Requirement of Cannabinoid Receptor Type 1 for the Basal Modulation of Hypothalamic-Pituitary-Adrenal Axis Function

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The endocannabinoid system affects the neuroendocrine regulation of hormone secretion, including the activity of the hypothalamus-pituitary-adrenal (HPA) axis. However, the mechanisms by which endocannabinoids regulate HPA axis function have remained unclear. Here we demonstrate that mice lacking cannabinoid receptor type 1 ($CB1^{-/-}$) display a significant dysregulation of the HPA axis. Although circadian HPA axis responsiveness is preserved, $CB1^{-/-}$ mice are characterized by an enhanced circadian drive on the HPA axis, resulting in elevated plasma corticosterone concentrations at the onset of the dark as compared with wild-type ($CB1^{+/+}$) littermates. Moreover, $CB1^{-/-}$-derived pituitary cells respond with a significantly higher ACTH secretion to CRH and forskolin challenges as compared with pituitary cells derived from $CB1^{+/+}$ mice. Both $CBL^{-/-}$ and $CB1^{+/+}$ mice properly respond to a high-dose dexamethasone test, but response to low-dose dexamethasone is influenced by genotype. In addition, $CB1^{-/-}$ mice show increased CRH mRNA levels in the paraventricular nucleus of the hypothalamus but not in other extrahypothalamic areas, such as the amygdala and piriform cortex, in which CB1 and CRH mRNA have been colocalized. Finally, $CB1^{-/-}$ mice have selective glucocorticoid receptor mRNA down-regulation in the CA1 region of the hippocampus but not in the dentate gyrus or paraventricular nucleus. Conversely, mineralocorticoid receptor mRNA expression levels were found unchanged in these brain areas. In conclusion, our findings indicate that CB1 deficiency enhances the circadian HPA axis activity peak and leads to central impairment of glucocorticoid feedback, thus further outlining the essential role of the endocannabinoid system in the modulation of neuroendocrine functions. (*Endocrinology* 148: 1574–1581, 2007)

C RH, SYNTHESIZED in the paraventricular nucleus (PVN) of the hypothalamus, represents the main driving force controlling hypothalamic-pituitary-adrenal (HPA) axis activation, the major hormone system responsible to maintain homeostatic balance in response to stressful stimuli (1, 2). Appropriate regulatory control of the HPA axis is critical for health and survival, and several limbic brain structures, such as the hippocampus, amygdala, and prefrontal cortex, are involved in the integration of the HPA hormonal response (3).

Several lines of evidence support a role for the endocannabinoid system as a modulator of the HPA axis (4, 5). The cannabinoid receptor type 1 (CB1) and its endogenous ligands (endocannabinoids) are widely present in brain areas regulating HPA axis function (6–8). Recent studies reported

that endocannabinoids are involved in mediating the acute negative fast-feedback effect of glucocorticoids on the HPA axis at the level of the PVN (9). Moreover, both glucocorticoids and endocannabinoids increase appetite and body weight (10, 11), and an important link exists between stress and obesity (10, 11). Interestingly, it has been suggested that endocannabinoid signaling negatively modulates HPA axis activation induced by stress in a context-dependent manner (12). In line with this latest finding, previous studies described the involvement of endocannabinoids in the regulation of anxiety-related behaviors, which represent part of the physiological responses to stressful stimuli (13–15). However, the differential contribution of CB1 in the regulation of extrahypothalamic, hypothalamic, and pituitary activity is still under debate (5).

Pharmacological studies using animal models have shown that the administration of endocannabinoids or CB1 agonists increases circulating ACTH and glucocorticoid levels as well as hypothalamic mRNA expression levels of CRH and proopiomelanocortin (16–18). A role for CB1 in HPA axis activation has been established by the ability of the CB1 antagonist SR141716A to reduce the stimulation of ACTH release induced by Δ^9-tetrahydrocannabinol (the main component of marijuana) (19). However, it has also been described that the administration of SR141716A is able *per se* to increase the

secretion of both ACTH and corticosterone (12, 13, 19, 20). Therefore, depending on a set of variables such as drug doses, environmental context, and genetic background of the animals used, cannabinoid compounds might differently affect HPA axis activity (21).

In the current study, we used mice lacking CB1 (CB1$^{-/-}$) as a model to determine, under basal conditions, the role of the endocannabinoid system in the regulation of the HPA axis at extrahypothalamic, hypothalamic, and pituitary levels to highlight the potential existence of a HPA axis-modulating endocannabinoid tone.

Materials and Methods

Animals

Female mice deficient for CB1 (CB1$^{-/-}$) and wild-type littermates (CB1$^{+/+}$) were used. The mice were in a mixed genetic background, with a predominant C57BL/6N contribution (seven back-crossings) as described earlier (22, 23). The animals were housed in groups of five in the animal facility of the Max-Planck-Institute of Psychiatry (Munich, Germany) under standard conditions with a 12-h light, 12-h dark cycle (light on, 0600 h) at 22 C. After monitoring the stage of the ovarian cycle, 16-wk-old mice were killed in the diestrus phase. Particular care was taken to kill the animals in unstressed conditions. All animal procedures complied with the guidelines for the care and use of laboratory animals of the Governments of the State of Bavaria, Germany.

Hormone measurements and dexamethasone (Dex) suppression test

Twenty-one CB1$^{+/+}$ and 21 CB$^{-/-}$ mice were used to measure circadian basal plasma corticosterone and ACTH. To verify whether the normal circadian activity of the HPA axis was preserved in the two genotypes, the hormones were measured both at the onset of the light (0600 h) and dark (1800 h) phase. Trunk blood was collected in 1.5-ml ice-cold EDTA-coated tubes containing Trasylol (10,000 Kallikrein inhibitor unit/ml; Bayer, Leverkusen, Germany). After 15 min of centrifugation at 2000 × *g* at 4 C, plasma samples were stored at −80 C. Measurements of plasmatic levels of ACTH and corticosterone were performed using commercial RIA kits (MP Biomedicals, Eschwege, Germany; ICN Biomedicals, Meckenheim, Germany) as described elsewhere (24), according to the manufacturer's instructions. For the Dex suppression test, 32 CB1$^{+/+}$ and 23 CB1$^{-/-}$ mice were injected ip with saline or dexamethasone 21-phosphate disodium salt (0.02 or 0.1 mg/kg) (ICN) during the light phase (1200 h). Six hours later (1800 h), mice were rapidly decapitated in an adjacent room, trunk blood was collected, and hormones were measured as described above.

Morphological analysis of adrenal glands

Adrenals from 11 CB1$^{+/+}$ and 11 CB1$^{-/-}$ mice were rapidly dissected, weighed, postfixed in a 10% formalin solution (Sigma-Aldrich, Schnelldorf, Germany), dehydrated, and embedded in paraffin for microtome sections (12 μm). Hematoxylin-eosin staining was performed and glands histology analyzed under the microscope.

Primary pituitary cell culture

Unless stated otherwise, materials and reagents were obtained from Sigma-Aldrich, Life Technologies, Inc. (Eggenstein, Germany), Nunc (Wiesbaden, Germany), and Falcon (Heidelberg, Germany). To establish primary pituitary cell cultures, 10 CB1$^{+/+}$ and 10 CB1$^{-/-}$ mice were killed and pituitaries were rapidly removed and immediately collected in buffer of HEPES 25 mM, NaCl 137 mM, KCl 5 mM, Na$_2$HPO$_4$ 0.7 mM, glucose 10 mM, amphotericin B 10 ml/liter, and penicillin/streptomycin 10 ml/liter. After mechanical and enzymatic dispersions, as described in (25), cells were washed by repetitive centrifugation and finally resuspended in DMEM (pH 7.3) supplemented as described elsewhere (26). Cell viability was consistently more than 90%, as assessed by acridine orange/ethidium bromide staining. Cells were plated in 48-well plates (100,000 cells/well in 0.5 ml culture medium) and incubated in a 5% CO$_2$ atmosphere at 37 C. After the cells had attached to the plate (48 h), the culture medium was replaced by stimulation medium (26). After a washout period of 24 h, fresh stimulation medium was added to the cells together with the drug treatment.

Pituitary hormone stimulation and measurement

CRH (Bachem, Heidelberg, Germany) was used at a concentration of 10^{-7} and 10^{-8} M/liter, and forskolin (a direct activator of the catalytic subunit of adenylate cyclase) was used at a concentration of 5 × 10^{-6} M/liter. The final volume of the stimulation medium was 0.5 ml/well. After 4 h of the incubation period, the supernatant was removed and the hormone content was determined. Mouse ACTH was measured by RIA as previously described (24). Cell number was counted at the end of the stimulation experiments using the cell proliferation reagent kit WST-1 (Roche, Mannheim, Germany), following the manufacturer's instructions. Values obtained for hormone secretion were normalized to cell number. All the experiments were performed in triplicate.

In situ hybridization (ISH) for CRH, mineralocorticoid receptors (MRs), and glucocorticoid receptors (GRs)

For the single ISH experiments, six CB1$^{+/+}$ and seven CB1$^{-/-}$ mice were killed at the onset of the dark phase and their brains were quickly removed and flash frozen. Coronal sections (20 μm) were cut on a cryostat (HM560; Microtome, Walldorf, Germany), mounted onto frozen SuperFrost/Plus slides (Menzer-Glaser, Braunschweig, Germany), dried, and stored at −20 C until further processed. CRH cDNA and correspondent riboprobe were obtained and ISH for CRH were performed as described (22). MR cDNA was a 742-bp fragment (nucleotides 1004–1745) of mouse MR coding sequence, and GR cDNA was a 610-bp fragment (nucleotides 1654–2263) of mouse GR coding sequence, cloned into the pPCRII-TOPO vector (Invitrogen, Karlsruhe, Germany), both kindly provided by J. Deussing (Max-Planck-Institute of Psychiatry). Restriction enzymes (New England Biolabs, Beverly, MA) used for linearization and RNA polymerases (Roche) used for the generation of each riboprobe were as follows: MR sense, SP6 polymerase and *Xba*I; MR antisense, T7 polymerase and *Bam*HI; GR sense, SP6 polymerase and *Xba*I; GR antisense, T7 polymerase and *Bam*HI. According to protocol design, riboprobes were labeled with ^{35}S and ISH was carried out as described in (22). In ISH experiments, sense RNA probes did not give any detectable signals (data not shown). Standards were included during film exposure to assure that the level of the analyzed signal was within the linear range.

Quantification of CRH, MR, and GR mRNA expression was performed on autoradiographic films, using National Institutes of Health Image program Scion Image (http://rsb.info.nih.gov/nih-image/). For the area analyzed [for CRH mRNA: PVN of the hypothalamus and amygdala complex; for MR and GR mRNAs: PVN of the hypothalamus, CA1, CA3, and dentate gyrus (DG) of the hippocampus], at least three sections per animal were quantified after background subtraction.

For CRH and CB1 double-ISH experiments, coronal sections of CB1$^{+/+}$ brains were used. CB1 cDNAs and the correspondent riboprobes were obtained as described (22). According to the protocol design, riboprobe for CRH was labeled with ^{35}S and CB1 riboprobe was labeled with digoxigenin, and double ISH for CRH and CB1 mRNA colocalization was performed as described (27). Different brain regions were chosen for numerical evaluation of coexpression based on the published distribution patterns of CB1 and CRH (6, 28). CB1-positive cells and CRH-positive cells were chosen in these regions on at least four different brain sections, and coexpression values were calculated as percentage of CB1-expressing cells per number of cells positive for CRH, as described in (22).

Statistical analysis

All values are reported as means ± SEM. Hormonal measurements were evaluated by one- or two-way ANOVA, with genotype or genotype and treatment as factors, depending on the experimental conditions examined. ANOVAs were followed by Tukey *post hoc* test to identify significant differences. Unpaired *t* test was used for the analysis of ISH

performed for CRH, MR, and GR. $P < 0.05$ denotes statistical significance.

Results

Lack of CB1 increases circadian drive on the HPA axis

To examine the basal activity of the HPA axis as well as the circadian changes in corticosterone and ACTH levels, plasma corticosterone and ACTH were measured both at the beginning of the light and dark phase. The circadian hormonal variation was maintained in both CB1$^{+/+}$ and CB1$^{-/-}$ mice and differed significantly between morning and evening in both genotypes (Fig. 1, A and B). However, at the onset of the dark, CB1$^{-/-}$ mice showed higher circulating corticosterone (Fig. 1A, $P < 0.05$) and a trend toward increased ACTH levels (Fig. 1B), compared with CB1$^{+/+}$ controls. Thus, to test the responsiveness of the HPA axis to corticosteroid feedback, animals were treated with a low (0.02 mg/kg) or high dose (0.1 mg/kg) of Dex. Statistical analysis of the Dex test response by two-way ANOVA revealed significant main effects of genotype ($P < 0.0001$ for both corticosterone and ACTH levels) and drug treatment ($P < 0.0001$ for both corticosterone and ACTH levels) and a significant interaction between the two factors ($P = 0.01$ for corticosterone levels; $P < 0.0001$ for ACTH levels). In all the conditions but the high Dex dose, CB1$^{-/-}$ mice had significantly higher plasma corticosterone and ACTH levels, compared with the CB1$^{+/+}$ littermates (Fig. 1, C and D). At the lowest Dex dose tested, CB1$^{+/+}$ littermates showed a nonsignificant trend to decreased circulating corticosterone and ACTH levels (Fig. 1, C and D). In contrast, corticosterone levels in CB1$^{-/-}$ mice did not respond to 0.02 mg/kg Dex. Furthermore, at this dose of Dex, a surprising significant increase was observed in ACTH levels of CB1$^{-/-}$ mice (Fig. 1D; $P < 0.05$). How-

ever, when animals received the highest Dex dose, both CB1$^{-/-}$ and CB1$^{+/+}$ mice responded with a significant suppression in circulating levels of corticosterone and ACTH (Fig. 1, C and D).

To determine whether possible chronic hyperactivity of the HPA axis with increased circulating corticosterone levels affected the structure of the adrenal glands, we investigated their morphology in CB1$^{+/+}$ and in CB1$^{-/-}$ mice. However, no difference in adrenal weight (CB1$^{+/+}$, 9.1 mg ± 0.5 *vs.* CB1$^{-/-}$, 8.5 mg ± 0.7; $P = 0.451$) and no apparent alterations in the histology of the adrenal cortex were observed (data not shown).

Modulation of pituitary ACTH secretion in CB1$^{-/-}$ mice

Steroids such as Dex penetrate the brain poorly and predominantly induce feedback regulatory mechanisms at the pituitary level (29). Notably, both rodent and human pituitary express CB1 and synthesize endocannabinoids (26, 30). Thus, to further elucidate whether the hyperactivity of the HPA axis observed in CB1$^{-/-}$ mice could be directly related to the lack of pituitary CB1, we decided to perform *in vitro* experiments to study basal and stimulated ACTH secretion, using primary pituitary cell cultures derived from CB1$^{+/+}$ and CB1$^{-/-}$ littermates.

Statistical analysis by two-way ANOVA revealed significant main effects of genotype ($P < 0.0001$) and drug treatment ($P < 0.0001$) and a significant interaction between the two factors ($P < 0.05$). We found that cells from pituitaries of CB1$^{-/-}$ mice were characterized by a basal, although not significant, ACTH hypersecretion, compared with pituitary cultures from CB1$^{+/+}$ mice (Fig. 2). CB1$^{+/+}$-derived cells treated with CRH 10^{-7} M/liter, CRH 10^{-8} M/liter, or forskolin 5 × 10^{-6} M/liter were characterized by the expected significant increase in the secretion of ACTH (Fig. 2). Simi-

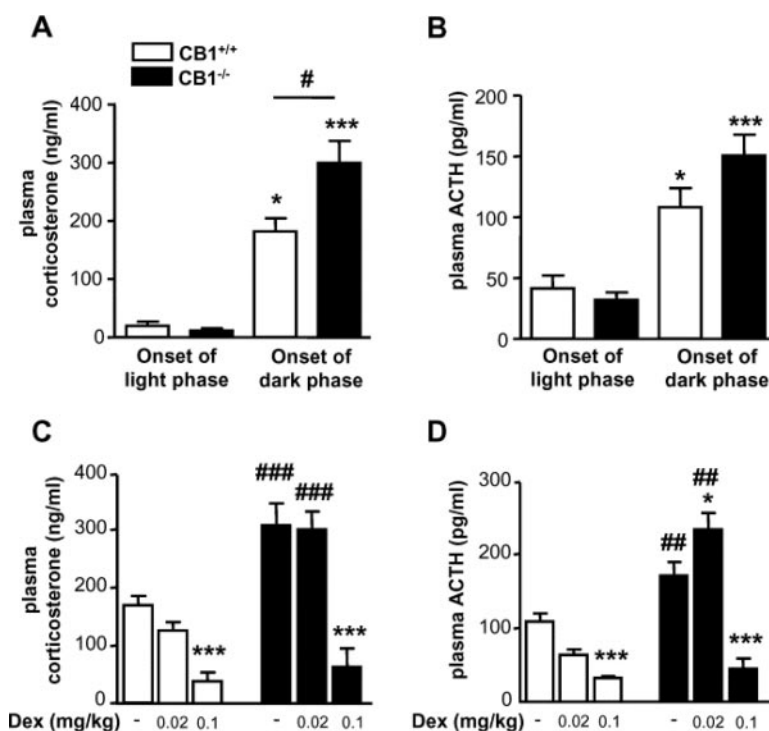FIG. 1. Plasma corticosterone and ACTH levels in CB1$^{+/+}$ and CB1$^{-/-}$ mice. A, Circadian changes of corticosterone levels. Values are mean ± SEM of eight to 13 animals per group. ***, $P < 0.001$ and *, $P < 0.05$ light phase *vs.* dark phase; #, $P < 0.05$ CB1$^{-/-}$ *vs.* CB1$^{+/+}$. B, Circadian changes of ACTH levels. Values are mean ± SEM of eight to 13 animals per group. ***, $P < 0.001$ and *, $P < 0.05$ light phase *vs.* dark phase. C and D, Corticosterone (C) and ACTH (D) levels before and after Dex test. Values are mean ± SEM of eight to 15 animals per group. ***, $P < 0.001$ and *, $P < 0.05$ Dex *vs.* saline; ###, $P < 0.001$ and ##, $P < 0.01$ CB1$^{-/-}$ *vs.* CB1$^{+/+}$ in the same treatment condition.

FIG. 2. ACTH secretion in primary pituitary cell cultures from CB1$^{+/+}$ and CB1$^{-/-}$ mice. ***, $P < 0.0001$, **, $P < 0.001$, and *, $P < 0.05$ vs. respective basal control; ###, $P < 0.001$ and ##, $P < 0.005$ vs. CB1$^{+/+}$ in the same treatment condition.
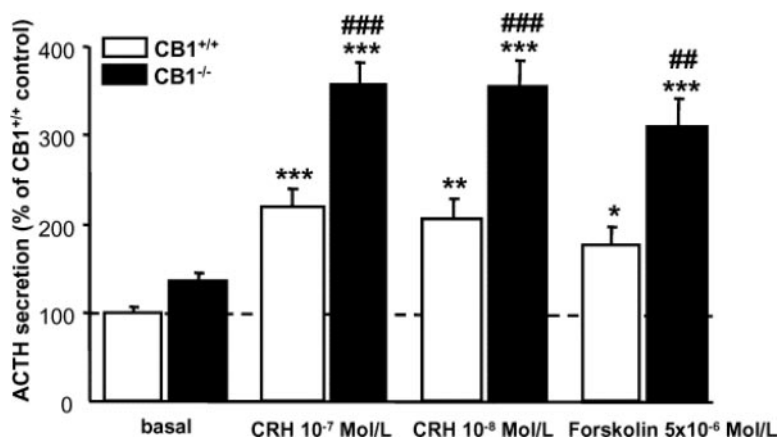
larly, after the stimulation with either CRH or forskolin, CB1$^{-/-}$-derived cells showed a significant increase of ACTH secretion, compared with basal CB1$^{-/-}$ ACTH values (Fig. 2). Remarkably, however, the CRH- or forskolin-induced ACTH secretion in CB1$^{-/-}$-derived cells was much higher than the one observed in CB1$^{+/+}$ cultures in the same experimental conditions (Fig. 2), thus suggesting an increased responsiveness of pituitary cells in absence of CB1 receptors.

Lack of CB1 affects CRH mRNA expression in the PVN but not other brain areas regulating HPA axis activity

The PVN of the hypothalamus and the CRH synthesized therein represent an essential driving force for the regulation of pituitary function. We recently described that male CB1$^{-/-}$ mice show, among other neuroendocrine modifications, higher CRH mRNA levels in the PVN (22). Female CB1$^{-/-}$ mice used in the current study also have significantly increased CRH mRNA expression in the PVN, compared with their CB1$^{+/+}$ littermates (Fig. 3, A and B; $P < 0.05$, one-tailed *t* test). To further investigate the involvement of extrahypothalamic areas that could possibly affect HPA axis activity, ISH experiments were performed on brain tissue

samples from CB1$^{+/+}$ mice to colocalize CB1 transcript with CRH mRNA. CB1 was found to be colocalized with CRH in several extrahypothalamic brain areas known to integrate the overall stress response, such as in the basolateral (BLA), basomedial (BMA), and central (CeA) nuclei of the amygdala, bed nucleus of the stria terminalis (BNST), piriform cortex, and prefrontal cortex (Fig. 4). In particular, as shown in Table 1, a large percentage of CRH neurons expressed CB1 mRNA in the amygdala (64.8% of CRH-positive cells in the BLA, 34.7% of CRH-positive cells in the BMA, and 32.3% of CRH-positive cells in the CeA-expressed CB1 mRNA, respectively). In the BNST and piriform cortex, 22.1% of CRH-positive cells and 19.0% of CRH-positive cells colocalized with CB1 mRNA, respectively. In the prefrontal cortex, 67.9% of CRH neurons expressed CB1 transcript. Therefore, we investigated the levels of expression of CRH mRNA in CB1$^{-/-}$ and CB1$^{+/+}$ mice focusing on the CeA, a structure considered to be a very important relay station in the stress response network (31) and known to have the highest CRH localization among the amygdaloid nuclei (28). However, we found no differences in the levels of expression of CRH between the two genotypes (data not shown). Similarly, no
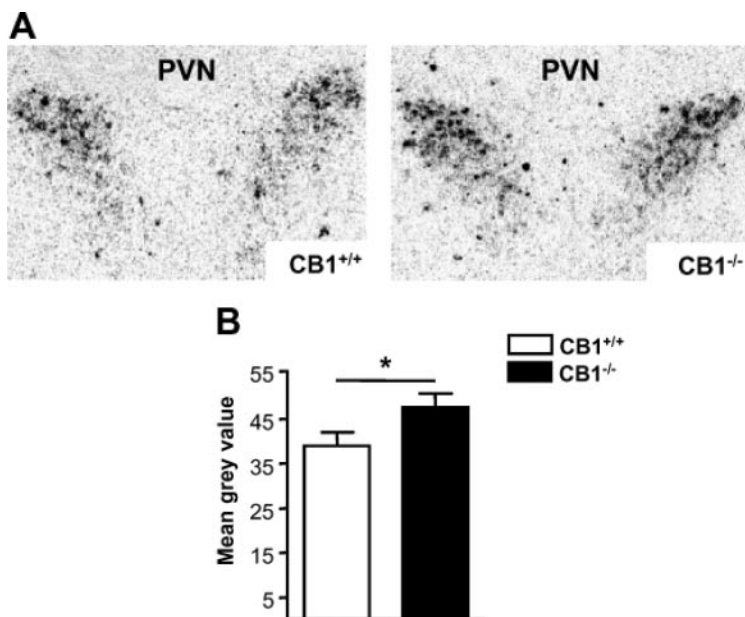
FIG. 3. Levels of CRH mRNA expression in the PVN of female CB1$^{+/+}$ and CB1$^{-/-}$ mice. A, Representative autoradiographic images showing the CRH mRNA expression in the PVN. B, Densitometric quantification by image analysis of CRH mRNA expression in the PVN. Values are mean ± SEM of six CB1$^{+/+}$ and seven CB1$^{-/-}$ mice. *, $P < 0.05$.
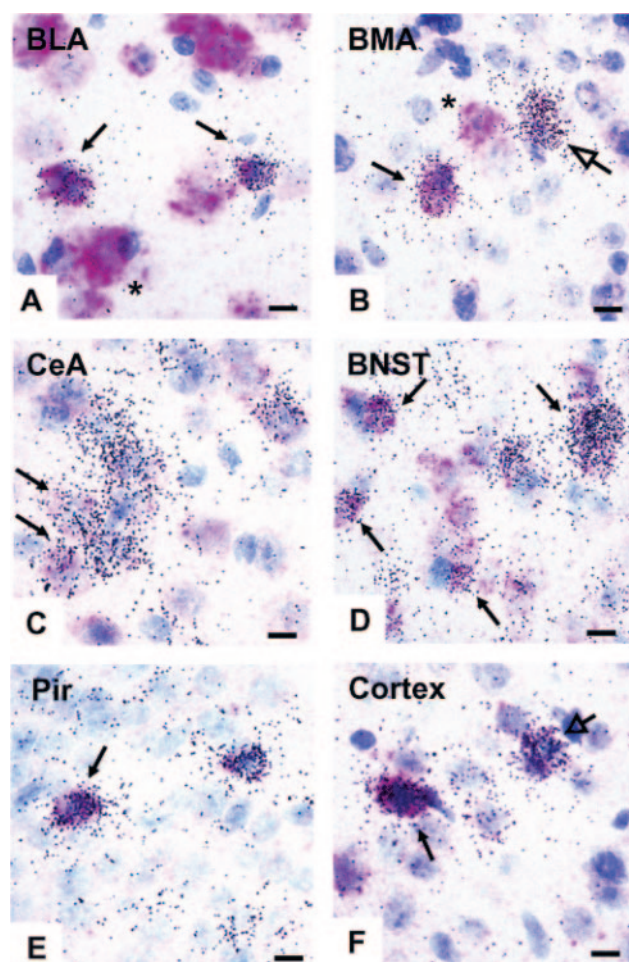
FIG. 4. Colocalization of CB1 and CRH mRNA in several extrahypothalamic brain regions. Bright-field micrographs. *Vector red staining*, CB1; *silver grains*, CRH. Colocalization of CB1 and CRH mRNA in the BLA (A), BMA (B), and CeA (C) nucleus of the amygdala and colocalization of CB1 and CRH mRNA in the BNST (D), piriform cortex (Pir) (E), and prefrontal cortex (F) are shown. *Filled arrow*, Cell coexpressing CB1 and CRH; *open arrow*, cell expressing only CRH mRNA; *asterisk*, cell expressing only CB1 mRNA. *Scale bars*, 10 μm.

alterations in CRH mRNA levels were noticed in the other extrahypothalamic brain areas examined during the studies of colocalization (data not shown).

CB1$^{-/-}$ mice have GR mRNA down-regulation in the CA1 region of the hippocampus

Corticosterone actions in the brain are mediated by GRs and MRs (29). Given the high circulating levels of corticosterone in CB1$^{-/-}$ mice at circadian peak, we investigated the possible dysregulation of GR and MR expression. No differences in MR mRNA levels were observed between CB1$^{+/+}$ and CB1$^{-/-}$ mice in either the PVN (Fig. 5A) or hippocampus (Fig. 5C). Interestingly, CB1$^{-/-}$ mice were characterized by a significant decrease in GR mRNA expression in the CA1 region of the hippocampus (Fig. 5, D and E; $P < 0.05$) and by a slight, nonsignificant down-regulation of GR mRNA in the DG (Fig. 5E). No differences in GR mRNA levels were noticed between genotypes at the level of the PVN (Fig. 5B).

Discussion

The appropriate regulation of the HPA axis is fundamental for the adequate response to internal and external challenges to maintain balance of homeostatic systems. Whereas the short-term activation of the HPA axis is highly adaptive for the survival of the organism, long-term HPA activity is often deleterious, leading to alterations of metabolic, cognitive, and behavioral functions and also being associated with body weight disorders (11) and neuropsychiatric diseases, such as anxiety and depression (32–34).

Here we describe that CB1$^{-/-}$ mice have increased circulating corticosterone levels at the circadian peak, increased *in vitro* responsiveness of their pituitary cells to CRH- and forskolin-induced ACTH secretion, dose-dependent responsiveness to Dex suppression test, increased CRH mRNA expression in the PVN, and decreased GR mRNA levels in the CA1 region of the hippocampus.

The present findings thus support the hypothesis that CB1 receptor signaling plays a critical role in regulating basal HPA axis activity because a reduced inhibitory tone on the HPA axis, particularly evident at the circadian peak, occurs in the absence of CB1 signaling.

In the central nervous system, endocannabinoids work as retrograde modulators of synaptic function and generally act as stress-recovery factors, produced in response to stressful stimuli reestablishing the steady state of neuropeptides, hormones, and neurotransmitters (35, 36). For instance, several lines of evidence suggest that hypothalamic endocannabinoid levels change in response to acute and chronic stress (5, 12). Moreover, endocannabinoids mediate the negative fast-feedback actions of glucocorticoids on CRH-containing neurons in the PVN by retrograde inhibition of glutamatergic transmission (9). Despite the fact that we did not test fast feedback mechanisms, the CB1$^{-/-}$ phenotype described in our study supports the hypothesis that CB1 signaling is a critical component of the regulatory glucocorticoid feedback that modulates HPA axis function.

Interestingly, the endocannabinoid system seems to be able to regulate the HPA axis by not only modulating the function of CRH-producing neurons at the hypothalamic level but also directly affecting ACTH secretion at the pituitary level. Indeed, the data obtained from our pituitary culture experiments clearly point to a direct involvement of CB1 in the modulation of ACTH secretion, suggesting that the absence of endocannabinoid signaling at the pituitary level may affect responsiveness to CRH. CB1$^{-/-}$-derived cells are hyperresponsive to stimuli, such as CRH and forskolin, which classically promote hypophyseal ACTH secretion. CRH stimulates ACTH secretion via a well-known G stimulating protein-coupled receptor pathway that leads to the activation of the adenylate cyclase enzyme and the increase of intracellular cAMP levels (37). CB1 is a seven-transmembrane G protein-coupled receptor whose effects are primarily due to the activation of the G$_i$ subunit, resulting in reduced adenylate cyclase activity and decreased intracellular cAMP (38). Therefore, in the corticotrope cells of the pituitary, the lack of CB1 might interfere with the normal regulation of adenylate cyclase activity, thus affecting ACTH secretion. However, in contrast to our results, a previous

TABLE 1. Coexpression of CB1 and CRH mRNA in extrahypothalamic brain regions

Area of the brain	Cells coexpressing CRH and CB1 (%)[a]	NC
Amygdala-BLA	64.8	871
Amygdala-BMA	34.7	106
Amygdala-CeA	32.3	320
BNST	22.1	590
Piriform cortex	19.0	466
Prefrontal cortex	67.9	3553

NC, Number of CRH-positive cells counted.
[a] Coexpression values were calculated as percentage of CB1-expressing cells per number of cells positive for CRH.

investigation on CB1$^{-/-}$ mice reported no alteration in the modulation of pituitary ACTH secretion (39), possibly because of differences in the inbred strains or, more likely, in the experimental procedure used.

CB1$^{-/-}$ mice respond properly with a decrease in ACTH and corticosterone secretion after the high-dose Dex treatment, thus suggesting that the feedback control on the HPA axis is still preserved. Nevertheless, it is worth mentioning that whereas CB1$^{+/+}$ mice tend to decrease both corticosterone and ACTH levels after a low dose of Dex, CB1$^{-/-}$ mice do actually show a further increase in ACTH levels and no reduction of corticosterone. The reasons for the paradoxical increase in ACTH levels found in CB1$^{-/-}$ mice after treatment with a low dose of Dex are currently unknown. Nevertheless, our findings suggest that the response to Dex treatment seems to be in part influenced by the lack of CB1.

GRs and MRs mediate corticosterone actions in the brain. Under low-corticosterone levels, only the high-affinity MR in the hippocampus is predominantly occupied. The low-affinity GR can be activated additionally to MR only when corticosterone levels are high, such as at the circadian peak or during stress (29). Thus, corticosterone action via MR exerts a tonic, permissive influence on hippocampus-associated functions, whereas occupancy of GR in this brain region mediates feedback actions aimed to terminate stress-induced HPA activation (29). Hence, corticosterone responses modulated by MR activation are themselves subject

to feedback action via GR (29). In CB1$^{-/-}$ mice, levels of GR and MR mRNA are unchanged in the PVN, and MR mRNA levels appear normal in various subregions of the hippocampus. However, GR mRNA is significantly decreased in the CA1 region of the hippocampus, possibly as a result of increased circulating glucocorticoids levels that may downregulate hippocampal GR expression.

Because CB1 is highly expressed in CA1, CA3, and DG of the hippocampus (6, 8), a question that remains unaddressed by the present findings is whether the prominent effects of glucocorticoids in this brain region also involve a CB1-dependent signal. Moreover, it is not known whether an interaction between CB1 and GR exists. Further experiments will be needed to clarify the possible role of the endocannabinoid system in the modulation of brain GR function.

CB1 and endocannabinoids are also present in limbic brain regions considered to be part of the emotional stress response circuitry and known to influence the HPA axis activity (3). Our double-ISH experiments demonstrate that CB1 and CRH mRNA colocalize in several brain structures, such as the amygdala, BNST, and prefrontal cortex. Nevertheless, under unstressed condition, the genetic deletion of CB1 does not affect the levels of CRH mRNA expression in these extrahypothalamic areas. On the other hand, it is well known that the exposure to psychological stress increases CRH mRNA levels in the amygdala, specifically in the CeA (31). Thus, it seems reasonable to hypothesize that CRH levels in the lim-

FIG. 5. Levels of MR and GR mRNA expression in the PVN and hippocampus of CB1$^{+/+}$ and CB1$^{-/-}$ mice. A and B, Densitometric quantification by image analysis of MR and GR mRNA expression in the PVN. C, Densitometric quantification by image analysis of MR mRNA expression in the CA1, CA3, and DG of the hippocampus. D, Representative autoradiographic images showing the GR mRNA expression in the CA1 of the hippocampus. E, Densitometric quantification by image analysis of GR mRNA expression in the CA1 and DG of the hippocampus. Values are mean ± SEM of six CB1$^{+/+}$ and seven CB1$^{-/-}$ mice. *, $P < 0.05$.
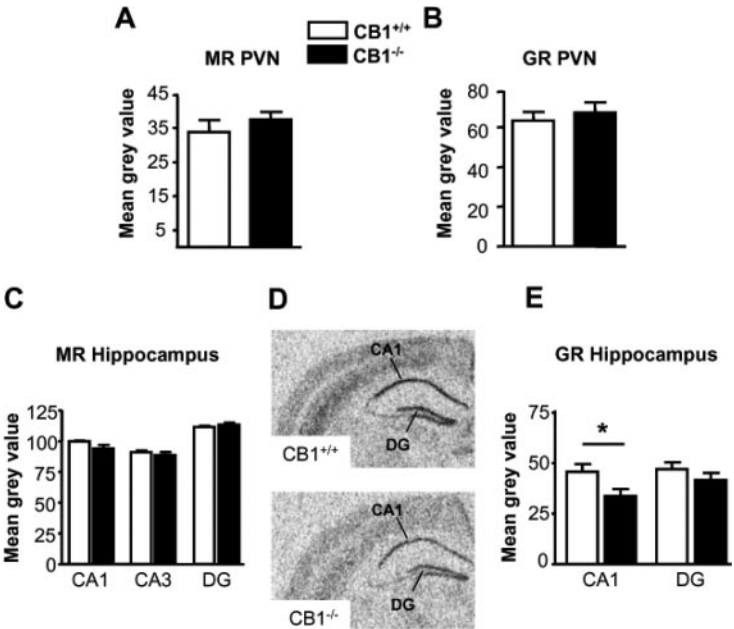
bic system could be altered in CB1$^{-/-}$ mice exposed to psychological stress. In support of this hypothesis, CB1$^{-/-}$ mice evaluated under different behavioral paradigms, exhibit increased aggressive, anxiogenic-like, and depressive-like responses (40–42), suggesting that absence of CB1 results in a greater vulnerability to stress (42).

Finally, caution should be used in administering weight-reducing CB1 antagonists to obese patients displaying an anxiety or depressive trait. In agreement with our conclusions, recent published trials indicate that a low but significant number of obese patients treated with the CB1 antagonist rimonabant discontinued the treatment due to increased occurrence of anxiety and depressed mood disorders (43–45).

Our current findings highlight an important role for the endocannabinoid system in the neuroendocrine network that regulates both ACTH and glucocorticoid secretion, implying that alterations of the central endogenous cannabinoid tone might be involved in the pathophysiology of stress-related diseases.

References

1. **De Kloet ER** 1995 Steroids, stability and stress. Front Neuroendocrinol 16:416–425
2. **Tsigos C, Chrousos GP** 2002 Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53:865–871
3. **Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE** 2003 Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24:151–180
4. **Murphy LL, Munoz RM, Adrian BA, Villanua MA** 1998 Function of cannabinoid receptors in the neuroendocrine regulation of hormone secretion. Neurobiol Dis 5:432–446
5. **Pagotto U, Marsicano G, Cota D, Lutz B, Pasquali R** 2006 The emerging role of the endocannabinoid system in endocrine regulation and energy balance. Endocr Rev 27:73–100
6. **Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC** 1991 Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. J Neurosci 11:563–583
7. **Bisogno T, Berrendero F, Ambrosino G, Cebeira M, Ramos JA, Fernandez-Ruiz JJ, Di Marzo V** 1999 Brain regional distribution of endocannabinoids: implications for their biosynthesis and biological function. Biochem Biophys Res Commun 256:377–380
8. **Marsicano G, Lutz B** 1999 Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur J Neurosci 11:4213–4225
9. **Di S, Malcher-Lopes R, Halmos KC, Tasker JG** 2003 Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. J Neurosci 23:4850–4857
10. **Dallman MF** 2003 Fast glucocorticoid feedback favors 'the munchies.' Trends Endocrinol Metab 14:394–396
11. **Dallman MF, Pecoraro N, Akana SF, La Fleur SE, Gomez F, Houshyar H, Bell ME, Bhatnagar S, Laugero KD, Manalo S** 2003 Chronic stress and obesity: a new view of "comfort food." Proc Natl Acad Sci USA 100:11696–11701
12. **Patel S, Roelke CT, Rademacher DJ, Cullinan WE, Hillard CJ** 2004 Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. Endocrinology 145:5431–5438
13. **Navarro M, Hernandez E, Munoz RM, del Arco I, Villanua MA, Carrera MR, Rodriguez de Fonseca F** 1997 Acute administration of the CB1 cannabinoid receptor antagonist SR 141716A induces anxiety-like responses in the rat. Neuroreport 8:491–496
14. **Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D** 2003 Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 9:76–81
15. **Wotjak CT** 2005 Role of endogenous cannabinoids in cognition and emotionality. Mini Rev Med Chem 5:659–670
16. **Weidenfeld J, Feldman S, Mechoulam R** 1994 Effect of the brain constituent anandamide, a cannabinoid agonist, on the hypothalamo-pituitary-adrenal axis in the rat. Neuroendocrinology 59:110–112
17. **Wenger T, Jamali KA, Juaneda C, Leonardelli J, Tramu G** 1997 Arachidonyl ethanolamide (anandamide) activates the parvocellular part of hypothalamic paraventricular nucleus. Biochem Biophys Res Commun 237:724–728
18. **Corchero J, Fuentes JA, Manzanares J** 1999 Chronic treatment with CP-55,940 regulates corticotropin releasing factor and proopiomelanocortin gene expression in the hypothalamus and pituitary gland of the rat. Life Sci 64:905–911
19. **Manzanares J, Corchero J, Fuentes JA** 1999 Opioid and cannabinoid receptor-mediated regulation of the increase in adrenocorticotropin hormone and corticosterone plasma concentrations induced by central administration of Δ(9)-tetrahydrocannabinol in rats. Brain Res 839:173–179
20. **Wade MR, Degroot A, Nomikos GG** 2006 Cannabinoid CB(1) receptor antagonism modulates plasma corticosterone in rodents. Eur J Pharmacol 551:162–167
21. **Gaetani S, Cuomo V, Piomelli D** 2003 Anandamide hydrolysis: a new target for anti-anxiety drugs? Trends Mol Med 9:474–478
22. **Cota D, Marsicano G, Tschöp M, Grübler Y, Flachskamm C, Schubert M, Auer D, Yassouridis A, Thone-Reineke C, Ortmann S, Tomassoni F, Cervino C, Nisoli E, Linthorst AC, Pasquali R, Lutz B, Stalla GK, Pagotto U** 2003 The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. J Clin Invest 112:423–431
23. **Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglgansberger W, Di Marzo V, Lutz B** 2002 The endogenous cannabinoid system controls extinction of aversive memories. Nature 418:530–534
24. **Karanth S, Linthorst AC, Stalla GK, Barden N, Holsboer F, Reul JM** 1997 Hypothalamic-pituitary-adrenocortical axis changes in a transgenic mouse with impaired glucocorticoid receptor function. Endocrinology 138:3476–3485
25. **Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W** 1998 Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat Genet 19:162–166
26. **Pagotto U, Marsicano G, Fezza F, Theodoropoulou M, Grübler Y, Stalla J, Arzberger T, Milone A, Losa M, Di Marzo V, Lutz B, Stalla GK** 2001 Normal human pituitary gland and pituitary adenomas express cannabinoid receptor type 1 and synthesize endogenous cannabinoids: first evidence for a direct role of cannabinoids on hormone modulation at the human pituitary level. J Clin Endocrinol Metab 86:2687–2696
27. **Hermann H, Marsicano G, Lutz B** 2002 Coexpression of the cannabinoid receptor type 1 with dopamine and serotonin receptors in distinct neuronal subpopulations of the adult mouse forebrain. Neuroscience 109:451–460
28. **Olschowka JA, O'Donohue TL, Mueller GP, Jacobowitz DM** 1982 The distribution of corticotropin releasing factor-like immunoreactive neurons in rat brain. Peptides 3:995–1015
29. **De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M** 1998 Brain corticosteroid receptor balance in health and disease. Endocr Rev 19:269–301
30. **Gonzalez S, Manzanares J, Berrendero F, Wenger T, Corchero J, Bisogno T, Romero J, Fuentes JA, Di Marzo V, Ramos JA, Fernandez-Ruiz J** 1999 Identification of endocannabinoids and cannabinoid CB(1) receptor mRNA in the pituitary gland. Neuroendocrinology 70:137–145
31. **Makino S, Shibasaki T, Yamauchi N, Nishioka T, Mimoto T, Wakabayashi I, Gold PW, Hashimoto K** 1999 Psychological stress increased corticotropin-releasing hormone mRNA and content in the central nucleus of the amygdala but not in the hypothalamic paraventricular nucleus in the rat. Brain Res 850:136–143
32. **Chrousos GP, Gold PW** 1992 The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 267:1244–1252

33. **de Kloet ER, Joels M, Holsboer F** 2005 Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6:463–475

34. **Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB** 1999 The role of corticotropin-releasing factor in depression and anxiety disorders. J Endocrinol 160:1–12

35. **Di Marzo V, Melck D, Bisogno T, De Petrocellis L** 1998 Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. Trends Neurosci 21:521–528

36. **Piomelli D** 2003 The molecular logic of endocannabinoid signalling. Nat Rev Neurosci 4:873–884

37. **Chalmers DT, Lovenberg TW, Grigoriadis DE, Behan DP, De Souza EB** 1996 Corticotrophin-releasing factor receptors: from molecular biology to drug design. Trends Pharmacol Sci 17:166–172

38. **Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG** 2002 International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol Rev 54:161–202

39. **Barna I, Zelena D, Arszovszki AC, Ledent C** 2004 The role of endogenous cannabinoids in the hypothalamo-pituitary-adrenal axis regulation: *in vivo* and *in vitro* studies in CB1 receptor knockout mice. Life Sci 75:2959–2970

40. **Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O** 2002 Involvement of CB1 cannabinoid receptors in emotional behaviour. Psychopharmacology (Berl) 159:379–387

41. **Uriguen L, Perez-Rial S, Ledent C, Palomo T, Manzanares J** 2004 Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors. Neuropharmacology 46:966–973

42. **Fride E, Suris R, Weidenfeld J, Mechoulam R** 2005 Differential response to acute and repeated stress in cannabinoid CB1 receptor knockout newborn and adult mice. Behav Pharmacol 16:431–440

43. **Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, Rossner S; RIO-Europe Study Group** 2005 Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. Lancet 365:1389–1397

44. **Despres JP, Golay A, Sjostrom L; RIO-Lipids Study Group** 2005 Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. N Engl J Med 353:2121–2134

45. **Pi-Sunyer FX, Aronne LJ, Heshmati HM, Devin J, Rosenstock J; RIO-North America Study Group** 2006 Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. JAMA 295:761–775