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Hippocampal dysfunction and cognitive impairments provoked by chronic early-life stress involve excessive activation of CRH receptors

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Abstract

Chronic stress impairs learning and memory in humans and rodents and disrupts long-term potentiation (LTP) in animal models. These effects are associated with structural changes in hippocampal neurons, including reduced dendritic arborization. Unlike the generally reversible effects of chronic stress on <u>adult</u> rat hippocampus, we have previously found that the effects of <u>early-life</u> stress endure and worsen during adulthood, yet the mechanisms for these clinically important sequelae are poorly understood. Stress promotes secretion of the neuropeptide corticotropin-releasing hormone (CRH) from hippocampal interneurons, activating receptors (CRF_1) located on pyramidal cell dendrites. Additionally, chronic CRF_1 occupancy negatively affects dendritic arborization in mouse organotypic slice cultures, similar to the pattern observed in middle-aged, early-stressed (CES) rats. Here we found that CRH-expression is augmented in hippocampus of middle-aged CES rats, and then tested if the morphological defects and poor memory performance in these animals involve excessive activation of CRF_1 receptors. Central or peripheral administration of a CRF_1 blocker following the stress period improved memory performance of CES rats in novel object recognition tests and in the Morris water maze. Consonant with these effects, the antagonist also prevented dendritic atrophy and LTP attenuation in CA1 Schaffer collateral synapses. Together, these data suggest that persistently elevated hippocampal CRH-CRF_1 interaction contributes importantly to the structural and cognitive impairments associated with early-life stress. Reducing CRF_1 occupancy *post-hoc* normalized hippocampal function during middle-age, thus offering potential mechanism-based therapeutic interventions for children affected by chronic stress.

Keywords

stress; memory; aging; dementia; LTP: Long-term potentiation; early-life stress; corticotropin releasing factor; CRF_1: CRF receptor type 1; glucocorticoids; dendrites; hippocampus

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Introduction

Chronic early-life stress (CES), including neglect, loss of parent or extreme poverty, affects the majority of children worldwide (UNICEF, 2005). Epidemiological studies have associated CES with enduring adverse consequences, including depression (Heim et al., 2008) and cognitive impairments (Nelson et al., 2007); these may emerge during adulthood and progress with age (Kaplan et al., 2001; Wilson, 2007). Elimination of global childhood stress is unlikely; therefore, effective therapies that can be employed *post-hoc* to prevent its long-term cognitive sequelae are needed to alleviate this significant problem.

The direct, causal role of early-life stress in subsequent memory dysfunction is difficult to study in humans. We therefore developed a rodent model of CES (during postnatal days 2-9) that utilizes fragmented maternal care to generate chronic stress in the pups (Avishai-Eliner et al., 2001b; Ivy et al., 2008). This model recapitulates the human condition in that it causes impaired memory later in life, and in addition, results in seriously impaired LTP, a variant of synaptic plasticity widely regarded as critical to the encoding of memory (Bliss and Collingridge, 1993). The LTP defects, which were selective to commissural / associational synapses in apical dendrites of fields CA1 and CA3 (Brunson et al., 2005), were accompanied by abnormal morphology of the corresponding neurons including impoverished dendritic arborization. However, the molecular mediators of these profound effects of early stress have remained unclear.

Glucocorticoids, the canonical stress hormones, mediate many actions of stress on hippocampus (Sapolsky, 1985; McEwen, 1999; de Kloet et al., 2007). More recently, the stress neuropeptide CRH has been implicated in stress-induced activation of neurons (Kovacs and Sawchenko, 1996; Reyes et al., 2008) including hippocampal pyramidal cells (Chen et al., 2006; Joels and Baram, 2009). CRH contributes to effects of acute stress on synaptic plasticity and memory (Lee and Davis, 1997; Blank et al., 2002; Chen et al., 2004b; Rainnie et al., 2004; Orozco-Cabal et al., 2006; Chen et al., 2010), and several lines of evidence support a role for CRH receptor occupancy in CES-induced hippocampal dysfunction: (1) Interneurons expressing CRH are abundant in hippocampus (Chen et al., 2001); (2) CRH is released into hippocampal intercellular space during stress (Chen et al., 2004a; Chen et al., 2006); (3) CRH receptor type 1 (CRF_1) resides on dendrites of CA1 cells, the same neurons sustaining dendritic atrophy after early-life stress (Chen et al., 2004b); (4) Chronic exposure to CRH provokes dendritic atrophy in a pattern similar to that found after early stress; (5) When administered into brains of immature rats, CRH led to persistent memory impairments reminiscent of those found after chronic early-life stress (Brunson et al., 2001b). Notably, CRH-induced impairments occurred even when glucocorticoid levels were 'clamped' at low physiological levels (Akana et al., 1988), suggesting that they were independent of glucocorticoid receptor activation (Brunson et al., 2001b).

Based on these findings, the current study examined if blocking CRF_1 immediately following the early-life stress period would eliminate subsequent memory and morphological defects. We further tested if CRF_1 activation <u>within the brain</u> contributed to these effects of early-life stress.

Methods

Animals

All experiments conformed to NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. *Rats:* Time-pregnant Sprague-Dawley rat dams gave birth in UCI vivaria, and dams and litters were

maintained on 12h light cycles with unlimited access to chow and water. Pups were mixed and adjusted to 12 per dam if necessary, to obviate the potential confounding effects of genetic variables and of litter size. Upon weaning on postnatal day (P) 21, only male rats were kept and were housed 2-3 per cage. *Transgenic mice:* B6.Cg-TgN transgenic mice expressing yellow fluorescent protein (YFP) under the *Thy1* promoter (Thy1-YFP; The Jackson Laboratory, Bar Harbor, ME) were born in UCI vivaria and were employed for organotypic hippocampal slice cultures on postnatal day 1, as described (Chen et al., 2008), and see below.

Early-life chronic stress (CES) paradigm

Chronic stress lasting for one week was generated in rat pups using a previously established model (Gilles et al., 1996; Avishai-Eliner et al., 2001b; Brunson et al., 2005; Ivy et al., 2008). This stress is a result of erratic and fragmented nurturing behaviors of rat dams, which stem from limited nesting material in the cage. Briefly, on P2, dams and pups of the CES group were placed in cages fitted with plastic-coated aluminum mesh bottoms (2.5cm above the floor, allowing collection of droppings), with no bedding material. Nesting material consisted of one paper towel that was shredded by the dam to construct a rudimentary nest. Control dams and their litters resided in bedded cages, containing ~0.33 cubic feet of sanitary chips. Control and experimental cages were undisturbed during P2-P9, and housed in a quiet room enclosed in a protective tent with strong laminar airflow, preventing ammonia accumulation. Maternal nursing behaviors were monitored during the week of CES as described (Ivy et al., 2008).

Administration of CRF_1 antagonist

The small-molecule selective CRF_1 blocker, NBI30775 (3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-N,N-dipropyl-pyrazolo[2,3-a]pyrimidin-7-amine) or vehicle solution were chronically infused via osmotic minipumps to control and CES male rats for one week, starting immediately after the stress period (P10-17). The administration of the CRF_1 blocker or vehicle was either subcutaneous (sc) for systemic release (Fenoglio et al., 2005), or intra-cerebroventricular (icv). The latter administration mode was conducted to exclude the potential confounder of blocking pituitary CRF_1 and downstream release of adrenal glucocorticoids. Briefly, NBI30775 was dissolved in warm distilled water and the pH was adjusted; vehicle solution was made using the same pH-adjusted distilled water without the compound. Drug solution (4 mg/ml) or vehicle was loaded into osmotic Alzet minipumps (model 1007D, Alzet Corp., Cupertino, CA), calibrated to release 0.5μl/h (~2.5mg/kg/day) over seven days. For systemic release of the CRF_1 blocker, pups were anesthetized with vaporized isofluorane and pumps implanted subcutaneously (sc) between the shoulder blades through a small incision made at the nape of the neck. The implantation surgery took < 5 minutes per animal, and, after recovery, pups returned to home cages. For icv infusions, pumps were implanted in the same manner and attached to a catheter connected to a 3.5mm cannula placed into the lateral cerebral ventricle using the following coordinates (with Bregma as landmark): AP - 0.7, L 2.0, V 3.3. For both systemic and icv routes, pumps were implanted on the morning of P10, and CRF_1 blocker or vehicle was continuously infused until P17. The pumps were removed upon weaning on P21 (Brunson et al., 2001b). Maternal care was monitored (Ivy et al., 2008; Rice et al., 2008), and there were no significant differences in dam interaction with implanted compared with non-implanted pups during P10-P21. Implantation of pumps did not significantly alter body weight at weaning in either type of surgical procedure ($p > 0.05$): naïve CTL = 42.3 ± 3.1, naïve CES = 40.32 ± 1.8, sc-implanted CTL = 41.3 ± 1.5, sc-implanted CES = 42.6 ± 1.4, icv-implanted CTL = 47.4 ± 2.3, icv-implanted CES = 40.8 ± 2.7.

Novel Object Recognition (OR)

Tests of learning and memory were conducted once rats reached middle-age (10-12 months); at this age early-life stressed rats were previously found to have significant memory impairments and abnormal LTP (Brunson et al., 2005). For the OR procedure, transparent plastic cages were coated with opaque paper except the front panel, to allow for live experimenter observation (Broadbent et al., 2004; Brunson et al., 2005). After 3 days of habituation to the cage, rats explored 2 objects in a familiarization session (day 1). In the test session on day 2, rats were presented with a duplicate of a previously encountered object from day 1 and a novel object. Preliminary object exposure tests were used in a separate cohort of rats (n = 14) to select the three objects used for the OR procedure, yielding exploration times for objects 1 (light bulb), 2 (square Lego brick), and 3 (padlock) of 29.8 ± 8.4, 27.1 ± 9.7 and 33.7 ± 8.3 seconds, respectively. Counter-balancing of the objects was carried out in an additional cohort of rats and did not influence the results (see Results). The duration of exploration of each object (sniffing with the animal's nose < 2 cm from objects) as well as total object exploration time were recorded during the 5 min sessions on the familiarization and testing days.

Morris Watermaze (MWM)

Rats were tested for hippocampus-dependent spatial memory using a variant of the Morris watermaze procedure with the least swim-time (and thus minimal potential stress; (Morris, 1984)), as described previously (Brunson et al., 2001b; Brunson et al., 2005). Briefly, rats were trained to find a submerged platform in a pool of opaque water over 6 trials per day, for two days. During the two days of training, the platform remained in the same quadrant and all rats were trained to the same platform position. In an additional cohort of rats, the goal quadrant was counter-balanced, and this did not influence the time to find the platform (two-way repeated measures ANOVA; effect of quadrant location $F_{1,23} = 0.48$, $p = 0.50$; interaction of quadrant location and trial $F_{11,253} = 1.23$, $p = 0.19$). For the reversal procedure, the platform was relocated to a new quadrant adjacent to the previous one. Rats entered the pool in one of three quadrants not containing the platform, and the start quadrant alternated between trials. At the start of each trial, rats were placed in the pool facing the wall and were allowed to swim for 60 sec or until the platform was found. If the rat did not mount the platform during the trial, he was guided to the platform and remained on it for 15 sec before removal from the pool. Rats were required to rely on spatial cues placed on the four walls of the room to learn the platform location during training and testing days, and short-term spatial memory was considered to have occurred if the latency time to finding the platform grew shorter upon repeated trials within the same day (acquisition of short-term memory does not exclude the possibility that long-term memory was being concurrently acquired). Long-term spatial memory was tested in a single, 60 sec "probe" trial with no platform in the pool. This was conducted in separate cohorts of rats either 24h after the second day of training or after the reversal procedure (fourth test day). A cued learning task was also conducted two weeks after watermaze test. This cued procedure does not rely on hippocampus and thus controls for potential differences in motivation or sensorimotor skills that could influence performance in the MWM. The cued procedure consisted of six trials, using a proximal cue (spherical object attached to the platform, raised 10cm above the water surface), while distal cues were removed. All trials were recorded, and data analyzed for escape latency, mean swim speeds and swim distances.

Tissue processing and immunocytochemistry (ICC)

Rats were anesthetized / euthanized with sodium pentobarbital and perfused with 0.9% saline (pH 7.4) for 5-8 minutes. Brains were removed and hemisected, and the left hemisphere was immersed in paraformaldehyde fixative for 24h followed by cryoprotection in 25% sucrose for 36-48h. The right hemisphere was used for Golgi impregnation studies

(see below). Left hemispheres were sectioned 20μm thick, and CRH ICC was performed on free-floating sections as previously described (Chen et al., 2001). Briefly, after washing (3 × 5 min) with 0.01M phosphate-buffered saline containing 0.3% Triton X-100 (PBS-T; pH 7.4), sections were treated for 30 min in 0.3% H_2O_2/PBS, followed by blockade of nonspecific sites with 5% normal goat serum in PBS for 30 min. After rinsing, sections were incubated for 2d at 4°C with rabbit anti-CRH antiserum (1:20,000; a gift from Dr. W. W. Vale, Salk Institute, La Jolla) in PBS containing 1% bovine serum albumin, and washed in PBS-T. Sections were incubated in biotinylated goat-anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA) in PBS for 2h at room temperature. After washing, sections were incubated in the avidin–biotin–peroxidase complex (ABC) solution (1:100; Vector) for 2h, rinsed (3 × 5 min PBS-T), and the reaction product visualized by incubating the sections in 0.04% 3,3″-diaminobenzidine (DAB) containing 0.01% H_2O_2, with or without 0.3% nickel chloride. Sections were mounted on poly-L-Lysine-coated slides and coverslipped with Permount (Fisher Scientific).

Golgi-Cox impregnation and quantification of CA1 pyramidal cell processes

Right hemispheres were immersed in Golgi-Cox solution (12-14 days), followed by 30% sucrose (3-5 days), both in the dark. Coronal sections (200μm) were collected in 6% sucrose, developed in 14% NH_4OH (30 min), and fixed (Kodak Fixative, Kodak). Individual, fully Golgi-impregnated CA1 neurons were imaged and reconstructed without knowledge of treatment group (n = 5-8 neurons per rat). Dendritic arborization was analyzed using the Sholl method (Sholl, 1953), that provides a quantitative description of the dendritic tree by counting the number of dendrites that cross virtual concentric circles drawn at fixed distances from the soma. The number of dendritic intersections at each concentric circle (20μm, 40μm, 60μm, etc.), as well as total dendritic length were measured and compared among groups.

Organotypic slice cultures

Hippocampal slice cultures were prepared from P1 YFP-expressing mice as described previously (Chen et al., 2008). Briefly, after decapitation, mouse brains were removed and placed in minimal essential medium (1X MEM; Invitrogen, Carlsbad, CA), and a block of tissue including entorhinal cortex and hippocampus was dissected from adhering tissue. Transverse slices (300μm) were cut (McIlwain tissue chopper; Mickle Lab, Gomshall, UK). Sections were placed onto Millicell-CM culture membranes (Millipore, Bedford, MA), and transferred to six-well plates filled with 1.2ml of culture medium (0.5X MEM, 25% horse serum, 25% HBSS, 30mM glucose, 7.5% $NaHCO_3$, and 0.01% ascorbic acid, pH 7.2). Cultures were incubated in a humidified, 5% CO_2 atmosphere at 36°C for 14 days in vitro (DIV), with medium changes every 2–3 days. To study the effects of chronic exposure to CRH on CA1 pyramidal cell dendrites, synthetic rat/human CRH (100nM; Bachem, King of Prussia, PA) was applied to the culture medium on DIV 2 as well as to refreshed medium. After 13d, cultures were fixed in fresh 4% paraformaldehyde for 30 min and processed for fluorescent ICC. Cultures were sectioned transversely (20μm) using a cryostat, and ICC was performed on free-floating sections (Chen et al., 2004a) using mouse anti-GFP (1:8000; Sigma, St. Louis, MO) followed by visualization with anti-mouse IgG conjugated to Alexa Fluor 488 (1:200; Invitrogen).

Long-term potentiation studies

Hippocampal slices were prepared as published (Brunson et al., 2005). Hippocampal slices (350μm) were collected into artificial CSF maintained at 32°C for ~1 hour in an interface chamber (95% O_2 / 5% CO_2; 31±1°C), superfused at 60-80ml/h with artificial CSF of the following composition (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4, 2.5 $MgSO_4$, 3.4 $CaCl_2$, 26 $NaHCO_3$). For LTP, a glass recording electrode (2M NaCl; 2MΩ), was positioned in CA1b

stratum radiatum (s.r.) to record field excitatory synaptic potentials (fEPSPs). Stimulating test pulses were delivered (0.05Hz) to the Schaffer collateral fibers in CA1c s.r. using a concentric bipolar stimulating electrode. Standard input-output curves were generated and test stimulation current was set to elicit a response = 50% of the maximum fEPSP amplitude. Baseline responses were recorded for 20 minutes, and unstable slices discarded. A train of high-frequency stimulation (HFS; 100Hz, 1s) was employed to induce LTP. Analysis of fEPSP amplitudes and slopes (20-80% fall) was performed on digitized files (NacGather 2.0; Theta Burst Corp., Irvine, CA). A post-recording correction factor for rising drift was applied to two CES+ANT slices before unblinding and did not affect statistical outcomes. Slope and amplitude analyses yielded identical results, and slope data are shown. Values from multiple slices were averaged for each rat, and statistical analysis performed by rat. One animal in the CES group failed to respond to the stress manipulations and was subsequently removed from analyses. Values are described in the text as % potentiation in means ± SD, whereas the figures demonstrate means ± SEM.

Image analysis of dendritic branching

Fixed organotypic cultures were imaged using a Zeiss (Oberkochen, Germany) LSM510 Meta or Bio-Rad (Hercules, CA) Radiance 2000 confocal scanning systems. Dendritic trees of individual YFP-expressing CA1 pyramidal cells were captured using "z-stack" three-dimensional projections (12–16 optical slices at 2μm each; 10–14 neurons per group) and files were converted to Adobe Photoshop. For both slice culture and Golgi impregnation techniques, neurons were reconstructed and drawn using Zeiss LSM Image Browser and Adobe Photoshop software.

Statistical analyses

Analysis of CRH levels was accomplished using Student's t-test and one-way ANOVA, as appropriate. Two-way repeated measures (RM) ANOVA was employed for the LTP experiments and Sholl analyses of dendrites. Early-life treatment group was used as the "between subjects" factor, and time after high-frequency stimulation (LTP) or distance away from soma (Sholl) were used for the "within subjects" factor.

The analysis of learning and memory in the watermaze employed three-way RM ANOVA, with two "between subjects" factors (control vs. CES, and vehicle vs. CRF_1 blocker treatment), and trial as the "within subjects" factor. This was followed by pair-wise comparisons among groups. To assess 24 hour memory in the watermaze, the percentage of total path length swam in the target quadrant was calculated, and these data were transformed (Arc-Sine) to meet the assumptions of parametric statistics. One-sample t-tests were then used to compare mean quadrant percentages to chance (25%), and Student's t-tests were used to compare these percentages directly across groups (*a priori* planned comparisons; CES vs. CTL, CES+ANT vs. CTL, CES vs. CES+ANT). For the novel object recognition tests, simple one-way ANOVA was conducted.

All ANOVAs were followed by Bonferroni's *post-hoc* Multiple Comparisons test, with the exception of LTP experiments (that were followed by Tukey's Honestly Significantly Different (HSD) *post-hoc* test). Significance levels were set at 0.05, and data are presented as mean ± SEM. Data were analyzed using the SPSS 16.0 (SPSS Inc, Chicago, IL) and GraphPad Prism 5.0 Software (San Diego, CA).

Results

CRH immunoreactivity is augmented in hippocampus of early-life stressed rats

CRH was expressed in basket-cell type interneurons in the pyramidal cell layer of hippocampus (Fig. 1*A,B*, see also insets), consistent with previous findings (Yan et al., 1998; Chen et al., 2001). Comparing CRH expression in hippocampal areas CA1 and CA3 of control and early-stressed middle-aged rats, we found that immunoreactive cells were significantly more abundant in CA1, CA3a and CA3c of the stressed rats (Fig. 1*C*; by *t* tests; CA1: $p = 0.049$; CA3a: $p = 0.028$; CA3c: $p = 0.012$). Whereas ICC may not be fully quantitative, these findings suggested that early-life stress augmented the expression levels of CRH chronically, so that the number of detectable cells was higher than that in controls.

Chronic exposure to nanomolar CRH levels reduces dendritic arborization *in vitro*

The ambient levels of CRH in extracellular space of control and early-life stressed rats are difficult to estimate. Available studies suggest that levels of peptide released within the hippocampus can reach ~200nM (Tringali et al., 2009), although lower stress-released CRH levels were measured in the amygdala (Merali et al., 2008). To evaluate the effects of similar levels of CRH on dendritic integrity, we employed organotypic hippocampal slice cultures where the presence of other stress hormones can be controlled, exposed for two weeks to 100 nM CRH. We took advantage of transgenic mice expressing yellow fluorescent protein (YFP) in pyramidal cells to directly visualize dendritic branching of CA1 neurons (Fig. 2*A*). Chronic exposure to CRH (Fig. 2*B*) resulted in reduced dendritic arborization in a pattern similar to that observed after early-life stress (Brunson et al., 2005). Sholl analysis revealed that exposure to CRH affected dendritic branching most severely in the proximal apical dendritic regions that receive input from the commissural-associational fibers (Fig. 2*C*; two-way RM-ANOVA, interaction: $F_{17,306} = 1.72$, $p = 0.04$). The number of Sholl intersections was also significantly lower in the basal dendritic region of pyramidal cells exposed to CRH (Fig. 2*D*; interaction: $F_{6,108} = 3.04$, $p = 0.008$).

Taken together, the results summarized in Figs. 1 and 2 suggested that hippocampal pyramidal cells of rats stressed early in life remain exposed to high levels of CRH beyond the stress period and even during middle age, and that these levels might suffice to stunt dendritic arborization. Therefore, we queried if interaction of hippocampal CRH with its principal hippocampal receptor, CRF_1, contributed to the dendritic atrophy and associated spatial memory and LTP defects found after early-life stress.

Chronic early-life stress leads to late-onset impairment in hippocampus-dependent cognitive functions that are prevented by *post-hoc* systemic administration of a CRF_1 antagonist

To test if a CRF_1 antagonist could prevent early-stress-provoked cognitive defects, hippocampus-dependent learning and memory were assessed in naive, vehicle-treated, or CRF_1 blocker-treated middle-aged CES rats, in comparison with controls. We employed the Morris watermaze test because learning in this task requires the integrity of dorsal hippocampus (Morris, 1984; Hollup et al., 2001; Broadbent et al., 2010). During the first two training days, all groups successfully learned to find the hidden platform (Fig. 3*A*, effect of trial: $F_{11,539} = 7.73$, $p < 0.001$). Early-life stress impaired acquisition of the watermaze (effect of stress condition: $F_{1,49} = 11.09$, $p < 0.005$), and CRF_1 blocker treatment improved performance regardless of stress exposure ($F_{1,49} = 5.7$, $p < 0.05$). Furthermore, planned comparisons revealed that by the end of each training day, CES rats did not find the platform as fast as controls (trial 6: $t_{40} = 2.13$, $p = 0.04$; trial 12: $t_{40} = 2.4$, $p = 0.02$). Antagonist treatment of CES rats improved their ability to find the platform on the final training trial (trial 12) (CES+ANT vs CES; $t_{23} = 2.43$, $p = 0.02$) such that their performance was

comparable to controls (CTL vs CES+ANT; $t_{31} = 0.98$, $p = 0.34$). On the third day (consisting of the reversal procedure), the location of the platform was changed, and rats were forced to rely on spatial cues to navigate to this new location (Fig. 3*B*). Although all groups reduced their escape latencies across subsequent trials ($F_{5,245} = 5.61$, $p < 0.001$), early-life stressed rats took significantly longer to reach the platform relative to controls (effect of stress condition: $F_{1,49} = 6.9$, $p < 0.012$). Again, escape latencies were significantly shorter in CRF$_1$ blocker treated rats regardless of stress exposure ($F_{1,49} = 11.12$, $p < 0.005$). Analysis of the last trial of the reversal procedure (trial 6) indicated that by the end of testing, CES rats had not learned the new platform location as well as controls ($t_{40} = 3.26$, $p < 0.05$), and CRF$_1$ antagonist treatment reversed this deficit (CES vs. CES+ANT: $t_{23} = 3.34$, $p < 0.005$; CES+ANT vs. CTL: $t_{31} = 1.33$, $p = 0.19$).

The increased latency to finding the platform in the stressed group was not a result of differences in motivation or physical ability, because hippocampus-independent cued platform learning (measured by escape latency), and distance traveled to platform did not differ among the four experimental groups (Supplemental Fig. 1*A,B*; $p > 0.05$). Notably, the memory of the early-stress group for platform location from the previous day was impaired, as tested in a probe trial on the post reversal day. In fact, untreated CES rats swam only 28% of their total path length within the target quadrant during the probe test. This performance was significantly worse than controls (43%; $t_6 = 2.76$, $p < 0.05$) and not statistically better than chance (one-sample t-test, $t_3 = 0.97$, $p > 0.05$) (Supplemental Fig.1*C*). Similar impairments in early-stressed rats were noted when the probe test was carried out immediately after the two days of training (CES vs. CTL, $t_{13} = 2.02$, $p = 0.06$). Furthermore, treatment with the CRF$_1$ blocker prevented the early stress-provoked difficulties in the probe test (CES+ANT vs. CTL, $t_4 = 0.86$, $p > 0.05$) (Supplemental Fig. 1*C*). Note that vehicle treatment did not influence spatial memory in control rats ($p = 0.22$) or CES rats ($p = 0.23$), so that data from naive and vehicle-treated groups were combined in Figs. 3*A* and 3*B*. Together, these data demonstrate a profound impairment in hippocampus-dependent spatial memory of the stressed rats, apparent from their failure to remember the location of the platform found in the previous trial as well as from their poor performance in the probe test. Notably, CRF$_1$ blocker treatment improved the affected memory functions in early-stress rats.

Although the version of the MWM used in this study was chosen to minimize stress (Morris, 1984), the results might be confounded by group-specific differences in "coping" with averse, potentially stressful components of the task (forced swimming in room temperature water). To exclude this possibility, we tested the ability of the CRF$_1$ antagonist to mitigate the effects of early stress on recognition memory using the novel object recognition (OR) task. This relatively stress-free test requires an intact limbic circuit including the hippocampus and related cortices (Broadbent et al., 2004; Winters et al., 2004; Squire et al., 2007; Broadbent et al., 2010). The OR task examined if rats could differentiate between a novel object and one encountered 24h previously, and the ratio of novel : familiar object exploration time was used as an index of memory. There was no significant difference in exploration times on day 1 of the test between naïve and vehicle-treated groups, for either the controls or the CES rats. (object 1: CTL vs CTL+VEH: $p = 0.29$; CES vs CES+VEH: $p = 0.30$; object 2: CTL vs CTL+VEH: $p = 0.47$; CES vs CES+VEH: $p = 0.41$). Therefore, data for naive and vehicle treated groups were combined. As shown in Fig. 3*C*, exploration durations of each object on day 1 did not differ significantly among any of the experimental groups ($F_{7,100} = 0.18$, $p = 0.986$). On the second (testing) day, control rats spent approximately twice as long exploring the novel object compared with the familiar one (Fig. 3*D*; Mean ratio ± SEM = 2.14 ± 0.18). In contrast, middle-aged CES rats failed to distinguish a novel object from one they had previously encountered (Ratio: 0.99 ± 0.04; $p < 0.0001$ compared to the control group). The cohort of middle-aged CES rats given the CRF$_1$

receptor blocker discriminated between the novel and familiar objects, spending significantly more time than untreated CES rats in exploring the novel object (Ratio: 1.84 ± 0.16; $p < 0.0001$), and this ratio was not significantly different from that of controls ($p = 0.26$). Total exploration times did not vary among groups on day 2 ($F_{3,50} = 0.62$, $p = 0.61$). In summary, the adverse effects of early-life stress on learning and memory, demonstrated in two independent tests, were abrogated by the systemic administration of a selective antagonist of CRF_1 during the week immediately after the stressful period.

CRF_1 antagonist treatment prevents LTP deficits in hippocampal CA3-CA1 synapses of middle-aged CES rats

To examine the cellular mechanisms responsible for the protective effects of the CRF_1 blocker on cognitive function in CES rats, electrophysiological studies evaluated LTP, generally considered to underlie memory functions in the hippocampal circuit (Roman et al., 1987; Bliss and Collingridge, 1993). Focusing on synaptic plasticity in CA3-CA1 apical dendritic synapses that is disrupted by early-life stress (Brunson et al., 2005), we found that CES had no significant effect on normal synaptic transmission within a range of stimulus currents that do not elicit a population spike; additionally, treatment with the CRF_1 antagonist during the week that followed the CES had no effect on this measure (Fig. 4*A*; $p = 0.64$; one-way ANOVA; n = 3-6 slices from 3-6 rats per group). However, high-frequency stimulation (HFS) demonstrated profound differences in synaptic plasticity among the four tested groups: In middle-aged control rats, HFS induced robust, enduring potentiation of field EPSPs that was stable for the 40 min duration of the recording (Fig. 4*C*; $41 \pm 6\%$ above baseline, n = 6 slices from 6 rats), and this potentiation was not significantly influenced by the administration of the CRF_1 blocker on postnatal days 10-17 ($47 \pm 10\%$, n = 3 slices from 3 rats). In contrast, hippocampal slices from middle-aged CES rats demonstrated attenuated LTP ($14 \pm 6\%$, n = 4 slices from 4 rats; $p = 0.02$ vs. controls, Tukey's HSD following two-way RM-ANOVA for minutes 30-40 post-HFS). In marked contrast to slices from vehicle-treated CES rats, potentiation in slices from CRF_1 antagonist-treated CES rats resembled that from controls ($44 \pm 11\%$; n = 5 slices from 4 rats) or blocker-treated controls, and differed significantly from non-treated CES slices ($p = 0.03$). These findings indicate that LTP in CA3-CA1 synapses was disrupted by chronic early-life stress in middle-aged hippocampus, concordant with the disruption of learning and memory. Notably, limiting CRF_1 occupancy during the week subsequent to the stress period abrogated these derangements of synaptic potentiation, in parallel with, and perhaps as a mechanism for, the prevention of cognitive deficits.

CRF_1 antagonist administration directly into the lateral ventricles of CES rats preserves their cognitive function

During stress, CRH is released from the paraventricular nucleus of the hypothalamus and binds to pituitary CRF_1, thus initiating a cascade of events ultimately leading to glucocorticoid release from the adrenal gland. Glucocorticoids cross the blood-brain barrier freely, and may reach the hippocampus to influence learning and memory through the mineralocorticoid (MR) or glucocorticoid (GR) receptors (Joels, 2007; Joels and Baram, 2009; Lupien et al., 2009). In the experiments described above, the small-molecule CRF_1 antagonist NBI30775, that crosses the blood-brain barrier (Heinrichs et al., 1995), was administered via subcutaneous osmotic minipump. Thus, the compound reached the systemic circulation and blocked peripheral (pituitary) CRF_1 receptors, and, after crossing the blood-brain barrier, also central CRF_1 receptors, including those on hippocampal pyramidal cells (Chen et al., 2000; Chen et al., 2004b). In view of these facts, it might be argued that systemic CRF_1 antagonist administration prevented the effects of CES on learning and memory by altering signaling through *peripheral* CRF_1 receptors and consequently modifying physiologic stress responses and levels of plasma glucocorticoids.

To examine if blocking CRH receptor within the brain or in the pituitary was responsible for the improved learning and memory, we administered the CRF_1 blocker directly into the lateral ventricles (icv) of control and early-stressed rats during P10-17, at doses found previously not to influence peripheral glucocorticoid release (Chen et al., 2006). We then tested for the effects of this treatment on hippocampus-dependent learning and memory using the watermaze.

During both the training (Fig. 5*A*) and reversal tests (Fig. 5*B*), all rats successfully learned to find the hidden platform (training: $F_{11,473} = 6.31$, $p < 0.001$; reversal: $F_{5,215} = 3.19$, $p = 0.008$). There were no overall differences in escape latencies between stress or drug treatment conditions ($p > 0.05$). However, when we compared untreated early-stressed rats directly to control rats, we confirmed our previously observed impairments in short-term spatial memory as a result of early-life stress in both tests (two-way ANOVA, main effect of group, training: $F_{1,36} = 5.230$, $p < 0.05$; reversal: $F_{1,36} = 13.986$, $p < 0.05$). The effects of icv administration of the CRF_1 blocker surfaced during the reversal testing (Fig. 5*B*). Indeed, planned comparisons revealed that by the last trial of reversal testing (trial 18), untreated CES rats did not find the platform as fast as controls ($t_{36} = 3.94$, $p < 0.001$). Importantly, icv antagonist administration significantly improved escape latencies of early-stressed rats (CES+ANT vs CES; $t_{18} = 2.52$, $p = 0.02$), such that their performance was comparable to controls (CTL vs CES+ANT; $t_{26} = 0.49$, $p = 0.63$). Note that in this analysis (similar to Fig. 3), mean escape latencies were comparable among naïve and vehicle-treated control rats as well as between naïve and vehicle-treated stressed rats ($p = 0.12$ and $p = 0.31$, respectively); therefore, data from naïve and vehicle-treated groups were collapsed in Fig. 5*A*. Taken together, these results are in line with the results of the systemic CRF_1 blocker administration, showing the effects of early-stress on specific memory measures, and the amelioration of these deficits by blocking CRH-CRH receptor signaling immediately after the early-life stress.

The novel object recognition task described above was also employed to assess the efficacy of intracerebral administration of the CRF_1 blocker. On day 1 of the task, total exploration times of objects 1 and 2 were similar among control and stressed groups that were either naïve or vehicle-treated ($p > 0.05$; t-tests), and the groups were collapsed. On this first day, exploration time for each of the two objects was not significantly different among the four major experimental groups (Fig. 5*C*: $F_{7,22} = 2.42$, $p > 0.05$). On the second, testing day, early-stressed rats failed to recognize a novel object, as apparent from the ratio of novel : familiar object exploration (Fig. 5*D*; Ratio: 1.2 ± 0.44), and there was a significant difference in these ratios across groups ($F_{3,9} = 4.59$, $p = 0.03$). Specifically, NBI30775-treated early-stressed rats had significantly higher novel : familiar exploration ratios than untreated early-stressed rats (Bonferroni's post-hoc tests, $p < 0.05$), and were comparable to both antagonist-treated and vehicle-treated controls ($p > 0.05$), indicating that CRF_1 blocker treatment prevented the early-stressed induced deficits in recognition memory. The longer exploration of the novel object on day 2 in the controls, and CRF_1-blocker treated control and stressed groups was not due to differences in motivation for exploring objects, because total exploration times were indistinguishable among groups ($p = 0.39$), and potential bias towards one of the objects was excluded using counterbalancing (Fig. *5E*). Together with the watermaze data, these findings demonstrate that selectively blocking *central* CRF_1 receptors during the week that followed the early-life stress period sufficed to abolish deficits in hippocampus dependent memory.

Dendritic atrophy of CA1 pyramidal neurons after chronic early-life stress is abrogated by *post-hoc* treatment with a CRF_1 antagonist

The cognitive deficits provoked by chronic early-life stress were associated with reduced arborization and total length (atrophy) of apical dendrites of CA1 pyramidal cells (Brunson

et al., 2005). This finding raised the possibility that the loss of dendritic spines and excitatory synapses that would normally reside on the missing dendrites might be the foundation of memory and LTP impairments. Therefore we tested if CRF_1-blocker administration that preserved hippocampal function in CES rats also protected the complexity of apical dendrites in CA1. Sholl analysis revealed a significant effect of group ($F_{3,62} = 6.79$, $p < 0.001$) and distance from soma ($F_{12,744} = 55.79$, $p < 0.0001$). Figure 6*B* illustrates that the early-stress related atrophy was most pronounced in proximal stratum radiatum, a region carrying the post-synaptic elements of Schaffer collateral axons synapses that were tested in the LTP experiments (between 60-120μm away from the soma; Bonferroni's *post-hoc* tests: $p < 0.05$, comparing CES to CTL, CTL+ANT and CES+ANT). In addition, there was a reduction in total length of apical dendrites in the early-stress cohort compared to all other treatment groups (Fig. 6*C*; $F_{3,22} = 4.6$, $p = 0.01$). In contrast, in basal dendrites, total length did not distinguish among groups (effect of group: $F_{3,42} = 1.28$, $p = 0.3$). In summary, the location of dendritic abnormalities resulting from CES was congruent with the loss of LTP, and these abnormalities were rescued by CRF_1 antagonist treatment. These findings suggest that prevention of excessive CRF_1 occupancy abrogated dendritic atrophy in selective regions of hippocampal CA1, which, in turn preserved LTP and memory function.

Post-hoc CRF_1 blocker treatment of early-life stressed rats blunts the augmented expression of hippocampal CRH

If persistent and excessive CRF_1 activation resulted from the enduringly increased levels of CRH in hippocampus and contributed to the dendritic atrophy in early-stressed rats, then did the CRF_1 blocker given after the stress prevent dendritic atrophy by abrogating the increase in CRH expression? Quantification of CRH-immunoreactive interneurons in anatomically matched sections from all groups revealed that vehicle-treated early-stressed rats had a significantly higher number of CRH-positive cells in areas CA1 and CA3, as shown in Fig. 7*A-C* using a cohort different from that of Fig. 1 ($p = 0.04$ (CA1) and $p < 0.0005$ (CA3), t-tests control vs stressed). Treatment of stressed rats with NBI30775 during the week following the stress period attenuated this effect. Thus, as shown in Fig. 7*C*, numbers of CRH-immunoreactive neurons in treated stressed rats were not significantly different from those of naïve and blocker-treated controls ($p > 0.05$, Bonferroni's *post-hoc* tests; CA1: $F_{3,13} = 5.20$, $p = 0.01$; CA3: $F_{3,13} = 13.58$, $p = 0.0003$, one-way ANOVA). Together, these data suggest that prevention of the increased numbers of ICC-detectable CRH cells, and of the presumed augmented release of CRH throughout life, might contribute to the mechanisms by which administration of the CRF_1 blocker during P10-17 prevented dendritic atrophy and loss of hippocampus-dependent memory.

Discussion

The current studies demonstrate that a selective blocker of the CRH receptor type 1, given during the week <u>after</u> early-life chronic stress, abolishes the deficits in hippocampus-dependent cognitive function provoked by this stress in middle-aged rats. The receptor antagonist was efficacious when given either systemically or directly into the brain, suggesting that blocking central CRH receptors contributed to the ameliorating effect of the blocker on hippocampal function. The cellular basis of the protective effects of the CRF_1 antagonist involved rescue of LTP in hippocampal synapses and preservation of dendritic branches of hippocampal pyramidal cells innervated by the projections tested in the electrophysiology experiments. These novel findings directly implicate CRF_1 activation in the enduring effects of early-life stress on hippocampal synaptic plasticity and learning and memory, and offer promising translational therapeutic opportunities.

Alternative mechanisms for the effects of CRF$_1$ blocker on early-life stress-induced defects of cognitive function and hippocampal structure

Chronic stress is associated with increased levels of plasma glucocorticoids. These may penetrate the blood brain barrier and act on receptors found in hippocampal neurons, resulting in dendritic atrophy (Magarinos and McEwen, 1995) and attenuated LTP (Pavlides et al., 2002; Joels et al., 2004). Involvement of glucocorticoids in the mechanisms by which early-life stress causes hippocampal defects could not be excluded by blocking these defects via systemic administration of a CRF$_1$ antagonist, because the antagonist blocked both pituitary and central CRH receptors (Brunson et al., 2002; Rivier et al., 2003). Blocking pituitary CRF$_1$ would attenuate ACTH and glucocorticoid release and interaction with hippocampal neurons. Therefore, it is conceivable that systemic administration of the CRF$_1$ blocker ameliorated the effects of early-life stress by reducing glucocorticoid levels and their subsequent effects on hippocampal neurons. To address this possibility, we also administered the blocker directly into the brain (Figs. 5-7), and found that doses of the blocker that did not affect plasma glucocorticoids (Chen et al., 2006) were still effective in preventing cognitive defects and dendritic atrophy in middle-age rats that had experienced early-life stress. These data support an alternative mechanism of action of the blocker, namely preventing directly the effects of CRH- presumably originating from within the hippocampus (Chen et al., 2001)- on hippocampal CRF$_1$ receptors (Chen et al., 2004b; Chen et al., 2004a; Chen et al., 2010). Whereas it is quite likely that glucocorticoids significantly contribute to any effects of stress, the efficacy of centrally-administered CRF$_1$ blocker is consistent with a critical role for local CRH receptors in the deleterious effects of early-life stress on hippocampal structure and function (Chen et al., 2008; Chen et al., 2010).

How might a CRF$_1$ blocker prevent disruption of synaptic plasticity by chronic early-life stress?

Synaptic plasticity, measured by LTP, is generally believed to underlie hippocampus-mediated learning and memory (Bliss and Collingridge, 1993). In the current studies, administration of the CRF$_1$ blocker during the week subsequent to the early-life stress restored both LTP and memory during middle age. LTP requires the functional integrity of dendritic spines (Segal, 2005; Chen et al., 2007; Harvey et al., 2008). For example, reduced spine number decreases total postsynaptic area of excitatory synapses, influencing receptor density, synaptic signaling (Harris et al., 2003; Kennedy et al., 2005), and the number of functional excitatory synapses (Segal, 2005). These changes, in turn, will affect synaptic plasticity via several routes, including a reduction in local density of NMDA-receptor gated synaptic currents (Engert and Bonhoeffer, 1999; Toni et al., 1999; Nagerl et al., 2004; Zhou et al., 2004). Early-life stress attenuated dendritic length and arborization with the associated loss of spines and excitatory synapses. The efficacy of the CRF$_1$ blocker in reversing these morphological defects suggests that endogenous hippocampal CRH contributes to their occurrence. In accord with this, early-life stress caused a persistent increase in the number of neurons expressing CRH, as assessed with ICC (Figs. 1,7), and the peptide is known to promote spine and dendrite loss (Chen et al., 2008). While the mechanisms by which early-life stress enhances CRH expression in hippocampus are not fully understood, CRH mRNA expression in hippocampal neurons of immature rats is augmented by glucocorticoids (Brunson et al., 2001a), and plasma levels of the latter were chronically elevated during the period of early-life stress (Gilles et al., 1996; Avishai-Eliner et al., 2001a).

How does a *transient* administration of a CRF$_1$ blocker result in enduring amelioration of hippocampal function in early-stressed rats?

Treatment with the CRF$_1$ blocker lasted for only one post-stress week (P10-P17) in our studies, yet resulted in enduring rescue of hippocampal LTP and memory function. This suggests that both the effects of early-life stress and the therapeutic actions of the CRF$_1$

blocker interact with developmental events within hippocampus. How might these two factors (i.e. CES and CRF_1 blockade) influence hippocampal development permanently? Stress causes release of CRH within immature hippocampus (Chen et al., 2004a; Chen et al., 2006). Therefore, persistent release of CRH during the chronic early-life stress and the consequent activation of CRF_1 receptors located on dendritic spines might promote dendritic spine loss, as found previously (Chen et al., 2008). Loss of CA1 and CA3 pyramidal cell spines would eliminate the post-synaptic targets of incoming axons, and interfere with the establishment and maturation of synaptic connections onto hippocampal pyramidal cells (Gall and Lynch, 1978; Avishai-Eliner et al., 2002). Whereas the axons of the granule cells ('mossy fibers') can overcome such disruption via a strong sprouting response that persists throughout adulthood (Pitkanen et al., 2000; Brunson et al., 2001b; Brunson et al., 2005), injured commissural / associational axons sprout poorly beyond a critical period terminating during the second postnatal week (Gall and Lynch, 1978).

Previous studies obtained evidence for the above argument: mossy fiber sprouting was found in middle-aged, early-stressed rats (Brunson et al., 2005), together with sparing of the electrophysiology of mossy fiber-CA3 pyramidal cell synapses. However, functional and structural disturbances were evident in the commissural-associational innervations of CA3, and in CA3-CA1 Schaffer collateral pathways (Brunson et al., 2005). Together, these data suggest that the efficacy of a transient administration of CRH receptor antagonist results from blocking the excessive activation of CRH receptors during a critical developmental period in which excess CRH irreversibly injures dendrites and spines. Protecting these elements allows for the anchoring of incoming axons and creation of a normal mature hippocampal network.

Similar critical windows for vulnerability and therapy in the developing hippocampus have been described for a number of other insults. For example, early-life seizures can disrupt hippocampus-mediated learning and these deficits can be restored via environmental enrichment that follows the seizures (Faverjon et al., 2002). Similarly, the consequences of suboptimal maternal care can be reversed by *post-hoc* enrichment of the environment (Bredy et al., 2003). The enduring efficacy of CRF_1-antagonist treatment that was initiated already during the second postnatal week indicates that the developmental window of opportunity for reversing early-stress-induced hippocampal defects extends beyond the stress period itself, and likely involves a number of age-specific aspects of hippocampal maturation (Swann et al., 1991).

A remaining conundrum involves the basis of the persisting effect of the CRF_1-antagonist treatment in abrogating the augmented expression of CRH in hippocampus of CES rats. The mechanisms by which CES leads to persistent upregulation of CRH expression are not yet known. Epigenetic modulation of this gene in response to early-life experience has recently been demonstrated in hypothalamus (Korosi et al., 2010), and might take place in hippocampus as well. In this scenario, blocking excessive CRF_1 occupancy might interfere with the mechanisms of CRH upregulation, consistent with previously demonstrated cross-regulation of CRH and its receptors (e.g., Bale et al., 2002; Brunson et al., 2002)

Translational, therapeutic relevance of the results of the current experiments

In summary, the long-lasting deleterious effects of chronic early-life stress on hippocampal anatomy, synaptic plasticity and learning were prevented by *post-hoc* administration of a CRF_1 blocker. Although the precise mechanisms underlying the rescue of hippocampal structure and function will require further investigation, the present results point to a clinically plausible molecular target for preventing the cognitive consequences of early life stress. It has now been established that the majority of the world's children grow up under chronic stress (UNICEF, 2005), and this cannot be prevented at present. Therefore,

interventions with translational and clinical potential will need to be effective when applied *post-hoc*, as is this the case for the treatments described here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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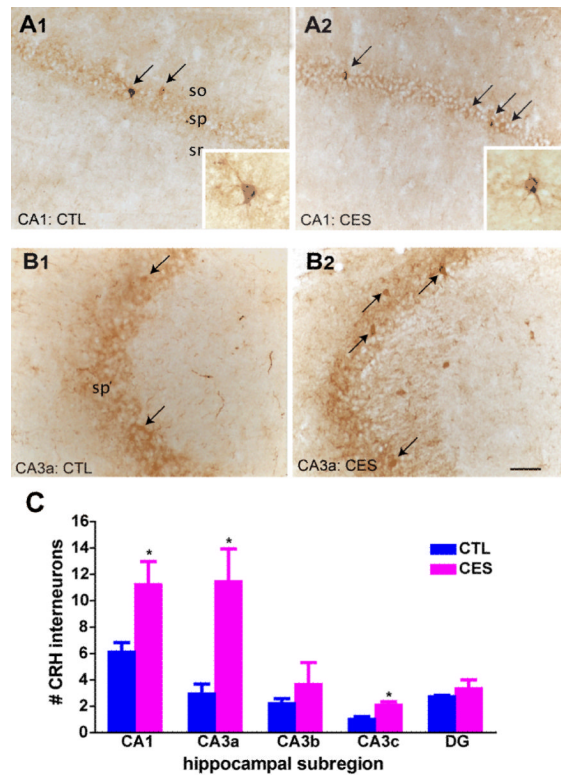
Fig. 1.
The number of corticotropin releasing hormone (CRH)-immunoreactive cells was augmented in middle-aged rats that had experienced chronic early-life stress (CES), compared to age-matched controls (CTL). Representative sections from control (*A1, B1*) and CES (*A2, B2*) demonstrate a larger number of basket cell-like neurons within the pyramidal cell layers of area CA1 (*A1, A2*) and CA3 (*B1, B2*) in 12 month-old early-stressed rats (arrows) (n = 3 rats per group). Insets show individual CRH-immunoreactive interneurons. *C,* Quantitative analyses demonstrate significantly larger numbers of CRH-immunoreactive neurons detectable by immunocytochemistry (ICC) in the CA1 and CA3 regions of hippocampi from the early-stressed rats. ICCs were carried out concurrently on all sections, and analyzed without knowledge of treatment group. (*$p < 0.05$, vs. controls). Error bars indicate SEM, scale bar = 100μm.
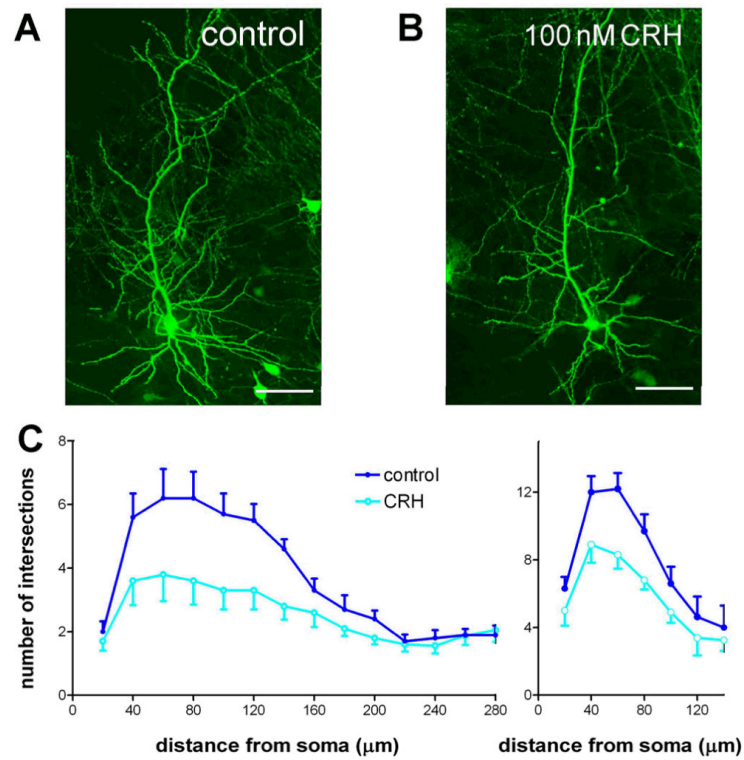
Fig. 2.
CRH application onto hippocampal organotypic slice cultures reduced dendritic complexity. Cultures were prepared from postnatal day 1 mice and grown in the presence of CRH (100nM) for two weeks. CA1 pyramidal neurons expressing YFP (n = 10 per group) are shown in *A,* control condition, and *B,* in cultures grown with CRH. *C,* Sholl analysis of both apical dendrites (left panel; $F_{1,16} = 26.99$; $p < 0.0001$) and basal dendrites (right panel; $F_{1,6} = 18.35$; $p < 0.0001$) revealed significant reductions in dendritic complexity induced by CRH exposure. Error bars indicate SEM. Scale bar = 70μm.
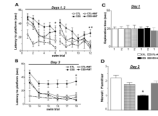
Fig. 3.

Hippocampus-dependent learning and memory were preserved in early-life stress rats treated systemically with a blocker of the CRH receptor type 1 (CRF$_1$), NBI30775, during postnatal days (P) 10-17. *A,* During the first two training days, all groups successfully learned to find the hidden platform (effect of trial: F$_{11,539}$ = 7.73, *p* < 0.001). Early-life stress impaired acquisition of the watermaze (effect of stress condition: F$_{1,49}$ = 11.09, *p* < 0.005), and CRF$_1$ blocker treatment improved performance regardless of stress exposure (F$_{1,49}$ = 5.7, *p* < 0.05). Compared to control middle-aged rats (CTL; n = 20), age-matched, chronic early-life stress (CES) rats (n = 13) required more time on the last trial of each watermaze training day (trials 6 and 12) to find a hidden platform (**p* < 0.05, CES vs. CTL). At the end of training (trial 12), this deficit was completely abolished in the stressed group that received CRF$_1$ antagonist (CES+ANT; n = 8; $^{\#}$*p* < 0.05, vs. CES; *p* > 0.05, vs. CTL). *B,* On Day 3 of the watermaze, the platform was moved to a new quadrant and rats relied on strategically-placed spatial cues to learn the new platform location. Untreated control rats, as well as control (CTL+ANT; n = 4) and early-stressed groups treated with a CRF$_1$ blocker, successfully learned the task, whereas untreated stressed rats had significantly higher escape latencies (**p* < 0.05, CES vs. all other groups). Analysis of the last trial of the reversal procedure indicated that by the end of testing, CES rats had not learned the new platform location as well as controls (*t*$_{40}$ = 3.26, *p* < 0.05), and CRF$_1$ antagonist treatment reversed this deficit (CES vs. CES+ANT: *t*$_{23}$ = 3.34, *p* < 0.005; CES+ANT vs. CTL: *t*$_{31}$ = 1.33, *p* = 0.19). *C,* On day 1 of the Object Recognition task, exploration times of both objects were similar among the four experimental groups. *D,* On day 2, preferential exploration of the novel object (apparent from the increased ratio of time spent with novel versus the familiar objects) was observed in untreated control rats (Ratio: 2.14), as well as CRF$_1$ blocker-treated controls (Ratio: 1.75). The cohort of middle-aged CES rats given the CRF$_1$ receptor blocker also discriminated between the novel and familiar objects (Ratio: 1.84), evidenced by a significantly higher mean exploration ratio compared to untreated CES rats (Ratio: 0.99; *p* < 0.0001) (**p* < 0.05, CES vs. all other groups). Error bars indicate SEM.
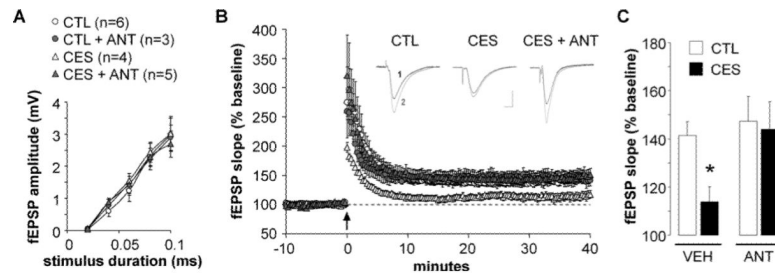
Fig. 4.
Hippocampal LTP was attenuated by chronic early-life stress and rescued by a CRF_1 blocker given during the week that followed the stress. Acute hippocampal slices were prepared from middle-aged rats sacrificed without any early-life manipulation (CTL) or exposed to chronic early-life stress (CES) without, or with CRF_1 blocker administration (ANT). *A,* Field EPSP amplitudes (±SEM) plotted as a function of input stimulus duration within a range subthreshold for eliciting a population spike revealed no differences between the three groups. *B, Inset:* Representative fEPSP traces collected during baseline (black, labeled '1') or 40 minutes after high frequency stimulation (HFS; gray overlay, labeled '2') from controls (left), CES (middle), and CES+ANT (right) rats illustrate the greater potentiation of the fEPSP in the control and CES+ANT rats as compared to the stressed rats. Calibration: 0.5mV, 5ms. Plot: fEPSP slopes measured from 10 minutes before to 1 hour after HFS (arrow). LTP in the stressed rats (open triangles) stabilized at significantly lower levels than did potentiation in controls (open circles; $p < 0.05$ vs. control; repeated measure ANOVA) or CES+ANT rats (gray triangles; $p < 0.05$); values from control and CES+ANT rats were not significantly different. Numbers denote time points of representative traces in *B*. *C,* Bar graph shows group mean slope values for the final 10 minutes of recording (*$p = 0.02$ vs. CTL).
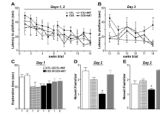
Fig. 5.

Administration of the CRF$_1$ antagonist directly into the cerebral ventricles (icv) sufficed to prevent late-onset cognitive impairments after chronic early-life stress. *A,* Deficits in spatial learning were found in early-life stressed (CES; n = 15) rats during the two days of Morris watermaze training, evidenced by higher escape latencies when compared to control ($p < 0.001$ vs. CTL; n = 23). *B,* The effects of icv CRF$_1$ antagonist treatment surfaced during the watermaze tests in which the platform was moved to a new quadrant (reversal). By the last trial of reversal testing (trial 18), the untreated CES rats took significantly longer to find the platform compared to control rats or early-stressed rats given icv CRF$_1$ blocker treatment (CES+ANT; n = 5) (*$p < 0.05$, CES vs. CTL, CES vs. CES+ANT). Antagonist treatment of control rats (CTL+ANT; n = 4) had no effect on escape latencies ($p > 0.05$, vs. CTL). *C,* In the novel object recognition task, rats from all groups explored two separate objects equally on day 1 of the task, eliminating the possibility of differences in motivation to explore. *D,* The ratio of time spent with a novel versus a familiar object, a measure of 24h recognition memory, was significantly lower in early-stressed rats compared to controls (*$p < 0.05$). CRF$_1$-blocker treatment significantly increased this ratio (*$p < 0.05$, vs. CES), and had little effect on recognition memory in control rats ($p > 0.05$, vs. untreated controls). *E,* A similar pattern of results was found when the novel objects were counterbalanced across groups. The ratio of time spent exploring a novel versus a familiar object was again lower in untreated early-stressed rats, and CRF$_1$-blocker treatment significantly increased this ratio (*$p < 0.05$, CES vs. all other groups). Data indicate mean ± SEM.
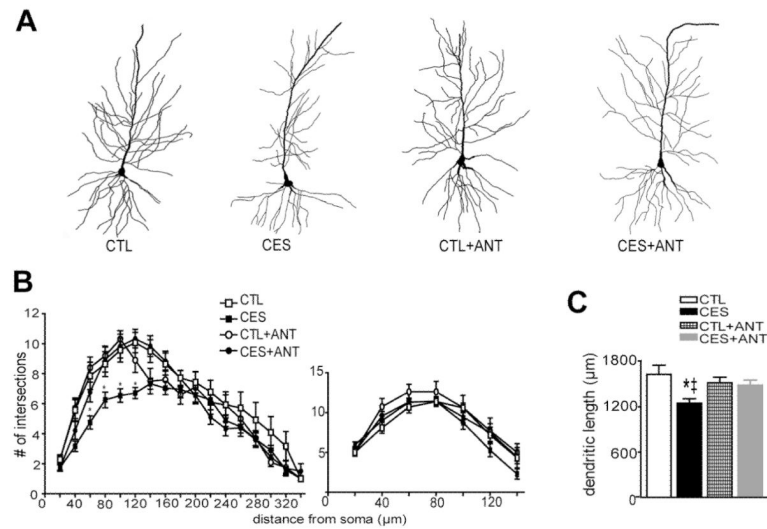
Fig. 6.
CA1 dendritic atrophy in middle-aged, early-life stressed rats was prevented by treatment with a blocker of CRF_1 during the week following the stress. *A,* Golgi-impregnated CA1 pyramidal cells from control, early-life stress (CES), CES+ANT and CTL+ANT groups were reconstructed using camera lucida. Representative neurons from each group illustrate the reduction in dendritic branching in neuron from the stress group, and the normalization of dendritic arborization of neurons from CES+ANT rats (n = 5-8 neurons per animal, 3-5 animals per group; Scale bar = 60μm). *B,* Complexity of dendritic arbors was quantified using Sholl analyses, counting the number of dendritic intersections in apical (left) and basal (right) dendritic regions. Significantly reduced dendritic complexity was found in the proximal stratum radiatum of neurons from early-stress rats when compared to either controls ($p < 0.05$) or CES+ANT ($p < 0.05$). *C,* Total apical dendritic length was significantly reduced in the early stress group, but not in stressed rats given the CRH receptor blocker NBI30775. Error bars indicate SEM. $*p < 0.05$ vs CTL, $‡p < 0.05$ vs. CES +A.
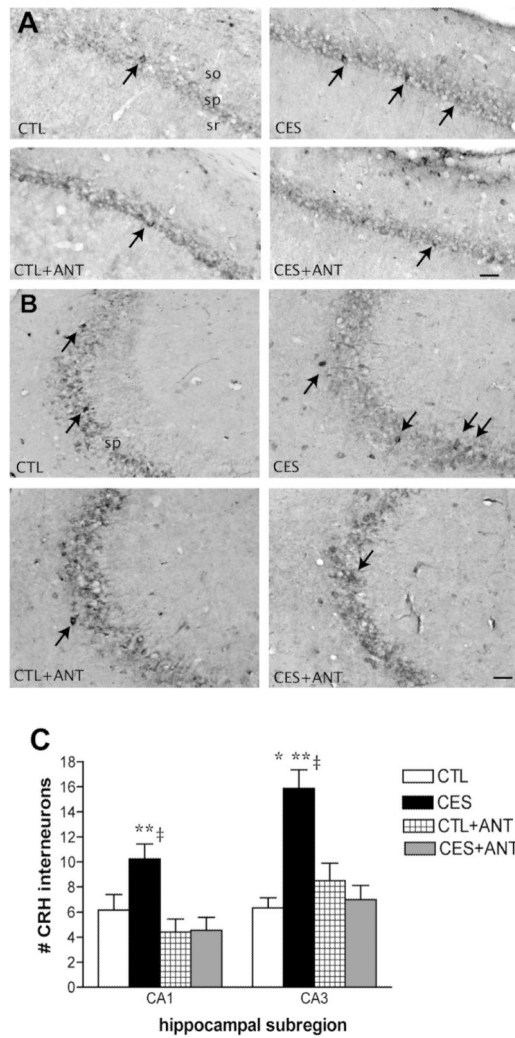
Fig. 7.
CRF$_1$ blocker treatment during P10-17 prevented augmentation of CRH-immunoreactive (IR) cells in middle-aged rats that had experienced chronic early-life stress (CES). *A,* CRH-IR neurons in hippocampal CA1 of control (CTL), early-stressed, antagonist-treated CTL (CTL+ANT), and antagonist-treated early=stressed (CES+ANT) rats. Arrows point to CRH-positive interneurons in the pyramidal cell layer. *B,* Representative images from hippocampal CA3 region of the same groups. *C,* Numbers of CRH-IR neurons in areas CA1 and CA3 of NBI30775 treated early-stressed rats were significantly lower than in untreated stressed littermates, and were comparable to controls. **p < 0.05* vs CTL, ***p < 0.05* vs. CTL+A, ‡*p < 0.05* vs. CES+A.