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Gonadal hormones, but not sex, affect the acquisition and maintenance of a Go/No-Go odor discrimination task in mice

T. Kunkhyen^a, E. Perez^a, M. Bass^b, A. Coyne^a, M.J. Baum^b, and J.A. Cherry^{a,*}

^aDepartment of Psychological and Brain Sciences, Boston University, Boston, MA 02215, United States

^bDepartment of Biology, Boston University, Boston, MA 02215, United States

Abstract

In mice, olfaction is crucial for identifying social odors (pheromones) that signal the presence of suitable mates. We used a custom-built olfactometer and a thirst-motivated olfactory discrimination Go/No-Go (GNG) task to ask whether discrimination of volatile odors is sexually dimorphic and modulated in mice by adult sex hormones. Males and females gonadectomized prior to training failed to learn even the initial phase of the task, which involved nose poking at a port in one location obtaining water at an adjacent port. Gonadally intact males and females readily learned to seek water when male urine (S+) was present but not when female urine (S–) was present; they also learned the task when non-social odorants (amyl acetate, S+; peppermint, S–) were used. When mice were gonadectomized after training the ability of both sexes to discriminate urinary as well as non-social odors was reduced; however, after receiving testosterone propionate (castrated males) or estradiol benzoate (ovariectomized females), task performance was restored to pre-gonadectomy levels. There were no overall sex differences in performance across gonadal conditions in tests with either set of odors; however, ovariectomized females performed more poorly than castrated males in tests with non-social odors. Our results show that circulating sex hormones enable mice of both sexes to learn a GNG task and that gonadectomy reduces, while hormone replacement restores, their ability to discriminate between odors irrespective of the saliency of the odors used. Thus, gonadal hormones were essential for both learning and maintenance of task performance across sex and odor type.

Keywords

urinary odors; gonadectomy; olfactometer; amyl acetate; estradiol; testosterone

*To whom correspondence should be addressed at: 64 Cummington Mall, Boston, MA 02215, jcherry@bu.edu.

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1. Introduction

Numerous mammalian species ranging from rodents to primates rely on the main olfactory system to detect and discriminate between different volatile environmental odorants that provide critical information about the presence of food as well as dangerous, toxic chemicals in the environment. Volatile chemosignals from conspecifics that are detected by the main olfactory system also complement the action of pheromones detected by a parallel, vomeronasal-accessory olfactory system in signaling the sex and social status of conspecifics. Over the past several decades a large literature has examined the existence of sex differences and related effects of circulating sex hormones on aspects of olfactory function (Dorries, 1992; Kass et al., 2017). For example, early studies by Carr and co-workers (Carr et al., 1962) used a thirst-motivated operant task to determine that prepubertal castration of male rats failed to disrupt their later ability to detect diminishing concentrations of urinary volatiles from estrous female rats or to discriminate between urinary volatiles from estrous vs anestrous females (Carr and Caul, 1962). In another early study using rats in a thirst motivated operant task (Pietras and Moulton, 1974) the ability of females to detect several non-social volatile odorants (e.g., eugenol) was maximal when subjects were in vaginal estrus and was much diminished at vaginal diestrus or after ovariectomy. In a pioneering series of studies Dorries and co-workers extended these early findings to the domestic pig by using a thirst-motivated operant task to show that gonadally intact (GI) females were significantly more sensitive than males to diminishing concentrations of the putative volatile boar pheromone, androstenone (Dorries, 1991; Dorries et al., 1995). A similar sex difference (female > male) in the capacity to detect very low concentrations of male as well as female urinary volatiles was seen in gonadectomized (GDX) mice that were tested using a simple habituation/dishabituation paradigm (Baum and Keverne, 2002). A similar sex difference (female > male) was also seen in a food motivated operant sand digging task in which ovariectomized female mice were better able than castrated males to detect diminishing concentrations of male urinary volatiles when estradiol was administered to both sexes (Sorwell et al., 2008). In several instances, these animal results have been extended to humans. Thus prepubertal children of both sexes were able to detect the volatile human male axillary secretion, androstenone, whereas after puberty women were significantly more likely than men to detect this odorant (Dorries et al., 1989). More recently, Dalton et al (Dalton et al., 2002) reported that the ability of diminishing concentrations of several odorants (e.g., benzaldehyde) to be detected after repeated exposure trials was significantly greater in young adult women than in men. No such sex differences were seen in prepubertal children or when post-menopausal women were compared to men, suggesting that circulating ovarian sex hormones may augment odorant detection.

Nearly all of the above-mentioned animal and human studies concerning the effects of subjects' sex and/or circulating sex hormones on main olfactory system function have assessed odorant detection thresholds instead of subjects' capacity to discriminate different odorants. An exception is a study (Wesson et al., 2006) that used a hunger motivated, Go/No-Go (GNG) task to compare odorant discrimination among male and female wild type mice as well as in mice with a null mutation of the CYP19 gene (aromatase knockout;

ArKO), which encodes aromatase, the enzyme that synthesizes estradiol from testosterone. In that study all mice were GDX and treated daily with estradiol throughout the experiment. The main behavioral findings were that wild type and ArKO males as well as ArKO females were significantly better than wild type females in discriminating pairs (male vs estrous female; testes-intact vs castrated male) of urinary odors as well as a pair of non-social odorants (amyl acetate vs butyl acetate). This outcome points to a possible early developmental role of estradiol, acting in the female, to disrupt brain mechanisms controlling olfactory discrimination. However, these behavioral results are surprising given earlier studies (reviewed above) showing that the capacity for odorant detection is normally greater in female than in male rodents. Also, the study of Wesson et al. (Wesson et al., 2006) did not assess the potential role of circulating sex steroids in modulating olfactory discrimination. We conducted the present experiments to assess more thoroughly the possible activational role of sex hormones in both male and female mice, first in the acquisition of a GNG task for assessing olfactory discrimination capacity and second in maintaining accurate discrimination of socially relevant as well as non-social pairs of volatile odorants.

We first compared the ability of adult GI vs GDX male and female mice to acquire a thirst-motivated GNG odor discrimination task. We then trained GI males and females to discriminate between pairs of urinary volatiles (male vs estrous female) followed by a pair of non-social odorants (amyl acetate vs peppermint) using the same GNG procedure. We subsequently assessed the ability of these same mice to discriminate these two types of odorants several weeks after GDX and then again several weeks after replacement hormones were given (males received testosterone propionate; females received estradiol benzoate).

2. Methods

2.1. Subjects

Male (n = 19) and female (n = 20) CFW mice were purchased at 5-7 weeks of age from Charles River Laboratories (Wilmington, MA, USA). All the procedures involving animals were approved by the Boston University Institutional Animal Care Use Committee (IACUC).

Animals were group-housed (4 per cage) in same-sex cages under a 12:12 h reversed light: dark cycle (lights off at 9 am). All behavioral tests were carried out during the dark phase of the cycle. All mice were sexually naïve and had no direct contact with members of the opposite sex after arriving in our vivarium. Food was given *ad libitum* but water was restricted beginning 24 hr prior to day 1 of training. During the training period, the amount of water reward for each mouse was recorded every day. Most animals met all of their water needs from two daily 20-minute training sessions. The few animals that performed poorly during any particular test (i.e., received <50 water rewards per test session) were given access to water for 20 minutes at the end of the 2nd training session. Subjects were weighed daily to ensure that body weight did not fall more than 20% below the pre-water deprivation baseline.

2.2. Odors

We speculated that in mice of both sexes circulating gonadal hormones may more readily influence subjects' ability to discriminate pairs of salient social odors (such as urinary odors from conspecifics) than pairs of non-social odors. Accordingly, we compared subjects' ability to discriminate between male vs female urinary volatiles in one set of tests and between amyl acetate and peppermint (Sigma Aldrich, St. Louis, MO) in another series of tests. Urine was collected in metabolic chambers from 4 testes-intact male and 4 ovary-intact female mice that were not otherwise included in the study. No effort was made to link females' estrous cycle stage to the collection of urine. Urine collected over multiple days was pooled and stored at -80°C . Male urine and amyl acetate were arbitrarily chosen as the rewarded (S+) odors; female urine and peppermint were used as the non-rewarded (S-) odors. Odors were placed in 25-ml glass vials at a total volume of 10 ml. Urine was diluted 1:10 in deionized water, while amyl acetate and peppermint were diluted 1:10 in mineral oil. At the end of each day's testing session the odors were discarded and fresh odors were prepared on the next day of testing.

2.3. Go/No-Go Test Box

The GNG testing box was a square Plexiglas box ($26.5\text{L} \times 20\text{W} \times 30\text{H}$ cm) that contained two ports (1.5 cm in diameter 5 cm apart: a port in which odors were presented and an adjacent port that dispensed water). Both ports were equipped with infrared beams so that when a nose poke occurred the beam was interrupted. Odors were presented using a custom built olfactometer (Verhagen et al., 2007) that was controlled by an Arduino UNO microcontroller using Arduino software. The olfactometer was made of stainless steel manifolds, Teflon tubing and dedicated connector lines to individual 25-ml glass odor vials to avoid cross-contamination of odor streams. House air under pressure was filtered through activated charcoal and used to deliver odors from the odor vial headspace. Odors were sent to a manifold into which subjects could insert the snout (nose-poke) through a port to access odorized air in the chamber. When animals were not nose-poking, a vacuum removed odors from the manifold, preventing odors from exiting through the port. However, once odors were introduced in phase 3, nose-poking turned off the vacuum, allowing subjects to sample the odor. Nose removal initiated introduction of the odor for the next trial (S+ or S-) into the manifold, and at the same time the vacuum was restored until the next nose-poke.

2.4. Procedure

All mice underwent bilateral removal of the gonads under 2% isoflurane anesthesia. Animals were given analgesic on the day of surgery and for the next two subsequent days (carprofen, 5 mg/kg, s.c.). Mice in Group 1 (n=7 males; n=8 females) were gonadectomized prior to any behavioral testing and given 3 weeks to recover before GNG training began. Mice in Group 2 (n=8 males and n=8 females) were initially left gonadally intact through olfactory discrimination training and testing, first with urinary odors followed by non-social odors. Mice were then gonadectomized, and after 3 weeks of washout from gonadal hormones, mice were retested in the GNG task again using the same pairs of urinary followed by non-social odors. After these tests, males began to receive daily, subcutaneous injections of either testosterone propionate (TP; males; 3 mg/kg in sesame oil) or 17β -

estradiol benzoate (EB; females; 1 µg in sesame oil) (Martel and Baum, 2009; Wesson et al., 2006). Injections were given for 7 days prior to resuming GNG testing, first with the pairs of non-social odors followed by the pairs of urinary odors. Daily steroid injections continued until testing was completed. Animals were given 2-3 day rest periods (when water was presented *ad libitum*) after testing with one set of odors was completed and before training began with the next set of odors. Some mice in Group 2 (1 male and 2 females) did not complete GNG testing, so GNG analyses were based on data from 7 males and 6 females.

2.5. Training

Animals were given 4 phases of training (see Fig. 1A for a summary of the training sequence). Subjects were always given 10 minutes to habituate to the apparatus prior to any formal testing. All testing was done in a dark room, with a white light above the testing chamber being illuminated to indicate the start of every new trial or to indicate a correct response, and with the light being turned off to indicate a time-out or false response. Every mouse was required to successfully complete criteria for each training phase (described below) before advancing to the next phase.

Phase 1 (P1 in Fig. 1A) : (Training to locate and receive water at the reward port)—Mice were first habituated to the test box while only the water port was open. Each time the mouse nose poked at the water port, ~20 µl of water was dispensed concurrent with a 3 sec tone. Each session lasted for either 20 minutes or 200 nose-pokes, whichever occurred first. Mice were trained twice per day for 3 days until the criterion of >50 pokes per session was reached.

Phase 2 (P2 in Fig. 1A): (Training to nose poke at the odor port in order to receive a water reward at the adjacent port)—Phase 2.1: An ‘odor’ port was made accessible for nose-poking alongside the water port. (Only clean air was presented at this port during phases 2.1 and 2.2). To begin each trial, animals were first required to nose-poke at the odor port, which would trigger a 15-sec auditory tone during which animals were allowed to visit the water port to receive water. This duration (and the tone duration) was gradually shortened to 5 sec in later sessions. Failing to nose-poke at the water port within the allotted time resulted in a 5-sec timeout during which the overhead white light was turned off and nose-poking at either port would result in 5 sec added to the timeout.

Phase 2.2: Once the animals successfully learned to nose-poke at the odor port followed by the water port within 5 sec, animals were trained to increase the nose-poke duration at the odor port. Only mice in Group 2, which were gonadally intact, learned this task. Animals were required to keep their snout in the odor port for 1.1 seconds before visiting the water port. Mice were trained twice per day (morning and afternoon) until a criterion of >50 pokes per session for three consecutive sessions was reached.

Phase 2.3: the S+ odor (male urine or amyl acetate) was introduced. Subjects were required to maintain a nose-poke for at least 1.1 sec. After this time, subjects had a 5-sec window in which to visit the water port; an IR beam break resulted in water delivery. Mice were trained in this phase for 2-3 days, and the auditory tone was phased out at this stage.

Phase 3 (P3 in Fig. 1A): (Odor discrimination training)—The S- odor (female urine or peppermint) was gradually introduced. Initially the S+ odor was presented in 80% of trials and the S- in 20% of trials. When the S- odor was detected, mice were required to remove their nose from the odor port and wait for 5 sec before going back to the odor port to sample the next odor. Failure to wait for 5 sec by either nose-poking either the water or odor port resulted in a 5-sec timeout. Animals were trained on this phase for 6 days during which the frequency of S- was incrementally increased to 40%. Testing carried out while subjects were GI as well as after GDX involved presentation of the pair of urinary odors followed later by the pair of non-social odors. Subsequently, this sequence of training with these 2 odor types was reversed while GDX subjects received replacement injections of gonadal steroids.

Phase 4 (P4 in Fig. 1A): (Odor discrimination test)—S+ and S- odors were presented at an equal frequency to test the ability of subjects to accurately discriminate between odors. Phase 4 tests were conducted over 4 consecutive days to insure that mice would receive a sufficient amount of water each day. Mice received a total of 100 trials per day.

The sequence of test phases (from P1 through P4), with pairs of either urinary or non-social odors being presented in P3 and P4 tests, together with the hormonal status of the male and female subjects from the start to the end of the experiment are shown in Fig. 1B. Note that mice in Group 1 were gonadectomized 3 weeks prior to the start of any behavioral testing whereas mice in Group 2 were left gonadally intact during test phases P1, P2.1-2.3, as well as during the initial sequence of P3 and P4 tests (Fig. 1B). Mice in Group 2 were subsequently gonadectomized and then given the sequence of P3 and P4 tests first without and then with daily injections of gonadal hormones, as shown in Fig. 1B.

2.6. Statistical Analysis

The mean total number of water rewards received per day in phase 1 and phase 2.1 were examined separately using 3-way repeated measures ANOVAs with sex (male or female) and gonadal status (GI or GDX) as between subjects factors and test day as repeated measures factors. For subjects that advanced to phase 4 testing, performance was scored in daily trials as total percentage of correct trials, which included trials performed correctly when the S+ was present (nose-poking at the water port) in addition to the trials performed correctly when the S- was present (not nose-poking at the water port). To determine whether animals performed at different levels across the 4 days of phase 4 testing, one-way repeated measures ANOVAs were carried out separately for each group in tests with each of the odor sets. Because no consistent differences in performance across days were seen for any group, scores were averaged for the 4 days of phase 4 testing for each group. Two-way repeated measures ANOVAs were then carried out separately for social and for non-social odors to compare the total percent correct discriminations scored by each sex across all three hormone conditions (GI, GDX only, GDX + hormone replacement). Student-Newman-Keuls were used to make *post hoc* group comparisons, where appropriate. Finally, to gauge whether there might be differences in odor processing or decision-making between groups, nose-poke durations in the S- trials were compared across groups. Due to programming

failure, this analysis was available only for only 7 subjects (4 female and 3 male subjects), so sex differences could not be examined. For each of these subjects, average nose-poke durations were obtained for the S– trials given during phase 4 testing, keeping separate tallies for trials performed correctly vs incorrectly. These data were then analyzed in a 3-way repeated measures ANOVA, with odor (chemical, urine), trial result (correct, incorrect) and hormonal condition as factors. Statistical calculations were performed using SigmaPlot11.0 (Systat Software, San Jose, CA) or IBM SPSS Statistics (Armonk, NY). Effect size statistics are reported as partial eta squared (η^2p) for main effects and interactions, and Cohen's *d* for pairwise comparisons.

3. Results

During training phase 1, GI and GDX mice of both sexes equivalently learned to locate the water reward port and steadily increased their water intake over the 3 days of testing [day: $F(2,54)=32.9$, $p<0.0001$, $\eta^2p=0.55$; sex: $F(1,27)=0.59$, $p>0.05$] (Figure 2; panels A and B). There were no effects of GDX on water intake [$F(1,27)=0.70$, $p>0.05$], although there was a statistically significant gonadal status X test day interaction [$F(2,54)=3.59$, $p=0.03$, $\eta^2p=0.12$], which reflected greater initial intake by castrated male subjects on day 1 and less intake on day 3 compared to GI males. By contrast, during training phase 2.1 GDX subjects failed to learn that nose-poking at the odor port signaled the availability of water at the reward port (Figure 2; panels C and D), which was revealed by highly significant effects of gonadal status [$F(1,27)=14.0$, $p<0.001$, $\eta^2p=0.34$] and gonadal status X test day interaction [$F(6,162)=6.89$, $p<0.001$, $\eta^2p=0.20$]. There were no significant effects involving sex (main effects or interactions).

Male and female mice in Group 2, which were GI at the beginning of training, readily learned the odor discrimination task as S+ and S– odors were introduced in phase 2.3. In P4 tests (when S+ and S– trials were given with an equal frequency) there was a significant effect of subjects' hormonal condition on their odor discrimination ability. For urinary odor tests, the total percent of correct discriminations was significantly affected by subjects' hormonal status [$F(2,22)=15.3$, $p<0.0001$, $\eta^2p=0.58$] (Fig. 3 top panel), but not by their sex [$F(1,11)=0.97$, $p=0.35$], and there was no sex X hormone condition interaction [$F(2,22)=0.161$, $p=0.85$]. *Post hoc* comparisons of different hormone groups showed that mice performed at a similar high level when they were either GI or GDX and given hormone replacement (HR), whereas performance was significantly lower when they were tested after GDX in the absence of hormone injections (GDX<GI, $p<0.001$, $d=1.66$; GDX<GDX+HR, $p<0.001$, $d=1.43$).

A similar, though not identical, profile of results was obtained when subjects were required to discriminate between volatile non-social odors. An overall ANOVA revealed a significant effect of hormonal condition [$F(2,22)=6.7$, $p=0.005$, $\eta^2p=0.38$] (Figure 3, bottom panel), but not sex [$F(1,11)=2.16$, $p=0.17$]. However, there was a statistically significant sex X hormonal condition interaction [$F(2,22)=3.8$, $p=0.04$, $\eta^2p=0.26$], which reflected reduced correct responding of GDX females compared to GDX males in discriminating non-social odors. *Post hoc* comparisons between hormone groups indicated that mice performed significantly better when they were GI than after GDX in the absence of hormone

replacement ($p < 0.004$, $d = 1.27$), but there was no significant difference in the performance of GDX mice during hormone replacement vs no hormone replacement.

A 3-way ANOVA comparison of nose-poke durations in a subset of subjects (4 female and 3 male) revealed statistically significant main effects of odor [non-social > urine, $F(1,6) = 51.4$, $p = 0.0004$, $\eta^2 p = 0.90$], trial result [incorrect > correct, $F(1,6) = 12.8$, $p = 0.01$, $\eta^2 p = 0.68$] and hormone condition [GI > GDX = GDX+hormone replacement, $F(2,12) = 5.47$, $p = 0.02$, $\eta^2 p = 0.48$], as well as a hormone condition X odor interaction [$F(2,12) = 37.4$, $p < 0.0001$, $\eta^2 p = 0.86$] (Table 1). Thus, longer nose-poke durations occurred during tests with non-social as opposed to urinary odors, in incorrectly compared to correctly performed trials, and in GI subjects compared to the other two endocrine conditions.

4. Discussion

The observed failure of GDX mice of both sexes to learn the GNG task is consistent with three previous studies suggesting that gonadal hormones facilitate thirst- as well as hunger-motivated operant learning in which either a urinary odor or a non-social odorant served as a discriminative stimulus. Thus, in an early study (Doty and Ferguson-Segall, 1989) castrated male rats were less capable than testes-intact males in learning to use ethyl acetate odor as a discriminative stimulus for obtaining water reward in a GNG task. More recently, it was found that GDX mice of both sexes failed to learn a GNG task for liquid food reward (personal communication, D.W. Wesson, M. Keller, J. Bakker, and M.J. Baum) whereas nearly all GDX subjects injected daily with EB did acquire the task that involved a discrimination between either urinary or non-biological odorants (Wesson et al., 2006). Finally, Sorwell et al. (Sorwell et al., 2008) examined odor detection thresholds in GDX male and female mice using a 2-choice, food-motivated task that required subjects to dig in a sand-filled cup scented with male urine as the S+ while not digging in a water-scented S–cup (Sorwell et al., 2008). In the absence of hormone treatments, only 4 out of 12 castrated males learned the task to criterion whereas a majority (7/10), although not all, of ovariectomized females learned the task. Additional past studies in rodents that did not involve olfactory stimuli also showed that learning was impaired by GDX, particularly in tasks involving spatial and/or working memory. Thus in a hunger-motivated eight arm radial maze task, ovariectomized female rats that were treated with EB acquired the task more readily than ovariectomized females given no hormone (Luine et al., 1998). Likewise, castrated male rats required almost twice the number of sessions as GI males to learn a thirst-motivated T-maze task (Kritzer et al., 2001). Finally, male rats that were castrated prior to training displayed deficits in acquiring a Barnes maze (Locklear and Kritzer, 2014), a T-maze (Kritzer et al., 2001) and a radial arm maze task (Daniel et al., 2003). By contrast, castration did not affect the ability of male rats to learn a non-spatial Sidman avoidance task (Gibbs, 2005).

In the present work it is possible that GDX subjects lacked sufficient thirst motivation to optimally perform the GNG task. There were no differences between water-deprived GI and GDX mice in the number of trials performed during training phase 1. This indicates that both groups received an equivalent amount of water and suggests that GDX did not diminish subjects' motivation to work for water. Nevertheless, there is published evidence suggesting

that gonadal hormones may affect thirst. Decreased renal blood flow stimulates the release of renin by the kidney, which in turn stimulates drinking behavior via the production of angiotensin II. In one study (Ellison et al., 1989) castration reduced renal angiotensinogen mRNA expression in male rats and this deficit was reversed by treatment with testosterone. In another study (Jones and Curtis, 2009) ovariectomized female rats took longer than ovariectomized, EB-primed females to begin drinking water after an infusion of hypertonic NaCl solution (which motivates subjects to seek water). These studies indicate that GDX may reduce thirst. Thus, despite the equivalent water-seeking seen in phase 1 when water was received with every nose-poke, it is possible that a reduction in subjects' motivation to drink was present during GNG testing when subjects were required to perform a spatial task in order to GDX mice to learn the GNG task.

Once GI subjects had acquired the GNG task in the present study, gonadectomy significantly reduced, but did not eliminate, the ability of subjects of both sexes to discriminate urinary odors. Subsequent administration of TP to castrated male mice and EB to ovariectomized females restored subjects' urinary odor discrimination performance up to the levels previously seen when these same mice were gonadally intact. A similar profile of results was seen when subjects were required to discriminate non-social odorants, although with these stimuli the effect of GDX was more striking in female than in male mice. Even so, no statistically significant, overall sex differences in odor discrimination were seen in our mice, with or without gonads or following hormone replacement. Previously, Sorwell et al. (Sorwell et al., 2008) found that administering EB to GDX mice of both sexes improved the ability of females, but not of males, to detect progressively lower concentrations of male urinary volatiles. In the present study we found that administration of sex-appropriate hormones (TP to males; EB to females) restored subjects' odor discrimination performance to pre-gonadectomy levels. A complete comparison of the effects of replacing the same hormone (e.g., estradiol) in GDX subjects of both sexes will require additional study.

There were no consistent group differences in percent correct discriminations during GNG testing in S+ vs S- trials, so these trials were combined for the statistical analyses. However, a comparison of nose-poke durations in S- trials revealed some group differences. In these trials, subjects were only required to nose-poke long enough to sample the odor stream and then wait briefly—without seeking a water reward—for the next trial to begin. One difference found was that nose-poke durations were longer in S- trials performed incorrectly compared to correctly performed trials. Also, urinary odors were sampled for less time than non-social odors, perhaps suggesting that the urinary odors, despite their similarity, were more easily discriminated than the non-social odors. While there were also differences in nose-poke durations between subjects in different hormone conditions, this effect was driven mainly by a large disparity in the nose-poke durations of GI subjects sampling urinary compared to non-social odors (i.e., urinary < non-social). The explanation for this may be simply that mice were tested first while GI, so they were less experienced in the task than during testing under the other hormonal conditions.

The absence of any significant overall sex difference in odor discrimination in mice tested while under the influence of circulating sex hormones contrasts with a previous study (Wesson et al., 2006) in which GDX male performed a hunger-motivated, GNG odor

discrimination task significantly better than GDX female mice while both sexes continuously received EB. This sex difference (male > female) was observed when subjects discriminated either urinary or non-social odors. In both the present and previous (Wesson et al., 2006) studies the rewarded stimulus (S+) included volatiles emitted from testes-intact male mouse urine while the unrewarded stimulus (S-) included volatiles emitted from estrous female mouse urine. Likewise, in both studies the non-social S+ stimulus presented was amyl acetate. Thus differences in the specific odorant stimuli presented in the two studies probably cannot account for the observation of a sex difference in the previous but not the present study. Procedural differences between the two studies involved the use of food (Wesson et al., 2006) vs water (present study) deprivation to motivate subjects to perform the GNG task as well as slightly different spatial dimensions of the task. Thus subjects in the Wesson et al. study kept their snout in one location in order to sample the discriminant odor stimulus and the liquid food reward, whereas in the present study the discriminative odor stimulus was presented in one port and the water reward was presented in an adjacent port. The 2-port arrangement was used because of future plans to exploit this separation of the sites of odor stimulus presentation and reward delivery, and it was necessary to demonstrate that mice could perform the task. It is possible that the 2-port task may have been more difficult to perform, which could contribute to differences in the two studies. However, as already explained, mice of both sexes that lacked circulating gonadal hormones failed to acquire the GNG task, regardless of whether the discriminative odor stimuli and reward were presented in the same (Wesson et al., 2006) or different locations (present study). For this reason we think it unlikely that procedural differences alone account for the different outcomes. A more likely explanation is that the specific circulating sex steroids present in the two sexes at the time of testing contributed to the different results. In the present study the presence of circulating testosterone (either of testicular origin or which was injected after castration) in male subjects and of circulating estradiol (either of ovarian origin or which was injected after ovariectomy) in females may somehow have obscured a male bias in olfactory discrimination otherwise seen when subjects of both sexes received estradiol after gonadectomy (Wesson et al. 2006). Again, this issue can only be resolved with a future study.

Another study (Dillon et al., 2013) suggests that estradiol facilitates olfactory memory formation equivalently in mice of both sexes. Thus GDX mice of both sexes failed to remember a non-social odor for a 30-minute period after its last presentation in a habituation/dishabituation sequence whereas both GDX males and females showed evidence of odor recall when they received estradiol either systemically or directly into the main olfactory bulb (MOB) (Dillon et al., 2013). Both alpha and beta type nuclear estradiol receptors as well as the membrane bound GPR30 estradiol receptors are expressed in the MOB (Hazell et al., 2009; Shughrue et al., 1997). Dillon et al. (Dillon et al., 2013) suggested that olfactory memory formation is enhanced by estradiol acting directly on MOB neurons that express beta type estradiol receptor. In another study (Pompili et al., 2010) the performance of female rats in a radial arm maze task was significantly better during proestrus (when circulating estradiol levels are high) compared to other stages of the estrous cycle. Other studies point to bulbar NMDA receptors or neuronal activity in the MOB to

explain the formation and retention of simple olfactory memories (Chaudhury et al., 2010; McNamara et al., 2008).

The adverse effects of GDX on odorant discrimination in the present study could reflect the withdrawal of some peripheral steroid action. In a previous study (Sorwell et al., 2008) the stimulatory effect of EB treatment on the ability of ovariectomized female mice to detect progressively lower concentrations of male urinary volatiles was correlated with a reduction in females' sniff frequency following hormone priming. These authors argued that EB somehow facilitated the access of male urinary odors to olfactory receptors in the main olfactory epithelium (MOE), which was reflected in a reduction in sniffing (odor seeking behavior). Support for this view derives from another study (Caruso et al., 2004) showing that nasal patency was enhanced in postmenopausal women by treatment with ovarian steroids, with this effect being correlated with enhanced detection of several odorants. There is also evidence (Massaro et al., 1996) that estradiol stimulates the surface area of alveoli in the rat lung, leading to enhanced gas exchange and a reduction in respiratory rate. Further evidence that sex steroids may somehow modulate the activity of the primary sensory neurons in the MOE derives from a recent study (Kass et al., 2017) in which the ability of non-social odorants to activate MOB glomeruli was studied in transgenic mice that expressed the fluorescent exocytosis indicator synaptopHluorin under the control of the olfactory marker protein promoter. In this way the ability of odorants to activate MOE sensory neurons could be compared in the two sexes that were either GI or GDX and thus lacked circulating sex steroids. Gonadally intact females showed a more rapid response to the nasal application of volatile odorants over a broader range of glomeruli located on the dorsal surface of the MOB than was seen in GI males. Gonadectomy (in the absence of hormone replacement) made these odor-induced glomerular signals slower and less discriminable in female mice whereas they became faster and more discriminable in males. Kass et al. (Kass et al., 2017) argued that these results imply that a sexually dimorphic response of sensory neurons in the olfactory epithelium to steroid hormones modulates subjects' initial responses to odor stimuli. More research is required along these lines to determine whether administration of sex steroids after GDX restores the original sex difference in odor-induced MOB glomerular activity that was originally seen in GI subjects.

Previous habituation-dishabituation studies show that GDX male and female mice can detect and reliably discriminate between urinary volatiles from male vs female conspecifics (Baum and Keverne, 2002; Karlsson et al., 2015). In these studies the motivation of mice to investigate urinary odors relied on subjects' intrinsic interest in these social stimuli. These results imply that a lack of circulating sex hormones does not eliminate the ability of mice to discriminate between different urinary odors. The same conclusion can be drawn from the present results in which GDX subjects of both sexes retained the ability to distinguish between two different urinary as well as non-social odors in order to obtain a water reward, albeit less reliably than when they were given replacement sex steroids.

In conclusion, our results show that circulating sex steroids (ovarian hormones in females; testicular testosterone in males) are required in order for male and female mice to learn a thirst-motivated GNG task, even before specific odorants are paired with the occurrence and non-occurrence of a water reward. Once the GNG task was acquired, GDX and the resulting

withdrawal of circulating steroid hormones significantly reduced the accuracy of male and female mice in discriminating between pairs of both urinary and non-social odorants. The detrimental effect of GDX on the discrimination of non-social odors was more dramatic in female than in male mice. Administration of testosterone to castrated male and of estradiol to ovariectomized female mice reversed the disruptive effect of sex hormone deprivation on the discrimination of both kinds of odorants. In the absence of any significant overall sex differences in odorant discrimination, the major conclusion is that the concurrent, activational actions of circulating sex hormones, as opposed to hard-wired, organizational actions of sex hormones in the main olfactory system, are primarily responsible for maximizing olfactory discrimination capacity.

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Highlights

- Gonad-intact, but not gonadectomized mice learn a go/no-go odor discrimination task
- Male and female performance is diminished following gonadectomy
- In general, mice perform the task equivalently with social or non-social odors

Figure 1.

A. A schematic representation is shown of the Go/No-Go (GNG) test box and the different phases of the training procedure. A detailed description of the 4 different training phases (P1, P2.1-P2.3, P3, and P4) is given in section 2.5 (*Training*). Abbreviations: WP = water port, S+ = rewarded odor, S- = unrewarded odor. **B.** The behavioral testing timeline is shown for male and female subjects that were trained to discriminate between pairs of urinary (yellow boxes) as well as pairs of non-social (green boxes) odors. Mice in Group 1 had been gonadectomized prior to the start of testing and successfully completed only phase

1 of testing. Mice in Group 2 were initially tested while gonadally intact, then while gonadectomized (GDX) and finally while GDX and given hormone replacement (HR, gonadectomy + administration of testosterone propionate to males and estradiol benzoate to females). See section 2.5 for further procedural details.

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Figure 2.

The number of water rewards received by gonadectomized (GDX, $n=7$ male, $n=8$ female) and gonadally intact (GI, $n=8$ male, $n=8$ female) mice is shown during training phases 1 and 2.1. Top: GI and GDX male (A) and female (B) mice learned to obtain a water reward by nose-poking at the water port (3-way ANOVA, $p > 0.05$ for main effects of sex and gonadal status). In phase 2.1 GI males (C) and females (D) were able to learn that nose-poking at an adjacent port was required before water was dispensed at the water port, but GDX subjects failed to learn the task within the 7 days of testing: 3-way ANOVA main effect of gonadal status (GI > GDX, $p < 0.001$) but no main effect of sex ($p > 0.05$).

Figure 3.

Effects of gonadectomy and sex steroid replacement on the performance of male and female mice on a go/no go (GNG) odor discrimination task. Results are presented as the percentage of correct choices during S+ (rewarded) and S- (non-rewarded) trials over 4 consecutive test days during which male (dark bars) and female (light bars) subjects discriminated between pairs of urinary odors (top) and non-social odors (bottom). Mice were tested while gonadally intact (GI), gonadectomized (GDX), and GDX with hormone replacement (HR; testosterone propionate to males and estradiol benzoate to females). Top panel: ANOVA revealed a

significant main effect of hormone condition ($p < 0.0001$) for urinary odors. *Post hoc* comparisons of this effect revealed that GDX reduced correct responding relative to both GI and HR. Bottom panel: ANOVA revealed a significant main effect of hormone condition ($p < 0.005$) for non-social odors. *Post hoc* comparisons indicated that performance was significantly better in the GI than in the GDX condition ($p < 0.004$).

TABLE 1**Average Nose-poke Durations in S– Trials During Go/No-Go Testing**

Hormone Condition:		GI		GDX		GDX + HR	
Trial Result:		Correct	Incorrect	Correct	Incorrect	Correct	Incorrect
Urinary Odors		0.96 (0.051)	1.12 (0.101)	1.07 (0.075)	1.19 (0.082)	1.01 (0.045)	1.38 (0.113)
Non-social Odors		1.52 (0.91)	1.61 (0.063)	1.15 (0.051)	1.26 (0.070)	1.02 (0.027)	1.24 (0.089)

Data shown are the mean number of seconds (SEMs shown under each mean) subjects nose-poked during S- trials that were performed correctly vs incorrectly while either urinary odors or non-social odors were used. Nose-poke durations were averaged for each of 7 subjects (4 females, 3 males) in trials given while subjects were gonadally intact (GI), following gonadectomy (GDX), and during hormone replacement (GDX + HR; estradiol benzoate for females; testosterone for males). Three-way repeated measures ANOVA showed statistically significant main effects for hormone condition, trial result, and odor type, as well as a hormone condition X odor type interaction ($p < 0.05$ for all).