Serotonin engages an anxiety and fear-promoting circuit in the extended amygdala

Catherine A. Marcinkiewicz1,8, Christopher M. Mazzone1,2,4, Giuseppe D’Agostino3, Lindsay R. Halladay4, J. Andrew Hardaway1, Jeffrey F. Diberto1, Montserrat Navarro3, Nathan Burnham3, Claudia Cristiano3, Cayce E. Dorrier1, Gregory J. Tipton1, Charu Ramakrishnan6, Tamas Kozic7,8, Karl Deisseroth6, Todd E. Thiele1,5, Zoe A. McElligott1,9, Andrew Holmes4, Lora K. Heisler3 & Thomas L. Kash1,2,5,10

Serotonin (also known as 5-hydroxytryptamine (5-HT)) is a neurotransmitter that has an essential role in the regulation of emotion. However, the precise circuits have not yet been defined through which aversive states are orchestrated by 5-HT. Here we show that 5-HT from the dorsal raphe nucleus (5-HT\textsuperscript{DRN}) enhances fear and anxiety and activates a subpopulation of corticotropin-releasing factor (CRF) neurons in the bed nucleus of the stria terminalis (CRF\textsuperscript{BNST}) in mice. Specifically, 5-HT\textsuperscript{DRN} projections to the BNST, via actions at 5-HT\textsubscript{2C} receptors (5-HT\textsubscript{2C Rs}), engage a CRF\textsuperscript{BNST} inhibitory microcircuit that silences anxiolytic BNST outputs to the ventral tegmental area and lateral hypothalamus. Furthermore, we demonstrate that this CRF\textsuperscript{BNST} inhibitory circuit underlies aversive behaviour following acute exposure to selective serotonin reuptake inhibitors (SSRIs). This early aversive effect is mediated via the corticotropin-releasing factor type 1 receptor (CRF\textsubscript{R1}, also known as CRHR1), given that CRF\textsubscript{R} antagonism is sufficient to prevent acute SSRI-induced enhancements in aversive learning. These results reveal an essential 5-HT\textsuperscript{DRN}→CRF\textsuperscript{BNST} circuit governing fear and anxiety, and provide a potential mechanistic explanation for the clinical observation of early adverse events to SSRI treatment in some patients with anxiety disorders\textsuperscript{1–3}.

Give the multiple converging lines of evidence pinpointing 5-HT as a crucial neuromodulator of pathological fear learning\textsuperscript{4–7}, we first interrogated the endogenous recruitment of the 5-HT\textsuperscript{DRN}→CRF\textsuperscript{BNST} circuit by an aversive footshock stimulus in mice. Using Fluro-Gold to retrogradely label BNST-projecting 5-HT neurons in the dorsal raphe nucleus (DRN), we found that c-fos, an immediate-early gene indicative of in vivo neuronal activation, was significantly elevated in 5-HT\textsuperscript{DRN}→BNST neurons after footshock (Fig. 1a–f). Using in vivo electrophysiology, we then probed the neuronal dynamics of the BNST during fear conditioning and recall, and found evidence for engagement during both conditioning and recall (Extended Data Fig. 1).

To decipher the role of this 5-HT\textsuperscript{DRN}→BNST circuit in aversive behaviour, Channelrhodopsin2 (ChR2)–eYFP was selectively expressed in 5-HT\textsuperscript{DRN} neurons through the delivery of a Cre-inducible viral vector in mice expressing Cre recombinase under the control of a serotonin transporter promoter (Sert\textsuperscript{Cre} (Sert is also known as Slc6a4) for both in vivo and ex vivo analysis. We observed eYFP\textsuperscript{+} (5-HT) cell bodies in the DRN and eYFP\textsuperscript{+} fibres in both the dorsal and ventral aspects of the BNST (Sert\textsuperscript{Cre}::ChR2\textsuperscript{DRN}–eYFP), confirming a direct projection of 5-HT neurons originating in the DRN to the BNST (Fig. 1g, h). Optical stimulation of these fibres in BNST slices evoked 5-HT release, as measured by fast-scan cyclic voltammetry (FSCV) (Fig. 1i, j). Furthermore, bath application of the SSRI fluoxetine reliably decreased the rate of 5-HT reuptake, confirming that photostimulation of SERT\textsuperscript{+} terminals in the BNST originating from the DRN induces 5-HT release (Fig. 1k, l).

We examined whether this 5-HT\textsuperscript{DRN}→BNST circuit is functionally relevant for fear and anxiety-like behaviour. To investigate this, Sert\textsuperscript{Cre}::ChR2\textsuperscript{DRN}→BNST mice were implanted with bilateral optical fibres and photostimulated in the BNST (473 nm, 20 Hz) using a standard tone-shock fear conditioning paradigm. Optogenetic stimulation of this pathway was paired with a tone that co-terminated with a scrambled footshock. Cued fear was assessed 24 h after, and contextual fear 48 h after, the initial fear acquisition session (Fig. 1m, n). Although no changes were observed during fear acquisition, both cued and contextual fear recall were significantly heightened in photostimulated Sert\textsuperscript{Cre}::ChR2\textsuperscript{DRN}→BNST mice (Fig. 1o–q). We next assessed anxiety-like behaviour using well-characterized assays: the elevated plus maze (EPM) and novelty-suppressed feeding (NSF) tests. Upon stimulation with light, Sert\textsuperscript{Cre}::ChR2\textsuperscript{DRN}→BNST mice exhibited enhanced anxiety-like behaviour in both the EPM and NSF tests (Fig. 1r, s and Extended Data Fig. 2a, b). Importantly, photostimulation did not induce hypolocomotion in the EPM or open field tests, nor did it alter home-cage feeding, thus confirming that hypophagia in the NSF assay was due to anxiety and not a reduction in appetitive drive (Extended Data Fig. 2c–e). One potential explanation of these results is that terminal stimulation in the BNST produces antidromic spikes in DRN cell bodies that release 5-HT in other brain regions, which could be also be driving these behaviours. Therefore we probing the mechanism more deeply using converging approaches.

To determine a receptor target through which 5-HT is signalling in the BNST, we then examined the impact of optogenetically evoked 5-HT\textsuperscript{DRN} release on postsynaptic neuronal excitability and found a 3.05 ± 0.59 mV depolarization that was blocked by a 5-HT\textsubscript{2C} antagonist (Fig. 1t, u). In contrast to previous reports demonstrating co-release of 5-HT and glutamate from DRN projections to the nucleus accumbens\textsuperscript{6}, we did not observe any time-locked light-evoked EPSCs in the BNST (data not shown). These results indicate that 5-HT\textsuperscript{DRN}→BNST projections have a predominantly excitatory effect that is dependent on 5-HT\textsubscript{2C}R signalling. To examine the role of 5-HT\textsubscript{2C}R containing neurons in anxiety-like behaviour, we took advantage of a Htr2\textsubscript{C}\textsuperscript{Cre} mouse line (Extended Data Fig. 3a, b).\textsuperscript{7} Using designer receptors exclusively activated by designer drugs (DREADDs) that are coupled to the G\textsubscript{q} signalling pathway (hM3Dq-DREADD\textsuperscript{8}), we found that activation of G\textsubscript{q} signalling in 5-HT\textsubscript{2C}R-expressing neurons in the BNST significantly delayed the onset of feeding in the NSF assay without affecting home cage feeding behaviour (Extended Data Fig. 3c–g), thus phenocopying the effect observed.

\textsuperscript{1}Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA. \textsuperscript{2}Curriculum in Neurobiology, School of Medicine, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, North Carolina 27599, USA. \textsuperscript{3}Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen AB25 2ZD, UK. \textsuperscript{4}National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland 20852-9411, USA. \textsuperscript{5}Department of Psychology & Neuroscience, College of Arts and Sciences, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA. \textsuperscript{6}Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA. \textsuperscript{7}These authors contributed equally to this work.
with 5-HT<sup>DRN→BNST</sup> fibre stimulation during NSF. Together, these results provide converging evidence that activation of 5-HT<sup>DRN→BNST</sup> inputs elicits anxiety-like behaviour via 5-HT<sub>2CR</sub> signalling.

We considered the neurochemical phenotype of these target 5-HT<sup>DRN→BNST</sup> neurons and hypothesized that 5-HT via 5-HT<sub>2CR</sub> modulates the activity of neurons expressing the neuropeptide...
CRF. This hypothesis was based on a previous analysis of 5-HT_{2C}R knockout mice, which exhibit an anxiolytic phenotype associated with a reduction of c-fos in CRF\textsuperscript{BNST} neurons\textsuperscript{9}. Initially, using CRF reporter mice to \textit{a priori} select CRF neurons for recordings, we found a heterogeneous 5-HT-induced response in CRF\textsuperscript{BNST} neurons (Extended Data Fig. 4a), with only a subset demonstrating a depolarization. Consistent with this, double fluorescence \textit{in situ} hybridization revealed that only a subset of CRF neurons within the dorsal BNST (~70%) and ventral BNST (~43%) express 5-HT\textsubscript{2}Rs (Extended Data Fig. 4b–d).

Although CRF signalling within the BNST is associated with anxiety-like behaviour\textsuperscript{10,11}, more recent studies using circuit-based tools have found that optogenetic stimulation of GABAergic projections (which include CRF\textsuperscript{BNST} neurons) to the ventral tegmental area (VTA) are anxiolytic\textsuperscript{12}. This led us to hypothesize the existence of functionally distinct subsets of CRF\textsuperscript{BNST} neurons that gate different behaviours and are differentially sensitive to 5-HT. We used fluorescent retrograde tracer beads to label CRF\textsuperscript{BNST} neurons as VTA-projecting or non-VTA-projecting (Fig. 2a), and found that VTA-projecting CRF neurons (CRF\textsuperscript{BNST}$\rightarrow$VTA neurons) were hyperpolarized by an average of 5.73 ± 1.24 mV and non-VTA-projecting CRF neurons were depolarized by an average of 2.74 ± 0.39 mV during 5-HT bath application. Moreover, the excitatory response to 5-HT in non-VTA-projecting CRF neurons was reversed in the presence of a 5-HT\textsubscript{2C} receptor antagonist (Fig. 2b). Furthermore, all CRF\textsuperscript{BNST}$\rightarrow$VTA neurons were non-responsive to the 5-HT\textsubscript{2}R agonist meta-chlorophenylpiperazine (mCPP), whereas all non-VTA projecting CRF neurons were depolarized by mCPP by an average of 3.26 ± 0.74 mV (Extended Data Fig. 4e–h). These findings suggest an anatomically distinct response to 5-HT by different subsets of CRF\textsuperscript{BNST} neurons. The subset of CRF\textsuperscript{BNST} neurons expressing 5-HT\textsubscript{2C}R do not project to the VTA and are depolarized by 5-HT, whereas the CRF\textsuperscript{BNST}$\rightarrow$VTA neurons are hyperpolarized by 5-HT, via actions at another 5-HT receptor.

To determine if this 5-HT-dependent mechanism extended to other anxiolytic efferents, we injected retrograde tracer beads into the lateral hypothalamus (LH) of CRF reporter mice and found 5-HT had similar bidirectional effects on non-LH-projecting and LH-projecting CRF\textsuperscript{BNST} neurons (Extended Data Fig. 5a–c). Noting the functional similarities between these two populations, we used retrograde tracing to determine that roughly ~58% of CRF\textsuperscript{BNST} neurons have projections to the LH or VTA (Extended Data Fig. 5d–f). Notably, ~20–31% of these CRF\textsuperscript{BNST} output neurons form parallel projections to these structures.

In light of recent reports that CRF\textsuperscript{BNST} neurons are exclusively GABAergic\textsuperscript{13}, we hypothesized that non-VTA-projecting CRF\textsuperscript{BNST} neurons may locally inhibit BNST$\rightarrow$VTA neurons to promote fear and anxiety. To test this hypothesis, we injected Cre\textsuperscript{Cre} mice with a Cre-inducible ChR2 into the BNST and retrograde tracer beads into the VTA. We then recorded light-evoked inhibitory postsynaptic potentials (IPSCs) from non-ChR2 (ChR2-negative, retrograde tracer-positive) VTA-projecting BNST neurons (Fig. 2c). Photostimulation produced action potentials in CRF\textsuperscript{BNST} neurons and light-evoked IPSCs in non-ChR2 VTA-projecting neurons, indicating that CRF\textsuperscript{BNST} neurons form local GABAergic synapses with BNST neurons that project to the VTA. Repeating these same experiments in Cre\textsuperscript{Cre}:ChR2\textsuperscript{BNST} mice with retrograde tracer beads in the LH, we found that we could evoke GABA currents using photostimulation in LH-projecting neurons as well (Extended Data Fig. 5g–i). Moreover, we observed that 5-HT increased GABAergic transmission to non-BNST$\rightarrow$VTA projecting neurons in a tetrodotoxin and 5-HT\textsubscript{2C}R antagonist dependent manner (Fig. 2d–f and Extended

Figure 2 | Serotonin activates a local population of CRF\textsuperscript{BNST} neurons that inhibits outputs to the midbrain. a, Recording scheme for CRF reporter mice injected with retrograde tracer beads in the VTA. b, 5-HT depolarizes local CRF neurons (t = 7.06, P < 0.001, one-sample t-test, n = 6 cells from 4 mice) in the BNST while hyperpolarizing CRF\textsuperscript{BNST}$\rightarrow$VTA neurons (t = 4.64, P < 0.01, one-sample t-test, n = 7 cells from 6 mice). Non-VTA-projecting CRF neurons are hyperpolarized by 5-HT in the presence of the 5-HT\textsubscript{2C}R antagonist RS-102221 (t = 4.74, P < 0.01, one-sample t-test, n = 5 cells from 3 mice). c, Top and middle, schematic depicting infusions and recording configuration for Cre\textsuperscript{Cre}:ChR2\textsuperscript{BNST} mice injected with retrograde tracer beads in the VTA. Bottom, representative trace of light-evoked IPSC in beaded (that is, VTA projecting), non-ChR2 expressing neurons in the BNST of Cre\textsuperscript{Cre}:ChR2 mice with retrograde tracer beads in the VTA (n = 8 cells from 3 mice) and blockade of this response by GABA\textsuperscript{Zine} (F (1,33) = 53.16, P < 0.001, repeated measures one-way ANOVA, n = 4 cells from 3 mice). d, Recording scheme for C57BL/6 mice with retrograde tracer beads in the VTA or LH. e, Representative traces of sIPSCs in BNST neurons that project to the VTA before and after 5-HT application for 5 cells from 4 mice. f, Bar graphs showing magnitude of 5-HT effect on average sIPSC frequency in BNST neurons that project to the VTA (t = 3.257, P < 0.05, one-sample t-test, n = 5 cells from 4 mice) and in BNST neurons that project to the LH (t = 3.027, P < 0.05, one-sample t-test, n = 6 cells from 3 mice) and blockade of these responses by tetrodotoxin (TTX) and RS-102221. Effects on amplitude were non-significant. g, Experimental scheme for experiments with Cre\textsuperscript{Cre}:Intresetct–ChR2\textsuperscript{BNST} mice. h, i, 5-HT significantly depolarizes non-projecting CRF (Intresetct) neurons in the BNST (t = 2.501, P < 0.05, one-sample t-test, n = 7 cells from 5 mice) and produces a significant change in membrane potential in CRF Intresetct neurons compared to all CRF neurons (t = 2.08, P < 0.05, Student’s unpaired two-tailed t-test, n = 21 cells from 14 mice for experiments in all CRF neurons and n = 7 cells from 5 mice for Cre\textsuperscript{Cre}:Intresetct–ChR2\textsuperscript{BNST} experiments). Data are mean ± s.e.m. * P < 0.05; ** P < 0.01; *** P < 0.001. § denotes P < 0.05 for the Student’s unpaired two-tailed t-test between all CRF neurons and CRF Intresetct neurons in h.
Data Fig. 5j–n). Similar effects of 5-HT on GABAergic transmission were found in BNST → LH projecting neurons (Extended Data Fig. 5o–v). Furthermore, slice recordings in a CRF reporter line indicates that 5-HT does not increase GABAergic transmission on to the general population of CRFBNST neurons nor does it directly excite non-CRF VTA projecting neurons (Extended Data Fig. 6). The 5-HT3R agonist mCPP also increased GABAergic but not glutamatergic transmission in the BNST (Extended Data Fig. 7). Finally, to test if optically evoked 5-HT can inhibit BNST outputs to the VTA, we performed slice recordings in the BNST of SerfCre::Chr2-eYFP viral construct in the BNST of mice and found that brief photostimulation of 5-HT terminals in the BNST increased spontaneous IPSCs (sIPSCs) on to VTA projecting neurons in a manner similar to bath-applied 5-HT (Extended Data Fig. 8a–c). Together, these experiments indicate that CRFBNST neurons inhibit at least two major BNST outputs to the VTA and LH that are reported to be anxiolytic providing mechanistic insight into the aversive actions of 5-HT signalling in the BNST.

We took advantage of a new combinatorial strategy called INTRonic Recombinase Sites Enabling Combinatorial Targeting or INTRSELECT that allows for direct visualization of these non-projecting, putatively local CRFBNST neurons in the BNST. By coupling retrograde Cre-dependent fliprases (HSV-LSL1-mCherry-RES-flopo) in the VTA and LH with a (Creon/flpoff)-Chr2-eYFP viral construct in the BNST of Cfx mice (Cfx::Intrsect-Chr2BNST mice), we were able to genetically isolate non-VTA/LH-projecting CRF neurons in the BNST. We also infused a Cre-dependent HSV-mCherry vector in a subset of Cfx::Intrsect-Chr2BNST mice as a control. In HSV-flpo infused Cfx::Intrsect-Chr2BNST mice, we observed a significant reduction in YFP+ cells in the ventral BNST (Extended Data Fig. 8d–f), indicating that a large proportion of VTA-projecting and LH-projecting CRFBNST neurons are located in the ventral BNST. We also found that 5-HT robustly depolarized these Cfx::Intrsect-Chr2BNST neurons compared to CRF neurons in general (Fig. 2g–i). Furthermore, we observed light-evoked IPSCs in the BNST of Cfx::Intrsect-Chr2BNST mice, confirming local GABA release from these neurons (Extended Data Fig. 8g). These results support the existence of a separate population of local CRFBNST neurons that is excited by 5-HT and increases local GABAergic transmission in the BNST, distinct from a population of CRFBNST neurons that project to and release GABA in the VTA or the LH (Extended Data Fig. 8h–j).

To probe the translational relevance of these BNST microcircuits, we adopted a pharmacological approach using SSRIs. SSRIs represent one of the most widely used classes of drugs for psychiatric disorders. One limitation of SSRIs is that acute administration can lead to negative behavioural states, a finding that is recapitulated in rodent models. Importantly, the BNST has been demonstrated to be an anatomical site of action for some of the aversive actions of SSRIs in rodents. This provided the opportunity to test our model that 5-HT

Figure 3 | Acute fluoxetine elicits aversive behaviour by engaging inhibitory CRF circuits in the BNST. a, Schematic of recording for in vivo fluoxetine experiments in CRF reporter mice. b, Representative traces of sIPSCs in VTA-projecting neurons in the BNST for 5 experiments in 2 saline-treated mice and 7 experiments in 2 fluoxetine-treated mice. c, d, Bar graphs showing that fluoxetine increases in sIPSC frequency (t0 = 2.55, P < 0.05, Student’s unpaired two-tailed t-test, n = 5 cells from 2 saline-treated mice, n = 7 cells from 2 fluoxetine-treated mice), but not amplitude (t0 = 0.4752, P > 0.05, Student’s unpaired two-tailed t-test, n = 5 cells from 2 saline mice, n = 7 cells from 2 fluoxetine mice) in VTA-projecting neurons in the BNST. e, Experimental configuration for assessment of anxiety in fluoxetine-treated Cfx::hM4DiBNST (Gi-coupled DREADD) mice and a coronal slice of the BNST expressing hM4Di-mCherry. Scale bar, 100 μm. f, Confocal microscopy in the BNST showing hyperpolarization of hM4Di-mCherry-expressing cells following bath application of CNO (t0 = 4.32, P < 0.01, one-sample t-test, n = 6 cells from 4 mice). g, h, Chemogenetic silencing of CRF neurons attenuates fluoxetine-induced anxiety-like behaviour on the elevated zero maze (F1,30 = 7.086, P < 0.05, two-way ANOVA, n = 10 fluoxetine and hM4Di and n = 8 for all other groups) without any concomitant locomotor effects. i, Experimental configuration for fear conditioning experiments in Cfx::hM4DiBNST mice. j, k, Chemogenetic silencing of CRFBNST neurons had no effect on freezing behaviour during fear learning but prevented fluoxetine enhancement of cued fear recall (F1,17 = 8.73, P < 0.01, two-way ANOVA, n = 6 mCherry and vehicle and n = 5 per group for all other groups). l, Experimental configuration for assessment of the role of BNST outputs to the VTA and LH in fluoxetine-induced aversive behaviour. m, Confocal image of the BNST from Cfx::hM3DqBNST mice. Scale bars, 500 μm. n, o, Chemogenetic activation of BNST neurons that project to the midbrain did not impact fear acquisition but attenuated fluoxetine-induced enhancement of cued fear recall (F1,17 = 7.541, P < 0.05, two-way ANOVA, n = 7 vehicle/hM3D and n = 8 for all other groups). Data are mean ± s.e.m.*P < 0.05; **P < 0.01.

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in the BNST drives aversive behaviour through inhibition of BNST outputs to the VTA. We observed that an acute systemic injection of the SSRI fluoxetine increased GABAergic transmission on to VTA projecting neurons in the BNST (Fig. 3a–d). We then interrogated the role of CRFBNST neurons in acute fluoxetine-enhanced anxiety using Cre+ mice transduced in the BNST using a Cre-inducible DREADD coupled to the Gqi signalling pathway (hM4Di–DREADD). We found that acute fluoxetine potentiated anxiety-like behaviour, and this effect was blocked by chemogenetic inhibition of CRFBNST neurons (Fig. 3e–i).

To evaluate directly whether endogenous 5-HT acts on CRFBNST neurons to enhance cued fear memory, we used the same chemogenetic approach to silence CRFBNST neurons during fluoxetine treatment and subsequent fear conditioning (Fig. 3i). Chemogenetic inhibition of CRFBNST neurons also significantly attenuated fluoxetine-induced enhancement of cued fear recall, providing proof of concept that augmentation of 5-HT via acute SSRI treatment recruits CRFBNST neurons to enhance fear-related behaviour (Fig. 3j, k). Using connectivity based chemogenetic approaches, we then tested whether inhibition of BNST outputs to the VTA and LH is a critical component of 5-HT→BNST-induced aversive states. We observed that activation of Gq signalling in VTA-projecting and LH-projecting BNST neurons, targeted by HSV-Cre–eYFP infused in the VTA and LH, potentially be involved in the early adverse events seen in clinical populations in acute SSRI-induced aversive behaviours in rodents, and could exacerbate symptoms, resulting in poor therapeutic compliance. Our results suggest that targeting this circuit may improve adverse symptoms during the initial weeks of SSRI treatment. Based on the critical role for CRFBNST neurons in fluoxetine-induced aversive behaviour, we examined the effect of a systemic CRF-R antagonist on SSRI enhancement of cued fear recall. Blocking the CRF system reduced this aversive state and abolished the increase in sPSCs in LH-projecting neurons in the BNST during bath application of 5-HT (Extended Data Fig. 9). This provides preclinical evidence that CRF-R antagonists given in concert with SSRIs could be a promising treatment for anxiety disorders.

Together, these data reveal a discrete 5-HT responsive circuit in the BNST that underlies pathological anxiety and fear associated with a hyperserotonergic state (Extended Data Fig. 10). SSRIs are currently a first-line treatment for anxiety and panic disorders, but can acutely exacerbate symptoms, resulting in poor therapeutic compliance. Our results strongly implicate 5-HT engagement of a local BNST-inhibitory microcircuit in acute SSRI-induced aversive behaviours in rodents, and could potentially be involved in the early adverse events seen in clinical populations, emphasizing the need to identify compounds that selectively target both genetically defined and pathway-specific cell populations.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 27 February 2015; accepted 20 July 2016.
Published online 24 August 2016.

Acknowledgements We acknowledge B. Roth for providing DREADD viral constructs and Ser1128:mCh mice, and B. Lowell for providing Cre+ mice. We also thank A. Lopez, D. Perron, and A. Kendra for technical assistance with stereotactic surgeries on mice. B. Geen for technical assistance with immunohistochemistry and E. Dankoski for technical assistance with the FSCV. This work was supported by NIH grants AA019454, AA01605 (T.L.K.), the Wellcome Trust (098012) and the Biotechnology and Biological Sciences Research Council grant (BB/K001418/1) (L.K.H.) and by NIH grant KO1AA023555 and the Alcohol Beverage Medical Research Fund (Z.A.M.). C.A.M. was supported by a postdoctoral NIAAA F32 (AA021319-02). C.M.M. is supported by a predoctoral NIAAA F31 fellowship (F31AA023440).

Author Contributions C.A.M., C.M.M., G.D., Z.A.M., L.K.H. and T.L.K. designed the experiments. A.H. and J.F.D. performed multiple feature chemogenetic manipulations in CRFBNST neurons that were used in fluoxetine fear conditioning experiments and C.A.M. performed behavioural and data analysis. C.E.D. performed surgical procedures and C.A.M. performed electrophysiological recordings and data analysis for fear conditioning experiments. M.N. and J.F.D. performed surgeries for chemogenetic manipulations in CRFBNST neurons that were used in fluoxetine fear conditioning experiments and N.B. and C.A.M. performed slice FSCV experiments and C.A.M. performed evoked 5-HT electrophysiology experiments. C.A.M. performed stereotactic surgeries, behavioural and data analysis for 5-HT7R–hM3D optogenetic experiments. C.A.M. performed all slice electrophysiology experiments and C.M.M. and C.A.M. performed all behavioral and data analysis. C.E.D. performed electrophysiological recordings and data analysis for fear conditioning experiments. M.N. and J.F.D. performed surgeries for chemogenetic manipulations in CRFBNST neurons that were used in fluoxetine fear conditioning experiments and N.B. and C.A.M. performed behavioural and data analysis. M.N. and J.F.D. performed surgeries for stereotactic manipulations in CRFBNST neurons that were used in fluoxetine fear conditioning experiments and N.B. and C.A.M. performed behavioural and data analysis. C.E.D. performed electrophysiological recordings and data analysis for fear conditioning experiments. M.N. and J.F.D. performed surgeries for chemogenetic manipulations in CRFBNST neurons that were used in fluoxetine fear conditioning experiments and N.B. and C.A.M. performed behavioural and data analysis. C.E.D. performed electrophysiological recordings and data analysis for fear conditioning experiments. M.N. and J.F.D. performed surgeries for chemogenetic manipulations in CRFBNST neurons that were used in fluoxetine fear conditioning experiments and N.B. and C.A.M. performed behavioural and data analysis. C.E.D. performed electrophysiological recordings and data analysis for fear conditioning experiments. M.N. and J.F.D. performed surgeries for chemogenetic manipulations in CRFBNST neurons that were used in fluoxetine fear conditioning experiments and N.B. and C.A.M. performed behavioural and data analysis. C.E.D. performed electrophysiological recordings and data analysis for fear conditioning experiments. M.N. and J.F.D. performed surgeries for chemogenetic manipulations in CRFBNST neurons that were used in fluoxetine fear conditioning experiments and N.B. and C.A.M. performed behavioural and data analysis. C.E.D. performed electrophysiological recordings and data analysis for fear conditioning experiments. M.N. and J.F.D. performed surgeries for chemogenetic manipulations in CRFBNST neurons that were used in fluoxetine fear conditioning experiments and N.B. and C.A.M. performed behavioural and data analysis.

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Reviewer Information Nature thanks A. Sahay and the other anonymous reviewer(s) for their contribution to the peer review of this work.
METHODS

Data reporting. Based on power analyses that assumed a normal distribution, a 20% change in mean and 15% variation, we determined that at least 9 mice per group would be needed for behavioral experiments. This was adhered to as far as possible, except in cases where mice had to be removed owing to misplaced injections or lost headcaps. Mice were randomly assigned to groups and attempts were made to balance groups according to variables such as age and housing condition. The investigators were not blinded to allocation during experiments, but were blinded to assessment for behavioral ex-periments.

Mice. Mice were used in all experiments. For experiments involving Cre lines, mice were crossed for several generations to C57 mice before using. All wild-type mice were C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbour, ME). For all behavioural experiments except those involving Htr2cCre mice, male mice ranging in age from 8–16 weeks were used. Female Htr2cCre mice were used in chemogenetic manipulations. Both male and female mice aged 6–20 weeks were used for slice electrophysiology and anatomical tracing experiments. All behav-ioral studies or tissue collection for ex vivo slice electrophysiology were performed during the light cycle.

All behavioural experiments in Htr2cCre mice were conducted at the University of Aberdeen and in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. All in vivo electrophysiology experiments were conducted in accordance with all rules and regulations at the National Institute for Alcohol Abuse and Alcoholism at the National Institutes of Health. All other procedures were conducted in accordance with the National Institutes of Health guidelines for animal research and with the approval of the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

All animals were group housed on a 12 h light cycle (lights on at 7 a.m.) with ad libitum access to rodent chow and water, unless otherwise described. Cre-ires-Cre (Creff) were provided by Dr. Brian Roth (Harvard University) and were previously described. C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbour, ME). To visualize CRF-expressing neurons, Creff and cre mice were crossed with either an Ai9 or a Cre-inducible L10-GFP reporter line (Jackson Laboratory)22 to produce Creff-Ai9 or CRF-L10GFP progeny, referred to throughout the manu-script as Cre reporters. Serff mice (from GENSAT) were a generous gift from Bryan Roth. Htr2cCre mice were supplied by Lora Heisler and are described in detail elsewhere.

Male mice were used for in vivo optogenetic behavioural experiments and for assessing the involvement of BNST CRF neurons on fluoxetine-induced enhance-ment of fear. Female 5-HT2C-Cre mice were used in chemogenetic manipulations. Both male and female mice were used for slice electrophysiology and anatomical tracing experiments. All behavioural studies or tissue collection for ex vivo slice electrophysiology were performed during the light cycle.

Viruses and tracers. All AAV viruses except INTRSECT constructs were produced by the Gene Therapy Center Vector Core at the University of North Carolina at Chapel Hill and had titres of >1012 genome copies per ml. For ex vivo and in vivo optical experiments, mice were injected with rAAV5-e11–DIO–hChR2(H134R)– eYFP or rAAV5-e11–DIO–eYFP as a control. Red IX retrobeads (Lumafluor) were used to fluorescently label LH- and VTA-projecting BNST neurons during the in vivo slice electrophysiology recordings. The retrograde tracer Fluoro-Gold (Fluorochrome) was used for anatomical mapping. Chelatoroxin B (CTB) 555 and CTB 657 retrograde tracers (Invitrogen; C34776, and C34777, respectively) diluted to 0.5% (w/v) in sterile PBS were used per injection site for anatomical mapping of collateral projections from BNST to LH and VTA. For chemogenetic manipulations, mice were injected with 400 nl of rAAV8-hsyn-DIO-hM3D(Gq)-mCherry, rAAV8-hsyn-DIO-hM4D(Gi)-mCherry, or rAAV8-hsyn-DIO-mCherry bilater-ally. Htr2ccre-Crfcre (supplied by Rachel Neve at the McGovern Institute for Brain Research at MIT) were back-crossed for several generations to C57 mice before using. All wild-type mice were C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbour, ME) and had titres of 1.7 × 1012 genome copies per ml. The INTRSECT construct AAVdj-hSyn-Con/Foff-hChR2(H134R)-EYFP was produced by the Gene Therapy Center Vector Core at the University of North Carolina at Chapel Hill and had titres of 1.012 genome copies per ml.

Surgical procedures. In vivo electrophysiological procedures. All behavioural studies or tissue collection for ex vivo slice electrophysiology were performed during the light cycle.

In vivo electrophysiological procedures. Surgical procedures. Male mice were anaesthetized with 2% isoflurane (Baxter Healthcare, Deerfield, IL) and implanted with 2 × 8 electrode (35 μm tungsten) micro-arrays (Innovative Neurophysiology, Durham, NC) targeted at the BNST (ML: 0.8 mm, AP: ± 0.5 mm, and DV: −4.15 mm relative to Bregma). Following surgery, mice were singly housed and allowed at least one week to recover before behavioural testing.

Fear conditioning. Fear conditioning took place in 27 × 27 × 11 cm conditioning chambers (Med Associates, St. Albans, VT), with a metal-rod floor (context A) and scented with 1% vanilla. Mice received 5 pairings of a pure tone CS with a 0.6 mA foot shock. 24 h following conditioning, mice underwent a CS recall test (10 presentations of the CS alone, 5 s ITI), which was conducted in a Plexiglas cylinder (20 cm diameter) and scented with 1% acetic acid (context B). Stimulus presentations for both tests were controlled by MedPC (Med Associates, St. Albans, VT). Cameras were mounted overhead for recording freezing behaviour, which was scored automatically using CinePlex Behavioural Research System software (Plexon, Dallas, TX).

Electrophysiological recording and single unit analysis. Electrophysiological recording took place during both fear conditioning and CS recall tests. Individual units were identified and recorded using Omniplex Neural Data Acquisition System (Plexon, Dallas, TX). Neural data was sorted using Offline Sorter (Plexon, Dallas, TX). Waveforms were isolated manually, using principal component analysis. To be included in the analyses, spikes had to exhibit a refractory period of at least 1 ms. Autocorrelograms from simultaneously recorded units were examined to ensure that no cell was counted twice. Single units were analysed by generating perievent histograms (3 s bins) of firing rates from 30 s before CS onset until 30 s after CS offset (NeuroExplorer 5.0, Nex Technologies, Madison, AL). Firing rates were normalized to baseline (30 s before CS onset) using z-score transformation. Analysis included a total of 139 cells over three days of recording. Data reported for raw firing rates include only putative principal neurons (>10 Hz).

The formula for computing the suppression ratio was: average freezing rate / (average freezing rate + average movement rate). Each cell was calculated individually. A value of 0.5 = no change in rate.

Ex vivo slice electrophysiology. Brains were sectioned at 0.07 mm per s on a Leica 1200 vibrotome to obtain 300 μm coronal slices of the BNST, which were incubated in a heated holding chamber containing normal, oxygenated aCSF (in mM: 124 NaCl, 4.4 KCl, 2.4 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 10.0 glucose, and 26.0 NaHCO3) maintained at 30 °C. Brains were cut horizontally into 200 μm sections and were incubated in a heated holding chamber containing normal, oxygenated aCSF (in mM: 124 NaCl, 4.4 KCl, 2.4 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 10.0 glucose, and 26.0 NaHCO3) maintained at 30 °C for at least 1 h before recording. Slices were transferred to a recording chamber (Warner Instruments) submerged in normal, oxygenated aCSF at a rate of 2 ml/min. Neurons of the BNST were visualized using infrared differential interference contrast (DIC) video-enhanced microscopy (Olympus). Borosilicate electrodes were pulled with a Flaming-Brown micropipette puller (Sutter Instruments) and had a pipette resist-ance between 3–6 MΩ. Signals were acquired via a Multiclamp 700B amplifier, digitized at 100 kHz and analysed with Clampfit 10.3 software (Molecular Devices, Sunnyvale, CA, USA).

Light-evoked action potentials. In Serff or Crfff mice, fluorescently labelled neurons expressing ChR2 were visualized and stimulated with a blue (470 nm) LED using a 1 Hz, 2 Hz, 5 Hz, 10 Hz, and 20 Hz stimulation protocol with a pulse width of 0.5 ms. Evoked action potentials were recorded in current clamp mode using a potassium gluconate based internal solution (in mM: 135 K+ gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.6 EGTA, 4 Na2ATP, 0.4 Na2GTP. pH 7.3, 285–290 °Cmosmol).

Light-evoked synaptic transmission. In Crfff mice with ChR2 in the BNST and retrograde tracer beads in the VTA or LH, we visualized non-ChR2-expressing, beaded neurons using green (532 nm) LED. Recordings were conducted in voltage
clamp mode using a caesium-methanesulfonate (Cs-Meth) based internal solution (in mM: 135 caesium methanesulfonate, 10 KCl, 1 MgCl₂, 0.2 EGTA, 2 QX-314, 4 MgATP, 0.3 GTP, 20 phosphocreatine, pH 7.3, 285–290 mOsmol) so that we could detect EPSCs (~55 mV) and IPSCs (~10 mV) in the same neuron. After confirming the absence of a light-evoked EPSC signal, we measured light-evoked IPSCs during a single 5-ms light pulse of 470 nm. In a subset of these experiments, SR95531 (GABAa/zine, 10 μM) was bath applied for 10 min to block IPSCs.

**Drug effects in CReNN neurons.** Cfpr-reporter mice were injected with retrograde tracer beads into the VTA (ML 0−0.5, AP −2.9, DV −4.6). We then transected from beaded (VTA-projecting) and non-beaded (non-projecting) CRe neurons in the BNST. Acute drug effects were determined in current clamp mode in the presence of PTX using a tetrode glutamate-based internal solution. After a 5-min stable baseline was established, 5-HT (10 μM) or mCPP (20 μM) was bath applied for 10 min while recording changes in membrane potential. The difference in membrane potential between baseline and drug application at peak effect (delta or Δ MP) was later determined. In a subset of mCPP experiments, slices were incubated with RS-102221 (5 μM) for at least 20 min before experiments began.

**Synaptic transmission.** Spontaneous inhibitory postsynaptic currents (sIPSCs) were assessed in voltage clamp using a potassium-chloride gluconate-based intracellular solution (in mM: 70 KCl, 65 K+−glucuronate, 5 NaCl, 10 HEPES, 0.5 EGTA, 4 ATP, 0.4 GTP, pH 7.2, 285–290 mOsmol). IPSCs were pharmacologically isolated by adding kynurenic acid (3 mM) to the aCSF to block AMPA and NMDA receptor-dependent postsynaptic currents. The amplitude and frequency of sIPSCs were determined from 2 min recording episodes at ~70 mV. The baseline was averaged from the 4 min preceding the application of 5-HT (10 μM) or mCPP (10 μM) for 10 min. In a subset of these experiments, RS-102221 (5 μM) was added to the aCSF and slices were incubated in this drug solution for at least 20 min before experiments began. For miniature IPSCs (mIPSCs), TTX was included in the aCSF to eliminate evoked IPSCs. Voltaammograms were digitally smoothed one time with a fast Fourier transform following data analysis. Voltammograms were averaged before optical stimulation for background subtraction. Voltammograms mediated EPSCs were pharmacologically isolated by adding 25 μM picrotoxin to the aCSF. sEPSC recordings were acquired in 2 min recording blocks at ~10 Hz. Photostimulation was applied.

For miniature IPSCs (mIPSCs), TTX was included in the aCSF to eliminate evoked IPSCs. Voltaammograms were digitally smoothed one time with a fast Fourier transform following data analysis. Voltammograms mediated EPSCs were pharmacologically isolated by adding 25 μM picrotoxin to the aCSF. sEPSC recordings were acquired in 2 min recording blocks at ~10 Hz. Photostimulation was applied. For miniature IPSCs (mIPSCs), TTX was included in the aCSF to eliminate evoked IPSCs. Voltaammograms were digitally smoothed one time with a fast Fourier transform following data analysis. Voltammograms mediated EPSCs were pharmacologically isolated by adding 25 μM picrotoxin to the aCSF. sEPSC recordings were acquired in 2 min recording blocks at ~10 Hz. Photostimulation was applied.

**Novelty-induced suppression of feeding.** 48 h before testing, mice were provided with access to a single piece of Froot Loops cereal (Kellogg’s) in their home cage. 24 h before testing, home cage chow was removed and mouse body weights were recorded. Water remained available ad libitum. Beginning at least one hour before testing, mice transferred to new clean cages so they were singly housed for the test session and body weights were recorded. During the test session mice were placed into an arena (25 × 25 × 25 cm) that contained a single Froot Loop on top of a piece of circular filter paper. Mice were monitored by a live observer and the latency (in min) for the mouse to begin eating was measured (allowing up to 10 min). All mice began eating within this time. Following the initiation of feeding, mice were removed from the arena and placed back into their home cages. Mice were then provided with 10 min of access to a pre-weighted amount of Froot Loops for a post-test feeding session. After this 10 min post-test, the remaining Froot Loops were weighed and mice were returned to ad libitum home cage chow. Mice were then returned to group housing at the end of this session. For optogenetic experiments, mice received constant 20 Hz optical stimulation during both the latency to feed assay and the 10 min post-test. During optogenetic experiments, one control mouse did not feed during the 10 min NSF session and was excluded from the results.

**Home cage feeding.** Sert+/− mice were food deprived for 24 h. On the day of the experiment, mice were acclimated to the behaviour room for 1 h. A single pre-weighted food pellet was placed in the home cage and the mice were allowed to eat for 10 min during optogenetic stimulation. At the end of the experimental session, the pellet was removed and weighed and mice were given ad libitum access to food. Htr2a+/− mice were acclimatized in metabolic chambers (TSE Systems, Germany) for 2 days before the start of the recordings. After acclimation, mice were food deprived for 24 h. Following fasting, mice received an i.p. injection of CNO 30 min before food presented again. Mice were recorded for 12 h with the following measurements being taken every 30 min: water intake, food intake, ambulatory activity (X and Z axes), and gas exchange (O₂ and CO₂) (using the TSE LabMaster system, Germany). Energy expenditure was calculated according to the manufacturer's guidelines (PhenoMaster Software, TSE Systems).

**Fear conditioning.** We used a three-day protocol to assess both cued and contextual fear recall. On the first day, mice were placed into a fear conditioning chamber (Med Associates) that contained a grid floor and was cleaned with a scented paper towel (19.5% ethanol, 79.5% H₂O, 1% vanila). After a 3 min baseline period, mice were exposed to a 30 s tone (3 kHz, 80 dB) that co-terminated with a 2 s scrambled foot shock (0.6 mA). A total of 5 tone-shock pairings were delivered with a random inter-tone interval (ITI) of 60–120 s. For optogenetic studies, light stimulation occurred only during the 30-s tones of this session. Following delivery of the last foot shock, mice remained in the conditioning chamber for a 2 min consolidation period. 24 h later, mice were placed into a separate conditioning box (Med Associates) that contained a white Plexiglas floor, a striped pattern on the walls, and was cleaned and scented with a 70% ethanol solution. After a 3 min baseline period, mice were presented with 10 tones (30 s, 3 kHz, 80 dB) with a 5 s ITI. Mice remained in the chamber after the last tone for a two-minute consolidation period. 24 h later (48 h after training), mice were returned to the original training chamber for 5 min. For each session, freezing behaviour was hand-scored every 5 s by a trained observer blinded to experimental treatments as described previously. Freezing was defined as a lack of movement except as required for respiration.

**Immunohistochemistry and histology.** All mice used for behavioural and anatomical tracing experiments were anesthetized with Avertin and transcardially perfused with 30 ml of ice-cold 0.01 M PBS followed by 30 ml of ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were extracted and stored in 4% PFA for 24 h at 4°C before being rinsed twice with PBS and stored in 30% sucrose and PBS until the brains sank. 45 μm slices were obtained on a Leica VT100S and stored in 50/50 sucrose/PBS. We blocked the sections for 1 h in 0.1% Triton/0.01 M PBS. We then proceeded directly to the c-fos Tryptophan hydroxylase/Fluoro-Gold/c-fos triple labelling.

**Letter research.** We confirmed the absence of a light-evoked EPSC signal, we measured light-evoked IPSCs during a single 5-ms light pulse of 470 nm. In a subset of these experiments, SR95531 (GABAa/zine, 10 μM) was bath applied for 10 min to block IPSCs. All mice used for behavioural and anatomical tracing experiments were anesthetized with Avertin and transcardially perfused with 30 ml of ice-cold 0.01 M PBS followed by 30 ml of ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were extracted and stored in 4% PFA for 24 h at 4°C before being rinsed twice with PBS and stored in 30% sucrose and PBS until the brains sank. 45 μm slices were obtained on a Leica VT100S and stored in 50/50 sucrose/PBS. We blocked the sections for 1 h in 0.1% Triton/0.01 M PBS. We then proceeded directly to the c-fos Tryptophan hydroxylase/Fluoro-Gold/c-fos triple labelling. We stained for free-floating dorsal raphe sections using indirect immunofluorescence sequentially for first tryptophan hydroxylase (TPH) and Fluoro-Gold (FG) and then c-fos. For TPH/FG, we washed sections 3 × 5 min with 0.01 M PBS, permeabilized them for 30 min in 0.5% Triton/0.01 M PBS, and washed the sections again 2 × with 0.01 M PBS. We blocked the sections for 1 h in 0.1% Triton/0.01 M PBS containing 10% (v/v) normal donkey serum and 1% (w/v) bovine serum albumin (BSA). We then added primary antibodies (1:500 mouse anti-TPH (Sigma Aldrich T0678) and 1:3,000 guinea-pig anti-Fluoro-Gold (Protos Biotech NM101)) to the sections overnight. The next day, we washed the sections 3 × for 5 min with 0.01 M PBS, then incubated them with 1:500 with Alexa Fluor 647-conjugated donkey anti-mouse and Alexa Fluor 488-conjugated donkey anti-guinea pig secondary antibodies for 2 h at room temperature, and washed the sections 4 × for 5 min with 0.01 M PBS. We then proceeded directly to the c-fos tyramide signal amplification based immunofluorescent staining. We permeabilized neurons.
Images were preprocessed using stitching and maximum intensity projection, and a 20 × tiled section of the BNST was obtained on a Zeiss 800 upright microscope and coverslipped using Vectashield. An image from the left and right hemispheres of reporter mice were injected with 200 nl of CTB 555 and CTB 647 bilaterally to the Choleratoxin retrograde tracer studies in CRF reporter mice. The sections in 50% methanol for 30 min, then quenched endogenous peroxidase activity in 3% hydrogen peroxide for 5 min. Followed by two 10 min washes in 0.01 M PBS, we blocked the sections in PBS containing 0.3% Triton X-100 and 1.0% BSA for 1 h. c-fos primary antibody (Santa Cruz Biotechnology, sc-52) was added to sections at 1:3,000 and sections were incubated for 48 h at 4°C. On day 3, we washed the sections in TNT buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 10 min, blocked in TNB buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% reagent – PerkinElmer FP1020) buffer for 30 min. We then incubated in the primary antibody (goat anti-rabbit HRP-conjugated PerkinElmer) 1:200 in TNT buffer for 30 min, washed the sections in TNT buffer 4 × for 5 min, and then incubated the sections in Cy3 dye diluted in TSA amplification diluents for 10 min. We washed the sections 2 × in TNT buffer, mounted them on microscope slides. We co-sliced the slides using Vectashield mounting medium. We acquired 4–5 of 2 × 2 tiled z-stack (5 optical slices comprising 7μm total) images of the dorsal raphe from each naïve and shock mouse on a Zeiss 800 upright confocal microscope. Scanning parameters and laser power were matched between groups. Images were preprocessed using stitching and maximum intensity projection and then analysed using an advanced processing module in Zeiss Zen Blue that allows nested analysis of multiple segmented fluorescent channels within parent classes. Double-labelled and triple-labelled cells were validated in a semi-automated fashion. At least 4 sections per mouse were counted in this way. One mouse was identified as a significant outlier in the shock group and was excluded from further analysis.

**Sert<sup>Cre</sup>-ChR2, and Cre<sup>Intresect</sup>-ChR2 validation.** To verify expression of ChR2-expressing fibres in the BNST originating from DRN serotoninergic neurons, 300 μm slices used for ex vivo electrophysiological recordings containing the DRN and BNST were stored in 4% paraformaldehyde at 4°C for 24 h before being rinsed with PBS, mounted, and coversliced with Vectashield mounting medium. Images showing eYFP fluorescence from the DRN and BNST were obtained on a Zeiss 800 upright confocal microscope using a 10 × objective and tiled z stacks. To validate the INTRSECT construct, mice received injections of HSV-hEFP<sup>Cre</sup>-mCherry or HSV-eYFP<sup>Cre</sup>-L51-L51-mCherry-IRE-Isfpo to both the LH and VTA bilaterally (n = 4 and 5, respectively). Both groups received AAVDJ-hSyn-Cre-on/Flp-off-hChR2(H134R)-EYFP to the BNST bilaterally. Six weeks following injection, mice were perfused and tissue was collected as described above. To visualize YFP expression in the BNST of Cr<sup>Cre</sup>-Intresect<sup>BNST</sup> mice, free-floating slices containing the BNST were rinsed three times with PBS for 5 min each. Slices were then incubated in 50% methanol for 30 min then incubated in 3% hydrogen peroxide for 5 min. Following three 10-min washes in PBS, slices were incubated in 0.5% Triton X-100 for 30 min followed by a 10 min PBS wash. Slices were blocked in 10% normal donkey serum/0.1% TNB buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 10 min, followed by a 10 min PBS wash. Slices were then washed with PBS, mounted, and coverslipped with Vectashield mounting medium. We then analysed 3 × 4 tiled z stack (10 optical sections comprising 14μm total) images of the entire 12 μm slice were obtained on a Zeiss 780 confocal microscope for assessment of CRF/2C colocalization. A single-plane 40 × tiled image of a CRF/2C slice was obtained on a Zeiss 800 upright confocal microscope for the magnified image shown in Extended Data Fig. 6b, right. 3 × 5 tiled z stack (7 optical sections comprising 18μm) images of 2C/Cre slices were obtained on a Zeiss 800 upright confocal microscope for the 2C/Cre validation. All images were preprocessed with stitching and maximum intensity projection. An image of the BNST from 3 mice in each condition was hand counted for each study using the cell counter plugin in FIJI (ImageJ). Cells were classified into three groups; probe 1−, probe 2+, or probe 1 and 2+. Only cells positive for a probe were considered. Results are plotted as average classified percentages across the three images.

**Group assignment.** No specific method of randomization was used to assign groups. Animals were assigned to experimental groups so as to minimize the influence of other variables such as age or sex on the outcome.

**Inclusion/exclusion criteria.** Pre-established criteria for excluding mice from behavioural analysis included (1) missed injections, (2) anomalies during behavioural testing, such as mice falling off the elevated plus maze, (3) damage to or loss of optical fibres, (4) statistical outliers, as determined by the Grubbs test.

**Sample size.** A power analysis was used to determine the ideal sample size for behaviour experiments. Assuming a normal distribution, a 20% change in mean and 15% variation, we determined that we would need 8 mice per group. In some cases, mice were excluded due to missed injections or lost optical fibres resulting in fewer than 8 mice per group. For electrophysiology experiments, we aimed for 5–7 cells from 3–4 mice.

**Statistics.** Data are presented as means ± s.e.m. For comparisons with only two groups, P values were calculated using paired or unpaired t-tests as described in the figure legends. Comparisons across more than two groups were made using a one-way ANOVA, and a two-way ANOVA was used when there was more than one independent variable. A Bonferroni post-test was used following significance with an ANOVA. In cases in which ANOVA was used, the post-hoc Tukey's test was used as a multiple comparison test. The null hypothesis was rejected if the p value was less than 0.05.

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Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | In vivo recordings in BNST neurons during fear conditioning reveal opposite patterns of activation during acquisition and recall. a, b, Representative neuronal firing rate (a) and population Z score of the firing rate (b) for BNST neurons (n = 45 cells from 7 mice) 30 s before conditioned stimulus (tone), during the conditioned stimulus (CS), and 30 s after the unconditioned stimulus. c, Percentage time spent freezing during fear acquisition, cued fear recall and contextual fear recall. d, Electrode placements for BNST recordings. e, Raw firing rates during freezing (blue) versus movement (red) epochs were averaged across all putative principal neurons (firing rate < 10 Hz). Acquisition: cells in BNST exhibited greater average firing rates during freezing epochs compared to movement epochs during CS3 (t_{44} = 2.88, P < 0.01, Student's unpaired two-tailed t-test), CS4 (t_{44} = 3.14, P < 0.01, Student's unpaired two-tailed t-test), and CS5 (t_{44} = 4.4, P < 0.001, Student's unpaired two-tailed t-test) (n = 45 cells from 7 mice). CS recall: average firing rates during freezing epochs decreased over CS presentations such that firing during block 5 was significantly less than block 1 (t_{44} = 3.44, P = 0.001, Student's unpaired two-tailed t-test). Freezing firing rates during block 5 were also significantly less than movement epochs during block 5 (t_{44} = 4.03, P < 0.001, Student's unpaired two-tailed t-test) (n = 42 cells from 7 mice). CX test: average firing rate was significantly greater during movement versus freezing epochs during minute 1 (t_{44} = 4.83, P < 0.001, Student's unpaired two-tailed t-test), minute 2 (t_{44} = 3.17, P < 0.01, Student's unpaired two-tailed t-test), and minute 5 (t_{44} = 4.36, P < 0.001, Student's unpaired two-tailed t-test) (n = 45 cells from 7 mice). f, Freezing-related changes in firing rates during the CS were determined by measuring the ratio of average firing rates during freezing versus movement epochs for each session. Acquisition: activity during freezing epochs increased significantly relative to movement epochs during CS4 (t_{45} = 3.26, P < 0.01, Student's unpaired two-tailed t-test) and CS5 (t_{45} = 2.17, P < 0.05, Student's unpaired two-tailed t-test) (n = 46 cells from 7 mice). CS recall: freezing significantly suppressed activity relative to movement epochs during the last two CS presentations (t_{47} = 5.29, P < 0.001, Student's unpaired two-tailed t-test) (n = 48 cells from 7 mice). CX test: freezing significantly suppressed activity during minutes 1 (t_{44} = 6.06, P < 0.001, Student's unpaired two-tailed t-test), minute 2 (t_{44} = 2.92, P < 0.01, Student's unpaired two-tailed t-test), and minute 5 (t_{44} = 3.55, P = 0.001, Student's unpaired two-tailed t-test) (n = 45 cells from 7 mice). g, Plots showing correlation between freezing behaviour and firing rate of BNST neurons across sessions and for all sessions. Data are mean ± s.e.m. *P < 0.05 **P < 0.01; ***P < 0.001. Scale bar, 100 µm.
Extended Data Figure 2 | Effects of optogenetic stimulation of 5-HT inputs to the BNST on feeding, anxiety and locomotion.

a–c, Sert*Cre:ChR2^DRN→BNST mice exhibited reduced probability (t15 = 2.67, P < 0.05, Student’s unpaired two-tailed t-test, n = 8 control, n = 9 ChR2) and latency (t15 = 1.003, P > 0.05, Student’s unpaired two-tailed t-test, n = 8 control, n = 9 ChR2) to enter the open arms of the EPM without exhibiting locomotor deficits. d, e, Photostimulation of 5-HT^DRN→BNST terminals had no effect on locomotor activity in the open field (d) (n = 9 control, n = 11 ChR2) or home cage feeding (e) (n = 4 control, n = 6 ChR2). Data are mean ± s.e.m. *P < 0.05.
Extended Data Figure 3 | Chemogenetic activation of 5-HT2C-R-expressing neurons in the BNST increases anxiety-like behaviour.

a, Confocal images of coronal BNST slices obtained from Htr2C-Cre mice following double fluorescence in situ hybridization for 5-HT2CR and Cre. Yellow arrows indicate cells in which there is co-localization, red arrows indicate cells in which only Cre is expressed and green arrows indicate cells in which only 5-HT2CR is expressed. b, Pie chart representing the distribution of genetic markers in BNST neurons. c, Experimental configuration in Htr2cCre::hM3DqBNST mice. d, Coronal images showing c-fos induction in 5-HT2C-R expressing neurons in the BNST of Htr2cCre::hM3DqBNST or Htr2cCre::mCherryBNST mice following CNO injection. e, Bath application of CNO depolarized 5-HT2C-R-expressing neurons expressing hM3Dq in slice (n = 3 cells from 3 mice). f, Chemogenetic stimulation of 5-HT2C-R expressing neurons in BNST increased latency to feed in the NSF (t11 = 2.591, P < 0.05, Student’s unpaired two-tailed t-test, n = 6; mCherry, n = 7 hM3Dq). g, Chemogenetic activation of 5-HT2C-R-expressing BNST neurons had no effect on home cage feeding (n = 5 mCherry, n = 6 hM3Dq). h, Confocal images from Htr2cCre::mCherryBNST mice showing mCherry expression in 5-HT2C-R-expressing soma in the BNST and fibres in the LH and VTA. Data are mean ± s.e.m. * P < 0.05. Scale bar, 100 μm.
Extended Data Figure 4 | Electrophysiological characterization of 5-HT responses and 5-HT receptor expression in CRF^{BNST} neurons.
a. A pie chart showing the distribution of CRF^{BNST} neurons that were depolarized, hyperpolarized, or had no response to 5-HT (n = 8 cells from 4 mice).
b. Coronal images of the BNST showing co-localization of 5-HT_{2C}R with CRF mRNA using double fluorescence in situ hybridization.
c, d. Histograms showing the percentage of 5-HT_{2C} neurons that express CRF and the percentage of CRF neurons that express 5-HT_{2C}Rs in the BNST (n = 3 slices from 3 mice).
e. Recording configuration in CRF^{BNST} neurons. f. Slice electrophysiology in BNST of Crf reporter mice showing depolarization of all (VTA-projecting and non-projecting) CRF neurons following bath application of the 5-HT_{2} receptor agonist mCPP (n = 12 cells from 6 mice) and blockade of this response by the 5-HT_{2C} receptor antagonist RS-102221 (n = 5 cells from 3 mice).
g. Change in membrane potential induced by mCPP (t_{12} = 2.18, P < 0.05, one-sample t-test, n = 13 cells from 6 mice) is blocked by a 5-HT_{2C}R antagonist (n = 5 cells from 3 mice).
h. mCPP selectively depolarizes non-VTA-projecting CRF^{BNST} neurons (n = 5 cells from 2 mice non-VTA-projecting CRF, n = 5 cells from 4 mice VTA-projecting CRF). Data are mean ± s.e.m. *P < 0.05.
Extended Data Figure 5 | 5-HT activates inhibitory microcircuits in the BNST that modulate outputs to the LH. a, Recording configuration in CRF reporter mice infused with retrograde tracer beads in the LH. b, Average traces of 5-HT induced depolarization in LH projecting versus non-projecting neurons. c, Histograms showing 5-HT induced depolarization in non-LH projecting BNST neurons (t = 4.425, P < 0.05, one-sample t-test, n = 5 cells from 3 mice) and hyperpolarization in LH-projecting neurons (t = 2.789, P < 0.05, one-sample t-test, n = 6 cells from 3 mice). d, Confocal image of retrogradely CTB-labelled VTA (red) and LH (green) outputs in a CRF-L10a reporter (blue). e, f, Pie charts depicting the percentage of LH-projecting only, VTA-projecting only, collateralizing, and CTB-negative (unlabelled) CRF in neurons in the dorsal and ventral aspects of the BNST (n = 6 hemispheres from 3 mice). g, Experimental schematic depicting viral infusions into the BNST and retrograde tracer bead infusions into the LH of CrfCre::ChR2BNST mice. h, Recording configuration in CrfCre::ChR2BNST mice with LH tracer beads. i, Representative trace of light evoked IPSCs in LH-projecting neurons (n = 7 cells from 4 mice) and blockade of this light evoked response by GABA\(\text{A}\)zine (n = 2 cells from 2 mice). j, Recording configuration in VTA-projecting neurons in the BNST of C57BL/6 mice. k, l, 5-HT has no effect on miniature IPSC frequency or amplitude in BNST→VTA projecting neurons (n = 7 from 4 mice). m, n, 5-HT has no effect on sIPSC frequency or amplitude in the presence of the 5-HT2CR antagonist RS-102221 (n = 5 cells from 4 mice). o, Recording configuration in LH projecting neurons in the BNST of C57BL/6 mice. p, Representative traces showing an increase in sIPSC frequency in the presence of 5-HT for 6 cells from 3 mice. q, r, 5-HT increases sIPSC frequency but not amplitude in BNST→LH projecting neurons (F_{11,55} = 11.65, P < 0.01, repeated measures one-way ANOVA, n = 6 cells from 3 mice). s, t, 5-HT has no effect on miniature IPSC frequency or amplitude (n = 5 cells from 3 mice). u, v, 5-HT has no effect on sIPSC frequency or amplitude in the presence of RS-102221 (n = 6 cells from 4 mice). Data are mean ± s.e.m. *P < 0.05.
Extended Data Figure 6 | 5-HT does not alter GABAergic transmission in CRF neurons nor does it directly excite non-CRF VTA-projecting neurons in the BNST. 

**a.** Recording configuration in CRF neurons in a CRF reporter. 

**b, c.** 5-HT has no effect on sIPSC frequency or amplitude in the total population of CRF neurons (n = 5 cells from 3 mice). 

**d.** Recording configuration in non-CRF, VTA-projecting neurons in the BNST and average trace of 5-HT effect on membrane potential in non-CRF, VTA-projecting neurons in the presence of tetrodotoxin. 

**e.** Histogram summarizing 5-HT effects on membrane potential in local and VTA-projecting CRF neurons and local CRF neurons in the presence of the 5-HT$_{2C}$ receptor antagonist RS-102221 (same data shown in Fig. 2b) juxtaposed with the lack of effect of 5-HT on membrane potential in non-CRF, VTA-projecting neurons ($t_{4} = 0.9381$, ns, one-sample $t$-test, n = 5 cells from 3 mice). Data are mean ± s.e.m. **$P < 0.01$; ***$P < 0.001$. 

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Extended Data Figure 7 | The 5-HT$_2$ agonist mCPP increases GABAergic but not glutamatergic transmission in the BNST. 

a, b, mCPP increases sIPSC frequency ($F_{15,30} = 1.863$, $P < 0.001$, Repeated measures one-way ANOVA, $n = 3$ cells from 3 mice) but not amplitude in the BNST of C57BL/6 mice. c, d, mCPP has no effect on spontaneous excitatory postsynaptic current (sEPSC) frequency or amplitude in the BNST of C57BL/6 mice ($n = 5$ cells from 3 mice). Data are mean ± s.e.m. *$P < 0.05$. 

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Extended Data Figure 8 | Optogenetic and intersectional characterization of 5-HT-CRF circuits in the BNST and outputs to the midbrain. a, Experimental design and recording configuration from Sert Cre::ChR2 DIO→BNST mouse with retrograde tracer beads in the VTA. b, Representative traces for 5 cells from 3 mice depicting the increase in sIPSCs in VTA-projecting neurons in the BNST following light-evoked 5-HT release. c, Histogram summarizing the effect of light evoked 5-HT release on sIPSC frequency in VTA-projecting neurons (t(4) = 4.90, P < 0.01, one-sample t-test, n = 5 cells from 3 mice). d, Experimental configuration in Crf Cre::Intrsect-ChR2 BNST mice. e, Representative images from 4 Crf Cre::HSV-LS1-mCherry-flpo VTA/LH mice and 4 Crf Cre::HSV-LS1-mCherry VTA/LH mice injected with Intrsect-ChR2-eYFP in the BNST. f, Cell counts of eYFP + neurons from HSV-LS1-flpo and HSV-LS1-mCherry injected Crf Cre::Intrsect-ChR2 BNST mice indicating the number of non-projecting CRF neurons compared to the total CRF population in the dorsal (top panel; t(14) = 1.95, ns, Student’s unpaired two-tailed t-test, n = 4 mice, 8 hemispheres per group) and ventral aspects of the BNST (bottom panel; t(7) = 2.43, P < 0.05, Student’s unpaired Welch’s corrected two-tailed t-test, n = 4 mice, 8 hemispheres per group). g, Recording configuration and light-evoked IPSC showing local GABA release from non-projecting CRF neurons in the BNST. h, Sterotaxic injection of ChR2 in Crf Cre mouse. i, j, Light evoked IPSCs in the VTA and LH indicating that CRF projections to these regions are GABAergic. Data are mean ± s.e.m. *P < 0.05; **P < 0.01.

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Extended Data Figure 9 | Pharmacological blockade of CRF1 receptors reduces fluoxetine-induced aversive behaviour and 5-HT enhancement of GABAergic transmission in the BNST. a, Experimental schedule of injections and behaviour. b, CRF1 antagonist does not modify fear acquisition but reduces fluoxetine enhancement of cued fear recall ($F_{1,20} = 13.70, P < 0.01$, two-way ANOVA, n = 6 per group). c, Recording configuration in BNST neurons that project to the LH in C57BL/6 mice. d, Bath application of a CRF1R antagonist blocks the 5-HT induced increase in sIPSC frequency in LH-projecting neurons in the BNST ($F_{10,30} = 0.2213$, ns, Repeated measures one-way ANOVA, n = 4 cells from 2 mice). e, There was a reduction in sIPSC amplitude during 5-HT bath application and CRF1R blockade ($F_{10,30} = 2.941, P < 0.05$, Repeated measures one-way ANOVA, n = 4 cells from 2 mice). Data are mean ± s.e.m. **P < 0.01.
Extended Data Figure 10 | Model of a serotonin-sensitive inhibitory microcircuit in the BNST that modulates anxiety and aversive learning. Serotonin inputs to the BNST activate 5-HT2CRs expressed in non-projecting 'local' CRF neurons. These local CRF neurons promote anxiety and fear by inhibiting anxiolytic outputs to the VTA and LH that are putatively GABAergic. Another discrete subset of CRF neurons, which are inhibited by 5-HT, send direct, inhibitory projections to the VTA and LH. These CRF_{BNST} output neurons are GABAergic and putatively anxiolytic and stress buffering. Blue dashed lines indicate hypothesized additional synapses between CRF_{BNST} neurons. Dashed red line indicates a putatively GABAergic synapse.