Figure S1, Related to Figure 1

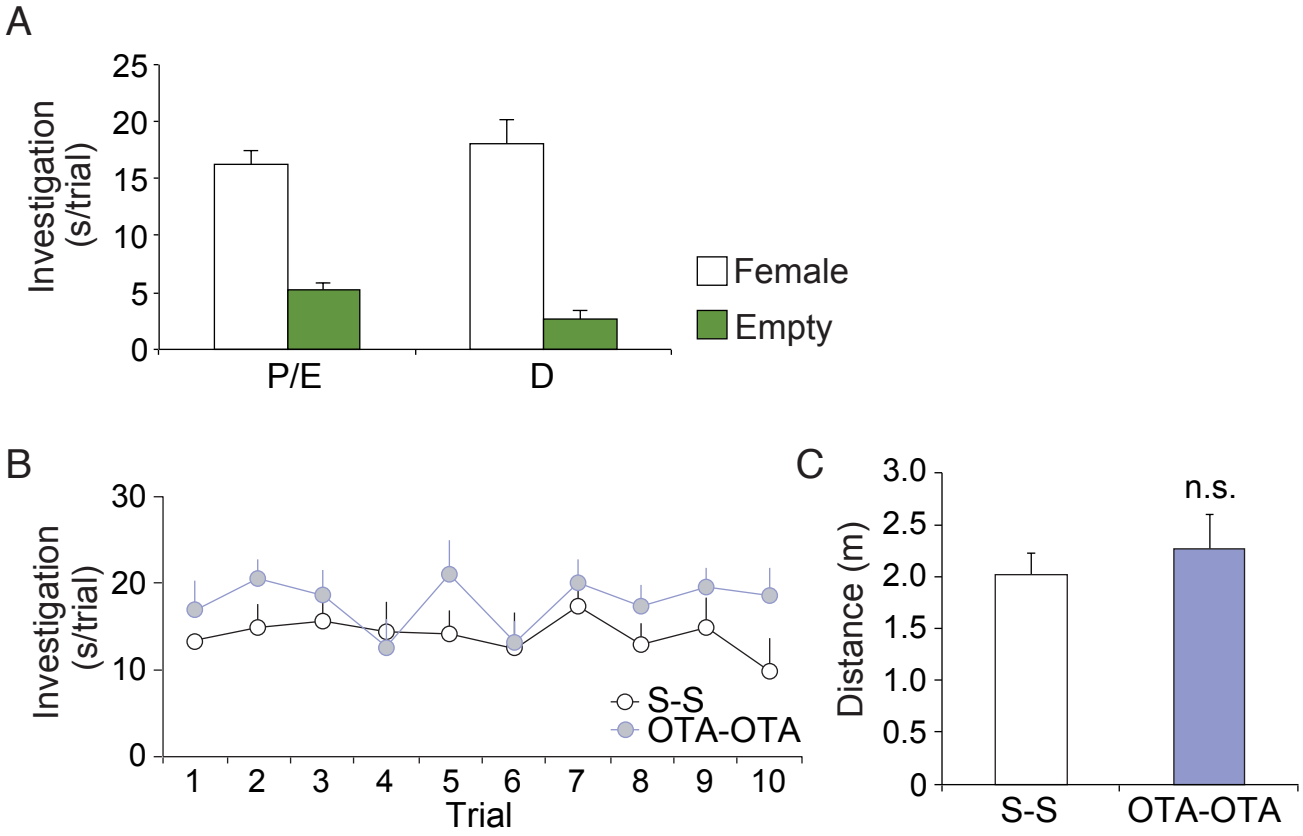


Figure S2, Related to Figure 3

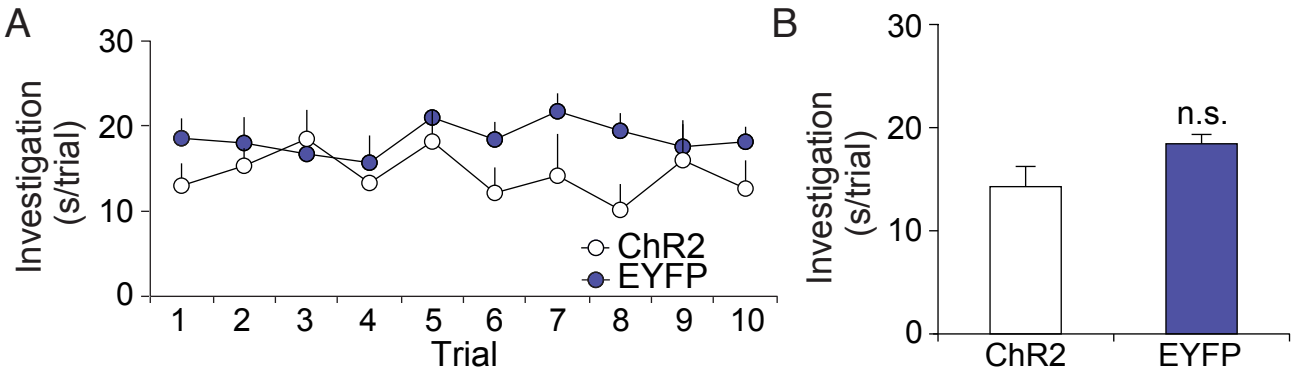


Figure S3, Related to Figure 4

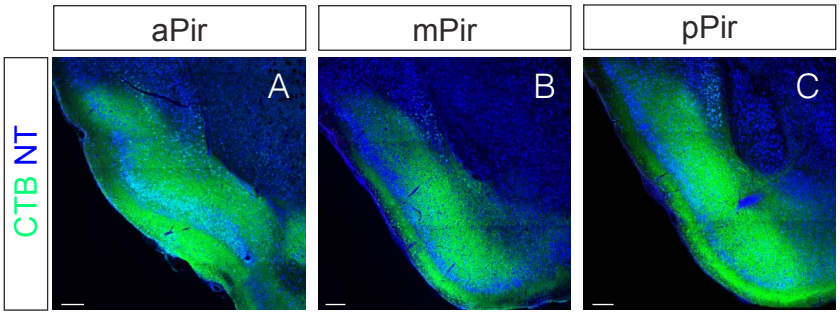


Figure S4, Related to Figure 5

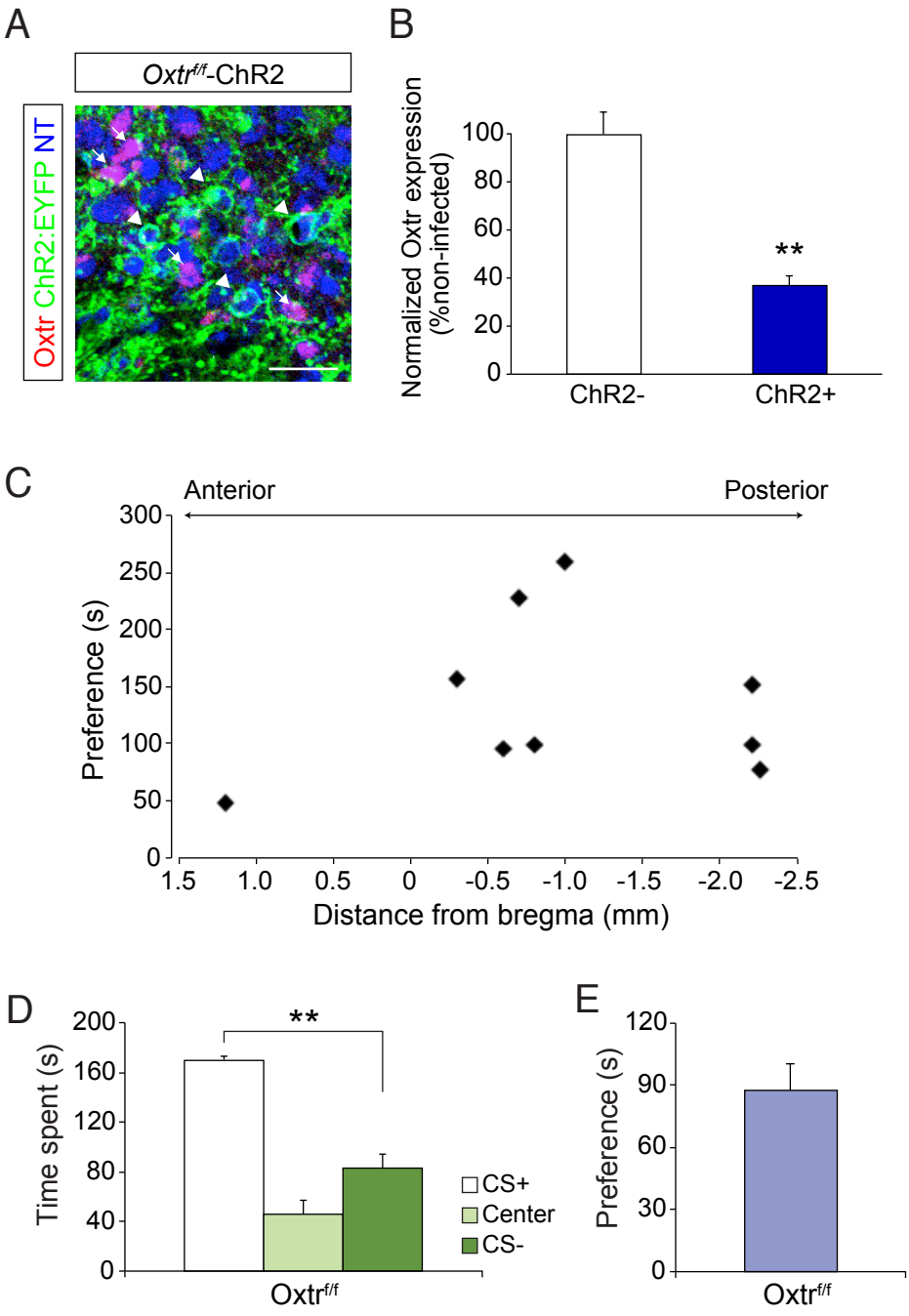
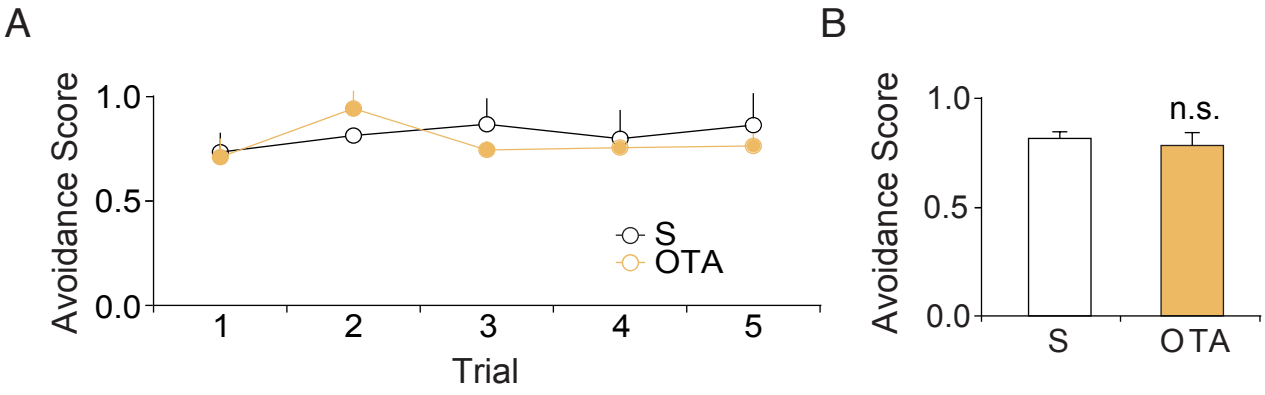


Figure S5, Related to Figure 6



Supplemental Figures and Legends

Figure S1. Investigation time toward the wire cages during training (A) Investigation time during training by subjects trained with proestrus/estrus (P/E) or diestrus (D) females. Average investigation time of the wire cage containing a female or the empty cage during training. (B and C) The effect of oxytocin receptor antagonist (OTA) on investigation time during training and distance traveled during testing. (B) Investigation time by subjects injected with saline (S-S) and OTA (OTA-OTA) before both training and testing. Investigation time of the wire cage with a female at each trial. (C) Total distance traveled during testing by subjects injected with S-S or OTA-OTA. P = 0.95 by Student's t-test. Data presented as mean ± SEM.

Figure S2. Investigation time during training by subjects supplemented with optogenetic activation of Oxt+ neurons. (A) Investigation time by $Oxt^{Cre/+}$ animals injected with Cre-dependent ChR2:EYFP or Cre-dependent EYFP. Investigation time of the wire cage with a diestrus female across trials. (B) Average investigation time of the wire cage housing a diestrus female. P > 0.05 by Student's t-test. Data presented as mean ± SEM.

Figure S3. Injection sites of cholera toxin B subunit (CTB) for retrograde tracing from the piriform cortex. (A-C) Coronal sections of (A) the anterior piriform cortex (aPir), (B) the middle piriform cortex (mPir), and (C) the posterior piriform cortex (pPir) in mice injected with CTB were stained for CTB (green) and counterstained with NT (blue). Scale bars in (A-C) represent 200 μm.

Figure S4. (A and B) Oxtr mRNA expression in *Oxtr*$^{f/f}$-ChR2 mice. (A) *In situ* hybridization for Oxtr mRNA (red) in the piriform cortex of *Oxtr*$^{f/f}$-ChR2 mice stained for EYFP (green) and counterstained with NT (blue). Arrowheads indicate neurons that express ChR2 (ChR2+). Arrows indicate neurons that do not express ChR2 (ChR2-). Scale bar represents 50 μm. (B) Quantification of *in situ* hybridization for Oxtr mRNA in ChR2- or ChR2+ neurons. Normalized by average intensity of ChR2-. **: $P < 0.01$ by Student's t-test. (C) Relationship between the preference score and the location of the ChR2-expressing ensembles of WT-ChR2 mice in social appetitive learning scheme. The positions of ChR2-expressing ensembles were plotted against preference scores. (D and E) Odor-driven social appetitive learning in *Oxtr*$^{f/f}$ mice. (D) Time spent in each chamber. **: $P < 0.01$ by Tukey HSD *post hoc* test. (E) Preference score for data presented in (D). Data presented as mean ± SEM.

Figure S5. The effect of oxytocin receptor antagonist (OTA) on avoidance of the CD1 male during training. (A) Avoidance score (fraction of total time spent away from the wire cage containing the CD1 male) for S or OTA injected animals. (B) Average avoidance score over all training trials for subjects injected with S or OTA. $P = 0.69$ by Student's t-test. Data presented as mean ± SEM.

Supplemental Experimental Procedures

Constructs and Viruses

Lentiviruses for *in vivo* injection were produced as previously described (Zhang et al., 2010). The following viruses were purchased from Penn Vector Core: AAV1.EF1α-DIO.ChR2:EYFP, AAV1.EF1α-DIO.EYFP, and AAV1.hSyn.EGFP. AAV1.EF1α-DIO.ChR2:tdTomato was purchased from the vector core at the University of North Carolina at Chapel Hill.

Generation of Oxt^{Cre} Knock-in Mice

The targeting construct was generated to insert an IRES sequence, a *Cre* recombinase, and *Frt*-flanked PGK-neomycin resistance cassette at the 3' end of *Oxt* coding sequence by homologous recombination. The 5' arm was amplified using the following primers: 5'-catggattaccttaggtgaga-3' and 5'-tcagcgctccgagaaggcaga-3'. The 3' arm was amplified using the following primers: 5'-gcccactttctgggaatacc-3' and 5'-gacttactgattctttggctg-3'. Constructs were electroporated into 129/SvEv ES cells (Hooper et al, 1987). Recombinant clones were determined using a 3' external probe on Southern blots of PacI and BglII digested ES cell genomic DNA, as described previously (Wang et al., 1998). Targeted clones were injected into C57BL/6 blastocysts to produce chimeras that transmitted the mutant allele through the germline.

Stereotaxic Injection and Fiber Optic Implantation into the Mouse Brain

Strains of mice used for experiments were wild-type C57BL/6J (Jackson Laboratory, Bar Harbor, ME), CD-1 (Charles River, Wilmington, MA), *Oxt*$^{Cre/+}$, and *Oxtr*$^{f/f}$. The surgeries were carried out using aseptic techniques. Mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Stereotaxic (Kopf Instruments, Tujunga, CA) injections of viruses were made through pulled glass pipettes (Drummond, 2-000-001, Broomall, PA) at the rate of <0.1 μl/min at the layer 2 and 3 of the piriform cortex (AP = -0.6 mm, ML = +/-3.8 mm from bregma, DV = -4.0 mm from brain surface; For the anterior piriform cortex: AP = +1.54 mm, ML = +/-2.80 mm, DV = -3.50 mm; For the posterior piriform cortex: AP = -2.06 mm, ML = +/-4.20 mm, DV = -4.10 mm) or the paraventricular nucleus of the hypothalamus (AP = -1.0 mm, ML = 0.0 mm , DV = -3.9 mm). The tip of the fiber optic implant (300-μm core size, Thorlabs, Newton, NJ) was subsequently placed 300-400 μm above the virus injection site. The cannula was fixed in place with a small amount of dental cement and the skin was glued back with Vetbond tissue adhesive (3M, St. Paul, MN). All animal procedures were approved by the Committee on Animal Care of Massachusetts Institute of Technology.

Tissue Slice Preparation and Immunohistochemistry

After being anesthetized by intraperitoneal injection of ketamine and xylazine, mice were perfused with 6 mL of PBS, followed by 7 mL of 4% paraformaldehyde (PFA). The brains were dissected out and post-fixed overnight in 4% PFA. The brains were cut coronally at 50-100 um thickness using a vibratome (Leica, Buffalo Grove, IL). The prepared slices were labeled with the following primary antibodies: chicken anti-GFP (Abcam, ab5450, 1:1000), goat anti-c-Fos (Santa Cruz, sc-52-G, 1:300), rabbit anti-c-Fos (Santa Cruz, sc-7270, 1:500), rabbit anti-DsRed (Clontech, 632496, 1:500), goat anti-CTB (List Biological laboratories, 703, 1:500), mouse anti-

oxytocin (Millipore, MAB5296, 1:1000), and rabbit anti-oxytocin (Millipore, AB911, 1:1000). The following fluorophore-conjugated secondary antibodies were used: Alexa 488 goat anti-chicken (Invitrogen, A11039), Alexa 633 donkey anti-goat (Invitrogen, A21082), Alexa 488 donkey anti-goat (Invitrogen A11055), Alexa 647 donkey anti-mouse (Invitrogen, A31571), Alexa 633 goat anti-rabbit (Invitrogen, A21071), Alexa 568 goat anti-rabbit (Invitrogen, A11036). All secondary antibodies were diluted 1:250. The slices were counterstained with NeuroTrace (NT) fluorescent Nissl stain (Invitrogen, N-21479 or N-21483). All images were taken using a Zeiss LSM-710 confocal microscope system (Zeiss, Oberkochen, Germany).

For quantitative analysis of photostimulation-dependent activation of ChR2- and EYFP-expressing neurons in *Oxt*$^{Cre/+}$ mice, animals were photostimulated (30 sec per min for 10 min) one hour before they were sacrificed. Brain slices were double-labeled for c-Fos and EYFP. The percentage of neurons expressing c-Fos was obtained by dividing the number of EYFP+ c-Fos+ cells by the number of EYFP+ cells within the area beneath the optical fiber placement.

Quantitative analysis of piriform ensemble images was carried out as previously described (Choi et al., 2011). Briefly, WT-ChR2 or *Oxtr*$^{f/f}$-ChR2 mice were photostimulated (30 sec per min for 10 min) one hour before they were sacrificed. Brain slices were double-labeled for c-Fos and DsRed. The percentage of neurons expressing ChR2 was obtained by dividing the number of ChR2+ NT+ cells by the number of NT+ cells within a 500 μm × 500 μm area around the center of injection site. The number of c-Fos+ ChR2+ neurons was counted within the same 500 μm × 500 μm area. The neurons were counted at least three different anterior-posterior levels and averaged for each animal.

Tracing Studies

For anterograde tracing, Cre-dependent ChR2 fused to EYFP was targeted to the PVH (AP: -1.0 mm, ML: 0.0 mm DV: -3.9 mm) of adult male *Oxt*$^{Cre/+}$ mice. Following a two-week survival time, 100-µm sections were cut and stained with antibodies against GFP and counterstained with NT. For retrograde tracing, we injected 0.5% cholera toxin B subunit (CTB) at three points along the anterior-posterior extent of the piriform of male wild-type animals. Injections were targeted to the anterior (AP: +1.5 mm, ML: +2.5 mm, DV: -3.5 mm), middle (AP: -0.7 mm, ML: +3.5 mm, DV: -4.4 mm), and posterior (AP: -1.8 mm, ML: +3.85 mm, DV: -4.1 mm) aspects of the piriform. After four days of survival time, 50-µm sections were cut and co-stained for CTB and Oxt for 48 hrs. Sections were counterstained with secondary antibodies and with NT for 24 hrs.

In Situ Hybridization

For fluorescent *in situ* hybridization, brains were dissected out of adult male mice after transcardiac perfusion with RNase-free 4% PFA. The brains were post-fixed in PFA overnight, incubated in RNase-free 30% sucrose in PBS for 48 hrs, then embedded in Tissue-Tek optimum cutting temperature compound (Sakura Finetek, Torrance, CA). The blocks were sectioned at a 16-µm thickness using a Cryostat (Leica). Fluorescent *in situ* hybridization was performed using branched DNA probe and amplification technology (ViewRNA ISH Tissue Assay kit, Panomics, Santa Clara, CA) according to manufacturer's protocol. Briefly, the sections were subject to proteinase K digestion for 10 min at 40°C, followed by fixation in 4% PFA. Probes were applied to the sections (1:50 dilution in hybridization buffer) and incubated for 6 hrs at 40°C. The probes were designed based on the following mRNA sequence: Oxtr (NM_001081147). After serial

hybridization with pre-amplification, amplification reagents, and alkaline phosphatase, the signal was developed with fast red.

Piriform cortical sections of *Oxtr*$^{f/f}$-ChR2 mice were for quantification of Oxtr mRNA intensity. Following *in situ* hybridization for Oxtr mRNA, the sections were stained for EYFP. All images were taken with identical imaging parameters at the same setup. A region of interest (ROI) was drawn around the soma of ChR2+ neurons and neighboring non-infected neurons. Background signal was defined as the intensity of the red channel in an area of the image that did not contain cells. The intensity of Oxtr mRNA for an individual neuron was defined as intensity of the red channel within the respective ROI after background subtraction. The normalized value was obtained by dividing the intensity of individual neurons by the average Oxtr mRNA intensity of non-infected neurons.

Behavioral analysis

Social Appetitive Learning Paradigm

Behavioral training and testing were carried out in a custom-built three-chambered arena with modifications from a previously established paradigm (Choi et al., 2011). Each chamber (35.5 × 17.75 × 17.75 cm) was constructed from 0.635 cm-thick acrylic Plexiglas. The middle chamber included two 10-cm-wide openings in each long wall, allowing free movement between all three chambers. One or two days before the experiment, subject males were singly caged and habituated for 15-20 min to the arena containing empty wire cages (11 cm height, 10.5 cm bottom diameter, bars spaced 1 cm apart; Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, Ohio), that were to be used later, in each side chamber. C57BL/6J females were transferred to a clean cage one day before the experiment and examined for their estrus phase by

vaginal smear examination on the experiment day. During training, a wire cage containing a C57BL/6J female was placed in one side chamber while an empty wire cage was placed in the opposite side chamber. An odor set comprised of orange and anise extracts or another set comprised of octanol and ethyl acetate were randomly chosen for experiments. Individual odors were randomly designated as either CS+ or CS- odors. At the beginning of each trial, subject males were placed in the middle chamber. Odor was perfused into the wire cage only when the center point of the male subject entered approximately 3-cm proximity (training zone) to the wire cage. Each female was used for 2 consecutive trials, and placement of the female-containing cage was alternated every other trial. Ten trials were completed during training, with an intertrial interval of 2.5 min. Wire cages and side chambers were thoroughly cleaned with paper towel and 70% ethanol in between trials. During testing, in the absence of a female, odor was delivered when the male was in one of the arbitrarily chosen side chambers (CS+ chamber). Odor presentation was controlled using custom-built olfactometers and automated tracking software (EthoVision XT, Noldus, Wageningen, Netherlands) during training and testing. Time spent in each chamber was quantified by the software and investigation time during training was manually counted. The preference score was obtained by subtracting the time spent in the CS- chamber from the time spent in the CS+ chamber. The subjects that spent more than half of testing session (150 sec) in the center chamber were excluded from analysis.

For optogenetic stimulation of Oxt+ neurons, $Oxt^{Cre/+}$ male mice were injected with virus encoding either Cre-dependent ChR2 fused to EYFP, or Cre-dependent EYFP, into the PVH. After two weeks of recovery, animals were trained in our social-appetitive learning paradigm. During training, laser stimulation (405 nm, 30 Hz, 30% duty cycle) (Knobloch et al., 2012) was triggered upon the subject animal entering the CS+ compartment containing a female in diestrus.

Photostimulation was controlled by a waveform generator (Keysight, 33220A, Santa Rosa, CA) and Ethovision XT. All other conditions were the same as described earlier for the appetitive social learning paradigm.

For ensemble-driven social appetitive learning, photostimulation (405 nm, 7.5-8 mW, 20 Hz, 50% duty cycle) was applied, instead of odor, when the center point of the male subject entered training zone. Oxytocin receptor antagonist (L-368,899) was purchased from Tocris (Bristol, United Kingdom) and prepared in saline as 25 mg/mL stock solution. The stock solution was further diluted with saline to make 0.625 mg/mL working solution. The subjects were briefly anesthetized with isoflurane for intraperitoneal (i.p.) injection of 5 mg/kg of antagonist.

Non-social Learning Paradigm

For entrainment to sucrose solution, subject males were water restricted for 2 weeks before the experiment. Two days before the experiment, subjects were singly housed. One day before the experiment, subjects were habituated for 15-20 min to the arena and to wire cages carrying 6 drops of tap water (20 μL total) on their wires. During training, a wire cage carrying 8-12 drops of 10% sucrose solution, instead of female, was placed in one side chamber while an empty wire cage was placed in the opposite side chamber. Pairing and testing were performed in the same way as with the appetitive social learning paradigm. For entrainment to food, males fed *ad lib* were trained with palatable foods (white chocolate (Kraft food, Northfield, IL), peanut butter (Supervalu, Eden Prairie, MN) mixed with nutella (Ferrero, Somerset, NJ), and NUTRI-CAL for ferrets (Tomlyn, Ft. Worth, TX)) applied on the wire cage. During the first three trials of training, one type of food was used for each trial. For the first six trials, each food was presented twice in random order. For the 7th and 8th trials, the two foods that were highly

investigated during previous trials were sequentially used as the US. If mice investigated both cues during the 7th and the 8th trials, the same foods were used during the 9th and the 10th trials in the same order. Otherwise, the food that was investigated the most during the 7th and the 8th trials was used for the final two trials. Testing was carried out for 4 min. Non-social aversive learning paradigm was performed as previously described (Choi et al., 2011).

Social Aversive Learning Paradigm

The three-chambered arena used for appetitive learning was modified for aversive learning. Side chambers were constrained (22.00 × 17.75 × 30.00 cm) and the center chamber was constrained (14.00 × 17.75 × 30.00 cm) using acrylic dividers. All subject mice were group housed prior to training. Prior to training, subject mice were exposed to a sexually experienced CD1 male for five minutes. If the CD1 did not initiate attack before 1.5 min, subject mice were placed in the home cage of a different CD1. Immediately following CD1 exposure, the subject mice were confined to the center of the three-chamber arena for five minutes before training began. Training consisted of 10 trials lasting 1 min each. During each trial, the subjects were confined to one side of the three-chamber arena with either the CS+ odor perfusing through the wire cage containing the previously encountered CD1, or the CS- odor perfusing through the empty wire cage. Each trial type alternated sides of the arena every trial. Immediately following training, animals were confined to the center chamber for five minutes. Animals were then assayed for odor preference in the same manner as in our appetitive social learning paradigm. Animals that spent greater than half of the test session (150 sec) in the center compartment were excluded from analysis. Avoidance scores during training were calculated by dividing the amount of time in the half of training compartment away from the wire cage by the total trial

duration. Trials in which photostimulation was used as a CS utilized a narrowed center chamber (5.00 × 17.75 × 30.00 cm). Side chambers were constrained (13.00 × 17.75 × 30.00 cm) during training using acrylic dividers. Training consisted of 16 trials lasting 40 sec each. Baseline and testing trials were unchanged. Animals that spent greater than 100 sec in the center compartment were excluded from analysis. The ROUT method (with Q set to 5%) was used to detect outliers (n = 2).

Real-Time Place Preference (RTPP)

We used a custom built chamber (10 × 10 cm, red acrylic sheet) divided into two equal compartments. Presence in one compartment triggered onset of laser stimulation. Presence in the other compartment was unstimulated. Trials lasted for 20 minutes. We used 30 Hz, 30% duty cycle laser stimulation. Tracking and laser stimulation triggering were accomplished using Ethovision XT software.

Statistics

Group differences in either social appetitive or aversive learning were evaluated by two-way analysis of variance (ANOVA) followed by Tukey HSD *post hoc* test. Group differences in preference toward CS+ were evaluated by Student's t-test or one-way ANOVA followed by Tukey HSD *post hoc* test. Investigation time during training along trial was evaluated by repeated-measures ANOVA (RM-ANOVA) followed by Bonferroni *post hoc* tests.

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