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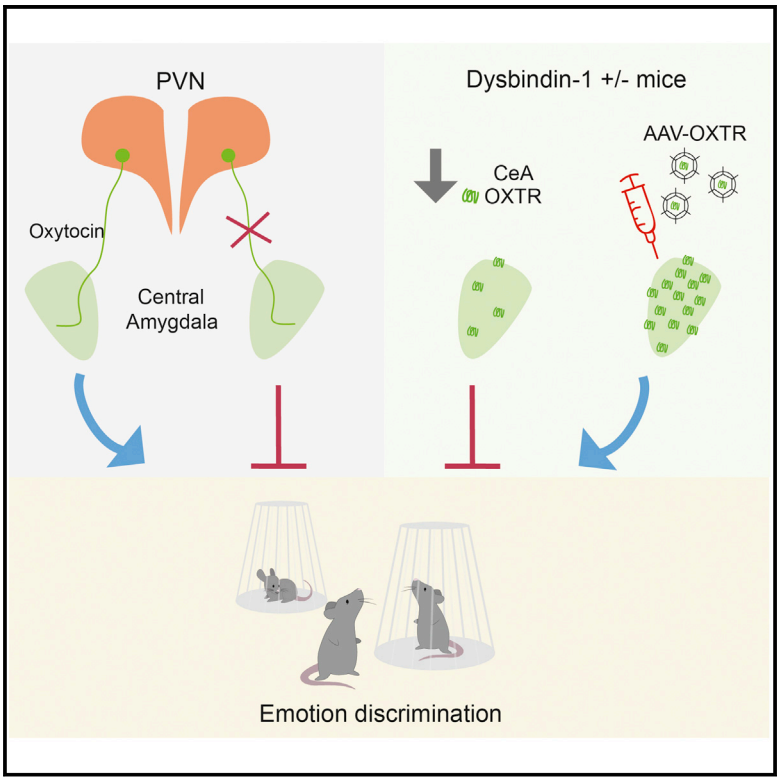
Oxytocin Signaling in the Central Amygdala Modulates Emotion Discrimination in Mice

Graphical Abstract



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In Brief

Is endogenous oxytocin implicated in emotion discrimination? Ferretti, Maltese et al. demonstrate that oxytocin signaling in the central amygdala plays a key role in the ability of mice to discriminate unfamiliar conspecifics based on their emotional state, both in physiological and genetically determined pathological conditions.

Highlights

- Mice discriminate unfamiliar conspecifics based on altered states (fear or relief)
- Endogenous OXT release from PVN to CeA is necessary for emotion discrimination
- Abolishing PVN OXT release to Nac, PFC, or CA2 does not affect emotion discrimination
- Dysbindin-dependent emotion discrimination deficits are caused by reduced CeA OXTRs

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Oxytocin Signaling in the Central Amygdala Modulates Emotion Discrimination in Mice

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SUMMARY

Recognition of other's emotions influences the way social animals interact and adapt to the environment. The neuropeptide oxytocin (OXT) has been implicated in different aspects of emotion processing. However, the role of endogenous OXT brain pathways in the social response to different emotional states in conspecifics remains elusive. Here, using a combination of anatomical, genetic, and chemogenetic approaches, we investigated the contribution of endogenous OXT signaling in the ability of mice to discriminate unfamiliar conspecifics based on their emotional states. We found that OXTergic projections from the paraventricular nucleus of the hypothalamus (PVN) to the central amygdala (CeA) are crucial for the discrimination of both positively and negatively valenced emotional states. In contrast, blocking PVN OXT release into the nucleus accumbens, prefrontal cortex, and hippocampal CA2 did not alter this emotion discrimination. Furthermore, silencing each of these PVN OXT pathways did not influence basic social interaction. These findings were further supported by the demonstration that virally mediated enhancement of OXT signaling within the CeA was sufficient to rescue emotion discrimination deficits in a genetic mouse model of cognitive liability. Our results indicate that CeA OXT signaling plays a key role in emotion discrimination both in physiological and pathological conditions.

INTRODUCTION

Social interactions are influenced by the ability to decipher expressions of emotions in others [1–3]. Disturbances in this capacity, defined as "social cognition" [2, 3], represent a distinctive feature of many psychiatric, neurodevelopmental, and neurodegenerative disorders [3]. For instance, abnormalities in social cue identification define autism spectrum disorders [4, 5]. Similarly, patients with schizophrenia show marked impairments in the processing of non-verbal social affective information while showing normal affect sharing and emotional experience [6]. Despite the deleterious impact on the everyday life of these subjects [7], social cognitive impairments still lack an effective treatment.

The oxytocin (OXT) system is considered a major player in social information processing and social cognition [8–10]. Rodent studies implicate the OXT system in a number of social domains, such as processing of sensory stimuli, social recognition [11–16], social memory [17, 18], consolation [19], social reward [20, 21], response to fear [22–24], and sexual and parental behaviors [25, 26]. Intranasal administration of OXT in humans, despite controversial and variable effects [27–32], has been reported to modulate recognition of emotions, empathy, and trust [8, 9, 33–39]. Genetic-association studies also support an implication of the OXT system in emotion processing [10, 40, 41]. However, the role of the endogenous OXT system and its potential modulation by genetic background in the perception and processing of other's emotions is still underexplored.

Here, in line with increasing evidence that higher-order social emotional processes can be studied in rodents [42], we implemented a behavioral setting that could approximate some features of human emotion recognition tasks [3, 43] or similar tasks used in non-human primates, dogs, sheep, and horses [44–47], to dissect the implication of the endogenous OXT system in emotion discrimination. In particular, using a chemogenetic approach, we explored the role of selected OXT projections from the paraventricular nucleus of the hypothalamus (PVN) in the ability of mice to discriminate unfamiliar conspecifics based on negatively or positively valenced emotional states. Furthermore, to start investigating the potential modulation by genetic background, we tested how a genetic variant (in dysbindin-1) with clinical relevance for cognitive and psychiatric liability [48–51] might modulate emotion discrimination through selective alterations in the endogenous OXT system.

Together, our results revealed an essential role of the PVN-CeA OXT pathway in the discrimination of the emotions of others

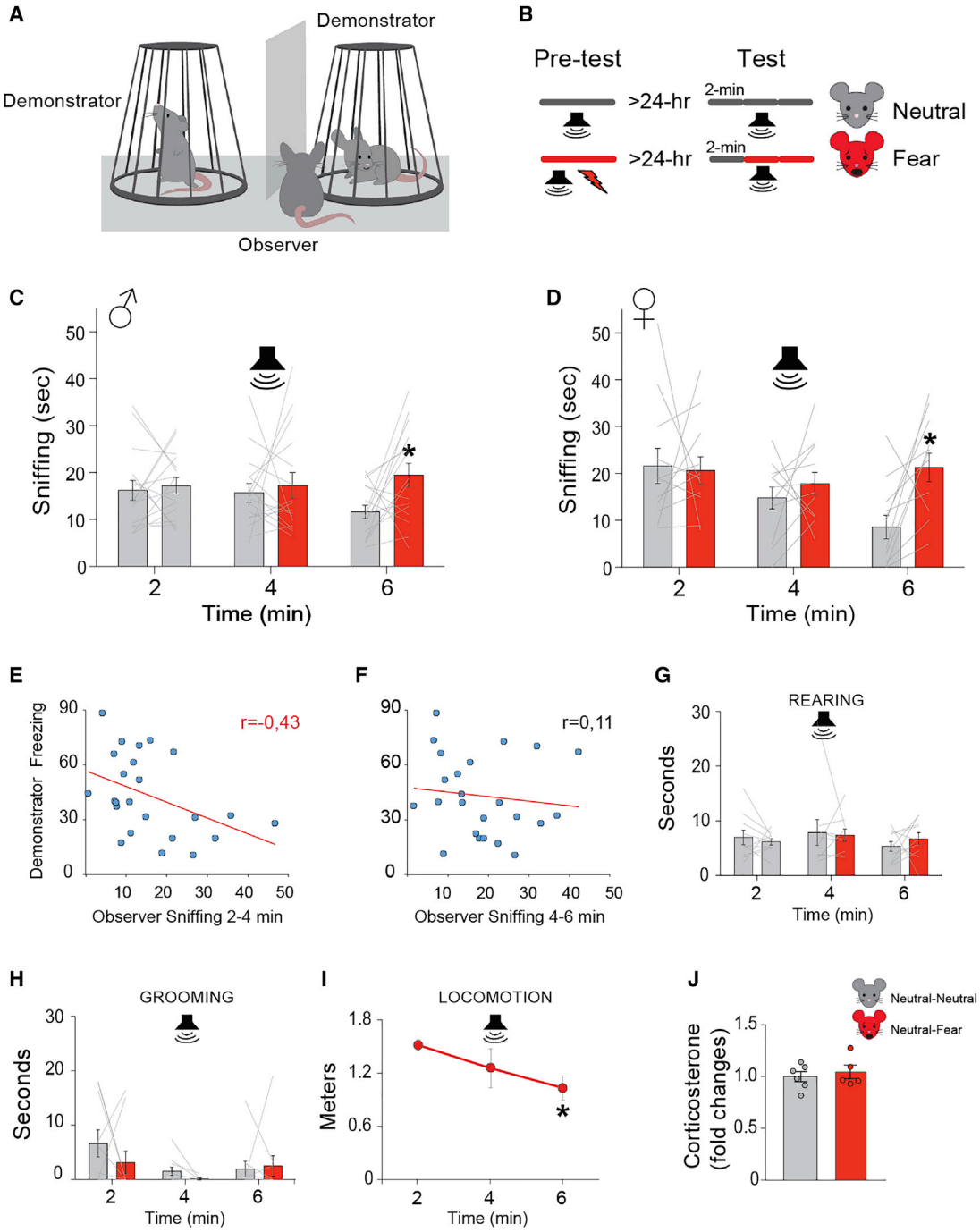

Figure 1. Mouse Emotion Discrimination for Fear

(A) Schematic drawing of the test setting.

(B) Timeline of pre-test and test procedures to evoke in one of the demonstrators a "fear" state by delivering the conditioned tone in the 2- to 4-min epoch.

(C and D) Time (in seconds) spent sniffing demonstrators in neutral (gray bars) or tone-induced fear (red bars) state displayed by (C) male and (D) female observer mice during the 6-min test, divided into three consecutive 2-min epochs (last 2-min repeated measurements [RM] ANOVA for males $F_{1,15} = 6.51$, $p = 0.022$, and females $F_{1,11} = 10.98$, $p = 0.006$; no significant differences in the 0- to 2-min and 2- to 4-min epochs). *$p < 0.05$ versus the exploration of the neutral demonstrator. n = 8/15 observers per group.

(E and F) Correlation analyses between the time the fear-conditioned demonstrator spent freezing (in y axis) and the time the observer spent sniffing the fear-conditioned demonstrator (in x axis; E) in the 2- to 4-min epoch or (F) in the 4- to 6-min epoch of the test ($r = -0.4310$ for 2–4 min; $r = -0.11$ for 4–6 min). n = 24 observers.

(G and H) Time (in seconds) spent (G) rearing and (H) grooming in proximity of the demonstrators in neutral (gray bars) or fear states (red bars) displayed by the same observer mice during the test (RM ANOVAs showed no significant differences).

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and that genetic background and variation in OXTR expression within the CeA can moderate these effects.

RESULTS

Mice Discriminate Unfamiliar Conspecifics based on Negatively Valenced Emotional States

To test whether mice could discriminate unfamiliar conspecifics expressing different emotional states, we placed an ''observer'' mouse in a cage containing two age- and sex-matched unfamiliar conspecifics (''demonstrators''). The demonstrators were placed in wire cups to allow visual, tactile, auditory, and olfactory communication while avoiding aggressive or sexual interactions (Figure 1A). The task was thus centered on behaviors initiated by the observer when simultaneously exposed to a neutral demonstrator and to a mouse in an altered emotional state.

In the ''fear'' manipulation, one of the two demonstrators was fear conditioned to a tone cue at least 1 day before the test (Figure 1B). Upon presentation during the test, the tone would then evoke a negatively valenced emotional state in the conditioned mouse [52]. In particular, the tone was delivered during the second 2-min epoch of the test (Figure 1B) in order to assess observers' responses before, during, and after the induction of the altered emotional state in the demonstrators. Consistently, we observed a freezing response in the fear demonstrator only during the 2-min tone presentation, associated with a reduction in rearing (Figure S1A). No other behavioral parameters differed between the two demonstrators during the 6-min test session (Figure S1A).

Both male and female observers increased their sniffing, but not any other observable behavior, toward the fear-conditioned demonstrator compared to the neutral one (Figures 1C, 1D, 1G–1I, and S2A–S2C). This effect became evident after the 2-min tone presentation (Figures 1C, 1D, S2A, and S2C). Although no discriminatory behavior was observed during the tone presentation, we found an inverse correlation between the time the fear demonstrator spent freezing and the time of observer sniffing, suggesting that freezing per se might influence observer discrimination (Figure 1E). However, we found no correlation between the demonstrator freezing and the observer sniffing after the tone presentation, suggesting that demonstrator's freezing did not affect the discriminatory behavior we observed (Figure 1F).

In light of previous evidence [23, 53, 54], we searched for signs of fear transfer from the emotionally altered demonstrator to the observer by quantifying freezing behavior, escape attempts, changes in locomotor activity, and other stress-related behaviors (i.e., rearing and grooming). During the 6-min test, we detected no sign of emotion contagion (Figures 1G–1I). Moreover, corticosterone levels of observer mice exposed to the fear paradigm or to two neutral demonstrators did not differ (Figure 1J).

These findings suggest that mice can detect and socially respond to unfamiliar conspecifics in a negatively valenced emotional state.

Mice Discriminate Unfamiliar Conspecifics based on Positively Valenced Emotional States

We next investigated whether observer mice could discriminate unfamiliar conspecifics by detecting a positively valenced state. In particular, we exposed observer mice to a neutral demonstrator and to a demonstrator that received 1-h *ad libitum* access to water after 23 h of water deprivation (Figures 2A and 2B). Water was selected as a rewarding stimulus to avoid odor-related cues that could differentiate the two demonstrators. We assumed that the relief from the distressing water deprivation would result in a positively valenced emotional state (''relief''). Consistently, we found that the 1-h *ad libitum* access to water resulted in a conditional place preference in mice that experienced the 23 h water deprivation, but not in mice in *ad libitum* water condition (Figures 2E and 2F). Moreover, 1-h *ad libitum* access to water after the 23-h deprivation reduced corticosterone levels in relief mice (Figure 2G). Furthermore, the relief manipulation induced no detectable behavioral alteration during the test compared to neutral demonstrators (Figure S1B).

Observers of both sexes showed increased social exploration toward the relief demonstrator compared to the neutral, selectively in the first 2 min of the task (Figures 2C, 2D, S2B, and S2D). No changes in rearing and grooming patterns toward the demonstrators and throughout the task were evident (Figures 2H and 2I). Moreover, observers showed the typical decrease in locomotor activity (Figure 2J) and did not show freezing behavior, escape attempts, or other stress-related behaviors during the entire test session. Furthermore, no alteration in corticosterone levels was detected between observers exposed to relief-neutral or neutral-neutral demonstrators (Figure S2I).

These findings indicate that mice can detect and socially respond to unfamiliar conspecifics in a positively valenced emotional state.

Sensory Modalities Implicated in Emotion Discrimination

Different sensory modalities might mediate social responses to emotional stimuli in different animal species [1, 55]. We explored the implication of visual, auditory, and olfactory cues in mice emotion discrimination in our setting.

Preventing visual cues can reduce emotion contagion responses induced by the observation of a partner in distress [53, 54]. However, when we performed the test in complete darkness (Figure 3A), mice were still able to discriminate between a neutral and an emotionally altered conspecific (Figures 3B and 3C), similarly to that observed in standard lighting conditions (Figures 1 and 2). This suggests that mice can discriminate emotions even in the absence of visual cues. Notably, removal of visual cues anticipated observer discrimination of the fear demonstrator to the tone epoch (Figure 3B). In light of the negative correlation described (Figure 1E), this finding further suggests that observing a mouse freezing might negatively influence observers' social approach.

(I) Locomotor activity displayed by the same observer mice during the test (RM ANOVA $F_{2,16} = 4.08$; $p = 0.03$). *$p < 0.05$ versus 0–2 min. n = 9 observers.
(J) Blood corticosterone levels displayed by observer mice immediately after being exposed to two neutral demonstrators (gray bar) or one neutral and one fear demonstrator (red bar). Data are expressed as fold changes compared to observers exposed to two neutral demonstrators (t test: df: 9; $p = 0.58$). n = 5/6 observers per group.
Error bars represent standard error of the mean. See also Figures S1 and S2.

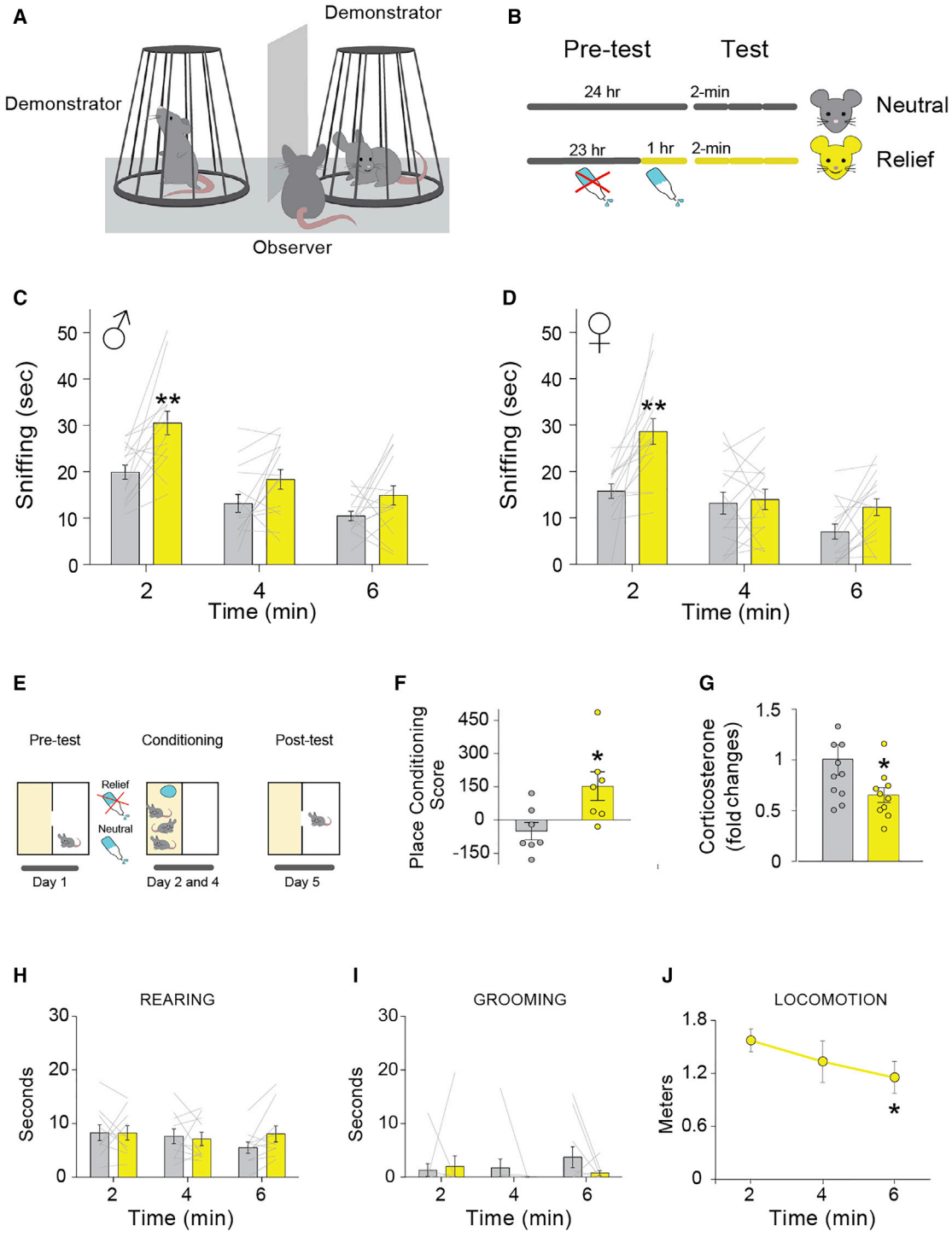

Figure 2. Mouse Emotion Discrimination for Relief

(A) Schematic drawing of the test setting.

(B) Timeline of pre-test and test procedures to evoke in one of the two demonstrators a "relief" state during the testing phase.

(C and D) Time (in seconds) spent sniffing demonstrators in neutral (gray bars) or water-induced relief (yellow bars) states displayed by (C) male and (D) female observer mice during the 6 min of the test, divided into three consecutive 2-min epochs (first 2-min RM ANOVA for males $F_{1,14} = 15.07$, $p = 0.001$ and females $F_{1,14} = 14.60$, $p = 0.001$; no significant differences for the 2- to 4-min and 4- to 6-min epochs). $^{**}p < 0.005$ versus the exploration of the neutral demonstrator. $n = 15$ observers per group.

(E) Place conditioning procedure used to assess whether the relief manipulation was associated with a negative-, neutral-, or positive-valence affective state.

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Auditory cues in the form of ultrasonic vocalizations (USVs) might be used by rodents for social communication [1, 55–58]. However, when we recorded USVs in the fear and relief conditions (Figure 3D), we detected very few vocalizations in a negligible number of mice (3/12) and with no differences in frequency, duration, or amplitude among the different conditions (Figure 3E). This was further confirmed when we individually recorded neutral, fear, and relief demonstrators (Figure S1C). Our data support previous evidence [58] that, in contrast to rat use of USVs [57, 59–61], adult mice might not use USVs as the main modality by which they communicate emotional states. Despite this, we cannot exclude the possibility that other unidentified auditory cues might be involved (e.g., [62]).

Finally, we tested the impact of olfactory cues presenting the observers with cotton balls after being swiped throughout the body, head, and anogenital areas of a neutral, fear, or relief demonstrator immediately after the manipulations (Figure 3F). Observer mice showed an avoidance for the odor of a fear mouse (Figure 3G), in agreement with previous evidence [63–65], and spent more time sniffing the relief odor compared to the neutral one (Figure 3H). These results confirm that olfactory cues convey information related to mouse emotional states but also indicate that observer responses are qualitatively different when demonstrators are physically present. Overall, this set of data indicates distinct implications of visual and olfactory social cues in the response to emotional stimuli.

Distinction between Emotion Discrimination and Sociability

The ability to discriminate between different states and the absolute quantity of social interaction (here referred to as "sociability") might be considered distinct constructs of social processes that are not necessarily interdependent [66]. Thus, we tested whether equivalent changes in sniffing behavior could be observable also in a context of no choice alternatives, presenting to observer mice a neutral, a fear, or a relief conspecific in a one-on-one free interaction setting. Social exploration levels did not differ between conditions (Figure 3I), showing the expected decrease of interaction over time [67]. Supporting this dichotomous effect, we revealed no correlation between emotion discrimination and the amount of social interaction in our setting (Figure S2H). Overall, this indicates that our setting (Figures 1 and 2) reveals aspects of social behavior not related to sociability.

Previous evidence measuring affective responses of an observer rat exposed to a demonstrator immediately after shock showed a similar decreased social exploration in one-on-one and one-on-two settings [16]. The discrepancy with our results, in which the one-on-one and one-on-two settings gave different results, might be due to the scalability feature of emotions [68], but not to mouse-rat differences. Indeed, using the same shock manipulations used in rats [16] in our setting (Figure 3J) recapitulated a similar general aversion during the whole test session (Figure 3K). This suggests that using higher-intensity emotional states might prove difficult to differentiate emotion discrimination from sociability measures.

Taken together, these data suggest that the paradigm used in this work (Figures 1 and 2) might reveal specific behavioral responses to mildly graded expression of emotions, possibly underestimated by previously used social interaction tests.

Endogenous Release of Oxytocin Is Necessary for Emotion Discrimination

The OXT system plays a pivotal role in social perception and cognition [8–10]. In particular, in humans, OXT has been associated with social cognitive functions, such as emotion recognition, empathy, and trust [8–10, 33, 35, 38, 39].

To assess whether the OXT system might be implicated in mouse emotion discrimination, we prevented OXT release from the PVN by bilateral injections of a recombinant adeno-associated virus (rAAV) expressing the inhibitory hM4D(Gi) DREADD (designer receptors exclusively activated by designer drugs) receptor under the control of an OXT promoter (Figures 4A, S3A, and S4A). In rodents, neurons in the PVN are the main source of OXT projections to the brain [22, 69]. We found that, in contrast to vehicle treatment (Veh), inhibition of PVN-OXT-projecting neurons (upon clozapine *N*-oxide [CNO] injection) abolished the ability of mice to discriminate either fear or relief states in conspecifics (Figures 4B, 4C, S3B, and S4F). This was equally evident in male (Figures 4B, 4C, and S4F) and female mice (Figure S3B) and was not associated with unspecific CNO effects, which suggests that CNO per se did not affect mouse responses during the test (Figures S3C and S3D). Notably, we were able to test the same mice in the different conditions as emotion discrimination ability was preserved when an observer was re-exposed to the same or to different paradigms (Figures S2E–S2G). Inhibiting PVN OXT projections produced selective effects on emotion discrimination. In fact, CNO administration had no effect on social exploration when the same mice were tested in a one-on-one free-interaction setting with an unfamiliar conspecific (Figure S4K). Overall, these data indicate a direct involvement of OXT release from PVN in the ability to discriminate different emotional states in conspecifics.

PVN OXT Projections to the Central Amygdala Are an Essential Neural Substrate for Emotion Discrimination

To investigate the selective OXTergic circuits involved in emotion discrimination, we first visualized PVN OXT projections, injecting

(F) Place conditioning scores (in seconds) displayed by mice conditioned during a neutral (gray bar) or relief (yellow bar) state. For each mouse, a place conditioning score was calculated as the post- minus the pre-conditioning time spent in the conditioning-paired compartment of the apparatus. A positive score indicates place preference, a negative score a place aversion, and 0 no place conditioning (t test: df = 12; p = 0.02). *p < 0.05 versus the neutral control group. n = 7 per group.

(G) Blood corticosterone levels displayed by demonstrator mice immediately after a period of 24-h water deprivation (gray bar) or after a period of 1 h *ad libitum* access to water following 23-h water deprivation (yellow bar; t test: df: 19; p = 0.05). *p = 0.05 versus water deprived mice. n = 11 per group.

(H and I) Time (in seconds) spent in (H) rearing and (I) grooming in proximity of demonstrators in neutral (gray bars) or relief (yellow bars) state displayed by observer mice during the test (RM ANOVAs showed no significant differences).

(J) Locomotor activity displayed by the same observer mice during the test (RM ANOVA $F_{2,18} = 4.35$; p = 0.04). *p < 0.05 versus minute 0–2. n = 10 observers. Error bars represent standard error of the mean. See also Figures S1 and S2.

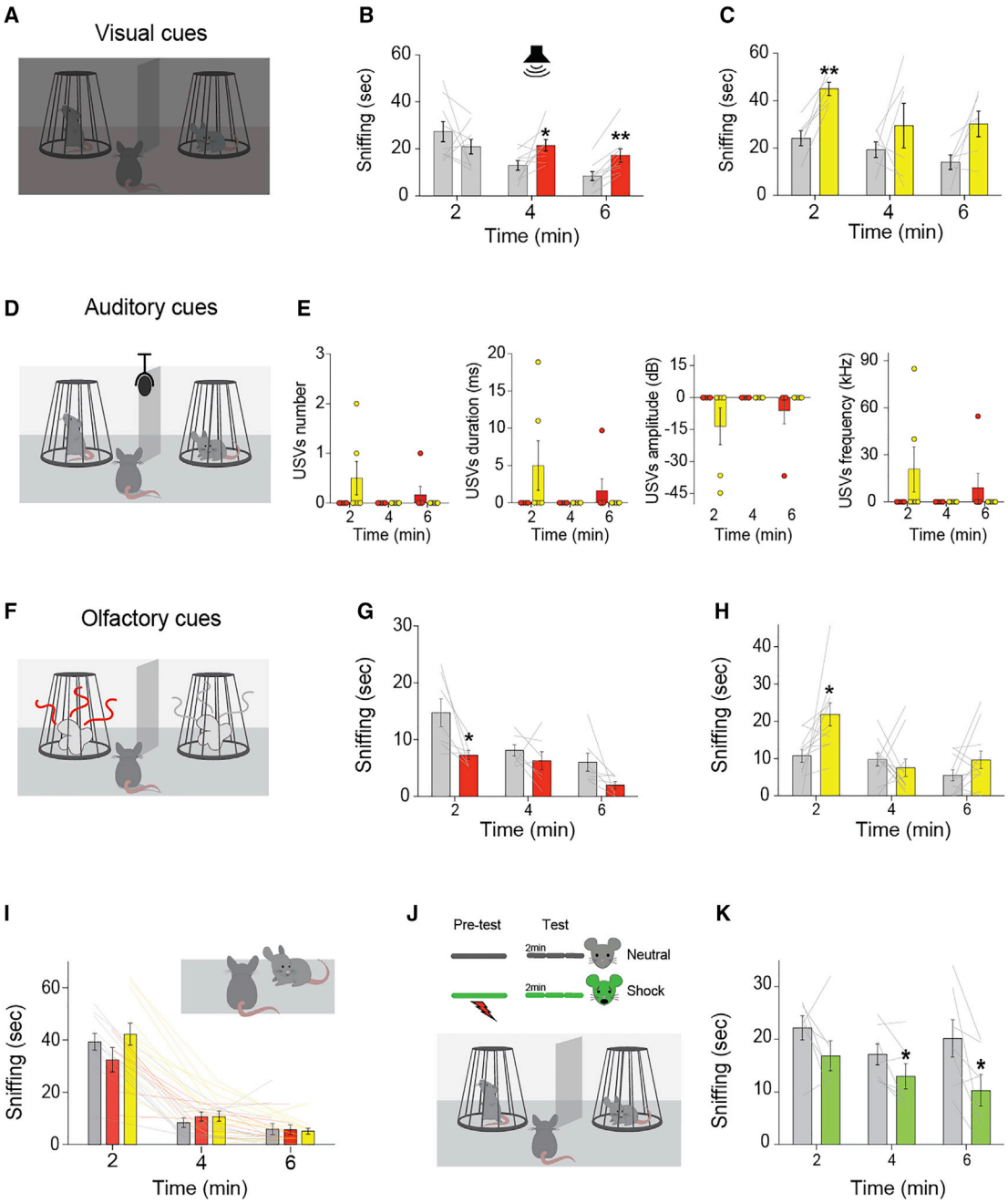

Figure 3. Analyses of Sensory Modalities and Distinction from Sociability

(A) Schematic drawing of the test setting performed in complete darkness.

(B and C) Time (in seconds) spent by observer mice sniffing demonstrators during the 6 min of the (B) fear and (C) relief manipulations of the emotion discrimination test. Time spent sniffing neutral demonstrators is depicted in gray. Time spent sniffing (B) fear or (C) relief demonstrators are depicted in red and yellow, respectively (RM ANOVAs for fear, 2–4 min: $F_{1,8} = 5.63$, $p = 0.04$; 4–6 min: $F_{1,8} = 28.08$, $p = 0.0007$; for relief, 0–2 min: $F_{1,5} = 33.32$, $p = 0.002$). *$p < 0.05$ and **$p < 0.005$ versus the exploration of the neutral demonstrator. n = 6/9 observers per group.

(D) Schematic drawing of the test setting to record USVs.

(E) USV mean of number of calls, duration in milliseconds, amplitude in decibel, and frequency in KHz emitted by mice during the fear and relief settings (two-way ANOVAs showed no significant differences). n = 6 observers per group.

(F) Schematic drawing of the test setting performed only with demonstrators' odors for fear and relief conditions.

(G and H) Time (in seconds) spent by observer mice sniffing the odors from neutral (gray), fear (red), or relief (yellow) demonstrators during the 6 min of the (G) fear or (H) relief test (RM ANOVA for the fear manipulation, 0–2 min: $F_{1,6} = 9.15$, $p = 0.02$. No significant differences for the 2- to 4-min and 4- to 6-min test periods. RM ANOVA for the relief manipulation, 0–2 min: $F_{1,11} = 6.89$; $p = 0.02$. Similarly, no significant differences for the 2- to 4-min and 4- to 6-min test periods). *$p < 0.05$ versus the exploration of the neutral odor. n = 7/11 observers per group.

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mice with a rAAV-expressing *Venus* under the control of an OXT promoter that allowed the fluorescent labeling of OXT-PVN neurons (Figures 4D and S5A). We focused on brain areas that have been identified as potential neuroanatomical substrates of emotion discrimination in humans [43, 70, 71] and that presented OXTergic innervation. We identified OXT-positive fibers in the central amygdala (CeA), nucleus accumbens (NAcc), hippocampal CA2, and medial prefrontal cortex (mPFC) (Figure 4E). Fewer fibers were evident in the insula, basolateral amygdala (BLA), and medial amygdala (MeA) (Figure S5B).

Next, to investigate the functional role of selective PVN OXT projections, we injected a retrogradely transported canine-adenovirus-2-expressing Cre recombinase (CAV2-Cre) into the target areas (CeA, NAcc, CA2 or mPFC) and also injected the PVN with a rAAV carrying a double-floxed inverted open reading frame (ORF) (DIO) of hM4D(Gi)DREADD receptor and mCherry under the control of the OXT promoter [69]. With this combination, we achieved DREADD(Gi)-mCherry expression exclusively in PVN OXT neurons projecting to the area injected with CAV2-Cre. We verified the regional and cell type specificity of virally mediated labeling of OXT neurons (Figures 5E and S5H–S5L). Due to the heterogeneity in fiber distribution in the different target areas, we controlled for the efficacy of DREADD-mediated inhibition in PVN back-labeled neurons from the different projection sites by performing *ex vivo* patch clamp electrophysiology recordings on PVN slices. We found a significant reduction in the number of evoked spikes after CNO application in back-labeled PVN neurons, which was equivalent for areas with intense OXTergic innervations (i.e., CeA and CA2) or with more sparse innervations (i.e., PFC; Figures 5F, 5G, and S5C–S5G).

In vivo, selective inhibition of OXT neurons projecting from the PVN to the CeA (Figures 5A, S4B, and S4G) was sufficient to recapitulate the deficits in emotion discrimination found by silencing all PVN projections (Figures 4B, 4C, and S3B). The same mice showed normal emotion discrimination abilities when treated with vehicle (Figures 5A and S4G). In contrast, selective inhibition of OXT neurons projecting from the PVN to the NAcc (Figures 5B, S4C, and S4H), the mPFC (Figures 5C, S4D, and S4I), and the CA2 (Figures 5D, S4E, and S4J) did not interfere with the ability to distinguish emotional states in conspecifics, indicating that OXT release from PVN to these brain regions is dispensable for emotion discrimination. Finally, none of the OXT pathways manipulations altered the ability to interact with an unfamiliar conspecific in a one-on-one free-interaction setting (Figures S4K–S4O), further supporting the distinction between emotion discrimination and sociability measures. Overall, these findings demonstrate a pre-eminent contribution of the CeA in emotion discrimination abilities in mice and indicate that PVN OXTergic projections to the CeA are an essential neural substrate of this social cognitive function.

Emotion Discrimination Depends on OXTR Levels in the CeA

Altered amygdala reactivity in emotion discrimination has been consistently reported in autism and schizophrenia in association with genetic liability [72, 73]. In heterozygous knockout mice for dysbindin-1 (Dys+/−), a clinically relevant mouse model of cognitive and psychiatric liability [48–50], we identified reduced expression levels of OXT receptors (OXTR) in the CeA, but not in the BLA or MeA compared to wild-type littermates (Dys+/+; Figures 6A and 6B). We then assessed Dys+/− mouse emotion discrimination abilities and observed deficits in both the fear and relief conditions (Figures 6C and 6D). In particular, we found that the impact of Dys mutation was selective for emotion discrimination, as Dys+/− sociability and social novelty in the classic 3-chamber test were similar to Dys+/+ controls (Figures S6A and S6B). These data unravel a clinically relevant genetic variation, which concurrently leads to deficits in emotion discrimination and to changes in the CeA OXT system.

To test whether reduced OXTR levels in the CeA were responsible for Dys+/− mouse emotion discrimination deficits, we increased the expression of OXTR selectively in the CeA of Dys+/− mice by bilateral injection of the AAV-EF1a-OXTR-IRES-EYFP, expressing OXTR and EYFP (enhanced yellow fluorescent protein) under the control of the EF1a promoter [24] (Figures 6E and S6D). Increased OXTR levels within the CeA were confirmed by receptor autoradiography quantification (Figures S6C–S6F). Increasing OXTR levels in CeA of Dys+/− mice was sufficient to rescue their emotion discrimination deficits (Figures 6F and 6G). Altogether, these findings strengthen the conclusion that appropriate OXTergic signaling within the CeA is critical to discriminate conspecifics based on their emotional state.

DISCUSSION

Combining behavioral, anatomical and genetic tools, this study reveals that the CeA, and in particular the OXTergic projections from the PVN to the CeA, is an essential neuronal substrate for the ability to socially respond to emotional states evoked in unfamiliar conspecifics. These mechanisms were revealed by a novel behavioral paradigm, which extends the opportunity to investigate previously unexplored aspects of social cognitive processes in mice.

Ethological Implications of Mice Emotion Discrimination

Using a two-choice setting, we reliably measured the ability of mice to distinguish conspecifics depending on their emotional state. This evidence supports and extends previous indications that rodents can perceive and react to altered emotional states in conspecifics [16, 19, 42, 53–55, 74–79]. Our data also extend

(I) Schematic drawing of the one-on-one test setting and time (in seconds) spent by observer mice sniffing a single demonstrator in a neutral (gray), fear (red), or relief (yellow) state during a 6-min free interaction test. The tone to which only the fear demonstrator was fear conditioned was delivered between 2 and 4 min of the test (ANOVAs revealed only a time effect with normal decreased exploration throughout the 6 min, $F_{2,56} = 132.01$; $p < 0.0001$). n = 12 observers.

(J) Schematic drawing of the task setting and timeline of pre-test and test procedures to trigger in one of the demonstrator a "shock" emotional state.

(K) Time (in seconds) spent by the observer mice sniffing demonstrators in neutral (gray bars) or shocked emotional state (green bars) during the test (RM ANOVAs, 0–2 min: $F_{1,6} = 2.40$, $p = 0.17$; 2–4 min: $F_{1,6} = 5.43$, $p = 0.05$; 4–6 min: $F_{1,6} = 8.11$, $p = 0.02$). *$p < 0.05$ versus the exploration of the neutral demonstrator. n = 7 observers.

Error bars represent standard error of the mean. See also Figure S1.

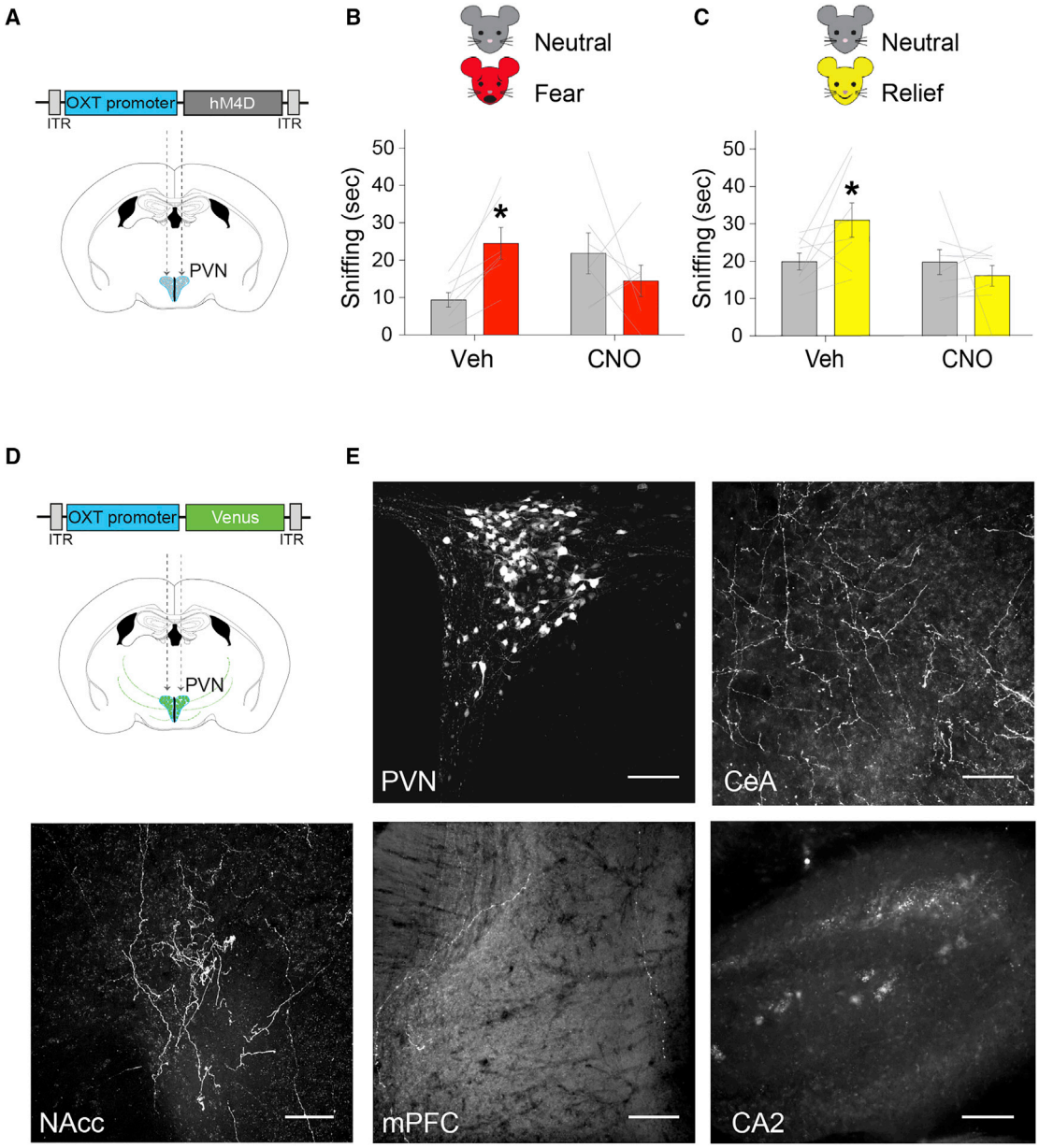

Figure 4. PVN OXT Release Is Necessary for Emotion Discrimination: Mapping of PVN OXT Projections in Mice

(A) Scheme of the viral vector used to infect the PVN OXT neurons. Injection placements in Figure S5.

(B and C) Time (in seconds) spent sniffing the two demonstrators during the last 2 min (for the fear) or the first 2 min (for the relief) epochs of the emotion discrimination test, displayed by the same observer mice treated with vehicle or CNO (intraperitoneal [i.p.], 3 mg/kg in a volume of 10 mL/kg, 30 min before the test) and shown separately for each demonstrator's state. The sniffing time of the different conditions throughout the whole 6-min period of the test are reported in Figure S4. Time spent sniffing (B) fear (last 2-min RM ANOVA veh: $F_{1,6} = 19.07$, $p = 0.004$; CNO: $F_{1,6} = 0.85$, $p = 0.39$) or (C) relief demonstrators (first 2-min RM ANOVA veh: $F_{1,7} = 7.24$, $p = 0.031$; CNO: $F_{1,7} = 0.50$, $p = 0.05$) is represented by red or yellow bars, respectively. *$p < 0.05$ versus the neutral demonstrator within the same observer treatment. n = 6/8 observers per group.

(D) Scheme of the viral vector used to infect the PVN OXT neurons.

(E) Anatomy of OXT projections from the PVN to the central amygdala (CeA), nucleus accumbens (NAcc), medial prefrontal cortex (mPFC), and hippocampus CA2 (CA2). Scale bars: 100 μm.

Error bars represent standard error of the mean. See also Figures S3–S5.

previous rodent emotion-based tests, which mostly detect behavioral responses between familiar conspecifics, which may vary depending on the sex of the tested subjects [19, 23, 54, 80]. Indeed, here, we found that emotion discrimination ability is exerted toward unfamiliar conspecifics and it is similarly evident in male and female mice. Intriguingly, although observer mice showed similar social responses toward fear or relief demonstrators, the ethological meaning underlying these behaviors

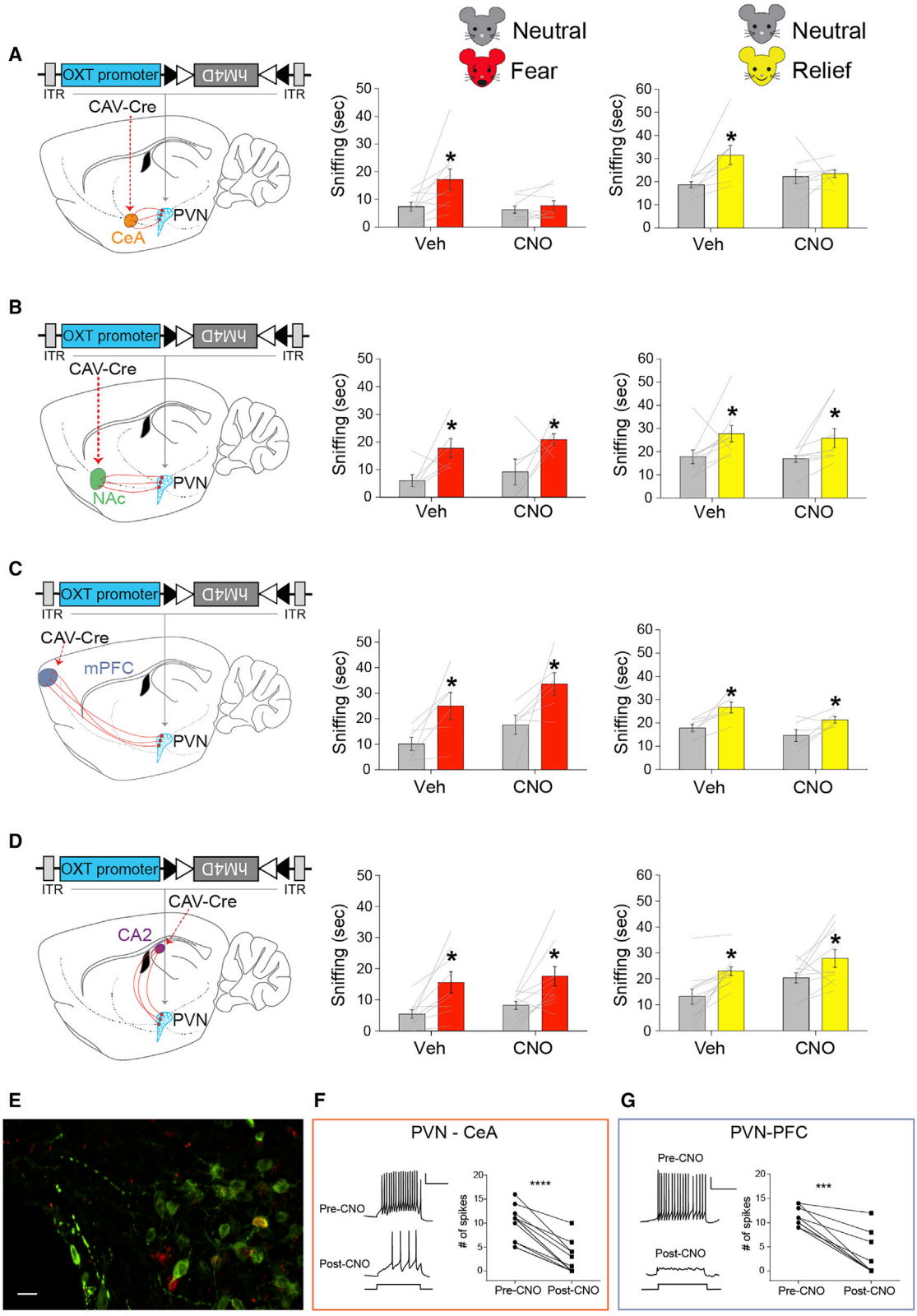

Figure 5. PVN-Central Amygdala OXT Projections Are Necessary for Emotion Discrimination in Mice

(A–D) Schemes showing the injection of viruses in the PVN and respective projection areas (CeA, NAcc, mPFC, or CA2). Placements are shown in Figure S5. Time (in seconds) spent sniffing the two demonstrators during the last 2-min (for the fear) or the first 2-min (for the relief) epochs of the emotion discrimination test is

(legend continued on next page)

might differ. The effects of the fear manipulation are in line with studies indicating that rodents are sensitive to the distress of others and can assimilate pain or fear responses expressed by conspecifics undergoing or recently exposed to pain or foot-shock challenge [16, 19, 23, 53–55, 75–80]. However, rodents have been also reported to actively escape from aversive stimuli [23, 81], USVs calls induced by heavy distress [16, 82], and odors emitted by shocked, stressed, defeated, or sick conspecifics [63–65]. The approach behavior observed toward fear demonstrators might then be potentially related to affective responses, such as helping or consolatory behaviors previously described in rats [74, 79] or prairie voles [19]. Indeed, in our setting, the lack of pain or the minimal levels of distress induced by the recall of a fear memory might not "alarm" the observer but promote a "pro-social" approach. In agreement, exposing the observer to a recently shocked demonstrator induced avoidance. Consistent with the fast and transient discriminatory behavior we observed (up to 2 min), we did not detect any behavioral or physiological transfer of responses from the demonstrators (e.g., freezing, escape behaviors, or altered corticosterone levels) to observer mice. This suggests that emotional contagion was not the motivation for the observers' social approach. However, based on previous reports [53, 55, 80], we cannot exclude that some sort of emotion contagion might take place at a later time point. The attraction exerted by the relief condition is instead consistently supported by several complementary findings: the approach toward the relief mouse, its odor, the induced place preference conditioning, and the reduced corticosterone levels, all point to an attractive signal of this positively valenced state. This is in agreement with a number of different rewarding stimuli in mice, such as pleasant odors, sexual signals, and the intrinsic reward valence of social interaction [57, 83–87]. However, the approach behavior we observed was clearly not correlated to the rewarding properties of social interaction per se. This is supported by the distinction between emotion discrimination and one-on-one social interaction (Figures 1 and 2 versus 3I), the lack of correlation between emotion discrimination and absolute quantity of social interaction (Figure S2H), the selective effects of OXT pathways manipulation in emotion discrimination, but not in social interaction (Figures 4 and 5 versus S4), and the effects of dysbindin-1 genetic variants in emotion discrimination, but not in the 3-chamber task (Figures 6C and 6D versus S6A and S6B). Altogether, our findings suggest that the two-choice emotion discrimination setting measures aspects of rodent social cognition that have been scarcely explored and that can complement currently available tools. Moreover, the ensemble of the presented results indicates the potential of this experimental setting to investigate a range of behavioral responses induced by emotional stimuli of different intensity and with potentially different ethological significance.

PVN-CeA OXT Projections as Key Modulator of Emotion Discrimination

We described the role of the endogenous OXT system, and specifically of OXT projections to CeA, NAcc, mPFC and hippocampal CA2, in the social response to emotional states evoked in unfamiliar conspecifics. Functional mapping of the selected PVN projections identified the CeA as a necessary site for OXT to allow discrimination of both negative and positive emotional states. These findings are in line with strong evidence across species, which implicate the amygdala as a critical hub in the processing of both positive and negative states [88–92]. In particular, given its access to primary sensory information, the CeA could orchestrate appropriate behavioral responses to salient stimuli with different valences [88, 93]. Consistently, a role for the CeA has been shown in modulating responses not just to threats or other aversive states [94–96] but also to reward-predictive cues and safety signals [97–101].

In humans, alterations in amygdala responses to positive and negative emotions have been reported in neuropsychiatric conditions, such as autism and schizophrenia [72, 73], in association with OXTR genetic variants [41, 102] or following intranasal OXT [33, 36, 38, 103]. However, the role of amygdala modulation, specifically in regard to the functionality of the endogenous OXT system, had not been elucidated. OXT has been shown to modulate emotion processing in rodents through an action in the anterior cingulate cortex, insula and lateral septum [16, 23, 24]. Our manipulations of endogenous PVN OXTergic projections add to this previous evidence, delineating OXT projections to the CeA as a neurobiological substrate for the ability to discriminate expression of emotions in others.

An intriguing question raised by our data is how the processing of both positive and negative states is achieved by OXT modulation in the CeA. The implication of CeA OXT signaling in processing threat responses [22, 94, 104] and social fear [105] would suggest a specific role in the detection of fear-mediated responses. The effect we found in the discrimination of both negative and positive states, however, supports the evidence of a generalized role of OXT in modulating CeA function in the response to socially communicated salient information, similar to evidence in humans [70, 90, 92, 106, 107]. Specific cell types and states of CeA neuronal subpopulations could be substrates

shown, displayed by the same observer mice treated with vehicle or CNO (i.p., 3 mg/kg in a volume of 10 mL/kg, 30 min before the test), and shown separately for each demonstrator's state. Time spent sniffing neutral demonstrators is depicted in gray. Time spent sniffing fear or relief demonstrators is represented by red or yellow bars, respectively. RM ANOVAs: (A) PVN-CeA (fear veh: $F_{1,8} = 5.76$, $p = 0.043$; CNO: $F_{1,8} = 1.57$, $p = 0.25$; relief veh: $F_{1,7} = 12.66$, $p = 0.009$; CNO: $F_{1,7} = 0.13$, $p = 0.73$); (B) PVN-NAcc (fear veh: $F_{1,5} = 6.02$, $p = 0.05$; CNO: $F_{1,5} = 7.40$, $p = 0.042$; relief veh: $F_{1,8} = 7.56$, $p = 0.025$; CNO: $F_{1,8} = 6.09$, $p = 0.039$); (C) PVN-mPFC (fear veh: $F_{1,5} = 7.25$, $p = 0.043$; CNO: $F_{1,5} = 6.80$, $p = 0.048$; relief veh: $F_{1,5} = 11.86$, $p = 0.02$; CNO: $F_{1,5} = 7.10$, $p = 0.044$); (D) PVN-CA2 (fear veh: $F_{1,10} = 11.39$, $p = 0.009$; CNO: $F_{1,10} = 11.15$, $p = 0.007$; relief veh: $F_{1,10} = 16.91$, $p = 0.002$; CNO: $F_{1,10} = 6.60$, $p = 0.04$). n = 6/11 observers per group. *$p < 0.05$ versus the exploration of the neutral demonstrator. The sniffing time throughout the whole 6-min period of the different conditions is reported in the Figure S4.

(E) Immunohistochemical staining for mCherry (red) and OXT (green) of back-labeled PVN neurons. Scale bar: 20 μm.

(F and G) Electrophysiological validation of hM4D(Gi) action in PVN back-labeled neurons from (F) CeA and (G) mPFC. Example traces of membrane potential changes (left) and quantification (right) of single-cell data points of the number of spikes evoked by a depolarizing current step (duration: 1 s; amplitude: 20 pA; I_{Hold}: 0 pA) in PVN neurons, pre- and post-bath application of CNO in artificial cerebrospinal fluid (ACSF). Two-tailed paired t test: (F) PVN-CeA, n = 12 from 3 mice ($t = 9.033$; $df = 11$; $p < 0.0001$); (G) PVN-mPFC, n = 9 from 3 mice ($t = 6.596$; $df = 8$; $p = 0.0002$). Scale bars are 40 mV and 500 ms. ****$p < 0.0001$.

Error bars represent standard error of the mean. See also Figures S3–S5.

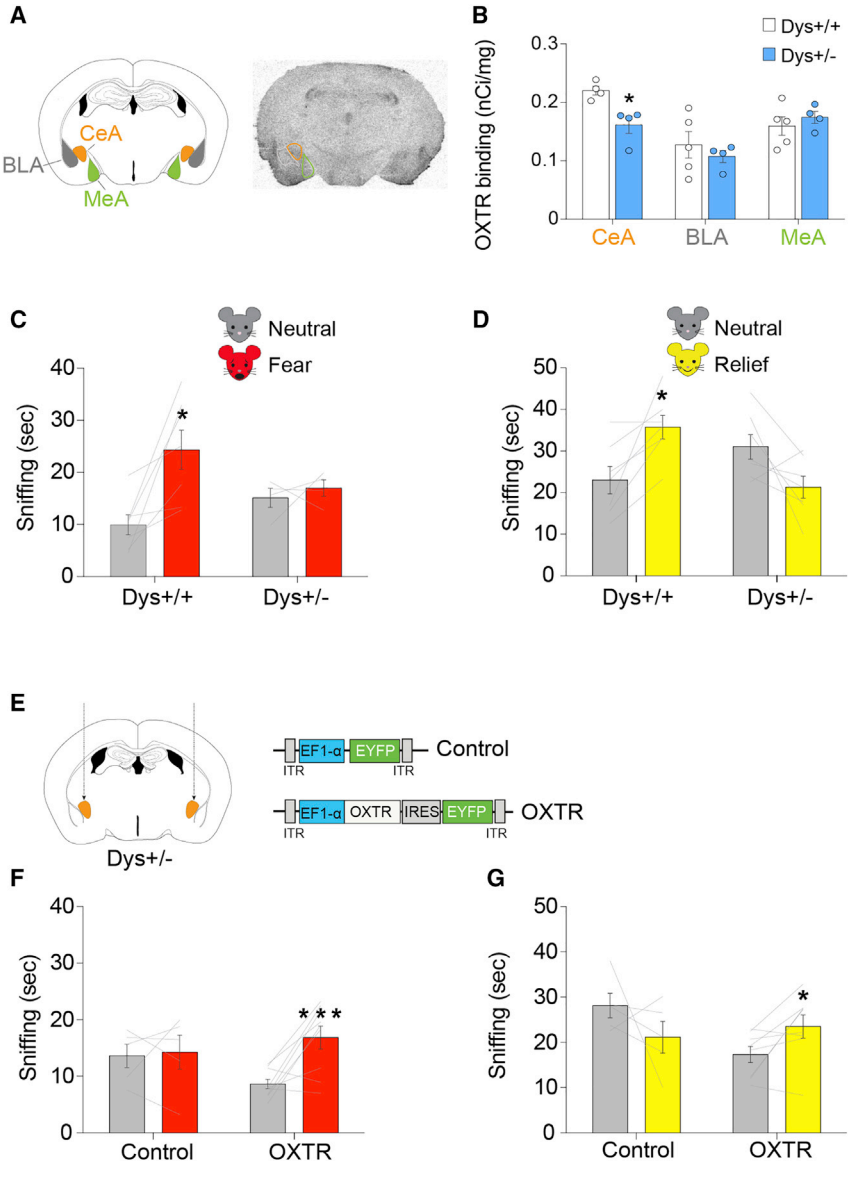

Figure 6. Emotion Discrimination Abilities Are Genetically Modulated and Depend on OXTR Levels in the CeA

(A) Representative drawing and autoradiograph showing the ligand binding of 20 pmol/L I^{125}-labeled OVTA, a potent and selective ligand for OXTR. Autoradiograms were obtained from coronal sections of brains of dysbindin-1 wild-type (Dys+/+) or heterozygous (Dys+/−) mice. CeA, central amygdala; BLA, basolateral amygdala; MeA, medial amygdala.

(B) OXTR binding sites are expressed as nCi/mg of tissue equivalent. One-way ANOVA for CeA, $F_{1,6}$ = 12.5035, p = 0.01; BLA, $F_{1,7}$ = 0.53027, p = 0.5; and MeA, $F_{1,7}$ = 0.5609, p = 0.48. *p < 0.05 versus Dys+/+ mice. n = 4/5 for each group.

(C and D) Time (in seconds) dysbindin-1 wild-type (Dys+/+) or heterozygous (Dys+/−) observer mice spent sniffing wild-type demonstrators in the two versions of the emotion discrimination test. Time spent sniffing neutral demonstrators is represented by gray bars. Time spent sniffing (C) fear demonstrators (only the last 2 min are displayed) or (D) water-induced relief state (only the first 2 min are displayed) are represented by red or yellow bars, respectively. RM ANOVAs: (C) fear last 2 min, Dys+/+: $F_{1,6}$ = 12.41, p = 0.012; Dys+/−: $F_{1,3}$ = 0.33, p = 0.61; (D) relief first 2 min, Dys+/+: $F_{1,6}$ = 12.24, p = 0.012; Dys+/−: $F_{1,6}$ = 5.11, p = 0.06. n = 4/9 observers per group. *p < 0.05 versus the exploration of the neutral demonstrator.

(E) Scheme of the viral vector used to infect the CeA of Dys+/− mice. Injection sites are shown in Figure S6.

(F and G) Time (in seconds) spent sniffing each of the two wild-type demonstrators during the test displayed by dysbindin-1 heterozygous (Dys+/−) observer mice bilaterally injected with AAV-EF1α-EYFP (control) or AAV-EF1α-OXTR-EYFP (OXTR) and shown separately for each demonstrator's emotion. Time spent sniffing neutral demonstrators is represented by gray bars. Time spent sniffing demonstrators with (F) tone-induced fearful state (only the last 2 min are displayed) or (G) water-induced relief state (only the first 2 min are displayed) is represented by red or yellow bars, respectively. RM ANOVAs: (F) fear last 2 min, Dys+/− control virus: $F_{1,4}$ = 0.050, p = 0.83; Dys+/− OXTR virus: $F_{1,8}$ = 13.63, p = 0.006; (G) relief first 2 min, Dys+/− control virus: $F_{1,4}$ = 1.44, p = 0.30; Dys+/− OXTR virus: $F_{1,7}$ = 6.11, p = 0.043. n = 5/9 observers per group. ***p < 0.0005 and *p < 0.05 versus the exploration of the neutral demonstrator.

Error bars represent standard error of the mean. See also Figure S6.

of the perception and response to stimuli with different emotional valences. Distinct neuronal populations in sub-regions of the amygdala have indeed been shown to control specific behavioral responses to fear [104, 108], although evidence of OXT modulation of positively valenced cues are still poor.

Mechanistically, OXT has been documented to increase neuronal firing rates, mainly through inhibition of interneuron activity, which results in an increase in signal-noise ratio and subsequently enhanced information transfer [13, 22, 109–111]. The close proximity of highly enriched PVN-OXT fibers to GABAergic CeA neurons expressing OXTR [22] suggests that a similar modulation might occur during emotion discrimination in the CeA. In particular, an interaction between the OXT and the corticotropin-releasing factor (CRF) system could be one of the possible substrates, considering the high levels of CRF expression in CeA [112, 113] and the reported CRF-dependent PVN plasticity in transfer of fear [80]. Projection-specific manipulations dissecting CeA connectivity, as previously reported [114, 115], could help to elucidate how CeA neurons process stimuli of positive or negative valence to direct behavior.

Genetic Modulation of Emotion Discrimination through CeA OXTR Levels

The OXT system is regarded as a promising target for the treatment of social cognitive dysfunctions [31]. Altered levels of OXT and OXTR in specific regions of the brain have been reported in a number of animal models of psychiatric and neurodevelopmental disorders [116–119]. However, how these alterations might be

involved in clinical manifestations and in particular in social cognitive endophenotypes remains unclear. The reduction in CeA OXTR levels in dysbindin-1 hypofunctioning mice has causally related OXT receptor-mediated mechanisms with the ability to discriminate negative and positive emotions in conspecifics. Genetic variants in dysbindin-1 are strongly associated with human intelligence [51]. Moreover, both mouse and human studies consistently indicate that reduced dysbindin-1 expression modulates higher-order cognitive functions [48–50]. Thus, our new findings extend the implication of dysbindin-1 genetics in the social cognitive domain and strengthen the evidence that OXT signaling within the CeA and, specifically, OXTR levels within this brain region constitute a crucial target to modulate emotion discrimination abilities.

From a therapeutic perspective, alterations in the endogenous OXT system can influence the response to exogenous OXT administration [40, 67, 120]. Notably, common functional genetic variants in dysbindin-1 have been recently shown to predict, in both humans and mice, cognitive responses to psychotropic drug treatments [50]. Thus, it is intriguing to speculate that dysbindin-1 genetic variants might also modulate social cognitive responses to exogenous OXT-related treatments. This aspect, together with the modulatory impact of endogenous and/or exogenous OXT on emotion discrimination, represents an important subject for future studies, which may explore OXT pathways in the context of other genetic variants.

Mouse Emotion Discrimination versus Human Emotion Recognition

The behavioral paradigm developed in this study was inspired by human emotion recognition tasks [3]. Emotion recognition tasks rely on the ability to discriminate basic expression of emotions in others and are the most extensively used paradigms to assess human social cognition [3, 43], with direct relevance for a number of pathologies, including autism and schizophrenia [5, 6]. Human emotion recognition paradigms include the presentation of positively and negatively valenced emotions [3]. Consistently, we adopted a two-choice discriminative setting, focused on behaviors initiated by the observer mouse and adopting manipulations that could induce both negative and positive emotional states in the demonstrators. Throughout the paper, we referred to "emotions" as subjective internal states of mice evoked by behavioral manipulations, aware that our definition is based on the assessment of behavioral output more than on the intangible and unmeasurable nature of emotions. Taking these important limitations in mind, our data provide some indication that, similar to human emotion recognition tasks [70, 90, 92, 121], mice can discriminate emotions in others in a way that is distinct from sociability. This ability shows strong test-retest reliability, and it is equally evident in male and female mice toward unfamiliar conspecifics. This ability appears to rely on a primary amygdala recruitment across all forms of emotion perception. Furthermore, we have demonstrated that emotion discrimination abilities in mice are dependent on the OXT system, in further agreement with human evidence where OXT has been associated with social cognitive functions, such as emotion recognition [8–10, 33–36, 38, 39]. Altogether, this indicates that our paradigm might approximate some features of human emotion recognition tasks [3, 43].

In conclusion, our data provide new insights into the role of endogenous OXT signaling in the ability to recognize emotions in unfamiliar conspecifics. Additionally, the evidence here reported also demonstrates an opportunity to reliably measure scarcely explored aspects of rodent social cognition. This could support more translational approaches between rodent and human social cognitive studies, with relevance to circuits, genetics, and neurochemical systems involved in different psychiatric disorders.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.04.070.

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AUTHOR CONTRIBUTIONS

Conceptualization, V.F. and F.P.; Methodology and Investigation, V.F., F. Maltese, G. Contarini, A.B., H.H., V. Gigliucci, M.N., G.M., G. Castellani, D.S., F. Managò, B.C., V. Grinevich, and F.P.; Validation, V.F., F. Maltese, G. Contarini, A.B., H.H., G.M., D.S., F. Managò, and F.P.; Resource, L.C., B.C., V. Grinevich, and F.P.; Writing, V.F., B.C., V. Grinevich, and F.P.; Visualization and Analysis, V.F., F. Maltese, G. Contarini, F. Managò, V. Grinevich, B.C., and F.P.; Supervision, F.P.; Project Administration, F.P.; Funding

Acquisition, B.C., V. Grinevich, and F.P. All of the authors revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
OXT monoclonal antibody	Provided by Dr. Harold Gainer	PS38
GFP polyclonal antibody	Invitrogen	Cat# A-11122; RRID: AB_221569
dsRED polyclonal antibody	Clontech Laboratories	Cat#632496; RRID: AB_10013483
Alexa Flour 488 goat anti-rabbit IgG (H+L) antibody	Thermo Fisher Scientific	Cat# A-11034; RRID: AB_2576217
Alexa Fluor 633 goat anti-rabbit IgG (H+L) antibody	Thermo Fisher Scientific	Cat# A-21071; RRID: AB_141419
Bacterial and Virus Strains		
AAV1/2- OXTp-Venus	[22]	N/A
AAV1/2-OXTp-DIO-hM4D(Gi)-mCherry	[22]	N/A
AAV1/2-EF1a-OXTR-IRES:EYFP	[24]	N/A
CAV2-Cre	[122]	N/A
Chemicals, Peptides, and Recombinant Proteins		
EDTA	Sigma-Aldrich	Cat#E5134; CAS: 6381-92-6
Clozapine n-oxide (CNO)	Tocris Bioscience	Cat#4936; CAS: 34233-69-7
Isopenthane	Sigma-Aldrich	Cat#M32631; CAS: 78-78-4
Gelatine	Sigma-Aldrich	Cat#G1890; CAS: 9000-70-8
Chromium potassium sulfate	Merck	Cat#1036; CAS: 7788-99-0
Paraformaldehyde	Sigma-Aldrich	Cat#158127; CAS: 30525-89-4
Bacitracin	Alfa Aesar	Cat#J62432; CAS: 1405-87-4
[125I]- ornithine vasotocin analog	Perkin Elmer	Cat#NEX254010UC
Critical Commercial Assays		
Detect X corticosterone enzyme-linked immunoassay	Arbor Assays	Cat#K014-H1
QuickTiter AAV Quantitation Kit	Cell Biolabs, Inc.	Cat#VPK-145
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	Charles River	Cat#027C57BL/6
Mouse: Dysbindin heterozygous	[49]	N/A
Mouse: OXTR knock-out	[123]	N/A
Software		
Statistica 13.2	Statsoft	http://www.statsoft.com
Anymaze	Stoelting	https://www.stoeltingco.com/anymaze
AVISOFT RECORDER 3.2	Avisoft Bioacoustics	https://www.avisoft.com
Avisoft SASLab Pro 4.40	Avisoft Bioacoustics	https://www.avisoft.com
pClamp 10.2	Molecular Devices	https://www.moleculardevices.com/
Fiji	NIMH	https://imagej.nih.gov
Adobe Photoshop CS5	Adobe	https://www.adobe.com
Other		
Cryostat	Reichert Jung	2007 Frigocut
Vibratome	Leica	VT1200S
Microtome	Leica	CM1900
Real Time PCR thermal cycler	Applied Biosystems	ABI 7700 cycler
Borosilicate glass capillaries	Sutter Instrument	B150-86-7.5
Galaxy Cup	Spectrum Diversified Designs, Inc.	https://www.spectrumdiversified.com/whs/products/Galaxy-Pencil-Utility-Cup
Monochrome camera	Imaging Source	DMK-22AUC03
Thermo camera	FLIR Systems	FLIR A315
Cubicles	TSE	https://www.tse-systems.com

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
UltraSoundGate condenser microphone	Avisoft Bioacoustics	CM16
Fear conditionig system	Ugo Basile SRL	Cat#46001
Patch-clamp amplifier	Molecular Devices	Multiclamp 700B
Digital converter	Molecular Devices	Digidata 1440A
Superfrost microscope slides	VWR	Cat#631-0909
BioMax MR Film	Carestream	Cat#870-1302

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Francesco Papaleo (francesco.papaleo@iit.it).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Males and females C57BL/6J mice, dysbindin-1 [49] heterozygous (Dys+/−) and their wild-type littermates (Dys+/+), and oxytocin receptor knockout (Oxtr−/−) [123], all 3-6 months-old, were used. Animals were housed two to four per cage in a climate-controlled (22 ± 2 C) and specific pathogen-free animal facility, with *ad libitum* access to food and water throughout, a standard environmental enrichment (material for nest and cardboard house), and with a 12-hour light/dark cycle (7pm/7am schedule). Experiments were run during the light phase (between 10am-5pm). All mice were handled on alternate days during the week preceding the first behavioral testing. Experimenters were blind to the mouse treatments and genotype during testing. Female mice were visually checked for estrus cycle immediately after the test and no correlation was found between estrus status and performance in the test. All procedures were approved by the Italian Ministry of Health (permits n. 230/2009-B and 107/2015-PR) and local Animal Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the European Community Council Directives.

METHOD DETAILS

Behavioral procedures

Emotion discrimination test

Habituation of the mice to the testing setting occurred on three consecutive days before the first experiment; each habituation session lasted 10 minutes. Test observer mice were habituated inside a Tecniplast cage (35.5x23.5x19 cm) with a separator and two cylindrical wire cups (10.5cm in height, bottom diameter 10.2cm, bars spaced 1 cm apart; Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH), around which they could freely move, as occurred during the test. A cup was placed on the top of the wire cups to prevent the observer mice from climbing and remaining on the top of them. The separator (11x14cm) between the two wire cups was wide enough to cover the reciprocal view of the demonstrators while leaving the observer mice free to move between the two sides of the cage. The wire cups, separators and experimental cages were replaced after each subject with clean copies to avoid scent carryover. Similarly, the rest of the apparatus was wiped down with water and dried with paper towels for each new subject. After each testing day, the wire cups, separators, and cubicles were wiped down with 70% ethanol and allowed to air-dry. Testing cages were autoclaved as standardly performed in our animal facility. Demonstrator mice – matched by age and sex to the observers – were habituated inside the same Tecniplast cage (35.5x23.5x19 cm), under the wire cups for three consecutive times, ten minutes each. During both habituation and behavioral testing, the cages were placed inside soundproof cubicles (TSE Multi Conditioning Systems) homogeneously and dimly lit (6 ± 1 lux) to minimize gradients in light, temperature, sound and other environmental conditions that could produce a side preference. Digital cameras (imaging Source DMK 22AUC03 monochrome, Ugo Basile) were placed facing the long side of the cage and on top of the cage to record the three consecutive two-minute epochs from different angles, using the Anymaze program (Stoelting, Ireland).

Behavioral scoring was performed *a posteriori* from videos by trained experimenters, blind to the manipulations of both the observers and demonstrators. Three independent persons scored the same data with an inter-rater reliability r score of 0.954. A sniffing event was considered when the observer touched with the nose the demonstrators' wire cup or when the observer's nose directly touched the demonstrator. The emotion discrimination experiments reported in this work were independently replicated by nine different researchers in three different laboratories.

Observers

Before the test, mice were habituated to the experimental setting as reported above. On the third day of habituation, mice were also habituated to the tone cue (4 kHz, 80 dB sound pressure level, three times for 30 s each with an intertrial interval of 90 s) without any

conditioning. One hour prior to behavioral testing, mice were placed in the testing cage, in an experimental setting (i.e., separator and two wire cups), in a room adjacent to the testing room. Five minutes before the experiment, the testing cages containing the observer mice were gently moved into the testing cubicles. The 6-minute experiment began after placing one emotionally 'neutral' and one "emotionally altered" demonstrator under the wire cups. The order of insertion of the neutral or emotionally-altered demonstrator was randomly assigned.

Neutral demonstrators

In the days before the test, all neutral mice were habituated to the experimental setting as reported above. For the relief condition, neutral demonstrators underwent no manipulation the day before the test. For the fear condition, the day before the test, neutral demonstrators were habituated to the tone cue inside the cups as for the experimental setting and as done for the observer mice. On the testing day, neutral demonstrators were brought inside their home cages in the experimental room one hour before the experiment began. Demonstrators were test-naive and used only once. In some cases, we re-used the same demonstrator for maximum two/three times, with always at least one week between each consecutive test. No differences were observed in the performance of the observer mice depending on the demonstrators' previous experience.

Relief demonstrators

In the days before the test, mice were habituated to the experimental setting as reported above. Relief demonstrators were then water deprived 23 hours before the experiment. One hour before the test, *ad libitum* access to water was reestablished, and mice were brought inside the experimental room in their home cages. Food was available *ad libitum* all the time and some extra pellets were put inside the home cage during the 1-hour water reinsertion.

Fear demonstrators

In the days before the test, mice were habituated to the experimental setting as reported above. Fear demonstrators were fear conditioned (from one day to one week before the test) using the parameters and context previously described [124], and using the same tone delivered to the observers and neutral demonstrators during their habituation process. In particular, the conditioned stimulus was a tone (4 kHz, 80 dB sound pressure level, 30 s) and the unconditioned stimulus were three scrambled shocks (0.7 mA, 2 s duration, 90 s intershock interval) delivered through the grid floor that terminated simultaneously with the tone (2 s). The day of the test these mice were habituated, inside their home cages, in a room adjacent to the testing room for one hour prior to the test; they were consequently brought inside the experimental room one by one, before placing them under their designated wire cup. Fear mice were conditioned only once, in a separate room and using distinct apparatus (Ugo Basile SRL, Italy) from the one where the emotion recognition task would be performed. Fear demonstrators were generally used only once. In the case of a second exposure to the test, these demonstrators were just re-exposed to the same conditioned tone, at least one week apart from the previous exposure and maximum 1 month from the initial conditioning.

Shock demonstrators

This manipulation was performed for direct comparison with a rat protocol and was performed as previously described [16]. In particular, these demonstrator mice were exposed to two footshocks (1 mA, 5 s duration, 60 s intershock interval) immediately before the 6-minute test session. All other procedures were identical to the other demonstrators as described above.

"Classic" social interaction test and 3-chamber social interaction test

Social interaction in freely interacting mice was performed as previously reported [67, 125]. Briefly, mice were individually placed in the testing cage 1 hour prior to testing. No previous single housing manipulation was adopted to avoid any home-cage territorial aggressive behaviors. Testing began when a stimulus mouse, matched for sex and age, was introduced into the testing cage for a 4-min period interaction.

Sociability and preference for social novelty in the 3-chamber task was tested as previously described [125]. In brief, the test consists of four phases of 10 minutes each. In *phase 1* the subject mouse is placed into the center chamber with both doors closed. Then, in *phase 2*, both doors are open and the subject can freely explore all three empty compartments for another 10 minutes. Next, *phase 3* consists of the "Sociability" test in which an empty wire cage is placed in one of the chambers and another wire cage with a stimulus mouse inside is placed in the other chamber. Finally, *phase 4* consists of the "Preference for Social Novelty" test in which the empty wire cage is replaced with a novel mouse.

One-on-one social exploration tests

This test was similarly performed as previously described [16]. One hour prior to behavioral testing, each experimental subject was placed into a Tecniplast cage (35.5x23.5x19 cm) with shaved wood bedding and a wire lid, in a room adjacent to the testing room. Five minutes before the experiment, the testing cages containing the observer mice were gently moved into the testing sound proof cubicles. To begin the test, a demonstrator mouse was introduced to the cage for 6 minutes (as for the emotion discrimination task), and exploratory behaviors initiated by the test subject were timed by two independent experimenters blind to the manipulations. Demonstrator mice were used only once. Each observer was given tests on consecutive days: once with an unfamiliar naive conspecific, once with an unfamiliar fear conspecific (fear conditioning exactly as above), and once with an unfamiliar relief conspecific (manipulated exactly as above). Test order was counterbalanced.

Sensory modality assessment

In the "complete darkness" experiments, mice were tested as above, but eliminating all sources of light within the testing cage as well as in the entire testing room. Videos were recorded for successive scoring either with an infrared thermal camera (FLIR A315, FLIR Systems) or with Imaging Source DMK 22AUC03 monochrome camera (Ugo Basile). The two camera settings gave the same experimental results.

For acoustic stimuli experiments, ultrasonic vocalisations (USVs) were recorded during the test phases performed as above in two different experimental settings: 1) exactly as reported above with one observer mouse and two demonstrators under the wire cups, and 2) with only one demonstrator present in the apparatus (and under the wire cup) for each emotional condition. This was done to make sure that the USVs recorded could be attributed to a single emotional state and/or to a communication between demonstrators and observer. Ultrasonic vocalizations recording and analysis were performed as previously described [67]. The ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM16, Avisoft Bioacoustics, Berlin, Germany), sensitive to frequencies between 10 and 180 kHz, was mounted 20 cm above the cage to record for subsequent scoring of USV parameters. Vocalisations were recorded using AVISOFT RECORDER software version 3.2. Settings included sampling rate at 250 kHz; format 16 bit. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (Version 4.40) and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated with an FFT-length of 1024 points and a time window overlap of 75% (100% Frame, Hamming window). The spectrogram was produced at a frequency resolution of 488 Hz and a time resolution of 1 ms. A lower cut-off frequency of 15 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. Call detection was provided by an automatic threshold-based algorithm and a hold-time mechanism (hold time: 0.01 s). An experienced user checked the accuracy of call detection and obtained a 100% concordance between automated and observational detection. Parameters analyzed included number of calls, duration of calls and quantitative analyses of sound frequencies measured in terms of frequency and amplitude at the maximum of the spectrum.

For odor stimuli experiments, observers were tested as described above, but replacing the "demonstrator" with cotton balls, which had been swiped throughout the body, head and anogenital areas of demonstrators. Odors were separately collected from neutral, relief (after the 1 hour *ad libitum* access to water) and fear (immediately after the delivery of the conditioned tone cue) demonstrators. Each odor was always taken fresh from one single mouse (which was not reused) and used only once.

Place conditioning

Mice were tested in a well-established place conditioning paradigm, able to assess either positive or negative affective states in mice [124, 126]. The place conditioning paradigm was performed in a rectangular Plexiglas box (length, 42 cm; width, 21 cm; height, 21 cm) divided by a central partition into two chambers of equal size (21 × 21 × 21 cm). One compartment had black walls and a smooth Plexiglas floor, whereas the other one had vertical black and white striped (2 cm) walls and a slightly rough floor. During the test sessions, an aperture (4 × 4 cm) in the central partition allowed the mice to enter both sides of the apparatus, whereas during the conditioning sessions the individual compartments were closed off from each other. To measure time spent in each compartment a video tracking system (Anymaze) was used. The place conditioning experiment lasted 5 days and consisted of three phases: pre-conditioning test, conditioning phase and post-conditioning test. On day 1, each mouse was allowed to freely explore the entire apparatus for 20 min, and time spent in each of the two compartments was measured (pre-conditioning test). Conditioning sessions took place on days 2 and 4. Mice were divided in two groups: neutral and relief. Mice of the same home- cage were assigned to the same group. Mice were then divided in the two experimental groups with similar preconditioning time values in the two sides of place conditioning apparatus. As for the same manipulation in the emotion discrimination test, the relief group was assigned to receive a 23-hour water deprivation period before the two conditioning sessions on the day 2 and 4, when they were confined with their cage mates in one of the two compartments for 1 hour with free access to water and food (conditioning). Food in the home cage was available all the time. Other than the two 23-hr deprivation periods, water was available all the time. The neutral group was exposed to the same procedure but without any water deprivation. The post-conditioning test was performed on day 5 in the same conditions as the preconditioning test. For each mouse, a conditioning score was calculated as the post-conditioning time minus the pre-conditioning time (in seconds) spent in the conditioned compartment of the apparatus.

Corticosterone assay

Corticosterone concentration was analyzed from mice plasma. Immediately after the behavioral test, each mouse was sacrificed by decapitation. The blood was quickly collected in EDTA(0,5M)-coated tubes and centrifuged at 3000 rpm for 10 min; the supernatant obtained was stored at −20°C until the assay. The corticosterone concentration was detected by a commercially available Detect X corticosterone enzyme-linked immunoassay (ELISA) kit (Arbor Assays, MI, USA; Cat N K014-H1) following the manufacturer's protocol. The level of corticosterone was expressed as fold changes compared to the control group average.

Viral vectors

The OXTp-Venus, OXTp-hM4D(Gi), OXTp-DIO-hM4D(Gi)-mCherry and EF1a-OXTR-IRES:EYFP AAV serotype 1/2 were cloned and produced as previously reported [22, 24, 69]. rAAV genomic titers were determined with QuickTiter AAV Quantitation Kit (Cell Biolabs, Inc., San Diego, California, USA) and RT-PCR using the ABI 7700 cycler (Applied Biosystems, California, USA). rAAVs titers were $\sim 10^{10}$ genomic copies per per μl. For the EF1a-OXTR-IRES:EYFP, AAV expression and spreading were assessed by injecting

0,5 μl in the CeA of OXTR deficient mice [123]. CAV2 equipped with Cre recombinase (titer: $2.5x10^{11}$ pp) was purchased from the Institute of Molecular Genetics in Montpellier CNRS, France [24, 122].

Stereotaxic Injections

Mice were anesthetized with 2% isoflurane in O_2 by inhalation and mounted onto a stereotaxic frame (Kopf) linked to a digital reader. Mice were maintained on 1.5 - 2% isoflurane during the surgery. Brain coordinates of injections were chosen in accordance to the mouse brain atlas [127]: PVN (AP: −0.9 mm; L: ± 0.2 mm; DV: −4.5), CeA (AP: −1 mm; L: ± 2.2 mm; DV: −4.5 mm), NAcc (AP: +1.7 mm; L: ± 0.5 mm; DV: −4 mm), mPFC (AP: +1.9 mm; L: ± 0.25 mm; DV: −2.5 mm). Mice that had been injected with AAVs and/or CAV2 were allowed 1 month to recover and for the viral transgenes to adequately express before undergoing behavioral experiments. The injected volume viruses (rAAV and CAV2) were 75-100 nL volume, depending on the brain region. CAV2 was pre-diluted at the $1x10^9$ ppl/ml concentration. Only mice with appropriate placements were included in the reported data (Figures S5H–S5L and S6F).

Drugs

At least 4 weeks after cerebral injections, we inhibited PVN OXT release by i.p. administration of Clozapine N-Oxide (CNO, #4936 Tocris Bioscience) dissolved in physiological saline (0.9% NaCl) at a dose of 3mg/kg in a volume of 10 ml/kg, 30 minutes before the emotion discrimination task. For control experiments, the same mice were injected with the same volume of saline.

Histology

At the end of the behavioral procedures mice were deeply anesthetized (urethane 20%) and transcardially perfused with 4% paraformaldehyde in PBS, pH 7.4. Brains were dissected, post fixed overnight and cryoprotected in 30% sucrose in PBS. 40-μm-thick coronal sections were cut using a Leica CM1900 microtome. For immunohistochemical studies free-floating sections of selected areas were washed in PBS three times for 10 minutes, permeabilized in PBS plus 0.4% Triton X-100 for 30 min, blocked by incubation in PBS plus 4% normal goat serum (NGS), 0.2% Triton X-100 for 1 h (all at room temperature) and subsequently incubated with a GFP polyclonal antibody (1:1000, rabbit, Invitrogen, CatNo. A-11122), a dsRED polyclonal antibody (1:1000, rabbit, Clontech, CatNo. 632496), or an OXT monoclonal antibody (1:1000, mouse, PS38, kindly provided by Dr. Harold Gainer). Primary antisera were diluted in PBS plus 2% NGS overnight at 4°C for GFP antibody and overnight at room temperature for dsRED and OXT antibodies. Incubated slices were washed three times in PBS plus 1% NGS for 10 minutes at room temperature, incubated for 2 h at room temperature with a 1:1000 dilution of an Alexa Flour 488 goat anti-rabbit IgG (H+L) (1:1000, Thermo Fisher Scientific, CatNo.A11034) and Alexa Fluor 633 goat anti-rabbit IgG (H+L) (1:1000, Thermo Fisher Scientific, CatNo.A21071) in 1% NGS in PBS and subsequently washed three times in PBS for 10 min at room temperature. The sections were mounted on slides and coverslipped.

Imaging

All images were acquired on a Nikon 1 confocal laser scanning microscope. Digitalized images were analyzed using Fiji (NIMH, Bethesda MD, USA) and Adobe Photoshop CS5 (Adobe, Montain View, CA).

Brain Autoradiography

A separate cohort of naive mice was handled as described above and their brains were rapidly explanted, snap-frozen in isopentane at −25°C and moved at −80°C for storage. 14 μm-thick coronal sections were then cut with a cryostat and mounted on chrome-alum-gelatin-coated microscope slides. All slides were stored at −80°C until receptor autoradiography. The binding procedure and quantification of the resulting autoradiographic images were performed as previously described [67]. Briefly, sections were fixed with 0.2% paraformaldehyde and rinsed twice with 0.1% bovine serum albumin in 50 mM Tris–HCl buffer (pH 7.4). OXT binding sites were detected by incubation (2 hours at room temperature in a humid chamber) with the radioiodinated OXTR antagonist ornithine vasotocin analog ([125I]-OVTA, Perkin Elmer, MA, USA) at 0.02 nM in a medium containing 50 mM Tris–HCl, 0.025% bacitracin, 5 mM MgCl2, 0.1% bovine serum albumin. Sections immediately adjacent to the ones used for [125I]-OVTA binding were used to determine non-specific binding by addition of 2 μM OXT to the incubation solution. The unbound excess of ligand was washed out and slides were quickly dried under a stream of cool air and exposed to Biomax MR Films (Carestream) in an autoradiographic cassette for 72 hours. The final autoradiograms were digitalized by grayscale high-resolution scanning (600x600 dpi) and analysis was carried out using the ImageJ 1.47v software (NIH, USA). Target regions were identified by comparison with a reference mouse brain atlas [127]. Specific densitometric gray intensity was calculated by subtraction of the gray level of the respective non-specific binding section. Autoradiographic ^{125}I microscales (Amersham International, UK) also were exposed for 72 hours and a reference standard curve was generated. Levels of gray intensity were then converted to nCi/mg tissue equivalent by interpolation with the standard curve.

Ex vivo electrophysiology

Ex vivo patch clamp electrophysiology recordings were performed on PVN virus-injected slices. Mice were anesthetized with isoflurane and transcardially perfused with an ice-cold cutting solution containing: 200 mM sucrose, 4 mM $MgCl_2$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM D-glucose (~300 mOsm, pH 7.4, oxygenated with 95% O_2 and 5% CO_2). Brains were removed and immersed in the cutting solution. Coronal slices (270 μm thick, VT1000S Leica Microsystem vibratome) were incubated for 2 min in a mannitol solution (225 mM mannitol, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 8 mM $MgSO_4$, 0.8 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM d-glucose (~300 mOsm, pH 7.4, oxygenated with 95% O_2 and 5% CO_2)) and then allowed to recover

for 1 hour at 35°C in a solution containing: 117 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose (~310 mOsm, pH 7.4, oxygenated with 95% O_2 and 5% CO_2). Recordings were performed in magnocellular and parvocellular neurons of the PVN at room temperature in artificial cerebrospinal fluid (ACSF) with the following composition: 117 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose (~310 mOsm, pH 7.4, oxygenated with 95% O_2 and 5% CO_2). Patch pipettes were made from thick-wall borosilicate glass capillaries (B150-86-7.5, Sutter Instrument). Pipettes (5-7 mΩ) were filled with an intracellular solution containing: 130 mM K-gluconate, 10 mM HEPES, 7 mM KCl, 0.6 mM EGTA, 4 mM Mg_2ATP, 0.3 Mm Na_3GTP, 10 mM Phosphocreatine. The pH was adjusted to 7.3 with HCl. Whole-cell recordings were performed on PVN neurons identified on a fluorescent-based approach. Once stable recording conditions were obtained (series resistances in the range of 10–25 mΩ), PVN neurons were identified electrophysiologically as magnocellular (presence of transient outward rectification) or parvocellular (lack of transient outward rectification) according to an established current-clamp protocol in literature [128]. Validation of iDREADDs was performed evoking spike firing in PVN neurons by injection of a small depolarizing current pulse (20 pA for 1 s) under current-clamp mode. Activation of iDREADDs was obtained using 10 μM Clozapine N-Oxide (CNO, #4936 Tocris Bioscience) applied in the bath for 15 min. Data, filtered at 0.1 Hz and 5 kHz and sampled at 10 kHz, were acquired with a patch-clamp amplifier (Multiclamp 700B, Molecular Devices) connected to a digital converter (Digidata 1440A, Molecular Devices) and analyzed using pClamp 10.2 software (Molecular Devices). All chemicals were purchased from Sigma, otherwise specified.

QUANTIFICATION AND STATISTICAL ANALYSIS

No statistical methods were used to predetermine sample sizes, although sample sizes were consistent with those of previous studies [19, 23, 80]. No explicit randomization method was used to allocate animals to different experimental groups, but samples always derived from mice of the same litter. Mice were tested and data were processed by investigators blinded to the treatment and genotype of the animals. All datasets were tested to fit a normal distribution using the Kolmogorov-Smirnov and Pearson's chi-square tests. All statistical parameters including the statistical tests used, exact value of n, what n represents, precision measures (mean ± SEM) and statistical significance are reported in the Figure Legends. Results are expressed as mean ± standard error of the mean (SEM) throughout. Each observer's behavior toward the two different demonstrator mice was analyzed using a within-groups Repeated-measures ANOVA (RM-ANOVA). The behavior of the two demonstrators and USVs recordings were analyzed by Two-Way ANOVAs with emotional state as between-subjects factors, and the within-session 2-min consecutive intervals as a repeated-measure within-subject factor. The behaviors of the observer mice in the one-on-one setting were analyzed by Two-Way ANOVAs with the emotional state of the demonstrator as between-subjects factors, and the within-session 2-min consecutive intervals as a repeated-measure within-subject factor. Two or One-Way ANOVAs were used for autoradiography and social interactions when different genotypes and treatments were involved. Newman–Keul's post hoc test with multiple comparisons corrections was used for making comparisons within groups when the overall ANOVA showed statistically significant differences. Two-tailed paired t test or Two tailed Wilcoxon matched pairs signed rank test were used for electrophysiological experiment. The accepted value for significance was $p < 0.05$. Statistical analyses were performed using Statistica 13.2 (StatSoft).