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Differential contribution of CBP:CREB binding to corticotropin-releasing hormone expression in the infant and adult hypothalamus

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Abstract

Corticotropin-releasing hormone (CRH) contributes crucially to the regulation of central and peripheral responses to stress. Because of the importance of a finely tuned stress system, CRH expression is tightly regulated in an organ- and brain region-specific manner. Thus, in the hypothalamus, CRH is constitutively expressed and this expression is further enhanced by stress; however, the underlying regulatory mechanisms are not fully understood. The regulatory region of the *crh* gene contains several elements, including the cyclic-AMP response element (CRE), and the role of the CRE interaction with the cyclic-AMP response element binding protein (CREB) in CRH expression has been a focus of intensive research. Notably, whereas thousands of genes contain a CRE, the functional regulation of gene expression by the CRE:CREB system is limited to ~100 genes, and likely requires additional proteins. Here, we investigated the role of a member of the CREB complex, CREB binding protein (CBP), in basal and stress-induced CRH expression during development and in the adult. Using mice with a deficient CREB-binding site on CBP, we found that CBP:CREB interaction is necessary for normal basal CRH expression at the mRNA and protein level in the nine-day-old mouse, prior to onset of functional regulation of hypothalamic CRH expression by glucocorticoids. This interaction, which functions directly on *crh* or indirectly via regulation of other genes, was no longer required for maintenance of basal CRH expression levels in the adult. However, CBP:CREB binding contributed to stress-induced CRH expression in the adult, enabling rapid CRH synthesis in hypothalamus. CBP:CREB binding deficiency did not disrupt basal corticosterone plasma levels or acute stress-evoked corticosterone release. Because dysregulation of CRH expression occurs in stress-related disorders including depression, a full understanding of the complex regulation of this gene is important in both health and disease.

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Declaration of interest
The authors have nothing to disclose.

Keywords

Corticotropin-releasing hormone; CREB binding protein; CRF; cyclic AMP; GR glucocorticoids; negative feedback; stress; TORC; transcription factor

Introduction

The neuropeptide corticotropin-releasing hormone (CRH) coordinates neuroendocrine, autonomic and behavioral responses to stress (Aguilera, 2011; Bale & Vale, 2004; Bonfiglio et al., 2011; Brunson et al., 2001a; Coste et al., 2001; de Kloet et al., 2005; Herman et al., 2003; Joëls & Baram, 2009; Lightman, 2008; Valentino & Van Bockstaele, 2008; Zoumakis & Chrousos, 2010), and dysfunctional CRH regulation is present in several stress-related affective disorders including anxiety and major depression (de Kloet et al., 2005; Flandreau et al., 2012; Lloyd & Nemeroff, 2011; Sink et al., 2012). CRH expression varies widely among tissues and brain regions, and the peptide is strongly expressed in the hypothalamic paraventricular nucleus (PVN), where its expression is regulated by a variety of factors including stress (Lightman, 2008; Swanson & Simmons, 1989; Watts, 2005). It is generally found that CRH release during stress is followed by a rapid increase in transcription of the gene, perhaps an adaptive response to establish ample peptide stores for future stress (Dent et al., 2000; Ginsberg et al., 2003; Liu et al., 2012; Ma et al., 1997; Osterlund & Spencer, 2011; Pace et al., 2009; Rivest et al., 1995; Tanimura & Watts, 1998; Watts, 2005; Yi & Baram, 1994a). Appropriate initiation and termination of both CRH synthesis and release is vital for physiological well-being (Aguilera & Liu, 2012; Bale et al., 2002; Coste et al., 2001; Liu & Aguilera, 2009). Therefore, elucidating the transcriptional regulation of *crh* is important for understanding normal stress mechanisms as well as stress-related disorders.

The *crh* gene is relatively small, and contains a limited number of regulatory elements (Adler et al., 1990; Itoi et al., 1998; Liu & Aguilera, 2009; Robinson et al., 1988; Seasholtz et al., 1988; Spengler et al., 1992; Van et al., 1990). A functionally defined glucocorticoid responsive element (GRE) at the *crh* promoter (Malkoski et al., 1997; Malkoski & Dorin, 1999; Van et al., 1990) interacts with glucocorticoid receptors, which are activated by the binding of glucocorticoids such as corticosterone (CORT). In accord, experiments employing steroid implants into the PVN demonstrated a direct negative regulation of *crh* expression by glucocorticoids (Kovács et al., 1986; Kovács & Mezey, 1987; Sawchenko, 1989). In contrast, glucocorticoids increase *crh* expression in other areas, such as the central amygdala (Brunson et al., 2001b; Makino et al., 1994) and the placenta (Robinson et al., 1988). The *crh* promoter contains an important, functional cyclic AMP response element (CRE) (Seasholtz et al., 1988; Spengler et al., 1992) that is activated by cyclic adenosine 3′, 5′ monophosphate (cAMP) stimulation (Itoi et al., 1998; Seasholtz et al., 1988; Spengler et al., 1992) and is required for stress-induced *crh* transcriptional activation in the hypothalamus in both mature (Itoi et al., 1998; Liu & Aguilera, 2009) and developing (Chen et al., 2001; Hatalski & Baram, 1997) brain. Cyclic AMP leads to phosphorylation of Ser^{133} of the cAMP response element-binding protein (CREB). Phospho-CREB binds to the CRE and facilitates transcription of CREB target genes, including CRH (Cardinaux et al., 2000; Wölfl et al., 1999). Interestingly, whereas a CRE is present on thousands of genes, only hundreds are actually regulated by this factor, and the specificity of the regulation is likely mediated by additional elements (Liu et al., 2008, 2012) including CREB binding protein (CBP). There is little information about the role of CBP in enabling CREB-mediated regulation of *crh* expression.

CBP is a transcriptional co-activator that has been implicated in neuronal signaling and plasticity (Flavell & Greenberg, 2008). CBP possesses both protein binding and histone

acetylation capabilities (Kwok et al., 1994). The transcription-factor binding domain of CBP, designated the KIX domain, binds the phosphorylated Ser^{133} within CREB's kinase-inducible domain (Parker et al., 1996; Radhakrishnan et al., 1997), and the CBP:CREB complex then activates the transcriptional machinery (Flavell et al., 2008; Kwok et al., 1994). In addition, stress increases CBP expression in the PVN in parallel with increased CRH expression (Sterrenburg et al., 2012). Thus, whereas CBP is well established as a contributor to crucial events in the expression of target genes, it is unknown if the protein is involved in the basal and stress-related regulation of hypothalamic CRH expression.

Here, we investigated the involvement of CBP in regulating hypothalamic *crh* gene transcription. Because the consequences of deficient CBP:CREB binding on *crh* expression might be compensated by actions of other transcriptional regulators, we sought to study this question also under conditions where *crh* expression is not responsive to glucocorticoids. Several groups have previously found that in the neonatal rodent (postnatal days 1–10), *crh* expression in the PVN was not regulated by glucocorticoids (Grino et al., 1989; Korosi & Baram, 2008; Vazquez et al., 2006; Yi et al., 1993), suggesting that during this developmental period, the regulatory role of CBP:CREB might be less likely to be compensated, and hence easier to discern. In addition, because the presence and *in vitro* function of a regulatory element might not necessarily reflect the physiological role in a given brain region (Adler et al., 1990; Itoi et al., 1998), we investigated this question *in vivo*. We employed mice with an impaired CBP KIX domain to examine CRH expression on postnatal day 9 and during adulthood (2–3 months), and examined both basal expression and stress-induced CRH synthesis in the PVN. The $Cbp^{KIX/KIX}$ mice have three point mutations at the KIX domain which restrict the ability of CBP to bind to phospho-CREB (Kasper et al., 2002), and thus provide a useful tool to look at the role of CBP:CREB interaction in gene regulation.

Methods

Animals

$Cbp^{KIX/KIX}$ mice were generated as previously described (Kasper et al., 2002; Wood et al., 2006). Briefly, the targeting vector for *Cbp* contained the point mutations Tyr^{650}Ala, Ala^{654}Gln, and Tyr^{658}Ala. The three mutations were introduced into the *Cbp* locus of 129P2/OlaHsd-derived E14 embryonic stem cells by homologous recombination (Figure 1). Mice carrying the mutant allele of the KIX-domain of *Cbp* (designated $Cbp^{KIX/KIX}$ for homozygous knock-in mice) were bred and backcrossed in a heterozygous state on a C57BL/6 genetic background for 10 generations. Male mice were used for all experiments. Mice for the experiments were generated from heterozygous matings and wild-type littermates were used as controls for all experiments; for the P9 ISH a few age-matched wild-type controls were not littermates. Neonatal mice were 8–10 days old, with average body weight of 4.8 g in $Cbp^{+/+}$ and 4.6 g in $Cbp^{KIX/KIX}$ mice. Adult mice were 2-3 months old. $Cbp^{+/+}$ mice on average weighed 27.6 g, with adrenal gland weight of 4.7 g. $Cbp^{KIX/KIX}$ mice on average weighed 28.9 g, with adrenal gland weight of 4.2 g. All animals had free access to food and water in their home cages. Lights were maintained on a 12 h light/12 h dark cycle, and all *in vivo* experiments were carried out between 08:00 and 10:00. All experiments were conducted according to National Institutes of Health guidelines for animal care and use and were approved by the Institutional Animal Care and Use Committee of the University of California-Irvine.

Stress paradigm

Whereas restraint is often used in adult rodents to activate the hypothalamic CRH-expressing neurons, other paradigms may be useful in adult and developing mice (Masini et

al., 2012; Schmidt et al., 2011) and might activate the hypothalamus differentially (Hatalski et al., 1998). Here we employed a combined physiological/psychological stress, consisting of restraint, jostling and loud music, modified from Chen et al. (2010). Briefly, adult male mice were individually restrained in 50-ml tubes, and two or more tubes were placed in a cage on a laboratory shaker in a brightly lit room bathed in loud music. The stress lasted for 15 minutes.

Immunohistochemistry (IHC)

Mice (n = 4 per genotype) were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused through the ascending aorta with 0.9% saline solution, followed by freshly prepared, cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4. Brains were collected and cryoprotected in 30% sucrose/0.1 M PB solution. Brains were coronally sectioned at 30 μm using a cryostat. To estimate the CRH-immunoreactive neurons in the PVN, consecutive sections throughout the entire PVN were collected as two series in six-well plates containing 0.1 M PB. One series of sections was subjected to IHC and the adjacent series was used for cresyl violet staining to verify the anatomy. Sections of $Cbp^{+/+}$ and $Cbp^{KIX/KIX}$ brains were processed concurrently in parallel wells.

Free-floating sections were subjected to standard avidinbiotin complex (ABC) methods as described previously (Fenoglio et al., 2006). Briefly, after several washes with 0.01 M PBS containing 0.3% Triton X-100, pH 7.4 (PBS-T), slices were treated for 30 min in 0.3% H_2O_2 in PBS, followed by blockade of nonspecific sites with 1% bovine serum albumin (BSA) and 2% normal goat serum in PBS for 30 min and incubation for 5 d at 4 °C with anti-CRH (1:40,000; gift from Dr. W. W. Vale, Salk Institute, La Jolla, CA) in PBS containing 1% BSA and 2% normal goat serum. After three 5 min washes in PBS-T, slices were incubated in biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories) in PBS containing 1% BSA for 2 h at room temperature. After washing in PBS-T (three times for 5 min), slices were incubated in avidin-biotin-peroxidase complex solution (1:250; Vector Laboratories) for 3 h at room temperature. Sections were then rinsed again in PBS-T (three times for 5 min). The reaction product was visualized by incubating the sections for 8–10 min in 0.04% 3,3-diaminobenzidine (DAB) containing 0.01% H_2O_2.

Quantification of IHC

To assess CRH expression in $Cbp^{+/+}$ versus $Cbp^{KIX/KIX}$ mice, we used dilute anti-CRH antiserum to visualize CRH-expressing neurons in the PVN and counted the numbers of cells that expressed sufficient levels of CRH by using unbiased stereological principles (West, 1999). IHC was performed on all sections from all groups simultaneously, in the same wells. To perform an unbiased determination, we employed a systematic random series of sections (West, 1999). The entire PVN was sliced and the consecutive sections were collected as two series (#1, 3, 5, 7 … and #2, 4, 6, 8 …). One series of sections was subjected to IHC for quantitative analysis yielding 9–10 sections per animal, and anatomically matched sections from each animal were subjected to cell counts without knowledge of genotype.

CRH-immunoreactive neurons were visualized using a Nikon E400 microscope and counted in three anatomically matched sections under a 100× oil-immersion objective (numerical aperture 1.4). To ascertain that each cell was counted once only, cell nuclei were counted in duplicate using the “optical dissector” technique (Gundersen et al., 1988; West, 1999) relying on the leading edges of nuclei in each section. Three matched sections (at the level of “coronal sections 241, 251 and 261”, Sidman et al., 1971) from four brains per genotype were analyzed, and neuronal density was expressed as the number of labeled neurons in a 30

μm thick PVN. Analyses were performed using Prism 5 (La Jolla, CA), and *n* = number of mice per group.

Semiquantitative *in situ* hybridization histochemistry (ISH)

Two independent methods were employed to measure CRH mRNA expression, ISH and quantitative real-time PCR (qPCR). ISH provides excellent regional resolution in an anatomically complex structure such as the PVN, whereas qPCR enables more precise analysis of mRNA levels. Notably, several groups have found a relative lack of sensitivity of qPCR to biologically important yet modest changes in CRH gene expression *in vivo* (cf. Shepard et al., 2005 *vs*. Chen et al., 2012). Basal levels of CRH mRNA were investigated as a measure of steady-state expression, and levels of unedited, heteronuclear CRH RNA were determined as a measure of direct activation of *crh* gene expression. For ISH studies, mice were killed by rapid decapitation and brains were quickly removed and frozen on dry ice. Forebrains were sectioned at 20 μm, mounted onto gelatin-coated glass slides, divided into series and stored at −80 °C. Expression levels of CRH mRNA and heteronuclear RNA (hnRNA) were examined using previously described methods (Fenoglio et al., 2006). Sections were air dried and postfixed in 4% paraformaldehyde for 20 min. Sections underwent a series of dehydration and rehydration steps through graded ethanol washes and were exposed to 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8, for 8 min. Adult sections were also exposed to chloroform for 5 min for defatting. After dehydration through graded ethanol washes, sections were incubated with prehybridization buffer for 1 h in a humidified chamber at hybridization temperature. Prehybridization and hybridization buffer contained 50% formamide, 5× SET (750 mM NaCl, 150 mM Tris, 12.5 mM EDTA), 0.2% SDS, 5× Denhart's solution, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast tRNA, 100 mM dithiothreitol, and 10% dextran sulfate. We employed 0.25×10^6 cpm of a 3′ ^{35}S-labeled 60 mer deoxyribonucleotide probe per section (Rice et al., 2008), and hybridized overnight at 42 °C. Sections were washed at 42 °C in 2× saline-sodium citrate (SSC) for two successive 30 min washes, and in 1× and 0.3× SSC at room temperature for 30 min each. Levels of unedited CRH heteronuclear RNA were determined using 0.8×10^6 cpm of ^{35}S-labeled ribonucleotide probe per section (courtesy of Drs. S. J. Watson, University of Michigan, Ann Arbor, MI, and S. Rivest, CHUL Research Center, Quebec, Canada). Sections were hybridized overnight at 55 °C, then rinsed in 4× and 2× SSC, subjected to a 30 min RNase wash at 37 °C, followed by a series of washes at 62 °C: 2× SSC (5 min), 1× SSC (5 min), 0.2× SSC (30 min), 0.1× SSC (30 min), and 0.03× SSC at 55 °C for 30 min.

Sections were dehydrated in graded ethanol solutions containing 0.3 M ammonium acetate, followed by 100% ethanol. Sections were air dried and apposed to film (Kodak BioMaxMR Film, MR-1; Eastman Kodak, Rochester, NY) for 5–14 d. The ISH signal was analyzed blindly on scanned, digitized images of sections at coronal levels corresponding at 3.8 to 3.5 mm anterior to bregma for PVN. The Image Tool software program (University of Texas Health Science Center, San Antonio, TX) was used after determining the linear range of optical densities (ODs) using ^{14}C standards. Values were only used if they fell in the linear range. Background was corrected for by subtracting the OD of signal over the thalamus. Because CRH expression is not homogenous throughout mouse PVN (e.g., Viau & Sawchenko, 2002), we focused on anatomically matched PVN regions rich in parvocellular CRH-expressing neurons and included two matched, CRH-expressing sections per mouse in the analysis. The OD from the two sections was averaged to generate an expression value for each PVN.

Quantitative polymerase chain reaction (qPCR)

Real time PCR was performed to examine CRH gene expression. Mice (*n* = 8–9 per genotype) were killed by rapid decapitation and brains were quickly removed and frozen on

dry ice. PVN tissue was collected by punching the region out of 2 mm thick sections. RNA was isolated using RNeasy kit (Qiagen, Carlsbad, CA). cDNA was reverse transcripted from individual PVN (1 μg total RNA), using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Sciences, Branford, CT). Primers were derived from the Roche Universal Probe Library: CRH left primer AAG AAG AGA AAG GAG AAG AGG AAG; CRH right primer CCG CAG CCG CAT GTT AG. CRH probes were conjugated to the dye FAM (Integrated DNA Technologies, San Diego, CA). GAPDH left primer AAT GGT GAA GGT CGGTGT G, GAPDH right primer GTG GAGTCA TACTGG AAC ATG TAG; conjugated to HEX 555 dye (Integrated DNA Technologies, San Diego, CA). The non-overlapping dyes and quencher on the reference gene allow for multiplexing in the Roche Lightcycler 480 II machine (Roche Applied Sciences, Branford, CT). All values were normalized to GAPDH expression levels. Analysis and statistics were performed using the Roche proprietary algorithms and REST 2009 based on the Pfaffl method (Pfaffl, 2001; Pfaffl et al., 2002).

Radioimmunoassay (RIA)

Basal plasma corticosterone (CORT) levels were measured in mice killed within 1 min of their disturbance, and stress-induced CORT levels were measured in mice killed following a 15 min stress described above. The RIA for plasma CORT levels was performed using a commercial kit (MP Biomedicals, Solon, OH) as previously described (Rice et al., 2008), and assay sensitivity was 5 ng/ml. Adult plasma samples were taken from the same mice used for CRH hnRNA quantification, and all adult samples were run in one assay. For postnatal day 9 mice, two samples from each genotype were pooled for RIA, and the reported data were derived from two separate assays, normalized using aliquots of adult rat plasma that were included, in three dilutions, as measures of inter-assay variability.

Data analysis

All experiments were analyzed without the knowledge of the group. When two groups only were compared, data were analyzed using Student's *t*-test or Kruskal-Wallis nonparametric test as appropriate (Prism 5.01, Graphpad Software, La Jolla, CA) at the 95% level of significance. CRH hnRNA ISH data were also analyzed using two-way ANOVA (stress and genotype). qPCR data were analyzed using one-way ANOVA. RIA data were analyzed using one-way ANOVA (basal levels by genotype, postnatal day 9 mice) and two-way ANOVA (stress and genotype) in adult mice. Values are reported as mean ±SEM.

Results

Basal CRH expression levels in PVN are reduced by CBP:CREB binding deficiency on postnatal day 9

CRH mRNA expression in the PVN was first examined in $Cbp^{KIX/KIX}$ and wild-type mice on postnatal day 9, prior to the onset of glucocorticoid-receptor mediated negative regulation (Grino et al., 1995; Yi et al., 1993). With the use of ISH, CRH mRNA was 74% lower in $Cbp^{KIX/KIX}$ mice compared to wild-type mice ($T(6) = 2.81$; $p<0.05$). When we included the heterozygous $Cbp^{KIX/+}$ mice in the analysis, we found a significant effect of genotype on CRH mRNA levels ($H = 6.89$, $p<0.05$, Figure 2A and B). Interestingly, there was a tendency towards a gene dose effect on CRH mRNA expression because CRH mRNA expression in heterozygous ($Cbp^{KIX/+}$) mice was intermediate between levels in wild-type and $Cbp^{KIX/KIX}$ mice. These findings suggested that CBP:CREB interaction contributed to basal mRNA CRH expression in the PVN of the developing rodent.

Whereas the use of ISH demonstrated changes in CRH mRNA levels that are often associated with biologically meaningful changes in the stress response, qPCR failed to demonstrate significant differences among the genotypes (wild-type 1.0±0.17, $Cbp^{KIX/+}$

1.15±0.18, and $Cbp^{KIX/KIX}$ 1.11±0.13, $p = 0.796$; $n = 8–9$ animals per group). These discrepancies between the ISH and qPCR methods have been previously encountered, with the ISH data often in line with functional and other quantitative measures of gene expression (e.g., Shepard et al., 2005 *vs.* Chen et al., 2012, as well as Marcelin et al., 2012 and Kanyshkova et al., 2009).

To examine if reduced CRH mRNA expression in the PVN of developing mice with deficient CBP:CREB interaction governs the peptide's expression, we studied CRH at the single cell resolution using immunohistochemistry (IHC; Figure 3). The number of cells expressing IHC-detectable CRH was not altered in $Cbp^{KIX/KIX}$ compared to wild-type mice (wild type *vs.* $Cbp^{KIX/KIX}$: 70.00±7.77 *vs*. 69.17±3.13; $n = 4$; $p = 0.92$). Together with the reduced mRNA expression found using ISH, the lack of reduction in the number of cells expressing CRH can be interpreted to suggest that expression of the peptide in individual cells was lower in the $Cbp^{KIX/KIX}$ mice compared to wild-type littermates. These IHC findings suggested that deficient CBP:CREB interaction affected CRH levels in cell groups within the PVN.

KIX mutation does not affect basal CORT levels in 9-day old mice

Basal plasma CORT levels averaged 3.6 ng/ml in $Cbp^{+/+}$, 5.0 ng/ml in $Cbp^{KIX/+}$ and 2.5 ng/ml in $Cbp^{KIX/KIX}$ mice (F (2,11) = 1.78, $p>0.05$, $n = 5$). These plasma levels are consistent with previous reports in developing mice (e.g., Rice et al., 2008; Schmidt et al., 2002). The absence of reduction of basal plasma CORT in $Cbp^{KIX/KIX}$ mice is consistent with reports of involvement of vasopressin in CORT regulation during the first week of life (Muret et al., 1992), or with a compensatory involvement of vasopressin in adrenal regulation under conditions of reduced CRH levels in PVN (Harbuz et al., 1994).

CRH mRNA levels at rest are unaffected by deficient CBP:CREB interaction in adult mice

As shown above, basal CRH mRNA levels were reduced in $Cbp^{KIX/KIX}$ on postnatal day 9 (Figure 2), when regulation of the hypothalamic *crh* gene by glucocorticoids may not be fully mature (Grino et al., 1995; Yi et al., 1993). Later in development, the *crh* gene is regulated by numerous factors in addition to CREB, including glucocorticoids (Brunson et al., 2001a; Grino & Burgunderf, 1992), CREM (Liu & Aguilera, 2009; Sassone-Corsi, 1998), and TORC (Liu et al., 2008, 2012). Therefore, we evaluated the contribution of CBP:CREB interaction to basal CRH expression in adult mice by measuring CRH mRNA levels in wild-type and $Cbp^{KIX/KIX}$ mice. Under stress-free conditions (i.e., mice sacrificed within 30–45 s of entering the vivarium), ISH revealed that CRH mRNA levels in the PVN were no longer reduced in $Cbp^{KIX/KIX}$ compared to wild-type mice (Figure 4A and B). CRH mRNA levels in the central nucleus of the amygdala were also not statistically distinguishable between wild-type and $Cbp^{KIX/KIX}$ mice (23.6±2.0 *vs.* 20.5±2.5, $n = 3$ per group).

Stress-induced transcription of the *crh* gene is impaired by deficient CBP:CREB interaction

The findings described above suggest that functional CBP:CREB interaction is not required to maintain basal CRH mRNA levels in adult mice. However, stress results in rapid initiation of CRH transcription in a phospho-CREB dependent manner (Chen et al., 2001; Kovács & Sawchenko, 1996a, b), and this is believed to enable replenishment of hypothalamic CRH stores depleted by stress-induced CRH release. Measuring unedited heteronuclear RNA (hnRNA) provides information about the levels of *de novo* transcription of *crh* (Herman et al., 1992; Shepard et al., 2005). To examine if CBP:CREB interaction was required for stress-induced CRH expression, we measured CRH hnRNA levels after imposition of stress, at 15 min from stress onset, within the time-frame of peak hnRNA

expression for this gene (Chen et al., 2001; Fenoglio et al., 2006; Gorton et al., 2007). Basal CRH hnRNA expression did not differ significantly between genotypes. Compared to stress-free controls, CRH hnRNA was increased by 51% in wild-type mice exposed to 15 min of combined physiological/psychological stress ($T(11) = 3.24$; $p<0.01$); in contrast, no significant increase was detected in $Cbp^{KIX/KIX}$ mice after stress (Figure 5). An additional two-way ANOVA analysis considered stress ($F(1,1) = 6.53$; $p = 0.019$), and genotype ($F(1,1) = 0.802$; $p = 0.38$), and found insignificant interaction ($F(1,1) = 1.58$; $p = 0.22$).

Taken together, these data suggest that functional CBP:CREB interaction, either directly or via the regulation of other genes or other upstream mechanisms, is not required to maintain basal CRH mRNA stores in the adult mouse PVN, and contributes to stress-evoked CRH expression. The lack of significant effect using the two-way ANOVA analysis might be a result of compensatory mechanisms involving TORC or other regulatory factors.

Stress-induced increase of plasma CORT is maintained in mice with deficient CBP:CREB interaction

Stress induces rapid release of CRH from median-eminence terminals of PVN neurons. This release is associated with, and might trigger, transcriptional activation of the gene. In view of the inability of $Cbp^{KIX/KIX}$ mice to initiate CRH-transcription in response to stress, we queried if CRH release was impaired in response to stress. CRH secretion into the hypothalamo-pituitary portal system initiates a cascade of events ultimately leading to glucocorticoid release from the adrenal glands. To obtain a general measure of the integrity this cascade in $Cbp^{KIX/KIX}$ mice, plasma corticosterone (CORT) levels were measured in adult mice during stress-free conditions and after a 15 min combined physiological/psychological stress ($n = 5$–6 per group). Basal plasma CORT levels were comparable in both wild-type and $Cbp^{KIX/KIX}$ mice, and CORT levels were augmented significantly by stress ($F(1,18) = 110.66$; $p<0.0001$), with minimal contribution of the genotype ($F(1,18) = 1.67$; $p = 0.212$; Figure 6). Whereas this single time-point measurement does not exclude problems with the duration or the magnitude of plasma CORT elevations, it suffices to infer that the loss of CBP:CREB interaction does not prevent the acute elevation of plasma CORT in response to stress.

Discussion

The principal findings of the current study are: (1) Impairment of CBP:CREB interaction reduces CRH expression in the hypothalamus on postnatal day nine, but does not disrupt steady-state CRH expression in the adult; (2) Stress-related transcriptional activation of the *crh* gene in PVN is altered by impaired CBP:CREB interaction in mature mice; (3) However, stress-induced increase in plasma CORT can take place in mice with deficient CBP:CREB interaction, suggesting that CRH is released in sufficient quantity or that compensatory mechanisms exist. Taken together, these findings support the notion that CBP:CREB contributes importantly to the repertoire of elements that regulate CRH expression and levels in diverse contexts.

Loss of functional CBP transcription factor binding domain alters basal CRH expression in the postnatal day 9 mouse

Disruption of CBP:CREB interaction reduced basal hypothalamic CRH expression at postnatal day nine but did not affect CRH expression in adulthood. This finding might be attributable, at least in part, to the differential maturation of mechanisms that regulate *crh* (Dent et al., 2000; Grino et al., 1989; Keegan et al., 1994; Muret et al., 1992). During the first week of life, basal CRE-binding activity and the phosphorylation of CREB in the PVN are high (Chen et al., 2001). In addition, exposure to stress increases levels of both (Hatalski

& Baram, 1997), with a larger effect on P9 compared to P6 (Baram & Hatalski, 1998). Thus, CRE-mediated gene transcription appears to be fully functional early in life (Dent, et al., 2000). Further support for the key role of CBP:CREB interaction in the regulation of CRH expression during development derives from closer analysis of the mice used here. While the knock-in KIX mutation is complete, CBP:CREB binding is reduced to ~30% of wild-type rather than completely eliminated (Kasper et al., 2002; Xu et al., 2007). Remarkably, this reduction sufficed to diminish CRH expression in PVN of immature mice, indicating a crucial role for CBP:CREB interaction in maintaining steady-state levels of CRH mRNA during the first weeks of life

Reduced CRH mRNA levels, detected using ISH, were reflected also in the immunohistochemical analyses. The latter revealed that the deficient CBP:CREB interaction did not diminish the number of CRH-expressing cells, but reduced the amount of protein produced, as measured using optical density of the immunoreactive peptide. Interestingly, qPCR did not detect these changes. The reasons for the discrepancy might be several. First, ISH and IHC measure CRH levels over anatomically defined and highly restricted cell populations. This resolution is not afforded by punching the PVN for qPCR (or Western blot). There are several CRH-expressing neuronal populations in PVN, and only a subset is regulated by stress (e.g., Swanson et al., 1987). Indeed, recent work using qPCR found significantly lower changes in CRH gene expression in response to stress compared to the use of ISH by the same investigators (Chen et al., 2012; Shepard et al., 2005). In addition, the authors and others have found excellent correlation of ISH and IHC with physiological measures of gene expression in restricted neuronal populations, whereas qPCR did not detect expression changes (Kanyshkova et al., 2009; Marcelin et al., 2012; Surges et al., 2006).

The reduction of CRH expression in developing $Cbp^{KIX/KIX}$ mice was no longer apparent in adult male mice. This may be attributable to maturational changes in the function of other *cis*- and *trans*-acting *crh* regulators. One *cis*-acting factor is the glucocorticoid response element (GRE). A non-canonic, functionally defined GR binding domain has been identified on the *crh* gene promoter, through which glucocorticoids reduce promoter activity *in vitro* (Malkoski et al., 1997; Malkoski & Dorin, 1999; Spengler et al., 1992; Van et al., 1990). In adult mice, glucocorticoids negatively regulate CRH expression in the PVN via binding of glucocorticoid receptors (GR), located in CRH-expressing PVN neurons (Liposits et al., 1987; Kovács & Sawchenko, 1996a,b). This hypothalamic GR-mediated repression may not be fully functional in early postnatal development, because GR antagonist administration into the PVN or pharmacological adrenalectomy did not affect CRH levels early in life (Baram & Schultz, 1992; Grino et al., 1989; Yi et al., 1993), but enhanced hypothalamic CRH expression in the adult (Kovacs et al., 1986; Kovacs & Mezey, 1987), and activation of GR failed to repress CRH expression prior to the second postnatal week (Avishai-Eliner et al., 1995; Fenoglio et al., 2004). It should be noted that GR is abundantly expressed in the rodent hypothalamus during the first week of life (van Oers et al., 1998; Yi et al., 1994b), and evidence from studies of adult rodents indicates the presence of GR in CRH-expressing PVN cells (Ginsberg et al., 2003; Lipositz et al., 1987; Osterlund & Spencer, 2011). The presence of the receptor throughout ontogeny suggests that the minimal effect of glucocorticoids on CRH expression in late fetal and earlypostnatal rodents (Baram & Schultz, 1992; Grino et al., 1989) is not a result of absent glucocorticoid receptors, but of immaturity of downstream mechanisms.

One important *trans*-acting factor that regulates *crh* is Transducer Of Regulated CREB (TORC). Upon activation by cAMP, TORC2 translocates to the nucleus and associates directly with CBP and CREB, stabilizing the CBP:CREB complex over the promoter (Ravnskjaer et al., 2007; Screaton et al., 2004). TORC2 is highly expressed in adult brain

and has been shown to facilitate transcription of *crh* in primary cultures of hypothalamic neurons (Aguilera & Liu, 2012; Liu et al., 2010), yet little is known about the developmental expression patterns of TORC2. Hence, it is plausible that in adult $Cbp^{KIX/KIX}$ mice, TORC may compensate for KIX insufficiency in regulating CRH expression. In addition to CBP, CREB and TORC, the cyclic-AMP pathway includes several known *trans*-acting regulators. For example, the transcriptional activator p300 (Ogryzko et al., 1996) is a CBP paralogue, and expression of p300 is undisturbed in $Cbp^{KIX/KIX}$ mice (Kasper et al., 2002). p300 is necessary for embryonic development, indicating that it is functionally expressed early in life. In the adult, p300 may bind to CREB to allow resumption of normal CRH transcription under stress-free conditions in $Cbp^{KIX/KIX}$ mice.

In summary, in immature mice, CBP:CREB binding is essential for maintaining basal CRH levels. In adult $Cbp^{KIX/KIX}$ mice, basal CRH expression is less CBP-dependent, possibly because of the presence of additional regulatory factors. However, CBP is involved in stress-induced *crh* transcription as discussed below.

Rapid transcription of CRH during stress involves CBP:CREB interaction

Stress led to an increase of over 50% in hnRNA in $Cbp^{+/+}$ mice, consistent with prior work from several groups (Chen et al., 2012; Dent et al., 2000; Fenoglio et al., 2006, but see Tanimura & Watts, 1998). This rapid, stress-induced transcriptional activation of the *crh* gene was blunted in $Cbp^{KIX/KIX}$ mice, suggesting that CBP is essential for this process. We measured heteronuclear CRH RNA at a single time-point, 15 min, based on previous studies by several groups who found maximal hnRNA levels at this time point (e.g., Baram & Hatalski, 1998; Chen et al., 2001; Dent et al., 2000; Liu et al., 2012; Ritter et al., 2003). In view of the single timepoint analysis, it is possible that the kinetics of transcription of *crh* in response to stress were delayed (or accelerated) in $Cbp^{KIX/KIX}$ mice. Indeed, the kinetics and overall ‘area under the curve’ of CRH transcription have been found to be dynamic and regulated, for example, by age (Baram & Hatalski, 1998), and might be further influenced by *crh*-regulating factors such as CREM (Liu & Aguilera, 2009; Shepard et al., 2005), TORC (Liu et al., 2008, 2012) or the repressor NRSF (Korosi et al., 2010; Seth & Majzoub, 2001).

CBP has several functional domains. In addition to binding to CREB, CBP acts as a histone acetyltransferase (HAT) (Bannister & Kouzarides, 1996; Ogryzko et al., 1996, Vo & Goodman, 2001), and histone acetylation is integral for transcriptional activation, because it contributes to unwinding of the chromatin and allows transcription factor binding (Kalkhoven, 2004). The triple mutation of $Cbp^{KIX/KIX}$ not only prevents CBP:CREB interaction, but also diminishes the acetylation capacity of CBP (Kasper et al., 2002), raising the possibility that this defect might account for reduced gene transcription. Histone acetylation by CBP can have a widely variable time course, ranging from 15 min to 9 h (Aoyagi & Archer, 2007; Katan-Khaykovich & Struhl, 2002; Levine et al., 2005; Shankaranarayanan et al., 2001). Whether histones adjacent to *crh* specifically are quickly acetylated is unknown: Such rapid acetylation would be required to account for the rapid effect of the KIX site mutation in attenuating stress-induced *crh* transcription, as found here. In the context of our findings, the most plausible explanation for the effect of the KIX mutation is through its blunting of the interaction of CBP with phospho-CREB, suggesting that this interaction is crucial for activating transcription of *crh* after stress.

Basal plasma CORT and stress-induced CORT elevation are not grossly impaired in mice with deficient CBP:CREB interactions

$Cbp^{KIX/KIX}$ mice had comparable basal plasma CORT to those of mice not carrying the mutations at both ages tested. This is not surprising, because regulation of CORT release

from the adrenal is carried out by several factors and at several levels, and a modest release of ACTH may enable appropriate plasma CORT levels during stress. In addition, vasopressin might be involved, especially during development (Muret et al., 1992). Finally, we employed a combined physiological/psychological stress that is fairly severe, and it is possible that a milder stress might elicit differences among genotypes. Here, we found elevation of plasma CORT in all genotypes in response to stress. We measured CORT at a single time-point, and therefore cannot exclude alterations of the kinetic profile of this elevation. Notably, we found dissociation between preserved plasma CORT elevation after stress and attenuated transcriptional responses of the *crh* gene. The time lapse between activation of CRH synthesis and new CRH mRNA is 1–2 h, and given that we found no deficit of CRH mRNA expression in $Cbp^{KIX/KIX}$ mice, it is not surprising that these animals possess sufficient stores of CRH for adequate release and downstream stimulation of CORT release (Watts, 2005). A similar dissociation was observed after several types of stress in normal rats (Pace et al., 2009; Watts et al., 2004). Furthermore, clinical studies have demonstrated that the stress hormone system responsiveness remains intact despite administration of CRH receptor antagonists (Holsboer & Ising 2008). Our data support the growing understanding the gene activation and release of CRH that take place after stress may not be coupled.

Conclusions

The transcriptional co-activator CBP is required for normal expression of CRH at the mRNA and protein levels during early postnatal life. In the adult, whereas basal expression normalizes, stress-induced *crh* transcription requires CBP:CREB interaction. Obviously, CBP:CREB interactions in the mouse strain used here is impaired throughout the brain. Therefore, the current approach cannot differentiate between an effect of deficient activation of *crh* in PVN cells from less direct mechanisms, in CRH-expressing cells or upstream cells and mechanisms that might account for some of the observed changes. Similarly, the potential full extent of deficient CREB:CBP interaction might be masked by compensatory mechanisms involving, for example, TORC, p300 or glucocorticoid receptor. With these considerations in mind, the current data support the idea that, in the mature organism, functional CBP:CREB binding via the KIX domain is necessary for stress-induced activation of the *crh* gene in rodent PVN.

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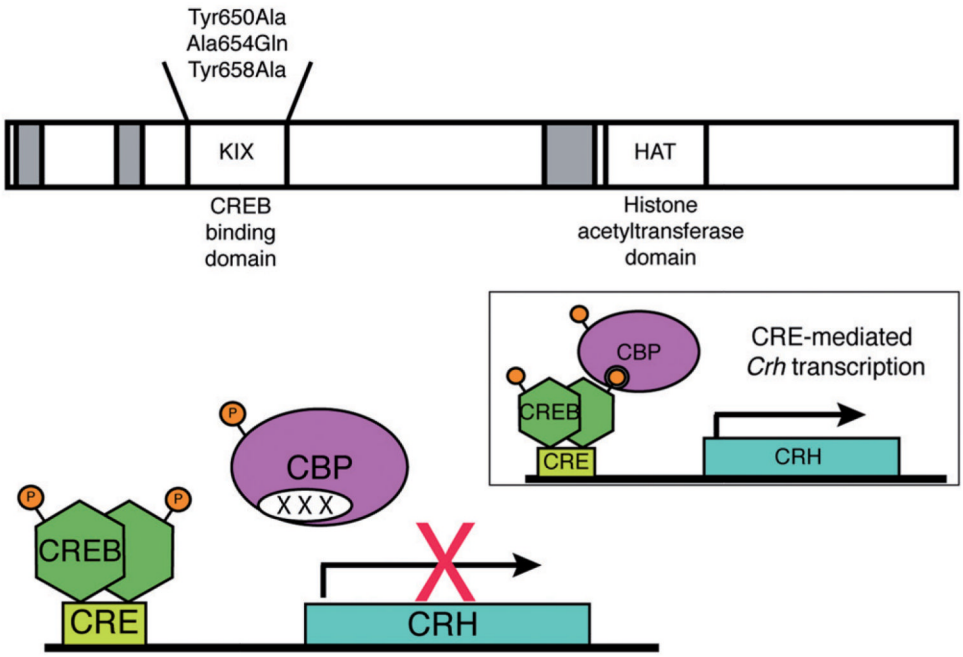

Figure 1.

Structural diagram of the KIX domain of the CREB-binding protein (CBP), and the functional consequences of mutating it. The *Cbp*^{KIX} allele generates a protein product carrying three point mutations in the KIX domain that binds phospho-CREB (Top). This mutation blocks CBP interaction with phospho-CREB, interfering with the role of CBP in initiating transcription of CRE-containing genes such as *crh*. Modified from Wood et al. 2006, with permission.

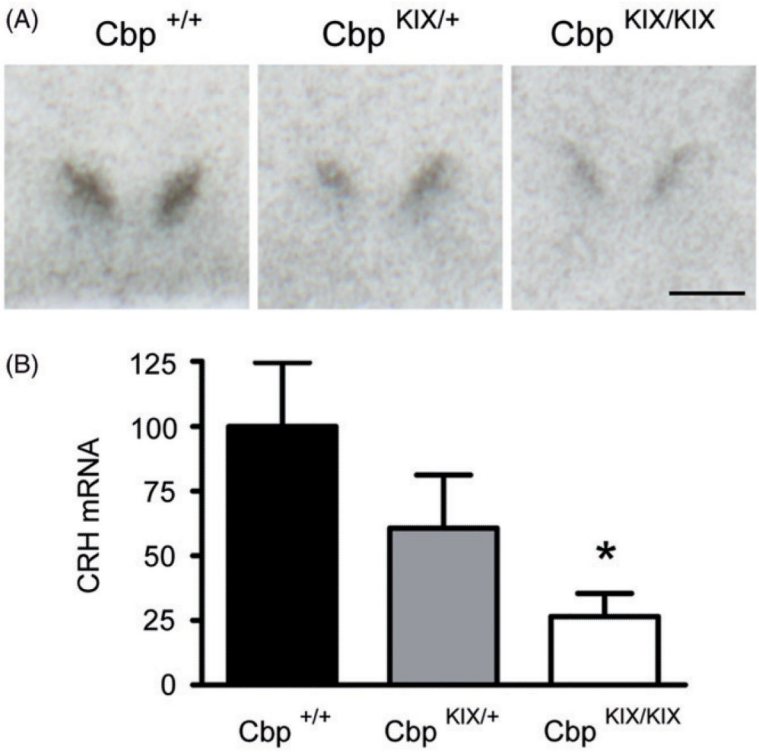

Figure 2.

In 9-day old mice, impaired functional interaction between CBP and CREB leads to reduction in CRH mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus. A, Representative bright-field photomicrographs of coronal sections at the level of the PVN from $Cbp^{+/+}$, $Cbp^{KIX/+}$ and $Cbp^{KIX/KIX}$ mice, subjected to ISH for CRH mRNA. Slides were exposed to film for 10 days. B, Quantification of mRNA levels, in reference to $Cbp^{+/+}$; $Cbp^{+/+}$ $n = 6$, $Cbp^{KIX/+}$ $n = 4$, $Cbp^{KIX/KIX}$ $n = 3$. A, *Scale bar* = 500 μm.

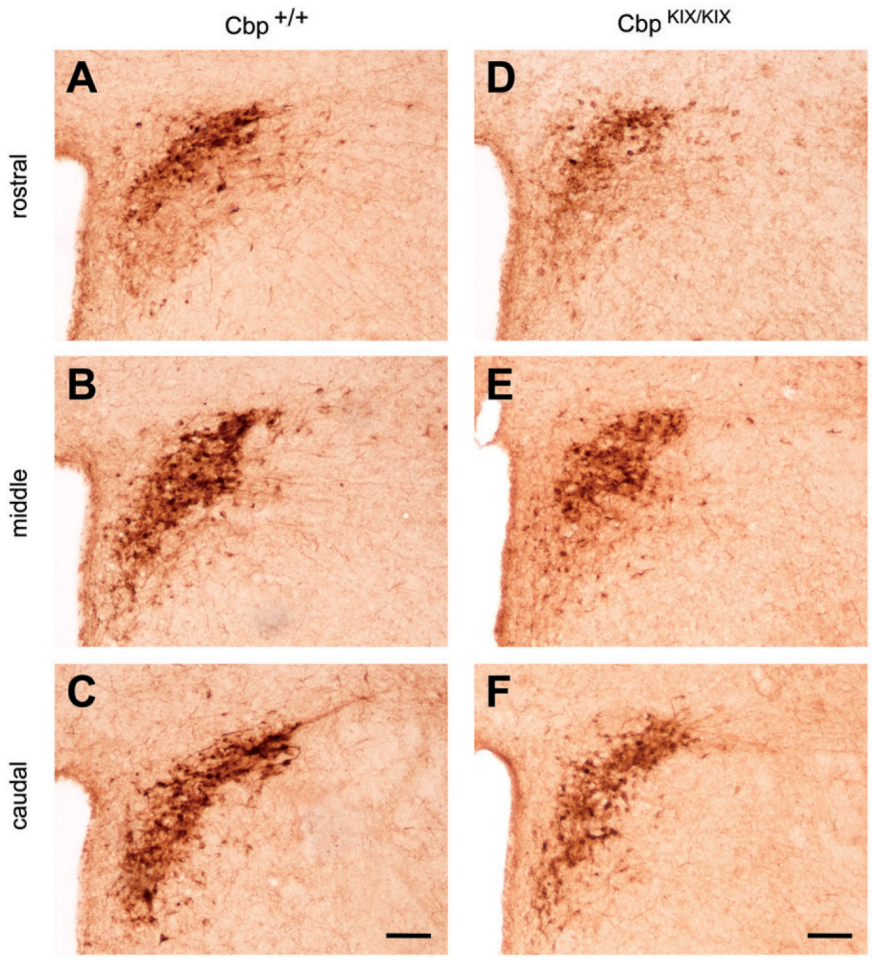

Figure 3.

CRH expression in the paraventricular nucleus (PVN) of the hypothalamus In 9-day old *Cbp*$^{+/+}$ and *Cbp*$^{KIX/KIX}$ mice. A–F, Serial photomicrographs of matched sections at three neuroanatomical levels (rostral, middle, and caudal) of the PVN of the hypothalamus. A–C = *Cbp*$^{+/+}$; D–F = *Cbp*$^{KIX/KIX}$. Brains were sectioned serially, and sections were run concurrently in the same IHC. C, F, *Scale bars* = 150 μm.

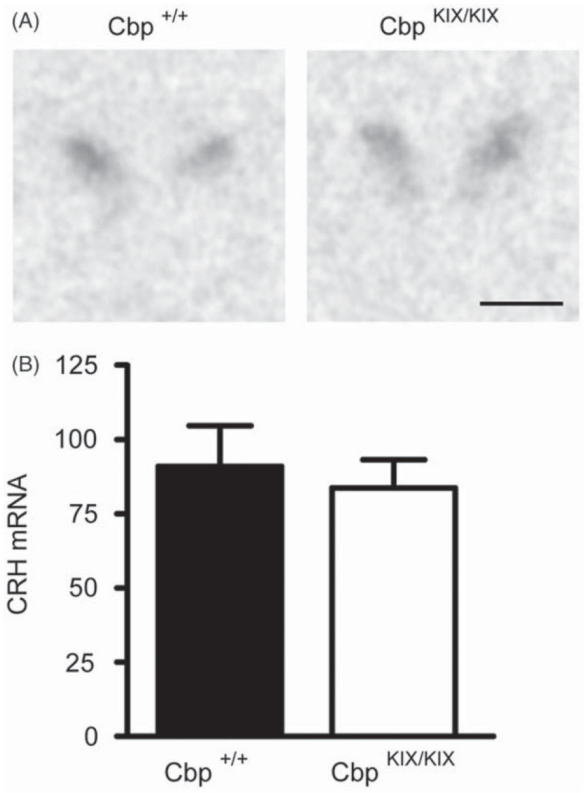

Figure 4.

In adult paraventricular nucleus (PVN), impaired CBP KIX-domain does not affect basal CRH mRNA expression. A, Representative bright-field photomicrographs of coronal sections from adult $Cbp^{+/+}$ and $Cbp^{KIX/KIX}$ mice subjected to ISH for CRH mRNA. B, Quantification of the mRNA levels, in reference to $Cbp^{+/+}$; $Cbp^{+/+}$ $n = 3$, $Cbp^{KIX/KIX}$ $n = 4$. Slides were exposed to film for 11–17 days. A, *Scale bar* = 500 μm.

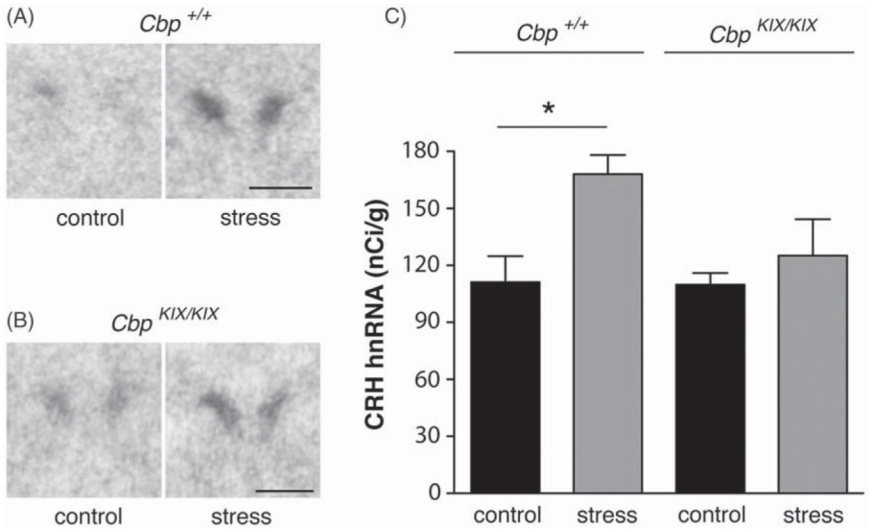

Figure 5.

Impaired CBP KIX-domain prevents stress-induced increase in CRH transcription in adult hypothalamus. A, Representative bright-field photomicrographs of coronal sections at the level of the paraventricular nucleus (PVN) from adult $Cbp^{+/+}$ mice in stress-free conditions (see Methods, left autoradiogram), and imediately after 15 min of stress. Sections were subjected to ISH for heteronuclear CRH RNA (CRH hnRNA). B, Representative bright-field photomicrographs of coronal sections at the level of the PVN from adult $Cbp^{KIX/KIX}$ mice in a stress-free condition (left) and at 15 min from the onset of stress. Sections were subjected to ISH for CRH hnRNA. C, Levels of CRH hnRNA were quantified in stress-free conditions and after 15 minutes of stress for $Cbp^{+/+}$ and $Cbp^{KIX/KIX}$ mice. (control $Cbp^{+/+}$ $n = 7$, Stress $Cbp^{+/+}$ n = 6, control $Cbp^{KIX/KIX}$ $n = 6$, stress $Cbp^{KIX/KIX}$ $n = 5$). CRH hnRNA levels were significantly higher in WT mice after stress as compared to non-stressed controls, but not different in stressed $Cbp^{KIX/KIX}$ mice compared to the basal state. A and B, *Scale bars* = 500 μm; *, $p<0.01$ compared to stress free levels. Two-way ANOVA analysis showed an effect of stress (F (1,1) = 6.53; $p = 0.019$), but not for genotype (F (1,1) = 0.802; $p = 0.38$), with insignificant stress × genotype interaction (F (1,1) = 1.58; $p = 0.22$).

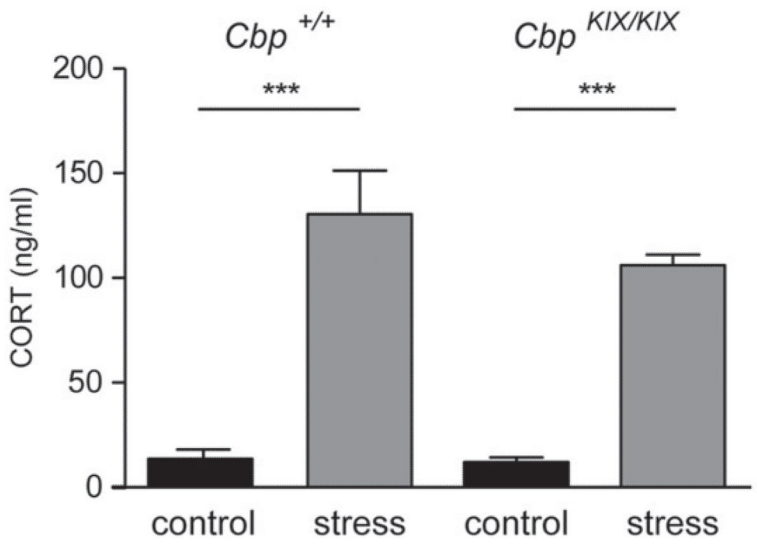

Figure 6.

Acute stress increases plasma corticosterone (CORT) levels in both $Cbp^{+/+}$ and $Cbp^{KIX/KIX}$ mice (n = 5–6 per genotype per group). Basal plasma CORT levels were comparable in wild-type and $Cbp^{KIX/KIX}$ mice and CORT levels were augmented significantly by 15 minutes of stress in both genotypes. ***, $p<0.0001$ compared to stress-free levels.