

SUPPLEMENTAL INFORMATION

EXTENDED EXPERIMENTAL PROCEDURES

Generation of *Crfr2* α -eGFPCre BAC transgenic mice

A mouse bacterial artificial chromosome (BAC clone RP23-78P13) containing the entire *Crfr2* gene was treated as follows to prepare it for the generation of transgenic mice. First, RecA-mediated homologous recombination (Yang et al., 1997) was used to disrupt expression of *Inmt*, a gene present on the BAC and known to be expressed in the brain; this step was taken to prevent a gain-of-function phenotype that could arise when a gene is overexpressed by a multicopy transgene. Disruption of *Inmt* was accomplished by inserting a bovine growth hormone polyadenylation sequence (BGHpA) cassette into the 5' end of the *Inmt* gene that removed the start codon; 5' and 3' homologous sequences (referred to as Box A and Box B, respectively) were amplified by PCR (Phusion DNA polymerase, New England Biolabs) and cloned into pBluescript (Stratagene) flanking the BGHpA cassette. Sequences used were as follows: Box A (nucleotides -698 to +9, relative to 5' end of *Inmt* exon I: forward primer, 5'-GATCTCTGGTCCTCCTACTTCC-3'; reverse primer, 5'-AATTCTAGCCAGCCCACACAGCT-3'; Box B (nucleotides +10 to +790): forward primer, 5'-AGGGCAAGGTATACATAGGAG-3'; reverse primer, 5'-AGTTCCTTGGCACCGAATGTCTGG-3'. The *Inmt*-mutant version of the BAC was then subjected to a second round of recombineering to target the *Crfr2* gene, for which two splice variants have been described in rodents: 1) a broadly expressed variant that is not highly expressed in brain (*Crfr2* β , ATG in exon I), and 2) a neuronally expressed form that is enriched in the brain and present at high levels in LS (*Crfr2* α , ATG in exon III) (Lovenberg et al., 1995; Chen et al., 2005). To simultaneously target *Crfr2* α and suppress expression *Crfr2* β from the BAC, two features were incorporated into the targeting vector: 1) a cassette containing an eGFPCre fusion gene followed by an SV40pA was inserted into the 5'UTR of the third exon of the *Crfr2* gene; 2) a STOP codon was introduced into exon IV (shared by both α and β splice forms) to terminate translation of any *Crfr2* β mRNA transcribed from the BAC. Sequences used were as follows: Box A (nucleotides -532 to +132, relative to 5' end of *Crfr2* exon III: forward primer, 5'-TTCCTAGATGTCCTCCTCTCACC-3'; reverse primer, 5'-CCCCGGAGCCGCGCG; Box B (nucleotides +149 to +790): forward primer, 5'-CGGCGCTGCTCCTCAGCCTGC-3'; reverse primer, 5'-ACTTCAACAAACAGCGTAGAGGAAGG-3'. The final recombineered BAC containing the desired modifications was linearized with NotI, purified away from smaller fragments released by digestion (vector backbone plus genomic sequences located more than 85kb upstream of the *Crfr2* α ATG) using a Sepharose CL4b column, integrity confirmed by pulsed field gel electrophoresis, and then microinjected into the pronuclei of FVB/N embryos to generate transgenic mice.

Animal maintenance

The *Crrf2* α -eGFPCre BAC transgene was maintained on an FVB/N (Taconic) background. For all behavior experiments, hemizygous transgenic FVB/N males were bred to C57BL/6N (Harlan Sprague Dawley) females, and FVBB6F1 male mice were used for testing. All animals were housed at 23°C with *ad libitum* access to food and water in a 13-h day/11-h night cycle, with the day starting at 21:00. All behavioral testing was done during the dark cycle, starting no earlier than 11:00 and no later than 20:00. Following surgery, mice were singly housed prior to behavioral testing. We note that quantitative differences in behavioral results might be obtained on an inbred C57BL/6 congenic background. All experiments were conducted in accordance with NIH guidelines and approved by the Caltech Institutional Animal Care and Use Committee.

In situ hybridization

Single color ISH for *Crrf2* was performed on 120 μ m thick free floating sections as described previously (Mongeau et al., 2003). In brief, brains were collected, cut in 3–4 mm coronal slabs using a block, and fixed overnight in 4% paraformaldehyde. Next, 120 μ m-thick coronal sections were made from the tissue slabs using a vibratome, followed by gentle digestion for 30 min using proteinase K, fixation with 4% paraformaldehyde, and hybridization at 60°C overnight with a cRNA digoxigenin-labeled probe against *Crrf2* mRNA. Nonhybridized probe was washed off at 60°C and digested with RNase A at 37°C for 30 min. Immunohistochemistry was performed using antidigoxigenin conjugated with alkaline phosphatase (Roche), and development performed with the alkaline phosphatase substrate NBT/BCIP. Double-label fluorescent ISH (dFISH) protocols have been previously described in detail (Thompson et al., 2008). In brief, dFISH was performed using a variation of the colorimetric protocol. Briefly, riboprobes were labeled with either digoxigenin-UTP or dinitrophenyl-11-UTP (DNP; Perkin Elmer). A DNP-labeled probe and a DIG-labeled probe were hybridized simultaneously to 25 μ m thick sections. Tyramide signal amplification was performed for each probe individually, using either anti-DIG-HRP with tyramide-biotin, or anti-DNP-HRP with tyramide-DNP for amplification. Visualization of signal was achieved using either streptavidin-Alexa Fluor 488 (Invitrogen/Molecular Probes) or anti-DNP-Alexa Fluor 555 (Invitrogen/Molecular Probes), and imaging performed on an Olympus FluoView-1000 confocal microscope. Regions used for probes are as follows: Cre recombinase, nucleotides 489-1441 (GenBank: X03453.1); *Crrf2*, nucleotides 459-1270 (NCBI Reference Sequence: NM_009953.3); *Esr1*, nucleotides 1123-2102 (GenBank: AB560752.1); *Gad2*, nucleotides 1069-2094 (NCBI Reference Sequence: NM_008078.2); *Penk*, nucleotides 312-1127 (NCBI Reference Sequence: NM_001002927.2); tdTomato, nucleotides 25-639 and 751-1365 (GenBank: AY678269.1); due to high homology, Cherry ISH was performed using the tdTomato probe.

Immunofluorescent labeling

For immunostaining of free floating sections, mice were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA) in 1x PBS. Brains were then removed and postfixed 16 hours at 4°C, washed in 1x PBS, and embedded in 3% low melting agarose for vibratome sectioning at 75-100µm. For immunostaining, sections were blocked in 'NDS block' (5% normal donkey serum, 0.1% Triton X-100 in 1x PBS) for a minimum of 30' at room temperature, and primary antibodies diluted in NDS block and incubated 16 hours at 4°C. Following this, sections were washed 3x in PBS-T (0.1% Triton X-100 in 1xPBS), and incubated for 1-2 hours at room temperature in fluorescent secondary antibodies (Jackson Immunoresearch) diluted 1:1,000 in PBS-T. After this incubation, sections were washed, counterstained with 4',6-diamidino-2-phenylindole (DAPI), mounted, and coverslipped using either ProLong Gold Antifade Reagent (Invitrogen) or Fluoro Gel (Electron Microscopy Sciences). Cryosection immunostaining was performed similarly, with the following differences. After perfusion, brains were postfixed in 4% PFA for 1-2 hours on ice, followed by cryoprotection overnight at 4°C in 15% sucrose in 1xPBS, embedding in OCT, cryosectioning at 20µm and collection onto Superfrost Plus slides (Ted Pella). Prior to immunostaining, sections were washed once in 1xPBS, and then postfixed for 5 minutes in cold methanol. Immunostaining then proceeding as detailed above for floating sections. Antibodies used were as follows: rabbit anti-Cre (Covance #PRB-106C *note: the lot used in this study is no longer available and newer lots have not worked for IHC), goat anti-CTB (List #703), chicken anti-eGFP (Abcam #ab13970), rabbit anti-ESR1 (Santa Cruz #sc-542), and rabbit anti-UCN3 (Cosmo Bio Co #YII-Y364-EX). Secondary immunoreagents were obtained from Jackson Immunoresearch and Invitrogen. Fluorescent nuclear counterstaining was performed using 4',6-diamidino-2-phenylindole (DAPI).

Cell counting

DAPI-counterstained sections were imaged on an Olympus FluoView-1000 confocal microscope, and cell counts performed using MetaMorph (Molecular Devices) in combination with manual scoring to ensure accuracy. Details on the specific regions quantitated are given in the relevant figure legends.

Cre-dependent viral vectors

Viral plasmids encoding Cre-dependent opsins were provided by the Deisseroth (AAV-EF1a-DIO-hChR2(H134R)-EYFP, AAV-EF1a-DIO-eNpHR2.0-EYFP) and Boyden (AAV-CAG Δ -ArchT-GFP-FLEX) laboratories and packaged in serotype AAV5 at the University of Pennsylvania (U Penn) Gene Therapy Vector Core (ChR2-YFP and ArchT-GFP) or Harvard Gene Therapy Initiative (HGTI, eNpHR2.0-YFP). AAV5 encoding Cre-dependent ChR2-tdTomato was purchased from U Penn Vector Core inventory (lot #V1345). Cre-dependent AAV plasmid expressing humanized renilla GFP (hrGFP) was a gift from Clif Saper and was packaged in AAV5 at HGTI. Cre-dependent AAV5 expressing eYFP was purchased from the University of North Carolina (UNC) Gene Therapy

Department stock inventory (AAV-EF1a-DIO-EYFP). AAV5 containing a CMV-Cre cassette was purchased from Vector Biolabs. A Cre-dependent nuclear-localized mCherry construct was generated by fusing a 3x nuclear localization sequence to mCherry and cloning this in place of ChR2-YFP in the vector EF1a-DIO-hChR2(H134R)-EYFP; packaging of this construct in AAV5 was done at U Penn. Construction, packaging, and validation of the Cre-dependent HSV129 variant HSV129 Δ TK-TT has been previously described (Lo and Anderson, 2011). HSV129 Δ TK-TT anterograde spread and labeling (tdT) are initiated only in Cre⁺ 'starter' cells; once rendered replication-competent in starter cells, the virus is able to propagate in the anterograde direction through downstream nodes of a given circuit.

Electrophysiological slice recordings

Standard mouse brain slice preparation and whole-cell recording were performed as follows: 3-5 month old mice were euthanized and coronal brain sections 250 μ m thick were cut on a Vibratome (VT1000S; Leica) in ice-cold glycerol-based artificial cerebrospinal fluid (composition in mM: 252 glycerol, 1.6 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.4 CaCl₂, 18 NaHCO₃, 11 glucose, oxygenated with 95% O₂/5% CO₂). Slices were allowed to recover for 90 minutes at 32°C in standard artificial cerebrospinal fluid (ACSF; composition in mM: 126 NaCl, 1.6 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.4 CaCl₂, 18 NaHCO₃, 11 glucose, oxygenated with 95% O₂/5% CO₂). After this recovery period, slices were then transferred to room temperature oxygenated ACSF where they remained until recording. Cells expressing GFP or YFP were detected by infrared differential interference contrast (IR-DIC) and fluorescence video microscopy (Olympus BX51). Whole-cell voltage and current clamp recordings were performed at 30°C with a MultiClamp 700B amplifier and Digidata 1440A (Molecular Devices). The patch pipette, with a resistance of 7–9M Ω , was filled with an intracellular solution containing (in mM) 135 potassium gluconate, 5 EGTA, 0.5 CaCl₂, 2MgCl₂, 10 HEPES, 2 MgATP and 0.1GTP, pH7.2, 280–300mosM. Series resistance was typically 20-30 M Ω . Cell attached recordings were performed in voltage clamp with holding current maintained at zero, and using pipettes filled with ACSF. Appropriate bridge balance was applied to all current clamp recordings. Data were sampled at 10kHz, filtered at 3kHz and analyzed with pCLAMP10 or Igor Pro (Wavemetrics) using the Neuromatic analysis software package written by Jason Rothman (<http://neuromatic.thinkrandom.com/>).

Stereotaxic surgery

Mice 2–5 months old were anaesthetized 2.5% isoflurane, placed into a stereotaxic frame (Kopf Model 940), and anesthesia maintained using 1-1.5% isoflurane. To enhance uptake of pressure injected solutions, animals were injected with 0.4 ml of 20% mannitol (Phoenix Pharmaceuticals). The skull was exposed and holes produced using with a hand-held drill (Roboz) with 0.7mm bits (Fine Science Tools). Injections were targeted using coordinates based on the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001), relative to bregma (LS, AP+0.5, ML \pm 0.59, DV-2.8; AHA, AP-0.8, ML \pm 0.3, DV-4.9;

LH, AP-1.6, ML±0.9, DV-5.25). Virus was backfilled into glass capillaries (tip diameter ~50µm) and delivered at a rate of 30nl per minute using a nanoliter injector (Nanoliter 2000, World Precision Instruments) controlled by an ultra microsyringe pump (Micro4, World Precision Instruments). Volumes delivered were between 50-100nl, amounts determined empirically to be the largest that would label cells in LS without spread to and recombination in adjacent brain regions. AAV molecular titers were on the order of ~3E+12, resulting in a total ~3E+8 total viral particles delivered per injection site. If needed, bilateral stainless steel guide cannulas (Plastics1) were implanted above the injection sites; lengths of tubing below pedestal were: LS, 2.6mm; AHA, 4.2mm. After implantation, bilateral stylets were inserted into the guides to prevent clogging. For optogenetic manipulations of LS *Crf2α*⁺ axons in the hypothalamus, bilateral stainless steel guide cannulas were implanted with tips located 1.5mm posterior and 1.5mm ventral to the virus injection sites in LS and which extended down to approximately 500µm above the medial hypothalamus at the level of the AHA. For postoperative care, mice were injected subcutaneously with ketoprofen (2 mg per kg body weight) and supplied for 7 days with drinking water containing 400 mg/L sulphamethoxazole and 200 mg/L ibuprofen. Mice were given four (eNpHR2.0 and ArchT) or six (ChR2) weeks after injection to allow for viral expression before being used for behavior experiments. All surgically manipulated animals underwent histological examination to ensure proper guide placement and/or that viral expression was restricted to the LS.

In vivo optogenetic control of neuronal activity

To enable in vivo delivery of light, custom bilateral fiber assemblies were built by inserting two 200µm optic fibers (0.37 NA multimode fiber, Thorlabs) through plastic bilateral guide pedestals (from which the steel tubing had been removed), gluing them in place, and then cutting the ends so that the tips of the fibers would be flush with the tips of implanted stainless steel guides in the brains of mice. On the day of testing, mice were anesthetized with isoflurane, stylets were removed and a bilateral fiber assembly was connected by inserting one end into the guide (held in place using dust caps (Plastics1)) and the other end connected via FC/PC to a 1x2 intensity division fiber optic rotary joint (Doric Lenses) that was suspended above the test arenas using a boom microphone stand (Proline). Two mice were tested at the same time by connecting two rotary joints to a beam splitter (1x2 intensity division minicube, Doric Lenses) that was in turn connected to lasers (473nm, 532nm, or 593nm, Shanghai Laser & Optics Century) located outside the testing room and which were controlled by a pulse stimulator (A310 Accupulser Signal Generator, World Precision Instruments). Light power at the tips of the fibers were measured prior to insertion into mice using a power meter (Thorlabs).

Behavioral testing

All behavioral testing was done using male *Crf2α*-eGFPCre mice on an FVBB6F1 background. Animals were lightly anesthetized with 2% isoflurane prior to testing to allow insertion of fiber optic cables, which was done in the

antechamber of the room where the animals were housed. To assay anxiety behavior, mice were tested sequentially in three standard assays, each lasting 10 minutes each: light-dark box (LDB), box dimensions 50x25x30cm; light side 31cm long, dark side 16cm long; doorway between light and dark sides, 5.25cm wide, 9cm high), mice introduced into dark side at start of assay, parameters measured included number of entries into and time spent in light side; open field (OF), 50x50x30 boxes constructed from white 1/8" ABS white plastic, mice introduced into a corner at start of assay, parameters measured included number of entries into and time spent in a 17x17cm square region in the 'center' of the open field, total distance moved, and time spent immobile; novel object (NO), a stainless steel cup (base diameter 3.5cm, lip diameter 5.75cm, base to lip height = 4.5cm) was placed in the center of the open field box, parameters measured included number of entries into and time spent exploring the object in a 17x17cm square region in the 'center' of the open field, total distance moved, and time spent immobile. Average velocity in the open field was calculated by dividing total distance moved by time spent mobile (Distance moved / Time in OF – time spent immobile). To avoid confounds from conditioning effects (i.e. conditioned fear instead of innate anxiety), separate groups of mice were injected and tested for each data set presented. The one exception to this is the data in Supplemental Figure 2H (retesting ChR2-injected mice 7 days after stimulation); in that experiment, different novel objects were used in the second assay (6x6x1.25cm plastic squares). For photoinhibition experiments involving stress, mice were subjected to 20 minutes of immobilization stress, an length of time determined empirically to be the minimum length necessary to elevate anxiety in FVBB6F1 mice (data not shown). Note that no differences were detected between groups in that experiment. All behavior experiments were captured in MPEG-2 (.m2v) format using Mediacruise (Canopus) and analyzed with Ethovision 3.1 (Noldus Information Technology). At the completion of behavioral testing, all mice were perfused and histologically analyzed to ensure viral expression was restricted to LS and guide cannulas were properly positioned. Procedures used for individual experiments were as follows:

Photostimulation of LS *Crfr2*⁺ cells using ChR2(H134R)-YFP during testing in naïve mice

Following insertion of optic fibers into guides, mice were placed in a new cage, transferred to the testing room and connected to a 1x2 rotary joint. After a 10 minute period to recover from anesthesia, mice were run sequentially through the LDB, OF, and NO (10 minutes each assay) in the presence of 473nm light (15 Hz, 20 ms pulse width, 5 sec ON, 5 sec off). For all ChR2 experiments, light power at the fiber tips was 380mW/mm², which is sufficient to yield 1mW/mm² (the minimum amount of light reported to be capable of efficient ChR2 activation (Aravanis et al., 2007)) at 1.4mm below the fiber tips and therefore should be capable of photostimulating the full dorsoventral span of LS *Crfr2*⁺ cell bodies.

Photostimulation of LS *Crfr2*⁺ cells using ChR2(H134R)-YFP prior to testing in naïve mice

Following insertion of optic fibers into guides, mice were transferred to a novel 'stimulation' room and placed in a clean cage inside an open box that had been sprayed with a novel odor (Simple Green). After connection to a 1x2 rotary joint, mice received photostimulation with 473nm light (15 Hz, 20 ms pulse width, 5 sec ON, 5 sec off) for 30 minutes. Following this, photostimulation was terminated, optic fibers were removed, and stylets reinserted into the guides. All animals were then moved out of the stimulation room and into the testing room, and run through the anxiety test battery (LDB,OF,NO) as described above.

Photostimulation of LS *Crf2 α* ⁺ cells using ChR2(H134R)-YFP during stress prior to testing

To minimize the potential confound of a ceiling effect (i.e. anxiety levels so high that no additional elevation is detectable in these assays), mice used for this experiment were habituated by handling 5 minutes per day for 3 days prior to testing in order to reduce sensitivity to stress. Following insertion of optic fibers into guides, mice were subjected to immobilization stress (IMS) by taping their limbs to a plastic tray using green lab tape (VWR); taping required ~30 seconds and was completed just as mice were awaking from isoflurane anesthesia. IMS was done in a biosafety cabinet for 30 minutes during which time mice received photostimulation with 473nm light (15 Hz, 20 ms pulse width, 5 sec ON, 5 sec off). Following this 30 minute period, optic fibers were removed, mice were released from IMS, and then transferred in a new cage to the testing room where they were then run through the anxiety test battery (LDB,OF,NO) as described above.

Photoinhibition of LS *Crf2 α* ⁺ cells using eNpHR2.0-YFP during stress prior to testing

Following insertion of optic fibers into guides, mice were subjected to immobilization stress (IMS) in a biosafety cabinet for 20 minutes during which time they received illumination with 593nm light (continuous light, 20 sec ON, 10 sec off). In all eNpHR2.0 experiments, light power at the fiber tips was 475mW/mm². Following this 20 minute period, optic fibers were removed, mice were released from IMS, and were then transferred in a new cage to the testing room where they were then run through the anxiety test battery (LDB,OF,NO) as described above.

Photoinhibition of LS *Crf2 α* ⁺ cells using eNpHR2.0-YFP after stress during testing

Following insertion of optic fibers into guides, mice were subjected to immobilization stress (IMS) in a biosafety cabinet for 20 minutes. Following this 20 minute period, mice were released from IMS and transferred in a new cage to the testing room. Fibers were connected to a 1x2 rotary joint, and mice were run through the anxiety test battery (LDB,OF,NO) as described above while receiving illumination with 593nm light (continuous light, 20 sec ON, 10 sec off) throughout testing.

Photoinhibition of LS *Crf2 α* ⁺ cells using eNpHR2.0-YFP during testing in naïve mice

Following insertion of optic fibers into guides, mice were transferred in a new cage to the testing room and connected to a 1x2 rotary joint. After a 10 minute period to recover from anesthesia, mice were run sequentially through the LDB, OF, and NO, mice were run through the anxiety test battery (LDB,OF,NO) while receiving illumination with 593nm light (continuous light, 20 sec ON, 10 sec off) throughout testing.

Photoinhibition of LS *Crf2 α* ⁺ projections to AHA using ArchT-GFP after stress during testing

Following insertion of optic fibers into guides, mice were subjected to IMS in a biosafety cabinet for 20 minutes. Following this 20 minute period, mice were released from IMS and transferred in a new cage to the testing room. Fibers were connected to a 1x2 rotary joint, and mice were run through the anxiety test battery (LDB,OF,NO) as described above while receiving illumination with 532nm light (continuous light, 20 sec ON, 10 sec off) throughout testing. Light power at the fiber tips was 318mW/mm².

Photostimulation of LS *Crf2 α* ⁺ terminals in AHA using ChR2(H134R)-YFP during testing in naïve mice

Following insertion of optic fibers into guides, mice were placed in a new cage, transferred to the testing room, and connected to a 1x2 rotary joint. After a 10 minute period to recover from anesthesia, mice were run sequentially through the LDB, OF, and NO (10 minutes each assay) in the presence of 473nm light (15 Hz, 20 ms pulse width, 5 sec ON, 5 sec off).

Statistics

Responses to stress can vary widely, with some individuals showing robust and persistent elevations in anxiety behavior while others are relatively resilient and maintain normal functioning even following exposure to severe trauma (Russo et al., 2012). We observed large variations in susceptibility to immobilization stress among the mice tested, including the presence of resilient outliers that resulted in datasets that were non-normally distributed (i.e. failed three different tests for normality: Kolmogorov-Smirnov, D'Agostino and Pearson omnibus normality test, and Shapiro-Wilk normality tests). For this reason, all data derived from stressed mice were plotted in box and whisker format (with whiskers representing minimum and maximum data points) and statistical significance assessed using nonparametric Mann-Whitney *U* tests. In contrast, data from unstressed mice generally passed at least one of the tests for normality and so were plotted as standard column bar graphs depicting mean \pm s.e.m. and analyzed for significance using unpaired t tests.

Channelrhodopsin-assisted circuit mapping (CRACM)

Four to six weeks after injecting Cre-dependent ChR2(H134R)-YFP AAV5 into LS, brain slices were prepared for electrophysiological recordings as described

above. Light from a 473nm laser (Shanghai Dream Laser) was delivered via a 200 μ m fiber optic cable positioned next to the region of interest using a micromanipulator. Light pulses (2ms pulse width) were triggered using pClamp; synchronous with the triggering of the laser, a 200 μ s depolarization of 1mV was done to create an artifact that enabled determination of the precise time when the light pulse was initiated. Cells in whole cell configuration were held in voltage clamp at -40mV and identification of currents as being GABA-A-mediated was done using 100 μ M picrotoxin. No postsynaptic excitatory currents were detected in response to stimulation of LS *Crfr2 α ⁺* neurons (0/66 cells). Onset of postsynaptic responses was determined using the Threshold method (Fedchyshyn and Wang, 2007), where IPSC onset was defined at the time point when the response reached 10% of its maximum. Latency to onset of a response was determined by subtracting the time point when the laser was triggered from the time point when onset of a PSC occurred.

Retrograde Tracing

For neuroanatomical studies using CTB, mice were prepared for stereotaxic surgery and CTB (0.5% in PBS, List) was iontophoretically delivered using alternating positive current (5 μ A, 7 sec ON, 7 sec OFF) for 3 minutes using a Midgard Precision Current source (Stoelting). Following survival times of 5-7 days, mice were perfused and processed for immunostaining. For experiments involving CRACM combined with retrograde tracing, Cre-dependent ChR2-YFP AAV was injected into the LS of *Crfr2 α -eGFPCre* mice. Following a 4 week viral incubation period, 20nl of red retrobeads (diluted 1:4) were injected into the PVN (AP-0.4, ML+0.25, DV-4.5 to 4.4), and recordings performed 5 or 14 days later.

Corticosterone measurements

Mice were injected and implanted as described above. To test effects of *Crfr2 α ⁺* neuronal stimulation in naïve mice on circulating corticosterone levels, *Crfr2 α -eGFPCre* male mice injected and implanted in LS with Cre-dependent ChR2-YFP AAV5 and guide cannulas respectively were anesthetized with 2% isoflurane and optic fibers inserted. Mice were transferred to a novel room and placed in a clean cage inside an open box. After connection to a 1x2 rotary joint, mice received photostimulation with 473nm light (15 Hz, 20 ms pulse width, 5 sec ON, 5 sec off) for 30 minutes. Light power at the fiber tips was 380mW/mm². Following this, photostimulation ceased and mice were immediately euthanized and decapitated (45-60 s following termination of photostimulation) for blood collection. To test the role of the LS *Crfr2 α ⁺* projection to the AHA in stress-induced elevation of corticosterone, *Crfr2 α -eGFPCre* male mice injected in LS with Cre-dependent ArchT-GFP AAV5 and implanted with guide cannulas over the AHA were anesthetized with 2% isoflurane and optic fibers inserted. Mice were subjected to IMS in a biosafety cabinet for 20 minutes during which time they received illumination with 532nm light (continuous light, 20 sec ON, 10 sec off). Light power at the fiber tips was 318mW/mm². Following this, illumination ceased and mice were immediately euthanized and decapitated (45-60 s following termination of illumination) for blood collection. For both groups, blood

was collected in 1.5ml eppendorf tubes and allowed to clot at room temperature for 10 minutes. Tubes were then spun at 2,000g for 15 minutes to pellet cells, and supernatant containing clear serum removed and frozen on dry ice. Corticosterone concentrations were measured using an enzyme immunoassay kit (Cat#900-097, Enzo Life Sciences).

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Additional characterization of *Crfr2* mRNA and BAC transgene expression in LS. (Related to Figure 2)

(A,B) dFISH for *Crfr2* (green) vs the glutamatergic marker *Slc17a6* (a.k.a. *vGlut2*, red).

(C) No overlap is observed between *Crfr2* and *Slc17a6* in LS: $Crfr2^+/DAPI^+ = 8.2\%$, $Slc17a6^+/DAPI^+ = 1.6\%$, $Crfr2^+/Slc17a6^+ = 0$, $Slc17a6^+/Crfr2^+ = 0$; $8.2\% \times 1.6\% \times 11,221$ DAPI⁺ cells counted ~ 15 $Crfr2^+Slc17a6^+$ cells expected if expression were random; 0 $Crfr2^+Slc17a6^+$ cells were observed.

(D-F) dFISH for *Crfr2* (green) vs the cholinergic marker *ChAT* (red). We detected no *ChAT* expression in lateral septum, despite observing numerous *ChAT*⁺ cells in other regions known to contain cholinergic neurons such as medial septum (MS in D,F), striatum, and basal forebrain (data not shown).

(G-O) Counts of eGFPCre⁺ cells at three rostrocaudal levels of LS (G-I, bregma+1.0; J-L, bregma+0.6; M-O, bregma+0.2) revealed that, similar to endogenous *Crfr2* mRNA (main Figures 2A-E), immunodetectable transgene expression is highest at middle LS levels (bregma+0.6), with fewer immunopositive neurons in more rostral and caudal regions. (I) Bregma+1.0, dorsal LS, eGFPCre⁺/DAPI⁺ = $1.3 \pm 0.5\%$, n=1,527 DAPI⁺ cells; ventral LS, eGFPCre⁺/DAPI⁺ = $0.9 \pm 0.6\%$, n=1,828 DAPI⁺ cells. (L) Bregma+0.6, dorsal LS, eGFPCre⁺/DAPI⁺ = $6.8 \pm 2.0\%$, n=1,932 DAPI⁺ cells; ventral LS, eGFPCre⁺/DAPI⁺ = $11.2 \pm 1.8\%$, n= 2,412 DAPI⁺ cells. (O) Bregma+0.2, dorsal LS, eGFPCre⁺/DAPI⁺ = $0.8 \pm 0.3\%$, n=1,881 DAPI⁺ cells; ventral LS, eGFPCre⁺/DAPI⁺ = $1.2 \pm 0.1\%$, n=2,525 DAPI⁺ cells. Counts are mean \pm s.e.m. from 3 separate *Crfr2* α -eGFPCre transgenic mice.

(P-S) Immunostaining for BAC transgenic eGFPCre (green) and GABA (red), and quantitation; (S) 306/354 GFPCre⁺ cells (86.4%) were GABA⁺, which is similar the percentage of *Gad2*⁺/*Crfr2*⁺ cells detected using dFISH (main Figure 2F).

Finally, we note that in our Channelrhodopsin-assisted circuit mapping (CRACM) experiments in which ChR2 was expressed in LS *Crfr2* α ⁺ cells and recordings performed to identify *postsynaptic* targets, monosynaptic inhibitory postsynaptic currents (IPSCs) were detected in 44/66 cells (15/18 in LS, 29/48 in hypothalamus). However, excitatory postsynaptic currents were never detected (0/66 cells). Taken together, these data demonstrate that *Crfr2* α ⁺ neurons are a predominantly GABAergic, inhibitory population.

Supplementary Figure 2. Injection of Cre-dependent AAVs employing the EF1 α promoter into *Crfr2 α -eGFPCre* mice yields specific expression in *Crfr2 α* ⁺ neurons. (Related to Figure 2)

(A) Stereotaxic injection into the LS of *Crfr2 α -eGFPCre* mice of a Cre-dependent AAV5 in which the EF1 α promoter drives expression of a nuclear-targeted mCherry. Recombination is strongest in laterally situated neurons of LSi and LSv, with scattered Cherry⁺ cells seen in medial LS. This pattern of recombination is similar to expression of endogenous *Crfr2* mRNA (Figure 1C in main text).

Volume of AAV injected = 100nl.

(B) Co-injection of the Cre-dependent Cherry construct together with a second AAV that constitutively expresses Cre recombinase was done to assess the labeling when recombination occurred in a large fraction of infected cells. In this case, no clear patterning is observed, with Cherry⁺ cells detected throughout the LS. Moreover, the amount of Cre-dependent Cherry AAV in this experiment (50nl) is half the volume injected in experiment shown in (A) and yet results in significantly more labeling. These data indicate that the pattern of recombination seen in (A) is a reflection of Cre activity and not due to an inability of AAV5 or the EF1 α promoter to infect or express in LS neurons in general.

(C-G) Assessment of the sensitivity and specificity of Cre-dependent AAVs injected into LS of *Crfr2 α -eGFPCre* mice. (C-F) Comparison of transgenically expressed eGFPCre (green) and virally expressed Cherry (red). Similar to what was observed with endogenous *Crhr2* mRNA (inset in Figure 1C of main text), the *Crfr2 α -eGFPCre* transgene showed a broad dynamic range of expression, driving both low (arrows in C-F) and high (arrowhead in C-F) expression of eGFPCre, both of which were sufficient to catalyze recombination of the injected AAV. (G) Counts were performed in regions containing Cherry⁺ cells:

eGFPCre⁺/DAPI⁺ = 15.1%, Cherry⁺/DAPI⁺ = 7.9%, Cherry⁺/eGFPCre⁺ = 34.7%, eGFPCre⁺/Cherry⁺ = 66.4%; total $n = 2,671$ DAPI⁺ cells

(H-K) Two factors complicate efforts to assess the specificity of recombination in transgenic or gene targeted mice that use cell type-specific promoters to express Cre. First, the ability of Cre to catalyze recombination even when present at very low levels can lead to labeling of cells in which the endogenous mRNA or protein under the regulation of the cell-specific promoter cannot be detected. This has been observed in gene targeted mouse lines (Madisen et al., 2010; Yizhar et al., 2011), suggesting it is a reflection of Cre sensitivity. Similarly and in the case of *Crfr2*, a second complicating factor is its broad dynamic range of expression and possibility of failure to detect message in low expressors when using dFISH (i.e. false negatives). We therefore used an indirect approach to further test specificity of recombination in *Crfr2 α -eGFPCre* mice. We reasoned that if recombination were occurring randomly, it should be detected in LS populations that are known to be *Crfr2*-negative. We defined two such populations as those that express *Esr1* or *Penk* (Figures 2G-2J in main text), and so used immunostaining (H,I) or dFISH (J,K) to determine if any recombination had occurred in *Esr1*⁺ (H,I) or *Penk*⁺ (J,K) cells. This analysis revealed that significant recombination had not occurred in either of these two populations; counts were performed in regions

containing Cherry⁺ cells: Cherry vs. ESR1: ESR1⁺/DAPI⁺ = 3.1%, Cherry⁺/DAPI⁺ = 7.8%, Cherry⁺/ESR1⁺ = 0, ESR1⁺/Cherry⁺ = 0; 3.1% \times 7.8% \times 1,645 cells counted = 4 Cherry⁺ESR1⁺ cells expected if recombination were random, 0 Cherry⁺ESR1⁺ cells were observed; Cherry vs. *Penk*: *Penk*⁺/DAPI⁺ = 9.4%; Cherry⁺/DAPI⁺ = 7.2%, Cherry⁺/*Penk*⁺ = 0.5%, *Penk*⁺/Cherry⁺ = 0.6%; 9.4% \times 7.2% \times 7,187 cells counted = 49 Cherry⁺/*Penk*⁺ cells expected if expression were random; 3 Cherry⁺*Penk*⁺ cells were observed.

(L-O) Absence of significant recombination in ESR1⁺ and *Penk*⁺ cells is not due to the inability of AAV5 or the EF1 α promoter to infect or express in these populations. Co-injection of the Cre-dependent Cherry AAV together with a CMV-Cre AAV resulted in Cherry expression in both ESR1⁺ (L,M) and *Penk*⁺ (N,O) cells near expected frequencies, counts were performed in regions containing Cherry⁺ cells. (M) Cherry vs. ESR1: ESR1⁺/DAPI⁺ = 5.3%, Cherry⁺/DAPI⁺ = 25.1%, Cherry⁺/ESR1⁺ = 29.1%, ESR1⁺Cherry⁺ = 6.2%; 5.3% \times 25.1% \times 2,780 cells counted = 37 Cherry⁺ESR1⁺ cells expected if recombination were random, 43 Cherry⁺ESR1⁺ cells were observed. (O) Cherry vs. *Penk*: *Penk*⁺/DAPI⁺ = 9.3%, Cherry⁺/DAPI⁺ = 27.3%, Cherry⁺/*Penk*⁺ = 36.7%, *Penk*⁺Cherry⁺ = 12.5%; 9.3% \times 27.3% \times 5,061 cells counted = 128 Cherry⁺/*Penk*⁺ cells expected if expression were random; 172 Cherry⁺*Penk*⁺ cells were observed.

Supplementary Figure 3. LS *Crf2 α* ⁺ neuronal responses to optogenetic stimulation, and ChR2 behavior controls. (Related to Figure 3)

(A,B) To determine the maximal rates at which LS *Crf2 α* ⁺ neurons are capable of efficient firing in response to photostimulation, whole cell recordings were performed on *Crf2 α* ⁺ChR2⁺ cells in acute slices from *Crf2 α* -eGFP-Cre mice injected 4 weeks earlier with Cre-dependent ChR2 AAV. (A) Cells (n=3) were photostimulated with 473nm light (20ms pulses) at three different frequencies (10, 15, or 20 Hz) in three sweeps (5 sec ON, 5 sec OFF). The number of missed spikes were counted and the fraction of successful action potentials was plotted (A'). Efficient firing was observed in response to photostimulation at 10 and 15 Hz, but larger numbers of failures occurred at 20 Hz. This suggests 15 Hz as the maximal practical photostimulation rate for *Crf2 α* ⁺ChR2⁺ cells, a frequency that also drove efficient picrotoxin (PTX)-sensitive postsynaptic inhibitory responses in downstream targets (B,B').

(C) Cell-attached recordings to test the ability of *Crf2 α* ⁺ChR2⁺ neurons to be repetitively photostimulated. *top*, a single 2ms flash of 473nm light induced a rapid current response (latency<1ms); (C') Photostimulation at 15Hz (2ms pulse, 5 sec ON) drove repetitive action currents (ACs) that were blocked by TTX. (C'') Extended photostimulation (15 Hz, 2ms pulse, 5 sec ON, 5 sec OFF for 30 minutes) drove efficient firing with low failure rates throughout the recording in 5/6 neurons tested (C''').

(D) Stimulating *Crf2 α* ⁺ neurons during testing increases anxiety in the elevated plus maze. hrGFP(n=14) vs. ChR2(n=16): entries in open arms (3.1 \pm 0.6 vs. 1.3 \pm 0.4, P <0.05), time in open arms (28.1 \pm 6.0 vs. 12.6 \pm 3.7, p <0.05).

(E) Stimulating *Crfr2* α^+ neurons during testing does not alter locomotor behavior. hrGFP($n=39$) vs. ChR2($n=38$): Average velocity in the open field when animals were mobile (14.9 ± 1.3 vs. 14.8 ± 0.8 , $P>0.9$).

(F) ChR2 expression in *Crfr2* α^+ neurons has no behavioral effects in the absence of 473nm light. hrGFP ($n=12$) vs. ChR2($n=12$): LDB, entries in light side (9.0 ± 2.1 vs. 9.3 ± 1.3 , $P>0.5$), time in light side (138.5 ± 42.7 vs. 160.0 ± 31.2 , $P>0.5$); OF, entries in center (18.7 ± 2.4 vs. 22.1 ± 3.7 , $P>0.4$), time in center (25.6 ± 4.7 vs. 31.4 ± 4.4 , $P>0.3$); NO, entries in center (34.3 ± 3.9 vs. 34.7 ± 2.8 , $P>0.9$), time in center (79.1 ± 13.3 vs. 86.7 ± 12.0 , $P>0.6$).

(G) Stimulating *Crfr2* α^+ neurons does not alter locomotor behavior during subsequent testing. eYFP($n=13$) vs. ChR2($n=12$): average velocity in the open field when animals were mobile (13.0 ± 0.3 vs. 12.9 ± 0.4 , $P>0.8$).

(H) Retesting of mice 1 week after stimulation revealed no significant behavioral differences between groups. eYFP($n=11$) vs. ChR2($n=11$): LDB, entries in light side (20.6 ± 2.7 vs. 18.2 ± 1.5 , $P>0.4$), time in light side (220.7 ± 22.0 vs. 257.8 ± 20.7 , $P>0.2$); OF(0-5'), entries in center (9.7 ± 1.0 vs. 7.5 ± 1.5 , $P>0.2$), time in center (14.0 ± 2.3 vs. 9.7 ± 1.9 , $P>0.15$); NO(0-5'), entries in center (12.0 ± 2.3 vs. 9.0 ± 2.3 , $P>0.35$), time in center (22.3 ± 7.7 vs. 18.5 ± 5.7 , $P>0.65$).

(I) Photostimulation of LS *Crfr2* α^+ neurons at 1 Hz is not sufficient to induce a persistent anxious state. eYFP($n=13$) vs. ChR2($n=14$): LDB, entries in light side (7.2 ± 1.8 vs. 4.5 ± 1.1 , $P>0.2$), time in light side (74.8 ± 23.6 vs. 44.9 ± 13.6 , $P>0.25$); OF(0-5'), entries in center (7.2 ± 1.4 vs. 4.5 ± 1.3 , $P>0.15$), time in center (7.2 ± 1.4 vs. 5.5 ± 1.5 , $P>0.4$); NO(0-5'), entries in center (12.9 ± 3.4 vs. 6.9 ± 2.5 , $P>0.15$), time in center (27.4 ± 7.6 vs. 11.3 ± 6.3 , $P>0.10$).

Values in (D-I) indicate mean \pm s.e.m.

Supplementary Figure 4. Photoinhibition of LS *Crfr2* α^+ neurons and controls for eNpHR2.0 behavior experiments. (Related to Figure 4)

(A-C) Whole cell voltage (A) or current (B) clamp recordings of eNpHR-YFP $^+$ *Crfr2* α^+ neurons in response to 593nm light (yellow lines) in LS slice preparations. (C) 593nm light drove (*left*) outward currents (peak = 47.5 ± 14.6 pA; steady state = 19.2 ± 5.2 pA) and (*right*) hyperpolarization (peak = -21.8 ± 4.4 mV; steady state = -10.9 ± 2.4 mV) of moderate amplitudes. Values indicate mean \pm s.e.m; $n = 8$ eNpHR-YFP $^+$ neurons recorded.

(D) To test whether repeated cycles of photoinhibition might lead to rebound firing (i.e. following hyperpolarization, membrane potential briefly rebounds to a more depolarized level than that prior to hyperpolarization, resulting in a transient increase in firing rate), *Crfr2* α^+ eNpHR-YFP $^+$ neurons in whole cell current clamp recording configuration were subjected to 40 sweeps of the following light protocol: 5 seconds light off, 20 seconds light ON, 5 seconds light OFF (*left*). In 3/3 cells, no significant rebound firing was observed, even after 20 minutes of repeated photoinhibition (*right*). Brief current injections performed at the completion of each sweep confirmed that the cells were healthy and capable of firing action potentials (not shown).

(E) Inhibiting *Crfr2* α^+ neurons during IMS does not alter locomotor behavior during subsequent testing in the open field. hrGFP($n=15$) vs. eNpHR ($n=17$), data presented Mann-Whitney U value, P value: ($U = 116.0$, $P > 0.65$).

(F) Inhibiting *Crfr2* α^+ neurons during testing does not alter locomotor behavior in the open field. hrGFP($n=11$) vs. eNpHR ($n=11$), data presented Mann-Whitney U value, P value: ($U = 46.5$, $P > 0.8$).

(G) To test if low anxiety behavior might be more prevalent during light ON periods, we pooled together animals that received photoinhibition during testing which were injected with either eNpHR (cell body inhibition, Figure 4L) or ArchT (axon terminal inhibition, Figure 6E) and counted the number of entries made into the light side of the light-dark box during light OFF periods versus the first 10 seconds of light-ON periods. Due to small group sizes, eNpHR- or ArchT-injected mice alone yielded trends but not significant increases in low anxiety behavior during light ON periods (not shown). In contrast, pooling both groups of mice together revealed a significant increase in entries made during light ON periods, indicating that the anxiolytic effects observed in response to optogenetic inhibition of *Crfr2* α^+ cell bodies or axons are not due to rebound effects during light OFF periods ($n=17$), Wilcoxon matched-pairs signed rank test ($P < 0.05$). No significant difference in entries during light OFF vs. ON periods was observed for control hrGFP expressing mice ($n=5$), Wilcoxon matched-pairs signed rank test ($P = 0.85$) (data not shown).

(H) In the absence of 593nm light, no differences between groups were observed hrGFP($n=10$) vs. eNpHR ($n=9$), data presented Mann-Whitney U value, P value: LDB, entries in light side ($U = 41.5$, $P > 0.75$), time in light side ($U = 34.5$, $P > 0.6$); OF, entries in center ($U = 42.5$, $P > 0.85$), time in center ($U = 40.0$, $P > 0.7$); NO, entries in center ($U = 35.0$, $P > 0.4$), time in center ($U = 36.0$, $P > 0.7$).

Supplementary Figure 5. LS *Crfr2* α^+ neurons send projections to multiple extraseptal targets, and make local inhibitory synapses within the LS.

(Related to Figure 5)

(A-L) Cre-dependent ChR2-YFP AAV was stereotaxically injected into the LS of *Crfr2* α -eGFPCre mice. After 4 weeks, analysis of YFP expression revealed robust fluorescence in the LS cell bodies and processes (A,B), as well as YFP $^+$ axons in anterior hypothalamic area (AHA, C,D), more caudal regions of medial hypothalamus (MH, E,F), medial amygdala (MEA, G,H), periaqueductal gray (PAG, I,J), and ventral hippocampus (vHIPP, K,L). All extra-septal images were imaged on a confocal using the identical laser settings. Numbers in parentheses indicate bregma level; boxed regions in top panels are shown at higher magnification in bottom panels. PVN, paraventricular nucleus; VMH, ventromedial hypothalamic nucleus; opt, optic tract; CA1, CA3, fields CA1 or CA3 of hippocampus; S, subiculum. Locations of cell nuclei and layers were determined using DAPI counterstaining (not shown).

(M) Schematic illustrating CRACM recordings, done by patching and recording photocurrents in ChR2-YFP-negative LS neurons that are near ChR2-YFP $^+$ fibers.

(N) Single 2ms pulses of 473nm light evoked strong IPSCs in 9/9 cells (B, *top left*); average response amplitude = 241.1 ± 54.2 pA; average responses latency = 4.7 ± 0.4 ms. *Bottom left*, 473nm light delivered at 15 Hz (2ms pulse width) was sufficient to inhibit induced firing of postsynaptic neurons. *Right*, both IPSCs (*top*) and IPSPs (*bottom*) were blocked by bath application of $100 \mu\text{m}$ picrotoxin.

Supplementary Figure 6. Photoinhibition and photostimulation of LS *Crfr2 α* ⁺ axon terminals in AHA. (Related to Figure 6)

(A) Cre-dependent ArchT-GFP AAV construct.

(B-E) Stereotaxic injection of (A) into the LS of *Crhr2 α -eGFPCre* mice yielded ArchT-GFP (green) expression in *Crfr2 α* ⁺ neurons (marked by Cre immunostaining, red). Boxed area in (D) shown at higher magnification in (E).

(F) Quantification of the specificity and sensitivity of recombination, counts were performed in regions containing ArchT-GFP⁺ cells: ArchT⁺/DAPI⁺ = $3.0 \pm 0.9\%$, Cre⁺/DAPI⁺ = $10.8 \pm 0.2\%$, Cre⁺/ArchT⁺ = $76.1 \pm 1.1\%$, ArchT⁺/Cre⁺ = $21.2 \pm 6.6\%$ (mean \pm s.e.m.). Total $n = 7,253$ DAPI⁺ cells from 2 injected mice.

(G,H) Whole cell recordings of ArchT-GFP⁺*Crfr2 α* ⁺ neurons in LS slice preparations. (H) 532nm light drove (*left*) outward currents (peak = $217. \pm 67.5$ pA; steady state = 150.3 ± 48.7 pA) and hyperpolarization (*right*) of large amplitude (peak = -39.9 ± 16.2 mV; steady state = -29.5 ± 13.3 mV). Values indicate mean \pm s.e.m; $n = 4$ ArchT-GFP⁺ neurons recorded.

(I) Diagram of experiment to test ArchT-GFP activity in axon terminals. Because no significant spontaneous activity of *Crfr2 α* ⁺ neurons was detected in slice recordings (data not shown), we used an approach based on CRACM that enables optogenetic control over presynaptic release. (I') Two Cre-dependent constructs were co-injected into the LS of *Crfr2 α -eGFPCre* mice to target expression of both ArchT-GFP and ChR2-tomato to *Crfr2 α* ⁺ neurons. (I'') Postsynaptic neurons in the AHA were then patched and photostimulation of ChR2-tomato⁺ArchT-GFP⁺ axons with 473nm light performed either in the absence or presence of 532nm light. If ArchT-GFP is functional in axon terminals, the expectation is that it should be capable of suppressing ChR2-mediated IPSCs recorded from postsynaptic neurons.

(J,K) Co-expression of ArchT-GFP (green) and ChR2-Tomato (red) in LS (J) and hypothalamus (K) in a *Crfr2 α -eGFPCre* mouse injected in LS with Cre-dependent AAVs encoding ArchT-GFP and ChR2-tomato.

(L) Voltage clamp recording of postsynaptic neuron in AHA from slice shown in (K). Photostimulation with a single 2ms pulse of 473nm light evoked IPSCs (left) that could be reversibly blocked by illumination with 532nm light (middle, right). Traces shown are averaged responses of 5 trials.

(M) Repetitive photoinhibition of the cell shown in (L) over a 20 minute period using the same 532nm illumination protocol as that applied in vivo (20 sec light ON, 10 sec light OFF) did not produce detectable rebound activity in axon terminals following cessation of light.

(N) Inhibiting LS *Crfr2α*⁺ axons in hypothalamus during testing does not alter locomotor behavior in the open field. hrGFP(*n*=12) vs. ArchT (*n*=10), data presented Mann-Whitney *U* value, *P* value: (*U* = 42.0, *P*>0.35).

(O) When light was not delivered through the guides, no significant differences in anxiety behavior were detected between groups. hrGFP(*n*=12) vs. ArchT (*n*=11), data presented Mann-Whitney *U* value, *P* value: LDB, entries in light side (*U* = 60.0, *P*>0.7), time in light side (*U* = 61.0, *P*>0.7); OF, entries in center (5-10') (*U* = 35.0, *P*>0.05), time in center (5-10') (*U* = 36.0, *P*>0.05); NO, entries in center (0-5') (*U* = 56.0, *P*>0.5), time in center (0-5') (*U* = 55.0, *P*>0.5).

(P) Stimulating LS *Crfr2α*⁺ axons in hypothalamus during testing does not alter locomotor behavior in the open field. eYFP(*n*=17) vs. ChR2 (*n*=15), presented as mean±s.e.m: average velocity in open field (11.73±0.2 vs. 13.1±0.8, *P*>0.1).

NOTE: In the NO assay, there was a trend towards increased anxiety (decreased number of entries on lighted side) that did not reach significance (Figure 6J). This may reflect a lower level of ChR2 present in nerve terminals (due to dilution during axonal transport). It is also possible that in the NO assay, a higher level of stimulation is required to overcome the appetitive influence exerted by the novel object, whereas no appetitive stimuli are present in the LDB and OF assays.

Supplementary Figure 7. Evidence that LS *Crfr2α*⁺ neurons monosynaptically inhibit 1) GABAergic neurons in AHA, and 2) AHA neurons that innervate the PAG. (Related to Figure 7)

(A-D) LS *Crfr2α*⁺ terminals overlap with GABAergic neurons in the AHA. Cre-dependent ChR2-YFP AAV was injected into the LS of *Crfr2α*-eGFPCre mice, yielding YFP labeling of axons (green, B,D) that are closely apposed to GABA⁺ cells (white, C,D) in the AHA.

(E-H) The polysynaptic anterograde tracer HSV129ΔTK-TT was injected into the LS of *Crfr2α*-eGFPCre mice, and expression of tdTomato (red) and GABA (white) was analyzed 2 days later. This is a time point when transsynaptic transfer first becomes detectable in the hypothalamus and many labeled cells are thus likely to be monosynaptic targets (labeling in the PVN is not detected until 3 days post-injection). Consistent with the ChR2-YFP tracing, we observed tdT⁺GABA⁺ cells (F-H), suggesting that GABAergic neurons in the AHA are a target of LS *Crfr2α*⁺ neurons.

(I-L) Combined CRACM and retrograde tracing demonstrate that LS *Crfr2α*⁺ neurons monosynaptically inhibit AHA neurons that project to the PAG. (I) Schematic of experiment, in which Cre-dependent ChR2-YFP was injected into the LS of *Crfr2α*-eGFPCre mice and Alexa647-conjugated CTB was injected into the PAG. (J) This yielded strong CTB labeling at the site of injection (PAG) as well as in scattered backlabeled neurons throughout the AHA. (K,L) Single 2ms pulses of 473nm light evoked strong IPSCs in 4/5 CTB⁺ cells in the AHA; average response amplitude = 74.7±25.8pA; average responses latency = 4.1±0.4 ms, consistent with monosynaptic connectivity.