Severe stress switches CRF action in the nucleus accumbens from appetitive to aversive

Julia C. Lemos1,2,3, Matthew J. Wanat1,2, Jeffrey S. Smith2, Beverly A. S. Reyes4, Nick G. Hollon1,2,3, Elisabeth J. Van Bockstaele4, Charles Chavkin2,3 & Paul E. M. Phillips1,2,3

Stressors motivate an array of adaptive responses ranging from ‘fight or flight’ to an internal urgency signal facilitating long-term goals. However, traumatic or chronic uncontrollable stress promotes a debilitating human condition characterized by an affective shift that coactivates both CRF1 and CRF2 receptors. Here, we report that corticotropin-releasing factor (CRF), a neuropeptide released in response to acute stressors, and other arousing environmental stimuli, acts in the nucleus accumbens of naive mice to increase dopamine release through coactivation of the receptors CRF1 and CRF2. Remarkably, severe-stress exposure completely abolished this effect without recovery for at least 90 days. This loss of CRF’s capacity to regulate dopamine release in the nucleus accumbens is accompanied by a switch in the reaction to CRF from appetitive to aversive, indicating a diametric change in the emotional response to acute stressors. Thus, the current findings offer a biological substrate for the switch in affect which is central to stress-induced depressive disorders.

CRF initiates neuroendocrine signalling in the hypothalamic-pituitary-adrenal axis and also regulates neurotransmission directly through two receptor subtypes, CRF receptor 1 (CRFR1) and CRFR2, which are distributed widely throughout the brain. In the nucleus accumbens, CRF facilitates cue-elicited motivation and social bonding, behaviours that are thought to be mediated by dopamine transmission. Therefore, we sought evidence for CRF–dopamine interactions in the nucleus accumbens, first using fluorescent immunohistochemistry. Dense CRF immunoreactivity was present throughout the rostro-caudal axis of the nucleus accumbens core and lateral shell, and in the most rostral portion of the medial shell in sparsely located large cell bodies (cholinergic interneurons, see Supplementary Fig. 1) and fibre terminals that were interdigitated with tyrosine-hydroxylase-immunoreactive fibres that are indicative of dopamine-containing axons (Fig. 1a). Immunoreactivity for the CRFR1 receptor displayed punctate staining with co-localization of tyrosine-hydroxylase-immunoreactive fibres that are indicative of dopamine-containing axons (Fig. 1a). Immunoreactivity for the CRFR2 receptor displayed punctate staining with co-localization of tyrosine-hydroxylase-immunoreactive fibres that are indicative of dopamine-containing axons (Fig. 1a).

Figure 1 | Cellular localization of CRF peptide, CRFR1 and CRFR2 in the nucleus accumbens. a–c, Immunoreactivity for CRF peptide (top), CRFR1 (middle) or CRFR2 (bottom) is shown in red and for tyrosine hydroxylase (TH) is shown in green. Arrows highlight examples of co-localization (yellow in the merged images). Scale bars, 10 μm. d, Transmission electron microscopy photomicrographs showing CRF receptors (labelled with immunogold particles; arrows) present on both TH-positive (immunoperoxidase labelled) and TH-negative profiles. Scale bars, 0.5 μm (top panel) and 1 μm (bottom panels).

1Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, Washington 98195, USA. 2Department of Pharmacology, University of Washington, Seattle, Washington 98195, USA. 3Program in Neurobiology and Behavior, University of Washington, Seattle, Washington 98195, USA. 4Department of Neuroscience, Farber Institute for Neurosciences, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, USA.
On days two and three, mice received CRF bilaterally into the nucleus accumbens (500 ng per side in 200 nl artificial cerebrospinal fluid; cannulae placements are shown in Supplementary Fig. 6) or vehicle infusions and were then isolated in one of the test chambers for 30 min. Four hours later they received the alternative infusion and were isolated in the other test chamber for 30 min. On day four, mice were again allowed free access to the apparatus. Following conditioning, mice exhibited a significant preference for the CRF-paired context, demonstrating that intra-accumbens CRF (500 ng) was an appetitive stimulus to these animals (conditioning by drug, $F_{1,12} = 6.435$, $P < 0.001$, two-way repeated-measures ANOVA; Fig. 3a). Similarly, unilateral infusions of CRF (500 ng in 200 nl) also produced conditioned place preference (conditioning by drug, $F_{1,12} = 11.77$, $P < 0.001$ two-way repeated-measures ANOVA; Fig. 3b and Supplementary Fig. 7a). This dose of CRF is within the range that produces selective effects in vivo, but it is difficult to ascertain the steady-state concentration at receptors as CRF undergoes both radial diffusion and active clearance.

Nevertheless, even at a lower dose of CRF (5 ng in 200 nl), conditioned place preference was observed (conditioning by drug, $F_{1,14} = 5.415$, $P < 0.05$, two-way repeated-measures ANOVA; Fig. 3b and Supplementary Fig. 7b). Taken together, these data indicate that CRF acts in the nucleus accumbens to produce a positive affective state.

To test whether this positive affective state is dependent upon CRF’s ability to increase dopamine release, we used the catecholaminergic-neuron-selective neurotoxin, 6-hydroxydopamine (6-OHDA). We compared unilateral CRF place conditioning in animals that had received ipsilateral infusions of 6-OHDA (2 μg in 500 nl) versus vehicle (0.09% NaCl, 0.1% ascorbate) into the nucleus accumbens 7 days earlier. CRF (500 ng in 200 nl) produced place preference in sham animals (conditioning by drug, $F_{1,18} = 6.95$, $P < 0.05$ two-way repeated-measures ANOVA; Supplementary Fig. 8a), of similar magnitude to controls (treatment by drug, $F_{1,10} = 0.35$, $P > 0.05$, two-way ANOVA). However, place preference to intra-accumbens CRF was absent in animals that received 6-OHDA (conditioning by drug, $F_{1,18} = 0.00$, $P > 0.05$, two-way repeated-measures ANOVA; Supplementary Fig. 8b) showing a significant change in the subjective effects of CRF ($P < 0.05$, unpaired t-test; Fig. 3b). This 6-OHDA treatment produced a significant dopamine depletion on the side of the injection ($P < 0.001$; Supplementary Fig. 8c), but did not alter locomotor activity ($P > 0.05$, unpaired t-test; Supplementary Fig. 8d); demonstrating that the unilateral lesions did not produce a general deficit in motor function. These data demonstrate that the positive affective state produced by CRF in the nucleus accumbens is dependent on its ability to increase dopamine release.

To ascertain the role of endogenously released CRF in the nucleus accumbens in mediating appetitive behaviours, we tested the effect of CRF antagonism on the response to an arousing stimulus by assaying novel object exploration, a behaviour that requires intact dopamine transmission. We bilaterally infused the CRF antagonist, α-helical CRF (500 ng in 200 nl per side) or vehicle (lactated ringer’s with 1% acetic acid), into the nucleus accumbens, placed animals into a arena, and then 15 min later introduced a novel object into the centre. Although α-helical CRF had no effect on baseline exploration of the centre of the arena compared to vehicle, it significantly attenuated the appetitive effects (that is, eliciting of approach and exploration) of the novel object (treatment by stimulus, $F_{1,18} = 4.62$, $P < 0.05$, two-way repeated-measures ANOVA; Fig. 3c). These data demonstrate that endogenous CRF in the nucleus accumbens is used under physiological conditions to mediate appetitive responses to arousing environmental stimuli.

Exposure to severe or chronic stress can produce profound alterations in normal stress signalling that can be detrimental to physical and mental health, predisposing individuals to depression. To model this phenomenon, we used a modified Porsolt paradigm in which mice are exposed to 2 days of repeated swim stress. Animals were placed in a vessel of water (29–31 °C) for 15 min followed by four additional
6-min swim sessions (separated by 6-min recovery periods) 24 h later. This protocol has been shown to produce escalating immobility across sessions, indicating a depression-like phenotype\textsuperscript{29}. We prepared coronal slices of the nucleus accumbens from these animals 30 minutes after the final stress exposure and found that the ability for CRF to potentiate dopamine release was completely abolished (stress exposure by drug, $\text{F}_{1,116} = 12.61$, $P < 0.001$ two-way ANOVA; Fig. 4a). Notably, we established that this change in the ability of CRF to regulate dopamine release was not a generalized change in stress-related peptide signalling as the effect of a $\kappa$-opioid agonist to reduce dopamine release was unaffected by the 2-day stress-exposure paradigm (Supplementary Fig. 9). Therefore, these data show that severe stress selectively abolishes CRF’s ability to modulate dopamine release in the nucleus accumbens. Surprisingly, there was no recovery of the action of CRF on dopamine release in the nucleus accumbens 7, 30 or even 90 days after stress exposure (stress exposure by drug, $\text{F}_{1,116} = 4.852$, $P < 0.01$, two-way ANOVA; Fig. 4a). This time period is consistent with the protracted course of stress-induced depressive disorders\textsuperscript{24}, and indeed, a depression-like phenotype was maintained across this 90-day post-stress period, as assessed by swim immobility (Supplementary Fig. 10). Importantly, the loss of the CRF response was not due to a baseline change in evoked dopamine release (Supplementary Fig. 11) and it was not simply an age-related phenomenon (Supplementary Fig. 12). Therefore, we have shown that severe stress produces a persistent dysregulation of CRF-dopamine interactions that normally produce a positive affective state.

Stress-induced depressive disorders are associated with altered levels of several neurochemicals that interact with the CRF system, including serotonin\textsuperscript{25}, dynorphin\textsuperscript{26} and glucocorticoids\textsuperscript{27,28}. Therefore, we targeted these systems to gain mechanistic insight into the stress-induced loss of CRF’s regulation of dopamine release. We pretreated animals (10 ml kg\textsuperscript{-1} intraperitoneal) with vehicle, fluoxetine (selective serotonin-reuptake inhibitor; 10 mg kg\textsuperscript{-1}), norBNI (k-opioid-receptor antagonist; 10 mg kg\textsuperscript{-1}) or RU486 (glucocorticoid-receptor antagonist; 30 mg kg\textsuperscript{-1}) before stress exposure on each of the swim-stress days. The animals were allowed to recover for 7 days, then slices were prepared and the CRF response was tested. Although acute regimens of fluoxetine do not alleviate pre-existing depression-related symptoms in patients or animal models, they have been shown to prevent the induction of some depression-like responses to stress\textsuperscript{29}. Nevertheless, this treatment did not affect the abolition of CRF modulation of dopamine release by stress ($P > 0.05$; Supplementary Fig. 13). Similarly, this stress-induced perturbation was not significantly affected by norBNI ($P > 0.05$; Supplementary Fig. 13); however, it was prevented by RU486 (30 mg kg\textsuperscript{-1}; $P < 0.001$; Fig. 4a and Supplementary Fig. 13), even at a lower dose (10 mg kg\textsuperscript{-1}; $P < 0.01$; Supplementary Fig. 13). These data show that glucocorticoid signalling is a critical component of the profound stress-induced dysregulation of CRF–dopamine interactions in the nucleus accumbens.

This robust loss of the neurochemical response to CRF in the nucleus accumbens after severe stress suggests a long-lasting alteration in its subjective qualities. To test this idea, we used the place-conditioning paradigm in animals that had been exposed to the 2-day swim-stress regimen. Mice that underwent repeated swim stress 7 days before conditioning spent significantly less time in the CRF-paired chamber than in the vehicle-paired chamber after conditioning, establishing that CRF in the nucleus accumbens is now aversive to these animals (conditioning by drug, $F_{1,10} = 5.824$, $P < 0.01$, two-way ANOVA, Supplementary Fig. 14a). Therefore, severe stress produces a diametric shift in the subjective qualities of CRF in the nucleus accumbens from positive to negative (Fig. 4b). Consistent with the enduring loss of CRF regulation of dopamine observed in vitro, the
Figure 4 | Stress exposure abolishes the CRF-mediated increase in evoked dopamine release and subsequent appetitive behaviours. a, Effect of CRF on dopamine release in naive mice (blue) and after swim stress (red) (left panel; n = 8–18), and in animals that were pretreated with the glucocorticoid-receptor antagonist, RU486 (30 mg kg^{-1}, intraperitoneal) or its vehicle before stress (right panel; n = 6–10). b, Mean place preferences for intra-accumbens CRF in naive (blue) and stress-exposed mice (red) (left panel; n = 6–8) and representative activity traces (right). c, Difference in the increased centre time during presentation of a novel object between vehicle and CRF-receptor antagonism in naive (blue) animals and in animals 7 days post stress (red) (n = 9–10). Error bars, s.e.m. NS, P > 0.05, *P < 0.05, **P < 0.01; $P < 0.05; §§P < 0.01 for interaction.

absence of CRF conditioned place preference persisted for at least 90 days after repeated stress exposure (F_{2,39} = 6.870, P < 0.05, one-way ANOVA with Dunnett’s post hoc; Fig. 4b and Supplementary Fig. 14b). Similarly, endogenously released CRF no longer stimulated exploration of a novel object when tested 7 days after stress exposure (stimulus by drug, F_{1,16} = 0.004, P > 0.05, two-way repeated-measures ANOVA; Supplementary Fig. 15) showing that severe stress abolished the function of CRF in the nucleus accumbens to stimulate appetitive responses to arousing stimuli (unpaired t-test, P < 0.05, Fig. 4c). Therefore, these findings demonstrate the long-term loss of a regulatory mechanism of motivated behaviour after severe stress.

Major depressive disorder has a lifetime prevalence of 17%, making it one the world’s greatest public-health concerns26; however, its neurobiological correlates.

METHODS SUMMARY

Subjects. Male C57BL/6 mice aged >50 days had ad libitum access to food and water. Mice housed together (two to four per cage) were subjected to the same behavioural treatments. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Neuroanatomy. Immunohistochemistry was carried out as described previously26. Sections were incubated for 24 h with a mixture of mouse anti-tyroside-hydroxylase 1:1,000 and rabbit anti-CRF (peptide) 1:150, and chicken anti-CHAT antibody 1:150 or rabbit anti-CFRR1 or goat anti-CFRR2 (1:100 to 1:500), then incubated in the appropriate fluorecently tagged secondary antibodies (1:500), and were imaged using epifluorescent and confocal microscopes. Transmission electron microscopy was carried out as previously described26.

Fast-scan cyclic voltammetry. 250-μm coronal slices containing the nucleus accumbens were continuously perfused (1.5–2.0 ml min⁻¹) with oxygenated artificial cerebrospinal fluid (aCSF) maintained at 31–33 °C. The potential at a carbon-fibre electrode was held at —0.4 V versus Ag/AgCl, ramped to +1.3 V and back to —0.4 V (400 V s⁻¹) every 100 ms. A single biphasic electrical pulse (2 ms per phase, 100–500 μA) was applied to the slice to evoke dopamine release.

Conditioned place preference. A three-compartment place-conditioning apparatus was used to measure preference as described previously26. On days 2 and 3, mice received two intra-accumbens microinjections per day: one injection of aCSF and one injection of CRF (500 ng in 200 nl per side) paired with different chambers. On day 4, mice were allowed free access to the apparatus for 30 min. At the end of behavioural testing, cannule placements were assessed.

Novel-object exploration. The novel object exploration assay was similar to an assay that has been described previously26. Animals received bilateral intra-accumbens microinjections of vehicle or x-helical CRF (500 ng in 200 nl) counterbalanced across 2 days of testing. On each testing day, the animal was exposed to a new novel object.

Full Methods and any associated references are available in the online version of the paper.

Received 13 May 2011; accepted 23 July 2012.

Published online 19 September; corrected online 17 October 2012 (see full-text HTML for details).

1. Korte, S. M., Koolhaas, J. M., Wingfield, J. C. & McEwen, B. S. The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. Neurosci. Biobehav. Rev. 29, 3–38 (2005).
2. Beck, A. T. The evolution of the cognitive model of depression and its neurobiological correlates. Am. J. Psychiatry 165, 969–977 (2008).
3. Clark, D. A. & Beck, A. T. Cognitive theory and therapy of anxiety and depression: convergence with neurobiological findings. Trends Cogn. Sci. 14, 418–424 (2010).
4. Nestler, E. J. et al. Neurobiology of depression. Neuron 34, 13–25 (2002).
5. Wang, B. et al. Cocaine experience establishes control of midbrain glutamate and dopamine by corticotropin-releasing factor: a role in stress-induced relapse to drug seeking. J. Neurosci. 25, 5389–5396 (2005).
6. Merali, Z., McIntosh, J. & Anisman, H. Anticipatory cues differentially provoke in vivo peptidergic and monoaminergic release at the medial prefrontal cortex. Neuropsychopharmacology 29, 1409–1418 (2004).
7. Gallagher, J. P., Orozco-Caball, L. F., Liu, J. & Shinnick-Gallagher, P. Synaptic physiology of central CRH system. Eur. J. Pharmacol. 583, 215–225 (2008).
et al.

8. Steckler, T. & Holsboer, F. Corticotropin-releasing hormone receptor subtypes and emotion. *Biol. Psychiatry* 46, 1480–1508 (1999).

9. Pecilta, S., Schulkin, J. & Berridge, K. C. Nucleus accumbens corticotropin-releasing factor increases cue-triggered motivation for sucrose reward: paradoxical positive incentive effects in stress? *BMC Biol.* 4, 8 (2006).

10. Lim, M. M. et al. CRF receptors in the nucleus accumbens modulate partner preference in prairie voles. *Horm. Behav.* 51, 508–515 (2007).

11. Aragona, B. J. et al. Nucleus accumbens dopamine differentially mediates the formation and maintenance of monogamous pair bonds. *Nature Neurosci.* 9, 133–139 (2006).

12. Lex, A. & Hauber, W. Dopamine D1 and D2 receptors in the nucleus accumbens core and shell mediate Pavlovian-instrumental transfer. *Learn. Mem.* 15, 483–491 (2008).

13. Waselus, M., Nazzaro, C., Valentino, R. J. & Van Bockstaele, E. J. Stress-induced redistribution of corticotropin-releasing factor receptor subtypes in the dorsal raphe nucleus. *Biol. Psychiatry* 66, 76–83 (2009).

14. Timpl, P. et al. Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nature Genet.* 19, 162–166 (1998).

15. Bale, T. L. et al. Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nature Genet.* 24, 410–414 (2000).

16. Cador, M., Ahmed, S. H., Koob, G. F., Le Moal, M. & Sinus, L. Corticotropin-releasing factor induces a place aversion independent of its neuroendocrine role. *Brain Res.* 597, 304–309 (1992).

17. Oldfield, E. H. et al. Active clearance of corticotropin-releasing factor from the cerebrospinal fluid. *Neuroendocrinology* 40, 84–87 (1985).

18. Fink, J. S. & Smith, G. P. Mesolimbic and mesocortical dopaminergic neurons are necessary for normal exploratory behavior in rats. *Neurosci. Lett.* 17, 61–65 (1980).

19. Bale, T. L. Stress sensitivity and the development of affective disorders. *Horm. Behav.* 50, 529–533 (2006).

20. Bruchas, M. R. et al. Stress-induced p38 mitogen-activated protein kinase activation mediates κ-opioid-dependent dysphoria. *J. Neurosci.* 27, 11614–11623 (2007).

21. Coryell, W. et al. The time course of nonchronic major depressive disorder. Uniformity across episodes and samples. National Institute of Mental Health Collaborative Program on the Psychobiology of Depression–Clinical Studies. *Arch. Gen. Psychiatry* 51, 405–410 (1994).

22. Torres, G., Horowitz, J. M., Lafamme, N. & Rivest, S. Fluoxetine induces the transcription of genes encoding c-fos, corticotropin-releasing factor and its type 1 receptor in rat brain. *Neuroscience* 87, 463–477 (1998).

23. Bruchas, M. R. & Chavkin, C. Kinase cascades and ligand-directed signaling at the kappa opioid receptor. *Psychopharmacology (Berl.)* 210, 137–147 (2010).

24. Iredale, P. A. & Duman, R. S. Glucocorticoid regulation of corticotropin-releasing factor1 receptor expression in pituitary-derived AtT-20 cells. *Mol. Pharmacol.* 51, 794–799 (1997).

25. Cryan, J. F. & Mombereau, C. In search of a depressed mouse: models of stress-induced p38 mitogen-activated protein kinase-dependent dysphoria. *Mol. Psychiatry* 9, 326–357 (2004).

26. Kessler, R. C. et al. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch. Gen. Psychiatry* 62, 593–602 (2005); erratum 62, 768 (2005).

27. Gelenberg, A. J. Depression symptomatology and neurobiology. *J. Clin. Psychiatry* 71, e02 (2010).

28. Refojo, D. et al. Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of CRHR1. *Science* 333, 1903–1907 (2011).

29. Chaki, S. et al. Anxiolytic- and antidepressant-like profile of a new CRF1 receptor antagonist, R278995/CRA0450. *Eur. J. Pharmacol.* 485, 145–158 (2004).

30. Reyes, B. A., Valentino, R. J. & Van Bockstaele, E. J. Stress-induced intracranial trafficking of corticotropin-releasing factor receptors in rat locus coeruleus neurons. *Endocrinology* 149, 122–130 (2008).

Supplementary Information is available in the online version of the paper.

Acknowledgements This work was supported by National Institutes of Health grants F31-MH086269 (J.C.L.), F32-DA026273 (M.J.W.), R01-DA009082 (E.J.V.B.), R01-DA030074 (C.C.), R01-MH079292 and R01-DA016782 (P.E.M.P.), the National Science Foundation (N.G.H.) and NARSAD (P.E.M.P.). We thank C. Zietz, M. Miyatake and P. Groblewski for assisting with histological verification of cannula placement, H. Gill for help with data analysis, D. Messinger for breeding and genotyping mice and N. Stella for use of a microscope. We thank M. Darvas and R. Palmiter for providing Y.1/1, ACRPR1A−/−, DbhTh/−/− mice. We thank R. Sapolsky, J. Day, S. Sesack, M. Soden, C. Walker and E. Horne for useful suggestions and insights.

Author Contributions J.C.L. performed immunohistochemistry. J.C.L. and N.G.H. carried out fast-scan cyclic-voltammetry experiments. J.C.L., M.J.W. and J.S.S. performed the behavioural experiments. B.A.S.R. and E.J.V.B. provided transmission electron microscopy data. J.C.L., M.J.W., C.C. and P.E.M.P. developed the conceptual and experimental framework, and J.C.L. and P.E.M.P. wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to P.E.M.P. (pemp@uw.edu).
METHODS

Subjects. Male C57BL/6 mice aged >50 days were maintained under a 12-h light–dark cycle (7:00 to 19:00 light) with access to standard food and water ad libitum. All procedures on animal subjects were approved by the University of Washington or Thomas Jefferson University Institutional Animal Care and Use Committee. Mice housed together (two per cage) were subjected to the same behavioural treatments.

Immunohistochemistry. We used perfusion, cryosectioning and immunohistochemistry procedures as described previously26. Sections (30 μm) were then incubated with a mixture of mouse anti-tyrosine hydroxylase 1:10,000 (Sigma) and either rabbit anti-CRF (peptide) 1:150 (Sigma) and chicken anti-ChAT antibody 1:150 (Invitrogen) or rabbit anti-CRFR1 or CRFR2 (Novus Biologicals) in blocking buffer for 24–36 h at room temperature. Sections were then washed with PBS, and detection was carried out using the fluorescent secondary antibody Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) 1:500, Alexa Fluor 550 goat anti-rabbit IgG and Alexa Fluor 633 goat anti-chicken IgG (Invitrogen) in blocking buffer for 2 h at room temperature. Sections were washed in PBS 3 times for 10 min and 0.1 M phosphate buffer twice for 10 min and mounted on Superfrost plus slides. Sections were imaged with epifluorescence (Nikon) and confocal microscopes (Leica).

Transmission electron microscopy. Mice were perfused and brains were sectioned as described previously. Sections (100 nm) were processed using standard transmission-electron-microscopy procedures30,31. Sections were incubated in mouse anti-TH (1:1,000; Immunostar) and rabbit anti-corticotropin-releasing factor receptor (1:1,000; Santa Cruz Biotechnology) overnight at room temperature. Immunoperoxidase detection of tyrosine hydroxylase and silver-intensified immunogold localization of CRFRs followed standard procedures32. Digital images were captured using the AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques). Only tissue sections with good preservation of ultrastructural morphology and with both tyrosine hydroxylase and CRFR immunoreactivity clearly apparent in the tissue were used for the analysis. For immunogold labelling, profiles with at least two immunogold-silver particles within a cellular compartment in a single thin section were considered immunolabelled33,34. The cellular elements were classified according to a method described previously33,34.

Fast-scan cyclic voltammetry. Mice were quickly decapitated and the head placed in pre-oxygenated ice-cold artificial cerebrospinal fluid (aCSF) in which sucrose (248 mM) was substituted for NaCl. The brain was rapidly removed and blocked to isolate the anterior forebrain. Coronal slices (250 μm) containing the nucleus accumbens were prepared using methods described previously35, placed in a recording chamber and continuously perfused (1.5–2.0 μl min⁻¹) with oxygenated aCSF (in mM: NaCl, 124; KCl, 2.5; NaH₂PO₄, 1.25; MgSO₄, 2.0; CaCl₂, 2.0; dextrose, 10; and NaHCO₃, 26) maintained at 31–33 °C. Carbon-fibre electrodes were fabricated using a Sutter P-97 puller. Carbon-fibre electrodes (working electrodes) were hand cut to approximately 100–150 μm past the capillary tip. The potential at a carbon-fibre electrode was held at −0.4 V versus Ag/AgCl and ramped to +1.3 V and back to −0.4 V (400 mV s⁻¹) every 100 ms. A single biphasic electrical pulse (2 ms per phase, 100–500 μA) was applied to the spike to evoke dopamine release.

Swim stress. Mice were subjected to either a single 15-min swim with a 24-h recovery period, or a 2-day swim stress in which they were exposed to a 15-min swim session on day 1, then 24 h later on day 2, were exposed to 4 swim sessions of 6 min separated by 6 min, conducted under bright light (690–700 lx) conditions. Water temperature was maintained at 29–31 °C. Animals were removed from the water if they became completely submerged for >1 s at any time during the paradigm. Some animals were killed at 30 min, 7, 30 or 90 days after the final swim session of the 2-day protocol, and nucleus accumbens slices were prepared.

Cannulations. Animals were anaesthetized with isoflurane and cannulation surgeries were carried out using a stereotactic alignment system, similar to methods described previously36. Double-guide cannulas (26 gauge, 3.5 mm from pedestal, 2 mm separation; Plastics One) were placed in the nucleus accumbens core at ±1 mm lateral, 1 mm posterior from bregma and 3.5 mm below the skull. Guide cannulas were anchored using dental cement, and dummy internal cannulas were placed inside until injection. Mice were injected intracerebroventricularly by placing a 33-gauge internal cannula (Plastics One) into the guide cannula.

Conditioned place preference. Animals were allowed to recover from surgery for at least 7 days. All animals were handled for 4 days before the pre-test day. Animals assigned to the stress-exposed group were subjected to the 2-day swim-stress paradigm after recovery; animals were not included if they did not show normal swimming responses. Stress-exposed animals began CRF conditioning 7 or 90 days after the final swim session. A three-compartment place-conditioning apparatus was used to measure preference as described previously37. On days 2 and 3, mice received 2 injections per day: 1 injection of aCSF and 1 injection of CRF (500 ng per 200 nl) paired with different chambers at 125 nl min⁻¹. On day 4, mice were once again allowed free access to the entire apparatus for 30 min. After the conclusion of behavioural testing, cannulae placements were assessed. Mice with cannula placements outside the accumbens were excluded from the study.

6-OHDA lesion and high-performance liquid chromatography. Mice were injected with either 6-OHDA (2 μg per 500 nl; Sigma) or vehicle (0.9% NaCl, 0.1% ascorbate). After the conclusion of behavioural testing, a tissue core (approximately 2 × 2 × 1 mm) of the ipsilateral and contralateral accumbens of each animal was microdissected, rapidly frozen in liquid nitrogen and stored in microcentrifuge tube at −80 °C until processed for tissue dopamine content. High-performance liquid chromatography (HPLC) was used to measure monoamine content by the Neurochemistry Core Laboratory at the Vanderbilt University Center for Molecular Neuroscience Research.

Novel object exploration. Mice were cannulated, allowed to recover from surgery and handled for 4 days before being subjected to a novel object exploration assay similar to previously described38. In brief, on test day 1, mice were given bilateral intra-accumbens microinjections of either vehicle (lactated ringer’s with 1% acetic acid) or α-helical CRF (2 μg) and were allowed to habituate in an open field for 15 min. Subsequently, a novel object was introduced and exploratory behaviour of the novel object was measured for an additional 15 min. On test day 2, the animals received the alternative pharmacological treatment to that which they received on day 1, were allowed to habituate again in the open field and then exposed to a second novel object. Both the pharmacological treatment and the novel objects were counter-balanced across test days. Identically to the place-conditioning experiments, 1 group of mice were exposed to swim stress 7 days before test day 1.

31. van Bockstaele, E. J., Sesack, S. R. & Pickel, V. M. Dynorphin-immunoreactive terminals in the rat nucleus accumbens: cellular sites for modulation of target neurons and interactions with catecholamine afferents. J. Comp. Neurol. 341, 1–15 (1994).
32. Reyes, B. A., Fox, K., Valentino, R. J. & Van Bockstaele, E. J. Agonist-induced internalization of corticotropin-releasing factor receptors in noradrenergic neurons of the rat locus coeruleus. Eur. J. Neurosci. 23, 2991–2998 (2006).
33. Peters, A., Palay, S. L. & Webster, H. D. The Fine Structure of the Nervous System (Oxford Univ. Press, 1991).
34. Peters, A. & Palay, S. L. The morphology of synapses. J. Neurocytol. 25, 687–700 (1996).
35. Bruchas, M. R., Land, B. B., Lemos, J. C. & Chavkin, C. CRF1-R activation of the dynorphin/kappa opioid system in the mouse basolateral amygdala mediates anxiety-like behavior. PLoS ONE 4, e8528 (2009).