BDNF and glucocorticoids regulate corticotrophin-releasing hormone (CRH) homeostasis in the hypothalamus

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Regulation of the hypothalamic–pituitary–adrenal (HPA) axis is critical for adaptation to environmental changes. The principle regulator of the HPA axis is corticotrophin-releasing hormone (CRH), which is made in the parventricular nucleus and is an important target of negative feedback by glucocorticoids. However, the molecular mechanisms that regulate CRH are not fully understood. Disruption of normal HPA axis activity is a major risk factor of neuropsychiatric disorders in which decreased expression of the glucocorticoid receptor (GR) has been documented. To investigate the role of the GR in CRH neurons, we have targeted the deletion of the GR, specifically in the parventricular nucleus. Impairment of GR function in the parventricular nucleus resulted in an enhancement of CRH expression and an up-regulation of hypothalamic levels of BDNF and disinhibition of the HPA axis. BDNF is a stress and activity-dependent factor involved in many activities modulated by the HPA axis. Significantly, ectopic expression of BDNF in vivo increased CRH, whereas reduced expression of BDNF, or its receptor TrkB, decreased CRH expression and normal HPA functions. We find the differential regulation of CRH relies upon the cAMP response-element binding protein coactivator CRTC2, which serves as a switch for BDNF and glucocorticoids to direct the expression of CRH.

knockout | cAMP response-element binding protein | CRTC2 | transcription

The hypothalamic–pituitary–adrenal axis (HPA) axis is regulated by the corticotrophin-releasing hormone (CRH), which controls the secretion of adrenocorticotropin (ACTH) from the anterior pituitary and glucocorticoids from the adrenal gland (1). Stress or threats activate the HPA axis via polysynaptic circuits that converge on the hypothalamic paraventricular nucleus (PVN) to activate CRH-producing neurons (2). Classically, endocrine feedback control is characterized by the down-regulation of CRH and ACTH by glucocorticoids to prevent the escalation of glucocorticoids to toxic levels (1, 3). Maintenance of the HPA axis, therefore, involves a homeostatic equilibrium between the activation and inhibitory feedback. Disruption of normal HPA axis is a major risk factor of neuropsychiatric disorders (4, 5). Elevated CRH levels have been documented in the cerebrospinal fluid of depressed individuals exhibiting hypercortisolism, indicating a close relationship between the production of glucocorticoids and CRH (4). Both activation and feedback regulation require the glucocorticoid receptor (GR), which occurs in brain structures that control HPA axis reactivity (2, 6).

We find that genetic disruption of GR in the PVN disinhibited both the HPA axis and the expression of hypothalamic BDNF. Neurotrophic factors, such as BDNF, are involved in regulating many functions carried out by the HPA axis (7). BDNF and its receptor, TrkB, are both expressed in the PVN (8, 9) and many other brain regions, and display dramatic effects upon neuroprotection and synaptic plasticity (10, 11). Despite studies that indicate that BDNF has a role in HPA axis activity that culminate in alterations in energy metabolism, eating behavior, obesity, and depression (9), integration between BDNF and glucocorticoid signals upon CRH regulation in the PVN has not been studied. To address the significance of BDNF and its receptor TrkB in the PVN, we have examined the critical role of hypothalamic BDNF signaling in the maintenance of HPA axis activity. We find that BDNF and glucocorticoid signaling intersect upon CRH. Our findings provide a unique mechanism involving cAMP response-element binding protein (CREB) and its coactivator protein, CRTC2, that explains how BDNF can balance the ability of glucocorticoids to influence CRH expression specifically in the PVN.

Results

Reduced Expression of the GR in the PVN Disinhibits the HPA Axis. The expression of CRH is sensitive to stress and glucocorticoids (12). Systemic administration of a selective GR agonist, dexamethasone (DEX), suppressed hypothalamic CRH protein levels (Fig. 1*A*). This finding is consistent with previous work showing that CRH mRNA is repressed by glucocorticoids (13). In primary rat hypothalamic neurons, CRH mRNA expression, as well as CRH promoter activation, are repressed by DEX, suggesting cell-autonomous regulation (Fig. 1*B*). GR is found in limbic regions, the hypothalamus, and the pituitary (6). Previous studies showed that brain-specific deletion of GR resulted in hypercortisolism (14) and a marked reduction in body weight because of hypophagia and imbalance in the expression of hypothalamic neuropeptides (15). However, the contribution of GR in the hypothalamus in vivo, particularly in CRH-producing neurons, has not been determined.

To address this question, we generated a mouse carrying a GR allele flanked by loxP sites (Fig. S1*A*) and used a *Sim1*-Cre recombinase BAC transgenic line (16) to excise the floxed allele. Sim1 promoter-driven expression of Cre starts during embryonic development and after birth in the PVN, supraoptic, and posterior hypothalamic nuclei, with only limited scattered expression in extrahypothalamic nuclei (16). We examined mice carrying a functional wild-type and floxed allele ($GR^{flox/+}$; Sim1Cre) because defective GR signaling rather than complete loss of function is observed in human pathologies. These

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hypomorphic mice demonstrated a decrease in body weight during adulthood (Fig. S1 *C* and *D*). Immunohistochemistry (Fig. 1*C*) and Western blot analysis showed a significant reduction of GR protein level in the PVN but normal levels in the pituitary and adrenal gland (Fig. S1*F*). Furthermore, these hypomorphic mice displayed enhanced CRH immunoreactivity in the PVN, with elevated plasma levels of ACTH and corticosterone (Fig. 1 *E*–*G*), indicating that reduced hypothalamic GR function contributed to CRH regulation and subsequent impaired maintenance of normal HPA axis function.

Maintenance of HPA Axis Reactivity by Hypothalamic BDNF Signaling. Neurotrophic factors can stimulate the HPA axis (9, 17). Intraventricular injection of BDNF raises CRH expression and activates the HPA axis (18). Virtually all hypothalamic CRH neurons coexpress GR and the BDNF receptor TrkB. Because both glucocorticoids and neurotrophic factors are known to affect the HPA axis, we monitored how a decrease in GR function would affect the neurotrophic pathway in the PVN. We found that BDNF protein, as well as TrkB phosphorylation, were elevated in the PVN of hypomorphic GR mice compared with control littermates (Fig. 2 *A* and *B*). Strikingly, the active form of TrkB (p-TrkB) was found in CRH neurons (Fig. 2*C*), where there was a strong positive correlation between the intensity of CRH immunoreactivity and the level of p-TrkB ($R^2 = 0.51$, $P < 0.001$, $n = 78$ cells). Conversely, suppression of CRH expression in mice given a bolus of DEX was correlated with the decrease of p-TrkB ($R^2 = 0.07$, $P = 0.0044$, $n = 111$ cells). Therefore, hypothalamic CRH expression may be balanced by glucocorticoid and BDNF signals.

BDNF employs TrkB signaling to activate the transcription factor CREB and regulate the expression of plasticity-related genes (7). Similar to TrkB phosphorylation, the levels of p-CREB increased when GR function was diminished in hypomorphic mice (Fig. 1*F*), and conversely, p-CREB levels were decreased upon GR activation in mice administered with DEX (Fig. 1*A*). To address the role of the TrkB receptor in CRH regulation and the maintenance of the HPA axis, we analyzed TrkB knockouts. Unlike TrkB-null mice, which are perinatal lethal, heterozygous mice survived into adulthood with hypothalamic defects (19). Lack of a single TrkB allele decreased TrkB protein levels in the PVN without affecting GR levels (Fig. 2*D*). In TrkB hypomorphic mice, there was a significant decrease of p-TrkB, p-CREB, and HPA axis parameters, such as corticosterone and ACTH levels (Fig. 2 *E*–*G*). We also assessed how the HPA axis of $TrkB^{+/-}$ mice functioned during negative feedback, stress, and circadian cycle. We found only evening levels of ACTH and corticosterone were decreased in $TrkB^{+/-}$ compared with wild-type controls (Fig. 2*G*). Stress raised corticosterone levels in both genotypes but corticosterone levels were lower 1 h after stress in $TrkB^{+/-}$ mice ($TrkB^{+/+}$ 216 ± 17 ng/mL vs. $TrkB^{+/-}$ 168 ± 20 ng/mL). Finally, an effect of genotype was not observed in the DEX suppression test, but an abnormal rebound of corticosterone levels was observed in $TrkB^{+/-}$ mice after coadministration of DEX and recombinant CRH (Fig. 2*H*). Our data therefore indicate that TrkB can exert an impact upon CRH and blood levels of ACTH and corticosterone.

Ectopic Expression of Hypothalamic BDNF Regulates CRH. To determine whether BDNF exerts a direct influence in the PVN, we increased BDNF using a Cre recombinase-mediated excision of a floxed STOP cassette inserted upstream of a *bdnf* transgene (20). Crossing these mice with the Sim1-Cre line produced BDNF-HA (Fig. 3*A*) and an 80% elevation of BDNF over that of control littermates in the PVN by Western blot analysis. With ELISA, we found that total hypothalamic BDNF levels increased by 30% (Fig. 3*B*), with no change in extrahypothalamic tissue, such as the parietal cortex (Cre^- 13.7 ± 0.9 pg/mg vs. Cre^+ 13.8 ± 1.1 pg/mg). Activation of hypothalamic TrkB by ectopic BDNF using Cre expression (1.74 ± 0.15-fold increase, $P = 0.01$) was similar to the homeostatic elevation of p-TrkB in hypomorphic GR mice (1.46 ± 0.1-fold increase, $P = 0.02$). Consistent with the

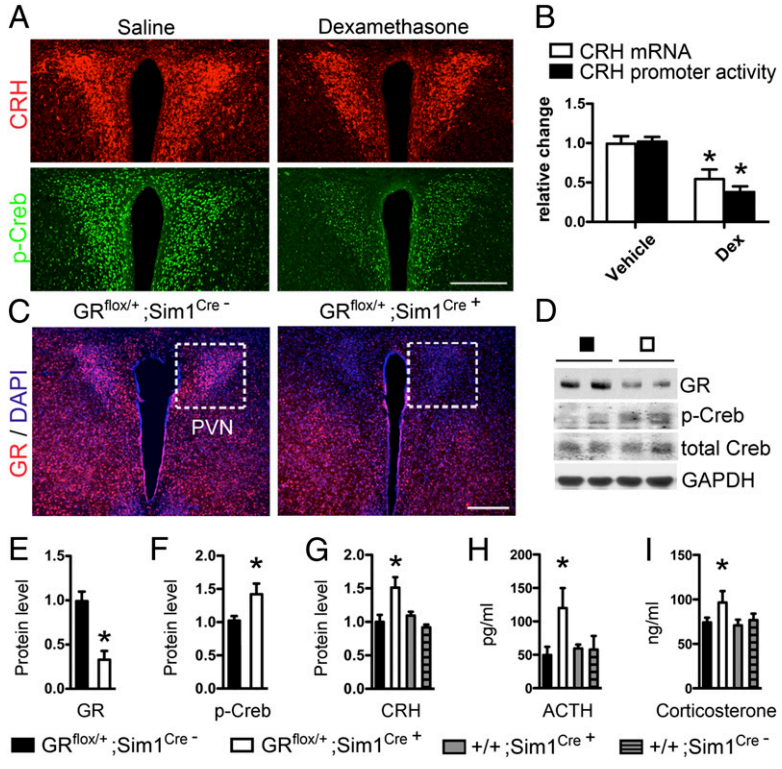

Fig. 1. Genetically reduced expression of the glucocorticoid receptor in the PVN disinhibits CRH and the HPA axis. (*A*) Hypothalamic CRH protein and CREB phosphorylation in mice given intraperitoneal administration of DEX 1 mg/kg. (Scale bar, 200 μm.) (*B*) CRH mRNA and promoter activity in primary rat hypothalamic neurons treated with 1 μM DEX for 3 h. Mean ± SEM of at least five independent experiments, $*P < 0.05$. (*C*) Reduced GR expression in the PVN using Sim1-Cre BAC transgenic mice. Epifluorescent images of hypothalamic GR (red) and DAPI stain (blue). (Scale bar, 200 μm.) (*D*) Expression of GR and CREB in PVN lysates of $GR^{flox/+}$;Sim1Cre mice. (*E*) GR protein in the PVN; $*P = 0.01$, $n = 6$–7 animals. (*F*) p-CREB levels in the PVN of $GR^{flox/+}$;Sim1Cre mice; $*P < 0.05$, $n = 6$–7 animals. (*G*) CRH protein in the PVN of GR^{flox};Sim1Cre mice; $*P < 0.05$, $n = 6$–10 mice per group. (*H*) Plasma ACTH level in GR^{flox};Sim1Cre mice; $*P < 0.05$, $n = 9$–11 mice per group. (*I*) Plasma corticosterone level in GR^{flox};Sim1Cre mice; $*P < 0.05$, $n = 9$–11 mice per group.

up-regulation of BDNF, there was a significant increase of p-CREB without affecting total GR levels (Fig. 3*C*). We found a significant increase of CRH protein in the PVN by this manipulation (Fig. 3*E*), but plasma levels of ACTH and corticosterone remained largely unaffected (Fig. 3 *F* and *G*). These results indicate that increased hypothalamic BDNF per se is not sufficient to affect blood levels of ACTH and corticosterone.

Mechanism of CRH Regulation Downstream of BDNF and Glucocorticoid Signaling. To understand the control of CRH expression downstream of TrkB and GR signaling, we treated primary rat hypothalamic neurons with BDNF and examined CRH expression. We found that BDNF raised the CRH mRNA level by threefold (Fig. 4*A*). In contrast, DEX repressed CRH mRNA regardless of BDNF treatment (Fig. 4*A*), indicating that glucocorticoid signaling dominated the neurotrophic effects. Unlike NGF or EGF, BDNF activated, whereas DEX repressed, a transfected CRH promoter construct (Fig. 4*B*). In addition, repression by DEX dominated the inductive action of BDNF upon the CRH promoter.

There are several possible mechanisms whereby DEX-mediated repression may override BDNF-dependent induction of CRH transcription. These mechanisms include: (*i*) the lack of CREB activating phosphorylation; (*ii*) loss of CREB DNA-binding; (*iii*) increase of GR DNA-binding; or (*iv*) nuclear translocation of transcriptional regulators. Using ChIP, we found that DEX treatment increased GR binding to the CRH promoter over basal occupancy, an effect that did not change upon cotreatment with BDNF (Fig. 4*C*). As previously reported (21), ligand-activated GR bound modestly to the *Crh* promoter compared with other known glucocorticoid-sensitive genes, like *Fkbp5* (10.91 ± 0.74-fold enrichment by DEX; 10.63 ± 1.05-fold enrichment by DEX+BDNF). The proximity between the GR and CREB DNA binding sites, distant of 22 base pairs, in the CRH promoter suggests possible interactions between both transcription factors. We found that BDNF increased CREB promoter occupancy, an effect that tended to diminish upon cotreatment with DEX (Fig. 4*C*).

Because DEX treatment did not result in dephosphorylation of CREB (Fig. 4*D*), we tested if CREB is required at all for DEX-mediated repression of CRH promoter in primary rat hypothalamic neurons. As expected, dominant-negative CREB blocked—whereas dominant-active CREB induced—CRH promoter activity like cAMP analogs. Despite the transfected dominant-active CREB, DEX-mediated repression of CRH promoter persisted (Fig. 4*E*). These observations do not fully explain how DEX-mediated repression of CRH occurs through CREB.

CRTC2 Phosphorylation-Dependent Subcellular Localization Determines CRH Expression Downstream of BDNF and Glucocorticoid Signaling. CREB activation is regulated by coactivator proteins (CRTC), which bind to the conserved arginine residue R314 of CREB to increase its transcriptional activity (22). Transfection of a CREB-R314A mutant in hypothalamic neurons diminished both induction and repression of CRH promoter afforded by BDNF and DEX, respectively (Fig. 4*E*), suggesting that CRTC proteins are involved in both BDNF and glucocorticoid actions through the CREB protein.

To test whether CRTC2 and p-CREB cooperate to regulate CRH expression, we manipulated CRTC2 in hypothalamic neurons. First, specific shRNA plasmids were used to knockdown the levels of CRTC2 (Fig. 4*F*), which resulted in a significant decrease in CRH promoter activity to DEX-repressed levels despite stimulation with cAMP analog and BDNF (Fig. 4*G*). Second, transfection of wild-type CRTC2 increased baseline and BDNF-stimulated CRH promoter activity (Fig. 4*H*). Third, we took advantage of a mutant of CRTC2 carrying a S171A mutation. Phosphorylation of S171 promotes cytoplasmic localization of CRTC2 (22). Transfection of a CRTC2 A171 mutant, which is mostly nuclear, increased CRH promoter activity above wild-type CRTC2 activity. Remarkably, DEX-mediated repression of CRH promoter was lost when the A171 mutant was overexpressed (Fig. 4*H*), suggesting that GR signaling can deactivate CREB-mediated CRH transcription by interfering with nuclear CRTC2.

Nuclear localization of CRTC2 is essential for its function. We found that systemic administration of DEX produced a change in the distribution of CRTC2 from the nucleus to the cytoplasm of hypothalamic CRH neurons (Fig. 5*A* and Fig. S2). Interestingly, CRTC2 phosphorylation and cytoplasmic localization were diminished in GR hypomorphs compared with control littermates (Fig. 5 *B* and *D*). Indeed, phosphorylation of CRTC2 (S171-P) was elevated in the PVN of mice exposed to a single forced-swim stress or after DEX treatment (Fig. 5 *C–E* and Fig. S3). These results suggest that phosphorylation and nuclear localization of hypothalamic CRTC2 can be modulated by GR signaling.

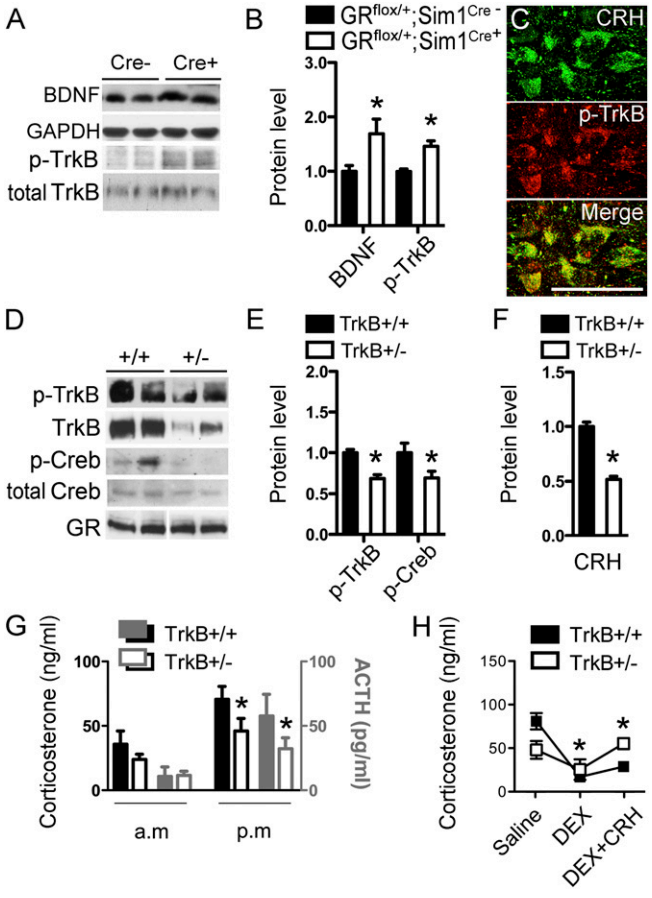

Fig. 2. BDNF signaling impacts the maintenance of CRH and the HPA axis. (*A*) Western blots of BDNF and TrkB in PVN lysates of $GR^{flox/+}$;Sim1Cre mice. (*B*) BDNF protein and TrkB phosphorylation relative to GAPDH and total TrkB in the PVN of $GR^{f/flox/+}$;Sim1Cre mice. Mean ± SEM of 6–7 mice, $*P < 0.05$. (*C*) Confocal images of adult mouse PVN neurons that coexpress CRH with the active form of TrkB, p-TrkB. (Scale bar, 20 μm.) (*D*) Western blots of TrkB, GR, and CREB in PVN lysates of TrkB knockouts. (*E*) TrkB and CREB phosphorylation relative to total TrkB and CREB in the PVN of TrkB knockouts. Mean ± SEM of 5–6 mice, $*P < 0.05$. (*F*) CRH protein in the PVN of TrkB knockouts. Mean ± SEM of 7–9 mice, $*P < 0.05$. (*G*) Basal morning and evening plasma levels of corticosterone and ACTH in TrkB knockouts; $*P < 0.05$, $n = 6–6$ mice. (*H*) Corticosterone level of TrkB knockouts administered with DEX (IP, 0.1 mg/kg) alone for 6 h or in combination with recombinant CRH (5 μg/kg i.p.) for an extra 1 h; $*P < 0.05$, $n = 7–6$ mice.

CRTC2 function and nuclear localization can be regulated by PKA, AMPK, salt inducible kinase (SIK), and MARK, and phosphatases, such as calcineurin, PP1 and PP2 (Fig. S4*A*). This finding raised the question of whether GR-mediated suppression of CRH transcription required specific protein kinase or phosphatase activity. Neither PKA (Fig. S4*B*) nor SIK family kinases appear to be involved in GR signaling. For example, GR-mediated suppression of CRH promoter activity was not affected by staurosporine, a general SIK inhibitor (Fig. 5*F*) (23). In contrast, inhibiting calcineurin by cyclosporine A decreased CRH promoter activity to DEX-repressed levels (Fig. 5*F*), despite normal p-CREB activation upon cAMP analog and BDNF treatment. Inhibition of other phosphatases, such as PP1/PP2, with okadaic acid robustly induced CRH promoter activity by increasing p-CREB levels, but DEX-mediated suppression did not change (Fig. 5*F*). This finding suggests that GR signaling targets calcineurin to affect CRTC2 phosphorylation and nuclear localization. Therefore, hypothalamic BDNF/TrkB and glucocorticoid/GR signaling pathways can differentially affect CREB and CRTC2 phosphorylation to balance CRH expression.

Discussion

We describe a molecular mechanism underlying the maintenance of HPA axis activity by glucocorticoid and neurotrophic signals. Using specific gain- and loss-of-function of GR, BDNF, and TrkB in the mouse PVN, we were able to distinguish GR and BDNF/TrkB signaling in hypothalamic CRH neurons. Cell-autonomous BDNF and glucocorticoid signaling was evident, which ensures a tight control over CRH in the PVN. Our results indicate that BDNF, through TrkB-CREB signaling, induces the expression of CRH, whereas glucocorticoids, via GR signaling, deactivate CREB-mediated CRH induction by neutralizing the function of the CREB coactivator CRTC2. The proximity between the GR and CREB DNA binding sites in the CRH promoter may present a more general mechanism to modulate the recruitment of CRTC2 at select CREB-occupied genes.

Many stimuli, including neuronal activity, excitatory neurotransmitters, neurotrophins, and cAMP analogs phosphorylate and activate CREB (24). Although required, phosphorylation of CREB is not sufficient to stimulate CRH promoter (25). The CREB coactivator CRTC2 must be actively transported to the nucleus to allow CREB-dependent transcription of *Crh*. Neuronal activity via calcium-permeable glutamate ion channels not only phosphorylates CREB but also triggers calcineurin-dependent dephosphorylation of CRTC2 that is necessary to activate CREB-mediated transcription. BDNF signaling and function, which require CRTC1 in cortical neurons (26), are gated by neuronal activity because BDNF alone cannot elicit nuclear translocation of CRTC. In contrast, GR signaling, which alone does not affect CREB phosphorylation, decreases CRTC2 nuclear localization in hypothalamic neurons. Glucocorticoids, when applied directly to the PVN, rapidly suppressed glutamate postsynaptic currents and signaling in CRH neurons (27), suggesting that glucocorticoid-mediated suppression of nuclear CRTC2 and CRH transcription may depend upon excitatory neurotransmission to modulate intracellular [Ca^{2+}], and CRTC2-kinases and CRTC2-phosphatases. We propose that GR signaling deactivates CREB function and *Crh* transcription by sequestering CRTC2 in the cytoplasm via increased phosphorylation. Accordingly, GR-mediated repression of CRH was countered when CRTC2 was forced into the nucleus with a phospho-deficient mutant.

Neurotrophins modulate neuronal connectivity and synaptic plasticity (28). Therefore, genetic disruption of BDNF/TrkB signaling may impair the remodeling, maintenance, and plasticity of synapses that contact CRH neurons in the PVN. Like TrkB hypomorphs, $BDNF^{+/-}$ mice displayed reduced hypothalamic CRH content and blood levels of ACTH and corticosterone (Fig. S5). In contrast, genetically elevated BDNF expression in the PVN induced p-CREB and raised CRH expression without altering normal HPA axis activity. The discrepancy between CRH and ACTH levels may reflect impaired CRH secretion because of an altered inhibition-excitation balance of PVN neurons. Indeed, BDNF regulates the formation and maintenance of both inhibitory and excitatory synapses (29). The homeostatic balance between synaptic inhibition and excitation may impact CRH secretion. In contrast, GR signaling in the PVN rapidly suppressed excitatory postsynaptic currents and signaling in CRH neurons (27). Therefore, reduced GR signaling in GR hypomorphs may increase excitation of CRH neurons, resulting in more CRH secretion.

Excess glucocorticoids associated with decreased BDNF levels have been linked to depression (3, 11). Conversely, heightened glucocorticoid levels of depressed individuals can be normalized by antidepressant treatment, which requires functional BDNF signaling (4, 30). Although this finding suggests interplay between glucocorticoid and BDNF pathways, the mechanisms are poorly understood. We observed that conditional reduction of GR levels in the PVN activated CRH neurons with elevated p-CREB and induction of the immediate early genes *fos* and *bdnf*.

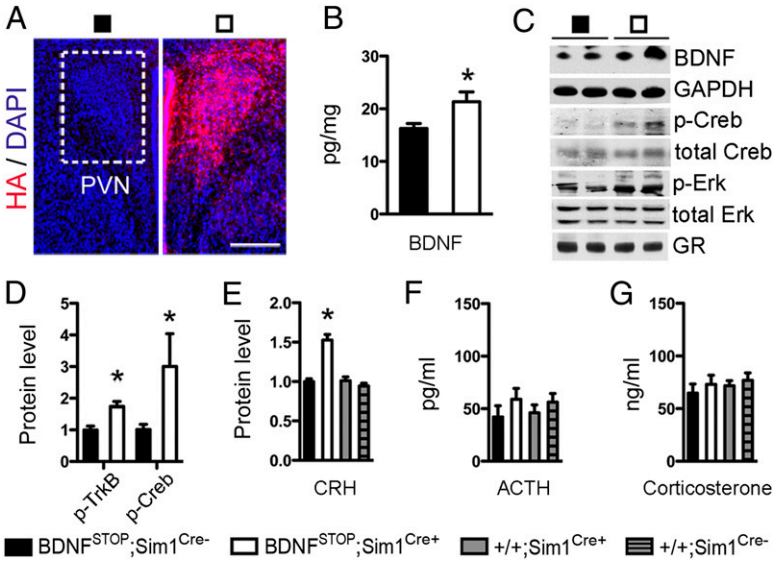

Fig. 3. Ectopic expression of BDNF in the PVN increases CRH. (*A*) Expression of BDNF in the PVN using CRE recombinase-mediated excision of a flox-STOP cassette upstream of a BDNF-HA tag transgene. Epifluorescent images showing hypothalamic HA immunoreactivity (red) and DAPI stain (blue). (Scale bar, 200 μm.) (*B*) Hypothalamic BDNF protein measured by ELISA. Mean ± SEM, **P* = 0.02, *n* = 6–6 mice. (*C*) Western blots of PVN lysates in BDNF-overexpressing mice. (*D*) TrkB and CREB phosphorylation relative to total TrkB and CREB in the PVN of BDNF-overexpressing mice. Mean ± SEM of 5–7 mice, **P* < 0.05. (*E*) CRH protein in the PVN of BDNF-overexpressing mice. Mean ± SEM of 8–14 mice per group, **P* < 0.05. (*F*) Plasma ACTH in BDNF-overexpressing mice. Mean ± SEM of 6–11 mice per group. (*G*) Plasma corticosterone in BDNF overexpressing mice. Mean ± SEM of 11–14 mice per group.

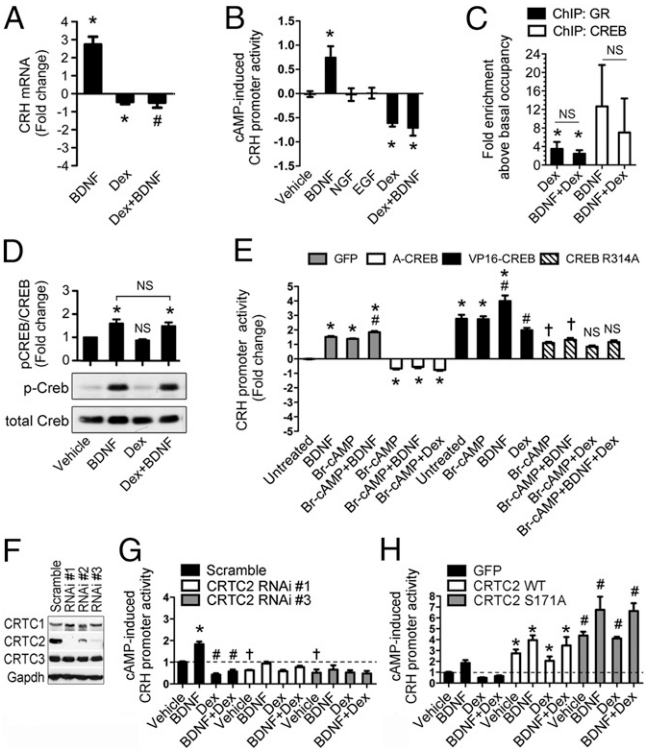

Fig. 4. The CREB coactivator, CRTC2, balances CRH expression downstream of glucocorticoid and BDNF signaling. (*A*) CRH mRNA in primary hypothalamic neurons treated with 50 ng/mL BDNF, 1 μM DEX, or both for 3 h. Quantitative PCR data are expressed as fold-change relative to vehicle-treated cells; $^{*}P < 0.05$, $n = 7$–9 experiments. (*B*) Recombinant CRH promoter activity induced by 1 mM Br-cAMP in transfected primary hypothalamic neurons. Treatments as indicated were applied once to the cells for 16 h (50 ng/mL BDNF and 1 μM DEX). Data are expressed as fold-change relative to vehicle-treated cells; $^{*}P < 0.05$, $n = 3$–5 experiments. (*C*) ChIP using GR or CREB antibodies in lysates of primary neurons treated with 50 ng/mL BDNF, 1 μM DEX, or both for 1 h. Data are expressed as fold-enrichement relative to basal occupancy in vehicle-treated cells. Mean ± SEM of at least four experiments $^{*}P < 0.03$. (*D*) p-CREB in primary hypothalamic neurons treated with 50 ng/mL BDNF, 1 μM DEX, or both for 3 h; $^{*}P = 0.0014$, $n = 11$ experiments. (*E*) CRH promoter activity in hypothalamic neurons transfected with mutants of CREB. Mean ± SEM of three to six experiments, $P < 0.05$ (#, *, and † represent significant change relative to Br-cAMP treated cells, untreated, and GFP controls, respectively). (*F*) Efficacy of CRTC2 sh-RNA in 293 cells transfected with flag-CRTC. (*G* and *H*) cAMP-induced CRH promoter activity in hypothalamic neurons transfected with various constructs. (*G*) Mean ± SEM of at least five experiments; vehicle vs. BDNF or DEX $^{*}P < 0.001$; BDNF vs. BDNF+DEX $^{\#}P < 0.001$; Scramble vs. RNAi #1 $^{\dagger}P < 0.01$; Scramble vs. RNAi #3 $^{\dagger}P < 0.001$. (*H*) Mean ± SEM of three to five experiments; GFP vs. WT $^{*}P < 0.01$; WT vs. A171 $^{\#}P < 0.05$.

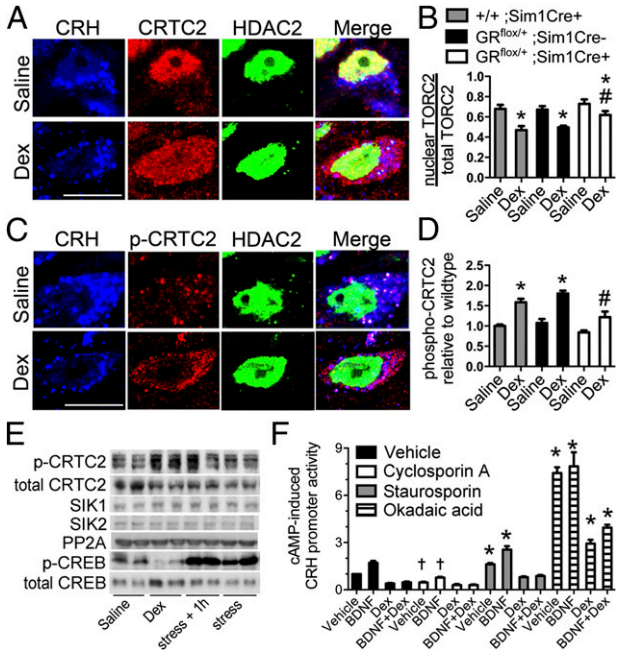

Fig. 5. CRTC2 phosphorylation, nuclear distribution, and function is regulated by GR signaling. (*A*) CRTC2 localization in CRH neurons of mice administered with DEX (10 mg/kg i.p.). (Scale bar, 10 μm.) (*B*) CRTC2 nuclear distribution in CRH neurons of GR^{flox};Sim1Cre mice. Nuclear/total somatic intensity ratio (Saline vs. Dex $^{*}P < 0.02$; Cre^{-} vs. Cre^{+} $^{\#}P < 0.02$, $n = 21$–40 cells from at least five mice per group). (*C*) CRTC2 phosphorylation in CRH neurons of GR^{flox};Sim1Cre mice administered with DEX. (Scale bar, 10 μm.) (*D*) p-CRTC2 intensity in the PVN of at least five mice per group. Saline vs. Dex $^{*}P < 0.001$; Cre^{-} vs. Cre^{+} $^{\#}P = 0.003$; $GR^{+/+}$;Sim1Cre^{+} vs. Cre^{+} $^{\#}P = 0.027$. (*E*) p-CRTC2 in PVN lysates of mice given DEX (10 mg/kg i.p.), exposed to a 10-min forced swim stress or 1 h after stress (one mouse PVN per lane). (*F*) cAMP-induced CRH promoter activity in rat hypothalamic neurons treated with cyclosporine A (1 μM), staurosporin (20 nM), and okadaic acid (1 μM) for 14 h. Mean ± SEM of at least five experiments. Vehicle vs. staurosporin $^{*}P < 0.001$; Vehicle vs. okadaic acid $^{*}P < 0.05$; vehicle vs. cyclosporin $^{\dagger}P < 0.001$.

Homeostatic regulation of BDNF levels was also observed in several brain regions of mice with genetically altered GR expression (31, 32). Because BDNF expression is sensitive to neuronal activity (27, 28), and is suppressed by high-dose glucocorticoids in the PVN (27, 28), tissue-specific change in GR and BDNF levels may calibrate neuronal activity.

Our findings revealed a homeostatic mechanism by which hypothalamic BDNF and glucocorticoid signaling maintain CRH and glucocorticoid bioavailability. When compromised, the HPA axis is not able to adequately respond to external stressors, and many maladaptative changes may occur that can set the stage for neuropsychiatric disorders (3). Future investigation will be necessary to understand how BDNF and glucocorticoid signaling changes plasticity of the HPA axis upon development, behavioral challenge, and pathological conditions.

Materials and Methods

Animals. Time-pregnant Sprague–Dawley rats, C57BL/6 mice (both from Charles River Laboratories), Sim1-cre (Jackson Laboratories), $BDNF^{STOP}$ (20) and GR^{flox} transgenic mice, and TrkB (33) knockouts were allowed ad libitum food access. Floxed GR mice were generated by inserting loxP sites to flank exon II (Fig. S1 and *SI Materials and Methods*). All studies were performed in 8- to 12-wk-old adult males. To monitor plasma levels of HPA axis parameters, trunk blood was collected at 8:00 AM or 6:00 PM in EDTA-coated tubes (BD Vacutainer) by decapitation. Systemic administration of Dexamethasone 21-phosphate disodium salt (Sigma–Aldrich) or saline 0.9% as vehicle at noon was for 6 h. All protocols complied with the New York University Medical Center institutional guidelines for the care and use of laboratory animals.

Reagents. Trophic factors were obtained from PeproTech. Recombinant human CRH and 8-bromo-cyclicAMP were obtained from Sigma–Aldrich. Antibodies used are listed in *SI Materials and Methods*. RNA was isolated using TRIzol (Invitrogen), cDNA amplified with First-Strand cDNA Synthesis Kit (Affimetrix) and quantitative PCR performed with SYBR Green Taq Ready Mix (Affimetrix) using MyiQ Single-Color Real-Time PCR Detection System from Bio-Rad. Primers used for amplification are described in *SI Materials and Methods*. Measurements of luciferase activity using Dual luciferase reporter assay and BDNF levels by ELISA were performed following the manufacturer's instructions (Promega). HPA axis parameters were assessed in plasma by ELISA following the manufacturer's instructions (ACTH from MDBioscience, corticosterone from AssayPro).

Cell Culture and Lysis. Primary neurons from embryonic day 18 rats were prepared from timed-pregnant Sprague-Dawley rats, cultured on poly-D-lysine, maintained in neurobasal medium containing B27 supplement, 0.5

mM L-glutamine, 5-fluoro-uridine, and uridine (10 mM each) and lysed in RIPA buffer, as previously described (34). Neurons were electroporated at day in vitro 0 using AMAXA (Lonza) for CRH promoter assay; 293 cells were grown in DMEM containing 10% FBS and transfected with lipofectamine (Invitrogen). For neurochemistry, the PVN were punched out of frozen brain sections on glass slides using a tissue punch set (Stoelting).

Plasmids. The human CRH promoter (−918 to +38 bp) and the CREB constructs were previously described (35–37). Short-hairpin sequences described in *SI Materials and Methods* were inserted into pLentiLox3.7 vector (ATCC). Flag-CRTC constructs were purchased from Addgene. Site-directed mutagenesis was performed using QuikChange kit from Stratagene. All constructs were verified by sequencing.

Immunohistochemistry and Microscopy. Transcardiac perfusion and post-fixation with 4% PFA was for 2 h. Sections were blocked Primary and AlexA fluor-conjugated secondary (Invitrogen) antibodies were incubated in 5% normal goat and horse sera, PBS, 0.1% TritonX-100. Images were captured with a LSM510 laser-scanning confocal microscope using LSM510 software (both Carl Zeiss Microimaging). Epifluorescence images were taken with a EclipseE800 microscope (Nikon Instruments) and a Axiocam HR camera using Axiovision software (both Carl Zeiss). Images stacks were deconvoluted and processed with ImageJ (National Institutes of Health). A mask obtained with the HDAC2 immunosignal served to delineate CRTC2 intensity confined in the nucleus. A second mask obtained with CRH immunosignal served to delineate somatic CRTC2 intensity. Nuclear/total somatic intensity ratio was obtained by dividing the first value by the second.

Chromatin Immunoprecipitation. Primary cortical neurons were analyzed by ChIP as described (38). Chromatin was sonicated using the Bioruptor (Diagenode; Twin UCD-400). Immunoprecipitation of GR was performed with 6 μg of a mixture of GR antibodies: MA1-510 and PA1-511A (Thermo Scientific), H-300 (Santa Cruz), or equivalent amount of rabbit and mouse IgG (Sigma-Aldrich). Following reversal of cross-linking, recovered DNA was purified using PrepEase DNA Clean-Up Kit (USB). Cycle threshold values were normalized to percent input and IgG.

Statistics. Statistical analysis (GraphPad Prism software, version 4.0) was carried out using paired Student *t* test and one-way ANOVA with post hoc Bonferroni's test. Statistical significance was defined at $P < 0.05$.

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