LETTER

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

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Stressors motivate an array of adaptive responses ranging from 'fight or flight' to an internal urgency signal facilitating long-term goals[1]. However, traumatic or chronic uncontrollable stress promotes the onset of major depressive disorder, in which acute stressors lose their motivational properties and are perceived as insurmountable impediments[2]. Consequently, stress-induced depression is a debilitating human condition characterized by an affective shift from engagement of the environment to withdrawal[3]. An emerging neurobiological substrate of depression and associated pathology is the nucleus accumbens, a region with the capacity to mediate a diverse range of stress responses by interfacing limbic, cognitive and motor circuitry[4]. Here we report that corticotropin-releasing factor (CRF), a neuropeptide released in response to acute stressors[5] and other arousing environmental stimuli[6], acts in the nucleus accumbens of naive mice to increase dopamine release through coactivation of the receptors CRFR1 and CRFR2. 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[7,8]. In the nucleus accumbens, CRF facilitates cue-elicited motivation[9] and social bonding[10], behaviours that are thought to be mediated by dopamine transmission[11,12]. 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 immunoreactivity on fibre segments in addition to localization on cell bodies within the nucleus accumbens (Fig. 1b and Supplementary Fig. 2). CRFR2 immunoreactivity had a more diffuse but still punctate pattern of staining, similar to that in other regions[13], with some co-localization with tyrosine-hydroxylase immunoreactivity (Fig. 1c and Supplementary Fig. 3). Expression of CRF receptors on subcellular profiles in the nucleus accumbens, including tyrosine-hydroxylase-positive terminals, was confirmed at higher spatial resolution using transmission electron microscopy (Fig. 1d; quantified in Supplementary Table 1). Together, these data indicate that the localization of CRF and its receptors in the nucleus accumbens is well-suited for modulation of dopamine release.

To directly test the functional effects of CRF on dopamine release in the nucleus accumbens, we selectively monitored dopamine release evoked by a single biphasic electrical pulse (2 ms per phase, 100–500 μA delivered once per minute) in acute coronal brain slices using fast-scan cyclic voltammetry at carbon-fibre microelectrodes (Fig. 2a and Supplementary Fig. 4). Vehicle or CRF (10, 100 or 1,000 nM) was applied to the slice for 15 min after 5 min of stable baseline, and the resultant effect was quantified by averaging the evoked dopamine current in the last 10 minutes. After application of vehicle, there was a modest (~7%) decrease in dopamine release (Fig. 2b), whereas CRF increased dopamine release in a concentration-dependent manner eliciting effects significantly greater than vehicle at 100 and 1,000 nM (27.8 ± 6.7 and 30.0 ± 8.4%, respectively, mean ± s.e.m.; $F_{3,\ 49} = 5.026$, $P < 0.01$, one-way analysis of variance (ANOVA) with Dunnett's post-hoc *t*-tests; Fig. 2b and Supplementary Fig. 5). Interestingly, this effect could be blocked by application of either the selective CRFR1 antagonist, antalarmin (1 μM), or the selective CRFR2 antagonist, anti-sauvagine 30 (ASVG 30; 250 nM), to the slice beginning 20 min before CRF application ($F_{2,\ 50} = 5.142$, $P < 0.01$, one-way ANOVA with Dunnett's post-hoc *t*-tests; Fig. 2c) indicating that coactivation of both receptors is required. Consistently, CRF (10,

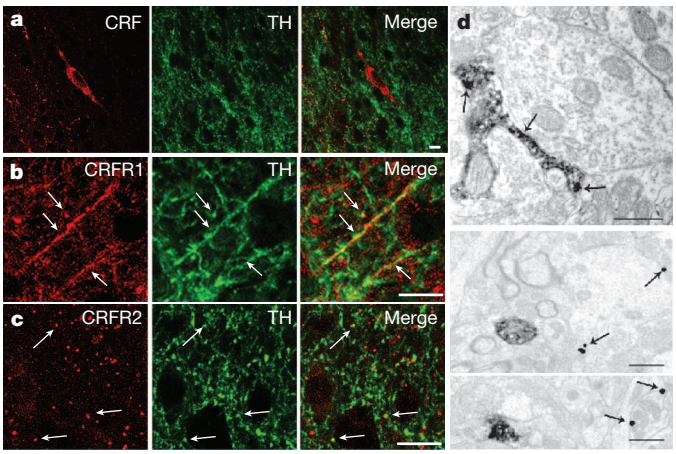

Figure 1 | Cellular localization of CRF peptide, CRFR1 and CRFR2 in the nucleus accumbens. **a**–**c**, Immunoreactivity for CRF peptide (top), CRFR1 (middle) or CRFR2 (bottom) is shown in red and for tyrosine hydroxylase (TH) is shown in green. Arrows highlight examples of co-localization (yellow in 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).

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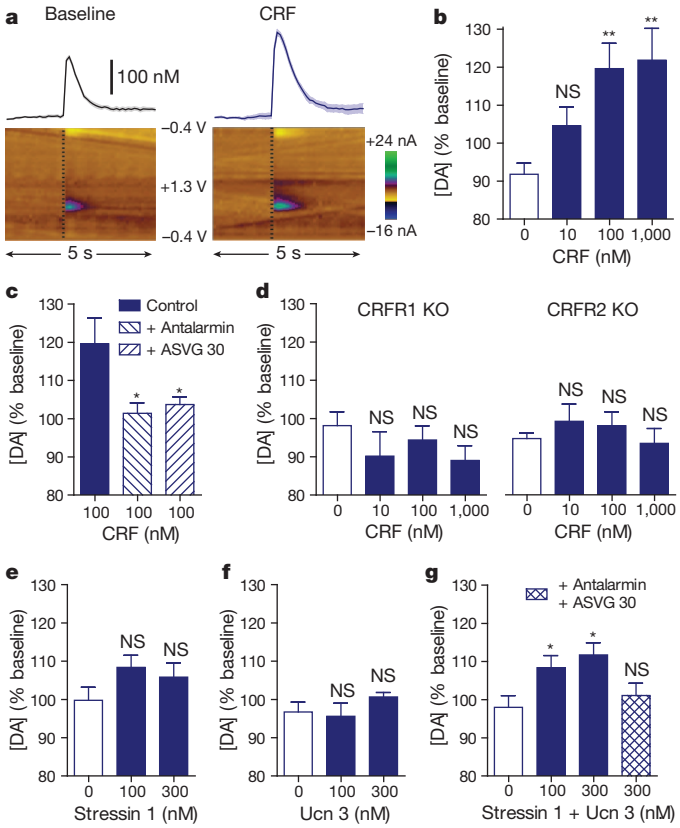

Figure 2 | CRF increases dopamine release in the nucleus accumbens through coactivation of CRFR1 and CRFR2. **a**, Representative dopamine release evoked by electrical stimulation (dashed lines) before (left) and after (right) application of 100 nM CRF (mean ± s.e.m. for 5 consecutive stimulations, top) and corresponding two-dimensional plots depicting changes in faradaic current (pseudocolour) with time as the abscissa and applied potential as the ordinate (bottom). **b**, Concentration response to CRF, $n = 11$–18. **c**, Effect of antagonists for CRFR1 (antalarmin, 1000 nM) or CRFR2 (antisauvagine 30 (ASVG 30), 250 nM), $n = 18$–20. **d**, CRF in mice lacking the gene encoding the CRFR1 (left) or CRFR2 (right) receptors, $n = 7$–13. **e**–**g**, Effect of the CRFR1 agonist, stressin 1, $n = 9$–15 (**e**), the CRFR2 agonist, urocortin 3 (100 or 300 nM), $n = 5$–8 (**f**) or their co-application, $n = 8$–15 (**g**). Error bars, s.e.m. DA, dopamine; NS, not significant (with $P > 0.05$); $^{*}P < 0.05$; $^{**}P < 0.01$ versus vehicle.

100, 1,000 nM) failed to increase dopamine release in the nucleus accumbens of mice with deletion of either the *Crfr1* (ref. 14) or *Crfr2* (ref. 15) gene (Fig. 2d). Application of the selective CRFR1 agonist stressin 1 (100 or 300 nM) or the selective CRFR2 agonist urocortin 3 (100 or 300 nM) failed to significantly increase dopamine release when applied individually ($P > 0.05$ compared to respective vehicles; Fig. 2e, f), but significantly increased dopamine release when applied together ($F_{3,36} = 3.528$, $P < 0.05$ versus vehicle, one-way ANOVA with Dunnett's post-hoc *t*-tests). The effect of the agonists together could be blocked by pre-treatment with antalarmin and ASVG 30 (unpaired *t*-test, $P > 0.05$; Fig. 2g). Together these data provide convergent evidence that CRF increases dopamine release in the nucleus accumbens through coactivation of CRFR1 and CRFR2.

If this ability for CRF to positively regulate dopamine in the nucleus accumbens has specific motivational relevance to the behaving animal, we would predict that it would cause conditioned place preference when restricted to the nucleus accumbens, even though centrally administered CRF elicits robust conditioned place aversion[16]. Therefore, we used a balanced place-conditioning apparatus consisting of two visually distinct test chambers separated by a smaller neutral compartment. On day one, mice were allowed to freely roam the apparatus, and the time they spent in each chamber was recorded. 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*[9], but it is difficult to ascertain the steady-state concentration at receptors as CRF undergoes both radial diffusion and active clearance[17]. 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, $F_{1,30} = 0.35$, $P > 0.05$, 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[18]. 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 an 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[19]. 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

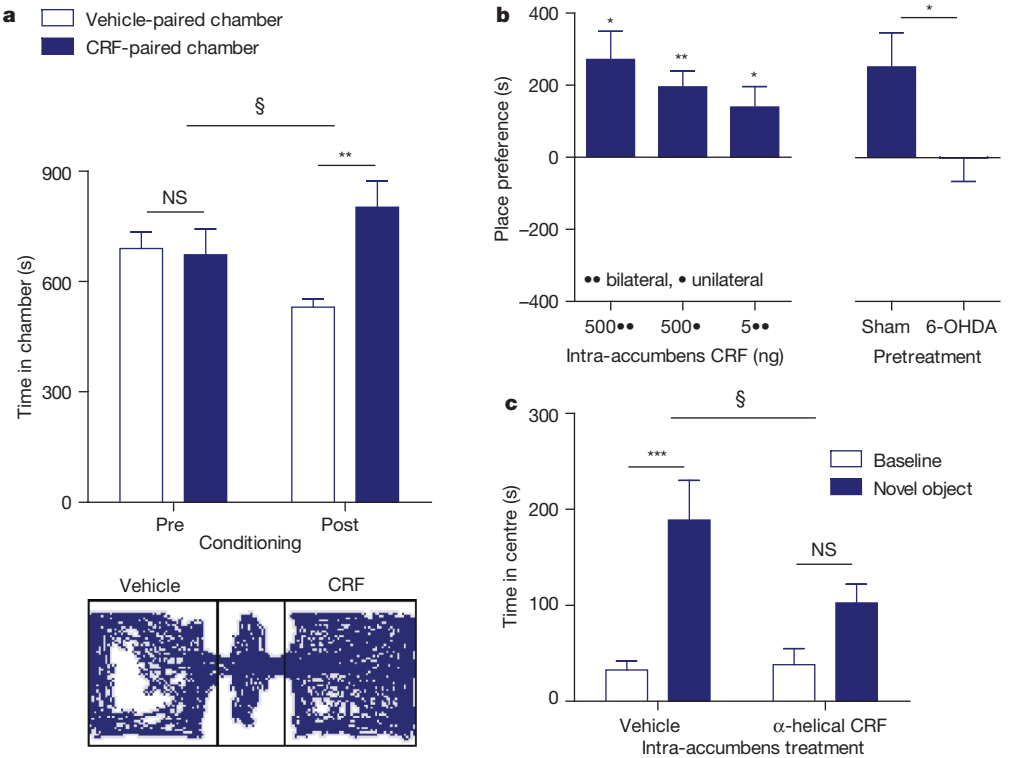

Figure 3 | CRF in the nucleus accumbens promotes appetitive behaviour. **a**, Mean difference in times spent in the CRF-paired chamber compared to the vehicle-paired chamber before and after conditioning (top panel; $n = 7$) and representative post-conditioning activity trace (bottom panel). **b**, Place preference (time in CRF-paired chamber minus the time spent in the vehicle-paired chamber post conditioning) for intra-nucleus accumbens injections of 500 ng CRF bilateral, 500 ng unilateral or 5 ng bilateral (left panel; $n = 7$–10). Place preference for 500 ng CRF (unilateral) in sham or 6-OHDA-treated mice (right; $n = 10$). **c**, Time spent in the centre of an open field before and during presentation of a novel object (placed in the centre of the field) after bilateral intra-accumbens infusion of the CRF-receptor antagonist α-helical CRF (500 ng) or its vehicle ($n = 10$). Error bars, s.e.m. NS, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; §$P < 0.05$ for interaction.

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[20]. 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, $F_{4,116} = 12.61$, $P < 0.001$ 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 κ-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, $F_{4,116} = 4.852$, $P < 0.01$, two-way ANOVA; Fig. 4a). This time period is consistent with the protracted course of stress-induced depressive disorders[21], 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[22], dynorphin[23] and glucocorticoids[4,24]. 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^{-1} intraperitoneal) with vehicle, fluoxetine (selective serotonin-reuptake inhibitor; 10 mg kg^{-1}), norBNI (κ-opioid-receptor antagonist; 10 mg kg^{-1}) or RU486 (glucocorticoid-receptor antagonist; 30 mg kg^{-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[25]. 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^{-1}; $P < 0.001$; Fig. 4a and Supplementary Fig. 13), even at a lower dose (10 mg kg^{-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

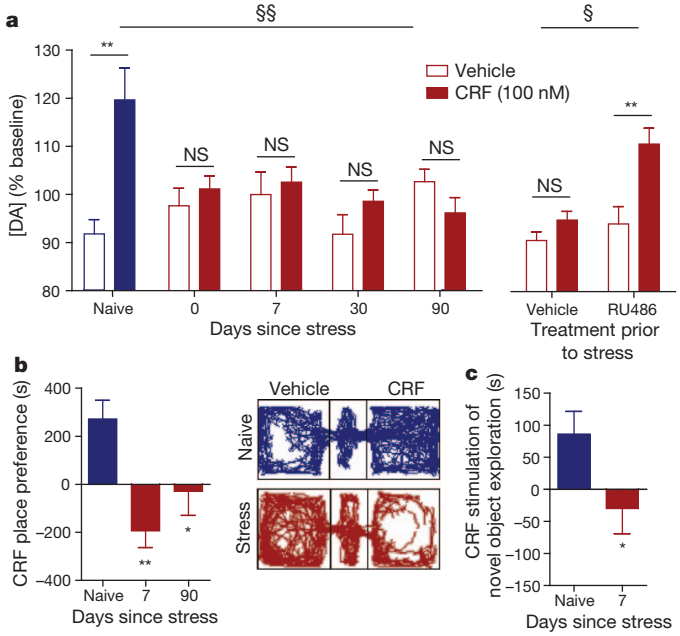

Figure 4 | Stress exposure abolishes the CRF-mediated increase in evoked dopamine release and subsequent appetitive behaviours. **a**, Effect of CRF on dopamine release in naive mice (blue) and after swim stress (red) (left panel; $n = 8$–18), and in animals that were pretreated with the glucocorticoid-receptor 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 ($F_{2,20} = 6.870$, $P < 0.05$, 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 concerns[26]; however, its molecular foundation has been elusive. Patients suffering from this disorder present with constellations of symptoms that include loss of affect, cognitive impairment and homeostatic imbalance[27]; symptoms that are presumably precipitated by dysregulation of several brain regions[4]. It is established that glucocorticoid-dependent hippocampal atrophy is a critical mediator of cognitive impairment in depression such as memory loss[4]. More recently, disruption of nucleus accumbens function has been implicated in the affective symptoms of depression[4]. In the current work, we studied the actions of CRF on neurotransmission within this brain region in an attempt to connect pathological stress-related neuroadaptation with the shift in affect observed in depressed patients.

CRF receptors are distributed widely throughout the brain[8] and mediate disparate effects (see Supplementary Discussion). Our data highlight the specificity of the local action of both exogenously applied and endogenously released CRF in the nucleus accumbens in producing a positive, rather than negative, subjective state by increasing dopamine release. Importantly, we show that severe stress disables this capacity of CRF to positively regulate dopamine, removing CRF's appetitive qualities, leaving a negative perceptual bias. This dysregulation is mediated by glucocorticoid, but not κ-opioid, receptors and is not ameliorated by acute prophylactic administration of a selective serotonin-reuptake inhibitor. Glucocorticoid signalling has been shown to have genomic repressive effects on the CRF system, in particular the downregulation of CRFR1[24]. Genetic deletion of the CRFR1 gene selectively from dopamine neurons increases anxiety-like behaviour[28], demonstrating further that disruption of CRF-dopamine interactions alone is sufficient to produce a negative affective state similar to that following severe stress[29].

Collectively, our data show a specific defect in the regulation of dopamine transmission in the nucleus accumbens as a consequence of exposure to stress that induces depression-like behaviour. Depressive disorders produce a profound change in the perception of, and behavioural response to, acute stressors and other arousing environmental stimuli that elicit CRF signalling. Taken together, our findings provide a neurobiological mechanism for the affective shift from engagement of the environment to withdrawal following severe stress, central to the manifestation of major depressive disorder.

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 previously[20]. Sections were incubated for 24 h with a mixture of mouse anti-tyrosine hydroxylase 1:1,000 and rabbit anti-CRF (peptide) 1:150, and chicken anti-ChAT antibody 1:150 or rabbit anti-CRFR1 or goat anti-CRFR2 (1:100 to 1:500), then incubated in the appropriate fluorescently tagged secondary antibodies (1:500), and were imaged using epifluorescent and confocal microscopes. Transmission electron microscopy was carried out as previously described[30].

Fast-scan cyclic voltammetry. 250-μm coronal slices containing the nucleus accumbens were continuously perfused (1.5–2.0 ml min^{-1}) with oxygenated artifical 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 previously[20]. 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, cannulae placements were assessed.

Novel-object exploration. The novel object exploration assay was similar to an assay that has been described previously[28]. Animals received bilateral intra-accumbens microinfusions of vehicle or α-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.

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

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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 to four per cage) were subjected to the same behavioural treatments.

Immunohistochemistry. We used perfusion, cryosectioning and immunohistochemistry procedures as described previously[20]. Sections (30 μm) were then incubated with a mixture of mouse anti-tyrosine hydroxylase 1:1,000 (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 555 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 procedures[30,31]. 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 procedures[30]. 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 immunolabelled[30,32]. The cellular elements were classified according to a method described previously[33,34].

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 previously[35], placed in a recording chamber and continuously perfused (1.5–2.0 ml min^{-1}) with oxygenated aCSF (in mM: NaCl, 124; KCl, 2.5; NaH_2PO_4, 1.25; $MgSO_4$, 2.0; $CaCl_2$, 2.0; dextrose, 10; and $NaHCO_3$, 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, 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.

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 stereotaxic alignment system, similar to methods described previously[20]. 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 previously[20]. 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 described[28]. In brief, on test day 1, mice were given bilateral intra-accumbens microinfusions 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.

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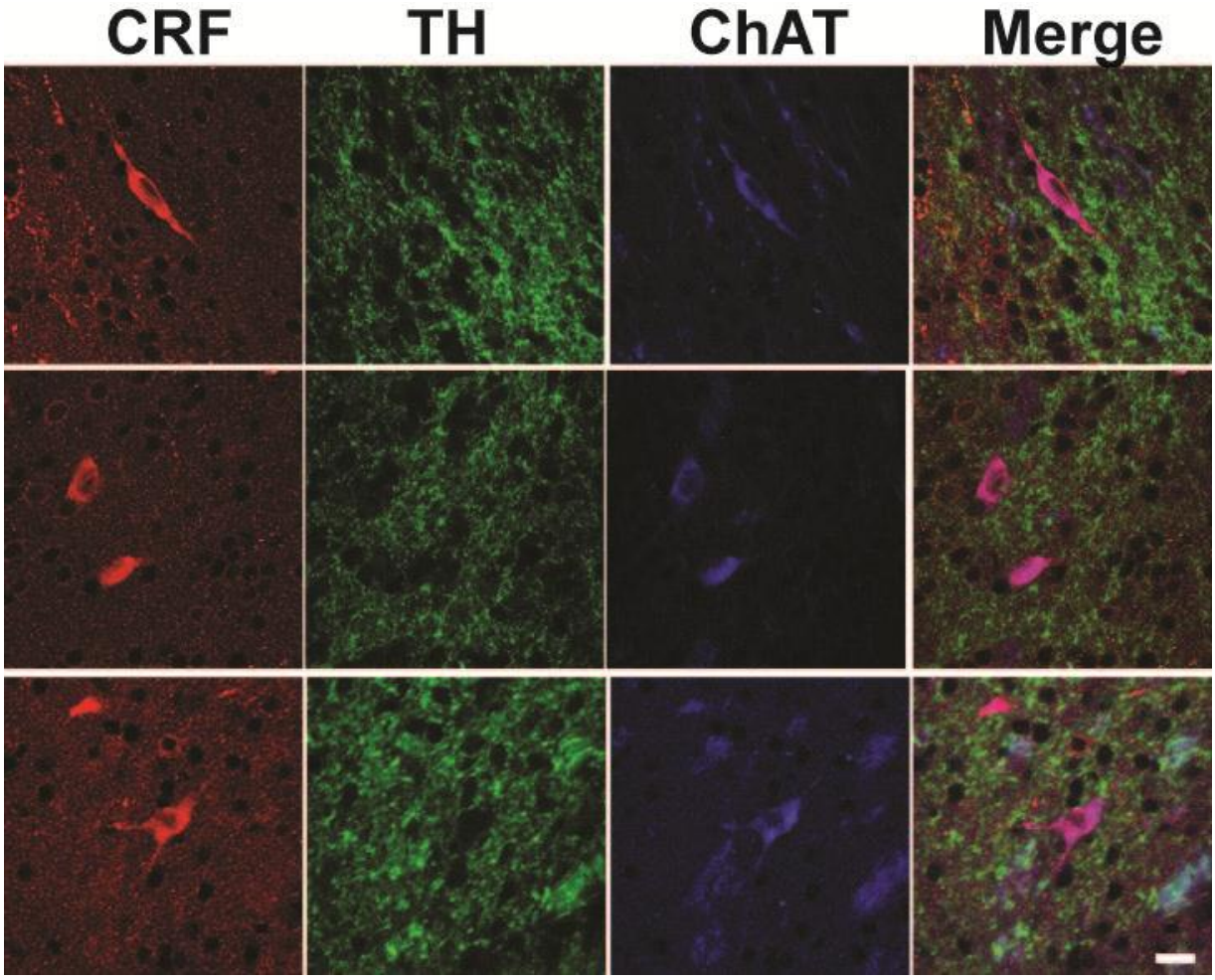
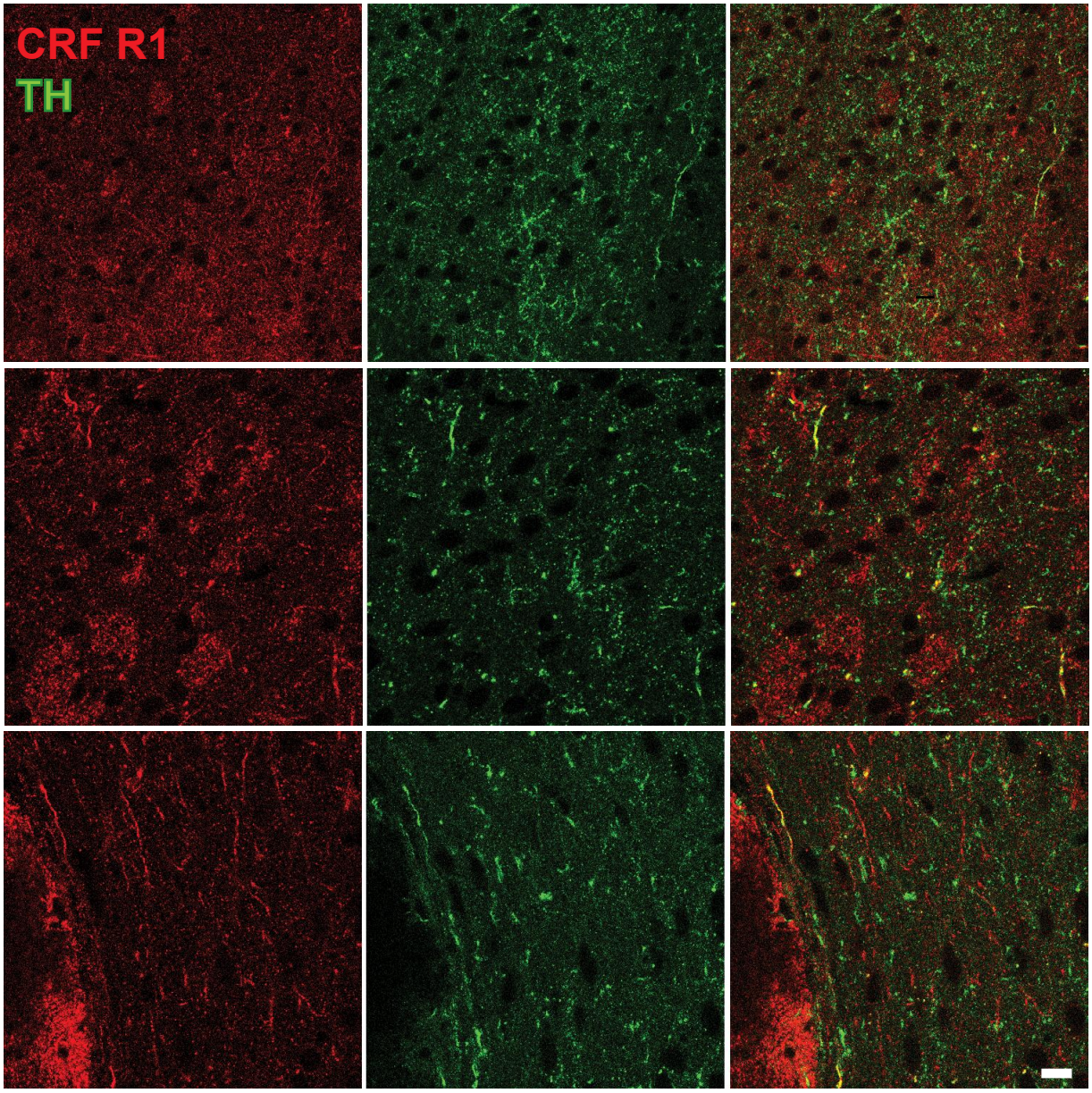
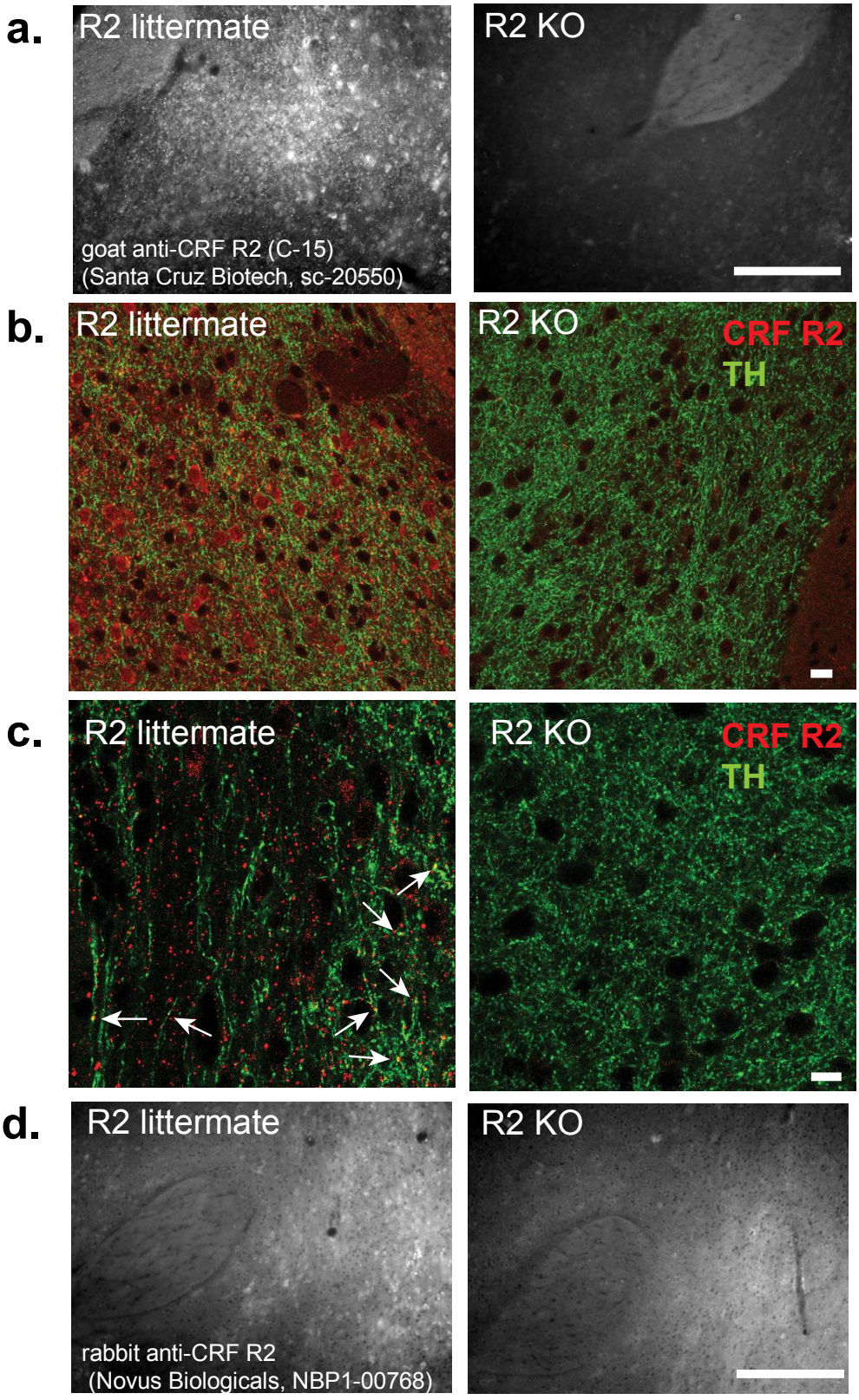
doi:10.1038/nature11436

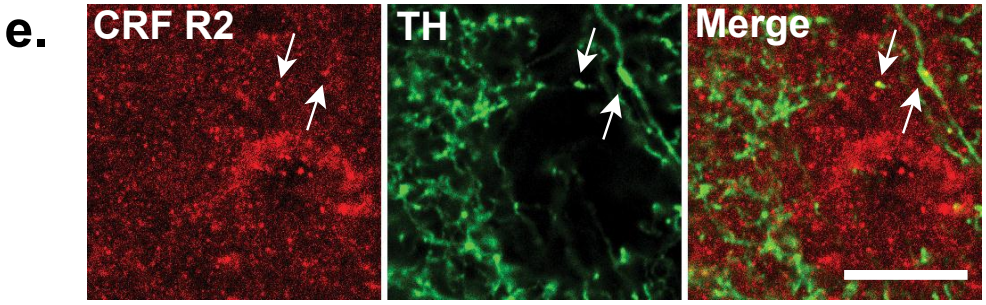


Supplementary Figure 1. CRF peptide is localized cholinergic interneurons throughout the nucleus accumbens. Immunofluorescent images demonstrating localization of CRF peptide, tyrosine hydroxylase (dopamine terminal marker) and choline acetyltransferase (ChAT) (acetylcholine marker) in the nucleus accumbens. CRF immunoreactivity was present in large cell bodies sparsely located throughout the nucleus accumbens. Three examples of confocal photomicrographs are shown, depicting CRF immunoreactivity (red) and ChAT immunoreactivity (blue) co-localization from three fields within the nucleus accumbens of three different mice. Scale bar = 20 μm.



Supplementary Fig 2. CRF R1 co-localization to TH positive fibers. Additional 60x images from two additional animals demonstrating co-localization of CRF R1-IR to TH-IR in the nucleus accumbens core. Scale bar = 10 μm.

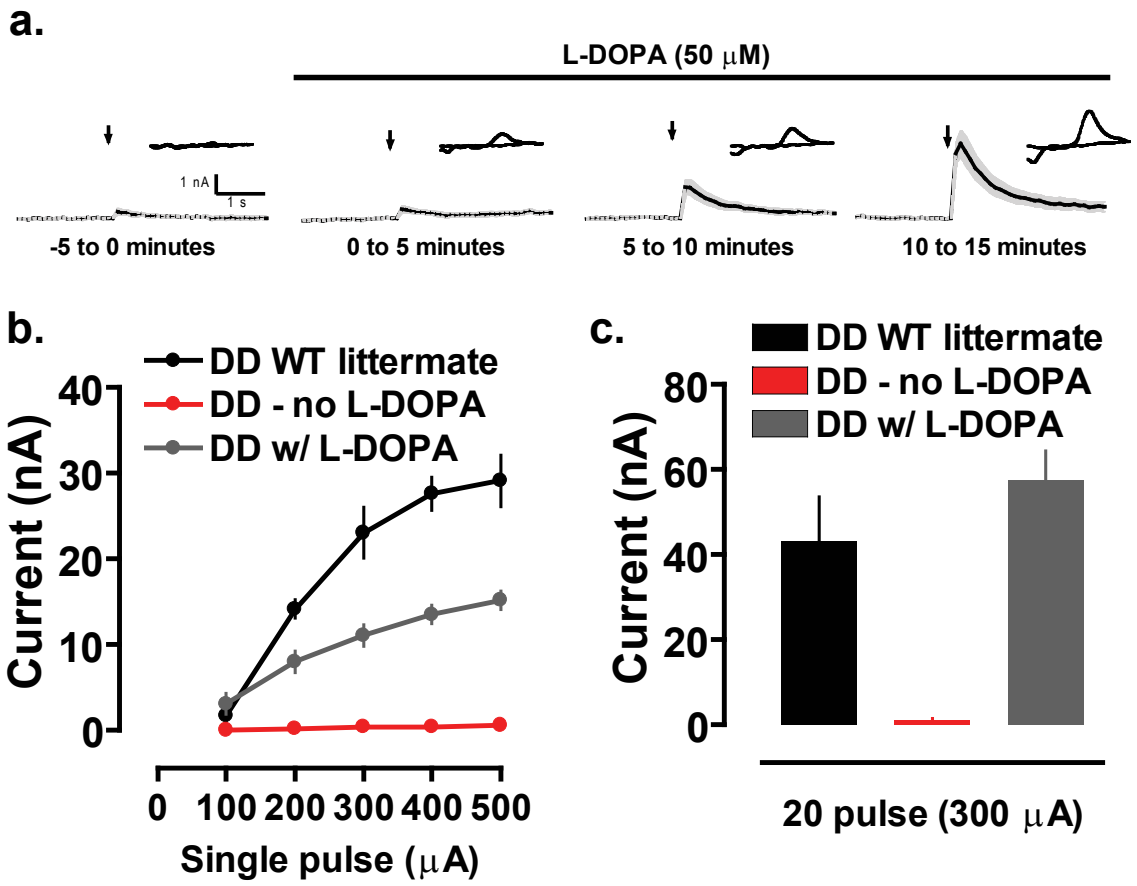

a.
R2 littermate
goat anti-CRF R2 (C-15)
(Santa Cruz Biotech, sc-20550)
R2 KO
b.
R2 littermate
R2 KO
CRF R2
TH
c.
R2 littermate
R2 KO
CRF R2
TH
d.
R2 littermate
rabbit anti-CRF R2
(Novus Biologicals, NBP1-00768)
R2 KO



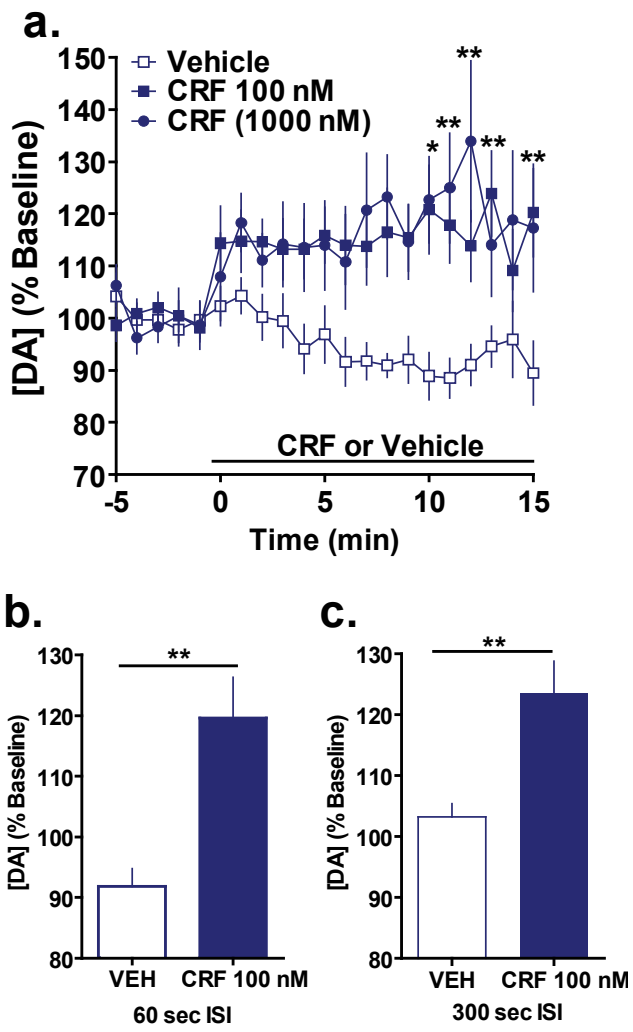
Supplementary Fig. 3. CRF R2 antibody validation and localization in the nucleus accumbens of WT littermate and R2 KO mice. The CRF R2 KO animals used in the study were derived from the original R2 KO line generated by Bale & Vale (2000) in which exons 10-12 have been deleted creating a functional CRF R2 KO mouse. Non-specific antibody staining in R2 KO mice may be a result of antibody recognition of an epitope on a non-functional truncated protein that has been translated or cross-reactivity of the antibody to another protein. Thus, we used antibodies that recognized an epitope of the c-terminal tail of the R2 protein (Santa Cruz sc-20550) that should be not translated and a Novus Biologicals antibody (NBP1-00768). Sections were prepared, processed and imaged in parallel (i.e. on the same days). **a**, Low power (20x) epifluorescent images of the nucleus accumbens of WT (left) and R2 KO (right) using the c-terminal R2 antibody (Santa Cruz sc-20550). Scale bar = 100 µm. **b**, High power (60x) confocal merged images demonstrating CRF R2 (red) and TH (green) localization in the nucleus accumbens of WT (left) and KO (right) animals. Using confocal imaging, there is virtually no detectable CRF R2 red immunofluorescence in the nucleus accumbens of KO mice. Scale bar = 10 µm. **c**, To eliminate false-positive identification of CRF R2, the threshold was set to a level that minimized labeling in the R2 KO. Under this stringent condition, red fluorescent puncta remain in the WT image. Scale bar = 10 µm. **d**, Low power (20x) epifluorescent images of the nucleus accumbens of WT (left) and R2 KO (right) using the Novus Biologicals R2 antibody (Novus Biologicals NBP1-00768). Scale bar = 100 µm. **e**, High power image (100x) demonstrating demonstrating co-localization of CRF R2 (Novus Biologicals NBP1-00768) with TH staining. The same pattern of staining seen in the c-terminal Santa Cruz antibody is also seen with the Novus Biologicals R2 antibody. Scale bar = 10 µm

	TH-axon terminals	CRFr in TH-at	Percentage
Mouse 1	299	41	13.71%
Mouse 2	367	38	10.35%
Mouse 3	390	75	19.23%
Total	1056	154	14.58%

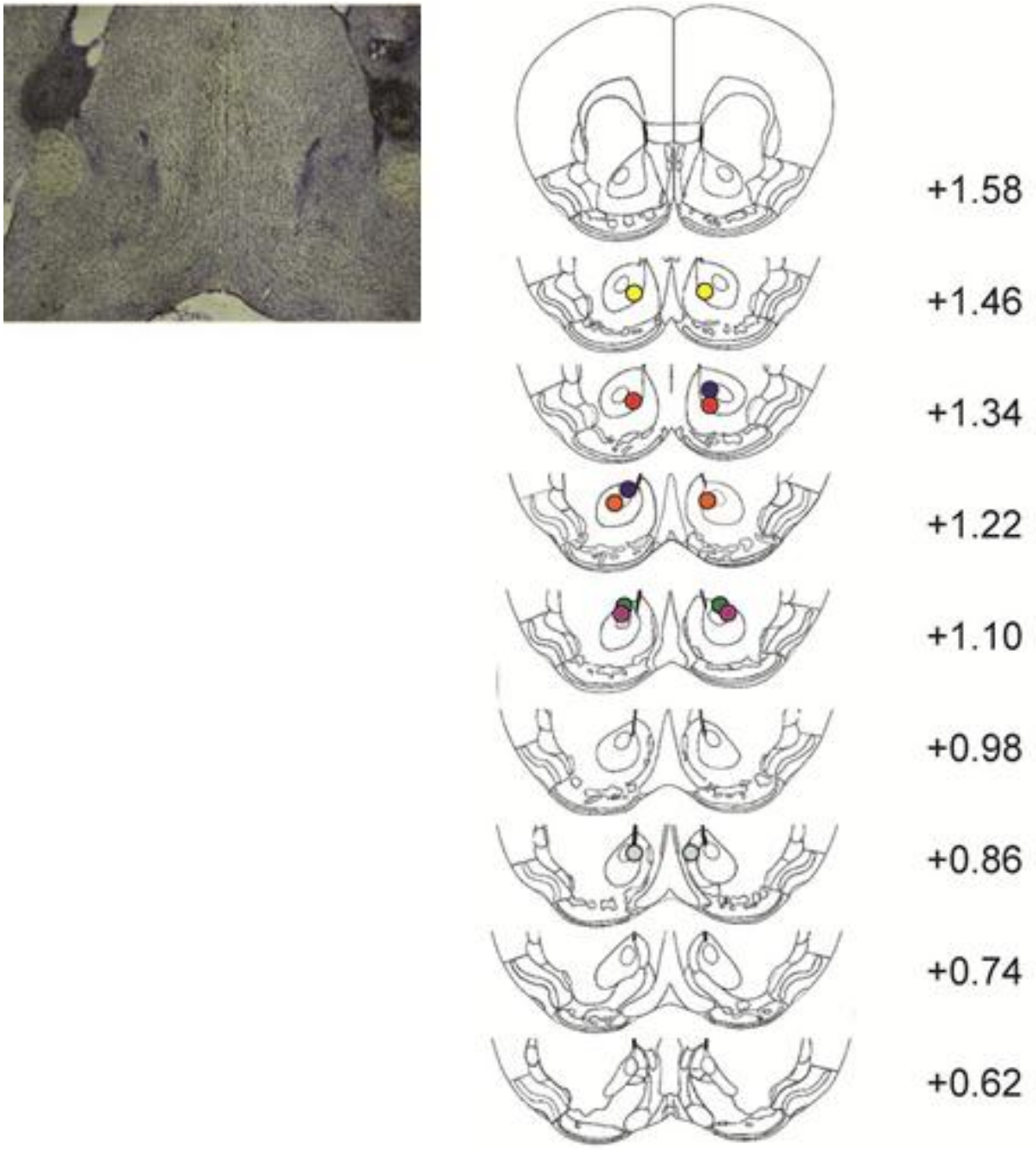
Supplementary Table 1. Quantification of EM labeling in 100-nm sections through the rostro-caudal axis of the nucleus accumbens to assess co-localization of CRF receptors and TH immunoreactivity. To estimate the proportion of dopamine terminals that express at least one CRF receptor, we used the equation $P = 1 - (1 - p)^n$ where p is the probability of observing a CRF-receptor immunogold particle per section of a TH-positive terminal and n is the number of sections per terminal. CRF receptor immunoreactivity was observed on 14.58 % of sections of TH-positive terminals (i.e., p = 0.1458). Given that dopamine terminals are approximately 1 μm in diameter, each total three-dimensional terminal profile occupies approximately ten 100-nm sections (i.e., n = 10). Therefore, we estimate that 80 % of dopamine terminals in the nucleus accumbens express CRF receptors.



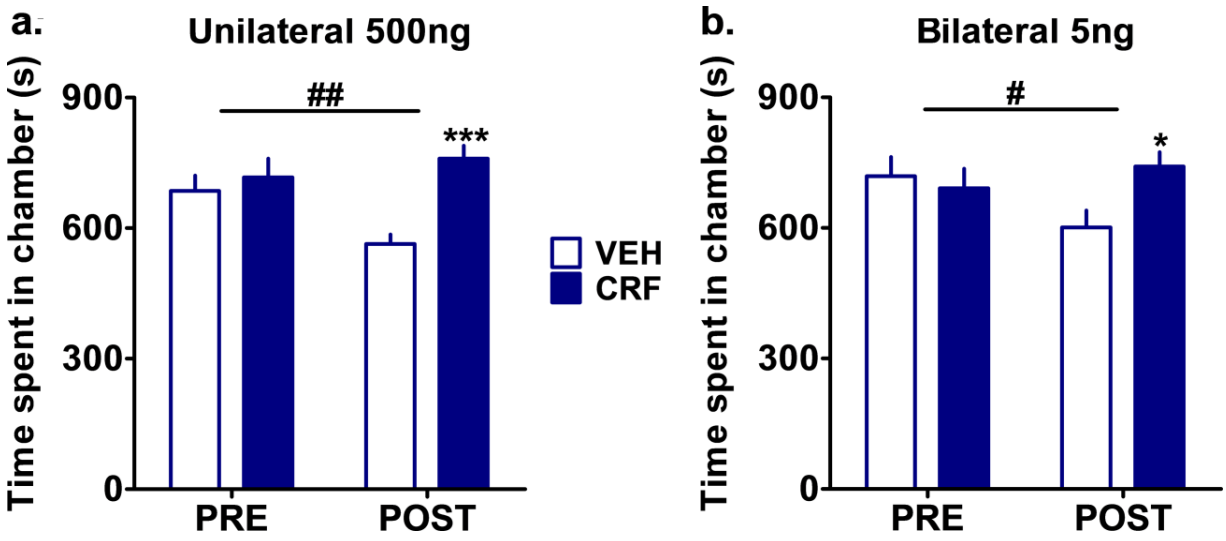
Supplementary Fig 4. Evoked electrical currents detected by carbon fiber electrodes placed in the nucleus accumbens core are solely attributable to dopamine release. Nucleus accumbens slices were prepared from "dopamine deficient" ($Th^{fs/fs}$; $Dbh^{Th/+}$) mice[36] or littermate control mice in parallel. Following a baseline input-output curve, a dopamine recovery experiment was carried out. Following this recovery experiment, another input-output curve was obtained. Following five baseline stimulations, stimulations once every minute continued while L-DOPA (50 μM) was applied to the slice. **a**, Averaged evoked responses before and after bath application of L-DOPA (50 μM). The insets are average cyclic voltammegrams (CV) corresponding to the averaged evoked response. As L-DOPA washes over the slice, the CV of the evoked response increasingly correlates with the stereotyped electrochemical fingerprint of dopamine. **b**, Input (electrical stimulation amplitude) – Output (subsequent current measured at the carbon fiber) for a single pulse stimulation in littermate control slices compared to slices from dopamine deficient mice before and after L-DOPA bath application. **c**, Evoked current elicited by a 20-p stimulation at 300 μA in slices prepared from littermate controls or slices from dopamine deficient mice either before or after L-DOPA bath application.



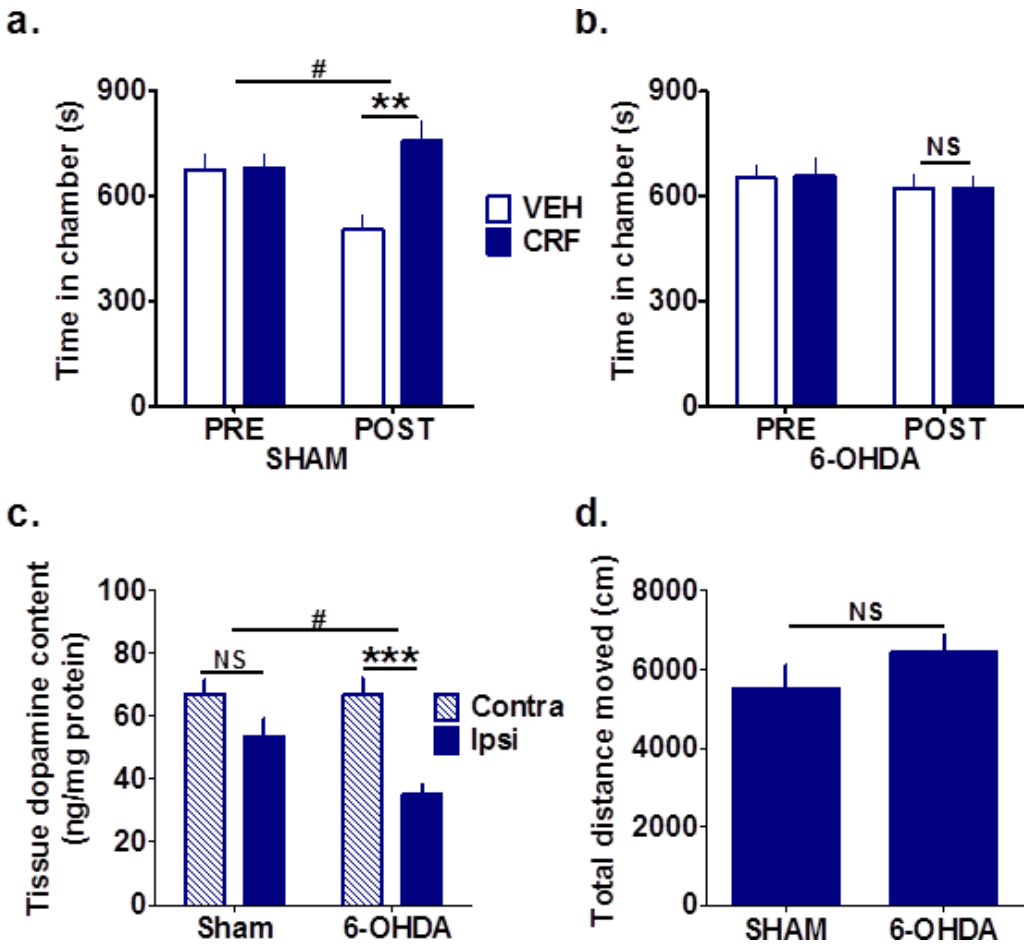
Supplementary Fig 5. Time course of CRF or vehicle effect on evoked dopamine release. a-b, A single bi-phasic stimulating pulse (100-500 μA) was applied to the slice once every minute, and dopamine release was measured at the carbon fiber electrode placed in the nucleus accumbens core. Following five stable baseline currents, CRF (100 nM, 1000 nM) or vehicle (0.07% acetic acid) was bath applied to the slice. There was a small (5-10%) depression in dopamine release over time apparent in the vehicle group ($p < 0.01$ vs. 100%). In contrast, CRF increased evoked dopamine release by 20% above baseline ($p < 0.001$ vs. 100%) and 27-30% above vehicle, n = 13 and 18 for vehicle and CRF experiments respectively (drug by time, $F_{20,560} = 2.994$, $p < 0.001$, two-way ANOVA). **c**, A significant increase of dopamine release (20.3%) following 100 nM CRF was also observed in the nucleus accumbens when the inter-stimulation interval was increased to 300 seconds, eliminating the run down under vehicle conditions ($p < 0.01$, t-test vs time-matched vehicle, n = 8-10).



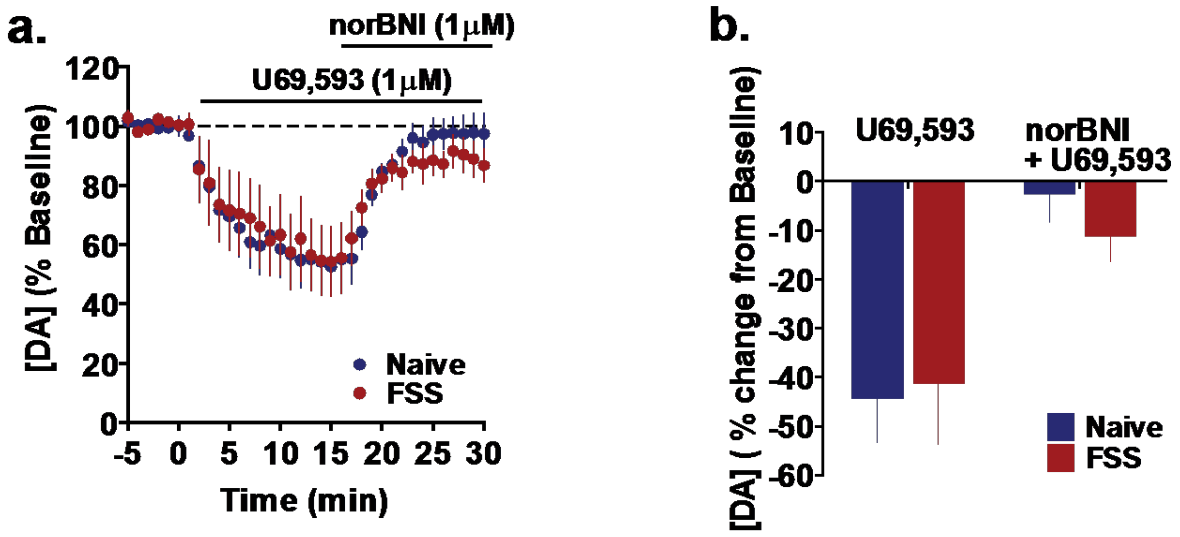
Supplementary Fig 6. Cannula placements for the place conditioning assay. Representative cresyl violet image from naïve cohort and cannula placements for each individual animal in the group.



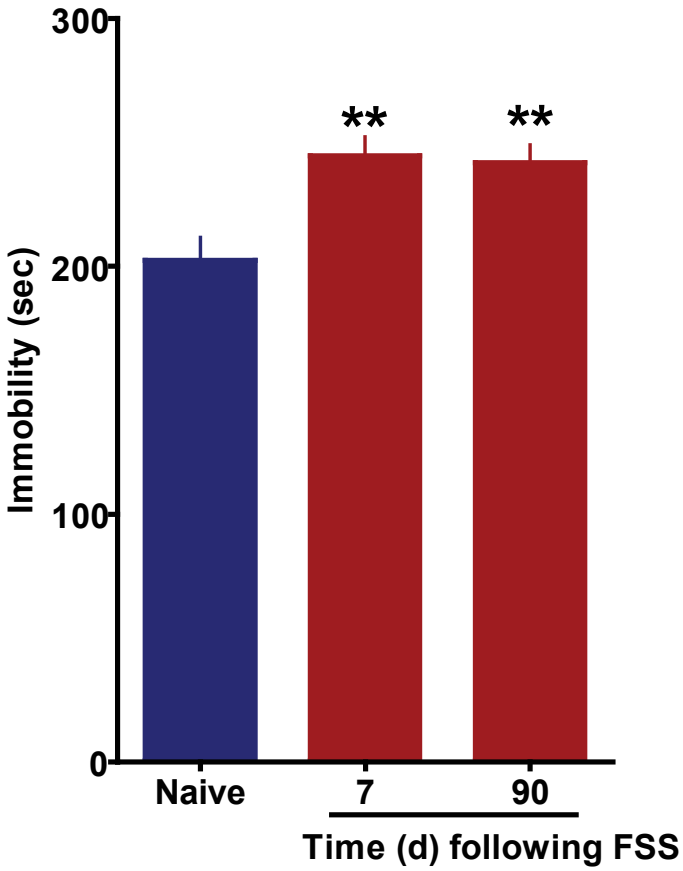
Supplementary Fig 7. Pre- and post-test times for CRF (500 ng) unilateral injections and CRF (5 ng) bilateral injections. a, CRF (500 ng) or VEH were administered unilaterally into the nucleus accumbens during conditioning days. Mice spent significantly more time in the CRF-paired chamber post-conditioning than the VEH paired chamber compared to pre-conditioning (conditioning by drug, $F_{1,12} = 11.77$, $p < 0.01$ two-way repeated measures ANOVA, n = 7 mice). **b**, CRF (5 ng) or VEH were administered bilaterally into the nucleus accumbens during conditioning days. Mice spent significantly more time in the CRF-paired chamber post-conditioning than the VEH paired chamber compared to pre-conditioning (conditioning by drug, $F_{1,14} = 5.415$, $p < 0.05$ two-way repeated measures ANOVA, n = 8 mice).



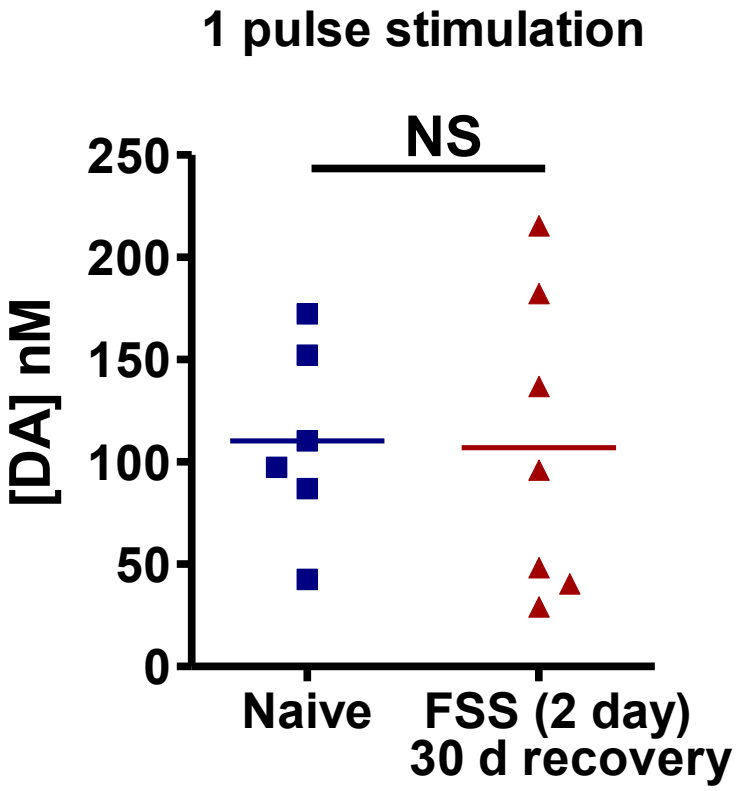
Supplementary Fig 8. Intra-accumbens dopamine depletion with 6-OHDA blocks conditioned place preference for Intra-accumbens CRF microinfusion. a, Sham vehicle injected animals significantly preferred the CRF paired chamber following conditioning (conditioning by drug, $F_{1,18} = 6.954$, $p < 0.05$, two-way repeated measures ANOVA with Bonferroni post-hoc tests). **b**, 6-OHDA injected animals did not demonstrate a preference for the CRF paired chamber following conditioning (conditioning by drug, $F_{1,18} = 0.004$, $p > 0.05$, two-way repeated measures ANOVA). **c**, Unilateral injection of 6-OHDA (2 µg/500 nl) into the nucleus accumbens significantly decreased tissue dopamine content compared to both the uninjected contralateral side assessed with HPLC on fresh frozen tissue in contrast to Sham vehicle (0.9% NaCl, 0.1% ascorbate) animals that did not show significant dopamine depletion (Drug by side, $F_{1,18}= 4.475$, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc tests). **d**, Unilateral 6-OHDA lesion did not effect locomotor activity compared to Sham injected animals ($p >0.05$, unpaired t-test). # $p < 0.05$ for interactions; NS $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ post-hoc tests. n = 10 for both Sham and 6-OHDA groups.



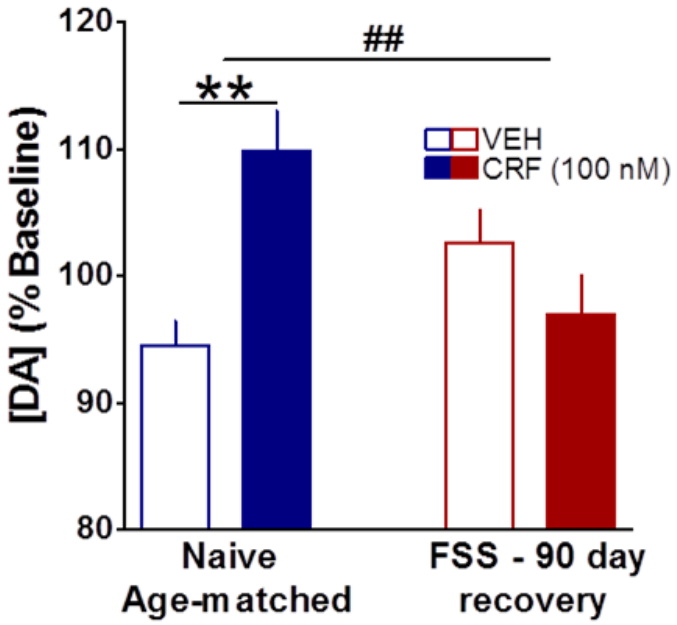
Supplementary Figure 9. Kappa opioid regulation of dopamine release in the nucleus accumbens is unaffected in mice exposed to swim stress. **a**, Time course demonstrating the effect of the kappa opioid receptor agonist U69,593 (1 μM) and subsequent reversal by the kappa opioid receptor antagonist norBNI (1 μM) on stimulated dopamine release in the nucleus accumbens core of naïve and stress-exposed mice. There were no significant differences in kappa opioid receptor mediated inhibition of dopamine release in slices from naïve versus stress-exposed animals (time by stress exposure, $F_{1,14} = 0.3508$, $p > 0.05$, two-way repeated measures ANOVA). **b**, Mean data showing the percent change in dopamine release from baseline in the last ten minutes following U69,593 application and the last ten minutes of norBNI reversal. U69,593 produced on average 44.08-% inhibition of dopamine release in nucleus accumbens of naïve animals and 41.12-% inhibition in stress-exposed animals, and in both cases was fully reversed by norBNI. There was no significant difference in mean responses between naïve and stressed groups ($p > 0.05$, Bonferroni post-hoc t-test, n = 6-10).



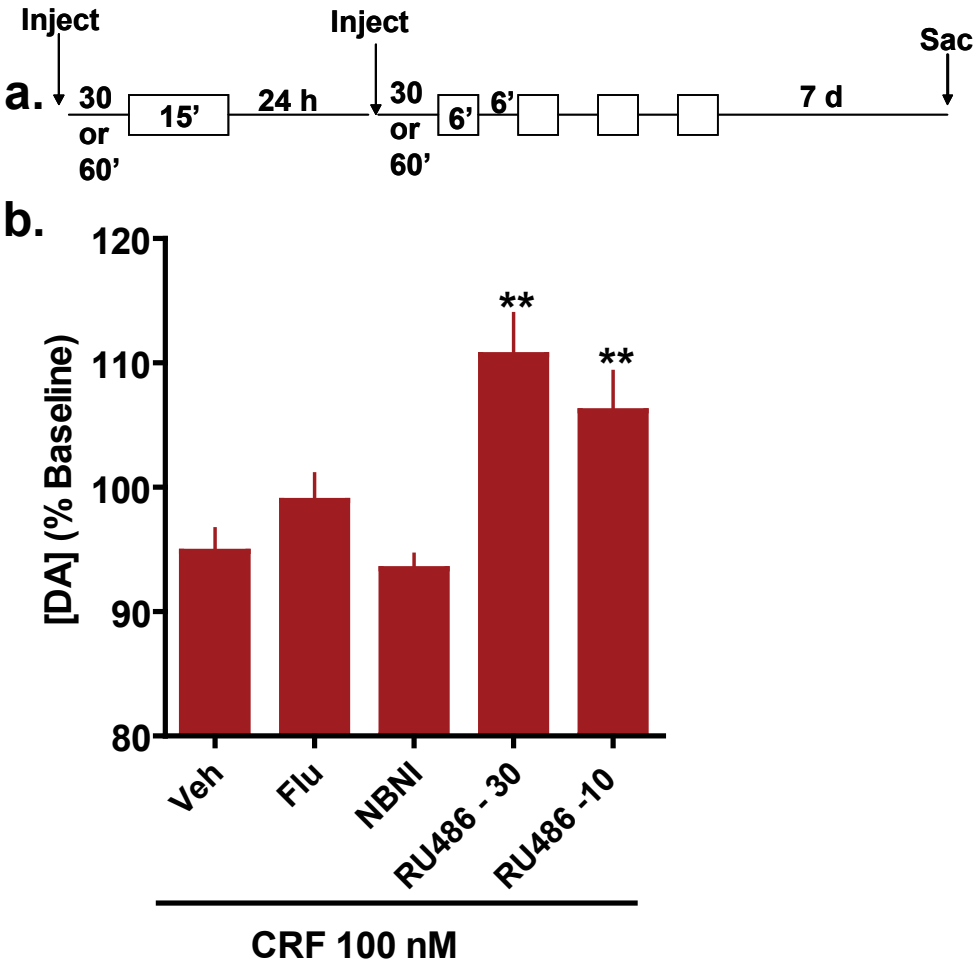
Supplementary Fig. 10. Animals displayed enhanced depression-like behavior compared to naïve animals up to 90 days following initial stressor exposure. Mice were given a five-minute forced-swim test either in the absence of prior stress history or 7 or 90 days following exposure to two-day swim stress. Compared to stress-naïve animals, animals exposed to swim stress 7 or 90 days prior showed significant immobility during the forced swim test indicating persistent depression-like behavior ($F_{2,25}$ = 8.287, $p < 0.01$, one way ANOVA with Neuman-Keuls.post-hoc t-test, n = 8-11).



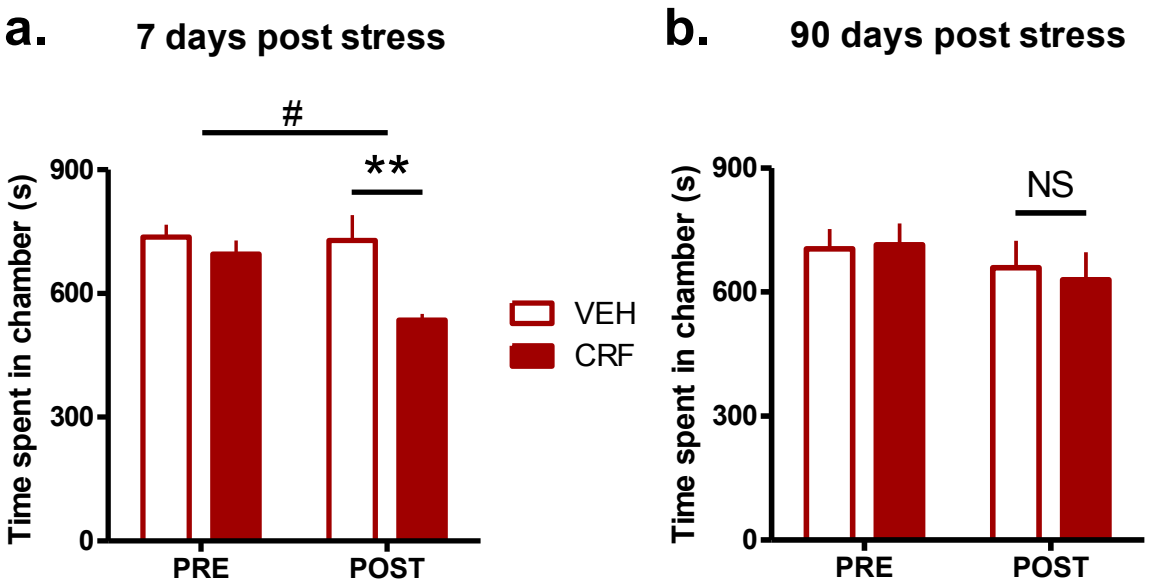
Supplementary Fig 11. Basal evoked dopamine release was not affected by stress exposure. Data were collected from nucleus accumbens slices prepared from naïve mice and stress-exposed mice that were allowed to recover for 30 days. A single-pulse electrical stimulation was delivered to three distinct sites in a nucleus accumbens slice and dopamine current was measured at each site. The evoked dopamine current for three sites was averaged. Following the conclusion of data collection, carbon fiber electrodes were calibrated using a flow cell system to 1 μM dopamine. Averaged current responses were converted to dopamine concentration. There were no differences in evoked dopamine release between naïve and stress-exposed animals (unpaired t-test, $p > 0.05$, $n = 6$-7).



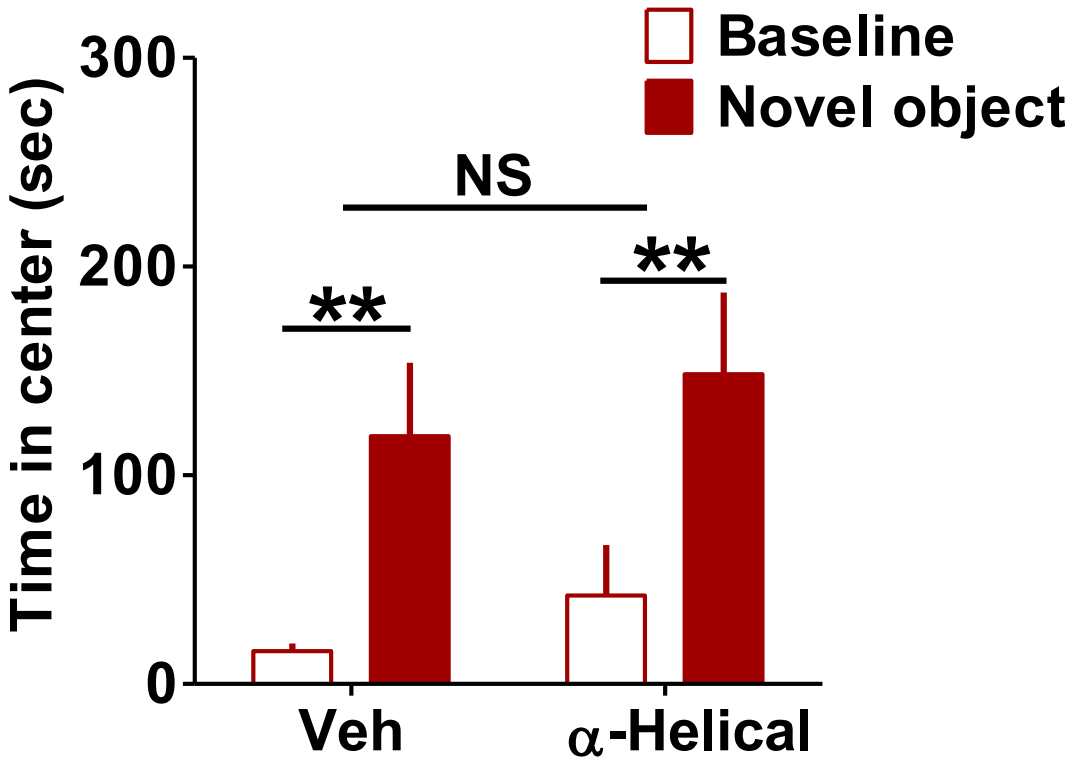
Supplementary Fig 12. Loss of CRF response following stress exposure is not age related. For all other experiments, animals were 60 -150 days old. However, stress-exposed animals allowed to recover for 90 days were >180 days old. To control for possible age-related effects on CRF responsivity, naïve-aged matched animals were interleaved (sacrificed and CRF response tested every other day) with stress-exposed animals allowed to recover for 90 days. Mice assigned to either the naïve or stress-exposed group were shipped on the same date and acclimated to the vivarium for the same amount of time. CRF significantly increased evoked dopamine release in nucleus accumbens slices from naïve age-matched mice compared to vehicle application, but had no effect on evoked dopamine release in the nucleus accumbens from stress-exposed animals that had recovered for 90 days compared to vehicle application (stress exposure by drug, $F_{1,42} = 10.97$, $p < 0.01$, two way ANOVA, n = 7-15).



Supplementary Fig 13. Pre-treatment with glucocorticoid receptor antagonist RU 486 prior to swim stress session protects CRF response. a, Mice were injected intraperitoneally (i.p.) with either vehicle (5-% DMSO, 20-% Cremophor dissolved in saline), norBNI (kappa-opioid-receptor antagonist, 10 mg/kg), fluoxetine (serotonin-selective reuptake inhibitor, 10 mg/kg) or RU 486 (glucocorticoid-receptor antagonist, 10 or 30 mg/kg) prior to each swim session. Mice were allowed to recover seven days following the last stressor exposure. **b**, CRF (100 nM) significantly increased dopamine release in slices prepared from mice pretreated with RU 486 (10 or 30 mg/kg) compared to mice pretreated with vehicle. ($F_{4,48}$ = 6.858, $p < 0.001$, one way ANOVA with Dunnett's post-hoc t-test compared to vehicle) but not mice pre-treated with fluoxetine or norBNI, ($F_{4,48}$ = 6.858, $p > 0.05$, one way ANOVA with Dunnett's post-hoc t-test compared to vehicle n = 10-11).



Supplementary Fig. 14. Pre- and post-test times for CRF conditioned place preference in mice exposed to two-day FSS. a, Mice that had been exposed to 2-day swim stress 7 days prior to conditioning did not show a conditioned place preference to the CRF-paired context but exhibited significant conditioned place aversion (conditioning by drug, $F_{1,10} = 5.824$, $p < 0.01$ two-way repeated-measures ANOVA with post-hoc Bonferonni t-tests, n = 6). **b**, Mice that had been exposed to 2-day swim stress 90 days prior to conditioning did not show a conditioned place preference to the CRF-paired context (conditioning by drug, $F_{1,14} = 0.1035$, $p > 0.05$, two-way repeated measures ANOVA, n = 8).



Supplementary Figure 15. Stress exposure abolishes CRF-dependent component of novel object exploration. Animals were exposed to two-day repeated swim stress 7 days prior to the first test day of the novel object exploration task. Identically to naïve animals, stress-exposed mice were given infusions of vehicle or α-helical CRF (500 ng) in a counter-balanced fashion across test days, prior to placement in an open field. While introduction of a novel object significantly increased center time in both drug conditions, there was not a significant drug interaction, indicating that stress exposure abolished the CRF-dependent component of novel object exploration (stimulus by drug, $F_{1,16} = 0.004$, $p > 0.05$, two-way repeated measures ANOVA, n = 9). ** $p < 0.01$ for Bonferroni post-hoc t-tests.

Supplementary Discussion

CRF receptors are distributed widely throughout the brain[8] and mediate disparate effects. For instance, CRF increases motor activity when administered locally into ventral tegmental area[37] or nucleus accumbens shell[38], but not the prefrontal cortex[39], nucleus accumbens core[38] or bed nucleus of the stria terminalis[40], and can even elicit freezing behavior when injected into the periaqueductal gray[41], basolateral or central nucleus of the amygdala[42]. Likewise CRF produces conditioned place aversion when infused into the bed nucleus of the stria terminalis[40] or following intracerebroventricular administration[16,43], yet we demonstrate that direct application to the nucleus accumbens produces conditioned place preference in naïve animals. This local effect of CRF in the nucleus accumbens is not surprising given the regulatory role on dopamine that we characterized, as dopamine agonist administration alone is sufficient to produce conditioned place preference[44-46]. Indeed, it was abolished by local dopamine depletion confirming the requirement for CRF to regulate dopamine in mediating this behavior. Furthermore, we demonstrate that endogenous CRF is present in the nucleus accumbens and promotes appetitive behavior towards arousing stimuli. Therefore, our data highlight the specificity of the local action of both exogenously applied and endogenously released CRF in the nucleus accumbens in producing a positive, rather than negative, subjective state.

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