Plasma Transcortin Influences Endocrine and Behavioral Stress Responses in Mice

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Glucocorticoids are released after hypothalamus-pituitary-adrenal axis stimulation by stress and act both in the periphery and in the brain to bring about adaptive responses that are essential for life. Dysregulation of the stress response can precipitate psychiatric diseases, in particular depression. Recent genetic studies have suggested that the glucocorticoid carrier transcortin, also called corticosteroid-binding globulin (CBG), may have an important role in stress response. We have investigated the effect of partial or total transcortin deficiency using transcortin knockout mice on hypothalamus-pituitary-adrenal axis functioning and regulation as well as on behaviors linked to anxiety and depression traits in animals. We show that CBG deficiency in mice results in markedly reduced total circulating corticosterone at rest and in response to stress. Interestingly, free corticosterone concentrations are normal at rest but present a reduced surge after stress in transcortin-deficient mice. No differences were detected between transcortin-deficient mice for anxiety-related traits. However, transcortin-deficient mice display increased immobility in the forced-swimming test and markedly enhanced learned helplessness after prolonged uncontrollable stress. The latter is associated with an approximately 30% decrease in circulating levels of free corticosterone as well as reduced Egr-1 mRNA expression in hippocampus in CBG-deficient mice. Additionally, transcortin-deficient mice show no sensitization to cocaine-induced locomotor responses, a well described corticosterone-dependent test. Thus, transcortin deficiency leads to insufficient glucocorticoid signaling and altered behavioral responses after stress. These findings uncover the critical role of plasma transcortin in providing an adequate endocrine and behavioral response to stress. (*Endocrinology* 151: 649–659, 2010)

Adaptive responses to stress are essential for life and involve activation of a complex repertoire of autonomic, neuroendocrine, and behavioral responses, originating both from the brain and the periphery, that work in concert to reinstate homeostasis. If these adaptive systems are overactive or fail to respond appropriately, psychiatric diseases, such as affective disorders, cognitive impairment, or vulnerability to drug addiction, may develop in vulnerable individuals (1–4). Important individual differences are observed in the ability of an individual to cope with stressful events. This variability depends on genetic, environmental (in particular during the perinatal period), and epigenetic factors (5, 6).

A major system involved in adaptive stress responses is the hypothalamus-pituitary-adrenal (HPA) axis (3, 7). Under normal physiological conditions, glucocorticoid (cortisol in humans, corticosterone in laboratory rodents) secretion follows a circadian rhythm entrained by light and food intake that stimulate the secretion of the hypothalamic peptide corticotrophin-releasing hormone

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Abbreviations: Bmax, CBG maximal binding capacity; CBG, corticosteroid-binding globulin; CRH, corticotrophin-releasing hormone; GR, glucocorticoid receptor; HPA, hypothalamus-pituitary-adrenal; MR, mineralocorticoid receptor.

(CRH), which, in turn, triggers pituitary secretion of ACTH that acts on adrenal glands to stimulate glucocorticoid release in the blood. In plasma, glucocorticoids bind with a high affinity but low capacity to transcortin, also called corticosteroid-binding globulin (CBG), and to albumin with a high capacity but low affinity. The free fraction of circulating glucocorticoids thus constitutes around 5% of the total glucocorticoid pool. Free glucocorticoids regulate negatively their own secretion by inhibiting CRH and ACTH release, and act on target tissues by binding to two nuclear receptors, the mineralocorticoid (MR) and the glucocorticoid receptors (GRs). Under stressful conditions, the rise in CRH levels results in transiently increased glucocorticoid secretion. The free fraction of circulating glucocorticoids is then more than proportionally increased because stress-induced glucocorticoid secretion overshoots transcortin binding capacity (8).

We have shown by genetic linkage analysis in pig models that the variability in stress-induced glucocorticoid levels depends strongly on a genomic locus containing the Cbg gene encoding transcortin (9). On further investigations, we have accumulated data favoring the hypothesis that Cbg gene polymorphism is indeed responsible for variability in stress-induced glucocorticoid levels in our pig models (10, 11). This genetic association between glucocorticoid stress levels and the genomic locus of Cbg gene has been replicated by an independent group in rat models (12). Thus, this set of data prompted us to create a rodent model of Cbg gene deficiency, in which Cbg gene expression is specifically modified, and to study, in more details than could be done in humans, its influence on HPA axis regulation and stress-induced behavioral responses. In this report, we present data showing the importance of CBG in defining glucocorticoid pool size as well as free glucocorticoid concentrations. Additionally, we show that these endocrinological alterations are associated with behavioral deficits.

Materials and Methods

Generation of Cbg-deficient mice

Cbg floxed mice were constructed at the Mouse Clinical Institute (Illkirch, France). Exon 2 of the mouse Serpina6 gene encoding CBG was flanked by loxP sites using homologous recombination in embryonic stem cells to obtain Cbg floxed mice. The Cbg floxed mice were then sent to our laboratory in Bordeaux and backcrossed with C57BL/6J mice from Charles River (L'Arbresle, France). For the inactivation of CBG in all cells, we crossed CMV-Cre transgenic mice (13) with Cbg floxed mice. Cbg+/+, Cbg+/−, and Cbg−/− mice were obtained by breeding Cbg+/− males and females. All mice used in the present work are males and have a C57BL/6J genetic background above 90%. Animals were maintained in an animal room (23 C) with a 12-h light-dark cycle (lights on at 0700 h) and with *ad libitum* access to food and water. All the experiments were conducted in strict compliance with current European Conventions and approved by Institutional Committee. The numbers of animals tested in each experiment were dependent on the availability of male mice of approximately the same age for each genotype.

Neuroendocrinological experiments

Mice were housed individually for a week before the experiments.

Circadian experiments

Blood samples were collected by tail nick every 4 h. Time from first handling to completion of this procedure did not exceed 2 min to obtain basal levels of corticosterone and CBG. A preliminary experiment done on eight C57Bl/6J male mice confirmed that this procedure allowed us to obtain basal levels because we got the same values as others who used different groups of animals at each time point. Blood was collected in EDTA solution (0.1% final concentration), and plasma, recovered after 10 min centrifugation of blood samples, was kept at −80 C. To measure free corticosterone, mice were anesthetized with a rapid isoflurane exposure (Aerrane, Baxter SA, Maurepas, France) and blood collected by cardiac puncture in less than 20 sec either between 0900 h and 1000 h for morning concentrations or between 1900 h and 2000 h for evening concentrations.

Restraint stress

The animals were placed in a 50-ml conical tube (with holes allowing breathing) for 20 min, blood was collected by tail nick, and then the animals were returned to their home cage.

Behavioral experiments

All the tests were conducted between 0900 h and 1300 h. The same groups of mice, 3–5 months of age, were used successively for activity cages, elevated-plus-maze, and open field with at least 1-wk interval between tests. Naive 4-month-old male mice were used for learned helplessness and locomotor response to cocaine experiments. The learned helplessness test was replicated on a second independent group of 3–3.5 months of age with blood samples collected before, during (just before putting the animal in the shuttle box), and right after the test for corticosterone measurements. An independent group of naive 3-month-old mice was used for the forced-swimming tests.

Details of the tests procedures and materials are provided in the supplemental data published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Corticosterone and ACTH measurements

Total corticosterone in plasma was measured with an in-house RIA using a highly specific antibody provided by H. Vaudry (University of Rouen, France). Cross reactivity with related compound such as cortisol was less than 3%. Intraassay and interassay variations were less than 10% and less than 15%, respectively. Plasma free corticosterone was estimated by isotopic dilution and plasma ultrafiltration as described (14) with some modifications detailed in supplemental data. The distribution of ^{3}H corticosterone between the different serum components (CBG and albumin) was calculated from measurements of the percentage of nonprotein bound corticosterone in treated and heat-treated serum (60 C for 1 h to eliminate CBG binding

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activity) as described previously (15). Variations for the entire assay was of 13% intraassays and less than 15% interassays. All animals from an experiment were measured in the same assay to avoid interassay variation. ACTH levels were measured using a commercial kit (ACTH ^{125}I RIA Kit, ref 24130; Diasorin, Antony, France).

CBG binding capacity assay

CBG maximal binding capacity (Bmax) was obtained by a saturation binding experiment as described (16) with some modifications detailed in supplemental data. Intraassay and interassay variations were less than 10% and less than 20%, respectively. All animals from an experiment were measured in the same assay to avoid interassay variation.

Western blot analysis of serum CBG and albumin

A total of 5 μg of protein from mice plasma was subjected to 10% SDS-PAGE (Ready gel; Bio-Rad, Marnes-la-Coquette, France) and electroblotted on a nitrocellulose membrane (Millipore, Molsheim, France). After 24 h of saturation at 4 C, membranes were incubated 1 h at room temperature with rabbit antimouse CBG antiserum diluted 1:1000 (gift from G. L. Hammond, University of British Columbia, Vancouver, Canada). After stripping of the bound CBG antiserum, the same membrane was incubated with rabbit antimouse albumin CLA3140 antiserum diluted 1:10000 (Cedarlane, Ontario, Canada). Specific antibody-antigen complexes were identified using a horseradish peroxidase-labeled antirabbit antibody (Santa Cruz Biotechnology, CA) and ECL+ detection reagents (PerkinElmer, Courtaboeuf, France). Chemiluminescence was captured by a Syngene detection system and quantified by Gene Tools software (Syngene, Cambridge, UK).

Gene expression by real-time PCR

Gene expression was evaluated by real-time PCR from reverse-transcribed total RNA. Detailed procedure and sequence of primers used are provided in supplemental Materials and Methods (see also supplemental Table S1). Total RNA was extracted from mice killed at 1900 h at the beginning of the dark phase for basal levels and from mice killed at the end of the learned helplessness test for stress levels. Relative quantification of target mRNA levels, normalized with 18S, was calculated with the SDS2.1 software (PerkinElmer, Courtaboeuf, France).

Statistics

Statistics were calculated with the software GraphPad Prism 5.0 (San Diego, CA). Data are presented as mean ± SEM. One-way ANOVA with Tukey post-hoc test was used for gene expression, CBG binding capacity, total and free corticosterone. Two-way ANOVA followed by Bonferoni post-hoc test was used for circadian variation of corticosterone in plasma and for kinetics of stress reactivity. Corticosterone data were log-transformed before analysis, and repeated measures parameter was used when appropriate. Nonparametric Kruskal-Wallis test was used for behavioral tests except for cocaine sensitization analyzed by two-way genotype × day ANOVA of repeated measures followed by paired *t* test within genotype. On graphs, *one symbol* (* or #) indicates a $P < 0.05$, *two symbols* $P < 0.01$, and *three symbols* $P < 0.001$.

Results

Generation of transcortin-deficient mice

Cre/loxP system was used to obtain Cbg floxed mice that were bred with the previously described CMV-Cre transgenic mice (13) to obtain Cbg knockout animals. Cbg+/+, Cbg+/−, and Cbg−/− mice were littermates obtained by breeding Cbg+/− males and females. Successful knockout of CBG gene was confirmed by several experiments (Fig. 1). Quantitative real-time PCR showed no expression of Cbg gene in liver of Cbg−/− mice and 50% of mRNA levels in Cbg+/− compared with wild type (ANOVA F = 21.5, $P < 0.0001$) (Fig. 1A). This was confirmed by Western blot analysis on plasma of three animals of each genotype using a specific mouse CBG antiserum (Fig. 1B). Albumin was revealed on the same membrane using a specific mouse albumin antiserum. No quantitative differences were found between genotype for albumin levels. Finally, CBG maximal binding capacity was assessed in each genotype. In wild-type males, values were found close to those previously reported : 136 ± 49 *vs.* 144 ± 21 (nM) in Cole *et al.* (17) for example. As expected, maximal binding capacity was found to be markedly decreased in Cbg−/− mice, and Cbg+/− animals show intermediate levels between wild type and Cbg−/− (ANOVA F = 33.6, $P < 0.0001$) (Fig. 1C). The distribution of corticosterone bound to either CBG or albumin was estimated as described (15), considering that albumin binding is resistant to a 60 C heating treatment of plasma, whereas CBG binding is inactivated at this temperature (Fig. 1D). The percentage of corticosterone bound to albumin as well as the free fraction rose dramatically in Cbg−/− and very moderately in Cbg+/− compared with Cbg+/+. In Cbg−/− mice, we found residual "CBG" binding (Fig. 1, C and D) that probably relates to unspecific binding to heat labile proteins in plasma.

HPA axis basal activity

The consequences of CBG deficiency on basal HPA activity were addressed by measuring total corticosterone during the diurnal cycle in animals fed *ad libitum* (Fig. 2A). By two-way analysis on log-transformed total corticosterone data, we found a genotype x time interaction not quite significant ($F_{8,71} = 2.0$, $P = 0.06$), a very significant genotype effect ($F_{2,71} = 18.4$, $P < 0.0001$) and time effect ($F_{4,71} = 26.8$, $P = 0.0001$). By using Bonferroni post-tests, no difference in total plasma corticosterone was detected between wild-type and heterozygous Cbg+/− mice at any time. However, Cbg−/− animals showed markedly reduced levels of plasma corticosterone at the end of the light and beginning of the dark phase compared with Cbg+/+ and Cbg+/− ($P < 0.01$). Total and free corticosterone levels were estimated in the plasma of new groups of mice

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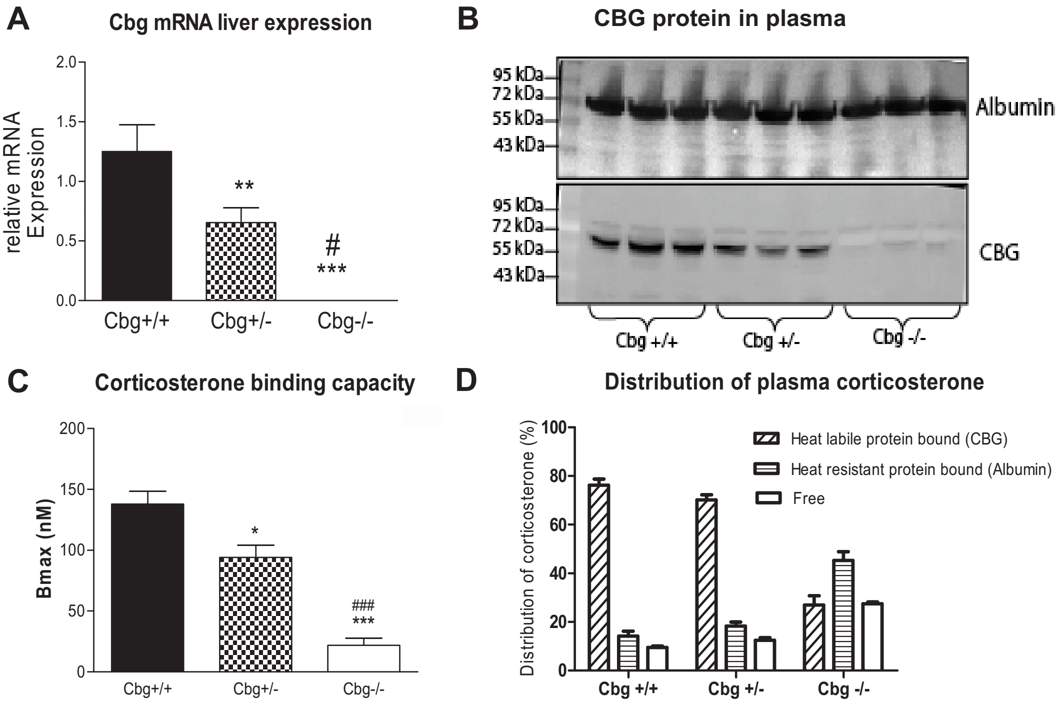

FIG. 1. Generation of mice deficient in transcortin. A, Cbg mRNA expression in liver of Cbg+/+, Cbg+/−, and Cbg−/− mice measured by real-time PCR (n = 6 per group). B, Western blots on plasma from each genotype using mouse albumin and Cbg antisera. C, Bmax to corticosterone estimated by saturation curve in plasma (n = 6 per genotype). D, Distribution of corticosterone binding in plasma estimated by isotopic dilution and ultrafiltration (n = 6 per genotype). Tukey post-hoc tests: *, *P* values of Cbg−/− or Cbg+/− *vs.* Cbg+/+; #, *P* values of Cbg−/− *vs.* Cbg+/−. *One symbol* indicates a $P < 0.05$, *two symbols* $P < 0.01$, and *three symbols* $P < 0.001$.

killed either in the morning (0900 h) or in the evening at the beginning of the dark phase (1900 h). Total corticosterone levels were similar to those reported in Fig. 2A. The free fraction of corticosterone, measured by isotopic dilution and plasma ultrafiltration, was 7.1 ± 0.4% in Cbg+/+, 8.7 ± 0.8% in Cbg+/−, and 20.5 ± 0.3% in Cbg−/− mice in the morning and 9.4 ± 0.6% in Cbg+/+, 12.3 ± 0.9% in Cbg+/−, and 26.0 ± 1.3% in Cbg−/− in the evening. Morning concentrations of free corticosterone, derived from the free fraction and the total corticosterone concentrations, were significantly higher in Cbg−/− compared with wild type and Cbg+/− (2.7 ± 0.1 nM *vs.* 1.1 ± 0.1 nM and 0.8 ± 0.1 nM, respectively, F = 108.0; $P < 0.0001$) as expected because there were no differences in total corticosterone levels. Conversely, in the evening, corresponding to the active phase of nocturnal animals such as mice, no variation in free corticosterone concentrations was detected between groups (F = 0.15, $P = 0.85$) (Fig. 2B). No significant differences were detected between groups for ACTH levels neither in the morning ($P = 0.09$) nor in the evening ($P = 0.70$) (Fig. 2C). The mRNA expression of some corticosteroid target genes [phosphoenolpyruvate carboxykinase (PEPCK), tyrosine aminotransferase (TAT), angiotensinogen, GR in liver]

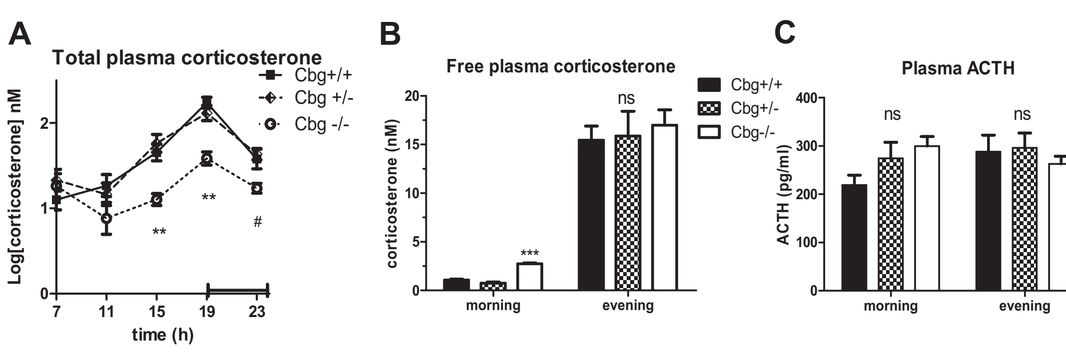

FIG. 2. Plasma corticosterone and ACTH levels at rest in transcortin-deficient mice. A, Total plasma corticosterone levels across the circadian cycle (n = 5–7 per group). *Thicker line* on x-axis indicates time when lights were off. B, Free plasma corticosterone levels in the morning (n = 5–6 per group) and in the evening (n = 7–8 per group) from mice of each genotype. C, Plasma ACTH levels (n = 8–10 per group). *, *Post hoc P* values of Cbg−/− or Cbg+/− *vs.* Cbg+/+; #, *P* values of Cbg−/− *vs.* Cbg+/− only. *One symbol* indicates a $P < 0.05$, *two symbols* $P < 0.01$. ns, Not significant.

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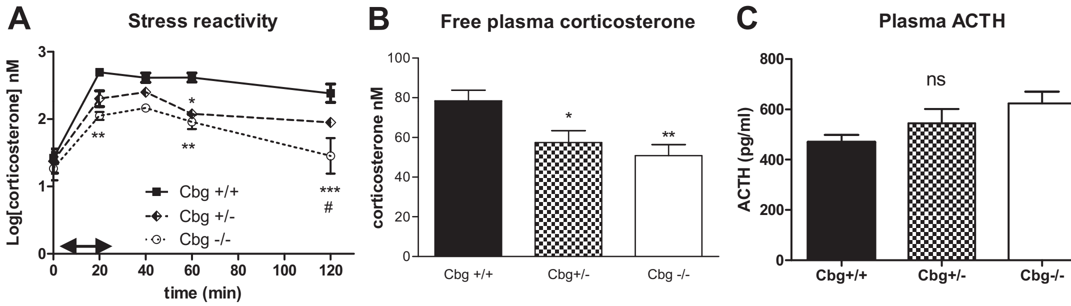

FIG. 3. Plasma corticosterone and ACTH levels after stress in transcortin-deficient mice. A, Total corticosterone levels at different time points (n = 5 per genotype and time point) after 20-min restraint stress. B, Free corticosterone levels after 20-min restraint stress (n = 7–8 per genotype). C, ACTH levels after stress (n = 5–6 per group). *, *Post hoc P* values of Cbg−/− or Cbg+/− *vs.* Cbg+/+; #, *P* values of Cbg−/− *vs.* Cbg+/− only. *One symbol* indicates a $P < 0.05$, *two symbols* $P < 0.01$, and *three symbols* $P < 0.001$. ns, Not significant.

and/or genes involved in the HPA axis regulation [CRH receptor 1 (CRHR1), proopiomelanocortin (POMC), GR in pituitary; CRH, GR in hypothalamus, CRHR1, GR, MR in hippocampus] was estimated by real-time PCR in tissues dissected from animals killed in the evening (supplemental Table S2). No significant difference was found between genotypes for any genes in any tissues tested.

HPA axis reactivity to stress

After a 20-min restraint stress, there was no significant genotype x time interaction by two-way ANOVA analysis on log-transformed data of total corticosterone values ($F_{8,55} = 1.6$; $P = 0.14$) (Fig. 3A). However, there were significant genotype ($F_{2,55} = 27.5$; $P < 0.0001$) and time ($F_{4,55} = 40.4$; $P < 0.0001$) effects. Cbg −/− and Cbg+/− mice showed altered levels of corticosterone response after stress with reduced peak levels compared with Cbg+/+ (115.9 ± 15.6 nM in Cbg−/−, 229.6 ± 54.6 nM in Cbg+/−, and 508.7 ± 67.4 nM in Cbg+/+ at $t = 20$ min). After 20-min stress, significant differences in free corticosterone levels were detected between genotypes: Cbg+/+ = 78.4 ± 5.5 nM, Cbg+/− = 57.5 ± 6.0 nM, and Cbg−/− = 50.9 ± 5.4 nM (ANOVA, F = 6.75, $P = 0.006$, Fig. 3B). Thus, free corticosterone levels increase after stress in all groups compared with basal levels (∼15-18 nM for each group at rest; Fig. 2) but to a lesser extent in transcortin-deficient mice. ACTH levels showed a tendency for higher levels in CBG-deficient mice as expected although the difference was not significant ($P = 0.07$) between groups (Fig. 3C).

Behavioral reactivity to mild stress

To evaluate the impact of CBG deficiency on behavioral reactivity, groups of mice from each genotype were submitted to a battery of moderately stressful behavioral tests. Cbg+/− and Cbg−/− mice exhibited behaviors indistinguishable from their wild-type littermates in the activity cage and open field tests that provide measures of locomotion and exploration under moderate stress (Fig. 4 and supplemental Fig. S1). In addition, mice from all genotypes showed similar anxiety-related behavior as measured in the time spent in the center of the open-field (supplemental Fig. S1) and the time or frequency of entries in the open arms of the elevated-plus-maze test (supplemental Fig. S2).

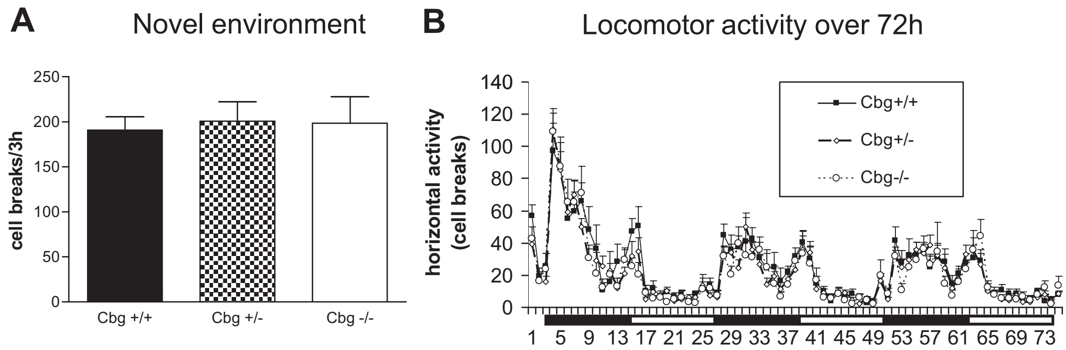

FIG. 4. Locomotor activity of transcortin-deficient mice. A, Locomotor activity in a novel environment was evaluated after the first exposure to activity cages during 3 h. B, Basal locomotor activity was measured during 3 d in activity cages after 3 h of habituation to the cages. n = 10–12 per group.

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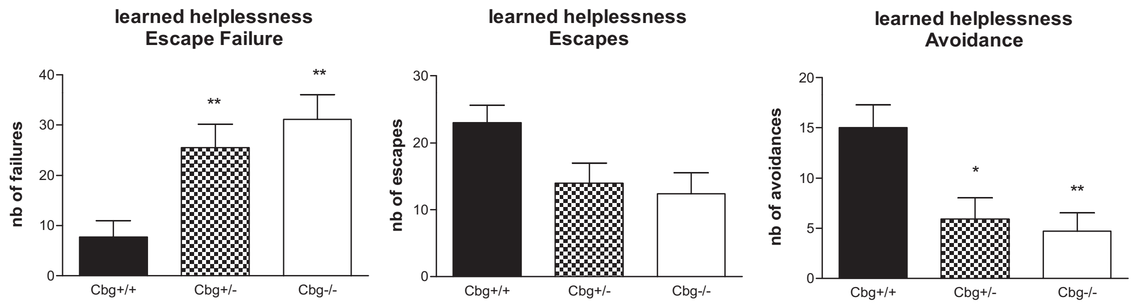

FIG. 5. Learned helplessness behavior in transcortin-deficient mice. Learned helplessness was evaluated in a shuttle box after exposure to two sessions of inescapable footshocks. The combined results of two independent groups are presented (n = 15–18 per genotype). *, *Post hoc P* values of Cbg+/− or Cbg−/− *vs.* Cbg+/+. *One symbol* indicates a $P < 0.05$, *two symbols* $P < 0.01$. nb, Number.

Depression-like behavior after intense and uncontrollable stress

We used the learned helplessness paradigm to evaluate despair-like behavior after an intense and uncontrollable stress. This paradigm is reported to show very good face validity and to have a good predictive validity for depressive states with both benzodiazepines and lithium being effective in reversing helplessness (18). After two sessions of unpredictable and uncontrollable footshocks performed on 2 consecutive days, learned helplessness behavior was assessed by the number of escape failures, escapes in reaction to the footshock, and avoidances in a shuttle box. The test was duplicated in two independent groups of mice and showed a similar pattern of responses. Thus, the data were combined for final analysis (Fig. 5). Compared with wild-type mice, Cbg mutant mice displayed a significantly increased number of escape failures (Kruskal-Wallis test, $P = 0.002$). In addition, there was a tendency for a lower number of escapes in reaction to the footshocks in both Cbg mutants mice ($P = 0.07$). Finally, the number of avoidances was significantly decreased in both Cbg+/− and Cbg−/− compared with wild types ($P = 0.0015$). On the second group of mice tested for learned helplessness, we have collected blood samples to evaluate corticosterone levels in the mice. Blood samples were collected by tail nick first just before the test for basal values, then the morning after the footshock sessions, *i.e.* just before the mice were submitted to the shuttle box, and finally by cardiac puncture at the end of the experiment when animals were killed. Free corticosterone and CBG were evaluated only at killing time because high plasma volume is required for the assays, and tissues were collected for gene expression analysis. The results are summarized in Table 1. Total corticosterone values before the test are in accordance with expected basal corticosterone morning levels. In the morning after the 2 d of footshocks, total corticosterone values were elevated in all groups. Just after the shuttle box test at the end of the whole experiment, total corticosterone levels were greatly elevated for all groups with lower levels for CBG-deficient mice ($F = 31.3$, $P < 0.0001$) as expected from Fig. 3. The free fraction of corticosterone was greatly increased in Cbg+/+ and Cbg+/− but moderately in Cbg−/− animals compared with the values observed in unstressed animals. In terms of concentrations, free corticosterone was found significantly reduced in Cbg-deficient mice compared with wild type ($F = 3.5$, $P < 0.05$), 22% decrease for Cbg+/−, and 31% for Cbg−/−. Additionally, we found a positive correlation between the number of avoidances observed in the learned helplessness test and the free corticosterone concentration ($r = 0.43$, $P < 0.05$; see supplemental Fig. S3). To evaluate whether this reduced free corticosterone in plasma translates into reduced glucocorticoid gene activation in the brain, we evaluated the expression of various genes in brain tissues of the mice dissected after the learned helplessness test (Table 1 and supplemental Fig. S3). Significant differences between genotypes were detected for Egr-1 mRNA in hippocampus with an approximately 40% decrease in transcortin-deficient mice compared with controls.

TABLE 1. Corticosterone, CBG during the learned helplessness test

	CBG+/+ *n* = 7	CBG+/− *n* = 8	CBG−/− *n* = 9	*P* (ANOVA)
Total [B] before LH test (d 1) (nM)	12.0 ± 2.3	9.2 ± 1.4	13.6 ± 1.1	NS
Total [B] before LH shuttle test (d 2) (nM)	25.2 ± 4.9	31.2 ± 7.9	19.6 ± 3.0	NS
Total [B] after LH test (d 3) (nM)	363.5 ± 20.2	253 ± 19.7	179.5 ± 9.1	*P* = 0.0001
% free B after LH test (d 3)	19.9 ± 0.7	22.2 ± 3.3	27.5 ± 2.0	*P* = 0.09
Free [B] after LH test (d 3) (nM)	72.0 ± 3.6	55.6 ± 8.5	49.7 ± 4.6	*P* = 0.03
CBG Bmax after LH test (d 3) (nM)	133.8 ± 9.1	91.5 ± 15.4	36.7 ± 2.3	*P* = 0.004
Egr-1 mRNA expression in hippocampus (relative mRNA abundance)	1.21 ± 0.16	1.06 ± 0.13	0.73 ± 0.11	*P* = 0.04

LH, Learned helplessness; NS, not significant.

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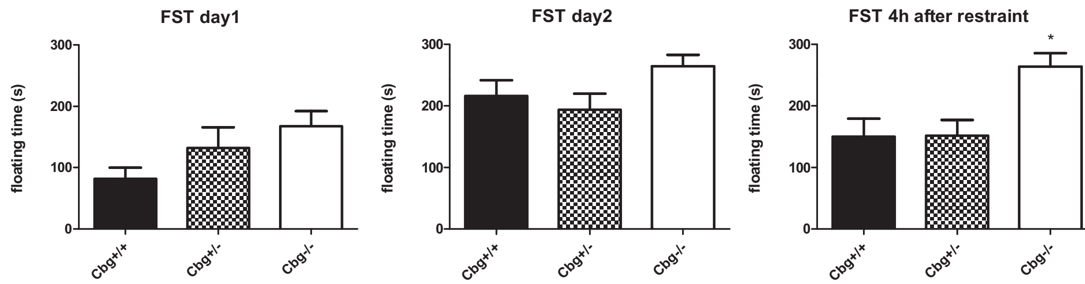

FIG. 6. Passive coping behavior of transcortin-deficient mice in the Porsolt forced-swimming test. Floating behavior was evaluated for 6 min in 25 C water (n = 7–10 per genotype) on two consecutive days and on d 4 after exposure to restraint stress. *, *Post hoc P* values of Cbg−/− *vs.* Cbg+/+. *One symbol* indicates a $P < 0.05$. FST, Forced swimming test.

To evaluate the helplessness behavior in a different paradigm, we have submitted new groups of mice to the forced-swimming test (Fig. 6). On the first day of test, there was no significant difference between groups by Kruskal-Wallis test, although a clear tendency for increased immobility of the Cbg−/− mice is observed. This nonsignificant result can be explained by a great dispersion of Cbg+/− mice scores as Cbg−/− immobility time is significantly different from Cbg+/+ by *t* test ($P < 0.05$). The second day of test, Cbg+/+ and Cbg+/− mice increased their levels of immobility to reach those of the Cbg−/− mice. Forty-eight hours later, all mice were submitted to a 1-h restraint stress, then left undisturbed for 3 h before submitting them to the forced-swimming test. This procedure had been used by others (19) to stimulate the CRF system before the behavioral test. In these conditions, a significant effect of genotype was found by Kruskal-Wallis test ($P = 0.02$), both the Cbg+/+ and Cbg+/− showed an active behavior with decreased immobility time, whereas the Cbg−/− remained with high levels of immobility time.

Cocaine-induced sensitization

Locomotor response to cocaine-induced sensitization is a phenomenon known to be corticosterone-dependent in rodents (4). Because our previous results suggested that CBG deficiency induces a glucocorticoid hyposignaling after stimulation of the HPA axis, we thought that cocaine-induced sensitization would be a good functional test of glucocorticoid signaling efficiency in the transcortin-deficient mice. To evaluate the role of CBG levels on cocaine sensitization, the locomotor response of mice after administration of saline or cocaine ip was measured in activity cages. Acute 20 mg/kg of cocaine administration produced a significant increase in locomotion in all groups with no difference between genotypes (Fig. 7A). However, sensitization to cocaine revealed differences between groups (two-way ANOVA, genotype x day interaction: $F_{1,12} = 7.7$, $P = 0.017$). After repeated administration of 20 mg/kg of cocaine over 5 d, locomotion increased progressively in wild-type and Cbg+/− mice, and a challenge given on d 14 produced a significant increase in locomotor activity in wild-type and a moderate rise in Cbg+/− animals (paired *t* test d 14 *vs.* d 1 within a genotype, $P = 0.005$ and $P = 0.038$, respectively). However, this sensitization to cocaine in terms of locomotor response was found totally suppressed in Cbg−/− mice ($P = 0.51$) (Fig. 7B).

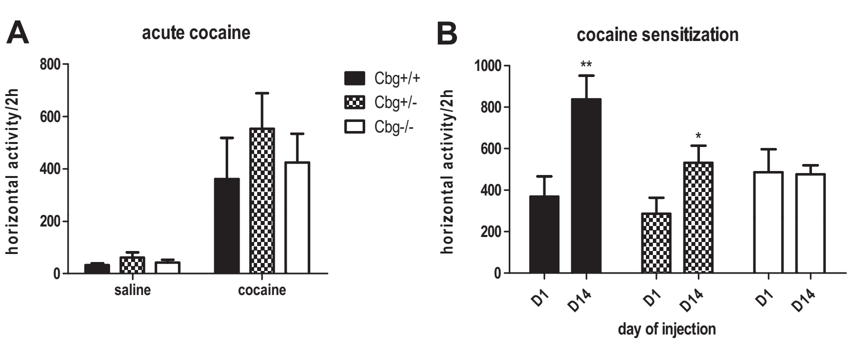

FIG. 7. Locomotor response to cocaine administration in transcortin-deficient mice. A, Locomotor response to a single 20 mg/kg injection of cocaine was evaluated in activity cages for 2 h in mice of each genotype (n = 6–7 per group). B, Sensitization of locomotor response to cocaine was evaluated in mice of each genotype after repeated injections for 5 d of 20 mg/kg of cocaine and a challenge with the same dose at d 14 (n = 6–9 per group). *, *t* test *P* values comparing d 14 to d 1 within a genotype. *One symbol* indicates a $P < 0.05$.

Discussion

In the present work, we produced a model of genetic transcortin variability with animals displaying 100, 50, or 0% of liver Cbg mRNA and plasma CBG protein. Our exploration of the HPA axis regulation under basal conditions showed data similar to those obtained in the few human patients described with heterozygous or homozygous null mutation in the Serpina6 gene encoding transcortin. Indeed, both in mice and human, absence of CBG leads to markedly decreased total glucocorticoid levels across the circadian cycle, elevated free

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fraction of glucocorticoids (~20–30%), elevated albumin bound fraction (~40%), and no apparent change in free glucocorticoid peak concentrations (*i.e.* morning in human and evening in mice) (20, 21). For mutant mice with only one Cbg-deficient allele like in human patients with 50% CBG levels, the free fraction of corticosterone is slightly increased (12.3 *vs.* 9.4% in Cbg+/+), but total glucocorticoid levels are comparable to controls in resting conditions. This absence of effect may be explained by the fact that CBG levels are in excess in resting conditions [68% of CBG is reported to circulate unbound in many species (8)]; thus, a 50% deficiency in CBG is still enough for normal basal glucocorticoid binding. As in human CBG-deficient patients, basal HPA axis regulation was not impaired in any mice groups. Indeed, free corticosterone levels at the beginning of the active phase were similar across genotypes. Second, no significant differences were found in the expression of glucocorticoid target genes or in genes involved in the regulation of the HPA axis in resting conditions. Third, the pattern of secretion of corticosterone over the circadian cycle was similar between genotypes. Thus, these data confirm that the basal corticosterone tone and the regulation of the HPA axis are driven by free and not total corticosterone. Contrary to interpretations by others, we therefore believe that Cbg −/− mice are a good model for the human condition of CBG deficiency (22). In their study of another mouse model of transcortin deficiency, Petersen *et al.* (23) defend the idea that their CBG knockout mice are unable to "sense" appropriately free corticosterone levels, based on their observation of elevated free corticosterone concentrations in Cbg−/− mice together with elevated ACTH levels. However, we have several concerns from the measures done in this study: blood was collected only in the morning, *i.e.* at the nadir of the circadian peak of corticosterone; the total corticosterone and ACTH values presented correspond to stress levels, and the free fractions of corticosterone are very low (~0.7% for Cbg+/+ and ~15% for Cbg−/−). In our hands, ACTH levels are on the high end compared with previous work that found concentrations around 100 pg/ml at rest in the morning, but we found no statistical difference between genotypes. Furthermore, we did not find differences between genotypes in CRH mRNA expression in hypothalamus.

After stress, the CBG-deficient mice showed a pattern of corticosterone secretion similar to the wild-type controls, *i.e.* a marked increase of total corticosterone levels followed by a progressive return to basal levels. However, the elevation of total corticosterone levels in CBG-deficient mice is moderate for Cbg+/− and weak for Cbg−/− compared with Cbg+/+. In these stress conditions, CBG levels are saturated with corticosterone binding and in a quicker way in Cbg+/− than Cbg+/+, explaining the difference in total corticosterone values. Furthermore, again in contrast to rest levels, the greater free fraction of corticosterone in transcortin-deficient mice does not compensate the smaller corticosterone rise after the 20-min stress. Consequently, free corticosterone levels are subnormal in the mutant mice. These results indicate the important role of CBG in determining glucocorticoid pool size (*i.e.* the mass of cortisol/corticosterone circulating) and are in agreement with previous data obtained in human subjects where CBG was shown to influence cortisol half-life, pool size, and volume distribution (24). Precise evaluation of corticosterone clearance is difficult to assess in small animals such as mice because it is very rapid. In their study, Petersen *et al.* (23) found that 5 min after injection of tritiated corticosterone, there was 10% left of the radioactive steroid in the plasma of Cbg+/+ *vs.* 2.5% in Cbg−/−, suggesting a 4-fold higher clearance in mutant mice. This higher clearance may explain our finding of similar kinetics of plasma corticosterone after stress despite subnormal free corticosterone concentrations in transcortin-deficient mice.

These results of altered rise in free corticosterone after stress in our Cbg-deficient mice prompted us to evaluate its possible impact on behavior in Cbg-deficient mice. Our behavioral data show that in situations of low or moderate stress Cbg−/−, Cbg+/−, and Cbg+/+ mice are indistinguishable with respect to exploration and anxiety-related traits. Petersen *et al.* (23) found diminished activity scores in Cbg−/− mice that they attribute to fatigue syndrome. It may be that they did their measures in the habituation phase and/or in more stressful conditions. Our results on 72 h of activity recording do not support the hypothesis of increased fatigue in our mutant mice, and anyway, we believe that fatigue syndrome cannot be recapitulated simply by a measure of locomotor activity. Similar observations of equivalent exploration and anxiety were made in mice with altered levels of GRs. Indeed, GR+/− mice showing 50% reduction in GR expression and YGR mice that have twice the amount of GR compared with wild-type animals, all display equivalent behavior in basal- or mild-stress situations (25). In addition, no differences in general activity or time spent in the center of the open field were observed in mice models with brain-specific GR or MR genetic manipulation (knockout or overexpression) (26–29). Collectively, these data suggest that variation in glucocorticoid signaling has no or little impact on exploratory and anxiety-related behaviors after a mild stress.

However, in paradigms of intense and uncontrollable stress, transcortin-deficient mice displayed clear altered behavior compared with wild-type mice. First, transcortin-deficient mice showed markedly increased learned

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helplessness with a higher number of escape failures and decreased number of avoided footshocks. Furthermore, we demonstrated that these behavioral responses are associated with decreased levels of free corticosterone in Cbg-deficient mice in plasma and decreased expression of Egr-1 gene in hippocampus. Recently, Egr-1 was shown to mediate stress-related behavioral effects of glucocorticoids in hippocampus (30). Its decreased expression is in accordance with reduced glucocorticoid signaling in the brain of our mutant mice, because the rapid (within 30 min) modification in Egr-1 expression is strictly regulated by glucocorticoid levels in brain independently from MAPK pathway signaling (30). There was no difference in the expression of the other genes tested. Because we killed the animals just after the test, it is not surprising that only immediate early genes such as Egr-1 were found affected. Basal levels of total corticosterone were altered in each group on d 2, showing that the mice had not recovered a normal HPA tone after 24 h. Whether CBG levels were suppressed in Cbg+/+ and Cbg+/− 24 h after the footshocks as reported in rat (31) could not be assessed because the blood volume collected at the tail of the animals was not sufficient.

Overall, our data are in good agreement with the literature, because low glucocorticoid levels have been associated with increased learned helplessness in rats. Indeed, both adrenalectomy and treatment with the glucocorticoid antagonist RU38486 enhance the development of learned helplessness in Sprague Dawley rats, an effect that is reversed by corticosterone (32). Additionally, the congenital learned helpless rat, genetically selected for susceptibility to learned helplessness behavior, has basal corticosterone levels similar to control animals, but exhibits corticosterone hyporesponsiveness to major stressors, similarly to our Cbg+/− mice (33). Finally, the GR+/− mice show increased learned helplessness, no differences in basal and slightly higher corticosterone levels after stress (25). The latter finding is not contradictory with our data because elevated corticosterone levels will not be effective in the brain of GR+/− mice. This higher despair-like behavior found in Cbg-deficient mice was confirmed in the forced-swimming test where Cbg−/− in particular showed a higher immobility time compared with Cbg+/+, especially if 1-h restraint is performed before the forced-swimming test. Second, our results on cocaine sensitization are in accordance with decreased brain glucocorticoid signaling in transcortin-deficient mice. Indeed, we found that sensitization of the locomotor response to cocaine is clearly suppressed in Cbg−/− mice and only a moderate sensitization is seen in Cbg+/− mice. Glucocorticoid facilitation of drug sensitization is a phenomenon described 20 yr ago in rats. Adrenalectomy resulting in depletion of glucocorticoids was shown to suppress amphetamine sensitization in rat but can be restored by dexamethasone replacement (34). More recently, cocaine sensitization was found suppressed in mice deleted for brain GR (35) and increased in mice overexpressing the GR in the central nervous system (28).

Therefore, our endocrine as well as behavioral results all converge to the finding of decreased glucocorticoid levels and signaling in Cbg-deficient mice after strong stress. Overall, our data are not contradictory with the findings reported in the study of Petersen *et al.* (23). The main difference between the two studies is the free glucocorticoid response after stress that we found reduced, whereas Petersen *et al.* (23) found it increased or equivalent compared with wild-types animals. Therefore, we conclude that Cbg deficiency leads to an insufficient glucocorticoid response to stress but normal resting levels, whereas Petersen *et al.* (23) believe that transcortin deficiency results in increased ACTH activity and hyporesponsiveness to glucocorticoids even in resting conditions. We cannot rule out that the apparent discrepancy in free corticosterone levels stem from the models themselves, for example the influence of the genetic background, but we favor the idea of methodological differences. To our benefit, our results fit better to the human condition of transcortin deficiency than Petersen *et al.* (23) data.

Both hypercortisolemia and hypocortisolemia have been reported to be associated with depressive states in human subjects (36–38). The hypocortisolism usually resulting from exhaustion of the HPA axis as a result of chronic stress is often associated with depression in fibromyalgia, burnout, and chronic fatigue syndromes (1, 39). Down-regulation of the HPA axis is also reported in atypical depression linked with fatigue and hyperphagia leading to increased body mass index (38). The increased learned helplessness associated with low corticosterone levels observed in our deficient mice is congruent with these depression subtypes. The role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders has been reviewed recently (40). According to the authors, both hypocortisolism and decreased glucocorticoids responsiveness are found associated with stress-related pathologies. As predicted by these authors, alteration in binding protein such as transcortin may lead to hypocortisolism and to decreased glucocorticoids signaling that will then influence the development of stress-related disorders. Thus, the data obtained on our transcortin-deficient mice fit with this hypothesis. Interestingly, most of the patients genetically deficient in CBG display a depressed mood, fatigue, and have a high body mass index (20, 21, 22). Such severe CBG mutations are rare, but variations in plasma transcortin levels are very frequent in

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human (41) and animal populations (11, 42). This variability may be genetic or secondary to intake of estrogen-containing contraceptives stimulating CBG production, or due to variations in insulin or IL-6 levels that inhibit transcortin (43–45).

In conclusion, we show that partial or total deficiency of plasma transcortin in mice does not affect the HPA axis functioning in resting conditions but leads to glucocorticoid hyposignaling after an intense stress and increased depression-like behaviors. The putative role of corticosterone-CBG complexes on membrane receptors proposed by some authors (46, 47) has not been studied and therefore cannot be excluded. Thus, transcortin plays a subtle but critical role in endocrine and behavioral stress responses that may explain the vulnerability to fatigue and depressive symptoms in transcortin-deficient patients. For more information, see the supplemental data published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

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