Oxytocin Mediates Entrainment of Sensory Stimuli to Social Cues of Opposing Valence

Highlights

- Oxytocin receptor signaling is specifically required for social learning
- Oxytocin neuron stimulation facilitates social learning
- Oxytocin directly impacts the piriform cortex to mediate social learning
- Both appetitive and aversive social learning require oxytocin receptor signaling

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In Brief

Choe, Reed et al. demonstrated that oxytocin receptor signaling is specifically required for learning with appetitive or aversive social cues through its direct impact on the piriform cortex. Oxytocin conveys saliency of social cues of opposing valence to sensory representations.
Oxytocin Mediates Entrainment of Sensory Stimuli to Social Cues of Opposing Valence

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SUMMARY

Meaningful social interactions modify behavioral responses to sensory stimuli. The neural mechanisms underlying the entrainment of neutral sensory stimuli to salient social cues to produce social learning remain unknown. We used odor-driven behavioral paradigms to ask if oxytocin, a neuropeptide implicated in various social behaviors, plays a crucial role in the formation of learned associations between odor and socially significant cues. Through genetic, optogenetic, and pharmacological manipulations, we show that oxytocin receptor signaling is crucial for entrainment of odor to social cues but is dispensable for entrainment to nonsocial cues. Furthermore, we demonstrate that oxytocin directly impacts the piriform, the olfactory sensory cortex, to mediate social learning. Lastly, we provide evidence that oxytocin plays a role in both appetitive and aversive social learning. These results suggest that oxytocin conveys saliency of social stimuli to sensory representations in the piriform cortex during odor-driven social learning.

INTRODUCTION

Animals continuously interact with conspecifics throughout their lifetime. Some of these interactions, like mating and aggression, are crucial for the survival of an individual and the propagation of species, while other interactions are behaviorally insignificant. However, how animals classify these social events as salient has not been extensively explored.

A set of sensory stimuli elicits stereotyped innate behaviors (Tinbergen, 1951). These stimuli are thought to be processed through developmentally determined, highly specific neural pathways connecting sensory inputs to behavioral outputs (Choi et al., 2005; Dulac and Wagner, 2006; Haga-Yamanaka et al., 2014; Holy et al., 2000; Hong et al., 2014; Li and Liberles, 2015; Stowers et al., 2002). Behavioral significance can also be imposed on sensory stimuli through experience. Learned behaviors, especially in social contexts, may be as important as innate behaviors, as they allow animals to maximize their chance of survival and reproduction (Beny and Kimchi, 2014; Pfaus et al., 2001). For instance, it would be crucial to remember cues predictive of encountering potential mates or aggressive individuals.

Oxytocin (Oxt) is a neuropeptide produced by neurons in the paraventricular nucleus (PVH), the medial preoptic nucleus (MPO), and the supraoptic nucleus (SON) of the hypothalamus (Landgraf and Neumann, 2004; Sofroniew, 1983; Swanson and Sawchenko, 1983). There is a single known oxytocin receptor (Oxtr), a seven-transmembrane protein, that is functionally coupled to G proteins (Gimpl and Fahrenholz, 2001). Oxt is released into the circulatory system and promotes contractions of the uterus during parturition and milk production. Oxt is also directly released within the brain, where it has been implicated in various social behaviors (Insel, 2010; Lee et al., 2009; Ross and Young, 2009; Stoop, 2012). Genetically removing or pharmacologically inactivating the Oxt-Oxtr pathway produces deficits in maternal behavior (Bosch and Neumann, 2012; Pedersen and Prange, 1979; Takayanagi et al., 2005), social recognition (Ferguson et al., 2000; Ferguson et al., 2001; Lee et al., 2008; Takayanagi et al., 2005), and social reward (Dölen et al., 2013). Despite its expansive involvement in social behaviors, whether Oxt plays a crucial role in associative learning in social contexts has not been addressed.

The ubiquity of olfactory-guided social behaviors across vertebrate (Isogai et al., 2011; Kaur et al., 2014; Leybold et al., 2002; Liberles, 2014; Lin et al., 2005; Pfaus et al., 2001; Stowers et al., 2002) and invertebrate species (Li and Liberles, 2015; Michener, 1974; Sokolowski, 2010; Suh et al., 2004) strongly suggests that olfactory systems are important for recording and processing social information. Thus, in this study, we used odor-driven behavioral paradigms that capture the essence of social learning—the pairing of an olfactory conditioned stimulus (CS) with a social unconditioned stimulus (US)—to investigate the role of Oxt in social learning. Our experiments reveal that Oxt is selectively required for social learning but is dispensable for learning tasks that do not involve social cues. Also, optogenetic activation of Oxt+ neurons promotes social learning with a nonsalient social stimulus. Furthermore, we show that Oxtr signaling in the piriform, the olfactory sensory cortex, is necessary to entrain initially neutral sensory representations to social cues. Finally, we demonstrate that Oxt is required for aversive as well as appetitive social learning. These results suggest that Oxt conveys saliency of social stimuli (US) to sensory representations (CS) in the piriform cortex during social learning.
RESULTS

Oxytocin Receptor Signaling Is Required for Social Learning

In order to assess the role of Oxt in social learning, we used an odor-driven appetitive social learning paradigm (Choi et al., 2011) in which an initially neutral odor served as a conditioned stimulus (CS) and was paired with a socially rewarding unconditioned stimulus (US). During training, male mice were allowed to explore a three-chambered arena, housing a female in a wire cage on one side and an empty wire cage on the other side (Figure 1A). CS+ odor or CS- odor was delivered when the subjects explored the female-containing wire cage or the empty cage, respectively. The subject’s preference for the CS+ or CS- odor was subsequently tested in the same arena in the absence of a female.

We initially asked if females in estrus phase serve as a more potent (attractive) social cue. Subject males were entrained to either females in reproductively receptive (proestrus or estrus, P/E) or nonreceptive phases (diestrus phase, D). During testing, males trained with proestrus/estrus females (P/E) preferred the chamber with the CS+ odor 3-fold more than the CS- chamber (Figures 1B and 1C) (CS+ chamber = 189.34 ± 20.74 s; center = 50.56 ± 13.22 s; CS- chamber = 60.13 ± 11.68 s, n = 6). On the other hand, males trained with diestrus females (D) spent an approximately equal amount of time in the CS+ and CS- chambers (Figures 1B and 1C) (CS+ chamber = 101.46 ± 13.62 s; center = 65.57 ± 9.58 s; CS- chamber = 132.88 ± 15.21 s, n = 7). This could not be attributed to any differences in the time spent investigating estrus versus diestrus females during training, since males spent a similar amount of time investigating females regardless of their estrus phase (see Figure S1A available online) (for P/E, female = 16.23 ± 1.25 s, empty = 5.26 ± 0.53 s, n = 4; for D, female = 18.10 ± 2.12 s, empty = 2.60 ± 0.78 s, n = 4). Thus, estrus phase does not influence innately driven approach behavior toward females, but impacts socially learned approach behavior toward the conditioned stimulus.

We next asked if oxytocin receptor (Oxtr) signaling is required for social learning using estrus females as a social cue. We blocked Oxtr signaling by administering L-368,899, a nonpeptidergic Oxtr antagonist that penetrates the blood-brain barrier (OTA, 5 mg/kg, intraperitoneally) (Boccia et al., 2007; Williams et al., 1994), before training and/or testing. Males injected with saline (Figures 1D and 1E) (S-S: CS+ chamber = 184.07 ± 13.79 s; center = 36.73 ± 8.79 s; CS- chamber = 79.10 ± 9.11 s, n = 6) or the antagonist before testing only (S-OTA: CS+ chamber = 163.61 ± 11.96 s; center = 52.58 ± 12.48 s; CS- chamber = 82.37 ± 4.99 s, n = 6) maintained a preference for the CS+ chamber. On the contrary, subject animals injected with Oxtr antagonist before both training and testing failed to exhibit a preference and spent a similar amount of time in the CS+ and CS- chambers (Figures 1D and 1E) (OTA-OTA: CS+ chamber = 109.26 ± 17.96 s; center = 78.69 ± 22.57 s; CS- chamber = 112.08 ± 20.19 s, n = 6). This deficit does not reflect potential effects of oxytocin receptor antagonist on innately driven approach behavior toward social cues. Animals treated with saline (S-S) or antagonist

Figure 1. Oxytocin Receptor Signaling Is Required for Social Learning
(A) Schematic of the odor-driven appetitive social learning paradigm. During training, the CS+ odor (purple shade) was presented when the male subject approached the wire cage containing a female (♀), while the CS- odor (orange shade) was presented when the subject approached the empty wire cage. During testing, the CS+ or the CS- odor was presented without females when the subject entered the randomly predetermined CS+/CS- chamber. (B) Time spent in each chamber during the 5 min testing period after training with proestrus and estrus (P/E) or diestrus females (D) as unconditioned stimuli. **p < 0.01 by Tukey HSD post hoc test. (C) Preference score (difference in time spent in the CS+ and CS- compartments) for data presented in (B). **p < 0.01 by Student’s t test. (D) Time spent in each chamber for subjects injected with saline before training and testing (S-S), with Oxtr antagonist (OTA) before training and testing (OTA-OTA), or with saline before training and OTA before testing (S-OTA). HBT, habituation. **p < 0.01 by Tukey HSD post hoc test. (E) Preference score for data presented in (D). *p < 0.05 and **p < 0.01 by Tukey HSD post hoc test. Data are presented as mean ± SEM.
(OTA-OTA) spent an approximately equal amount of time investigating females (Figure S1B) (average investigation time per trial: for S-S, female = 14.15 ± 1.85 s/trial, empty = 4.67 ± 0.71 s/trial; for OTA-OTA, female = 16.58 ± 1.08 s/trial, empty = 4.38 ± 0.54 s/trial, n = 6 each). Animals in both groups also traveled a similar distance during testing (Figure S1C) (for S-S, 2.01 ± 0.23 m; for OTA-OTA, 2.12 ± 0.37 m, n = 6 each). These data demonstrate that Oxtr signaling is necessary for association of an initially neutral odor with a rewarding social cue.

**Oxytocin Receptor Signaling Is Dispensable for Nonsocial Learning**

We next asked if Oxtr signaling is also required for association of odor with a rewarding nonsocial cue. The social learning paradigm was modified such that males were exposed to the CS+ odor in the presence of a nonsocial reward: sucrose solution or palatable food (Figure 2A). In the scheme with sucrose solution as the reward, saline-injected animals spent 2.5-fold more time in the CS+ chamber than in the CS− chamber (Figures 2B and 2C) (S-S: CS+ chamber = 172.22 ± 26.15 s; center = 58.41 ± 22.29 s; CS− chamber = 68.17 ± 13.23 s, n = 7). This preference was maintained in animals treated with Oxtr antagonist (OTA-OTA: CS+ chamber = 160.78 ± 11.43 s; center = 53.06 ± 8.61 s; CS− chamber = 86.17 ± 12.30 s, n = 7). Similarly, in the scheme with palatable food as the reward, the treatment with Oxtr antagonist did not impair the learned preference toward the CS+ chamber (Figures 2D and 2E) (S-S: CS+ chamber = 172.22 ± 26.15 s; center = 58.41 ± 22.29 s; CS− chamber = 68.17 ± 13.23 s, n = 7; OTA-OTA: CS+ chamber = 123.92 ± 19.59 s; center = 64.10 ± 18.50 s; CS− chamber = 52.18 ± 6.25 s, n = 5).

We also asked if Oxtr signaling is required for association of odor with an aversive nonsocial cue. During training, delivery of the CS+ odor was followed by a mild foot shock presented only on the side where the subject was located, allowing the subject to escape toward the opposite side of the chamber (Figure 2F). During testing, flight behavior in response to the CS+ odor was measured in the absence of shock. Both saline- and
OTA-injected animals robustly avoided the CS+ odor (Figure 2G) (S-S: 86.0% ± 7.1%, n = 10; OTA-OTA: 75.6% ± 8.7%, n = 9). These results collectively suggest that Oxtr signaling is required for social learning but is dispensable for other associative learning tasks that do not involve social cues.

Optical Activation of Oxt+ Neurons in the PVH Promotes Social Learning

We next asked if stimulation of Oxt+ neurons is capable of promoting social learning. We generated a knockin mouse line to gain genetic access to Oxt+ neurons, by targeting Cre recombinase to the 3’ end of the Oxt coding sequence (Figure 3A). To exogenously activate Oxt+ neurons in these OxtCre+ mice, we then delivered adeno-associated virus (AAV) driving Cre-dependent ChR2 fused to EYFP (ChR2:EYFP) under control of the EF1α promoter into the PVH (Figure 3B), a major source of centrally released Oxt (Landgraf and Neumann, 2004). Double labeling experiments confirmed that Cre expression recapitulates endogenous expression of Oxt; the majority of ChR2:EYFP-expressing neurons coexpressed OXT (Figures 3C and 3D) (91.5% of 255 cells from two mice). Furthermore, analysis of c-Fos expression, a marker of neuronal activation, confirmed photostimulation-dependent activation of ChR2-expressing neurons. We observed that 42% of cells expressing ChR2:EYFP also expressed c-Fos (Figures 3D and 3E) (ChR2: 42.46% ± 5.23%, n = 6), whereas 9% of cells expressing EYFP were positive for c-Fos in control animals (EYFP: 9.72% ± 7.98%, n = 4).

We asked if diestrus females, which are normally incapable of driving learning in our behavioral scheme (Figures 1B and 1C), can serve as an effective US when supplemented with optogenetic
stimulation of Oxt+ neurons in subject animals. In this experiment, photostimulation of Oxt+ neurons was applied during training when animals were exposed to a diestrus female in the CS+ chamber. Testing was performed in the absence of both the female and photostimulation (Figure 3F). ChR2-expressing animals spent substantially more time in the CS+ chamber than in the CS− chamber (Figures 3G and 3H) (ChR2: CS+ chamber = 181.53 ± 18.57 s; center = 39.21 ± 7.03 s; CS− chamber = 78.73 ± 12.69 s, n = 8). In contrast, EYFP-expressing animals failed to exhibit a significant preference for the CS+ chamber (Figures 3G and 3H) (EYFP: CS+ chamber = 126.95 ± 10.84 s, center = 54.77 ± 9.97 s; CS− chamber = 112.30 ± 14.99 s, n = 8). Stimulation of Oxt+ neurons did not enhance investigation time toward diestrus females during training (Figure S2) (ChR2: female = 14.35 ± 1.97 s/trial, empty = 5.40 ± 1.44 s/trial, n = 7; EYFP: female = 18.53 ± 0.90 s/trial, empty = 5.47 ± 1.17 s/trial, n = 6). Furthermore, photostimulation of Oxt+ neurons did not generate reward-related effects in a real-time place preference test (RTPP) (Stamatakis and Stuber, 2012). In the RTPP arena, Oxt+ neuron stimulation occurred when animals were in one, but not in the neighboring, compartment (Figure 3I). Both ChR2-and EYFP-expressing animals spent a similar amount of time in the stimulated and unstimulated compartments (Figure 3J) (ChR2: stimulated = 640.00 ± 55.75 s, unstimulated = 560.04 ± 55.75 s, n = 10; EYFP: stimulated = 696.27 ± 68.73 s, unstimulated = 503.12 ± 68.62 s, n = 8), suggesting that stimulation of Oxt+ neurons in the PVH does not elicit approach or avoidance behavior. The foregoing experiments demonstrate that stimulation of Oxt+ neurons in the PVH facilitates entrainment of odor to a nonsalient social cue.

Oxt Neurons Project to the Piriform Cortex

Anatomical, physiological, and behavioral studies have suggested that the piriform cortex serves as a likely neural substrate for odor-driven associative learning (Choi et al., 2011; Ghosh et al., 2011; Illig and Haberly, 2003; Miyamichi et al., 2011; Poo and Isaacson, 2009; Sosulski et al., 2011; Stettler and Axel, 2006). We carried out fluorescent in situ hybridization for Oxtr mRNA to determine if Oxt acts on the piriform cortex. Dense Oxtr expression was observed in all three layers of the piriform cortex (Figure 4A) (layer 1 = 24% ± 6% of total DAPI+ nuclei; layer 2 = 64% ± 4%; layer 3 = 48% ± 2%, n = 3), in accordance with a previous autoradiography study (Yoshida et al., 2009). Oxtr expression was also evident in other olfactory centers, such as the glomerular layer of the main olfactory bulb (MOB) (Figure 4B), the olfactory tubercle (Tub) (Figure 4C), and the cortical amygdala (CoA) (Figure 4D).

We next asked if Oxt+ neurons in the hypothalamus directly project to the piriform cortex. We labeled Oxt+ neurons by injecting AAV encoding Cre-dependent ChR2:EYFP into the PVH of OxtCre+ mice (Figure 4E) and examined the piriform cortex for the presence of labeled fibers. In these animals, sparse EYFP+ fibers originating from Oxt+ neurons were found in the piriform cortex (Figures 4F and 4G). We also injected cholera toxin B subunit (CTB), a retrograde tracer, into the piriform (Figure S3). CTB was detected in Oxt+ neurons in the anterior PVH (aPVH) and the medial preoptic area (MPO) (Figure 4H), but not in Oxt+ neurons in the posterior PVH (pPVH) (Figure 4I) or the supraoptic nucleus (SON) (Figure 4J). These results demonstrate that the piriform cortex contains a dense population of Oxtr-expressing neurons and receives direct input from Oxt+ neurons in the aPVH/MPO.

Oxtr Expression in Piriform Ensembles Is Necessary for Appetitive Social Learning

Based on the foregoing observations in conjunction with the suggested role of the piriform in odor-driven learning, we hypothesized that Oxtr signaling in the piriform is involved in entrainment of odor to social cues. However, the large size of the brain structure and lack of piriform-specific Cre driver lines pose a technical challenge to interrogating its function in social learning. Thus, we used exogenously activated piriform neural ensembles (Choi et al., 2011) to ask if Oxtr expression is required in the piriform cortex for odor-driven social learning. Activation of these ensembles expressing ChR2, like odors, can be entrained to elicit appetitive or aversive behavior when associated with reward or punishment.
We virally introduced ChR2 in layer 2 and 3 of the piriform cortex. We coinjected AAV driving Cre-dependent ChR2:tdTomato with lentivirus encoding Cre from the human Synapsin1 promoter. This dual virus strategy was applied to either wild-type (WT) or Oxtrf/f mice, in which the Oxtr coding region is conditionally deleted in a Cre-dependent manner (Lee et al., 2008). This generated Oxtr-intact ChR2-expressing ensembles in WT (WT-ChR2) and Oxtr-deficient ChR2-ensembles in Oxtrf/f mice (Oxtrf/f-ChR2), respectively (Figure 5A). In Oxtrf/f-ChR2 mice, the level of Oxtr mRNA was significantly reduced in ChR2-expressing neurons (ChR2+) compared to neighboring noninfected neurons (ChR2−) (Figures S4A and S4B) (normalized intensity of Oxtr mRNA: ChR2− = 100.00 ± 9.24; ChR2+ = 37.08 ± 3.84, n = 167 cells from three mice for ChR2− and 72 cells from three mice for ChR2+). ChR2 expression was detected in approximately 5%–10% of the neurons in a localized region (Figures 5B and 5C) (percentage of ChR2-expressing neurons: WT = 6.32% ± 1.35%; Oxtrf/f = 4.53% ± 1.18%, n = 11 for WT and 6 for Oxtrf/f). Analysis of c-Fos expression revealed that ChR2 ensembles both in WT and Oxtrf/f mice were activated upon photostimulation (Figures 5B and 5C) ([c-Fos+ ChR2+]/[ChR2+] = 60.73% ± 4.52%; Oxtrf/f-ChR2 = 66.26% ± 5.06%, n = 11 for WT-ChR2 and 6 for Oxtrf/f-ChR2).

We first confirmed that ChR2-expressing piriform ensembles, just like odorants, require Oxtr signaling for social learning by
entRAINING THESE ENSEMBLES IN THE PRESENCE OR ABSENCE OF OXTR ANTAGONIST. DURING TRAINING, WE REPLACED THE CS+ OdOR WITH PHOTOSTIMULATION such that ChR2 ensembles were activated when males investigated the females (Figure 5D). Males injected with saline prior to training spent significantly more time in the CS+ chamber (Figures 5E and 5F) (CS+ chamber = 220.53 ± 22.16 s; center = 25.94 ± 9.31 s; CS− chamber = 53.08 ± 15.24 s, n = 5). The observed preference for CS+ chamber was independent of the anterior-posterior position of the ChR2-ensemble within the piriform (Figure S4C). Males injected with Oxtr antagonist, however, failed to exhibit preference for the CS+ chamber and spent a similar amount of time in the CS+ and CS− chambers (Figures 5E and 5F) (CS+ chamber = 108.62 ± 35.03 s; center = 44.17 ± 14.15 s; CS− chamber = 147.23 ± 30.52 s, n = 6). Our results are not explained by deficits intrinsic to Oxtr-deficient mice, since Oxtr−/− animals injected with control virus encoding EGFP were still capable of odor-driven social learning (Figures S4D and S4E) (CS+ chamber = 170.15 ± 3.18 s; center = 45.40 ± 11.19 s; CS− chamber = 82.87 ± 11.43 s, n = 4). In contrast to entrainment to social cues, entrainment of ChR2-expressing ensembles to nonsocial cues (i.e., palatable food) was affected neither by systemic Oxtr signaling blockade nor by ChR2-ensemble specific knockout of Oxtr (Figures 5G and 5H) (WT-ChR2-S: CS+ chamber = 121.09 ± 5.78 s; center = 42.62 ± 8.53 s; CS− chamber = 76.29 ± 8.21 s, n = 8; WT-ChR2-OTA: CS+: 125.97 ± 13.84 s; center = 53.66 ± 11.78 s; CS− chamber = 60.37 ± 7.40 s, n = 7; Oxtr−/−ChR2-S: CS+: 135.06 ± 15.74 s; center = 30.56 ± 8.45 s; CS− chamber = 74.38 ± 11.43 s, n = 5). Therefore, these results demonstrate that representations in the piriform cortex require Oxtr expression specifically for entrainment to social cues.

**Oxytocin Receptor Signaling Is Required for Aversive Social Learning**

Our data suggest that Oxt is crucial for appetitive social learning. We next asked if Oxt is also required for learning with aversive social cues. We designed an odor-driven aversive social learning paradigm, in which odor served as a CS and was paired with an aversive social US (Figure 6A). Prior to training, male mice were introduced into the home cage of a CD1 male, an aggressive strain of mouse, and allowed to interact in a resident-intruder scheme. This social encounter was characterized by frequent attacks directed toward the intruding subject mouse. During training, subjects were confined to one of the side compartments in the three-compartment arena, while they were exposed to odor in the presence or absence of the previously encountered CD1 male. After interleaved CS+ (CS+ odor with the CD1 male in a wire cage) and CS− trials (CS− odor with an empty wire cage), the subjects were tested in the same arena, which contained only the CS+ and CS− odors. Subjects that directly interacted with an aggressive CD1 prior to training avoided the CS+ chamber (Figures 6B and 6C) (CS+ chamber = 125.97 ± 13.84 s; center = 101.58 ± 10.66 s; CS− chamber = 138.37 ± 12.65 s, n = 7), while the subjects that had not been exposed to the CD1 prior to training spent an approximately equal amount of time in the CS+ and CS− chambers (Figures 6B and 6C) (CS+ chamber = 93.52 ± 15.47 s; center = 96.05 ± 17.31 s; CS− chamber = 100.74 ± 10.05 s, n = 6). Thus, odor can be entrained to an aversive social stimulus to produce avoidance behavior, and this learning requires an aggressive encounter prior to training in our behavioral scheme.

We next asked if Oxtr signaling is required for aversive social learning. Subjects were injected with either saline or Oxtr antagonist after interaction with the CD1, but before training. While subjects injected with saline avoided the CS+ chamber (Figures 6D and 6E) (CS+ chamber = 63.57 ± 4.10 s; center = 91.04 ± 9.34 s; CS− chamber = 144.39 ± 11.00 s, n = 7), animals injected with antagonist did not exhibit avoidance of the CS+ chamber (Figures 6D and 6E) (CS+ chamber = 108.96 ± 14.95 s; center = 80.74 ± 8.43 s; CS− chamber = 106.18 ± 18.01 s, n = 6). We quantified the fraction of time the subject spent away from the pencil cup to assess avoidance of the CD1 during training. Avoidance of the CD1 was indistinguishable between animals injected with saline or Oxtr antagonist (Figure S5) (S: avoidance score = 0.82 ± 0.03, n = 7; OTA: avoidance score = 0.78 ± 0.06, n = 6). These results suggest that Oxtr signaling does not influence the avoidance of the CD1, but rather it is necessary for entrainment of odor to an aversive social cue.

To test whether Oxtr signaling in the piriform is involved in aversive social learning, we entrained Oxtr-intact (WT-ChR2) and Oxtr-deficient (Oxtr−/−-ChR2) ChR2 ensembles to a CD1. Males injected with saline avoided the CS+ chamber (Figures 6F and 6G) (CS+ chamber = 110.37 ± 6.73 s; center = 44.12 ± 7.63 s; CS− chamber = 144.02 ± 7.37 s, n = 10), although to a lesser extent than what was observed with odor. However, subjects injected with Oxtr antagonist, rather than avoiding the CS+ chamber, spent more time in the CS+ chamber (CS+ chamber = 148.45 ± 12.12 s; center = 32.70 ± 8.82 s; CS− chamber = 94.49 ± 9.78 s, n = 6). Furthermore, Oxtr−/− animals harboring Oxtr-deficient ensembles failed to avoid the CS+ chamber (Figures 6F and 6G) (CS+ chamber = 133.92 ± 7.91 s; center = 48.18 ± 6.83 s; CS− chamber = 115.67 ± 6.93 s, n = 7). These results demonstrate that representations in the piriform cortex require Oxtr expression for entrainment to aversive social stimuli.

**DISCUSSION**

We asked if Oxt plays a crucial role in promoting odor-driven social learning by pairing an initially neutral olfactory stimulus with social cues. We show that Oxt is required for entrainment of odor to salient social cues, and capable of promoting entrainment of odor to nonsalient social cues. Oxt signaling carries out these functions by acting on the piriform cortex. Furthermore, we show that Oxt signaling is required for aversive as well as for appetitive social learning. Taken together, these results suggest that Oxt conveys social saliency of unconditioned stimuli to sensory representations in the piriform cortex during odor-driven social learning (Figure 7).

Oxt has been widely viewed as a molecule promoting prosocial behaviors or transmitting positive value of social interactions (Insel, 2010; Lee et al., 2009; Ross and Young, 2009). Consistent
with these notions, we show that Oxt is required for entrainment of an odor to an appetitive social cue. The role of Oxt is not limited to appetitive social learning, but rather it is also involved in entrainment of an odor to an aversive social cue. Our results indicate that Oxt mediates social learning of opposing valence, depending on the context. This is consistent with previous observations that Oxt is released by both mating and aggressive encounters in rodents (Ebner et al., 2000; Engelmann et al., 1999; Waldherr and Neumann, 2007).

Behavioral changes as a consequence of learning occur following conspicuous social interactions; females in estrus, but not in diestrus, served as an effective US in our learning paradigm. Furthermore, CD1 stimulus males, when presented without prior resident-intruder encounter, failed to drive learning in our aversive social scheme. However, females in diestrus, when supplemented with optogenetic activation of Oxt+ neurons in the subject animals, were capable of producing learned approach behavior. These data, taken together with the observation that Oxt mediates social learning of opposing valence, suggest that Oxt may convey saliency of social encounters. This model may explain the paradoxical observations made in humans that exogenous administration of Oxt improves social cognition and increases prosocial behavior in positive contexts (Guastella et al., 2008; Kosfeld et al., 2005; Rimmele et al., 2009) but promotes antisocial effects in negative contexts (Bartz et al., 2011; Bartz and Hollander, 2006; Heinrichs et al., 2009).

The notion that Oxt may facilitate opposing behavioral responses dependent upon the appetitive or aversive nature of the US raises the question of how Oxt accomplishes this context-dependent function. In one model, context of social encounter may be conveyed through distinct neural pathways (Gunaydin et al., 2014), parallel to the Oxt system. In an alternative but not mutually exclusive model, Oxt itself may provide contextual information by acting on distinct brain regions. For instance, the resident-intruder encounter assay has been shown

**Figure 6. Oxt Signaling Is Required for Aversive Social Learning**

(A) Schematic of odor-driven aversive social learning. After resident-intruder interaction, subjects were confined to one side chamber and presented with the CS+ odor and the wire cage containing the CD1 (elongated) that served as resident. The CS – odor was presented with an empty wire cage. For testing, the CS+ or the CS – odor was presented, without the CD1, when the subject entered the randomly predetermined CS+/CS– chamber.

(B) Time spent in each chamber during testing, with resident-intruder pretraining (RI), or without pretraining (No RI). ***p < 0.001 by Tukey HSD post hoc test.

(C) Preference score for data presented in (B). **p < 0.01 by Student’s t test.

(D) Time spent in each chamber for subject mice injected with saline (S) or with OTA (OTA). (E) Preference score for data presented in (D). *p < 0.05 by Student’s t test.

(F) Time spent in each chamber for WT-ChR2 mice injected with S or OTA, and Oxtr/f–/f-ChR2 mice injected with S. *p < 0.05, **p < 0.01 by Tukey HSD post hoc test.

(G) Preference score for data presented in (F). *p < 0.05 by Tukey HSD post hoc test.
to induce Oxt release into the septum (Ebner et al., 2000), while Oxt in the ventral striatum has been shown to encode positive value of social interactions (Dölen et al., 2013). In the latter scenario, the Oxt+ neuronal population could be comprised of heterogeneous subpopulations that differentially process either positive or negative social stimuli.

If Oxt conveys saliency of social encounters, it may be dispensable for learning that involves nonsocial unconditioned stimuli. This stimulus selectivity is observed upon inhibiting Oxt signaling; Oxt antagonist treatment impairs entrainment of odor to males and females, but not to sucrose solution, palatable food, or mild foot shock. These results are consistent with social-specific effects of Oxt previously reported in other rodent studies (Dölen et al., 2013; Ferguson et al., 2000). Moreover, in humans, intranasal administration of Oxt improved memory of faces, but not of nonsocial objects (Rimmele et al., 2009). These observations suggest that the social-specific effect of Oxt may be conserved across mammalian species.

Where does Oxt operate to permit odor-driven social learning? Anatomical and physiological studies have suggested that the piriform cortex, one of the recipients of olfactory information, is a neural substrate for odor-driven associative learning (Ghosh et al., 2011; Illig and Haberly, 2003; Miyamichi et al., 2011; Poo and Isaacson, 2009; Sosulski et al., 2011; Stettler and Axel, 2009; Giessel and Datta, 2014). Consistent with this notion, optogenetic activation of arbitrarily chosen ensembles of piriform neurons, just like odorants, could be entrained to various unconditioned stimuli to produce both appetitive and appetitive learned behaviors (Choi et al., 2011). These results demonstrate that representations in the piriform cortex are sufficient to drive learned behaviors. On the other hand, another recipient of olfactory information, the cortical amygdala, recently has been identified as a neural substrate for odor-driven innate behaviors (Root et al., 2014). We show here that ChR2-expressing piriform ensembles require Oxt expression for entrainment to social cues. This observation, in conjunction with the tracing experiments (Figures 4E–4J), suggests that Oxt directly impacts the piriform cortex to permit odor-driven social learning. Considering the neuromodulatory action of Oxt and the distribution of oxytocin receptor reported in other brain regions (Owen et al., 2013; Marlin et al., 2015), Oxt’s role in social learning may be generalized across different sensory modalities.

Oxt is expressed at multiple nodes along both the main and accessory olfactory pathways, as well as in brain regions implicated in generation of motivated behaviors (Stoop, 2014). It is plausible that Oxt operates at additional nodes of the olfactory pathways to support odor-driven social learning. For example, Oxt has been shown to act on the medial amygdala to mediate social recognition (Ferguson et al., 2001). In general, Oxt is poised to influence social learning at multiple loci from perception of both conditioned and unconditioned stimuli to behavioral output. How Oxt coherently orchestrates these distributed circuits to produce social learning remains to be determined.

**EXPERIMENTAL PROCEDURES**

**Stereotaxic Injection and Fiber Optic Implantation into the Mouse Brain**

Strains of mice used for experiments were wild-type C57BL/6J, CD-1, Oxt<sup>Cre</sup>−/+ and Oxt<sup>Δf</sup>. The surgeries were carried out using aseptic techniques. Stereotaxic injections of viruses were made at the rate of <0.1 μl/min at layers 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 PVH (AP = −1.0 mm, ML = 0.0 mm, DV = −3.9 mm). The tip of the fiber optic implant was subsequently placed 300–400 μm above the virus injection site. All animal procedures were approved by the Committee on Animal Care of Massachusetts Institute of Technology.

**Tissue Slice Preparation and Immunohistochemistry**

The prepared slices were labeled with the following primary antibodies: chicken anti-GFP (Abcam, ab5450, 1:1,000), 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, AB5296, 1:1,000), and rabbit anti-oxytocin (Millipore, AB911, 1:1,000). 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 A1055), Alexa 647 donkey anti-mouse (Invitrogen, A31571), Alexa 633 goat anti-rabbit (Invitrogen, A21071), and Alexa 568 goat anti-rabbit (Invitrogen, A10136). 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.

For quantitative analysis of photostimulation-dependent activation of ChR2- and EYFP-expressing neurons in Oxt<sup>Cre</sup>−/+ mice, animals were photostimulated (30 s per min for 10 min) 1 hr 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 Oxt<sup>Δf</sup>−/ChR2 mice were photostimulated (30 s per min for 10 min) 1 hr 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 × 500 μm area around the center of injection site. The number of c-Fos+ ChR2+ neurons was counted within the same 500 × 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 ChrR2 fused to EYFP was targeted to the PVH (AP, −1.0 mm; ML, 0.0 mm; DV, −3.9 mm) of adult male OxtCre+/− mice. Following a 2 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 4 days of survival time, 50 μm sections were cut and costained for CTB and Oxt for 48 hr. Sections were counterstained with secondary antibodies and with NT for 24 hr.

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). 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), that were to be used later, in each side chamber. C57BL/6J females were transferred to a clean cage 1 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 was 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 two 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) 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 s) in the center chamber were excluded from analysis.

For optogenetic stimulation of Oxt+ neurons, OxtCre+/− male mice were injected with virus encoding either Cre-dependent ChrR2 fused to EYFP, or Cre-dependent EYFP, into the PVH. After 2 weeks of recovery, animals were trained in our social-appetitive learning paradigm. During training, larval 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 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. Oxtacin receptor antagonist (L-368,899) was purchased from Tocris 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.

Nonsocial 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 six 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 libitum were trained with palatable foods (white chocolate, peanut butter mixed with Nutella, and NUTRI-CAL for ferrets) 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 seventh and eighth trials, the two foods that were highly investigated during previous trials were sequentially used as the US. If mice investigated both cues during the seventh and the eighth trials, the same foods were used during the ninth and the tenth trials in the same order. Otherwise, the food that was investigated the most during the seventh and the eighth trials was used for the final two trials. Testing was carried out for 4 min. Nonsocial appetitive 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 5 min. 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 5 min before training began. Training consisted of ten 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 5 min. 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 s) in the center compartment were excluded from analysis. Avoidance scores during training were calculated by dividing the amount of time in the half of the 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 s each. Baseline and testing trials were unchanged. Animals that spent greater than 100 s 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
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 min. 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.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.neuron.2015.06.022.

AUTHOR CONTRIBUTIONS
H.K.C. and M.D.R. designed the experiments, conducted the experiments, analyzed the data, and wrote the manuscript. N.B., D.M., N.S., and Y.S.Y.
conducted the experiments. G.B.C. conceived the project, designed the experiments, conducted the experiments, and wrote the manuscript.

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