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Short communication

The impact of early-life stress on corticosteroid carrier protein levels and 11β-hydroxysteroid dehydrogenase 1 expression in adolescent rats

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ABSTRACT

Background: Corticosteroid-binding globulin (CBG), albumin and 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes play crucial roles in the bioavailability of glucocorticoids. Downstream of the adrenal glands, these proteins affect glucocorticoid levels in target tissues. Early-life stress (ELS) is known to program glucocorticoid action at many levels. The effects of ELS on the concentrations and synthesis of CBG and albumin and on the expression of 11β-HSD remain unclear.

Methods: The maternal separation (MS) procedure in rats on postnatal days 1–14 was used as a model of ELS. On postnatal day 35 (adolescence), the serum corticosterone, CBG and albumin concentrations of male rats were measured by ELISA, while the mRNA and protein levels of CBG, albumin and 11β-HSD1 in the liver and brain were examined by RT-qPCR and Western blot, respectively.

Results: Under basal conditions, MS rats displayed lower levels of serum CBG and albumin. However, MS did not affect CBG or albumin synthesis in the liver, suggesting that the half-life and/or secretion of these proteins were influenced by MS. Additionally, MS rats showed increased protein expression of 11β-HSD1, specifically in the medial prefrontal cortex.

Conclusions: These results indicate that ELS may potentially program glucocorticoid action through its effects on glucocorticoid bioavailability in tissues.

Introduction

Clinical data show that early-life stress (ELS) increases the risk of mood and anxiety disorders in both young subjects and adults [1]. ELS is known to alter brain development and maturation [2]. However, not all subjects exposed to ELS develop psychopathology [2]. Vulnerability or resilience to the effects of ELS can be programmed on many different physiological and molecular levels [3]. One such level is glucocorticoid (GC) signaling in the brain and periphery [3]. GCs affect brain maturation and many crucial brain functions, especially in the hippocampus (HP) and medial prefrontal cortex (mPFC), i.e., in the regions implicated in stress regulation and cognitive function, as well as the pathophysiology of mood and anxiety disorders [3,4]. GCs mediate their cellular action through two types of receptors, glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) [4]. The effects of GR and MR signaling depend on the level of GC receptors expression and the availability of GCs in tissue [4]. Although GC secretion is the main regulatory process that determines tissue GC concentration, many downstream factors may also affect GC levels, e.g., the carrier proteins that bind GCs in the blood [4] or 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes engaged in GC metabolism at the intracellular level [5]. Under basal conditions, 80–90% of GCs in the blood are bound to corticosteroid-binding globulin (CBG), and 10–15% are bound to albumin. Importantly, 5% of GCs are unbound, and only this fraction can reach the tissues and interact with GC receptors [4,6]. CBG and albumin are produced and secreted by the liver [4,6].

Among the two forms of 11β-HSD, 11β-HSD1 is ubiquitously expressed in multiple tissues, including the brain and liver [5]. *In vivo*, 11β-HSD1 reduces inactive 11-keto derivatives of GCs into active forms, e.g., dehydrocorticosterone and cortisone into corticosterone and cortisol, respectively. In this way, 11β-HSD1 increases the availability of active GCs in tissues [5]. The liver is the main organ involved in GC metabolism. 11β-HSD1 action in the liver contributes 20–40% of the daily GC production, thus it strongly influences the half-life of GCs [5].

Considerable amounts of data have accumulated showing that ELS affects the activity of the hypothalamic-pituitary-adrenal (HPA) axis and the expression and function of GC receptors [7–10]. In the present study, we hypothesize that ELS may also influence

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GC signaling *via* mechanisms independent of HPA axis activation and GC receptor expression. Therefore, we applied the maternal separation (MS) procedure in rats, which mimics ELS, and studied its effects on serum CBG and albumin concentrations, as well as CBG, albumin synthesis and 11β-HSD1 expression in the liver. Furthermore, we studied the impact of MS on CBG and 11β-HSD1 expression in the mPFC and HP. The experiments were carried out in adolescent rats because during adolescence, stress-sensitive brain areas, especially the mPFC, undergo substantial structural and functional remodeling [11,12]. Unbalanced GC signaling during adolescence may enhance the risk for early-onset psychopathologies [11,13].

Materials and methods

Animals

All experimental procedures were approved by the Local Committee for Laboratory Animal Welfare and the Ethics Committee in Krakow, Poland and met the requirements of the European Council Guide for the Care and Use of Laboratory Animals (86/609/EEC). Adult Wistar rats were purchased from Charles River Laboratories (Sulzfeld, Germany). All animals were housed under controlled conditions with an artificial 12 h light/dark cycle (lights on from 07:00 to 19:00), humidity at 55 ± 10% and temperature of 22 ± 2 °C. Food and tap water were freely available. The rats were mated at the Institute Animal Facility. Before delivery, the dams were housed individually in standard plastic cages (38 × 24 × 19 cm). The day of birth was designated postnatal day (PND) 0. On PND 1, the litter size was standardized to eight pups per litter (four males and four females), and the litters were randomly assigned to one of the following rearing conditions: maternal separation (MS) or animal facility rearing (AFR), i.e., control conditions.

Maternal separation procedure

The maternal separation procedure has been previously described [14–16]. On PNDs 1–14, the dams and pups were removed from the maternity cages for 3 h (09:00–12:00) daily. The mothers were placed individually in the holding cages (38 × 24 × 19 cm), while each litter was placed in a plastic container (22 × 16 × 10 cm) lined with fresh bedding material, moved to an adjacent room and placed in an incubator (34 °C). After the 3 h separation, the pups and dams were returned to the maternity cages. AFR animals were left undisturbed with their mothers except during the weekly cage cleaning. On PND 22, the animals were weaned, sexed and randomly distributed and designated to the specific experimental groups. The rats were housed in standard plastic cages (57 × 33 × 20 cm) in same-sex groups of five unrelated subjects and under the same treatment protocol until adolescence (PND 35). Male offspring from 12 AFR and 12 MS litters were used in the study. The final experimental groups consisted of unrelated subjects (n = 5–9).

Tissue collection

On PND 35, rats from each experimental group were sacrificed *via* decapitation between 8 a.m. and 12 a.m. in counterbalanced, alternating order. Trunk blood was collected, and the brains were immediately removed from the skulls, whereas the livers were dissected from the trunks. Samples of the mPFC (including the prelimbic and infralimbic regions) were dissected from 1-mm-thick coronal slices using a rodent brain matrix (Ted Pella, Inc., CA, USA), whereas the HP was collected whole. The liver samples were collected from both lobes (ca. 60 mg/lobe). Brain and liver tissues were quickly frozen in liquid nitrogen and stored at −80 °C for later use. Blood was centrifuged (10000 ×*g*, 5 min), and the serum was stored at −20 °C until the day of the assay.

Enzyme-linked immunosorbent assay (ELISA)

Commercially available ELISA kits were used to determine the serum concentrations of corticosterone (LDN, Labor Diagnostika Nord GmbH & Co., Germany), albumin (GenWay Biotech, Inc., USA) and CBG (Cloud-Clone Corp., USA) according to the manufacturer's instructions.

RT-qPCR

The RT-qPCR method used in this study has been previously described in detail [15]. Briefly, the total RNA was extracted from the brain and liver samples using the QuickGene-810 Nucleic Acid Isolation System and the QuickGene RNA tissue kit S II (Kurabo, Japan). RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). qPCR was performed using the QuantStudio 12 K Flex system (Applied Biosystems). TaqMan® Gene Expression Assays (Thermo Fisher Scientific) were used, including Rn00592480_m1 for Alb (albumin), Rn01517119_m1 for Cbg, Rn00567167_m1 for Hsd11b1, and Rn99999916_s1 for glyceraldehyde-3-phosphate dehydrogenase (Gapdh), which was used for assay normalization. The abundance of RNA was calculated according to the following equation: abundance = $2^{-(\text{threshold cycle})}$. The results were normalized to Gapdh expression levels.

Western blot analysis

The Western blot method used in this study has been previously described in detail [14]. Briefly, tissue samples from the brain and liver were run on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto a nitrocellulose membrane (BioRad, USA). The blots were probed with the following primary antibodies at the specified dilutions: mouse anti-serpin A6 (CBG) (1:1000; R&D System, USA), rabbit anti-albumin (1:1000, Santa Cruz Biotechnology, Inc., USA), rabbit anti-11β-HSD1 (1:500, Abcam), and mouse anti-GAPDH (1:1000, Abcam). The blots were visualized using a Lumi-LightPLUS Western Blotting Kit (Roche Diagnostics, Switzerland) and evaluated using a luminescent image analyzer (LAS-4000, Fujifilm, USA) and Image Gauge software (Fujifilm, USA). The results were normalized to GAPDH expression levels.

Statistical analysis

The data are presented as the group means ± SEM. Statistical analysis was performed using the Statistica 12 package (StatSoft Inc., OK, USA). Data were analyzed by one-way ANOVA with the rearing conditions as the independent variable. P values < 0.05 were considered significantly different.

Results

The effects of MS on serum corticosterone, CBG and albumin levels under basal conditions

MS did not significantly affect basal serum corticosterone levels in adolescent rats ($F(1,22) = 0.38$, $p = 0.542$) (Fig. 1A). Concurrently, under basal conditions, MS rats showed reduced serum concentrations of both CBG ($F(1,14) = 8.97$, $p = 0.01$) (Fig. 1B) and albumin ($F(1,16) = 18.13$, $p = 0.001$) (Fig. 1C) compared to those of AFR rats.

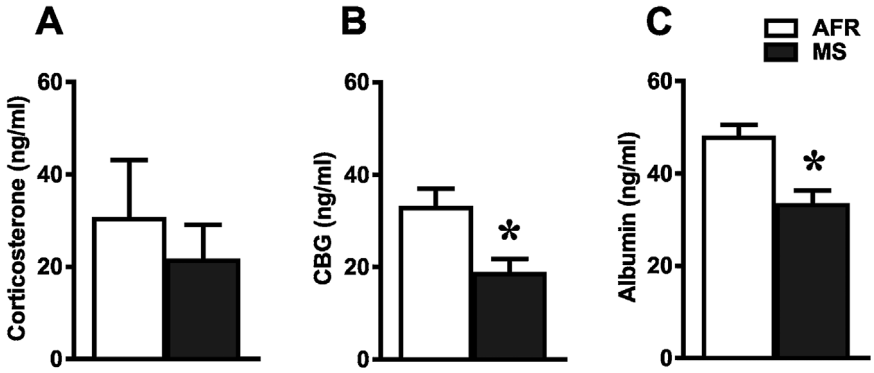

Fig. 1. The effects of MS on serum corticosterone (A), CBG (B) and albumin (C) levels under basal conditions determined by ELISA. The data are presented as the group mean ± SEM, $n = 8–9$. $^{*}p < 0.05$ compared with AFR rats.

The effects of MS on CBG and albumin synthesis and 11β-HSD1 expression in the liver

Statistical analysis did not reveal any significant effects of MS on either CBG mRNA or protein expression in the liver; however, a trend toward lower CBG protein expression was observed in MS rats (mRNA: $F(1,11) = 0.537$, $p = 0.479$; protein: $F(1,12) = 3.533$, $p = 0.085$) (Table 1; Fig. 2A). MS did not affect the synthesis of albumin (mRNA: $F(1,11) = 1.276$, $p = 0.283$; protein: $F(1,12) = 0.498$, $p = 0.496$) and 11β-HSD1 expression (mRNA: $F(1,10) = 0.544$, $p = 0.478$; protein: $F(1,12) = 0.004$, $p = 0.947$) in the liver as well (Table 1, Fig. 2B–C).

The effects of MS on CBG and 11β-HSD1 expression in the brain

Our study revealed undetectable levels of CBG mRNA in the mPFC and HP (Ct > 38). Conversely, 11β-HSD1 mRNA and proteins were abundant in the mPFC and HP. Statistical analysis revealed that MS specifically increased the expression of the 11β-HSD1 protein ($F(1,10) = 7.35$, $p = 0.021$) but not 11β-HSD1 mRNA ($F(1,8) = 0.026$, $p = 0.875$) in the mPFC (Table 2, Fig. 2D). However, MS did not affect 11β-HSD1 mRNA or protein expression in the HP (mRNA: $F(1,10) = 0.915$, $p = 0.361$; protein: $F(1,10) = 0.005$, $p = 0.944$) (Table 2, Fig. 2E).

Discussion

Our study showed that MS decreased the serum concentrations of GC carrier proteins and increased 11β-HSD1 protein expression in the mPFC of adolescent rats.

Sparse data have revealed that chronic stress decreases CBG levels in adult rats [17]. Additionally, prenatal stress has been shown to reduce serum CBG levels both in adolescent and adult rats [18]. One study also reported that MS decreased the plasma CBG concentration in juveniles [19]. These and our data strengthen the hypothesis that regulation of GC carrier protein levels can be one of the mechanisms by which chronic stress, especially ELS, may program GC bioavailability. CBG and albumin act as delivery molecules and as reservoirs and buffers for GCs [6]. They also regulate the metabolic clearance of GCs [6]. Reduction in the serum levels of GC carrier proteins may lead to increases in the concentration of unbound corticosterone that can be easily subjected to degradation in the liver. On the other hand, free corticosterone negatively regulates its own secretion [6]. CBG deficiency has been widely studied in human patients and in CBG knockout mice [6,20,21]. These studies revealed that CBG

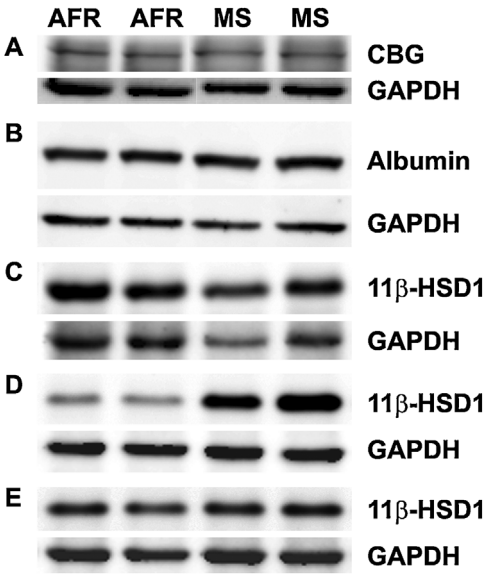

Fig. 2. Representative immunoblots showing the impacts of MS on CBG, albumin and 11β-HSD1 protein expression in the liver (A–C) and 11β-HSD1 protein levels in the mPFC (D) and HP (E).

Table 1
The effects of MS on CBG, albumin and 11β-HSD1 expression in the liver.

Marker		AFR	MS
CBG	mRNA	1.00 ± 0.063	0.94 ± 0.061
	protein	1.00 ± 0.130	0.75 ± 0.038
Albumin	mRNA	1.00 ± 0.043	0.92 ± 0.054
	protein	1.00 ± 0.053	1.08 ± 0.108
11β-HSD1	mRNA	1.00 ± 0.058	0.90 ± 0.115
	protein	1.00 ± 0.080	1.01 ± 0.154

mRNA levels were quantified by RT-qPCR, whereas protein expression was determined by Western blots. Data are presented as the group mean ± SEM (n = 6–7) and expressed as the fold change relative to the AFR rat values.

Table 2
The effect of MS on 11β-HSD1 expression in the brain.

Region		AFR	MS
mPFC	mRNA	1.00 ± 0.149	0.97 ± 0.051
	protein	1.00 ± 0.255	2.20 ± 0.362*
HP	mRNA	1.00 ± 0.119	0.88 ± 0.025
	protein	1.00 ± 0.170	0.98 ± 0.166

mRNA levels were quantified by RT-qPCR, whereas protein expression was determined by Western blots. Data are presented as the group mean ± SEM (n = 5–7) and expressed as the fold change relative to the AFR rat values. $^{*}p < 0.05$ compared with AFR rats.

deficiency is characterized by either a reduction or no change in total cortisol/corticosterone concentrations in basal conditions, depending on the extent of CBG deficiency (homozygotes *vs.* heterozygotes for specific Cbg mutation, respectively) [20,21]. Additionally, homozygous Cbg knockout mice showed an elevation in the concentration of free corticosterone at rest; however, this occurred only in the morning and not in the evening [20,21]. After stress, both homozygous and heterozygous Cbg knockout mice displayed reduced levels of total and free corticosterone [21].

Our study did not reveal a significant effect of MS on basal serum corticosterone levels. This may result from the relatively small extent of GC carrier protein deficiency. Nevertheless, these results do not exclude the possibility that MS affected GC availability in the brain. In CBG-deficient mice, not only homozygotes but also heterozygotes showed symptoms of insufficient GC signaling and altered behavioral responses after stress [21].

In our study, lower serum levels of CBG and albumin in MS rats were not a result of attenuation of their synthesis in the liver. Only a trend toward reduced expression of CBG proteins was observed in the liver of MS rats. We speculate that MS may have affected the half-lives of CBG and albumin and/or the regulation of their secretion from the liver.

We did not observe reliably detectable levels of CBG mRNA in the HP and mPFC of adolescent rats. Low levels of CBG mRNA and protein have been detected by others, mainly in the hypothalamus, pituitary gland and HP; however, these studies were performed in adult animals [22,23]. Thus, we cannot exclude that CBG expression in the brain differs depending on the developmental stage of the animal.

Interestingly, we found that MS increased 11β-HSD1 protein expression specifically in the mPFC. Thus, MS may potentially increase the availability of active GCs in this brain region of adolescent rats. During adolescence, the mPFC undergoes intensive structural and functional remodeling [12,13]. Unbalanced GC levels within the mPFC can be detrimental for mPFC maturation and synaptic plasticity processes, which underlie learning and memory [12]. Forebrain overexpression of 11β-HSD1 has been shown to predispose subjects to cognitive problems [24].

On the other hand, many reports showed a downregulation of GRs in the cortex of animals subjected to ELS procedures [7,8]. Therefore, we cannot exclude the possibility that increased 11β-HSD1 expression and potentially greater GC availability in the mPFC of MS rats are beneficial adaptations helping to balance GC signaling locally, especially when GC carrier protein levels are concurrently reduced.

In conclusion, we found that MS affects serum levels of GC carrier proteins and 11β-HSD1 expression in the mPFC of adolescent rats, which may potentially influence the GC bioavailability and, in consequence, GC signaling in the brain. This effect of ELS may affect the maturation of brain functions during the adolescent period and determine vulnerability or resilience to psychopathology.

Conflict of interest

The Authors declare no conflict of interest.

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