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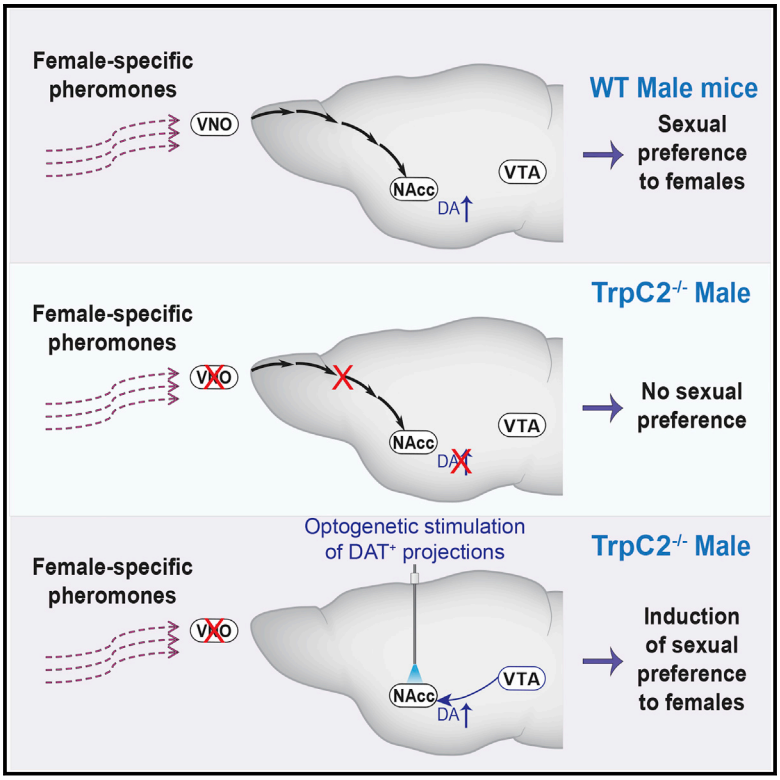
Nucleus Accumbens Dopamine Signaling Regulates Sexual Preference for Females in Male Mice

Graphical Abstract



Highlights

- NAcc DA release in response to female mice is necessary for sexual preference
- Olfactory preference for female pheromones requires NAcc D1R signaling
- Optogenetic activation of the mesolimbic DA pathway induces sexual preference

Authors

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In Brief

Beny-Shefer et al. find that female-specific pheromone signals detected by the vomeronasal organ induce dopamine release in the nucleus accumbens core (NAcc) of male mice. Optogenetic stimulation of the mesolimbic dopamine system in males during female exposure promotes sexual preference and behavior, whereas blocking NAcc dopamine receptor type 1 obstructs sexual preference.

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Nucleus Accumbens Dopamine Signaling Regulates Sexual Preference for Females in Male Mice

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SUMMARY

Sexual preference for the opposite sex is a fundamental behavior underlying reproductive success, but the neural mechanisms remain unclear. Here, we examined the role of dopamine signaling in the nucleus accumbens core (NAcc) in governing chemosensory-mediated preference for females in *TrpC2*$^{-/-}$ and wild-type male mice. *TrpC2*$^{-/-}$ males, deficient in VNO-mediated signaling, do not display mating or olfactory preference toward females. We found that, during social interaction with females, *TrpC2*$^{-/-}$ males do not show increased NAcc dopamine levels, observed in wild-type males. Optogenetic stimulation of VTA-NAcc dopaminergic neurons in *TrpC2*$^{-/-}$ males during exposure to a female promoted preference response to female pheromones and elevated copulatory behavior toward females. Additionally, we found that signaling through the D1 receptor in the NAcc is necessary for the olfactory preference for female-soiled bedding. Our study establishes a critical role for the mesolimbic dopaminergic system in governing pheromone-mediated responses and mate choice in male mice.

INTRODUCTION

Sexual preference is a complex trait presented in a sexually dimorphic manner, across animal species, including humans (Yang and Shah, 2014; Zilkha et al., 2016). Sexual stimuli are part of a few natural rewards necessary for reproduction (Beny and Kimchi, 2014; Hu, 2016). In rodents, sexual behavior is commonly divided into two distinct and progressive phases: the appetitive (precopulatory) and consummatory phases (Hashikawa et al., 2016). The precopulatory phase is mainly characterized by sexual preference for the opposite sex, manifested in the motivation to approach and engage in extensive olfactory investigation. These actions lead to the consummatory phase, which, in males, includes mounting, intromissions (i.e., pelvic thrusts), and ejaculation (Veening and Coolen, 2014).

Dopamine (DA) release in the nucleus accumbens (NAc) was suggested to be involved in mediating sexual motivation and behavior (Brom et al., 2014; Pfaus and Phillips, 1991). Specifically, female urine or female-soiled bedding, considered to be sources of pheromones (Choi et al., 2005; Isogai et al., 2011), have been shown to be intrinsically rewarding and strongly attractive to males (Ago et al., 2015; Beny and Kimchi, 2016; Malkesman et al., 2010). These pheromone sources were also shown to activate the mesolimbic DA system, even prior to expression of copulatory behavior (Kim et al., 2015; Malkesman et al., 2010; Robinson et al., 2001), and can serve as unconditioned natural reinforcers that induce associative learning (Beny and Kimchi, 2016; Griffiths and Brennan, 2015). Importantly, it was recently shown that activation of mesolimbic DA neurons increases same-sex social interaction in mice, mainly in females (Gunaydin et al., 2014). Mesolimbic DA has also been shown to regulate monogamous pair bonding in prairie voles (Aragona et al., 2003; Wang et al., 1999). However, the role of NAc DA in pheromone-mediated sexual preference is unclear.

Female pheromones play a key role in the regulation of innate reproductive responses in male mice (reviewed in Hashikawa et al., 2016; Stowers and Liberles, 2016). Pheromones are largely detected by sensory neurons in the vomeronasal organ (VNO) and main olfactory epithelium (Stowers and Kuo, 2015). These sensory neurons relay the information to higher-order brain areas including the accessory and main olfactory bulb, amygdala nuclei, hypothalamic nuclei, and piriform cortex (reviewed in Dulac and Wagner, 2006; Koyama, 2016; Liberles, 2014). Various studies have shown that these brain regions, implicated with processing of sexual pheromone signals, send direct and indirect projections to the mesolimbic dopaminergic pathway (Beier et al., 2015; Beny and Kimchi, 2014; Dulac and Wagner, 2006; Iyilikci et al., 2016; Liberles, 2014; Sánchez-Catalán et al., 2017; Watabe-Uchida et al., 2012).

The primary signal transduction pathway of VNO neurons is mediated by the transient receptor potential cation channel 2 (TrpC2). Studies have shown that genetic ablation of the *TrpC2* gene in male mice results in a lack of aggressive behavior, as well as in displays of sexual behavior toward both males and females indiscriminately (Kimchi et al., 2007; Leypold et al., 2002; Stowers et al., 2002). In addition, we have recently shown that *TrpC2*$^{-/-}$ males do not display an innate olfactory preference toward female chemosignals. However, using olfactory aversive conditioning we have demonstrated that *TrpC2*$^{-/-}$ males are actually able to identify and distinguish between male and female

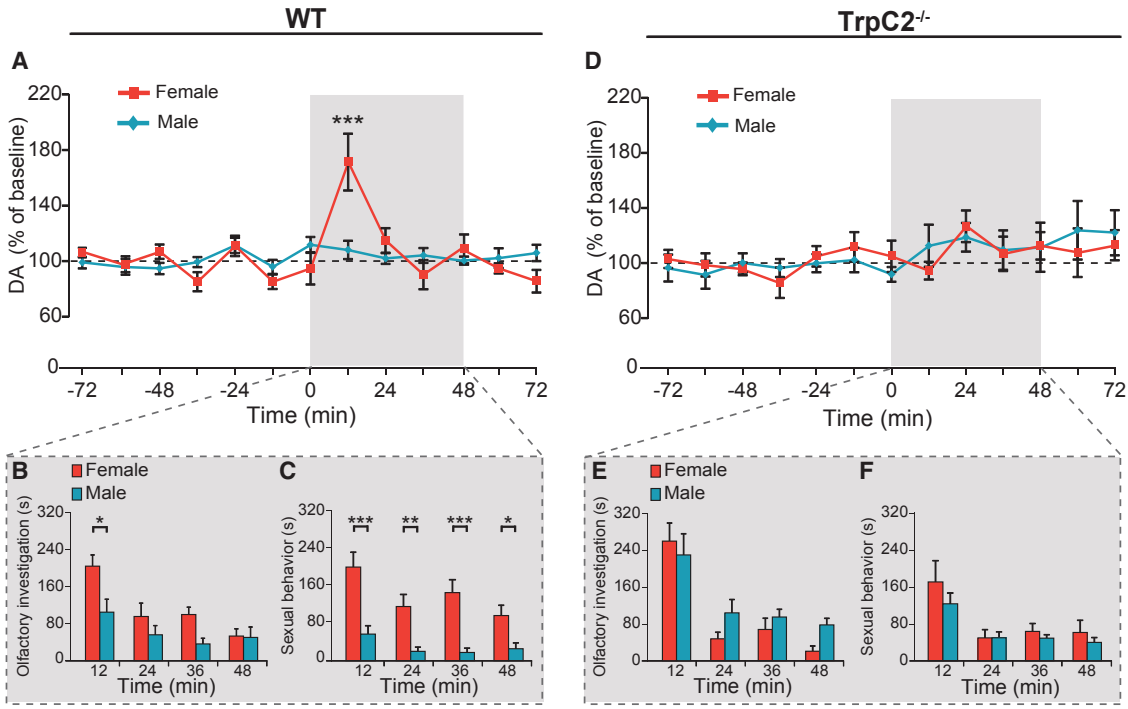

Figure 1. Female-Induced NAcc DA Release in Males Requires TrpC2 Signaling

(A and D) Extracellular dopamine (DA) levels in the NAcc of wild-type (WT, A) and *TrpC2*$^{-/-}$ (D) males during social interaction, as measured by *in vivo* microdialysis in freely behaving animals (WT: n_{female} = 6, n_{male} = 7; *TrpC2*$^{-/-}$: n_{female} = 6, n_{male} = 7). Gray boxes indicate the time of female or male intruder presence. Data are shown per 12-min time bin.

(B and E) Duration of olfactory investigation of female or male conspecific in 48 min of social interaction by WT (B) and *TrpC2*$^{-/-}$ (E) male mice.

(C and F) Duration of sexual behavior toward an unfamiliar female or male mouse in WT (C) and *TrpC2*$^{-/-}$ (F). Values are displayed as mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05.

conspecific pheromones (Beny and Kimchi, 2016). Thus, we hypothesize that the lack of sexual preference for females in *TrpC2*$^{-/-}$ males is a result of deficiencies in processing the rewarding properties of conspecific female signals.

In this study, we investigated the role of the ventral tegmental area (VTA)-NAc DA signaling in directly mediating the mating and olfactory preferences of male mice. Several studies specifically linked the core subregion of the NAc (NAcc) to assigning the reward value of stimuli in general (Chaudhri et al., 2010; Day et al., 2007; Lim et al., 2012; Saunders and Robinson, 2012; Shiflett and Balleine, 2010) and sexual stimuli, in particular (Dölen et al., 2013; Hedges et al., 2009; Robinson et al., 2001, 2002; Ubeda-Bañon et al., 2007). Hence, we focused on the NAcc in our experiments. First, we used *in vivo* microdialysis to measure natural DA secretion in the NAcc during exposure to females and males, in freely behaving wild-type (WT) and *TrpC2*$^{-/-}$ males. Then, using optogenetic manipulations to activate VTA DA projections to the NAcc of *TrpC2*$^{-/-}$ males we examined whether sexual preference toward female stimuli can be restored. Finally, we assessed the specific involvement of DA receptor type 1 (D1R) in the NAcc in encoding the rewarding value of female chemosignals. Our results show that female-specific DA signaling in the NAcc is absent in *TrpC2*$^{-/-}$ males. We also found that optogenetic activation of DA projections to the NAcc in *TrpC2*$^{-/-}$ males during exposure to a receptive female induced mating preference as well as olfactory preference toward female over male conspecifics. Finally, we found that NAcc D1R plays a key role in mediating the reinforcing properties of female stimuli to promote olfactory preference of males toward female chemosignals.

RESULTS

TrpC2$^{-/-}$ Males Do Not Display the Typical Increase in NAcc DA in the Presence of Females

In order to examine whether the lack of olfactory preference and mating preference of *TrpC2*$^{-/-}$ males might be related to a deficit in encoding the sexual incentive of female stimuli, we measured the changes in extracellular NAcc DA levels in *TrpC2*$^{-/-}$ and WT males during social interaction with either female or male intruders (Movie S1). In WT males, we revealed a significant "sample" × "stimuli" interaction ($F_{(12,132)}$ = 4.38, p < 0.001, two-way repeated-measures ANOVA), and *post hoc* analysis indicated a significant increase in DA levels of about 70% from baseline during the first 12 min of social interaction with a female intruder (n = 6, p < 0.001; Figure 1A). No changes in NAcc DA levels were detected during interaction with a male intruder among the WT group (n = 7, Figure 1A). In contrast, in *TrpC2*$^{-/-}$ males, NAcc DA levels remained unchanged during social interaction with either female or male conspecifics (n_{female} = 6; n_{male} = 7, $F_{(12,132)}$ = 0.39, p = 0.96; Figure 1D).

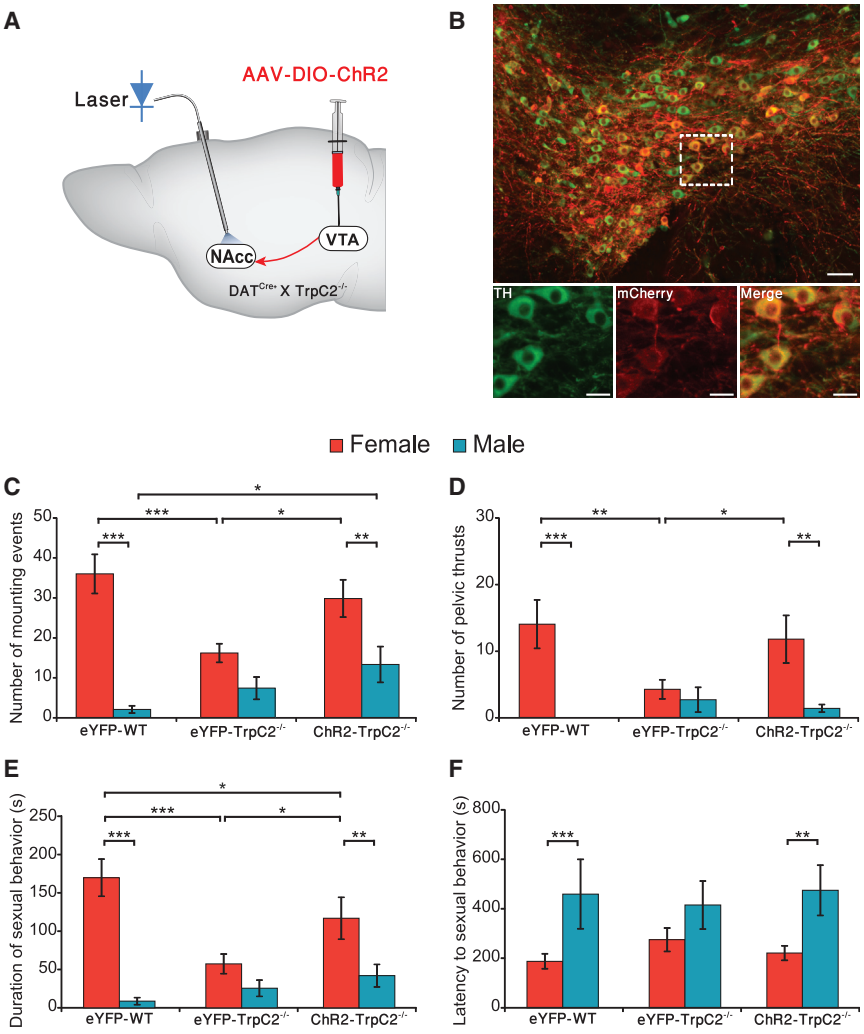

Figure 2. Optogenetic Activation of VTA DA Projections to the NAcc Resumes Sexual Preference toward a Female Intruder in *TrpC2*$^{-/-}$ Males

(A) Schematic illustration of the procedure: AAV-DIO-ChR2 was injected into the VTA of DAT^{Cre+} × *TrpC2*$^{-/-}$ mice, followed by implantation of an optic fiber above the NAcc region.

(B) Representative confocal images of the VTA showing cell-specific ChR2-mCherry (red) expression in TH-expressing neurons (green) in the VTA. Scale bar for upper image, 50 μm, and for lower images, 20 μm.

(C–F) Number of mounting events (C), number of pelvic thrust bouts (D), total duration of sexual behavior (E), and latency to present sexual behavior (F) for eYFP-WT (n = 9), eYFP-*TrpC2*$^{-/-}$ (n = 9), and ChR2-*TrpC2*$^{-/-}$ (n = 8) groups during the resident intruder assay. Values are displayed as mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05.

Behavior quantification during DA measurements showed that, in both WT and *TrpC2*$^{-/-}$ groups, the olfactory investigation of the conspecific intruders was highest during the first 12-min interval of the encounter with the conspecifics (two-way repeated-measures ANOVA; "time" main effect, WT: $F_{(3,30)}$ = 12.79, p < 0.001; *TrpC2*$^{-/-}$: $F_{(3,30)}$ = 33.28, p < 0.001; Figures 1B and 1E). WT males spent significantly more time olfactory investigating the female intruder compared to the male intruder (p < 0.05; Figure 1B), while *TrpC2*$^{-/-}$ males spent similar amounts of time olfactory investigating both sexes (p = 0.81; Figure 1E).

Moreover, during all social interaction intervals, WT males spent significantly more time displaying sexual behavior toward the female intruder ("stimuli" effect, $F_{(1,10)}$ = 32.42, p < 0.001; Figure 1C), while *TrpC2*$^{-/-}$ males spent similar amounts of time engaging in sexual behavior toward female and male intruders (stimuli effect, $F_{(1,10)}$ = 1.02, p = 0.33; Figure 1F). Also, throughout the social encounter, WT males presented robust aggressive behavior toward male intruders, but not female intruders (Kruskal-Wallis; $H_{(1,13)}$ = 9.95 3, p < 0.01; Figure S1A), while *TrpC2*$^{-/-}$ males did not present any aggression toward either of the intruders (Figure S1B).

To ensure the difference in the dopaminergic response is specific to sexual stimuli, we measured changes in NAcc DA levels induced by a non-social rewarding stimulus, i.e., consumption of a sucrose solution. In both WT and *TrpC2*$^{-/-}$ males, the levels of NAcc DA increased significantly during consumption of the reward (repeated-measures ANOVA "sample" effect for WT: $F_{(10,50)}$ = 4.1, p < 0.001, n = 6; for *TrpC2*$^{-/-}$: $F_{(10,40)}$ = 2.84, p < 0.01, n = 5; Figures S1C and S1D). The two genotypes exhibited no differences in baseline DA levels prior to presentation of the sucrose reward (p = 0.43) or in a measurement of NAc DA content (ng/mL) performed on a separate cohort of naive mice (Student's t test; $t_{(8)}$ = −1.16, p = 0.27, n = 5 per group; Figure S1E). Also, there were no differences in mRNA expression of DA receptors in the NAc ($t_{D1R(12)}$ = −0.68, p = 0.5, $t_{D2R(12)}$ = −0.56, p = 0.58, n = 7 per group; Figure S1F).

Activation of VTA-NAcc Dopaminergic Neurons in *TrpC2*$^{-/-}$ Males during Exposure to a Female Promotes Mating and Olfactory Preference

Next, we examined whether optogenetic activation of VTA-NAcc dopaminergic neurons paired with exposure to a receptive female will reinstate sexual preference for female chemosignals in *TrpC2*$^{-/-}$ males. We injected a Cre-dependent adeno-associated virus (AAV) carrying channelrhodopsin-2 into the VTA of DAT^{Cre+} × *TrpC2*$^{-/-}$ mice (ChR2-*TrpC2*$^{-/-}$; n = 8; Figures 2A and 2B). To selectively activate VTA-NAcc DA projections, optic fibers were implanted bilaterally into the NAcc (Figures 2A, S2B, and S2C). Control groups were DAT^{Cre+} × *TrpC2*$^{+/+}$ males (eYFP-WT, n = 9), as well as DAT^{Cre+} × *TrpC2*$^{-/-}$ males

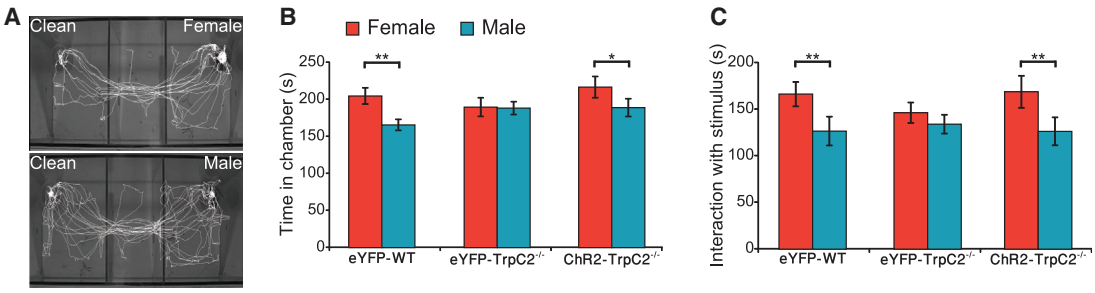

Figure 3. ChR2-*TrpC2*$^{-/-}$ Males Present Olfactory Preference for Female Chemosignals following VTA-NAcc Optogenetic Activation

(A) Representative automated path tracking (white line; Ethovision software) of an eYFP-WT male during the olfactory preference assay with female- (top) and male- (bottom) soiled bedding.

(B and C) Time spent in the chamber containing female- and male-soiled bedding (B) and time spent interacting with female- and male-soiled bedding (C) during the olfactory preference assay for the eYFP-WT (n = 9), eYFP-*TrpC2*$^{-/-}$ (n = 9), and ChR2-*TrpC2*$^{-/-}$ (n = 8) groups. Values are displayed as mean ± SEM. **p < 0.01, *p < 0.05.

(eYFP-*TrpC2*$^{-/-}$, n = 9), injected with an eYFP-expressing virus and implanted with optic fibers similarly to the ChR2-*TrpC2*$^{-/-}$ group. In order to induce an increase in DA signaling in the NAcc, during male-female encounters, we paired phasic photostimulation of DA neuronal projections with a 5-min exposure to a receptive female placed in a perforated box (Figure S2A). Stimulation was conducted for 3 consecutive days using bursts of blue light delivered at 20 Hz (473 nm, 8 bursts, 15 ms each) every 5 s throughout the 5-min encounter (Movie S2). Analysis of behavior during optogenetic activation showed no significant difference between the groups in either olfactory investigation of the female stimuli (p = 0.31, Figure S2D), time spent in proximity to the female (p = 0.79; Figure S2E), or locomotion parameters (p > 0.3 Figures S2F–S2H).

Following optogenetic activation of the VTA-NAcc DA projections, we assessed the olfactory preference and reproductive behavior in all 3 experimental groups (Figure S2A). In the resident-intruder assay, we introduced either a female or a male intruder into the home cage of each mouse and measured sexual behavior (Figures 2C–2F) and aggressive behavior (Figure S3A), as well as additional social parameters (grooming, olfactory investigation, avoidance, and non-social duration; Figures S3B–S3D). We found significant "group" × "sex" interactions in the number of mounting events ($F_{(2,23)}$ = 5.3, p < 0.05, two-way repeated-measures ANOVA), number of pelvic thrusts ($F_{(2,23)}$ = 4.06, p < 0.05), and in the total duration of sexual behavior ($F_{(2,23)}$ = 8.08, p < 0.01). Moreover, ChR2-*TrpC2*$^{-/-}$ males that received optogenetic stimulation exhibited significantly more mounting (p < 0.05; Figure 2C) and more pelvic thrusts (p < 0.05; Figure 2D) toward a female intruder and engaged more time in sexual behavior toward a female intruder (p < 0.05; Figure 2E) compared to eYFP-*TrpC2*$^{-/-}$ control males. Also, both eYFP-WT and ChR2-*TrpC2*$^{-/-}$ males presented significantly more mounting and more pelvic thrusts and spent more time engaging in sexual behavior with the female compared to the male intruder, and with shorter latencies (sex main effect, $F_{\text{mounting}(1,23)}$ = 36.06, p < 0.001; $F_{\text{pelvic thrusts}(1,23)}$ = 21.43, p < 0.001; $F_{\text{sexual duration}(1,23)}$ = 42.86, p < 0.001; $F_{\text{latency to sexual behavior}(1,23)}$ = 11.32, p < 0.01, *post hoc* for eYFP-WT and ChR2-*TrpC2*$^{-/-}$, p < 0.05, Figures 2C–2F), while eYFP-*TrpC2*$^{-/-}$ did not present any such difference (p > 0.1). None of the groups presented any aggression toward the female intruders. Significant differences were found between the groups in the number of aggressive events directed toward the male intruders (Kruskal-Wallis test; $H_{(2.26)}$ = 15.01, p < 0.001; Figure S3A). Moreover, eYFP-*TrpC2*$^{-/-}$ males presented significantly lower aggressive behavior toward the male intruders compared to eYFP-WT males (p < 0.01), consistent with previous reports (Leypold et al., 2002; Stowers et al., 2002). Notably, ChR2-*TrpC2*$^{-/-}$ males did not differ from eYFP-*TrpC2*$^{-/-}$ males in this respect and displayed significantly less male-directed aggression compared to eYFP-WT males (p < 0.01). Similarly to aggression, none of the mice displayed any grooming behavior toward the female intruders. No differences were noticed between the groups in their allogrooming toward the male intruders ($F_{(2,23)}$ = 1.29, p = 0.3; Figure S3B). Likewise, no differences were found in the duration of non-social behavior ($F_{(2,23)}$ = 1.29, p = 0.3; Figure S3D), or in avoidance from the intruders (p = 0.39). Finally, analysis of olfactory investigation of the female and male intruders revealed significant main effects of group ($F_{(2,23)}$ = 8.83, p < 0.01) and sex ($F_{(1,23)}$ = 43.28, p < 0.001; Figure S3C); however, no differences were found between the groups of ChR2-*TrpC2*$^{-/-}$ and eYFP-*TrpC2*$^{-/-}$ (p > 0.4).

In the olfactory preference assay, we used a sexual stimulus of female- or male-soiled bedding (Figures 3 and S2A; Movie S3). A two-way repeated-measures ANOVA showed a significant main effect for "stimuli" in both time spent in chamber ($F_{(1,28)}$ = 11.12, p < 0.01, Figure 3B) and in time interacting with the bedding ($F_{(1,28)}$ = 18.049, p < 0.001, Figure 3C). As expected, the eYFP-WT males (Figure 3A) presented a significant preference for the female chamber and for interacting with female bedding compared to the male (p < 0.01), unlike eYFP-*TrpC2*$^{-/-}$ males, which did not display any preference (p_{chamber} = 0.9, p_{bedding} = 0.34). Importantly, ChR2-*TrpC2*$^{-/-}$ males presented a significant preference for female versus male bedding in the olfactory preference assay (p_{chamber} < 0.05, p_{bedding} < 0.01; Figures 3B and 3C). Notably, these differences were not due to any effect of locomotion (Figure S3E).

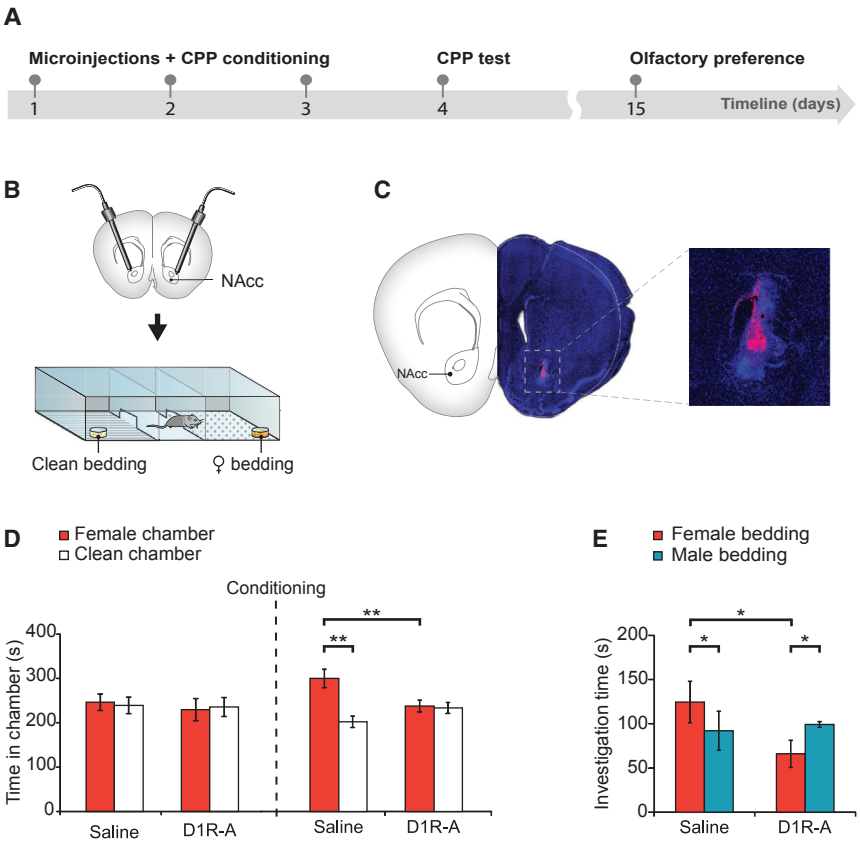

Figure 4. D1R-Mediated Signaling in the NAcc Is Essential for Conditioned Place Preference and Olfactory Preference for Female Chemosignals

(A) Timeline (days) for the conditioned place preference (CPP) protocol followed by the olfactory preference assay.

(B) Schematic illustration of the procedure.

(C) Coronal section of the mouse brain with a confocal image depicting the injection site into the NAcc (red).

(D) Time spent in the female-associated chamber versus neutral chamber before and after the conditioning, in the saline- and D1R-A-treated males (n_{saline} = 9, $n_{D1R\text{-}A}$ = 8).

(E) Time spent interacting with female and male bedding in the olfactory preference assay. Values are displayed as mean ± SEM. **p < 0.01, *p < 0.05. D1R-A, dopamine receptor type 1 antagonist; NAcc, nucleus accumbens core.

D1 Receptor in the NAcc Mediates the Reinforcing Properties of Female Chemosignals

D1R signaling in the NAc has previously been implicated in reward-seeking behavior (Chaudhri et al., 2010; Lim et al., 2012; Pina and Cunningham, 2014; Stuber et al., 2011; Young et al., 2014) and in the reinforcing effects of copulatory behaviors (Domínguez-Salazar et al., 2014; Pitchers et al., 2013), as well as in mediating same-sex social behavior (Gunaydin et al., 2014). We reasoned that D1R activation might have a role in encoding the rewarding properties of female pheromones, leading to sexual preference. To test this, we utilized the conditioned place preference (CPP) paradigm, where daily encounters with the reward in one specific location induce preference for the location itself through associative learning (Roberts et al., 2012; Tzschentke, 2007). During the conditioning session, WT males were bilaterally injected with either saline (n = 9) or a specific D1R antagonist (D1R-A, n = 8) into the NAcc, before being placed in the 3-chamber apparatus containing clean and female-soiled bedding in opposing chambers (Figures 4A, 4B, and S4D). Before conditioning, neither group presented any side preference for a specific chamber ($p > 0.05$, two-way repeated-measures ANOVA; Figure 4D). CPP was tested with clean bedding in both chambers, 24 hr following the last conditioning day. In the test phase, we revealed significant effects of side ($F_{(1,16)} = 5.48$, $p < 0.05$, two-way repeated-measures ANOVA) and side × group interaction for time spent in each chamber ($F_{(1,16)} = 6.41$, $p < 0.05$). *Post hoc* comparisons revealed that saline-treated males spent significantly more time in the chamber previously associated with female-soiled bedding ($p < 0.01$), while the D1R-A group spent similar amounts of time in both chambers ($p = 0.89$; Figure 4D). Importantly, during the conditioning phase, the D1R-A and saline-treated groups spent similar amounts of time olfactory investigating the female-soiled bedding (Figure S4A). Additionally, no differences were observed in locomotion between the two groups during the CPP test (Figures S4B and S4C). Following CPP, we employed the olfactory preference assay and discovered differences in the preference presented by mice from the saline and D1R-A group toward female versus male-soiled bedding (Figure 4E); a two-way repeated-measures ANOVA revealed a significant stimuli × group interaction ($F_{(1,15)} = 16.03$, $p < 0.01$). *Post hoc* comparisons showed that the D1R-A males spent significantly less time investigating female-soiled bedding than male-soiled bedding ($p < 0.05$), unlike the saline-treated males, which preferred to interact with the female-soiled bedding ($p < 0.05$; Figure 4E).

DISCUSSION

Pheromone-evoked sexual behavior and the preference of males to seek out and interact with female over male chemosignals are critically important determinants of reproductive success across mammalian species. However, the neural circuits governing this pattern of behavior are not understood. We have recently shown that *TrpC2*$^{-/-}$ male mice can successfully distinguish between male and female pheromones (Beny and Kimchi, 2016), suggesting that their lack of sexual preference might be a result of deficiencies in processing the intrinsic rewarding properties of conspecific female signals.

Numerous studies imply that DA plays a central role in the expression of male sexual behavior, both in terms of motivation

and of copulatory ability (Hull et al., 2004; Love, 2014; Veening and Coolen, 2014). Moreover, VTA dopaminergic neurons projecting to the NAc were shown to play a key role in the pre-copulatory phase and in sexual motivation (Brom et al., 2014; Fiorino et al., 1997; Pfaus and Phillips, 1991). Generally, an increase in extracellular DA in the NAc is thought to encode reward predications (i.e., represent an expectation of reward), enhance reinforcement learning, and signal the motivational salience of a stimulus (Cohen et al., 2012; Eshel et al., 2015; Schultz et al., 1997; Watabe-Uchida et al., 2017). This is postulated to promote goal-directed approach behavior in order to obtain the rewarding stimulus (Salamone and Correa, 2012). Previous studies in rodents show that DA is elevated in the NAc of males following exposure to a female stimulus and mainly during the initiation of the sexual act (Fiorino et al., 1997; Robinson et al., 2001, 2002; Wenkstern et al., 1993). Of the two subregions of the NAc (i.e., core and shell), previous studies indicate a more dominant role to the core in assigning the rewarding value to sexual and sexually associated stimuli (Damsma et al., 1992; Hedges et al., 2009; Kippin et al., 2003; Robinson et al., 2001, 2002; Ubeda-Bañon et al., 2007).

Much of the evidence linking DA systems to the control of mammalian sexual behavior comes from pharmacological analyses in rats and to a lesser extent also in mice. Here, we used male mice with a null mutation in the *TrpC2* gene, the primary signal transduction channel of VNO sensory neurons, together with both pharmacological and optogenetic manipulations to demonstrate that NAcc DA signaling is required for the control of pheromone-induced sexual preference of males to conspecific females. First, by using *in vivo* microdialysis we have identified a robust transient increase in DA levels in the NAcc of WT males while they encounter unfamiliar conspecific females, but not males. In contrast, we demonstrated that social encounters of *TrpC2*$^{-/-}$ males with either female or male conspecifics did not induce any NAcc DA elevation, even though these males presented intense olfactory investigation and mounting behavior toward both sexes. Thus, our findings indicate that, in sexually naive males, female pheromonal signals detected by the VNO are necessary to induce an increase in NAcc DA levels and suggest that *TrpC2*$^{-/-}$ males are deficient in their expectation for reward upon initial encounter with a receptive female. More specifically, our data suggest that *TrpC2*$^{-/-}$ males have similar expectations for reward from both males and females, which should drive them to approach and investigate both male and female stimuli indiscriminately. Next, we hypothesized that VNO-mediated DA secretion in the NAcc is crucial for assigning an incentive salience to the female stimulus, which is necessary for the preference to approach females over males. To test this hypothesis, we used optogenetic stimulation to induce DA secretion in the NAcc (Adamantidis et al., 2011; Eban-Rothschild et al., 2016; Stuber et al., 2010) in DAT^{Cre+} × *TrpC2*$^{-/-}$ males during exposure to a female stimulus. Our results demonstrate that optogenetic activation of DA projections to the NAcc, occurring while a *TrpC2*$^{-/-}$ male is exposed to a female (in a perforated cage; see Movie S2), can induce olfactory preference for female- over male-soiled bedding, to a similar level of control males. Moreover, optogenetic activation of dopaminergic terminals in the NAcc of *TrpC2*$^{-/-}$ males during exposure to a female stimulus promoted sexual behavior toward a receptive female, similar to that of eYFP-WT males. Importantly, activation of the VTA-NAcc dopaminergic pathway during exposure to a female did not influence the behavior toward male conspecifics, as ChR2-*TrpC2*$^{-/-}$ and eYFP-*TrpC2*$^{-/-}$ males presented similar levels of inter-male aggression and male-directed sexual behavior.

DA signaling through D1R-type expressing neurons in the NAc is thought to possess a crucial role in reward-seeking behavior (Lim et al., 2012; Stuber et al., 2011), in mediating the reinforcing effect of male ejaculation (Domínguez-Salazar et al., 2014; Pitchers et al., 2013) and in modulating reward-related same-sex social encounters (Gunaydin et al., 2014). Thus, we next tested whether DA signaling through D1R is necessary for assigning rewarding characteristics to female pheromones by using the CPP paradigm. This assay was previously employed to demonstrate that sexual behaviors and sex pheromones are powerful rewarding stimuli (reviewed in Beny and Kimchi, 2014; Pfaus et al., 2012).

We found that three consecutive sessions of a 10-min association with female-soiled bedding were sufficient to induce CPP to the chamber previously associated with the female stimuli, consistent with previous findings. Notably, we showed that administration of a D1R antagonist into the NAcc during the conditioning phase blocked the development of a preference for the chamber previously associated with female pheromones. Our findings are in line with other studies employing the CPP paradigm in male mice, which have demonstrated that D1R mediates the acquisition of place preference induced by both natural and artificial rewards (Domínguez-Salazar et al., 2014; Lim et al., 2012; Pina and Cunningham, 2014). Moreover, we have demonstrated that D1R blockage in the NAcc during the conditioning phase reduced the olfactory preference of males toward female chemosignals more than 10 days later. The reinforcing effects of sexual stimuli are mediated by long-lasting neuroplasticity changes in the NAc (Beloate et al., 2016; Pitchers et al., 2012; Staffend et al., 2014), which are dependent on D1R signaling and its down-stream effects (Pitchers et al., 2013; Staffend et al., 2014). Our results suggest that blocking D1R signaling during the initial interactions with female chemosignals prevented the occurrence of female-induced neuroplasticity in the NAcc, and this altered the olfactory preference of males days later. It should be emphasized, though, that D1R-A treatment altered olfactory preference only by effecting the attraction to the female bedding, without effecting male-directed behavior (unlike the common phenotype of *TrpC2*$^{-/-}$ males). Taken together, these findings suggest that signaling through D1R in the NAcc is necessary for the development of female-induced place preference as well as olfactory preference to female chemosignals.

Researchers have dissected reward processing into 3 separate components: ‘‘liking,’’ ‘‘wanting,’’ and ‘‘learning,’’ while DA has been shown to be involved in the wanting aspect of reward, assigning incentive salience to a stimulus and driving reward-seeking behavior (Berridge and Kringelbach, 2015; Berridge et al., 2009; Hu, 2016). Our results are consistent with studies demonstrating a role for DA in the wanting aspect of reward (Berridge, 2007) and postulate that activation of dopaminergic

neuronal projections in *TrpC2*$^{-/-}$ males led to attribution of an incentive salience to the associated female stimuli, which became the object of desire and motivationally wanted over other unassociated stimuli. Our data support the notion that DA is not involved in the liking aspect of reward (Berridge and Kringelbach, 2015), as both WT and *TrpC2*$^{-/-}$ males spent longer times interacting with the stimulus bedding (either male or female) over clean bedding during olfactory preference assay. Additionally, manipulating DA levels in the NAcc had no influence on the immediate consumption of female reward. These results are in line with recent findings using similar methodologies to describe the involvement of VTA-NAc DA neurons in promoting same-sex social interactions in WT females, through D1R (Gunaydin et al., 2014). Here, we show that VTA-NAc DA signaling depends on VNO-mediated pheromone inputs, to facilitate the regulation of olfactory preference toward females and pheromone-mediated mating behavior.

Moreover, the current findings can be integrated into the known circuitry of primary and high-order pheromone signaling. This circuitry includes several components of the pathways connecting the vomeronasal olfactory system to the mesolimbic DA system (reviewed in Beny and Kimchi, 2014; Chamero et al., 2012; Hashikawa et al., 2016). First, it has been broadly established that pheromone signals detected by the VNO largely activate several subregions of the amygdala, including the medial amygdala and basolateral amygdala (Bergan et al., 2014; Moncho-Bogani et al., 2005) (Cádiz-Moretti et al., 2013; Gutiérrez-Castellanos et al., 2014; Kang et al., 2009). These amygdaloid areas project to the medial hypothalamus (Kollack-Walker and Newman, 1997; Romero-Carbente et al., 2007; Simerly and Swanson, 1986), a relay crucial for sexual behavior (Baum and Everitt, 1992; Choi et al., 2005; Sokolowski and Corbin, 2012). Finally, the medial hypothalamus, and specifically the medial preoptic area (Beier et al., 2015; Watabe-Uchida et al., 2012) and paraventricular nucleus (Xiao et al., 2017), directly innervates the VTA, and these pathways have been shown to direct social and sexual behavior (Hung et al., 2017; Iyilikci et al., 2016; Mogenson et al., 1980; Stolzenberg and Numan, 2011). Our results indicate that, by signaling through D1R in the NAcc, intrinsic positive rewarding properties (incentive salience) are assigned to female chemosignals, and this promotes reward-seeking behavior and sexual preference for female. In the absence of intact VNO function, DA secretion does not occur, and sexual preference for females is not presented. Taken together, in this study, we managed to artificially ''bypass'' the circuitry leading from the VNO to the VTA-NAcc and showed that the intrinsic incentive salience of female pheromones can be modified by direct induction of female-specific activity in the mesolimbic DA pathway, independently of VNO-mediated signaling. These findings demonstrate the critical role of the dopaminergic system in the innate reinforcing properties of female stimuli and provide a circuit-level understanding of olfactory and mating preference for females in male mice.

EXPERIMENTAL PROCEDURES

Further details and an outline of resources used in this work can be found in Supplemental Experimental Procedures.

Animals

Adult sexually naive male mice aged 10–18 weeks at the beginning of experimental procedures were used in all the experiments. Mice were kept under standard pathogen-free laboratory conditions at the animal facility of the Weizmann Institute of Science, with food and water *ad libitum*.

The Institutional Animal Care and Use Committee of the Weizmann Institute of Science approved all experimental procedures.

Behavioral Procedures

Two weeks before the initiation of behavioral assays, mice were housed in single cages and were transferred to a reversed 12/12-hr light/dark cycle. All behavioral procedures were performed during the dark phase under dim red light.

For *in vivo* microdialysis, WT and *TrpC2*$^{-/-}$ male mice were implanted with a unilateral CMA7 guide cannula into the NAcc, and cerebrospinal fluid (CSF) was collected at 12-min intervals during an encounter with either a female or a male intruder. Extracellular DA levels were analyzed as diethyl-labeled derivatives with an ultra- performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) instrument.

In the optogenetics experiment, DAT-Cre × *TrpC2*$^{+/+}$ (WT) and DAT-Cre × *TrpC2*$^{-/-}$ male mice were injected with a ChR2-DIO-mCherry or a control EYFP AAV into the VTA, and optic fibers were implanted into the NAcc. Optogenetic activation was conducted during exposure to a receptive female, and the behavior toward male and female stimuli was assessed by an olfactory preference and a resident-intruder assay as previously detailed (Beny and Kimchi, 2016).

In the pharmacology experiment, WT male mice were implanted with a 24G guide cannula into the NAcc and received infusions of either a D1R antagonist or saline during the conditioning phase of the CPP procedure, where one chamber was associated with female-soiled bedding. Their preference for the female-associated chamber was then measured, as well as their later olfactory preference for female versus male chemosignals.

Statistical Analysis

All statistical analyses, unless stated otherwise, were analyzed by repeated-measures ANOVA, followed by Fisher's least significant difference (LSD) *post hoc* analysis. Locomotion parameters (i.e., mean velocity, distance moved, body rotation frequency, etc.) during optogenetic activation and during behavioral assays were analyzed using one-way ANOVA followed by Fisher's LSD *post hoc* analysis. Percentage of olfactory investigation during CPP conditioning and velocity and distance traveled during the CPP test were all analyzed using a Student's t test. Assumption for homogeneity of variances and normal distributions were presumed based on prior experience. Aggressive and grooming behavior were analyzed only with respect to male intruders, as none of the groups displayed these behaviors toward female intruders and were analyzed using the Kruskal-Wallis test, followed by multiple comparisons of mean ranks. The number of mice used is indicated by "*n*" throughout the text and figures. All statistical analyses were performed using the Statistica software (Statsoft, Tulsa, OK). Significant results were considered for $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three movies and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.062.

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AUTHOR CONTRIBUTIONS

Y.B.-S., N.Z., and T.K. wrote the manuscript. Y.B.-S. performed the optogenetic and pharmacology experiments with assistance from N.Z. and N.B. Y.L.-A. performed the microdialysis experiments with assistance from M.D., A.B., and I.R. T.K. and Y.B.-S. conceived and designed the experiments.

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