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Impairment of synaptic plasticity by the stress mediator CRH involves selective destruction of thin dendritic spines via RhoA signaling

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Abstract

Stress is ubiquitous in modern life and exerts profound effects on cognitive and emotional functions. Thus, whereas acute stress enhances memory, longer episodes exert negative effects through as yet unresolved mechanisms. We report a novel, hippocampus-intrinsic mechanism for the selective memory defects that are provoked by stress. CRH (corticotropin-releasing hormone), a peptide released from hippocampal neurons during stress, depressed synaptic transmission, blocked activity-induced polymerization of spine actin and impaired synaptic plasticity in adult hippocampal slices. Live, multiphoton imaging demonstrated a selective vulnerability of thin dendritic spines to this stress hormone, resulting in depletion of small, potentiation-ready excitatory synapses. The underlying molecular mechanisms required activation and signaling of the actin-regulating small GTPase, RhoA. These results implicate the selective loss of dendritic spine sub-populations as a novel structural and functional foundation for the clinically important effects of stress on cognitive and emotional processes.

Keywords

CRF; CRF receptor; spine; synapse; synaptic plasticity; memory

Introduction

Stress, generally defined as a signal conveying potential threat,[1–6] is a powerful and evolutionarily conserved modulator of synaptic plasticity and memory.[1–3,5] Stress influences the hippocampus, with consequences that contribute to a number of stress-related cognitive and affective disorders.[4,6–11] Within hippocampus, whereas acute stress (lasting seconds to a few minutes) enhances memory and long-term potentiation (LTP), stress lasting longer generally impairs learning and memory tasks considered hippocampus dependent.[1–3,12]

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Conflict of interest
The authors declare no conflict of interest.

Intensive studies into the causes of these effects have focused primarily on the roles of adrenal-derived steroid stress hormones and their signaling via glucocorticoid receptors and, more recently, mineralocorticoid receptors.[1–3,5,13–15] Interestingly, mineralocorticoid receptor activation enhances rather than depresses synaptic plasticity,[3,13] whereas glucocorticoid receptors are only weakly expressed in key hippocampal subdivisions.[16,17] These observations have prompted a search for additional factors that may contribute to the effects of stress on hippocampus-dependent cognitive functions.

The neuropeptide CRH (corticotropin-releasing hormone) is synthesized within interneurons residing in the pyramidal cell layer of adult hippocampus,[18] a brain region crucial for several types of memory.[19–21] The peptide is released during stress to activate receptors located on dendritic spines of pyramidal cells (as well as on somata and dendrites).[22] Here we report that short treatments with CRH at concentrations occurring during stress produce profound but selective effects on the structure and plasticity of hippocampal neuron connectivity. CRH impaired synaptic plasticity and selectively reduced the density of small PSD-95 (postsynaptic density protein 95)-expressing synapses. Live multiphoton imaging of yellow fluorescent protein (YFP)-expressing adult hippocampi indicated that, rather than shifting the size/shape profile of populations of dendritic spines, CRH *directly* affected thin spines in a time-dependent manner. The selective disruptive actions of CRH on thin spines depended on the activation of a specific signaling cascade, the RhoA pathway, which exerts a potent regulatory effect on the sub-synaptic cytoskeleton. These results point to a new mechanism for the impaired operation of hippocampal networks during episodes of stress, suggesting novel targets for preventing stress-related cognitive problems.

Materials and methods

Animals

C57BL as well as B6.Cg-TgN transgenic mice, 3–4 months old, expressing YFP under the *Thy1* promoter (Thy1-YFP; Jackson Lab, Bar Harbor, ME, USA) were used. Electrophysiological studies were performed using 6–10-week-old Sprague-Dawley rats (Harlan, Placentia, CA, USA). Only males were used to avoid cyclic fluctuations of spine densities that occur in females.[23] Animals were maintained in a quiet, uncrowded, National Institutes of Health (NIH)-approved facility on a 12-h light/dark cycle, with *ad libitum* access to lab chow and water. All experiments were approved by University Animal Care Committees and conformed to the NIH guidelines.

Slice preparation and infusion of CRH and ROCK blocker

Hippocampal slices were prepared as described previously.[24,25] Briefly, after decapitation, brains were removed and placed in artificial cerebral spinal fluid (room temperature) containing 126 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose and 20 mM sucrose. A block of tissue including entorhinal cortex and hippocampus was dissected from adhering tissue. Transverse slices (300 μm) were cut (McIlwain tissue chopper; Mickle Lab, Gomshall, UK), and maintained in an infusion chamber (at 32 °C with 95% O_2/5% CO_2) with constant 30–40 ml min^{-1} artificial cerebral spinal fluid perfusion. Synthetic rat/human CRH (100 nM; Bachem, King of Prussia, PA, USA), the ROCK (Rho-associated protein kinase) blocker H1152 (100 nM, EMD, Gibbstown, NJ, USA), CRH + H1152 or vehicle were infused for 60 min (0.5 ml min^{-1}). After a brief normal medium washout, the slices were quickly frozen and collected for pull-down analysis or fixed in fresh 4% paraformaldehyde (45–60 min) for immunocytochemistry. Each experiment included equal numbers of slices for each group.

Immunocytochemistry

Hippocampal slices were fixed in fresh 4% paraformaldehyde for 45 min and processed for sub-sectioning (20 μm) using a cryostat. Immunocytochemistry with or without tyramide signal amplification was performed on free-floating sections.[18,22,26] Antibodies included mouse anti-GFP (1:8000; Sigma, St Louis, MO, USA), mouse anti-PSD-95 (1:2000; Affinity BioReagents, Golden, CO, USA) and rabbit GluR1 (1:1000; PC246, Calbiochem, San Diego, CA, USA). The latter recognizes the extracellular moiety of the receptor subunit. Antibody binding was visualized with anti-mouse IgG conjugated to Alexa Fluor 488 (1:200; Molecular Probes, Eugene, OR, USA) or Cyanine 3 Tyramide (1:150; PerkinElmer, Waltham, MA, USA). Sections from all experimental groups were run concurrently in the same conditions, and analyzed without knowledge of treatment group.

Electrophysiology

Transverse hippocampal slices were cut at 350 μm using a McIIwain chopper and placed in ice-cold cutting solution containing 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 5 mM $MgSO_4$, 26 mM $NaHCO_3$ and 10 mM dextrose. Slices were then transferred and maintained in an interface recording chamber (at 32 1C with 95% O_2/5% CO_2) with constant 60–70 ml min^{-1} perfusion with artificial cerebral spinal fluid containing 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM dextrose.[27] Field electrophysiology was performed using a single bipolar stimulating electrodes (65 μm twisted nichrome wire) placed in mid-proximal stratum radiatum of CA1a to stimulate independent populations of synapses recorded with a glass electrode (2 M NaCl) in CA1b.[27] Baseline responses were set at 40–50% of maximum spike-free field excitatory postsynaptic potentials (fEPSPs). LTP was induced using theta burst stimulation (TBS; 10 bursts of four pulses at 100 Hz with 200-ms interburst intervals). The fEPSP slope values in figures and text are measured as 10–90% fall and are normalized to 10 min before drug infusion or the TBS. Slices used for microscopic analysis were collected 10 min after TBS and fixed in 4% paraformaldehyde in 0.1 M of sodium phosphate buffer. The n values given in the figures represent slices per group.

Live imaging studies

To study the dynamic effects of CRH on dendritic spines, YFP-expressing slices were transferred to a superfusion chamber maintained at 36 °C via a heating platform (Warner Instruments, Hamden, CT, USA) to avoid hypothermia-related spine loss; this resulted in a tissue temperature of ~34 °C. The chamber was mounted on an upright Zeiss microscope, and slices were superfused with culture medium saturated with 95% O_2 and 5% CO_2. CRH (100 nM) or vehicle was infused for up to 45 min (1 ml min^{-1}) followed by a normal medium washout. When used, the ROCK blocker H1152 (100 nM) was infused 5–10 min before CRH application. In addition, the potential effects of H1152 itself on spines were evaluated by infusing the compound for 30 min. To avoid phototoxicity and enable imaging deep within the tissue, dendrites of YFP-expressing CA1 pyramidal neurons were imaged using a Zeiss LSM710 Meta two-photon scanning system with excitation wavelength 920 nm (Zeiss, Oberkochen, Germany). Based on the established localization of the effects of stress and CRH,[18,25] we focused on the apical dendrites of CA1 pyramidal cells and obtained *z* stacks (12–20 optical slices, 2 μm per slice) of 100 μm long dendritic segments of the main and oblique branches starting around 30–100 μm within stratum radiatum.

Spine analysis

The analysis was performed using two independent methods. (1) Image analysis of YFP-labeled spines (Figure 2b).[22,25] Briefly, YFP-expressing individual CA1 pyramidal neurons were reconstructed and drawn using a Zeiss LSM Image Browser and Adobe Photoshop (V.

CS5). Neurons were chosen using systematic unbiased sampling from the rostral third of the anterior/posterior extent of the hippocampus (9–15 neurons per group; 3–5 independent experiments). Dendritic protrusions including spines and filopodia were identified[28–32] and reconstructed. Spine density was quantified comparing dendritic branches of the same order. Spine density was expressed as the number of spines per 10 μm of dendrite length. No correction factors were applied to the spine counts because high-magnification neuronal reconstruction permitted all spines of a given dendritic segment to be visualized. (2) Deconvolution analysis of wide-field three-dimensional images.[33] Briefly, z-series (0.2 μm steps) images were captured from the stratum radiatum in CA1 area at ×63 (NA 1.4) using a Leica microscope (DM6000, Wetzlar, Germany) and a charge-coupled device camera (Orca ER; Hamamatsu Photonics, Shizuoka, Japan). These images were collected from six sections per group per experiment in 3–5 experiments. Iterative deconvolution (99% confidence) was performed using the Volocity 4.1 software (PerkinElmer). Labeled synaptic structures were measured and counted from a 136 × 105 3 μm (x, y, z) sampling zone. Object area (> 0.04 and < 1.2 μm^3) and eccentricity criteria were applied to eliminate artifacts.

In situ *labeling of F-actin*

Alexa Fluor 568–phalloidin (6 mM in pipette; Invitrogen, Grand Island, NY, USA) was applied to slices (four 3-min intervals) at the end of the experiment, as described.[27,34] Values in the figure and text are presented per 550 μm^2 from collapsed 10 μm *z* stacks.

Hippocampal neuronal cultures

Primary hippocampal slice cultures were prepared as described in detail previously.[35] Briefly, hippocampi from postnatal 0 (P0) Sprague-Dawley rats were quickly dissected, removed from adherent meninges and incubated for 30 min in buffered salt solution containing 10 U ml^{-1} papain (Worthington, Lakewood, NJ, USA). After removal of papain, cells were mechanically triturated and plated at a density of 400–600 cells μm^{-2} on 12 μm coverslips that were precoated with poly-D-lysine (Sigma). Cultures were initially maintained in Neurobasal Medium (NBM) with B-27 supplement (Gibco, Grand Island, NY, USA) at 36 °C, 5% CO_2. At 3–4 h after plating, half of the culture medium was replaced with Neurobasal/B-27-based medium that was preconditioned for 24 h by 1- to 2-week-old nonneuronal cell culture prepared from P3–P4 rat cortices. Cultures were subsequently refreshed every 3–4 days with the conditioned medium. On the third day *in vitro*, 1 mM Cytosine-Arabinoside (Invitrogen) was added to the culture media to inhibit glial proliferation. The effects of CRH on dendritic spine density were assessed on 17–21 days *in vitro*. Spine density was assessed after immunocytochemistry for PSD-95 as described above.

Pull-down assays for activated Rho GTPases

Samples (6–10 pooled slices) were homogenized in Mg^{2+} lysis buffer (Millipore) with complete protease inhibitor cocktail, normalized and then assayed using RhoA or Rac/cdc42 pull-down kits (Millipore, Billerica, MA, USA), as described.[33] Samples were incubated with assay reagents for Rac/cdc42 (PAK1 PAK-PBD agarose) or Rho (Rhotekin-RBD agarose) and rocked for 2 h. Rhotekin binds strongly to RhoA and RhoC (absent in brain), so that RhoA is the only form detected in assays employed here. Agarose beads were collected by centrifugation (for 10 s at 14 000 g at 4 °C), washed, resuspended in Laemmli buffer and boiled for 5 min. Western blot analysis (12% SDS-polyacrylamide gel electrophoresis) used mouse anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Rac (Millipore) or rabbit anti-cdc42 (Millipore). GTP and GDP loading controls were incubated with 100 mM GTP-γS or 1 mM GDP for 30 min at 30 °C.

Western blot analysis

Samples (two to three pooled slices) were homogenized in radioimmunoprecipitation assay buffer containing 10 mM Tris, pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, 1% Triton X-100, 1× complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and phosphatase inhibitor cocktails 1–2 (Sigma-Aldrich, St Louis, MO, USA).[33] Samples were normalized by Bio-Rad (Hercules, CA, USA), protein assay and processed for western blot analysis (12% SDS-polyacrylamide gel electrophoresis) using rabbit antisera to RhoA, Rac 1 and cdc42 (Cell Signaling Technology, Beverly, MA, USA) and the ECL Plus detection system (GE Healthcare, Piscataway, NJ, USA). Blots were re-blotted (Millipore) and probed for β-tubulin and β-actin (Sigma-Aldrich). Immunoreactive bands were measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). The *n* values represent the numbers of samples tested.

Statistical analysis

All values in figures, figure captions and text represent group means ± s.e.m. For quantification of immunopositive elements and western blots, statistical significance ($P \leq 0.05$) was determined by analysis of variance followed by Tukey's *post hoc* test or two-tailed Student's *t*-test using SPSS 15.0 (SPSS, Chicago, IL, USA) or PRISM 5 (La Jolla, CA, USA). Curve fitting and statistical tests were performed using Matlab 7 (Natick, MA, USA).

Results

Prolonged treatment with 'stress levels' of CRH impairs synaptic function

Stress lasting longer than seconds or a few minutes generally leads to memory problems that involve the hippocampus[1–3,6,12,14,22] and to related abnormal synaptic plasticity.[1,14,22] Therefore, we tested if treatment with CRH for similar durations produces comparable effects, cognizant of the fact that *in vivo*, generation of high concentrations of CRH at dendritic spines likely requires sustained stress-induced release for some time, unlike the *in vitro* condition. Infusion of the hormone onto adult hippocampal slices had pronounced effects on synaptic physiology. Field EPSPs began to decline ~75 min into the infusion period by 21% (to 79% of preinfusion baseline values), whereas responses in slices from the same animals treated with a control peptide (adrenocorticotropic hormone (ACTH) 100 nM) and in non-treated slices were stable across this period (Figure 1a). Notably, the waveforms of the fEPSPs in the CRH-treated slices were not noticeably different from those recorded before hormone infusion. Short-term potentiation induced by one train of naturalistic TBS[36] was greatly reduced in the CRH-treated slices (Figure 1a). The initial enhancement of fEPSPs at 2 min after TBS, normalized to the fEPSP values collected for 10 min before delivery of the stimulation bursts, was 133 ± 5% (mean ± s.e.m.) in control slices and 95 ± 5% in the CRH-treated cases ($P = 0.0015$, two-tailed *t*-test). A comparable difference was found at 10 min after TBS: potentiation was 90 ± 6% above baseline for controls and 56 ± 5% for the CRH slices ($P = 0.006$). The control peptide, ACTH, did not influence potentiation. Thus, the STP process leading to persistent potentiation was severely impaired in the CRH-treated group.

Polymerization of actin within dendritic spines carrying excitatory synapses occurs within 2 min of the patterned stimulation that promotes synaptic potentiation, and contributes critically to maintaining the potentiated state.[24,37,38] Therefore, we examined actin polymerization following TBS and found that it was greatly reduced in CRH-treated slices relative to controls (Figure 1b). This suggested that the observed actions of CRH on synaptic responses and potentiation were associated with disruption of the actin cytoskeleton.

Selective reduction of thin dendritic spine density by CRH

To investigate the basis of the depression of fEPSPs in CRH-treated slices, we evaluated the actions of the peptide on dendritic spines that carry the postsynaptic elements of excitatory synapses in hippocampal CA1 (Figures 1c–e). We initially carried out a dose-finding study of CRH, aiming to approximate levels considered by others to be released during stress.[39,40] Exposure to 30 n_M CRH had an insignificant effect (not shown), whereas 60 and 100 n_M CRH reduced spine density of hippocampal neurons in a dose-dependent manner (Figure 2a). Therefore, we employed 100 n_M CRH for the subsequent studies. A 1-h infusion of 100 n_M CRH onto hippocampal slices prepared from adult YFP-expressing mice caused a significant decrease in the overall density of spines on pyramidal cell dendrites in CA1 stratum radiatum (Figures 1c–e), consistent with reports for field CA3.[22] Closer analysis showed that this effect was pronounced for the large apical branches (Figure 1c), and also involved the oblique branches (Figure 1d). The net effect across the entire sampling field was ~22% ($P < 0.01$; Figure 1e).

It is reported that morphological and functional changes that accompany memory processes take place in specific types of dendritic spines, and especially in thin spines.[30–32,41] Therefore, we asked if the CRH-provoked losses were restricted to particular subtypes of spines. For consistency across measurements, we focused the analysis on apical oblique branches of comparable size. CRH caused a strongly selective loss of thin spines in YFP-expressing adult mice (Figures 2b–d). The selective reduction of thin spines was reflected in a change of the percent contributions of thin and mushroom-type spines to the overall spine population (Figure 2e). Whereas mushroom-type spines were minimally affected by CRH (Figure 2d), there was a significant increase in the relative contribution of these spines, which are not considered to undergo further changes upon LTP induction, to the overall spine population. Commensurately, there was a major reduction in the availability of thin spines. The surprisingly discrete effect on thin dendritic spines also confirmed that the application of CRH does not produce global injury in adult pyramidal neurons.

CRH directly affects discrete sub-populations of dendritic spines

Spine shape and size are dynamic.[30–32] Thus, the above results, showing reduced numbers of thin spines after exposure to the stress-hormone CRH, could arise from either a CRH-induced transformation of thin spines into mushroom-type spines[30–32] or from a direct destruction of thin spines. To distinguish between these possibilities, we conducted experiments using live, multiphoton microscopy of hippocampal slices from YFP-expressing adult mice, spanning the duration in which CRH exerts its physiological effects. These experiments demonstrated the basic stability of both thin (arrows) and mushroom-type (arrowheads) spines under the conditions of the experiment (~75 min; Figures 3a and b). CRH (100 n_M) led to selective and progressive disappearance of thin spines over the same time course (Figure 3c). These data indicate that rather than potentially shifting the size/shape profile of populations of dendritic spines, CRH directly affected a distinct sub-population.

Loss and size shift of synapses accompany disappearance of thin dendritic spines

If the loss of thin spines, found using direct visualization of YFP-expressing adult mice, accounts for the observed depression of synaptic responses, then it should be accompanied by a measurable loss of synapses. We tested this prediction using an independent method, wide-field three-dimensional deconvolution microscopy followed by immunostaining for PSD-95, an integral protein of glutamatergic synapses.[22,24,30,41] Previously published work has demonstrated the identity of PSD-95-labeled puncta of precisely defined size and shape as synapses in hippocampal stratum radiatum.[24,33,42] CRH caused an ~28% reduction in excitatory synapses (Figure 4b), a value similar to that for the net loss of spines in the same

dendritic subfield (Figure 2d). Plotting the size distribution of the PSD-95-expressing puncta demonstrated a prominent reduction of the small (0.1–0.2 μm) puncta in CRH-treated slices (Figure 4c), and a distinct preservation of the large ones (Figure 4c, inset). This suggests that the population remaining after CRH application was enriched with spines with large heads and thus large synapses.[24,30,32,41,43] Together, the results from two very different measurements support the conclusion that prolonged exposure to nanomolar concentrations of the hippocampal stress hormone CRH reduces the number of thin spines carrying small synapses.

It is generally considered that thin spines, and small synapses, are relatively poor in the amino-3-hydroxy-5-methyl-4-isoxazole propionic (AMPA) receptor subunit GluR1.[44–47] When these synapses and spines receive patterned afferent stimulation, they may undergo structural and functional plasticity that contributes to the process of memory;[48,49] that is, synapses expand[24,30,31,41,43] and GluR1 receptors are added,[30,44,46,47] concomitant with growth and transformation of thin spines into larger, mushroom-type spines.[31,32,41] These facts predict that selective removal of thin spines, as described here, should have relatively little effect on GluR1-immunoreactive clusters in the size range of synapses. We tested this point using antisera selective to surface GluR1 followed by quantitative immunofluorescence microscopy. We found that the ratio of GluR1-labeled/PSD-95-labeled puncta was ~0.5 (Figures 4b and d), consistent with the percentage of mushroom-type spines (Figure 2e). In accord with the apparent selective effects of CRH on thin spines found above, a 1-h infusion of the peptide onto adult hippocampal slices did not reduce the number of visible GluR1-labeled puncta in the size range of synapses (Figure 4d), nor did it alter the size profile of these puncta (Figure 4e). These results provide additional evidence that CRH has little influence on the spines and synapses that remain after elimination of thin, GluR1-poor entities.

Loss of thin spines induced by CRH requires RhoA signaling

What type of mechanism underlies the selective spine losses caused by CRH? One possibility is suggested by the observation that the peptide blocks theta burst-driven actin polymerization. This polymerization was previously shown to depend on stimulation of an actin signaling pathway involving the small GTPase RhoA and its downstream effector cofilin, a protein involved in actin filament assembly.[24,25,33,50] Testing if CRH affects the level of RhoA activation, we discovered that a 60-min infusion led to a marked (threefold) increase in the concentration of active, GTP-bound RhoA as assessed in immunoblots (Figure 5a). These effects were relatively selective in that CRH led to only a modest reduction (~25%) in active Rac1 and little change in the activation of cdc42 (Figures 5b and c), two other members of the Rho family of small GTPases. Because the Rho GTPases may compete for the same pool of GTP, major increases in the activation of one member are not uncommonly associated with modestly reduced activation of a second member.[33] If abnormal RhoA activation contributes importantly to the effects described here, then inhibitors of RhoA kinase (ROCK), the principle effector of this GTPase, should protect thin spines from the deleterious effects of CRH. We therefore infused a highly selective inhibitor of the kinase (H1152) along with CRH onto adult hippocampal slices from YFP-expressing mice, and carried out the analyses of spine numbers and types. The results were dramatic; in the presence of H1152, CRH had no effect on the number or size distribution of spines (Figures 5d and e).

Discussion

Stress has profound and time-dependent effects on the function of specific hippocampal neuronal populations.[1–3,13–15] These effects are important, because they may result in either enhancement or attenuation of the memory of stressful experiences, which play cardinal

roles in affective and cognitive health, as well as in stress-related neuropsychiatric diseases.[1,3,5,7,13] A large body of literature has focused on the role of steroid stress hormone on the structure and function of hippocampal neurons, dendritic spines and synapses,[1–3,5,6,9,11,51,52] leading to the discovery of novel molecular mechanisms.[53,54]

The peptide CRH has a dual role: when released from the hypothalamus, it activates the release of glucocorticoids from the adrenals, and these, in turn, prepare the body to potential threats.[3,5,55] CRH is also synthesized and released from specific populations of hippocampal interneurons, including parvalbumin-positive basket cells and chandelier cells,[18,56] and is thus positioned to enable fine tuning of synaptic function and plasticity within hippocampus in response to stress.[3,10,18,57,58] Indeed, brief application of CRH primes and augments LTP[59,60] through CRH receptor 1 ($CRHR_1$) signaling and enhances memory.[3,58,59,61] Here we find that longer exposure to presumed stress levels[39,40] of this intrinsic hippocampal peptide produces effects on hippocampal networks that likely contribute to stress-related memory problems.[1,2,13,15,22] Notably, whereas during stress, generation of high concentrations of CRH at dendritic spines likely requires sustained stress-induced release for some time, infusion of the hormone onto adult hippocampal slices may result in these concentrations more quickly. The data provided here illustrate the likely mechanisms by which CRH contributes to the loss of hippocampal dendritic spines that is observed after chronic stress. Whereas a canonical role for glucocorticoids in these changes has been established, recent studies using forebrain-specific knockout of $CRHR_1$ suggest that, even in the presence of a functional glucocorticoid system, the absence of $CRHR_1$ attenuates the detrimental effects of chronic stress on dendritic arborization and spatial memory.[62] Similarly, pharmacological blockade of $CRHR_1$ prevented loss of hippocampal CA1 pyramidal cell dendrites and attenuation of LTP and hippocampus-dependent memory functions provoked by chronic stress early in life.[63]

Hour-long treatments with CRH depressed fast excitatory synaptic responses and attenuated theta burst-induced potentiation. These effects can be reasonably assumed to severely disturb hippocampal contributions to the processing and encoding of information and thus to account (at least in part) for the negative influence of prolonged stress on memory and cognition. The basis of these impairments was striking: the stress peptide caused a rapid and highly selective elimination of thin dendritic spines, with a commensurate reduction in synapse numbers. Remarkably, the reductions of thin spines and synapses were unequally distributed across main and oblique elements of the dendritic tree, and other categories of spines and receptor populations were unaffected.

How does CRH produce effects that are severe and yet discrete to specific spine sub-populations and synapses? Because the vast majority of synapses in the affected zones have a common input—the commissural/associational system arising within field CA3—selective actions of CRH on different types of afferents are unlikely. Previous pharmacological and genetic approaches have demonstrated that the effects of CRH on dendritic spines are mediated via $CRHR_1$.[22,25] Different concentrations of CRH receptors ($CRHR_1$) in dendritic subfields and spine types[22] might account for the observed selectivity. However, our current data do not suggest that $CRHR_1$ is preferentially located on thin spines.[22,40] An alternative possibility derives from the common assumption that thin spines are relatively immature and more labile.[31,32] In this scenario, CRH would act in a uniform fashion across spines but have toxic consequences only on the less-stable spines.

Searching for the mechanisms responsible for observed spine losses we built on work showing that this effect required signaling through $CRHR_1$, as selective antagonists of the receptor abolished the actions of CRH on dendritic spines,[25] and these were not found in mice lacking $CRHR_1$.[25] Downstream of this G-protein-coupled receptor, the current work

produced the first evidence that CRH triggers an intense activation of RhoA, whereas other members of the Rho family of small GTPases are less affected.

It remains to be studied how ligand binding to $CRHR_1$ provokes RhoA activation. Likely candidates include factors (guanine nucleotide exchange factors) that interact with receptors on the spine head and activate the Rho GTPases.[64,65] RhoA has complex effects on spines;[49,65–67] chronic overexpression of RhoA impairs maturation and maintenance of spines in hippocampus,[67] whereas brief activation of the enzyme following TBS initiates the actin restructuring needed for consolidation of LTP.[49] Here we found that a well-characterized inhibitor of the RhoA effector ROCK abrogated the spine losses produced by CRH. Previous work showed that the inhibitor, while preventing LTP consolidation, leaves baseline synaptic physiology and initial expression of LTP intact,[49] and here we found that the inhibitor itself had no effect on the size or number of thin spines. Therefore, we propose that modest and short-lasting activation of RhoA facilitates the depolymerization of F-actin required for the rearrangement of the spine's actin that contributes to spine enlargement and synaptic potentiation. In contrast, prolonged and intense stimulation of RhoA, such as what takes place during long application of CRH (and during severe, long stress), destabilizes the spine cytoskeleton and thereby compromises the integrity of thin spines.

In summary, the present findings delineate a novel and highly selective process that explains how a relatively small loss of synapses and spines accounts for the profound effects of extended stress episodes on cognitive functioning. The model also defines previously unrecognized quantitative constraints for enzymes that govern spine structure; modest activation of RhoA is required for spine plasticity, so that moderate local release of hippocampal CRH during mild or acute stress may augment spine cytoskeletal modifications needed for the consolidation of newly encoded information.[3,58,59,61,68] In contrast, strong sustained activation of the CRH receptor and the GTPase impairs thin spine integrity. Therefore, attempts to reduce the pathological consequences of extended stimulation of CRH receptors must not interfere with the learning-related functions of the same RhoA-mediated actin signaling cascades. New approaches enabling graded inhibition of the RhoA molecular signaling cascade should be promising therapeutic strategies for stress-related defects of synaptic function and plasticity.

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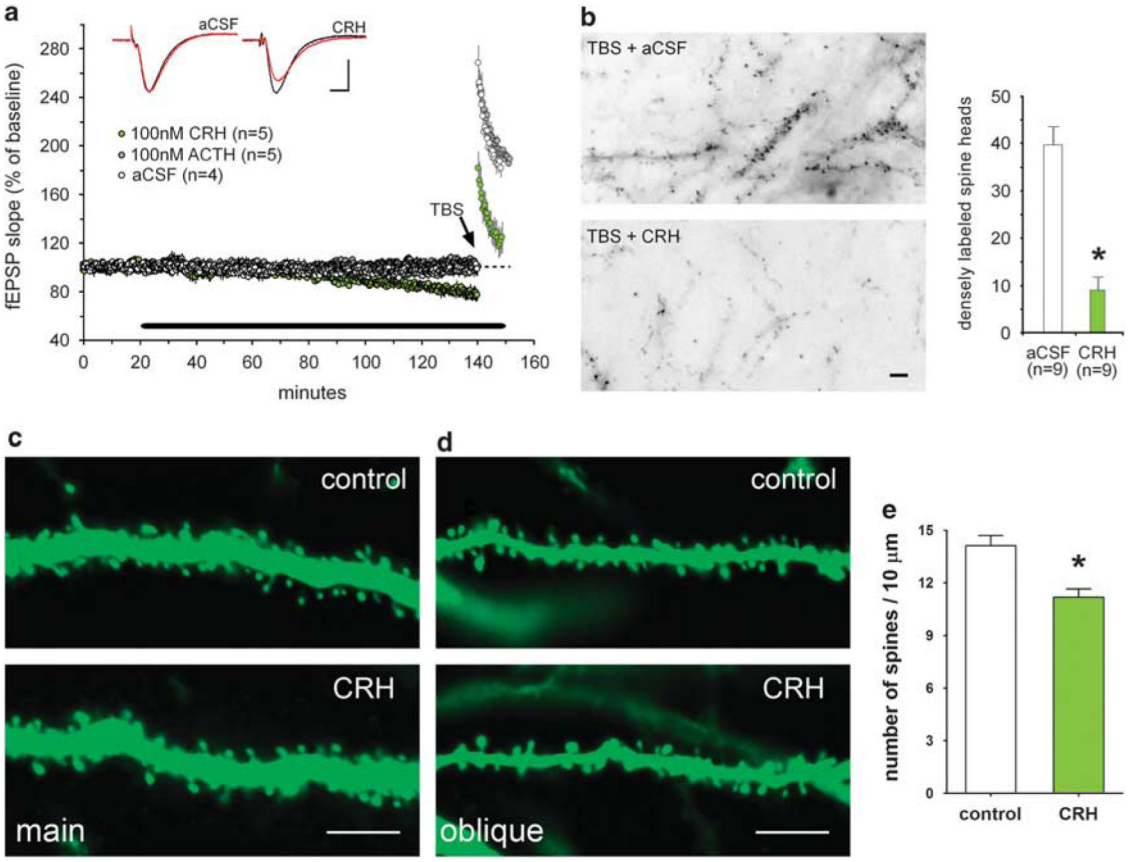

Figure 1.

Corticotropin-releasing hormone (CRH) impairs synaptic function and reduces spine density. (**a**) Reduced synaptic responses, short-term plasticity and actin polymerization in mature hippocampal slices after prolonged application of CRH. (Top) Field excitatory postsynaptic potentials (fEPSPs) declined significantly in CRH-treated slices ($n = 5$) as compared with artificial cerebral spinal fluid (aCSF) controls ($n = 4$). Values indicate percent change from baseline (10 min before drug infusion or the theta burst stimulation (TBS)). Values of mean slope ± s.e.m.: Control slices = –2 ± 3%, adrenocorticotropic hormone (ACTH) slices = +3 ± 2%; CRH slices = –21 ± 4% ($P = 0.01$). There was no apparent change in EPSP wave form. (*Inset*) Representative traces collected before (black) and at the end (red) of infusion; scale bar = 1 mV per 5 ms. (Bottom) Short-term potentiation induced by the naturalistic TBS paradigm was greatly reduced in the CRH-treated slices (green filled circles) compared with those treated with a control peptide that is also involved in stress regulation, ACTH ($n = 5$; gray filled circles), and aCSF control slices ($n = 4$; empty circles). The initial enhancement of fEPSPs at 2 min after TBS, normalized to the fEPSP values collected for 10 min before delivery of the stimulation bursts, was 133 ± 5% (mean ± s.e.m.) in control slices, and 95 ± 5% in the CRH-treated cases ($P = 0.0015$, two-tailed *t*-test). A comparable difference was found at 10 min after TBS; potentiation was 90 ± 6% above baseline for controls and 56 ± 5% for the CRH slices ($P = 0.006$). (**b**) TBS-induced actin polymerization was significantly reduced in slices infused with CRH. (Left) Representative images of phalloidin incorporation into dendritic spines in control slices (top) and in slices infused with CRH. (Right) Quantitative analysis of densely phalloidin-labeled spine heads, denoting polymerization and remodeling of actin, in control and CRH-treated slices (*$P = 0.01$). (**c**) CRH (100 nM), infused for 60 min onto hippocampal slices from 4-month-old yellow fluorescent protein (YFP)-expressing mice, drastically reduced the density

of spines on apical dendritic branches in CA1 stratum radiatum. (**d**) Reduced spine density was also detected in oblique dendrite segments. (**e**) Overall, CRH reduced dendritic spin density by 22% (**P* = 0.0005). A total of 2950 μm of dendrites was imaged and analyzed in 6 experiments, 2–4 neurons each. Scale bar = 1 μm (**b**) and 5 μm (**c, d**).

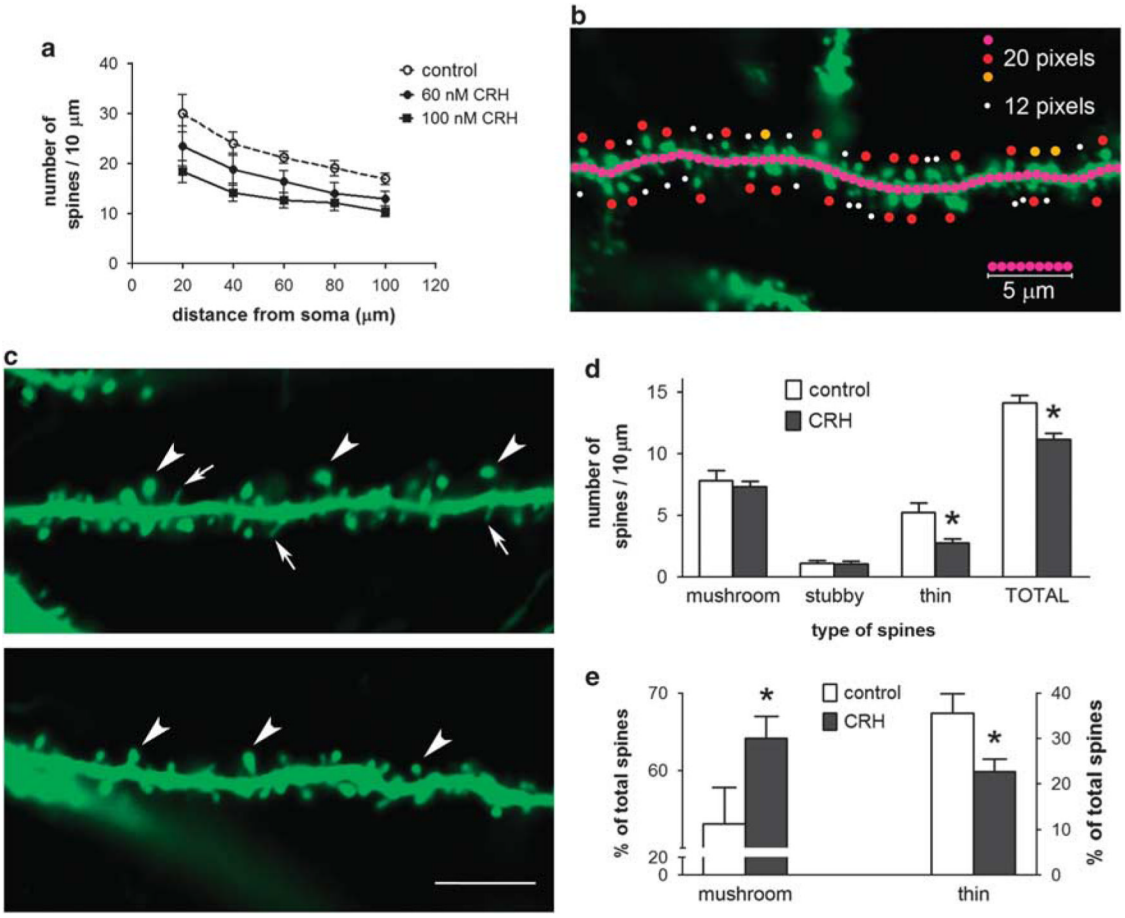

Figure 2.

Corticotropin-releasing hormone (CRH) preferentially affects the integrity of thin spines. (**a**) A dose-response curve of the effects of CRH on dendritic spine density was carried out in hippocampal neurons. Whereas exposure to 30 nM of the peptide did not influence spine density in dendrites significantly (not shown), 60 and 100 nM application led to a dose-dependent reduction of spine density as measured using PSD-95 (postsynaptic density protein 95)-immunoreactive puncta (see Materials and methods). (**b**) Classification and quantification of dendritic spines on apical dendrites of CA1 pyramidal neurons. Spines are classified by size and shape as mushroom-type, thin and stubby. Mushroom-type spines have a bulbous head diameter exceeding 0.6 µm and a narrow neck (red circle; diameter = 20 pixels). Thin spines (white circles, 12 pixels) lack a definable head or possess a head diameter < 120% of the stem's. Stubby spines (yellow circles; 20 pixels) lack a clear neck, and their overall length roughly equals their width. Confocal image of oblique apical dendritic branch is from a 4-month-old yellow fluorescent protein (YFP)-expressing transgenic mouse. (**c**) Oblique dendritic branches in stratum radiatum from 4-month-old YFP-expressing transgenic mice. In a control dendrite (top panel), both thin (arrows) and mushroom-type (arrowhead) spines are visible. In the CRH-treated slice, the density of thin spines is reduced. Control and CRH-treated slices were always derived from the same mouse. (**d**) Thin spines on CA1 pyramidal neurons were selectively eliminated in response to CRH infusion (100 nM, 60 min), whereas the density of mushroom-type spines was unaffected after CRH application. Quantitative analysis of spine density and spine types is based on 9–12 CA1 pyramidal neurons that were imaged and analyzed without knowledge of treatment group (*$P < 0.05$; analysis of variance (ANOVA) with Bonferroni's *post hoc*

tests). (**e**) Analysis of the relative contributions of different spine types to the overall population before and following exposure to CRH. Whereas mushroom-type spines were minimally affected by CRH (**d**), there is a significant increase in the contribution of these spines, which are not considered to undergo further changes upon long-term potentiation (LTP) induction, to the overall spine population. Commensurately, there is a major reduction in the availability of thin spines (*$P < 0.05$). Scale bar = 5 μm (**c**).

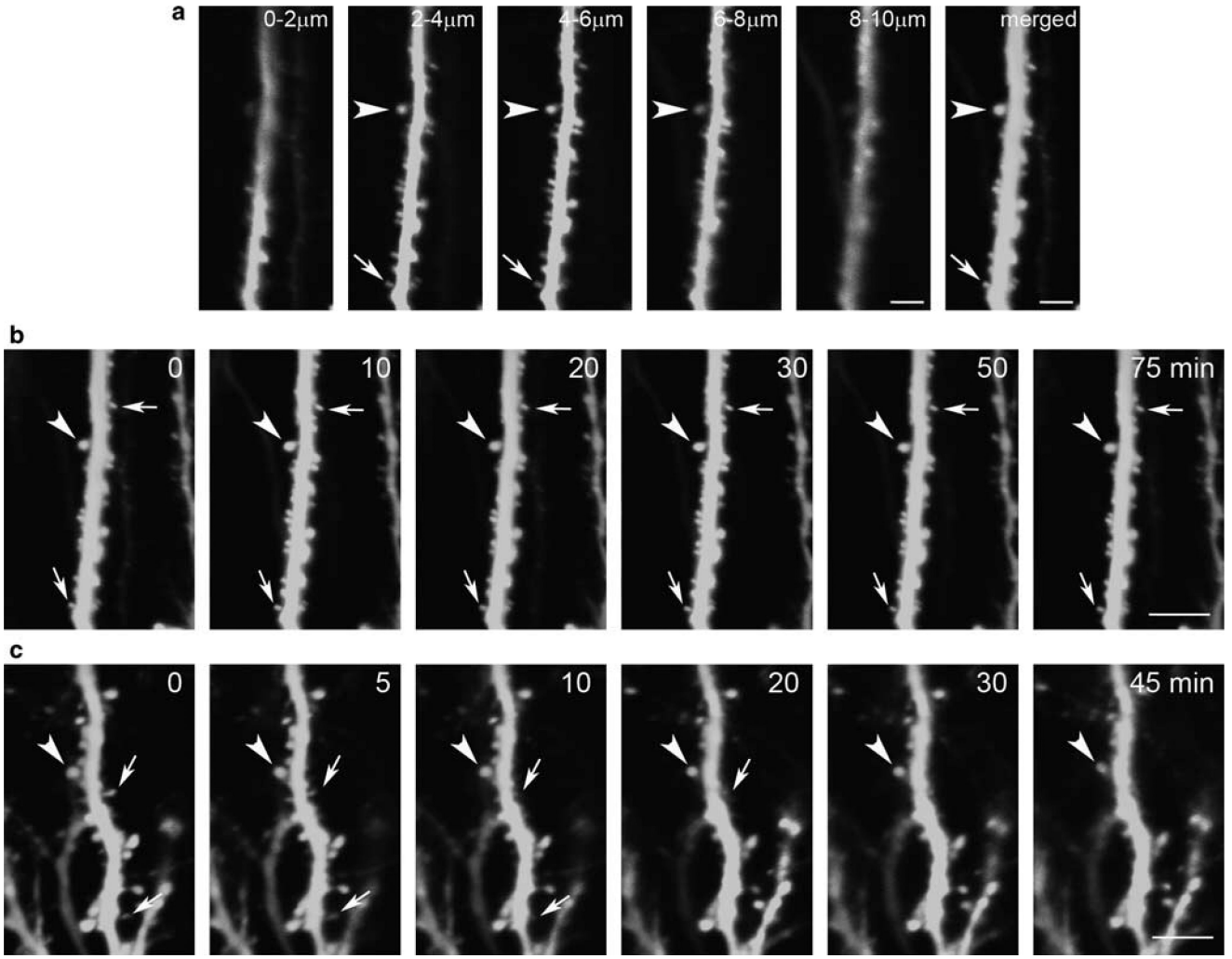

Figure 3.

Corticotropin-releasing hormone (CRH) directly influences thin dendritic spines, rather than shifting the size profile of the spine population. (**a**) Live time-lapse multiphoton imaging of adult mouse hippocampal slices. A series of contiguous 2 μm two-photon images that was used to generate the *z* stacks shown in panels **b** and **c**. These series enable unambiguous identification of both thin (arrow) and mushroom-type (arrowhead) spines along the thickness of apical dendrites. (**b**) Time-lapse two-photon imaging shows that both thin and mushroom-type spines remained constant under the control experimental conditions over 75 min of imaging. (**c**) Infusion of CRH (100 n_M) resulted in a selective elimination of thin spines, with little change in mushroom-type spines. Scale bars = 2 μm (**a**) and 3 μm (**b, c**).

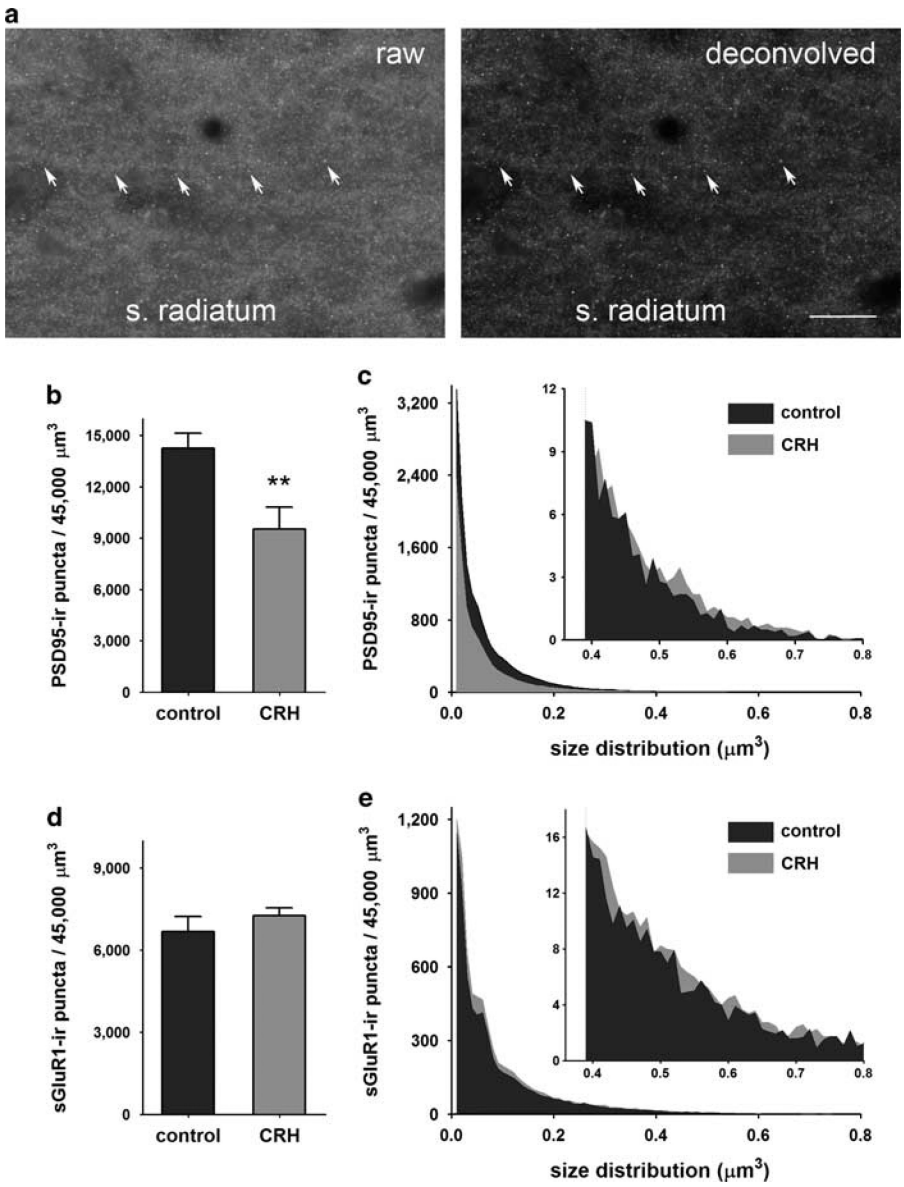

Figure 4.

Numbers of excitatory synapses are reduced by exposure to stress levels of corticotropin-releasing hormone (CRH). (**a**) A wide-field image (raw), and the same image after restorative three-dimensional deconvolution (deconvolved) show PSD-95 (postsynaptic density protein 95)-immunoreactive (ir) puncta (arrows). These represent mature spines with well-developed excitatory synapses. The image of the hippocampal CA1 stratum radiatum (s. radiatum) was obtained from a 4-month-old C57/Black mouse. (**b**) PSD-95-ir puncta were less abundant in the CRH-treated (100 n_M, 60 min) group versus control. The above wide-field microscopy followed by three-dimensional restorative deconvolution analysis was employed in 12 slices per group from 4 independent experiments (** $P < 0.01$ vs control; Student's *t*-test). (**c**) The size-frequency distributions of PSD-95-labeled puncta in CRH-treated slices (gray) indicates a reduction in the number of small puncta (between 0.1 and 0.2 μm^3), and little change in the numbers of large puncta (between 0.4 and 0.8 μm^3) compared with control slices (black); this suggests that CRH infusion resulted in a selective reduction of small synapses. (**d, e**) Application of CRH (100 n_M, 60 min) did not alter the

number (**d**) or size distribution (**e**) of surface GluR1-ir puncta in the stratum radiatum of hippocampal CA1. *n* = 12 slices from 4 independent experiments. Scale bar = 15 μm (**a**).

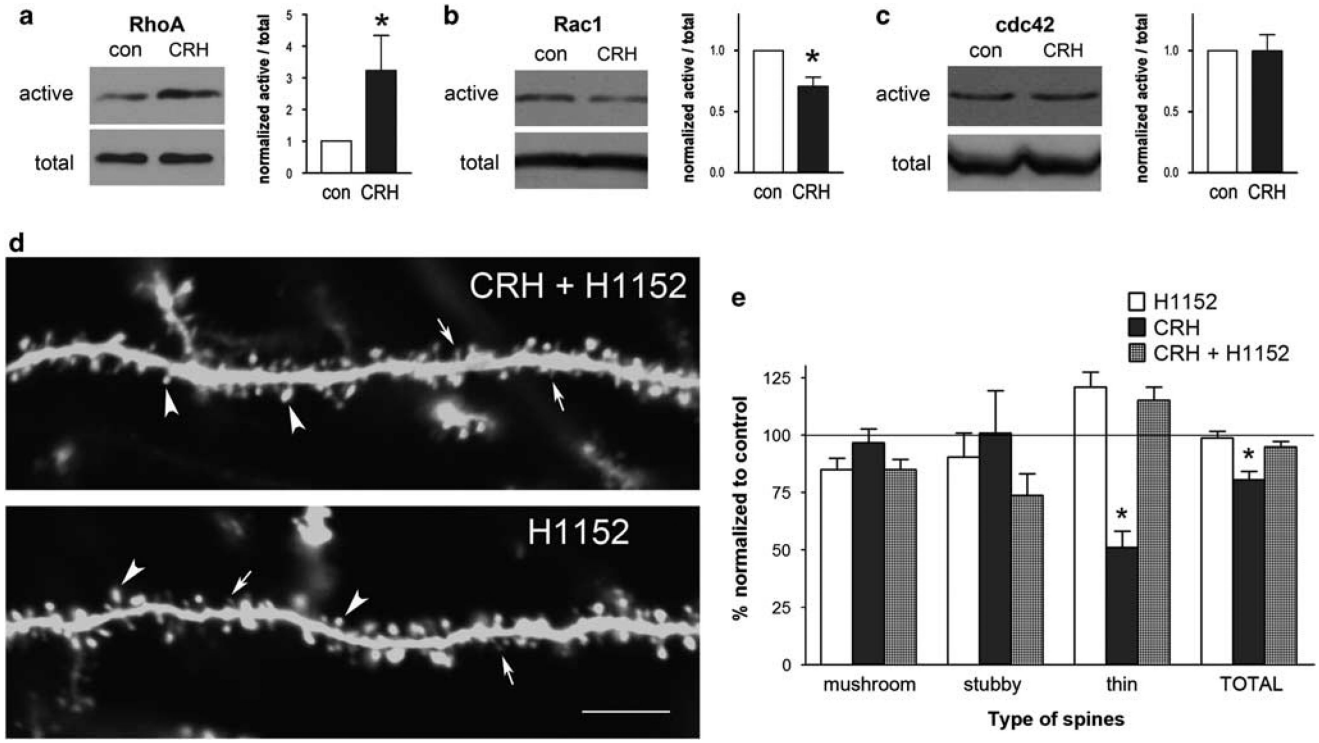

Figure 5.

RhoA signaling mediates the destructive effects of corticotropin-releasing hormone (CRH) on thin dendritic spines. (**a**) Exposure to CRH (100 nM) significantly increased RhoA activation. Active RhoA was detected by pull-down assays using GST-Rhotekin binding domains, and the same samples were probed for total RhoA protein. Shown are group band optical density measures of the levels of the active GTPase, normalized to the respective total protein level (*$P < 0.05$; $n = 5$ independent experiments; 12 mice total). (**b, c**) CRH infusion had no effect on active cdc42, and modestly decreased active Rac1. Active Rac1 and cdc42 were detected by pull-down assays and the optical densities of bands corresponding to active Rac1 and cdc42 were normalized to their respective total protein levels (*$P < 0.05$, $n = 3$ independent experiments; 9 mice total). (**d, e**) Treatment with a blocker of the RhoA effector, ROCK (Rho-associated protein kinase), H1152, abrogates the CRH-induced loss of thin spines (*$P < 0.05$), and normalizes the population profile of spines on CA1 apical dendrites. The blocker had no effect when applied alone. Scale bar = 5 μm.