SUPPLEMENTARY INFORMATION

MJAG Henckens, Y Printz, U Shamgar, J Dine, M Lebow, Y Drori, C Kuehne, A Kolarz, M Eder, JM Deussing, NJ Justice, O Yizhar and A Chen. *CRF receptor type 2 neurons in the posterior bed nucleus of the stria terminalis critically contribute to stress recovery.*
Supplementary Figure 1. Sites of Cre-recombinase expression visualized in brain sections (50 µm) of CRFR2-tdTomato⁺ mice. Loci nicely overlap with brain regions known to express CRFR2: (a, b) the olfactory bulb, (c) lateral septal nucleus, (d) pBNST, (e) medial amygdala, and (f) mesencephalic raphe nuclei. amygdalohippocampal area, anterolateral part (AHIAL); accessory olfactory bulb (AOB); bed nucleus of the stria terminalis intraamygdaloid division (BSTIA), medial division posterolateral part (BSTMPL), posterointermediate part (BSTMPI), posteromedial part (BSTMPM); dorsomedial hypothalamic nucleus (DM); dorsal raphe (DR); external plexiform layer of the accessory olfactory bulb (EPIA); lateral septal nucleus, dorsal part (LSD), intermediate part (LSI), ventral part (LSV); medial entorhinal cortex (MEnt); medial amygdaloid nucleus, posterodorsal part (MePD), posteroverentral part (MePV); mitral cell layer of the accessory olfactory bulb (Mia); periaqueductal gray (PAG); posterolateral (PLCo), posteromedial (PMCo) cortical amygdaloid nucleus; ventromedial hypothalamic nucleus (VMH).
Supplementary Figure 2. Coronal brain section of a CRFR2-tdTomato’ mouse showing strong tdTomato expression in the pBNST. Bed nucleus stria terminalis medial division posterior part (BSTMP); stria medullaris of the thalamus (sm). Inset shows selected section in 2.5x magnification.
Supplementary Figure 3. Coronal brain sections depicting the expression of CRFR2 mRNA in the pBNST of a wild type (CRFR2_Ctrl) and GABAergic CRFR2 conditional knockout mouse line (CRFR2_{GABA-CKO}). Bed nucleus stria terminalis medial division posterior part (BSTMP); lateral ventricle (LV); third ventricle (3V).
Supplementary Figure 4. Projection sites of pBNST CRFR2-expressing neurons as identified using Cre-inducible AAV5-EF1α-DIO-eYFP (see also Figure 1d). Projections were observed in (a) the paraventricular thalamic nucleus (PV), (b) brain stem around the locus coeruleus (LC), and (c) running through the stria terminalis (st). Other projection sites were (d) the periaqueductal gray (PAG), (e) lateral septal nucleus (LS), and (f) paraventricular nucleus of the hypothalamus. third ventricle (3V); fourth ventricle (4V); anterior hypothalamic area, central part (AHC), posterior part (AHP); aqueduct (Aq); Barrington’s nucleus (Bar); dorsal third ventricle (D3V); fimbria of the hippocampus (fi); lateral septal nucleus, intermediate part (LSI), ventral part (LSV), dorsal part (LSD); lateral ventricle (LV); medial habenular nucleus (MHb); medial parabrachial nucleus (MPB); periaqueductal gray, medial (MPAG), lateral (LPAG), dorsolateral (DLPAG), ventrolateral (VLPAG); paraventricular hypothalamic nucleus, dorsal cap (PaDC), lateral magnocellular part (PaLM), medial magnocellular part (PaMM); periventricular hypothalamic nucleus (Pe); superior cerebellar peduncle (scp); uncinate fasciculus (unc); ventral spinocerebellar tract (vsc).
Supplementary Figure 5. Immunohistochemistry in pBNST sections of CRFR2-tdTomato+ mice for potential ligands for CRFR2. (a) CRF, (b) urocortin 1 (Ucn1), and (c) urocortin 3 (Ucn3). Inset shows selected section in 2x magnification.
Supplementary Figure 6. Correlation between spiking probability of recorded pBNST ChR2⁺-neurons and the photocurrent amplitude under 475 nm light. The strong correlation observed suggests that in cells with smaller photocurrents, the depolarizing current was not sufficient to evoke action potentials.
Supplementary Figure 7. Unilateral photoactivation of pBNST CRFR2-expressing neurons in CRFR2-ChR2 mice induced expression of the immediate early gene c-Fos. (a) Unilateral photoactivation (c) induced a significant increase in c-Fos expression ($T_{60} = 3.54, P = 0.012$), when (b) the stimulated side was compared to the (d) unstimulated one. Error bars indicate SEM. *: $P < 0.05$
Supplementary Figure 8. Behavioral effects of photostimulation (either activation or inhibition) of pBNST CRFR2-expressing neurons. (a) Photostimulation did not affect locomotor activity as measured by total distance traveled in the open field test (ChR2: n_{cre+} = 10, n_{cre-} = 10; eNpHR3.0: n_{cre+} = 11, n_{cre-} = 10). (b) Compulsive behavior as measured in the marble burying test (ChR2: n_{cre+} = 13, n_{cre-} = 15; eNpHR3.0: n_{cre+} = 13, n_{cre-} = 11) and (c) depressive-like behavior as assessed by the tail suspension test (ChR2: n_{cre+} = 8, n_{cre-} = 9; eNpHR3.0: n_{cre+} = 13, n_{cre-} = 11) was also not affected by manipulation of pBNST CRFR2-neuronal activation. Error bars indicate SEM.
Supplementary Figure 9. Corticosterone responses to acute stress exposure combined with photoactivation of pBNST CRFR2-expressing neurons. Light-induced activation did not affect the corticosterone response when applied either (a) during or (c) 30 min after stressor onset. However, stimulation in (b) the immediate aftermath of stress exposure significantly reduced the incline in corticosterone levels ($n_{cre} = 5$, $n_{cre-} = 5$). Error bars indicate SEM. *: $P < 0.05$
Supplementary Figure 10. The PTSD-induction protocol caused a PTSD-like phenotype in part of the mice, whereas others were resilient. PTSD-like mice displayed hypervigilance as assessed by a shortened latency to peak startle ($T_{(13)} = 2.05, P = 0.031$), impaired risk assessment ($T_{(12)} = 1.88, P = 0.042$), a tendency towards increased compulsive behavior as measured in the marble burying test ($T_{(13)} = 1.15, P = 0.127$), and displayed heightened activity during the inactive (i.e., light) phase ($T_{(13)} = 2.88, P = 0.007$). The reduction in pre-pulse inhibition failed significance in this cohort ($T_{(13)} < 1$). Error bars indicate SEM. *: $P < 0.05$, **: $P < 0.01$, ~: $P = 0.1$.
Supplementary Figure 11. Corticosterone response to restraint stress in PTSD-like and Resilient animals. (a) PTSD-like animals tended to show an increased corticosterone stress response ($n_{\text{Resilient}} = 6$, $n_{\text{PTSD-like}} = 8$), which was (b) significantly correlated to their PTSD symptom score. Error bars indicate SEM. ‘‘ $P = 0.06$
Supplementary Figure 12. Locations of fiber optic cannula placement. At the end of all behavioral testing, accuracy of fiber optic cannula placement was checked, and only animals with the cannula place < 200 µm from the target site were included into analyses.
**SUPPLEMENTARY TABLES**

<table>
<thead>
<tr>
<th>Behavioral measure</th>
<th>Score</th>
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<td>% Risk assessment behavior</td>
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<tr>
<td>Latency to peak startle amplitude</td>
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<td>Total PPI disruption</td>
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<td>% Marbles buried</td>
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**Inclusion criterion**

- **PTSD-like**: Total score $\geq 4$
- **Resilient**: Total score $= 0$

**Supplementary Table 1.** Application of inclusion criteria to qualify as either PTSD-like or Resilient animal.

Scores assigned to each behavioral test – if the mouse was in the top 20% or bottom 20% the behavioral response depending on the test – were added up and animals with a PTSD-score $\geq 4$ were categorized as PTSD-like, whereas animals with a total score of zero qualified as Resilient animals.
SUPPLEMENTARY MATERIALS AND METHODS

Generation of CRFR2-cherry-2A-Cre recombinase BAC

Engineering of the CRFR2-cherry-2A-Cre recombinase (CRFR2-chy-Cre) BAC was performed essentially as described in Liu et al. A sequence containing a mCherry-f2A-Cre recombinase cassette along with an f3-PGK-EM7-neoR-f3 selection cassette was used as a template for PCR. Primers used in the PCR contained homologous sequences for targeting the cassette for insertion in the third exon of Crfr2, at the initiation of translation (i.e., ATG site) of the Crfr2a transcript (5’: GAGAGGGCAGAGGAAGTCTTCTAACATCGCGGTGACGT GGAGGAGATCCCGGCTATGGTGAGCAAG, and 3’: GGATTCTCTCCACGTCA CCGCATGTAGAGACTTCCCTCGCCCTCCTCCGTCCATCCCATCTTCCAGCAG GCGCC). The PCR products were purified and transformed into bacterial cells carrying a BAC containing the entire mouse Crfr2 genomic locus (RP24-351G5, Children’s Hospital Oakland, bacpac.chori.org), that had been induced to express recombination enzymes. Recombinants were identified based on kanamycin resistance and screened for homologous recombination by colony PCR. CRFR2-chy-f2a-Cre BAC DNA was purified (QIAGEN, Hilden, Germany) before being injected into single-celled mouse oocytes to generate transgenic mouse lines.

Animal maintenance

Animals were group housed (2-5 animals per cage) in a pathogen-free temperature-controlled (22 °C ± 1) mouse facility on a reverse 12/12 h light/dark cycle (lights on at 20:00 h) with ad libitum access to food and water, according to institutional guidelines.
**Characterization Cre-recombinase expression**

For visualization and characterization of Cre-recombinase expression heterozygote CRFR2-Cre transgenic males were crossbred with homozygote tdTomato conditional females (Ai9, B6.Cg-Gt(ROSA)26SoRtm9(CAG-tdTomato)Hze/J, Jackson Laboratory, Bar Harbor, Maine, USA). For visualization purposes, adult, male CRFR2-tdTomato^+^ offspring was transcardially perfused with ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were fixed overnight in 4% PFA and then equilibrated in 30% sucrose in PBS. The entire brain was cut into 50 μm-thick coronal sections on a freezing microtome and every third section was mounted on a microscope slide with Immu-Mount (Thermo Scientific Shandon, Fisher Scientific UK Ltd, Loughborough, UK).

**Generation of CRFR2^GABA-CKO^ transgenic mice**

For the generation of conditional knockout mice lacking CRHR2 in GABAergic forebrain neurons, the embryonic stem (ES) cell clone EPD0229_5_E04 was obtained from the EUCOMM/KOMP consortium. This ES cell clone harbors a modified Crfr2 allele following the standard EUCOMM/KOMP targeting strategy, i.e. a targeted knockout first, reporter-tagged insertion with conditional potential (Crfr2^tm1a(KOMP)Wtsi^). For the generation of chimeric mice ES cells were injected into BALB/c blastocysts. Germline transmission of the modified Crfr2 allele was achieved by breeding chimeras to Flpe-Deleter mice.\(^5\) Simultaneously the frt site flanked reporter selection cassette was removed resulting in a floxed Crfr2 allele (Crfr2^loxP; Crfr2^tm1c(KOMP)Wtsi^) in which exon 4 is flanked by loxP sites. Finally, mice lacking CRFR2 in GABAergic forebrain neurons (CRFR2^GABA-CKO^) were obtained by breeding with Dlx5/6-Cre mice.\(^6\) For the detection of conditional CRFR2 deletion (Figure 1b), *in situ* hybridization procedures were performed as previously described.\(^7,8\) Briefly, mice (10-week old) were sacrificed in the morning (10:00 h) with an overdose of isoflurane. Brains were
carefully removed and immediately snap-frozen on dry ice. Frozen brains were cut on a cryostat into 20 \( \mu m \)-thick coronal sections and mounted on Superfrost Plus slides (Thermo Scientific Shandon, Fisher Scientific UK Ltd, Loughborough, UK) and processed for in situ hybridization (ISH) as previously described.\(^7\) A riboprobe covering the \( \text{loxP} \) flanked exon 4 was used (nucleotides 400-595 of accession number NM_001288618). Hybridization was performed as described before.\(^8\) Dark-field photomicrographs were taken from autoradiographs with a Zeiss Axioplan2 microscope (ZEISS, Göttingen, Germany). Images were digitalized using Axio Vision 4.9 (ZEISS, Göttingen, Germany), and afterwards integrated into plates using image-editing software. Only sharpness, brightness and contrast were adjusted. For an adequate comparative analysis in the corresponding mutant and wild type sections the same adjustments were undertaken. Brains slices were digitally cut out and set on to an artificial white background.

**Double in situ hybridization**

Double ISH procedures were performed as previously described.\(^8\) CRFR2-tdTomato\(^+\) male mice (10-week old) were sacrificed in the early afternoon (14:00 h) by decapitation. Brains were carefully removed and immediately snap-frozen on dry ice. Frozen brains were cut on a cryostat into 20 \( \mu m \)-thick sections and mounted on SuperFrost Plus slides (Thermo Scientific Shandon, Fisher Scientific UK Ltd, Loughborough, UK). All sections were processed for ISH as described before.\(^8\) The following riboprobes were used: GAD65: nucleotides 753-1600 of GenBank accession no. NM_008078; GAD67: nucleotides 984-1940 of GenBank accession no. NM_008077; tdTomato: nucleotides 337-1026 of GenBank accession no. NM_205769. Specific DNA fragments coding for the riboprobes were generated by PCR applying T7 and T3 or SP6 primers using plasmids containing the above-mentioned cDNAs as templates. Antisense and sense cRNA probes were synthesized and labeled with \(^{35}\)S-UTP or dioxygenin
(DIG) by *in vitro* transcription with 200 ng of respective PCR product used as templates. For DIG detection anti-DIG-POD (Fab) antibody was used 1/400. Tiramide-biotin signal amplification (TSA) was performed using the NEL700A Kit (Perkin Elmer, Waltham, MA, USA) following the manufacturer’s instructions. Dipping, development, and acquisition of the autoradiograms were performed as described before.\(^8\)

Bright-field photomicrographs were captured with a Zeiss Axioplan2 microscope (ZEISS, Göttingen, Germany). Images were digitalized using Axio Vision 4.9 (ZEISS, Göttingen, Germany), and photomicrographs were integrated into plates using image-editing software. Only sharpness, brightness and contrast were adjusted. Brain slices were digitally cut out and set onto an artificial white background. tdTomato positive cells were counted on 39 photomicrographs taken with a 40X objective around Bregma -0.22 ± 0.11 and categorized as either GAD65/67 positive or negative. Absolute cell counts are reported.

**Slice immunohistochemistry**

For identification of the ligand of CRFR2-expressing neurons in the pBNST, anesthetized (35% chloral hydrate) adult, male CRFR2-tdTomato\(^+\) mice were transcardially perfused with ice-cold 4% PFA in PBS. Brains were fixed overnight in 4% PFA and then equilibrated in 30% sucrose in PBS. 30 μm-thick coronal sections were cut on a freezing microtome (Leica Microsystems GmbH, Wetzlar, Germany) and stored in PBS at 4ºC until processed for immunohistochemistry. Free-floating sections of the pBNST were next washed in PBS and then incubated for 60 min in PBS containing 0.3% Triton and 10% normal horse serum (NHS) to prevent non-specific binding. Primary antibody incubations were performed overnight at 4 °C in 0.3% triton/PBS (rabbit anti-CRF 1:1000, rabbit anti-Ucn1 1:5000, rabbit anti-Ucn3 1:5000, kindly provided by Wylie Vale, Salk Institute for Biological Studies, La Jolla, CA, USA); only one type of antibody per slice was used. Sections were then washed
and incubated with secondary antibodies (goat anti-rabbit; 1:200) conjugated to Cy2 (Chemicon-Millipore, Schwalbach, Germany) for 90 min at room temperature. Lastly, sections were washed in PBS and mounted on gelatin-coated microscope slides with Immumount (Thermo Scientific Shandon, Fisher Scientific UK Ltd, Loughborough, UK). Confocal fluorescence images were acquired on a Zeiss laser scanning microscope (LSM 700 Axio Examiner, ZEISS) using a 20X/1.0 water immersion objective and Zen Microscope and Imaging Software (ZEISS).

**Anterograde tracing**

Projection sites of pBNST CRFR2-expressing neurons were identified by unilateral injection of AAV5-EF1α-DIO-eYFP (UNC Vector Core, Chapel Hill, NC, USA) into the pBNST. Male CRFR2-Cre mice (2–3 months old) were first subcutaneously injected with analgesic (0.2 mg/kg buprenorphine) for pain relief, and half an hour later anaesthetized using 4.0% isoflurane, and placed on a computer-guided stereotaxic instrument (Angle Two Stereotaxic Instrument, myNeurolab, Leica Microsystems Inc., Bannockburn, IL, USA), which was fully integrated with the Franklin and Paxinos mouse brain atlas through a control panel. Anesthesia was reduced to 1.5-2.5% isoflurane, a midline incision was made across the top of the skull, the periosteum was cleaned, and the brain was leveled. The targeted injection site was 0.1 mm more ventral than for fiber-optic cannula placement to reach the center of the pBNST, on coordinates (relative to Bregma): AP -0.22, ML 1.60, DV -3.90. 0.3 µL of cold AAV5-EF1α-DIO-eYFP was injected using a Hamilton syringe connected to a motorized nanoinjector at a rate of 0.1 µL/min. To allow diffusion of the solution into the brain tissue, the needle was left for an additional 5 min after the injection, and then very slowly removed. The skin was stitched and animals were left undisturbed for 3 weeks for the virus to spread. Then, animals were anesthetized (35% chloral hydrate) and transcardially perfused with ice-
cold 4% PFA in PBS. Brains were fixed overnight in 4% PFA and then equilibrated in 30% sucrose in PBS. The entire brain (covering Bregma ~4.00:-6.00 mm) was cut into 50 μm-thick coronal sections on a freezing microtome (Leica Microsystems GmbH, Wetzlar, Germany), which were divided over three sets. Two sets were stored in PBS at 4°C and one set was used for immunohistochemistry. Free-floating sections were washed in PBS and then blocked for 60 min in PBS containing 0.3% Triton and 10% NHS. Primary antibody incubation was performed overnight at RT in 0.3% triton/PBS with goat anti-GFP biotinylated (1:200, Abcam, Cambridge, England). Sections were then washed and incubated with secondary antibodies (Cy2 streptavidin 1:200, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 90 min at RT, after which they were washed and mounted on gelatin-coated microscope slides with Immu-Mount (Thermo Scientific Shandon, Fisher Scientific UK Ltd, Loughborough, UK). The brain was scanned for projection sites (i.e., eYFP-expression) using a confocal microscope (LSM 700 Axio Examiner, ZEISS) with a 10X/0.3W water immersion objective and Zen Microscope and Imaging Software (ZEISS). Confocal fluorescence images of representative sections of projection sites were acquired using a 20X/1.0 water immersion objective.

**In vitro electrophysiological recording**

**Ligand infusion.** Adult (8-10 week old) male mice were anaesthetized with isoflurane (Abbott, Abbott Park, IL, USA) and decapitated. The brain was gently removed from the skull and chilled in ice-cold choline chloride-based cutting solution containing (in mM): 120 choline chloride, 3 KCl, 27 NaHCO3, 2 MgCl2, 17 D-glucose, pH 7.4, saturated with carbogen (95% O2/5% CO2). The brain was trimmed in a large block and afterwards sliced using a vibratome (HM650V, ThermoFisher Scientific, Waltham, MA). 300 μm-thick coronal slices were cut through the full extent of the BNST (anterior + posterior). Slices were incubated for 30 min at
34°C in artificial cerebrospinal fluid (aCSF). Subsequently, slices were maintained in aCSF at room temperature (23–25°C) for at least 60 min prior to patch-clamp recordings. All experiments were conducted at room temperature. Neurons of the posterior part of the BNST expressing CRFR2-tdTomato were identified by epifluorescence microscopy. Afterwards, the cell bodies of these neurons were visualized by infrared videomicroscopy and the gradient contrast system. The patch-clamp electrodes were pulled from borosilicate glass capillaries on a DMZ-Universal puller (Zeitz-Instruments, Munich, Germany). Somatic whole-cell patch-clamp recordings (seal resistance >1 GΩ) were performed in bridge mode using a discontinuous single-electrode voltage-clamp amplifier (SEC-10L, npi electronics, Tamm, Germany). Only measurements of the access resistance were done in voltage-clamp mode (holding potential -70 mV). The current/potential was low-pass filtered at 3 kHz, digitized at 9 kHz via an ITC-16 interface board, and stored with the standard software Pulse 8.31 (HEKA Elektronik, Lambrecht/Pfalz, Germany). Offline analysis was performed using the Pulse Software and statistical evaluation with SigmaStat 3.5.

Photostimulation. Mice were deeply anesthetized with sodium pentobarbital (130 mg/kg, IP) and perfused transcardially with ice-cold oxygenated sucrose solution (in mM: 234 sucrose, 11 glucose, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, 0.5 CaCl₂; 340 mOsm/kg). Brains were rapidly harvested and coronal slices (300 μm thick) were obtained using a vibratome (speed 0.1 mm/s, amplitude 1 mm, Leica VT 1200S). Slices were allowed to recover in high osmolarity artificial cerebrospinal fluid (aCSF; in mM: 3.24 KCl, 11.88 glucose, 132.8 NaCl, 28.1 NaHCO₃, 1.35 NaH₂PO₄, 1.08 MgCl₂, 2.16 CaCl₂; 320 mOsm/kg) at 32-34 °C for 20-25 min, then transferred to iso-osmotic aCSF (in mM: 3 KCl, 11 glucose, 123 NaCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂; 300 mOsm/kg) at 32-34 °C for an additional 30-40 min, and finally stored at room temperature in aCSF until use. All solutions were continuously oxygenated (95% O₂ / 5% CO₂).
Whole-cell patch-clamp recording and optogenetic stimulation. Whole-cell patch-clamp recordings were obtained under visual guidance using infrared differential interference contrast (DIC) microscopy (Olympus BX51WIF, Scientifica, Uckfield, UK) and an Andor Neo sCMOS camera (Andor, Belfast, UK). Recordings were carried out using a Multiclamp 700B amplifier (Axon Instruments / Molecular Devices, LLC, Sunnyvale, CA, USA).

For photocurrent measurement, the membrane potential was clamped to $-70$ mV and ten consecutive 0.5 s light pulses were delivered. The peak current during the first pulse was considered the peak photocurrent, whereas the last 100 ms of the last light pulse were considered as steady-state. For success rate calculation (Figure 3a), light pulse trains were delivered for ten consecutive 5 s periods. The success rate was calculated only for the last 5 s period, presumed to reflect the steady-state response of the cell to the stimulation regimen.

For firing rate calculations (Figure 4a), ten consecutive epochs of 3.5 s current injection with intermittent 0.5 s light stimulation were carried out. Firing rate with and without light was calculated over the entire time of current injection in all ten recordings.

Stereotactic surgery

Adult CRFR2-ChR2/eNpHR3.0 male mice (2–6 months old) were subcutaneously injected with analgesic (0.2 mg/kg buprenorphine) for pain relief. Half an hour later, the animals were anaesthetized using 4.0% isoflurane, and placed on a computer-guided stereotaxic instrument (Angle Two Stereotaxic Instrument, myNeurolab, Leica Microsystems Inc., Bannockburn, IL, USA), which was fully integrated with the Franklin and Paxinos mouse brain atlas through a control panel. Anesthesia was reduced to 1.5-2.5% isoflurane, a midline incision was made across the top of the skull, the periosteum was cleaned, and the brain was leveled. Injection sites were targeted using coordinates based on the Paxinos and Franklin mouse brain atlas,\textsuperscript{10} relative to Bregma (AP -0.22, ML ±1.60, DV -3.80),\textsuperscript{9} which defined the most dorsal part of
the pBNST (very close to the lateral ventricle) to allow light penetration to the entire region. To allow for bilateral fiber implantation, two fiber-optic cannulas (Doric lenses, DRC-MFC_200/260/900FLT, 200 µm thick, pre-cut at a length of 4 mm using a diamond blade) were inserted under an angle of ±10 degrees. Cannulas were secured using the C&B-Metabond kit (Parkell Inc., Edgewood, NY, USA) and Jet acrylic dental cement (Lang Dental Manufacturing Co., Wheeling, IL, USA). Mice were given one week of recovery from surgery, followed by one week (5 sessions) of habituation to fiber-optic cable attachment. During each habituation session, the mouse was held still to connect fiber-optic cables to the cannulas, and then was allowed to freely explore a new cage (with the cables attached) for a few minutes afterwards. By the end of the habituation protocol, this procedure resulted in a significant reduction in struggling during cable attachment and freezing behavior of the animals immediately afterwards.

After completion of all behavioral experiments, the surgically manipulated animals were overdosed with 35% chloral hydrate and perfused intracardially. The brains were removed and sectioned in the coronal plane at 50 µm thickness for histological examination to ensure proper optic fiber placement (Supplementary Figure 12). Only animals with the optic fibers inserted at a distance < 200 µm from the target site were included in the analyses.

**Opsin activation validation and immunohistochemistry**

To validate *in vivo* neuronal activation in response to photostimulation, seven adult, male CRFR2-ChR2+ mice were exposed to 15 min of unilateral *in vivo* light stimulation and then anesthetized (35% chloral hydrate) and transcardially perfused with ice-cold 4% PFA in PBS 90-100 min after termination of stimulation. Brains were fixed overnight in 4% PFA and then equilibrated in 30% sucrose in PBS. Before cutting 30 µm-thick coronal sections on a freezing microtome (Leica Microsystems GmbH, Wetzlar, Germany), brains were marked (i.e.,
horizontal cut with razor blade) at the side of stimulation, and cut and stored in PBS at 4°C until the sections containing the pBNST were processed for immunohistochemistry. Free-floating sections were washed in PBS and then blocked for 1 h with PBS containing 0.3% Triton and 10% NHS. Primary antibody incubations were performed overnight at RT with rabbit anti-c-Fos (1:1000, c-Fos sc-52, Santa Cruz Biotechnology, Dallas, TX, USA), and goat anti-GFP biotinylated (1:200, Abcam, Cambridge, England). Sections were then washed and incubated with secondary antibodies (goat anti-rabbit Alexa Fluor® 594, 1:200 (Life Technologies, Eugene, OR, USA), and Cy2 streptavidin 1:200 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 90 min at RT. Sections were washed and mounted on gelatin-coated microscope slides with Immu-Mount (Thermo Scientific Shandon, Fisher Scientific UK Ltd, Loughborough, UK). Confocal fluorescence images of representative sections of the pBNST (Bregma -0.22 ± 0.08), bilaterally, were acquired on a Zeiss laser scanning microscope (LSM 700 Axio Examiner, ZEISS) using a 20X/1.0 water immersion objective and Zen Microscope and Imaging Software (ZEISS). Image analysis was performed blind to the experimental condition. Both the total number of ChR2⁺ cells and the number of ChR2⁺ cells positive for c-Fos in the pBNST of both hemispheres were counted, and the proportion of c-Fos expression was calculated and tested for an effect of photostimulation.

**Behavioral assessment**

Group sizes for the behavioral assessment of the effects of pBNST CRFR2-neuronal activation were based on previous studies investigating the modulatory actions of specific neuronal subclasses on stress and anxiety using optogenetics,¹¹-¹⁴ as similar effect sizes were expected. Prior to behavioral testing, all mice were habituated in a dark room adjacent to the testing room for > 90 min prior to task onset.
Elevated Plus Maze. The test apparatus comprised a central part (5 x 5 cm), two opposing open arms (30.5 x 5 cm), and two opposing Plexiglas closed arms (30.5 x 5 x 15 cm). The apparatus was elevated at a height of 53.5 cm and the open arms were illuminated with 6-9 lux. Mice were placed in one of the closed arms facing the center to initiate a 15 min test session. The session consisted of a first 5 min time bin without light stimulation (i.e., light off), followed of a 5 min time bin with light stimulation (i.e., light on), and ended with a 5 min time bin without light stimulation (i.e., light off). Time spent in the open arms, distance traveled in the open arms, and number of visits to the open arms were quantified using a camera mounted above the apparatus and analyzed by TSE software VideoMot2 (TSE Systems GmbH, Bad Homburg, Germany) and Ethovision software (Noldus, Wageningen, Netherlands).

Open Field Test. The open-field apparatus consisted of a white Plexiglas box (50 x 50 x 40 cm) lightened with 120 lux. Each mouse was placed in the corner of the apparatus to initiate a 15 min test session. The session consisted of a first 5 min time bin without light stimulation (i.e., light off), followed of a 5 min time bin with light stimulation (i.e., light on), and ended with a 5 min time bin without light stimulation (i.e., light off). Time spent in the center (the inner 25 x 25 cm), distance traveled in the center, number of visits to the center, and total distance traveled were quantified using a camera mounted above the apparatus and analyzed by TSE software VideoMot2 (TSE Systems GmbH, Bad Homburg, Germany).

Marble Burying. Mice were placed in a compartment illuminated by 10 lux with dimensions (30 x 27 x 26 cm) containing 5 cm autoclaved bedding with 20 marbles centrally arranged 4 by 5. Mice were then filmed for 30 min. Videos were scored by counting the number of unburied marbles every 5 minutes until the end of the test by an experimenter blind to the experimental group of the animal.
**Tail Suspension.** The tail suspension test was performed in the TSE Tail Suspension Monitor (TSE Systems GmbH, Bad Homburg, Germany). Each mouse was taped by the tip of its tail to initiate a 6 min test session. Time spent immobile and time spent struggling were calculated and recorded by the software based on pre-set thresholds.

**Acoustic Startle and Pre-Pulse Inhibition.** The acoustic startle response (StartleResponse, TSE-systems, Bad Homburg, Germany) protocol was adapted from Neufeld-Cohen *et al.*\(^\text{15}\) Briefly, mice were placed in a small Plexiglas and wire mesh cage on top of a vibration-sensitive platform in a sound-attenuated, ventilated chamber. A high-precision sensor, integrated into the measuring platform, detected movement. Two high-frequency loudspeakers inside the chamber produced all of the audio stimuli. The acoustic startle response (ASR) session began with 5 min acclimation to white background noise (65 dB) maintained through the whole session. Twenty-four startle stimuli (120 dB, 40 ms in duration with a randomly varying ITI of 12–30 ms) were presented interspersed with an additional thirty-six startle stimuli randomly preceded by 20 ms prepulses of either 74 dB, 78 dB, or 82 dB. Maximal ASR and latency to peak startle amplitude were measured both in response to individually presented startle stimuli and in response to startle stimuli preceded by pre-pulses. Percentage pre-pulse inhibition (PPI) was calculated as the percentage difference between the maximal ASR to startle stimuli preceded by pre-pulses compared to that without averaged overall pre-pulse intensities.

**Foot shock Stress.** Foot shocks were given in a fear-conditioning apparatus (TSE Systems GmbH, Bad Homburg, Germany). Mice were placed in a transparent, Plexiglas cage (21 × 20 × 36 cm) with a metal grid floor and 10 lux illumination, where they received 5 inescapable foot shocks of 0.7 mA for 1 s.
Restraint Stress. In order to combine acute stress exposure with simultaneous photostimulation we referred to a restraint stress protocol. Mice were manually held in restraint for 15 min while receiving stimulation.

Re-exposure to Stress Context. Mice were placed back for a 5 min-session in the fear-conditioning apparatus (TSE) in which they received 5 inescapable foot shocks six weeks earlier. The context was identical to that of initial shock exposure (Plexiglas cage with a metal grid floor and 10 lux illumination), but no shocks were delivered upon re-exposure. To assess fear memory, freezing behavior (defined as periods of < 1% mobility) throughout the session was scored using Ethovision software (Noldus, Wageningen, Netherlands).

PTSD Induction and Evaluation. We used a previously validated mouse PTSD model⁹ (based on the rat model¹⁶,¹⁷) to induce a PTSD-like phenotype in susceptible animals. The model began on day 1, in context A, in which mice received 14 shocks of 1 mA, 1 s in duration with a continuous pulse over 85 min at variable intervals, representing the “trauma”. On day 2, the same mice received 5 pulsed shocks of 0.7 mA (20 Hz), 1 s in duration over 5 min in fixed intervals representing a “trigger” in context B. Shocks were given in a fear-conditioning apparatus (TSE Systems GmbH, Bad Homburg, Germany). Context A consisted of a transparent, Plexiglas cage (21 × 20 × 36 cm) with a metal grid floor and 10 lux illumination. Habituation on day 1 took place in a room adjacent to the experiment room with red light. Animals were transferred to the experiment room in darkness and in the experiment room lights were kept off. Between animals, the grid and cage were cleaned with 1% acetic acid solution. Context B consisted of a black, opaque cage (21 × 20 × 36 cm) with a metal grid floor, a black plastic tray under the metal grid, 70 dB background noise, and no illumination. Habituation on day 2 took place in a room slightly further away from the experiment room than habituation of context A. Mice were transferred to a brightly lit experiment room in small carton cages. Between animals, cages and the metal grid were cleaned with 10% ethanol solution.
solution. Mice were then tested in five behavioral tests: percentage risk assessment (on day 8), marble burying (day 10), latency to peak startle amplitude (day 12), pre-pulse inhibition (day 12), and total light activity (days 13-14).

**Percentage Risk Assessment.** Risk assessment was measured using the dark/light transfer test. The test apparatus consisted of a box divided by a partition into two environments: a dark covered compartment (15 x 20 x 25 cm) and a brightly illuminated (1000–1100 lux) light compartment (30 x 25 x 25 cm). The compartments were connected by a small passage in the bottom center of the partition. The mice were placed in the dark compartment to initiate a 5-minute test session. Time spent in the light zone, number of visits to the light zone and the latency on entering the light zone were quantified using a camera mounted above the apparatus and analyzed by TSE software VideoMot2 (TSE Systems GmbH, Bad Homburg, Germany). An additional arena of 3 cm in length by 6 cm in width was programmed into the software tracking measurements surrounding the opening of the light area. Time spent in the risk assessment area and the number of visits to the risk assessment area were measured. Percentage risk assessment time was calculated as the amount of time spent in the risk assessment arena as a percentage of total time spent in the light area outside of the risk assessment zone.

**Latency to Peak Startle Amplitude and Pre-Pulse Inhibition.** To assess the animals’ latency to peak startle and the amount of pre-pulse inhibition (PPI) the acoustic startle protocol was used as described above. The amount of PPI was defined as the percent difference between the maximal ASR to startle stimuli preceded by pre-pulses compared to that without averaged overall pre-pulse intensities.

**Marble Burying.** The marble burying protocol was executed as described above. Videos were scored by counting the number of unburied marbles after 25 min, as previously described. 18
**Homecage Locomotion.** Homecage locomotion was assessed using the InfraMot system (TSE Systems GmbH, Bad Homburg, Germany). Mice were housed individually for 72 h, in which the first 24 h were considered habituation to the individual housing conditions. Measurements of general locomotion consisted of two light and two dark cycles in the last 48 h collected at 10 min intervals.

**Evaluation of PTSD-like Behavior.** Mice were subcategorized as PTSD-like or Resilient based on their behavioral results in the aforementioned tests. Results on each behavioral test were sorted from highest to lowest and the 25% (6 mice) with the most extreme scores (i.e., lowest risk assessment, most marbles buried, shortest latency to peak startle amplitude, smallest PPI, and highest light activity) were attributed points. The amount of points attributed to each behavioral phenotype was based on a previously established factor analysis in which tests were clustered in three separate groups: (1) latency to peak startle amplitude and percentage risk assessment, (2) percentage PPI, and (3) marble burying and total light activity. As a result, 3 points were contributed to extreme latencies to peak startle amplitude and abnormal risk assessment, 2 points to extremely low PPI, and 1 point to high burying and total light activity. The points per animal were tallied. Mice that had totals of 4 or more points were termed PTSD-like, whereas only mice with zero points (no extreme behavior in any of the tests) were termed resilient (Supplementary Table 1).

**Corticosterone collection and measurement**

To monitor the corticosterone response to stress, plasma was extracted from blood samples that were collected by tail bleed before (basal, t = 0 min), immediately after stress (at t = 15 min), and 30 and 90 min after stress initiation. All blood samples were collected at least 4 h after beginning of the dark phase, when basal corticosterone levels are relatively stable. Blood samples were briefly stored on ice and then centrifuged (3500 rpm for 20 min at 4 °C).
Extracted plasma was stored at -80 °C until assayed for corticosterone using a Corticosterone Double Antibody RIA Kit (MP Biomedicals, Orangeburg, NY, USA).

**Brain tissue collection**

Approximately 4 weeks following PTSD-induction (i.e., following the assessment of a potential PTSD-like phenotype), animals were sacrificed by decapitation. The brain was removed immediately and placed in a steel brain matrix (1.0 mm, Coronal, Stoelting, Wood Dale, IL, USA) to acquire a 1.0 mm slice specifically covering the pBNST (Bregma -0.10:-1.10 mm). Slices were taken using standard razor blades and were snap-frozen on dry ice. The pBNST was punched out bilaterally using a 16 G microdissecting needle. Tissue was immediately stored at -80 °C until RNA extraction.

**RNA extraction and real-time PCR**

RNA extraction was performed using mirNeasy RNA extraction kit (QIAGEN, Hilden, Germany). RNA preparations were reverse transcribed to generate cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Waltham, MA, USA). The cDNA products were used as templates for real-time PCR analysis. Sense and antisense primers were selected to be located on different exons to avoid false-positive results caused by genomic DNA contamination. Next, expression of the immediate early genes (c-Fos, Arc, Npas, Egr1) was tested using a custom-designed TaqMan® Gene Expression Assay (Applied Biosystems, Life Technologies, Waltham, MA, USA). Real-time PCRs were performed on a 7500 Real-Time PCR system using fluorescent SYBR Green technology (Applied Biosystems, Life Technologies, Waltham, MA, USA). Reaction protocols had the following format: 10 min at 95°C for enzyme activation followed by 40 cycles of 15 s at 94 °C and 60 s at 60 °C. Melting curve analysis checked the specificity of the
amplification products. All reactions contained the same amount of cDNA, 10 μL of master mix, and 250 nm primers to a final volume of 20 μL. For the analysis of immediately early gene mRNA expression levels, Hprt was used as housekeeping gene, to which expression levels were normalized.
SUPPLEMENTARY REFERENCES


PTSD-like behavior is mediated by corticotropin-releasing factor receptor type 2 levels in the bed nucleus of the stria terminalis. *J Neurosci* 2012; **32**: 6906-6916.


