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Vasopressin receptor 1a, oxytocin receptor, and oxytocin knockout male and female mice display normal perceptual abilities toward non-social odorants

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Abstract

The genetic knockout of the vasopressin receptor 1a (A*vpr1a*), oxytocin receptor (*Oxtr*), or oxytocin peptide (*Oxt*) gene in mice have helped cement the causal relationship between oxytocin (OXT) and vasopressin (AVP) signaling and various social behaviors (e.g., social investigation, recognition, and communication, as well as territoriality and aggression). In mice, these social behaviors depend upon the olfactory system. Thus, it is critical to assess the olfactory capabilities of these knockout models to accurately interpret the observed differences in social behavior. Prior studies utilizing these transgenic mice have sought to test for baseline deficits in olfactory processing; predominantly through use of odor habituation/dishabituation tasks, buried food tests, or investigation assays using non-social odorants. While informative, these assays rely on the animal's intrinsic motivation and locomotor behavior to measure olfactory capabilities and thus, often yield mixed results. Instead, psychophysical analyses using operant conditioning procedures and flow-dilution olfactometry are ideally suited to precisely quantify olfactory perception. In the present study, we used these methods to assess the main olfactory capabilities of adult male and female *Avpr1a, Oxtr*, and *Oxt* transgenic mice to volatile non-social odorants. Our results indicate that homozygous and heterozygous knockout mice of all three strains have the same sensitivity and discrimination ability as their wild-type littermates. These data strongly support the hypothesis that the observed social deficits of these global knockout mice are not due to baseline deficits of their main olfactory system.

Introduction

Oxytocin (OXT) and vasopressin (AVP) are well-established neuromodulators of social behavior in a variety of vertebrate species including rodents and humans (Choe et al., 2015; Guastella et al., 2011; Insel, 2010; Mitre et al., 2018; Rigney et al., 2022; Wacker and Ludwig, 2012; Young et al., 2011). To investigate the contribution of the OXT and AVP systems to social behavior, transgenic mice with selectively reduced or abolished expression of the AVP receptor 1a (*Avpr1a*), oxytocin receptor (*Oxtr*), or oxytocin (*Oxt*) genes have been used. Studies inactivating these genes or blocking OXT or AVP signaling have yielded deficient maternal behaviors, (Gross et al., 1998; Nishimori et al., 1996; Young et al., 1996;

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Yu et al., 1996), decreased social communication in infancy via ultrasonic vocalizations (Takayanagi et al., 2005; Winslow et al., 2000), altered infant social learning (Hammock et al., 2013), deficient social recognition and investigation behavior (Bielsky et al., 2004; Bielsky et al., 2005; Ferguson et al., 2000; Lee et al., 2008; Oettl et al., 2016; Pobbe et al., 2012; Sala et al., 2011; Sala et al., 2013; Tobin et al., 2010; Takayanagi et al., 2005; Yao et al., 2017), and differences in aggression and anxiety-like behavior (Bielsky et al., 2004; Sala et al., 2013; Takayanagi et al., 2005; Wersinger et al., 2007; Winslow and Insel 2002); indicating that the OXT and AVP systems affect a wide variety of social processes throughout development and adulthood in mice.

Accurate assessments of olfactory capabilities are critical to interpret any deficits in mouse social behavior (Oettl and Kelsch, 2017; Silverman et al., 2010; Yang and Crawley, 2009). In fact, many behavioral tasks designed for mice, rely on olfactory cues (Bielsky and Young, 2004; Kavaliers et al., 2003; Oettl and Kelsch, 2017; Pobbe et al., 2012). For example, the behavioral assays used to assess social recognition in these transgenic mice operationalize social memory as olfactory investigation of a familiar versus an unfamiliar conspecific (Bielsky et al., 2004; Bielsky et al., 2005; Choe et al., 2015; Ferguson et al., 2000; Ferguson et al., 2001; Lee et al., 2008; Oettl et al., 2016; Oettl and Kelsch, 2017; Stoop, 2016; Tobin et al., 2010; Wacker and Ludwig, 2012). Thus, baseline deficits in olfactory capabilities could be interpreted as abnormal social behavior. This potential confound is even more important when assessing global knockout strains of either *Oxtr* or *Avpr1a*, as these neuropeptide receptors are prevalent within multiple olfactory brain regions (Bester-Meredith et al., 2015; Ferguson et al., 2001; Hammock and Levitt, 2013; Levasseur et al., 2004; Lukas et al., 2019; Mitre et al., 2018; Newmaster et al., 2020; Oettl et al., 2016; Oettl and Kelsch, 2017; Quintana et al., 2019; Sun et al., 2021; Tobin et al., 2010; Vaccari et al., 1998; Wacker et al., 2010; Wacker et al., 2011; Wacker and Ludwig, 2018; Yao et al., 2017), and the selective increase of these neuropeptides in the main olfactory bulb alters social interactions (Dluzen et al., 1998; Gheusi et al., 1994; Sun et al., 2021; Winslow and Camacho, 1995; Winslow and Insel, 2002). Thus, it is critically important to assess the impact of globally removing these genes on baseline olfactory functioning.

Prior studies investigating social behavior within *Avpr1a, Oxtr*, and *Oxt* knockout mice have sought to test for baseline deficits in olfactory processing but have yielded conflicting results. Wersinger et al. (2007) found that $Avpr1a^{-/-}$ mice exhibited a longer latency and were less likely to complete the hidden-cookie test compared to wild-type mice. This study also observed an inability of $Avpr1a^{-/-}$ mice to discriminate between a social (urine) and a non-social (almond extract) odor (Wersinger et al., 2007). While these results would seemingly suggest that the genetic deletion of *Avpr1a* influences baseline olfactory processing, another study also utilizing the odor habituation/dishabituation assay with anise extract found no differences in olfactory ability between $Avpr1a^{+/+}$and $Avpr1a^{-/-}$ male littermates (Bielsky et al., 2004). Similarly, studies testing the contribution of the oxytocin receptor to baseline olfactory function have also yielded conflicting results. Lee et al. (2008) found that $Oxtr^{-/-}$ mice displayed normal olfactory discrimination ability to a non-social odor (almond) as measured by the habituation/dishabituation assay. However, in another study both heterozygous ($Oxtr^{+/-}$) and homozygous ($Oxtr^{-/-}$) knockout mice displayed muted avoidance behavior during the initial encounter with a non-social odor (butyric acid;

Osada et al., 2018), suggesting abnormal olfactory processing. Interestingly, while wild-type and heterozygous mice habituated to this odorant, *Oxtr* $^{-/-}$ mice failed to habituate and instead avoided the location of this odorant for the following three days (Osada et al., 2018). Lastly, Ferguson et al. (2000) found that *Oxt* $^{-/-}$ mice did not differ in their ability to find a chocolate chip placed in the cage nor in their ability to discriminate between two non-social odors (lemon extract and vanilla extract) in the habituation/dishabituation task. However, these *Oxt* $^{-/-}$ mice did display reduced olfactory investigation towards these non-social odors compared to wild-type mice (Ferguson et al., 2000). While these odor investigation assays provide a quick measure of basic olfactory capabilities, they are not optimal to accurately quantify any potential deficits. Instead, psychophysical analyses using operant conditioning procedures and flow-dilution olfactometers are ideally suited to define relative olfactory capabilities in a consistent and robust manner (Bodyak and Slotnick, 1999).

The goal of the current study was to determine whether the genetic deletion of the vasopressin 1a receptor (*Avpr1a*), oxytocin receptor (*Oxtr*), or the oxytocin peptide (*Oxt*) influences non-social olfactory processing. To accomplish this goal, we used our established operant conditioning method to assess the sensitivity, discriminatory ability, and learning ability of mice towards non-social odorants. Our behavioral method has resulted in estimates of olfactory sensitivity that are internally consistent (across individuals and different cohorts), and able to identify olfactory detection deficits in transgenic mouse models (Dewan et al., 2018; Jennings et al., 2022; Williams and Dewan, 2020). Our results provide definitive evidence in support of the marked social deficits of the *Avpr1a*, *Oxtr*, and *Oxt* knockout mice, by serving to rule out hyposmia via dysfunction of the main olfactory system as a potential cause for the aberrant social behavior of these knockout mice. These results will hopefully provide additional clarity for researchers investigating the social deficits related to the oxytocin and vasopressin systems.

Materials and Methods

Animals

The generation of the oxytocin receptor knockout strain (*Oxtr* $^{tm1.1Knis}$), vasopressin receptor 1a (*Avpr1a* ^{tm1Sbhu}), and oxytocin peptide (*Oxt* ^{tm1Zuk}) were previously described (Hu et al., 2003; Nishimori et al., 1996; Takayanagi et al., 2005). Mice carrying these alleles were originally obtained from Larry Young at Emory University and subsequently backcrossed to and maintained on a C57BL/6J background using the Speed Congenics Service from Jackson Laboratories. All protocols and procedures were performed under the approval of the Animal Care and Use Committee at Florida State University, and in accordance with state and federal guidelines.

All mice (36 M; 36 F) were generated at the Florida State University animal facilities from heterozygous breeder pairs and weaned at post-natal day 21 to same-sex cages. Mice were maintained on 12 light/12 dark cycle and were tested during the dark phase of their circadian cycle. Sex assignment at weaning was determined by anogenital distance. Each strain of mice was tested as a cohort, with each cohort consisting of 24 mice: 8 age matched littermates (4 male and 4 female) for each genotype (wild-type, heterozygous, and homozygous) of a specific knockout strain (*Avpr1a*, *Oxtr*, or *Oxt*). Prior to surgery,

mice were housed in wire-top shoe box cages with woodchip bedding in the vivarium and provided ad libitum food (standard rodent chow, LabDiet, PMI Nutrition International, LLC, Brentwood, MO) and water. Mice (12-24 weeks old) were anesthetized with isoflurane (2-3%) in oxygen and secured in a stereotaxic head holder with non-rupture ear bars throughout the duration of the head bar surgery. Mice were administered buprenorphine (0.1 mg/kg) as an analgesic and lidocaine (2 mg/kg) as a local anesthetic. A custom titanium head bar (<1g) and 2 or 3 micro-screws were affixed to the skull and secured with dental cement. We utilize head-fixation to precisely standardize odor delivery across animals and facilitate behavioral monitoring (Dewan et al., 2018; Jennings et al., 2022; Williams and Dewan, 2020). After surgery, mice were individually housed, given three days to recover, then baseline weights were recorded. Mice were then put on a water restriction protocol for two weeks; wherein they received 1-2 mL of water daily depending on their body weight (maintained above 80% of baseline weight). Mice remained on this water restriction protocol throughout the duration of the experiments. One mouse (n = 1 out of 72) was excluded because it died prior to the completion of the experiment.

Odor Stimuli

For the initial behavioral training and odor thresholding experiment, hexyl acetate (CAS# 142-92-7) was used. This odorant was chosen because sensitivity measures of C57BL/6J mice are available (Jennings et al., 2022) and it presumably lacks social relevance. For the odor discrimination experiment, the ability of mice to discriminate between pentyl acetate (CAS# 628-63-7) and eight odorants was assessed. This odorant panel consisted of four esters and four structurally similar odorants of different chemical classes: pentyl acetate (CAS# 628-63-7), butyl acetate (CAS# 123-86-4), isoamyl acetate (CAS# 123-92-2), hexyl acetate (CAS# 142-92-7), pentanal (CAS# 110-62-3), 2-pentanone (CAS# 107-87-9), amylamine (CAS# 110-58-7), and 1-pentanol (CAS# 71-41-0). All odorants (Millipore-Sigma) were of the highest available purity (>98%) and were stored within a vented chemical storage cabinet (Air Science) under nitrogen to minimize odorant oxidation. All odorants were pipetted in a laboratory fume hood with filtered pipette tips and diluted in mineral oil (CAS# 8042-47-5). The actual vapor-phase concentration (ppm) of each odorant dissolved in mineral oil was determined according to method described in Jennings et al. (2022). For the sensitivity assay, the maximum liquid concentration of hexyl acetate tested was a 1:100 dilution in mineral oil with a 10-fold flow dilution. For the discrimination assay, each odorant was diluted in mineral oil to 10 ppm and flow diluted to 1 ppm. This concentration was chosen because it has previously been used to assess olfactory discriminatory ability in mice (Laska et al., 2008).

Stimulus Delivery

Diluted odorants and mineral oil solvent in disposable 40 mL amber glass vials were attached to the olfactometer manifolds and delivered via an eight-channel, flow-dilution olfactometer (Williams and Dewan, 2020). A manual needle valve between the two sets of manifolds allowed vials connected to different manifolds to be pressure matched. During the experiment, the manifolds switched between a pressure-balanced empty carrier (dummy) vial through normally open solenoid valves, and one of the seven odorant/solvent vials through normally closed solenoid valves. Two mass flow controllers (MFCs; Alicat

Scientific) regulated the flow of nitrogen gas (100 mL/min MFC) and clean air (900 mL/min MFC) through the manifolds, resulting in a 10-fold flow dilution. Nitrogen was used in the odorized line to minimize the oxidation of the odorant. A dual-synchronous three-way valve (final valve) connected the olfactometer and a purified air line (~1000 mL/min) to an exhaust line and the odor port. Care was taken to ensure that both lines were impedance matched to limit pressure spikes. During stimulus delivery, the final valve swapped the flow to the animal from clean air to the stimulus. The selected vial within the olfactometer was actuated 600ms prior to stimulus delivery to allow the odor concentration to reach equilibrium prior to delivery to the animal. The distance between the odor port and the animal's nose was standardized via micromanipulator.

At the end of each training or experimental day, the olfactometer (including the manifolds and all tubing) were flushed with acetone, followed by 70% isopropanol, then Nanopure water, and dried with pressurized clean air overnight. The vial caps and tubing were also cleaned with acetone, then isopropanol, followed by Nanopure water and dried overnight.

Operant Conditioning

After two weeks of water restriction, mice were trained to report the detection of an odorant in a Go/No-go task in a custom-built behavioral apparatus, described in detail previously (Dewan et al., 2018; Jennings et al., 2022; Williams and Dewan, 2020). Mice were placed in a custom holder with their nose 1 cm from the odor port. The odor port was mounted on a concave base that housed both the lick tube and a vacuum connection to remove excess odor. Licks were detected electronically via a lick circuit and water delivery was controlled by a solenoid valve connected to a small water reservoir. A python script (https://github.com/olfactorybehaviorlab/) sent trial parameters to an Arduino-based behavioral controller that coordinated the trial structure and monitored licks, actuated the olfactometer, and stored the response data.

Behavioral training consisted of two stages. During the first stage of training (lasting 4-7 days), mice learned to lick a metal tube to receive a water reward (1.8 – 2.2 μL) during the 2 second stimulus period (signaled by LED). The intertrial interval (ITI) was steadily increased from 2 seconds on the first day to 8 seconds over the course of several sessions. After the second training session, mice were exposed to clean air (1000 mL/min) from either the purified air line (during the intertrial interval) or the olfactometer (stimulus period). Each stage 1 training session lasted 30-45 minutes, resulted in 250-1000 trials depending on the ITI of a particular training day, and occurred only once per day.

In stage 2 of training (lasting 4-8 days), mice learned to report the detection of the odorant in a Go/No-go task by licking for a water reward during a Go stimulus trial (15 mL mineral oil blank) and refraining from licking during a No-go stimulus trial (15 mL of 1:100 liquid dilution of hexyl acetate). Correct responses during the 2 second stimulus period were immediately rewarded with water and/or short intertrial intervals (8-10 seconds). Incorrect responses were punished with longer intertrial intervals (13-18 seconds). ITI duration was randomized within these ranges to prevent mice from anticipating trial start times. Since over-motivation from increased thirst can mask true olfactory sensitivity (Berditchevskaia et al., 2016), the first ten trials of every session were Go trials and not included in any analyses.

Training sessions typically lasted 200-300 trials and were terminated if mice missed 3 Go trials in a row or reached 350 trials. Behavioral performance was determined by the number of correct responses (hits + correct rejections) divided by the total number of trials (excluding the initial Go trials). Upon reaching the completion criterion (two consecutive training sessions with ≥90% accuracy), mice were tested in the odor thresholding assay. Stage 2 training does not include a cheating check (see Odor Thresholding Assay below), so the maximal behavioral performance of a training session was 100% (compared to 85% for the thresholding experiment).

Odor Thresholding Assay

To accurately determine behavioral thresholds, mice were only tested at one concentration per day. This approach eliminated any masking/adaptation effects resulting from the contamination of the olfactometer by higher concentrations of the target odorant. Each session, the olfactometer was loaded with 3 blank solvent (Go) vials, 3 diluted odorant (No-go) vials, and a single blank solvent (No-go) vial. Each vial was replaced daily, and their positions were randomized. The first session used a 1:100 liquid dilution of hexyl acetate, whereas each subsequent session (7 total) presented the mice with a 10-fold decrease of the liquid dilution of the odorant. Again, mice typically performed 200–300 trials per session, and each session was terminated if the mice missed 3 Go trials in a row or when they reached 350 trials. Mice were tested in a random order each day.

The total flow rate (but not flow dilution factor) from the olfactometer was fluctuated (970, 980, 990, or 1000 mL/min) on a per-trial basis to limit mice from using slight variations in air pressure (likely associated with small differences in the resistivity of each solenoid/vial combination) to solve the task. The blank No-go vial (or “cheating check”) served to test whether the mice were using cues other than the presence or absence of the target odor to maximize performance. This blank No-go vial should be indistinguishable from other blank Go vials unless the animal is using non-odor cues to maximize behavioral performance. Thus, mice are “cheating” at this task if they can reject (i.e., not lick) the blank No-go vial at a frequency higher than the percentage of misses (i.e., not licking during a blank Go vial). If this occurred, the session was excluded from the analysis. Because this check is included in our thresholding analysis, the maximum performance a mouse can attain using only odor cues in this experiment is approximately 85% (in contrast to stage 2 training and discrimination in which mice can achieve 100% behavioral performance). After the completion of all odor concentrations, the mouse’s ability to discriminate between vials using non-odor cues was again tested by loading the olfactometer with only blank vials. These data are included in each figure.

Odor Discrimination Assay

Upon the completion of the thresholding assay, mice were trained to discriminate 1ppm of pentyl acetate (Go stimulus) from a mineral oil blank (No-go stimulus). Training was terminated once mice reached criterion: ≥90% accuracy for two consecutive training days. For the discrimination assay, mice were tested with one odor-pair per day utilizing the same session criteria listed above. The olfactometer was loaded with 3 pentyl acetate (Go) vials and 3 (No-go) vials containing either one of the eight previously listed diluted odorants (see

Odor Stimuli) or the mineral oil solvent, depending on the session. To ensure mice were not maximizing their performance by utilizing non-odor cues, the ability of each mouse to discriminate between identical concentrations of pentyl acetate was also tested. These data are included in each figure. Our discrimination assay does not include an additional cheating check, so the maximal behavioral performance was 100%.

Data analysis

Data collection and analysis were performed blind to the animal's genotype. Behavioral performance data for each odor were fitted with a Hill function.

$$R = R_{min} + \frac{R_{max} - R_{min}}{\left[1 + \left(\frac{C_{1/2}}{C}\right)^{n}\right]}$$

where R is the behavioral accuracy, C is odor concentration, $C½$ is the concentration at half-maximal performance, and n is the Hill coefficient. We defined olfactory detection thresholds (ODTs) in the standard psychophysical manner as the concentration at which mice discriminate the odor from blank with 50% accuracy ($C½$), typically represented by the inflection point of the psychometric curve (for a more detailed description, see Harvey, 1986). ODTs were compared between cohorts using independent sample t-tests. The influence of genotype and sex was evaluated for odor thresholding and odor learning with two-way ANOVA analyses, while odor discrimination results were analyzed via repeated measures two-way ANOVA with multiple comparisons. To assess the similarity of these congenic strains to C57BL/6J mice, we compared the ODT and learning dataset from *Avpr1a*, *Oxtr*, and *Oxt* mice to a previously published dataset using C57BL/6J mice (Jennings et al., 2022). Learning criteria (trials to reach 90% behavioral accuracy and maintain ≥90% for the duration of learning) of each cohort was compared by genotype to C57BL/6J learning via one-way ANOVA (Prism GraphPad and SPSS).

Results

To measure olfactory detections thresholds (ODTs), we used a head-fixed Go/No-go operant conditioning assay (Figure 1a). The repeated actuation of a single odorant vial resulted in consistent odor pulses throughout a session (Figure 1b). The behavioral sensitivity to hexyl acetate of the wild-type littermates of each of the three transgenic mouse lines was not statistically different from the behavioral performance of C57BL/6J mice (Jennings et al., 2022, Figure 1c–e, Table 1). For the *Avpr1a* stain, the wild-type ($Avpr1a^{+/+}$) ODT did not statistically differ from the published C57BL/6J ODT ($t(14) = 2.18$, $p = 0.34$, $d = 1.09$; Figure 1c, Table 1). For the *Oxtr* stain, the wild-type ($Oxtr^{+/+}$) ODT also did not statistically differ from the published C57BL/6J ODT ($t(14) = -0.29$, $p = 0.78$, $d = -0.15$; Figure 1d, Table 1). Additionally, the *Oxt* stain wild-type ($Oxt^{+/+}$) ODT was not statistically different from the C57BL/6J ODT ($t(14) = -0.29$, $p = 0.78$, $d = -0.15$; Figure 1e, Table 1). These data provide further evidence that our behavioral approach is not only consistent between individuals, but even across cohorts of animals tested months apart, by different experimenters using different behavioral setups and olfactometers.

The behavioral sensitivity to hexyl acetate did not differ according to sex or genotype for any of the transgenic mouse lines tested (Figure 2a–d). Within the *Avpr1a* strain, behavioral sensitivity to hexyl acetate did not differ by genotype or sex (*Avpr1a* | genotype: $F(2, 18) = 1.23$, $p = 0.32$, $\eta^2p = 0.12$; sex: $F(1, 18) = 3.45$, $p = 0.08$, $\eta^2p = 0.16$; interaction: $F(2, 18) = 0.69$, $p = 0.52$, $\eta^2p = 0.07$, two-way ANOVA with multiple comparisons, Figure 2a, Table 1). For the *Oxtr* strain, hexyl acetate sensitivity did not differ by genotype or sex (*Oxtr* | genotype: $F(2, 18) = 0.49$, $p = 0.62$, $\eta^2p = 0.05$; sex: $F(1, 18) = 1.05$, $p = 0.32$, $\eta^2p = 0.06$; interaction: $F(2, 18) = 0.71$, $p = 0.51$, $\eta^2p = 0.07$, two-way ANOVA with multiple comparisons, Figure 2b, Table 1). For the *Oxt* strain, hexyl acetate sensitivity also did not differ across genotype or sex (*Oxt* | genotype: $F(2, 18) = 0.43$, $p = 0.66$, $\eta^2p = 0.05$; sex: $F(1, 18) = 0.002$, $p = 0.97$, $\eta^2p = 0.0001$; interaction: $F(2, 18) = 1.29$, $p = 0.3$, $\eta^2p = 0.13$, two-way ANOVA with multiple comparisons, Figure 2c, Table 1).

Deletion of the *Avpr1a*, *Oxtr*, or *Oxt* genes did not influence the ability of these mice to discriminate between pentyl acetate and other structurally or chemically similar odorants (Figure 3, Table A.1). For the *Avpr1a* strain, there was a significant effect of odorant, due to the control pentyl acetate versus pentyl acetate experiment, but no significant effect for genotype or sex (*Avpr1a* | odor: $F(8, 128) = 519.66$, $p < 0.001$, $\eta^2p = 0.97$; genotype: $F(2, 16) = 0.10$, $p = 0.90$, $\eta^2p = 0.01$; sex: $F(1, 16) = 2.26$, $p = 0.15$, $\eta^2p = 0.12$; genotype × sex interaction: $F(2, 16) = 1.46$, $p = 0.26$, $\eta^2p = 0.15$; repeated measures two-way ANOVA with multiple comparisons, tests of within and between subjects, sphericity assumed). We observed a similar result for the *Oxtr* strain (*Oxtr* | odor: $F(8, 136) = 1072.01$, $p < 0.001$, $\eta^2p = 0.98$; genotype: $F(2, 17) = 1.62$, $p = 0.23$, $\eta^2p = 0.16$; sex: $F(1, 17) = 1.36$, $p = 0.26$, $\eta^2p = 0.07$; genotype × sex interaction: $F(2, 17) = 1.36$, $p = 0.28$, $\eta^2p = 0.14$; repeated measures two-way ANOVA with multiple comparisons, tests of within and between subjects, sphericity assumed), and *Oxt* strain (*Oxt* | odor: $F(8, 144) = 686.17$, $p < 0.001$, $\eta^2p = 0.97$; genotype: $F(2, 18) = 0.87$, $p = 0.44$, $\eta^2p = 0.09$; sex: $F(1, 18) = 3.58$, $p = 0.08$, $\eta^2p = 0.17$; genotype × sex interaction: $F(2, 18) = 0.005$, $p = 0.10$, $\eta^2p = 0.001$; repeated measures two-way ANOVA with multiple comparisons, tests of within and between subjects, sphericity assumed).

To assess whether animals lacking the *Avpr1a*, *Oxtr*, or *Oxt* gene differed in their abilities to learn the operant conditioning task, we analyzed the behavioral performance of these animals during their initial stage 2 training (Figure 4, Table 1). For the *Avpr1a* strain, the average number of trials necessary to reach criterion (≥90% behavioral performance maintained for the duration of learning) was not statistically different across either genotype or sex (*Avpr1a* | genotype: $F(2, 18) = 0.02$, $p = 0.98$, $\eta^2p = 0.002$; sex: $F(1, 18) = 3.96$, $p = 0.06$, $\eta^2p = 0.18$; interaction: $F(2, 18) = 0.48$, $p = 0.62$, $\eta^2p = 0.05$, two-way ANOVA with multiple comparisons). The average number of trials to reach criterion did not differ for the *Avpr1a* genotype when compared to C57BL/6J ($F(3, 28) = 0.05$, $p = 0.98$, $\eta^2 = 0.006$, one-way ANOVA). For the *Oxtr* strain, the average number of trials to reach criterion was also not statistically different across genotype or sex (*Oxtr* | genotype: $F(2, 18) = 2.31$, $p = 0.13$, $\eta^2p = 0.20$; sex: $F(1, 18) = 0.10$, $p = 0.75$, $\eta^2p = 0.006$; interaction: $F(2, 18) = 0.40$, $p = 0.68$, $\eta^2p = 0.04$, two-way ANOVA with multiple comparisons, Figure 4b). The average number of trials to reach criterion did not differ by *Oxtr* genotype when compared to C57BL/6J ($F(3, 28) = 2.05$, $p = 0.13$, $\eta^2 = 0.18$, one-way ANOVA). Lastly, for the

Oxt strain, the average number of trials to reach learning criterion was also not statistically different across genotype or sex (*Oxt* | genotype: $F(2, 18) = 0.39$, $p = 0.68$, $\eta^2 p = 0.04$; sex: $F(1, 18) = 0.25$, $p = 0.63$, $\eta^2 p = 0.01$; interaction: $F(2, 18) = 2.40$, $p = 0.12$, $\eta^2 p = 0.21$, two-way ANOVA with multiple comparisons). The average number of trials to reach criterion did not differ by *Oxt* genotype when compared to C57BL/6J ($F(3, 28) = 0.28$, $p = 0.84$, $\eta^2 = 0.03$, one-way ANOVA).

Discussion

We found that mice with a global deletion of the vasopressin 1a receptor (*Avpr1a*), oxytocin receptor (*Oxtr*), or oxytocin peptide (*Oxt*) gene did not differ in their sensitivity to a non-social odorant, nor their ability to discriminate between structurally similar non-social odorants. Additionally, their ability to learn an odor-based operant task was not affected by their genotype or sex. These results provide strong evidence that the social behavioral effects attributed to *Avpr1a, Oxtr*, and *Oxt* knockout mice are not the result of baseline deficits in main olfactory system processing in adulthood.

Estimates of perceptual abilities in animal models can be influenced by a number of factors including the behavioral assay used and the method of odor delivery (please see Williams and Dewan, 2020 for a full discussion regarding how our method compares to other approaches). Our approach has yielded highly consistent estimations of behavioral sensitivity (Dewan et al., 2018; Jennings et al., 2022; Williams et al., 2020). In the current study, we observed that the wild-type mice from each of these three transgenic strains (C57BL/6J background) did not differ in their sensitivity to hexyl acetate as compared to C57BL/6J mice, even though these mice were tested in a different operant chamber, with a different olfactometer, by a different experimenter, more than six months later (Figure 1, Jennings et al., 2022). Previously, we have also demonstrated this level of consistency in our method for different cohorts of mice tested with the same odorant (Jennings et al., 2022; Williams et al., 2020), and across the wild-types of different transgenic mouse strains (Dewan et al., 2018). Further, we have yet to observe any sex differences in odor sensitivity, discrimination, or initial learning ability using this method (Current study; Jennings et al., 2022; Williams and Dewan, 2020). Similarly, Kunkhyen et al. (2018) found that the sex of an animal does not influence the acquisition of an odor discrimination task. However, sex may influence odor discrimination ability in gonadectomized animals (under the influence of circulating sex hormones; Wesson et al., 2006); although, this result was not observed in a later study (Kunkhyen et al., 2018). Our behavioral approach has successfully detected even small deficits in sensitivity (~2.6-7.9 fold) attributed to the genetic deletion of single olfactory receptors (Dewan et al., 2018). Thus, it is highly unlikely that we would be unable to detect significant olfactory deficits associated with either sex or genotype; however, we cannot rule out minor deficits that are masked by individual behavioral variability.

To assess the baseline olfactory capabilities of these transgenic mouse lines, this project was limited to non-social odorants. However, it is quite possible that further research using operant conditioning with social odors would uncover significant deficits in social processing in these transgenic strains. In fact, a similar operant conditioning approach was used to determine that *Avpr1a*$^{-/-}$ mice have a reduced ability to discriminate between

male and female mouse urine (Wersinger et al., 2007). While more research is needed, the available data provide support for the hypothesis that the genetic removal of components of the vasopressin or oxytocin systems influences the processing of socially relevant stimuli without severely impacting baseline olfactory function. However, our study does not preclude a functional role for these neuropeptides in olfactory processing. The oxytocin and vasopressin systems are present in multiple olfactory regions and previous studies have developed several hypotheses regarding the contribution of these neuropeptides to olfactory function. For example, oxytocin is thought to facilitate social recognition in mice via oxytocin receptors in the anterior olfactory nucleus by inhibiting the primary projection neurons from the olfactory bulb and ultimately increasing the salience of the social signal (Ferguson et al., 2000; Oettl et al., 2016). Additionally, oxytocin may also facilitate social memory formation via pheromone signaling in the accessory olfactory bulb (AOB), which could influence social recognition behaviors (Fang et al., 2008). The salience of social stimuli is also thought to be modulated through the action of vasopressin in the main olfactory bulb (Tobin et al., 2010). While the proposed role of these neuropeptides in the processing of social odors does not conflict with our results, it is important to note that vasopressin and oxytocin show significant binding promiscuity between their respective receptors (Grinevich and Stoop, 2018; Pierce et al., 2020; Song et al., 2016; Song and Albers, 2017; Winslow and Insel, 2002). Thus, it is possible that compensatory neural mechanisms in these global knockout mouse strains have minimized the impact of these neuropeptides on baseline olfactory function.

In conclusion, our data provide evidence in support of the selective social deficits found within *Avpr1a, Oxtr*, and *Oxt* knockout mice, and serve to bolster the claim that these deficits are due to differences in the processing of social stimuli, rather than a ubiquitous functional deficit in olfactory processing. We hope that our data can provide additional clarity for the role of the oxytocin and vasopressin systems in social processing and olfaction, as well as provide support for the marked social deficits observed in these knockout animals.

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Appendix

Appendix

Table A.1.

Non-social odor discrimination ability for *Avpr1a*, *Oxtr*, and *Oxt* transgenic mice.

Strain	5ATE v. BLANK	5ATE v. 4ATE	5ATE v. I5ATE	5ATE v. 6ATE	5ATE v. 5AL	5ATE v. 5ONE	5ATE v. 5A	5ATE v. 5OL	5ATE v. 5ATE
***Avpr1a*$^{+/+}$**	93.9 ± 2.3	94.1 ± 1.8	95.7 ± 3.8	96.7 ± 1.9	92.8 ± 7.1	96.1 ± 2.6	94.9 ± 2.9	94.5 ± 6.0	49.0 ± 2.7
***Avpr1a*$^{+/-}$**	92.5 ± 2.2	92.3 ± 2.1	95.5 ± 3.5	96.0 ± 4.1	95.8 ± 3.3	96.1 ± 3.1	94.8 ± 3.6	97.8 ± 3.0	49.8 ± 3.4
***Avpr1a*$^{-/-}$**	94.2 ± 3.1	92.3 ± 2.6	93.4 ± 4.4	94.6 ± 4.8	93.4 ± 4.7	97.0 ± 2.3	93.5 ± 3.8	95.9 ± 2.9	51.9 ± 2.4
***Oxtr*$^{+/+}$**	93.6 ± 3.0	92.9 ± 3.6	94.2 ± 3.5	95.4 ± 2.4	94.1 ± 3.0	95.1 ± 2.5	95.1 ± 2.9	96.1 ± 2.8	50.4 ± 2.6
***Oxtr*$^{+/-}$**	93.2 ± 2.7	94.5 ± 2.9	94.7 ± 2.1	97.6 ± 1.6	94.7 ± 2.1	94.9 ± 3.5	93.8 ± 2.8	97.4 ± 2.8	47.9 ± 3.5
***Oxtr*$^{-/-}$**	94.6 ± 2.6	95.4 ± 1.9	94.1 ± 2.2	97.7 ± 2.1	95.8 ± 2.2	95.4 ± 2.0	96.2 ± 2.5	98.0 ± 1.1	50.3 ± 2.0
***Oxt*$^{+/+}$**	93.3 ± 2.9	93.2 ± 2.9	94.8 ± 3.1	93.7 ± 3.5	93.1 ± 4.8	93.0 ± 2.1	93.9 ± 4.6	95.4 ± 2.0	50.1 ± 2.0
***Oxt*$^{+/-}$**	94.5 ± 2.5	93.5 ± 2.9	93.6 ± 3.2	93.2 ± 2.4	92.6 ± 3.1	93.3 ± 3.7	93.9 ± 3.4	97.5 ± 1.9	51.6 ± 2.5
***Oxt*$^{-/-}$**	95.5 ± 1.3	92.6 ± 4.3	95.4 ± 2.5	94.7 ± 3.0	93.4 ± 3.9	94.4 ± 3.0	94.9 ± 2.9	97.4 ± 2.5	52.7 ± 2.8

Data represent mean behavioral performance (%) with standard deviation for the discrimination of pentyl acetate (5ATE) from mineral oil (BLANK), butyl acetate (4ATE), isoamyl acetate (I5ATE), hexyl acetate (6ATE), pentanal (5AL), 2-pentanone (5ONE), amyl amine (5A), 1-pentanol (5OL), and pentyl acetate (5ATE) for *Avpr1a*, *Oxtr*, and *Oxt* transgenic mice. There is no statistical difference in behavioral performance by genotype or sex across all three cohorts ($p>0.05$, repeated measures two-way ANOVA with multiple comparisons).

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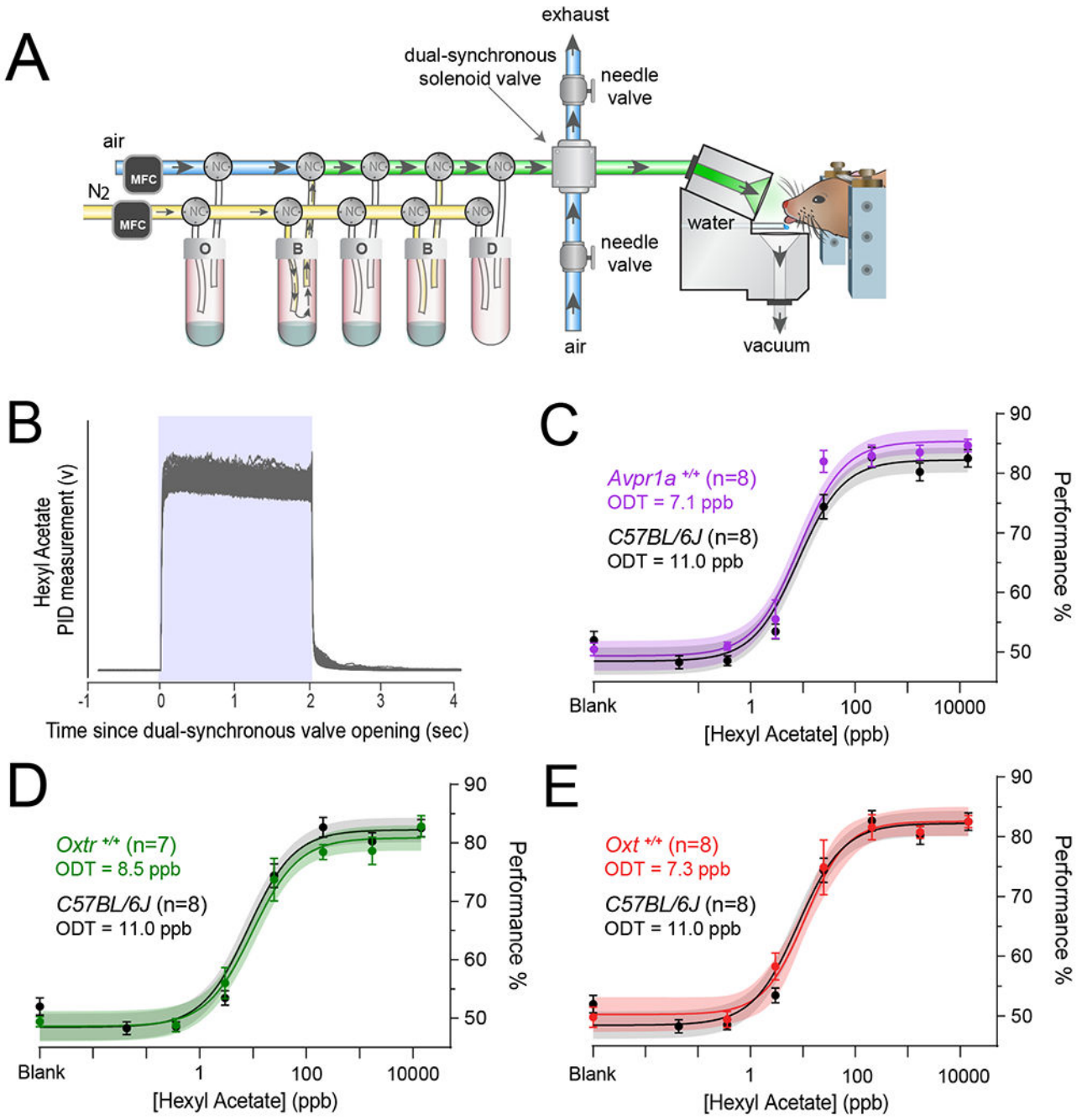

Figure 1. Wild-type mice from each transgenic strain do not differ from C57BL/6J mice in their behavioral sensitivity to hexyl acetate.

To measure odor detections thresholds (ODTs), we used a head-fixed Go/No-go operant conditioning assay combined with well-controlled and highly reproducible stimulus delivery. (**A**) Odorants are delivered using an eight-channel, flow dilution olfactometer that switches between a pressure-balanced dummy (D) vial (via normally open valves, NO) and either odor (O) or blank (B) vials containing only the solvent (via normally closed valves, NC). Odorized air is directed to exhaust to allow the stimulus to reach equilibrium prior to

stimulus delivery. During stimulus application, a dual-synchronous solenoid valve re-directs pressure-balanced, odorized air from exhaust to the animal. At the conclusion of the trial, the dual-synchronous solenoid valve returns the pressure-balanced clean air to the animal. (**B**) Photoionization detector (PID) traces of 250 stimulus presentations of hexyl acetate. Shaded area signifies 2 second stimulus period. (**C-E**) The wild-type mice from each transgenic strain did not differ from a published C57BL/6J odor detection threshold for hexyl acetate (Jennings et al., 2022; $p>0.05$, independent samples t-test). Data were fitted using a Hill function. Maximal behavioral performance for each odorant concentration is limited to ~85% (see methods). Plots show mean +/− SE with shaded 95% confidence interval.

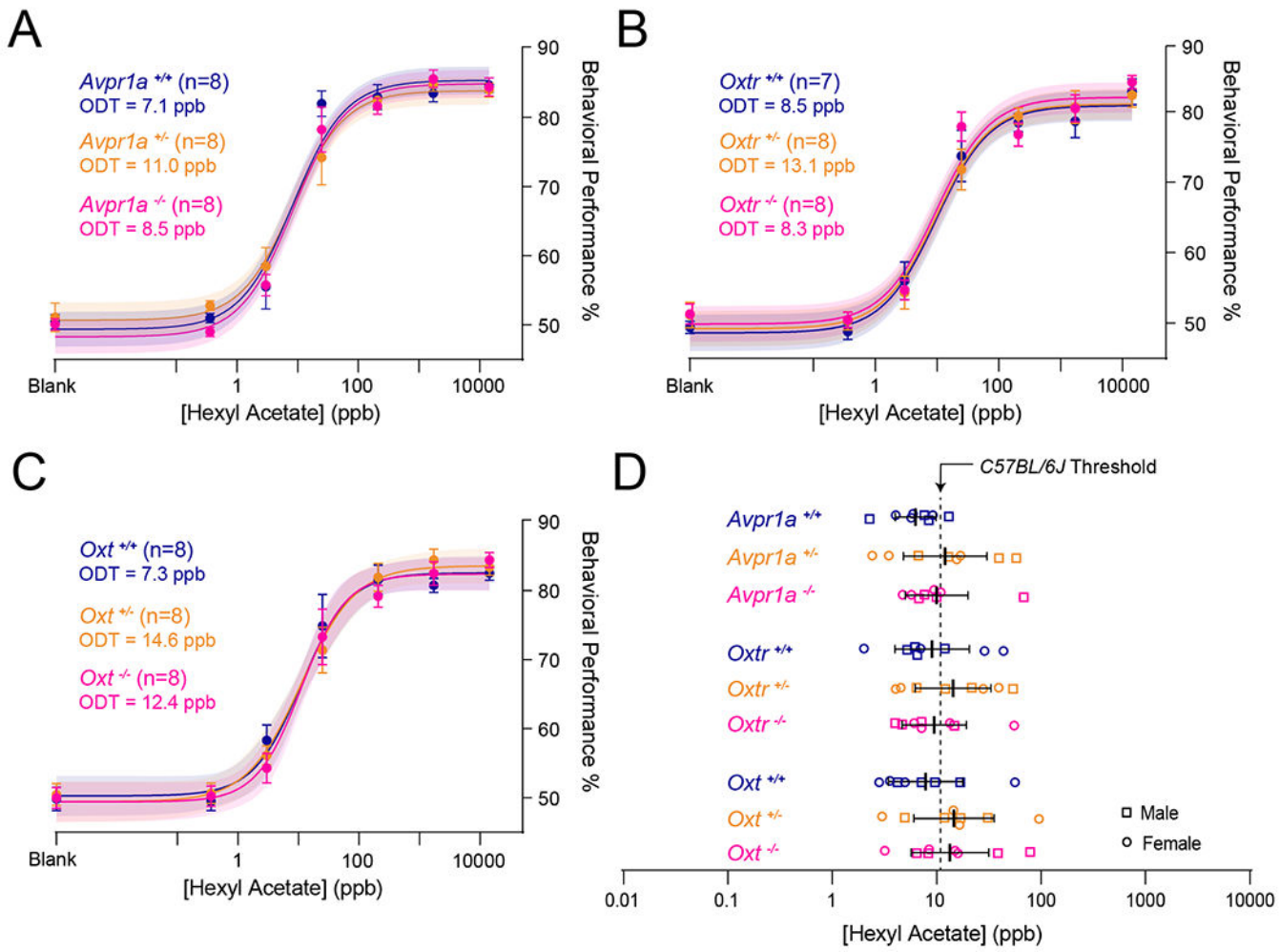

Figure 2. *Avpr1a, Oxtr*, and *Oxt* knockout and heterozygous mice have normal odor detection thresholds to hexyl acetate.

Genetic deletion of the vasopressin receptor 1a (*Avpr1a*), oxytocin receptor (*Oxtr*), or oxytocin (*Oxt*) gene does not impact behavioral sensitivity to a non-social odor. (**A-C**) Psychometric curves for wild-type (blue), heterozygous (orange), and homozygous knockouts (pink) of the (**A**) vasopressin 1a receptor, (**B**) oxytocin receptor and (**C**) oxytocin genes to the non-social odor, hexyl acetate. Plots show mean ± SE with shaded 95% confidence interval. Data were fitted using a Hill function. Maximal behavioral performance for each odor concentration is limited to ~85% (see methods). Olfactory detection threshold (ODT) is defined as the odor concentration at half-maximal behavioral performance. There was no statistical difference in ODT by genotype or sex across the three cohorts ($p>0.05$, two-way ANOVA with multiple comparisons). (**D**) Summary of behavioral sensitivity for all transgenic strains. Plots show mean with a 95% confidence interval. Individual thresholds for each genotype are denoted with open symbols. Squares denote males while circles denote females. Dashed line signifies the published threshold for C57BL/6J mice.

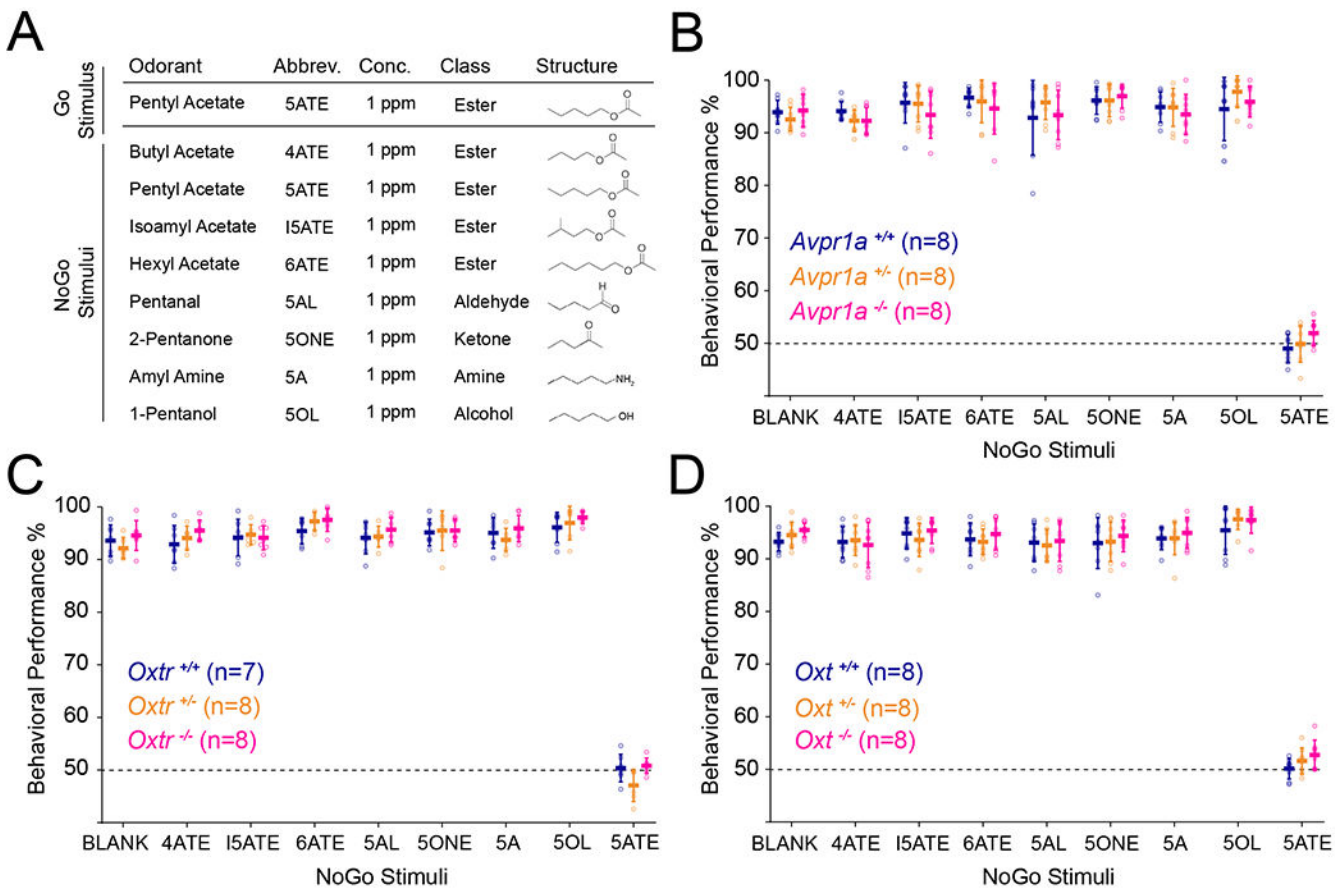

Figure 3. *Avpr1a*, *Oxtr*, and *Oxt* knockout and heterozygous mice can discriminate between similar non-social odorants.

Genetic deletion of the *Avpr1a*, *Oxtr*, or *Oxt* gene does not impact the animals' ability to discriminate between chemically or structurally similar non-social odorants. (**A**) Summary of the odorants tested in a Go/No-go odor discrimination assay. Odorants were diluted in mineral oil and then further flow diluted (10-fold) to reach the desired 1 ppm vapor concentration. (**B-D**) Behavioral performance of wild-type (blue), heterozygous (orange), and homozygous knockouts (pink) of the (**B**) vasopressin 1a receptor, (**C**) oxytocin receptor, and (**D**) oxytocin genes in an odor discrimination task. Pentyl acetate served as the Go stimulus for all sessions, while the No-go stimulus was either the mineral oil solvent (blank), one of the seven structurally similar odorants, or pentyl acetate (to test the animal's ability to use non-odor cues to maximize their behavioral performance, see methods). Individual mice are plotted with open circles while plots show mean ± SD. There was no statistical difference in behavioral performance by genotype or sex across the three cohorts ($p>0.05$, repeated measures two-way ANOVA with multiple comparisons).

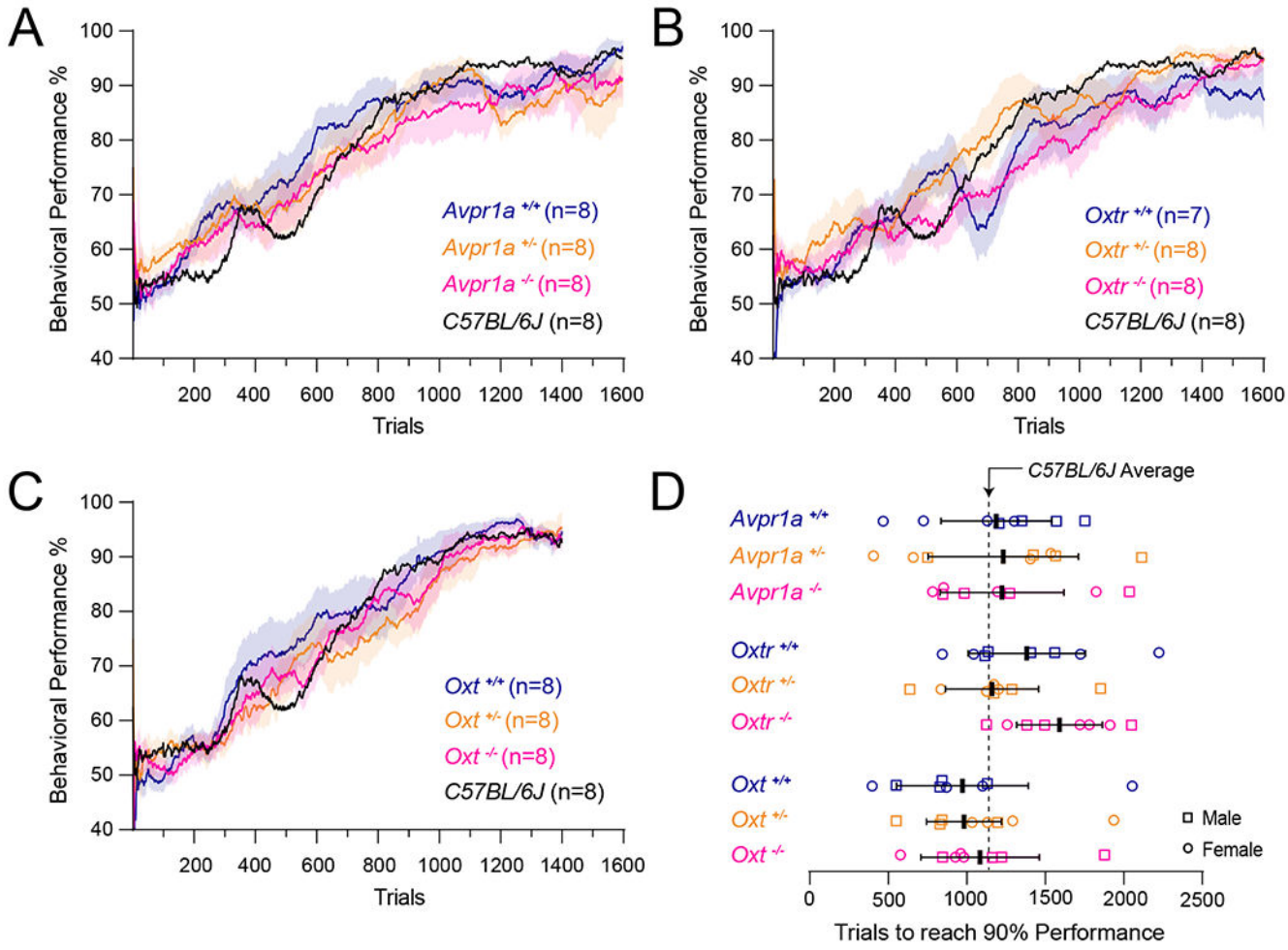

Figure 4. *Avpr1a, Oxtr,* and *Oxt* knockout and heterozygous mice can learn an odor-based operant conditioning task

Genetic deletion of the *Avpr1a*, *Oxtr*, or *Oxt* gene does not impact an animal's ability to learn a basic odor-based Go/No-go operant conditioning task. (**A-C**) Running average (100-trial sliding window) of trial-by-trial behavioral performance of wild-type (blue), heterozygous (orange), and homozygous knockouts (pink) of the (**A**) vasopressin receptor 1a, (**B**) oxytocin receptor, and (**C**) oxytocin genes; and behavioral performance of C57BL/6J mice (black, **A-C**; Jennings et al., 2022) in an odor learning task. Mineral oil solvent served as the Go stimulus, while the No-go stimulus was hexyl acetate (1:100 liquid dilution, diluted 10-fold). (**D**) Learning data from A-C replotted with the number of trials necessary for an individual mouse to reach learning criterion: ≥90% behavioral accuracy maintained for the duration of all learning sessions. Individual mice are plotted with open circles (females) or open squares (males) while plots show mean with a 95% confidence interval. There was no statistical difference in the number of trials needed to reach learning criteria by genotype compared to C57BL/6J ($p>0.05$, one-way ANOVA), or by genotype or sex across cohorts ($p>0.05$, two-way ANOVA with multiple comparisons).

Table 1.

Avpr1a, *Oxtr*, *Oxt*, and C57BL/6J mice odor detection thresholds (ODTs) and odor learning for hexyl acetate.

	Thresholding (ppb)		Learning (# of trials)	
Strain	**ODT**	**95% CI**	**Trials**	**95% CI**
***Avpr1a*$^{+/+}$**	7.1	4.7 – 10.8	1188	767 – 1609
***Avpr1a*$^{+/-}$**	11.0	6.3 – 19.2	1232	660 – 1804
***Avpr1a*$^{-/-}$**	8.5	5.7 – 12.6	1225	754 – 1696
***Oxtr*$^{+/+}$**	8.5	4.8 – 15.7	1382	936 – 1828
***Oxtr*$^{+/-}$**	13.1	7.6 – 22.5	1161	807 – 1515
***Oxtr*$^{-/-}$**	8.3	5.3 – 13.0	1590	1264 – 1916
***Oxt*$^{+/+}$**	7.3	4.1 – 13.3	972	470 – 1474
***Oxt*$^{+/-}$**	14.6	8.6 – 24.2	1157	738 – 1576
***Oxt*$^{-/-}$**	12.4	7.3 – 21.4	1061	677 – 1445
C57BL/6J [1]	11.0	7.0 – 16.0	1143	630 – 1656

ODTs and the number of trials to reach learning criteria (≥90% behavioral accuracy maintained for the duration of learning) are listed with 95% confidence intervals (CI), and do not statistically differ by genotype or sex across the three cohorts, or when compared to C57BL/6J data ($p>0.05$, two-way ANOVA with multiple comparisons).

[1] C57BL/6J data were originally published in Jennings et al., (2022)