Dissecting the Roles of GABA and Neuropeptides from Rat Central Amygdala CRF Neurons in Anxiety and Fear Learning

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SUMMARY

Central amygdala (CeA) neurons that produce corticotropin-releasing factor (CRF) regulate anxiety and fear learning. These CeA^{CRF} neurons release GABA and several neuropeptides predicted to play important yet opposing roles in these behaviors. We dissected the relative roles of GABA, CRF, dynorphin, and neurotensin in CeA^{CRF} neurons in anxiety and fear learning by disrupting their expression using RNAi in male rats. GABA, but not CRF, dynorphin, or neurotensin, regulates baseline anxiety-like behavior. In contrast, chemogenetic stimulation of CeA^{CRF} neurons evokes anxiety-like behavior dependent on CRF and dynorphin, but not neurotensin. Finally, knockdown of CRF and dynorphin impairs fear learning, whereas knockdown of neurotensin enhances it. Our results demonstrate distinct behavioral roles for GABA, CRF, dynorphin, and neurotensin in a subpopulation of CeA neurons. These results highlight the importance of considering the repertoire of signaling molecules released from a given neuronal population when studying the circuit basis of behavior.

Graphical Abstract

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In Brief

Pomrenze et al. demonstrate that CRF neurons of the central amygdala differentially regulate fear and anxiety through the release of GABA and different neuropeptides.

INTRODUCTION

Genetic tools that permit cell-type and pathway-specific targeting of tracers and actuators have provided unprecedented insight into how neural circuits control behavior (Yizhar et al., 2011). Cre-driver mouse lines and viral tools are typically used to manipulate the activity of neuronal subpopulations (Daigle et al., 2018). However, a pitfall of this approach lies in the temptation to attribute the actions of the manipulated subpopulation to the gene product, usually a neuropeptide or neurotransmitter, whose promoter was used to drive Cre recombinase expression while overlooking the contributions of other signaling molecules produced by the targeted neurons.

This complexity is particularly evident when deciphering circuit effects of neuropeptides. Essentially all neuropeptide neurons express more than one and release a fast-acting neurotransmitter such as glutamate or γ-aminobutyric acid (GABA) (Nusbaum et al., 2017; van den Pol, 2012). Compared with fast-acting neurotransmitters, neuropeptides may require higher-frequency stimulation and larger increases in intracellular calcium for release. Neuropeptides can also signal over longer distances because of extrasynaptic release, local diffusion, and the requirement of extracellular proteolytic cleavage as opposed to reuptake

for signal termination. An interesting question is how multiple peptides released by a single neuron interact, particularly when they evoke initially opposing responses. Furthermore, determining how multiple neurotransmitter signals are integrated by postsynaptic cells to generate flexible physiological and behavioral outputs remains a significant challenge.

One brain structure rich in neuropeptides is the central amygdala (CeA), which contains a large population of GABAergic cells that express the stress-responsive neuropeptide corticotropin-releasing factor (CRF). CeA[CRF] neurons also express other neuropeptides such as dynorphin (DYN) and neurotensin (NTS), and when activated, they promote anxiety-like behavior and fear learning (Asok et al., 2018; Kim et al., 2017; McCall et al., 2015; Pliota et al., 2018; Pomrenze et al., 2015; Sanford et al., 2017). Despite CeA[CRF] neurons having established roles in fear and anxiety, the relative contribution of the individual neurotransmitters that they release has only been explored for CRF (McCall et al., 2015; Regev et al., 2012; Sanford et al., 2017).

In this study, we examined the question of how CeA[CRF] neurons control and fine-tune behavior through the release of diverse signaling molecules, some of which are predicted to have opposing actions. We examined the roles of CRF, GABA, and the co-expressed neuropeptides DYN and NTS by using RNAi in a rat line that expresses Cre recombinase under control of the CRF promoter (Pomrenze et al., 2015). Our results demonstrate that CeA[CRF] neurons play a multimodal role in regulating these behaviors through the coordinate actions of different neurotransmitters. These findings highlight the importance of considering the spectrum of signaling molecules expressed by a subpopulation of neurons when studying brain physiology and behavior.

RESULTS

GABA in CeA[CRF] Neurons Regulates Baseline Anxiety-like Behavior

An important question is whether GABA and neuropeptides released from CeA[CRF] neurons (Dabrowska et al., 2013; Pomrenze et al., 2015) cooperate to regulate behavior or play distinct roles. Because CRF is anxiogenic (Liang et al., 1992; Swerdlow et al., 1986) and activation of CeA[CRF] neurons can produce anxiety-like behavior in mice (McCall et al., 2015; Pliota et al., 2018; Regev et al., 2012), we hypothesized that GABA released from rat CeA[CRF] neurons would synergize with CRF to generate anxiety-like behavior. To test this hypothesis, we reduced vesicular GABA levels in these neurons (Figure S1G) by viral delivery of a Cre-dependent short hairpin RNA (shRNA) that targets the 3′ UTR of the transcript encoding the vesicular GABA transporter (*Vgat*) (Yu et al., 2015). After 4–6 weeks to allow adeno-associated virus (AAV) expression and knockdown, we tested rats for anxiety-like behavior. Surprisingly, we observed increased anxiety-like behavior in both elevated plus maze (EPM) and open-field (OF) tests in animals with *Vgat* knockdown compared with animals expressing a control shRNA (Figures 1B and 1C). There was no effect of *Vgat* knockdown on locomotion (Figures S2A and S2B). This finding suggests that GABA release from CeA[CRF] neurons is anxiolytic under baseline conditions.

Knockdown of *Vgat* in CeA[CRF] neurons could promote anxiety-like behavior through disinhibition of downstream circuits. To investigate this possibility, we challenged a separate

group of rats with *Vgat* knockdown by placing them in the OF and used Fos as a readout for neural activity engaged by OF exposure (Figure 2A) (Heisler et al., 2007). Control rats exposed to the OF showed low levels of Fos in the CeA and the oval bed nucleus of the stria terminalis (BNST), a structure that is known to modulate anxiety and is strongly connected with the CeA as part of the extended amygdala (Swanson and Petrovich, 1998). In contrast to controls, rats with *Vgat* knockdown displayed a large induction of Fos in both structures (Figures 2B–2E). Several Fos^{+} neurons in the CeA and oval BNST expressed protein kinase C δ (PKCδ), a marker for a subpopulation of non-CRF neurons that when activated can drive anxiety-like behaviors in mice (Botta et al., 2015). Altogether, these data suggest that CeA[CRF] neurons release GABA to dampen baseline anxiety-like behavior through inhibition of other subpopulations of neurons in the extended amygdala.

CeA[CRF] Neuron Neuropeptides and Baseline Anxiety

We have shown previously that rat CeA[CRF] neurons express several neuropeptides besides CRF (Pomrenze et al., 2015), consistent with other reports (Kim et al., 2017; Marchant et al., 2007). Pharmacological studies indicate that CRF and DYN are anxiogenic (Crowley et al., 2016; Knoll et al., 2011; Regev et al., 2012). However, because neuropeptide release typically requires high-frequency stimulation (van den Pol, 2012), we hypothesized that CRF and DYN play minor roles in baseline anxiety. To examine this question, we designed Cre-dependent shRNAs against the pro-peptides for CRF, DYN, and NTS (Figures S1A and S1B). The most effective shRNA sequences were packaged into AAV8 vectors and injected bilaterally into the CeA of *Crh*-Cre rats. After 4 weeks, we verified *in vivo* knockdown of respective mRNAs by qPCR (Figures S1D and S1E).

Rats expressing these shRNAs were tested for baseline anxiety-like behavior. Compared with control rats, knockdown of each peptide modestly increased the percentage of open-arm entries but did not alter the percentage of time in the open arms of the EPM (Figure 1D). Knockdown also did not alter the time spent in the center or the number of entries into the center of the OF (Figure 1E). Locomotor activity was not altered in either test (Figures S2C and S2D). These findings indicate that CRF, DYN, and NTS in CeA[CRF] neurons play minor roles compared with GABA in setting the level of baseline anxiety.

Activation of CeA[CRF] Neurons Promotes Anxiety-like Behavior through CRF and DYN

CeA[CRF] neurons can evoke anxiety-like behavior when stimulated (McCall et al., 2015; Pliota et al., 2018), and overexpression of CRF in the CeA of rats and primates is anxiogenic (Kalin et al., 2016; Keen-Rhinehart et al., 2009). Therefore, we investigated whether CRF in CeA[CRF] neurons is necessary for the increased anxiety-like behavior observed when CeA[CRF] neurons are activated. We transduced CeA[CRF] neurons with a Cre-dependent excitatory designer receptor hM3Dq, together with the Cre-dependent shRNA against CRF (Figure 3A). All animals received an injection of the hM3Dq-specific ligand CNO (2 mg/kg intraperitoneally [i.p.]), which causes depolarization and large increases in spontaneous firing in CRF neurons that express hM3Dq (Pomrenze et al., 2019). Rats with CRF knockdown showed less anxiety-like behavior in the EPM compared with controls but no differences in the OF (Figures 3B and 3C). To investigate this unexpected result, we systemically administered the CRF1 receptor antagonist R121919 (20 mg/kg subcutaneously

[s.c.]) to rats expressing Cre-dependent hM3Dq 30 min before a CNO injection (2 mg/kg i.p.). Again, we observed that compared with controls, rats treated with CNO alone exhibited anxiety-like behavior in both tests, but rats treated with CNO and R121919 showed less anxiety-like behavior only in the EPM (Figure S3). These data indicate that CRF released from CeA^{CRF} neurons promotes anxiety-like behavior on the EPM, but not the OF.

The anxiogenic effect of central CRF administration depends on DYN signaling (Bruchas et al., 2009). To investigate whether anxiety-like behavior upon stimulation of CeA^{CRF} neurons depends on DYN, we repeated the preceding hM3Dq experiment but injected rats with the Cre-dependent shRNA targeting DYN. Compared with control animals, rats with DYN knockdown showed reduced anxiety-like behavior in both EPM and OF tests (Figures 3D and 3E).

Injection of an NTS receptor antagonist into the oval BNST prevents anxiety evoked by chronic unpredictable stress (Normandeau et al., 2018b). To determine whether NTS in CeA^{CRF} neurons contributes to hM3Dq-evoked anxiety, we activated CeA^{CRF} neurons in rats previously injected with Cre-dependent hM3Dq and shRNA against NTS. Knocking down NTS in CeA^{CRF} neurons did not alter behavior in the EPM or OF tests after stimulation with CNO (Figures 3F and 3G). Knockdown of these peptides did not alter locomotor activity in either test (Figures S2E–S2J), except for a slight reduction in closed-arm entries in rats with CRF knockdown (Figure S2E). These results indicate that CRF and DYN, but not NTS, in CeA^{CRF} neurons regulate hM3Dq-evoked anxiety-like behavior.

CRF, DYN, and NTS, but Not GABA, in CeA^{CRF} Neurons Modulate Fear Learning

CeA^{CRF} neurons contribute to fear learning in mice (Sanford et al., 2017) and rats (Asok et al., 2018). We confirmed this role by expressing the inhibitory designer receptor hM4Di in the CeA of *Crh*-Cre rats to silence the activity of CeA^{CRF} neurons. Rats were administered CNO (2 mg/kg i.p.) before or immediately after fear conditioning or before retrieval trials (Figures 4B and S4). All rats exhibited shock-induced freezing, but only those administered CNO during conditioning showed reduced freezing in subsequent contextual and cued retrieval trials (Figure 4B). Rats with CeA^{CRF} neuron inhibition immediately after conditioning or before retrieval trials exhibited no differences in contextual or cued freezing (Figure S4). These data confirm that CeA^{CRF} neurons contribute to fear learning and are relatively dispensable during expression tests once fear memory has been formed.

The contribution of CeA-derived CRF to fear learning is unclear. In mice, knockdown of CRF in the CeA had little effect on fear behavior (Regev et al., 2012), yet CRF knockout via Cre-mediated gene deletion disrupted fear acquisition to low unconditioned stimulus (US) intensities (Sanford et al., 2017). In rats, knockdown of CRF in the CeA impaired contextual fear memory consolidation (Pitts et al., 2009). Using our Cre-dependent shRNA targeting CRF, we found that CRF knockdown reduced freezing to the fear context and cues without altering shock-induced freezing during the conditioning session (Figure 4C). Therefore, in rats, CeA^{CRF} neurons are a major source of CRF that mediates fear learning.

DYN and NTS also influence fear learning but do so in opposite directions. Blockade of κ-opioid receptors in the amygdala decreases conditioned fear in rats (Fanselow et al., 1991;

Knoll et al., 2011). In contrast, NTS1 receptor knockout mice display enhanced fear expression (Yamada et al., 2010), and in rats, NTS receptor agonists reduce while antagonists increase conditioned fear behavior (Prus et al., 2014; Toda et al., 2014). The source of DYN and NTS involved in these responses is not known. Using our Cre-dependent shRNAs, we found that similar to knockdown of CRF, knockdown of DYN in CeA[CRF] neurons disrupted contextual and cued fear retrieval (Figure 4C). In contrast, knockdown of NTS in CeA[CRF] neurons enhanced cued fear retrieval without altering shock-induced freezing or contextual fear retrieval (Figure 4C). These results indicate that CeA[CRF] neurons are a major source of DYN and NTS that differentially regulate fear learning.

Because we found that GABA in CeA[CRF] neurons regulates baseline anxiety, we next asked whether it also plays a role in fear learning. Surprisingly, knockdown of *Vgat* in CeA[CRF] neurons had little effect on fear learning or expression (Figure 4D).

DISCUSSION

We were surprised to find that CRF neurons in the lateral CeA produce neurotransmitters with opposing roles on anxiety-like behavior. Under non-stressful conditions, GABA in these neurons was critically important for limiting baseline anxiety, while CRF, DYN, and NTS had little effect. However, CRF and DYN mediated anxiety-like behavior evoked by chemogenetic stimulation of these neurons. Both CRF and DYN are anxiogenic (Crowley et al., 2016; Knoll et al., 2011; McCall et al., 2015; Regev et al., 2012), and our results indicate that their co-release from CeA[CRF] neurons is a mechanism by which they synergize to increase anxiety (Bruchas et al., 2009). In contrast, knockdown of NTS had no detectable effect on baseline or evoked anxiety, in agreement with prior work (László et al., 2010). Altogether, our results indicate that GABA and neuropeptides produced by CeA[CRF] neurons differentially regulate anxiety-like behavior under basal versus stimulated conditions. Studies are needed to determine whether increased basal anxiety in rats with GABA-deficient CeA[CRF] neurons is mediated by homeostatic increases in CRF or DYN tone.

We found that CRF knockdown or blockade of CRF1 receptors prevented hM3Dq-induced anxiety on the EPM, but not in the OF, whereas knockdown of DYN prevented anxiety-like behavior in both. The EPM might be less anxiogenic than the OF; the closed arms represent safety areas, whereas the OF offers less protection. We speculate that in animals with CRF knockdown, DYN in these neurons was sufficient to maintain anxiety in the OF, but not in the EPM.

When we used chemogenetics to inhibit CeA[CRF] neurons during fear conditioning, we found that these neurons contribute to fear learning, but not to fear retrieval, consistent with recent studies in mice (Sanford et al., 2017) and rats (Asok et al., 2018). Moreover, we found that knockdown of CRF or DYN in CeA[CRF] neurons disrupted contextual and cued fear retrieval, whereas knockdown of NTS enhanced cued retrieval. Our results are consistent with reports showing that CRF knockout or knockdown, or CRFR1 antagonism in the amygdala (Pitts et al., 2009; Sanford et al., 2017), disrupts fear learning. Our results are also consistent with reports showing that antagonists of κ-opioid receptors reduce fear behavior (Fanselow et al., 1991; Knoll et al., 2011) and antagonists of NTS1 receptors enhance fear

behavior (Prus et al., 2014; Steele et al., 2017; Yamada et al., 2010). In these pharmacological studies, the source of DYN and NTS was not identified. Here, using the CRF gene as a cellular entry point, we have identified CeA[CRF] neurons as an important source of DYN and NTS involved in fear learning.

Our results reveal an anxiolytic role for CeA[CRF] neurons in the basal state that is mediated by GABA, which switches to an anxiogenic role mediated by CRF and DYN when these neurons are activated. Several mechanisms may explain how GABA and cotransmission of these neuropeptides could produce divergent behavioral responses. Unlike GABA, neuropeptides are stored in dense core vesicles and can be released somatodendritically (Iremonger and Bains, 2009) or stored and released axonally at non-synaptic sites (Atasoy et al., 2014). There is a firing-rate dependence to co-release, whereby neuropeptide release tends to require higher firing rates than are needed for small-molecule neurotransmitters like GABA (Nusbaum et al., 2017). These peptides may reside in different dense-core vesicles and may be localized in terminal fields different from those harboring small vesicles containing GABA. For example, activation of CRF neurons from the amygdala to the locus coeruleus can increase activity of noradrenergic neurons through a CRF-dependent process that is independent of fast neurotransmitter release (McCall et al., 2015). Moreover, differential distribution of GABA and neuropeptide receptors and their coupling to different signaling pathways can lead to divergent or convergent circuit responses. For example, CRF and NTS enhance presynaptic GABA release onto oval BNST neurons (Normandeau et al., 2018b), whereas DYN and NTS exhibit opposing control over evoked GABA release from CeA inputs to the oval BNST (Normandeau et al., 2018a). In addition, co-expressed receptors can have cooperative or antagonistic effects on synaptic transmission in target neurons, similar to what has been observed in dopamine neurons co-expressing CRF and α1-adrenergic receptors (Tovar-Díaz et al., 2018). How the co-release of multiple neurotransmitters coordinates circuit activity to achieve a specific behavioral outcome is clearly a complex and important question for future study.

In summary, our study demonstrates the versatility of CeA[CRF] neurons in regulating fear and anxiety through different neurotransmitters. CRF and DYN promote fear and anxiety, whereas NTS suppresses fear and GABA constrains baseline anxiety in non-stressed conditions. These results indicate that CeA[CRF] neurons are not restricted to subserving just one type of behavioral response. Furthermore, our findings suggest that CeA[CRF] neurons, and perhaps the CRF system, interacts strongly with other neuropeptide systems. This property may have contributed to negative results from clinical trials evaluating effects of CRF receptor antagonists on stress-related alcohol craving and negative emotions associated with post-traumatic stress disorder (PTSD) (Dunlop et al., 2017; Kwako et al., 2015; Schwandt et al., 2016). It is our expectation that investigations like ours will improve understanding of how brain neuropeptide systems interact to regulate limbic circuits and behavior and contribute to the development of more effective combined therapeutic strategies for treating emotional disorders.

STAR★METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Robert O. Messing (romessing@austin.utexas.edu). Plasmids generated in this study have been deposited to Addgene for distribution to the research community.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Subjects—All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee. We used male hemizygous *Crh*-Cre rats (Pomrenze et al., 2015) outcrossed to wild-type Wistar rats (Envigo, Houston, TX), aged 5–6 weeks at the start of the surgical procedures and 10–14 weeks at the start of experimental procedures. Rats were group housed and maintained on a 12-hr light:dark cycle with food and water available *ad libitum*. Cre$^+$ rats were randomly assigned to either experimental or control groups within each litter.

METHOD DETAILS

Drugs and viral vectors—Clozapine-N-oxide (CNO) was supplied through the NIMH Chemical Synthesis and Drug Supply Program. CNO (2 mg/kg body weight) was dissolved in 5% dimethyl sulfoxide (DMSO) and then diluted to 2 mg/mL with 0.9% saline. Systemic injections were administered at 1 mL/kg. The selective CRF1 receptor antagonist 3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-*N,N*-dipropyl-pyrazolo[2,3-a]pyrimidin-7-amine (R121919) was provided by Dr. Kenner Rice (Drug Design and Synthesis Section, NIDA, Bethesda, MD) and dissolved in a 1:1 solution of 0.9% saline and 1N HCl before adding 25% hydroxypropyl-β-cyclodextrin (HBC; Sigma Aldrich, St. Louis, MO) to yield a final concentration of 10 mg/mL R121919 in 20% HBC, pH 4.5. R121919 injections were administered at 2 mL/kg.

The Cre-dependent viral vector AAV8-hSyn-DIO-hM3Dq-mCherry was obtained from Addgene (Cambridge, MA). AAV constructs containing shRNAs targeting *Vgat, proCrh, prodynorphin, neurotensin*, and a control were packaged by the University of North Carolina Chapel Hill viral vector core. All AAVs were injected at 4–6×10^{12} infectious units per mL.

Stereotaxic surgery—Rats weighing 200–250 g were anesthetized with isoflurane (5% v/v) and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Viruses were injected bilaterally into the CeA (AP: −2.2; ML: ± 4.5; DV: −8.0 from skull) at a rate of 150 nL min^{-1} for 5 min (750–800nL total volume per hemisphere) with a custom 32-gauge injector cannula coupled to a pump-mounted 2μL Hamilton syringe. Injectors were slowly retracted after a 5 min diffusion period. Rats were group housed to recover for 4–6 weeks before experiments began.

Generation of shRNAs—We designed shRNAs targeting the 3′ untranslated regions (UTR) of rat *proCrh, prodynorphin*, or *neurotensin* and subcloned them into a pPRIME vector containing a modified microRNA (*miR30*) cassette (Addgene #11657) for *in vitro*

validation of knockdown. The 22-mer oligonucleotide 3′-untranslated sequences used to generate shRNAs were:

shCrh1: AACACAGTATTCTGTACCATAC

shCrh2: AAGTGTGTTTCTTTGTAGTAAC

shDyn1: TACACTGAGCCTCGTTCTCCAT

shDyn2: AGCTCTTCATGTGTTCTGAAAT

shNts1: ACATGTGATTCTCATCCTTTAC

shNts2: TACCTGTTATCTGGATACACAT

The *miR30* cassette with the most effective shRNA sequence for each peptide was then subcloned into a Cre-dependent (*flex*) pAAV vector (Addgene #67845). A previously validated Cre-dependent shRNA targeting the 3′-UTR of the vesicular GABA transporter (*Vgat*) and a control shRNA targeting luciferase within the same *miR30* cassette were gifts from Dr. William Wisden (Imperial College, London, UK; Yu et al., 2015). These constructs were packaged into AAV8 by the UNC Viral Vector Core. AAV8-hSyn-*flex*-eGFP-sh*Crh*, AAV8-hSyn-*flex*-eGFP-sh*Dyn*, AAV8-hSyn-*flex*-eGFP-sh*Nts*, AAV8-hSyn-*flex*-eGFP-sh*Vgat*, and AAV8-hSyn-*flex*-eGFP-sh*Control* were injected at $2–3 \times 10^{12}$ particles per mL. *In vivo* knockdown was verified using RT-qPCR from AAV-infected CeA tissue punches.

RT-qPCR—*Crh*-Cre rats were bilaterally injected into the CeA with AAVs carrying sh*Crh*, sh*Dyn*, sh*Nts*, or sh*Con*. After 4 weeks, rats were euthanized and their brains flash frozen in isopentane on dry ice and stored at −80°C. Brains were then equilibrated to −20°C in a cryostat for 1hr and the CeA sectioned coronally at 250 μm and mounted onto cold Superfrost Plus slides (Fisher Scientific). Tissue punches (2 mm) spanning the CeA of both hemispheres were collected on dry ice and snap frozen in liquid nitrogen. RNA was extracted immediately using RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany). Purified RNA samples were reverse transcribed using the High Capacity cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using a TaqMan Gene Expression Assay Kit (Applied Biosystems, Foster City, CA). All TaqMan probes were purchased from Applied Biosystems: *Crh* (Rn01462137_m1), *pDyn* (Rn00571351_m1), *Nts* (Rn01503265_m1), and *GusB* (Rn00566655_m1). Target amplification was performed using a ViiA 7 Real-Time PCR System (Applied Biosystems). Relative mRNA expression levels were calculated using a comparative threshold cycle (C_t) method with *GusB* as an internal control: $\Delta C_t = C_t$ (gene of interest) – C_t (*GusB*). The gene expression fold change was normalized to the control sample and then was calculated as $2^{-\Delta\Delta C_t}$.

Western blotting—shRNA-mediated knockdown was tested *in vitro* using HEK293 cells. Cells were plated at a density of 3×10^5 cells/well in 12-well plates. Twenty-four hours after plating, cells were co-transfected with a pPRIME vector and a corresponding transgene encoding CRF or DYN (including their 3′-UTRs) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Sixty hours after transfection, media was aspirated and cells were lysed by incubating with 200μl of ice-cold RIPA buffer at 4°C for 30 min. The lysate was then

centrifuged at $10,000 \times g$ for 15 min at 4°C. Supernatant was collected and protein concentrations were measured using the bicinchoninic assay method (Life Technologies). Samples (40 μg) were resolved on a 10% SDS polyacrylamide gel and proteins were then transferred onto a nitrocellulose membrane. After transfer, the membrane was blocked with 5% milk in Tris-buffered saline containing 0.01% Tween-20 (TBST). The blot was probed with 1:200 dilution of goat anti-CRF antibody (Santa Cruz Biotechnology, Dallas, TX, sc-1761) or 1:1000 dilution of guinea pig anti-DYN antibody (Neuromics, GP10110) in 5% milk overnight at 4°C with shaking. Blots were washed three times in 1X TBST and probed with 1:2500 dilution (in 5% milk) of horseradish peroxidase conjugated anti-goat or anti-guinea pig secondary antibodies (Santa Cruz Biotechnologies) for 1 hr at room temperature followed by chemiluminescent detection (Super-signal West, Life Technologies). Blots were stripped (Restore buffer, Life Technologies) and probed with anti-rabbit GAPDH (1:10,000 dilution in 5% milk, Cell Signaling Technologies, 5174S). Immunoreactive bands were quantified using Fiji (Schindelin et al., 2012). CRF and DYN levels were normalized to GAPDH and percent knockdown was calculated.

Behavior—We used two assays to evaluate anxiety-like behavior: the elevated plus maze (EPM) and the open field (OF) tests. The EPM consisted of two open arms (50×10 cm) and two enclosed arms ($50 \times 10 \times 40$ cm) connected by a central area measuring 10×10 cm, 50 cm above the floor. At the beginning of each trial, rats were placed in the center facing one open arm. Trials lasted for 5 min and were performed under red lighting. The OF consisted of an open topped arena ($100 \times 100 \times 50$ cm) situated on the floor. The center zone measured 55×55 cm. Rats were placed into a corner of the arena at the beginning of each trial. Each trial lasted 10 min and was performed under red lighting. All testing equipment was cleaned with 70% ethanol between trials. Behaviors were tracked with EthoVision (Noldus Information Technology, Leesburg, VA, USA).

Rats were subjected to a typical fear conditioning protocol with 3 CS-US (tone-shock) pairings (75 dB, 5 kHz, 20 s tones co-terminating with 0.7 mA, 500 ms shocks, variable ITI (average 180 s)) for delay conditioning (Monfils et al., 2009; Schafe et al., 1999). Twenty-four hours later rats were tested for contextual fear retrieval by being placed back into the original fear context for 5 min. Another 24 hr later rats were tested for cued fear retrieval in a distinct context with the presentation of 4 CS tones. The distinct context consisted of pinstripe and checkered walls, smooth floors, and the scent of 1% acetic acid.

Histology—Rats were anesthetized with isoflurane and perfused transcardially with PBS followed by 4% paraformaldehyde in PBS, pH 7.4. Brains were extracted, post-fixed overnight in the same fixative and cryoprotected in 30% sucrose in PBS at 4°C. Brains were sectioned at 40μm on a cryostat and collected in PBS. Free-floating sections were washed three times in PBS with 0.2% Triton X-100(PBST)for 10 min at 27°C and then incubated in PBST with 3% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, Cat. No. 017-000-121) for 1 hr. Sections were next incubated in goat anti-cFos (1:2000, Santa Cruz Biotechnology, sc-52-G) and rabbit anti-PKCδ (1:2000, Santa Cruz Biotechnology, sc-213) in blocking solution rotating at 4°C for 18–20 hr. After three 10 min washes in PBST, sections were incubated in species-specific secondary antibodies Alexa Fluor 594 and 647

(1:700, Invitrogen, Carlsbad, CA, A-11058 and A-31573) in blocking solution for 1h at 27°C. Finally, sections were washed three times for 10 min in 1X PBS, mounted in 0.2% gelatin onto SuperFrost Plus glass slides, and coverslipped with Fluoromount-G with DAPI (Southern Biotech, Birmingham, AL, 0100–20). Fluorescent images were collected on a Zeiss 710 confocal microscope or a Zeiss AxioZoom stereo microscope. Quantification of fluorescence was performed on 3–6 sections per rat from approximately Bregma −1.90 to −3.00 in the CeA and Bregma +0.2 to −0.2 in the oval BNST using the cell-counter plugin in Fiji (Schindelin et al., 2012).

Fluorescence *in situ* hybridization—For examination of *Vgat* expression in the CeA, coronal sections were processed for fluorescent *in situ* hybridization by RNAscope according to manufacturer's guidelines. Genes examined in the CeA were *Vgat* (ACDBio cat# 424541), *Crh* (ACDBio cat# 318931), and viral-mediated *egfp* (ACDBio cat# 409971), and hybridization was performed using RNAscope Fluorescent Multiplex Kit (Advanced Cell Diagnostics). Slides were coverslipped with Fluoromount-G with DAPI (Southern Biotech, 0100–20) and stored at 4°C in the dark before imaging. *Vgat* puncta were counted selectively in *egfp*+ cells using the cell-counter plugin in Fiji (Schindelin etal., 2012).

QUANTIFICATION AND STATISTICAL ANALYSIS

We calculated sample sizes of n = 8–12 animals per condition using SD values measured in pilot studies of anxiety-like behavior, α = 0.05, and power = 0.80, with the goal of detecting at least a 25% difference in mean values for treated and control samples, using G*Power (Faul etal., 2007). All results were expressed as mean ± SEM values and analyzed using Prism 7.0 (GraphPad Software, San Diego, CA). Data distribution and variance were tested using Shapiro-Wilk normality tests. Normally distributed data were analyzed by unpaired, two-tailed t tests, or one or two factor ANOVA with post hoc Tukey's or Bonferroni's tests. Data that were not normally distributed were analyzed by Mann-Whitney U tests when comparing two conditions. Differences were considered significant when p < 0.05.

DATA AND CODE AVAILABILITY

The published article includes all data generated or analyzed during this study. This study did not generate code.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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Highlights

- Central amygdala CRF neurons release GABA and several neuropeptides

- Preventing GABA release in these neurons suppresses basal anxiety-like behavior

- Knockdown of CRF or dynorphin prevents anxiety evoked by chemogenetic activation

- CRF or dynorphin knockdown reduces but neurotensin knockdown enhances fear learning
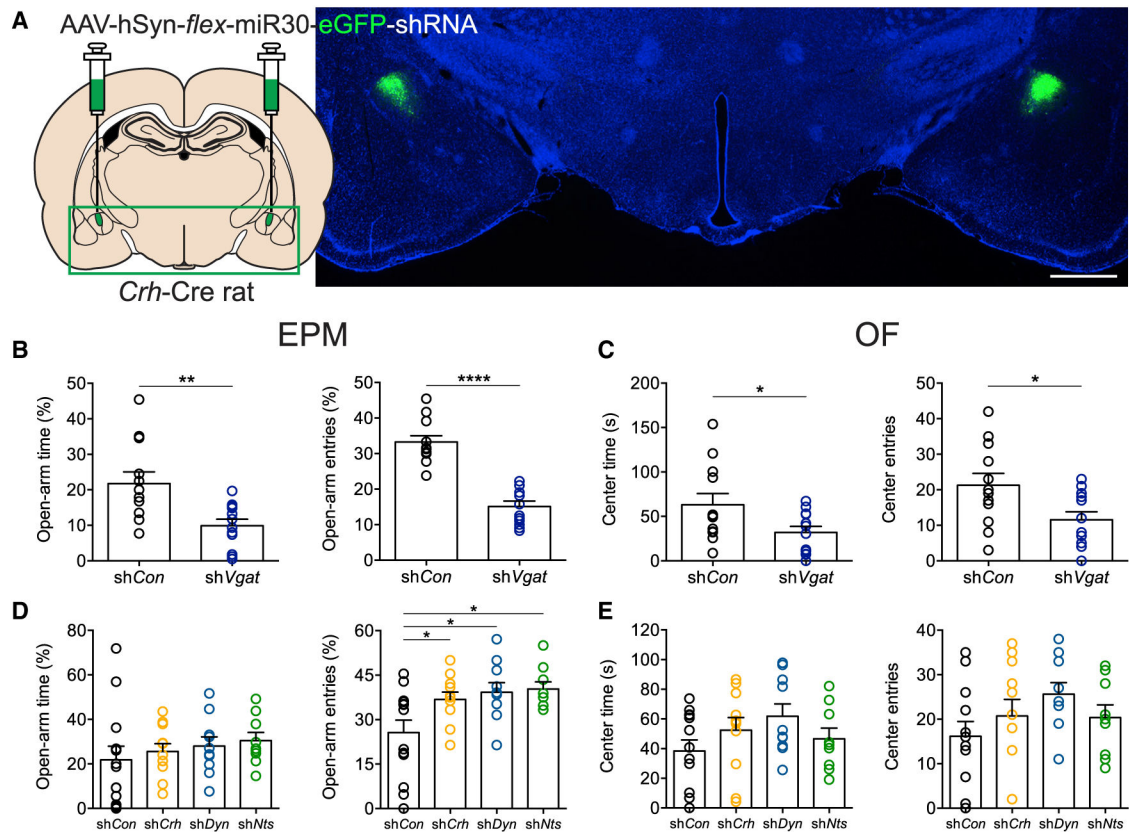
Figure 1. Knockdown of VGAT, but Not Neuropeptides, in CeA^CRF Neurons Increases Anxiety-like Behavior

(A) Injection schematic and representative image of bilateral AAV infection in CeA^{CRF} neurons (DAPI counterstain). Scale bar, 500 μm.

(B) Knockdown of VGAT in CeA^{CRF} neurons reduced time spent in the open arms (t(22) = 3.158, **p = 0.0046; n = 12 for both groups) and entries into the open arms (t(22) = 7.858, ****p < 0.0001; n = 12 for both groups) on the elevated plus maze (EPM).

(C) Knockdown of VGAT reduced time spent in the center(t(22) = 2.156, *p = 0.0423; n = 12 for both groups) and entries into the center(t(22) = 2.407, *p = 0.0249; n = 12 for both groups) of the open field (OF).

(D) Knockdown of CRF, DYN, or NTS did not change time spent in the open arms of the EPM (F(3,39) = 0.5965, p = 0.6211, one-way ANOVA; n = 13 shCon, 11 shCrh, 10, shDyn, and 9 shNts) but did increase the number of entries into the open arms (F(3,39) = 2.139, p = 0.0092, one-way ANOVA; n = 13 shCon, 11 shCrh, 10, shDyn, and 9 shNts; *p < 0.05 compared with shCon by Dunnett's test).

(E) Knockdown of CRF, DYN, or NTS did not change baseline anxiety-like behavior in the open field (time in the center: F(3,39) = 1.610, p = 0.2026; entries into the center: F(3,39) = 1.477, p = 0.2356; n = 13 shCon, 11 shCrh, 10, shDyn, and 9 shNts).

Data are represented as mean ± SEM.
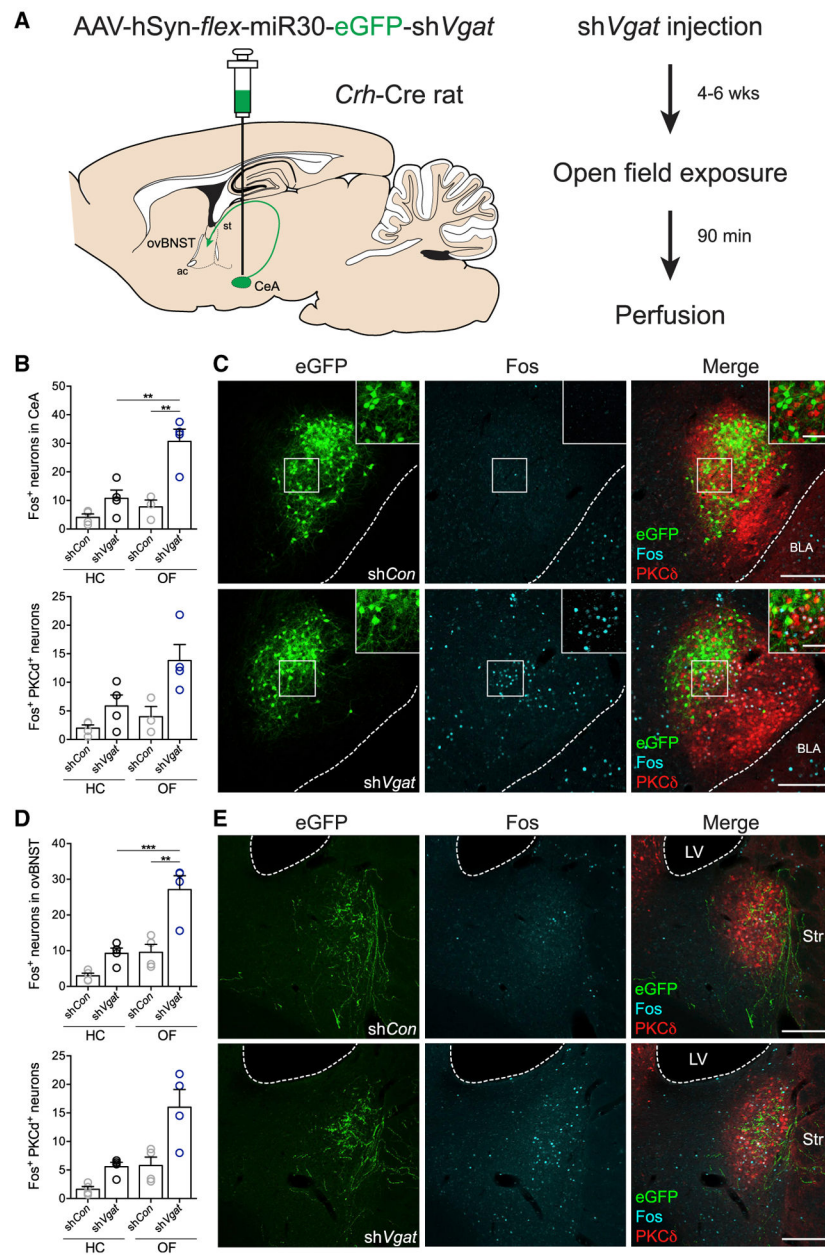
Figure 2. Knockdown of VGAT in CeA[CRF] Neurons Disinhibits Activation of the Extended Amygdala during Open-Field Exposure

(A) Left, viral injection schematic. Right, experimental protocol.

(B) Top, knockdown of VGAT in CeA[CRF] neurons increased Fos expression in the CeA after open-field exposure ($F_{sh\,Vgat \times OF}(1,11) = 5.604$, $p = 0.0212$; n = 3–4; **p = 0.0025 for sh*Vgat* in the home cage [HC] compared with shVgat in the open field [OF] and **p = 0.0015 for sh*Con*:OF compared with sh*Vgat*:OF by Tukey's tests). Bottom, several Fos+ neurons also expressed PKCδ.

(C) Representative images demonstrating increased Fos expression in the CeA of rats expressing sh*Vgat* in CeA[CRF] neurons. Scale bars, 200 and 50 μm in the insets.

(D) Top, knockdown of VGAT in CeA^{CRF} neurons increased Fos expression in the oval BNST after open-field exposure ($F_{sh\,Vgat\,\times\,OF}$(1,12) = 5.604, p = 0.0356; n = 4; ***p = 0.0009 for sh*Vgat*:HC compared with sh*Vgat*:OF and **p = 0.0011 for sh*Con*:OF compared with sh*Vgat*:OF by Tukey's tests). Bottom, several Fos$^+$ neurons also expressed PKCδ.

(E) Representative images demonstrating increased Fos expression in the oval BNST of rats expressing sh*Vgat* in CeA^{CRF} neurons. EGFP$^+$ axons emerging from CeA^{CRF} neurons are visible in the oval nucleus. Scale bars, 200 μm.

Data are represented as mean ± SEM.
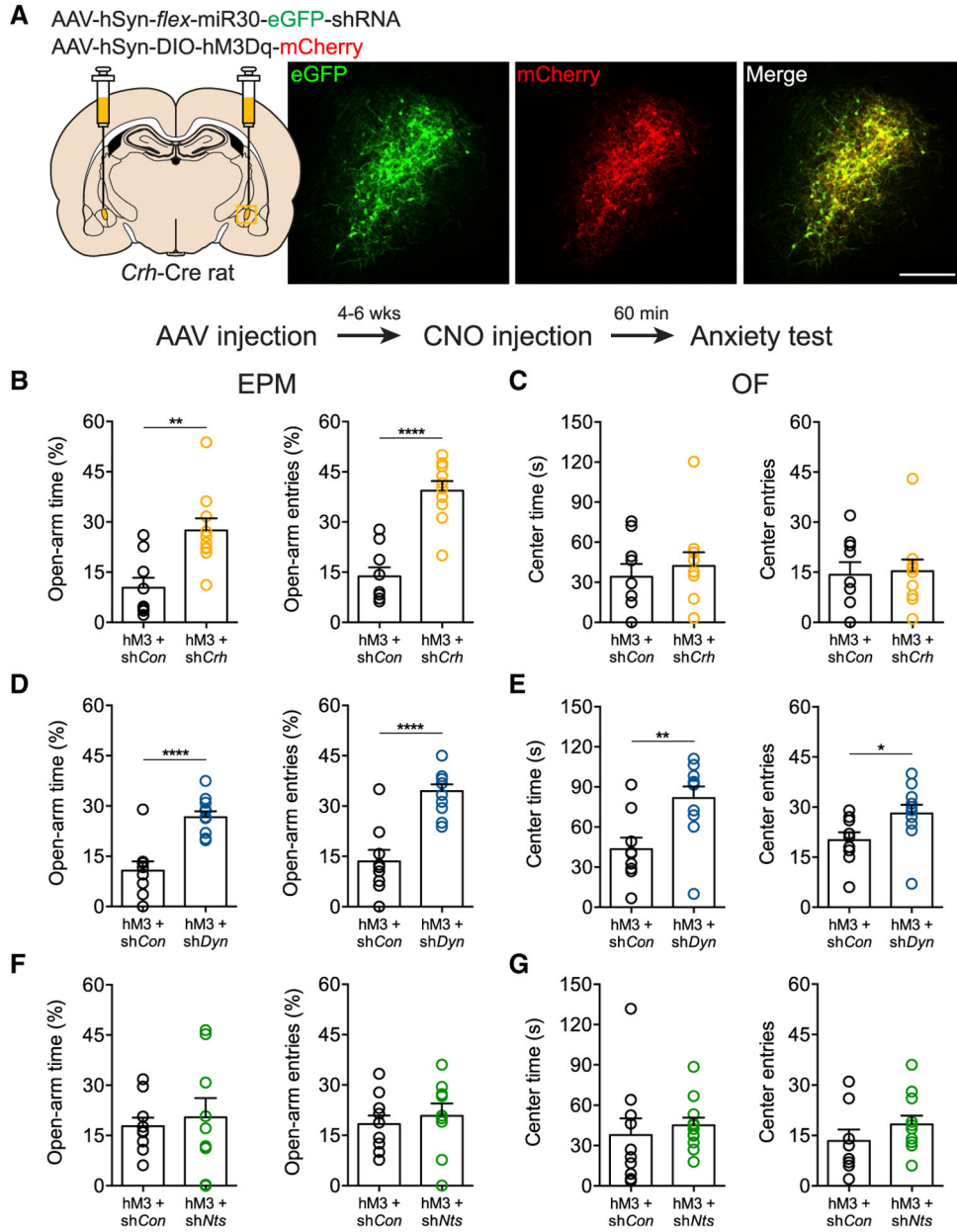
Figure 3. CRF and Dynorphin, but Not Neurotensin, in Activated CeA^{CRF} Neurons Mediate Anxiety-like Behavior

(A)Top, example image of dual infection of CeA^{CRF} neurons with a cocktail of AAVs carrying shRNA and hM3Dq. Scale bar, 200 μm. Bottom, experimental protocol.

(B) Knockdown of CRF led to more time spent on the open arms (t(17) = 3.613, **p = 0.0021; n = 9 sh*Con* and 10 sh*Crh*) and more entries into the open arms (t(17) = 6.468, ****p < 0.0001; n = 9 sh*Con* and 10 sh*Crh*) of the elevated plus maze after activation of CeA^{CRF} neurons with hM3Dq and CNO (2 mg/kg i.p.).

(C) Knockdown of CRF did not alter anxiety-like behavior in the open field (center time: t(17) = 0.854, p = 0.5669; center entries: t(17) = 0.208, p = 0.8376; n = 9 sh*Con* and 10 sh*Crh*) after activation of CeA^{CRF} neurons with hM3Dq and CNO (2 mg/kg i.p.).

(D) Knockdown of DYN led to more time spent on the open arms ($t(18) = 5.151$, ****$p < 0.0001$; n = 9 sh*Con* and 11 sh*Dyn*) and more entries into the open arms ($t(18) = 5.589$, ****$p < 0.0001$; n = 9 sh*Con* and 10 sh*Dyn*) of the elevated plus maze after activation of CeA^{CRF} neurons.

(E) Knockdown of DYN led to more time spent in the center ($U = 15$, **$p = 0.0074$; n = 9 sh*Con* and 11 sh*Dyn*) and more entries into the center ($U = 17.5$, *$p = 0.013$; n = 9 sh*Con* and 11 sh*Dyn*) of the open field after activation of CeA^{CRF} neurons.

(F) Knockdown of NTS did not change the time spent on the open arms ($t(17) = 0.4315$, $p = 0.6716$; n = 10 sh*Con* and 9 sh*Nts*) or entries into the open arms ($t(17) = 0.5536$, $p = 0.5871$; n = 10 sh*Con* and 9 sh*Nts*) of the elevated plus maze after activation of CeA^{CRF} neurons.

(G) Knockdown of NTS did not change the time spent in the center ($U = 30$, $p = 0.2428$; n = 10 sh*Con* and 9 sh*Nts*) and more entries into the center ($t(17) = 1.319$, $p = 0.2046$; n = 10 sh*Con* and 9 sh*Nts*) of the open field after activation of CeA^{CRF} neurons.

Data are represented as mean ± SEM.
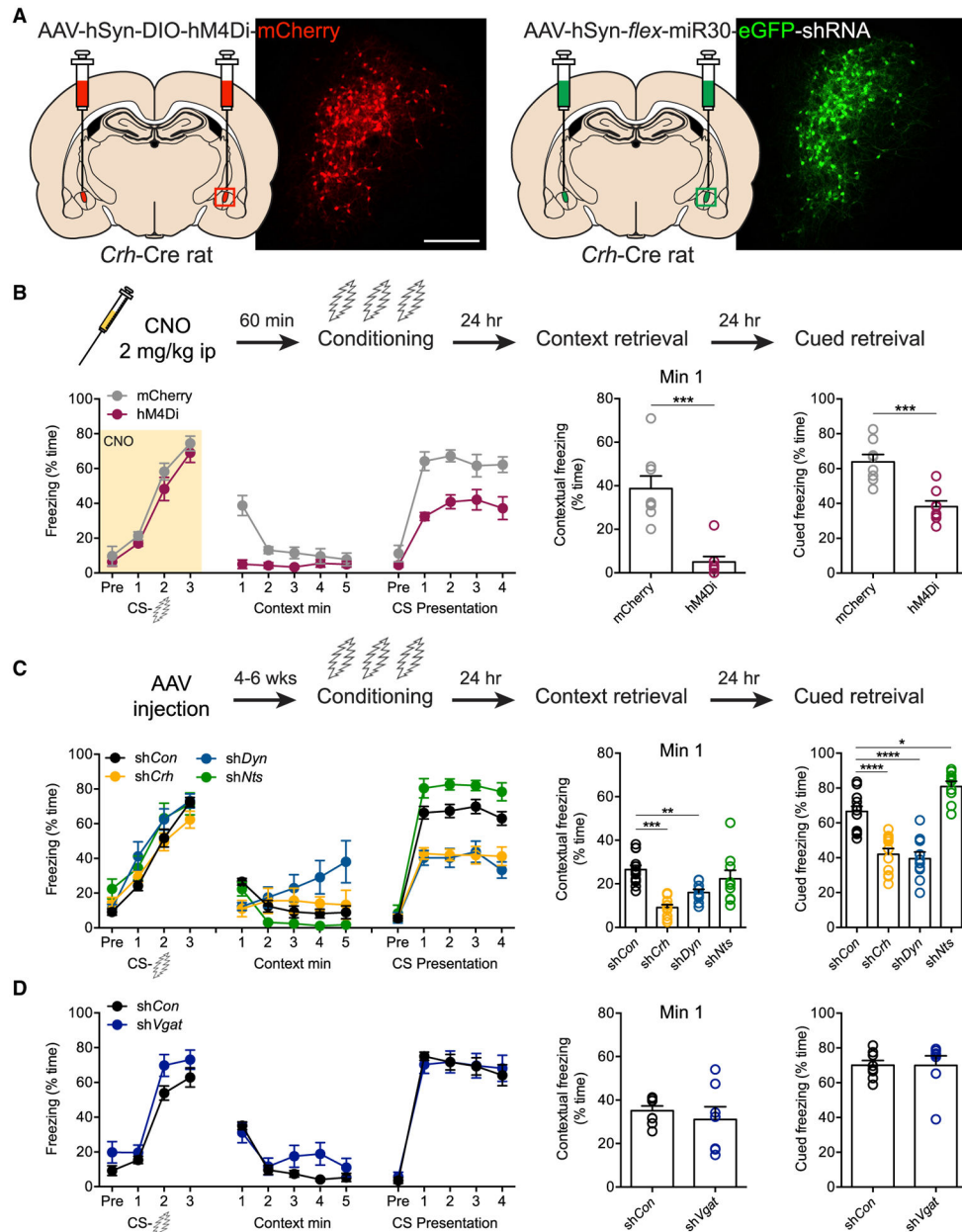
Figure 4. CRF, Dynorphin, and Neurotensin in CeA^{CRF} Neurons Modulate Fear Learning

(A) Injection schematics and example of viral expression in CeA^{CRF} neurons infected with hM4Di-mCherry DREADD (left) and shRNA with EGFP (right). Scale bar, 200 μm.

(B)Top, experimental protocol for fear conditioning. Bottom, chemogenetic inhibition of CeA^{CRF} neurons with hM4Di and CNO(2 mg/kg i.p.) did not affect freezing during fear conditioning but disrupted contextual fear retrieval during the first minute (U = 1, ***p = 0.0003; n = 8 for both groups), as well as cued fear retrieval (t(14) = 4.846, ***p = 0.0003; n = 8 for both groups).

(C) Top, experimental protocol. Bottom, shRNA-mediated knockdown of CRF and DYN, but not NTS, in CeA^{CRF} neurons blunted contextual fear retrieval during the first minute (F(3,38) = 12.53, p < 0.0001, one-way ANOVA; n = 13 sh*Con*, 11 sh*Crh*, 9 sh*Dyn*, and 9

sh*Nts*; ***p<0.0001 sh*Crh* compared with sh*Con* and **p = 0.0054 sh*Dyn* compared with sh*Con* by Dunnett's test). Knockdown of CRF and DYN also reduced cued freezing, yet knockdown of NTS enhanced cued freezing (F(3,39) = 34.13, p < 0.0001; n = 13 sh*Con*, 11 sh*Crh*, 10 sh*Dyn*, and 9 sh*Nts*; ****p < 0.0001 sh*Crh* compared with sh*Con*, ****p < 0.0001 sh*Dyn* compared with sh*Con*, and *p = 0.0115 sh*Nts* compared with sh*Con* by Dunnett's test).

(D) Knockdown of Vgat in CeA^{CRF} neurons did not affect contextual fear learning (t(13) = 0.6684, p = 0.5156; n = 8 sh*Con* and 7 sh*Vgat*) or cued fear learning (t(13) = 0.0125, p = 0.9902; n = 8 sh*Con* and 7 sh*Vgat*).

Data are represented as mean ± SEM.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat polyclonal anti-cFos	Santa Cruz Biotechnology	Ca# sc-52-G; RRID:AB_2629503
Rabbit polyclonal anti-PKCdelta	Santa Cruz Biotechnology	Cat# sc-213; RRID:AB_632228
Goat polyclonal anti-CRF	Santa Cruz Biotechnology	Cat# sc-1761; RRID:AB_631299
Guinea pig polyclonal anti-Dynorphin	Neuromics	Cat# GP10110; RRID:AB_1621439
Donkey anti-goat Alexa Fluor 594	Invitrogen	Cat# A-11058; RRID:AB_142540
Donkey anti-rabbit Alexa Fluor 647	Invitrogen	Cat# A-31573; RRID:AB_2536183
Anti-rabbit GAPDH	Cell Signaling	Cat# 5174S; RRID:AB_10622025
Bacterial and Virus Strains		
AAV8-hSyn-DIO-hM3Dq-mCherry	Addgene	44361
AAV8-hSyn-flex-miR30-eGFP-shVgat	Yuetal., 2015	N/A
AAV8-hSyn-/tex-miR30-eGFP-shCrh	This paper	N/A
AAV8-hSyn-flex-miR30-eGFP-shDyn	This paper	N/A
AAV8-hSyn-ffex-miR30-eGFP-shNts	This paper	N/A
AAV8-hSyn-flex-miR30-eGFP-shControl	Yuetal., 2015	N/A
Chemicals, Peptides, and Recombinant Proteins		
Clozapine-N-Oxide	NIMH Chemical Synthesis and Drug Supply Program	C-929
R121919	Chemical Biology Research Branch- NIAAA-NIDA	N/A
Hydroxypropyl-β-cyclodextrin	Sigma Aldrich	H107
Lipofectamine 2000	Invitrogen	11668019
Anti-goat secondary antibody, HRP conjugate	Santa Cruz Biotechnology	Cat# sc-2020; RRID:AB_631728
Anti-guinea pig secondary antibody, HRP conjugate	Santa Cruz Biotechnology	Cat# sc-2903; RRID:AB_650493
Fluoromount-G with DAPI	Southern Biotech	0100–20
Normal donkey serum	Jackson Immunoresearch	017-000-121
Critical Commercial Assays		
RNAscope Fluorescent Multiplex Kit	Advanced Cell Diagnostics (ACD)	320851
RNeasy Lipid Tissue Mini Kit	QIAGEN	74804
High Capacity cDNA Synthesis Kit	Invitrogen	4368814
TaqMan Gene Expression Assay Kit	Applied Biosystems	4331182
Pierce BCA Protein Assay Kit	Thermo Scientific	23227
Experimental Models: Cell Lines		
HEK293FT cells (female)	Thermofisher Scientific	R70007
Experimental Models: Organisms/Strains		
Crh-Cre rats	Pomrenze et al., 2015	N/A
Oligonucleotides		
TaqMan Crh RNA probe	Applied Biosystems	Rn01462137_m1
TaqMan Pdyn RNA probe	Applied Biosystems	Rn00571351_m1
TaqMan Nts RNA probe	Applied Biosystems	Rn01503265_m1

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TaqMan GusB RNA probe	Applied Biosystems	Rn00566655_m1
RNAscope Vgat probe	ACD	424541
RNAscope egfp probe	ACD	409971
sh*Crh*1: AACACAGTATTCTGTACCATAC	This paper	N/A
sh*Crh*2: AAGTGTGTTTCTTTGTAGTAAC	This paper	N/A
sh*Dyn*1: TACACTGAGCCTCGTTCTCCAT	This paper	N/A
sh*Dyn*2: AGCTCTTCATGTGTTCTGAAAT	This paper	N/A
sh*Nts*1: ACATGTGATTCTCATCCTTTAC	This paper	N/A
sh*Nts*2: TACCTGTTATCTGGATACACAT	This paper	N/A
Recombinant DNA		
pPRIME-CMV-GFP-recipient	Addgene	11657
pAAV-hSyn-flex-dsRed-shVgat	Addgene	67845
Software and Algorithms		
FIJI/ImageJ	NIH	https://imagej.nih.gov/ij
EthoVision XT	Noldus	http://www.noldus.com/
Prism 7	GraphPad Software	https://www.graphpad.com/
G*Power	Faul et al., 2007	N/A