 0.05$	$T_{(1,22)} = 2.760$, $p < 0.05$	$T_{(1,22)} = 3.443$, $p < 0.01$	
Two-way ANOVA repeated measures	Time (week) effect $F_{(7,154)} = 13.855$, $p < 0.001$								
	Time (week) × genotype effect $F_{(7,154)} = 3.396$, $p < 0.01$								
	Genotype effect $F_{(1,22)} = 23.807$, $p < 0.001$								

N = 12/group. A: significantly different from week 1; B: significantly different from week 2; C: significantly different from week 3; D: significantly different from week 4; E: significantly different from week 5; F: significantly different from week 6; G: significantly different from week 7–15; all p-values are < 0.05; ns: non significant.

FIGURE 2 | Mean ejaculation frequencies (±SEM) over 10 weeks of training of male Wistar rats of group two (selection for slow ejaculating rats). Added is also the mean ± SEM of the saline data from S15535 experiment (week 11–14). a: significantly different ($p < 0.05$) from week 1; b: significantly different ($p < 0.05$) from week 2; c: significantly different ($p < 0.05$) from week 3; d: significantly different ($p < 0.05$) from week 4; e: significantly different ($p < 0.05$) from week 5; f: significantly different ($p < 0.05$) from week 6; g: significantly different ($p < 0.05$) from week 7; h: significantly different ($p < 0.05$) from week 8; i: significantly different ($p < 0.05$) from week 9; j: significantly different ($p < 0.05$) from week 10; *significantly different ($p < 0.05$) from SERT$^{+/+}$. Detailed statistical analyses are provided in **Table 3**.

Figure 2 we also included the saline data gathered in the S15535 dose-response experiment (Exp. 3) of the second group (week 11–14). Again, an enhanced baseline level of sexual behavior was found in the saline treated animals during the S-155355 treatment weeks.

Dose-Response of F15599 (Figure 3 and Supplementary Table 1)

In the dose-response experiment significant dose ($F_{(4,88)} = 8.75$; $p < 0.001$) and genotype ($F_{(1,22)} = 22.278$; $p < 0.001$) effects, but no interactions, were found for the number of ejaculations. Comparable significances were found for ejaculation latencies and intromission ratios (see **Supplementary Table 1** for statistics of all behavioral parameters). Further analysis revealed that the lowest and intermediate doses of F15599 (0, 0.01, 0.04, and 0.16 mg/kg) had no significant effects on sexual behavior in either genotype (**Figure 3** and **Supplementary Table 1**). Compared to saline ($p < 0.001$), 0.01 ($p < 0.01$), and 0.04 ($p < 0.001$) mg/kg doses of F-15599, the highest dose (0.64 mg/kg) significantly increased the ejaculation frequency. Moreover, ejaculation latencies were significant shorter in 0.64 mg/kg F15599 compared to saline ($p < 0.01$), 0.01 mg/kg ($p < 0.05$), and 0.04 mg/kg ($p < 0.01$) F15599 (**Figure 3** and **Supplementary Table 1**) in both SERT$^{+/+}$ and SERT$^{-/-}$ animals; the 0.64 mg/kg F15599 dose also significantly increased the efficiency of the animals to ejaculate (IR; $p < 0.05$; **Figure 3** and **Supplementary Table 1**) compared to saline ($p < 0.01$), 0.01 mg/kg ($p < 0.05$), and 0.04 mg/kg ($p < 0.05$) of F15599.

TABLE 3 | Sexual Behavior performance during training weeks of male SERT$^{+/+}$ and SERT$^{-/-}$Wistar rats from group 2 (selection for slow ejaculating rats).

SERT	Week											
	1	2	3	4	5	6	7	8	9	10	11–14	One Way ANOVA Time effect
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	
	A	B	C	D	E	F	G	H	I	J	K	
+/+	0.0 ± 0.0	0.0 ± 0.0	0.20 ± 0.13	0.10 ± 0.10	0.20 ± 0.13	0.20 ± 0.20	0.10 ± 0.10	0.10 ± 0.10	0.10 ± 0.10	0.20 ± 0.20	0.70 ± 0.39	
−/−	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.09 ± 0.09	0.0 ± 0.0	0.18 ± 0.12	0.0 ± 0.0	0.09 ± 0.09	0.0 ± 0.0	0.09 ± 0.09	0.54 ± 0.24	
t-test genotype per week	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	$F_{(10,220)}$ = 3.418, $p < 0.001$
											A,B,C,D,E,F,G,H,I,J	
Two-way ANOVA repeated measure	Time (week) effect $F_{(10,180)} = 3.453$, $p < 0.001$											
	No time (week) × Genotype effect $F_{(10,180)} = 0.147$, n.s.											
	No genotype effect $F_{(1,18)} = 1.976$, n.s.											

N = 10 and 11, respectively. A: significantly different from week 1; B: significantly different from week 2; C: significantly different from week 3; D: significantly different from week 4; E: significantly different from week 5; F: significantly different from week 6; G: significantly different from week 7; H: significantly different from week 8; I: significantly different from week 9; J: significantly different week 10; all p-values are < 0.01; NA: non applicable.

A significant decrease in the number of ejaculations of SERT$^{-/-}$ rats was found compared to SERT$^{+/+}$ rats in the saline treatment ($p < 0.05$), and in the 0.01 mg/kg ($p < 0.05$), 0.04 mg/kg ($p < 0.05$), 0.16 mg/kg ($p < 0.05$), and 0.64 mg/kg ($p < 0.05$) F15599 treatment. Similarly, an increase in ejaculation latency was found for SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats in saline treatment ($p < 0.001$), and in 0.01 mg/kg ($p < 0.01$), 0.04 mg/kg ($p < 0.001$), 0.16 mg/kg ($p < 0.05$), and 0.64 mg/kg ($p < 0.001$) F15599 treatment. For the intromission ratio, a significant decrease was found for SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats in saline treatment ($p < 0.05$), and in the 0.01 mg/kg ($p < 0.01$), 0.04 mg/kg ($p < 0.05$), and 0.16 mg/kg ($p < 0.05$) F15599 treatment.

Dose-Response of F13714 (Figure 4 and Supplementary Table 2)

Overall, F13714 induced pro-sexual effects in both genotypes, although the dose-effect curves for both genotypes differed considerably (**Figure 4** and **Supplementary Table 2**). Considering ejaculations, significant dose ($F_{(4,88)} = 3.287$, $p < 0.05$), genotype ($F_{(1,22)} = 20.649$, $p < 0.001$), and genotype × dose interactions ($F_{(4,88)} = 4.810$, $p < 0.01$) were found. Comparable significances were found for ejaculation latencies (see **Supplementary Table 2** for statistics of all behavioral parameters). In SERT$^{+/+}$ rats, F13714 stimulated sexual behavior significantly, illustrated (compared to saline) in the increase in ejaculation frequencies at 0.0025 mg/kg ($p < 0.01$), 0.01 mg/kg (tendency; $p = 0.06$), and 0.04 mg/kg ($p < 0.05$) mg/kg F13714. In the SERT$^{-/-}$ rats, pro-sexual effects were observed only at the highest dose (0.16 mg/kg) compared to saline ($p < 0.05$) and 0.025 mg/kg ($p < 0.05$) of F13714. Although the ejaculation latency was decreased at this high dose for both genotypes, the difference was not statistically significant. The number of mounts was equally decreased in SERT$^{+/+}$ and SERT$^{-/-}$ rats at 0.16 mg/kg F13714 compared to saline ($p < 0.01$), 0.025 mg/kg ($p < 0.01$), 0.01 mg/kg ($p < 0.001$) and 0.04 mg/kg ($p < 0.05$) F13714. In SERT$^{+/+}$, but not in SERT$^{-/-}$, rats, the intromission latency was enhanced at the highest dose ($F_{(4,55)} = 4.203$, $p < 0.01$). The intromission latency at the highest dose (0.16 mg/kg) of F13714 was significant longer compared to saline ($p < 0.01$), 0.0025 mg/kg ($p < 0.001$), 0.01 mg/kg ($p < 0.01$), and 0.04 mg/kg ($p < 0.05$) F13714. Lastly, the number of intromissions was significantly decreased in SERT$^{+/+}$ rats only ($F_{(4,55)} = 8.194$; $p < 0.001$). Intromissions were significant reduced in animals treated with 0.16 mg/kg F13714 compared to saline ($p < 0.001$), 0.0025 mg/kg ($p < 0.01$) and 0.01 mg/kg ($p < 0.01$) F13714 treated SERT$^{+/+}$ rats. In addition, 0.04 mg/kg F13714 treated SERT$^{+/+}$ rats had a significant reduced number of intromissions compared to those treated with saline ($p < 0.001$), 0.0025 mg/kg ($p < 0.01$), and 0.01 mg/kg ($p < 0.05$) F13714.

SERT$^{-/-}$ rats had significant lower ejaculation frequencies compared to SERT$^{+/+}$ rats after treatment with saline ($p < 0.05$), and after treatment with 0.0025 mg/kg ($p < 0.01$), 0.01 mg/kg ($p < 0.01$), and 0.04 mg/kg ($p < 0.01$) F13714. For mounts, only at a dose of 0.04 mg/kg F13714 SERT$^{-/-}$ rats showed a

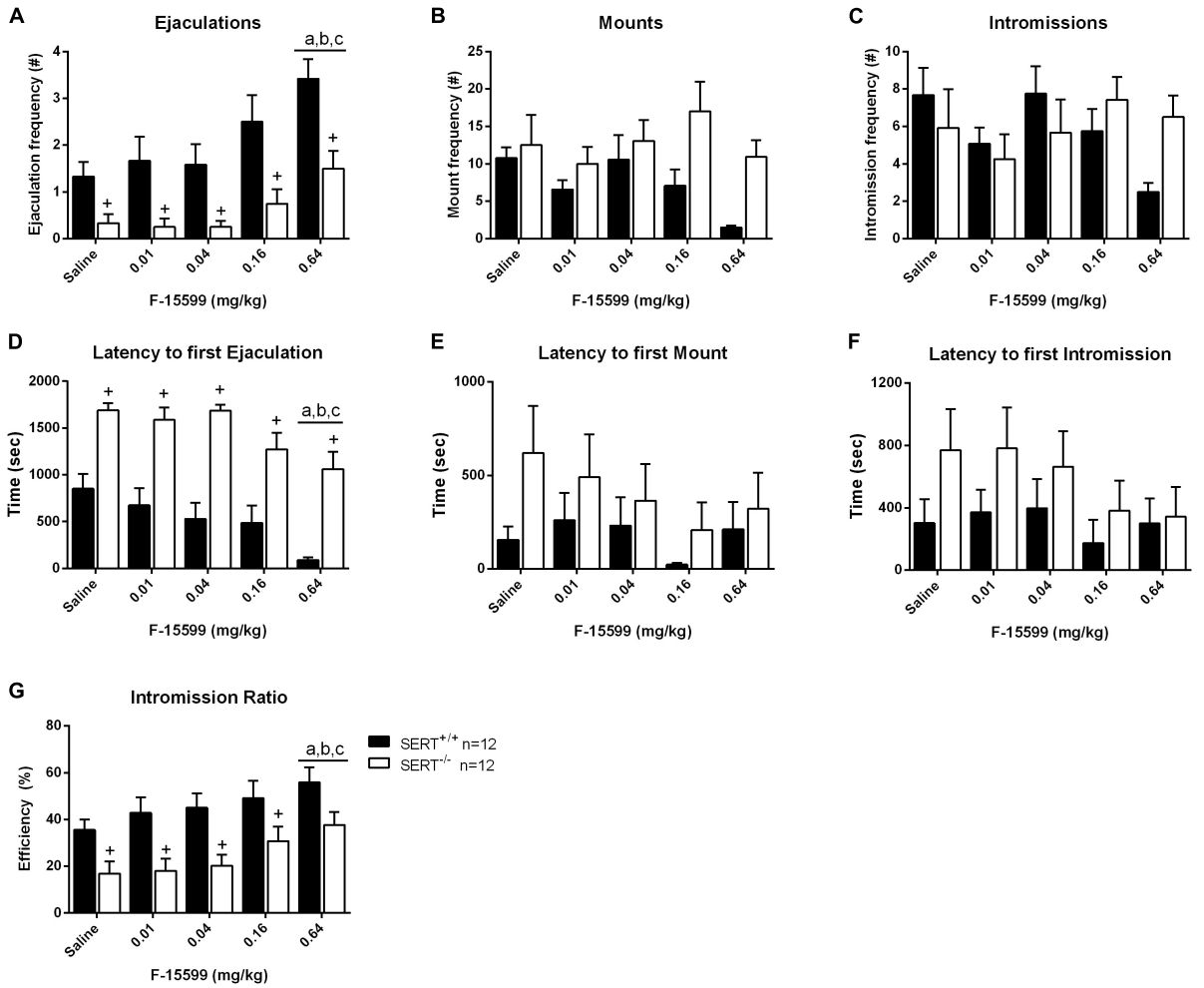

FIGURE 3 | Sexual behavior of male rats treated with 0, 0.01, 0.04, 0.16, or 0.64 mg/kg, IP of F-15599. The number and latency of ejaculations per 30 min **(A,D)**, number and latency of Mounts **(B,E)**, number and latency of Intromissions **(C,F)** and Intromission Ratio **(G)** of the first Ejaculation Series are displayed. Detailed statistical analyses are provided in **Supplementary Table 1**. a: significant difference ($p < 0.05$) compared to saline group, b: significant difference ($p < 0.05$) compared to 0.01mg/kg group, c: significant difference ($p < 0.05$) compared to 0.04/mg/kg group. +Significant difference between SERT$^{+/+}$ and SERT$^{-/-}$ ($p < 0.05$).

significant higher mount frequency ($p < 0.01$) compared with SERT$^{+/+}$ rats. At the same dose SERT$^{-/-}$ rats also showed a higher intromission frequency compared to SERT$^{+/+}$ rats. For latency to the first ejaculation a significant increase was found for SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats for all doses (all p-values < 0.01). The latency to the first mount was significantly higher for SERT$^{-/-}$ rats compared to SERT$^{+/+}$ after saline treatment ($p < 0.05$) and the latency to the first intromission was also significantly higher for SERT$^{-/-}$ rats compared to SERT$^{+/+}$ at 0.0025 mg/kg F13714 ($p < 0.05$).

Dose-Response of S15535 (Figures 5, 6 and Supplementary Tables 3, 4)

S15535 (0.25, 1, and 4-mg/kg) had no significant effects on sexual behavior in SERT$^{+/+}$ and SERT$^{-/-}$ (**Figures 5**, **6**) compared to saline in either group of animals (Exp. 2 and 3). In the normal ejaculating rats (Exp. 2), a significant genotype effect for ejaculation frequencies was found ($F_{(1,22)} = 21.167$, $p < 0.001$; **Figure 5A**). SERT$^{+/+}$ rats had significant higher ejaculation frequencies after treatment with saline ($p < 0.001$), 0.25 mg/kg ($p < 0.001$), 1 mg/kg ($p < 0.05$), and 4 mg/kg ($p < 0.001$) S15535 in comparison with SERT$^{-/-}$ rats. Similar effects were found for the ejaculation latency ($F_{(1,22)} = 25.627$, $p < 0.001$; **Figure 5D** and **Supplementary Table 3**) where there was an increase for SERT$^{-/-}$ versus SERT$^{+/+}$ animals after saline treatment ($p < 0.001$), and after treatment with 0.25 mg/kg ($p < 0.05$), 1 mg/kg ($p < 0.05$), and 4 mg/kg ($p < 0.001$) S15535, and to some extent in the intromission ration, although this was only significant in the saline treated ($p < 0.05$) and 4 mg/kg ($p < 0.05$) S155355 treated group.

In the slow ejaculating rats (Exp. 3), no significant differences were found in the majority of parameters measured, although a significant dose effect was found for the number of mounts

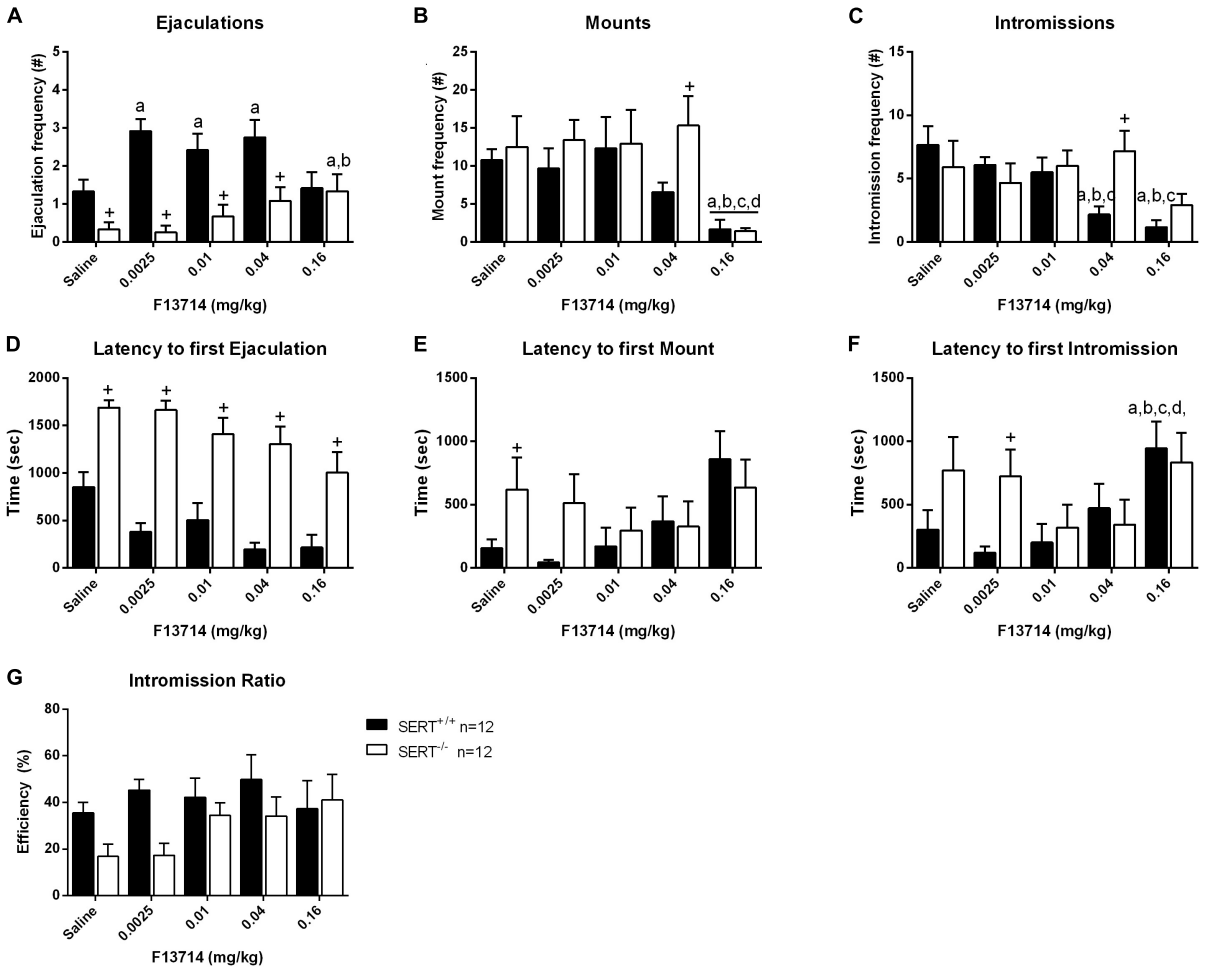

FIGURE 4 | Sexual behavior of male rats treated with 0, 0.0025, 0.01, 0.04, or 0.16 mg/kg F13714. The number and latency of ejaculations per 30 min **(A,D)**, number and latency of Mounts **(B,E)**, number and latency of Intromissions **(C,F)**, and Intromission Ratio **(G)** of the first Ejaculation Series are provided. Detailed statistical analyses are displayed in **Supplementary Table 2**. a: significant difference ($p < 0.05$) compared to saline group, b: significant difference ($p < 0.05$) compared to 0.0025mg/kg group, c: significant difference ($p < 0.05$) compared to 0.01/mg/kg group. +Significant difference between SERT$^{+/+}$ and SERT$^{-/-}$ ($p < 0.05$).

($F_{(3,54)} = 3.077$, $p < 0.05$). Analysis revealed a significant reduction in the number of mounts between saline and 0.025 mg/kg ($p < 0.05$), between saline and 4 mg/kg S-155355 ($p < 0.05$) and between 0.025 and 1 mg/kg S-155355 ($p < 0.05$). In addition, a genotype effect was found in the latency to the first intromission ($F_{(1,18)} = 5.786$, $p < 0.05$). Compared to SERT$^{+/+}$, SERT$^{-/-}$ displayed a shorter latency to the first intromission ($p < 0.05$; see **Figure 6** and **Supplementary Table 4**).

Comparison Between F15599 and F13714 (Supplementary Figure 3)

A fit curve plot for SERT$^{+/+}$ and SERT$^{-/-}$ rats on a log scale (see **Supplementary Figure 3**) was made where data were normalized against the saline treated group. The ED_{50} was calculated for SERT$^{+/+}$ (F15599, ED_{50} = 0.21 mg/kg; F13714, ED_{50} = 0.0065 mg/kg) and SERT$^{-/-}$ (F15599, ED_{50} = 0.165 mg/kg; F13714, ED_{50} = 0.00178 mg/kg) and illustrated that F13714 was more potent compared to F15599 in both SERT$^{+/+}$ and SERT$^{-/-}$ rats. The curved fit plot also showed that SERT$^{-/-}$ rats were sensitive to both compounds, as they were able to increase the percentage of ejaculations compared with the saline treated group.

DISCUSSION

In the present study, after extensive training of the two genotypes studied (SERT$^{+/+}$ and SERT$^{-/-}$), animals showed two different but stable sexual phenotypes, confirming earlier findings (Chan et al., 2011) where male SERT$^{+/+}$ rats performed sexual behavior at a higher level than SERT$^{-/-}$ rats. Permanent changes in serotonergic processes in the central nervous system by removing the SERT protein from conception on (Chan et al., 2011; Olivier et al., 2011), apparently leads to permanent changes in overt male sexual behavior in rats. The male rat sexual behavior paradigm

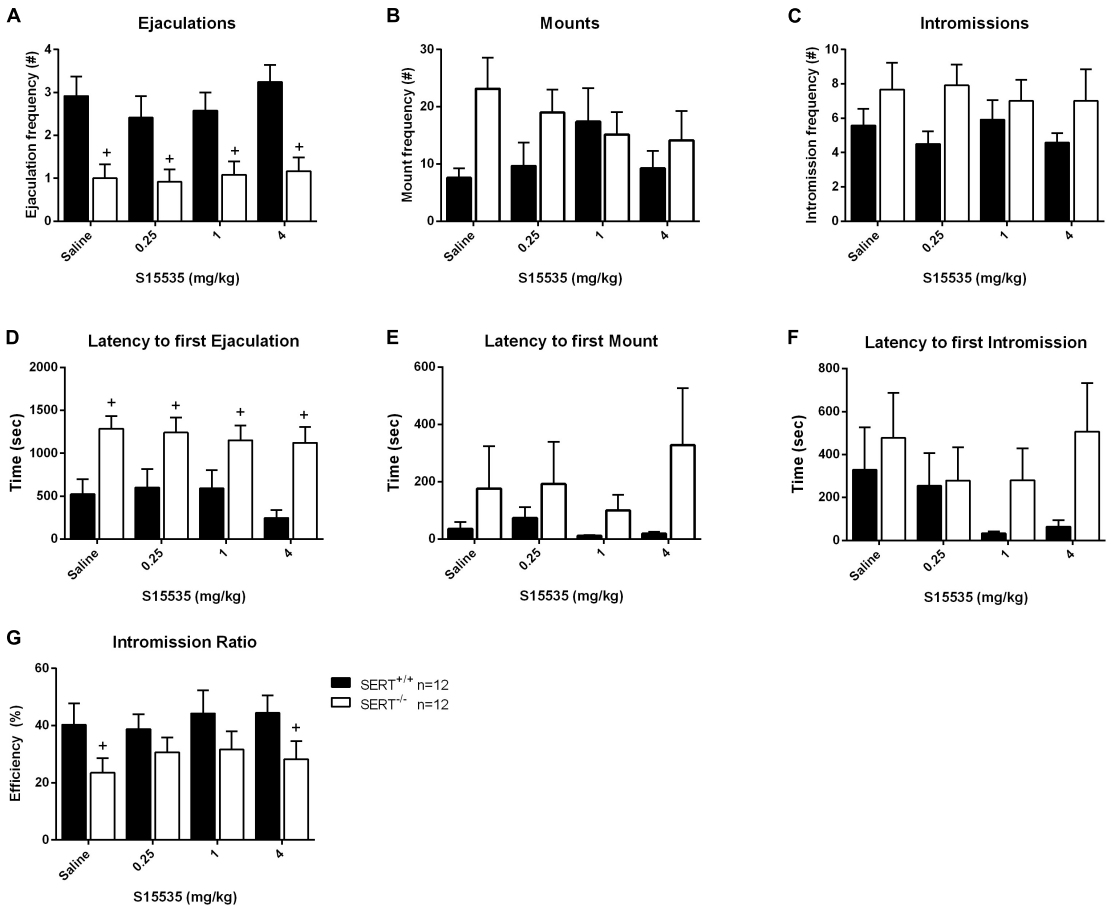

FIGURE 5 | Sexual behavior of male rats from normal ejaculating rats treated with 0, 0.25, 1, or 4 mg/kg S15535. The number and latency of ejaculations per 30 min **(A,D)**, number and latency of Mounts **(B,E)**, number and latency of Intromissions **(C,F)**, post-ejaculatory interval **(G)**, and Intromission Ratio **(H)** of the first Ejaculation Series are provided. Detailed statistical analyses are displayed in **Supplementary Table 3**. +Significant difference between SERT$^{+/+}$ and SERT$^{-/-}$ groups.

used in the present studies has been developed over the last decades (Pattij et al., 2005; Chan et al., 2008; Olivier et al., 2011), specifically to test the effects of psychoactive drugs, including antidepressants (Waldinger and Olivier, 2005; Chan et al., 2010; Heijkoop et al., 2018). The paradigm is able to distinguish acute effects of drugs like the pro-sexual effects of 5-HT$_{1A}$ receptor agonists (Pattij et al., 2005), but also the chronic inhibitory effects of SSRI antidepressants (Chan et al., 2010, 2011; Bijlsma et al., 2014). Pro-sexual effects of drugs in male rat sexual behavior are reflected in the speed of onset of sexual activity toward a newly introduced female in behavioral estrus; reflected in a shorter interval to reach ejaculation (Andersson and Larsson, 1994), including reduced number of mounts and intromissions to reach ejaculation and enhanced number of ejaculations over a certain test period (in our case 30 min). Reduction of sexual behavior, e.g., by chronic antidepressants (Chan et al., 2010; Bijlsma et al., 2014) has reversed effects. This chronic SSRI (antidepressant)-induced profile of reduced male sexual behavior is comparable to the sexual behavior of SERT$^{-/-}$ rats and supports the hypothesis that male SERT$^{-/-}$ rats are modeling the sexual effects of chronic SSRI administration (Chan et al., 2011; Olivier et al., 2011). Several studies on SERT-genotypes in sexual behavior have been performed in at least three different labs (Utrecht, Groningen, Netherlands) and Hefei (China: Geng et al., 2019). In all three independent studies male SERT$^{-/-}$ rats display a significantly lower level of sexual behavior than SERT$^{+/+}$ rats (Olivier et al., 2011; Geng et al., 2019; Olivier and Olivier, 2019). It can be suggested that full absence of the SERT reduces the level of each individual rat's sexual behavior. It can be speculated that the resulting sexual phenotype of a SERT$^{-/-}$ rat may be derived from a certain basic sexual behavior that in some way is permanently inhibited when the SERT is absent from conception on. This can also be illustrated by the comparable ejaculation curves (# ejaculations after training) for both genotypes of which the SERT$^{-/-}$ rats are shifted to the left compared to the SERT$^{+/+}$ rats (Olivier and Olivier, 2019). The typical distribution patterns of the # of ejaculations (or the 1st ejaculation latency times) in a large cohort of SERT-genotypes (Olivier and Olivier, 2019) supplies us with the possibility to behaviorally match animals with a certain genotype, e.g., only high versus low sexually performing animals, as we did previously in the study of tramadol effects on male sexual behavior (Esquivel-Franco et al., 2018).

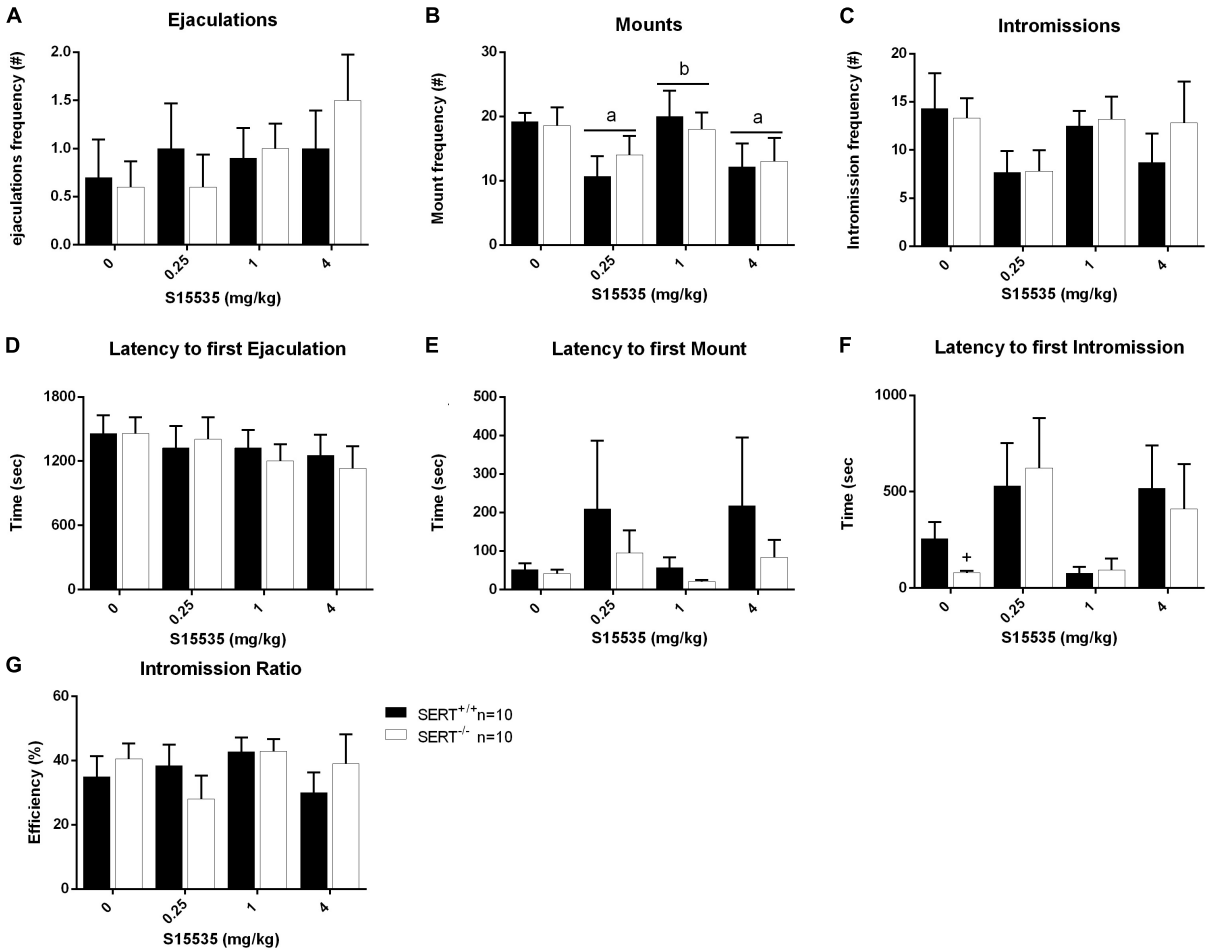

FIGURE 6 | Sexual behavior of male rats from slow ejaculating treated with 0, 0.25, 1, or 4 mg/kg S15535. The number and latency of ejaculations per 30 min **(A,D)**, number and latency of Mounts **(B,E)**, number and latency of Intromissions **(C,F)** and Intromission Ratio **(G)** of the first Ejaculation Series are given. Detailed statistical analyses are shown in **Supplementary Table 4**. a: significant difference ($p < 0.05$) compared to saline group, c: significant difference ($p < 0.05$) compared to 1/mg/kg group. +Significant difference between SERT$^{+/+}$ and SERT$^{-/-}$ ($p < 0.05$).

In the present experiments we intentionally created (Exp. 2) two groups of SERT$^{+/+}$ and SER$^{-/-}$ rats with low (not statistically different) levels of sexual behavior in order to circumvent possible interference of high versus low rates of behavior.

Two biased 5-HT$_{1A}$ receptor agonists, the preferential 5-HT$_{1A}$ auto-receptor agonist F13714 (Assié et al., 2006; Becker et al., 2016) and the preferential 5-HT$_{1A}$ heteroreceptor agonist F15599 (Newman-Tancredi et al., 2009; Becker et al., 2016) were tested in SERT$^{+/+}$ and SERT$^{-/-}$ rats. Both compounds induced pro-sexual activity in SERT$^{+/+}$ and SERT$^{-/-}$ rats (for overview see **Table 4**). F13714 is considerably more potent than F15599 in eliciting the pro-sexual effects, but the similarity of the response of both compounds on male sexual behavior suggests that both compounds share comparable mechanisms of action in evoking sexual behavior. This may point to an autoreceptor-mediated effect. Unfortunately, full dose-response curves of this pro-sexual effect were not available for both compounds making definite conclusions impossible. In F13714-treated SERT$^{-/-}$ rats the dose-response curve of pro-sexual activity was shifted to the right compared to SERT$^{+/+}$ rats, but this was not the case in F15599 treated rats where the sexual inhibiting doses were comparable in both genotypes. 5-HT$_{1A}$ receptor stimulation by "non-selective" (with regard to pre- and post-synaptic receptors) 5-HT$_{1A}$ receptor agonists like 8-OH-DPAT, flesinoxan, buspirone, ipsapirone, and others

TABLE 4 | Overview of ejaculatory responses to 5-HT$_{1A}$ receptor agonists in SERT$^{+/+}$ and SERT$^{-/-}$-/ rats.

	F15599	F13714	S15535	S15535
			Normal ejaculating rats	*Slow ejaculating rats*
SERT$^{+/+}$ rats	↑	↑ at lower dose	No effect	No effect
SERT$^{-/-}$ rats	↑	↑ at higher dose	No effect	No effect

↑*: increased ejaculation frequency.*

(Olivier et al., 1999) have pro-sexual effects in wildtype rats (Snoeren et al., 2014 for review), but no studies were performed before where the specific contributions of 5-HT$_{1A}$ auto-receptors or 5-HT$_{1A}$ heteroreceptors (or both) are investigated. S15535, an auto-receptor selective 5-HT$_{1A}$ receptor agonist and heteroreceptor-selective 5-HT$_{1A}$ receptor antagonist, did not have any effects on male sexual behavior of SERT$^{+/+}$ and SERT$^{-/-}$ rats, neither in normal ejaculating (on average 1–2 ejaculations/30 min; group 1) nor in slow ejaculating (0–1 ejaculations/30 min; group 2) rats. We conclude that S15535 behaves as a "silent" 5-HT$_{1A}$ receptor ligand in male rat sexual behavior.

The prototypal 5-HT$_{1A}$ receptor agonist (±) or (+)-8-OH-DPAT, a non-selective auto-receptor and heteroreceptor agonist (Larsson et al., 1990), has strong and dose-dependent pro-sexual effects (Mos et al., 1991; Chan et al., 2011; Snoeren et al., 2014). This pro-sexual effect can be fully antagonized by the 5-HT$_{1A}$ receptor antagonist WAY100,635, a behaviorally silent compound (de Jong and Neumann, 2015). In male SERT$^{-/-}$ rats (Chan et al., 2011) 8-OH-DPAT had pro-sexual effects, although (like the biased agonist F13714 in the present study) the dose-response curve was shifted to the right compared to SERT$^{+/+}$ rats. The lack of any behavioral effect of S15535 in either SERT$^{+/+}$ or SERT$^{-/-}$ rats is rather puzzling. Apparently, 5-HT$_{1A}$ receptor antagonistic activity on 5-HT$_{1A}$ heteroreceptors in SERT$^{-/-}$ rats did not cause inhibition of male sexual behavior like WAY100,635 treatment (Chan et al., 2011). The stimulating effect of F13714 and F15599 in male sexual behavior in both SERT$^{+/+}$ and SERT$^{-/-}$ rats is also quite puzzling, because it makes explanations in term of pre- or post-synaptic 5-HT$_{1A}$ receptor mechanisms involved, troublesome. However, it remains possible that the preferential post-synaptic 5-HT$_{1A}$ receptor agonist F15599, at higher doses (like in this experiment) also displays some presynaptic autoreceptor agonistic activity. In that case F15599 does not appear the specific tool to selectively activate post-synaptic 5-HT$_{1A}$ heteroreceptors.

How do the sexual data obtained with these three serotonergic ligands compare to their effects in other behavioral systems? The research group of De Boer (de Boer and Newman-Tancredi, 2016) has tested these (and other) ligands extensively in male rat models of offensive aggression in Wildtype Groningen (WTG) rats. In male rat offensive aggression (de Boer et al., 1999, 2000) 8-OH-DPAT potently and dose-dependently reduced offensive aggression but also induces strong sedative-like behaviors. Because 5-HT$_{1A}$ receptor agonists induce a so-called serotonin-5-HT$_{1A}$ syndrome, characterized by Lower Lip Retraction (LLR), Forepaw Treading (FPT), and Flat Body Posture (FBP), it is not completely clear whether this sedative-like activity is similar to these serotonergic behaviors. These anti-aggressive and other effects of 8-OH-DPAT can be fully antagonized by WAY100,635 (de Boer et al., 1999, 2000), a silent antagonist in offensive aggression. F13714, F15599, and S15535 all reduce offensive aggression (de Boer and Newman-Tancredi, 2016). Both F13714 and F15599 induce a serotonergic-5-HT$_{1A}$ syndrome in rats (Newman-Tancredi et al., 2009; Assié et al., 2010; Jastrzębska-Więsek et al., 2018). S15535 does not induce the serotonergic-5-HT$_{1A}$ syndrome at all (de Boer and Newman-Tancredi, 2016; Jastrzębska-Więsek et al., 2018) and also has no sedative-like activity in offensive aggression (de Boer et al., 2000). WAY100,635 antagonized the anti-aggressive action of S15535, F15599, and F13714 (de Boer and Newman-Tancredi, 2016).

If the mechanisms of action of the three 5-HT$_{1A}$ ligands as extensively investigated by various research groups are true, mechanistic interpretations of the behavioral effects found in male sexual behavior are rather difficult to make. Serotonergic 5-HT$_{1A}$ auto-receptors in the raphe nuclei are generally considered as, upon activation, leading to inhibition of cell firing and consequently a decrease of serotonin release. Subsequently, all post-synaptic 5-HT (hetero) receptors (including 5-HT$_{1A}$ heteroreceptors) receive diminished or no stimulation by serotonin and depending on the coupling of the post-synaptic receptor to different transduction mechanisms the neuron involved will be activated or inhibited. Serotonin is also known to crosstalk with non-serotonergic systems which may exert effects on (sexual) behavior as well (e.g., Blier, 2001). In case of a non-selective 5-HT$_{1A}$ receptor agonist like 8-OH-DPAT, next to its inhibiting action on the serotonergic neuron, direct 5-HT$_{1A}$ heteroreceptor stimulation still occurs leading to post-synaptically mediated effects, like the serotonergic-5-HT$_{1A}$ behavioral syndrome (Berendsen et al., 1990; Jastrzębska-Więsek et al., 2018). In the case of F13714, a relatively selective (compared to heteroreceptor) 5-HT$_{1A}$ auto-receptor agonist (Assié et al., 2006) potently facilitated sexual activity in male SERT$^{+/+}$ rats suggesting that pro-sexual activity is related to activation of 5-HT$_{1A}$ auto-receptors. The relatively selective 5-HT$_{1A}$ heteroreceptor agonist F15599 also facilitated male sexual activity in SERT$^{+/+}$ rats. The difference in potency (factor 256 difference) to obtain the pro-sexual activity (at the lowest effective dose) can possibly be explained by the difference of the *in vitro* and *in vivo* affinity and efficacy of both compounds on 5-HT$_{1A}$ receptors (Assié et al., 2010; Newman-Tancredi, 2011; Jastrzębska-Więsek et al., 2018). This might be taken as suggestive that both compounds exert pro-sexual activity via activation of 5-HT$_{1A}$ auto-receptors. Strangely enough, both compounds also activate the serotonergic-5-HT$_{1A}$ syndrome (Newman-Tancredi, 2011; Becker et al., 2016). The 5-HT$_{1A}$ auto-receptor agonist S15535 does not induce pro-sexual behavior, neither in normal nor in sexually slow ejaculating rats. Whether blocking of post-synaptic 5-HT$_{1A}$ heteroreceptors antagonizes the expected pro-sexual effect of the auto-receptor stimulation is rather difficult to envisage. This would assume a rather high level of basal activity of 5-HT$_{1A}$ heteroreceptors involved in sexual behavior. Interestingly, Pattij et al. (2005) showed that slow, normal and rapid ejaculating rats showed increased ejaculations after treatment with 8-OH-DPAT; however, when rats were re-tested 1 week after this 5-HT$_{1A}$ receptor agonist administration all phenotypes returned to ejaculatory behavior levels found before the 8-OH-DPAT treatments. In the present study we found that during the weeks where treatment with S155355 were administered, the saline groups (and thus baseline levels) showed significant higher ejaculation frequencies compared to the ejaculation frequencies during the training weeks. This might suggest that pro-sexual effects due to 5-HT$_{1A}$ receptor agonist can be long-lasting, most likely

due to alterations in the 5-HT$_{1A}$ receptors. Further research is warranted to investigate how long this effect would persist and whether it is, 1 week after all treatments with 5-HT$_{1A}$ receptor agonists, and without saline treatment, still present.

SERT$^{-/-}$ rats, a model of permanently changed serotonergic activity in the brain (Homberg et al., 2007) and associated with an altered sexual phenotype (Chan et al., 2011) may be helpful in explaining the behavioral effects found for the three compounds. Chan et al. (2011) have found that 8-OH-DPAT has pro-sexual effects in male SERT$^{-/-}$ rats, although the dose-response curve has been shifted to the right compared to SERT$^{+/+}$ rats. Remarkably, WAY100,635, a non-selective 5-HT$_{1A}$ receptor antagonist and without any behavioral effects in SERT$^{+/+}$ males, was (dose-dependently) inhibitory in SERT$^{-/-}$ rats. WAY100,635 was able to completely antagonize the pro-sexual effects of 8-OHDPAT in SERT$^{+/+}$ rats but only partially in SERT$^{-/-}$ rats (Chan et al., 2011). We concluded from these data that complete absence of SERT molecules had led to alterations in 5-HT$_{1A}$ receptor functioning, hypothesizing that one pool of 5-HT$_{1A}$ receptors mediates pro-sexual effects of 5-HT$_{1A}$ receptor stimulation and is not (de)sensitized, whereas another pool of 5-HT$_{1A}$ receptors, mediating the inhibitory effects of antagonized 5-HT$_{1A}$ receptors seems sensitized in the SERT$^{-/-}$ rats. The hypothesis of two differentially regulated 5-HT$_{1A}$ receptor pools in SERT$^{-/-}$ rats has also been found in autonomic regulation of body temperature and stress (Olivier et al., 2008). The findings with F15599 and F13714 in the SERT$^{-/-}$ rats cannot be explained in terms of action on different 5-HT$_{1A}$ receptor pools. If any, both compounds seem to activate the pool mediating the pro-sexual effects. The 5-HT$_{1A}$ heteroreceptor antagonistic effects of S15535 do not lead to inhibition of male sexual behavior in the better performing (normal ejaculating) SERT$^{-/-}$ rats, as was the case for WAY100,635 in the Chan et al. (2011) study.

Our expectation that biased 5-HT$_{1A}$ receptor agonists and a mixed 5-HT$_{1A}$ presynaptic receptor agonist and post-synaptic antagonist might help to reveal the potential contribution of these different 5-HT$_{1A}$ receptors was too optimistic. The mechanisms of action of the respective molecules are probably to complex, especially *in vivo* in complicated networks, where 5-HT$_{1A}$ receptors interact with various other neurotransmitter systems in the modulation of male sexual behavior.

CONCLUSION

The data collected with the pharmacological experiments show that selective (preferential) pre- and postsynaptic 5-HT$_{1A}$ receptor agonists possess pro-sexual effects in SERT$^{+/+}$ and SERT$^{-/-}$, although the response is diminished in SERT$^{-/-}$ animals, most likely due to desensitization of 5-HT$_{1A}$ receptors. The pharmacological experiment with S15535 compared with previous experiments performed in aggression lacked any sexual behavioral effect. Further experiments are needed to explore whether separate neurobiological substrates at the 5-HT$_{1A}$ receptors level exist.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the principles of the EU Directive 2010/63/EU.

AUTHOR CONTRIBUTIONS

DE-F, BO, and JO contributed with conception and design of the work. DE-F carried out all the experimental work, data collection and analysis, and draft work. DE-F, BO, SB, and JO contributed to the interpretation of the data and results, made sure all parts of the work were appropriately investigated and resolved. DE-F, BO, SB, MW, and JO contributed on revising critically the intellectual content, accountability and accuracy of the work, and gave approval for the publication of the content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.00040/full#supplementary-material

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Mating Enhances Expression of Hormonal and Trophic Factors in the Midbrain of Female Rats

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Among female rats, mating enhances neurosteroid formation in the midbrain ventral tegmental area (VTA; independent of peripheral steroid-secreting glands, ovaries, and adrenals). The sources/targets for these actions are not well understood. In Experiment 1, proestrous rats engaged in a mating paradigm, or did not, and the midbrains had been assessed *via* the Affymetrix rat genome microarrays. In Experiment 2, the influence of gonadal and adrenal glands on the expression of these genes was assessed in rats that were proestrous, ovariectomized (OVX), or OVX and adrenalectomized (ADX). The microarrays revealed 53 target genes that were significantly up-regulated (>2.0-fold change) in response to mating. Mating significantly enhanced the midbrain mRNA expression of genes involved in hormonal and trophic actions: Gh1, S100g, and Klk1b3 in proestrous, but not OVX and/or ADX, rats; Fshb in all but OVX/ADX rats; and lutenizing hormone β and thyroid-stimulating hormone (TSH) β in all rats. Thus, mating enhances midbrain gene expression independent and dependent of peripheral glands.

Keywords: calbindin, follicle-stimulating hormone, growth hormone, kallikrekin peptidase, luteinizing hormone, thyroid-stimulating hormone

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INTRODUCTION

In rats, copulatory behavior is complex, and the manner in which mating occurs can affect the reproductive outcomes. Mating in rats involves a series of mounts, intromissions, and ejaculations (Beach and Jordan, 1956). A mount refers to the male rat positioning his two front paws on the back of the female rat, without penile penetration (Gilbert et al., 1983). Intromissions refer to a slight penetration of the penis into the vagina, which lasts approximately 0.25 s (Peirce and Nuttall, 1961a,b). An ejaculation refers to a deeper intromission, during which the male deposits a sperm plug in the vaginal canal of the female (Matthews and Adler, 1977). A series of mounts and intromissions that ends in ejaculation is called an ejaculatory series. Typically, laboratory rats need 10–20 intromissions before they attain their first ejaculation (Beach and Jordan, 1956).

These standard measures of copulatory behavior are all defined in terms of male behaviors: mounting, intromissions, and ejaculations. However, the rate, timing, and position of all mating behaviors are a result of the interactions between the male and the female of any species (Beach, 1976). Female rats exhibit a species-specific mating behavior. A female rat orients her body toward

the male rat as he approaches; she often does this in a hopping manner and then darts away. This type of solicitation, or proceptive behavior, was first described by McClintock and Adler (1978) in a study of a semi-natural environment. The female rat was sometimes observed to put a great deal of space between herself and the male. In a semi-natural environment, the time between intromissions seem to be controlled by female solicitations of males (McClintock et al., 1982). When the size of the cage or a chance for escape allows the female rats to control her interaction with the male rats, her time away is a function dependent upon the preceding behavior(s) of the male (Erskine, 1985). Her time away (i.e., latency period) is the greatest after ejaculations and the shortest after mounts, but in between for intromissions. The percentage of exits is also a result of an antecedent male behavior in that the female will try to get away a fewer times when she receives a mount, as opposed to when she received an ejaculation or intromission (Erskine, 1985).

In 1982, Erskine and Baum found that not only is inter-intromission time dependent upon the preceding male behavior but also the interintromission periods are longer when the female can control the interaction. This phenomenon of female rats being able to escape a male rat by some kind of divided chamber has been termed "pacing" behavior (Gilman and Hitt, 1978). With paced mating, fewer intromissions are necessary to bring about the neuroendocrine response of luteal functioning (Gilman and Hitt, 1978), which results in progesterone secretion, the *sine qua non* for pregnancy. Indeed, paced mating is rewarding for females as it induces a conditioned place preference (Paredes and Alonso, 1997).

Near three scores ago, for my undergraduate thesis, I received a research undergraduate award from NSF to work with Dr. Mary Erskine, a pioneer in the field. I examined the effects of paced or non-paced mating and the time of day of mating in the morning (7–9 am) or in the evening (4–6 pm) to ascertain effects on measures of fertility (number of days in luteal function/pseudopregnancy/pregnancy) and fecundity. Female rats that paced their contacts and mated in the evening were much more likely to become pregnant and have the most pups (Frye and Erskine, 1990; Frye et al., 1998). I went on to show that these effects were due to progesterone's actions in the midbrain ventral tegmental area (VTA), where there is transient expression of non-estrogen-induced progestin receptors perinatally that remit shortly after birth (Frye, 2001; Blaustein, 2003). Notably, this region is largely devoid of intracellular progestin receptors in adults (Frye, 2001; Blaustein, 2003), but $GABA_A$ and NMDARs, co-localized to calbindin-expressing dopaminergic neurons, are prominent (Willick and Kokkinidis, 1995; Westerink et al., 1996; Olson and Nestler, 2007). In hamsters, mating alters gene expression in the diencephalon (i.e., striatum and nucleus accumbens; Bradley et al., 2005).

Progestogens, including progesterone (P) and its metabolites, are pleiotropic factors that can have diverse actions to influence development and/or behavioral processes throughout life. Progesterone and its 5α-reduced metabolite, dihydroprogesterone (DHP), can be secreted from peripheral sources, such as the gonads and the adrenals, to travel through circulation and passively diffuse into target cells in the periphery or brain. The endocrine actions of P or DHP can occur *via* binding to cognate, intracellular steroid receptors (Shughrue et al., 1997) *via* "traditional" actions that modulate gene transcription and translation to protein (Pfaff et al., 1976) in a process that can take approximately 5–10 min to days. These traditional actions of progestogens for reproduction and maintaining pregnancy are well known (Boling and Blandau, 1939; Robson, 1940; Hall, 1956; Erskine, 1989). Moreover, progestogen formation throughout the lifespan may confer protection from neurodegeneration or later central insults (Schumacher et al., 2004; De Nicola et al., 2009; Paris et al., 2011; Garay et al., 2012). Thus, progestogens are important neurotrophic factors that can be naturally enhanced *via* engagement in reproductive behavior.

Some of P's trophic and behavioral effects may be due to the actions of its neuroactive metabolite that can have rapid actions occurring at "non-traditional" receptor sites (such as membrane-relegated neurotransmitter targets). Progesterone's 5α-reduced metabolite, DHP, can be further metabolized by 3α-hydroxysteroid dehydrogenase to form 5α-pregnan-3α-ol-20-one (3α, 5α-THP, a.k.a., allopregnanolone). Neurosteroids, such as 3α, 5α-THP, can be synthesized *de novo* in astrocytic and/or neural cells, even in the absence of peripheral steroid sources (gonads and/or adrenals; Baulieu, 1980; Paul and Purdy, 1992; Mellon, 1994). Unlike P and DHP, 3α, 5α-THP is a potent allosteric modulator of $GABA_A$ receptors (Majewska et al., 1986; Callachan et al., 1987; Fodor et al., 2005), where it can promote rapid (<10 min) effects (Baulieu, 1980; Gee et al., 1995; Frye and Vongher, 1999). 3α, 5α-THP may also allosterically modulate glutamatergic N-methyl-D-aspartate receptors (NMDARs; Frye and Paris, 2011) and has less well-defined actions through other non-steroidal, ligand-gated, ion channel, and/or G protein-coupled receptors (Rupprecht and Holsboer, 1999). Thus, P can have rapid, non-traditional actions in the brain *via* conversion to its metabolite, 3α, 5α-THP.

In rodents, mating is utilized as a bioassay to determine the mechanisms that underlie the steroids' effects. Female rodents are dependent upon the central actions of P, and/or its metabolites, to promote lordosis (a stereotypical posture that allows mating to occur). In hamsters, mating alters gene expression in the diencephalon (i.e., striatum and nucleus accumbens; Bradley et al., 2005). We have utilized a paced mating paradigm, wherein female rats are given free access to a chamber containing a confined male and an empty chamber. In this paradigm, females can temporally pace their mating contacts (Erskine, 1985). Engaging in this type of mating enhances 3α, 5α-THP content *via* neurosteroidogenesis in the midbrain, hippocampus, frontal cortex, and diencephalon (Frye and Rhodes, 2006; Frye et al., 2007). Moreover, these effects have been localized to the midbrain VTA, an important region for motivated behavior and reward (Bain and Kornetsky, 1989; Agars and Kokkinidis, 1992; Frye et al., 1992; McBride et al., 1993). Notably, this region is largely devoid of estradiol-induced intracellular progestin receptors (Frye, 2001; Blaustein, 2003), but $GABA_A$ and NMDARs, co-localized to calbindin-expressing dopaminergic neurons, are prominent (Willick and Kokkinidis, 1995; Westerink et al., 1996; Olson and Nestler, 2007). Blocking

3α, 5α-THP formation, or these neurotransmitter receptors and their downstream signal transduction targets, within the VTA attenuates lordosis (Frye et al., 2008a; Frye and Paris, 2011; Frye, 2011). Thus, engagement in paced mating is an important stimulator of neurosteroidogenesis in the midbrain VTA among female rats, and mating-induced enhancement of 3α, 5α-THP in the VTA is critical for the maintenance of rodent reproduction.

The mechanisms that may underlie mating behavior *via* non-traditional actions in the midbrain remain to be elucidated. We hypothesized that engagement in paced mating would alter the gene expression of traditional, and/or non-traditional, steroid targets in the midbrain, the latter of which may include GABAergic, dopaminergic, and glutamatergic substrates and/or their downstream signal transduction processes. Further, we hypothesized that gonadal and/or adrenal steroid production might play an important role in the expression of these and/or other important trophic factors: there are rapid changes in the midbrain VTA in response to progestogens, and mating can rapidly increase progestogens (Frye, 2011). As such, RNA microarrays were performed on sexually receptive (proestrous) female rats that engaged in paced mating with a male or proestrous rats that were exposed to an empty, clean paced mating chamber in the absence of a male. To ascertain the roles of peripheral progestogen sources, some targets were followed up with quantitative polymerase chain reaction (qPCR) in midbrain tissues from proestrous rats that were gonadally intact, had their ovaries removed (OVX), or had their ovaries and adrenals (OVX/ADX) removed.

MATERIALS AND METHODS

These methods were approved by the Institutional Animal Care and Use Committee at The University at Albany-SUNY and were conducted in accordance with the ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85-23).

Study Procedure

Rats that were gonadally intact and had regular 4- to 5-days estrous cycles ($n = 6$) underwent paced mating for one ejaculatory sequence ($n = 3$) or were yoked controls that were exposed only to a clean paced mating chamber ($n = 3$). The rats were euthanized immediately following behavioral testing as described below. Frozen midbrain tissue was assessed *via* RNA microarrays, as described below, at the Center for Functional Genomics, The University at Albany. RNA expression differences in some targets that were of interest were verified *via* qPCR. Some changes that occur in midbrain gene expression with mating may be due to the actions of progestogens; however, given that neural cells can synthesize progestogens in the absence of peripheral production (King, 2008) and may serve as important trophic factors, it was important to include extirpation groups that had minimal circulatory progestogens. In order to ascertain the degree to which peripheral progestogen secretion influenced changes in RNA expression, some rats were OVX ($n = 6$) or were OVX/ADX ($n = 6$), estradiol-benzoate (EB)-primed (10 μg, SC), and tested 10 days after surgery. Therefore, the influence of peripheral progestogens on central changes in gene expression that occurred with mating among extirpation groups is assumed to be limited. Ovariectomized or OVX/ADX rats were primed with EB (10 μg, SC) and tested 44 h later in the paced mating task or as yoked controls. This EB priming regimen has been utilized to facilitate mating and produce physiological estradiol and P in OVX or OVX/ADX rats previously (Frye et al., 2008b; Frye and Paris, 2011) and is necessary so that comparisons could be completed in rats that engaged in mating, which otherwise would not occur without EB priming. Midbrain was collected from rats that were OVX and/or ADX, and RNA was isolated in these tissues as described. qPCR was run in these tissues to assess the ovarian and/or adrenal contributions to up-regulation of targets delineated in the microarray experiments.

Animals and Housing

Adult (50–60 days old) Long–Evans female rats ($N = 25$) were bred in the Life Sciences Laboratory Animal Care Facility at The University at Albany-SUNY (original stock obtained from Taconic Farms, Germantown, NY, USA). The rats were housed in polycarbonate cages (45 × 24 × 21 cm) with woodchip bedding in a temperature-controlled room (21 ± 1°C) and were maintained on a 12:12-h reversed light cycle (lights off at 08:00 h) with continuous access to Purina rat chow and tap water in their home cages.

Determination of Estrous Cycle Phase in Gonadally Intact Rats

Vaginal epithelium was collected from gonadally intact rats ($n = 13$) for 21–22 consecutive days by inserting an eye dropper with distilled water into the vaginal canal and squeezing. Epithelial samples were assessed under a light microscope to determine the phase of the estrous cycle as per previously described methods (Long and Evans, 1922; Frye et al., 2000; Marcondes et al., 2002). Briefly, samples characterized by numerous epithelial cells that were nucleated were considered to be of the proestrous phase; the time when circulatory E levels are elevated, progestogen levels are rising, and female rats are sexually receptive. The samples that were characterized by the presence of many cornified cells were considered to be of the estrous phase. A lack of nucleated or cornified cells, combined with the presence of leukocytes, was indicative of the diestrous phase. The presence of all three cell types, in similar proportions, was considered to be indicative of the meta-estrous phase. Six of the 13 gonadally intact rats in the present study had typical 4- to 5-days estrous cycles across the 21- to 22-days period and were utilized for behavioral testing.

Surgical Protocol for Ovariectomized and/or Adrenalectomized Rats

The rats underwent surgery using xylazine (12 mg/kg) and ketamine (70 mg/kg) anesthesia. Ovariectomy was performed on some rats ($n = 12$) as previously described (Frye et al., 2008b). Briefly, a ∼3-cm incision was made in the dorsal region of the flank, just anterior to the kidney. The ovary was isolated from the surrounding adipose tissue, ligated with surgical silk (4–0 USP, 1.5 m), and extirpated. The muscle wall and skin were closed with 2–3 silk sutures, and the procedure was repeated for the ovary

on the alternate side. Some rats also underwent ADX (n = 6) at the time of OVX as per prior methods (Rhodes et al., 2004). For ADX, the adrenal gland was located *via* proximity to the kidney; the gland was isolated with a forceps and extirpated. One OVX/ADX rat died prior to surgical completion. Following surgery and prior to testing, the animals were monitored for changes in weight, righting response, flank stimulation response, and/or muscle tone (Marshall and Teitelbaum, 1974). No rats failed these assessments in the present study. Given that ADX rats are rendered sodium deficient, they were provided continuous access to a bottle of sodium chloride (0.9%) in addition to drinking water. All rats that had surgery were allowed 10 days for recovery and hormonal washout prior to testing.

Behavioral Outcome Measures

Paced mating was conducted as per previous methods (Erskine, 1985). In brief, the paced mating apparatus (37.5 × 75 × 30 cm) was equally divided by a Plexiglass partition, which contained a small (5 cm in diameter) hole in the bottom center, allowing the female (but not the stimulus male) free access to both sides of the apparatus. The frequency of mating contacts (intromissions) was recorded, and a lordosis quotient was calculated [(frequency of female dorsiflexion during a sexual contact/total sexual contacts by a male) * 100] during a 15-min test. The frequency of proceptive/solicitation behavior (ear wiggling, hopping, and darting) prior to intromission was recorded and calculated as a proceptivity quotient [(frequency of proceptive behaviors/total sexual contacts by a male) * 100]. The percentage of defensive aggressive behaviors (vocalizing and attack) that females displayed in response to male intromission was calculated as a defensive aggression quotient [(frequency of defensive aggression/total sexual contacts by a male) * 100]. Following intromission, the percentage of times the experimental female left the chamber containing the male (% exits) following sexual contacts was recorded. The effects of ovarian and/or adrenal gland extirpation to reduce the sexual response of female rodents to male mounting have been established (Davidson et al., 1968; Komisaruk and Diakow, 1973; Feder et al., 1974), and the present behavioral data is confirmatory in this regard.

Tissue Collection and Dissection

Immediately following the behavioral testing, the rats were euthanized *via* rapid decapitation in an RNase/DNase-free environment. All surfaces, tools, and personal protective equipment that were utilized for euthanasia were decontaminated with quatricide and RNase/DNase cleansing solution (RNase AWAY, Laboratory Products Sales, Rochester, NY, USA) prior to termination of each subject. For each subject, whole brains were removed at the time of death, and the brain was positioned ventral side up for gross dissection of the midbrain immediately after the brain was extracted from the skull. The optic chiasm was utilized as the anterior border of the dissection, the pontine regions were utilized as the posterior border, and the cerebral aqueduct was utilized as the ventral border. The borders were ∼1.5 mm from the anterior, posterior, and midline as previously reported (Frye et al., 2007). Dissected midbrains were placed in RNase/DNase-free tubes and immediately flash-frozen on dry ice and maintained at −80°C until microarray.

Microarray Analyses

Tissues from gonadally intact rats (N = 6; chamber-exposed, n = 3, paced mated, n = 3) were sent to the microarray core at the Center for Functional Genomics at the University at Albany-SUNY to have the RNA extracted. Then, they were sent along to UCLA Microarray Core Facilities to the consortium coordinators, Brandy Hamill and Stanley Nelson, to complete the Affymetrix Rat Genome 230 2.0 gene chip as described below. See the following files from the UCLA Microarray Consortium for the raw data of Experiment 1 comparing the intact paced mated and the non-mated rats (frye-affy-rat-483660). The effects on mating of the midbrain gene expression of OVX and OVX/ADX hormone-primed rats (Experiment 2) are on file at UCLA Microarray Consortium files frye-affy-rat-584452 and 584783, respectively. All of the data are available at the following website: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127272.

In brief, tissue preparation was performed as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA, USA). RNA was isolated from tissue using an RNeasy RNA Isolation kit (Qiagen, Valencia, CA, USA), which included an on-column Dnase step. The integrity of isolated RNA was assessed by using a nanodrop spectrophotometer and an Agilent Bioanalyzer. RIN between 8.5 and 10 for all samples and RNA between 50 and 200 μg/sample were detected. RNA was converted to single-stranded cDNA using Superscript II reverse transcriptase and the GeneChip T7 promoter primer kit (Affymetrix, Santa Clara, CA, USA). The single-stranded cDNA was converted to double-stranded cDNA using DNA polymerase I, DNA ligase, and RNase H from *Escherichia coli*. Double-stranded cDNA was cleaned up and *in vitro*-transcribed to biotin-labeled cRNA. There were 35–200 base fragments generated by metal-induced hydrolysis and hybridized to Affymetrix Rat Genome 230 2.0 oligonucleotide arrays. After hybridization, the chip was washed and stained with streptavidin–phycoerythrin before being scanned. An antibody amplification staining protocol that uses biotinylated goat IgG, followed by a second streptavidin–phycoerythrin staining, increases the sensitivity of the assay. The chip was then scanned, and images were analyzed qualitatively using the Affymetrix GeneChip Operating System software. Further analyses of the data were done in GeneSpring v7.3, wherein the data were normalized using GC robust multi-array average (GCRMA). The signals were also baseline-transformed to the median of all samples, following which the probe sets were filtered to exclude those with signal values less than the 20th percentile across all conditions. This list was subjected to an ANOVA ($p < 0.05$) with a Benjamini–Hochberg false discovery rate correction to identify statistical differences between all conditions vs. control. The statistically significant genes were subjected to a twofold change filter to identify genes that were differentially expressed in paced vs. in non-paced animals. The *raw data* from our microarray analyses is accessible at (Accession: GSE127272) a public data repository through the UCLA Microarray Consortium reference

files (frye-affy-rat-483660, 584783, and 584452) for intact, OVX, and OVX/ADX rats, respectively.

Quantitative Real-Time Polymerase Chain Reaction

Some genes that were revealed to be significantly different in paced and non-mated rats in Experiment 1 were followed up with qPCR in gonadally intact, OVX, and OVX/ADX tissues. In order to avoid the inclusion of tissues from another sample, the same tissues that were utilized for the microarray were utilized for qPCR analyses, yielding n = 3/condition. RNA was isolated and converted to cDNA as described above. qPCR assays were run using the iTaq SYBR green supermix with ROX (Bio-Rad, Hercules, CA, USA) on a 7900HT platform (Applied Biosystems). The final reaction volume was 20 μl, comprised of iTaq SYBR green master mix (10 μl), nuclease-free dH_2O (4.96 μl), forward and reverse primers (0.02 μl each), and 20 ng cDNA template (5 μl). Nuclease-free dH_2O was utilized in place of cDNA for a non-template control (NTC). Reactions were run in triplicate. Each plate had represented a negative (NTC) and positive control (β-actin or Gapdh reference gene). These normalizing control/housekeeping genes were selected because their expression did not change per microarray data. Reactions were incubated for 10 min at 95°C to activate iTaq polymerase, followed by 40 cycles consisting of 15 s at 95°C and elongation at 60°C for 10 s. After each cycle, fluorescence was measured.

Primers

The primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) and were validated *via* BLAT for specificity and selectivity. All primers were synthesized by Eurofins MWG Operon (Huntsville, AL, USA) and were reconstituted to a 100-μM concentration. The forward and reverse primers are indicated in **Table 1**.

TABLE 1 | mRNA sequences that were used forward and backward (1st and 2nd lines) and were investigated with quantitative real-time polymerase chain reaction (qPCR).

GenBank Accession #	Gene Symbol	mRNA sequences first line forward/second line backward
NM_022384	Ascl1	5′-GGGGGCGGTCACAAGTCAGC-3′
		5′-ACTTGACCCGGTTGCGCTCG-3′
NM_053918	Cga	5′-CCCACTCCCGCCAGGTCCAA-3′
		5′-TCAGCAGTCGTCAGCGCAGC-3′
D00577	$Fshb_1$	5′-GCCCGCCACTCAGACTCCCT-3′
		5′-GCCCTGGCACTCCCAGTCCT-3′
M36804	$Fshb_2$	5′-AGATTGCCTGGCTGTGCCGC-3′
		5′-GCCCCAGTGCAGAGCAGACC-3′
NM_012858	Lhb	5′-TACAAAGAGTTCGAGCGTGCC-3′
		5′-AATGGAATAGCGCTGTCCCTC-3′
NM_031038	Gnrhr	5′-ATCGCTCCCTGGCCGTCACT-3′
		5′-AAACTGCTGGCCCAGAGCCG-3′
NM_057187	Grifin	5′-AAGGTGCTCCAGTTCCCACAT-3′
		5′-TCTGGTGATGGTAGCTAGCGGT-3′
NM_031523	Klk1b3	5′-GCTGCTCACTGCGCAACCGA-3′
		5′-CCGAGGCAAGGCAGGTGCTC-3′
NM_012858	Lhb	5′-GAATGGAGAGGCTCCAGGGGCT-3′
		5′-GAATGGAGAGGCTCCAGGGGCT-3′
NM_012629	Prl	5′-CAGCCAAGTGTCAGCCCGGA-3′
		5′-GTGTCTGGCAGTCGCCACCA-3′
AI230625	S100g	5′-TGGCAGCACTCACTGACAGCA-3′
		5′-TGGACAGCTGGTTTGGATCGCC-3′
M10902	Tshb	5′-CACCACCATCTGCGCTGGGT-3′
		5′-TTGCAGCTCAGGGCAACGGG-3′

Statistical Analyses

Independent student's t-tests were used to assess differences of extirpation condition between groups (gonadally intact, OVX, and OVX/ADX) on behavioral endpoints as compared to intact conditions. For each qPCR analyte, the results were calculated with the Applied Biosystem's Sequence Detection Software and calculated *via* the ΔΔCt method, with the mean of control rats utilized as the calibrator for each experimental rat. The qPCR results were analyzed by student's t-tests on extirpation (gonadally intact, OVX, and OVX/ADX) and mating condition (chamber-exposed and paced-mated) to assess between-groups differences. All data are expressed as mean ± SEM. The effects were considered as significant when $p < 0.05$.

RESULTS

Behavioral Endpoints

Extirpation of the ovaries and/or adrenals significantly reduced engagement in lordosis ($t_{OVX(7)} = 6.11$, $p < 0.05$; $t_{OVX/ADX(7)} = 8.17$, $p < 0.05$; **Figure 1**, top), reduced proceptive solicitations ($t_{OVX(7)} = 10.64$, $p < 0.05$; $t_{OVX/ADX(7)} = 10.64$, $p < 0.05$; **Figure 1**, middle), and increased defensive aggression ($t_{OVX(7)} = -14.08$, $p < 0.05$; $t_{OVX/ADX(7)} = -2.84$, $p < 0.05$; **Figure 1**, bottom) in response to sexual contact by a male compared to gonadally intact proestrous rats.

Microarray Endpoints

Differences in RNA expression were detected among several genes in gonadally intact, proestrous rats. There were 53 genes that had greater than 2-fold enhancement of RNA expression among paced mated rats compared to chamber-exposed controls (**Table 3**).

qPCR Among Gonadally Intact, OVX, and/or ADX Rats

Of the genes that were up-regulated, the most robust enhancement was observed in growth hormone 1 (Gh1). The extirpation status significantly influenced the mRNA expression, such that Gh1 expression was greater among gonadally intact ($t_{(10)} = 2.99$, $p < 0.05$) or OVX ($t_{(10)} = 2.26$, $p < 0.05$) rats compared to OVX/ADX rats (**Figure 2**, top). Similarly, kallikrekin 1-related peptidase b3 (Klk1b3, a.k.a. Ngfg) was significantly greater among gonadally intact rats compared to OVX ($t_{(10)} = 1.87$, $p < 0.05$) or OVX/ADX rats ($t_{(10)} = 1.88$, $p < 0.05$; **Figure 2**, bottom). The extirpation condition significantly influenced the expression of the follicle-stimulating hormone β polypeptide (Fshb). The expression of Fshb was greater among gonadally intact ($t_{(10)} = 1.14$, $p < 0.05$ or $t_{(10)} = 0.72$, $p < 0.05$) rats compared to OVX/ADX rats (**Table 2**).

Engagement in paced mating was also observed to significantly enhance two genes irrespective of peripheral gland extirpation. The expression of lutenizing hormone β (Lhb; $t_{(16)} = -2.71$, $p < 0.05$) and thyroid-stimulating hormone β

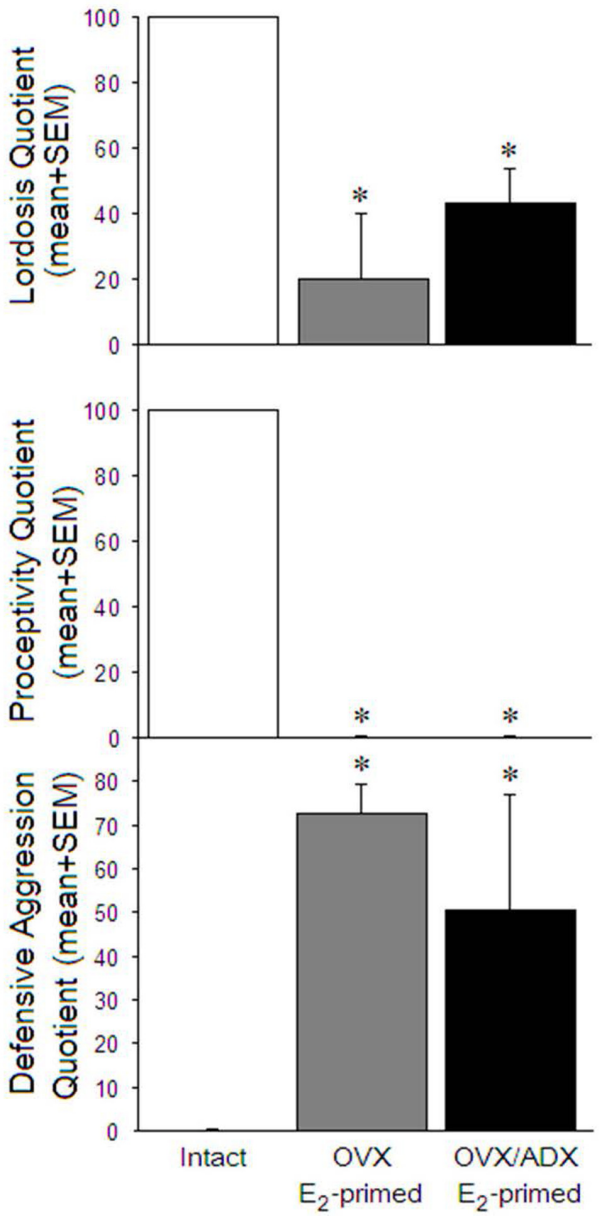

FIGURE 1 | Lordosis quotients (top) and proceptivity quotients (middle) were significantly higher, and defensive aggression quotients (bottom) significantly lower, among naturally receptive rats (left), compared to ovariectomized estradiol primed (middle) and ovariectomized and adrenalectomized estradiol primed (right) sexually-naïve male rats. GH1 was elevated most among naturally mated rats, and was increased similarly among estradiol primed rats whether mated or not. *$p < 0.05$.

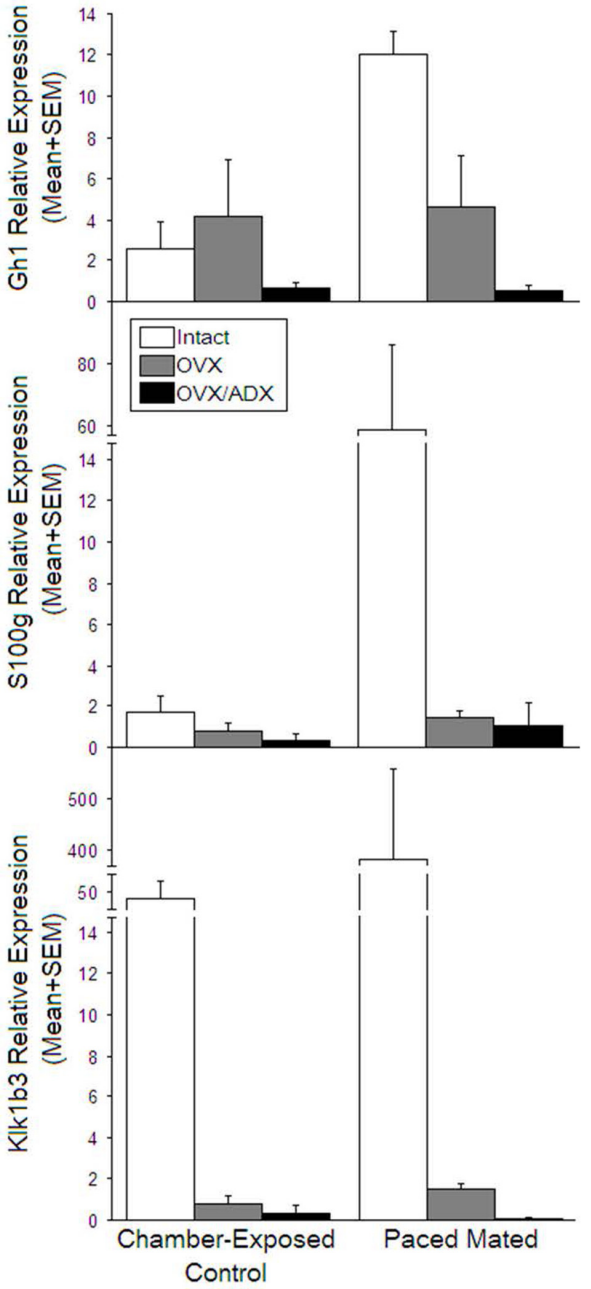

FIGURE 2 | Relative expression among among naturally receptive rats (open bars), ovariectomized estradiol primed (gray bars) and ovariectomized and adrenalectomized estradiol primed (black) that were just exposed to the mating chamber left side of graph or were actually mated (right side of graph). Top panel: GH1 was elevated most among naturally mated rats, and was increased similarly among estradiol primed rats whether mated or not. Middle panel S100 expression was increased most among naturally mated rats. Bottom panel kallikrekin 1-related peptidase b3 (Klk1b3, a.k.a.Ngfg) was increased in naturally receptive rats irrespective of mating.

(Tshb; $t_{(16)} = -2.28$, $p < 0.05$) were significantly enhanced among mated, compared to chamber-exposed, rats (**Table 2**). Notably, the latter effect was not due to an enhancement in Tshb among the mated OVX/ADX rats (**Table 2**). Fshb expression did not significantly differ among the mated rats compared to the chamber-exposed rats.

Gonadotropin-releasing hormone receptor and achaete-scute complex homolog 1 did not significantly differ among groups (**Table 2**). Other genes that were probed for validation, prolactin, glycoprotein hormone α polypeptide, and galectin-related inter-fiber protein, did not significantly differ between extirpation and mating groups (**Table 2**).

TABLE 2 | mRNA that were significantly (>2-fold change) up-regulated in the midbrain among rats that were paced mated compared to yoked control rats that were only exposed to the paced mating chamber (n = 3/group).

Up-regulated Genes			
GenBank Accession #	**Gene Symbol**	**Gene Name**	**Fold Change in Paced Mated vs. Chamber-Exposed**
V01238	Gh1	growth hormone 1	162.6
NM_012629	Prl	prolactin	58.7
NM_012521	S100g	S100 calcium binding protein G	32.0
D00577	$Fshb_1$	follicle-stimulating hormone, β polypeptide	21.4
M36804	$Fshb_2$	follicle-stimulating hormone, β polypeptide	16.1
NM_012858	Lhb	luteinizing hormone β	10.3
NM_031038	Gnrhr	gonadotropin releasing hormone receptor	9.0
NM_031523	Klk1b3	kallikrein 1-related peptidase b3	8.3
NM_022384	Ascl1	achaete-scute complex homolog 1 (Drosophila)	7.3
NM_053918	Cga	glycoprotein hormones, α polypeptide	6.0
M10902	Tshb	thyroid stimulating hormone, β	4.6
NM_057187	Grifin	galectin-related inter-fiber protein	2.9
BG669096	Mpz	myelin protein zero	9.2
NM_133563	Giot1	gonadotropin inducible ovarian transcription factor 1	6.5
NM_017027	Mpz	myelin protein zero	5.2
AF260741	Gpha2	glycoprotein hormone α 2	5.1
NM_053572	Pcdh21	protocadherin 21	4.7
NM_024388	Nr4a1	nuclear receptor subfamily 4, group A, member 1	4.5
NM_031796	Galnt5	UDP-N-acetyl-α-D- galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)	3.5
AF200684	Slc7a7	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	3.4
NM_022526	Dap	death-associated protein	3.4
AI230625	LOC6848 71	similar to Protein C8orf4 (Thyroid cancer protein 1; TC-1)	3.2
NM_031972	Aldh3a1	aldehyde dehydrogenase 3 family, member A1	3.2
NM_012999	Pcsk6	proprotein convertase subtilisin/kexin type 6	3.0
NM_031808	Capn6	calpain 6	2.8
AA819329	RGD130 5347	similar to RIKEN cDNA 2610528J11	2.8
AF214568	Enpep	glutamyl aminopeptidase	2.7
NM_019237	Pcolce	procollagen C-endopeptidase enhancer	2.7
AA819629	Ifi44l	interferon-induced protein 44-like	2.6
BI278379	Rcn3	reticulocalbin 3, EF-hand calcium binding domain	2.6
NM_053744	Dlk1	Delta-like 1 homolog (*Drosophila*)	2.6
NM_053750	Nppc	natriuretic peptide precursor C	2.6
NM_052805	Chrna3	cholinergic receptor, nicotinic, α 3	2.5
M83681	Rab3d	RAB3D, member RAS oncogene family	2.5
NM_013069	Cd74	Cd74 molecule, major histocompatibility complex, class II invariant chain	2.5
AI029410	Fndc3c1	fibronectin type III domain containing 3C1	2.4
NM_053819	Timp1	tissue inhibitor of metalloproteinase 1	2.4
Y00480	RT1-Da	RT1 class II, locus Da	2.4
AF065147	Cd44	Cd44 molecule	2.4
NM_031334	Cdh1	cadherin 1	2.4
NM_021663	Nucb2	nucleobindin 2	2.3
NM_033237	Gal	galanin prepropeptide	2.2
NM_057194	Plscr1	phospholipid scramblase 1	2.2
NM_031817	Omd	Osteomodulin	2.2
NM_019296	Cdk1	Cyclin-dependent kinase 1	2.1
NM_012760	Plagl1	pleiomorphic adenoma gene-like 1	2.1
NM_031511	Igf2	insulin-like growth factor 2	2.1
L07646	Gnrhr	gonadotropin releasing hormone receptor	2.1
NM_019354	Ucp2	uncoupling protein 2 (mitochondrial, proton carrier)	2.1
M23995	Aldh1a7	aldehyde dehydrogenase family 1, subfamily A7	2.0
NM_012949	Eno3	enolase 3, β, muscle	2.0
AF419342	Sytl4	synaptotagmin-like 4	2.0
NM_022232	Dnajc3	DnaJ (Hsp40) homolog, subfamily C, member 3	2.0

*Those highlighted in gray were followed up on with qPCR (see **Table 3**).*

TABLE 3 | qPCR results for nine genes that were significantly (>2-fold change) up-regulated in the midbrain of rats that were paced mated compared to yoked control rats that were only exposed to the paced mating chamber (n = 3/group).

Relative expression differences *via* qPCR (mean ± SEM) vs. mean of control rat expression						
	Chamber-exposed			Paced mated		
Gene symbol	Gonadally intact	OVX	OVX/ADX	Gonadally intact	OVX	OVX/ADX
Prl	373.5 ± 373.0	0.8 ± 0.4	0.3 ± 0.3	1276.7 ± 1018.9	1.5 ± 0.3	5.0 ± 2.8
$Fshb_1$	19.4 ± 18.8	0.7 ± 0.6	0.3 ± 0.3	150.2 ± 95.1	3.6 ± 2.4	19.7 ± 19.3
$Fshb_2$	0.8 ± 0.4^	0.8 ± 0.4^	0.3 ± 0.3	2.1 ± 0.7^	1.5 ± 0.3^	0.2 ± 0.2
Lhb	1.3 ± 0.8	1.2 ± 0.7	0.3 ± 0.3	10.1 ± 6.5*	8.4 ± 5.7*	6.2 ± 3.1*
Gnrhr	2.6 ± 2.1	0.8 ± 0.4	0.3 ± 0.3	34.0 ± 19.4	1.5 ± 0.3	3.4 ± 3.1
Ascl1	1.0 ± 0.2	0.8 ± 0.4	0.3 ± 0.3	5.5 ± 2.8	1.5 ± 0.3	0.2 ± 0.2
Cga	3.1 ± 1.9	2.3 ± 1.6	0.3 ± 0.3	2.1 ± 0.7	1.9 ± 0.9	0.1 ± 0.1
Tshb	0.4 ± 0.3	0.5 ± 0.3	0.3 ± 0.3	1.4 ± 0.2*	2.2 ± 0.9*	0.4 ± 0.4*
Grifin	3.9 ± 1.7	4.4 ± 2.9	2.0 ± 1.5	10.8 ± 3.0	2.4 ± 0.7	4.2 ± 3.6

*Probes were conducted in gonadally intact rats, ovariectomized (OVX) rats, and OVX/adrenalectomized (OVX/ADX) rats that were chamber-exposed or paced mated. *Indicates a main effect for paced mated rats significantly differing from chamber-exposed control rats; ^Indicates a main effect for groups significantly differing from OVX/ADX rats, $p < 0.05$.*

DISCUSSION

Our hypothesis that engagement in paced mating would alter the gene expression of steroid targets in the midbrain, including those that may be involved in GABAergic, dopaminergic, and glutamatergic signaling and/or their downstream signal transduction processes, was upheld. Further, among rats that engaged in paced mating, the mRNA of 53 genes was significantly up-regulated in the midbrain compared to those of the chamber-exposed controls. Engaging in paced mating enhances neurosteroidogenesis in the midbrain (Frye and Rhodes, 2006; Frye et al., 2007). As such, GABAergic, dopaminergic, and glutamatergic signaling may be influenced by these changes in gene expression, albeit it must be noted that this represents only a few aspects of the functions that are mediated by the genes that were up-regulated. The present results support and extend those of previous studies to suggest some of the hormonal factors for these effects of paced mating. Our hypothesis that mating can enhance trophic factors was upheld. An unexpected finding was that the most highly up-regulated genes in the midbrain (by microarray analysis) were pituitary hormone genes [alpha glycoprotein, TSHb, LHb, FSHb, growth hormone (GH), and prolactin (PRL)] and GnRH. There was no downregulation of gene expression with mating.

Gh1

Paced mating may enhance GH expression in the midbrain, and these actions may contribute to progestogens' effects to maintain mating. In the present investigation, an interaction was observed, wherein Gh1 was enhanced by paced mating among gonadally intact rats, but not among OVX or OVX/ADX rats. Moreover, ADX rats had the lowest expression of Gh1 compared to the other groups. It is notable that 3α, 5α-THP content is co-expressed with GnRH (which promotes GH secretion) *in vitro* (Buchanan et al., 2000), 3α, 5α-THP promotes GnRH secretion (El-Etr et al., 1995; Sim et al., 2001), and infusion of GnRH facilitates lordosis of E-primed OVX rats *via* inhibition of VTA neurons (similar to 3α, 5α-THP; Sirinathsinghji et al., 1995; Suga et al., 1997). Lesioning dopamine neurons in the VTA *via* infusion of 6-hydroxydopamine (which preferentially depletes dopaminergic and noradrenergic neurons) exacerbates this effect (Sirinathsinghji et al., 1995), suggesting that the actions of GnRH neurons in this region may present a separate, but necessary, component mediating the maintenance of lordosis within the midbrain. In particular, in the midbrain central gray, β-endorphin has been demonstrated to inhibit lordosis, but this effect is overcome by the prior infusion of GnRH (Sirinathsinghji et al., 1995), supporting the notion that tonic inhibition of GnRH neurons in this region is upstream of opioid effects on lordosis. As such, the enhancement of midbrain Gh1 mRNA, *via* engagement in paced mating, may play an important role in the feedback from ovarian-derived, and adrenally derived, sex steroids that maintain mating once initiation has occurred.

The enhancement of Gh1 mRNA may also confer some of progestogens' neuroprotective effects. Gonadal steroids, such as central progestogens and peripheral E, mediate pulsatile GH secretion from pituitary in people and rodent models (Hohmann et al., 1998; Veldhuis, 1998). These effects are typically considered to occur *via* the actions of steroids at target sites within the hypothalamus and pituitary. In particular, E can act at growth hormone-releasing hormone receptor to promote GH and PRL release (Simard et al., 1986; Martel et al., 1990). Growth hormone is trophic and, throughout development, plays an important role in the maintenance of skeletal mass (Harris and Heaney, 1969; Stuart and Lazarus, 1975). Innervation of GnRH neurons *via* neurosteroid targets is enhanced cyclically. Among young adult rats in proestrous (the high-progestogen phase of the estrous cycle), the number of vesicular GABA transporter terminals that synapse on GnRH neurons is decreased and the number of vesicular glutamate transporter-2 terminals is increased compared to those in diestrous (the low progestogen phase of the estrous cycle; Khan et al., 2010). This variation is attenuated among middle-aged rats, suggesting that hormone decline drives these plastic effects (Khan et al., 2010). Thus, Gh1 mRNA enhancement, *via* exposure to paced mating, may promote trophic actions in the brain and may be partly dependent on peripheral ovary and adrenal signaling.

Lhb, Tshb, and Fshb

Engaging in paced mating may enhance the secretion of gonadotropin β subunits independent of peripheral steroidogenesis. Irrespective of whether the animals were intact, OVX, or OVX/ADX, Lhb and Tshb were significantly up-regulated in the midbrain with mating. Others have demonstrated that 3α, 5α-THP dose-dependently enhances luteinizing hormone and follicle-stimulating hormone secretion in OVX E-primed rats, effects that could be attenuated by pretreatment with a $GABA_A$, but not progestin receptor, antagonist (Murphy and Mahesh, 1984; Brann et al., 1990). While Lhb is typically considered with respect to its pulsatile secretion from the anterior pituitary to stimulate P production from ovaries (Dalkin et al., 2001; Ascoli et al., 2002), these data demonstrate Lhb mRNA enhancement in a gonad-independent manner in the midbrain (albeit this effect was greatest among gonadally intact rats). While steroid-independent examples of gonadotropin-modulating genes exist (Matagne et al., 2009), such expression is developmentally regulated. The activational effects of E to negatively regulate Lhb are critical in rodent hypothalamus and are thought to occur *via* classic steroid–DNA interactions (Glidewell-Kenney et al., 2008). However, in the midbrain, LH synthesis in response to mating may be gonad/adrenal independent and/or insufficiently repressed *via* systemic E-priming. Apart from the well-studied actions in the hypothalamus, LH may interact with important effectors at the level of the midbrain. Iontophoresis of LH-releasing hormone (LHRH) in the midbrain central gray enhances the neuronal membrane sensitivity among OVX E-primed rats (Schiess et al., 1987). Noradrenaline is also shown to stimulate LH secretion, in part, *via* actions in the dorsal noradrenergic tracts of the midbrain (Sar and Stumpf, 1981). Stimulating these midbrain tracts reduces LH secretion, supporting the importance of LH modulation at the level of the midbrain (Bergen and Leung, 1988). Similarly, Tshb was enhanced with paced mating irrespective of peripheral gland extirpation. [^{3}H]thyroid-stimulating hormone (TSH) accumulates in the midbrain (Bhargava et al., 1989), and noradrenergic projections from the locus coeruleus to the hypothalamus have been suggested to play a lesser role in TSH secretion (Jaffer et al., 1990). The nigrostriatal dopamine system has been demonstrated to modulate TSH secretion (Männistö et al., 1981), and the present data support the notion that engagement in paced mating can facilitate actions at Tshb in the midbrain. Unlike Lhb and Tshb, Fshb was reduced among OVX/ADX rats compared to that in intact or OVX rats. Fshb feedback is also independent of classic E actions in the mouse hypothalamus (Glidewell-Kenney et al., 2008). As such, these data support the notion that E may also play an important role in sensitizing gonadotropin β subunits *via* noradrenergic stimulation in the midbrain, and these actions may be facilitated by engagement in mating to further promote progestogen biosynthesis to maintain mating.

The gonadotropin substrates revealed in the present study likely demonstrate an interaction between the midbrain and the hypothalamic processes. The sequential actions of E and P in the ventromedial hypothalamus are critical for the initiation of lordosis and appear to be upstream of steroids' actions in the midbrain, which maintain lordosis and mediate the appetitive aspects of mating (solicitations and proceptive behavior; Mong and Pfaff, 2004). Prolactin cell bodies, localized to the arcuate and ventromedial nuclei of the hypothalamus, project to the midbrain central gray (Harlan et al., 1989). Luteinizing hormone releasing hormone, PRL, and TSH excite spontaneous activity of periaqueductal gray neurons in the midbrain (Ogawa et al., 1992). Further, administration of $GABA_A$ agonists modulates these effects (Ogawa et al., 1992). Thus, gonadotropins in the midbrain and the hypothalamus act as important mediators of central signaling and influence the effects of peripherally, and centrally, derived sex steroids.

S100g and Klk1b3

The enhanced mRNA of neurotrophic factors with paced mating may confer some of progestogens' neuroprotective effects. Engaging in paced mating enhances the expression of S100g mRNA in the midbrain among intact, but not OVX or OVX/ADX rats. S100g (calbindin D28k) is a calcium-binding protein that is best studied for its presence in non-degenerating midbrain dopaminergic neurons in Parkinson's disease (Lavoie and Parent, 1991; Lavoie et al., 1991; Mouatt-Prigent et al., 1994). In owl monkeys, calbindin-immunoreactive neurons are characterized by noradrenergic innervation (Gaspar et al., 1992). In rats, dopaminergic calbindin-positive cells are expressed in VTA, can be GABAergic, and are resistant to depletion *via* the 6-hydroxydopamine toxin (Sarabi et al., 2001). Similarly, in the present study, Klk1b3 mRNA up-regulation was observed markedly among gonadally intact rats, but not among OVX or OVX/ADX rats. Klk1b3 (Ngfg) protein is a trophic factor and may partly underlie the neuroprotective effects of progestogens in the brain. In rat models of traumatic brain injury or ischemic insult, the administration of P, or 3α, 5α-THP, reduces edema and tissue degeneration following the insult and improves the morphological and functional outcomes (He et al., 2004; Schumacher et al., 2004; Sayeed et al., 2007). Progesterone treatment reduces the mRNA and the protein markers of apoptosis, such as Bax and Bad in the cerebral cortex of rats that have had traumatic brain injury (Yao et al., 2005). Thus, the present data showing changes in S100g and Klk1b3 mRNA with paced mating suggest that these factors may be involved in the effects of paced mating to promote neuroprotective effects, partly *via* the neurosteroidogenesis of progestogens, in a peripheral gland-dependent manner.

Limitations of These Findings

As in all scientific work, there are limitations. Those that have limited our study are as follows: first, we were limited by the number of microarray analyses provided (n = 18). As such, we have small samples, which is typically not a problem in gene-related studies that generates volumes of data. However, this precluded ANOVAs for behavioral studies or attributional statistics about particular aspects of behavior and how it influenced gene expression. Indeed we were fortunate

to find a manageable number of factors (n = 53), many of which relate to our prior investigation that were identified in the midbrain, as well as some new and interesting targets. Second, among our study's novel findings was that most of the highly up-regulated genes found were for pituitary glycoprotein hormones. Indeed the most highly up-regulated genes in the midbrain (by microarray analysis) were pituitary hormone genes (alpha glycoprotein, TSHb, LHb, FSHb, GH, and PRL). However, there was no change in GnRH gene expression as detected by microarray (although the receptor did increase slightly). The expression of these genes in the midbrain, while consistent with some data on peripheral expression/secretion, is curious. We discuss how changes in midbrain gene expression among intact and/or those with ovaries and/or adrenals may deplete peripheral progestogen sources to influence these effects, albeit these glands are not purely progestogen secreting. For example, PRL expression is completely ablated by ovariectomy, an effect known to be dependent on estrogens. As such, we cannot rule out the role of estrogens, glucocorticoids, and/or mineralocorticoids in these processes. As discussed above, we propose that the PRL neurons in the hypothalamus project to the midbrain central gray, wherein there are many connections to the VTA below. Alternatively, there could be pituitary secretion of the said hormones in response to lordosis and modulation of midbrain neuronal activity. In order to address this, up-regulated proteins for pituitary glycoprotein hormones could be confirmed in another way, such as immunohistochemistry. However, there are just as many challenges with that technique, and given the number of factors to investigate, it is beyond the scope of the present work but will be an important topic of future investigations.

CONCLUSION

Engagement in paced mating is dependent on progestogens' actions in the midbrain and involves gonadotropin signaling, which may be important for neurosteroidogenesis, maintenance of reproduction, and/or trophic signaling. However, it must be noted that changes in gene expression cannot be solely attributed to the pacing aspects of mating in the present study. The changes observed in gene expression could be due to mating in any condition and are not necessarily associated with the pacing of the mating contacts. Future investigations should aim to assess the effects of non-paced vs. paced mating on gene expression in the brain in order to parse out the influence of consummatory vs. appetitive aspects of mating. Moreover, the direct influence of 3α, 5α-THP in these effects remains implied but unknown. Microarrays of rat midbrain tissue confirmed the presence of the pregnane xenobiotic receptor (PXR; albeit the RNA expression of PXR was not mating dependent). Ongoing work is focused on investigating the role of PXR, which may act upstream of mitochondrial mechanisms to enhance *de novo* 3α, 5α-THP production, and the subsequent effects on mating (Frye, 2011; Frye et al., 2011). Future investigations should aim to assess the influence of intra-VTA PXR and/or other biosynthetic factors upstream of 3α, 5α-THP formation in the midbrain. Despite these uncertainties, the present report demonstrates a mating-enhanced mRNA expression of gonadotropin β subunits (Lhb, Tshb, and Fshb) and neurotrophic factors (Gh1, S100g, and Klk1b3) in the midbrain. These targets may present novel substrates for neurosteroids' reproductive and/or neuroprotective effects.

The most important results of the present study were the changes in the expression of the midbrain neurosteroid genes in relation to the mating rate of females. Using microarray methods, as many as 53 genes, involved in the expression of neurosteroids, trophic factors, and pituitary hormones, differed in the proestrous paced mating group compared to the OVX/ADX groups who mated less. Thus, data on the importance of mating in relation to the greatest changes among neurosteroid, trophic, and pituitary genes were observed.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the UCLA GSE microarray consortium.references files (frye-affy-rat-483660, 584783, 584452) for intact, ovx and ovx adx rats, respectively.

ETHICS STATEMENT

The animal study was reviewed and approved by UAlbany IACUC (Institutional Animal Care and Use Committee).

AUTHOR CONTRIBUTIONS

SC assisted Jason Paris with running the microarray samples in his laboratory. CF acquired funding for the project as a supplement to her R01 examining the role of neurosteroids in the midbrain VTA in social, cognitive, and reward behavior and assisted Jason Paris through all phases of the project.

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Altered Sexual Behavior in Dopamine Transporter (DAT) Knockout Male Rats: A Behavioral, Neurochemical and Intracerebral Microdialysis Study

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Central dopamine plays a key role in sexual behavior. Recently, a Dopamine Transporter knockout (DAT KO) rat has been developed, which displays several behavioral dysfunctions that have been related to increased extracellular dopamine levels and altered dopamine turnover secondary to DAT gene silencing. This prompted us to characterize the sexual behavior of these DAT KO rats and their heterozygote (HET) and wild type (WT) counterparts in classical copulatory tests with a sexually receptive female rat and to verify if and how the acquisition of sexual experience changes along five copulatory tests in these rat lines. Extracellular dopamine and glutamic acid concentrations were also measured in the dialysate obtained by intracerebral microdialysis from the nucleus accumbens (Acb) shell of DAT KO, HET and WT rats, which underwent five copulatory tests, when put in the presence of an inaccessible sexually receptive female rat and when copulation was allowed. Markers of neurotropism (BDNF, trkB), neural activation (Δ-FosB), functional (Arc and PSA-NCAM) and structural synaptic plasticity (synaptophysin, syntaxin-3, PSD-95) were also measured in the ventral tegmental area (VTA), Acb (shell and core) and medial prefrontal cortex (mPFC) by Western Blot assays. The results indicate that the sexual behavior of DAT KO vs. HET and WT rats shows peculiar differences, mainly due to a more rapid acquisition of stable sexual activity levels and to higher levels of sexual motivation and activity. These differences occurred with differential changes in dopamine and glutamic acid concentrations in Acb dialysates during sexual behavior, with lower increases of dopamine and glutamic acid in DAT KO vs. WT and HET rats, and a lower expression of the markers investigated, mainly in the mPFC, in DAT KO vs. WT rats. Together these findings confirm a key role of dopamine in sexual behavior and provide evidence that the permanently high levels of dopamine triggered by DAT gene silencing cause alterations in both the frontocortical glutamatergic neurons projecting to the Acb and VTA and in the mesolimbic dopaminergic neurons, leading to specific brain regional changes in

trophic support and neuroplastic processes, which may have a role in the sexual behavior differences found among the three rat genotypes.

Keywords: sexual behavior, DAT knockout rats, dopamine, glutamic acid, D-FosB, BDNF/trkB, synaptic proteins, Arc

INTRODUCTION

Brain dopamine is involved in both motivational and consummatory aspects of male sexual behavior. Among brain areas that mediate the sexual roles of dopamine, the most studied are hypothalamic nuclei, as the paraventricular nucleus of the hypothalamus (PVN) and the medial preoptic area, the ventral tegmental area (VTA), the nucleus accumbens (Acb) and the prefrontal cortex (PFC). While the PVN receives the synapses of the incertohypothalamic dopaminergic neurons (originating in the catecholaminergic A3 and A4 groups, see Dahlström and Fuxe, 1964), the VTA contains the cell bodies of mesolimbic and mesocortical dopaminergic neurons, which send their projections to the Acb and PFC (Everitt, 1990; Pfaus and Phillips, 1991; Hull et al., 1995; Melis and Argiolas, 1995, 2011; Pfaus and Everitt, 1995; Argiolas and Melis, 1995, 2005, 2013; Pfaus, 2010; Sanna et al., 2011, 2012; Hull and Dominguez, 2015). Accordingly, alterations/differences in dopamine function in these areas can substantially affect several aspects of sexual behavior. For instance, we have recently reported (Sanna et al., 2014a) significant differences in both motivational and performance aspects of sexual behavior between Roman High- and Low-Avoidance (RHA and RLA) rats, which are two lines of rats psychogenetically selected for their extremely divergent acquisition of the active avoidance response in the shuttle box, and which display opposite biobehavioral traits (Giorgi et al., 2007, 2019). The sexual differences between the two Roman rat lines seem to be related, at least in part, to differences in dopamine function (Sanna et al., 2013, 2014b), in particular in the tone of mesolimbic and mesocortical dopaminergic neurons at the level of the Acb (Sanna et al., 2015b) and of the medial PFC (mPFC; Sanna et al., 2017b). Accordingly, both naïve (i.e., never exposed before to sexual stimuli) and sexually experienced RHA rats (i.e., exposed to five preliminary copulatory tests), which displayed higher dopamine increases in the Acb and mPFC when exposed to a sexually receptive female rat, displayed also higher sexual motivation and better copulatory performances (i.e., higher ejaculation frequency and intromission ratio and shorter post-ejaculatory interval and latencies to mount, intromit and ejaculate) when compared to their RLA counterparts. Sexually naïve and sexually experienced RHA and RLA rats showed also significant differences in the expression of molecules considered as markers of neural activation (i.e., C-Fos and Δ-FosB) and plasticity [Brain-Derived Neurotrophic Factor (BDNF), the tyrosine kinase receptor B (trkB), and Arc] in the VTA, Acb (shell and core) and mPFC after the exposition to, and sexual interaction with, a sexually receptive female rat (Sanna et al., 2019). In particular, RHA rats displayed higher levels of C-Fos, Δ-FosB and Arc after sexual activity than their RLA counterparts and these differences were very evident in naïve animals being reduced, although not completely, in the experienced ones (Sanna et al., 2019).

Worth noting, Roman RHA and RLA rat lines are not the only ones that display different patterns of sexual behavior concomitant to a different monoaminergic (i.e., dopaminergic and/or noradrenergic) tone. This has been found also in high and low novelty exploration responders (bNEHR and bNELR) rat lines (Cummings et al., 2013) and the High- and Low-yawning (HY and LY) rat lines (Eguibar et al., 2016) (for a review on the specific features of sexual behavior of these rat lines see Melis et al., 2019). Together these findings not only confirm the involvement of dopamine in sexual behavior (Pfaus, 2010) but also show that differences in dopamine function at the level of specific brain areas (e.g., VTA, Acb, and mPFC) may be responsible for differences in several aspects of physiological and pathological sexual behavior as well as, more in general, in motivated behavior (Melis et al., 2019).

Recently, a novel strain of knockout rats for the plasma membrane dopamine transporter DAT (DAT KO rats) has been developed by silencing the gene encoding DAT by using zinc finger nuclease technology (Leo et al., 2018b). DAT KO rats (with total DAT gene silencing) develop normally but weigh less than heterozygote (HET; with partial DAT gene silencing) and wild type (WT; with no DAT gene silencing) rats and show pronounced spontaneous locomotor hyperactivity associated with impairments in cognition (i.e., working memory) and sensory-motor gating. These rats display also impulsive/compulsive traits, stereotypies, anhedonia, asocial profile, alterations in facing novelty and in motivation (Adinolfi et al., 2018, 2019; Cinque et al., 2018; Apryatin et al., 2019; Mariano et al., 2019). To date, these behavioral alterations have been mainly related to a dysfunctional striatal dopamine turnover due to the total or partial DAT gene silencing and to alterations in frontostriatal BDNF, trkB and post-synaptic density protein 95 (PSD-95) levels, leading to consider these animals as a new model for the study of pathological hyperdopaminergic conditions ranging from the attention-deficit/hyperactivity disorder (ADHD) to autism and psychosis spectrum disorders (Leo et al., 2018a).

The availability of these new DAT KO rats prompted us to characterize the sexual behavior of these animals and their HET and WT counterparts in classical copulatory tests with a sexually receptive female rat, to get further insights on the role of dopamine in the male rat sexual behavior. We have first characterized the copulatory pattern of DAT KO rats compared to their HET and WT counterparts and verified if and how sexual behavior changes in following (up to five) copulatory tests done at 3-day intervals from each other, with a sexually receptive

female rat. After the characterization of their copulatory patterns, these DAT KO, HET, and WT rats were then implanted with an intracerebral microdialysis probe to measure the extracellular concentration of dopamine in the dialysates from the shell of the Acb, a key area involved in the motivational aspects of sexual behavior (Fiorino et al., 1997; Sanna et al., 2015b) and, more in general, in the transposition of the motivational drive in goal-directed behaviors (Goto and Grace, 2005 and references therein), during both the appetitive (motivation) and consummatory (motivation and performance) phases of sexual behavior. Extracellular glutamic acid concentration was also measured in the same dialysate aliquots used for dopamine measurement, due to the key role of this excitatory amino acid in modulating dopamine activity in the Acb (Britt et al., 2012; Quiroz et al., 2016). Finally, since it has been shown: (i) that dopamine neurotransmission is related to the proper expression of products of the immediate early gene as Δ-FosB (Pitchers et al., 2013) and Arc (Fosnaugh et al., 1995; Managò et al., 2016); (ii) that the dopaminergic dysfunction caused by DAT gene silencing may hamper brain maturation and cause long-lasting impairment of cortico-striatal expression of molecules involved in neurotrophic support and synaptic plasticity such as BDNF, trkB and PSD-95, leading to a persistent reduction in neuronal plasticity and subsequent behavioral alterations (Fumagalli et al., 2003; Yao et al., 2004; Efimova et al., 2016; Leo et al., 2018b); and (iii) that RHA rats, which have a higher dopaminergic tone than RLA rats in the Acb and mPFC, also displayed higher levels of C-Fos, Δ-FosB and Arc after sexual activity than their RLA counterparts together with differential changes in BDNF-trkB system in the VTA, mPFC and Acb (Sanna et al., 2019); we measured not only the expression of the neurotrophic molecules BDNF and trkB (Cunha et al., 2010) and of Δ-FosB, a marker of neural activation (Nestler, 2008; Pitchers et al., 2010b), but also of Arc and PSA-NCAM, two markers of functional synaptic plasticity (see Muller et al., 2000; Bonfanti, 2006; Gascon et al., 2007; Bramham et al., 2010; Korb and Finkbeiner, 2011), and of synaptophysin, syntaxin-3 and PSD-95, markers of structural synaptic plasticity (see El-Husseini et al., 2000; Minzer et al., 2004; Yoon et al., 2007), respectively, by means of Western Blot assays in *ex vivo* tissues of the VTA, mPFC and Acb shell and core of DAT KO, HET and WT rats that underwent intracerebral microdialysis. We hypothesized that: (i) DAT KO, HET, and WT rats should display behavioral differences in several aspects of sexual behavior (e.g., acquisition of sexual experience, motivation, performance) with DAT KO rats displaying a more rapid acquisition of sexual experience (i.e., a stable level of sexual activity) and higher levels of sexual motivation and performance than HET and WT rats, in particular, shorter latencies to mount, intromit and ejaculate, and a higher intromission ratio and ejaculatory frequency; and (ii) these differences should occur concomitantly with differences in the activity of dopamine and/or glutamic acid neurotransmission at the level of the Acb shell, in particular, a higher dopamine and/or glutamic acid activity in DAT KO rats compared to their HET and WT counterparts, and in the expression of one or more markers of neurotrophism, neural activation, functional and structural synaptic plasticity in limbic brain areas relevant for sexual behavior as the VTA, the mPFC and/or the Acb [e.g., DAT KO should be expected to display greater differences than HET compared to WT rats and, in particular, higher levels of Δ-FosB and Arc (Sanna et al., 2019) but lower levels of BDNF, trkB, and PSD-95 (Leo et al., 2018b)].

MATERIALS AND METHODS

Animals

The DAT KO rat line was created in the outbred Wistar Han background at SAGE Labs. The procedures used for the construction, validation, selection, and breeding of the colony have been described in detail elsewhere (Leo et al., 2018b). Male DAT knockout (DAT KO; $N = 8$), heterozygote (HET; $N = 10$) and wild type (WT; $N = 8$) rats (weighing 250–300 g at the beginning of the experiments) were from the colony established at the Italian Institute of Technology, Genoa, Italy. Genotyping was performed by PCR followed by enzymatic digestion with BtsI MutI (New England Biolabs, Milan, Italy). Primers used for PCR amplification were the following: Slc6a3 Cel-1 F 5′-TCCTGGTCAAGGAGCAGAAC-3′, Slc6a3 Cel-1 R 5′-CACAGGTAGGGAAACCTCCA-3′ (Leo et al., 2018b).

Ovariectomized stimulus female rats ($N = 30$, weighing 250–300 g at the beginning of the experiments) used in all the experiments, were obtained from Envigo (San Pietro al Natisone, Italy). Animals were kept 2–4 per cage (38 cm × 60 cm × 20 cm) and were acclimated to the housing facilities of the Department of Biomedical Sciences of the University of Cagliari for at least 10 days before the beginning of the experiments under controlled environmental conditions (24°C, 60% humidity, reversed 12 h light/dark cycle, with lights off from 08:00 to 20:00 h) and with water and standard laboratory food *ad libitum*. To limit the stress due to manipulation during the experiments, each animal was daily handled for approximately 1–2 min throughout the habituation period; also, contact with the animal house maintenance personnel was limited to a single attendant and bedding in the home cages was never changed either the day before or on the day of the experiment. The experiments were performed between 10:00–18:00 h according to the guidelines of the European Communities Directive of September 22, 2010 (2010/63/EU) and the Italian Legislation (D.L. March 4, 2014, n. 26), and approved by the Ethical Committee for Animal Experimentation of the University of Cagliari.

Experimental Groups

Male DAT KO, HET, and WT rats were used in classical 60 min copulatory tests with an ovariectomized sexually receptive female rat. Oestrus was induced by subcutaneous injections of oestradiol benzoate (200 μg/rat in peanut oil) and progesterone (0.5 mg/rat in peanut oil), 48 and 6 h before the behavioral tests, respectively, and ascertained by May-Grunwald-Giemsa coloration and microscopical examination of vaginal smears 1 h before the experiments (Contini et al., 2018). All DAT KO, HET and WT rats underwent five consecutive copulatory tests at 3 days intervals from each other with an always new sexually receptive female rat (Sanna et al., 2014a,b, 2015a,b, 2017b, 2019). Two days after the last copulatory tests, DAT KO, HET and WT

rats underwent stereotaxic surgery for the implantation of the microdialysis probe in the Acb shell.

Sexual Behavior

The following sexual responses were recorded during the first series of copulatory activity (e.g., from the first mount/intromission to the first intromission after the first ejaculation) of the five preliminary copulatory tests and the microdialysis experiment, by an observer who was not aware of the rat line used: mount and intromission latency (ML and IL, timed from the moment in which the receptive female rat is directly accessible to the male until the first mount and/or the first intromission, respectively); mount and intromission frequency (MF and IF, the number of mounts and intromissions in the first series of copulatory activity, respectively); ejaculation latency (EL, timed from the first intromission in the first series until ejaculation) and post-ejaculatory interval (PEI, timed from the first ejaculation until the next intromission). Intromission ratio (IR, the number of intromissions in the first series divided by the sum of the number of mounts and intromissions in the same series) and the inter-intromission interval (III, the ratio between the ejaculation latency of the first series and the number of intromissions in that series) were also calculated. In addition to the above parameters, the total number of mounts (TMF), of intromissions (TIF) and of ejaculations (EF) in the whole 60 min period of copulation, the total copulatory rate (TCR), calculated by dividing the sum of all the activity periods of the male with the female rat (an activity period was defined from the first mount/intromission in a series of copulatory activity until ejaculation in that series) by the sum of all mounts and intromissions of the whole test and the total intromission ratio (TIR) calculated as the TIF divided by the sum of TIF and TMF were also calculated. Moreover, the number of noncontact penile erections (NCPEs), counted during the 30 min period in which the receptive female rat was inaccessible to the male during the microdialysis experiment (see below), was also recorded (Sachs and Barfield, 1976; Melis et al., 2003; Sanna et al., 2014a,b, 2015a,b, 2017b, 2019; Le Moëne and Ågmo, 2019). Finally, since substantial differences in genital self-grooming, a centrally-mediated, self-directed highly stereotyped behavior that in the context of copulation may indicate self-cleaning and/or self-stimulation (Sachs et al., 1988; Berridge et al., 2005), were observed among the three rat lines during the five copulatory tests, the percent of mounts, intromissions, and ejaculations followed by genital grooming as well as the frequency and duration of genital grooming episodes after mounts, intromissions and ejaculation were recorded for each animal in the first series of copulatory activity during the microdialysis experiment (Sachs et al., 1988).

Microdialysis in the Acb Shell During Sexual Behavior

The day before the microdialysis experiment, DAT KO, HET and WT rats were stereotaxically implanted (Stoelting Co., Wood Dale, IL, USA), under isoflurane anesthesia (1.5–2%; Harvard Apparatus, Holliston, MA, USA), with a microdialysis probe with a U-shaped dialysis membrane (approximately 2 mm of free surface for dialysis), prepared as previously described (Melis et al., 2003), and aimed unilaterally at the Acb shell (coordinates: 2.0 mm anterior and 0.8 mm lateral to bregma, and 8.0 mm ventral to dura; Paxinos and Watson, 2004). On the day of the experiment, during the dark phase of the cycle, the rats were transferred to a mating cage (45 cm × 30 cm × 24 cm) located in a soundproof room lit by a dim red light. The mating cage contained another small Plexiglas cage (15 cm × 15 cm × 15 cm) with 25 holes (Ø 2 mm) in each vertical wall to allow for visual, olfactory and acoustic communication (Sanna et al., 2015b, 2017b). After a 2 h habituation period, the microdialysis probe was connected *via* polyethylene tubing to a CMA/100 microinfusion pump (Harvard Apparatus, Holliston, MA, USA) and perfused with Ringer's solution, containing 147 mM NaCl, 3 mM KCl and 1.2 mM $CaCl_2$, pH 6.5, at a constant flow rate of 2.5 μl/min. After a 2 h equilibration period, four aliquots of 37.5 μl of Acb dialysates were collected every 15 min in polyethylene tubes kept on ice for the measurement of dopamine and glutamic acid concentrations, as described below. A sexually receptive female rat was then introduced into the small cage located inside the mating cage for 30 min, during which two more dialysate aliquots were collected and NCPEs counted (NCPEs are pheromone-mediated penile erections that male rats show in the presence of an inaccessible sexually receptive female rat that they can see, hear, smell but not touch, and are considered an index of sexual arousal; Sachs et al., 1994; Melis et al., 2003; Sanna et al., 2009). Thirty minutes after the introduction of the female rat, the small cage was removed from the mating cage and sexual interaction/copulation allowed for 60 min, during which four more dialysate aliquots were collected and sexual parameters recorded (see above). After 60 min of copulation, the female rat was removed from the mating cage and one additional dialysate aliquot collected (Pfaus and Everitt, 1995; Melis et al., 2003; Sanna et al., 2015b, 2017b).

Determination of Dopamine Concentration in Dialysates From the Acb Shell

Dopamine concentration in the dialysate from the Acb shell was measured by high-pressure liquid chromatography (HPLC) on a 7.5 cm × 3.0 mm i.d., Supelcosil C18, 3 μm particle size column (Supelco, Supelchem, Milan, Italy) coupled to electrochemical detection (Coulochem II, ESA, Cambridge, MA, USA) using a 4011 dual cell, as already described (Sanna et al., 2015b, 2017b). Detection was performed in reduction mode with potentials set to +350 and −180 mV. The mobile phase was 0.06 M citrate/acetate pH 4.2, containing methanol 20% v/v, 0.1 mM EDTA, 1 μM triethylamine and 0.03 mM sodium dodecyl sulfate at a flow rate of 0.6 ml/min. The sensitivity of the assay was 0.125 pg.

Determination of Glutamic Acid Concentration in Dialysates From the Acb Shell

The glutamic acid concentration in the dialysate from the Acb shell was measured in the same dialysate aliquots used

for the measurement of dopamine as previously described (Succu et al., 2011; Bratzu et al., 2019). Briefly, glutamic acid concentration was measured in 5 μl aliquots of dialysate added to 5 μl of $HClO_4$ 100 mM after pre-column derivatization with orto-phtalaldialdehyde and 2-mercaptoethanol by HPLC. The chromatograph was equipped with an automatic injector, a 15 × 0.4 cm Supelco C18 column, 5 μm particle size, and coupled to fluorescence detection (excitation wavelength: 318 nm; emission wavelength: 452 nm; SFM 25 spectrofluorimeter, Kontron, Milan, Italy). The mobile phase was phosphate buffer 0.1 M, pH 6.2 containing methanol 30% v/v and the flow rate 1 ml/min. The column temperature was maintained at 35°C. The sensitivity of the assay was 10 nM.

Western Blot

After sacrifice by guillotine, rat brains were rapidly dissected and cooled in dry ice for 15 s, placed in a brain matrix and cut in 2 mm thick coronal slices using the stereotaxic coordinates of the rat brain atlas of Paxinos and Watson (2004) as a reference. Unilateral punches of the Acb shell and core (diameter 1.5 mm), contralateral to the side implanted with the microdialysis probe, and bilateral paramedian punches of the mPFC (diameter 2.5 mm) and the VTA (diameter 3 mm) were collected as previously described (Sanna et al., 2019). For each rat, tissue punches were rapidly frozen at −80°C and homogenized in distilled water containing 2% sodium dodecyl sulfate (SDS; 300 μl/100 mg of tissue) and a cocktail of protease inhibitors (cOmplete^{TM}, Mini Protease Inhibitor Cocktail Tablets, Cat# 11697498001, Roche, Basel, Switzerland).

Protein concentrations were determined using the Lowry method (Lowry et al., 1951) with bovine serum albumin as the standard. Proteins, 40 μg for each tissue homogenate, diluted 3:1 in 4× loading buffer (NuPAGE LDS Sample Buffer 4×, Novex ThermoFisher Scientific, Waltham, MA, USA), were heated to 95°C for 7 min and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using precast polyacrylamide gradient gel (NuPAGE 4–12% Bis-Tris Gel Midi, Novex, ThermoFisher Scientific, Waltham, MA, USA) in the XCell4 Sure-Lock Midi-Cell chamber (ThermoFisher Scientific, Waltham, MA, USA). Internal molecular weight (MW) standards (Precision Plus Protein WesternC Standards, Bio-Rad, Hercules, CA, USA) were run in parallel. Two gels at a time were run for Coomassie staining and immunoblotting, respectively. Proteins for immunoblotting were electrophoretically transferred on a polyvinylidene fluoride membrane (Amersham Hybond-P, GE Healthcare, Little Chalfont, UK) using the Criterion Blotter (Bio-Rad). Blots were blocked by immersion in 20 mM Tris base and 137 mM sodium chloride (TBS), containing 0.1% Tween 20 (TBS/T) and 5% milk powder, for 60 min, at room temperature. The primary antibodies were rabbit polyclonal antibodies against BDNF (Cat# N-20 sc-546, Santa Cruz Biotechnology, Dallas, TX, USA), and trkB [Cat# (794) sc-12, Santa Cruz Biotechnology, Dallas, TX, USA], both diluted 1:1,000, and syntaxin-3 (Cat#ab133750, AbCam, Cambridge, UK), diluted 1:500; rabbit monoclonal antibodies against Δ-FosB (Cat#14695; Cell Signalling Biotechnology, Netherlands) and synaptophysin (Cat#5461; Cell Signalling Biotechnology), both diluted 1:1000; and mouse monoclonal antibodies against PSA-NCAM (Cat# MAB5324, RRID: AB_95211, Merck Millipore, Darmstadt, Germany), PSD-95 (Cat# MAB1596; Merck Millipore) both diluted 1:1,000, and Arc (Cat# sc-17839, RRID: AB_626696; Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:300 in TBS/T containing 5% milk powder and 0.02% sodium azide. Incubations with primary antiserum were carried out for one night at 4°C. After rinsing in TBS/T, blots were incubated at room temperature, for 60 min, with peroxidase-conjugated goat anti-rabbit serum (Cat#9169, Sigma Aldrich, St. Louis, MO, USA), diluted 1:10,000, and anti-mouse serum (AP124P, Merck Millipore), diluted 1:5,000 in TBS/T. Controls for equal-loading of the wells were obtained by immunostaining the membranes, as above, using a mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; MAB374, Merck Millipore), diluted 1:1,000, as the primary antiserum, and a peroxidase-conjugated goat anti-mouse serum (AP124P, Merck Millipore), diluted 1:5,000, as the secondary antiserum. To control for non-specific staining, blots were stripped and incubated with the relevant secondary antiserum. To check for antibody specificity and cross-reactivity, the anti-BDNF antibody was challenged with 200 ng of rhBDNF (Cat# B-257, Alomone Labs, Jerusalem, Israel), while the anti-PSA-NCAM antibody was preabsorbed with 500 ng of the alfa-2–8-linked sialic polymer colominic acid (Cat# sc-239576, Santa Cruz Biotechnology, USA). After rinsing in TBS/T, protein bands were developed using the Clarity Max ECL Substrate (Cat# 1705062, Bio-Rad), according to the protocol provided by the manufacturer, and visualized using the ImageQuant LAS-4000 (GE Healthcare). Approximate MW and relative optical density (O.D.) of the labeled protein bands were evaluated by an examiner who was not aware of the rat line from which the tissue analyzed was obtained. The ratio of the intensity of the BDNF-, trkB-, PSA-NCAM-, Arc-, Syntaxin-3-, Synaptophysin-, PSD-95- and Δ-FosB-positive bands, to the intensity of the GAPDH-positive ones was used to compare the relative expression levels of these proteins in the DAT KO, HET, and WT rats. Image Studio Lite Software (RRID: SCR_014211, Li-Cor[1]) was used to quantify the O.D. of each sample.

Histology

During the tissue sampling procedure (see above) the hemi-slice containing the track of the microdialysis probe in the Acb shell was collected and immediately stored in 4% aqueous formaldehyde for 12–15 days. Forty micrometre transverse brain sections were then prepared using a freezing microtome, stained with Neutral Red and inspected on a phase-contrast microscope. The position of the tip of the microdialysis probe in the Acb shell was localized by following the tract of the microdialysis probe through a series of brain sections (**Figure 1**). Only animals with the dialyzing membrane of the microdialysis probe positioned correctly in the Acb shell (eight WT, 10 HET, and eight DAT KO rats) were considered for the statistical evaluation of the results.

[1]https://www.licor.com/bio/image-studio-lite/download

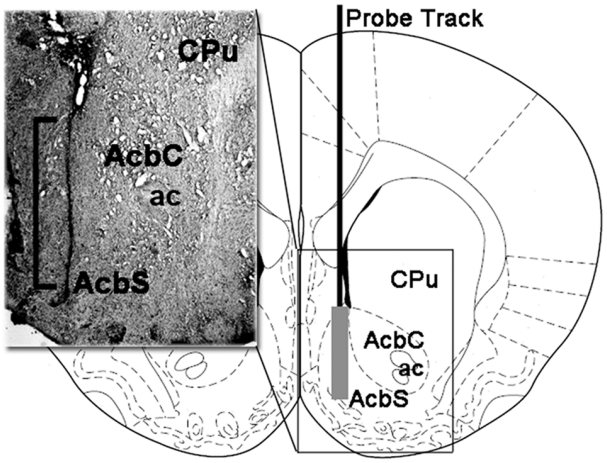

FIGURE 1 | Schematic representation of a coronal section of the rat brain showing the track of the microdialysis probe in the Acb shell (Paxinos and Watson, 2004). Insert: the square bracket in the micro-photograph indicates the portion of the Neutral Red-stained section showing the active part of the dialyzing membrane of the microdialysis probe into the Acb shell. Abbreviations: CPu, caudate-putamen; ac, anterior commissure; AcbS, nucleus accumbens shell; AcbC, nucleus accumbens core.

Statistics

Data reported in **Figures 2**, **6A** are presented as a percent of the scored sexual responses and were analyzed using the Chi-square (χ^2) test. All the other data, reported in **Figures 3–8**, are presented as mean values ± SEM and were analyzed using one- or two-way ANOVAs for repeated measures with the rat line as a between-subjects factor and the time (i.e., copulatory test or dialysate fraction depending on the data set) as a within-subjects factor. When ANOVAs revealed statistically significant main effects and/or interactions, pairwise comparisons were performed by using the Tukey's multi comparison test or Bonferroni's corrected multiple t-tests.

Before performing ANOVAs, data sets of each experimental variable were checked for the normal distribution of the values with the Shapiro–Wilk's test and the homogeneity of variances with the Brown–Forsythe test. When significant differences in the variances of a data set were found, these data were analyzed by means of ANOVAs with the Brown-Forsythe or the Geisser–Greenhouse correction for one- and two-way ANOVAs, respectively.

Animals that did not mount or intromit or ejaculate with the available female rat were assigned the respective full range scores: 900 s for ML and IL when male rats did not mount or intromit within 15 min; 1,800 s for EL when male rats did not ejaculate within 30 min from the first intromission and 600 s for PEI when male rats did not intromit within 10 min after the first ejaculation (Sanna et al., 2014a,b, 2015a,b, 2017b, 2019).

Data from Western blot assays were first analyzed by using the ROUT test (Q = 10%) to exclude outliers due to technical problems in collecting/processing the brain tissues (which can lead to erroneous values interfering with the sum-of-the-squares calculation, leading to misleading results) and then analyzed using one-way ANOVAs as described above.

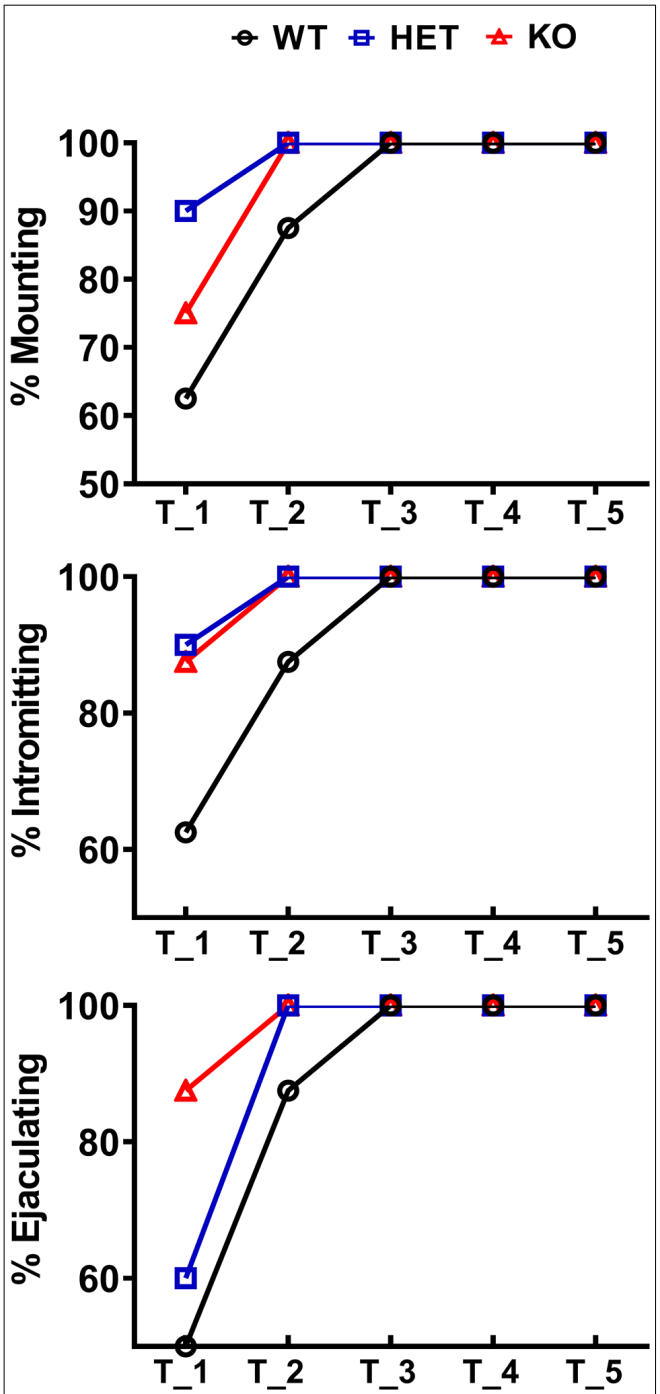

FIGURE 2 | Percentage of male WT, HET and DAT KO rats (8–10 per group) engaged in sexual behavior in copulatory tests 1–5. During each test, male rats were put together with a sexually receptive female rat for 60 min as described in the "Materials and Methods" section. The percent of rats showing mounts, intromissions and that reached ejaculation is reported (Chi-square test, not significant).

Statistical analyses were all carried out with PRISM, Graph Pad 8 Software (San Diego, USA) with the significance level set at $p < 0.05$.

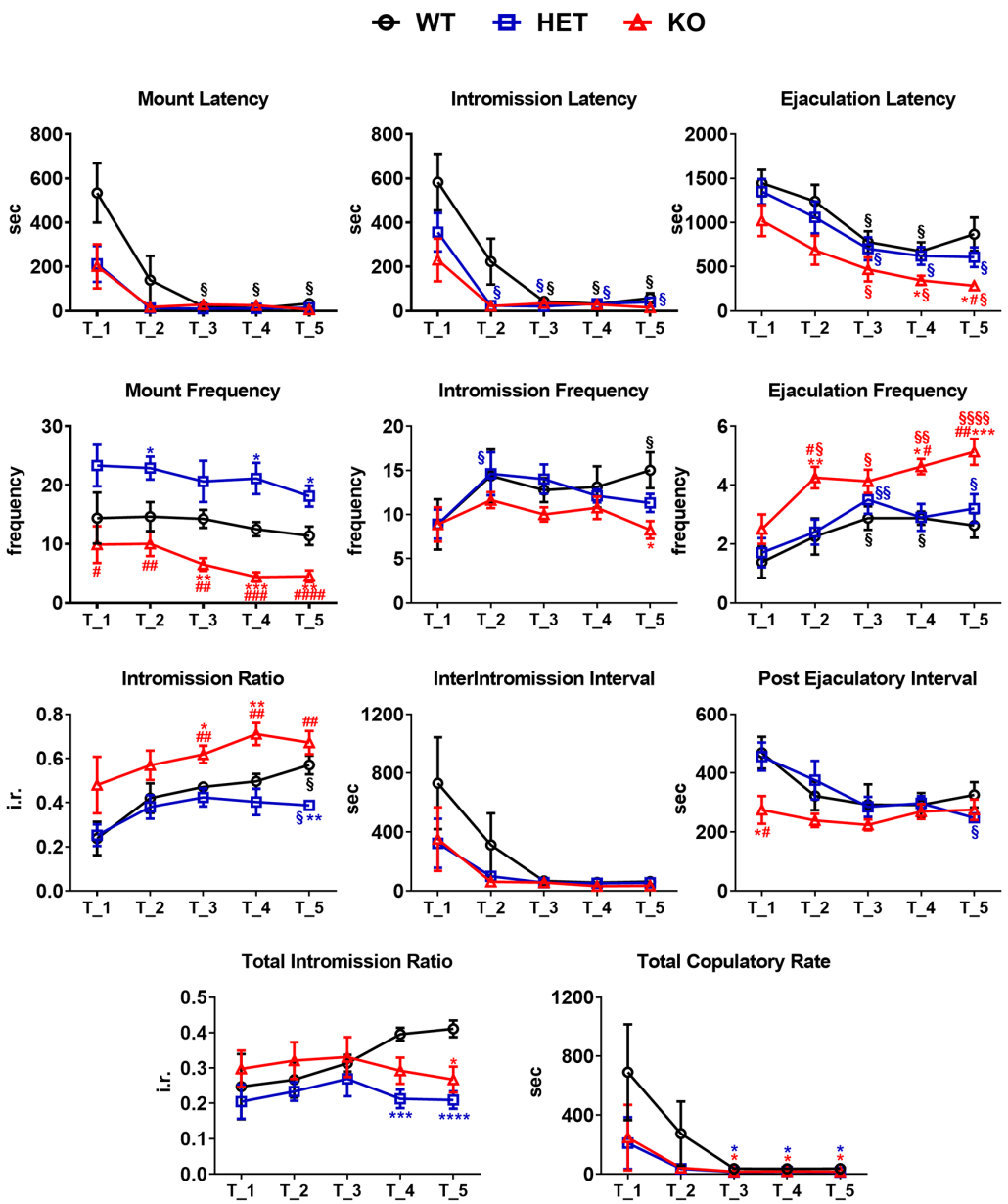

FIGURE 3 | Sexual behavior of male WT, HET and DAT KO rats in the first series of the copulatory test 1, 2, 3, 4 and 5. Male rats were put together with a sexually receptive female rat and observed in order to measure copulatory parameters as described in the "Materials and Methods" section. Copulatory parameters were measured directly or calculated as described in the "Material and Methods" section. Values are means ± SEM of 8/10 rats per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ with respect to WT; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, DAT KO with respect to HET; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S\S}P < 0.0001$, with respect to T1 (two-way ANOVA for repeated measures followed by Tukey's or Bonferroni's pairwise comparisons).

RESULTS

Male DAT KO, HET and WT Rats Display Differences in Sexual Behavior When Exposed to a Sexually Receptive Female Rat

As shown in **Figure 2**, in the first copulatory test more than 80% of DAT KO and HET rats engaged in copulatory behavior with a sexually receptive female rat, showing mounts and intromissions, but with about 85% of DAT KO rats achieving ejaculation compared to the 60% of HET rats. These values were higher than those of WT rats since only 50–60% of these rats engaged in copulatory behavior with the receptive female rat and achieved ejaculation in the first test. The percent of DAT KO and HET rats showing mounts, intromissions and achieving ejaculation raised to 100% in the second test, as did the WT rats in the third test (**Figure 2**). Irrespective

TABLE 1 | F values and significance levels from two-way ANOVAs for repeated measures (*df* = 2, 4, 8, 92) performed on data reported in **Figure 3**.

Parameter	F values		
	Line	Test	Line × Test
ML	3.570*	19.15****	2.394*
IL	5.000*	27.04****	2.279*
EL	4.683*	24.87****	0.3438
MF	22.56****	2.376	0.2151
IF	1.487	3.970**	0.8752
EF	8.002**	12.02****	0.9428
PEI	4.394*	4.962**	1.341
III	1.560	7.871**	0.7993
IR	11.73***	9.781****	0.7833
TCR	2.013	5.492*	0.9628
TIR	3.510*	1.115	1.790

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

of the rat line considered, the differences observed in the first and, to a lesser extent, second test among the three rat lines, disappeared in the fourth and fifth tests. However, these differences did not reach statistical significance probably due to the relatively low number of animals used (Chi-square test, Test 1, mounts: $\chi^2 = 1.918$, intromissions: $\chi^2 = 2.501$, ejaculations: $\chi^2 = 2.693$; Test 2, mounts: $\chi^2 = 2.340$, intromissions: $\chi^2 = 2.340$, ejaculations: $\chi^2 = 2.340$, all $Ps > 0.05$). Perhaps more importantly, the above differences occurred together with changes in the copulatory patterns of DAT KO, HET and WT rats (**Figure 3**) along the five copulatory tests. Accordingly, two-way ANOVA analyses of the parameters recorded in the first series of copulatory activity of the five copulatory tests, revealed significant differences in ML, IL, EL, IF, EF, IR, III, PEI, and TCR along the five tests, and in ML, IL, EL, MF, EF, IR, TIR and PEI among the three rat lines, differences that support a higher level of sexual activity of DAT KO and HET rats compared to WT rats. With the exclusion of the ML and IL, no Line × Test interactions were detected for the other parameters analyzed, a finding that shows that most of the differences observed among the three rat lines during the first copulatory test tended to be conserved along the subsequent ones (**Table 1**). Moreover, pairwise comparisons (main effect "Test") also showed that some of the copulatory parameters of the first series of copulatory activity of the first test of DAT KO, HET and WT rats underwent significant changes when compared with those of the first series of copulatory activity of the subsequent tests. Such changes were very evident in all three rat lines when moving from the first to the second and third test, after which no further change was observed in the fourth and fifth test. Infact, when moving from the first to the fifth test, ML, IL, EL and III values decreased and EF and IR values increased in all rat lines, while PEI decreased and IF increased depending on the rat line (see **Figure 3** for single points of significance for each rat line). Irrespective of the similar trends found along the five tests among DAT KO, HET and WT rats, pairwise comparisons (main effect "Line") revealed also that DAT KO rats displayed: (i) a shorter EL and a higher EF, which became both statistically significant in the last two (4th and 5th) tests, and a shorter PEI during the first test when compared to both WT and HET rats; (ii) a significantly lower, and HET rats a significantly higher, MF than WT rats along the five tests; (iii) a significantly higher, and HET rats a significantly lower IR, which became both statistically significant in the last test(s) (tests 3–5), than that of WT rats (see **Figure 3** for pairwise single points of significance between rat lines).

Basal Concentrations of Extracellular Dopamine and Glutamic Acid in Acb Shell Dialysates From DAT KO, HET and WT Rats

Under the used experimental conditions, the amounts of dopamine and glutamic acid in the dialysate obtained from DAT KO ($N = 8$), HET ($N = 10$) and WT ($N = 8$) rats that underwent five copulatory tests, and with the microdialysis probe correctly implanted in the Acb shell, were ≅54.7, 15.2 and 6.1 pg of dopamine in 20 μl of dialysate, respectively, and 0.44, 0.51 and 0.59 ng of glutamic acid in 5 μl of dialysate, respectively. These values correspond to a concentration of ≅18.70, 5.30 and 2.00 nM for extracellular dopamine, respectively, and of ≅0.60, 0.70 and 0.80 μM for extracellular glutamic acid, respectively, in DAT KO, HET and WT rats (**Table 2**). The above values were obtained after a 2 h perfusion period to equilibrate the Ringer's solution with the Acb shell extracellular fluid. Since the recovery of authentic dopamine and glutamic acid of the dialysis probes was approximately 20%, extracellular dopamine and glutamic acid concentrations may be estimated to be close to ≅93.50, 26.50 and 10.00 nM for dopamine and ≅3.00, 3.50 and 4.00 μM for glutamic acid in the Acb shell of DAT KO, HET and WT rats, respectively. One-way ANOVA detected significant differences in the basal values of dopamine but not of glutamic acid concentrations among the three rat lines (calculated as the mean of the dopamine/glutamic acid values of the last four dialysate aliquots for each rat before the introduction of the female rat in the small cage of the mating cage; **Table 2**).

The Extracellular Dopamine and Glutamic Acid Concentrations in the Acb Shell Dialysates From DAT KO, HET and WT Rats Change Differentially During Sexual Activity

As shown in **Figure 4A**, the presence of the inaccessible receptive female rat and subsequent copulation with her led to an increase in extracellular dopamine concentrations in the Acb shell dialysate from DAT KO, HET and WT rats, but with significant differences among the three rat lines. These differences are due mainly to the differences found in the absolute values of dopamine in the Acb dialysate of the three rat lines (dopamine in DAT KO > HET > WT rats) rather than to differences in the temporal patterns of dopamine release, as these were found very similar among the three rat lines. Accordingly, two-way ANOVA analysis of the dopamine values of the three rat lines revealed significant effects of Line, Time and a significant Line × Time interaction

TABLE 2 | Basal concentrations of extracellular dopamine and glutamic acid in the dialysate from the Acb shell of WT, HET and DAT KO rats.

Parameter	Line			One-way ANOVA	
	WT (*N* = 8)	HET (*N* = 10)	DAT KO (*N* = 8)	$F_{(2,23)}$	*p*
Dopamine (nM)	1.98 ± 0.18	5.27 ± 0.40	18.72 ± 3.64$^{****\#\#\#}$	19.38	>0.0001
Glutamic Acid (μM)	0.80 ± 0.12	0.71 ± 0.07	0.59 ± 0.06	1.39	0.2692

$^{****}P < 0.0001$ vs. WT. $^{\#\#\#}P < 0.001$ DAT KO vs. HET. One-way ANOVAs followed by Tukey's pairwise comparisons. Values are means ± SEM of the means of the last four dialysate aliquots of 8/10 rats/group collected before the introduction of the female rat in the mating cage.

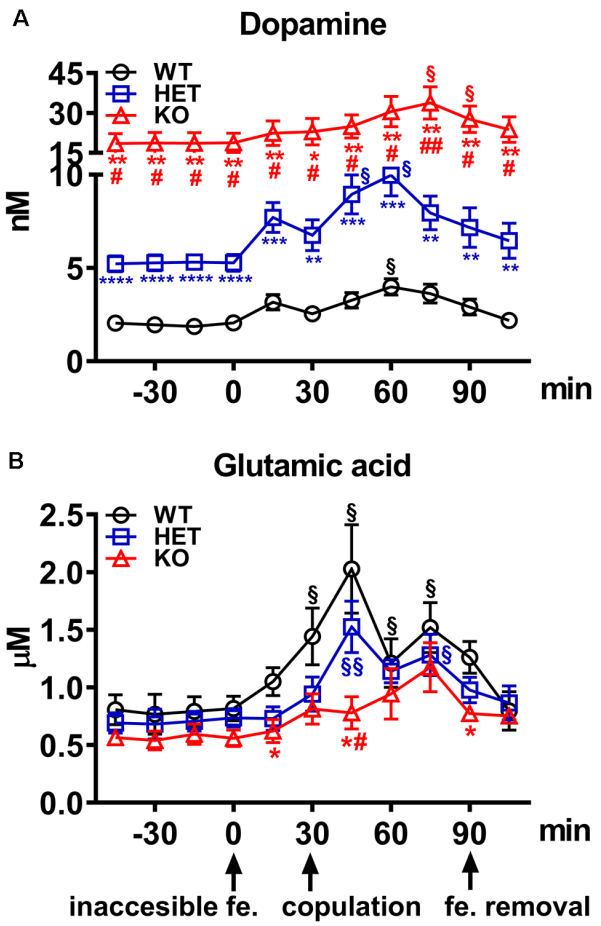

FIGURE 4 | **(A)** Extracellular dopamine and **(B)** glutamic acid concentrations in the Acb shell dialysates obtained from WT, HET and DAT KO rats during sexual activity. Rats from each line, which underwent five copulatory tests with a sexually receptive female rat in the 3 weeks preceding the experiment, stereotaxically implanted with a microdialysis probe aimed at the Acb shell, were placed individually into the mating cage and perfused with the dialysis buffer as described in the "Materials and Methods" section. An inaccessible receptive female rat was then placed inside the small cage of the mating cage (time = 0). After 30 min, copulation was allowed by removing the small cage for 60 min, after which the female rat was removed from the mating cage. During the experiment, NCPEs were counted and copulatory parameters measured, and dialysate aliquots collected every 15 min and analyzed for dopamine and glutamic acid as described in the "Materials and Methods" section. Values are means ± SEM of the values obtained by 8/10 rats per group. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, with respect to basal values (no female rat); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ with respect to WT; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, DAT KO with respect to HET rats (two-way ANOVA for repeated measures followed by Tukey's or Bonferroni's pairwise comparisons).

(see **Table 3** for F values and significance level). Moreover, pairwise comparisons showed: (i) significant differences in extracellular basal dopamine values among the three rat lines as well as during the exposition to the receptive female rat (main effect "Line"; see **Figure 4A** for pairwise single points of significance between rat lines); and (ii) a significant increase in the dopamine release during sexual interaction with the receptive female rat at 45 min in HET, 60 min in WT and at 75 min in DAT KO rats (with peaks of 101%, 89% and 80% above basal values, at 60 min in WT and HET and 75 min in DAT KO rats, respectively; main effect "Time"). The increments in dopamine release persisted throughout the entire copulation period, decreasing slowly to values similar to the basal ones after removal of the female rat in WT rats and, to a lesser extent, in HET and DAT KO rats (see **Figure 4A** for single points of significance during the experiment).

As shown in **Figure 4B**, the concentrations of glutamic acid also increased above the basal values in the Acb shell dialysate from DAT KO, HET, and WT rats when exposed to the receptive female rat. However, although the basal values of the amino acid were found similar among DAT KO, HET and WT rats, the temporal pattern of glutamic acid release was significantly different among the three rat lines. Accordingly, two-way ANOVA analysis of glutamic acid values of DAT KO, HET and WT rats revealed significant effects of Line, Time and a significant Line × Time interaction (see **Table 3** for F values and significance level). Moreover, pairwise comparisons (main effect "Time") revealed a first significant increase in glutamic acid concentration after the introduction of the female rat in the mating cage, but only in WT rats (80% above basal values), whereas a higher increase in WT and also in HET rats was detected during copulation, with peak values in the first 15 min for both lines (154% and 115% above basal values, in WT and HET rats, respectively). In contrast, while no increase in glutamic acid concentration was detected in DAT KO rats either when the female rat was inaccessible or in the first 30 min of copulation, an increase in the concentration of the amino acid was found in DAT KO rats only in the second half period of copulation with a peak value at 75 min (100% above basal values; see **Figure 4B** for single points of significance during the experiment). Pairwise differences (main effect "Line") in glutamic acid values among the three rat lines were also detected during copulation (from 45 up to 90 min), in particular between DAT KO and WT rats and, to a lesser extent, between DAT KO and HET rats (see **Figure 4B** for pairwise single points of significance among rat lines).

TABLE 3 | F values and significance levels of two-way ANOVAs for repeated measures performed on the results shown in **Figures 4**, **5**.

Parameter	F Values			
	Line	Time	Line × Time	*df*
Dopamine	22.60****	11.98****	4.103****	2,10, 20, 230
Glutamic acid	3.479*	15.23****	1.874*	2,10, 20, 230
NCPEs	6.911**	3.338	4.507*	2,1, 2, 23
Mounts	23.83****	1.890	4.574***	2,3, 6, 23
Intromissions	1.385	32.18****	1.616	2,3, 6, 23
Ejaculations	6.369**	11.29****	0.8957	2,3, 6, 23

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

The Differences in Extracellular Dopamine and Glutamic Acid Concentrations in Acb Shell Dialysates From DAT KO, HET and WT Rats Occur Concomitantly With Differences in Sexual Behavior

The differences in extracellular dopamine and glutamic acid concentrations in the dialysate from the Acb shell found in DAT KO, HET and WT rats (**Figure 4**) occurred concomitantly with differences in the number of NCPEs recorded when the female rat was inaccessible and in several copulatory parameters (MF, IF, EF) recorded during copulation with the available female rat (**Figure 5**). Accordingly, point to point analyses by two-way ANOVA (see **Table 3** for F values and significance level) followed by pairwise comparisons revealed significant differences among DAT KO, HET and WT rats in all the above parameters except for the IF. Briefly, pairwise comparisons (main effect "Line") revealed that: (i) HET rats showed more NCPEs than their DAT KO counterparts and WT rats as well; (ii) HET and, to a lesser extent, DAT KO rats showed more mounts compared to WT rats; (iii) DAT KO rats showed more ejaculations (e.g., higher EF) compared to WT and, to a lesser extent, HET rats (see **Figure 5** for statistical significance of single points among lines).

Moreover, one-way ANOVA analyses of the sexual parameters recorded in the first series of copulatory activity confirmed the results of the last (fourth/fifth) copulatory tests performed before the microdialysis experiment, that is: (i) a lower ML, IL and EL in DAT KO and HET rats compared to

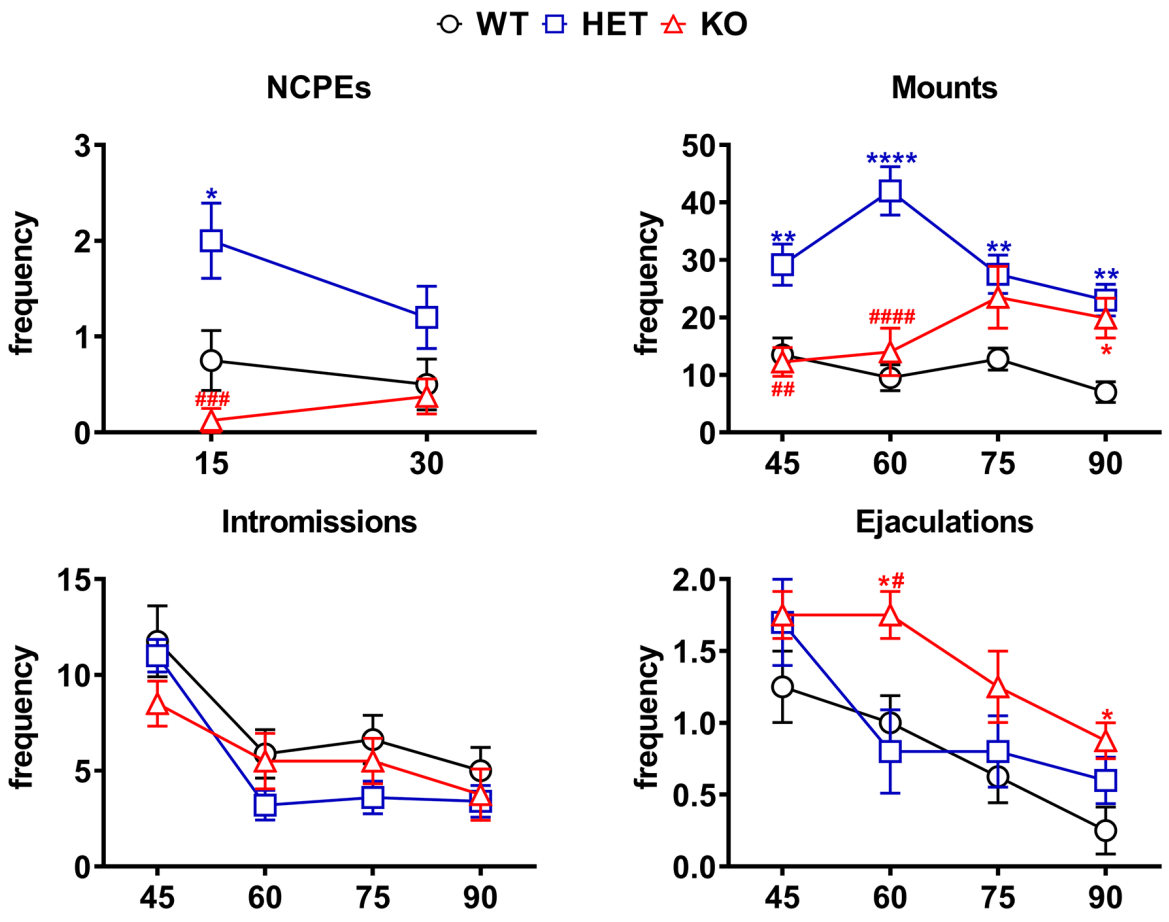

FIGURE 5 | Differences in the number of NCPEs, mounts, intromissions, and ejaculations recorded from WT, HET and DAT KO rats during the microdialysis experiments reported in **Figure 4**. The experimental conditions were identical to those described in the legend of **Figure 4**. Values are means ± SEM of the values obtained by 8/10 rats per group. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ with respect to WT; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$, DAT KO with respect to HET rats (two-way ANOVA for repeated measures followed by Tukey's or Bonferroni's pairwise comparisons).

TABLE 4 | Non-contact penile erections (NCPEs), copulatory parameters measured in the first series of copulatory activity (ML, IL, EL, MF, IF, III, IR, and PEI) and the total number of mounts (TMF), intromissions (TIF) and ejaculations (TEF), total copulatory rate (TCR) and total intromission ratio (TIR) of DAT KO, HET and WT rats during the entire microdialysis experiment.

Behavioral parameters	Line			One-way ANOVA	
	WT (*N* = 8)	HET (*N* = 10)	DAT KO (*N* = 8)	$F_{(2,23)}$	*p*
NCPEs	1.25 ± 0.53	3.20 ± 0.68*	0.50 ± 0.19##	6.911	0.0045
ML (1st series)	41.00 ± 14.16	11.50 ± 1.61*	13.25 ± 2.97	4.404	0.0240
IL (1st series)	59.13 ± 24.73	15.00 ± 2.32	16.00 ± 2.56	3.464	0.0484
EL (1st series)	700.5 ± 176.1	478.6 ± 44.77	302.4 ± 16.94*	3.790	0.0378
MF (1st series)	11.88 ± 1.93	22.00 ± 2.56**	5.62 ± 0.88####	16.83	>0.0001
IF (1st series)	10.25 ± 1.15	8.90 ± 0.69	6.25 ± 0.88*	4.790	0.0182
III (1st series)	78.78 ± 23.71	55.45 ± 6.01	60.85 ± 15.80	0.604	0.5552
IR (1st series)	0.477 ± 0.03	0.297 ± 0.02*	0.533 ± 0.07##	7.983	0.0023
PEI (1st series)	323.6 ± 41.27	306.8 ± 15.17	226.6 ± 10.93*	4.104	0.0299
TMF	42.75 ± 6.59	121.7 ± 7.11****	69.63 ± 11.30***	23.83	>0.0001
TIF	29.25 ± 3.91	21.20 ± 2.13	23.25 ± 4.69	1.385	0.2704
TEF	3.12 ± 0.58	3.90 ± 0.50	5.62 ± 0.32**#	6.369	0.0063
TCR	46.54 ± 10.32	18.44 ± 1.82**	22.93 ± 2.61*	6.682	0.0052
TIR	0.422 ± 0.05	0.150 ± 0.02***	0.260 ± 0.04*	12.31	0.0002

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs. WT. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$, DAT KO vs. HET. One-way ANOVAs followed by Tukey's pairwise comparisons. Values are means ± SEM of 8/10 rats/group.

WT rats; (ii) a higher MF in HET and lower in DAT KO rats compared to WT rats; (iii) a lower IR in HET compared to both DAT KO and WT rats; and (iv) a lower IF and higher EF in DAT KO than WT and, to a lesser extent, HET rats (**Table 4**). Additionally, at variance from the IR (which is calculated in the first series of copulatory activity), the total IR (TIR; obtained by dividing the number of total intromissions by the sum of total mounts and total intromissions) displayed lower values in DAT KO and HET compared to WT rats. Finally, while no difference was observed in the III during the first series of copulatory activity among DAT KO, HET and WT rats, the analysis of the TCR (which considers the number of both mounts and intromissions during the whole test) detected significantly lower values (approximately half) in DAT KO and HET compared to WT rats, a finding that indicates a general higher rate of approaching behavior to the female rat during the entire experiment in these two rat lines than that of WT rats (**Table 4**).

DAT KO, HET and WT Rats Display Significant Differences in Genital Self-grooming During Copulation

As shown in **Figure 6**, during the microdialysis experiment with the available female rat, in the first series of copulatory activity DAT KO, HET and WT rats showed also genital self-grooming after mounts, intromissions and ejaculation as expected (Sachs et al., 1988). However, the percent of mounts followed by genital self-grooming was significantly higher in DAT KO than in HET and WT rats (Chi-square test, DAT KO vs. WT: $\chi^2 = 4.503$; DAT KO vs. HET: $\chi^2 = 3.905$, both *P*s < 0.05; **Figure 6A**), despite the lower frequency of this behavior due to the lower number of mounts displayed by DAT KO rats (**Figure 6B**). This did not occur after intromissions or ejaculation, when all rats always showed genital grooming (**Figure 6A**). DAT KO rats had also significantly longer genital self-grooming episodes after mounts, intromissions and ejaculations compared to WT and, to a lesser extent, HET rats, which displayed intermediate time values between DAT KO and WT rats (**Figure 6C**). Accordingly, one-way ANOVA analyses followed by pairwise comparisons confirmed significant differences in the frequency and duration of genital self-grooming episodes among the three rat lines (see **Table 5** for F values and significance level).

Differences in the Expression of trkB, BDNF, Δ-FosB, Arc, Synaptophysin, Syntaxin-3, PSD-95 and PSA-NCAM in the VTA, mPFC and Acb Among DAT KO, HET and WT rats

The antibodies against trkB, BDNF, Δ-FosB, Arc, synaptophysin, syntaxin-3, PSD-95, and PSA-NCAM recognized protein bands with a relative MW of ≅140, 13, 36, 55, 39, 33, 80 and 206 kDa, respectively (**Figures 7**, **8**), consistent with the expected MWs (see Carta et al., 2018; Serra et al., 2018; Sanna et al., 2019 and references therein).

As shown in **Figures 7**, **8**, significant differences were found in the expression of trkB and BDNF, and markers of neuronal activation (Δ-FosB), synaptic function (synaptophysin, syntaxin-3, PSD-95) and plasticity (Arc and PSA-NCAM) in the VTA, the mPFC and the Acb (shell and core), limbic areas relevant for the motivational aspects of sexual behavior, among the three rat lines, in particular between WT and KO rats, although tendencies were also observed in HET rats (see **Table 6** for F values and significance level from one-way ANOVAs). Indeed, in the mPFC, except Arc whose levels were higher (+66.5%) and PSA-NCAM whose lower levels did not reach statistical significance, all the other markers showed lower levels in DAT KO than WT rats [with HET rats displaying a similar trend and lower levels of PSA-NCAM (−43.6%) and syntaxin-3 (−21.1%) compared to WT rats], a finding suggestive of the presence of a lower prefrontal activation, synaptic function and plasticity in DAT KO animals

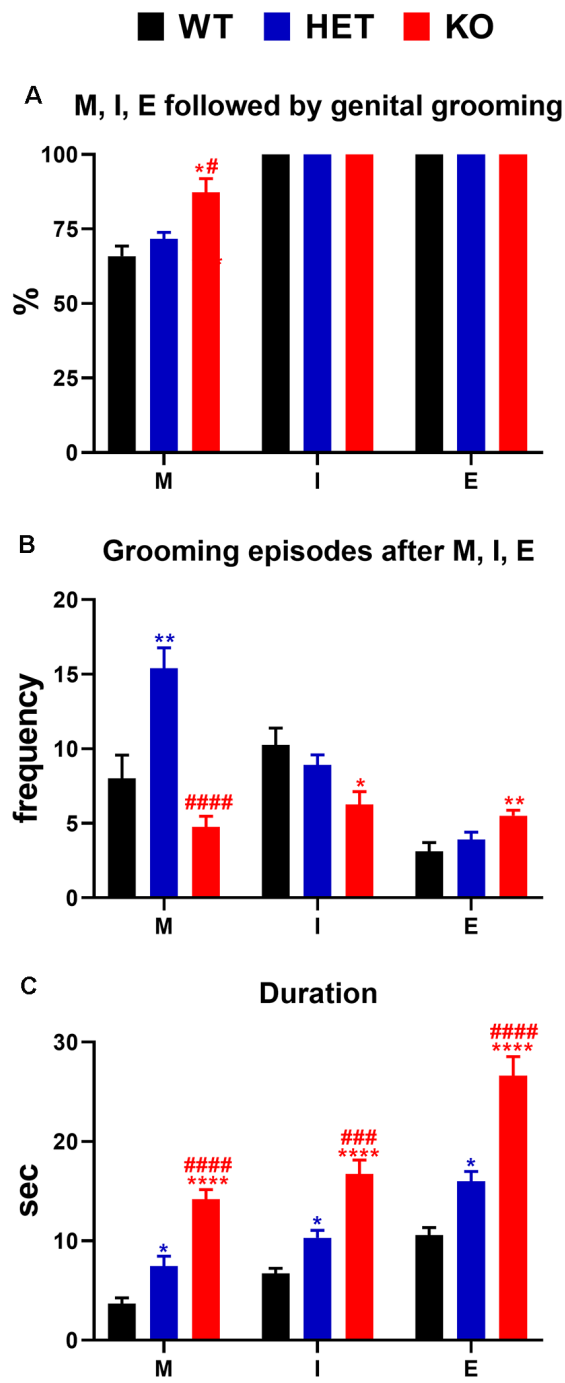

FIGURE 6 | **(A)** Percent of mounts, intromissions, and ejaculations followed by genital self-grooming; **(B)** frequency (i.e., number of episodes of genital self-grooming after mounts, intromissions and ejaculations) and **(C)** duration (s) of genital self-grooming episodes in WT, HET and DAT KO rats after mounts, intromissions and ejaculations in the first series of sexual activity during the microdialysis experiment reported in **Figure 4**. The experimental conditions were identical to those described in the legend of **Figure 4**. Values are means ± SEM of the values obtained by 8/10 rats per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ vs. WT rats; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, DAT KO with respect to HET rats (**A**: Chi-square test; **B,C**: one-way ANOVA followed by Tukey's pairwise comparisons). M, mounts; I, intromissions; E, ejaculations.

TABLE 5 | F values and significance levels of one-way ANOVAs performed on the results shown in **Figures 6B,C**.

Genital self-grooming parameter	One-way ANOVA	
	$F_{(2,23)}$	p
No. of genital grooming episodes after mounts	18.50	> 0.0001
No. of genital grooming episodes after intromissions	4.79	0.0182
No. of genital grooming episodes after ejaculations	5.455	0.0115
Duration of genital grooming episodes after mounts	32.92	> 0.0001
Duration of genital grooming episodes after intromissions	26.67	> 0.0001
Duration of genital grooming episodes after ejaculations	38.42	> 0.0001

(−66.9%, −58.6%, −74.8%, −51.8%, −21.2%, −86.4%, for trkB, BDNF, Δ-FosB, synaptophysin, syntaxin-3, and PSD-95, respectively; **Figure 7**). Moreover, in the VTA, DAT KO rats showed lower BDNF levels (−46.9%) accompanied by a higher expression of its high-affinity receptor trkB (+57.1%), lower levels of syntaxin-3 (−29.3%), and higher levels of Δ-FosB, PSD-95 and PSA-NCAM (+64.2%, +72.2%, and +72.9%, respectively) compared to WT rats, with HET rats displaying a similar trend and lower levels of BDNF (−65.7%) and syntaxin-3 (−25.1%) than WT rats (**Figure 7**). Conversely, lower levels of Δ-FosB (−56, 5%), synaptophysin (−60.9%), syntaxin-3 (−58.4%) and PSD-95 (−37.3%), and higher levels of Arc (+124.9%) were observed in the Acb shell of DAT KO compared to WT rats, with HET rats displaying a similar trend and lower levels of PSD-95 (−33.6%) than WT rats (**Figure 8**). Finally, compared to WT rats, a higher expression of synaptophysin (+84.2%), Δ-FosB (+116%) and PSA-NCAM (+98.3%) together with lower levels of Arc (−30.6%) and PSD-95 (−42.9%) was observed in the Acb core of HET rats, with DAT KO rats displaying a similar trend and lower levels of Arc (−28.4%) than WT rats (**Figure 8**; see **Table 6** for F values and significance levels).

DISCUSSION

This study shows for the first time that DAT KO rats (Leo et al., 2018b) exhibit patterns of sexual behavior different from those of their matched HET and WT counterparts when put together with a sexually receptive (ovariectomized and primed with estradiol and progesterone) female rat. The differences in the sexual patterns and associated copulatory parameters in DAT KO, HET and WT rats are more evident in the first copulatory test and tend to stabilize, but not disappear, after five copulatory tests. This study also shows that once stabilized, the patterns of sexual behavior in DAT KO, HET, and WT rats are related to differential changes among the three rat lines in the release of dopamine and glutamic acid in the shell of the Acb, and differences in the expression of Δ-FosB, synaptophysin, syntaxin-3, BDNF, trkB, PSD-95, PSA-NCAM and Arc in the medial PFC, in the VTA and the Acb shell and core.

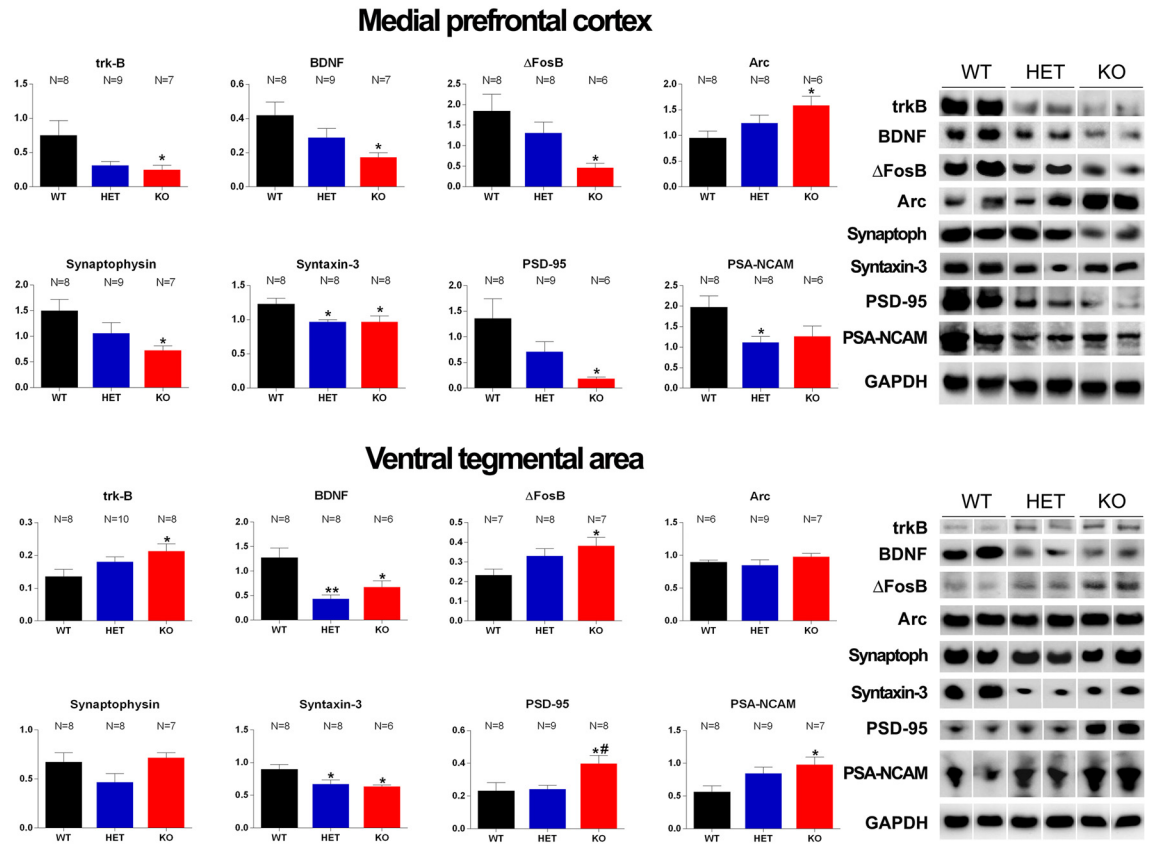

FIGURE 7 | Western Blot analysis of trkB, BDNF, Δ-FosB, Arc, synaptophysin, syntaxin-3, PSD-95 and PSA-NCAM in the mPFC and VTA of WT, HET and DAT KO rats after the microdialysis experiment. Histograms on the left are the densitometric analyses of the marker/GAPDH band gray optical density (O.D.) ratios. Blot lines on the right are representative samples of two animals from each experimental group. Values are means ± SEM of the values obtained by the reported number of rats per group. *$P < 0.05$, **$P < 0.01$ with respect to WT rats; $^{\#}P < 0.05$ DAT KO with respect to HET rats (one-way ANOVA followed by Tukey's pairwise comparisons).

DAT KO, HET and WT Rats Show Different Patterns of Sexual Behavior With a Sexually Receptive Female Rat

DAT KO, HET and WT rats showed significant differences in the first copulatory test when put together with a sexually receptive female rat. Accordingly, while 60% of WT rats engaged in sexual behavior with mounts and intromissions, 75% of HET rats and 90% of DAT KO rats did so with 90% of DAT KO and 60% of HET rats achieving ejaculation already in the first test against the 50% of the WT counterpart. The differences between DAT KO and HET rats disappeared in the second test, while those between DAT KO and HET rats and WT rats disappeared in the third test when, irrespective of the belonging rat line, all rats (e.g., 100%) engaged in sexual activity and reached ejaculation. Although the Chi-square (χ^2) test failed to reveal any statistical significance on these differences due to the relatively low number of animals used, these findings seem to indicate a faster approach directed to and/or a higher ability to sexually interact stably and efficiently with the female rat in DAT KO rats compared to HET rats and, even more, to WT rats. This occurred together with the typical significant improvements in sexual parameters recorded during the first series of copulatory activity during the five tests in DAT KO, HET and WT rats, e.g., a decrease of the ML, IL, and EL, an increase of the EF, and an improvement of the IR. However, this general trend apart, significant differences occurred among DAT KO, HET, and WT rats, with DAT KO rats constantly displaying higher EF and IR values, and lower MF and shorter ML, IL and EL values already during the first test and in the subsequent ones compared to WT and also to HET rats, which displayed values similar to those of WT rats in the majority of parameters during the first and following tests, except for minor improvements in IR and higher MF values among all the five tests, compared to WT rats. This last result together with the differences in the IF values (lower for DAT KO rats, similar between HET and WT rats) is responsible for the significantly lower IR values of HET compared to DAT KO and WT rats in the last test. HET and DAT KO rats showed also a lower TIR [(total intromission ratio) = TIF / (TIM + TIF)] and a significantly shorter (approximately half) TCR (total copulatory rate). Together with a higher level of sexual motivation and sexual activity in DAT KO rats inferred the first by the lower ML and IL, and the second by the higher EF and IR shown in the first and following tests as well, compared to

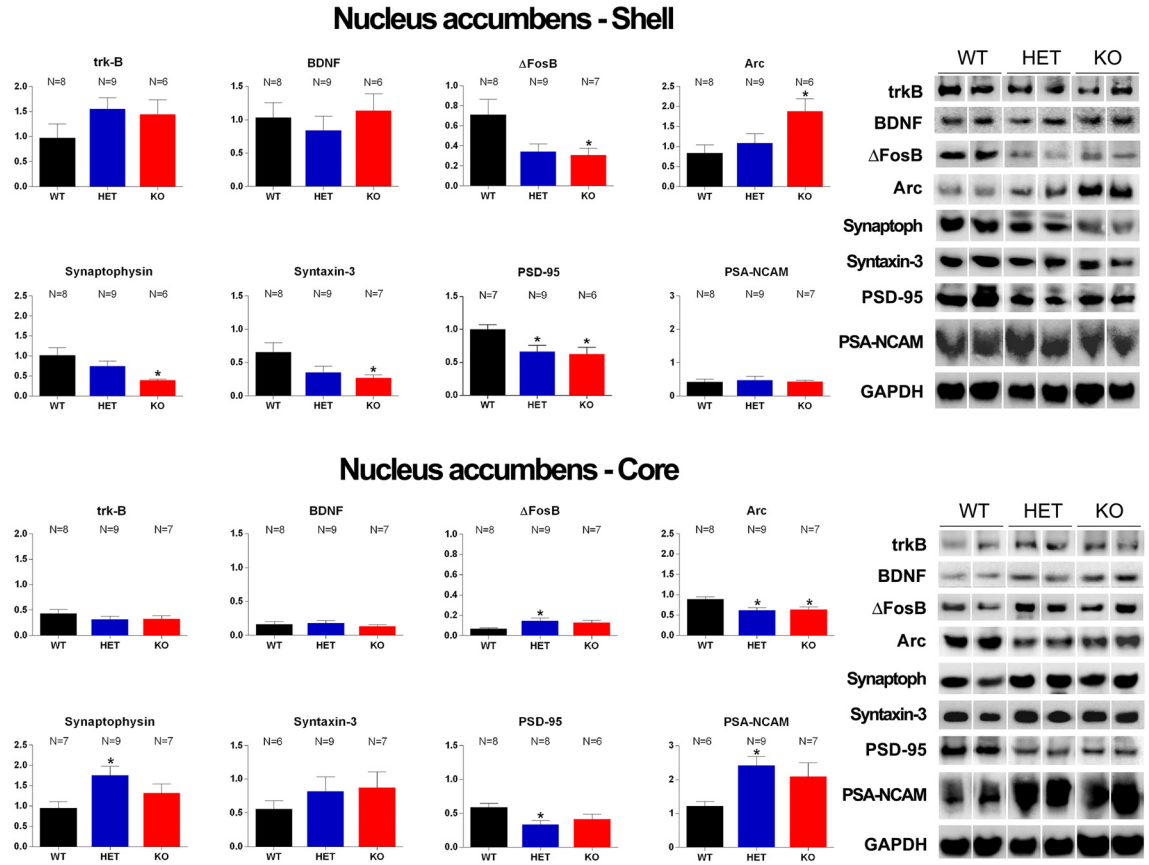

FIGURE 8 | Western Blot analysis of trkB, BDNF, Δ-FosB, Arc, synaptophysin, syntaxin-3, PSD-95 and PSA-NCAM in the Acb shell and Acb core of WT, HET and DAT KO rats after the microdialysis experiment. Histograms on the left are the densitometric analyses of the marker/GAPDH band gray optical density (O.D.) ratios. Blot lines on the right are representative samples of two animals from each experimental group. Values are means ± SEM of the values obtained by the reported number of rats per group. *$P < 0.05$ with respect to WT rats (one-way ANOVA followed by Tukey's pairwise comparisons).

WT and HET rats, these results account for faster acquisition of the ability to sexually interact stably and efficiently with a receptive female rat, of DAT KO rats compared to HET and mainly WT rats.

In line with the results of this study, sexual patterns similar to those observed in DAT KO rats are seen in rats treated with drugs that block DAT function, mainly cocaine (reviewed in Frohmader et al., 2010; Pfaus et al., 2010). Accordingly, acute cocaine treatment facilitates penile erection, reduces the number of intromissions, increases the number of mounts and shortens the ejaculation latency (that sensitizes with chronic administration of cocaine over time leading to higher ejaculation frequency). These data suggest that cocaine facilitates ejaculation when given acutely and even more after chronic administration and, although acute cocaine increases ML and IL, tolerance develops to these effects. Moreover, the lower number of intromissions together with the higher number of mounts account for a lower intromission rate in cocaine-treated rats. Although cocaine inhibits not only DAT but also noradrenaline (NET; see Tatsumi et al., 1997) and serotonin (SERT) transporters (see Rudnick and Sandtner, 2019), together, the findings of this study and those of cocaine-treated rats, apparently confirm that the absence (as in DAT KO rats) or the inactivation of DAT function (as in cocaine-treated rats) in areas involved in sexual behavior, leads to alterations of sexual behavior by impacting, in particular, the latency and the frequency of ejaculation (which become the first shorter and the latter higher, respectively) and the intromission rate [i.e., number of intromissions/(number of mounts + number of intromissions)], due to changes in the number of mounts and intromissions, the first being increased and the latter decreased in both DAT KO and in cocaine-treated rats.

According to previous reports (Sachs et al., 1988), this study also shows that mounts, intromissions and ejaculations were followed by bouts of genital grooming in DAT KO, HET and WT rats. These bouts were seen more frequently in DAT KO compared to HET and WT rats after mounts, but not after intromissions and ejaculation, and lasted longer in DAT KO rats and, to a lesser extent in HET rats, when compared to WT rats. The reason why a higher percent of mounts are followed by genital self-grooming episodes in DAT KO rats as well as why DAT KO rats are those that show the longest

TABLE 6 | F values and significance levels of one-way ANOVAs performed on the results shown in **Figures 7**, **8**.

Brain area	Marker	One-way ANOVA		
		F	*d.f.*	*p*
mPFC	BDNF	4.209	2,21	0.0290
	Trk-B	4.293	2,21	0.0273
	Arc	3.891	2,19	0.0383
	Δ-FosB	4.599	2,19	0.0235
	Synaptophisin	4.021	2,21	0.0332
	Syntaxin-3	4.365	2,21	0.0260
	PSD-95	4.495	2,20	0.0244
	PSA-NCAM	4.469	2,19	0.0257
VTA	BDNF	9.731	2,19	0.0012
	Trk-B	3.787	2,23	0.0379
	Arc	0.9958	2,19	0.3879
	Δ-FosB	3.972	2,19	0.0362
	Synaptophisin	2.607	2,20	0.0986
	Syntaxin-3	5.290	2,19	0.0149
	PSD-95	4.872	2,22	0.0177
	PSA-NCAM	4.217	2,21	0.0289
Acb Shell	BDNF	0.4327	2,20	0.6547
	Trk-B	1.471	2,20	0.2535
	Arc	4.319	2,20	0.0276
	Δ-FosB	4.224	2,21	0.0287
	Synaptophisin	4.116	2,20	0.0318
	Syntaxin-3	3.730	2,21	0.0411
	PSD-95	4.979	2,19	0.0183
	PSA-NCAM	0.0946	2,21	0.9100
Acb Core	BDNF	0.4706	2,21	0.6311
	Trk-B	0.8223	2,21	0.4531
	Arc	5.469	2,21	0.0123
	Δ-FosB	3.719	2,21	0.0414
	Synaptophisin	3.636	2,20	0.0450
	Syntaxin-3	0.5830	2,19	0.5679
	PSD-95	4.853	2,19	0.0198
	PSA-NCAM	4.076	2,19	0.0337

duration of genital self-grooming after mounts, intromissions and ejaculation among the three rat lines, is unknown at the moment. However, since excessive grooming has been considered a putative symptom of compulsive behavior (Berridge et al., 2005; Feusner et al., 2009) when associated to conditions of elevated dopamine or higher serotonin activity (Bagdy et al., 1992; Berridge et al., 2005; Taylor et al., 2010), given the tendency of DAT KO rats to show compulsive behavioral traits (Adinolfi et al., 2018, 2019; Cinque et al., 2018), the long-lasting genital self-grooming found in DAT KO rats after mounts, intromissions and ejaculation, may be interpreted as a further compulsive trait.

Extracellular Dopamine and Glutamic Acid Concentrations in the Dialysate Obtained From the Acb Shell of DAT KO, HET and WT Rats With Stable Levels of Sexual Behavior During Sexual Activity With a Receptive Female Rat

This study also shows that DAT KO, HET, and WT rats, which underwent five copulatory tests and show different patterns of sexual behavior and associated values of copulatory parameters (see above), have different basal concentrations of extracellular dopamine in the Acb shell dialysate (e.g., when no sexual receptive female rat was present, extracellular dopamine concentration in DAT KO rats was 9-fold and in HET rats 2–3 fold higher, respectively, than that of WT rats). This finding resembles the higher levels of extracellular dopamine found in the dialysate obtained from the dorsal striatum of DAT KO rats compared to those of HET and WT rats (Leo et al., 2018b). Irrespective of the different basal levels of extracellular dopamine in the Acb dialysate among DAT KO, HET, and WT rats, dopamine levels increased in the dialysate of the Acb shell of DAT KO, HET and WT rats when a sexually receptive female rat was introduced in the mating cage, either when the female rat was inaccessible (e.g., the male can see, hear and smell, but not interact with her) or when copulation was allowed, as expected (see Fiorino et al., 1997; Sanna et al., 2015b). Most importantly, the patterns of release in response to the presence of the inaccessible female rat first, and then to the direct interaction with her, were very similar among the three lines, with peak increases of 22%, 46%, and 59%, respectively, at 15 min with the inaccessible female rat, and of 80%, 89%, and 101%, respectively, after 30–45 min of direct sexual interaction with the receptive female rat, in DAT KO, HET and WT rats. These increases lasted, although reduced, for the entire experiment, and decreased after the removal of the female rat from the mating cage.

At variance from dopamine, this study failed to reveal significant differences in the basal levels of extracellular glutamic acid in the same dialysate obtained from the Acb shell used for dopamine measurement in DAT KO, HET and WT rats. Nevertheless, the patterns of release in response to the presence of the inaccessible sexually receptive female rat first, and then to the direct interaction with her, were also similar between WT and HET rats, but with significant differences in DAT KO rats. Accordingly, glutamic acid increased of 32.8% and 80.4%, respectively, at 30 min with the inaccessible female rat, and of 114.9% and 153.5%, respectively, after 15 min of direct sexual interaction with the receptive female rat, respectively, in HET and WT rats. These increases also lasted, although substantially reduced, for the entire experiment and decreased after the removal of the female rat from the mating cage. Surprisingly, at variance from HET and WT rats, no increase in glutamic acid levels was found either during the presence of the inaccessible female rat and up to 45 min of copulation in DAT KO rats, after which a peak increase of 99.3% was observed that lasted, although reduced, until the end of the copulatory test and removal of the female rat from the mating cage.

Perhaps more importantly, the increases in extracellular dopamine and glutamic acid levels, which occurred in the Acb shell dialysate when the sexually receptive female rat was introduced in the mating cage, were parallel to differential changes in the values of the copulatory parameters (i.e., MF, IF and EF) similar to those found after stabilization of copulatory behavior in the forth/fifth copulatory test, among DAT KO, HET, and WT rats. Indeed, also in this experiment, HET rats showed higher MF than DAT KO and WT rats mainly in the first 30 min of copulation, and a lower EF (similar to that of WT rats) than DAT KO rats, despite a similar IF among the three rat lines. Likewise, HET and DAT KO rats (which displayed

more mounts than WT rats) showed also a lower TIR [total intromission ratio = TIF / (TIM + TIF)] and a significantly shorter (approximately half) total copulatory rate (TCR; i.e., they displayed a higher number of approaches to the female rat per time unit) compared to WT rats. As expected, HET and WT rats showed also NCPEs, with HET rats showing more NCPEs than WT rats, but surprisingly these penile erections did not occur in DAT KO rats.

The above differences in the pattern of release of dopamine and glutamic acid in the Acb shell and NCPEs and copulatory parameters among DAT KO, HET and WT rats, deserve some comment. First, the higher basal levels of dopamine found in the Acb shell dialysate from DAT KO rats compared to those of HET and WT rats raise the possibility that a higher basal dopaminergic tone exists in the Acb shell of DAT KO rats and that this enhanced tone may be responsible, at least in part, for the higher sexual motivation and for the higher levels of sexual activity found in these animals compared to their HET and WT counterparts (see above). Indeed, a higher dopaminergic tone may contribute to making these animals more prone to interact with the receptive female rat, thereby facilitating the first interaction with her and, as a consequence, accounting for faster acquisition of the ability to stably sexually interact with the receptive female rat than their HET and WT counterparts. In line with the above hypothesis, we have recently reported that a different mesocorticolimbic dopaminergic tone correlates with differences in several aspects of sexual behavior, from the acquisition of sexual experience to sexual motivation and performance, in RHA and RLA rats (see the "Introduction" section; Sanna et al., 2015b, 2017b). RHA rats exhibit higher levels of sexual motivation and better performances than RLA rats (Sanna et al., 2014a) both in naïve and sexually experienced conditions, and these sex differences are secondary to the more robust functional dopaminergic tone occurring in RHA compared to RLA rats (Sanna et al., 2013, 2014b, 2015b, 2017b).

However, the explanation given above that a higher dopaminergic tone of the mesolimbic system is responsible for a higher level of sexual motivation and sexual activity in DAT KO rats compared to HET and WT rats is complicated to some extent by a few findings. First, at variance from DAT KO, HET and WT rats, the higher dopamine release of RHA vs. RLA rats occurs without any difference in the basal levels of extracellular dopamine in the Acb and mPFC dialysate of these two rat lines (e.g., basal extracellular dopamine levels are similar in the Acb and mPFC dialysate from RHA and RLA rats; Giorgi et al., 2007; Sanna et al., 2015b, 2017b). Second, there is no correlation between the dopamine concentrations found in the Acb dialysate from DAT KO, HET and WT rats and the number of concomitant NCPEs shown by the three lines when exposed to the inaccessible female rat. NCPEs are pheromone-induced penile erections which a male rat displays when put in the presence of an inaccessible receptive female rat and are considered an index of sexual arousal (Sachs et al., 1994). NCPEs usually occur concomitantly with an increase in extracellular dopamine levels in areas relevant for a role of dopamine in sexual behavior, e.g., the paraventricular nucleus of the hypothalamus (Melis et al., 2003), the Acb shell (Sanna et al., 2009, 2015b) and the mPFC (Sanna et al., 2017b). This led to suggest that male rats that display more NCPEs, display also a higher dopamine release in these areas and the Acb in particular, compared to rats displaying less NCPEs (Sanna et al., 2009, 2015b). In line with the above hypothesis, DAT KO rats, which show the lower dopamine increases (22% above basal values) among the three rat lines, fail to display NCPEs; however, against the above hypothesis, HET and WT rats, which show dopamine peaks of 46% and 59% above basal levels, respectively, showed a mean of 2 and 0.8 NCPEs, respectively, that is HET rats showed 2-fold more NCPEs than WT rats, irrespective of the fact that WT rats had the highest dopamine release and should be then expected to show the highest number of NCPEs. Further complications also arise from the different basal levels of extracellular dopamine in the Acb dialysate from DAT KO, HET and WT rats. Indeed, since basal dopamine levels are about 3/4-fold higher in DAT KO rats than those in HET rats and 9-fold higher than those in WT rats, one should also expect that DAT KO rats would have shown more NCPEs than HET rats, and HET rats more NCPEs than WT rats. In contrast to this hypothesis and as discussed above, DAT KO rats failed to show NCPEs and HET rats showed more NCPEs than WT rats. Although further work is necessary to clarify whether NCPEs appearance is secondary to the increase in dopamine output more than to the absolute amount of extracellular dopamine in the extracellular fluid, the finding that DAT KO rats do not show NCPEs together with the finding that these rats show the lowest dopamine increases among the three rat lines, suggest that the increase in dopamine release rather than the basal levels of the neurotransmitter in the extracellular fluid may play a major role in mediating this sexual response. Finally, DAT KO and HET rats (both having basal dopamine levels higher, and displaying more mounts than WT rats, see **Figure 5**), compared to WT rats, show a lower TIR [total intromission ratio = TIF / (TMF + TIF)] that occurs despite what seen in ML, IL, EL and EF (the first three decrease and the latter increases more in DAT KO and HET than WT rats). Indeed, this finding suggests that the higher dopaminergic tone is related to the total number of approaches (i.e., the sum of mounts and intromissions during the copulatory test) performed by HET and DAT KO rats compared to WT rats rather than to their level of performance, usually measured by the IR (intromission ratio). Accordingly, both DAT KO and HET rats display not only a reduced TIR but also a significantly shorter (approximately half) total copulatory rate (TCR; they display a higher number of approaches to the female rat per time unit) compared to WT rats.

As to the increase in Acb glutamic acid output found in DAT KO, HET and WT rats, these findings provide evidence for a role of this excitatory amino acid in sexual behavior in the Acb shell. To our knowledge, this study is the first to show that: (i) extracellular glutamic acid levels increase in the Acb shell dialysate during sexual behavior; (ii) this increase occurs concomitantly to an increase in extracellular dopamine and (iii) it is associated to differences in the pattern of sexual behavior in DAT KO, HET and WT rats. So far, only a few other studies provided evidence for a role of Acb glutamic acid in sexual behavior. Among these, one shows that the

acquisition of sexual experience causes long-term alterations in glutamate receptor expression and function (i.e., a decrease in AMPA/NMDA receptors ratio) and that changes in the NMDA (NR1 subunit) and AMPA (GluA1 and GluA2 subunits) receptors are differentially associated to experience acquisition, reward and abstinence, pointing out to a complex fine-tuning (i.e., changes in receptor subunits trafficking) at the synaptic level of the neuroplastic processes occurring during the acquisition of sexual experience in male rats (Pitchers et al., 2012). Another one shows that in female Syrian hamsters a glutamatergic projection from the medial PFC to the Acb is activated during sexual behavior and that silencing these mPFC glutamatergic afferents (with DREADD techniques) to the Acb prevents C-Fos expression in the Acb due to sexual interaction, although this silencing did not affect female sexual behavior expression, thus excluding a role in sexual performance and pointing out on a possible role in the motivational (i.e., anticipatory and rewarding) aspects of sexual activity (Moore et al., 2019). The other available studies support a facilitatory role of glutamic acid in sexual behavior in brain areas such as the PVN and the medial preoptic area of male rats. Accordingly, extracellular glutamic acid (and aspartic acid), was found to be increased in the paraventricular dialysate during the exposition to an inaccessible receptive female rat and even more during copulation (Melis et al., 2004; here the excitatory amino acid facilitates male sexual behavior by activating central oxytocinergic neurotransmission, see Melis and Argiolas, 2011; Argiolas and Melis, 2013) and in the medial preoptic area dialysate, where the excitatory amino acid increased maximally at ejaculation (Dominguez et al., 2006). However, at variance from Acb extracellular dopamine, whose levels increase during sexual behavior and for which an increased tone has been already involved in the different patterns of sexual behavior of several rat lines (see Fiorino et al., 1997; Sanna et al., 2015b, 2017b; Melis et al., 2019), allowing us to suggest that the different sexual patterns found in DAT KO, HET and WT rats may be related to the differential changes in the Acb dopamine output (see above), further studies are necessary to clarify if and how the different patterns of release of glutamic acid found in the Acb of DAT KO, HET, and WT rats are related to the differences in sexual patterns and copulatory parameters of these three rat lines. Nevertheless, it is reasonable to assume that some, if not all, of the above differences in sexual behavior among DAT KO, HET and WT rats may be mediated, at least in part, by an altered interaction between Acb dopamine and glutamic acid. Infact, it is well accepted that: (i) the function of mesolimbic and mesocortical dopaminergic neurons, which play a key role in motivated behavior (Goto and Grace, 2005) and have their cell bodies mainly in the VTA, is finely modulated by the activity of glutamic acid neurons, which originate, although not exclusively, from the PFC and project to the Acb and to the VTA (Carlsson et al., 2001; Carlezon and Thomas, 2009; Beloate and Coolen, 2017; Bamford et al., 2018); (ii) an intact glutamatergic function in the PFC is required for cortical control over the activity of the dopaminergic mesolimbic system to modulate, together with dopamine release, the activity of the GABAergic medium spiny neurons at the level of the Acb and motivated behavior (Carlsson et al., 2001; Kalivas, 2009); and (iii) dopamine and glutamic acid, together with the neuropeptide oxytocin, are key neurotransmitters in the functioning of a complex brain circuit, interconnecting hypothalamic, limbic and cortical areas, involved in both the motivational and performance aspects of male rat sexual behavior (Melis et al., 2007, 2009, 2010; Succu et al., 2007, 2008, 2011; Melis and Argiolas, 2011; Sanna et al., 2017a; Bratzu et al., 2019).

Differences in the Expression of trkB, BDNF, Δ-FosB, Arc, Synaptophysin, Syntaxin-3, PSD-95 and PSA-NCAM in the VTA, mPFC and Acb of DAT KO, HET and WT Rats Are Related to the Differences in the Patterns of Sexual Activity Among the Three Rat Lines

This study also shows for the first time that the relative protein levels of markers of neurotropism (BDNF, trkB), neuronal activation (Δ-FosB), synaptic function (synaptophysin, syntaxin-3, and PSD-95) and plasticity (Arc and PSA-NCAM; Carta et al., 2018; Serra et al., 2018; Sanna et al., 2019 and references therein) are differentially expressed in the VTA, mPFC and Acb of DAT KO, HET and WT rats, which underwent five copulatory tests and a final session of sexual activity performed during intracerebral microdialysis with a sexually receptive female rat. The most significant differences were detected mainly in the mPFC, where a lower expression of almost all the markers investigated, except for Arc (whose expression was higher) and PSA-NCAM (whose lower levels did not reach statistical significance), was found in DAT KO compared to WT rats, with HET rats usually showing intermediate changes between the other two. Differences in the expression of the majority of the above markers were also detected in the VTA and the Acb shell and core; however, only in a few cases, these differences were in the same direction of those found in the mPFC among the three rat lines. Thus, comparing DAT KO rats to WT ones, BDNF was lower and trkB higher in the VTA, with no differences in the Acb core and shell; Δ-FosB was higher in the VTA, but lower in the Acb shell; synaptophysin was lower in the Acb shell; syntaxin-3 was lower in the VTA and the Acb shell; PSD-95 was higher in the VTA, and lower in the Acb shell; PSA-NCAM was higher in the VTA but did not differ in the Acb shell; Arc, which was higher in the mPFC and Acb shell, was lower in the Acb core and did not differ in the VTA.

Interestingly, in line with our previous observations in the limbic system of Roman rats (Sanna et al., 2015b, 2017b, 2019 and references therein), the differences in the markers' expression in the VTA, mPFC and Acb among DAT KO, HET and WT rats parallel the different sexual patterns and the differential changes found during sexual activity among the three rat lines in the activity of the mesolimbic dopamine and frontocortical glutamic acid neurons, which interconnect these brain areas, with the higher difference in extracellular dopamine and glutamic acid levels in the Acb shell dialysate found between DAT KO and WT rats. Since DAT KO rats lack one of the most important regulators of dopamine function at synaptic level (Kuhar et al., 1990), and thus bear permanent alterations in the processes of

neuronal plasticity and tropism driven by it (Fosnaugh et al., 1995; Fasano et al., 2013; Pitchers et al., 2013; Collo et al., 2014), and DAT KO rats are also those that show the majority of differences compared to WT rats in the expression of the markers investigated in the VTA, mPFC and Acb, it is then likely that (i) these differences are due to the absence of DAT, which leads to a reduced/altered neuronal activation and synaptic function and plasticity in the above brain areas of DAT KO compared to HET and WT rats, and (ii) a causal relationship may exist among the distinct tissue expression profiles of these markers in DAT KO, HET and WT rats and the differences in behavioral sexual patterns, copulatory parameters, and dopamine and glutamic acid release in the Acb shell. Accordingly, and in line with previous studies in mice and rats with the DAT KO genotype (Fumagalli et al., 2003; Yao et al., 2004; Leo et al., 2018b), we found a lower expression of most of the markers investigated mainly in the mPFC of DAT KO vs. WT rats. These differences would cause in DAT KO rats a weakened/altered prefrontal control over the mesolimbic systems dedicated to the translation of motivational drives into goal-directed behaviors (Goto and Grace, 2005), and lead in turn, to minor flexibility in response to changes in environmental demands such as those by sexual behavior. Thus, as already discussed above (see "Discussion" section "Extracellular Dopamine and Glutamic Acid Concentrations in the Dialysate Obtained From the Acb shell of DAT KO, HET and WT Rats With Stable Levels of Sexual Behavior During Sexual Activity With a Receptive Female Rat"), the different sexual responses of DAT KO vs. WT rats may be secondary to the altered activity of both prefrontal glutamatergic neurons and mesolimbic dopaminergic neurons, which may determine, at least in part, the different markers' expression found in the mPFC, VTA, Acb shell and core among the three rat lines.

Mechanisms involved apart, the significance of the differences among markers' expression across the three rat lines is also unknown, as only a few studies have investigated their expression concerning sexual behavior, mainly focusing on BDNF, trkB, Δ-FosB, and Arc (Pitchers et al., 2010b, 2013; Sanna et al., 2019; Turner et al., 2019).

As discussed above, in line with a weakened prefrontal control of DAT KO rats vs. their HET and WT counterparts, our results are in agreement with lower levels of BDNF and trkB in the mPFC reported in DAT KO mice (Fumagalli et al., 2003; Yao et al., 2004) and rats (Leo et al., 2018b). They further show that a decrease in BDNF expression also occurred in the VTA of DAT KO rats, though accompanied by an increase rather than a decrease in trkB expression when compared to HET and WT lines, while revealed no significant differences in both BDNF and trkB levels in the Acb shell and core among the three rat lines. If this decrease in the expression of both BDNF and trkB in the mPFC of DAT KO rats is related to the fact that these rats are those showing the higher basal levels of extracellular dopamine, or the lowest dopamine release during sexual activity, in the Acb shell is unknown. No help in this circumstance is provided by our previous findings on the expression of BDNF and trkB in RHA and RLA rats (Sanna et al., 2019). Indeed similar levels of BDNF were found in the mPFC, VTA and Acb, shell and core, of RHA and RLA rats after five copulatory tests, even though RHA rats have a higher dopaminergic tone in the mPFC and the Acb shell than RLA rats. Likewise, no significant differences were detected in trkB levels between RHA and RLA rats in the above brain areas except for the mPFC, in which higher levels of trkB levels were found in RLA vs. RHA rats (Sanna et al., 2019).

As to Δ-FosB, a truncated form of C-Fos, its expression pattern higher in the VTA and Acb core but lower in the mPFC and in the Acb shell of DAT KO rats, which occurred even though among the three lines the DAT KO one had the highest levels of extracellular dopamine in the Acb shell, is certainly surprising. Indeed, this marker of neuronal activation is believed to play a key role in mediating the rewarding properties of sexual behavior and in the acquisition of sexual experience, as it has been found robustly increased in the Acb shell and the mPFC, either in response to the rewarding properties of sexual activity (Pitchers et al., 2010b) or in male rats with a higher dopaminergic tone in the Acb shell and mPFC (e.g., RHA compared to RLA rats; Sanna et al., 2019). Thus, in line with the above results, DAT KO rats should be those with the highest levels of Δ-FosB in the Acb shell, as in the striatum of DAT KO mice, where basal levels of extracellular dopamine were reported to be 5-fold higher than their WT counterparts (Cyr et al., 2003). One explanation for this discrepancy may be that DAT KO rats are also those that show the lowest increase in dopamine release in the Acb shell during sexual activity when compared to WT rats. Together, the lower Δ-FosB expression and dopamine increases suggest that it is the dopamine output itself rather than the absolute dopamine amount in the synaptic cleft to drive the increase in Δ-FosB expression. However, it is also possible that Δ-FosB expression in DAT KO rats is lower because these rats are also those that show the lowest levels of glutamic acid release in the Acb shell during sexual activity and this could have interfered with its dopamine-induced activation (Beloate and Coolen, 2017). Interestingly, in this regard, inhibition of the mPFC glutamatergic input to the Acb has been found to prevent C-Fos expression in this area during sexual activity in the female hamster (Moore et al., 2019). The above hypotheses may also be not mutually exclusive.

As to the expression of Arc, a marker of neural activity and structural plasticity (Bramham et al., 2010; Korb and Finkbeiner, 2011), this was found to be higher in the mPFC and Acb shell, but lower in the Acb core of DAT KO rats compared to WT rats. These findings are in contrast with the minor difference found in RHA and RLA rats after five copulatory tests, that is after reaching a stable level of sexual activity as done for DAT KO, HET and WT rats used in this study. However, they resemble the higher increases in RHA vs. RLA rats, after the first copulatory test, a finding that is in line with the fact that Arc protein is synthesized and then transported at postsynaptic sites where it accumulates to be used when appropriate stimuli linked to new relevant experiences, as it may be considered the first exposition to a sexually receptive female and copulation, occur. Further studies are necessary to explain why, at variance from the Roman lines, DAT KO, HET and WT rats that have reached stable levels of sexual activity have different levels in Arc expression

in the mPFC and Acb shell. Since the Arc protein expression pattern is modulated by changes in AMPA receptor-mediated glutamic acid transmission (Bramham et al., 2010; Korb and Finkbeiner, 2011; but see also Pitchers et al., 2012), and reduction in Arc levels as in Arc KO mice leads to changes in both AMPA receptor trafficking (Chowdhury et al., 2006) and mesocortical/mesolimbic dopamine activity (which are the first inhibited and the second activated by Arc gene silencing; Managò et al., 2016; Managò and Papaleo, 2017), it can be assumed that the differences in both glutamic acid and dopamine release, as detected in the Acb shell of DAT KO, HET, and WT rats, might be involved in the brain regional differences of Arc expression levels among the three DAT rat lines as well as between the DAT KO (present data) and the Roman lines (Sanna et al., 2019).

A lower glutamic acid release may be also involved in the lower expression of PSD-95 found in the mPFC and the Acb shell in DAT KO compared to WT rats. Accordingly, this protein, which plays a role in synaptic plasticity and the dendritic spine remodeling (together with BDNF; El-Husseini et al., 2000; de Bartolomeis et al., 2014), has been identified as a regulator of dopamine-mediated synaptic and behavioral plasticity and its expression is markedly reduced in the presence of a decreased activity of frontocortical glutamatergic projections to the Acb in DAT KO mice (Yao et al., 2004; Efimova et al., 2016). Likewise, the higher expression of PSA-NCAM in the VTA and Acb core and its lower expression in the mPFC of DAT KO and HET rats may also be related to the altered dopamine/glutamic acid function of these animals when compared to WT rats. Accordingly, the enzymatic removal of PSA (polysialic acid) from PSA-NCAM was found able to decrease spine density and to reduce the expression of vesicular glutamate transporter-1 in apical dendrites of mPFC pyramidal neurons without affecting their inhibitory innervation in male rats (Castillo-Gómez et al., 2016).

Although additional studies are necessary to ascertain if and how the different levels of expression of the above markers across the VTA, mPFC, Acb shell and core are directly related to DAT gene silencing as well as to the differential changes found between DAT KO and WT rats in the dopamine and/or glutamic acid output in the Acb shell and to the different patterns of sexual behavior, this study provides strong evidence that DAT KO and, to a lesser extent, HET rats, compared to WT rats, which have reached stable levels of sexual activity, show differences at several levels in the plastic processes occurring in these brain areas. Since sexual behavior, although highly stereotyped and instinct-guided, is also influenced by learning as other rewarding behaviors (see Pfaus et al., 2012), and learning activates plastic processes which, in turn, cause a broad array of functional and structural changes that involve neurotransmitters and their receptors, immediate early genes and their products and other molecules controlling neuronal tropism and synaptic plasticity as well (Leuner et al., 2010; Pitchers et al., 2010a, 2012; Sanna et al., 2015b, 2017b, 2019; Turner et al., 2019), these changes may have a pervasive impact on sexual behavior and, more in general, on the motivated behavior of these animals.

CONCLUSIONS

In conclusion, this study shows that DAT KO rats and, to a lesser extent, HET rats, present differences in the motivational and consummatory aspects of sexual behavior when compared to WT matched controls. DAT KO rats needed a lower number of copulatory tests to reach a stable level of sexual activity with a sexually receptive female rat and presented higher levels of sexual activity as indicated by the changes in copulatory parameters across the copulatory tests, mainly shorter latencies to mount, intromit and ejaculate and higher ejaculation frequencies when compared to WT rats. DAT KO rats showed also a lower number of NCPEs when put in the presence of an inaccessible receptive female rat. These sexual differences among DAT KO, HET and WT rats that have reached a stable level of copulatory activity occurred concomitantly to significant differences in the basal levels of extracellular dopamine in the dialysate obtained by intracerebral microdialysis from the Acb shell (DAT KO rats show dopamine levels much higher than HET and, even more, WT rats), but not to basal levels of extracellular glutamic acid (which were similar among the three rat lines), and to significant differences in extracellular dopamine and glutamic acid release during sexual activity. Indeed, although both dopamine and glutamic acid concentrations increased above basal values in WT more than in DAT KO rats during sexual activity, with dopamine concentration increased during the entire period of sexual activity in all the three rat lines, glutamic acid increased in DAT KO rats only in the last period of sexual activity, at variance from WT and HET rats, in which it increased significantly already in the first period of sexual activity. It is reasonable to assume that these differential changes in dopamine and glutamic acid outputs are involved in the differences in sexual behavior among the three rat lines, since dopamine and glutamic acid, together with the neuropeptide oxytocin, are key neurotransmitters of a complex brain circuit that interconnects hypothalamic, limbic and cortical areas involved in both the motivational and performance aspects of the male rat sexual behavior (Melis and Argiolas, 2011; Argiolas and Melis, 2013; Sanna et al., 2017a; Bratzu et al., 2019; Melis et al., 2019). Finally, differences were also detected among DAT KO, HET and WT rats in the expression of BDNF and its trkB receptor, markers of neural activation (Δ-FosB) and plasticity (Arc and PSA-NCAM) and synaptic structural proteins (synaptophysin, syntaxin-3, PSD-95) in the VTA, mPFC, and Acb (shell and core), which are all brain areas relevant for sexual motivation and sexual performance. The expression of the majority of these markers, excluding Arc, is decreased in the mPFC, in line with the assumption of a reduced function of this brain area in DAT KO rats (Leo et al., 2018b).

Taken together, the results of this study confirm a key role of dopamine in both the motivational and performance aspects of sexual behavior, and confirm that conditions characterized by permanently high levels of dopamine (i.e., "hyperdopaminergia"), like those due to DAT gene silencing or to chronic treatment with psychostimulants (e.g., cocaine and amphetamines) in laboratory animals, may be useful for modeling the behavioral, neurochemical and molecular

features not only of psychopathological conditions such as psychotic states and attention-deficit/hyperactivity disorders (ADHD) but also to characterize the neural substrates underlying conditions such as hypersexuality and altered sexual behavior.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study and the experiments were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Cagliari.

AUTHOR CONTRIBUTIONS

FS, AA, and MM designed the study. FS and JB designed, performed and analyzed the data from sexual behavior and microdialysis experiments. MS, MQ, and MB performedthe Western Blot experiments and analyzed Western Blot data. RG, DL, and SE bred and selected DAT KO, HET and WT rats. FS, AA, MM, and MQ supervised the study. FS, AA, MM, and MQ wrote the manuscript. All authors discussed the results and commented on the manuscript.

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