Male ejaculation phasically activates dorsal raphe serotonin neurons in mating female mice

Eileen L. Troconis[1], Changwoo Seo[2,3], Akash Guru[2,3], and Melissa R. Warden[2,3,4*]

[1]Department of Biological and Biomedical Sciences, Cornell University, Ithaca, NY 14853 USA
[2]Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853 USA
[3]Cornell Neurotech, Cornell University, Ithaca, NY 14853 USA
[4]Lead Contact

*Correspondence: mrwarden@cornell.edu

SUMMARY

Sexual stimulation triggers species-dependent changes in female physiology and behavior, including preparing the uterus for pregnancy and inducing sexual satiety. Serotonin is an important regulator of female reproductive physiology and sexual receptivity, but the relationship between sexual stimulation and serotonin neural activity in females is poorly understood. Here, we investigated the relationship between dorsal raphe serotonin neural activity and sexual behavior in female mice. We found that serotonin neural activity in mating females rapidly and specifically increased at the moment of male ejaculation, and remained elevated until males disengaged. Artificial intravaginal mechanical stimulation was sufficient to elicit increased activity, but delivery of ejaculatory fluids was not. Distal penis erectile enlargement ("penile cupping") at ejaculation and forceful expulsion of ejaculatory fluid each provided sufficient mechanical stimulation to elicit serotonin neuron activation, and simultaneous disruption of both was required to block activation. Ejaculation is a key mating event required for the induction of pregnancy-related neuroendocrine changes in female mice. Our study identifies a neuromodulatory signal specific to this event and reveals its underlying mechanisms.

Keywords: serotonin, dorsal raphe, female, mating, ejaculation, calcium imaging, mechanosensory, chemosensory, sexual stimulation, interoception

INTRODUCTION

For species with sexual reproduction and internal fertilization, mating facilitates the transfer of sperm into the female body. During the appetitive and consummatory phases of mating, the female receives multimodal sexual stimulation that is independent of sperm transfer but just as critical for successful reproduction (Hashikawa et al., 2016; Lorenz, 1981; Lenschow and Lima, 2020; Wei et al., 2021; Yin and Lin, 2023). Visual, acoustic, and olfactory stimuli in the appetitive phase are necessary for conspecific recognition, evaluation of mate quality, and courtship (Coen et al., 2014; Egnor and Seagraves, 2016; Ishii et al., 2017; Osakada et al., 2018; Ribeiro et al., 2018). Here we focus on the consummatory phase, in which chemosensory and mechanosensory signals provided by the male elicit profound changes in female physiology and behavior.

Chemical signaling through the ejaculatory fluid is a common mechanism by which female post-copulatory reproductive function is regulated across species. For example, after mating *Drosophila melanogaster* females become sexually unreceptive and begin laying eggs

(Auer and Benton, 2016). This critical behavioral switch is primarily triggered by sex peptide—a short peptide produced by the male accessory glands which binds a receptor in the female's reproductive tract and central nervous system (Wang et al., 2020; Yapici et al., 2008). In addition, seminal fluid in *D. melanogaster* (Heifetz and Wolfner, 2004) contains signaling molecules that condition the environment in the female reproductive tract. In mammals, cytokines and prostaglandins synthesized in male accessory glands induce changes in female gene expression that modify the structure of the endometrium to facilitate embryo development and implantation (Robertson, 2007).

In addition to chemosensory stimulation, male genitalia provide mechanical stimulation that influences physiology and behavior in the female. Vaginocervical stimulation received during mating induces ovulation in cats (Concannon et al., 1980), rabbits (Jones et al., 1976), and other reflexively ovulating species (Bakker and Baum, 2000). In rats, both natural and artificial vaginocervical mechanical stimulation trigger pseudopregnancy and prolactin release, which is required to support luteal function in early pregnancy (Edmonds et al., 1972; Erskine, 1995; Greep and Hisaw, 1938; Gunnet and Freeman, 1983; Smith et al., 1976). Mechanical sexual stimulation is also critical for regulating female post-copulatory behavior. Vaginocervical stimulation induces conditioned place preference in rats (Meerts and Clark, 2009; Parada et al., 2010; Paredes and Alonso, 1997; Trezza et al., 2011), and reduces sexual receptivity in golden hamsters (Huck and Lisk, 1986) and garter snakes (Mendonca et al., 2003). In the damselfly *Calopteryx Haemorrhoidalis*, the reproductive organ of the male that delivers sperm also stimulates mechanoreceptors in the female reproductive tract, which causes the sperm of rival males to be ejected from female sperm stores (Cordoba-Aguilar, 1999). In *D. melanogaster,* the copulation effect—a short-term reduction in sexual receptivity—is independent of the male ejaculate and is instead mediated by mechanosensory innervations of the female vaginal plate (Shao et al., 2019).

How does the female brain recognize sexual stimulation during mating and induce post-mating changes in female physiology and behavior? Serotonin (5-HT) is an important modulator of female sexual function. In clinical studies, more than 25% of women administered selective serotonin reuptake inhibitors (SSRIs) report sexual dysfunction, including decreased libido and difficulty achieving sexual arousal and orgasm (Lorenz et al., 2016). Mating-related 5-HT neural activity in females has not been described, but could provide insight into the role of 5-HT in regulating reproductive behavior. Neural activity in female mice that is associated with male ejaculation is of particular interest, because in mice ejaculation is required for the induction of pregnancy and pseudopregnancy, and initiates a neuroendocrine process that supports luteal function and uterine preparation for implantation (Yang et al., 2009).

Here, we describe DRN 5-HT neural dynamics in female mice during the consummatory phase of mating, which includes mounting, intromission, ejaculation, and post-ejaculation

freezing. We use fiber photometry to record DRN 5-HT population neural activity in female mice during the complete mating sequence and in response to artificial sexual stimulation. Our findings reveal a pronounced increase in female DRN 5-HT neural activity that occurs at the moment of male ejaculation, and which persists until the male disengages. Using newly-developed surgical interventions to perturb specific aspects of ejaculatory physiology in males, we show that mechanical intravaginal stimulation provided by distal penis expansion ("penile cupping") or forceful fluid expulsion are each sufficient to support ejaculation-related DRN 5-HT neural activity in recipient females without chemosensory cues.

RESULTS

Female DRN 5-HT neural activity increases at ejaculation

We used fiber photometry (Gunaydin et al., 2014) to monitor DRN 5-HT population neural activity in freely behaving female mice during mating. We injected AAVdj-EF1α-DIO-GCaMP6m (Chen et al., 2013) into the DRN of female SERT-Cre mice (Zhuang et al., 2005) and implanted an optical fiber over the DRN (**Figure 1A**). To control the timing and extent of female sexual receptivity during behavioral testing, we ovariectomized females and used a standard hormonal priming protocol. The consummatory phase of mating can be subdivided into events defined by male behavior, and here we investigate four—mount, intromission, ejaculatory shudder, and post-ejaculatory freeze (**Figure 1B**). Mount is contact between the male abdomen and the back of the female, intromission is intravaginal penis insertion, ejaculatory shudder is high-frequency thrusting that ends with ejaculation (**Video S1)**, and post-ejaculatory freeze is prolonged immobile male grasping of the female following ejaculation (McGill, 1962).

Ejaculation was associated with a robust increase in female DRN 5-HT activity (GCaMP, $n = 14$ females, $p < 0.001$; GFP, $n = 3$ females, $p = 0.75$; Wilcoxon signed rank test, **Figure 2 and Video S1**). This increase in neural activity was maintained for several seconds until a reduction was seen at the end of the post-ejaculatory intromission ($n = 8$ females, $p < 0.05$, paired t-test, **Figures S1A and S1B**). Female DRN 5-HT activity did not increase on pre-ejaculatory mounts and intromissions (mount, $n = 7$ females, $p = 0.06$; intromission, $n = 7$ females, $p = 0.44$; ejaculation, $n = 8$ females, $p < 0.01$; Wilcoxon signed rank test, **Figures S1C and S1D**). We also recorded DRN 5-HT neural activity in naturally cycling females during mating and found activation at ejaculation, therefore this result did not depend on ovariectomy and hormonal priming ($n = 4$ females, $p < 0.05$, paired t-test, **Figure S2)**. Thus, DRN 5-HT neural activity in female mice is specifically elicited by male ejaculation during the consummatory phase of mating.

Artificial mechanical intravaginal stimulation elicits increased DRN 5-HT neural activity

As ejaculation is required for the initiation of neuroendocrine processes in female mice that support pregnancy and pseudopregnancy (Yang et al., 2009), we sought to distinguish between ejaculation-specific stimuli that may be detected intravaginally by the female. In other species, sperm are accompanied by fluid that contains a variety of signaling molecules including prostaglandins and sex peptides (Wyatt, 2017), and chemical stimulation in these species is necessary for normal female reproductive behavior (Aprison and Ruvinsky, 2019; Chen et al., 1988; Yapici et al., 2008). Alternatively, mechanical stimulation may be provided by ejaculation-specific changes in the shape of the mouse penis, or by the forceful expulsion of ejaculatory fluid or copulatory plug (McGill and Coughlin, 1970; Sachs, 1980).

To investigate whether chemical or mechanical stimulation might underlie the increase in female DRN 5-HT neural activity upon ejaculation, we used a syringe to pseudo-randomly deliver 5 different stimuli into the vaginal canal of gently restrained female mice (**Figure 3A**). To test chemical stimulation, we used two solutions: ejaculatory fluid (including the homogenized copulatory plug) collected from a female uterus immediately after ejaculation, and homogenized accessory sex glands from a male. As controls, we delivered equivalent volumes of air or saline. To test mechanical stimulation, we used a piece of rubber attached to the end of a syringe which could be inflated into a small balloon (McGill, 1970). We observed a significant increase in DRN 5-HT neural activity in response to balloon inflation, but not in response to any of the other stimuli (GCaMP: balloon, $n = 6$ females, $p < 0.05$; air, $n = 6$ females, $p = 0.09$; saline, $n = 6$ females, $p = 0.09$; homogenized glands, $n = 5$ females, $p = 0.06$; homogenized ejaculate, $n = 5$ females, $p = 0.13$; Wilcoxon signed ranked test, **Figures 3B-3D**. GFP: balloon, $n = 3$ females, $p = 0.59$; air, $n = 3$ females, $p = 0.82$, paired t-test, **Figures S3A and S3B**). DRN 5-HT neural activity rose at the moment the syringe plunger was depressed and fell when the plunger was pulled to deflate the balloon (balloon, $n = 6$ females, $p < 0.05$, paired t-test, **Figures S3C and S3D**), supporting a strong association between DRN 5-HT neural activity and mechanical stretch caused by intravaginal balloon inflation. These data are consistent with the hypothesis that mechanical but not chemical intravaginal stimulation elicits activity in DRN 5-HT neurons in female mice.

Separate blocking of penile cupping or ejaculatory fluid/copulatory plug expulsion does not disrupt female DRN 5-HT neural activity at ejaculation

Ejaculation is a dynamic process comprising several intravaginal steps. One step of potential relevance is the striking morphological change that occurs in the mouse penis at the moment of ejaculation (McGill and Coughlin, 1970). 2-3 seconds after the onset of the ejaculatory shudder, the diameter of the distal end of the penis dramatically increases, forming a “penile cup” that

lasts 5-12 seconds. This phenomenon has also been observed during *ex copula* induction of penile reflexes (Sachs, 1980). Following penile cupping, the ejaculatory fluid is forcefully expelled and a large copulatory plug is shot from the penis with such force that it embeds deeply into the cervix and occasionally penetrates the uterus (McGill and Coughlin, 1970). These ejaculation-specific mechanical stimuli are thought to induce vaginal/cervical stretching, and have been hypothesized to play a role in the induction of pseudopregnancy in mice (McGill and Coughlin, 1970). We sought to determine if these stimuli might contribute to the rise in female DRN 5-HT neural activity on male ejaculation.

We first investigated whether intravaginal mechanical stimulation during ejaculation might be provided by penile cupping (McGill and Coughlin, 1970). Transection of the paired bulbocavernosus (BC) muscles at the base of the penis has been shown to damage penis erectile function in mice, including *ex copula* penile cup display (Elmore and Sachs, 1988). To test whether penile cupping elicits the rise in female DRN 5-HT neural activity at ejaculation, we performed unilateral transections of the BC muscle in male mice (cup males, **Figure 4A**). Unilateral transections were selected over bilateral ones because we observed that male mice with complete transection of the BC muscles were unable to perform intromissions and ejaculations (data not shown). Males with unilateral BC transections displayed normal mating behavior, but did exhibit a greater number of "intromission breaks"—periods after intromission when the penis leaves the vagina and is reinserted without a dismount (Elmore and Sachs, 1988) (**Figure S4**). This behavioral phenotype results from damage to the penis glans erectile function, which is supported by the BC muscles and required for both continuous intromission and penile cup formation at ejaculation. We recorded DRN 5-HT neural activity from females mating with males with unilateral BC transections, and found a significant increase in neural activity at ejaculation, similar to the increase observed in the same females during mating sessions with unmodified control males (cup, $n = 5$ females, $p < 0.05$; control, $n = 5$ females, $p < 0.05$; paired t-test, **Figures 4B and 4C**). Thus, blocking penile cupping alone does not disrupt female DRN 5-HT neural activity during ejaculation.

Another possible source of mechanical stimulation is the forceful expulsion of the ejaculatory fluid and copulatory plug characterized in *ex copula* observations of the mouse ejaculatory sequence (McGill and Coughlin, 1970). To test whether ejaculatory fluid/copulatory plug expulsion elicits the rise in female DRN 5-HT neural activity at ejaculation, we surgically modified male mice to re-route urine and ejaculate through an alternative, non-penile exit (fluid-plug males, **Figure 4D and Video S2**). To generate these males, we transected the urethra caudal to the prostate glands and reconnected the cranial urethral segment to the abdominal wall, leaving a stoma through which urine and ejaculatory fluid could be diverted. Additionally, the bulbourethral and preputial glands—two pairs of accessory sex glands connected to the caudal urethral segment that also contribute components to the ejaculatory fluid—were resected. At the

end of every mating session we verified that no copulatory plug was left inside the female vagina after shudder and freezing behavior. In a few instances, the copulatory plug was found on the female's flank and perineal fur (**Figure S5**). We recorded DRN 5-HT neural activity from females mating with males with diverted ejaculatory fluid/copulatory plug, and found that there was significant DRN 5-HT neural activation at the time of ejaculation, similar to the signal observed in the same females during mating sessions with unmodified males (fluid-plug, $n = 7$ females, $p < 0.05$; control, $n = 7$ females, $p < 0.05$; Wilcoxon signed rank test, **Figures 4E and 4F**). Thus, blocking ejaculatory fluid/copulatory plug expulsion alone does not disrupt female DRN 5-HT neural activity during ejaculation.

Simultaneous blocking of both penile cupping and ejaculatory fluid/copulatory plug expulsion disrupts female DRN 5-HT neural activity at ejaculation

We considered the possibility that neither penile cup formation or ejaculatory fluid/copulatory plug expulsion would provide sufficient intravaginal mechanical stimulation to independently elicit female DRN 5-HT neural activation upon ejaculation, but that they might in combination. To test this hypothesis, we generated males with both surgeries—unilateral transection of the BC muscle to disrupt cupping, and urethral redirection to divert ejaculatory fluid/copulatory plug expulsion (cup/fluid-plug males, **Figure 5A**). When females mated with these males, we found no significant DRN 5-HT activation upon ejaculation (cup/fluid-plug, $n = 7$ females, $p = 0.30$; control, $n = 7$ females, $p < 0.05$; Wilcoxon signed rank test, **Figures 5B and 5C**). Male mating behavior was otherwise unchanged and indistinguishable from that of intact males mated with the same females, except for the expected display of intromission breaks (**Figure S4**). Thus, penile cupping or forceful expulsion of ejaculatory fluid each provide sufficient mechanical stimulation to elicit 5-HT neural activity during ejaculation, and simultaneous disruption of both was required to block this rise in neural activity.

One question that emerges from our findings is how the expulsion of the ejaculatory fluid exerts mechanical force on the walls of the female reproductive tract. A feature of mouse ejaculatory fluid is its coagulation into a copulatory plug (Schneider, 2016), thus we sought to test whether the copulatory plug was necessary for female DRN 5-HT neural activation at ejaculation by interfering with coagulation. To block copulatory plug formation, we resected the coagulating glands and seminal vesicles, which produce a critical ingredient for copulatory plug formation, seminal vesicle secretion 2 (Gotterer et al., 1955; Kawano, 2014). We combined this with unilateral transection of the BC muscle to additionally block penile cupping (cup/plug males, **Figure 5D**). We verified at the end of every mating session that no copulatory plug was left in the female vagina. Females mated with these modified males exhibited robust DRN 5-HT neural activation at ejaculation (cup/plug, $n = 5$ females, $p < 0.05$; control, $n = 5$ females, $p <$

0.05; paired t-test, **Figures 5E and 5F**), indicating that forceful ejaculatory fluid expulsion alone, without copulatory plug formation or penile cupping, provides enough mechanical stimulation to elicit female DRN 5-HT activity upon male ejaculation. Consistent with this finding, we observed that forceful delivery of saline into the vaginal canal is sufficient to elicit female DRN 5-HT neural activity (**Figure S6**). Together, these data reveal that mechanical stimulation provided by either male penile cupping or forceful fluid expulsion are sufficient to elicit an increase in female DRN 5-HT neural activity upon ejaculation.

DISCUSSION

Here, we sought to investigate how female DRN 5-HT neurons respond to sexual stimulation during the consummatory phase of mating. Fiber photometry recordings from freely behaving females revealed pronounced DRN 5-HT neural activation elicited specifically by male ejaculation. Artificial intravaginal mechanical stimulation was sufficient to activate DRN 5-HT neurons, but intravaginal delivery of ejaculatory fluids was not. To identify the source of mechanical intravaginal stimulation responsible for female DRN 5-HT neural activation during natural mating, we developed surgical techniques to perturb two principal aspects of male ejaculatory physiology: forceful ejaculatory fluid expulsion and distal penis expansion (cupping) upon ejaculation. We found that both factors were independently capable of eliciting activity in female DRN 5-HT neurons, and that combined disruption of both was required to block the rise in female DRN 5-HT neural activity upon ejaculation.

Reproductive roles of vaginocervical stimulation

Interoceptive detection of mechanical vaginocervical stimulation triggers multiple behavioral, autonomic, and neuroendocrine responses that have critical reproductive roles. In rodents, mechanical vaginocervical stimulation induces behavioral and sensory changes that include immobility, lordosis, and analgesia (Komisaruk and Diakow, 1973; Naggar and Komisaruk, 1977; Komisaruk and Wallman, 1977), and rodents exhibit conditioned place preference for a context paired with vaginocervical stimulation (Meerts and Clark, 2009). Mechanical vaginocervical stimulation is also essential for inducing physiological changes that initiate and support early pregnancy and pseudopregnancy in rodents. In rats, repeated intromission without ejaculation is sufficient to induce pseudopregnancy, and the likelihood of pseudopregnancy induction depends on the number and pacing of intromissions. In mice, ejaculation is required for the induction of pseudopregnancy (McGill and Coughlin, 1970). If mating mice are separated shortly after the onset of the ejaculatory shudder, just before penile cupping and fluid expulsion, most females do not become pseudopregnant. If separation happens just a few seconds later,

however, most females do become pseudopregnant. The vaginocervical stimulation induced specifically by male ejaculation in mice thus has profound neuroendocrine consequences for recipient females. The neural mechanisms that underlie the translation of this brief mechanosensory stimulus into a persistent hormonal state change are currently unclear, but the the rise in DRN 5-HT neural activity specifically at the moment of ejaculation in female mice raises the possibility that serotonin may play a role.

Copulatory plug formation is not required for pseudopregnancy induction (McGill et al., 1968), and our findings show that it is not necessary for DRN 5-HT activation upon ejaculation. Separately, ejaculatory fluid expulsion or penile cupping each provide enough intravaginal mechanical stimulation for both DRN 5-HT activation and pseudopregnancy. In humans, Masters and Johnson estimated that the pressure exerted by the ischiocavernosus and bulbospongiosus muscles is sufficient to expel the ejaculate 30 to 60 cm (Masters and Johnson, 1966). In mice, the expulsion of the ejaculatory fluid/copulatory plug observed in *ex copula* penis behavior has been characterized as a "forceful ejection" (McGill and Coughlin, 1970), and the pressure inside the mouse penis corpus spongiosum at ejaculation is more than twice the pressure during pre-ejaculatory intromissions (Soukhova-O'Hare et al., 2007). Further quantification is necessary to determine the force that the ejaculate fluid alone exerts on the walls of the female reproductive tract.

Mechanisms of vaginocervical stimulus detection

Vaginocervical information may be carried to the brain via both spinal and non-spinal pathways, since the rodent vagina and cervix are innervated by terminations of the pelvic, hypogastric, and vagus nerves (Hubscher and Berkley, 1995; Peters et al., 1987; Steinman et al., 1992). Transection of the pelvic nerve prevents the induction of pseudopregnancy by vaginocervical stimulation (Kollar, 1953), and combined vagus and pelvic nerve transection is required to abolish pupil dilation on vaginocervical stimulation (Komisaruk et al., 1996). Single-cell RNA sequencing transcriptomic analysis of vagal nodose and lumbar dorsal root ganglia has revealed a vast diversity of neuron types consistent with chemoreceptors, nociceptors, and mechanoreceptors, including neuron types that express the mechanically sensitive channel *Piezo2* (Kupari et al., 2019; Usoskin et al., 2015). Nevertheless, how pelvic, hypogastric, and/or vagus nerves detect and convey mechanosensory information from the female reproductive tract remains unclear. The vaginal epithelium expresses Piezo1 channels (Dalghi et al., 2019), which are mechanically activated (Coste et al., 2010), but their involvement in the effects of vaginocervical stimulation is unknown.

DRN 5-HT neural activity and female sexual behavior

The DRN 5-HT system modulates a wide spectrum of animal behaviors outside the context of female sexual function and mating. 5-HT has been associated with behavioral inhibition, waiting, and patience (Soubrié, 1986; Miyazaki et al., 2012; Miyazaki et al., 2014; Fonseca et al., 2015), and recent reports have linked 5-HT neural activity with emotional behaviors such as freezing in threatening contexts (Teissier et al., 2015; Seo et al., 2019; Paquelet et al., 2022). At the moment of ejaculation, female rodents maintain a stationary receptive posture that facilitates appropriate transfer of sperm and placement of the copulatory plug, which influences reproductive outcome (Adler and Toner Jr, 1986; Dean, 2013; Elmore and Sachs, 1988; Matthews and Adler, 1977). The DRN 5-HT neural activation characterized here could play a role in facilitating this behavioral response to sexual stimulation. The locomotor pause and receptive posture seen at ejaculation are also observed during other phases of mating that are not associated with significant DRN 5-HT neural activation, however, such as pre-ejaculatory intromissions, suggesting that these mating phases may be regulated by other neural circuits. DRN 5-HT neurons have also been implicated in physiological and sensory responses such as pupil dilation and analgesia (Cazettes et al., 2021, Dugué et al., 2014), which can likewise be elicited by vaginocervical stimulation (Komisaruk et al., 1996). Measurements of other physiological quantities such as muscle tone, respiratory rate, or heart rate may reveal additional DRN-5-HT contributions related to sexual behavior.

The function of DRN 5-HT in female post-copulatory behavior and physiology may be mediated in part by projections to hypothalamic nuclei involved in these functions (Muzerelle et al., 2016). For example, previous pharmacologic work has implicated 5-HT in the regulation of female lordosis (Uphouse et al., 2006), which is traditionally considered a measure of sexual receptivity (Erskine et al., 2004). Infusion of 5-HT2 receptor agonists into the mediobasal hypothalamus facilitated lordosis behavior in rats, and antagonists of these receptors reversed the effect (Uphouse, 2014; Uphouse et al., 1996; Wolf et al., 1998). In contrast, infusion of 5HT1a receptor agonists in the same region reduced lordosis (Gonzalez et al., 1997; Uphouse et al., 1992, 1993). While these findings suggest a role for tonic 5-HT in regulating female sexual behavior, causal manipulation of 5-HT neural activity on a fine timescale would inform our understanding of the specific functional role of 5-HT release during ejaculation.

Activity in other female neural circuits elicited by ejaculation

The requirement for ejaculation to induce pseudopregnancy in mice suggests that detection of ejaculation by the female mouse brain is critical in initiating subsequent reproductive programs. In female mice, ejaculation elicits changes in neural activity in a number of brain regions that have been implicated in the regulation of reproductive behavior and physiology, and which receive dense projections from DRN 5-HT neurons. Mating stimulation sufficient to induce

pseudopregnancy elicits cFos expression in the posterodorsal medial amygdala (MeApd), the ventrolateral part of the ventromedial hypothalamus (VMHvl), and the bed nucleus of the stria terminalis (BNST) in female mice (Yang et al., 2009). Progesterone receptor-expressing VMHvl neurons in female mice exhibit phasic responses to mounts with intromission and ejaculation (Inoue et al., 2019), and cholecystokinin A receptor-expressing VHMvl neurons in female mice are persistently suppressed immediately following male ejaculation (Yin et al., 2022). BNST GABAergic neurons in female mice exhibit phasic responses specific to ejaculation (Bayless et al., 2019), and hypothalamic gonadotropin-releasing hormone (GnRH) neurons become persistently active following ejaculation in female mice (Wu et al., 1992). Neuromodulatory systems other than serotonin, such as dopamine and norepinephrine, have also been shown to respond to ejaculation in female mice (Etgen and Morales, 2002; Cameron et al., 2004; Dai et al., 2022). Investigation of the role of projection-specific DRN 5-HT neural activity in modulating ejaculation-related neural activity in these brain regions would be informative.

Overall, our findings support the hypothesis that a key regulator of DRN 5-HT activity is intravaginal mechanical stimulation provided by ejaculatory fluid expulsion and distal penis expansion. The surgical techniques developed here provide a novel methodology for the study of the functional impact of distinct aspects of ejaculation receipt on female physiology and behavior. Our results highlight the importance of exploring sex-specific functions of 5-HT for deepening our understanding of this neuromodulatory system.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Virus Strains		
AAV-DJ-EF1α-DIO-GCaMP6m	Stanford Vector Core	N/A
AAV5-CAG-Flex-GFP	UNC Vector Core	N/A
Chemicals		
Estradiol	Sigma-Aldrich	Cat # E8875; CAS 50-28-2
Progesterone	Sigma-Aldrich	Cat # P0130; CAS 57-83-0
DABCO	Sigma-Aldrich	Cat # 290734; CAS 280-57-9
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	The Jackson Laboratory	RRID:IMSR_JAX:000664
Mouse: B6.129(Cg)-Slc6a4$^{tm1(cre)Xz}$/J	The Jackson Laboratory	RRID:IMSR_JAX:014554
Software and Algorithms		
MATLAB	MathWorks	https://www.mathworks.com/products.html; RRID:SCR_001622
GraphPad Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/; RRID:SCR_002798

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents may be directed to the Lead Contact, Melissa R. Warden (mrwarden@cornell.edu).

Materials Availability

This study did not generate new reagents.

Data and Code Availability

Data and source code supporting the current study will be made available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All procedures conformed to guidelines established by the National Institutes of Health and have been approved by the Cornell University Institutional Animal Care and Use Committee. SERT-Cre female mice (The Jackson Laboratory, Bar Harbor, ME) between 2 and 12 months of age were used for 5-HT-specific viral vector expression. All Cre driver lines were fully backcrossed to C57BL/6J mice. All mice were maintained on a 12-hour reverse light-dark cycle with *ad libitum* access to food and water, and experiments were conducted during the dark portion of the cycle.

METHOD DETAILS

Viral vector injection and fiber implantation

Mice were anesthetized using isoflurane (5%). Fur was shaved and mice were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). A heating pad was placed under the mice to prevent hypothermia. Isoflurane (1-2 %) was delivered via a nose cone throughout the surgery. Ophthalmic ointment was used to protect the eyes. Baytril (5 mg/kg, subcutaneous) and Ringer's lactate solution (0.5 mL, subcutaneous) were given at the start of surgery. The scalp was disinfected with betadine scrub and 70% ethanol. A 1:1 mixture of 0.5 % lidocaine and 0.25% bupivacaine (0.1 mL) was injected intradermally along the incision line. An incision was made

using a scalpel along the scalp midline. The exposed skull was thoroughly cleaned and a craniotomy was made above the DRN. The viral vector was targeted to the DRN (-4.5 AP, 0 ML, -3.3 & -2.9 DV), and pressure injected at 100 nl/min using a 10 µL Hamilton syringe (Nanofil; WPI, Sarasota, FL), 33 gauge beveled needle (Nanofil; WPI, Sarasota, FL), and micro-syringe pump controller (Micro 4; WPI, Sarasota, FL). After each injection, the needle was left in place for 10 minutes and then slowly withdrawn. A total of 800 nL (400 nL at each DV site) of viral vector was injected. A 400 µm diameter, 0.48 or 0.53 NA optical fiber (Doric Lenses, Quebec, Canada) embedded in a metal ferrule was implanted at 15° (-4.5 AP, 0.72 ML, -2.75 DV). A layer of metabond (Parkell, Inc., Edgewood, NY) and dental acrylic (Lang Dental Manufacturing, Wheeling, IL) was applied to firmly hold the implant in place. The surrounding skin was closed with a simple interrupted suture pattern. Subcutaneous buprenorphine (0.05 mg/kg), carprofen (5 mg/kg), and Ringer's lactated solution (0.5 mL) were administrated post-operatively. Viral vector expression was allowed for a minimum of 2 weeks before behavioral testing.

Ovariectomy

General surgical procedures were performed as described above. The ovariectomy protocol was conducted as reported previously (Souza et al., 2019), during the same surgical session as the viral vector injection and fiber implantation. Briefly, the dorsal fur between the last rib and the cranial pelvic edge was shaved on both sides. In each side, an incision was made into the peritoneal cavity, and the ovarian fat pad was carefully pulled out. The oviduct was clamped with hemostats, and the ovary was excised after cauterization of the tissue and blood vessels. The skin was sutured and covered with topical antibiotic (Water-Jel Technologies Llc., Carlstadt, NJ). Females were allowed to recover for at least 1 week before starting hormonal priming and behavioral testing.

Urethrostomy

General surgical procedures were performed as described above. The ventral fur between the last rib and the penis base was shaved. An incision was made along the *linea alba* to access the peritoneal space. Using blunt dissection, the urethral segment caudal to the prostate gland was identified and pulled ventrally. The urethra was incised transversely, between the prostate and the dorsal penis curvature, taking care to not damage the pair of cavernous nerves running on both sides of the urethra. The caudal urethral end connected to the penis was cauterized and returned to the abdominal cavity. The sides of the cranial urethral wall connected to the bladder were sutured to the abdominal muscle and skin layers creating a stoma. The incision cranial and caudal to the stoma was closed, making sure that the urethral lumen remained continuous with the outside. Post-operatively, mice were kept on absorbent underpads to track normal urination

for 3 days before being placed in normal bedding. Mice were allowed to recover for at least one week before behavioral testing. During this time, mice were checked daily, and common complications were monitored and treated appropriately, such as inflammation, infection, wound dehiscence, and urethral blockage. If animals presented signs of uroabdomen, they were euthanized immediately.

Resection of accessory sex glands

General surgical procedures were performed as described above. The ventral fur between the last rib and the penis base was shaved, as well as the fur covering the scrotal sac. A midline incision was performed through the scrotal sac. Blunt dissection was used to identify the bulbourethral glands bilaterally. Each gland was pulled out, clamped at the base with a hemostat, and excised as close to its urethra connection as possible. The skin was sutured and covered with topical antibiotic.

Removal of the preputial glands was performed by making a midline incision into the skin layer ~1 cm cranial to the penis base. After blunt dissection of the subcutaneous tissue, the preputial glands were pulled bilaterally. Each gland was clamped at the base with a hemostat, and excised. For removal of the seminal vesicles and associated coagulating glands, a midline incision along the *linea alba* was performed ~1 cm cranial to the penis base. Blunt dissection was used to identify the seminal vesicles and coagulating glands, which were transected at their base connection to the bladder. All layers of the abdominal wall incision were sutured and covered with topical antibiotic.

Partial bulbocavernosus (BC) muscle transection

General surgical procedures were performed as described above. The scrotal fur was shaved, and a midline incision was performed through the scrotal sac. Blunt dissection was used to identify the BC muscles and isolate them from the ischiocavernosus muscles. The right BC muscle was transected, and absorbable suture was placed in the cranial muscle end connected to the penis base. The scrotal skin incision was sutured and covered with topical antibiotic.

Hormone priming

Estrus was induced in ovariectomized females with a subcutaneous injection of 10 μg β-estradiol 3-benzoate (Sigma) in 0.05 mL sesame oil ~48 hours before testing. This was followed by a subcutaneous injection of 500 μg progesterone (Sigma) in 0.05 mL sesame oil 4 to 6 hours before testing. All females were ovariectomized and hormonally primed unless indicated otherwise.

Staging of estrous cycle using vaginal cytology

Intact females were gently restrained to expose their vaginal opening. A 200 uL pipet tip connected to a rubber bulb was filled with ~100 uL of saline solution kept at room temperature. The solution was flushed in and out of the vagina several times, making sure to not insert the pipet tip. Samples were placed on a microscope slide and allowed to air-dry. Samples were then stained using JorVet Dip Quick Stain (Jorgensen Labs, Loveland, CO) and examined under 10X magnification in a light microscope. The relative quantities of neutrophils, nucleated and anucleated epithelial cells were used to determine the estrous cycle stage (Cora et al., 2015). Female estrous cycle stage was scored for at least 2 weeks prior to behavioral testing during proestrus/estrus.

Mating assay

Mating sessions were recorded at 30 frames/second under infrared illumination. Females were group housed and males were single housed for at least 1 week prior to testing. All mating sessions took place in the stud's home cage, to which the female was transferred at the beginning of the session. The pair was allowed to interact for 15 min or until the stud performed the first successful intromission. If no intromission took place during this time, the pair was separated and the session was cancelled. After the first successful intromission, the female was connected to the fiber photometry system and recording started. Video and photometry data was recorded for up to 1 hour or until 3 minutes after successful ejaculation.

Videos were manually annotated using Bento (Segalin et al., 2020). Mounts were defined as the time when the female tail was between the male's hindlegs and his abdomen was directly over the female dorsum. Intromissions scored from a side view video were defined as the time of low-frequency thrusting during mounts. Intromissions scored from bottom view videos were defined as the time when the penis was seen inside the vagina. Shudders were defined as the time of high-frequency thrusting that preceded freezing, where the male was completely immobile and connected to the female with all four limbs. Ejaculation was defined as the shudder offset. "Start to shudder on" latency was the time from the beginning of the photometry recording to the shudder onset. "Mount to shudder on" latency was the time from the onset of the last mount to the shudder onset. Failed mounts were defined as mounts with no intromission.

Artificial vaginal stimulation

Six artificial stimuli were prepared and delivered 5-12 times each, in pseudorandom order. The combination of stimuli delivered in a given session varied by experiment. For all stimuli, a 1 mL

tuberculin slip tip syringe connected to a ~1.5 cm segment of PVC tubing (1/16" outer diameter) was inserted in the female vaginal canal. Care was taken to not exert pressure on the syringe beyond the natural resistance point of the caudal cervical wall. In the air condition, 0.1 mL of air were delivered. In the saline condition, 0.1 mL of saline were delivered at ~0.2 mL/s. In the "fast saline" condition, 0.7 mL of saline were delivered at ~0.7 mL/s. For the gland fluid condition, all accessory sex glands and testes from a male mouse were collected, homogenized, and stored in saline solution at -80 degrees (Dean et al., 2009). In preparation for the artificial stimulation session, the solution was thawed to room temperature. For the uterus fluid condition, females were sacrificed immediately after mating, and their entire reproductive tract was extracted. The copulatory plug was extracted and homogenized in saline along with fluid stripped from the uterus. The solution was frozen at -80 degrees (Dean et al., 2011) and thawed to room temperature at the time of the artificial stimulation session. For the balloon condition, a ~1x1 cm piece of balloon rubber was tied to the end of the tubing using suture material. The apparatus design was based on the one described in McGill, 1970. The syringe was filled with glycerol and labeled at the mark that resulted in inflation of a balloon with 6 mm diameter.

Females were gently restrained to expose their vaginal opening while artificial stimuli were delivered. Females were allowed at least 14 seconds of unrestrained rest in between deliveries, and at least 20 seconds in between stimuli. For all stimuli, the tip of the syringe tubing was inserted in the vaginal opening for ~5 seconds before pressing the syringe plunger. In the empty, saline, gland fluid, and uterus fluid conditions, the syringe tubing was held inside the vagina for ~10 seconds after pressing the plunger and before removal of the apparatus. In the balloon condition, the balloon was kept inflated inside the vagina for ~10 seconds. After retracting the plunger, the syringe tubing with the deflated balloon was held in the vagina for ~5 seconds before removing the apparatus. Artificial stimulation sessions were recorded at 30 frames/second under low lighting conditions, taking care to capture clear views of the female vaginal opening and the syringe plunger. Videos were manually annotated using Bento (Segalin et al., 2019).

Perfusion and histological verification

Animals were deeply anesthetized with Fatal Plus at a dose of 90mg/kg, and transcardially perfused with 20 mL of phosphate-buffered saline (PBS), followed by 20mL of 4% paraformaldehyde (PFA) solution. Brains were extracted and stored overnight at 4°C in 4% PFA solution. After 24 hours, brains were transferred to 30% sucrose solution and allowed to equilibrate for a day. Brains were sectioned coronally (100 um) on a freezing microtome. Sections were washed in PBS and mounted on slides with PVA-DABCO. Images were acquired using a Zeiss LSM 800 confocal scanning laser microscope with a 20X air objective.

Fiber photometry

The fiber photometry system used LEDs to deliver 405 nm and 473 nm light. These light streams were sinusoidally modulated at 211 Hz and 531 Hz respectively, combined into a minicube (Doric Lenses, Quebec, Canada), and coupled to an optical patch cord (400 µm; Doric Lenses). The optical patch cord was connected to the animal using a zirconia sleeve (2.5 mm; Doric Lenses). Emitted fluorescence was received through the same optical patch cord and collected by a femtowatt photodetector (New Focus 2151, Newport Corporation, Irvine, CA). This signal was processed using Doric Photometry Software. Photometry data analysis and statistical tests were performed using custom code in MATLAB (MathWorks, Natick, MA). Data were collected at 12 kHz, and low-pass filtered at 12 Hz. The calcium activity-independent 405 nm reference channel was fit to the calcium activity-dependent 473 nm channel using linear least squares. Relative fluorescence changes, reported as ΔF/F, were calculated using the following equation:

$$\frac{\Delta F}{F} = \frac{\text{473 nm signal} - \text{fitted 405 nm signal}}{\text{mean 473 nm signal}} \times 100$$

Mean ΔF/F before and after an event or behavior was calculated by calculating the mean signal in a 5 s window pre- and post-timepoint.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics

Data are presented as mean ± standard error of mean (SEM), and statistical significance was considered for $p < 0.05$. Statistical analysis was performed using GraphPad PRISM (GraphPad Software Inc., CA). Sample distribution was assessed with a D'Agostino-Pearson omnibus normality test, and used to select parametric vs. non-parametric statistical tests for each dataset.

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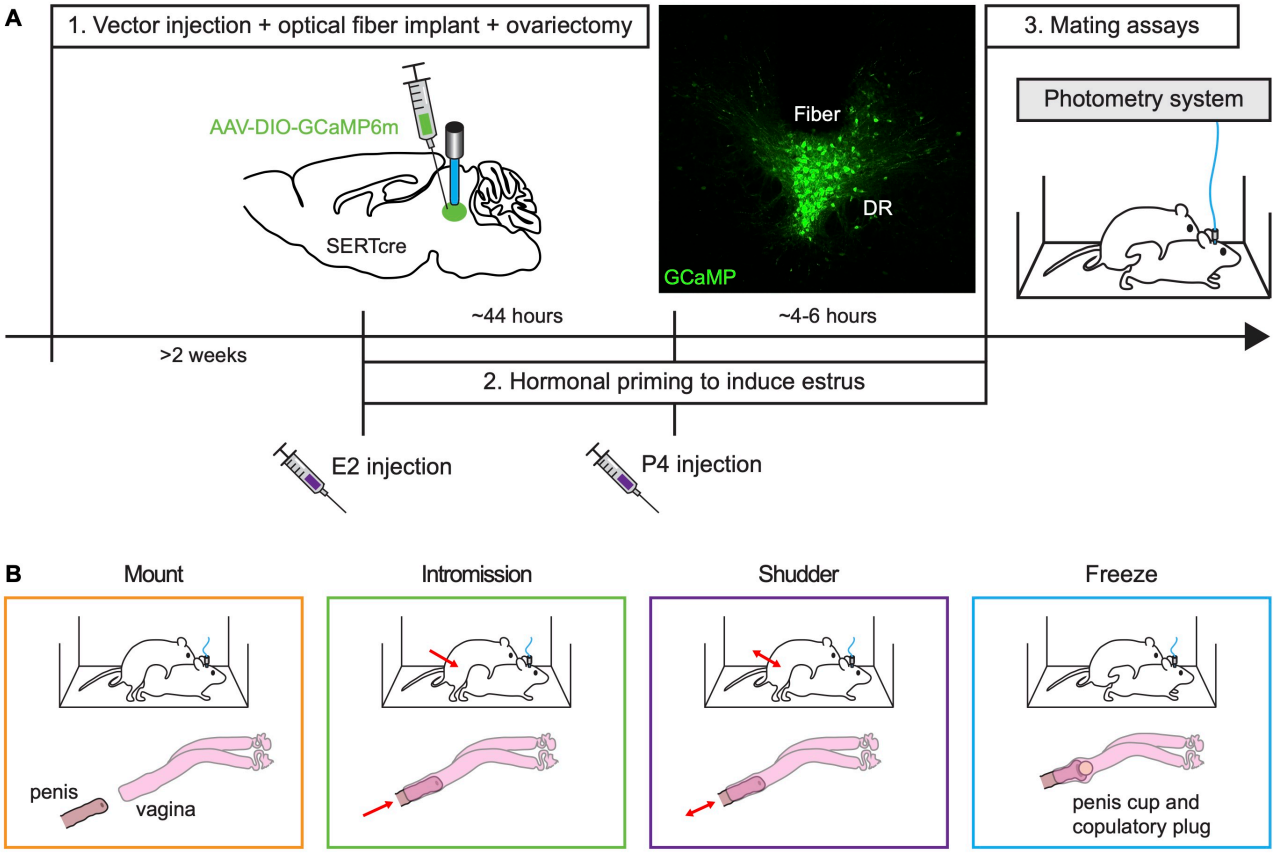

Figure 1. DRN 5-HT neural activity recorded with fiber photometry during mating
(A) Experimental timeline and GCaMP expression in DRN 5-HT neurons of a SERT-Cre female mouse. (B) Schematic of male sexual behaviors during the consummatory phase of mating. Mount is male abdominal contact with the back of the female. Intromission is insertion of the penis into the vagina, repeated at low frequency during mount. Shudder is the short period of high-frequency thrusting that ends in ejaculation. Freeze is the post-ejaculatory period during which the male grasps the female hind body and is immobile.

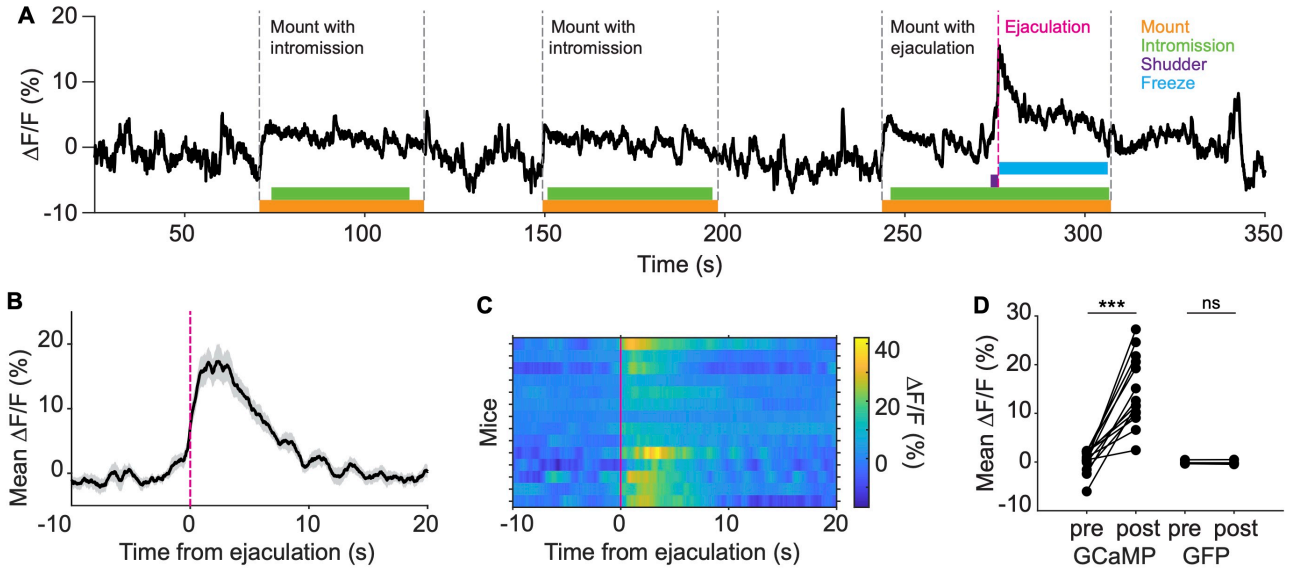

Figure 2. Female DRN 5-HT neural activity increases at ejaculation.
(A) Example female DRN 5-HT GCaMP ΔF/F during mating. Colored boxes indicate scored male behaviors. Grey dotted lines indicate the onset and offset of male mounts. Magenta dotted line indicates ejaculation.
(B) Mean DRN 5-HT GCaMP ΔF/F aligned to ejaculation (n = 14 females). Shaded area indicates SEM.
(C) Heatmap of DRN 5-HT GCaMP ΔF/F for all animals, aligned to ejaculation (n = 14 females).
(D) Mean DRN 5-HT ΔF/F 5 s before and after ejaculation in GCaMP (n = 14 females) and GFP females (n = 3 females) (Wilcoxon signed rank test, ns = non-significant, ***$p < 0.001$).
See also Figures S1 and S2 and Video S1.

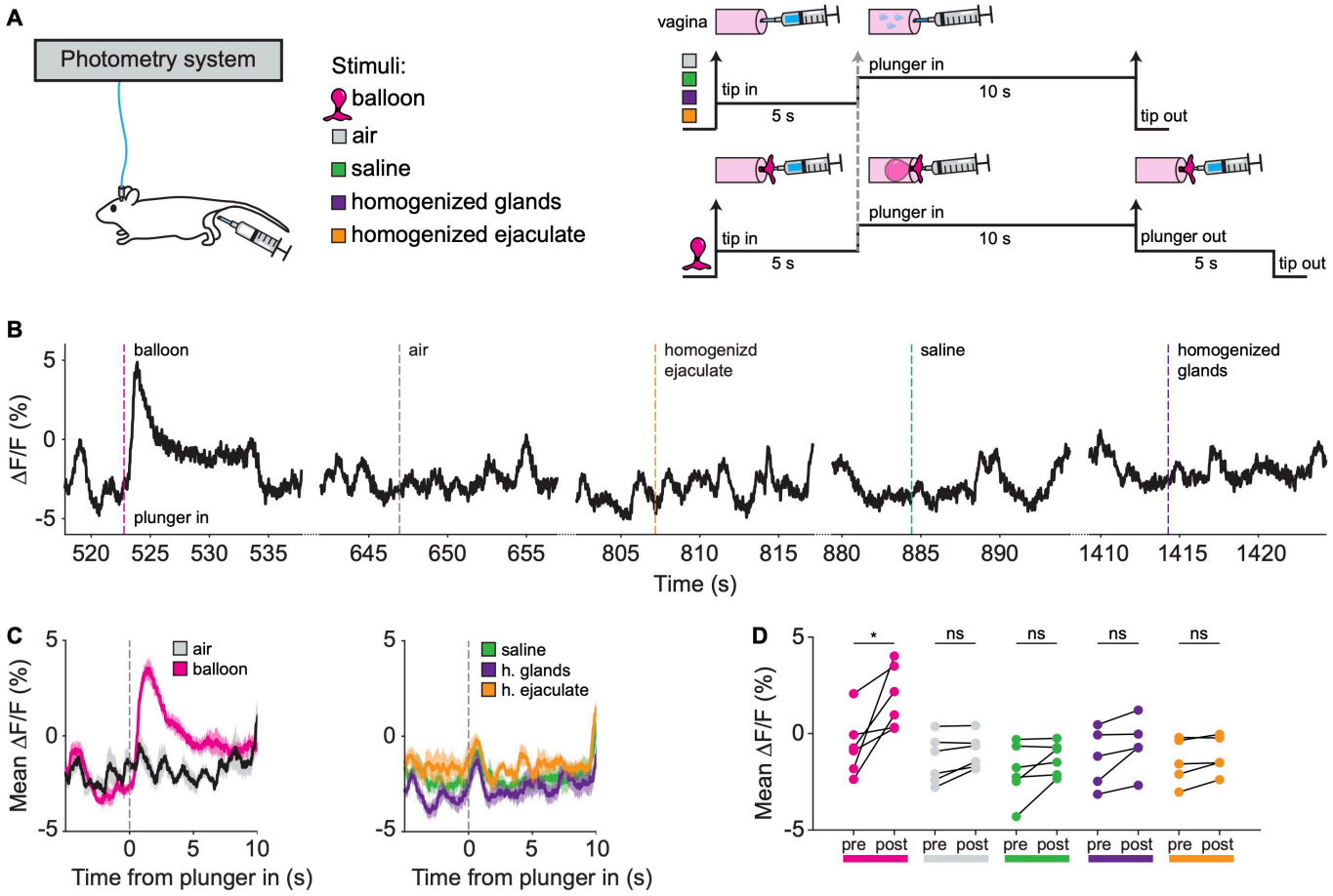

Figure 3. Artificial mechanical intravaginal stimulation elicits increased DRN 5-HT neural activity.
(A) Experimental design. Artificial stimuli were delivered into the vagina of gently restrained females using a syringe. In each session, each stimulus was delivered 5-10 times, pseudo-randomly, every >20 s. For all conditions, after stimulus delivery the syringe was held in place for ~10 s.
(B) Example DRN 5-HT GCaMP ΔF/F during artificial stimulation. Dotted lines for each condition indicate when the syringe plunger was pushed in.
(C) Mean DRN 5-HT GCaMP ΔF/F aligned to plunger in for the same mouse for all conditions (n = 10 trials per condition). Shaded area indicates SEM.
(D) Mean DRN 5-HT GCaMP ΔF/F calculated in a 5 s window before and after plunger in (balloon, air, saline, n = 6 females; homogenized glands, homogenized ejaculate, n = 5 females) (Wilcoxon signed ranked test, ns = non-significant, *$p < 0.05$).
See also Figure S3.

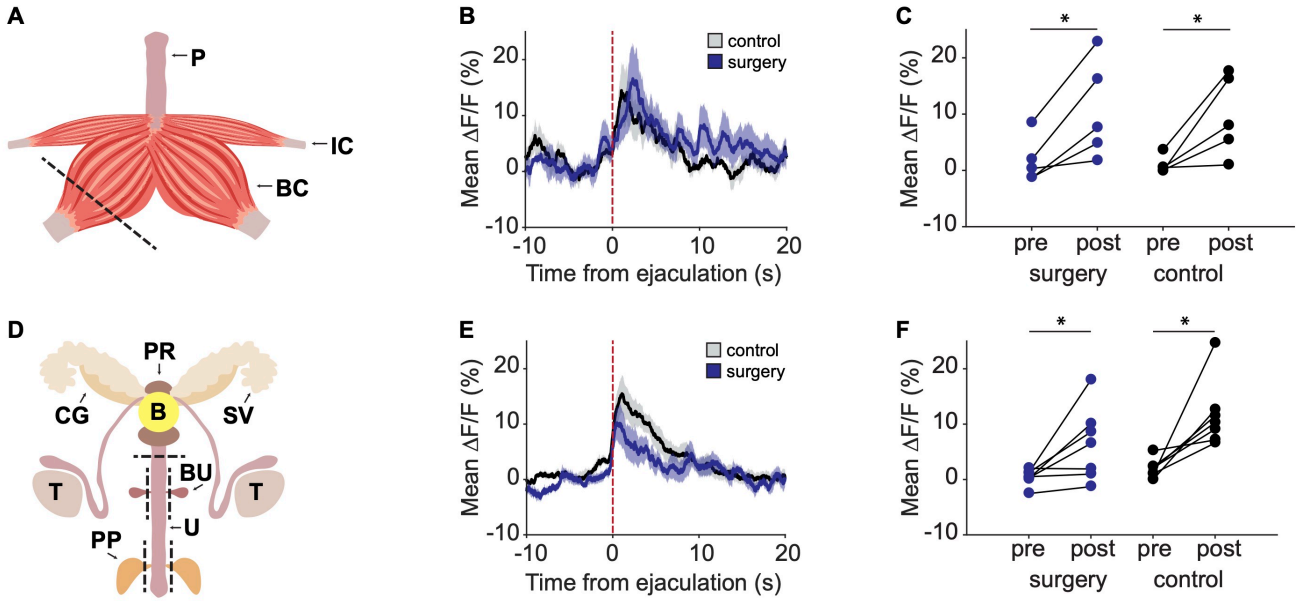

Figure 4. Separate blocking of penile cupping or ejaculatory fluid/copulatory plug expulsion does not disrupt female DRN 5-HT neural activity at ejaculation

(A) Surgical strategy to block penile cupping (cup males). The male right bulbocavernosus muscle located at the base of the penis was transected. BC = bulbocavernosus muscle, P = penis, IC = ischiocavernosus muscle.

(B) Mean DRN 5-HT ΔF/F aligned to ejaculation in females (n = 5) mated with cup males (blue) and control males (black). Shaded area indicates SEM.

(C) Mean DRN 5-HT ΔF/F in a 5 s window before and after ejaculation in females (n = 5) mated with cup males (blue) and control males (black) (paired t-test, *$p < 0.05$; data normally distributed, D'Agostino-Pearson, $p = 0.08$).

(D) Surgical strategy to divert ejaculatory fluid/copulatory plug expulsion (fluid-plug males). The male urethra was transected caudal to the prostate glands, and the cranial urethral end was sutured to a stoma in the abdominal wall so that all fluids exited via the stoma. Bulbourethral and preputial accessory sex glands caudal to urethral transection were resected. B = bladder, T = testes, U = urethra, PR = prostate glands, BU = bulbourethral glands, PP = preputial glands, SV = seminal vesicles, CG = coagulating glands.

(E) Mean DRN 5-HT ΔF/F aligned to ejaculation in females (n = 7) mated with fluid-plug males (blue) and control males (black). Shaded area indicates SEM.

(F) Mean DRN 5-HT ΔF/F in a 5 s window before and after ejaculation in females (n = 7) mated with fluid-plug males (blue) and control males (black) (Wilcoxon signed rank test, *$p < 0.05$; data not normally distributed, D'Agostino-Pearson, **$p < 0.01$).

See also Figures S4 and S5.

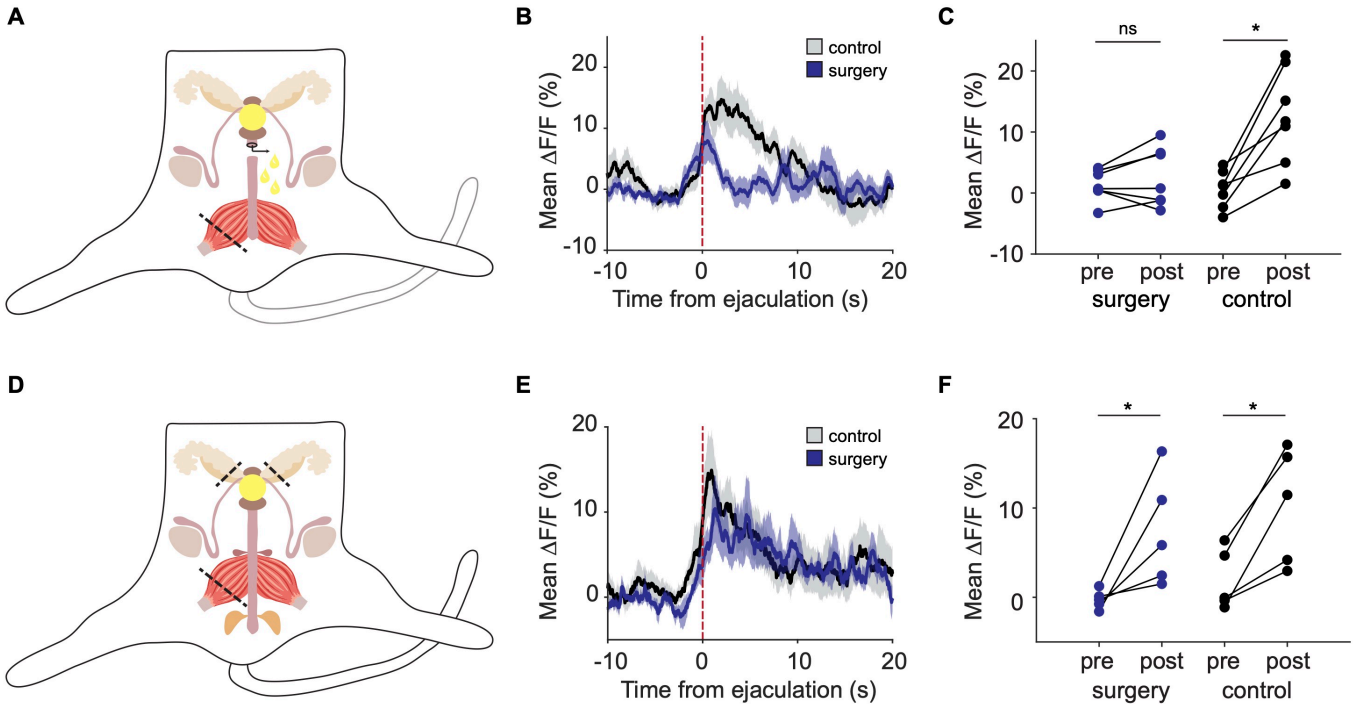

Figure 5. Simultaneous blocking of both penile cupping and ejaculatory fluid/copulatory plug expulsion disrupts female DRN 5-HT neural activity at ejaculation

(A) Surgical strategy to simultaneously block penile cupping and ejaculatory fluid/copulatory plug expulsion (cup/fluid-plug males). Procedures in Figures 4a and 4d were performed in the same males.

(B) Mean DRN 5-HT ΔF/F aligned to ejaculation in females (n = 7) mated with cup/fluid-plug males (blue) and control males (black). Shaded area indicates SEM.

(C) Mean DRN 5-HT ΔF/F 5 s before and after ejaculation in females (n = 7) mated with cup/fluid-plug males (blue) and control males (black) (Wilcoxon signed rank test, ns = non-significant, *$p < 0.05$; data not normally distributed, D'Agostino-Pearson, **$p < 0.01$).

(D) Surgical strategy to block penile cupping and copulatory plug expulsion, while preserving ejaculatory fluid expulsion (cup/plug males). The right bulbocavernosus muscle was transected as in Figure 4, and the seminal vesicles and coagulating glands were resected bilaterally.

(E) Mean DRN 5-HT ΔF/F aligned to ejaculation in females (n = 5) mated with cup/plug males (blue) and control males (black). Shaded area indicates SEM.

(F) Mean DRN 5-HT ΔF/F 5 s before and after ejaculation in females (n = 5) mated with cup/plug males (blue) and control males (black) (paired t-test, *$p < 0.05$; data normally distributed, D'Agostino-Pearson, $p = 0.17$).

See also Figure S6.

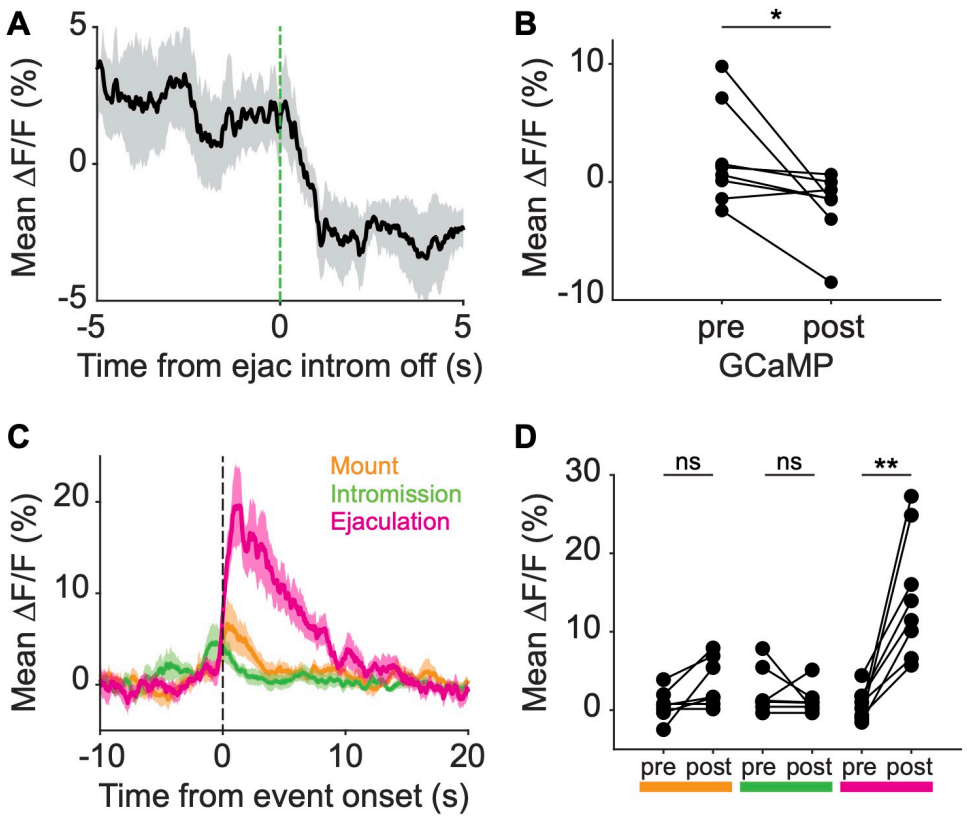

Figure S1. Female DRN 5-HT neural activity decreases at post-ejaculatory intromission offset, and DRN 5-HT neural activity is not elicited by pre-ejaculatory mounts and intromissions, related to Figure 2

(A) Mean DRN 5-HT ΔF/F aligned to post-ejaculatory intromission offset (n = 8 females). Shaded area indicates SEM.

(B) Mean DRN 5-HT ΔF/F 5 s before and after male post-ejaculatory intromission offset (n = 8 females) (paired t-test, *$p < 0.05$; data normally distributed, D'Agostino-Pearson, $p = 0.08$).

(C) Mean DRN 5-HT ΔF/F aligned to mount onset (orange, n = 7 females), intromission onset (green, n = 7 females), and ejaculation (magenta, n = 8 females). Shaded area indicates SEM.

(D) Mean DRN 5-HT ΔF/F 5 s before and after mount onset (n = 7 females), intromission onset (n = 7 females), and ejaculation (n = 8 females) (Wilcoxon signed ranked test, ns = non-significant, **$p < 0.01$; data not normally distributed, D'Agostino-Pearson, $p < 0.001$).

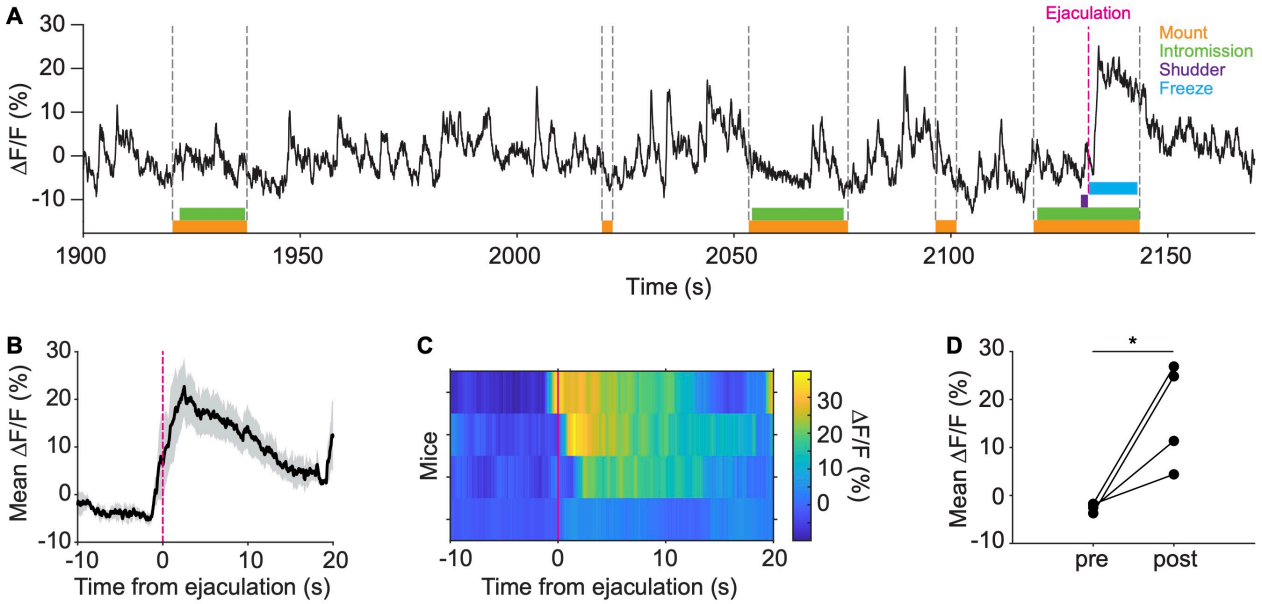

Figure S2. DRN 5-HT neural activity of naturally cycling females increases at ejaculation, related to Figure 2

(A) Example DRN 5-HT ΔF/F for a naturally cycling female during mating. Color boxes indicate scored male behaviors. Grey dotted lines indicate the onset and offset of male mounts. Magenta dotted line indicates the moment of ejaculation.

(B) Mean DRN 5-HT ΔF/F aligned to ejaculation for naturally cycling females (n = 4 females). Shaded area indicates SEM.

(C) Heatmap of DRN 5-HT ΔF/F (n = 4 females) aligned to ejaculation.

(D) Mean DRN 5-HT ΔF/F 5 s before and after ejaculation in naturally cycling females (n = 4 females) (paired t-test, *$p < 0.05$; data normally distributed, D'Agostino-Pearson, $p = 0.39$).

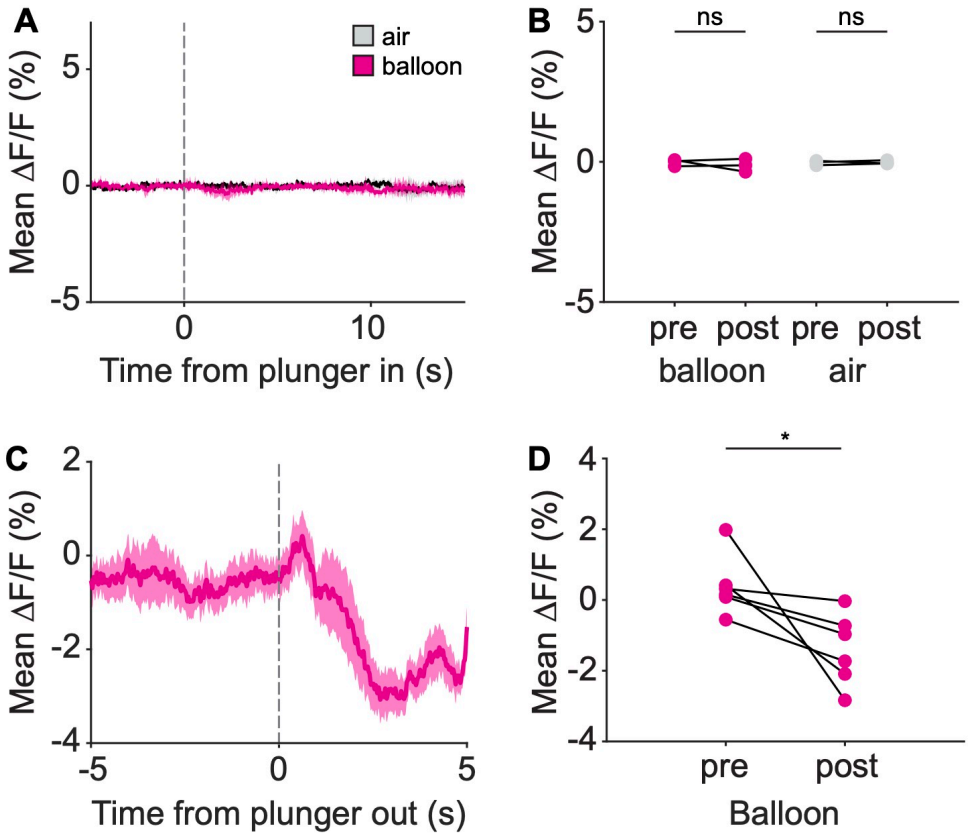

Figure S3. GFP ΔF/F during artificial mechanical intravaginal stimulation, and GCaMP ΔF/F during balloon deflation, related to Figure 3

(A) Mean DRN 5-HT GFP ΔF/F aligned to plunger in (n = 3 females). Shaded area indicates SEM.
(B) Mean DRN 5-HT GFP ΔF/F 5 s before and after plunger in for balloon (n = 3 females) and air (n = 3 females) (paired t-test, ns = non-significant; data normally distributed, D'Agostino-Pearson, p = 0.06).
(C) Example mean DRN 5-HT GCaMP ΔF/F aligned to balloon plunger out (n = 10 trials).
(D) Mean DRN 5-HT GCaMP ΔF/F 5 s before and after plunger out (n = 6 females) (paired t-test, *p < 0.05; data normally distributed, D'Agostino-Pearson, p = 0.88). Shaded area indicates SEM.

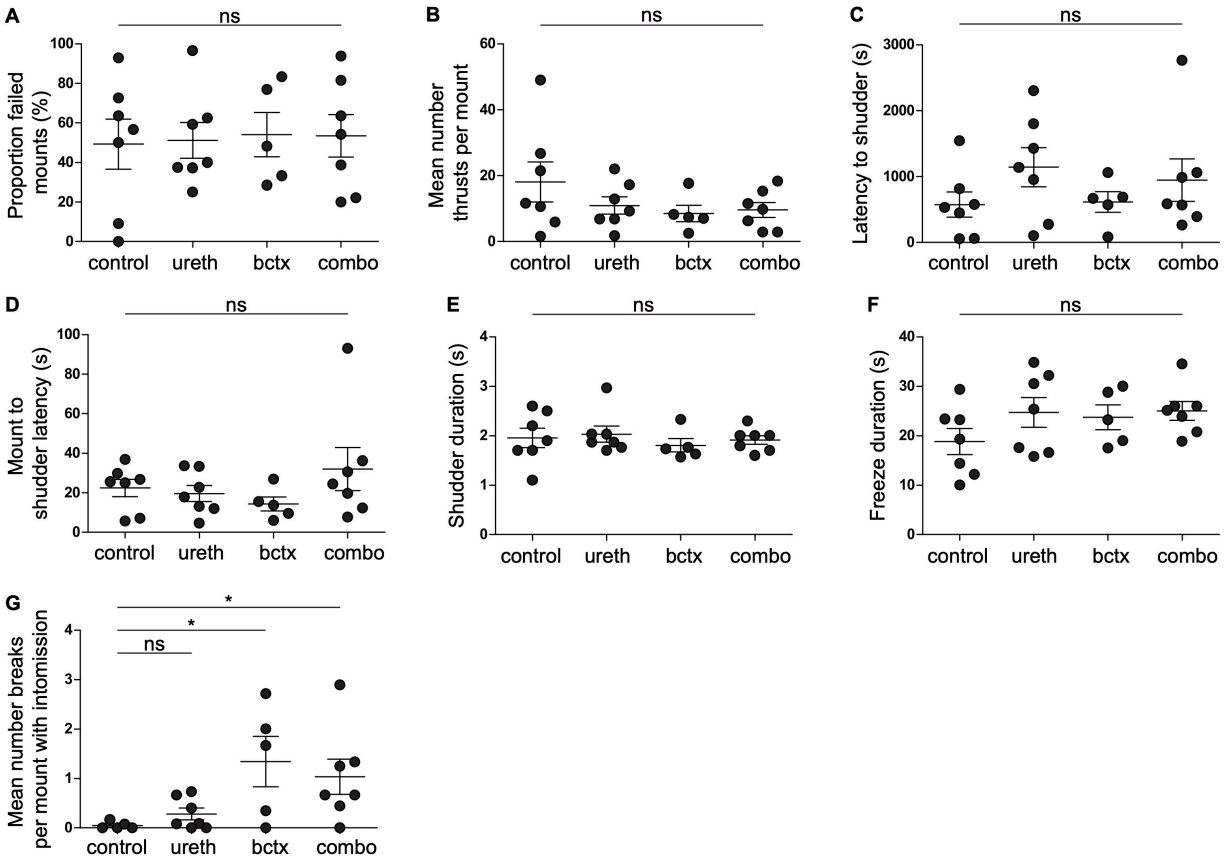

Figure S4. Surgically modified males exhibit normal mating behavior except for intromission breaks, related to Figures 4 and 5

(A) Percent mounts that did not result in intromission (one-way ANOVA, $p = 0.99$). Control ($n = 7$ males); ureth = urethrostomy to redirect ejaculatory fluids ($n = 7$ males); bctx = bulbocavernosus transection to disrupt penile cupping ($n = 5$ males); combo = both surgeries ($n = 7$ males). Each data point is a mating session.

(B) Mean number of intromission thrusts per mount (one-way ANOVA, $p = 0.32$).

(C) Time from start of session to shudder onset (one-way ANOVA, $p = 0.39$).

(D) Time from start of final ejaculatory mount to shudder onset (one-way ANOVA, $p = 0.37$).

(E) Duration of shudder (one-way ANOVA, $p = 0.80$).

(F) Duration of post-ejaculatory freeze (one-way ANOVA, $p = 0.29$).

(G) Mean number of intromission breaks per mount (one-way ANOVA, $*p < 0.05$). Only mounts with intromission were included.

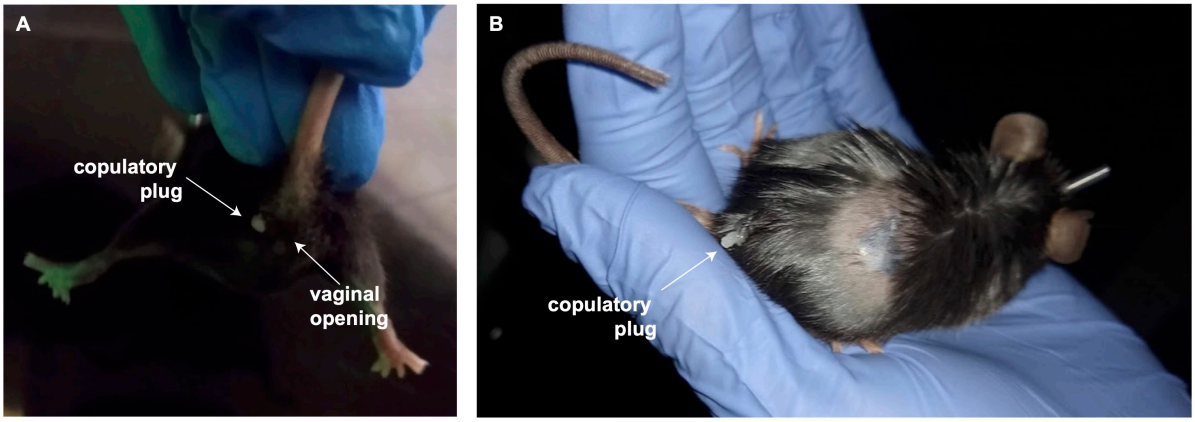

Figure S5. Copulatory plug found outside the vagina after mating sessions with urethrostomized males, related to Figure 4

(A) Copulatory plug found lateral to the vaginal opening in first example female immediately after mating session with urethrostomized male.

(B) Copulatory plug found lateral to the tail base in second example female immediately after mating session with urethrostomized male.

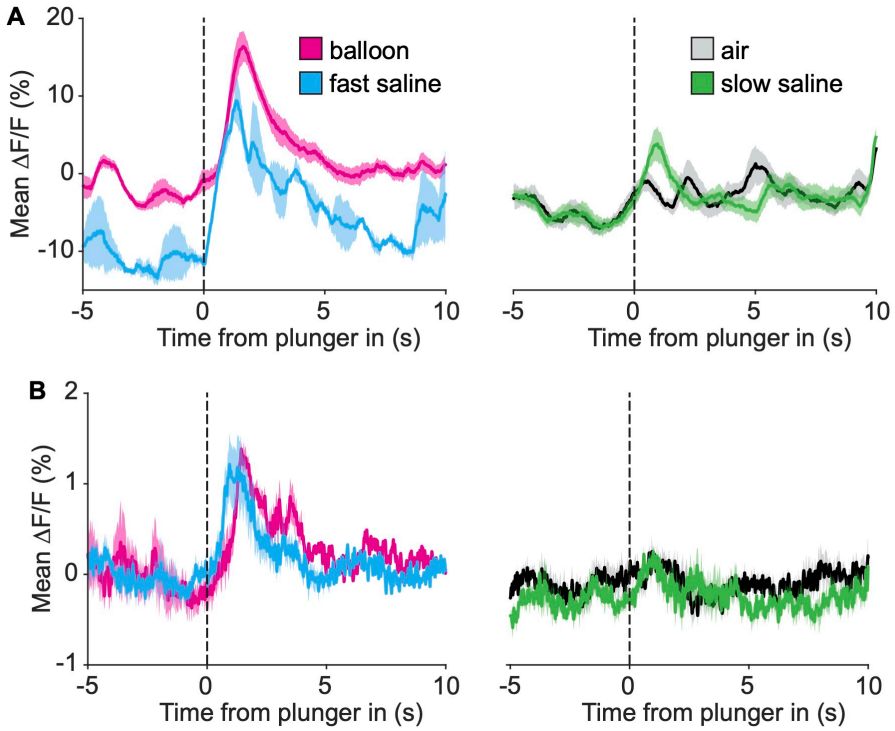

Figure S6. Fast intravaginal delivery of saline can elicit female DRN 5-HT neural activation comparable to balloon inflation, related to Figure 5

(A) Mean DRN 5-HT ΔF/F aligned to plunger in for balloon inflation and fast saline in first example female, left. Air and slow saline, right. Shaded area indicates SEM.

(B) Mean DRN 5-HT ΔF/F aligned to plunger in for balloon inflation and fast saline in second example female, left. Air and slow saline, right. Shaded area indicates SEM.

Video S1. Female DRN 5-HT neural activity increases at ejaculation, related to Figure 1.
DRN 5-HT neurons in SERT-Cre female show increase in GCaMP6 fluorescence at ejaculation, but not during pre-ejaculatory intromissions. Video frames are aligned to red line in female DRN 5-HT photometry trace. Color boxes indicate scored male behaviors.

Video S2. Bladder expression in example urethrostomized male, related to Figure 4.
Gentle pressure applied externally to the bladder of a urethrostomized male results in urine release via the artificial stoma instead of the penis.