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Selective Activation of the Hypothalamic Vasopressinergic System in Mice Deficient for the Corticotropin-Releasing Hormone Receptor 1 Is Dependent on Glucocorticoids*

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ABSTRACT

Deficiency of CRH receptor 1 (CRHR1) severely impairs the stress response of the hypothalamic-pituitary-adrenocortical (HPA) system and reduces anxiety-related behavior in mice. Intriguingly, in mice deficient for the CRHR1 (*Crhr1*$^{-/-}$), basal plasma levels of ACTH are normal, suggesting the presence of compensatory mechanisms for pituitary ACTH secretion. We therefore studied the impact of the hypothalamic neuropeptides arginine vasopressin (AVP) and oxytocin (OXT) on HPA system regulation in homozygous and heterozygous Crhr1 mutants under basal and different stress conditions. Basal plasma AVP concentrations were significantly elevated in *Crhr1*$^{-/-}$ mice. AVP messenger RNA expression was increased in the paraventricular nucleus of *Crhr1*$^{-/-}$ mutants together with a marked increase in AVP-like immunoreactivity in the median eminence. Administration of an AVP V_1-receptor antagonist significantly decreased basal plasma ACTH levels in mutant mice. After continuous treatment with corticosterone, plasma AVP levels in homozygous *Crhr1*$^{-/-}$ mice were indistinguishable from those in wild-type littermates, thus providing evidence that glucocorticoid deficiency is the major driving force behind compensatory activation of the vasopressinergic system in *Crhr1*$^{-/-}$ mice. Neither plasma OXT levels under several different conditions nor OXT messenger RNA expression in the paraventricular nucleus were different between the genotypes. Taken together, our data reveal a selective compensatory activation of the hypothalamic vasopressinergic, but not the oxytocinergic system, to maintain basal ACTH secretion and HPA system activity in *Crhr1*$^{-/-}$ mutants. (*Endocrinology* **141:** 4262–4269, 2000)

CRH IS THE primary hypothalamic hypophysiotropic factor regulating basal and stress-induced release of pituitary ACTH and mediating the mammalian stress response by activation of the hypothalamic-pituitary-adrenocortical (HPA) system (for review, see Ref. 1). In addition to its central role in coordinating and integrating the responses to a variety of physiological and stress-associated stimuli, recent clinical data implicate CRH in the etiology and pathophysiology of several psychiatric (*e.g.* major depression and anorexia nervosa), endocrine (*e.g.* Cushing's disease) and inflammatory disorders (*e.g.* rheumatoid arthritis) (for review, see Refs. 2 and 3). The biological actions of CRH are mediated by specific, high affinity, G protein-coupled seven *trans*-membrane domain receptors. To date, two distinct receptor subtypes have been characterized [CRH receptor 1 (CRHR1) and CRH receptor 2 (CRHR2)]. CRHR1 and CRHR2 display a markedly different tissue distribution and pharmacological specificity (for review, see Ref. 4). CRHR1 has been proposed to mediate the effects of CRH on HPA system function and anxiety-related behavior (5–8), whereas CRHR2 might be predominantly involved in the regulation of feeding behavior (9).

Recently, mice deficient for the CRHR1 (*Crhr1*$^{-/-}$) have been created (10, 11), and their phenotype confirms the obligatory role of CRHR1 in both the stress-associated response of the HPA system and anxiety; in particular, *Crhr1*$^{-/-}$ mutants display a severe impairment of stress-induced HPA system activation and marked glucocorticoid deficiency and exhibit significantly reduced anxiety-related behavior. However, despite the lack of functional CRHR1 on pituitary corticotropes, basal plasma ACTH concentrations in homozygous *Crhr1*$^{-/-}$ mutants are similar to those found in wild-type controls (10), suggesting that basal ACTH secretion is regulated or compensated for by signaling pathways other than CRH/CRHR1.

The neuropeptides arginine vasopressin (AVP) and oxytocin (OXT) are mainly synthesized in the magnocellular neurons of the hypothalamic paraventricular (PVN) and supraoptic nuclei that project to the posterior pituitary. In addition, parvocellular neurons of the PVN coexpressing AVP and CRH coordinate HPA system activity and project to the external layer of the median eminence, where AVP and CRH are released into the portal blood (for review, see Refs. 12 and 13). Numerous investigations have consistently shown that AVP potently syn-

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ergizes with CRH to stimulate pituitary ACTH release *in vitro* and *in vivo* (for review, see Ref. 12). A recent study provided indirect evidence that vasopressin might be activated to maintain HPA system activity in *Crhr1*$^{-/-}$ mutants (14). OXT, although less effectively than AVP, has also been shown to markedly potentiate CRH-induced ACTH release from pituitary corticotropes in rodents (15, 16).

Although the ACTH secretagogue potency of both AVP and OXT has been extensively described, little information is available about 1) whether one or both neuropeptides may compensate for disrupted CRH/CRHR1 signaling in mice congenitally deficient for the CRHR1, and 2) whether such a compensatory role of AVP and OXT, having been characterized mainly in the rat (17–20), is also relevant in mice. Therefore, we investigated the roles of AVP and OXT in compensatorily maintaining HPA system activity in the absence of functional CRHR1, both under basal conditions and in response to ethologically relevant stressors.

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.

Homozygous and heterozygous transgenic mice deficient for the CRHR1 and wild-type controls were generated as originally described by Timpl *et al.* (10). The present experiments were performed with 8- to 12-week-old male mice, weighing 25–35 g. No significant difference in body weight was observed between homozygous *Crhr1*$^{-/-}$ mutants and heterozygous and age-matched wild-type animals. In addition, homozygous *Crhr1*$^{-/-}$ mutants do not show any significant difference in their total amount of water and food intake (21). Measurement of plasma sodium concentrations revealed no significant difference between male wild-type mice and homozygous *Crhr1*$^{-/-}$ mutants (wild-type mice, 157 ± 4.7 mmol/liter; homozygous *Crhr1*$^{-/-}$ mutants, 161 ± 5 mmol/liter; n = 8). 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 0600–1800 h; 22 ± 1 C; 40–60% humidity) and received standard pelleted food and water *ad libitum*.

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 AVP, OXT, ACTH, CORT, and sodium, mice (n = 6–9/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 sec. Blood was sampled into prechilled EDTA tubes.

Poststress levels of hormones.

Forced swim test. Animals were subjected to the forced swim stress as a predominantly physical stressor. On the day of testing, between 0800–1200 h, each mouse (n = 6–8/genotype) was placed for 5 min in a glass beaker filled with tap water (21 C; diameter, 12.5 cm; height, 14 cm). Subsequently, the animals were returned to their homecages, and blood collection was performed by retroorbital bleeding 5 min after the end of stress exposure.

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 homecage. Usually, within three tests the resident reliably attacked the intruder within less than 2 min (adapted with modifications from Ref. 22). Social defeat stress consisted of introducing a naive, singly housed, experimental mouse (intruder; male homozygous *Crhr1*$^{-/-}$, heterozygous mutants, and wild-type mice; n = 6–8/group) into the resident's homecage. 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 homecage. 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 5 min. Subsequently, the experimental mouse was returned to the homecage, and blood collection was performed by retroorbital bleeding 5 min after the end of the stressor.

Administration of an AVP receptor (V_1) antagonist

After 1 week of daily handling, male homozygous and heterozygous *Crhr1*$^{-/-}$ mutants and wild-type littermates (8–12 weeks; n = 6/group) were sc injected with either vehicle or 5 μg of a selective V_1 receptor antagonist (dP[Tyr(Me)$_2$]AVP, provided by Dr. M. Manning, Toledo, OH) (23, 24) dissolved in 50 μl 0.9% saline (injection time, 0800–0830 h). One hour after the injection, the animals were killed by rapid decapitation, and trunk blood was collected for determination of plasma ACTH levels.

Measurement of plasma AVP after corticosterone (CORT) treatment

Male homozygous *Crhr1*$^{-/-}$ mutants and wild-type littermates (8–12 weeks; n = 8/group) were housed singly and received standard pelleted food and water *ad libitum*.

Oral administration of CORT was performed by adding water-soluble CORT 21-sulfate (Sigma, Deisenhofen, Germany; 13.5 mg/liter) to the drinking water. After the animals had been given CORT 21-sulfate continuously for 2 weeks, they were left undisturbed throughout the night before the experiment. Blood collection was performed between 0700–0800 h by rapid retroorbital bleeding, and blood samples for measurements of plasma AVP and CORT concentrations were collected.

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 and −20 C until assay. Plasma ACTH (50 μl) and CORT (10 μl) levels were measured using commercially available kits (Biochem, Freiburg, Germany) according to the respective protocols. AVP and OXT contents were measured in lyophilized plasma samples after extraction by highly sensitive and selective RIAs [detection limit, 0.1 pg/sample; cross-reactivity of the antisera with other related peptides, including AVP (for the anti-OXT antiserum) and OXT (in case of the anti-AVP antiserum, respectively), <0.7%] (25). Synthetic AVP and OXT (Ferring Pharmaceuticals Ltd., Malmo, Sweden) were used as standards, and iodinated nonapeptide (SA, 2,200 Ci/mmol; NEN Life Science Products, Boston, MA) was used as a tracer. Rabbit antibodies raised in our laboratory were used at a dilution of 1:350,000.

AVP and OXT in situ hybridization histochemistry

Animals (n = 5) were killed with an overdose of halothane. Thereafter, brains were quickly removed. Tissue was frozen on dry ice and stored at −80 C. For subsequent *in situ* hybridization experiments, brains (n = 5/genotype) were sectioned in a cryostat. All brains were cut in five parallel series (20 μm, coronal sections, spanning the region of the PVN from bregma −0.70 mm to bregma −1.22 mm), with every fifth section being thaw-mounted on the same glass slide. Sections were stored at −20 C until use.

The following oligonucleotide DNA probes were used for *in situ* hybridization: AVP (48-mer), 5′-GCA GAA GGC CCC GGC CGG CCC GTC CAG CTG CGT GGC GTT GCT CCG GTC-3′ (directed against the last 16 amino acids of the glycoprotein that are not shared with oxytocin) (26); and oxytocin (48-mer), 5′-CTC GGA GAA GGC AGA CTC AGG GTC GCA GGC GGG GTC GGT GCG GCA GCC (directed against nucleotides 890–937 of the rat oxytocin sequence) (26). The specificity of these oligonucleotides has been described in detail previously (27).

All *in situ* hybridization experiments were carried out as previously described in detail (28), and for each oligonucleotide probe all sections

were run in the same experiment under identical conditions. Briefly, the synthesized oligonucleotides were labeled at the 3′-end with [α-^{35}S]deoxy-ATP (NEN Life Science Products-DuPont, Boston, MA) using terminal dexoynucleotidyl transferase (Roche, Mannheim, Germany). Radiolabeled probe (10^6 cpm/200 μl/slide) was diluted into hybridization buffer consisting of 1 × Denhardt's solution, 0.25 mg/ml yeast transfer RNA (Sigma), 0.5 mg/ml salmon sperm DNA (Sigma), 10% dextran sulfate, 10 mM dithiothreitol, and 50% formamide; applied to the slides; and incubated for 20 h at 42 C. After hybridization, the slides were washed in 1 × SSC (55 C) four times for 15 min each time, dehydrated in ethanol, and air-dried. Finally, the slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 in distilled water, exposed for 5 weeks at 4 C, and then developed in Kodak D19 solution. The developed slides were lightly counterstained with cresyl violet and examined using a Leica Corp. microscope (Bensheim, Germany) with both bright- and darkfield condensers.

Immunohistochemistry

Animals (n = 4/genotype) were deeply anesthetized with phenobarbital and transcardially perfused with phosphate-buffered 4% paraformaldehyde. Brains were removed from the skull, postfixed for 6 h in 4% paraformaldehyde, and then transferred to 15% sucrose in PBS (pH 7.4). Serial 30-μm coronal frozen sections were cut in a cryostat into PBS and processed as free floating sections. For each animal, all sections spanning the region of the median eminence (between bregma −1.58 mm and bregma −2.30 mm) were analyzed to allow for exact comparison 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, preincubation with 5% normal goat serum for 2 h was 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) at 4 C. The specificity of this antibody has been tested by the manufacturer. The sections were then incubated with a biotinylated goat antirabbit 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 (dilution, 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), washed, mounted on glass slides, air-dried, and lightly counterstained with hematoxylin. Appropriate negative controls were performed by omission of the primary antibody.

Optical densitometry

Representative areas of the hypothalamic PVN were scanned by a digital camera under dark-light conditions. Care was taken to scan all images under identical light conditions. Quantitative analysis of messenger RNA (mRNA) expression was performed blind to the animal's genotype as previously described (28), using the Macintosh-based public domain image analysis program NIH image, version 1.6.1 (developed at the NIH and available on the internet at http://rsb.info.nih.gov./nih-image). At least three parallel tissue sections per animal and region were analyzed, and the mean values for each animal and region were calculated.

AVP mRNA. Levels of mRNA expression (optical density) were determined by measuring the mean gray value on inverted (*in situ* hybridization signal, black), automatically thresholded images in the PVN, analyzing the left and right hemispheres separately. The region of interest was outlined according to adjacent sections that had been stained with cresyl violet to determine orientation and the precise anatomical localization and borders of the nuclei. As the three-dimensional structure of these hypothalamic nuclei varies from rostral to caudal, at least three parallel sections per animal and region were analyzed, spanning the section containing the highest signal intensity.

OXT mRNA. As neurons expressing OXT mRNA are scattered throughout the PVN, quantitative analysis of OXT mRNA expression was performed as 1) an automatic count of labeled objects (=cells) on thresholded and binary images, and 2) an automatic measurement of the optical density over each object (cell) automatically outlined, representing the density of silver grains per labeled cell. The means for both the number of labeled cells and the optical density per cell were calculated for each animal.

Statistical analysis

Results are presented as the mean ± SEM. Statistical analysis was performed with a software package (GB Stat, version 6.5 PPC, Dynamic Microsystems, Inc., Silver Spring, MD). Statistical significance of differences between groups was determined by ANOVA (one-way ANOVA, factor genotype, or two-way ANOVA, factors genotype and treatment, where appropriate), followed by *post-hoc* Newman-Keuls test. A significant difference in plasma sodium concentration was determined by Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Results

Basal plasma AVP levels are significantly increased in homozygous Crhr1$^{-/-}$ mice

Two-way ANOVA revealed a significant effect of both genotype ($P < 0.0001$) and treatment condition ($P = 0.0086$) with a significant interaction of both factors ($P < 0.0001$; Fig. 1). *Post-hoc* analysis revealed significantly higher basal morning plasma AVP levels in *Crhr1*$^{-/-}$ mice compared with wild-type mice ($P < 0.01$) and heterozygous mutants ($P < 0.01$). After forced swim stress or social defeat, plasma AVP in homozygous *Crhr1*$^{-/-}$mice fell to levels indistinguishable from those in heterozygous mutants or wild-type controls. Significant differences in poststress plasma AVP levels compared with basal morning plasma AVP concentrations were detected in homozygous *Crhr1*$^{-/-}$ mice only (forced swim stress, $P < 0.01$; social defeat, $P < 0.01$).

Plasma OXT levels do not differ significantly among the genotypes

Two-way ANOVA revealed a significant effect of treatment condition ($P < 0.0001$; Fig. 1). Basal morning plasma oxytocin levels did not differ significantly among the three genotypes. In all three groups and in both stress paradigms, poststress plasma OXT levels were significantly increased compared with the respective basal morning levels (forced swim: wild-type, $P < 0.05$; heterozygous mutants, $P < 0.01$; homozygous *Crhr1*$^{-/-}$ mutants, $P < 0.01$; social defeat: wild-type, $P < 0.01$; heterozygous mutants, $P < 0.01$; homozygous *Crhr1*$^{-/-}$ mutants, $P < 0.01$). In wild-type mice only, plasma OXT levels after social defeat were significantly higher than the respective plasma concentrations after forced swim stress ($P < 0.01$).

The stress-induced increase in plasma ACTH and CORT levels is severely impaired in homozygous Crhr1$^{-/-}$ mutants: differential effects of emotional vs. combined physical and emotional stressor on ACTH release

Plasma ACTH levels. Two-way ANOVA revealed a significant effect of both genotype (P = 0.01) and treatment condition ($P < 0.0001$), with a significant interaction between both factors ($P < 0.0001$; Fig. 1). Under basal conditions, no significant differences in plasma ACTH levels were observed. Both forced swim stress and social defeat induced a significant increase in plasma ACTH in wild-type mice compared with basal levels (forced swim, $P < 0.01$; social defeat, $P < 0.01$). In heterozygous mutants and homozygous *Crhr1*$^{-/-}$ mutants, a significant increase in plasma ACTH was ob-

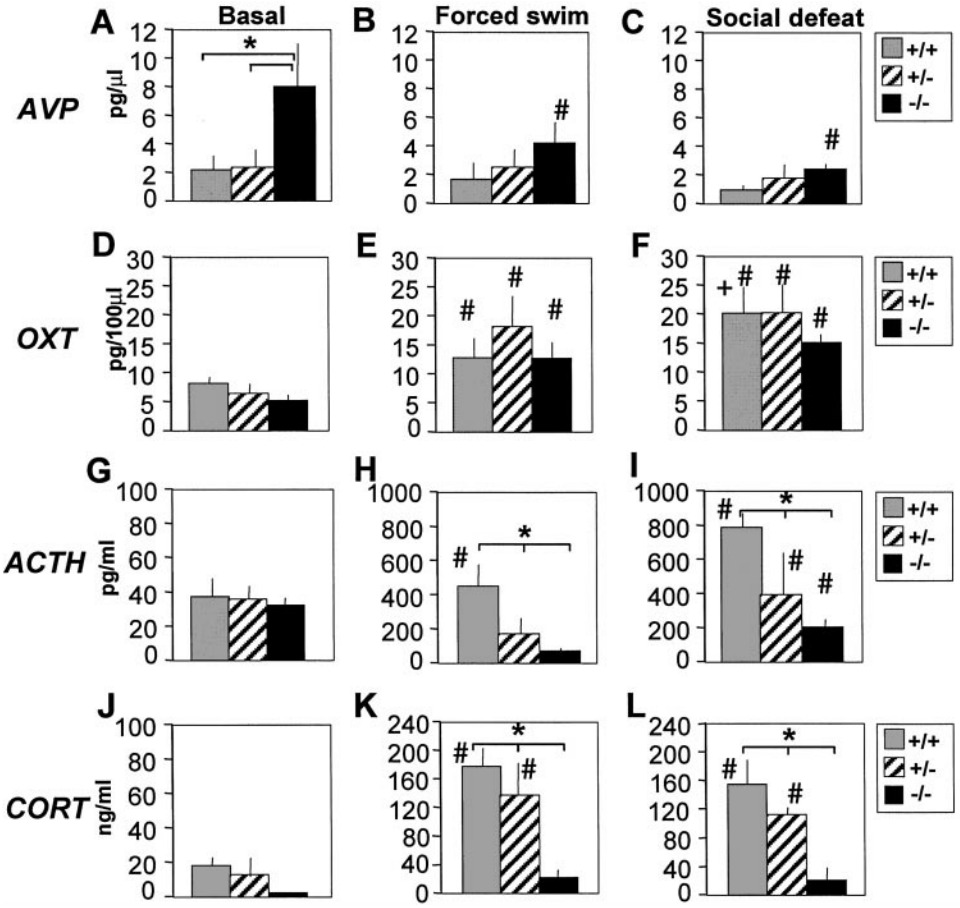

FIG. 1. Plasma AVP, OXT, ACTH, and CORT concentrations in homozygous *Crhr1*$^{-/-}$ mutants (−/−), heterozygous mutants (+/−), and wild-type littermates (+/+) under basal conditions (A, D, G, and J) and after forced swim stress (B, E, H, and K) and social defeat (C, F, I, and L). Data are expressed as the mean ± SEM (n = 6–9). *, Statistically significant differences between the genotypes; #, significant effect of the experimental condition *vs.* basal levels within the same genotype; +, significant effect between the different types of stress within the same genotype ($P < 0.05$). Note different scales between basal and stress-induced plasma ACTH and CORT concentrations.

served after social defeat only (heterozygous mutants, $P < 0.01$; homozygous *Crhr1*$^{-/-}$ mutants, $P < 0.05$).

Plasma CORT levels. Two-way ANOVA revealed a significant effect of both genotype ($P < 0.0001$) and treatment condition ($P < 0.0001$) as well as a significant interaction of both factors ($P < 0.0001$). No significant differences between the groups could be detected in basal morning plasma CORT levels; however, basal morning levels of CORT in homozygous *Crhr1*$^{-/-}$ mutants were consistently below the assay detection limit. Both heterozygous mutants and wild-type mice showed a significant increase in plasma CORT levels after forced swim stress (heterozygous mutants, $P < 0.01$; wild-type mice, $P < 0.01$; Fig. 1) and social defeat stress (heterozygous mutants, $P < 0.01$; wild-type mice, $P < 0.01$; Fig. 1). In homozygous *Crhr1*$^{-/-}$ mutants, in contrast, plasma CORT concentrations did not increase significantly after any of the stress conditions. No differential effects of the different stressors on plasma CORT levels were observed in either wild-type or heterozygous mice.

Plasma ACTH concentrations decrease after administration of a V_1 receptor antagonist in homozygous Crhr1$^{-/-}$ *mutants*

Administration of a selective V_1 receptor antagonist under resting conditions significantly decreased plasma ACTH levels in homozygous *Crhr1*$^{-/-}$ mice compared with those in both vehicle-treated mutants ($P < 0.01$) and antagonist-treated wild-type mice ($P < 0.01$; Fig. 2). There was no statistical difference between the two vehicle-treated groups. The V_1 receptor antagonist did not exert any significant effect on plasma ACTH levels in the wild-type controls.

Increased expression of AVP mRNA in the PVN of homozygous Crhr1$^{-/-}$ *mutants*

One-way ANOVA with *post-hoc* Newman-Keuls tests revealed that AVP mRNA expression in the hypothalamic PVN was significantly increased in homozygous *Crhr1*$^{-/-}$ mutants compared with wild-type controls ($P < 0.05$). No significant differences could be observed between either wild-type mice and heterozygous mutants or heterozygous mutants and homozygous *Crhr1*$^{-/-}$ mutants (Figs. 2 and 3).

Increased AVP-like immunoreactivity in the median eminence in homozygous Crhr1$^{-/-}$ *mutants*

Light microscopic analysis revealed a marked increase in AVP-like immunoreactivity in both the zona interna and the zona externa of the median eminence in homozygous *Crhr1*$^{-/-}$ mutants (Fig. 3) compared with wild-type littermates. No obvious difference was found between heterozygous mutants and wild-type controls (data not shown).

In homozygous *Crhr1*$^{-/-}$ mice, the zona externa displayed many prominent and strongly immunoreactive axons, whereas in wild-type mice, only weakly immunoreactive structures were present in the zona externa. No obvious difference in the number of AVP-immunoreactive neurons in the PVN could be detected between the different genotypes,

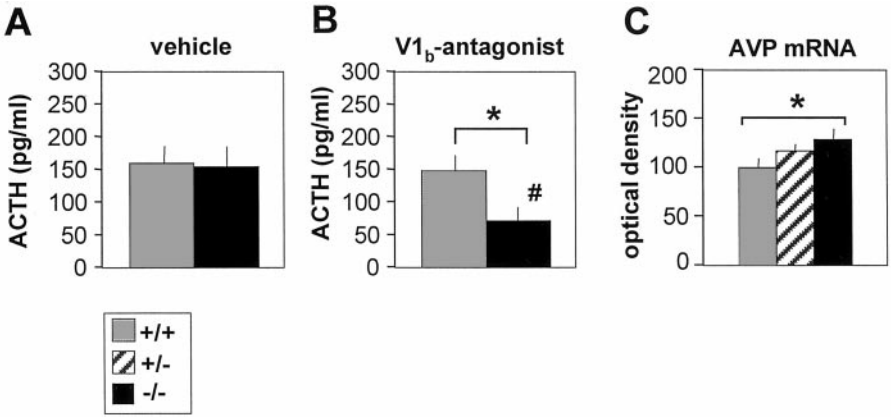

FIG. 2. Effect of a selective V_1 receptor antagonist on basal plasma ACTH levels in homozygous *Crhr1*$^{-/-}$ mutants (−/−) and wild-type mice (+/+). Groups (n = 6) were sc injected with vehicle (A) or 5 μg of the V_1 receptor antagonist (B) and were decapitated 1 h later. Data are expressed as the mean ± SEM. *, Statistically significant differences between the genotypes; #, significant effect of the V_1 receptor antagonist *vs.* vehicle within the same genotype. The expression of AVP mRNA in the PVN is significantly increased in homozygous *Crhr1*$^{-/-}$ mutants (−/−) compared with wild-type controls (+/+; C; n = 5).

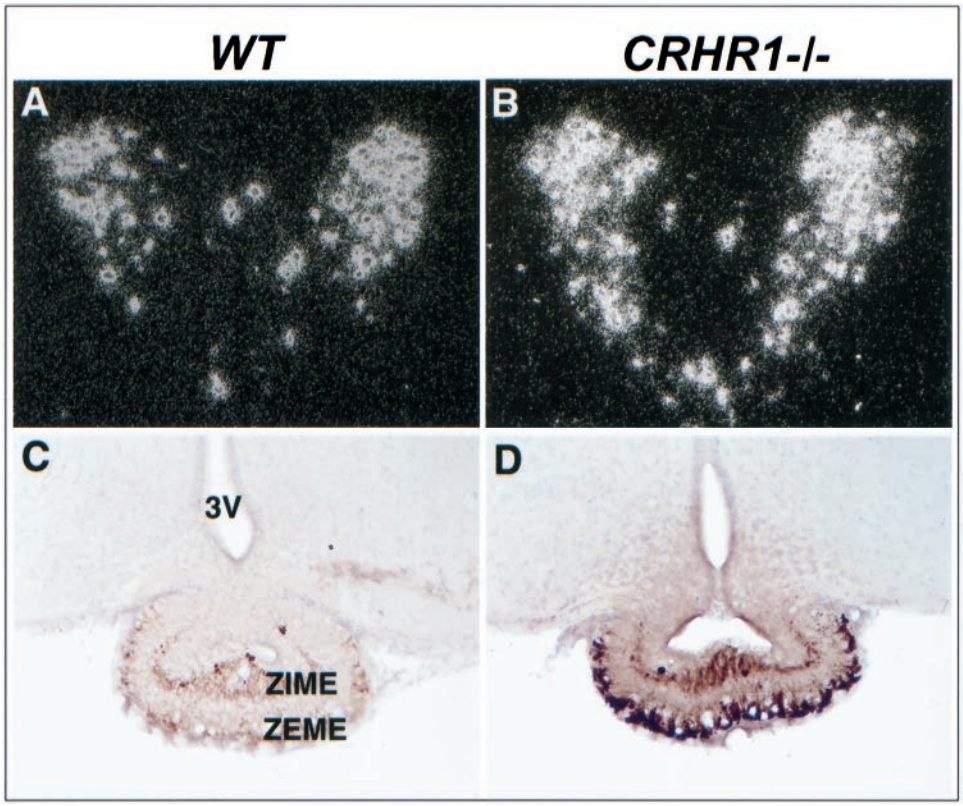

FIG. 3. Darkfield photomicrographs showing a significantly increased expression of AVP mRNA in the PVN of homozygous *Crhr1*$^{-/-}$ mutants (B) compared with wild-type mice (A). Immunostaining with an antibody against AVP revealed a marked increase in AVP-like immunoreactivity in the median eminence of homozygous *Crhr1*$^{-/-}$ mutants (D) compared to that in wild-type controls (C). Note the strong increase in AVP-like immunoreactivity in the external zone of the median eminence (ZEME) in homozygous *Crhr1*$^{-/-}$ mutants. ZIME, Internal zone of the median eminence; 3V, third ventricle.

nor was there any clear difference in the distribution pattern or projection sites of paraventricular neurons in homozygous *Crhr1*$^{-/-}$ mutants (data not shown).

Administration of CORT normalizes basal plasma AVP levels in homozygous Crhr1$^{-/-}$ *mutants*

After continuous oral treatment with CORT 21-sulfate for 2 weeks, basal plasma CORT levels were indistinguishable between homozygous *Crhr1*$^{-/-}$ mutants and wild-type controls (wild-type mice, 22.3 ± 6.3 ng/ml; homozygous *Crhr1*$^{-/-}$ mutants, 18.7 ± 4.1 ng/ml; Fig. 4). Concomitantly, basal plasma AVP concentrations decreased to levels indistinguishable from those in control mice (wild-type mice, 2.5 ± 1.3 pg/μl; homozygous *Crhr1*$^{-/-}$ mutants, 2.1 ± 0.6 pg/μl; Fig. 4).

No differences in the expression of OXT mRNA in the PVN

No significant differences in either the number of oxytocin mRNA-expressing neurons in the paraventricular nucleus (wild-type mice, 66.7 ± 9.6; heterozygous mutants, 62.9 ± 4.1; homozygous *Crhr1*$^{-/-}$ mutants, 62.2 ± 6.7) or the mean optical density per cell (wild-type mice, 134.5 ± 7.1; het-

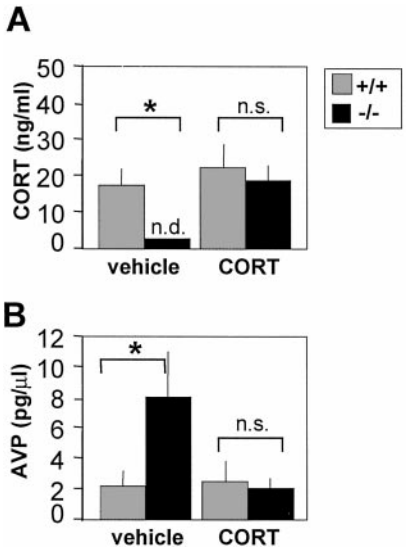

FIG. 4. Effects of oral administration of CORT 21-sulfate on basal plasma CORT (A) and AVP (B) levels in homozygous *Crhr1*$^{-/-}$ mutants and wild-type littermates [data for vehicle treatment (=water) are derived from results shown in Fig. 1, A and J]. Under vehicle conditions, plasma CORT levels were below the detection limit of the assay in homozygous *Crhr1*$^{-/-}$ mutants (detection limit, 2.7 ng/ml). After 2 weeks of continuous CORT 21-sulfate administration (CORT), plasma CORT levels were indistinguishable between homozygous *Crhr1*$^{-/-}$ mutants and wild-type littermates (A). Concomitantly, plasma AVP levels decreased to wild-type levels in CORT-treated homozygous *Crhr1*$^{-/-}$ mutants (B). *, Statistically significant differences between the genotypes. n.d., Nondetectable; n.s., nonsignificant.

erozygous mutants, 126.1 ± 6.1; homozygous *Crhr1*$^{-/-}$ mutants, 131.2 ± 2.2) could be detected between the different genotypes (one-way ANOVA; number of cells/factor genotype, $P = 0.72$; mean optical density/factor genotype, $P = 0.3$).

Discussion

The present study reveals a selective activation of the hypothalamic vasopressinergic system in homozygous *Crhr1*$^{-/-}$ mutants. Significantly elevated levels of hypothalamic AVP mRNA together with a marked increase in AVP-like immunoreactivity in both the zona interna and the zona externa of the median eminence and significantly elevated basal plasma AVP concentrations strongly suggest that AVP plays a key role in compensatorily stimulating pituitary ACTH secretion in homozygous *Crhr1*$^{-/-}$ mice. Further, by administration of a selective V_1 antagonist we could provide evidence that this activation of the vasopressinergic system is, indeed, functionally relevant. In addition, we could show that hypothalamic AVP overexpression in *Crhr1*$^{-/-}$ mutants is most likely dependent on glucocorticoid deficiency.

Selective activation of the vasopressinergic system maintains basal HPA system activity in the absence of CRHR1

Since the discovery of CRH by Vale *et al.* (29), it was rapidly established that AVP potently synergizes with CRH to stimulate pituitary ACTH release; when CRH and AVP are given together, hormone output is well above the added effects of the two peptides alone in both rodents and humans (30, 31). This CRH/AVP synergism is known to be functionally relevant under both physiological (19) and pathophysiological conditions, such as stress (17, 20) or glucocorticoid deficiency (18, 32). AVP is predominantly synthesized in magno- and parvocellular neurons of the hypothalamic supraoptic nucleus and PVN. Distinct neuronal entities and anatomical subnuclei have been studied in detail in the rat hypothalamus (33); however, only sparse information is available for the mouse to date. In addition, considerable species differences in hypothalamic AVP expression have been described in rodents (34). Compared with the rat, the mouse vasopressinergic system has been found to be strikingly more profuse (35), and AVP/CRH colocalization studies revealed that all parvocellular CRH-positive nerve endings are also immunoreactive for AVP (34). Evidence that AVP derived not only from the parvocellular part of the PVN, but also from the magnocellular neurosecretory system released *en passant* from axons within the internal zone of the median eminence influences pituitary ACTH release has become considerably strong (12). Recently, Turnbull and colleagues provided indirect evidence that vasopressin might be compensatorily activated to maintain HPA system activity in *Crhr1*$^{-/-}$ mutants (14); mice were injected with a polyclonal antiserum against AVP before measurements of plasma ACTH. However, because of the structural homology between the two neuropeptides, AVP and OXT, a polyclonal antiserum raised against AVP is likely to cross-react with OXT. Therefore, this experiment cannot sort out whether only AVP or both neuropeptides might be compensatorily activated in *Crhr1*$^{-/-}$ mutants.

Several studies have shown that immunohistochemical detection of AVP in the median eminence may be considered a reliable indicator of hypothalamic vasopressinergic activity, allowing for a distinction between predominantly parvocellular (axons passing through the external zone of the median eminence) and magnocellular (axons passing through the internal zone of the median eminence) contributions to the hypothalamic vasopressinergic system (36, 37). The strong increase in AVP-like immunoreactivity in homozygous *Crhr1*$^{-/-}$ mutants in both the external and internal zones of the median eminence is in complete accordance with our observation that AVP mRNA is significantly increased in the PVN. No significant difference in plasma sodium concentration could be observed between homozygous *Crhr1*$^{-/-}$ mice and wild-type littermates. It is, therefore, most likely that in *Crhr1*$^{-/-}$ mutants chronically exposed to increased plasma vasopressin levels, a marked desensitization of renal vasopressin V_2 receptors occurs to maintain water and electrolyte homeostasis.

To test the hypothesis that the observed activation of the vasopressinergic system is functionally relevant, we injected the mice with a selective V_1 receptor antagonist. In *Crhr1*$^{-/-}$ mutants, the plasma ACTH concentration decreased significantly after administration of the V_1 receptor antagonist, providing evidence that activation of the vasopressinergic system is critically involved in maintaining basal pituitary ACTH release in the absence of functional CRHR1. However, it is likely that additional factors besides AVP might be

activated to maintain HPA system activity in *Crhr1*$^{-/-}$ mutants, and studies to identify other potential ACTH secretagogues in *Crhr1*$^{-/-}$ mutants are currently underway.

In the present investigation, stress-induced plasma AVP levels were lower than baseline values, confirming previous results in rats, in which AVP release into the blood has been described to be unaffected or even suppressed in response to emotional stressors (38, 39).

Glucocorticoid deficiency is the major driving force behind activation of the vasopressinergic system in Crhr1$^{-/-}$ *mutants*

It is well known that AVP expression is markedly increased under conditions of glucocorticoid deficiency, such as adrenalectomy (18, 32, 40). Accordingly, very low plasma CORT levels in *Crhr1*$^{-/-}$ mutants (10), leading to reduced negative feedback on the activity of neurosecretory neurons, may be considered an additional and potent stimulus for increased hypothalamic AVP expression. To investigate whether glucocorticoid deficiency causally contributes to hypothalamic vasopressinergic stimulation in homozygous *Crhr1*$^{-/-}$ mutants, we treated homozygous *Crhr1*$^{-/-}$ mutants with CORT 21-sulfate. Mutant mice reached basal plasma CORT levels indistinguishable from those of wild-type littermates after continuous administration of CORT 21-sulfate added to the drinking water (13.5 mg/liter). Concomitantly, basal plasma AVP concentrations decreased to wild-type levels in CORT-treated homozygous *Crhr1*$^{-/-}$ mutants. We can therefore conclude that glucocorticoid deficiency is the major driving force behind the selective activation of the hypothalamic vasopressinergic system.

The oxytocinergic pathway is not activated in Crhr1$^{-/-}$ *mutants*

OXT, although less effectively than AVP, has also been shown to potentiate CRH-induced ACTH release from pituitary corticotropes (15, 16) and was therefore considered a second candidate to maintain HPA system activity in homozygous *Crhr1*$^{-/-}$ mutants. In contrast to AVP, basal as well as increased poststress plasma OXT levels were indistinguishable among wild-type mice and heterozygous and homozygous mutants. Accordingly, no differences in either the number of OXT mRNA-expressing cells in the PVN or the mean optical density per cell could be observed among the genotypes. Our data therefore suggest that despite their close structural, functional, and anatomical relationship, AVP and OXT are distinctly regulated and independently involved in different mechanisms of HPA system regulation and activity.

The stress-induced increase in plasma ACTH and CORT levels is severely impaired in homozygous Crhr1$^{-/-}$ *mutants: differential effects of social defeat vs. forced swim stress on ACTH release*

Forced swim stress, a combined physical and emotional stressor, was previously shown not to elicit a significant increase in plasma ACTH and CORT levels in homozygous *Crhr1*$^{-/-}$ mutants (10). However, it is well known that different types of stressors (*e.g.* predominantly physical, emotional, and combined physical-emotional stressors) are accompanied by specific patterns of neuroendocrine activation. Most recently, a widespread distribution of CRHR1 in the mouse brain and, in particular, in autonomic pathways of the central nervous system has been described (41). CRHR1 deficiency may therefore lead to significant effects not only on the neuroendocrine system, but also on the sympathetic nervous system and autonomic regulation. Social defeat by a male conspecific can be considered one of the most severe stressors among a number of laboratory stressful stimuli in terms of neuroendocrine activation with respect to both the magnitude and the quality of the stress response (for review, see Ref. 42). Moreover, the stress response by social defeat in particular is characterized by a strong sympathetic dominance, with both plasma epinephrine and norepinephrine being significantly increased (43). Therefore, we subjected *Crhr1*$^{-/-}$ mutants to social defeat stress to examine whether this severe emotional stressor might lead, in contrast to the forced swim stress paradigm, to a significant activation of the HPA system. Indeed, social defeat induced a significant increase in plasma ACTH in homozygous *Crhr1*$^{-/-}$ mutants, whereas plasma ACTH levels after forced swim stress remained indistinguishable from basal levels, herewith confirming our previous findings (10). The differential effects of these two stressors on pituitary ACTH release in homozygous *Crhr1*$^{-/-}$ mutants might be explained by the strong sympathetic activation accompanying the social defeat stress paradigm. Adrenergic receptors have been localized on pituitary corticotrophs (44), and a stimulatory effect of central catecholamines on pituitary ACTH release has been shown both *in vitro* and *in vivo* (45; for review, see Ref. 46).

Taken together, we could demonstrate a selective activation of the hypothalamic vasopressinergic, but not the oxytocinergic, system and its critical involvement in the maintenance of basal HPA system activity in homozygous *Crhr1*$^{-/-}$ mutants. Heterozygous mutants, in contrast, are normal (that is, indistinguishable from their wild-type littermates) with respect to plasma AVP levels, hypothalamic AVP expression, and lack of responsiveness to the administration of a V_1 receptor antagonist. Our data add to the complexity of HPA system regulation and stress hormone homeostasis. In addition, these findings are of particular importance for the question of potential side-effects of a long-term administration of selective CRHR1 receptor antagonists, the potential antidepressant and anxiolytic properties of which are currently under investigation in clinical trials (3, 47).

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