Corticosteroid-binding globulin contributes to the neuroendocrine phenotype of mice selected for extremes in stress reactivity

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Abstract

Increasing evidence indicates an important role of steroid-binding proteins in endocrine functions, including hypothalamic–pituitary–adrenal (HPA) axis activity and regulation, as they influence bioavailability, local delivery, and cellular signal transduction of steroid hormones. In the plasma, glucocorticoids (GCs) are mainly bound to the corticosteroid-binding globulin (CBG) and to a lesser extent to albumin. Plasma CBG levels are therefore involved in the adaptive stress response, as they determine the concentration of free, biologically active GCs. In this study, we investigated whether male mice with a genetic predisposition for high-reactivity (HR), intermediate-reactivity (IR), or low-reactivity (LR) stress-induced corticosterone (CORT) secretion present different levels of free CORT and CORT-binding proteins, basally and in response to stressors of different intensity. Our results suggest a fine control interaction between plasma CBG expression and stress-induced CORT release. Although plasma CBG levels, and therefore CBG binding capacity, were higher in HR animals, CORT secretion overloaded the CBG buffering function in response to stressors, resulting in clearly higher free CORT levels in HR compared with IR and LR mice (HR > IR > LR), resembling the pattern of total CORT increase in all three lines. Both stressors, restraint or forced swimming, did not evoke fast CBG release from the liver into the bloodstream and therefore CBG binding capacity was not altered in our three mouse lines. Thus, we confirm CBG functions in maintaining a dynamic equilibrium between CBG-bound and unbound CORT, but could not verify its role in delaying the rise of plasma free CORT immediately after stress exposure.

Key Words

- ▶ corticosteroids
- ▶ HPA axis
- ▶ hormone transport
- ▶ mouse
- ▶ behavior

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Introduction

Steroid hormones such as glucocorticoids (GCs), i.e. cortisol and corticosterone (CORT), and sex hormones, e.g. estradiol, progesterone, and testosterone, are generally transported in the blood, bound to carrier proteins such as albumin, sex hormone-, and corticosteroid-binding globulin (CBG, also called transcortin). CBG is a monomeric glycoprotein synthesized and stored mainly by the liver (Rothschild *et al.* 1972, Weiser *et al.* 1979, Kuhn *et al.* 1986, Hammond *et al.* 1987, Henley & Lightman 2011). Evidence for an intracellular location of CBG in the CNS and the pituitary has been reported, but its role within the brain remains to be determined (de Kloet *et al.* 1984, Mopert *et al.* 2006, Sivukhina *et al.* 2012). CBG is capable of binding GC with high affinity but with low capacity, in contrast to albumin, which binds GC with high capacity but with low affinity (Slaunwhite & Sandberg 1959, Sandberg *et al.* 1964, Burton & Westphal 1972, Breuner & Orchinik 2002, Moisan 2010). The association of steroids with CBG is more than three orders of magnitude tighter than with albumin, and the interaction is highly responsive to temperature but not to pH variations (Cameron *et al.* 2010). While 80–90% of circulating GCs are bound to CBG, 10–15% are bound to albumin, leaving only about 5% of biologically active unbound free GC available to diffuse through cell membranes and to bind to glucocorticoid receptors and mineralocorticoid receptors (Mendel 1989, Lewis *et al.* 2005, Henley & Lightman 2011, Cizza & Rother 2012). Despite the relatively weak binding affinity of GC to albumin, the physiological concentrations of albumin are such that it must be taken into consideration. The distribution between CBG-bound GC, albumin-bound GC and the free GC fraction in the plasma is challenged when a surge of GC is released from the adrenal cortex in response to stressors. Although only free GCs are able to exert biological effects, little is known about this regulation during stress.

CBG also plays an important role in GC action. It is discussed that CBG regulates the amount of free GC by buffering the elevations of GC during a secretory surge. In contrast, during periods of rest or reduced secretion, CBG provides a reservoir of circulating protein-bound GC that is biologically inactive but becomes active after dissociation of the CBG–GC complex, for example at the sites of inflammation where the serine protease elastase specifically cleaves CBG due to its SERPIN structure (Mendel 1989, Breuner & Orchinik 2002). Interestingly, CBG acts as a protein thermocouple that is exquisitely sensitive to temperature changes, and here too it will act as

a GC delivery molecule that will release cortisol in response to fever or external sources of heat (Cameron *et al.* 2010). Moreover, CBG regulates GC clearance, as the concentration of CBG in human plasma correlates inversely with metabolic clearance rates for cortisol, and CBG-deficient patients and CBG knockout mice showed increased GC clearance (Siiteri *et al.* 1982, Bright 1995, Petersen *et al.* 2006).

Previous reports have suggested that CBG may have an important role in the stress response. In some studies, following certain types of acute or chronic stressors, CBG expression was found to be downregulated, thereby increasing free GC levels (Neufeld *et al.* 1994, Fleshner *et al.* 1995, Spencer *et al.* 1996). Recently, the analysis of a mouse model of targeted CBG deficiency (CBG knockout) has shown that CBG is necessary for mice to mount a normal CORT response and to display adequate adaptive behaviors (Richard *et al.* 2010, Minni *et al.* 2012, Moisan 2013). These studies have highlighted the major role of CBG in maintaining a reservoir of GC readily available in case of emergencies, such as stress responses. Furthermore, Qian *et al.* (2011) have shown that in rats the rise of free CORT in the target compartments such as the brain and subcutaneous tissue is delayed by 20–30 min compared with the total CORT rise in the blood. Moreover, they discovered that CBG is a key player in this delay, as both moderate and strong stressors (i.e. restraint and forced swimming) evoked a fast and significant rise in CBG protein and binding capacity in the blood through a release of CBG from the liver. Thus, the increase in circulating CBG levels restrains the rise of free CORT concentrations for ~20 min in face of mounting total GC levels in the circulation. These results suggest a highly dynamic role for CBG in regulating free GC concentrations in response to acute stressors.

Considering the critical role of steroid-binding proteins in mounting adequate neuroendocrine and behavioral responses to stressors, and their eminent role in CORT bioavailability, local delivery, and cellular signal transduction, the overall aim of this study was to investigate whether mice with a genetic predisposition for extremes in HPA axis reactivity, and therefore in the stress-induced secretion of CORT, would differ in the expression and release of free CORT and CORT-binding proteins, i.e. albumin and CBG, in the liver and in plasma, both basally and in response to stressors. To achieve this, we used the recently established stress reactivity (SR) mouse model (Touma *et al.* 2008). This genetic animal model of affective

disorders consists of three mouse lines selectively bred for high reactivity (HR), intermediate reactivity (IR) or low reactivity (LR) of the HPA axis in response to stressors.

Materials and methods

Animals and housing conditions

From weaning until 4 weeks of age, mice were housed in same-sex groups of four. Housing and experimental rooms were maintained under standard laboratory conditions (12 h light:12 h darkness cycle, lights on at 0800 h; temperature, 22 ± 1 °C; relative humidity, $55 \pm 10\%$). Commercial mouse diet (Altromin no. 1324, Altromin GmbH, Lage, Germany) and tap water were available and the animals were allowed to eat and drink *ad libitum*. In total, 132 male mice derived from generations XV, XVI, and XX of the HR, IR, and LR breeding lines of the SR mouse model (Touma *et al.* 2008) were used in this experiments.

In order to avoid potential litter effects, the mice were selected from a minimum of six different litters per line. Animals were between 3 and 5 months of age during the experiments. All experiments were performed during the trough of the circadian rhythm of GC secretion (between 0900 and 1200 h, Touma *et al.* (2009)) and the order of testing was counterbalanced across the different breeding lines. At least 10 days before the experiments, the animals were housed singly in transparent polycarbonate cages (standard Makrolon cages type II, Bayer MaterialScience GmbH, Darmstadt, Germany; $23 \times 16 \times 14$ cm). The presented work complies with current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC). All experiments were announced to the appropriate local authority and were approved by the 'Animal Welfare Officer' of the Max Planck Institute of Psychiatry (Az. 55.2-1-54-2531-64-07).

Experimental designs

To investigate whether genetic alterations of HPA axis reactivity, i.e. dysregulated stress-induced CORT secretion, would interfere with the expression and release of free CORT and CORT-binding proteins, we performed three sets of experiments (see below). The experimental design is also depicted in [Supplementary Figure 1](#), see section on [supplementary data](#) given at the end of this article.

Experiment 1: basal and restraint-stress-induced total CORT, CBG, and albumin To assess total CORT, CBG, and albumin concentrations in the plasma

and liver under basal conditions and after stress exposure, a total of 54 male mice were subjected to a psychological stressor in the SR test (SRT). The SRT consists of a 15-min restraint period and two tail blood samples immediately before ($t=0$) and after restraint ($t=15$) (Touma *et al.* 2008). After the SRT, animals were transferred back to their home cages for 15 min. Thirty minutes after the beginning of the restraint period ($t=30$), the mice ($n=10$ per line) were decapitated after isoflurane anesthesia and trunk blood was collected. Livers were carefully dissected without the gall bladder, immediately frozen on dry ice and stored at -80 °C until further analysis. Animals of the basal group ($n=8$ per line) were killed under unstressed conditions. Trunk blood and livers were harvested following the same protocol. All blood samples were treated with the protease inhibitor, Trasylol (Bayer Vital GmbH, Leverkusen, Germany), centrifuged for 10 min ($4000g$ at 4 °C), and the plasma was stored at -80 °C until further analysis, when plasma total CORT concentrations were determined (see section 'Plasma total CORT'). Plasma and liver samples were used for CBG and albumin immunoblottings (see section 'Immunoblotting').

Experiment 2: basal and restraint-stress-induced free CORT and CBG Bmax To evaluate plasma free CORT and CBG maximal binding capacity (CBG Bmax) in unstressed and stressed animals, 24 male mice ($n=8$ per line) were subjected to the same 15 min restraint stressor as in Experiment 1. Immediately after restraint ($t=15$), animals were decapitated and trunk blood was collected. Animals of the basal group ($n=8$ per line) were killed under unstressed conditions; trunk blood was collected following the same protocol as described above. Plasma free CORT concentrations and CBG Bmax were determined (see sections 'Plasma free CORT' and 'CBG binding capacity').

Experiment 3: total CORT, free CORT, CBG, and albumin in response to forced swimming In order to assess total and free CORT, CBG, and albumin levels before and after exposure to a more severe (physical + psychological) stressor, 30 male mice ($n=10$ per line) were subjected to a 10-min open field (OF) test, directly followed by a 6-min forced swim test (FST). The animals were killed by decapitation immediately after the FST and trunk blood was collected as described earlier. Three days before the start of the experiment, a basal blood sample of each animal was obtained by tail nick. Plasma samples were used for CBG and albumin immunoblotting and total

and free CORT measurements (see sections 'Plasma total CORT' and 'Immunoblotting').

The OF test and FST are described in detail elsewhere (Touma *et al.* 2008). Briefly, the OF apparatus consisted of a black, open topped PVC cylinder (60 cm diameter and 40 cm height), and was dimly illuminated (15 lux). The animals' locomotor activities and exploratory drives were assessed utilizing the ANY-maze video tracking software (Stoelting Co., Wood Dale, IL, USA). In the FST, the animals were placed in a glass beaker filled with 23 °C water, where they are unable to reach the bottom with their hind paws or tail. The duration of struggling, swimming, and floating behavior was scored by a trained observer blind to mouse line using the EVENTLOG Software (Event Recorder, EMCO Maier GmbH, Hallein-Taxach, Austria).

Neuroendocrine measures

Plasma total CORT Plasma total CORT concentrations were measured in duplicate using a commercially available CORT RIA Kit (DRG Instruments GmbH, Marburg, Germany) following the manufacturer's protocol with slight modifications as described previously (Touma *et al.* 2008). The intra and inter-assay coefficients of variation (CV) were both below 10%.

Plasma free CORT Plasma free CORT was estimated by isotopic dilution and plasma ultrafiltration as described previously (Minni *et al.* 2012). Free CORT percentage was calculated as the ratio of c.p.m. filtrate (free CORT)/c.p.m. retentate (bound CORT). Free CORT concentration was obtained as percentage of free CORT multiplied by total CORT concentration measured by prior RIA.

CBG binding capacity CBG maximal binding capacity (B_{max}) was assessed by a saturation binding assay as described previously (Hammond & Lahteenmaki 1983, Richard *et al.* 2010). The intra-assay CV was below 10%. All samples from an experiment were measured in the same assay to avoid inter-assay variation.

Immunoblotting

Whole livers were homogenized for 2 min using a tissue homogenizer (VDI 12, VWR International GmbH, Darmstadt, Germany) in 5 ml ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, and 0.5% sodium deoxycholate) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, Roche Applied Sciences). Homogenates were then sonified

with 20 strokes (Branson Sonifier 250, G. Heinemann, Schwäbisch Gmünd, Germany). The samples were subsequently incubated with continuous rolling for 30 min at 4 °C to accomplish cell lysis. Homogenates were centrifuged for 30 min (11 350 *g* at 4 °C) and the transparent supernatants were saved. Protein concentration was quantified in triplicates using a BCA protein assay (Thermo Scientific, Rockford, IL, USA) read at 550 nm (Dynatech MR 7000, (Dynatech, Denkendorf, Germany)).

Ten nanograms to ten micrograms of protein (depending on the target protein) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany). The membranes were blocked for 1–2 h in 5% (w/v) fat-free milk (Carl Roth GmbH, Karlsruhe, Germany) in Tris-buffered saline 0.1% Tween 20 (TBST). Primary antibodies were diluted in 2.5% milk-TBST solution and incubated overnight at 4 °C: rabbit anti-human CBG 1:2000 (Affiland (Liege, Belgium), as previously applied in rodents studies (Qian *et al.* 2011)), rabbit anti-mouse albumin 1:50 000 (Cedarlane, Burlington, ON, Canada), mouse anti-glyceraldehyde-3-phosphate-dehydrogenase 1:2000, and rabbit anti-TF 1:1000 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Following incubation with the respective HRP-conjugated secondary antibody anti-rabbit or anti-mouse (Sigma-Aldrich and Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK) for 1 h at room temperature, membranes were incubated with ECL solution, protein bands were detected by the ChemiDoc MP imaging system (Bio-Rad Laboratories GmbH) and analyzed by Image Lab Software (version 4.0.1 build 4, Bio-Rad).

Statistical analysis

As normality and variance homogeneity could not always be assumed, all data were exclusively analyzed using nonparametric statistics (Siegel & Castellan 1988). All tests were applied two-tailed and were calculated using the software package SPSS (version 18.0). Two dependent samples were compared using the Wilcoxon test (*W*-test). Differences between more than two dependent samples were calculated with the Friedman test (*F*-test). Two independent samples were compared using the Mann-Whitney *U* test (MWU-test), while differences between more than two independent samples were calculated with the Kruskal-Wallis *H*-test (KWH-test). In the case of significant variation proven by the *F*- or KWH-tests, *post-hoc* pairwise comparisons between the groups were done using multiple *W*- or MWU-tests respectively. Here, significance levels were corrected according to the

sequential Bonferroni's technique (Rice 1989). Differences were considered significant if their probability of occurring by chance was $<5\%$ ($P \leq 0.05$).

Results

Free CORT mirrors total CORT in response to restraint stress

Plasma samples were obtained from animals under basal conditions or after restraint, according to the methods for Experiments 1 and 2 (see sections 'Experiment 1: basal and

restraint-stress-induced total CORT, CBG, and albumin' and 'Experiment 2: basal and restraint-stress-induced free CORT and CBG Bmax'; [Supplementary Figure 1](#)). As expected, plasma total CORT concentrations were low in all three lines of the basal group, although a between-line comparison showed that HR animals had significantly higher total CORT concentrations compared with mice of the LR line ([Fig. 1A](#)). Similarly, in the blood sample collected immediately before restraint ($t=0$), plasma total CORT concentrations were low ([Supplementary Figure 2A](#), see section on [supplementary data](#) given at the end of this article), but restraint ($t=15$) markedly increased plasma

Figure 1

Effect of 15 min restraint on total and free corticosterone (CORT) concentrations and corticosteroid-binding globulin (CBG) binding capacity (Bmax) in the plasma of high (HR), intermediate (IR), and low (LR) reactivity mice. Data are given as box plots showing medians (lines in the boxes), 25 and 75% percentiles (boxes), 10 and 90% percentiles (whiskers) as well as outliers (circles). (A) Plasma total CORT levels under basal conditions (KWH-test_{basal}: $n=8/8/8$, $H=6.8$, $df=2$, $P=0.032$; *post-hoc* MWU-tests_{basal}: HR vs IR, $U=19$, $P=0.172$; HR vs LR, $U=9$, $P=0.048$; and IR vs LR, $U=17$, $P=0.230$). (B) 15-min restraint stress ($t=15$) caused a significant increase in total CORT levels in the plasma, total CORT kept increasing to significantly higher levels up to 30 min ($t=30$) after the onset of restraint (F -test _{$t=0$ vs $t=15$ vs $t=30$} : HR/IR/LR, $n=10/10/10$, all $\chi^2=20$, all $df=2$, all $P \leq 0.001$; *post-hoc* W -tests: $t=0$ vs $t=15$, all $Z=-2.8$, all $P=0.01$; $t=0$ vs $t=30$, all $Z=-2.8$, all $P=0.005$; and $t=15$ vs $t=30$, all $Z=-2.8$, all $P=0.015$). (C) Plasma free CORT concentrations under basal conditions (KWH-test_{basal}: $n=8/8/8$, $H=7.0$, $df=2$, $P=0.030$; *post-hoc* MWU-tests_{basal}: HR vs IR, $U=17$, $P=0.230$; HR vs LR, $U=9$, $P=0.048$; and IR vs LR, $U=18$, $P=0.141$). (D) Free CORT

levels followed a similar pattern to total CORT in response to the 15-min restraint stressor (MWU-tests: HR_{basal} vs HR _{$t=15$} , IR_{basal} vs IR _{$t=15$} , LR_{basal} vs LR _{$t=15$} , all $U=0$, all $P=0.001$). (E) CBG binding capacity (CBG Bmax) was significantly lower in LR mice under basal conditions (KWH-test_{basal}: $n=8/8/8$, $H=12.2$, $df=2$, $P=0.002$; *post-hoc* MWU-tests_{basal}: HR vs IR, $U=31$, $P=0.916$; HR vs LR, $U=1$, $P=0.003$; and IR vs LR, $U=6$, $P=0.012$). (F) CBG Bmax was not significantly changed by the 15-min restraint stressor (MWU-tests: HR_{basal} vs HR _{$t=15$} , $U=24$, $P=0.401$; IR_{basal} vs IR _{$t=15$} , $U=13$, $P=0.083$; and LR_{basal} vs LR _{$t=15$} , $U=26$, $P=0.529$). Basal, unstressed animals; $t=0$, sampling immediately before restraint; $t=15$, sampling immediately after the restraint; $t=30$, sampling 30 min after beginning of restraint stressor. Significant differences are indicated at the top of each panel by bold lines and bold symbols (KWH- or F -tests), pairwise group comparisons (*post-hoc* MWU- or W -tests) are indicated below with thinner lines spanning between the two compared groups ($P > 0.05$ NS, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$).

total CORT concentrations in all three mouse lines, and CORT levels were even further elevated 30 min ($t=30$) after the onset of the restraint stressor (Fig. 1B). Furthermore, there was a significant difference in the magnitude of the CORT increase between the three lines, both at $t=15$ and 30, with HR mice displaying a greater rise in

plasma total CORT concentrations than that of IR mice, while LR animals showed the lowest increase (HR>IR>LR, Supplementary Figure 2B).

Similar to the total CORT measurements, plasma free CORT levels were low in all three lines of the basal group and significant differences could be detected between HR and LR animals (HR>LR, Fig. 1C). After the 15-min restraint ($t=15$), plasma free CORT concentrations were significantly increased in all three mouse lines (Fig. 1D). As observed for plasma total CORT, there was a significant difference in the magnitude of the free CORT increase between the three lines (HR>IR>LR, Supplementary Figure 2C). CBG Bmax also differed between the three lines. It was significantly lower in LR mice under basal conditions (Fig. 1E), as well as after the restraint stressor ($t=15$) (Supplementary Figure 2D). Stress exposure did not affect CBG Bmax in any of the lines (Fig. 1F).

Differential expression of CBG but not albumin

Plasma and liver samples from restrained animals were analyzed as described for Experiment 1 (see section 'Experiment 1: basal and restraint-stress-induced total CORT, CBG, and albumin'; Supplementary Figure 1).

Figure 2

Effects of restraint stress on corticosteroid-binding globulin (CBG) protein expression in the plasma and liver of high (HR), intermediate (IR), and low (LR) reactivity mice. Representative western blot images of CBG, transferrin (TF), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) in the plasma (A) and liver (B) of mice killed under unstressed conditions (basal) or 30 min after the onset of a 15-min restraint stressor ($t=30$) are presented above each panel. Data are given as box plots (for description see legend of Fig. 1). (A) CBG plasma concentrations were significantly different between the three mouse lines under both conditions (KWH-tests: HR/IR/LR_{basal}, $n=8/7/8$, HR/IR/LR _{$t=30$} , $n=10/10/10$, $H=13.6-18.9$, $df=2$, all $P\leq 0.001$; *post-hoc* MWU-tests: basal, HR vs IR, $U=5$, $P=0.016$; HR vs LR, $U=3$, $P=0.006$; IR vs LR, $U=9$, $P=0.028$; $t=30$, HR vs IR, $U=11$, $P=0.006$; HR vs LR, $U=0$, $P=0.003$; and IR vs LR, $U=18$, $P=0.016$). The strong band observed below TF is probably albumin, which due to its high abundance was constantly present in the TF blots but did not interfere with TF quantification. (B) CBG protein expression in the liver was not different between the lines under basal conditions and remained unchanged after restraint (KWH-tests: all HR/IR/LR, $n=8/8/8$, all $df=2$, basal: $H=2.7$, $P=0.249$; $t=30$: $H=0.1$, $P=0.949$). The expression of CBG relative to the sample loading controls (TF or GAPDH) was normalized to the mean of the IR group. Significant differences between the three lines are indicated at the top of each panel by bold lines and bold symbols (KWH-test), pairwise group comparisons (*post-hoc* MWU-tests) are indicated below with thinner lines spanning between the two compared groups ($P>0.05$ NS, $*P\leq 0.05$, $**P\leq 0.01$, and $***P\leq 0.001$). Molecular weights (kDa): CBG = 55, TF = 79, GAPDH = 37, MWM, molecular weight marker. (C) Correlation between plasma CBG relative protein expression and plasma total CORT levels 30 min after the onset of a 15-min restraint stressor ($t=30$). Spearman's rank correlation coefficient (r_s), $n=10/10/10$, $**P\leq 0.01$.

The three mouse lines differed significantly regarding their plasma CBG protein expression in both the basal and the stressed groups (Fig. 2A). HR mice showed clearly increased plasma CBG levels compared with IR animals,

while animals of the LR line presented the lowest amount of plasma CBG (Fig. 2A). Moreover, a positive correlation was found between plasma CBG protein expression and plasma total CORT levels 30 min after the onset of the restraint stressor (Fig. 2C).

The within-line comparison, however, did not reveal significant differences between basal and restrained animals of either line (Supplementary Figure 3A, see section on supplementary data given at the end of this article). The analysis of CBG expression in the liver revealed no significant differences between HR, IR, and LR mice (Fig. 2B). Furthermore, there was no significant effect of restraint stress on liver CBG expression in IR and LR mice (Supplementary Figure 3B). Similarly, the expression of albumin did not differ significantly neither in the plasma (Fig. 3A) nor in the liver (Fig. 3B) of HR, IR, and LR animals under basal conditions as well as after restraint stress exposure (Supplementary Figure 4A and B, see section on supplementary data given at the end of this article).

Psychological and physical stressors do not modulate CBG plasma levels

Plasma samples collected after 15-min restraint (SRT, Experiment 1) or after exposure to the FST (Experiment 3) were analyzed (see sections 'Experiment 1: basal and restraint stress-induced total CORT, CBG, and albumin', and 'Experiment 3: total CORT, free CORT, CBG, and albumin in response to forced swimming'; Supplementary

Figure 3

Effects of restraint stress on albumin protein expression in the plasma and liver of high (HR), intermediate (IR), and low (LR) reactivity mice. Representative western blot images of albumin, transferrin (TF), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) in the plasma (A) and liver (B) of mice killed under unstressed conditions (basal) or 30 min after the onset of a 15-min restraint stressor ($t=30$) are presented above each panel. Data are given as box plots (for description see legend of Fig. 1). (A) Albumin concentrations in the plasma (KWH-tests: HR/IR/LR_{basal}, $n=8/7/7$, $H=0.3$, $df=2$, $P=0.826$; and HR/IR/LR _{$t=30$} , $n=10/10/10$, $H=0.1$, $df=2$, $P=0.946$) and (B) in the liver (KWH-tests: all $n=8/8/8$, HR/IR/LR_{basal}, $H=5.4$, $df=2$, $P=0.064$; and HR/IR/LR _{$t=30$} , $H=4.8$, $df=2$, $P=0.087$) were not significantly different between the three mouse lines under basal conditions and also remained not significantly different between the lines after exposure to the restraint stressor. The strong band observed below TF is probably albumin, that due to its high abundance was constantly present in the TF blots but did not interfere with TF quantification. The expression of albumin relative to the sample loading controls (TF or GAPDH) was normalized to the mean of the IR group. Significant differences between the three lines are indicated at the top of each panel by bold lines and bold symbols (KWH-test) ($P>0.05$ NS). Molecular weights (kDa): albumin, 69; TF, 79; GAPDH, 37; MWM, molecular weight marker.

Figure 4

Effects of different stressors on total corticosterone (CORT) increase free CORT levels and CBG and albumin protein expression in the plasma of high (HR), intermediate (IR), and low (LR) reactivity mice. Representative western blot images of CBG, albumin, and transferrin (TF) in the plasma from mice sampled under unstressed conditions (basal), after 15-min restraint stress (SRT), and after a 10-min open field test plus 6-min forced swim test (FST). Data are given as box plots (for description see legend of Fig. 1). (A) Plasma total CORT increases after the FST was significantly higher than after the SRT (MWU-tests: for each line $n = 10/10$, HR_{SRT} vs HR_{FST}, $U = 8$, $P = 0.001$; IR_{SRT} vs IR_{FST}, $U = 0$, $P \leq 0.001$; and LR_{SRT} vs LR_{FST}, $U = 5$, $P = 0.001$). (B) Plasma free CORT after the FST was significantly higher than after the SRT (MWU-tests: HR_{SRT} vs HR_{FST}, $n = 8/7$, $U = 1$, $P = 0.002$; IR_{SRT} vs IR_{FST}, $n = 7/8$, $U = 0$, $P = 0.001$; and LR_{SRT} vs LR_{FST}, $n = 8/8$, $U = 0$, $P = 0.001$). (C) CBG expression was significantly higher in the plasma of HR mice compared with IR and/or LR animals for all three conditions (KWH-test: HR/IR/LR_{basal}: $n = 5/5/5$, $df = 2$, $H = 7.4$, $P = 0.024$; and HR/IR/LR_{SRT}: $n = 5/5/5$, $df = 2$, $H = 6.6$,

$P = 0.037$; HR/IR/LR_{FST}: $n = 10/10/10$, $df = 2$, $H = 7.1$, $P = 0.028$; post-hoc MWU-tests: basal, HR vs IR, $U = 3$, $P = 0.094$; HR vs LR, $U = 0$, $P = 0.027$; IR vs LR, $U = 11$, $P = 0.754$; SRT, HR vs IR, $U = 3$, $P = 0.094$; HR vs LR, $U = 1$, $P = 0.016$; IR vs LR, $U = 11$, $P = 0.754$; FST, HR vs IR, $U = 14$, $P = 0.021$; HR vs LR, $U = 29$, $P = 0.224$; and IR vs LR, $U = 39$, $P = 0.406$). (D) Albumin plasma concentrations were not significantly different between the three lines for all three conditions (KWH-test: HR/IR/LR_{basal}: $n = 5/5/5$, $df = 2$, $H = 5.0$, $P = 0.080$; HR/IR/LR_{SRT}: $n = 5/5/5$, $df = 2$, $H = 2.0$, $P = 0.368$; and HR/IR/LR_{FST}: $n = 10/10/10$, $df = 2$, $H = 2.7$, $P = 0.247$). The CBG and albumin relative expressions to the sample loading control (transferrin (TF)) were normalized to the mean of the IR group. Significant differences between the three lines are indicated at the top of each panel by bold lines and bold symbols (KWH-test), pairwise comparisons (MWU-tests) are indicated with thinner lines spanning between the two compared groups ($P > 0.05$ NS, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$). Molecular weights (kDa): CBG, 55; albumin, 69; TF, 79; MWM, molecular weight marker.

Figure 1). As expected, plasma total and free CORT values after the FST were significantly higher than those after the SRT (Fig. 4A and B). However, plasma CBG levels after the FST remained similar to those observed under basal conditions and after the SRT (Fig. 4C and Supplementary Figure 5C). A between-line comparison revealed significant differences in the swim-stress-induced plasma total and free CORT concentrations, with HR mice showing clearly higher plasma total and free CORT levels compared with the IR line, while LR animals presented the lowest CORT increase (Supplementary Figure 5A and B). Regarding plasma albumin expression, no differences were found between the three mouse lines under basal conditions, as well as after stress exposure in the SRT or FST (Fig. 4D). The within-line comparison revealed a tendency toward increased albumin levels after the FST only in HR animals (Supplementary Figure 5D).

Regarding the behavioral response of the animals in the OF test and the FST, significant differences were observed between HR, IR, and LR mice. The results of the OF test assessing the locomotor activity and exploratory drive are summarized in Supplementary Table 1, see section on supplementary data given at the end of this

article. The FST also revealed significant differences in stress-coping behavior between the three lines, with HR animals showing clearly increased struggling and decreased floating compared with IR animals, while LR mice showed more passive coping behavior with less struggling and more floating (Fig. 5A, B and C).

Discussion

The emerging role of steroid-binding proteins in endocrine functions has received increasing attention (Nakhla *et al.* 1999, Breuner & Orchinik 2002, Henley & Lightman 2011, Perogamvros *et al.* 2012). Plasma CBG levels are important for the adaptive stress responses as they determine the GC pool size and consequently the concentration of free GC (Minni *et al.* 2012, Moisan 2013). In this study, three mouse lines selectively bred for HR, IR, or LR stress-induced secretion of CORT were used to elucidate whether the differences in HPA axis reactivity are influenced by differential free CORT and CBG availability and release. We performed experiments involving stressors with different intensity and analyzed plasma and liver samples for the abundance of

Figure 5

Stress-coping behavior of high (HR), intermediate (IR), and low (LR) reactivity mice in the forced swim test (FST). Data are given as box plots (for description see legend of Fig. 1). (A) HR mice showed significantly increased struggling time compared with the other two lines. (B) Total swimming time was not significantly different between the three mouse lines. (C) HR mice showed clearly decreased floating behavior, while LR mice show increased floating compared with IR animals. Significant differences between the three lines (KWH-test: all $n = 10/10/10$, all $df = 2$,

HR/IR/LR_{struggling}: $H = 13.0$, $P = 0.001$; HR/IR/LR_{swimming}: $H = 3.8$, $P = 0.147$; and HR/IR/LR_{floating}: $H = 16.5$, $P \leq 0.001$) are indicated at the top of the panel by bold lines and bold symbols, pairwise group comparisons (*post-hoc* MWU-tests: struggling: HR vs IR, $U = 17$, $P = 0.013$; HR vs LR, $U = 7$, $P = 0.003$; IR vs LR, $U = 28$, $P = 0.096$; floating: HR vs IR, $U = 10$, $P = 0.004$; HR vs LR, $U = 6$, $P = 0.003$; and IR vs LR, $U = 19$, $P = 0.019$) are indicated below with thinner lines spanning between the two compared groups ($P > 0.05$ NS, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$).

CORT, CBG, and albumin. Our results revealed that 15-min restraint as well as 6-min forced swimming did not trigger a fast CBG release from the liver into the circulation, although we could observe significant line differences regarding plasma total and free CORT as well as plasma CBG expression and CBG B_{max} (HR > IR > LR).

The currently accepted model of steroid action proposes that only steroids that are not bound to plasma proteins can gain access to target tissues and intracellular receptors (Siiteri *et al.* 1982, Mendel 1989). Hence, we evaluated plasma free CORT concentrations before and after exposure to restraint stress (Fig. 1C and D). As expected, the stress-induced surge of CORT exceeds CBG's binding capacity, resulting in significantly increased, but different, free plasma CORT concentrations (HR > IR > LR), thus paralleling the line-specific differences in plasma total CORT (see Fig. 1B and D). Interestingly, we were able to demonstrate significantly increased plasma free CORT concentrations as early as 15 min after stress initiation (Fig. 1D). Heinzmann *et al.* (2010), using *in vivo* microdialysis, reported that the rise in free CORT levels in the

hippocampus is delayed for about 30 min and peaks at around 60 min after the onset of the restraint in HR, IR, and LR mice. This is in line with other studies showing a delayed increase in free CORT levels in the brain and other GC target tissues in mice (Thoeringer *et al.* 2007, Tronche *et al.* 2010) and rats (Droste *et al.* 2008, 2009, Qian *et al.* 2011). Recently, Qian *et al.* (2011) have shown that, in rats, this 20–30 min delay in the increase in free CORT in the plasma (jugular vein), as well as in other body compartments (subcutaneous tissue and hippocampus), is associated with a fast rise of CBG in the plasma in response to stressors, i.e. forced swimming and exposure to novel environment. However, applying a similar experimental design in our SR mice, we observed elevated plasma free CORT concentrations after 15-min of restraint stress without changes in plasma CBG concentrations or CBG binding capacity (see Figs 1D and F, 2A). Therefore, it remains to be clarified whether the regulatory interactions of CBG and CORT are different in mice and rats or whether the timeframe of these physiological processes follows different patterns in these two rodent species.

It is conceivable that increased CORT clearance might contribute to the low plasma free CORT values observed in LR mice (Fig. 1D), as indicated by results from Cbg knockout (Cbg^{-/-}) animals (Petersen *et al.* 2006) and humans (Siiteri *et al.* 1982). Plasma half-life of injected [³H]corticosterone in Cbg^{-/-} animals is significantly shortened, while after 5 min of injection there was 10% of the radioactive steroid left in the plasma of WT mice, there was only 2.5% in Cbg^{-/-} animals, suggesting a fourfold higher clearance due to the CBG knockout (Petersen *et al.* 2006). In human plasma, CBG concentrations inversely correlate with metabolic clearance rates of cortisol (Siiteri *et al.* 1982). Although there is clear evidence indicating that the adrenal size and sensitivity of the CORT-secretion response to ACTH are significantly different between HR, IR, and LR mice (diminished in LR and increased in HR mice; J-M Heinzmann, S Kloiber, G E Mattos, M Bielohuby, M V Schmidt, R Palme, F Holsboer, M Uhr, M Ising & C Touma, 2013, unpublished observations), the CORT clearance rates could contribute to the observed differences in HPA axis reactivity and should be therefore considered in future studies. In addition, the expression and possible functional alterations of liver enzymes involved in steroid catabolism (e.g. cytochrome p450 2c39, hydroxysteroid 17 β dehydrogenase 9, and carboxyl-esterase 1) should also be investigated, as these enzymes were found to be upregulated in a gene expression profiling study with Cbg^{-/-} mice (Petersen *et al.* 2006).

Moreover, it is noteworthy that CBG acts as a protein thermocouple (Burton & Westphal 1972). Studies by Cameron *et al.* (2010) have shown that the affinity of cortisol for CBG drops approximately by 16-fold as the temperature increases from 35 to 42 °C, while the binding of cortisol to albumin remains unaffected. Accordingly, a rise in body temperature from 36.5 to 38 °C, as observed in HR, IR, and LR animals subjected to the stress-induced hyperthermia test (Touma *et al.* 2008), would induce a 50% reduction in CBG binding affinity for CORT. As a 15-min restraint period would, in theory, induce a similar or even greater change in body temperature, this would result in a decreased CBG binding affinity and consequently proportional CORT release. Thus, we speculate that the difference in CBG levels between the lines could contribute to the increased plasma free CORT after restraint stress by this temperature-dependent process, as the CBG and GC pool is higher in HR than that in IR and LR animals.

Plasma CBG levels in HR, IR, and LR mice already showed significant differences under basal conditions, with the highest abundance of CBG in HR mice, while LR animals presented the lowest amounts (see Fig. 2A).

The first conclusion that can be drawn from this observation is that the significant differences in plasma CBG between the three mouse lines might already affect plasma total and free CORT concentrations under resting conditions. Moreover, in response to stressors such as restraint and forced swimming, the observed differences in plasma total and free CORT levels between the three lines are further enhanced (HR > IR > LR, see Fig. 4A and B). However, plasma CBG levels remained unaffected by the stressors, as well as CBG maximal binding capacity and liver CBG expression (see Figs 1F, 2A and B). Assuming that i) there is no differential storage of CBG per liver cell, even though total CBG storage could be smaller in HR due to a smaller relative liver mass (HR < IR = LR, see Supplementary Figure 6, see section on supplementary data given at the end of this article), ii) there are no differences in CBG half-life in the blood, and iii) CBG mRNA is consistently translated into CBG protein in hepatocytes, we suggest that the observed differences in plasma CBG between the HR, IR, and LR mouse lines are brought about by differences in CBG synthesis rate. Thus, the origin of this differences synthesis levels remains to be determined.

Our findings are not in accord with those published by Qian *et al.* (2011) who suggested that in rats moderate and strong stressors evoke a fast (within 5 min) rise in CBG protein and binding capacity in the blood through a massive release of CBG from the liver. Although we have used a very similar experimental design, we could not observe significant differences in stress-induced CBG protein and binding capacity in the blood of HR, IR, and LR mice. This discrepancy may simply relate to species differences between rats and mice, however, in order to evaluate the clinical relevance of a CBG response to stress, future studies should first address whether the human physiological response to stress is more similar to the pattern observed in rats or in mice.

In order to also consider the other major steroid transport protein, we investigated the role of albumin. Albumin's main functions are the regulation of the osmotic pressure in the blood and the transport of lipophilic compounds including steroids, fatty acids, retinoids, thyroid hormone, and prostaglandins (Kragh-Hansen 1981, 1990, Peters 1985, Baker 2002). Despite its evident role in steroid transport, we did not detect any line differences or stress-induced alterations in albumin protein expression in the plasma or liver of HR, IR, or LR mice (Fig. 3). This might be due to the high abundance of this protein in these two tissues (albumin concentration in the plasma, measured by ELISA $\approx$ 35 mg/ml, see Supplementary Figure 7, see section on supplementary

data given at the end of this article), implicating that small changes would be difficult to detect in face of the high total amounts.

Regarding plasma total and free CORT (Fig. 1A, B, C and D) and the stress-coping behavior in the FST (Fig. 5), our results are in agreement with the findings reported by Richard *et al.* (2010). In particular, the neuroendocrine and behavioral endophenotypes of our LR animals, which have lower plasma levels of CBG, clearly parallel those of homozygous CBG knockout mice. LR and *Cbg*^{−/−} animals have normal free CORT values at rest but a markedly blunted surge after restraint stress, reflected by the reduced increase in total CORT. Moreover, LR mice and *Cbg*^{−/−} present no changes in anxiety-related behaviors (Touma *et al.* 2008, Richard *et al.* 2010) but display a depression-like behavioral phenotype with increased passive stress-coping behavior (floating/immobility) in the FST (Fig. 5C). Thus, the phenotypic characteristics of LR mice may stem in part from an increased GC clearance due to lower CBG levels.

Previous studies revealed distinct differences between HR, IR, and LR animals not only regarding their CORT secretion but also their sleep architecture, activity rhythms, emotional behavior, cognitive abilities, and neuroendocrine functions as well as neurobiological alterations in the hippocampus and prefrontal cortex resembling several endophenotypes observed in depressed patients, particularly when the melancholic and atypical subtypes of major depression are considered (Touma *et al.* 2008, 2009, Knapman *et al.* 2010a,b, 2012). J-M Heinzmann, S Kloiber, G Ebling-Mattos, M Bielohuby, M V Schmidt, R Palme, F Holsboer, M Uhr, M Ising and C Touma (unpublished observations) provided further evidence that the differences in SR between HR, IR, and LR mice are brought about by functional alterations at all levels of the HPA axis (peripheral, central, and regulation). HR mice present enhanced adrenal sensitivity while LR animals show blunted adrenal responsiveness to ACTH. In addition, significantly altered ACTH protein and proopiomelanocortin mRNA expression suggested differential activation of the anterior pituitary. In the brain, acute stress exposure triggered significant differences in neuronal activation of the paraventricular nucleus of the hypothalamus. Therefore, we believe that the differences in plasma CBG expression levels (HR > IR > LR) reported in this study add another point to the list of molecular changes underlying the behavioral and physiological alterations observed in the SR mouse model.

Taken together, our study has revealed that i) the free CORT increase resembles the pattern of total CORT increase

in HR, IR, and LR mice, although with lower concentrations; ii) there is a strong positive correlation between CBG plasma levels and SR, i.e. CORT release, across the three SR mouse lines; iii) although CBG plasma levels, and therefore CBG binding capacity, are higher in HR animals, CORT secretion overshoots the CBG buffering function in response to moderate and strong stressors, resulting in clearly higher free CORT levels in HR compared with IR and LR mice; iv) we did not observe the stress-induced fast CBG increase as described for rats (Qian *et al.* 2011), indicating a different regulatory mechanism in mice; and v) the lower CBG expression in LR mice could also implicate a faster CORT clearance in these animals and therefore might contribute to the lower CORT levels. However, in HR mice, the high levels of CBG retain a high pool of total CORT in the plasma, at the same time allowing the release of large amounts of free CORT provided by the dynamic equilibrium between CBG-bound and unbound CORT. This process assures an adequate amount of circulating free CORT under basal conditions and a more pronounced effect in response to stressors in HR compared with IR and LR mice.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0255>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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