ORIGINAL ARTICLE

Corticotropin-releasing factor and urocortin regulate spine and synapse formation: structural basis for stress-induced neuronal remodeling and pathology

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Dendritic spines are important sites of excitatory neurotransmission in the brain with their function determined by their structure and molecular content. Alterations in spine number, morphology and receptor content are a hallmark of many psychiatric disorders, most notably those because of stress. We investigated the role of corticotropin-releasing factor (CRF) stress peptides on the plasticity of spines in the cerebellum, a structure implicated in a host of mental illnesses, particularly of a developmental origin. We used organotypic slice cultures of the cerebellum and restraint stress in behaving animals to determine whether CRF *in vitro* and stress *in vivo* affects Purkinje cell (PC) spine density. Application of CRF and urocortin (UCN) to cerebellar slice cultures increased the density of spines on PC signaling via CRF receptors (CRF-Rs) 1 and 2 and RhoA downregulation, although the structural phenotypes of the induced spines varied, suggesting that CRF-Rs differentially induce the outgrowth of functionally distinct populations of spines. Furthermore, CRF and UCN exert a trophic effect on the surface contact between synaptic elements by increasing active zones and postsynaptic densities and facilitating the alignment of pre- and post-synaptic membranes of synapses on PCs. In addition, 1 h of restraint stress significantly increased PC spine density compared with those animals that were only handled. This study provides unprecedented resolution of CRF pathways that regulate the structural machinery essential for synaptic transmission and provides a basis for understanding stress-induced mental illnesses.

Keywords: stress; spine; synapse; cerebellum; maturation; dendritic development

INTRODUCTION

Stress-induced mental illnesses such as anxiety and depression exact an immense health-care burden at the individual and societal levels.[1] A pathological hallmark of stress-induced mental illnesses is remodeling of neuronal connections,[2] with alterations in the density and morphology of dendritic spines appearing to be central to the process.[3–5] Dendritic spines are structurally, functionally and neurochemically distinct compartments on which the majority of excitatory synapses occur and, as such, they are essential cogs in the machinery of synaptic transmission and coordinated brain activity,[6] with their alterations being integral to the pathogenesis of stress-induced mental illnesses. Identifying the molecular pathways engaged in the stress-induced remodeling of dendritic spines will serve to elucidate the subcellular basis of stress-induced mental illnesses and hopefully unearth potential therapeutic targets.

Chronic stress decreases spine numbers in hippocampal and prefrontal cortex neurons, yet increases spine density in the amygdala.[2] Cerebellar Purkinje cells (PCs) exhibit the most exuberant dendritic tree as well as spine density and morphological heterogeneity[7] of all the neurons in the central nervous system, yet the effect of stress on PC spine dynamics has yet to be explored. This is partly because of the cerebellum being considered primarily to be involved in motor coordination.[8] However, emerging evidence highlights its role in cognition[9] as well as emotive behaviors[10] and thus could be an important loop in stress circuitry responsible for stress-induced disorders.[11,12]

The endocrine and cognitive loops of the stress response are, in part, mediated by the family of corticotropin-releasing factor (CRF) peptides[13] and are central to the development of stress-induced psychiatric disorders.[14] Apart from their homeostatic roles, CRF peptides also have a direct effect on dendritic architecture of different types of neurons.[15–17] In the current study, we adopt a combinatorial approach to investigate the effects of CRF peptides and acute stress on the structure and function of PC spines.

MATERIALS AND METHODS

Approval to conduct the study on animals was obtained from the Ethics Committee on Animal Experimentation, University of Groningen, and the University of Portsmouth, UK.

Organotypic slice cultures of rat cerebellum

In total, 72 postnatal day 8 black-hooded Lister rat pups were used. The preparation of cerebellar slices was performed according to previously

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published protocols.[17] See Supplementary Information for reagents and concentrations.

Dye loading of PCs in culture and semiquantitative nonstereological planar 2 data analyses of spine density and morphology

Cells were patched with pipettes containing Alexa Fluor 488 hydrazide (Molecular Probes) as described,[18] and the spines imaged with a confocal microscope according to the criteria detailed in Supplementary Information.

Analysis of synapses

Electron microscopical images of PCs were identified on the basis of morphological criteria. Care was taken to avoid double counting of single synaptic profiles. The length of active zone (AZ) and postsynaptic density (PSD) per synapse was measured on digital electron micrographs using analySIS Soft Imaging System (Münster, Germany). A total of 169 synapses (8 slices, 3 rats) were measured (53 synapses from CTRL, 59 synapses from CRF-treated slices and 57 synapses from urocortin (UCN)-treated slices).

Western blotting

Following treatment, the slice cultures were collected and the lysates probed for a range of proteins according to previously published protocols that are detailed in the Supplementary Information.[19]

Acute stress paradigm, assessment of anxiety and *in vivo* PC spine density estimation

Adult animals ($\sim$9 weeks old) were either handled by the investigator (control, $n=4$) or exposed to 1 h of restraint stress in a Plexiglas cone ($n=4$). Levels of anxiety and PC spine density were then investigated according to the methods described in Supplementary Information.

Statistical analysis

Where relevant, the data are presented as mean $\pm$ s.e.m. as they were not normally distributed. One-way analysis of variance with Bonferroni's *post hoc* test was used for comparing means for three or more groups. Values of $P<0.05$ were considered significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

RESULTS

CRF and UCN differentially increase PC spine density via CRF receptors (CRF-Rs) 1 and 2

The effects of exogenously applied CRF and UCN were assessed with respect to the density of PC spines, the main sites of excitatory synaptic input. CRF and UCN significantly increased the density of dendritic spines compared with untreated controls (Figure 1a). Quantification of spine density revealed that CTRL cells had 0.8 ± 0.2 spines per μm length of dendrites. In contrast, CRF-treated cells showed 100% increase of spine density (2.0 ± 0.2 spines per μm length of dendrite) with UCN-treated cells exhibiting $\sim$63% increase in density (1.3 ± 0.2 spines per μm length of dendrite; Figure 1b and Supplementary Table S1; $P<0.01$ in both cases).

CRF-R antagonists were used to confirm the specificity of CRF and UCN effects in terms of increasing spine density (Supplementary Table S1 and Figure 1b). The application of a selective CRF-R1 antagonist (NBI) and a selective CRF-R2 antagonist (AS30) alone did not significantly affect spine number when compared with CTRL cells. As the blockade of CRF-Rs, on its own, did not alter spine number, all the observed effects are because of the applied CRF and UCN. Blockade of the CRF-R1 with NBI significantly attenuated the enhancing effect of CRF on spine density without significantly affecting the effect of UCN. In contrast, blockade of the CRF-R2 with AS30 dramatically reduced the effects of both CRF

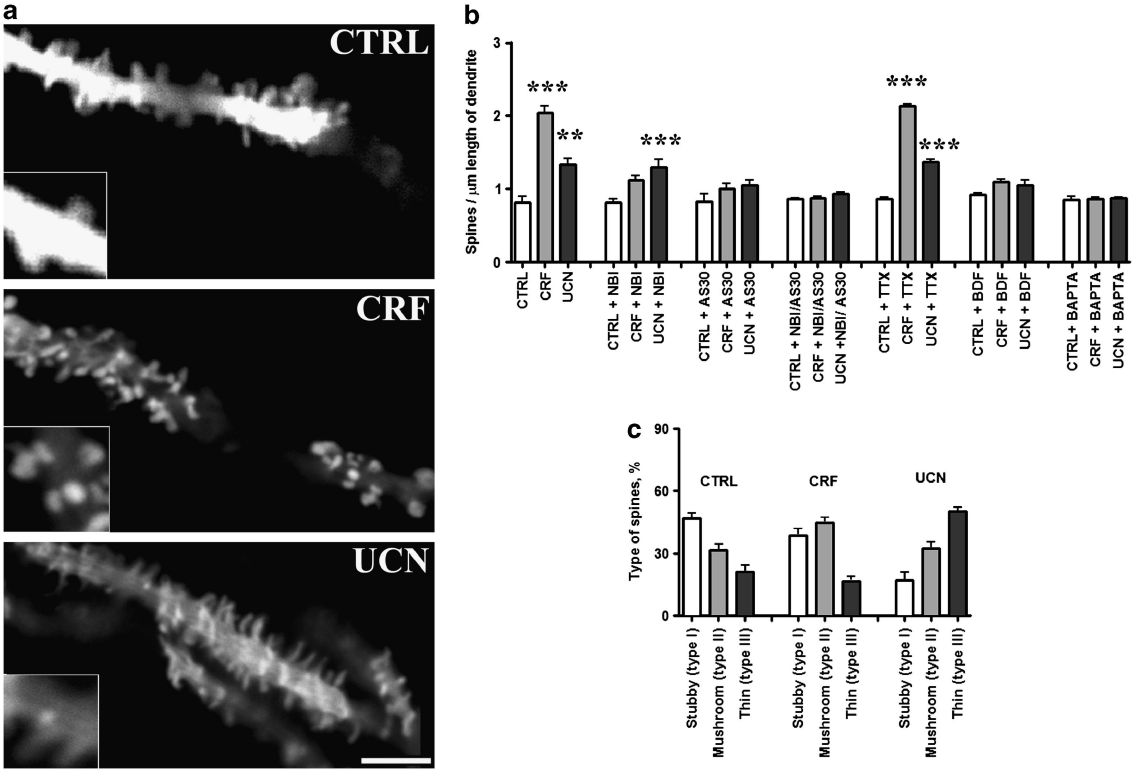

Figure 1. Corticotropin-releasing factor (CRF) and urocortin (UCN) induce spine outgrowth via CRF receptors. (**a**) Confocal image of Purkinje cell (PC) spines obtained following the loading of cells *in vitro* with Alexa Fluor 488. (**b**) Graphical representation of the numbers of spines with and without CRF and UCN treatment. The abscissa indicates the various pharmacological treatments. Bars represent means with lines indicating s.e.m. **$P<0.01$; ***$P<0.001$. (**c**) The proportion of mushroom and thin spines. Scale bar, 5 μm.

and UCN. Finally, the combined blockade of CRF-R1 and CRF-R2 completely prevented the CRF- or UCN-induced spine formation of PCs (Figure 1b and Supplementary Table S1). The differences in the magnitudes of CRF and UCN effects could be because of the varying affinities that CRF and UCN have for the different CRF-Rs[20] and the disparate expression patterns of the receptors within the cerebellar circuitry.[21] CRF-R1 is located on PC dendrites, granule cells and radial glia, whereas CRF-R2 is located on PC somata and inhibitory interneurons.[21] UCN has equimolar affinities for both receptors, and thus blocking the CRF-R1 receptor could still allow it to signal via CRF-R2. However, CRF has an $\sim$30 times higher affinity for the CRF-R1 compared with CRF-R2. Thus, blockade of CRF-R1 is likely to abolish most of its effects at a certain concentration.

The effects of CRF and UCN on spine formation are not activity dependent

To determine whether the effects of CRF and UCN on spine number and morphology require neuronal activity, we treated the slices with CRF and UCN in the presence of tetrodotoxin, a blocker of voltage-gated sodium channels that prevents action potential propagation. Tetrodotoxin failed to prevent the CRF- or UCN-induced increase in spine number (Supplementary Table S1). The effects of CRF and UCN on spines, however, required the mobilization of calcium stores as the presence of the calcium chelator BAPTA-AM in the patch pipette prevented the formation of spines after CRF or UCN treatment (Figure 1b and Supplementary Table S1). This is in keeping with current evidence of CRF releasing calcium stores and CRF-Rs being coupled to calcium-activated potassium channels.[22] Treatment of slices with CRF or UCN together with Bdf, an agent that can stop transmitter release and vesicle cycling,[23] prevented the CRF- and UCN-induced spine formation (Supplementary Table S1). This observation suggests that the well-known G-protein cascade signaling pathways utilized by CRF and UCN[13] could include additional pathways with links to synaptic vesicle signaling.

CRF and UCN induce distinct spine morphological phenotypes

PC spines exhibit a range of morphologies that correlate with their distinct inputs,[7] inferring a close correlation between the shape or morphology of the spines and their function. We characterized the effects of CRF and UCN with respect to individual spine morphology according to the relationship between the length (L), diameter of neck (d_n) and diameter of head (d_h) of spine. The occurrence of the different spines shapes was expressed as a proportion of the total number of spines and classified as follows; type I (shorter stubby spines with $L \approx d_n \approx d_h$), type II (mushroom spines with $d_n << d_h$) and type III (thin spines that typically have $L >> d_n$). In untreated PCs, the majority of the spines ($46.75 \pm 1.25\%$) belonged to the type class I followed by type II (31.75 ± 2.75) and then type III ($21.25\% \pm 2.75$). In contrast, in CRF-treated cells, the majority of spines belonged to type II ($44.75\% \pm 2.08$) followed by type I ($38.5\% \pm 2.33$) and then type III ($16.75\% \pm 0.63$). In the UCN-treated cells, the majority of the spines belonged to type III ($50.25 \pm 0.63\%$), followed by type II ($32.50 \pm 0.96\%$) and then type I ($17.25 \pm 1.25\%$) (Figure 1c). The specific phenotypes of spines induced by CRF and UCN appear relatively permanent as the characteristic morphologies induced by CRF and UCN persisted even after a 3-day washout period during which time the cultures were left untreated (Supplementary Figure S2). Taken together, CRF and UCN induce distinct spine morphologies that could be important in terms of influencing synaptic plasticity.

CRF and UCN enhance synapse formation and alignment of pre- and post-synaptic elements

To determine whether the CRF- and UCN-induced increase in spine density also resulted in altered synapse formation, we determined the number of spines that were innervated by presynaptic terminals compared with 'free' spines that did not have any apparent input. In the cerebellum, the only terminals to synapse on PC spines are the axons of granule cells. These are distinguished by their expression of VGluT1,[24] and this marker was used to analyze the number of parallel fibers (PFs)/PC spine appositions (Figure 2a). The number of free spines per dendrite decreased following the application of CRF (15.42 ± 1.21) and UCN (8.31 ± 1.03) compared with untreated cells (21.43 ± 1.97) (NS: control 10 cells, 5 slices, 3 different platings; CRF: 7 cells, 4 slices, 4 different platings; UCN: 5 cells, 3 slices; 3 different platings; Figure 2b). These data suggest that CRF or UCN have varying potencies with respect to their ability to induce synapse formation as both CRF and UCN increase the number of spines contacted by PFs; however, UCN did this to a greater extent (Figure 2b).

CRF and UCN increase the lengths of PC synaptic AZs and PSDs at excitatory synapses

Electron microscopy and calbindin immunostaining were used to determine the effect of CRF and UCN on the structure of the AZs and PSDs of synapses, two subcellular domains integral to the docking of synaptic vesicles[25] and the clustering of receptors,[26] respectively. Synapses in which the AZ/PSD was easily discernable were selected to compare the lengths of AZs and PSDs following treatment by CRF or UCN (Figure 3a). CRF or UCN application resulted in a significantly larger AZ as well as a longer PSD (Figure 3b) compared with untreated control synapses.

To further support the argument that CRF and UCN promote the alignment of pre- and post-synaptic elements,[27] we calculated the proportion of synapses in which the lengths of the AZs and

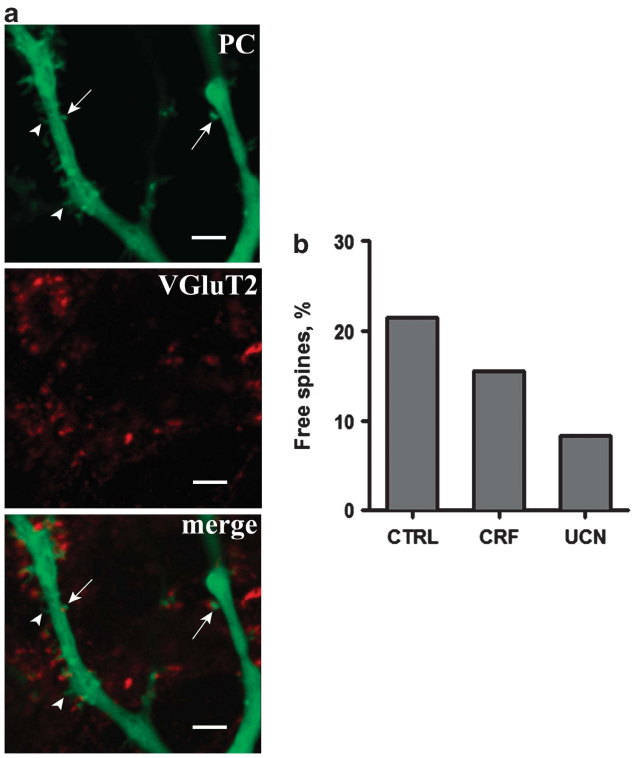

Figure 2. Corticotropin-releasing factor (CRF) and urocortin (UCN) enhance the alignment of pre- and post-synaptic elements. (**a**) Immunofluorescence image showing VGluT1 immunoreactivity (red), a marker of parallel fibers. Arrows indicate occupied spines with arrowheads indicating 'free' spines that lack any VGluT1 contacts. (**b**) Quantification of the percentage of 'free' spines following treatment with CRF and UCN. Scale bars, 3 μm.

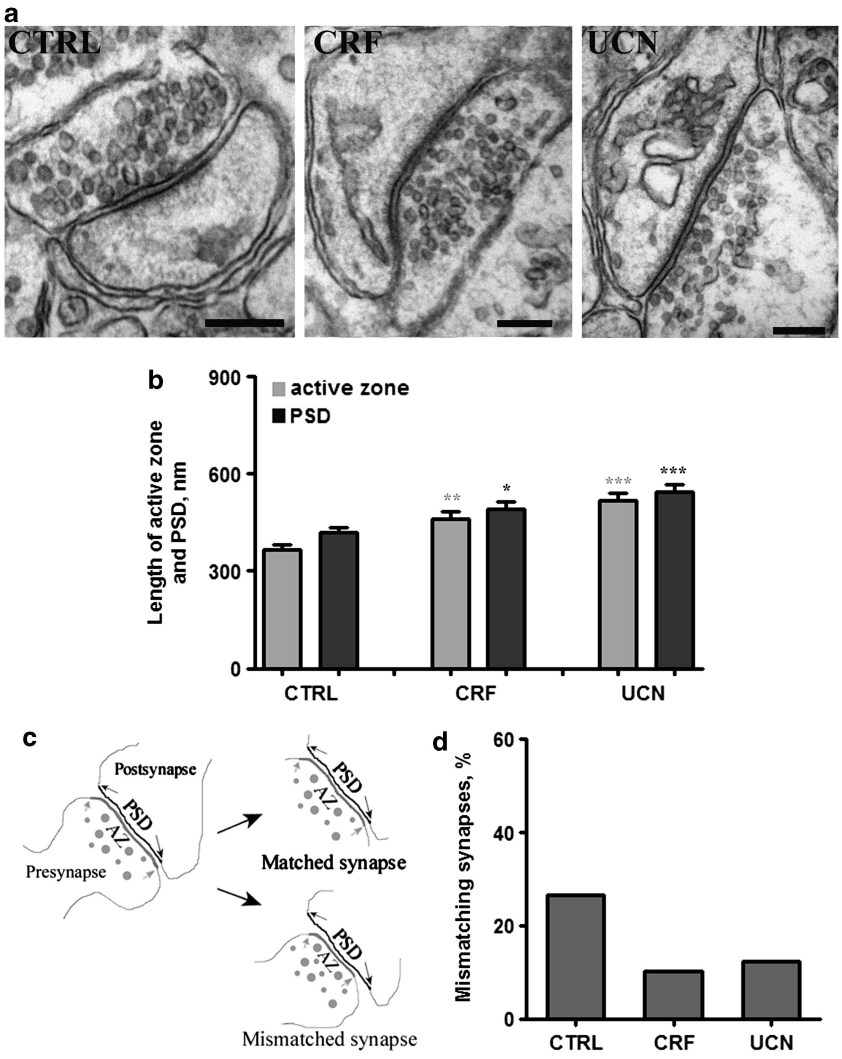

Figure 3. Ultrastructural evidence for the effect of corticotropin-releasing factor (CRF) and urocortin (UCN) on synapse organization. (**a**) Representative electron micrographs of synapses between Purkinje cell (PC) and parallel fibers showing that CRF and UCN application resulted in synapses that exhibited longer active zones (AZs) and postsynaptic densities (PSDs). (**b**) Quantification of the lengths of the AZs and PSDs. The abscissa indicates the pharmacological treatments. Bars represent means with lines indicating s.e.m. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with CTRL values. (**c**) A model illustrating two types of relationships between AZ and PSD in synapses. The upper right represents an idealized, matched synapse, where the lengths of AZ and PSD are approximately equal in length. The lower right represents a mismatched synapse where the length of the PSD is noticeably larger than the AZ. (**d**) Graphical representation of the percentage of mismatched synapses among all synapses. Scale bars, 200 nm.

PSDs closely correlated or 'matched' according to the criteria of McEwen.[28] In a 'matching' synapse, the length of the AZ and that of the PSD are comparable and there is an exact apposition of the pre- and post-synaptic components. For quantitative comparison, we defined a synapse as mismatching when the edges of the AZ and PSD were >70 nm apart (half the length of the synapse with a minimum length of the AZ; Figure 3c). Under this criterion, we found that in CTRL slices, 25% of synapses were mismatched (Figure 3d). In contrast, CRF treatment resulted in only 10% of mismatched synapses (Figure 3d), and 12% following UCN treatment, suggesting that CRF and UCN treatment increases the area of contact between pre- and post-synaptic elements, possibly facilitating synaptic transmission.

Effect of CRF and UCN on functional synaptic transmission

To determine whether the increase in the number of spines, the larger AZ and PSDs, and the enhanced matching of pre- and post-synaptic elements correlated with increases in synaptic function, we recorded spontaneous miniature excitatory postsynaptic currents in slices treated with CRF, UCN or control. In CRF- and UCN-treated slices, miniature excitatory postsynaptic current frequency in PCs was significantly higher compared with CTRL (Figures 4a and c), suggesting an increased number of excitatory inputs. No change was seen in miniature excitatory postsynaptic current amplitude in any of the conditions compared with CTRL (Figures 4a and b).

The effect of acute restraint stress on PC spine density

To determine whether stress *in vivo* has any effect on PC spine density, adult rats were either handled or exposed to an acute stressor in the form of 1 h of restraint stress, following which the animals were evaluated for levels of anxiety using the elevated plus maze, and the brains were then perfusion-fixed in order to estimate the effect of stress on PC spine density *in vivo* using

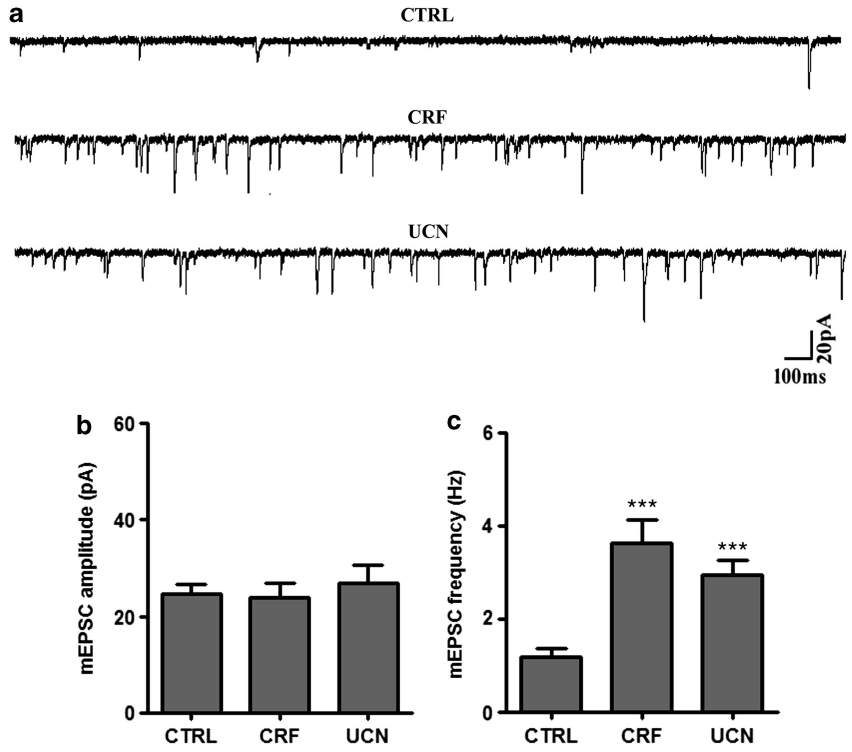

Figure 4. Corticotropin-releasing factor (CRF) and urocortin (UCN) application results in an increased number of functionally active synapses. (**a**) Representative trace of a whole cell voltage-clamp recording showing miniature excitatory postsynaptic currents (mEPSCs) in untreated CTRL slices exposed to CRF and UCN. (**b**) The mean ± s.e.m. amplitude of mEPSCs following treatment with CRF and UCN. (**c**) Quantification of the mean ± s.e.m. frequency of mEPSCs following treatment with CRF and UCN. ***$P < 0.001$.

nonstereological confocal microscopy (Figure 5). Animals exposed to stress spent on average less time in the open arm compared with those only handled (mean ± s.d. in s, handled vs stress, 66 ± 11 vs 52 ± 28), more time in the closed arms (120 ± 31 vs 139 ± 36) and made marginally less entries into the open arm (17 ± 3 vs 15 ± 3), although the differences were not statistically significant ($P = 0.248$, 0.386 and 0.554 for time in open arms, closed arms and entries to open arms, respectively, un-paired *t*-test). The mean PC spine density in stress animals was significantly greater compared with handled animals (handled, 6063 spines per $1000 \pm 210\,\mu m^2$ vs stress, 7679 spines per $1000 \pm 245\,\mu m^2$, $P = 0.0001$, Mann–Whitney).

CRF and UCN signal in the cerebellum via RhoA

As CRF and UCN appear to exert divergent effects on spinogenesis in PCs, we investigated the potential intracellular pathways associated with CRF signaling. We have previously shown that CRF signaling in the locus coeruleus is associated with RhoGTPase pathways.[19] The levels of RhoGTPase proteins were probed in CRF- and UCN-treated cultures using western blotting techniques. RhoA was consistently downregulated by CRF and to a larger degree by UCN compared with control (Supplementary Figure S1). No other members of the RhoGTPase family tested (RhoB, RhoC and Rac 1, 2, 3) showed any consistent changes in response to CRF/UCN treatment (data not shown).

DISCUSSION

Stressful life events have a direct effect on neuronal architecture.[28] Offspring subjected to different stress paradigms show increased spine density in adulthood, although these effects vary with respect to gender and the brain regions examined.[29–32] This study shows for the first time that stress pathways affect dendritic spines of neurons in the cerebellum. CRF and UCN, when applied *in vitro*, increased the numbers of spines on cerebellar PCs. In addition, exposing animals to an acute stressor led to an increase in PC spine density *in vivo*. This is in contrast to the CRF-induced decrease in spine number in the excitatory pyramidal cells of the hippocampus, via CRF-Rs.[16] Other molecular members involved in the endocrine response to stress, such as glucocorticoids, are invariably also involved in spine plasticity.[33] Therefore, the varying expression patterns of the different stress pathways within different brain regions and cell types probably result in CRF acting in direct and indirect mechanisms, and this most likely accounts for the varying structural effects of CRF reported throughout the brain. There is currently no clear functional rationale for the role of CRF in cerebellar function. The functional consequences of this stress-induced increase in PC spine number *in vivo* are difficult to predict in light of the general role of the cerebellum in motor learning and thus future functional studies are essential. Long-term depression (LTD) of glutamatergic PF/PC synapses, which should disinhibit PC input onto deep cerebellar nuclei, is thought to underlie motor learning.[34] It has been demonstrated that CRF contained in climbing fibers is essential for PF/PC LTD.[35] However, if the additional spines infer additional excitatory input onto PCs, this will result in decreased cerebellar output because of the enhanced PC-mediated inhibition of deep cerebellar nuclei. The eventual effect could be determined by the degree or time course of the stress with acute episodes favoring PF/PC LTD and thus motor learning or coping during such short-lived stressful events. However, sustained or chronic stress could shift the role of CRF toward structural plasticity in the form of increased spine number, potentially impairing motor learning or coping skills via decreased cerebellar output, and thus provide the structural correlates that underlie various stress-induced behavioral phenotypes or disease states.[36,37]

Spine density and structure are dynamic and alter in response to synaptic function,[38] changes in cognition and memory[39] and

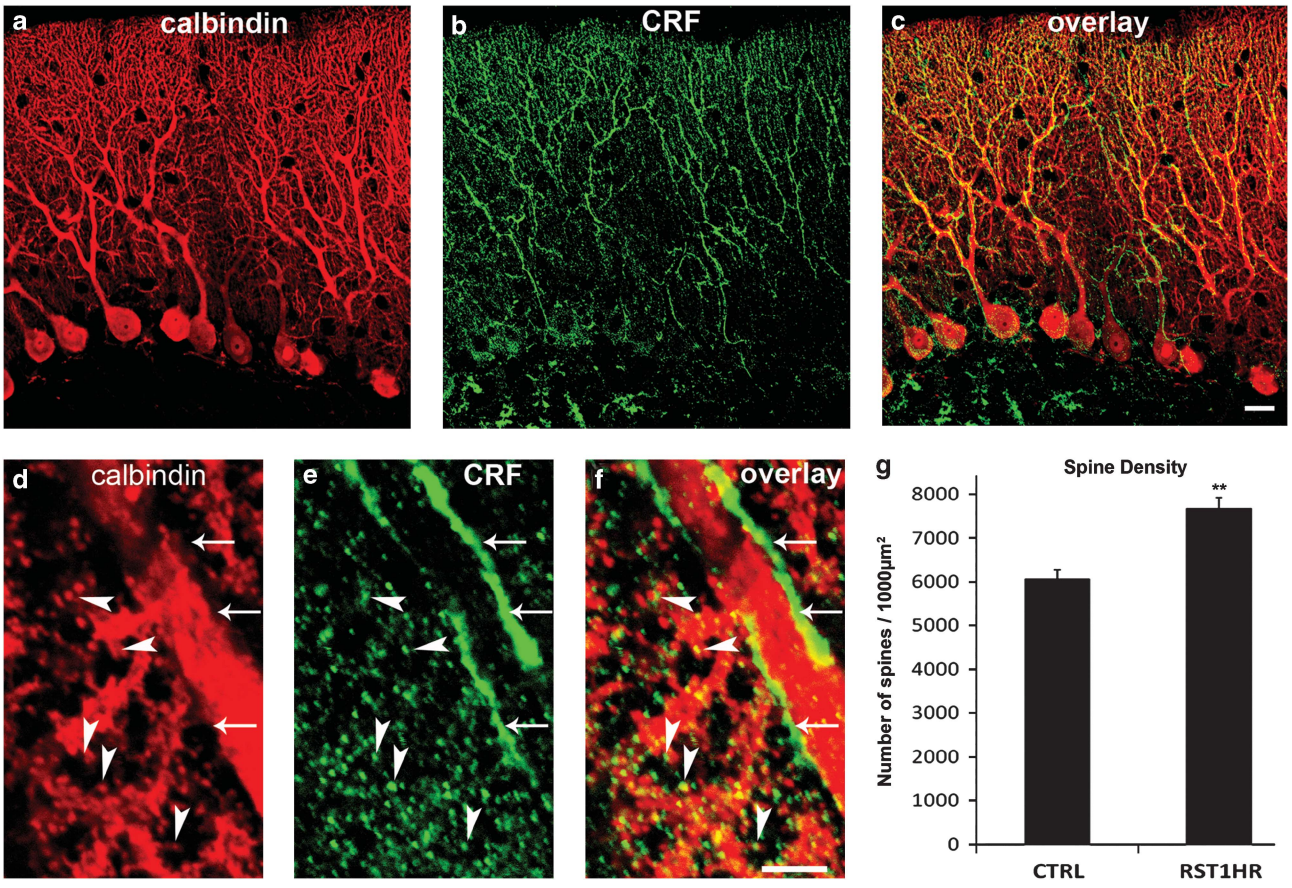

Figure 5. Acute restraint stress increases Purkinje cell (PC) spine density. (**a**) Calbindin immunoreactivity identifies PC processes within the molecular layer (ML) of the cerebellum. (**b**) Corticotropin-releasing factor (CRF) immunoreactivity within the ML is selectively contained within climbing fibers. (**c**) An overlay of (**a**) and (**b**). (**d**) A magnified region of the ML highlighting a PC dendritic shaft (arrows) and dendritic spines (arrowheads). (**e**) CRF immunoreactivity is located within climbing fiber arbors (arrows) and in boutons (arrowheads). (**f**) An overlay of (**d**) and (**e**). (**g**) Summary data of PC spine density quantification (mean ± s.d.) in animals either handled (CTRL) or exposed to 1 h of restraint stress ($n = 3$ animals, 18 optical sections for each group). ***P*-value of 0.001. Scale bar: **a**–**c**, 20 μm, **d**–**f**, 5 μm.

mental illness.[40] Thus, experience directly influences the nature of synaptic connections. However, the specific pathways involved in relaying external factors to the level of the synapse are diverse. The precise mechanism by which CRF peptides regulate spine density and structure is still unclear; however, a role in influencing the actin cytoskeleton is most likely. Spine structure and synapse function are modulated by the actin cytoskeleton,[41] which in dendrites is highly regulated by small RhoGTPases.[42] Members of the RhoGTPases have opposing roles in neurite formation with the Racs enhancing and the Rhos inhibiting neurite growth.[43] In the locus coeruleus, we have shown that CRF peptides regulate dendritic arborization by upregulating Rac1 and downregulating RhoA.[19] In this study, we are able to confirm that both CRF and UCN downregulate RhoA, although UCN did so to a greater degree, and appeared to have no effect on Rac pathways. This difference in the magnitude of RhoA downregulation by CRF and UCN, coupled with their varying affinities for the two CRF-Rs, might provide a parsimonious explanation as to why CRF and UCN appear to induce unique morphological spine phenotypes. This link between CRF signaling and RhoGTPase pathways in the cerebellum is in agreement with recent studies in the hippocampus.[44] Although in contrast to our data that show that CRF downregulates RhoA in the cerebellum, Chen *et al*.[44] demonstrate that CRF upregulates RhoA in the hippocampus. This once again highlights the cell-specific nature of CRF in terms of structural plasticity. The growing number of pharmacological agents that interact with Rac/Rho function[45] provide potential for the use of novel agents targeted against stress-induced brain disorders.

In conclusion, our results suggest that CRF and UCN induce specific and detailed morphological changes in synapses at spines of PCs. Although fundamental in its nature, the study provides unprecedented resolution of CRF pathways that regulate the structural machinery essential for synaptic transmission and could provide a basis for understanding pathologies arising from early-life stress-induced psychiatric disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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