Published in final edited form as:

Behav Brain Res. 2014 November 1; 0: 243–247. doi:10.1016/j.bbr.2014.08.024.

6-HYDROXYDOPAMINE LESIONS OF THE ANTEROMEDIAL VENTRAL STRIATUM IMPAIR OPPOSITE-SEX URINARY ODOR PREFERENCE IN FEMALE MICE

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Abstract

Rodents rely upon their olfactory system to perceive opposite-sex pheromonal odors needed to motivate courtship behaviors. Volatile and nonvolatile components of pheromonal odors are processed by the main (MOS) and accessory olfactory system (AOS), respectively, with inputs converging in the medial amygdala (Me). The Me in turn targets the mesolimbic dopamine system, including the nucleus accumbens core (AcbC) and shell (AcbSh), the ventral pallidum (VP), medial olfactory tubercle (mOT) and ventral tegmental area (VTA). We hypothesized that pheromone-induced dopamine (DA) release in the ventral striatum (particularly in the mAcb and mOT) may mediate the normal preference of female mice to investigate male pheromones. We made bilateral 6-OHDA lesions of DA fibers innervating either the mAcb alone or the mAcb +mOT in female mice and tested estrous females' preference for opposite-sex urinary odors. We found that 6-OHDA lesions of either the mAcb alone or the mAcb+mOT significantly reduced the preference of sexually naïve female mice to investigate breeding male urinary odors (volatiles as well as volatiles+nonvolatiles) vs. estrous female urinary odors. These same neurotoxic lesions had no effect on subjects' ability to discriminate between these two urinary odors, on their locomotor activity, or on their preference for consuming sucrose. The integrity of the dopaminergic innervation of the mAcb and mOT is required for female mice to prefer investigating male pheromones.

Keywords

Dopamine; sociosexual behavior; olfactory; nucleus accumbens; olfactory tubercle

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The display of courtship behaviors in female rodents depends on the perception of chemical cues released by male conspecifics [1]. These salient chemosignals (often referred to as pheromones) are detected by the main (MOS) and accessory olfactory systems (AOS) and relayed to the medial amygdala (Me). The Me is critical for odor-guided courtship and reproductive behaviors in female rodents [2–4]. It sends axonal projections to several downstream targets including the bed nucleus of the stria terminalis (BNST), the medial preoptic area (MPA), ventromedial (VMHvm), and ventrolateral (VMHvl) divisions of the ventromedial hypothalamus, and to numerous ventral striatal targets, including the nucleus accumbens core (AcbC) and shell (AcbSh), ventral pallidum (VP), medial olfactory tubercle (mOT) and islands of Calleja (ICj) [5–7].

Female mice display a hardwired attraction to and preference for male urinary odors [8]. We hypothesized that the saliency attributed to these odors arises from pheromone-induced activation of the mesolimbic dopamine ‘reward’ system, yet little is known about the neural pathways via which pheromonal stimuli access the mesolimbic dopamine system. Male and female mice will form a conditioned place preference for opposite-sex urinary odors [9,10], suggesting that these odors are rewarding. Additionally, immediate early gene studies have shown that opposite-sex (but not same-sex) conspecific body odorants activate several segments of the brain’s reward circuitry including the mOT (unpublished observations), AcbC, AcbSh, and the ventral tegmental area (VTA) [8,11–12]. More recently, electrolytic lesions of the ventral striato-pallidum, a region that includes the mOT, disrupted opposite-sex odor preference in female mice [13], whereas 6-OHDA lesions of the dopamine (DA) fibers innervating the mAcb did not affect this preference [14]. These latter authors suggested that pheromonal reward is DA-independent, which conflicts with earlier studies using *in vivo* voltammetry and microdialysis techniques showing that exposing male rats to estrous female odors (both volatiles alone and volatiles+nonvolatiles) causes significant increases in DA release from the Acb [15,16].

We asked whether the modulatory influence of DA in the ventral striatum, specifically in the mAcb and mOT, is necessary for the normal preference of female mice for male urinary odors. We made 6-OHDA lesions of the DA fibers innervating either the mAcb alone or the mAcb+mOT and subsequently assessed females’ odor preference behavior compared to Sham-operated subjects. Because of prior studies indicating DA release in the mAcb in response to investigating opposite-sex pheromones, we created one group of subjects with 6-OHDA lesions confined to the mAcb. Given the recently discovered involvement of the mOT in pheromone reinforcement [13], we also created a group of subjects with 6-OHDA lesions centered on the mOT. In this group of subjects, leakage of the neurotoxin almost always spread to the mAcb. Hence we considered this group of subjects to be ‘mAcb+mOT-lesioned.’ It should be noted that mAcb+mOT Lesion subjects did not have larger DA lesions than mAcb Lesion subjects, but rather had lesions of the same size that were centered more ventrally, destroying DA fibers in the mAcb as well as the mOT.

Thirty-seven adult female Swiss Webster mice (Charles River Laboratories, Wilmington, MA, USA), were purchased at 6 weeks of age and maintained on a reversed 12:12h light:dark cycle with food and water available *ad libitum*. All procedures were approved by the Boston University Charles River Campus Institutional Animal Care and Use Committee.

Females were housed 4 per cage until 48 hours prior to the start of behavioral testing, whereupon they were housed individually. All behavioral testing was conducted under red light during the dark phase of the photoperiod. Five days after arrival in the animal colony, female subjects underwent bilateral ovariectomy under 2% isoflurane anesthesia and were allowed 1 week to recover. Subjects were given injections of the anti-inflammatory analgesic carprofen (5 mg/kg, s.c.) for two days after surgery and were implanted subcutaneously at the back of the neck with SILASTIC silicone capsules (inner diameter, 1.57mm; outer diameter, 2.41mm; length, 5mm) packed with estradiol (E₂; diluted 1:1 with cholesterol) at the time of ovariectomy. Urine used for odor preference and odor discrimination testing was collected from testes-intact male (n=8) and ovariectomized, estrogen and progesterone-primed female (n=8) donor mice using metabolic cages. Pooled urine was then aliquotted into 1 ml vials according to sex and stored at -20°C until use.

Mice were anesthetized under continuous 2% isoflurane vapor, and the head was secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Small holes were drilled bilaterally over each lesion site (coordinates: anterior-posterior: 5.3mm from interaural line, Medial-Lateral: 0.7mm from sagittal suture, Depth: 4.7mm from dura for Lesion groups, and 4.0mm for Shams) and 250nl of 20mM 6-OHDA hydrobromide in 0.01% ascorbic acid (Sigma, Saint Louis, MO, USA) was delivered using a 5µl syringe with a 30-gauge needle (Hamilton Company, Reno, NV, USA). To protect noradrenergic neurons from the neurotoxic effect of 6-OHDA, all subjects received an i.p. injection of desipramine (Sigma, Saint Louis, MO, USA; 25mg/kg) 30 min prior to surgery. Subjects received carprofen (5mg/kg, s.c.) for two days after surgery and were allowed 1 week to recover before commencing behavioral testing.

To ensure that destruction of DA terminals in the ventral striatum had no effect on locomotor activity, subjects' movements were tracked for 20 min in Plexiglas boxes (57x14x19 cm) using a digital video camera and Any-Maze software (Stoelting Co., Wood Dale, IL, USA). Two days later, subjects were brought into estrus with an injection of progesterone (500µg, s.c.). Four hr after injection subjects were given a 5-min odor preference test in their homecage in which testes-intact male and estrous female urinary volatile odors were presented simultaneously (see [2] for details). This procedure was repeated 4 days later with the location of urinary cues reversed, and direct nasal access to the urinary stimuli permitted. Two days following this test, subjects underwent a homecage habituation/dishabituation test to verify that subjects could discriminate between testes-intact male- and estrous female urinary volatiles [2]. Twenty-four hours later, animals were given a 48hr sucrose preference test [17] to determine whether ventral striatal DA depletion caused hedonic deficits leading to a decrement in sucrose consumption [18,19]. Briefly, subjects were given access to two bottles, one of which contained water only and the other 5% sucrose. Halfway through testing (at 24hr) the location of the bottles was switched to avoid a potential side bias in liquid consumption. At the conclusion of behavioral testing, subjects were sacrificed, and brains were removed and processed for histology as previously described [2]. The presence, location, and extent of 6-OHDA fiber lesions in the ventral striatum were visualized via the immunohistochemical detection of tyrosine hydroxylase (TH), (ImmunoStar, mouse antiTH, 1:2500) using standard procedures [5]. Except where noted, behavioral data were analyzed by 2-way repeated measures ANOVAs, with Urinary

Stimulus and Lesion Location as main effects, followed by Student-Newman-Keuls (SNK) *post hoc* tests where appropriate.

Lesion damage was assessed by determining the number of tissue sections containing the mAcb and mOT in which bilateral depletion of TH-immunoreactivity was observed. Boundaries of the mAcb and mOT, spanning over 1 mm in the rostral to caudal dimension, were defined using a stereotaxic atlas [20] as a guide. Subjects included in the lesion groups were those in which >70% of the sections through these structures bilaterally exhibited depletion of TH-immunoreactivity. Using these criteria, subjects were assigned to one of three groups: Sham (n=8), bilateral medial accumbens shell lesion (mAcb Lesion; n=7), or bilateral medial accumbens shell+medial olfactory tubercle lesion (mAcb+mOT Lesion; n=7). Mice with inaccurate DA lesion placement (n=3), spread of the neurotoxin into adjacent nuclei (n=5), or no observable lesion or small lesions (<50% of sections) on one or both sides (n=7) were not used.

Tissue examination revealed substantial bilateral reduction in TH-immunoreactive fibers specific to the mAcb (Fig. 1D–F) and to the mAcb+mOT (Fig. 1G–I). While DA depletion was specific to the mOT in more rostral sections (Fig. 1G), the neurotoxin often spread into the adjacent mAcb more caudally (Fig. 1H–I). This absence of DA terminals in 6-OHDA-lesioned subjects was not present in Sham-operated animals, which received vehicle (saline) injections (Fig. 1A–C). Several effects of lesions on odor investigation were noted. First, in tests with intact male vs. estrous female urinary volatiles, analysis of mean investigation time revealed a main effect of Urinary Stimulus ($F_{1,18}=19.9$; $p<0.001$), but not of Lesion Location ($F_{2,18}=2.22$; $p>0.05$), as well as a Urinary Stimulus $\times$ Lesion Location interaction ($F_{2,18}=5.44$; $p<0.02$; Fig. 2A). Similar results were obtained when subjects were allowed direct nasal contact with the stimulus: a main effect of Urinary Stimulus ($F_{1,19}=32.6$; $p<0.001$), but not of Lesion Location ($F_{2,19}=3.36$; $p>0.05$), and a Urinary Stimulus Lesion Location interaction ($F_{2,19}=11.52$; $p<0.001$). *Post hoc* analysis showed that mAcb Lesion and mAcb+mOT Lesion subjects investigated intact male urine less in comparison to Shams ($p<0.02$; Fig. 2B). A 1-way ANOVA comparing difference scores (time investigating male urine minus time spent investigating female urine) revealed an overall effect of Lesion Location in both the volatiles only test ($F_{2,21}=6.87$; $p<0.007$; Fig. 2C) and volatiles +nonvolatiles test ($F_{2,21}=11.52$; $p<0.001$; Fig. 2D). *Post hoc* tests revealed that subjects with mAcb or mAcb+mOT lesions lost their preference for male vs female urinary odors in comparison to Shams ($p<0.01$). In the odor discrimination test (Fig. 3A) all groups dishabituated from the final presentation of water to the first presentation of estrous female urine, as well as from the final presentation of estrous female urine to the first presentation of intact male urine (Student's *t*-tests; all $p<0.05$). Although no group differences were found in the amount of time investigating the first presentation of estrous female urine (1-way ANOVA, $F_{2,21}=1.82$; $p>0.18$), mAcb+mOT Lesion subjects showed a significant reduction in the first investigation of intact male urine compared to the other groups ($F_{2,21}=4.30$; $p<0.03$; Fig. 3A). Meanwhile, all groups strongly preferred a 5% sucrose solution to water (main effect of Liquid Type, $F_{1,18}=195.93$, $p<0.001$, but not Lesion Location, $F_{2,18}=0.84$, $p>0.44$; Fig. 3B). Lastly, there were no significant differences in mean

distance traveled across the 3 lesion groups in the open field locomotion test ($F_{2,19}=1.23$; $p>0.31$; Fig 3C).

Dopaminergic neurons innervating the ventral striatum have been implicated in numerous reward processes [21,22]. Here, we show that DA in the anteromedial ventral striatum establishes salience for opposite-sex pheromonal odors: 6-OHDA lesions of DA terminals in this region abolished the hardwired preference of female mice for breeding male over estrous female urinary odors (volatiles and volatiles+nonvolatiles), while leaving subjects' ability to discriminate between the two odors intact. Furthermore, DA lesions had no effect on locomotor/ambulatory activity or on preference for consuming sucrose over water, another hedonic behavior that requires DA in the VTA [18,19]. Females with mAcB or mAcB+mOT lesions showed similar reductions in their preference for male urinary odors, although there was one difference between these two lesioned groups: subjects with DA lesions that included the mOT displayed a significant decrease in investigation time for male urine in the odor discrimination test, even though they could still perceive the odor, as indexed by a robust dishabituation response. However, there was also a trend toward reduced investigation by mAcB+mOT Lesion subjects in the first dishabituation response to estrous female urine, suggesting that DA lesions that include the mOT may result in a generalized amotivation to investigate socially relevant odors. More work is needed to test this possibility.

The odor preference results are consistent with previous data showing DA release in the AcB during investigation of opposite sex odors [15,16], but differ from those reported by Martinez-Hernandez et al., 2012 [14], who found that 6-OHDA lesions of the mAcB had no effect on opposite-sex odor preference in female mice. There are several possible explanations for this discrepancy. Martinez-Hernandez and colleagues measured time spent in proximity to the odor stimulus in ovary-intact (non-hormone primed) female mice, rather than the time spent sniffing (actively investigating) the stimulus in estrous (hormone-primed) female mice, as in the current study. Thus other behaviors, such as grooming or marking in proximity to the odor, may have been registered in addition to investigating the pheromonal stimulus. Female subjects may have been at different stages of the estrous cycle during testing, which could have an effect on the level of arousal and/or motivation to investigate opposite-sex pheromones, since females display different odor-evoked behaviors relative to estrous cycle stage [23]. Additionally, the odors tested in the previous study were clean bedding (a familiar, non-biologically relevant odor) vs. male-soiled bedding (a novel, biologically relevant odor). Given this choice, it is not surprising that female mice would prefer the male odor due both to its novelty and its sexual relevance to the animal. Comparing differences in investigation between same-sex and opposite-sex urinary odors, by contrast, provides an assessment of females' sexual vs. social motivation because both odors are socially relevant to the animal, but only the opposite sex odor is sexually relevant.

Opposite sex urinary odors are natural, reinforcing stimuli. DA innervation of the anteromedial ventral striatum originates predominantly from cell bodies in the posterior VTA [24], and in estrous female mice we have observed a selective activation (increased FOS expression) of neurons in the posterior VTA that project to the mOT specifically in response to male (but not female) urinary volatiles (unpublished observations). Pheromonal

information reaches the Me via both the main (volatiles) and accessory (nonvolatiles) olfactory bulbs. This information is processed by the Me and subsequently directed to ventral striatal nuclei (and particularly to the mOT) via direct and indirect pathways, likely involving the BNST and VTA [5]. VTA-originating DA release from terminals in the mAcb and mOT in response to opposite-sex pheromones likely plays a role in the attribution of saliency to these odors, driving females to seek out male odors, without which the motivation to find a mate and reproduce would be compromised.

Acknowledgments

Supported by NIH grant DC008962 awarded to JAC.

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Highlights

- 6-OHDA lesions of the anteromedial ventral striatum impair urinary odor preference
- Subjects with 6-OHDA lesions could discriminate male vs. female odors
- 6-OHDA lesioned animals showed no locomotor or hedonic (sucrose intake) impairments
- Anteromedial ventral striatal dopamine modulates innate odor preference

Fig. 1.

Photomicrographs of coronal brain sections through three levels (from rostral to caudal) of the ventral striatum that were immunostained for tyrosine hydroxylase (TH) in representative subjects that received Sham (A–C) or 6-OHDA lesions of DA fibers innervating the medial accumbens (mAcb Lesion, D–F) or medial accumbens+medial olfactory tubercle (mAcb+mOT Lesion, G–I). Areas of significant depletion of DA fibers are outlined using dashed red lines. Numerical values above each column represent the approximate distance anterior to bregma for each coronal histological plate in that column. Acb, nucleus accumbens; OT, olfactory tubercle; aca, anterior commissure.

Fig. 2.

6-OHDA lesions of the DA fibers innervating the mAcb and medial mAcb+mOT disrupted the preference of ovariectomized, estradiol and progesterone-primed female mice for testes-intact male- over estrous female urinary odors presented simultaneously in the subjects' homecage. On the left, mean ($\pm$ SEM) times are shown for investigation of estrous female- and testes-intact male urinary volatiles (A) or volatiles+nonvolatiles (B). Asterisks indicate statistically significant differences ($p < 0.05$; 2-way ANOVA followed by SNK *post hoc* test). On the right, the mean ($\pm$ SEM) differences in time spent investigating intact male vs estrous female urinary volatiles (C) or volatiles+nonvolatiles (D) are shown. Bars with different letters are significantly different from each other ($p < 0.05$; 1-way ANOVA followed by SNK *post hoc* test). The number of subjects in each group is given in parentheses.

Fig. 3.

A, odor discrimination test. Estrous female vs. testes-intact male urinary volatiles were reliably discriminated by all groups (Student's *t*-test comparisons of mean investigation times of third water vs. first female urine, and third female urine vs. first male urine; *, +, #, $p < 0.001$). There was less investigation by the mAcb+mOT group during the first presentation of testes-intact male urine (** $p < 0.05$, 1-way ANOVA SNK *post hoc* test). The number of subjects in each group is given in parentheses. B, sucrose preference. All groups consumed more of the 5% sucrose solution compared to water alone in a two-choice 48hr preference test (* $p < 0.001$; 2-way ANOVA followed by SNK *post hoc* test). C, locomotion. Subjects with dopaminergic lesions of either the mAcb or mAcb+mOT showed no locomotor impairments: mean ($\pm$ SEM) distances traveled in a 20 min open field test did not differ between groups ($p > 0.3$; 1-way ANOVA).