

HPA axis changes during the initial phase of psychosocial stressor exposure in male mice

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Abstract

Chronic subordinate colony (CSC) housing for 19 days results in unaffected basal morning corticosterone (CORT) levels despite a pronounced increase in adrenal mass, likely mediated by an attenuation of adrenal corticotropin (ACTH) responsiveness. Given that the pronounced increase in basal morning plasma CORT levels returns to baseline as early as 48 h after the start of CSC, it is likely that the attenuated ACTH responsiveness develops already during this initial phase. This was tested in the present study. In line with previous findings, basal morning plasma CORT levels were elevated following 10 h, but not 48 h, of CSC exposure. Basal morning plasma ACTH concentrations and relative *in vivo* adrenal CORT content were increased following 10 h and to a lesser extent following 48 h of CSC exposure, positively correlating. Relative *in vitro* adrenal CORT secretion in response to ACTH (100 nM) and kidney protein expression of 11 β -hydroxysteroid dehydrogenase type 2 (HSD11B2) were unaffected following both time points. Adrenal mRNA expression of key steroidogenic enzymes was unaffected/decreased following 10 h and unaffected/increased following 48 h of CSC exposure. Together, our findings suggest that basal plasma hypercorticism during the initial CSC phase is mainly prevented by an attenuation of pituitary ACTH release. An increased absolute adrenal weight following 10 h, but not 48 h, of CSC exposure indicates that restoration of normal adrenal mass also adds to a lesser extent to prevent basal hypercorticism. A contributing role of alterations in enzymatic CORT degradation and steroidogenic enzyme availability is likely, but has to be further addressed in future studies.

Key Words

- ▶ psychosocial stress
- ▶ chronic subordinate colony (CSC) housing
- ▶ HPA axis
- ▶ adrenal glands
- ▶ ACTH
- ▶ hypercorticism
- ▶ corticosterone

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Introduction

Exposure to acute stressful stimuli leads to the activation of both the sympathetic nervous system and the hypothalamo-pituitary-adrenal (HPA) axis and, consequently, to the systemic release of catecholamines and glucocorticoids (GCs). These stress hormones, in turn, trigger physiological alterations enabling an organism to adjust to the new situation and to exhibit an adequate behavioral response (for a review, see Chrousos (1998) and

Charmandari *et al.* (2005)). This indicates that the acute stress response is generally beneficial and increases an individual's chance of survival. However, if the stress systems are activated over a prolonged period of time, chronically elevated levels of plasma GCs can promote the development of somatic and affective disorders (for a review, see Sapolsky (1996), McEwen (2000) and Vanitallie (2002)). Thus, it is adaptive for an individual to habituate

as fast as possible to prolonged and not life-threatening homotypic stressors (Kudielka *et al.* 2006, Sasse *et al.* 2008).

Although it is generally accepted that habituation to a prolonged homotypic stressor does not occur if the stressor is of social nature (for review, see Bartolomucci (2007)), we have just recently provided the first evidence that this does not hold true for the chronic subordinate colony (CSC) housing paradigm, an adequate and clinically relevant mouse model of chronic psychosocial stress (Reber *et al.* 2007, Reber & Neumann 2008, Slattery *et al.* 2012, Uschold-Schmidt *et al.* 2012). CSC mice exhibit unaffected basal morning plasma corticosterone (CORT) levels, despite significantly enlarged adrenal glands following 19 days of CSC compared with single-housed control (SHC) mice. A general breakdown of HPA axis functionality in chronically stressed mice, explaining this controversy, can be excluded here, as CSC mice compared with the SHC mice exhibit an even more pronounced CORT response to an acute heterotypic stressor. The latter finding, indicating functionally not compromised adrenal glands in CSC mice, is further supported by data showing increased availability and mobilization capacity of the CORT precursor molecule cholesterol in CSC mice compared with the SHC mice, contributing to their increased *in vivo* CORT response during acute heterotypic stressor exposure (Füchsl *et al.* 2013). Moreover, relative adrenal mRNA expression of the key steroidogenic enzymes: steroidogenic acute regulatory protein (*Star*), side-chain cleavage enzyme (*Cyp11a1*), and 11 β -hydroxylase (*Cyp11b1*) (for a review, see Miller (1988) and BIASON-LAUBER (1998)) is not affected or even increased following 19 days of CSC (Uschold-Schmidt *et al.* 2012).

In concert with the lower relative adrenal *in vitro* ACTH responsiveness following 19 days of CSC, these data strongly suggest that CSC mice prevent the health-compromising consequences of enlarged and fully functional adrenal glands, namely increased basal CORT levels, by reducing relative adrenal sensitivity to the main CORT secretagogue ACTH.

Although elevated basal morning plasma CORT levels (observed following 10 and 24 h) in CSC mice return to baseline as early as 48 h after the start of stressor exposure (Reber *et al.* 2007, 2011), the reduction in adrenal ACTH responsiveness has been investigated and described only after 19 days of CSC so far. It is, thus, the aim of the present study to reveal that CSC-induced attenuation of relative *in vitro* adrenal ACTH responsiveness develops already during the initial 48 h of CSC, the time period in which elevated morning basal plasma CORT levels return to baseline.

To test this in the present study, we investigated the effects of 10 h, as basal morning plasma CORT levels have

been found to be strongly increased at this time point (Reber *et al.* 2011), and 48 h of CSC on i) basal morning plasma CORT and ACTH concentrations, ii) relative *in vivo* adrenal CORT content, iii) relative *in vitro* adrenal ACTH responsiveness, iv) absolute and relative adrenal weights, and v) relative adrenal mRNA expression of the key steroidogenic enzymes *Star*, *Cyp11a1*, and *Cyp11b1*. Given that the attenuated ACTH responsiveness after 19 days of CSC exposure developed in both the left and right adrenal glands (Uschold-Schmidt *et al.* 2012), relative *in vivo* adrenal CORT content, relative *in vitro* adrenal ACTH responsiveness, and relative adrenal mRNA expression levels of *Star*, *Cyp11a1*, and *Cyp11b1* were assessed only in the left adrenal gland of an experimental mouse. Nevertheless, absolute and relative adrenal weight as assessed in both the left and right adrenal glands. Furthermore, vi) relative protein expression levels of the enzyme 11 β -hydroxysteroid dehydrogenase type 2 (HSD11B2), mediating the inactivation of CORT (for review, see Seckl & Walker (2001)), were assessed following 10 and 48 h of CSC exposure in the kidneys of both the SHC and CSC mice.

Materials and methods

Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19–22 g (experimental mice) were individually housed in standard polycarbonate mouse cages (16×22×14 cm) for 1 week before starting the CSC paradigm. As described previously (Füchsl *et al.* 2013), the male offspring (weighing 30–35 g) of high-anxiety-related behavior female mice (kindly provided by Prof. Dr R Landgraf, Max Planck Institute of Psychiatry, Munich) and C57BL/6 male mice (Charles River) were used as dominant animals. All mice were kept under standard laboratory conditions (12 h light:12 h darkness cycle, lights on at 0600 h, 22 °C and 60% humidity) and had free access to tap water and standard mouse diet. All experimental protocols were approved by the Committee on Animal Health and Care of the local government and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Experimental procedures

Experimental mice were either stressed by exposure to the CSC paradigm or SHC in a weight-matched setup.

Experimental mice were killed in the morning between 0800 and 1000 h following either 10 h (start of CSC

between 2200 and 2400 h) or 48 h (start of CSC between 0800 and 1000 h) of CSC exposure and body weight was assessed. To reveal the effects of CSC on absolute and relative adrenal weights, *in vivo* adrenal CORT content (only the left adrenal gland), *in vitro* adrenal ACTH responsiveness (only the left adrenal gland), and relative adrenal mRNA expression levels of *Star*, *Cyp11a1*, and *Cyp11b1* (only the left adrenal gland), the left and right adrenal glands were removed, pruned of fat, and treated according to the respective readout parameter as described below. To assess the effects of CSC on relative protein expression levels of HSD11B2, the left kidney was removed and treated as described below. Furthermore, trunk blood was collected from the SHC and CSC mice for the determination of basal morning plasma CORT and ACTH concentrations following 10 and 48 h of CSC exposure.

CSC paradigm

The CSC paradigm was conducted as described previously (Reber *et al.* 2007, Reber & Neumann 2008, Singewald *et al.* 2009, Slattery *et al.* 2012, Uschold-Schmidt *et al.* 2012). Briefly, 1 week after arrival, experimental mice were weighed and in a weight-matched manner assigned to either the SHC or the CSC group. The SHC mice remained undisturbed in their home cages except for change of bedding once a week. The CSC mice were housed in groups of four together with a dominant male for either 10 or 48 h, in order to induce psychosocial stress. The SHC and CSC mice were again weighed in the morning between 0800 and 1000 h following 10 or 48 h of CSC exposure, immediately before being killed by decapitation.

Trunk blood sampling

In the morning between 0800 and 1000 h following either 10 or 48 h of CSC exposure, the SHC and CSC mice were rapidly killed by decapitation under CO₂ anesthesia within 3 min after entering the animal room. Trunk blood was collected in EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) on ice and centrifuged at 4 °C (4200 g, 10 min). Plasma samples were stored at –20 °C until assayed for plasma CORT and ACTH concentrations using commercially available ELISA kits for CORT and ACTH (IBL International, Hamburg, Germany).

Determination of adrenal weight

After decapitation, the left and right adrenal glands of each mouse were removed, pruned of fat, and weighed

separately. The left adrenal glands were then used for the determination of *in vivo* adrenal CORT content, *in vitro* ACTH stimulation, or assessment of mRNA expression levels of *Star*, *Cyp11a1*, and *Cyp11b1*. Values represent absolute adrenal weight (mg) or relative adrenal weight (mg/body weight in g). In addition, the sum of left and right absolute and relative adrenal weights was calculated for each mouse. Owing to methodological problems, three 48-h SHC mice and one 48-h CSC mouse had to be excluded from the analysis of absolute and relative adrenal weights. These mice were, therefore, also excluded from the analysis of body weight.

Determination of *in vivo* adrenal CORT content

After removal, pruning of fat, and weighing, the left adrenal gland of each mouse was immediately frozen in liquid nitrogen. Further processing was performed as described previously (Uschold-Schmidt *et al.* 2012). Briefly, the left adrenal glands were homogenized with 20% ethanol in 1× PBS on ice and centrifuged at 4 °C (2688 g, 5 min), and supernatants were collected and stored at –20 °C until assayed for CORT concentrations using a commercially available ELISA (IBL International, Hamburg, Germany).

ACTH stimulation of adrenal explants *in vitro*

ACTH stimulation was performed as described previously (Uschold-Schmidt *et al.* 2012). Briefly, after removal, pruning of fat, and weighing, the left adrenal gland of each mouse was stored in ice-cold DMEM/F-12 (Life Technologies, Inc.) containing 0.1% BSA until all mice were killed and adrenals removed. Afterwards, each left adrenal gland was cut into two halves each containing cortical and medullary tissue. The halves were then weighed and pre-incubated in 200 µl DMEM/F-12 for 4 h (37 °C, 95% O₂ and 5% CO₂) before any further treatment. Culture medium was then replaced, and each half of one adrenal gland was incubated with either a medium containing saline (basal) or a medium containing ACTH (100 nM) for 6 h at 37 °C (95% O₂ and 5% CO₂). Afterwards, the supernatant of each adrenal explant was carefully removed and stored at –20 °C until assayed for CORT concentrations using a commercially available ELISA (IBL International).

ELISA for CORT and ACTH

Plasma and supernatant samples were analyzed using commercially available ELISA kits for CORT (analytical

sensitivity <1.631 nmol/l and intra-assay and inter-assay coefficients of variation (CV) \leq 6.35%; IBL International) and ACTH (plasma samples only; analytical sensitivity 0.22 pg/ml and intra-assay and inter-assay CV \leq 7.1%; IBL International). Supernatant CORT concentrations (ng/ml) were calculated in relation to the respective left adrenal explant weights (ng/ml per mg; *in vitro* adrenal ACTH stimulation; equal to relative *in vitro* CORT secretion) or in relation to the respective left adrenal gland weights (ng/ml per mg; *in vivo* adrenal CORT content; equal to relative adrenal *in vivo* adrenal CORT content). Owing to methodological problems, one 10-h SHC mouse, five 10-h CSC mice, and two 48-h CSC mice had to be excluded from the analysis of plasma CORT concentrations. Furthermore, for the same reason, one 10-h SHC mouse and two 10-h CSC mice had to be excluded from the analysis of relative *in vivo* adrenal CORT content and three 10-h SHC mice from the analysis of relative *in vitro* adrenal CORT secretion.

Western blotting for the assessment of HSD11B2 protein levels in the kidney

Protein expression analysis was performed as described recently (Füchsl *et al.* 2013). The left kidney of each mouse was removed and immediately shock-frozen in liquid nitrogen and stored at -80°C until assayed. For protein extraction, the frozen kidneys were homogenized separately in EDTA lysis buffer (50 mM EDTA, 250 mM NaCl, 0.5 mM HEPES, 0.5% Igepal, and 10% Complete Mini Protease Inhibitor; Roche Diagnostics GmbH), and total protein concentration was determined using a commercially available detection kit (Bicinchoninic Acid Protein Assay Kit, Thermo Scientific, Rockford, IL, USA). Western blotting was carried out using 40 μg of protein/kidney. Samples were loaded on SDS-polyacrylamide gels (10%) and subsequently transferred onto nitrocellulose membranes. The membranes were then blocked for 1 h at RT in 5% milk powder diluted in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBST; Sigma-Aldrich) before being probed with primary rabbit anti-HSD11B2 antibody (1:400; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) overnight at 4°C . Visualization was performed using HRP-conjugated goat anti-rabbit antibody (1:2000; Cell Signaling Technology, New England Biolabs GmbH, Frankfurt am Main, Germany) and later using ECL Western Blotting Detection Reagents (GE Healthcare, Freiburg, Germany). Immunoblots were digitized using Molecular Imager ChemiDoc XRS+ system and analyzed using Image the Lab Software (Bio-Rad Laboratories GmbH). Afterwards, the membranes were stripped using the Re-Blot Plus Mild Antibody Stripping

Solution (Millipore GmbH, Schwalbach, Germany), blocked twice with 5% milk powder in TBST for 5 min, and probed with primary rabbit anti- β -tubulin antibody (1:1000, Cell Signaling Technology, New England Biolabs GmbH, Frankfurt am Main, Germany) for 1 h at RT. Visualization and digitization were performed as described above (HRP-conjugated goat anti-rabbit antibody, 1:2000). Bands were detected at \sim 40 kDa for HSD11B2 and \sim 50 kDa for the loading control β -tubulin, as specified by the manufacturers. The expression of HSD11B2 was normalized to the respective β -tubulin protein expression and averaged per group.

Quantitative real-time PCR for *Star*, *Cyp11a1*, and *Cyp11b1* using TaqMan technology

The quantitative real-time PCR (qPCR) was performed as described recently (Reber *et al.* 2011, Uschold-Schmidt *et al.* 2012). Briefly, total RNA was prepared from the left adrenal tissue using the RNeasy Mini Kit (Qiagen) and reverse-transcribed into first-strand cDNA (25 ng/ μl RNA for each mouse, AffinityScript Multiple Temperature cDNA Synthesis Kit, Agilent Technologies, Waldbronn, Germany). The expression levels of murine *Star*, *Cyp11a1*, and *Cyp11b1* were quantified by TaqMan qPCR (7500 Fast Real-Time PCR System; Applied Biosystems) in single-tube reactions (20 μl) in 96-well plates. Primers and probes used were as follows: *Star* forward, GGA GCT CTC TGC TTG GTT CTC A; *Star* reverse, CAC CTC TCC CTG CTG GAT GTA G; *Star* probe, TCT ATA GTG ACC AGG AGC TGT; *Cyp11a1* forward, CCG GAG CGG TTC CTT GTG CC; *Cyp11a1* reverse, CAG GAC CCC AAT GGG CCT CTG A; *Cyp11a1* probe, CTG GGT GGC CTA TCA CCA GTA; and *Cyp11b1* forward, GCA GAG ATG ATG CTC CTG C; *Cyp11b1* reverse, CCG CAC ATC CTC TTT CTC TTG; *Cyp11b1* probe, TGT GCT GAA ATC CTT CCA CGT. The probes were labeled 5' with 6-carboxyfluorescein (FAM) and 3' with 6-carboxytetramethylrhodamine (TAMRA). TaqMan qPCR was performed using 2 μl cDNA, 0.15 μl forward and reverse primers (each 18 μM), 1.4 μl probe (5 μM), 10 μl Mastermix+0.3 μl ROX reference dye (Brilliant III Ultra-Fast QPCR Master Mix, Agilent Technologies, Waldbronn, Germany), and 0.6 μl glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) Mix (served as a reference; Applied Biosystems) and made up to the final volume of 20 μl with sterile H_2O . Cycling was as follows: 95°C for 3 min followed by 40 repeats of 95°C for 15 s and 60°C for 30 s. Expression value normalized to *Gapdh* mRNA expression was quantified for each mouse and averaged per group. SHC values were set to 100%, and the percentage change in mRNA expression following 10 or 48 h of CSC was calculated.

Statistical analysis

For statistical comparisons, the software package SPSS statistics (version 19.0) was used. Data of the two experimental groups (SHC vs CSC) were compared employing the Student's *t*-test. The absolute and relative left and right adrenal weights (factors CSC and body side), plasma CORT and ACTH concentrations, left *in vivo* adrenal CORT content, HSD11B2 protein expression in the kidney, left adrenal mRNA expression of *Star*, *Cyp11a1*, and *Cyp11b1* (factors CSC and duration), and left *in vitro* adrenal CORT secretion (factors CSC and ACTH) were compared using a two-way ANOVA followed by a *post hoc* Bonferroni's test when appropriate. In addition, for statistically significant differences, the effect size (Cohen's *d* values; standardized mean difference) as a measure of the magnitude of the difference between the groups was calculated. Cohen's $d=0.2-0.5$ is conceived as a small effect, $d=0.5-0.8$ as a medium effect, and $d \geq 0.8$ as a large effect. Correlation analysis of plasma ACTH concentrations with left *in vivo* adrenal CORT content in the CSC mice was carried out employing Pearson's correlation. Data represent means + s.e.m. Significance was taken at $P < 0.05$.

Results

Plasma CORT concentrations are increased following 10 h, but not 48 h, of CSC exposure

Basal morning plasma CORT concentrations following 10 and 48 h of CSC exposure were found to be dependent on the factors CSC ($F_{1,120} = 32.24$; $P < 0.001$) and duration ($F_{1,120} = 38.46$; $P < 0.001$) and also on the interaction between both the factors ($F_{1,120} = 26.97$; $P < 0.001$) (Fig. 1A). *Post hoc* analysis revealed significantly increased plasma CORT concentrations in the CSC mice compared with the SHC mice following 10 h ($P < 0.001$; $d = 1.42$), but not 48 h, of CSC exposure. Consequently, basal morning plasma CORT concentrations were significantly lower in the CSC mice at the 48-h time point compared with the 10-h time point ($P < 0.001$; $d = 1.56$) (Fig. 1A).

Plasma ACTH levels are increased following both 10 and 48 h of CSC exposure

Basal morning plasma ACTH concentrations following 10 and 48 h of CSC exposure were found to be dependent on the factor CSC ($F_{1,127} = 23.63$; $P < 0.001$) as well as the factor duration ($F_{1,127} = 11.63$; $P = 0.001$) (Fig. 1B). Although *post hoc* analysis revealed a significant increase

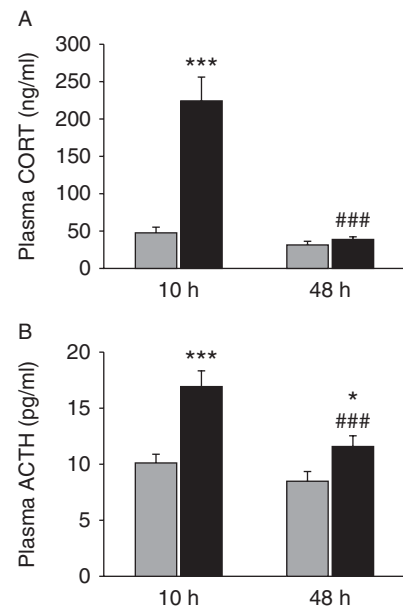


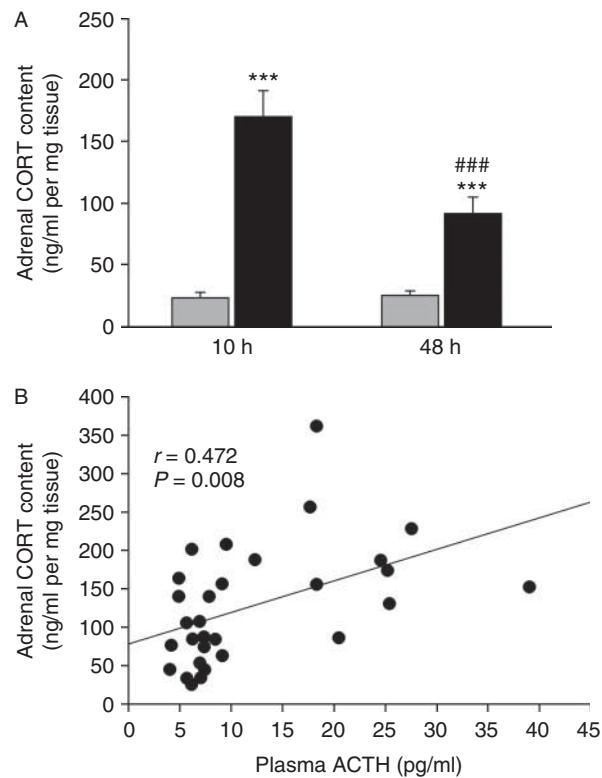
Figure 1

Effects of 10 and 48 h of CSC exposure on basal morning plasma CORT and ACTH concentrations. SHC and CSC mice were decapitated in the morning between 0800 and 1000 h following either 10 h (A and B; left panel) or 48 h (A and B; right panel) of CSC exposure and trunk blood was collected. Afterwards, plasma CORT (ng/ml; SHC, $n = 29-30$; CSC, $n = 31-34$; A) and plasma ACTH (pg/ml; SHC, $n = 30$; CSC, $n = 35-36$; B) concentrations were determined. Gray bar, SHC; black bar, CSC. Data represent means + s.e.m. * $P < 0.05$, *** $P < 0.001$ vs respective SHC mice, and ### $P < 0.001$ vs respective 10-h time point.

in plasma ACTH levels in the CSC mice compared with SHC mice following both 10 h ($P < 0.001$; $d = 1.04$) and 48 h ($P = 0.032$; $d = 0.62$) of CSC exposure, the increase was less pronounced at the latter time point (10 vs 48 h CSC, $P < 0.001$; $d = 0.78$; Fig. 1B).

Relative *in vivo* adrenal CORT content is increased following both 10 and 48 h of CSC exposure

Relative (per mg tissue) *in vivo* adrenal CORT content following 10 and 48 h of CSC exposure was found to be dependent on the factors CSC ($F_{1,57} = 74.49$; $P < 0.001$) and duration ($F_{1,57} = 9.57$; $P = 0.003$) as well as on the interaction between both the factors ($F_{1,57} = 10.57$; $P = 0.002$) (Fig. 2A). Although *post hoc* analysis revealed an increased relative *in vivo* adrenal CORT content in the CSC mice compared with the SHC mice following both 10 h ($P < 0.001$; $d = 2.60$) and 48 h ($P < 0.001$; $d = 1.69$) of CSC exposure, it was less pronounced at the latter time point (10 vs 48 h CSC, $P < 0.001$; $d = 1.17$; Fig. 2A). Interestingly, statistical analysis further revealed

**Figure 2**

Effects of 10 and 48 h of CSC exposure on relative *in vivo* adrenal CORT content. SHC and CSC mice were killed following either 10 h (SHC, $n = 15$; CSC, $n = 14$) or 48 h (SHC and CSC, $n = 16$) of CSC exposure. The left and right adrenal glands were removed, pruned of fat, and weighed. Afterwards, the left adrenal glands were homogenized and relative adrenal CORT content (ng/ml per mg tissue) was determined in the supernatants (A). Gray bar, SHC; black bar, CSC. Data represent means \pm s.e.m. *** $P < 0.001$ vs respective SHC mice and ### $P < 0.001$ vs respective 10-h time point. Additionally, Pearson's correlation analysis was performed for basal morning plasma ACTH concentrations (pg/ml) with relative *in vivo* adrenal CORT content (ng/ml per mg tissue) in the CSC mice (10 and 48 h; $n = 30$; B).

a positive correlation for basal morning plasma ACTH concentrations with relative *in vivo* adrenal CORT content in the CSC mice (10 and 48 h; Pearson's $r = 0.472$; $P = 0.008$; Fig. 2B).

Relative *in vitro* adrenal CORT secretion in response to ACTH is not affected following both 10 and 48 h of CSC exposure

Relative (per mg tissue) *in vitro* adrenal CORT secretion was found to be dependent only on the factor ACTH treatment following both 10 h ($F_{1,58} = 19.51$; $P < 0.001$) and 48 h of CSC exposure ($F_{1,64} = 19.74$; $P < 0.001$). *Post hoc* analysis revealed that relative *in vitro* adrenal CORT secretion in both the SHC and CSC mice was significantly increased

following ACTH treatment compared with the respective basal values at both time points (for each $P \leq 0.004$; $d \geq 0.98$; Fig. 3A and B).

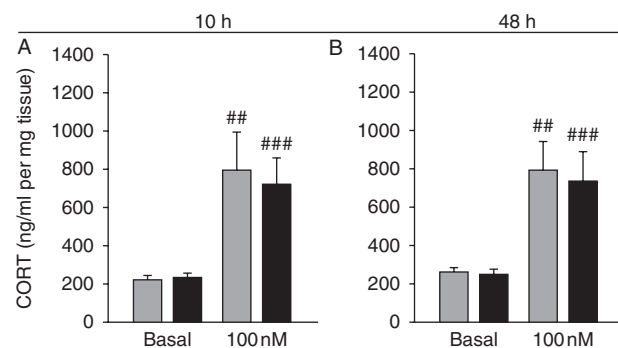
Body weight is decreased following 10 and 48 h of CSC exposure

Following both 10 h ($P = 0.006$; $d = 0.70$; Fig. 4B) and 48 h ($P < 0.001$; $d = 1.02$; Fig. 4D) of CSC exposure, body weight was significantly decreased in the CSC mice compared with the SHC mice. Importantly, the body weights of the SHC and CSC mice were neither different before the 10-h CSC exposure nor before the 48-h CSC exposure (Fig. 4A and C).

Relative left and right adrenal weight is increased following 10 and 48 h of CSC exposure

Following 10 and 48 h of CSC exposure, relative adrenal weight of both adrenal glands (the left and right adrenal weights of each mouse was summed up) was significantly increased in the CSC mice compared with the SHC mice (10 h, $P \leq 0.001$; $d = 1.38$; 48 h, $P \leq 0.001$; $d = 0.92$; Fig. 5B and D).

Statistical analysis, considering the factors body side and CSC exposure, revealed a significant main effect of both the factors following 10 h (body side, $F_{1,128} = 41.92$; $P < 0.001$; CSC, $F_{1,128} = 55.56$; $P < 0.001$) and 48 h (body

**Figure 3**

Effects of 10 and 48 h of CSC exposure on *in vitro* adrenal CORT secretion in response to ACTH. SHC and CSC mice were killed following either 10 h (A) or 48 h (B) of CSC exposure. The left and right adrenal glands were removed, pruned of fat, and weighed separately. Afterwards, the left adrenal glands were cut into two halves. These left adrenal halves of the SHC (10 h, $n = 11$; 48 h, $n = 14$) and CSC (10 h, $n = 20$; 48 h, $n = 20$) groups were weighed again and incubated with a medium containing either saline (basal) or 100 nM ACTH for 6 h. Afterwards, CORT concentrations (ng/ml per mg tissue) were determined in the supernatants. Gray bar, SHC; black bar, CSC. Data represent means \pm s.e.m. ## $P < 0.01$ and ### $P < 0.001$ vs respective basal values.

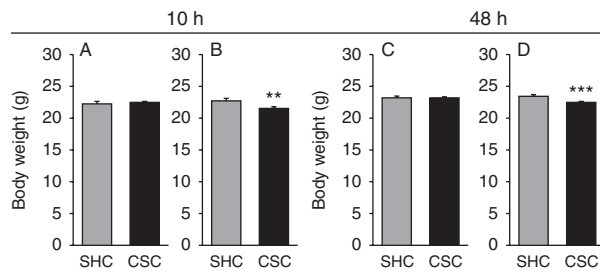


Figure 4

Effects of 10 and 48 h of CSC exposure on body weight. Body weight (g) was assessed in the SHC (10 h, $n=30$; 48 h, $n=27$) and CSC (10 h, $n=36$; 48 h, $n=35$) mice before the start of CSC exposure (A and C) and following 10 h (B) and 48 h (D) of CSC exposure before the mice were killed by decapitation. Gray bar, SHC; black bar, CSC. Data represent means \pm s.e.m. ** $P<0.01$ and *** $P<0.001$ vs respective SHC mice.

side, $F_{1,120}=93.20$; $P<0.001$; CSC, $F_{1,120}=19.71$; $P<0.001$) of CSC exposure (Fig. 5A and C). *Post hoc* testing further revealed that relative weight of both the left (10 h, $P<0.001$; $d=1.31$; 48 h, $P<0.001$; $d=1.01$) and right (10 h, $P<0.001$; $d=1.30$; 48 h, $P=0.020$; $d=0.59$) adrenal glands was increased in the CSC mice compared with the SHC mice following 10 and 48 h of CSC exposure. Moreover, relative weight of the left adrenal gland was increased compared with that of the right adrenal gland in both the SHC and CSC mice (for each $P<0.001$ and $d \geq 1.07$; Fig. 5A and C) following 10 h as well as 48 h of CSC exposure.

Absolute left and right adrenal weight is increased following 10 h, but not 48 h, of CSC exposure

Following 10 h of CSC exposure, absolute weight of both the adrenal glands (the left and right adrenal weights of each mouse were summed up) was significantly increased in the CSC mice compared with the SHC mice ($P<0.001$; $d=1.33$; Fig. 5F), an effect that was absent after 48 h of CSC exposure (Fig. 5H).

Statistical analysis, considering the factors body side and CSC exposure, revealed a significant main effect of both the factors following 10 h of CSC exposure (body side, $F_{1,128}=80.27$; $P<0.001$; CSC exposure, $F_{1,128}=46.57$; $P<0.001$) and of only the factor body side ($F_{1,120}=116.75$; $P<0.001$) following 48 h of CSC exposure. *Post hoc* analysis further revealed that absolute weight of both the left ($P<0.001$; $d=1.23$) and right ($P<0.001$; $d=1.15$) adrenal glands was increased in the CSC mice compared with the SHC mice following 10 h of CSC exposure. Moreover, absolute weight of the left adrenal gland was increased compared with that of the right adrenal gland in both the

SHC and CSC mice following both 10 and 48 h of CSC exposure (for each $P<0.001$; $d \geq 1.46$; Fig. 5E and G).

Relative protein expression levels of 11 β -HSD2 in the kidney are not affected following 10 and 48 h of CSC exposure

Relative (per 40 μ g of total protein) 11 β -HSD2 protein expression in the kidney was affected neither by the factor CSC nor by the factor duration (Fig. 6A and B).

Relative adrenal mRNA expression levels of *Star*, *Cyp11a1*, and *Cyp11b1* are unaffected or decreased following 10 h and unaffected or even increased following 48 h of CSC exposure

Relative adrenal mRNA expression of *Star* was affected neither by the factor CSC nor by the factor duration following both time points (Table 1). However, relative adrenal mRNA expression of *Cyp11a1* following 10 and 48 h of CSC exposure was found to be dependent on

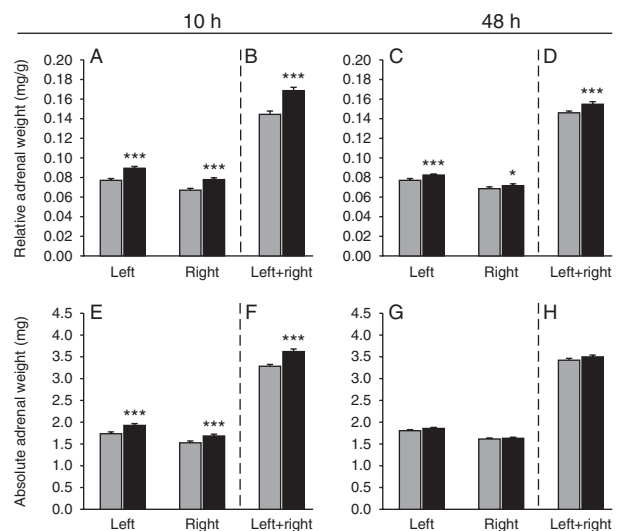


Figure 5

Effects of 10 and 48 h of CSC exposure on relative and absolute adrenal weights. SHC and CSC mice were killed following either 10 h (A, B, E and F) or 48 h (C, D, G and H) of CSC exposure. The left and right adrenal glands were removed, pruned of fat, and weighed separately. The relative (mg/g; A, B, C and D) and absolute (mg; E, F, G and H) weights of the left and right adrenal glands (A, C, E and G) of the SHC (10 h, $n=30$; 48 h, $n=27$) and CSC (10 h, $n=36$; 48 h, $n=35$) mice are shown. In addition, the sum of relative (B and D) and absolute (F and H) left and right adrenal weights of the SHC and CSC mice is shown for both time points. Symbols indicating significant differences are only shown for effects between the groups. For significant within-group differences, see detailed information given in the Results section. Gray bar, SHC; black bar, CSC. Data represent means \pm s.e.m. ** $P<0.05$ and *** $P<0.001$ vs respective SHC mice.

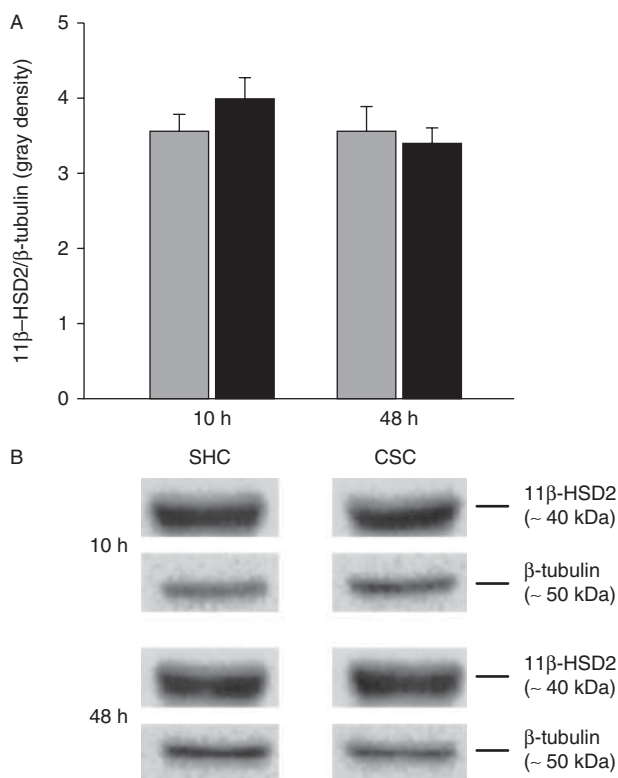


Figure 6

Effects of 10 and 48 h of CSC exposure on the relative protein expression levels of HSD11B2 in the kidney. SHC and CSC mice were killed following either 10 h (SHC and CSC, $n=8$; A, left panel) or 48 h (SHC, $n=8$; CSC: $n=7$; A, right panel) of CSC exposure. The left kidney was removed and protein was extracted for the determination of HSD11B2 protein expression (gray density) normalized to the expression of the loading control β -tubulin. Gray bar, SHC; black bar, CSC. Data represent means \pm S.E.M. In addition, representative images of the bands detected for HSD11B2 (~ 40 kDa; B) and the respective loading control β -tubulin (~ 50 kDa; B) are shown for the SHC and CSC mice following both time points.

the factors CSC ($F_{1,27}=9.05$; $P=0.006$) and duration ($F_{1,27}=28.90$; $P<0.001$) as well as on the interaction between both the factors ($F_{1,27}=28.90$; $P<0.001$), and adrenal mRNA expression of *Cyp11b1* was dependent on the factor duration ($F_{1,27}=80.68$; $P<0.001$) and on the interaction between the factors CSC and duration ($F_{1,27}=80.67$; $P<0.001$). *Post hoc* analysis further revealed that mRNA expression of *Cyp11a1* was significantly decreased in the CSC mice compared with the SHC mice following 10 h ($P<0.001$; $d=2.63$), but not 48 h, of CSC. Consequently, mRNA expression of *Cyp11a1* was significantly higher in the CSC mice at the 48-h time point compared with the 10-h time point ($P<0.001$; $d=4.40$). *Cyp11b1* mRNA expression in the CSC mice compared with the SHC mice was significantly decreased following 10 h ($P<0.001$; $d=3.58$) and significantly increased following 48 h ($P<0.001$; $d=3.05$) of CSC exposure. Consequently,

mRNA expression of *Cyp11b1* was also significantly higher in the CSC mice at the 48-h time point compared with the 10-h time point ($P<0.001$; $d=5.39$; Table 1).

Discussion

The findings of the present study indicate that basal hypercorticism during the initial phase of CSC exposure is not prevented by the development of an attenuated ACTH responsiveness of the adrenal glands. They rather suggest that the normalization of initially increased basal morning plasma CORT concentrations following 48 h of CSC exposure is mediated by an attenuated pituitary ACTH release and the restoration of normal functional adrenal mass. Although the data of the present study further suggest an enhanced enzymatic inactivation of CORT and a normalization of steroidogenic enzyme expression to at least partly contribute to the normalization of basal plasma CORT levels following 48 h of CSC exposure, future studies need to address this in detail.

To investigate basal HPA axis activity during the initial phase of CSC exposure, mice were killed in the morning following either 10 or 48 h of psychosocial stressor exposure. In line with previous findings (Reber *et al.* 2007, 2011), basal morning plasma CORT concentrations were significantly increased in the CSC mice compared with the SHC mice following 10 h (eight- to ninefold), but not 48 h, of CSC exposure. By contrast, basal morning plasma ACTH concentrations were increased following 10 h as well as 48 h of CSC, with the increase being less pronounced following the latter. Together, these findings at first glance support our initial hypothesis that the attenuated adrenal ACTH responsiveness observed following 19 days of CSC exposure (Uschold-Schmidt *et al.* 2012) already develops during the initial phase of psychosocial stressor exposure to prevent chronic basal hypercorticism.

However, although basal morning plasma CORT levels were increased following 10 h, but not 48 h, of CSC exposure, relative *in vivo* adrenal CORT content was found to be increased in the CSC mice compared with the SHC mice at both time points. Interestingly, this increase was again less pronounced following 48 h than following 10 h of CSC, well in line with the changes observed in plasma ACTH concentrations and, thus, clearly arguing against our initial hypothesis. The idea of fully ACTH-sensitive adrenal glands during this initial CSC phase is further supported by the fact that the statistical analysis even revealed a positive correlation of *in vivo* adrenal CORT content with plasma ACTH

Table 1 Effects of 10 and 48 h of CSC exposure on adrenal mRNA expression of *Star*, *Cyp11a1*, and *Cyp11b1*. SHC and CSC mice were killed following either 10 or 48 h of CSC exposure. The left and right adrenal glands were removed and pruned of fat. Afterwards, total RNA was extracted from each left adrenal gland of the SHC and CSC mice and reverse-transcribed into cDNA for the determination of adrenal mRNA expression of *Star*, *Cyp11a1*, and *Cyp11b1* via qPCR using TaqMan technology normalized to mRNA expression of the housekeeping gene *Gapdh*. SHC values were set to 100%, and the percentage change in mRNA expression following 10 or 48 h of CSC exposure was calculated. Numbers in parentheses indicate group sizes. Data represent means + s.e.m.

	10 h		48 h	
	SHC (8)	CSC (8)	SHC (8)	CSC (7)
<i>Star</i>	100.00+3.76	83.41+3.67	100.00+7.66	106.29+8.38
<i>Cyp11a1</i>	100.00+5.91	58.75+5.16*	100.00+4.65	111.65+3.05 [†]
<i>Cyp11b1</i>	100.00+3.52	69.89+2.29*	100.00+1.99	137.78+6.49* [†]

* $P < 0.001$ vs respective SHC mice and [†] $P < 0.001$ vs respective 10-h time point.

concentrations in the CSC mice in the present study. Furthermore, relative *in vitro* CORT secretion in response to ACTH (100 nM) was comparable between the SHC and CSC mice following both 10 and 48 h of CSC exposure, also suggesting an unaffected adrenal responsiveness to the main CORT secretagogue.

Given that plasma CORT concentrations were comparable between the SHC and CSC mice, despite a still increased relative *in vivo* adrenal CORT content following 48 h of CSC exposure, this suggests an enhanced enzymatic inactivation of CORT at that time point, contributing at least partly to the prevention of prolonged basal hypercorticism. Enzymatic degradation of CORT in its biologically inactive 11-keto form is mainly mediated by HSD11B2, most highly expressed in aldosterone target tissues such as the kidney (for review, see Seckl & Walker (2001)). Importantly, although relative protein levels of HSD11B2 in the kidney were unaffected following both 10 and 48 h of CSC exposure, the activity of this enzyme might still be enhanced in the CSC mice compared with the SHC mice and needs to be addressed in future studies.

To investigate whether alterations in the expression levels of key steroidogenic enzymes are likely to contribute to the changes in basal morning plasma CORT concentrations during the initial phase of CSC exposure, we further analyzed the mRNA expression levels of *Star*, *Cyp11a1*, and *Cyp11b1* following both 10 and 48 h of CSC exposure. In contrast to what one would expect if changes in steroidogenic enzyme expression indeed contribute to the changes in basal plasma CORT concentrations during the initial CSC phase, the expression levels of these enzymes were unaffected (*Star*) or significantly decreased (*Cyp11a1* and *Cyp11b1*) following 10 h of CSC

exposure and either unaffected (*Star* and *Cyp11a1*) or even increased (*Cyp11b1*) following 48 h of CSC exposure. However, it has been repeatedly shown for adrenal tissue that mRNA and protein expression levels of steroidogenic enzymes do not necessarily correspond to each other (Lehoux *et al.* 1992, 1998). Therefore, a divergent regulation of protein expression of these enzymes in the present study is not unlikely, probably contributing to the changes in basal morning plasma CORT concentrations during the initial phase of CSC exposure.

In one of our previous studies, we showed that relative adrenal mass is significantly increased during CSC at all time points assessed, beginning as early as 24 h after the start of chronic psychosocial stressor exposure (Reber *et al.* 2007). In the present study, we confirmed the increase in relative adrenal weight following 48 h of CSC exposure and extended these findings even to the 10-h CSC time point. However, in contrast to relative adrenal weight, absolute weight of both the left and right adrenal glands was increased only after 10 h, but not 48 h, of CSC exposure. Considering the CSC-induced reduction in body weight following both 10 and 48 h of CSC exposure, which is in line with previous findings (Slattery *et al.* 2012), this clearly indicates that the increase in relative left and right adrenal weights observed following 48 h of CSC is exclusively due to changes in body weight and not to changes in the adrenal weight itself. Interestingly, these findings seem to be in line with those of our above-mentioned study assessing the time course of relative adrenal weight during CSC exposure. Although relative adrenal weight of the CSC mice was increased compared with the SHC mice at all the time points assessed, a closer look at this parameter during the initial CSC phase clearly

indicates a pronounced drop in relative adrenal weight at the 48-h time point (Reber *et al.* 2007). Taken together, the changes in absolute adrenal weight during the initial phase of CSC seem to run in parallel with the changes in basal morning plasma CORT levels. Following 10 h of CSC exposure, both parameters are significantly increased, whereas following 48 h of CSC exposure, both parameters return to baseline values. Thus, it seems that reversing the early increase in adrenal mass in the CSC mice (10 h) contributes, at least partly, to prevent them from prolonged exposure to elevated plasma CORT levels. This is supported by previous studies showing a positive correlation between plasma CORT concentrations and adrenal weight under stress conditions in rats (Schwartz *et al.* 1997, Baranyi *et al.* 2005). However, future studies are needed to clarify whether these early changes in absolute adrenal weight are mediated by hyper/hypotrophy or by hyperplasia/apoptosis of adrenal cells.

In summary, the data of the present study indicate that elevated basal morning plasma CORT levels following 10 h of CSC exposure seem to be due to a concurrent increase in the release of pituitary ACTH and functional adrenal tissue in both the left and right adrenal glands and not to changes in adrenal ACTH responsiveness. Whether and to what extent an altered mRNA turnover of key steroidogenic enzymes is involved in the CSC-induced initial changes in plasma CORT levels need to be addressed in future studies. Furthermore, in contrast to the situation faced following 19 days of CSC exposure, at which hypercorticism seems to be prevented by a reduction in adrenal ACTH responsiveness, basal hypercorticism during the initial phase of CSC seems to be prevented by an attenuated release of pituitary ACTH and, most likely to a lesser degree, by restoration of normal functional adrenal mass. Whether and to what extent an enhanced enzymatic inactivation of CORT, which is clearly suggested by the findings of the present study, plays a crucial role also has to be addressed in future studies. Thus, together with previous findings (Uschold-Schmidt *et al.* 2012), our data suggest different mechanisms to be involved in the restoration of normal basal plasma CORT concentrations and, consequently, in the prevention of basal hypercorticism during either the initial or chronic phase of homotypic psychosocial stressor exposure.

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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Author contribution statement

N U-S was responsible for the conception and design of experiments, collection, analysis and interpretation of data, and manuscript writing. D P and A M F were responsible for the collection and analysis of data. S O R was responsible for the conception and design of experiments, revision of the manuscript, and supervision of the project.

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