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# Regulation of the Hypothalamic-Pituitary-Adrenocortical System in Mice Deficient for CRH Receptors 1 and 2

JENS PREIL*, MARIANNE B. MÜLLER*, ANGELA GESING, JOHANNES M. H. M. REUL, INGE SILLABER, MARCEL M. VAN GAALEN, JOBST LANDGREBE, FLORIAN HOLSBOER, MARY STENZEL-POORE, AND WOLFGANG WURST

*Max Planck Institute of Psychiatry (J.P., M.B.M., A.G., J.M.H.M.R., I.S., M.M.V.G., J.L., F.H., W.W.), 80804 Munich, Germany; GSF Research Center, Institute for Mammalian Genetics (W.W.), 85764 Munich, Germany; and Department of Molecular Microbiology and Immunology, Oregon Health Sciences University (M.S.-P.), Portland, Oregon 97201*

**Recent investigations in mouse lines either deficient for the CRH receptor 1 (CRHR1) or 2 (CRHR2) suggest that the CRH neuronal system may comprise two separate pathways that can be coordinately and inversely activated in stress-induced hypothalamic-pituitary-adrenal (HPA) response and anxiety-like behavior. We generated mice deficient for both CRHR1 (*Crhr1*$^{-/-}$) and CRHR2 (*Crhr2*$^{-/-}$) to investigate the HPA system regulation in the absence of known functionally active CRH receptors under basal conditions and in response to different ethologically relevant stressors. To elucidate possible gene dose effects on the action of both CRH receptors, our analysis included heterozygous and homozygous CRHR1- or CRHR2-deficient mice, mutants lacking both CRH receptors, compound mutants with homozygous and heterozygous deficiency for either of the receptors, and their wild-type littermates. Both male and female *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants were viable, fertile, and indistinguishable in size from wild-type littermates. We show that the endocrine phenotype of mice lacking both CRHRs is dominated by the functional loss of CRHR1. CRHR2 does not compensate for CRHR1 deficiency, nor does the lack of CRHR2 exacerbate the CRHR1-dependent impairment of the HPA system function. Within the intraadrenal CRH/ACTH system, our data suggest different roles for CRHR1 and CRHR2 in fine-tuning of adrenocortical corticosterone release. (*Endocrinology* 142: 4946–4955, 2001)**

CRH IS INVOLVED in the mediation of complementary neuroendocrine, autonomic, and behavioral responses to stress. One important function of CRH is its role in activating the hypothalamic-pituitary-adrenal (HPA) system by initiating stress-induced ACTH secretion, which results in the adrenocortical release of corticosterone (CORT) (for reviews, see Refs. 1 and 2). In addition to its endocrine function, CRH may act as a neurotransmitter or neuromodulator in extrahypothalamic circuits to integrate the multisystem response to stress. Clinical studies indicate that alterations in the CRH neuronal systems contribute to the pathophysiology of several psychiatric (*e.g.* major depression, anxiety disorders, and anorexia nervosa), endocrine (*e.g.* Cushing's disease), autoimmune (*e.g.* rheumatoid arthritis), and neurodegenerative disorders (*e.g.* Alzheimer's disease) (for reviews, see Refs. 3 and 4). Urocortin (UCN) and UCN II have been described as neuropeptides of the CRH family (5, 6). Although UCN has been demonstrated to produce behavioral and physiological effects qualitatively similar to those of CRH, it appears to be a more potent suppressor of ingestive behavior (7) and a less potent inducer of anxiety-like behavior than CRH (for review, see Ref. 8). In contrast to UCN, the expression of UCN II in the paraventricular, supraoptic, and arcuate nuclei of the hypothalamus and the locus coeruleus suggests potential capacities for participating in stress-related functions (6).

The biological actions of CRH, UCN, and UCN II are mediated by specific, high affinity, G protein-coupled seven *trans*-membrane receptors, which mediate ligand-dependent stimulation of intracellular cAMP. The cloning of distinct receptor subtypes, CRHR1, CRHR2$\alpha$, and CRHR2$\beta$, facilitates the separation of the many physiological effects of these peptides. CRHR1 and CRHR2 display markedly different pharmacological specificity and tissue distribution. Although the expression of CRHR1 is high in neocortical, cerebellar, and sensory relay structures, the expression of CRHR2 mRNA is generally confined to subcortical structures, in particular within the choroid plexus, specific septal and amygdaloid nuclei, and various hypothalamic nuclei (for review, see Ref. 9). Differences in ligand affinities of CRH and UCN for CRHR1 and CRHR2 support the proposition that UCN/UCN II and CRHR2$\alpha$ may represent a functionally different circuit to the CRH/CRHR1 system (6, 8). Especially UCN II may be of value in dissociating functions mediated by the two receptors, as it binds selectively to CRHR2 (6).

CRHR1 has been proposed to mediate the effects of CRH on HPA system function and anxiety-related behavior (10–14). Mice deficient for CRHR1 display a severe impairment of stress-induced ACTH release from pituitary corticotropes, marked glucocorticoid deficiency, and significantly reduced anxiety-like behavior (15, 16). In line with these findings in rodents, a clinical study using a selective CRHR1 antagonist found alleviation of psychopathology in patients with severe major depression (17). The neuroendocrine analyses of CRHR2-deficient mice suggest that CRHR2 has a modulatory function on the HPA stress response involving the maintenance of ACTH release during stress and the recovery of plasma CORT after the end of the stressor (18, 19).

Abbreviations: CORT, Corticosterone; CRHR, CRH receptor; HPA, hypothalamic-pituitary-adrenal; UCN, urocortin.

Thus, the detailed investigations in mouse lines deficient for either CRHR1 or CRHR2 together with recent data from rat models (20) suggest that the CRH neuronal system may comprise two separate, but interrelated, subdivisions that can be coordinately and inversely activated in stress-induced HPA response and anxiety-like behavior.

We generated compound CRHR1/CRHR2 mutant mice to investigate the HPA system regulation in the absence of known CRHRs under basal conditions and in response to different ethologically relevant stressors. To elucidate possible gene dose effects in the action of both CRHRs, our analysis included all nine different genotypes, *i.e.* heterozygous and homozygous CRHR1- or CRHR2-deficient mice, mice lacking both CRH receptors, compound mutants with homozygous and heterozygous deficiency for either of the receptors and their wild-type littermates.

## Materials and Methods

### *Animals*

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany.

Heterozygous CRHR1-deficient male mice (129Ola/CD1) originally generated by Timpl *et al.* (15) and heterozygous CRHR2-deficient female mice (129SvJ/C57BL/6J) created by Coste *et al.* (19) were chosen as the first generation parents. Pairs of their $Crhr1^{+/-}Crhr2^{+/-}$ offspring were mated to produce the $F_2$ generation. To obtain a relevant number of all possible genotypes within the same age range, the following pairs of $F_2$ were mated: male $Crhr1^{-/-}Crhr2^{+/-}$ mutants with female $Crhr1^{+/-}Crhr2^{-/-}$ mutants, male $Crhr1^{+/-}Crhr2^{-/-}$ and $Crhr1^{-/-}Crhr2^{+/-}$ mutants with female $Crhr1^{+/-}Crhr2^{+/-}$ mutants, male $Crhr1^{+/-}Crhr2^{+/-}$ mutants with female $Crhr1^{+/-}Crhr2^{+/-}$ mutants, and male $Crhr1^{+/-}Crhr2^{+/-}$ mutants with female $Crhr1^{+/-}Crhr2^{+/-}$ mutants.

The present experiments were performed with 8- to 12-wk-old male and female mice, weighing between 25–35 g. The animals were housed four to six per cage in the breeding unit of the Max Planck Institute of Psychiatry under standard conditions with a 12-h light, 12-h dark cycle (lights on from 0600–1900 h, 22 ± 1 C, 40–60% humidity) and received standard pelleted food and water *ad libitum*.

### *Measurement of food intake*

To assess basal feeding, 24-h food consumption was measured for 7 consecutive d in male mice (n = 10/genotype). Mice were singly housed and were fed *ad libitum* with a standard purified, water-based liquid diet (Lieber-DeCarli diet, Dyets, Inc., Bethlehem, PA), that allowed exact quantification of food intake in each animal (21). Basal food intake (milliliters of daily prepared liquid diet in graduated feeding tubes) was measured once a day (0630 h).

### *Blood collection and stress experiments*

Two weeks before the experiments, animals were separated and housed singly to avoid uncontrolled stress reactions.

### *Basal hormone levels*

To determine the basal morning plasma levels of ACTH and CORT, naive male and female mice (n = 6–10/genotype) were left undisturbed throughout the night before the experiment. Blood sampling was performed in the early morning (0700–0800 h) by rapid retroorbital bleeding, with time from first handling of the animal to completion of bleed not exceeding 45 s.

### *Poststress levels of hormones*

*Restraint stress.* Male and female animals were subjected to restraint stress as a combined emotional and physical stressor (22). On the day of testing, between 0800–1200 h, each mouse (n = 6–10/genotype) was placed into a restrainer (diameter, 3 cm; length, 11 cm) for 2, 5, and 10 min, respectively. Blood samples were obtained by retroorbital bleeding immediately after stress exposure. The study was performed in three independent experiments (for each duration of stress) using male and female mice.

*Social defeat.* Male, singly housed mice served as resident stimulus animals for the experimental subjects. In preparation of the social defeat stress procedure, male resident mice were evaluated for their display of aggressive behavior by placing a group-housed intruder male into the resident's home cage. Usually within three tests the resident reliably attacked the intruder within less than 2 min (adapted with modifications from Ref. 23). Social defeat stress consisted of introducing a naive, singly housed experimental mouse (intruder; male $Crhr1^{-/-}Crhr2^{+/+}$, $Crhr1^{+/+}Crhr2^{-/-}$, and $Crhr1^{-/-}Crhr2^{-/-}$ mutants and wild-type mice; n = 6–10/group) into the resident's home cage. Immediately after being attacked by the resident for the first time, the intruder was separated from the resident by wire mesh within the resident's home cage. The resident continued to attack and threaten the intruder while the latter was protected from physical injury but was exposed to auditory, visual, and olfactory stimulation for 15 min. Blood collection was performed by retroorbital bleeding immediately after the end of the stressor.

### *CRH challenge*

After 1 wk of daily handling, male $Crhr1^{-/-}Crhr2^{+/+}$ and $Crhr1^{-/-}Crhr2^{-/-}$ mutants and wild-type littermates (age, 8–12 wk; n = 6–9/group) were sc injected with either vehicle or 1 μg CRH (both delivered from Ferring Pharmaceuticals Ltd., Malmo, Sweden; injection time, between 0800–0830 h). Blood samples were taken by rapid retroorbital bleeding 30 min after the injection for determination of plasma ACTH and CORT levels.

### *Treatment of blood samples and hormone analysis*

Blood samples were collected in prechilled tubes containing EDTA and a protease inhibitor (10 μl aprotinin; Trasylol, Bayer Corp., Leverkusen, Germany) and centrifuged (10 min, 3500 rpm, 4 C). Plasma samples were stored at −80 C (ACTH) and −20 C (CORT) until assay. Plasma ACTH (50 μl sample) and CORT (10 μl sample) levels were measured using commercially available kits (Biochem, Freiburg, Germany) according to the respective protocols. All samples were measured twice. The inter- and intraassay variabilities were less than 7%.

### *Histological and immunohistochemical analyses*

Animals (n = 3/genotype; wild-type mice, $Crhr1^{-/-}Crhr2^{+/+}$, $Crhr1^{+/+}Crhr2^{-/-}$, and $Crhr1^{-/-}Crhr2^{-/-}$ mutants) were deeply anesthetized with phenobarbital and transcardially perfused with phosphate-buffered 4% paraformaldehyde. Brains were removed from the skull, postfixed for 3 h in 4% paraformaldehyde, and then transferred to 20% sucrose in PBS (pH 7.4). Serial 30-μm coronal frozen sections were cut in a cryostat, transferred into PBS, and processed as free-floating sections. For each animal, all sections spanning the respective region of interest were analyzed to allow for exact comparisons of the different genotypes. All of the following steps were interposed by copious washes in PBS, and all reagents for immunohistochemistry were diluted in PBS with 1% BSA unless otherwise specified. After blocking endogenous peroxidase in absolute methanol with 0.01% hydrogen peroxide for 15 min, preincubations with 5% normal goat serum (AVP) and normal donkey serum (CRH), respectively, for 2 h were performed. The sections were then incubated with the primary antibody diluted 1:10,000 (polyclonal rabbit-anti AVP antibody; IHC 8103, Peninsula Laboratories, Inc., Belmont, CA) and 1:5,000 (polyclonal goat-anti-CRH antibody; Sc 1761, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), respectively, at 4 C. The specificities of these antibodies have been tested by the manufacturer. The sections were then incubated with a biotinylated goat antirabbit (AVP) or donkey antigoat (CRH) secondary antibody diluted 1:300 for 45 min at room temperature (Vector Laboratories, Inc., Burlingame, CA), followed by incubation with avidin biotinylated horseradish peroxidase complex (ABC Elite universal kit, Vector Laboratories, Inc.) for 45 min at room temperature (1:300). Finally, the sections were

developed in a substrate solution of 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 M Tris-HCl, pH 7.6. CRH staining was intensified with heavy metals according to the method described by Adams (24). The slides were then washed in PBS, mounted on glass slides, air-dried, and lightly counterstained with hematoxylin. Appropriate negative controls were performed by omission of the primary antibody. For histological analysis, peripheral organs (pituitary gland, thymus, lung, blood vessels, heart, liver, spleen, adrenal gland, and testes) were dissected after perfusion with phosphate-buffered 4% paraformaldehyde. Subsequently, tissue was postfixed, dehydrated, and embedded in paraffin, and 8-$\mu$m sections were cut on a microtome. Sections were mounted on glass slides and air dried. After deparaffination and hydration, routine hematoxylin-eosin staining was performed.

### *Statistical analysis*

Statistical analysis was performed with a software package (SigmaStat, version 2.03, Statistical Solutions Ltd., Boston, MA). Statistical significance of differences between groups was determined by ANOVA (one-way ANOVA, factor: genotype; or two-way ANOVA, factors: genotype and duration of stress, where appropriate), followed by *post-hoc* Newman-Keuls test. $P < 0.05$ was considered statistically significant.

## Results

### *Mice completely deficient for both CRHRs are viable, but exhibit adrenal gland histopathology*

Both male and female *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants are viable, fertile, and indistinguishable in size and weight from wild-type littermates. Except in the adrenal gland, we found no abnormalities in organ histology (brain, pituitary gland, thymus, lung, blood vessels, heart, liver, spleen, and testes). In *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants, the adrenal gland was considerably reduced in size compared with that in age-matched wild-type controls. In particular, the zona fasciculata of the adrenal cortex was markedly reduced in size, whereas the zona glomerulosa and medullary cells appeared relatively unaffected (Fig. 1). We observed no differences in adrenal gland size and structure between male CRHR2-deficient mice and their wild-type littermates. In contrast, *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mice showed a marked adrenocortical atrophy, yet no obvious difference in adrenal gland pathology could be observed between male *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants (Fig. 1). No sexual dimorphism in the degree of adrenocortical atrophy was detected within *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants. Furthermore, no obvious differences in adrenal gland pathology could be observed between female *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants (data not shown).

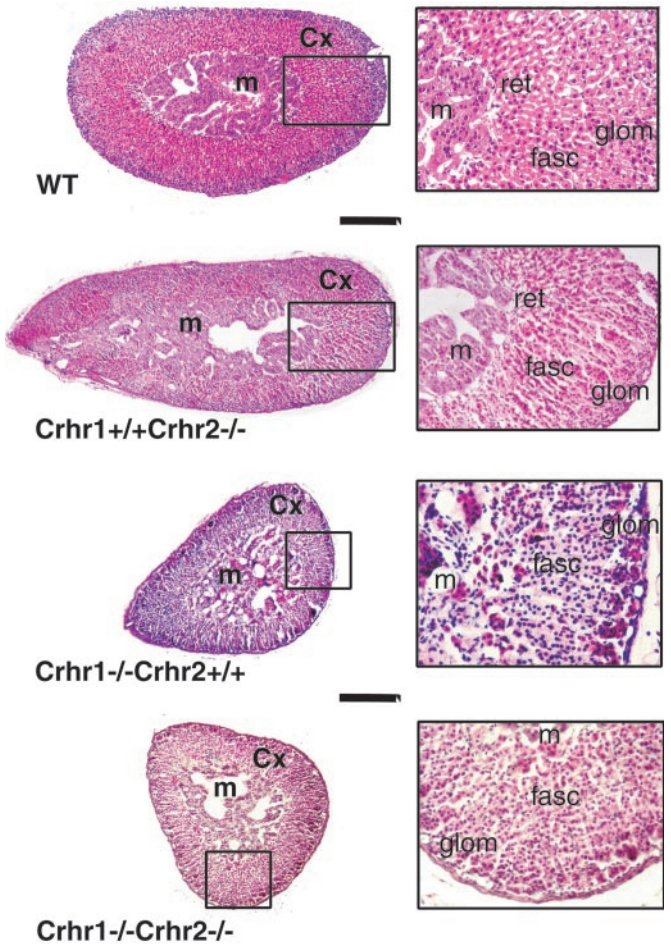


FIG. 1. Histological analysis of the adrenal gland in wild-type mice, single CRHR knockouts, and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants. Note the reduced size of the gland and the hypotrophic zona fasciculata in both CRHR1-deficient mice. In contrast to wild-type animals and CRHR2 mutants, a clear layering of the cortex into three zones disappeared in many regions of the adrenal gland in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ and *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mice (high magnification, *right panels*). Cx, Adrenal cortex; m, medulla; glom, zona glomerulosa; fasc, zona fasciculata; ret, zona reticularis. *Bars*, 400 $\mu$m.

### *Body weight and food intake*

No significant difference in body weight was observed among *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants, compound heterozygous, and wild-type animals. In addition, *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutant mice did not show any significant difference in their total amount of basal food intake compared with wild-type controls. During 24 h male wild-type animals consumed 22.2 ± 2.1 ml liquid diet (female wild-type animals, 17.1 ± 1.0 ml) compared with 20.1 ± 1.2 ml in male *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants (female mutants, 17.0 ± 1.4 ml).

### *Neuroendocrinology*

To investigate the differential effects of CRHR1 and CRHR2 on HPA system function as well as its gene dose dependence, we examined nine different genotypes in male animals, *i.e.* heterozygous and homozygous CRHR1- or CRHR2-deficient mice (*Crhr1*$^{+/-}$*Crhr2*$^{+/+}$, *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$, *Crhr1*$^{+/+}$*Crhr2*$^{+/-}$, *Crhr1*$^{+/-}$*Crhr2*$^{-/-}$), mice lacking both CRH receptors (*Crhr1*$^{-/-}$*Crhr2*$^{-/-}$), compound mutants with homozygous and heterozygous deficiency for either of the receptors (*Crhr1*$^{+/-}$*Crhr2*$^{+/-}$, *Crhr1*$^{+/-}$*Crhr2*$^{-/-}$, and *Crhr1*$^{-/-}$*Crhr2*$^{+/-}$), and their wild-type littermates (*Crhr1*$^{+/+}$*Crhr2*$^{+/+}$) and four different genotypes in females (wild-types, mice deficient for either CRHR1 or CRHR2, and mice lacking both CRHRs) under basal conditions and in response to different ethologically relevant stressors. Male and female animals (all genotypes) were subjected to different durations of restraint stress as a combined emotional and physical stressor. In addition, male mice (wild-type, mice deficient for either CRHR1 or CRHR2, and mice lacking both CRHRs) were subjected to social defeat as a predominantly emotional stressor (for review, see Ref. 25).

**TABLE 1.** Plasma ACTH and CORT levels in male CRHR-deficient mice and wild-type littermates under basal conditions

| CRHR type 1 | Type 2 +/+ | | +/− | | −/− | |
|---|---|---|---|---|---|---|
| | ACTH (pg/ml) | CORT (ng/ml) | ACTH (pg/ml) | CORT (ng/ml) | ACTH (pg/ml) | CORT (ng/ml) |
| +/+ | 83.2 ± 10.0 | 4.6 ± 1.4 | 49.8 ± 7.3 | 1.8 ± 1.1 | 111.6 ± 9.1 | 13.8 ± 3.1 |
| +/− | 41.7 ± 5.6 | 1.3 ± 0.8 | 58.2 ± 7.5 | 0.7 ± 0.1 | 90.8 ± 9.7 | 9.0 ± 2.4 |
| −/− | 88.9 ± 8.4 | 2.2 ± 1.0 | 102.6 ± 9.3 | 0.5 ± 0.4 | 81.6 ± 7.0 | 0.4 ± 0.2 |

Data are expressed as the mean ± SEM (n = 6–10). Two-way ANOVA including all tested genotypes did not reveal significant differences between any of the genotypes.

*Basal levels of ACTH and CORT in male and female animals*

Two-way ANOVA including all tested genotypes did not reveal significant differences between any of the genotypes in basal morning plasma ACTH and CORT levels either in male (Table 1) or female (Fig. 2) animals.

*Poststress levels of plasma hormones*

*Plasma ACTH levels after restraint stress in female animals.* Two-way ANOVA revealed a significant effect of both genotype ($P = 0.001$) and duration of stress ($P < 0.001$), with a significant interaction between both factors ($P < 0.001$; Fig. 2A). No significant increase in plasma ACTH was observed in either *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants following any of the restraint procedures ($P > 0.8$). Two minutes of restraint stress induced a significant increase in plasma ACTH in wild-type mice and *Crhr1*$^{+/+}$*Crhr2*$^{-/-}$ mutants compared with basal levels ($P < 0.001$). In *Crhr1*$^{+/+}$ *Crhr2*$^{-/-}$ mutants, plasma ACTH peaked after 5-min restraint stress and significantly decreased after a 10-min period of stress ($P < 0.05$). In contrast, wild-type animals showed a continuous increase in plasma ACTH with increasing durations of stress.

*Plasma CORT levels after restraint stress in female animals.* Two-way ANOVA revealed a significant effect of both genotype ($P < 0.001$) and duration of restraint stress ($P < 0.001$) as well as a significant interaction of both factors ($P < 0.001$; Fig. 2B). No significant differences between the genotypes could be detected in plasma CORT levels after 2 min of restraint stress. In wild-type mice and *Crhr1*$^{+/+}$*Crhr2*$^{-/-}$ mutants, plasma CORT levels significantly increased after 5-min stress compared with basal levels ($P < 0.01$). Both wild-type animals and *Crhr1*$^{+/+}$*Crhr2*$^{-/-}$ mutants showed a further increase in plasma CORT after 10-min restraint stress ($P < 0.01$). In contrast to plasma ACTH levels, CORT response curves were not significantly different between wild-type animals and *Crhr1*$^{+/+}$*Crhr2*$^{-/-}$ mutants. A significant increase in plasma CORT levels was observed in female *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutants after 5- and 10-min restraint stress ($P < 0.01$). However, female mice deficient for both CRHRs did not show elevated plasma CORT levels compared with baseline after any of the stress conditions.

*Plasma ACTH levels after restraint stress in male animals.* In male mutants lacking functional CRHR1, plasma ACTH and CORT concentrations did not increase significantly after any of the stress conditions (different lengths of restraint stress and 15-min social defeat stress; see Fig. 3 and 4).

Two-way ANOVA revealed a significant effect of both genotype ($P < 0.001$) and duration of stress ($P < 0.001$), with a significant interaction of both factors ($P < 0.001$; Fig. 3). All genotypes other than mutants lacking CRHR1 showed a significant increase in plasma ACTH after restraint stress ($P < 0.001$). Plasma ACTH response curves did not significantly differ among animals having two intact alleles for CRHR1. Among heterozygous CRHR1-deficient mice, however, an earlier onset of ACTH release was observed in *Crhr1*$^{+/-}$*Crhr2*$^{-/-}$ mutants. These animals showed significantly higher plasma ACTH levels after 2-min restraint stress ($P < 0.05$) compared with *Crhr1*$^{+/-}$*Crhr2*$^{+/+}$ and

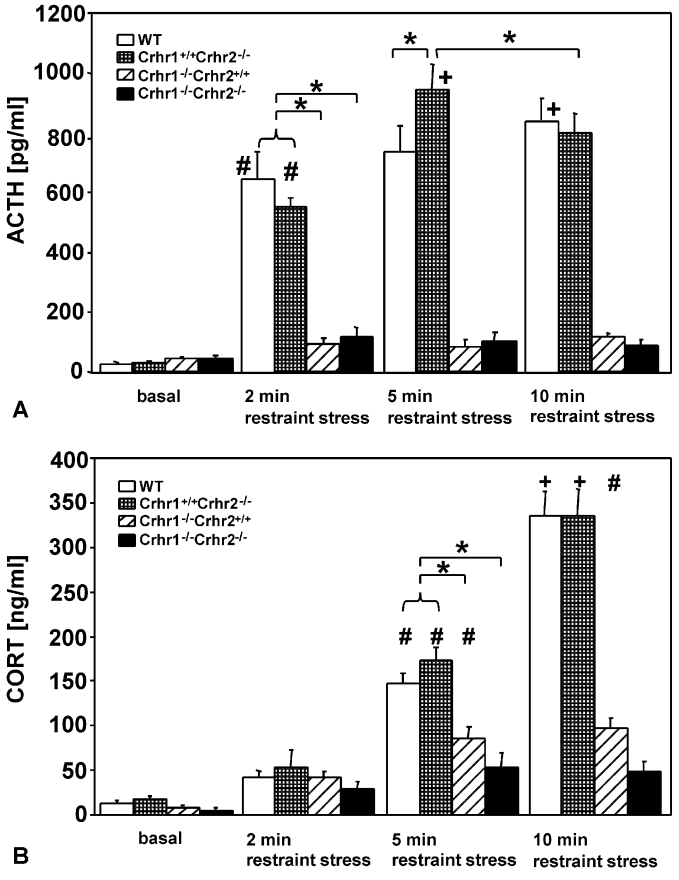


FIG. 2. Plasma ACTH (A) and CORT (B) levels in female CRHR-deficient mice and wild-type littermates under basal conditions and after different durations of restraint stress. Data are expressed as the mean ± SEM (n = 6–10). *, Statistically significant differences between genotypes or different durations of stress within the same genotype; #, significant effect of the experimental condition *vs.* basal levels within the same genotype; +, significant effect of the experimental condition *vs.* hormone levels after 2 min (A) and 5 min (B) of the restraint procedure, respectively.

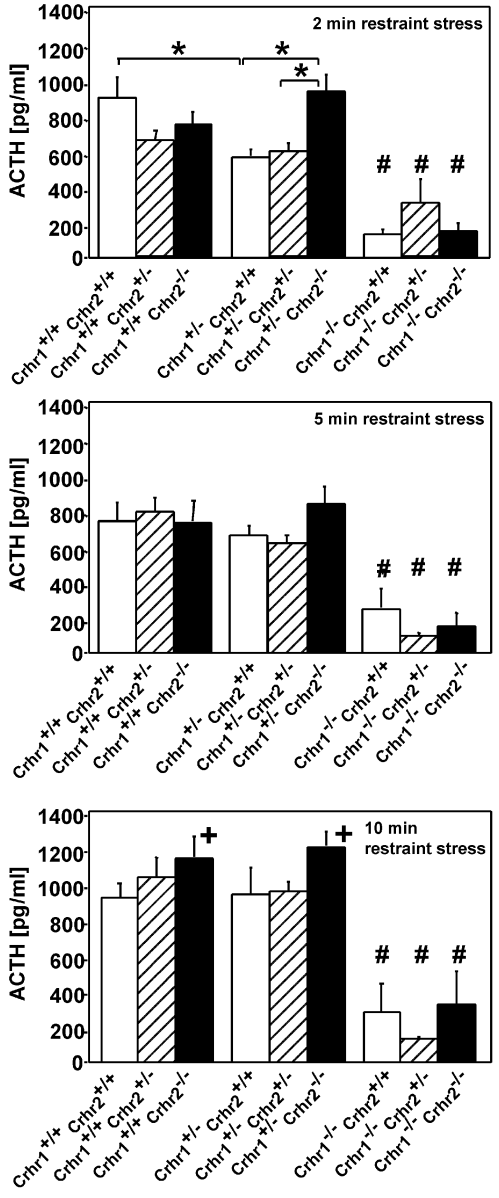


FIG. 3. Plasma ACTH levels in male CRHR-deficient mice and wild-type littermates after different durations of restraint stress. Data are expressed as the mean ± SEM (n = 6–10). □, $Crhr2^{+/+}$ genotypes ▨, $Crhr2^{+/-}$ mice; ■, $Crhr2^{-/-}$ genotypes. Each triple set of columns represents a particular CRHR1 genotype group. *, Statistically significant differences between genotypes; #, no significant effect of the experimental condition *vs.* basal levels within the same genotype; +, significant effect of the experimental condition *vs.* hormone levels after 5 min of the restraint procedure.

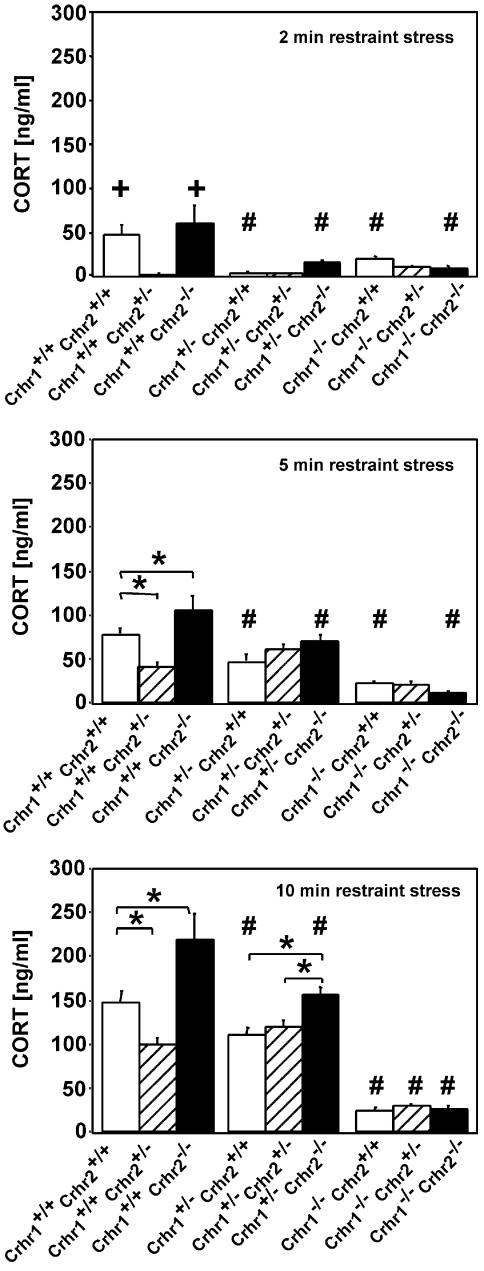


FIG. 4. Plasma CORT levels in male CRHR-deficient mice and wild-type littermates after different durations of restraint stress. Data are expressed as the mean ± SEM (n = 6–10). □, $Crhr2^{+/+}$ genotypes; ▨, $Crhr2^{+/-}$ mice; ■, $Crhr2^{-/-}$ genotypes. Each triple set of columns represents a particular CRHR1 genotype group. +, Significant effect of the experimental condition *vs.* basal levels; #, significant difference between a particular $Crhr1^{+/-}$ or $Crhr1^{-/-}$ genotype group and the respective $Crhr1^{+/+}$ genotype group; *, statistically significant differences between genotypes.

$Crhr1^{+/-}Crhr2^{+/-}$ mutants. However, plasma ACTH concentrations in all three $Crhr1^{+/-}$ genotypes were similar after 5- and 10-min stress (see Fig. 3). In contrast to females, ACTH response curves in male $Crhr1^{+/+}Crhr2^{-/-}$ mutants did not show a rapid peak and early decline. No significant differences were observed among the six genotypes with at least one copy of the CRHR1 gene after 5- and 10-min stress, respectively.

*Plasma CORT levels after restraint stress in male animals.* Two-way ANOVA revealed a significant effect of both genotype ($P < 0.001$) and duration of stress ($P < 0.001$), with a significant interaction of both factors ($P < 0.001$; Fig. 4). In wild-type animals and $Crhr1^{+/+}Crhr2^{-/-}$ mutants only, a significant increase in plasma CORT concentrations was observed after 2-min restraint stress compared with basal levels ($P < 0.001$). $Crhr1^{+/+}Crhr2^{+/-}$ mutant mice did not show elevated CORT levels after 2-min stress. Among the three genotypes with two copies of the CRHR1 gene, the plasma

CORT response curve in homozygous CRHR2-deficient mice showed the most rapid increase in plasma CORT levels (see Fig. 4). In contrast, $Crhr1^{+/+}Crhr2^{+/-}$ mutants showed a delayed onset of CORT release and slowly increasing plasma CORT levels after different lengths of stress. In heterozygous CRHR1-deficient mice, plasma CORT levels rose after 5-min, but not 2-min, restraint stress and were further increased after a 10-min period of stress ($P < 0.001$). After increasing length of stress, $Crhr1^{+/-}Crhr2^{-/-}$ mutant mice showed significantly elevated CORT concentrations compared with $Crhr1^{+/-}Crhr2^{+/+}$ and $Crhr1^{+/-}Crhr2^{+/-}$ mutants (see Fig. 4). Apart from the impaired CORT release in $Crhr1^{+/+}Crhr2^{+/-}$ mutant mice, plasma CORT levels in mice with two copies of the CRHR1 gene were significantly higher than CORT levels in $Crhr1^{+/-}$ mutants after any of the stress conditions ($P < 0.05$).

### *Stress hormone response after social defeat stress in male animals*

*Plasma ACTH levels (Fig. 5A).* Two-way ANOVA revealed a significant effect of both genotype ($P < 0.001$) and treatment condition ($P < 0.001$), with a significant interaction of both factors ($P < 0.001$). As observed after restraint stress, social defeat induced a significant increase in plasma ACTH in wild-type mice and $Crhr1^{+/+}Crhr2^{-/-}$ mutants compared with basal levels ($P < 0.001$). In $Crhr1^{-/-}$ mutants, however, plasma ACTH did not increase significantly after either social defeat or restraint stress compared with basal levels. In contrast to restraint stress, $Crhr1^{+/+}Crhr2^{-/-}$ mutants showed a significantly greater increase in plasma ACTH compared with wild-type mice after social defeat ($P < 0.05$).

*Plasma CORT levels (Fig. 5B).* Two-way ANOVA revealed a significant effect of both genotype ($P < 0.001$) and treatment conditions ($P < 0.001$), with a significant interaction of both factors ($P < 0.001$). Both $Crhr1^{+/+}Crhr2^{-/-}$ mutants and wild-type mice showed a significant increase in plasma CORT levels after social defeat ($P < 0.001$). Consistent with ACTH levels, plasma CORT concentrations were significantly higher in $Crhr1^{+/+}Crhr2^{-/-}$ mutants compared with wild-type mice ($P < 0.05$). However, no effects of the different stressors on plasma CORT levels were observed in either $Crhr1^{-/-}Crhr2^{+/+}$ or $Crhr1^{-/-}Crhr2^{-/-}$ mutants.

### *CRH challenge*

In wild-type mice only, administration of CRH under basal conditions significantly increased plasma ACTH and CORT levels compared with those in vehicle-treated animals ($P < 0.01$; Fig. 6). There was no statistically significant difference among the three vehicle-treated groups. CRH did not exert any significant effect on plasma ACTH and CORT levels in mice lacking CRHR1 ($Crhr1^{-/-}Crhr2^{+/+}$ and $Crhr1^{-/-}Crhr2^{-/-}$ mutants).

### *Increased CRH-like immunoreactivity in the PVN and median eminence in mice lacking both CRHRs*

Light microscopic analysis revealed an obvious difference in the expression level of CRH-like immunoreactivity in PVN neurons in $Crhr1^{-/-}Crhr2^{-/-}$ mutants compared with wild-

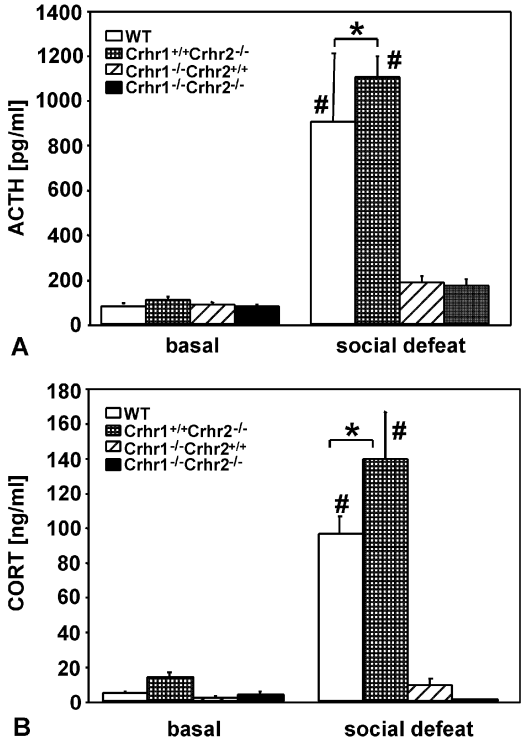


FIG. 5. Plasma ACTH (A) and CORT (B) levels in male CRHR1-deficient mice and wild-type littermates under basal conditions and after 15 min of social defeat stress. Data are expressed as the mean ± SEM (n = 6–10). *, Statistically significant differences between genotypes; #, significant effect of the experimental condition *vs.* basal levels within the same genotype.

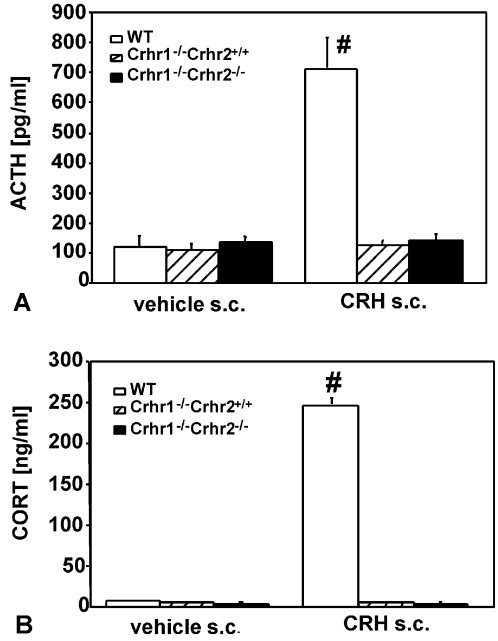


FIG. 6. Effects of CRH on plasma ACTH (A) and CORT (B) levels in male CRHR1-deficient mice and wild-type littermates 30 min after the administration of either CRH or vehicle. Data are expressed as the mean ± SEM (n = 6–9). #, Significant effect of the CRH challenge *vs.* levels after vehicle injection within the same genotype.

type littermates (Fig. 7A). In mice homozygously deficient for CRHR1, the zona externa of the median eminence exhibited a considerable increase in CRH-like immunoreactivity compared with wild-type littermates (Fig. 7B). This region displayed many prominent and strongly immunoreactive axons accumulating in the neurosecretory vesicles, whereas in wild-type mice and *Crhr2*$^{-/-}$ mutants, only weakly immunoreactive structures were present in the zona externa. No difference in CRH-like immunoreactivity was observed between CRHR2-deficient mice and wild-type animals.

*AVP-like immunoreactivity is increased in the hypothalamic neurosecretory system in mice lacking both CRHRs*

Light microscopic analysis revealed a marked increase in AVP-like immunoreactivity in both the PVN and SON in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants compared with wild-type littermates (Fig. 8). In *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutant mice, both nuclei contained a higher number of AVP-positive neurons that displayed many strongly labeled axons. However, in wild-type mice, a smaller number of less intensely stained axons were observed along the different projection pathways.

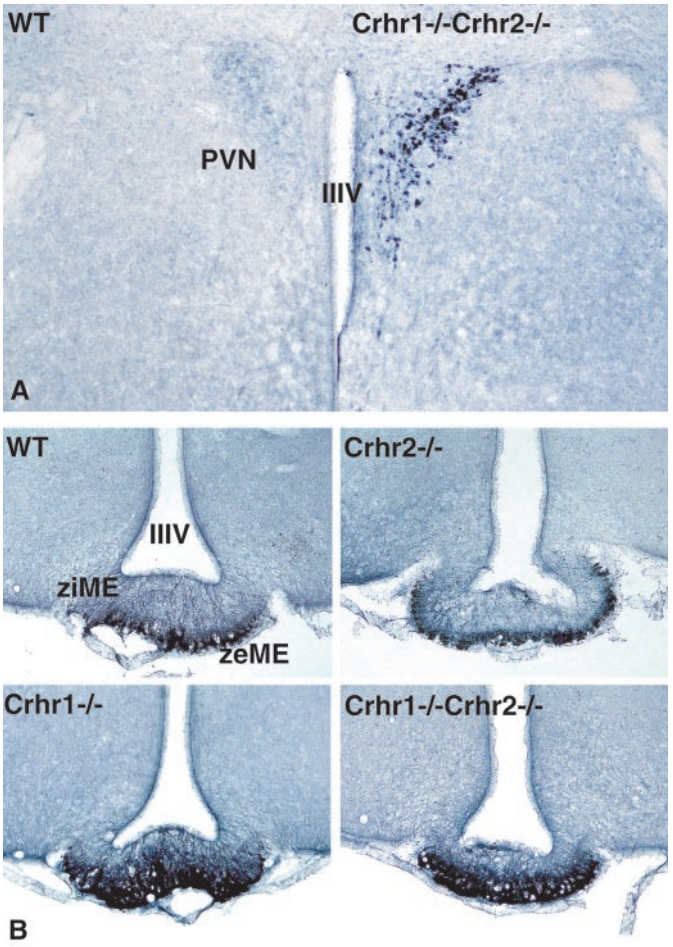


FIG. 7. CRH-like immunoreactivity in the paraventricular nucleus (PVN; A) and the median eminence (B). CRH-like immunoreactivity is clearly increased in the PVN and the external zone of the median eminence in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants and CRHR1-deficient mice. No obvious difference in staining intensity is observed between *Crhr1*$^{+/+}$*Crhr2*$^{-/-}$ mutants and wild-type animals. ziME, Zona interna; zeME, zona externa of the median eminence; IIIV, third ventricle.

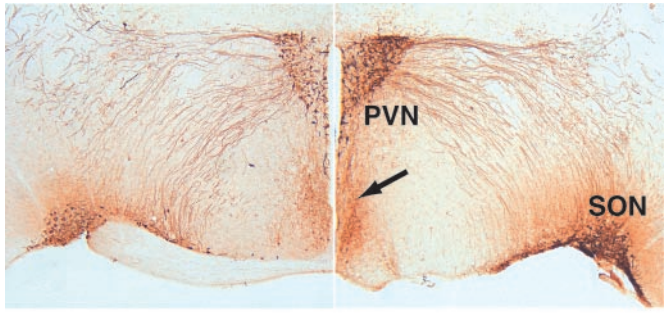


FIG. 8. AVP-like immunoreactivity in the hypothalamic neurosecretory system. AVP immunohistochemistry revealed a marked increase in the neuropeptide in both paraventricular nucleus (PVN) and supraoptic nucleus (SON). Note the prominent axon bundles apparently originating in the PVN along the third ventricle (*arrow*). In the *right panel*, the optical tract was lost upon removal.

## Discussion

*Neonatal survivability is affected by a functional lack of CRHR1*

In this study we bred mice with a targeted disruption in both CRHR1 and CRHR2. Male and female *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants were viable and fertile. *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutant offspring from heterozygous matings showed normal neonatal survivability, whereas the progeny from homozygous matings died within hours after birth. This observation is consistent with findings in CRH-deficient mice (26), mice with a targeted disruption in the GR gene (27) and in CRHR1-deficient mice (16), all showing similar neonatal mortality due to lung dysplasia. However, the survival of the offspring of females lacking CRH and functional CRHR1, respectively, could be restored by CORT supplementation beginning *in utero* (16, 26). This indicates that a CRHR1 deficiency-dependent lack of maternal CORT leads to inadequate fetal/neonatal lung maturation. Obviously, CRHR2 deficiency produced no additional effect on neonatal survival in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants compared with *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mice. Accordingly, no abnormalities were reported for *Crhr1*$^{+/+}$*Crhr2*$^{-/-}$ mutant mice (18, 19).

*Deficiency of CRHR1 and CRHR2 does not affect regulation of basal food intake and body weight*

Because CRH and both UCN and UCNII have been shown to influence feeding behavior (6, 7, 28), we measured weight and basal food intake in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants and wild-type animals. In the present series of experiments *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants did not show any significant difference in body weight compared with age-matched wild-type littermates. Accordingly, the total food intake per 24 h did not differ between the genotypes. These findings are consistent with previous reports of feeding regulation in CRHR1-deficient mice (29) and mice lacking a functional CRHR2 (18, 19). Thus, neither CRHR is likely to play a critical role in the basal regulation of body weight and the total amount of food intake. Rather, they may be involved in biphasic control of

UCN-mediated feeding behavior (19, 30) as well as in cross-talk among central leptin, melanocortin, and CRH pathways (31, 32).

### *The endocrine phenotype of Crhr1$^{-/-}$Crhr2$^{-/-}$ mutant mice is determined by the functional lack of CRHR1*

CRHR1 was found to be absolutely required for a normal endocrine stress response. Mice lacking a functional CRHR1 display a severe impairment of stress-induced HPA system activation and marked glucocorticoid deficiency (15, 16). In CRHR2-deficient mice, changes in the stress-induced time course of ACTH and CORT recovery indicate a role for CRHR2 in adaptation of the stress response initiated by CRHR1 (18, 19). The present study shows that the phenotype of mice lacking both CRHR1 and CRHR2 is similar to that of mice deficient for CRHR1.

No significant differences between wild-type and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutant mice were detected in basal plasma levels of ACTH and CORT in male or female animals. Recently, we demonstrated a selective activation of the hypothalamic vasopressinergic system to maintain basal ACTH release in CRHR1-deficient mice (29). The increased AVP-like immunoreactivity in the hypothalamic neurosecretory system, in particular in the PVN, suggests a similar compensatory regulation of stress hormone homeostasis in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants under basal conditions.

In contrast to the basal situation, where AVP possibly maintains ACTH secretion, none of the stressors applied (different durations of restraint stress and social defeat) led to an increase in circulating ACTH and CORT in male *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutant mice. This confirms the fundamental role of CRHR1 in initiating the stress-induced HPA response (15, 16).

CRH-like immunoreactivity in the PVN and the zona externa of the median eminence was clearly increased in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ and *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutant mice, whereas no difference in hypothalamic CRH-like immunoreactivity could be detected between wild-type animals and CRHR2-deficient mice. Consistent with this finding, an increase in CRH-like immunoreactivity (15) as well as in the expression of CRH mRNA (16) was detected in the PVN of CRHR1 deficient mice, whereas no change in the CRH mRNA level was seen in the PVN of nonstressed *Crhr2*$^{-/-}$ mutants (18, 19). As only single CRHR1-deficient mice and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants display a significant glucocorticoid deficiency with basal plasma CORT levels being hardly detectable, the increased CRH expression in the PVN may be due to the reduced negative feedback effects of corticosteroids. Supporting this hypothesis, adrenocortical steroids are known to feedback directly on the paraventricular neurons predominantly regulating CRH release (33).

It is well known that different types of stressors exert specific patterns of neuroendocrine activation (for review, see Ref. 25). Social defeat, for example, induces a stress response pattern that almost exclusively activates the sympathetic nervous system, reflected by elevated plasma epinephrine and norepinephrine concentrations (34). To compare the effects of different stressors, we subjected male *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ and *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutants to social defeat stress and examined whether a different neural stress response might lead, in contrast to the restraint paradigm, to significant HPA activation. However, social defeat failed to induce a significant increase in plasma ACTH and CORT levels in either *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ or *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutant mice.

Despite this severe impairment of HPA system function in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants, physical and vegetative adaptations promote normal growth and body weight in response to at least mild environmental stressors such as novel cage stress, reproduction, or social life. Therefore, it would be of particular interest whether other stress response systems, in particular the locus coeruleus/noradrenergic-sympathetic system, might compensate for the impairment of the CRH system in animals lacking functional CRH receptors.

### *CRHR1 and CRHR2 may be indirectly involved in fine-tuning of adrenocortical CORT release*

It is important to note that plasma ACTH and CORT concentrations did not differ between male *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ and *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutants after any of the stress conditions. Thus, neither CRHR2 is likely to compensate for a functional loss of CRHR1, nor does its deficiency exacerbate the CRHR1-dependent impairment of the HPA system function in male animals.

However, female *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutants were still able to elicit a rudimentary CORT response after 5 min of restraint stress, whereas female *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants did not show a significant hormone increase after any of the restraint periods. Indeed, there is evidence that CRHR1 deficiency may be at least partly compensated in female mice. In agreement with our results, significant CORT release in response to stress was also reported for female mice lacking CRHR1 and CRH, respectively (16, 26). As the ACTH response is markedly blunted in both the *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ and *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutant mice, the more distinct impairment of CORT response in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants might suggest an additional effect of CRHR2 on CORT release. In male *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ and *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutants, however, CRH administration failed to illicit a CORT response. Thus, at least in males, CRHR2 alone is not likely to stimulate CORT release at the adrenocortical level, which is consistent with our observations in male compound heterozygous mutants. Rather, the sexual dimorphism in CORT response may suggest a role for sex steroids in regulation of the HPA system that is not mediated through CRH (26). Furthermore, a different degree of adrenocortical atrophy between female *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants might be another reason for the different CORT response. In accordance with this hypothesis, in CRH-deficient mice, a sexually dimorphic adrenal response to stress has been correlated with a different degree of adrenocortical atrophy between the genders (26). However, we observed no sexual dimorphism in the degree of adrenocortical atrophy within the groups of *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ and *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutants. Corresponding to male animals, no obvious differences in adrenal gland pathology could be detected between female *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ and *Crhr1*$^{-/-}$

*Crhr2*$^{-/-}$ mutants. Consistent with our findings, no differences in adrenal gland size or structure between male and female *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutants have been reported, although both genders show different CORT responses to stress (15, 16). Apparently, there is no constant correlation between the degree of adrenocortical atrophy and CORT release in CRHR-deficient mice.

It is well known that CRH may influence adrenocortical steroidogenesis independently of pituitary function (for review, see Ref. 35). Recently, CRHR1 and CRHR2 mRNA expression has been demonstrated in the adrenal gland of humans (36). Accordingly, within the adrenal gland itself, CRH-like immunoreactivity with ACTH-releasing activity has been detected in the adrenal glands of humans (37), rats (38), and other species (39, 40). The possibility of adrenocortical CRH/CRH receptor signaling is further supported by our findings in male compound heterozygous mice. Although plasma ACTH concentrations did not significantly differ among the six genotypes with at least one copy of CRHR1, CORT concentrations clearly depended on the gene dose of CRHR1 and CRHR2.

In heterozygous CRHR1 mutant mice, the CORT response curve showed a later onset compared with those in wild-type controls and *Crhr1*$^{+/+}$*Crhr2*$^{-/-}$ mutants. This shift was independent of the gene dose of CRHR2. In addition, plasma CORT levels were significantly lower in *Crhr1*$^{+/-}$*Crhr2*$^{+/+}$ mutants compared with wild-type mice as well as in *Crhr1*$^{+/-}$*Crhr2*$^{-/-}$ mutants compared with *Crhr1*$^{+/+}$*Crhr2*$^{-/-}$ mutant animals after any of the stress conditions applied. In contrast, plasma CORT levels were significantly increased in CRHR2-deficient mice compared with animals having both copies of the CRHR2 gene despite having one or two copies of CRHR1. Additionally, CORT and ACTH response curves in *Crhr1*$^{+/-}$*Crhr2*$^{-/-}$ mutants confirm the initial description of the single CRHR2 knockout with a rapid increase, but early decline, of ACTH release in these mutants (19). In summary, our data suggest that CRH is likely to stimulate adrenocortical CORT release through CRHR1, whereas CRHR2 might mediate inhibitory CRH actions on adrenocortical function.

To further investigate whether CORT can be released through adrenal CRHR2, we compared the ACTH and CORT responses to a CRH challenge in wild-type, *Crhr1*$^{-/-}$ *Crhr2*$^{-/-}$, and *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutant mice. CRH did not exert any significant effect on circulating ACTH and CORT in *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutants. Thus, a direct effect of CRH via CRHR2 on adrenocortical steroidogenesis seems unlikely.

Most recently, a third novel CRHR has been cloned from catfish that is highly homologous to its mammalian counterparts. The novel receptor has been found to be highly expressed in pituitary and brain (41). However, the failure to activate the HPA system after CRH administration in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ mutant mice suggests that a third receptor binding CRH with high affinity is not likely to be involved in stress hormone release, at least not via direct effects on pituitary corticotropes or adrenocortical cells.

### *CRH signaling may play a role in adrenal gland development*

In our previous study (15) no significant difference in the size of the adrenal cortex could be observed between CRHR1-deficient mice and their wild-type littermates. Here, we report a similar decrease in size of the CORT-producing zona fasciculata in *Crhr1*$^{-/-}$*Crhr2*$^{-/-}$ and *Crhr1*$^{-/-}$*Crhr2*$^{+/+}$ mutants. Considering the well known influence of genetic background on the morphological phenotype of transgenic mice (for review, see Ref. 42), it is likely that the difference in adrenocortical size between our *Crhr1*$^{-/-}$ mouse line (129Ola/CD1 background) (15) and the single CRHR1-deficient progeny from our breeding of CRHR double mutants (mixed 129Ola/CD1–129SvJ/C57BL/6J background) is attributed to their different genetic background. Correspondingly, Smith *et al.* (16) observed significant adrenocortical atrophy in their CRHR1-deficient mouse line (129SvJ/C57BL/6J background) compared with wild-type animals. These researchers could restore normal gland morphology by ACTH replacement during early postnatal life, suggesting that a functional hypothalamic-pituitary system is required for normal development of the adrenal gland. According to this finding, one reason for the histopathological defect in the adrenal cortex in mice lacking functional CRHR1 might be a marked impairment of ACTH secretion during the neonatal period. Correspondingly, no adrenocortical atrophy could be detected in CRHR2-deficient mice (18, 19). On the other hand, there is evidence that intraadrenal CRH and ACTH exert trophic effects on adrenocortical cells. Their actions may be mediated via locally produced peptide growth factors, such as members of the fibroblast growth factor and IGF families (43). The loss of CRHR-dependent pathways might also lead to a severe impairment of normal adrenocortical growth and differentiation, suggesting an indirect involvement of the CRHR system in adrenal gland development.

In summary, we show that the endocrine phenotype of mice lacking both CRHR1 and CRHR2 is dominated by the functional loss of CRHR1. CRHR2 does not appear to compensate for CRHR1 deficiency, nor does the lack of CRHR2 synergistically exacerbate the CRHR1-dependent impairment of HPA system function. Pointing to a dual organization of the CRHR system, our results confirm the fundamental role of CRHR1 in initiating the stress-induced HPA response and the modulation of its recovery by CRHR2. Delineation of compensatory contributions of other neural stress systems, in particular maintenance of physical and vegetative homeostasis, will require a precise analysis of their components in mice with mutations in CRH signaling pathways. In addition, our data add to the complexity of adrenocortical function (44) and suggest different roles of CRHR1 and CRHR2 in fine-tuning adrenocortical CORT release.

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Address all correspondence and requests for reprints to: Wolfgang Wurst, Ph.D., Max Planck Institute of Psychiatry, Molecular Neurogenetics Kraepelinstrasse 2-10, 80804 Munich, Germany. E-mail: wurst@mpipsykl.mpg.de.

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* J.P. and M.B.M. contributed equally to this work.

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