An intra-amygdala circuit specifically regulates social fear learning

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Adaptive social behavior requires transmission and reception of salient social information. Impairment of this reciprocity is a cardinal symptom of autism. The amygdala is a critical mediator of social behavior and is implicated in social symptoms of autism. Here we found that a specific amygdala circuit, from the lateral nucleus to the medial nucleus (LA–MeA), is required for using social cues to learn about environmental cues that signal imminent threats. Disruption of the LA–MeA circuit impaired valuation of these environmental cues and subsequent ability to use a cue to guide behavior. Rats with impaired social guidance of behavior due to knockout of Nrxn1, an analog of autism-associated gene NRXN, exhibited marked LA–MeA deficits. Chemogenetic activation of this circuit reversed these impaired social behaviors. These findings identify an amygdala circuit required to guide emotional responses to socially significant cues and identify an exploratory target for disorders associated with social impairments.

The reciprocal exchange of information by social interaction is a key ability that promotes survival. This ability allows an observer to deduce the intentions and emotions of others by interpretation of social cues, such as facial expression and body posture. One consequence is a capacity to avoid threats by observing the association between others’ social cues and co-occurring events. Previous studies have implicated several interconnected brain regions as a social network that contributes to the transmission and reception of social information. The amygdala is a key component of this network1–5 and may serve as a link between recognition of social cues and the production of socially motivated affective responses. In support of this, the amygdala displays functional abnormalities in individuals with impaired social comprehension, such as people with autism6,7.

The nuclei of the amygdala work together to orchestrate a range of affective behaviors. However, intra-amygdala connections that guide learned social behavior are not known. The lateral nucleus (LA) of the basolateral complex is implicated in guidance of affective behavior by environmental cues. Outputs from the LA to the central amygdala, bed nucleus of stria terminalis and nucleus accumbens guide autonomic and behavioral aspects of fear and appetitive behavior in response to learned cues4–10. The posterior medial amygdala (MeA) also receives input from the LA11, is an essential mediator of social behavior4,12–15 and exhibits patterns of activation consistent with a role in social learning16. Therefore, interaction between LA and MeA might be required for successfully linking social cues with an appropriate learned affective response. Little is known about the LA and MeA interaction or how their interaction may be impaired in aberrant social comprehension. The purpose of this study was to test the importance of the LA–MeA circuit in social behaviors and to identify whether the LA–MeA path can serve as a link between comprehension of a social behavior and appropriate use of that information to guide behavior.

RESULTS

Amygdala circuit in social learning

To test whether the LA–MeA path can serve as a link between comprehension of a social behavior and the appropriate use of that information to guide responses, we employed a social fear conditioning paradigm. The premise of this approach is that a rat that values social cues produced by a conspecific can use those cues to guide their immediate affective behavior and can also imbue associated environmental cues and contexts with affective significance. The valuation of social cues can be inferred from the use of associated conditioned cues and contexts to guide behavior. In social fear conditioning, a ‘demonstrator’ undergoes classical fear conditioning (footshock paired with conditioned stimulus (CS+)) with a conspecific ‘observer’ in close proximity17. In these experiments, rats are separated only by a mesh barrier (Fig. 1a). The behavior of these rats is measured over the entire course of fear conditioning and again in response to the CS+ after 48 h. An appropriate affective response to the CS+ in this task requires the observer rat to successfully recognize and process a social cue and associate this social cue with environmental cues. Observer rats readily demonstrated social approach (Fig. 1b) during initial phases of this task and developed freezing in response to the CS+ tone (Fig. 1c), despite no direct experience with footshock. When tested after 48 h, observer rats exhibited contextual freezing in the same chamber (Fig. 1d) and cued freezing to the CS+ in a novel chamber (Fig. 1e). To verify that this was a result of social transmission, this same conditioning model was repeated with an anesthetized demonstrator. When conditioning was performed with an anesthetized demonstrator, the observing rat displayed less approach behavior (Fig. 1b; time proximal Cohen’s d = 1.17, 95% confidence interval (C.I.) = −26.8 to −2.5; time nose poking Cohen’s d = 1.27, 95% C.I. = −35.9 to −4.9, two-tailed unpaired t-test) and did not display freezing during conditioning (Fig. 1c; η² = 0.10, one-way

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Figure 1 Fear learning through social transmission. (a) The social fear conditioning apparatus (left) was divided down the middle to allow footshock delivery paired with a tone to the animal on the right side (demonstrator), while the animal on the left received no footshock (observer). The animals were separated by a mesh divider (right). (b) Observer rats (n = 14) displayed exploratory behavior directed toward the demonstrator, measured as amount of time spent proximal to the divider that separated the rats (left) and amount of time spent with its nose in close proximity or poking into the divider (right). If the rat on the other side of the divider was anesthetized, the observer rat (n = 7) displayed significantly less time spent with its nose in close proximity or poking into the divider (right). If the rat on the other side of the divider was anesthetized, the observer rat (n = 7) displayed significantly less time engaged in this social exploratory behavior (time proximal, P = 0.0206, t = 2.524, degrees of freedom (d.f.) = 19, Cohen’s d = 1.17, 95% C.I. = −26.8 to −2.5, two-tailed unpaired t-test; time nose poking, P = 0.0128, t = 2.749, d.f. = 19, Cohen’s d = 1.27, 95% C.I. = −35.9 to −4.9, two-tailed unpaired t-test). (c) Observer rats (n = 14) displayed increased freezing over the course of social fear learning (6 trials; P = 0.0108, F6.91 = 5.423, r² = 0.29, one-way RM-ANOVA) if the demonstrator rat was awake, despite no physical contact with shock. However, if the demonstrator rat was anesthetized during the social fear conditioning, the observer rats (n = 7) displayed no significant freezing (P = 0.5875, F6.42 = 0.6337, r² = 0.10, one-way RM-ANOVA), resulting in a significant difference in freezing between groups (i.e., difference in the effect of social fear conditioning; trial x anesthesia interaction, P = 0.0446, F7.114 = 2.237, r² = 0.031, two-way RM-ANOVA; main effect of anesthesia, P = 0.0108, F1.19 = 7.988, r² = 0.19, two-way RM-ANOVA). (d) When replaced in the same chamber after 48 h, rats that had been paired with an awake demonstrator (n = 14) displayed contextual freezing, while awake rats that had been paired with an anesthetized demonstrator (n = 7) displayed significantly less contextual freezing (P = 0.0045, t = 3.219, d.f. = 19, Cohen’s d = 1.49, 95% C.I. = 5.9 to 28.0, two-tailed unpaired t-test). (e) Similarly, when placed in a novel chamber and presented with the conditioned tone (CS+; 12 trials), rats that had been paired with an awake demonstrator displayed conditioned freezing to the CS+ (n = 14) while rats that had been paired with an anesthetized demonstrator (n = 7) displayed significantly less freezing (main effect of treatment, P = 0.0038, F1.19 = 15.15, r² = 0.21; treatment x trial interaction, P = 0.0013, F1.228 = 2.810, r² = 0.043, two-way RM-ANOVA). Data are means ± 95% confidence intervals. *P < 0.05, main effect of group in two-way RM-ANOVA; †P < 0.05, main effect of treatment in two-way RM-ANOVA.

emitted by the demonstrator drive the social learning. To determine whether the MeA or LA are required for social fear learning, we used a chemogenetic approach, bilaterally inactivating nuclei during social fear conditioning by first injecting them with an adeno-associated virus (AAV) vector containing a calcium/calmodulin-dependent protein kinase (CaMKIIa) promoter, a human influenza hemagglutinin (HA) epitope tag, a modified human muscarinic M4 receptor linked to an inhibitory G protein receptor (hM4D(Gi)), and the fluorescent protein mCitrine following an internal ribosome entry site (IRES) (AAV-CaMKIIa-HA-hM4D(Gi))-IRES-mCitrine; hereafter, DREADD (designer receptors exclusively activated by designer drugs)-Gi)19,20 and then injecting 1 mg/kg clozapine-N-oxide (CNO) intraperitoneally 40 min before conditioning: Fig. 2a,b). Control rats expressed a reporter without DREADD-Gi (AAV-CaMKIIa-YFP; n = 8). We administered CNO to both control and DREADD-expressing rats and found that it decreased the activity of MeA or LA neurons in DREADD-Gi-expressing rats but not in control rats (Fig. 2c–f). MeA firing rate Cohen’s d = 0.85, 95% C.I. = −2.9 to −1.2; LA firing rate Cohen’s d = 1.16, 95% C.I. = −0.69 to −0.22, two-tailed unpaired t-test), and this effect was specific for the targeted region (Fig. 2g). This demonstrates the utility of this chemogenetic approach to decrease LA and MeA activity. DREADD expression and recording loci were confirmed to lie within the MeA or LA (Supplementary Figs. 1 and 2).

Inactivation of the LA during social fear conditioning had no effect on social approach behavior (Fig. 3a) but attenuated freezing during social fear conditioning (Fig. 3b; r² = 0.11, two-way RM-ANOVA). When tested in a novel context after 48 h, conditioned freezing to the CS+ was attenuated (Fig. 3d; r² = 0.18, two-way RM-ANOVA), while freezing to the original context in the absence of the CS+ was spared (Fig. 3c). The LA, then, was a key mediator of learning to associate an environmental cue (but not context) with a paired social cue, and its inactivation led to a deficit in conditioned responding to that environmental cue when tested later. To test whether the MeA is similarly required for social fear learning, (posterior) MeA was bilaterally inactivated during social fear conditioning by the same DREADD-based approach. Observer rats with MeA inactivation during social fear conditioning exhibited both reduced social approach (Fig. 3a; time nose poking r² = 0.33 and time proximal r² = 0.35, one-way ANOVA) and reduced freezing in response to the conspecific that received foot shocks during conditioning (Fig. 3b). When tested after 48 h, prior MeA inactivation reduced conditioned freezing to both the CS+ and the context (Fig. 3c; d; r² = 0.36, one-way ANOVA). This is consistent with a role for the MeA in general recognition of social cues or responding to social cues. Overall, this demonstrated a specific role for LA in social learning about an important cue and an additional, more general role for the MeA in social approach and freezing responses to conspecific footshock during social fear conditioning.

To determine the precise role of the LA–MeA connection in social fear learning, we used a functional disconnection approach, whereby the LA in one hemisphere and the MeA in the other hemisphere were chemogenetically inactivated during social fear conditioning (Fig. 2b; functional disconnection by inactivation instead of anatomical disconnection). This approach allowed for one fully functional LA and MeA to send and receive information from other brain structures during this task. The LA–MeA functional disconnection did not decrease social approach during social fear conditioning (Fig. 3e), demonstrating that the LA–MeA connection is not required for normal social approach, recognition or responding to social cues per se. Despite normal social approach behavior, the LA–MeA functional disconnection caused reduced freezing in the observer rats to the

repeated-measures (RM) ANOVA and showed less conditioned freezing during contextual and cued testing (Fig. 1d,e; contextual freezing Cohen’s d = 1.49, 95% C.I. = 5.94 to 28.0, two-tailed unpaired t-test; cued freezing r² = 0.21, two-way RM-ANOVA). This is similar to the absence of social fear conditioning seen when the demonstrator receives no footshock (‘tone-only’ control)18. This indicates that social cues
CS+ tone over the course of conditioning (Fig. 3f; \(\eta^2 = 0.047\), two-way RM-ANOVA), consistent with decreased association of the CS+ tone with conspecific social cues. When tested after 48 h, rats with prior LA–MeA functional disconnection displayed impaired freezing to the CS+ (Fig. 3h; \(\eta^2 = 0.22\), two-way RM-ANOVA) but not contextual freezing (Fig. 3g), which indicated impaired ability to learn the association between an environmental cue paired with a social cue. This recapitulated the effect of either bilateral LA inactivation or bilateral MeA inactivation. Inactivation of LA and MeA within one hemisphere, leaving a functional LA–MeA connection in the other hemisphere, did not significantly impair social fear conditioning (Fig. 3f–h). This demonstrated that at least one functional connection between the LA and MeA was necessary and sufficient for social fear learning. The importance of the direct LA–MeA connection was further verified by selective inactivation of LA neurons that project to the posterior MeA. This was accomplished by an intersectional approach with MeA infusion of a retrogradely transported viral vector that transduces expression of Cre (CAV2-Cre) and LA infusion of Cre-dependent DREADD-Gi (AAV-hSyn-DIO-hM4D(Gi)-mCherry), leading to specific expression of DREADD-Gi only in those LA neurons that project to MeA. Similarly to the disconnection experiments above, inactivation of the LA–MeA circuit
impaired social fear conditioning (Fig. 3i and Supplementary Fig. 3; \( \eta^2 = 0.16 \), two-way RM-ANOVA). Off-target infusions did not impair social fear conditioning (Fig. 3j–l). Thus, disruption of the LA–MeA pathway (caused by bilateral inactivation of either node, functional disconnection or specific inactivation) resulted in failure to use social information to imbue predictive environmental cues with emotional salience.

The specificity of the LA–MeA path in social learning of paired cues was demonstrated by intact social learning of contextual fear, which indicates that (i) the LA–MeA path was not required for the ability to freeze and form other associations based on social information, (ii) the LA–MeA path was not required for recognition of social cues and (iii) the LA–MeA path was needed to link social cues with environmental cues and to use those environmental cues to guide social behavior during conditioning. This is also consistent with other studies that demonstrate that cue-specific information is processed by the LA, while context-specific information may be processed by the basolateral nucleus of the amygdala (BA)\(^9\). Indeed, in experiments that included inactivation of the BA–MeA path, social learning of contextual fear was impaired (Fig. 3k).

To determine the specificity of the LA and MeA functional connectivity to social fear learning, we tested their role in mediating the association between a nonsocial aversive outcome and a novel sensory cue in classical fear conditioning. Bilateral inactivation of the LA during classical (nonsocial) fear conditioning impaired conditioned freezing to a CS+ (Supplementary Fig. 4a; \( \eta^2 = 0.27 \), two-way RM-ANOVA), consistent with prior studies\(^9,21\) and supporting the effectiveness of inactivation by DREADD-Gi. However, bilateral inactivation of the MeA or LA–MeA disconnection during classical fear conditioning did not impair conditioned freezing in response to a CS+ (Supplementary Fig. 4b). Thus, the LA–MeA connection was selectively required for social fear learning but not for nonsocial associative learning.

The LA–MeA path may be necessary for other behaviors that contribute to social fear learning. To determine the specificity of this pathway’s role in social fear learning, we measured nonlearned social interaction with a novel rat in an open field. There was no gross abnormality in total time interacting or number of interactions upon bilateral LA or MeA inactivation or upon LA–MeA ipsilateral or crossed inactivation (Supplementary Fig. 5a–d). A decrease in the duration of interaction events was observed upon bilateral MeA inactivation (Supplementary Fig. 5b; \( \eta^2 = 0.39 \), one-way ANOVA). This indicates that the LA–MeA path itself is not required to drive spontaneous unlearned social exploration, but the MeA contributes to sustained social interaction. No abnormalities in exploration of the open field or elevated plus maze were observed with any of the inactivation conditions (Supplementary Fig. 5e,f), demonstrating that general, unconditioned anxiety behaviors are unlikely to rely on this circuit.

To further link the LA–MeA pathway with learned fear, the strength of this pathway was measured in vivo after a modified social fear learning procedure. In this modified social fear learning procedure, fewer CS+ trials were performed (3 trials; Fig. 4a) to produce greater interindividual variability in social learning. This resulted in a wider range of conditioned freezing responses to the CS+, including ‘bad learners’, which displayed little detected conditioned freezing to the CS+ during an abbreviated test session (3 trials, < 15% time freezing), and ‘good learners’, which displayed conditioned freezing during this test session (> 15% time freezing; Fig. 4a). The in vivo strength of the LA–MeA pathway was measured in good learners and bad learners by recording the posterior MeA local field potential response upon
stimulation of the LA in anesthetized rats (1.5 g per kg urethane). The input–output curve of the MeA response to LA was quantified (Fig. 4b). There was a significant correlation between the strength of the LA–MeA path and the amount of socially conditioned freezing to the CS+ (Fig. 4c). As a control, we measured the strength of the LA–MeA path after a weak classical fear conditioning procedure (2 trials, 0.3 mA) that produced variability in nonsocial conditioned freezing. There was no significant association between classical conditioned freezing (3 trials) and the strength of the LA–MeA path (Fig. 4c). Taken together, these data demonstrate the necessity of the LA–MeA path in the ability to use social cues to assign value to an environmental cue that predicts threat, and importantly, this ability scaled as a function of the strength of the pathway.

The LA–MeA circuit in a condition with impaired social behavior
The acute deficits in responding to social cues and assigning value to socially relevant cues that was produced by inactivation of nodes in the LA–MeA circuitry is reminiscent of social deficits observed in many patients with autism. Impaired function of the LA–MeA path may contribute to social deficits in this and other disorders. Indeed, imaging studies demonstrate hyper- or hypoactivity of the amygdala associated with abnormal social behavior in autism. The next goal of this study was to test whether a dysfunction of the LA–MeA path could underlie deficits in animals that display impaired social learning. Deletions and copy number variations of the neurexin gene (NRXN) and mutations of the neuligin gene (NLGN), which encodes neurexin's binding partner, are associated with autism and neurexin has an important role in neurotransmission in several brain regions, leading to the expectation of abnormal social behavior in rodents with neurexin mutations. We therefore tested whether LA–MeA circuitry is impaired in rats with a knockout of the neurexin-1 gene (Nrxn1), which impairs production of neurexin-1 (Nrxn rats) and whether this impairment is associated with a deficit in social learning. Nrxn rats were bred from homozygous pairs. Age-matched wild-type (WT) rats of the same genetic background were bred at the same facility, housed on the same rack in the same room and shipped at the same time. As expected, Nrxn rats expressed significantly lower neurexin-1 mRNA and protein levels compared to WT controls (Supplementary Fig. 6a,b; relative protein Cohen's d = 3.34, 95% C.I. = −1.23 to −0.44; relative mRNA Cohen's d = 5.25, 95% C.I. = −1.21 to −0.65, two-tailed unpaired t-test).

In vivo measurement of the LA–MeA connection in Nrxn rats revealed critical impairments consistent with those that explain aberrant responses to conspecific behavior observed in chemogenetic
tended to display a greater local field potential than bad learners. (c) of stimulation intensities to produce an input–output curve. Good learners the strength of the LA–MeA local field potential. There was a significant individual rats (quantified as average percent time freezing during test; the strength of the LA–MeA local field potential. There was a significant difference in paired pulse facilitation and the coefficient of synaptic drive (an indication of reduced presynaptic reliability of excitatory membrane responsiveness (input resistance; two-way RM-ANOV A), measured as action potential firing. This was in vivo experiments (above). MeA activity was recorded in anesthetized rats (urethane, 1.5 g per kg) while LA was activated by a stimulation electrode. Activation of the LA evoked rapid responses in MeA of WT rats (Fig. 5a and Supplementary Fig. 7a–c). This response had all the characteristics of a monosynaptic input rather than an antidromic or polysynaptic input. (The latency jitter was between 0.5 and 3 ms, with <2 ms shift in latency with increasing stimulation intensity. Evoked action potentials did not collide with preoccurring spontaneous action potentials.) The reciprocal in vivo LA activity was also measured while the MeA was stimulated in anesthetized rats. The strength of the LA–MeA path was measured as the probability of an MeA neuron firing in response to LA stimulation and was significantly stronger than the relatively weak reciprocal MeA–LA path (Supplementary Fig. 7b,c) indicating that the flow of information in the LA-to-MeA direction is more efficacious ($\eta^2 = 0.16$, two-way RM-ANOVA). The in vivo response of MeA neurons to LA input was attenuated in Nrxn rats (Fig. 5b–d), whether measured as MeA neuronal response probability or as MeA local field potential response. Moreover, spontaneous firing of MeA neurons in Nrxn rats was significantly reduced compared to WT (Fig. 5e); measured in vivo from anesthetized rats; Cohen’s $d = 0.76$, 95% C.I. = −5.82 to −1.67, two-tailed t-test). These data point to a deficit in LA-to-MeA transmission and a more global MeA deficit that impacted firing activity. To test the proximal basis for the impaired responsiveness of MeA neurons, we measured membrane excitability and synaptic drive in vitro. The neuronal excitability of MeA neurons was significantly impaired in Nrxn rats (WT, 5.6 ± 0.9 Hz, n = 36 neurons from 8 rats; Nrxn, 1.9 ± 0.6 neurons, n = 59 neurons from 9 rats; $P = 0.0005$, t = 3.583, d.f. = 93, Cohen’s $d = 0.76$, 95% C.I. = −5.82 to −1.67, two-tailed t-test). Data are means ± 95% confidence intervals except where noted.

**Figure 4** LA–MeA strength is associated with social fear learning. (a) A modified, weaker version of social fear conditioning (3-trial conditioning, left; n = 9 rats) produces substantial variability between rats in conditioned freezing, with some rats displaying robust conditioned freezing (good learners, n = 5 rats) and others displaying minimal conditioned freezing (bad learners, n = 4 rats). (b) Stimulation of LA caused a local field potential response in the posterior MeA, measured as the slope of the initial deflection. The slope was measured across a range of stimulation intensities to produce an input–output curve. Good learners tended to display a greater local field potential than bad learners. (c) The slope of the LA–MeA input–output curve was quantified as a measure of the strength of the LA–MeA local field potential. There was a significant correlation between the LA–MeA strength and social fear learning in individual rats (quantified as average percent time freezing during test; Pearson $r = 0.78$, $R^2 = 0.64$, $P = 0.01$). In control experiments we found no correlation between LA–MeA strength and classical fear conditioning in individual rats ($n = 6$ rats, Pearson $r = 0.22$, $R^2 = 0.049$, $P = 0.67$). Data are means ± 95% confidence intervals except where noted.

**Figure 5** Disruption of intra-amygdala path in Nrxn rats. (a) Stimulation of LA caused increased firing in posterior MeA neurons in vivo ($n = 16$ neurons from $5$ rats, defined as > 3 times baseline firing rate in a 5-ms time window within 10 ms of LA stimulation). Responses were included in analysis only if they met criteria for monosynaptic connections (average latency 4.9 ms, range 3.7–5.8 ms, latency jitter average = 1.37 ms, range 0.66–2.21 ms). Shown here are frequency histograms of the distribution of latency and latency jitter. (b) The average response was significantly weaker in Nrxn rats at the same stimulation intensity (WT, n = 16 neurons from 5 rats; Nrxn, n = 11 neurons from 5 rats; 0.6 mA, mean ± 95% confidence interval; genotype × time interaction, $P < 0.0001$, $F_{7,1,950} = 2.744$, $\eta^2 = 0.053$, two-way RM-ANOVA). (c) The response of these same MeA neurons was significantly weaker in Nrxn rats over a range of LA stimulation intensities (main effect of genotype, $P < 0.0001$, $F_{1,125} = 35.94$, $\eta^2 = 0.070$; genotype × stimulation interaction, $P = 0.0002$, $F_{4,125} = 6.05$, $\eta^2 = 0.047$, two-way RM-ANOVA). (d) The field potential in posterior MeA evoked by LA stimulation was significantly reduced in Nrxn rats ($n = 16$ rats) compared to those evoked in WT rats ($n = 11$ rats; slope of the first negative peak; main effect of genotype on negative peak, $P = 0.011$, $F_{1,25} = 7.50$, $\eta^2 = 0.087$; genotype × stimulation interaction, $P = 0.0043$, $F_{4,100} = 4.06$, $\eta^2 = 0.041$, two-way RM-ANOVA). (e) The firing rate of posterior MeA neurons in vivo was significantly decreased in Nrxn compared to that in WT rats (WT, 5.6 ± 0.9 Hz, n = 36 neurons from 8 rats; Nrxn, 1.9 ± 0.6 neurons, n = 59 neurons from 9 rats; $P = 0.0005$, t = 3.583, d.f. = 93, Cohen’s $d = 0.76$, 95% C.I. = −5.82 to −1.67, two-tailed t-test). Data are means ± 95% confidence intervals except where noted.
synaptic input. There was little difference in spontaneous excitatory postsynaptic currents (miniature EPSCs: Fig. 6e,f) perhaps because spontaneous EPSCs are induced by different presynaptic mechanisms than EPSCs evoked by action potentials.\(^{28,29}\) Overall, these results demonstrate that the LA–MeA circuit was impaired in Nrxn rats due to reduced input strength and the attenuated responsiveness of MeA neurons. In contrast, LA neuron responsiveness was not significantly different in Nrxn rats despite similar abnormality of synaptic function (excitability \(\eta^2 = 0.0027\), two-way RM-ANOVA; paired-pulse ratio Cohen’s \(d = 1.26, 95\%\) C.I. = -0.32 to -0.097; coefficient of variance CV Cohen’s \(d = 0.86, 95\%\) C.I. = 0.0086 to 0.13, two-tailed unpaired \(t\)-test) (Supplementary Fig. 8a–e).

To test whether the weakness in the LA–MeA connection in Nrxn rats was associated with similar outcomes as LA–MeA disconnection, we measured social behaviors. Gross social interaction behaviors and social preferences of neurexin knockout mice have been found to be fairly normal in some studies\(^{30}\) but abnormal in others.\(^{31}\) Social interaction with a novel WT rat was measured to assess whether Nrxn rats demonstrate preference for social interaction. Nrxn rats did not display significant abnormalities in the frequency or total time of social interactions with a novel rat (Fig. 7a). However, there was a significant decrease in the duration of each social interaction (Fig. 7a; Cohen’s \(d = 1.59, 95\%\) C.I. = -1.36 to -0.50, two-tailed \(t\)-test), similar to the previous result we observed in bilateral MeA-inactivated animals. There was no difference in exploration of a novel object between WT and Nrxn rats (Fig. 7b). However, when given the choice between social interaction with a novel rat or exploration of a novel object, Nrxn rats displayed a significantly diminished preference for the novel rat (Fig. 7c; Cohen’s \(d = 0.98, 95\%\) C.I. = -1.44 to -0.22, two-tailed unpaired \(t\)-test). Taken together, these results are consistent with a relatively low significance of social interaction for Nrxn rats. To test the ability of Nrxn rats to learn by social transmission, we measured social fear conditioning. The Nrxn rats displayed significantly reduced approach to the conspecific during social fear conditioning (Fig. 7d; time proximal Cohen’s \(d = 1.34, 95\%\) C.I. = -25.4 to -7.4; time nose poking Cohen’s \(d = 1.77, 95\%\) C.I. = -36.3 to -15.1, two-tailed unpaired \(t\)-test) and significantly smaller responses to the socially conditioned CS+ (Fig. 7e; \(\eta^2 = 0.18\), two-way RM-ANOVA). This was associated with reduced conditioned freezing to the CS+ cue and context after 48 h, consistent with impaired MeA function, including impaired guidance of MeA by LA (Fig. 7f,g; contextual freezing Cohen’s \(d = 1.27, 95\%\) C.I. = -24.2 to -6.5, two-tailed unpaired \(t\)-test; cued freezing \(\eta^2 = 0.13\), two-way RM-ANOVA). This reduced social engagement and learning did not appear to be due to differences in the behavior of the demonstrators, which displayed similar freezing and social interactive behavior regardless of observer genotype (Supplementary Fig. 9a). Previous studies have demonstrated that social fear transmission can utilize auditory and olfactory components.\(^{32,34}\) Nrxn rats displayed normal freezing to a loud unconditioned tone (95 dB, 1.5 kHz, 1s; Supplementary Fig. 9b), indicative

**Figure 6** Intrinsic and synaptic properties of MeA neurons. Properties of MeA (posterior) neurons were measured in vitro using whole cell recordings. (a) Posterior MeA neurons (Type I neurons based on firing pattern) recorded in vitro from Nrxn rats had significantly lower membrane excitability compared to those in WT rats (WT, n = 11 neurons from 11 slices; Nrxn, n = 12 neurons from 12 slices; main effect of genotype, \(P = 0.0004, F_{1,21} = 17.6, \eta^2 = 0.21\); genotype \(\times\) current interaction, \(P < 0.001, F_{1,84} = 18.8, \eta^2 = 0.15\), two-way RM-ANOVA). (b) MeA neurons from Nrxn rats displayed significantly lower input resistance (\(R_{\text{in}}\)) than those from WT rats (\(P = 0.0004, t = 3.875, \text{d.f.} = 35\), Cohen’s \(d = 1.28, 95\%\) C.I. = -112.2 to -35.1, two-tailed unpaired \(t\)-test; WT, n = 19 neurons from 14 slices; Nrxn, n = 18 neurons from 13 slices). (c) The resting membrane potential of these same MeA neurons was not significantly different between WT and Nrxn rats (\(P = 0.129, t = 1.556, \text{d.f.} = 35\), Cohen’s \(d = 0.51, 95\%\) C.I. = -4.10 to 0.54, two-tailed unpaired \(t\)-test; WT, n = 19 neurons; Nrxn, n = 18 neurons). (d) The paired-pulse ratio of EPSCs in MeA neurons was significantly lower in Nrxn rats (WT, n = 10 neurons from 10 slices; Nrxn, n = 12 neurons from 12 slices; \(P = 0.015, t = 2.66, \text{d.f.} = 20\), Cohen’s \(d = 1.14, 95\%\) C.I. = -0.60 to -0.07, two-tailed unpaired \(t\)-test). CVs of EPSCs in MeA neurons were significantly higher in Nrxn rats (WT, n = 22 neurons; Nrxn, n = 23 neurons; \(P = 0.0049, t = 2.965, \text{d.f.} = 43\), Cohen’s \(d = 0.88, 95\%\) C.I. = 0.012 to 0.065, two-tailed unpaired \(t\)-test). (e) Miniature EPSCs (mEPSCs) were recorded from MeA neurons (WT, n = 17 neurons from 14 slices; Nrxn, n = 19 neurons from 14 slices). The mEPSC frequency (left; \(P = 0.397, t = 0.859, \text{d.f.} = 34\), Cohen’s \(d = 0.29, 95\%\) C.I. = -3.39 to 1.38, two-tailed unpaired \(t\)-test) and amplitude (right; \(P = 0.457, t = 0.753\), Cohen’s \(d = 0.25, 95\%\) C.I. = -3.39 to 1.38, two-tailed unpaired \(t\)-test) were not significantly different between WT and Nrxn rats. *\(P < 0.05\), two-tailed unpaired \(t\)-test; #\(P < 0.05\), main effect of group in two-way RM-ANOVA. Data are means ± 95% confidence intervals.
of unimpaired hearing ability. Nrxn rats also displayed normal ability to locate grape-flavored sucrose pellets that were buried in bedding (Supplementary Fig. 9c) and normal discrimination between male and female pheromones, as demonstrated by time spent exploring used bedding from female and male rats (Supplementary Fig. 9d), though they demonstrated less preference for the bedding from female rats than WT rats did (Cohen’s $d = 1.12$, 95% C.I. = 1.09 to −0.02, two-tailed unpaired $t$-test). Overall, this is consistent with intact olfaction. However, this does not completely rule out more subtle forms of sensory deficits and does not conclusively demonstrate the functionality of these modalities in the transfer of social information. Nevertheless, Nrxn rats displayed normal or enhanced classical fear conditioning with similar footshock sensitivity (Supplementary Fig. 9e–g), consistent with a previous study. Together, these data indicate that Nrxn rats exhibit adequate hearing and olfactory function, as well as the ability to learn in associative tasks that utilize an auditory CS+, yet still do not adequately use social cues to direct learning behavior. Further evidence for abnormal social processing was observed during social interaction in the open field. A proportion of Nrxn rats (5 of 16 rats) displayed mounting behavior directed toward the male conspecific in the open field. None of the WT rats displayed this behavior (0 of 16 rats; Supplementary Fig. 10a,b). This behavior was also observed to occur in pairs of Nrxn rats in their home cage, but was rarely observed in WT pairs (Cohen’s $d = 1.74$, 95% C.I. = 1.15 to 3.85, two-tailed unpaired $t$-test). Overall, this is consistent with intact olfaction.
unpaired $t$-test). The Nrxn rats also displayed normal exploration behavior in the elevated plus maze and open field (Supplementary Fig. 9h,i), demonstrating that abnormally elevated anxiety is unlikely to underlie impaired social learning.

The physiological and behavioral data argue for a deficit of MeA function in Nrxn rats that leads to impairment of behaviors that rely on the LA–MeA path. Therefore, increased activation of the MeA may alleviate deficits of social fear conditioning in Nrxn rats. To test this, MeA was bilaterally chemogenetically activated in WT and Nrxn rats using a construct that activates stimulatory G (Gs) protein (AAV-CaMKIIa-HA-rM3D(Gs)-ires-mCitrine; DREADD-Gs35; Supplementary Fig. 11a–d) during social fear conditioning. Control Nrxn rats were transduced with a reporter only (AAV-CaMKIIa-EYFP). The effectiveness of DREADD-Gs at activating MeA neurons was assessed using in vivo extracellular recordings from anesthetized rats (as above). Administration of CNO caused an increase in the overall firing rate and number of spontaneously firing neurons recorded in the MeA of anesthetized DREADD-Gs rats, whereas vehicle had no significant effect (Supplementary Fig. 11a–c; $\eta^2 = 0.38$, two-way RM-ANOVA).

The MeA was activated by CNO during social fear conditioning. Activation of MeA improved social fear conditioning in Nrxn rats, as demonstrated by increased freezing during conditioning (Fig. 8a; $\eta^2 = 0.19$, two-way RM-ANOVA) and in response to the socially conditioned context and CS+ cue when tested after 48 h (Fig. 8b; contextual freezing $\eta^2 = 0.62$, one-way ANOVA; cued freezing $\eta^2 = 0.12$, two-way RM-ANOVA). This increased freezing was absent if the experiment was repeated with an anesthetized demonstrator (Fig. 8c), indicating that MeA activation itself did not induce spurious freezing to tone or context (Fig. 8c). Furthermore, DREADD-Gs activation of the MeA did not significantly increase conditioned freezing upon classical fear conditioning (Fig. 8e), indicating that DREADD-Gs did not lead to abnormal elevation of fear behaviors that do not rely on MeA circuitry. This remarkable improvement in use of social information to guide behavior upon MeA activation was observed despite a lifetime with genetic abnormality and a history of MeA dysfunction.
DISCUSSION

These results demonstrate that an intra-amygdala path between the LA and MeA underlies the ability to link the affective content of social cues with other external predictive cues. Specifically, the MeA was required to value a social cue emitted by a conspecific, while the LA–MeA circuit was required to assign value to environmental cues associated with social cues and to subsequently use that environmental cue to guide behavior. The interpretation most readily supported by observed effects of LA–MeA pathway inactivation is impaired social learning rather than impairment limited to expression during learning. The reason for this is that if social fear learning was intact but expression was transiently impaired due to DREADD inactivation, we would expect to observe conditioned freezing when the rats were tested after 48 h, but we did not. This is congruent with a role for the amygdala in processing social signals from faces and recognition of facial expression, and further adds the role of the LA–MeA in learning about and initiation of an affective response to the learned social cue. This places the LA–MeA as a specialized circuit parallel to the basolateral amygdala (BLA)–central amygdala path, BLA–bed nucleus of the stria terminalis path and BLA–accumbens path in the production of a coordinated response to conditioned cues. The MeA is important for species-specific social behaviors; LA inputs to the MeA help select and guide appropriate social behaviors and participate in broadening the importance of an associated social cue with environmental contingencies.

In addition to impairments in social learning caused by acute disruption of the LA–MeA path and of the correlation between social learning and LA–MeA path strength, this specific amygdala circuit was abnormal in a model of impaired social learning, the Nrxn rat. Previous studies that used social interaction and social preferences behaviors of Nrxn mice have found mixed results, consistent with the relatively subtle differences in social interaction observed here. Instead, a more robust social deficit of Nrxn rats appears in the processing of emotionally relevant social cues that signal environmental threats and in the assignment of value to a cue with social importance. These impairments in Nrxn rats map closely to the impairments found upon disconnection between the LA and MeA, parallel the impaired function of this circuit and share similarities with abnormalities observed after isolation rearing. Activation of MeA by DREADD-Gs reduced these impairments, though this is not necessarily due to repair of the underlying pathophysiology. In addition, the observed pathophysiology in Nrxn rats may not reflect the etiology of social deficits in autism spectrum disorders, which are associated with loss-of-function heterozygous mutations instead of knockout mutations. Surprisingly few studies have tested the importance of the amygdala in genetic models associated with social abnormalities, such as those found in autism. While informative, those few studies largely focused on the basolateral amygdala complex and did not test the impact on intra-amygdala function. Furthermore, to our knowledge no previous studies have tested the importance of the LA–MeA intra-amygdala circuit in social behavior. The current study found very discrete, targetable, abnormalities in this circuitry associated with impaired social learning.

Use of DREADD-based manipulations causes prolonged but temporary changes in neuronal activity. However, it is not the same as a true inactivation, and the functional disconnection induced here is unlikely to be as complete as disconnections produced by pharmacological or anatomical approaches. In addition, while use of DREADDs here was demonstrated to cause a change in neuronal activity, DREADD-based manipulations exert their effects by modulating intracellular signaling, which may contribute to the observed outcomes upon DREADD-induced inactivation. DREADD expression was under the control of a CaMKIIα or human synapsin 1 promoter. While CaMKIIα may be expressed in several BLA neuronal types, CaMKIIα expression is limited to BLA projection neurons. Therefore, use of the CaMKIIα promoter is expected to limit DREADD expression to projection neurons, as previously demonstrated, and use of this promoter with DREADD-Gi has been demonstrated to impact the physiology of BLA projection neurons. In addition, its expression was observed in fibers in regions that are LA projection targets, such as entorhinal cortex. While CaMKIIα expression can be observed in sections that include the posterior MeA, it is not entirely clear in which MeA neurons it is expressed. However, fibers were observed in hypothalamus and stria terminalis, indicating that DREADD expression included MeA projection neurons and these neurons may overlap with specific populations of MeA neurons that modulate social behavior.

Treatments for autism often rely on behavioral interventions that are less effective in severe autism. Our current results provide a rationale for pursuing approaches that augment MeA function in combination with behavioral intervention. This may produce benefits in the development of social abilities despite social impairments from an early age.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.E.V. designed experiments to measure mRNA and protein; R.C.T. and J.A.R. designed the remaining experiments. R.C.T., J.E.V., S.L., M.P. and J.A.R. performed all experiments. R.C.T., S.L., J.E.V. and J.A.R. performed statistical analyses. J.A.R. wrote the manuscript with input from all authors. R.C.T. and J.E.V. provided critical revisions.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animal model. All procedures were approved by the Rosalind Franklin University Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Mice with neurexin-1 knockout (Nxrr) rats and wild type (WT) control rats (Sprague-Dawley background, Sage Labs/Horizon Discovery, Boyertown, PA) and standard Sprague-Dawley rats (Harlan, Indianapolis, IN) were obtained at 9–12 weeks old. The sex (male) and age (9–12 weeks) were the same across all experiments. The number of animals is indicated for each experiment. Xnr rats were bred from homozygous pairs. Age-matched WT rats of the same genetic background were bred at the same facility, housed on the same rack in the same room and shipped at the same time. Rats were acclimated to the animal facility for at least 10 days before use. The rats were housed 2 or 3 to a cage in a climate-controlled facility with ad libitum access to food and water. Lights in the housing room were on a 12:12-h reversed light–dark schedule. All rats were naive at the initiation of experiments. All experiments were performed during the dark cycle.

Validation of neurexin-1α knockout in rats. A subset of rats (4 Nxrr and 5 WT controls) were deeply anesthetized with 5% isoflurane (Sigma-Aldrich, St. Louis, MO) and decapitated. Brains were quickly extracted, flash frozen in bromobutane and methylbutane on dry ice, and subsequently stored at −80 °C until RNA or protein extraction.

For protein extraction, brains were homogenized in RIPA buffer with the addition of a proteasine inhibitor cocktail (Roche Diagnostic, Indianapolis, IN). Homogenates were centrifuged at 15,000g for 20 min at 4 °C then stored at −80 °C. Protein (30 μg) was separated by electrophoresis on an 8–16% MiniPROTEAN TGX gel (Bio-Rad Laboratories, Hercules, CA) for 15 min at 90 V followed by 1.5 h at 150 V, then transferred to a nitrocellulose membrane for 1.5 h at 110 V. The membrane was blocked in 5% BSA and 0.1% Tween-20 in Tris-buffered saline (TBST) for 1 h at room temperature. The membrane was washed a final time with TBST (4 times for 10 min each time) and then protein bands were developed with HRP-conjugated goat anti-mouse antibody (Bethyl Laboratories, Montgomery, TX) at 1:5,000 and visualized with ultraviolet light (FOTO/UV2) and images were acquired (12-megapixel resolution, iPhone 6S, Apple Inc, Cupertino, CA) from 12 in (30.5 cm) above the gel, without flash. Band densities were obtained from these images using ImageJ software (NIH) and normalized to GAPDH CDNA.

Chromogenic surgical procedure. To induce DREADD expression in the MeA or LA, adeno-associated viral (AAVs) vectors with plasmids for DREADD receptors (Dr. Bryan Roth, UNC Vector Core, Chapel Hill, NC) were injected intracranially. Rats were randomly assigned to the different groups. Rats were anesthetized with either an intraperitoneal (i.p.) cocktail of ketamine (80–100 mg/kg, Webster Veterinary Supply, Devens, MA) and xylazine (10–20 mg/kg; Webster Veterinary Supply) or isoflurane (5% induction, 2–3% maintenance; Sigma-Aldrich). After confirmation of anesthesia, meloxicam (Metacam, 1 mg/kg, s.c.) was injected, each rat was placed in a stereotaxic apparatus with blunt ear bars (David Kopf Instruments, Tujunga, CA), and its scalp was shaved and wetted with Betadine and ethanol (70%). A small incision was made and 1% procaine was placed on the skull. Burrs were drilled over the LA (A–P −3.3 mm, M–L 5.0 mm, D–V −8.0 mm), MeA (A–P −3.0 mm, M–L 3.3 mm, D–V −9.0 mm) or both, based on the atlas of Paxinos and Watson. DREADD-Gi (AAV-CaMKIIa-HA-m4D8(Gi)-IRES-Citrine), DREADD-Gs (AAV-CaMKIIa-HA-rm3D(Gs)-IRES-mCitrine), AAV-CaMKIIa-EYFP or both Cre-dependent DREADD-Gi (AAV-hSyn-DIO-m4D8(Gi)-mCherry) and CAV2-Cre (Plateforme de Vectorologie de Montpellier, Institut de Génétique Moléculaire de Montpellier) was infused (0.5 μL/jouc) through a small-bore stainless steel cannula (33 gauge), driven by an infusion pump at 50–100 nL/min (PHD 2000 infusion pump, Harvard Apparatus, Holliston, MA). The cannula remained in place for 5 min before withdrawal. The incision was cleaned and stapled, and the rat was returned to its home cage after full mobility was regained. For 48 h after surgery, rats were administered a daily analgesic (1 mg/kg meloxicam, s.c.). Behavior procedures began after 2–3 weeks.

Immunohistochemical validation of infusion sites. At the conclusion of experiments that used rAAV-HA-DREAD, immunostaining was performed for the HA tag of the plasmid. Brains were fixed in 4% paraformaldehyde at 4 °C for 12–24 h, then transferred to 30% sucrose in phosphate-buffered saline (PBS) for at least 2 d. Brains were sectioned (50 μm), permeabilized (0.4% Triton in PBS for 1 h at room temperature) and blocked (1% bovine serum albumin/5% normal goat serum in 0.4% Triton/PBS). Sections were incubated with the primary antibody (HA-tag rabbit monoclonal antibody, 1:500; Cell Signaling Technology, catalog #3724; western blot validation at https://www.cellsignal.com/products/primary-antibodies/h-a-tag-c994-rabbit-mab/3724) overnight at 4 °C, washed (0.4% Triton/PBS, 10 min, 3 times), then incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit, 1:250; Invitrogen, Thermo-Fisher Scientific catalog #A-11008; immunohistochemistry validation at https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-antibody-Polyclonal-A-11008 and peer-reviewed citations for immunohistochemistry at https://www.biorxiv.org/lookup/0%20fluor%20488%20goat%20anti%20rabbit%20IgG%20product?utf8={8}&r=4.10&uq=Goat%20Anti-Chicken%20Secondary%20Antibodies) and then a final set of washes (4 times for 10 min each time), before being developed with chemiluminescent detection reagent (Amersham ECL Select) and exposed on autoradiographic film (Geneseel Scientific, San Diego, CA). Blots were subsequently washed (4 times for 10 min each time), then reprobed with chicken anti-α-tubulin (1:10,000, Sigma-Aldrich, catalog #SAB100023; western blot validation at http://www.sabiosciences.com/catalog/product/sigma/sab100023?flag=en&region=US and peer-reviewed citations at https://www.biorxiv.org/lookup/0%20fluor%20488%20goat%20anti%20rabbit%20IgG%20product?utf8={8}&r=4.10&uq=Goat%20Anti-Chicken%20Secondary%20Antibodies) and a final set of washes (4 times for 10 min each time), before developing with chemiluminescent detection reagent (Amersham ECL Select) and exposed on autoradiographic film. Individual band densities were obtained with ImageJ software (NIH), and normalized to corresponding α-tubulin band densities.

Brain tissues were also separately processed for RNA extraction with Trizol Reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA) according to the manufacturers’ protocol. Total RNA was treated with DNase I enzyme (Thermo Fisher Scientific) and reverse transcribed using recombinant M-MuLV reverse transcriptase (Thermo Fisher Scientific) into cDNA template. Jumpstart Taq ReadyMix (Sigma) was used to amplify cDNA using the following primers: GAPDH forward (5′-GACATGCCGCTTGGAGAA-3′), GAPDH reverse (5′-AGGCCAGGTGCTCTTGTAGT-3′); and neurexin-1α forward (5′-ATCTAGAGCTCGTCTGGCCGAC-3′), neurexin-1α reverse (5′-ATTTCTGCGCTGGCTTTG-3′).

PCR cycling conditions for both GAPDH and neurexin-1α were as follows: 94 °C for 2 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, followed by 5 min at 72 °C. PCR products were sequenced on a 2.5% agarose gel, visualized with ultraviolet light (FOTO/UV2) and images were acquired (12-megapixel resolution, iPhone 6S, Apple Inc, Cupertino, CA) from 12 in (30.5 cm) above the gel, without flash. Band densities were obtained from these images using ImageJ software (NIH) and normalized to GAPDH CDNA.

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incubation in rabbit anti-Cre-recombinase (1:750, Biolegend, catalog #908001; validation at http://www.biolegend.com/purified-anti-cre-recombinase-antibody-11331.html) with 0.4% Triton X-100/0.1 M PBS at 4 °C. The following day, sections were washed (6 times for 10 min) and incubated in Alexa Fluor 488 goat anti-rabbit (1:750, Life Technologies, catalog #A11008, validation as described for Alexa Fluor 488 above) for 2 h at RT. Tissue was washed again (6 times for 10 min each time), then sections were mounted on coverslips and were applied with Fluromount mounting media (Sigma). Sections were imaged (Nikon E600 microscope, Melville, NY) and Cre-recombinase and mCherry expression was mapped to identify the location of neurons transduced with CAV2-Cre-recombinase and AAV8-hSyn-DIO-hM4D(Gi)-mCherry respectively. Only animals with successful viral transduction were included in data analysis. Images were acquired (Olympus Fluoview 10 Scanning Laser confocal microscope or Nikon E600 microscope) and postacquisitional analysis was performed using Fluoview software (Olympus America, Center Valley, PA) and Adobe Photoshop (Adobe Systems, San Jose, CA).

Behavior. All behavioral activity was filmed with IR-sensitive cameras (Fire-i, Unibrain, San Ramon, CA), fed to a computer (Dell E6500, Round Rock, TX) and saved for offline analysis using AnyMaze software (Wood Dale, IL). Where indicated, rats were injected with saline vehicle or clozapine-N-oxide (CNO, 1 mg/kg, i.p.) 40 min before the behavioral test. The number of animals used for behavioral experiments was determined by sample size calculations based on the expected effect size in a previous study.18 For all experiments that used DREADDs or Nrxn rats, the experimenter performing the behavioral study was blind to the treatment groups or genotype of the rat. Rats were randomly assigned to groups. When animals underwent multiple behavioral tests, they occurred in this order: open field, social interaction test, novel object exploration test, novel object–versus–novel rat exploration. Rats were excluded from the study if there was a failure of equipment.

Open field. Open field exploration was conducted in a dimly lit room (20–25 lx) with computer-generated white noise (65–70 dB). A rat was placed individually into the open field (black opaque, 24 × 35 in or 60.96 × 88.90 cm) for 5 min. Total distance, speed and time spent in the center and periphery of the field were quantified.

Social interaction. After the open field exploration, a novel rat was added to the field for 5 min. The novel rat was a WT Sprague-Dawley within 50 g body-weight of the subject rat. The novel rats had all had a minimum of 10 min prior exposure to the open field. The number and durations of social interactions were quantified. The type of social interaction was noted (aggressive or exploratory).

Rats with DREADD expression underwent the social interaction test two times (48 h apart), once with vehicle and once with CNO in a counterbalanced manner. In a separate experiment, cohabitating WT pairs and Nrxn pairs were observed in their home cages in the housing room (15 min observation, each pair observed 4 times). The number of aggressive episodes (mounting) and the number of play episodes were quantified.

Novel object exploration. One to three days after the social interaction test, the rat was returned to the open field (same conditions as above). During this test, the open field contained a novel object. Novel object exploration was defined as physical manipulation of the object or sniffing of the object. The number and durations of times spent exploring the novel object were quantified.

Novel object versus novel rat exploration. During this test, subject rats were placed in the open field (conditions as above), which for this tests contained a novel object suspended from a string (2–3 cm above the floor of open field) and a novel rat. The novel rat was a WT Sprague-Dawley within 50 g bodyweight of the subject rat and had had a minimum of 10 min prior exposure to the open field. The numbers and durations of times spent investigating the novel object and the novel rat were quantified.

Odor exploration. To verify that the WT and Nrxn rats had sufficient olfactory ability, the following tests were performed:

- Sucrose pellet detection: rats were given grape-flavored sucrose pellets in their home cage 1 d before testing to produce familiarity before the test. During the test, rats were placed in a novel polycarbonate container (17 × 8.5 × 8 height, in inches; or 43.18 × 21.59 × 20.32 height in cm) with fresh bedding. Under the bedding were six grape-flavored sucrose pellets. The latency to find the first pellet was measured.
- Female odor approach: during this test, subject rats were placed in the open field (conditions as above), with a glass dish (10 cm diameter, 2 cm height) of bedding in opposite corners. One dish of bedding was from a cage of novel, cycling adult female rats; the other dish of bedding was from a cage of novel male adult rats. The numbers and durations of times investigating each tray were quantified.

Standard cued fear conditioning. Cued fear conditioning and testing were performed in different chambers with distinct contexts (wall pattern and color, odors, flooring) as described previously.18 Rats were placed in a chamber (10.625 in × 10.625 in × 14.125 in in height (26.99 cm × 26.99 cm × 35.88 cm height) or 13.5 in × 10 in × 12 in in height (34.29 cm × 25.40 cm × 30.48 cm height), counter-balanced across groups) with stainless steel grid floor. The chamber was housed inside a sound attenuating cabinet (UGO Basile, VA, Italy; 21 in × 17.5 in × 21.25 in height or 53.34 cm × 44.45 cm × 53.98 cm height) with dim light (~20 lx) with an additional infrared LED light, and constant white noise (70 dB) produced by a fan. A ceiling-mounted digital camera sensitive to light in the IR range was used to record behavior. Chambers were cleaned with ethanol (50%) before behavioral procedures and between each subject. Rats were allowed to explore the chamber freely. After a 180-s habituation period, a tone (2 kHz, 85 dB, 10 s duration) was presented. At the last second of the tone, a footshock was presented (1 s duration, 0.4–0.5 mA intensity depending on chamber), such that the shock and the tone coterminated. This was repeated 4 times at 60-s intertrial intervals. Rats remained in the chamber for 1 min after the end of the last conditioning trial, and were then returned to their home cage. After 48 h, conditioned freezing was tested in the same context, and cued conditioned freezing and within session extinction were tested in a novel context. The contextual freezing test lasted 5 min. The cued conditioned freezing consisted of a 3-min habituation period followed by 15 trials of tone presentation (20 s, 85 dB) at a 60-s intertrial interval. No footshock was presented during testing trials. Freezing was quantified using AnyMaze software based on a threshold of change in video image pixels. A freezing episode had to last a minimum of 1 s to be included in the software analysis. These criteria were compared against visually confirmed freezing (behavioral immobility except for movement associated with respiration). Total freezing during each trial (entire 60 s) was used as an index of conditioned fear and converted to a percentage (% of time of freezing/60 s) × 100) for analysis. Data from one animal were excluded due to mechanical failure.

Footshock intensity was determined from a separate group of rats. Footshock was delivered through the floor grid in 0.1 mA increments from 0.2 mA (0.2 mA, 0.3 mA, 0.4 mA, 0.5 mA) until a forepaw withdrawal with an avoidance response (for example, backpedaling) was noted. Based on analysis of this response, a footshock intensity of 0.4 mA was found to be appropriate for one conditioning chamber, and 0.5 mA was found to be appropriate for the other. There was no significant difference between groups in this threshold response to footshock for each chamber (see Results). Conditioning chambers were counterbalanced across groups.

Social-cued fear conditioning. Social fear conditioning was performed similarly to a previous study.18 The conditioning chamber (as above) was separated in half with a vinyl coated wire mesh divider (3/4-in or 1.91-cm mesh) in a Plexiglas frame. The floor of one half was a stainless steel grid; the other half was a Plexiglas panel. The demonstrator rat was placed into the side with the exposed stainless steel grid. The demonstrator rat was always a WT rat. The observer rat was placed into the side with the Plexiglas floor. Upon placement in the chamber, rats were given 180 s to habituate. Following habituation, a tone (10 s, 2 kHz, 85 dB) was presented to the chamber. At the end of the tone, footshock (0.5–0.6 mA, 1 s, coterminating with the tone) was delivered through the stainless steel floor grid to the demonstrator rat. The observer rat was fully isolated from the footshock. This was repeated 6 times, at an interval of 60 s. After 48 h, contextual freezing was measured from individual rats by placing the rat into the same chamber that was used for conditioning for 5 min. The rat was then placed into a novel chamber (contextually distinct in wall pattern, size, shape and odor) for 120 s of habituation followed by tone presentation (20 s, 2 kHz, 85 dB). The tone was repeated 12 times at an interval of 60 s. Freezing during each trial was measured (as above), along with nose poking through the mesh divider, location of the observer rat and social interaction during conditioning. Social cued conditioning was performed once per rat, unless otherwise noted. In those instances, the second conditioning was performed in a context that was distinct from the original context.
Electrophysiology. The numbers of rats and neurons for electrophysiological experiments were determined by sample-size calculations with an expected effect size estimated from preliminary data.

In vivo electrophysiology. Rats were anesthetized with urethane (1.5 g/kg, i.p.; Sigma-Aldrich) and their ears were infiltrated with 2% lidocaine hydrochloride jelly. Upon verification of anesthesia, rats were placed into a stereotaxic apparatus (David Kopf Instruments or Stoelting Instruments, Wood Dale, IL). Core body temperature was monitored rectally and maintained near 37 °C with a heating pad (TC-1000 Temperature Controller, CWE Inc., Ardmore, PA). An incision was made on the scalp and bore holes were drilled over the LA (A-P −3.3 mm, M−L 5.0 mm, D−V −8.0 mm) and posterior MeA (A−P −3.0 mm, M−L 3.3 mm, D−V −9.0 mm). A concentric bipolar stimulation electrode (0.25-mm outer diameter; Rhodes Medical Instrument) was lowered into LA or MeA. This electrode was used to record intra-amygdala spontaneous field potentials to gauge anesthesia state and to deliver electric stimulation. A glass microelectrode (2.0-mm outer diameter borosilicate glass) was heat-pulled (PE-2 microelectrode puller, Narishige Group, Tokyo, Japan) and filled with 2% pontamine sky blue (Alfa Aesar, Ward Hill, MA) in 2 M NaCl (Thermo Fisher Scientific), with an in situ resistance of 10−20 MΩ. The glass electrode was lowered slowly to the amygdala by a hydraulic microdrive (Model MO-10, Narishige Group). Signals were filtered and amplified (2400 Extracellular Preamplifier, Dagan Corp., Minneapolis, MN; or Model 1800 amplifier, A-M Systems, Carlsborg, WA) and monitored audially (AM10 amplifier, Grass Technologies, Warwick, RI). Signals were digitized (5-10 kHz; Instrutech TIC-18, HEKA Instruments, Bellmore, NY), fed to a computer (Mac Pro, Apple Inc.), visualized online (AxoGraph X, Australia) and saved for later analysis. Electrical stimulation was delivered (S88 Stimulator and PSI/6 Stimulation Isolation Unit, Grass Technologies) through the bipolar electrode with an intensity range of 0.1 to 0.9 mA, 0.2 ms duration, repeating at 0.2 Hz. Responses that displayed polysynaptic or antidromic characteristics were not included in analysis. To minimize the spread of stimulation current between LA and MeA and reduce the risk of measurement of a nonspecific response, three approaches were used: (i) stimulation intensity was kept below 1.0 mA, (ii) the distance between stimulation electrodes was consistently >2.0 mm and (iii) the latency of monosynaptic responses had to be >3.0 ms. The stimulation site had to be histologically confirmed to lie within the LA. Further confirmation that current spread was not likely to underestimate the obtained results was provided by the finding that stimulation sites that were mispositioned medially (i.e., closer to MeA and in the central amygdala) did not evoke mono- and/or corticostriatal excitatory responses (0 of 18 neurons from 4 animals).

At the conclusion of experiments, pontamine was iontophoresed (~30 μA) from the recording electrode using constant current for 30−45 min. The rat was euthanized and the brain was placed in 4% formaldehyde (Electron Microscopy Sciences) in 2 M NaCl (Electron Microscopy Sciences) in 0.1 M phosphate buffer, an active brain region, then cryoprotected in 30% sucrose (Sigma-Aldrich) in 0.1 M phosphate buffer. Brains were sectioned (60 μm thick) with a freezing microtome (Leica Microsystems, Buffalo Grove, IL) and stained with cresyl violet (Sigma Aldrich). Recording and stimulation sites were verified by light microscopy. Data were excluded if recording or stimulation sites were outside the borders of the MeA or LA.

In vitro electrophysiology. Rats were anesthetized with a cocktail of ketamine (80−100 mg/kg; Webster Veterinary Supply) and xylazine (10−20 mg/kg; Webster Veterinary Supply). Upon confirmation of anesthesia, rats were intracardially perfused with ice-cold, aerated (95% O2/5% CO2), high-sucrose artificial cerebrospinal fluid (ACSF) containing (in mM) 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 7 dextrose, 7 MgCl2, 0.5 CaCl2, 210 sucrose, 1.3 ascorbic acid, 3 sodium pyruvate. Osmolarity of high sucrose ACSF was approximately 290 mOsm. The rate of perfusion was approximately 4 mL/min with a total volume of 20−30 mL. Rats were decapitated and the brain was removed quickly. The brain was sectioned coronally at 300 μm in a vibratome (Ted Pella, Inc., Redding, CA) in ice-cold high sucrose ACSF; and brain slices were placed for approximately 1 h at 34 °C in physiological ACSF containing (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 10 dextrose, 1 MgCl2 and 2 CaCl2, with the addition of 1.3 mM ascorbic acid and 3 mM sodium pyruvate. Recordings were performed at 32−34 °C in submerged slices in physiological ACSF (as above, without ascorbic acid or sodium pyruvate). We added (+)-bicuculline (10 μM; Ascent Scientific, Princeton, NJ; dissolved in dimethyl sulfoxide), picrotoxin (10 μM; dissolved in ethanol), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) disodium salt (10 μM; Ascent Scientific, Princeton, NJ; dissolved in ddH2O) and DL-2-amino-5-phosphopentanoic acid (DL-AP5) sodium salt (50 μM; Abcam Biochemicals, Cambridge, MA; dissolved in 100 mM NaOH) to the ACSF as noted to block GABAa-receptor-, AMPA-receptor- and NMDA-receptor-mediated currents. Final solvent concentrations were <0.1% of the total ACSF volume. Solutions were continuously aerated with 95% O2/5% CO2.

Electrodes (1.8−11.5 MΩ open tip resistance) for recording of voltage were filled with an intracellular solution containing (in mM) 120 potassium glutonate, 20 KCl 0.2 EGTA, 10 HEPES, 2 NaCl, 4 ATP-Mg, 0.3 GTP-Tris, 7 Tris-phosphocreatine and 0.2% neurobiotin (Vector Laboratories, Inc., Burlingame, CA), with a pH of 7.3. Electrodes for recording of currents were filled with an intracellular solution containing (in mM) 150 CsCl, 0.2 EGTA, 10 HEPES, 2 NaCl, 4 ATP-Mg, 0.3 GTP-Tris, 7 Tris-phosphocreatine, 5 QX314 chloride (Ascent Scientific, Princeton, NJ) and 0.2% neurobiotin (Vector Laboratories, Inc.). Whole-cell recordings were performed in voltage-clamp or bridge mode from visually identified neurons within LA or posterior MeA (AxoClamp 2B, Molecular Devices, Inc., Sunnyvale, CA). Signals were low-pass filtered at 3−5 kHz and digitalized at 10−20 kHz (Instrutech TIC-18, HEKA Instruments). Mean series resistance for each group was below 25 MΩ. All electrophysiology data were monitored with AxoGraph X software and stored on a computer (Mac Pro, Apple Inc.) for offline analysis.

After recordings, slices were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for up to 4 weeks at 4 °C. Sections were rinsed three times with PBS, treated with Triton X-100 (VWR International, Radnor, PA; 1% in PBS) for 6 to 8 h and then incubated in Vectastain ABC Reagent (Vector Laboratories) in PBS at room temperature overnight. After three rinses with PBS, sections were reacted with diaminobenzidine (DAB) and H2O2 (Peroxidase Substrate Kit DAB, Vector Laboratories) in water to visualize the neurobiotin-filled neurons. Sections were washed in PBS repeatedly to stop the reaction. Sections were mounted, dried and coverslipped. Stained sections were used to confirm principle neuron morphology and localize the recording sites.

Statistical analysis. Data analysis was performed using GraphPad Prism software (La Jolla, CA). Significance was set at P < 0.05. Data were tested for normal distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett’s test). All data that were compared with parametric statistical tests met these assumptions. Planned comparisons between two groups were performed with two-tailed unpaired t-tests, and between three groups with one-way ANOVA (for example, time proximal toDiv, time peak divider, contextual freezing, firing rate, number of neurons per track, input resistance, EPSC frequency, paired-pulse ratio, CV of EPSC, auditory-induced freezing, latency to find sucrose pellets, shock threshold, time in open arm lengths of elevated plus maze). Comparisons between two or more groups on multiple factors were performed with two-way ANOVA or two-way repeated measures (RM)-ANOVA, if repeated measures from the same subject were obtained (for example, fear conditioning freezing, cued freezing, social interaction (vehicle and CNO), slope of field potential, excitability, time in open field center (high versus low lux), novel bedding exploration (male versus female)). Significance in the ANOVA test was followed by Holm-Sidak’s multiple comparisons test to compare groups. Significant effect sizes (Cohen’s d and confidence interval (C.I.) for t tests and η2 for ANOVA) are included in text, with full comparison details in figure legends. Data are presented as group means ± 95% confidence intervals, unless otherwise indicated. A Supplementary Methods Checklist is available.

Data availability. The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Gpdl and Nrxn1 are available at Genbank under accession codes NM_017088.4 and NM_021767.2.

51. National Research Council. Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011).