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Regulation of 11β-Hydroxysteroid Dehydrogenase Type 1 and 7α-Hydroxylase CYP7B1 during Social Stress

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Abstract

11β-hydroxysteroid dehydrogenase type 1 (11HSD1) is an enzyme that amplifies intracellular glucocorticoid concentration by the conversion of inert glucocorticoids to active forms and is involved in the interconversion of 7-oxo- and 7-hydroxy-steroids, which can interfere with the activation of glucocorticoids. The presence of 11HSD1 in the structures of the hypothalamic-pituitary-adrenal (HPA) axis suggests that this enzyme might play a role in the regulation of HPA output. Here we show that the exposure of Fisher 344 rats to mild social stress based on the resident-intruder paradigm increased the expression of 11HSD1 and CYP7B1, an enzyme that catalyzes 7-hydroxylation of steroids. We found that social behavioral profile of intruders was significantly decreased whereas their plasma levels of corticosterone were increased more than in residents. The stress did not modulate 11HSD1 in the HPA axis (paraventricular nucleus, pituitary, adrenal cortex) but selectively upregulated 11HSD1 in some regions of the hippocampus, amygdala and prelimbic cortex. In contrast, CYP7B1 was upregulated not only in the hippocampus and amygdala but also in paraventricular nucleus and pituitary. Furthermore, the stress downregulated 11HSD1 in the thymus and upregulated it in the spleen and mesenteric lymphatic nodes whereas CYP7B1 was upregulated in all of these lymphoid organs. The response of 11HSD1 to stress was more obvious in intruders than in residents and the response of CYP7B1 to stress predominated in residents. We conclude that social stress induces changes in enzymes of local metabolism of glucocorticoids in lymphoid organs and in brain structures associated with the regulation of the HPA axis. In addition, the presented data clearly suggest a role of 11HSD1 in modulation of glucocorticoid feedback of the HPA axis during stress.

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Introduction

A large number of studies have shown that different stressors produce profound physiological and behavioral disturbances that may contribute to psychopathology [1,2] and alterations in immune system functionality [3]. Stress activates the sympatho-adrenomedullar and the hypothalamic-pituitary-adrenocortical (HPA) systems. The HPA axis is self-regulatory, utilizing its end products, cortisol and corticosterone, to control its own activation and responsiveness through a negative feedback mechanism. The neurons of the paraventricular nucleus represent the central coordinator of the HPA axis that is not only driven by negative corticosteroid feedback signals, but also by central stress excitatory and inhibitory circuits that are activated by stressors in both intrahypothalamic (arcuate nucleus, dorsomedial hypothalamus) and extrahypothalamic structures, in particular, the limbic structures (medial prefrontal cortex, hippocampus, amygdala) [4,5]. These structures express glucocorticoid receptors that contribute to feedback control of the HPA axis [6].

The response of the target cells to the glucocorticoid signal does not only depend on the plasma level of the hormone and the density of corticosteroid receptors, but also on the intracellular concentration of glucocorticoids, which is predominantly determined by the local pre-receptor steroid metabolism. This metabolism depends on 11β-hydroxysteroid dehydrogenase (11HSD), an enzyme that exists in two forms - type 1 (11HSD1) and type 2 (11HSD2). 11HSD1 generally catalyses the reduction of biologically inactive 11-oxo-steroids cortisone and 11-dehydro-corticosterone to cortisol and corticosterone and thus increases the local concentration of active corticosteroids. In contrast, 11HSD2 operates as a strict dehydrogenase that oxidizes corticosterone and cortisol to 11-oxo-derivatives [7]. 11HSD1 is expressed in the brain [8–10], pituitary [11,12], adrenal gland [13], and many peripheral organs including the spleen, thymus, and lymphatic nodes [14,15].

The presence of 11HSD1 in the structures of the HPA axis suggests that the enzyme might play a role in the regulation of HPA output. Indeed, 11HSD1 knock-out mice exhibit attenuated glucocorticoid feedback on the HPA axis and exaggerated glucocorticoid response to stress [16], however, the genetic background of the mice significantly modulates their response to 11HSD1 deletion [17]. These findings indicate a potential role of

11HSD1 in stress-induced alterations of the HPA axis. However, previous studies investigating the effect of stress and glucocorticoids on 11HSD1 have been incomplete and contradictory [18–21]. They differed in the type and duration of stress applied, animal species and type of cells in which 11HSD1 was assessed. Until now, only one study has addressed the topic of chronic social stress on 11HSD1 in the hippocampus [20], even though social stressors have a profound influence on behavior, immunity, and physiology [22,23].

As the most common stressors for humans are psychosocial in nature, we used a model of social stress based on a resident-intruder paradigm with the aim of evaluating the consequences of repeated mild/moderate stress on the regulation of 11HSD1 expression in brain structures associated with the HPA axis and in the adrenal glands. As social stress has been shown to have a profound influence on immune and inflammation responses [24,25], the effect of social stress on 11HSD1 was also assessed in primary and secondary lymphoid organs. In addition, we investigated the effect of repeated social stress on the expression of CYP7B1, which catalyzes the 7α-hydroxylation of C_{19} and C_{21} steroids, and is expressed in various tissues including the brain. These 7-hydroxy-steroids interact with 11HSD1 and their presence can interfere with the activation of 11-hydroxy-steroids from 11-oxo-steroids catalyzed by 11HSD1 [26].

Materials and Methods

Animals

The experiments were performed on 65-day-old male (n = 21) Fisher 344 rats (Charles River, Germany). Animals were housed in groups of three to four in a temperature-controlled room on a 12/12-h light/dark cycle with *ad libitum* access to food and water throughout the entire study. They were left for three weeks to acclimatize before any experimental procedures. The animals were randomly assigned to one of three groups: (1) controls (2) residents, and (3) intruders, each consisting of seven animals. The protocol of the experiments was approved by the animal Care and Use Committee of the Institute of Physiology to be in agreement with the Animal Protection Law of the Czech Republic, which is fully compatible with the guidelines of European Community Council Directive 86/609/EEC. All efforts were made to minimize the animal suffering and to reduce the number of animal used.

Resident-intruder Paradigm

The general design of the test was adapted from [27]. Briefly, if an unfamiliar conspecific intruder is introduced into the home cage of an isolated resident, intense social behavior arises. Such behavior is mainly initiated by the resident animal, indicating territorial advantage. This territorial advantage is obvious after a few days of the resident being isolated. The test relies on the concept of the ethological analysis of rodent behavior and can be used as a model of "social anxiety". At the beginning of the experiment the resident rats were housed individually for one week, the intruders were housed in groups of three or four. Following the seven-day isolation period of the residents, the social encounter was performed for seven consecutive days, and arranged to ensure that each intruder rat met each of the corresponding residents for 30 minutes. The resident rats remained isolated in their home cages throughout the experiment, while the intruders were returned to their respective groups. There was no difference in the body weight of the residents (222.1±1.0 g) and intruders (218.5±1.5 g) after exposure to social stress and the weights were similar to control unstressed animals of the same age.

Behavioral Testing

To compare social behavior between the first and the final social interaction (Session 1 vs. Session 7) the behavior was video-recorded for 15 minutes and the test was performed under low light intensity (35–45 lx) between 9.00 and 12.00 AM. The social behavioral patterns displayed were subsequently analyzed in detail by two trained experimenters using the computerized behavioral analysis system Observer (Noldus Information Technology, Wageningen, The Netherlands). Behavior was scored separately for each member of a pair (resident and intruder) except for wrestling, as this pattern involved two animals concurrently performing the same behavior. The number and the duration of the following exhibited patterns were evaluated: following/chasing (the pursuit of one rat by another) grabbing (rat grabbing the fur of another in the region of the neck), wrestling (both animals roll and tumble with one another), on-top posture (one rat positioned over another with forepaws placed on it), and digging (moving substrate forward with front paws and nose, or backwards with hind paws), a non-social behavior observed only in intruders.

Brain Sampling and Processing

Intact animals (controls) and the rats after recording the last social interaction session (Session 7) were immediately anesthetized with isoflurane and blood was collected by cardiac puncture. Then the rats were killed by decapitation and the brain and selected other tissues were removed and immediately frozen in liquid nitrogen.

Brain specimens of hypothalamic paraventricular nucleus (PVN), central (CeA) and lateral amygdala (LA), prelimbic (plPFC) and infralimbic prefrontal cortex (ilPFC), hippocampal CA2 and CA3 regions, and ventral (vCA1) and dorsal (dCA1) parts of CA1 region, were prepared by laser microdissection and RNA analysis was performed as previously described, with some modifications [28]. Briefly, coronal brain sections (20 μm) were serially cut with a cryostat at −19°C. The regions were identified based on standard anatomical landmarks and stereotaxic coordinates (see Table 1, Fig. 1) according to Paxinos and Watson [29]. The sections of the studied structures were mounted onto slides coated with polyethylene naphthalate membrane (Leica Microsystems, Wetzlar, Germany), fixed in 95% ethanol, stained with 4% cresyl violet acetate and washed three times in 95% ethanol. The studied brain structures were dissected using a LMD6000 Laser Microdissection System (Leica) and captured into the caps of the microcentrifuge tubes. Microdissected tissues were homogenized in 75 μl RLT buffer (Qiagen, Hilden, Germany) and stored at −80°C until RNA isolation.

Total RNA was isolated from the captured tissue using an RNeasy Micro Kit (Qiagen, Hilden, Germany) and evaluated with a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA). The RNA samples were reverse-transcribed to cDNA with Enhanced Avian Reverse Transcriptase (Sigma-Aldrich, St. Louis, MO, USA). Because the RNA yield of cytokine transcripts was low, an aliquot of the cDNA sample was amplified with TaqMan PreAmp Master Mix Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA samples were analyzed by real-time PCR on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan Gene Expression Master Mix and TaqMan Assays (Life Technologies) specific for rat 11HSD1 (cat.no. Rn01461862_m1), 7-hydroxylase (CYP7B1; cat.no. Rn00567167_m1), glucocorticoid receptor (GR; cat.no. Rn00561369_m1), interleukin 1β (IL-1β; cat.no. Rn01514151_m1), tumor necrosis factor α (TNFα; cat.no. Rn 99999017_m1), osteopontin (OPN; cat.no. Rn01449972_m1),

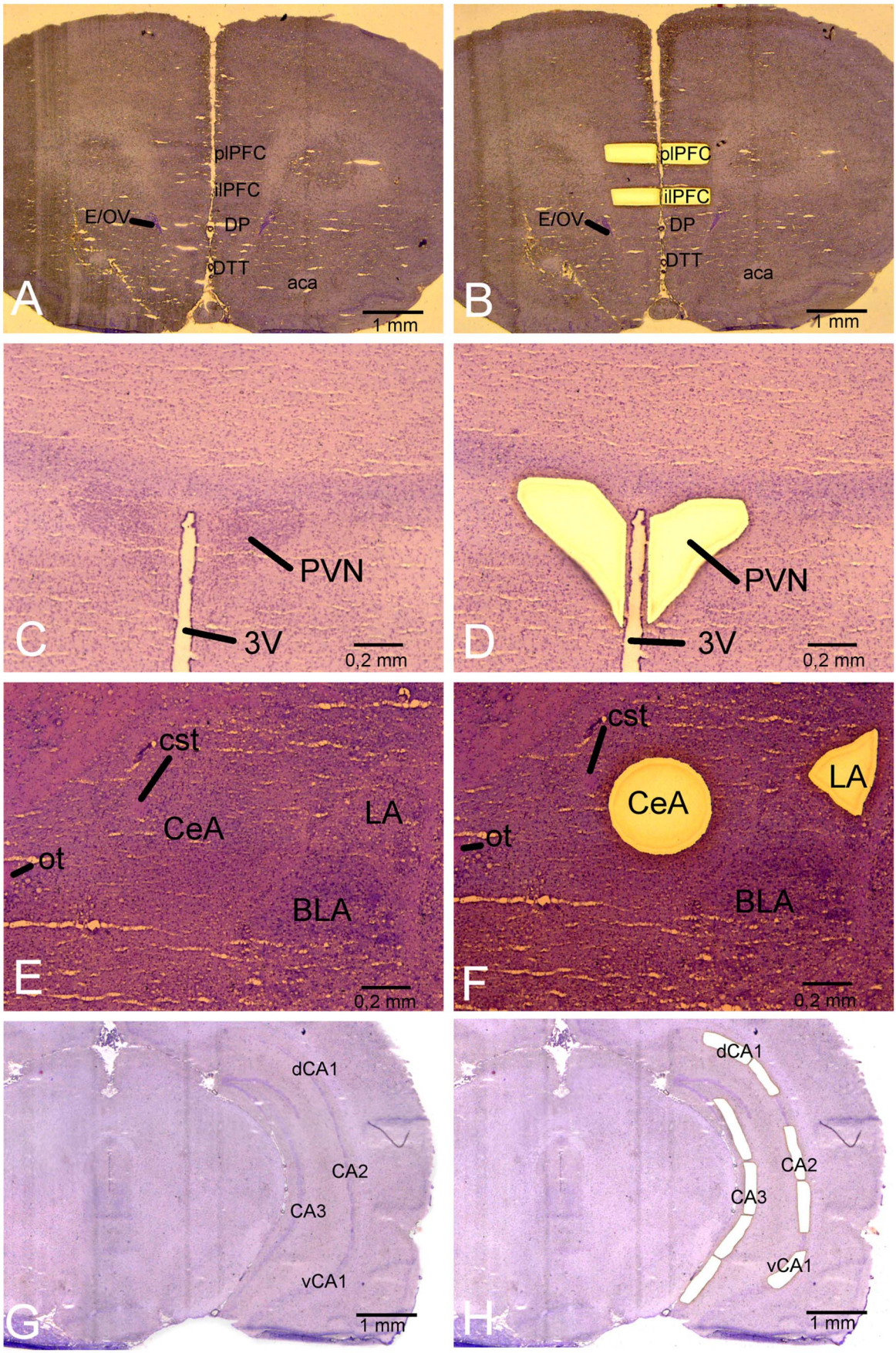
A
plPFC
ilPFC
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Figure 1. Laser capture microdissection of cell populations from the prelimbic and infralimbic cortex (A, B), hypothalamic paraventricular nucleus (C, D), central and lateral nucleus of amygdala (E, F) and CA1, CA2 and CA3 regions of hippocampus (G, H). Representative 20-μm-thick coronal sections of unfixed, frozen rat brain stained with cresyl violet are shown before (left panel) and after capturing (right panel). The dissected regions were captured into the microcentrifuge tubes and used for total RNA isolation and RT-PCR analysis. *Abbreviations*: aca, anterior commissure; BLA, basolateral amygdala; CA2 and CA3, hippocampal CA2 and CA3 regions; CeA, central nucleus of amygdala; cst, commissural stria terminalis; dCA1, dorsal part of CA1 hippocampus; DP, dorsal peduncular cortex; DTT, dorsal tenia tecta; E/OV, ependymal and subependymal layer of olfactory ventricle; ilPFC, infralimbic prefrontal cortex; LA, lateral nucleus of amygdala; ot, optic tract; plPFC, prelimbic prefrontal cortex; PVN, paraventricular hypothalamic nucleus; vCA1, ventral part of CA1 hippocampus; 3V, 3rd ventricle;
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corticotropin-releasing hormone (CRH; cat. no. Rn01462137_m1) and CRH receptor 1 (CRHR1; cat. no. Rn00578611_m1). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; TaqMan Endogenous Control cat. No. 4352338), was measured to normalize the mRNA expression in each sample, as its transcript is not changed in the rat brain during stress [30]. A single PCR reaction was performed in a final volume of 30 μl using target gene probes labeled with FAM in duplex with a GAPDH probe (VIC/MGB). The quantity of the transcript was determined using the standard curve method with 10-fold dilutions of the mixed cDNA sample.

Peripheral Tissues Collection and Processing

Tissue samples of the anterior pituitary, adrenal gland, thymus, spleen and mesenteric lymphatic nodes (MLN) were snap-frozen and stored in liquid nitrogen. To separate the adrenal cortex and medulla, the samples of the adrenal gland were laser-microdissected and the samples of cortex and medulla were processed identically to the brain samples mentioned above. RNA from the pituitary and lymphoid organs was isolated using a GeneElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich) and 0.1 μg of pituitary RNA and 1.0 μg of spleen, thymus and MLN RNA were reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit and random hexamers (both Life Technologies). The mRNA levels were quantified by real-time PCR with TaqMan Gene Expression Master Mix and the TaqMan Assays mentioned above in a LightCycler 480 (Roche, Mannheim, Germany) or ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The reactions were performed in 30 μl aliquots on a 96-well optical reaction plate containing TaqMan Gene Expression Master Mix with AmpErase UNG (Applied Biosystems), cDNA and TaqMan probes as mentioned above. The standard curve method was used to analyze the relative gene expression and the genes of interest were normalized to GAPDH.

Table 1. Rat brain regional coordinates.

Region	Bregma coordinates	ML	DV	Figure no.
plPFC	3.00 to 2.50 mm	±(0 to 1.0)	3.2 to 4.5	10–12
ilPFC	3.00 to 2.50 mm	±(0 to 0.9)	4.5 to 5.2	10–12
CeA	−2.56 to −2.76 mm	±(3.8 to 4.8)	7.5 to 8.6	54–56
LA	−2.56 to −2.76 mm	±(5.2 to 5.8)	7.4 to 8.4	54–56
dCA1	−4.92 to −4.97 mm	±(3.5 to 5.5)	2.8 to 4.0	74
vCA1	−4.92 to −4.97 mm	±(4.8 to 6.2)	6.8 to 8.8	74
CA2	4.92 to 4.97 mm	±(5.3 to 5.9)	5.3 to 5.9	74
CA3	4.92 to 4.97 mm	±(3.5 to 5.0)	5.0 to 7.0	74
PVN	−1.72 to −1.80 mm	±(0 to 0.5)	7.8 to 8.6	47–48

plPFC, prelimbic prefrontal cortex; ilPFC, infralimbic prefrontal cortex; CeA, central amygdala; LA, lateral amygdala; dCA1 and vCA1, dorsal and ventral parts of hippocampal CA1 region; CA2, hippocampal CA2 region; CA3, hippocampal CA3 region; PVN, paraventricular nucleus. The extent of the dissected regions is characterized according to the mediolateral (ML) and dorsoventral (DV) axis. Coordinates and figure numbers are based on the atlas ''The Rat Brain In Stereotaxic Coordinates. 6th Edition by G. Paxinos & C. Watson. Elsevier, 2007''. The thickness of the brain coronal sections was 20 μm and the total area of the isolated brain structure was 0.12 to 0.60 mm^2.
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Plasma Corticosterone Measurement

Blood was collected in tubes containing K_2EDTA and centrifuged for 10 min at 3,000 g. Plasma was aliquoted and kept frozen at −80°C until the assay. Corticosterone was measured using a commercial RIA kit (MP Biomedicals, Solon, OH, USA).

Statistical Analysis

Data are presented as means ± SEM. The behavioral variables were analyzed with a two-way repeated measure ANOVA followed by Student-Newman-Keuls post-hoc test, where the type of condition (resident vs. intruder) was the between-subject factor and the time of measurement (session) was the within-subject factor. Digging behavior was analyzed by a one-way ANOVA because this behavior was observed only in intruder rats. Similarly, a one-way ANOVA and a *post hoc* multiple comparison test (Student-Newman-Keuls) were used for studies involving single comparisons of mRNA levels and plasma levels of corticosterone. All analyses were carried out using the software Statistica 6.1. (StatSoft Inc., Tulsa, OK, USA). The significance level was set at $P<0.05$.

Results

Effect of Repeated Social Stress on Behavior

The differences in the behavioral profile of resident and intruder rats are illustrated in Fig. 2. To simplify the text, only statistically significant differences were stated. ANOVA showed a significant effect on social interaction in the number as well as in the duration of all evaluated social behavioral patterns ($F_{1,12} = 29.52$, $P<0.001$; $F_{1,12} = 10.18$, $P<0.01$, respectively). In the first social session, the *post hoc* test revealed a significant decrease in both the number and the duration of all social behaviors displayed by intruders compared to residents (Fig. 2A,B). In the final encounter (Session 7; Fig. 2A,B), the intruder rats had a significant decrease in the number but not in the duration of social behavior. Further, the number of all behavioral patterns displayed by residents in the final seventh session was lower that in the first session.

The analysis of individual behavioral patterns revealed the condition (resident vs. intruder) to have a major effect on the number and the duration of following ($F_{1,12} = 37.14$, $P<0.001$; $F_{1,12} = 26.91$, $P<0.001$, respectively) and on the number as well as the duration of grabbing ($F_{1,12} = 7.99$, $P<0.01$; $F_{1,12} = 5.03$, $P<0.05$, respectively). The overall analysis did not reveal any significant differences in either the number or the duration of the on-top posture. In intruders, the *post hoc* test showed a decrease in the number of following and grabbing behaviors but only in the duration of following in both sessions. As for wrestling, no difference was detected between Session 1 and Session 7. Finally, digging (Fig. 2E, F), an index of anxiety-like behavior, was

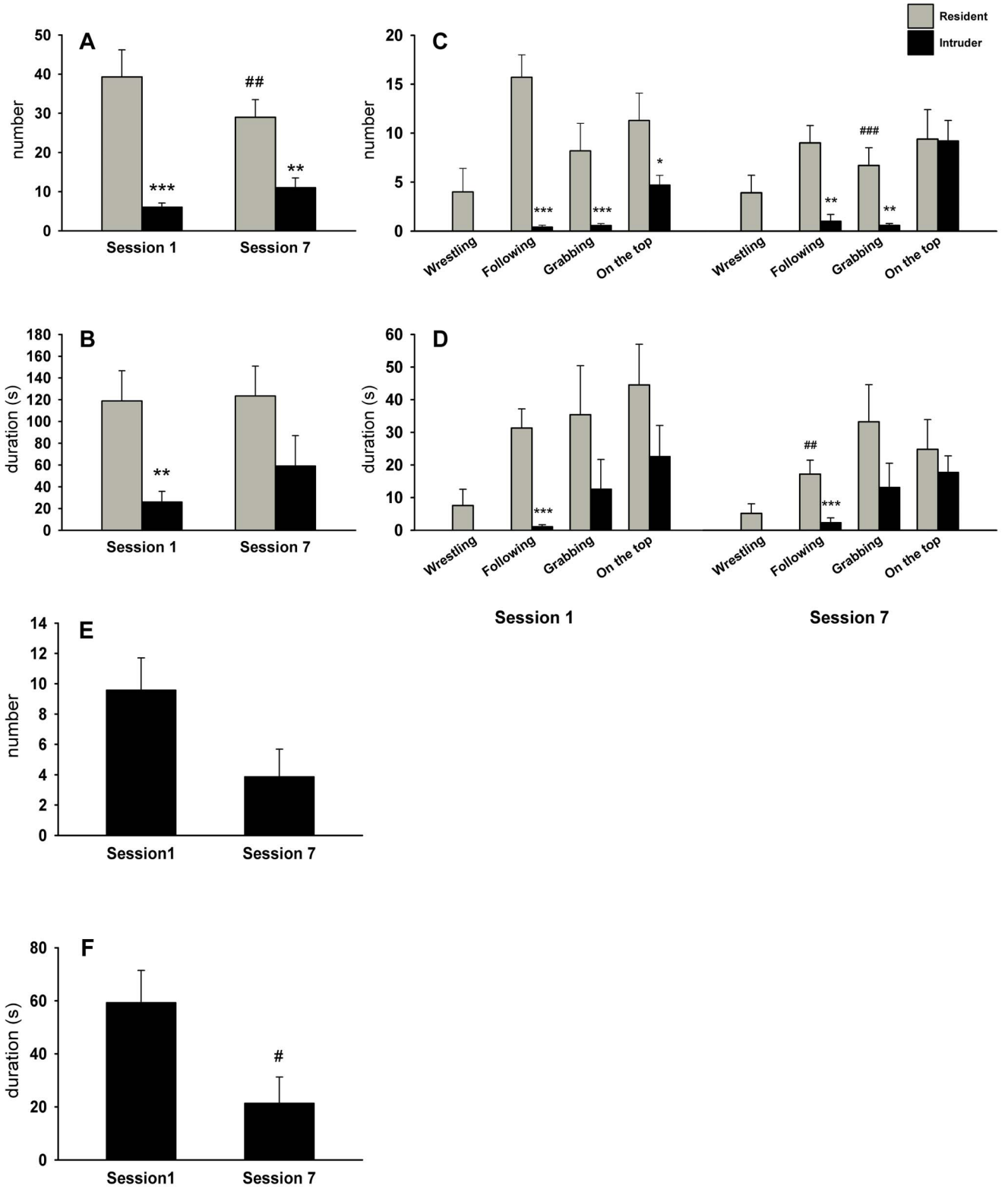

Figure 2. Behavioral differences in Fisher 344 rats on the resident-intruder test. Left side up: Total number (**A**) and total duration (**B**) of all social behavioral patterns displayed by resident and intruder rats. Right side up: Total number (**C**) and total duration (**D**) of individual behavioral patterns exhibited during social interaction. Left side down: Total number (**E**) and total duration (**F**) of digging patterns displayed only by intruder rats. Session 1: the first day, session 7: the last day. The values are expressed as means ± SEM. Significant differences between residents and intruders: *P<0.05, **P<0.01, ***P<0.001, and between Session 1 and Session 7: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.
doi:10.1371/journal.pone.0089421.g002

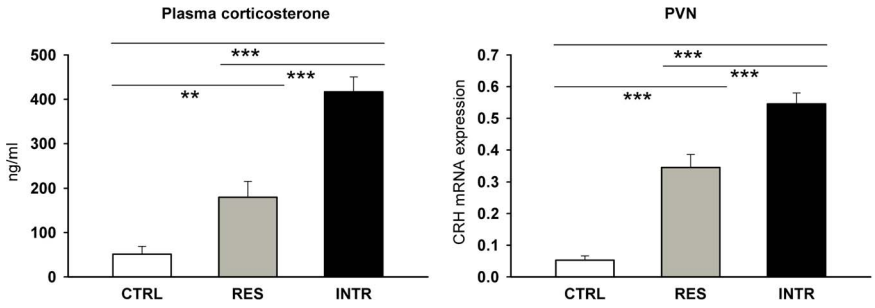

Figure 3. Plasma level of corticosterone and expression of corticotropin-releasing hormone (CRH) in hypothalamic paraventricular nucleus of control (CTRL), resident (RES) and intruder rats (INTR) after the last social session. All values are means ± SEM. **P<0.01, ***P<0.001.
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observed only in intruder rats and its duration was significantly decreased in Session 7 compared to Session 1 ($F_{1,6} = 8.49$, $P < 0.05$).

Effect of Repeated Social Stress on Plasma Corticosterone and Expression of 11HSD1, GR and Cytokines

The repeatedly stressed rats had significantly elevated plasma corticosterone compared to intact control animals and the plasma levels in intruders were significantly higher than in residents (Fig. 3; $F_{2,18} = 40.03$, $P<0.0001$). Similarly, stress significantly increased the expression of CRH in PVN and the levels were significantly higher in intruders than in residents (Fig. 3; $F_{2,13} = 52.53$, $P < 0.0001$). As social status plays an important role in determining the impact of stress on brain cytokines [31,32], the expression of proinflammatory cytokines IL-1β, TNFα and OPN was measured in the PVN and CA1 region of the hippocampus. In intact controls, the expression of cytokines was absent or very low in both the PVN and CA1. Social interactions were followed by the upregulation of TNFα, IL-1β and OPN, and the effect did not differ between resident and intruder rats (Table 2). For comparative reasons we measured also expression of IL-1β in MLN (Table 2) and found out that stress did not upregulate cytokine expression in this tissue.

Given that stress activates HPA axis that is self-regulated through negative feedback mechanism utilizing its end products, cortisol and corticosterone, we hypothesized that stress might modulate the feedback signaling in the HPA axis. To test this hypothesis, we analyzed the effect of stress on the expression of 11HSD1 and GR in PVN, prefrontal cortex, amygdala and hippocampus. As shown in Fig. 4, the expression of 11HSD1 was affected by social stress in some brain structures associated with regulation of the HPA axis. In particular, the 11HSD1 transcript was upregulated in the CeA and LA ($F_{2,12} = 11.57$; $P<0.01$ and $F_{2,17} = 4.80$; $P<0.05$, respectively), in the plPFC ($F_{2,14} = 3.95$; $P < 0.05$), in the vCA1 ($F_{2,15} = 8.38$; $P<0.001$), but not dCA1 subfield of hippocampus, and in the CA2 hippocampus ($F_{2,15} = 12.67$; $P < 0.0001$). Stress did not change the expression of 11HSD1 in the CA3 hippocampal region and PVN, even if the effect in PVN was just shy of statistical significance. Similarly, expression of GR (Table 3) was not changed by social stress in any investigated brain area with the exception of the vCA1 region of the hippocampus ($F_{2,18} = 5.20$; $P<0.01$).

Similar to PVN, 11HSD1 expression was neither changed in the pituitary nor in the adrenal gland (Fig. 5) that constitute the principle components of the HPA axis. However, the social stress provoked changes in the expression of GR in the pituitary ($F_{2,18} = 5.17$; $P<0.001$) and upregulated it more in intruders than in residents (Fig. 6). In contrast, the expression of CRHR1 was not changed, even if the stimulatory action of stress-induced CRH release is mediated primarily through binding to this receptor and CRH expression in PVN was significantly upregulated in both residents and intruders (Fig. 3).

Social stress has profound influence on immune responses [24,25] and thus we measured also the expression of 11HSD1 in primary and secondary lymphoid organs of resident and intruder rats. As summarized in Fig. 5, the repeated social stress significantly modulated 11HSD1 in the lymphoid organs. 11HSD1 was upregulated in the spleen ($F_{2,17} = 5.44$; $P<0.05$) and MLN ($F_{2,17} = 14.83$; $P<0.001$) and this effect was more intensive in intruders than in resident rats. In the thymus, the effect was the opposite ($F_{2,17} = 53.96$; $P<0.0001$). The weights of

Table 2. Expression of pro-inflammatory cytokines tumor necrosis factor α (TNFα), interleukin 1β (IL-1β) and osteopontin (OPN) in paraventricular nucleus (PVN), CA1 region of ventral hippocampus (vCA1) and in mesenteric lymphatic nodes (MLN).

	PVN		vCA1		MLN	
	CTRL	Experimental	CTRL	Experimental	CTRL	Experimental
TNFα	n.f.	0.25±0.12	n.f.	0.38±0.14	n.m.	n.m.
IL-1β	0.02±0.01	0.23±0.10*	n.f.	n.f.	5.03±0.42	4.51±0.35
OPN	0.03±0.02	5.83±1.84**	0.01±0.00	2.03±0.65**	n.m.	n.m.

Data are means ± SEM (CTRL, n = 5–7; Experimental = 12–14); as upregulation of the transcripts was not significantly different in residents and intruders, both groups were merged; n.f., no signal of the transcript was found either in standard or in preamplified samples; n.m., the transcript was not measured in the samples. Quantitative PCR was measured in standard (MLN) or preamplified samples (PVN, vCA1) of intact controls and experimental animals (residents, intruders) as mentioned in Materials and Methods. *P<0.05, **P<0.01.
doi:10.1371/journal.pone.0089421.t002

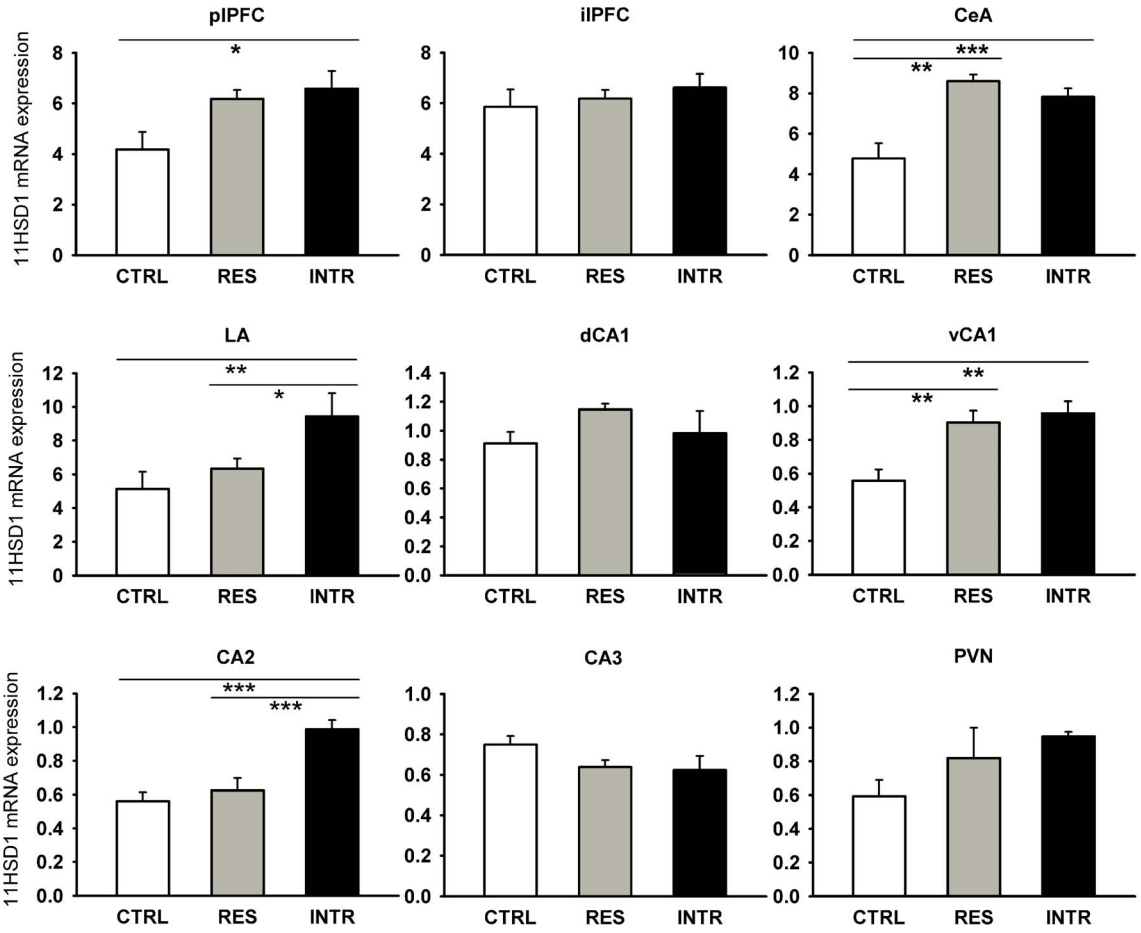

Figure 4. Effect of repeated social stress on expression of 11HSD1 in brain structures associated with the HPA axis. CTRL, control rats; RES, resident rats; INTR, intruder rats; plPFC, prelimbic prefrontal cortex; ilPFC, infralimbic prefrontal cortex; CeA, central amygdala; LA, lateral amygdala; dCA1 and vCA1, dorsal and ventral parts of CA1 hippocampus; CA2 and CA3, hippocampal CA2 and CA3 regions; PVN, paraventricular nucleus. All values are means ± SEM. *P<0.05, **P<0.01, ***P<0.001.
doi:10.1371/journal.pone.0089421.g004

Table 3. A comparison of glucocorticoid receptor mRNA expression in brain structures associated with the HPA axis in residents, intruders and unstressed control rats.

	Residents	Intruders	Controls
plPFC	0.15±0.01	0.16±0.02	0.13±0.03
ilPFC	0.17±0.01	0.16±0.01	0.17±0.02
CeA	0.31±0.06	0.34±0.01	0.26±0.03
LA	0.11±0.02	0.10±0.01	0.11±0.02
dCA1	0.70±0.07	0.92±0.07	0.78±0.07
vCA1	0.49±0.06**	0.41±0.04**	0.26±0.04
CA2	0.23±0.03	0.26±0.06	0.25±0.04
CA3	0.19±0.01	0.17±0.03	0.17±0.02
PVN	0.31±0.02	0.25±0.01	0.22±0.05

plPFC, prelimbic prefrontal cortex; ilPFC, infralimbic prefrontal cortex; CeA, central amygdala; LA, lateral amygdala; dCA1 and vCA1, dorsal and ventral parts of hippocampal CA1 region; CA2, hippocampal CA2 region; CA3, hippocampal CA3 region; PVN, paraventricular nucleus. All values are means ± SEM. **P< 0.01.
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the spleen, thymus, and adrenal glands in the controls were not different from the groups of residents and intruders (data not shown).

Effect of Repeated Social Stress on Expression of Cytochrome CYP7B1

It has been postulated that 7-hydroxy-metabolites of C_{21} and C_{19} steroids, such as 7-hydroxy-dehydroepiandrosterone, can decrease local glucocorticoid levels by interaction with 11HSD1 [26]. In order to determine whether metabolism of 7-hydroxy-steroids may modulate 11HSD1 during stress, we examined the effect of social stress on CYP7B1, an enzyme, which catalyzes 7α-hydroxylation of steroids. The social stress used here lead to the upregulation of CYP7B1 in some brain structures, specifically, in the CeA ($F_{2,12} = 4.51$; $P<0.05$), ilPFC ($F_{2,12} = 4.69$; $P<0.05$), PVN ($F_{2,13} = 5.67$; $P<0.05$) and dCA1 ($F_{2,15} = 19.31$; $P<0.0001$), vCA1 ($F_{2,15} = 7.58$; $P<0.001$) and CA3 hippocampus ($F_{2,15} = 4.93$; $P< 0.05$). As shown in Fig. 7, stress significantly increased CYP7B1 expression in the residents, and in the case of the PVN, dCA1, and vCA1 also in intruders. Moreover, stress stimulated the expression of CYP7B1 in the pituitary ($F_{2,17} = 14.69$; $P<0.0001$) and this effect was found in both groups of stressed animals (Fig. 8). These findings gave rise to the hypothesis that social stress might also modulate CYP7B1 expression in the lymphoid organs. The data

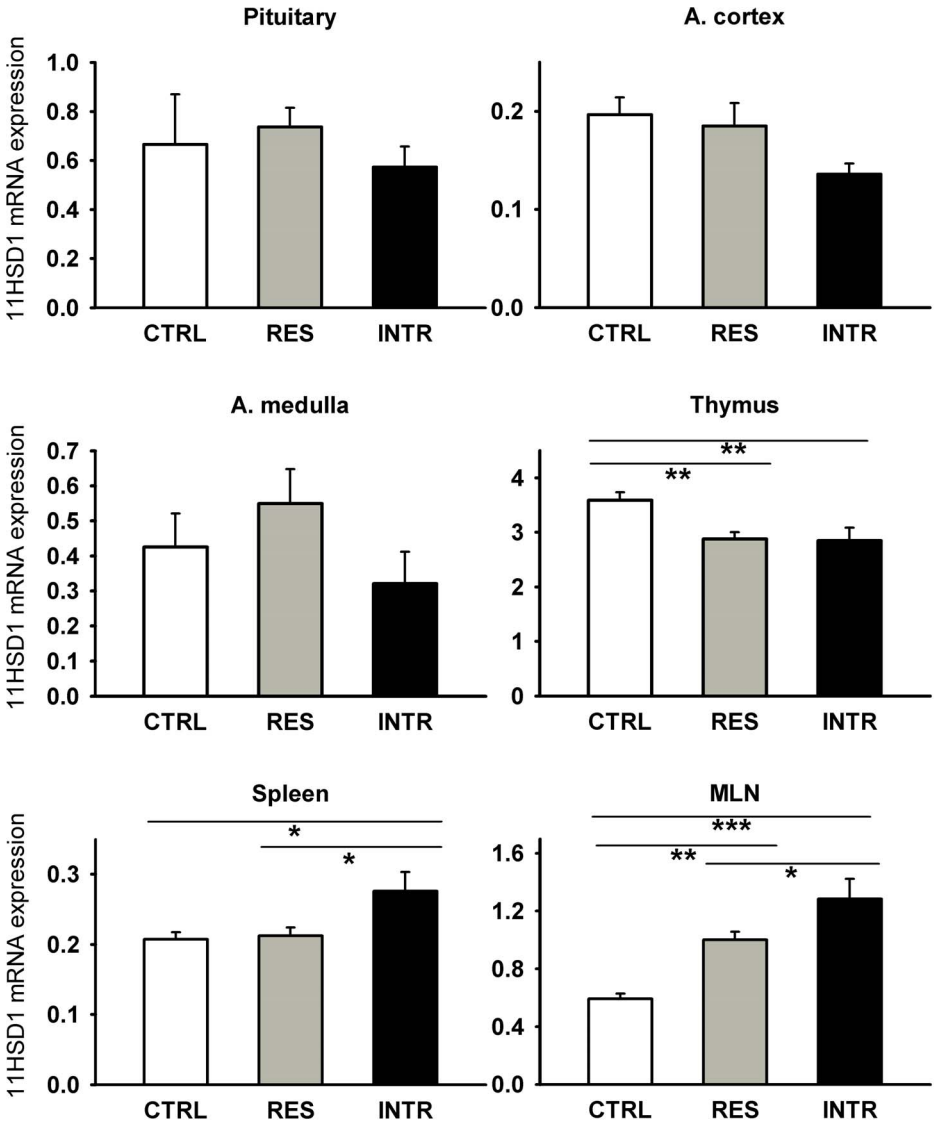

Figure 5. Effect of repeated social stress on expression of 11HSD1 in lymphoid organs, pituitary and adrenal cortex and medulla. CTRL, control rats; RES, resident rats; INTR, intruder rats; MLN, mesenteric lymphatic nodes. Data represent means ± SEM. *P<0.05, **P<0.01, ***P< 0.001.
doi:10.1371/journal.pone.0089421.g005

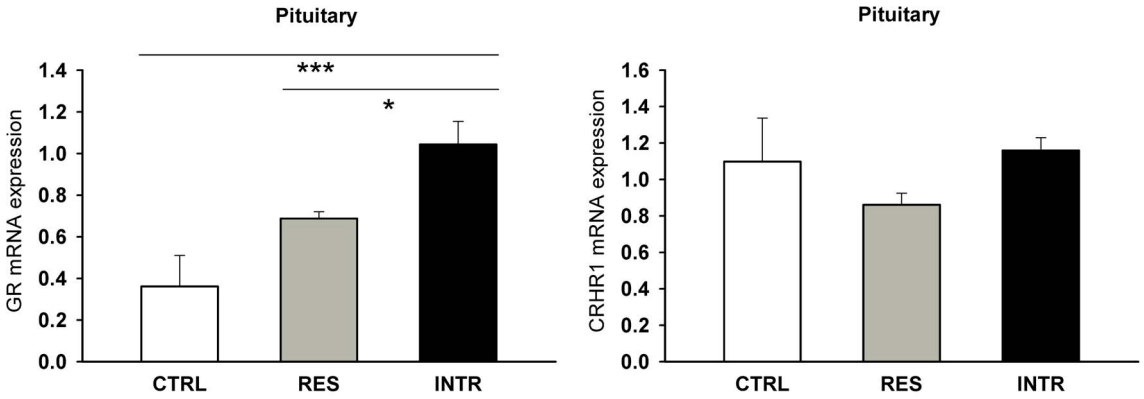

Figure 6. Expression of glucocorticoid (GR) and CRH receptors (CRHR1) in pituitary of control (CTRL), resident (RES) and intruder rats (INTR) after repeated social stress. All values are means ± SEM. *P<0.05, ***P<0.001.
doi:10.1371/journal.pone.0089421.g006

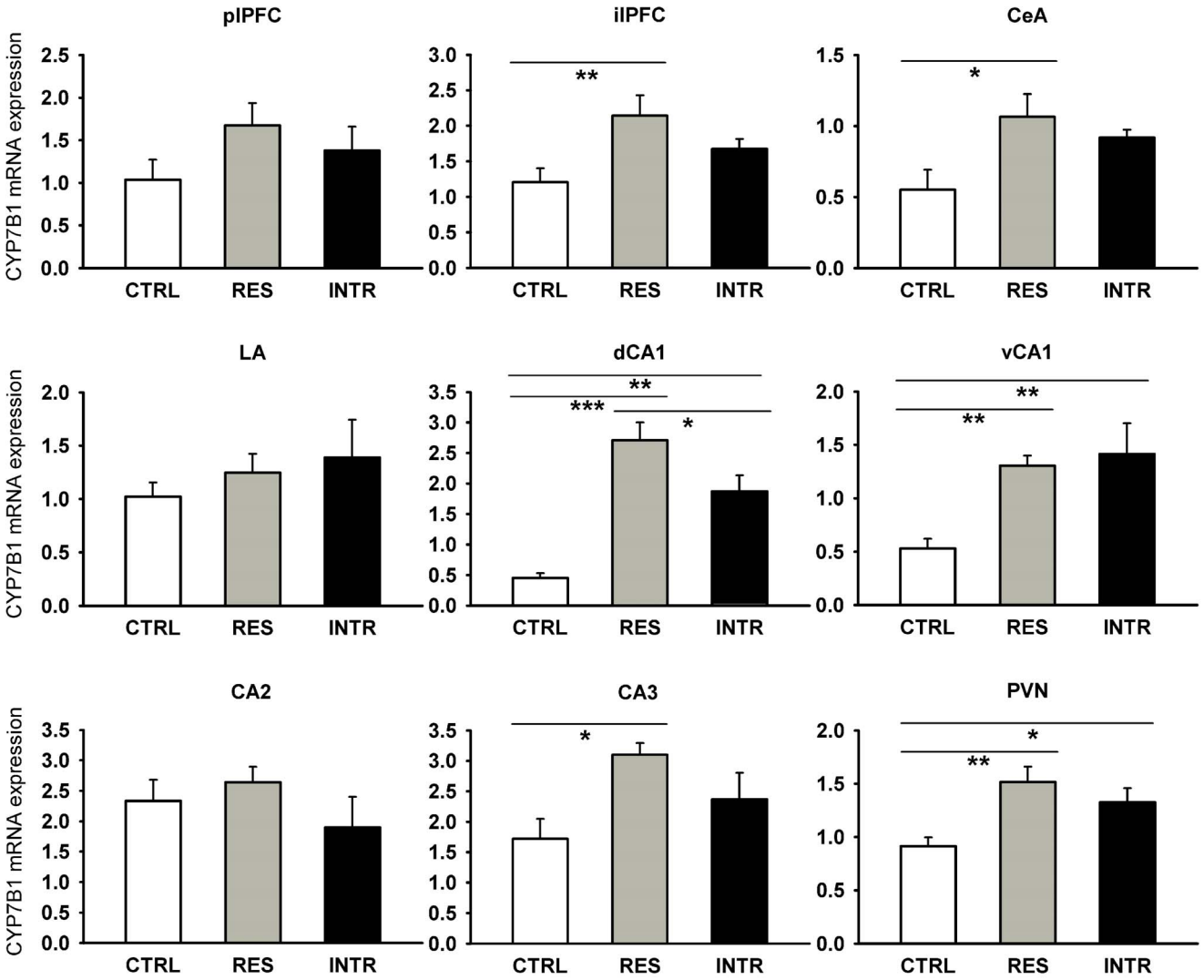

Figure 7. Effect of repeated social stress on expression of CYP7B1 in brain structures associated with the HPA axis. CTRL, control rats; RES, resident rats; INTR, intruder rats; plPFC, prelimbic prefrontal cortex; ilPFC, infralimbic prefrontal cortex; CeA, central amygdala; LA, lateral amygdala; dCA1 and vCA1, dorsal and ventral parts of CA1 hippocampus; CA2 and CA3, hippocampal CA2 and CA3 regions; PVN, paraventricular nucleus. All values are means ± SEM. *P<0.05, **P<0.01, ***P<0.001.
doi:10.1371/journal.pone.0089421.g007

summarized in Fig. 8 show that social stress caused a significant increase of CYP7B1 expression in both the primary (thymus: $F_{2,17} = 11.67$; $P<0.0001$) and secondary lymphoid organs (spleen: $F_{2,17} = 4.54$; $P<0.05$; MLN: $F_{2,17} = 19.89$; $P<0.0001$). Both residents and intruders exhibited a higher expression of CYP7B1 than the intact control rats.

Discussion

In the present study, we report that chronic social stress upregulated the expression of enzymes that are able to modulate local concentration of glucocorticoids. Using the resident-intruder paradigm, we showed that manipulation with the social status of the animals for several consecutive days suppressed the social behavioral profile of the intruders and increased physiological stress markers (plasma corticosterone, CRH expression in PVN) in both intruders and residents, however, the intruders were stressed more than the residents. The results also demonstrate that short-term moderate repeated social stress did not increase the regulation of 11HSD1 mRNA in the principal components of the axis itself, even when 11HSD1 was previously detected in the PVN, anterior pituitary and adrenal glands [8–13]. In the adrenal glands, 11HSD1 expression was not influenced by the social stress, neither in the adrenal cortex nor in the medulla, where glucocorticoids are required for the normal functioning of chromaffin cells and their capacity to produce epinephrine [33]. However, the confrontation of resident and intruder increased the 11HSD1 mRNA in the secondary lymphoid organs and in the amygdala, prelimbic cortex and some regions of the hippocampus – the limbic structures that are activated by psychosocial stressors and are associated with the regulation of the HPA axis [5,34]. Our findings are in agreement with the known role of the limbic structures in regulation of the HPA axis and with the sensitivity of these structures to glucocorticoids. Prelimbic cortex inhibits the HPA axis [4] and its activation reduces glucocorticoid secretion after stress [35] similar to corticosterone implants to this region [36]. Amygdala also appears to process glucocorticoid information, although there is functional differentiation among the

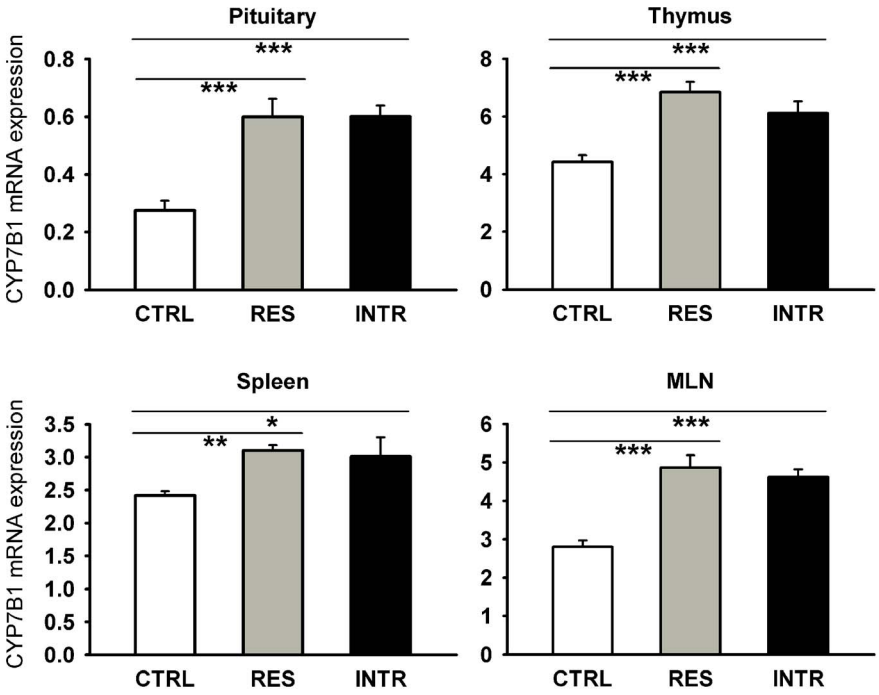

Figure 8. Effect of repeated social stress on expression of CYP7B1 in pituitary and lymphoid organs. CTRL, control rats; RES, resident rats; INTR, intruder rats. Data represent means ± SEM. *P<0.05, **P<0.01, ***P<0.001.
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individual amygdalar regions [5,6]. Additionally, numerous studies indicate that hippocampus is involved in inhibiting the HPA axis response and expresses high levels of glucocorticoid receptors [37].

The stimulatory effect of stress in the vCA1 and CA2 regions of the hippocampus is consistent with a previous study of the effect of arthritic stress in rats on the whole hippocampus [19] but not with the finding of chronic psychosocial stress in tree shrews [20]. The reason for this discrepancy might reflect species-specific control of 11HSD1 or the type and duration of stress applied. As hippocampal cells reactivate inactive 11-dehydrocorticosterone to active corticosterone [38], we suggest that an increase in 11HSD1 expression after repeated social stress could modulate the local corticosterone concentration. The regional differences in the response of 11HSD1 mRNA to stress among CA subfields are difficult to reconcile with the hippocampal functions. Emerging evidence indicates that the dorsal hippocampus performs primarily cognitive functions, whereas the ventral part is connected to stress and emotion [39] and that corticosteroids have been shown to act as structural and functional modulators of limbic areas, including learning and memory [34,40]. Comparison of the current findings with previous works indicates that corticosterone-sensitive neurons in limbic structures play a role in the feedback regulation of stress responses and thus the amplification of glucocorticoid signals due to upregulation of 11HSD1 might facilitate this feedback.

The action of glucocorticoids is predominantly mediated through intracellular lower-affinity glucocorticoid receptors (GR) that are activated by large amounts of glucocorticoids secreted during stress [34,40]. We studied, therefore, not only the effect of chronic stress on 11HSD1 but also on GR. However, the expression of GR did not differ between the stressed and unstressed rats in all studied brain regions with the exception of the vCA1 hippocampus, even if the expression of 11HSD1 was upregulated in the prelimbic cortex, amygdala, and vCA1 and CA2 hippocampus. As GR are highly expressed in the hippocampus, prelimbic cortex and amygdala, which are critically involved in mediating stress-related behavior and modulating hippocampal functions [5,40], the absence of changes in expression of GR together with upregulation of 11HSD1 indicates that these limbic structures undergo an adaptive corticosteroid-signaling reaction during repeated social stress based on 11HSD1 and not on GR. This reaction does not occur in the principal components of the HPA axis, such as the pituitary and adrenal gland, or in the intrahypothalamic regulatory nuclei such as the PVN, even if all of these structures express 11HSD1. In light of these facts, it can be hypothesized that the upregulation of 11HSD1 in amygdala, prelimbic cortex and some areas of hippocampus might intensify the glucocorticoid signal via activating GR due to conversion of plasma 11-dehydrocorticosterone to corticosterone and thus might subsequently attenuate the HPA axis via the activation of stress-inhibitory and damping of stress-excitatory regions of limbic structures [4].

Besides the brain, the social stress also modulated 11HSD1 expression in primary and secondary lymphoid organs. The stimulatory effect of stress on 11HSD1 in the spleen and MLN is in agreement with the suppression of immune responses as the most-reported consequence of stress [3]. Mucosal immunosuppression paralleled by epithelial barrier defects were found in murine social-stress-induced colitis [25] and we have recently shown an upregulation of 11HSD1 in the spleen and lymphatic nodes during colitis [15]. This amplification of 11HSD1 might be related to the action of proinflammatory mediators, since exposure to TNFα and IL-1β increases 11HSD1 [41,42] and social stress upregulates plasma level and tissue gene expression of IL-1 cytokines [43]. Splenic and lymphatic node 11HSD1 upregulation and increased glucocorticoid regeneration might be part of the immunosuppresive effects of social stress, even if the reason for the discrepant observations between the secondary lymphoid organs and thymus are not clear.

How exactly social stress modulates 11HSD1 mRNA expression remains unknown. We can only speculate about a feasible mechanism based on the knowledge of regulation of 11HSD1 gene expression and neurohumoral and humoral factors secreted during stress. Traditionally, CRH, ACTH, catecholamines and glucocorticoids have been attributed to stress acclimation. However, studies investigating the effect of these humoral factors are limited and their results contradictory [19,21,50,51]. Sequence analysis of 11HSD1 revealed several putative binding sites for various transcription factors, particularly CCAT/enhancer binding proteins (C/EBPs), AP1 (Fos/Jun), and NF-κB [44–47] and several studies have linked the regulation of 11HSD1 to these factors. Overexpression of AP1, C/EBPα and C/EBPβ potently increases 11HSD1 promoter activity, whereas overexpression of NF-κB rather inhibits this activity [44,45]. Depending on cell types, several studies have linked TNFα to 11HSD1 upregulation via C/EBP-, AP1-, NF-κB-, or MAPK-signaling pathways [45,46,48] and OPN has been shown to activate NF-κB via degradation of NF-κB inhibitor IKβ [49]. In addition, recent data provide evidence for an indirect interaction of GR with 11HSD1 promoter via C/EBPβ transcription factor [52,53].

Adding to the complexity of 11HSD1 regulation during social stress, we demonstrated here that the same stress protocol is able to enhance the expression of CYP7B1 both in brain and peripheral tissues. This cytochrome P450 catalyses the 7α-hydroxylation of steroids that subsequently interact with 11HSD1 and may direct the fine tuning of glucocorticoids [26]. An interesting hypothesis is that the upregulation of CYP7B1 by stress increases local concentration of 7α-derivatives and thus transformation of inactive 11-dehydrocorticosterone into active corticosterone may be modulated when 11HSD1 is faced with 7α-derivatives at the same time. This hypothesis is supported by several findings. First, a positive correlation was shown between increased CYP7B1 mRNA/CYP7B1 enzyme activity and the progression (severity) of murine arthritis [54]. Second, elevated IL-1β increased CYP7B1 activity [54]. Third, 7-OH metabolites of dehydroepiandrosterone counteracted glucocorticoid-induced apoptosis of murine splenocytes [55], and fourth, the directionality of CYP7B1 reaction depended crucially on the level of pyridine dinucleotide cosubstrates in endoplasmic reticulum [56], which can be modulated during psychosocial stress.

In summary, we provide evidence for the role of social stress in the regulation of the enzymes of local metabolism of glucocorticoids in specific brain structures and in lymphoid organs. The role of stress on expression of 11HSD1 and CYP7B should be kept in mind when studying the neuronal regulation of the stress reaction and the stress-associated changes in immune and inflammation responses.

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Author Contributions

Conceived and designed the experiments: MV PE AM JP. Performed the experiments: MV PE AM LR PK J. Makal MS J. Musílková. Analyzed the data: PE AM PZ JP. Contributed reagents/materials/analysis tools: MV PE AM JP. Wrote the paper: AM JP.

References

1. McEwen BS (2007) Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol Rev 87: 873–904.
2. Krugers HJ, Lucassen PJ, Karst H, Joëls M (2010) Chronic stress effects on hippocampal structure and synaptic function: relevance for depression and normalization by anti-glucocorticoid treatment. Front Synaptic Neurosci 2: 24. Available: http://www.frontiersin.org/Synaptic_Neuroscience/10.3389/fnsyn.2010.00024/full. Accessed 2013 October 1.
3. McEwen BS, Biron CA, Brunson KW, Bulloch K, Chambers WH, et al. (1997) The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. Brain Res Brain Res Rev 23: 79–133.
4. Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, et al. (2003) Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24: 151–180.
5. Ulrich-Lai YM, Herman JP (2009) Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 10: 397–409.
6. Myers B, McKlveen JM, Herman JP (2012) Neural regulation of the stress response: the many faces of feedback. Cell Mol Neurobiol 32: 683–694.
7. Wyrwoll CS, Holmes MC, Seckl JR (2011) 11β-hydroxysteroid dehydrogenases and the brain: from zero to hero, a decade of progress. Front Neuroendocrinol 32: 265–286.
8. Moisan MP, Seckl JR, Edwards CR (1990) 11β-hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus, and cortex. Endocrinology 127: 1450–1455.
9. Sakai RR, Lakshmi V, Monder C, McEwen BS (1992) Immunocytochemical localization of 11 β-hydroxysteroid dehydrogenase in hippocampus and other brain regions of the rat. J Neuroendocrinol 4: 101–106.
10. Bisschop PH, Dekker MJHJ, Osterthun W, Kwakkel J, Anink JJ, et al. (2013) Expression of 11β-hydroxysteroid dehydrogenase type 1 in the human hypothalamus. J Neuroendocrinol 25: 425–432.
11. Korbonits M, Bujalska I, Shimojo M, Nobes J, Jordan S, et al. (2001) Expression of 11β-hydroxysteroid dehydrogenase isoenzymes in the human pituitary: induction of the type 2 enzyme in corticotropinomas and other pituitary tumors. J Clin Endocrinol Metab 86: 2728–2733.
12. Hanafusa J, Mune T, Tanahashi T, Isomura Y, Suwa T, et al. (2002) Altered corticosteroid metabolism differentially affects pituitary corticotropin response. Am J Physiol Endocrinol Metab 282: E466–E473.
13. Shimojo M, Condon J, Whorwood CB, Stewart PM (1996) Adrenal 11β-hydroxysteroid dehydrogenase. Endocr Res 22: 771–780.
14. D'Elia M, Patenaude J, Bernier J (2009) Regulation of glucocorticoid sensitivity in thymocytes from burn-injured mice. Am J Physiol Endocrinol Metab 296. E97–E104.
15. Ergang P, Vytáčková K, Švec J, Bryndová J, Mikšík I, et al. (2011) Upregulation of 11β-hydroxysteroid dehydrogenase 1 in lymphoid organs during inflammation in the rat. J Steroid Biochem Mol Biol 126: 19–25.
16. Harris HJ, Kotelevtsev Y, Mullins JJ, Seckl JR, Holmes MC (2001) Intracellular regeneration of glucocorticoids by 11β-hydroxysteroid dehydrogenase (11β-HSD)-1 plays a key role in regulation of the hypothalamic-pituitary-adrenal axis: analysis of 11β-HSD-1-deficient mice. Endocrinology 142: 114–120.
17. Carter RN, Paterson JM, Tworowska U, Stenvers DJ, Mullins JJ, et al. (2009) Hypothalamic-pituitary-adrenal axis abnormalities in response to deletion of 11β-HSD1 is strain-dependent. J Neuroendocrinol 21: 879–887.
18. Monder C, Sakai RR, Miroff Y, Blanchard DC, Blanchard RJ (1994) Reciprocal changes in plasma corticosterone and testosterone in stressed male rats maintained in a visible burrow system: evidence for a mediating role of testicular 11β-hydroxysteroid dehydrogenase. Endocrinology 134: 1193–1198.
19. Low SC, Moisan MP, Noble JM, Edwards CR, Seckl JR (1994) Glucocorticoids regulate hippocampal 11β-hydroxysteroid dehydrogenase activity and gene expression in vivo in the rat. J Neuroendocrinol 6: 285–290.
20. Jamieson PM, Fuchs E, Flugge G, Seckl JR (1997) Attenuation of hippocampal 11β-hydroxysteroid dehydrogenase type 1 by chronic psychosocial stress in the tree shrew. Stress 2: 123–132.
21. Pelletier G, Luu-The V, Li S, Bujold G, Labrie F (2007) Localization and glucocorticoid regulation of 11β-hydroxysteroid dehydrogenase type 1 mRNA in the male mouse forebrain. Neuroscience 145: 110–115.
22. Blanchard RJ, McKittrick CR, Blanchard DC (2001) Animal models of social stress: effects on behavior and brain neurochemical systems. Physiol Behav 73: 261–271.
23. Kiecolt-Glaser JK, Glaser R (2002) Depression and immune function: central pathways to morbidity and mortality. J Psychosom Res 53: 873–876.
24. Bailey MT, Kinsey SG, Padgett DA, Sheridan JF, Leblebicioglu B (2009) Social stress enhances IL-1β and TNF-α production by Porphyromonas gingivalis lipopolysaccharide-stimulated $CD11b^+$ cells. Physiol Behav 98: 351–358.
25. Reber SO, Peters S, Slattery DA, Hofmann C, Schölmerich J, et al. (2011) Mucosal immunosuppression and epithelial barrier defects are key events in murine psychosocial stress-induced colitis. Brain Behav Immun 25: 1153–1161.
26. Muller C, Hennebert O, Morfin R (2006) The native anti-glucocorticoid paradigm. J Steroid Biochem Mol Biol 100: 95–105.
27. Mitchell PJ, Redfern PH (2005) Animal models of depressive illness: the importance of chronic drug treatment. Curr Pharm Des 11: 171–203.

28. Švec J, Ergang P, Mandys V, Kment M, Pácha J (2010) Expression profiles of proliferative and antiapoptotic genes in sporadic and colitis-related mouse colon cancer models. Int J Exp Pathol 91: 44–53.
29. Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates. Amsterdam: Elsevier.
30. Porterfield VM, Zimomra ZR, Caldwell EA, Camp RM, Gabella KM, et al. (2011) Rat strain differences in restraint stress-induced brain cytokines. Neuroscience 188: 48–54.
31. Barnum CJ, Blandino P, Deak T (2008) Social status modulates basal IL-1 concentrations in the hypothalamus of pair-housed rats and influences certain features of stress reactivity. Brain Behav Immun 22: 517–527.
32. Audet MC, Jacobson-Pick S, Wann BP, Anisman H (2011) Social defeat promotes specific cytokine variations within the prefrontal cortex upon subsequent aggressive or endotoxin challenges. Brain Behav Immun 25: 1197–1205.
33. Zuckerman-Levin N, Tiosano D, Eisenhofer G, Bornstein S, Hochberg Z (2001) The importance of adrenocortical glucocorticoids for adrenomedullary and physiological response to stress: a study in isolated glucocorticoid deficiency. J Clin Endocrinol Metab 86: 5920–5924.
34. de Kloet ER, Joëls M, Holsboer F (2005) Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6: 463–475.
35. Jones KR, Myers B, Herman JP (2011) Stimulation of the prelimbic cortex differentially modulates neuroendocrine responses to psychogenic and systematic stressors. Physiol Behav 104: 266–271.
36. Dorio D, Viau V, Meaney MJ (1993) The role of the medial prefrontal cortex (cingulated gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J Neurosci 13: 3839–3847.
37. de Kloet ER, Vreugdenhil E, Oitzl MS, Joëls M (1998) Brain corticosteroid receptor balance in health and disease. Endocr Rev 19: 269–301.
38. Rajan V, Edwards CR, Seckl JR (1996) 11β-Hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. J Neurosci 16: 65–70.
39. Fanselow MS, Dong HW (2010) Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 65: 7–19.
40. Herbert J, Goodyer IM, Grossman AB, Hastings MH, de Kloet ER, et al. (2006) Do corticosteroids damage the brain? J Neuroendocrinol 18: 393–411.
41. Escher G, Galli I, Vishwanath BS, Frey BM, Frey FJ (1997) Tumor necrosis factor α and interleukin 1β enhance the cortisone/cortisol shuttle. J Exp Med 186: 189–198.
42. Cooper MS, Bujalska I, Rabbitt E, Walker EA, Bland R, et al. (2001) Modulation of 11β-hydroxysteroid dehydrogenase isozymes by proinflammatory cytokines in osteoblasts: an autocrine switch from glucocorticoid inactivation to activation. J Bone Miner Res 16: 1037–1044.
43. Engler H, Bailey MT, Engler A, Stiner-Jones LM, Quan N, et al. (2008) Interleukin-1 receptor type 1-deficient mice fail to develop social stress-associated glucocorticoid resistance in the spleen. Psychoneuroendocrinology 33: 108–117.
44. Iwasaki Y, Takayasu S, Nishiyama M, Tsugita M, Taguchi T, et al. (2008) Is the metabolic syndrome an intracellular Cushing state? Effects of multiple humoral factors on the transcriptional activity of the hepatic glucocorticoid-activating enzyme (11β-hydroxysteroid dehydrogenase type 1) gene. Mol Cell Endocrinol 285: 10–18.
45. Tsugita M, Iwasaki Y, Nishiyama M, Taguchi T, Shinahara M, et al. (2008) Differential regulation of 11β-hydroxysteroid dehydrogenase type-1 and -2 gene transcription by proinflammatory cytokines in vascular smooth muscle cells. Life Sci 83: 426–432.
46. Ignatova ID, Kostadinova RM, Goldring CE, Nawrocki AR, Frey FJ, et al. (2009) Tumor necrosis factor-α upregulates 11β-hydroxysteroid dehydrogenase type 1 expression by CCAAT/enhancer binding protein- β in HepG2 cells. Am J Physiol Endocrinol Metab 296: E367–E377.
47. Williams LJ, Lyons V, MacLeod I, Rajan V, Darlington GJ, et al. (2000) C/EBP regulates hepatic transcription of 11β-hydroxysteroid dehydrogenase type 1. A novel mechanism for cross-talk between the C/EBP and glucocorticoid signaling pathways. J Biol Chem 275: 30232–30239.
48. Ahasan MM, Hardy R, Jones C, Kaur K, Nanus D, et al. (2012) Inflammatory regulation of glucocorticoid metabolism in mesenchymal stromal cells. Arthritis Rheum 64: 2404–2413.
49. Wang KX, Denhardt DT (2007) Osteopontin: role in immune regulation and stress responses. Cytokine Growth Factor Rev 19: 333–345.
50. Friedberg M, Zoumakis E, Hiroi N, Bader T, Chrousos GP, et al. (2003) Modulation of 11β-hydroxysteroid dehydrogenase type 1 in mature human subcutaneous adipocytes by hypothalamic messengers. J Clin Endocrinol Metab 88: 385–393.
51. Schmid J, Ludwig B, Schally AV, Steffen A, Ziegler CG, et al. (2011) Modulation of pancreatic islets-stress axis by hypothalamic releasing hormones and 11β-hydroxysteroid dehydrogenase. Proc Natl Acad Sci USA 108: 13722–13727.
52. Yang Z, Guo C, Zhu P, Li W, Myatt L, et al. (2007) Role of glucocorticoid receptor and CCAAT/enhancer-binding protein α in the feed-forward induction of 11β-hydroxysteroid dehydrogenase type 1 expression by cortisol in human amnion fibroblasts. J Endocrinol 195: 241–253.
53. Sai S, Esteves CL, Kelly V, Michailidou Z, Anderson K, et al. (2008) Glucocorticoid regulation of the promoter of 11β-hydroxysteroid dehydrogenase type 1 is indirect and requires CCAAT/enhancer-binding protein-β. Mol Endocrinol 22: 2049–2060.
54. Dulos J, Verbraak E, Bagchus WM, Boots AM, Kaptein A (2004) Severity of murine collagen-induced arthritis correlates with increased CYP7B activity: enhancement of dehydroepiandrosterone metabolism by interleukin-1β. Arthritis Rheum 50: 3346–3353.
55. Sterzl I, Hampl R, Sterzl J, Votruba J, Stárka L (1999) 7β-OH-DHEA counteracts dexamethasone induced suppression of primary immune response in murine splenocytes. J Steroid Biochem Mol Biol 71: 133–137.
56. Nashev LG, Chandsawangbhuwana C, Balazs Z, Atanasov AG, Dick B, et al. (2007) Hexose-6-phosphate dehydrogenase modulates 11β-hydroxysteroid dehydrogenase type 1-dependent metabolism of 7-keto- and 7β-hydroxy-neurosteroids. PLoS ONE 2: e561. Available: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0000561. Accessed 2013 October 1.

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Differential impact of stress on hypothalamic—pituitary—adrenal axis: Gene expression changes in Lewis and Fisher rats

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KEYWORDS
11β-Hydroxysteroid dehydrogenase type 1;
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Summary The aim of the present work was to study the influence of variable stress on the expression of 11β-hydroxysteroid dehydrogenase type 1 (11HSD1) and the neuropeptides corticotropin-releasing hormone (CRH), urocortins 2 and 3(UCN2, UCN3), arginine vasopressin (AVP), oxytocin (OXT) and adenylate cyclase-activating polypeptide (PACAP) in two inbred rat strains: stress hypo-responsive Lewis (LEW) and hyper-responsive Fisher 344 (F344) rats. We found site-specific and strain-dependent differences in the basal and stress-stimulated expression of 11HSD1, CRH, UCN2, UCN3 and PACAP. In LEW rats, stress upregulated 11HSD1 in the prefrontal cortex and lateral amygdala, whereas in F344 rats 11HSD1 was upregulated in the central amygdala and hippocampal CA2 and ventral but not dorsal CA1 region; no effect was observed in the paraventricular nucleus, pituitary gland and adrenal cortex of both strains. The expression of glucocorticoid receptors did not parallel the upregulation of 11HSD1. Stress also stimulated the expression of paraventricular OXT, CRH, UCN3 and PACAP in both strains but amygdalar CRH only in LEW and UCN2/UCN3 in F344 rats, respectively. The upregulation of PACAP and CRH was paralleled only by increased expression of PACAP receptor PAC1 but not CRH receptor type 1. These observations provide evidence that inbred F344 and LEW rats exhibit not

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only the well-known phenotypic differences in the activity of the HPA axis but also strain- and stress-dependent differences in the expression of genes encoding 11HSD1 and neuropeptides associated with the HPA axis activity. Moreover, the differences in 11HSD1 expression suggest different local concentration of corticosterone and access to GR in canonical and noncanonical structures of the HPA axis.

1. Introduction

Stress is a common aspect of modern life that produces profound physiological and behavioral disturbances and may contribute to many psychiatric disorders, including depression and post-traumatic stress disorders (de Kloet et al., 2005). Exposure to stressors triggers activation of the nervous, endocrine and behavioral systems to promote physiological adaptations and maintain homeostasis (Herman et al., 2003). The principal endocrine component of the stress response is the activation of the hypothalamic–pituitary–adrenocortical (HPA) axis, a self-regulatory pathway that utilizes its end products (cortisol and corticosterone) to control its own activation and responsiveness through a negative feedback mechanism. The HPA axis is controlled by neurons located in the paraventricular nucleus (PVN) of the hypothalamus but also by central stress excitatory and inhibitory circuits that are activated by stressors in both intrahypothalamic and extrahypothalamic structures (Ulrich-Lai and Herman, 2009). Various neurotransmitters, neuromodulators and steroid stress mediators are released to stress response and can influence distinct neuronal circuits. As summarized by Joëls and Baram (2009), a number of neuropeptides are released by stress in specific populations of neuronal cells and contribute to the activations of the stress response or counteract it. The canonical stress-activated neuropeptides include vasopressin (AVP) and corticotropin-releasing hormone (CRH), which is expressed together with its receptors CRHR1 and CRHR2 not only in the PVN but also in other brain structures. Similar to CRH, stress upregulates the expression of two other members of the CRH family, the urocortins UCN2 and UCN3, which have much higher potency to bind CRHR2 than CRHR1 (Tanaka et al., 2003; Jamieson et al., 2006), the oxytocin (OXT), a neuropeptide that reduces physiological and behavioral indices of stress (Lee et al., 2009), and the adenylate cyclase-activating polypeptide (PACAP), a pleiotropic neuropeptide that is an important regulator of neuroendocrine stress response pathways (Lezak et al., 2014) and has a less-appreciated modulatory role in biosynthesis and secretion of some pituitary hormones (Halvorson, 2014). In addition, the effects of individual mediators on the HPA axis are modulated by glucocorticoids whose release is triggered by stress and receptors are ubiquitously expressed in brain (Joëls and Baram, 2009).

The response of the target cells to glucocorticoids is not dependent merely on the level of free hormones in blood or the activity of multidrug resistance efflux pumps and receptor density in target cells but also on the prereceptor metabolism of glucocorticoids that determines the intracellular concentration of the hormone. In the majority of cells and tissues, this metabolism depends on 11β-hydroxysteroid dehydrogenase type 1 (11HSD1), an enzyme, which converts in vivo biologically inactive 11-oxo-steroids (cortisone, 11-dehydrocorticosterone) to cortisol and corticosterone and thus amplifies cellular glucocorticoid action (Tomlinson et al., 2004; Wyrwoll et al., 2011). This enzyme is expressed in the brain (Wyrwoll et al., 2011; Bisschop et al., 2013; Vodička et al., 2014), pituitary gland (Hanafusa et al., 2002), adrenal gland (Shimojo et al., 1996) and many other peripheral organs (Tomlinson et al., 2004). Another enzyme in the 11HSD family is 11HSD type 2 (11HSD2), which catalyzes the oxidation of cortisol and corticosterone to the inactive cortisone and 11-dehydrocorticosterone, thereby reducing the local glucocorticoid signals (Wyrwoll et al., 2011). It is expressed predominantly in mineralocorticoid target tissues but also in the adrenal gland (Shimojo et al., 1996), where it plays a role in regulation of basal and stimulated adrenal steroid secretion and modulates the expression of phenylethanolamine-N-methyltransferase, a glucocorticoid-dependent enzyme, which catalyzes the conversion of norepinephrine to epinephrine (Musajo et al., 1996; Shimojo et al., 1996). Moderate levels of 11HSD2 expression were also found in some loci of brain (Wyrwoll et al., 2011).

The expression of 11HSD1 in the principal components of the HPA axis and in brain areas that are responsible for the positive and negative regulation of this axis suggests that 11HSD1 might modulate the activity of the HPA axis. Two findings support this hypothesis. First, targeted inactivation of enzyme hexose-6-phosphate dehydrogenase, which regenerates NADPH required for 11HSD1 catalyzed reduction of 11-dehydrocorticosterone to corticosterone, is associated with decreased negative feedback of the HPA axis in spite of elevated circulating levels of corticosterone (Rogoff et al., 2007). Second, 11HSD1 knock-out mice have elevated corticosterone levels and exaggerated ACTH and corticosterone responses to stress (Harris et al., 2001), but the HPA axis phenotype is dependent on the background strain of the mice (Carter et al., 2009). These findings suggest that the regeneration of glucocorticoids by 11HSD1 may be an important regulator of glucocorticoid feedback of HPA axis in vivo and that the genetic background may influence the interaction between 11HSD1 and HPA axis.

Some data indicate that stressful situations modulate the expression of 11HSD1 in the brain and some peripheral organs, but the results are contradictory (Low et al., 1994; Monder et al., 1994; Jamieson et al., 1997; Sesti-Costa et al., 2012; Vodička et al., 2014). In addition, little is known regarding whether genetic background determines the effect of stress on 11HSD1 in specific brain areas associated with the regulation of the HPA axis and in pituitary and adrenal glands. One approach used to investigate these questions is testing animals with different genotypically determined HPA axes. For such study, can be used the histocompatibly similar Lewis (LEW) and Fisher 344 (F344) inbred rat strains, which differ in their responses of both the HPA axis and the immune system to stressogenic stimuli (Sternberg et al., 1989). Generally, LEW rats display a markedly smaller reaction to a wide range of stressors compared with F344 rats, even if there is no difference in the GR level between both strains in the hippocampus and HPA axis (Dhabhar et al., 1993; Grota et al., 1997).

As outlined above, there is evidence that (1) 11HSD1 is expressed in brain structures of HPA axis, (2) stress modulates the expression of 11HSD1 and (3) the ratio of corticosterone/11-dehydrocorticosterone is significantly different among various brain structures (Cobice et al., 2013). These results, taken together with evidence indicating the role of 11HSD1 in control of HPA axis (Harris et al., 2001; Carter et al., 2009) and the different HPA axis phenotype in LEW and F344 rats, suggest that 11HSD1 could participate in different stress response of LEW and F344 rats. We examined, therefore, whether the activation of the HPA axis in response to stressors observed in LEW and F344 rats will translate into differences of 11HSDs and glucocorticoid receptors in canonical structures of HPA axis and in brain structures involved in positive or negative alterations of this axis. To explore further the possible role of 11HSD1 in activation of HPA axis, we investigated the impact of stress on expression of neuropeptides and receptors, which are known to be modulated by glucocorticoids namely, CRH (Kageyama and Suda, 2010), urocortins (Chen et al., 2003; Tillinger et al., 2013), AVP (Kim et al., 2001), OXT (Uchoa et al., 2009) and PACAP receptor (Lezak et al., 2014).

2. Materials and methods

2.1. Animals

Male Fisher 344 (F344) and Lewis (LEW) rats (Charles River, Germany) that were 60—65 days old at the beginning of the experiments were used. Animals were housed in groups of three to four in Plexiglas cages in a temperature-controlled room ($23 \pm 1\,^{\circ}C$) on a 12/12-h light/dark cycle with ad libitum access to food and water throughout the entire study except for stress sessions. They were left for three weeks to acclimatize before any experimental procedures. The animals were randomly assigned to four groups: (1) naïve F344 rats, (2) F344 rats subjected to variable stress, (3) naïve LEW rats and (4) LEW rats subjected to variable stress. Control rats were left undisturbed in their home cages. Studies were performed in accordance with the guidelines of the Animal Protection Law of the Czech Republic and approved by the animal Care and Use Committee of the Institute of Physiology.

2.2. Stress procedure

The short-term variable stress protocol according to Ilin and Richter-Levin (2009) was used. On Day 1, the animals were exposed to forced swim (predominantly physical stressor) for 15 min in an opaque circular water tank (water temperature $22 \pm 1\,^{\circ}C$). On Day 2, the animals were placed on an elevated platform (12 cm × 12 cm at a height of 70 cm above floor level, emotional stressor) in brightly lit room for 30 min. This trial was repeated three times, with a 60 min interval between trials. On day 3, the rats underwent a 2-h restraining stress in an opaque plastic box (emotional/physical stressor) that prevented the free movement of the animal. These protocols were applied simultaneously to all rats in the cage. The advantage of this paradigm of subchronic short-term variable stress was that (i) it combined three different stressors, which utilize various neural pathways, and (ii) the variability and unpredictability of stressors minimized habituation, compared to repeated homotypic stress.

2.3. Tissue sampling and processing

The rats were anesthetized with isoflurane immediately after the third stress session (a two-hour restrain stress), blood was collected by cardiac puncture and the animals were killed by decapitation without delay. The brain, pituitary and adrenal gland were removed, immediately frozen, and stored in liquid nitrogen. Isoflurane was used because no significant in vivo gene transcriptional response was detected in brain within short-term anesthesia (Pan et al., 2006). Brain specimens and samples of the adrenal cortex and medulla were prepared by laser microdissection and RNA analysis was performed as previously described (Vodička et al., 2014). Briefly, coronal brain sections (20 μm) were serially cut with a cryostat at $-19\,^{\circ}C$. The regions were identified based on standard anatomical landmarks and stereotaxic coordinates according to Paxinos and Watson (2007). The sections of the studied structures were mounted onto slides coated with polyethylene naphthalate membrane (Leica Microsystems, Wetzlar, Germany), stained with cresyl violet, and dissected using a LMD6000 Laser Microdissection System (Leica). Microdissected tissues (average area = 0.25 mm^2) were homogenized in 75 μl RLT buffer (Qiagen, Hilden, Germany) and stored at $-80\,^{\circ}C$ until RNA isolation. The following brain regions were studied: prelimbic prefrontal cortex (plPFC), infralimbic prefrontal cortex (ilPFC), central nucleus of amygdala (CeA), lateral nucleus of amygdala (LaA), paraventricular nucleus of hypothalamus (PVN), and ventral and dorsal CA1 (vCA1, dCA1), CA2 and CA3 subfields of hippocampus. Total RNA from the captured microsamples was isolated using an RNeasy Micro Kit (Qiagen, Hilden, Germany) and from the pituitary gland using a GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA).

Single-strand cDNA was prepared from total RNA isolated from tissue microsamples and macrosamples using random hexamers and either Enhanced Avian Reverse Transcriptase (Sigma-Aldrich, St. Louis, MO, USA) or High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA), respectively. The cDNA samples were analyzed by real-time PCR on an Viia 7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using 5× Hot Firepol Probe QPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) and TaqMan Assays (Life Technologies, Carlsbad, CA, USA) specific for rat 11HSD1 (cat. no. Rn00567167_m1), 11HSD2 (cat. no. Rn00492539_m1), glucocorticoid receptor (GR; cat. no. Rn00561369_m1), CRH (cat. no. Rn01462137_m1), UCN2 (cat. no. Rn00591306_s1), UCN3 (cat. no. Rn02091611_s1), CRH receptor type 1 (CRHR1; cat. no. Rn00578611_m1), CRH receptor type 2 (CRHR2; cat. no. Rn00575617_m1), PACAP (cat. no. Rn00566438_m1), PACAP type 1 receptor (PAC1: cat. no. Rn00591653_m1), AVP (cat. no. Rn00566449_m1), OXT (cat. no. Rn00564446_g1), tyrosine hydroxylase (TH; cat. no. Rn00562500_m1), L-aromatic amino acid decarboxylase (DDC; cat. no. Rn00561113_m1), dopamine β-hydroxylase (DBH; cat. no. Rn00565819_m1) and phenylethanolamine-N-methyltransferase (PNMT; cat. no. Rn01495588_m1). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; TaqMan Endogenous Control, cat. no. 4352338E) was measured to normalize the mRNA expression in each sample, as its transcription does not change during stress in the rat brain (Porterfield et al., 2011). A single PCR reaction was performed in a final volume of 20 μl using target gene probes labeled with FAM in duplex with a GAPDH probe (VIC/MGB). The quantity of the PCR product was determined using the standard curve method with 10-fold dilutions of the mixed cDNA sample.

Table 1 Effect of stress on plasma level of corticosterone and adrenal, thymus and spleen weights in Fisher 344 and Lewis rats. CTRL, control unstressed rats.

	Fisher 344		Lewis		Two-way ANOVA		
	CTRL	Stress	CTRL	Stress	Strain	Stress	Strain × stress
Corticosterone (ng/ml)	51 ± 18	361 ± 35***	78 ± 5	213 ± 35$^{**,+++}$	<0.05 (4.83)	<0.001 (65.26)	<0.01 (10.06)
Adrenal weight (mg/100 g BW)	7.9 ± 0.7	8.6 ± 0.4	7.0 ± 0.5	9.9 ± 0.6**	ns	<0.01 (9.14)	ns
Thymus weight (mg/100 g BW)	111 ± 3	91 ± 3***	89 ± 5^{+++}	87 ± 2	<0.01 (10.80)	<0.01 (8.19)	<0.05 (5.62)
Spleen weight (mg/100 g BW)	242 ± 3	221 ± 2*	166 ± 6^{+++}	156 ± 4^{+++}	<0.001 (83.75)	<0.01 (20.90)	ns

Results are expressed as means ± SEM. Seven animals in each group. *F* values are given in parentheses; $^{*}P < 0.05$ or $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. control group; $^{+}P < 0.05$ or $^{++}P < 0.01$ or $^{+++}P < 0.001$ vs. Fisher 344 rats.

Plasma corticosterone was assayed using commercial RIA kit (MP Biomedicals, Solon, OH, USA).

2.4. Statistical analysis

The results are presented as the means ± SEM. All analyses were conducted in Statistica 6.1. (StatSoft Inc., Tulsa, OK, USA) using a two-way analysis of variance. The independent variables were treatment and strain consisting of two levels: naïve or stressed and F344 or LEW, respectively. Post hoc analyses were performed using Fisher's Protected Least Significant Difference method when overall significant main effects were observed. The results were considered significant if $P < 0.05$.

3. Results

3.1. Effect of stress on organ weights and on plasma level of corticosterone

Repeated exposure to stressors resulted in a significant effect of stress on weight of adrenal glands, thymus and spleen as reflected by changes in the absolute weights as well as the values adjusted relative to body weight. Two-way ANOVA revealed an effect of stress on absolute weights of adrenal gland ($F = 5.46$, $P < 0.05$) and spleen ($F = 50.29$, $P < 0.001$) and on relative weights of adrenal gland, thymus and spleen (Table 1). The effect of strain on absolute weight was identified in adrenal gland ($F = 20.48$, $P < 0.001$), thymus ($F = 16.38$, $P < 0.001$) and spleen ($F = 9.83$, $P < 0.01$) and on relative weight in thymus and spleen (Table 1). Significant stress x strain interactions were found in case of absolute values in all three organs (adrenal gland: $F = 5.39$, $P < 0.05$; thymus $F = 16.13$, $P < 0.001$; spleen: $F = 5.02$, $P < 0.05$), whereas in case of relative weights only in thymus (Table 1). Post hoc analysis of relative organ weights revealed significant adrenal hypertrophy following stress in LEW rats, whereas in F344 rats the increase in adrenal weight was not significant. In contrast, stressed F344 rats showed significant thymic and splenic atrophy relative to the unstressed group (Table 1). Post hoc analysis of absolute weights (not shown) gave similar results. Stress induced hypertrophy of adrenal gland in LEW ($P < 0.01$) but not F344 rats and atrophy of thymus and spleen in both strains (thymus: F344, $P < 0.001$, LEW, $P < 0.05$; spleen: F344, $P < 0.001$, LEW, $P < 0.01$).

Resting corticosterone levels were similar in both strains. In response to stressors, we observed a significant effect of strain and stress (Table 1). Consistently with previously published data, F344 rats had significantly higher plasma corticosterone following exposure to stress compared to LEW rats. These differences were also supported by a significant interaction between strain and stress exposure for the variable plasma level of corticosterone (Table 1).

3.2. Effect of stress on the canonical structures of the HPA axis

To establish the impact of stress on multiple members of the glucocorticoid and neuropeptide signaling families and the catecholamine biosynthesis, we examined their expression in the PVN and pituitary and adrenal gland. As shown in Fig. 1, exposure to stress significantly increased the expression of CRH and UCN3, but not UCN2, in the PVN (CRH: $F = 27.82$, $P < 0.001$; UCN3: $F = 7.17$, $P < 0.05$) without any effect of the genotype background and stress by strain interaction. The CRHR1 receptor, which has been suggested together with CRH as critical for initiating stress response, was not different between both strains and was not influenced by stress (Table 2). The expression of CRHR2, which has much higher potency to bind UCN2 and UCN3 than CRH, was upregulated in stressed animals of both strains (Table 2). Similar to CRH, stress significantly upregulated the expression of OXT ($F = 36.57$, $P < 0.001$; Fig. 1), PACAP ($F = 39.76$, $P < 0.001$; Fig. 1) and its receptor PAC1 (Table 2) and these effects were strain-independent except for PACAP ($F = 7.31$; $P < 0.05$). Expression of AVP was not influenced by stress (Fig. 1). Additionally, ANOVA did not reveal any inter-strain differences and strain x stress interactions of CRH, UCN2/UCN3, AVP and OXT expression. No significant effect of stress and strain x stress interaction was observed also in the expression of 11HSD1 even if the naïve F344 rats had lower expression of 11HSD1 than LEW rats ($F = 8.06$, $P < 0.01$) (Fig. 1). Expression of 11HSD2 (Fig. 1) and GR (Table 3) was similar in both strains and was not modulated by stress.

Within the pituitary gland, stress upregulated the expression of GR of LEW but not F344 rats (Table 3) and was

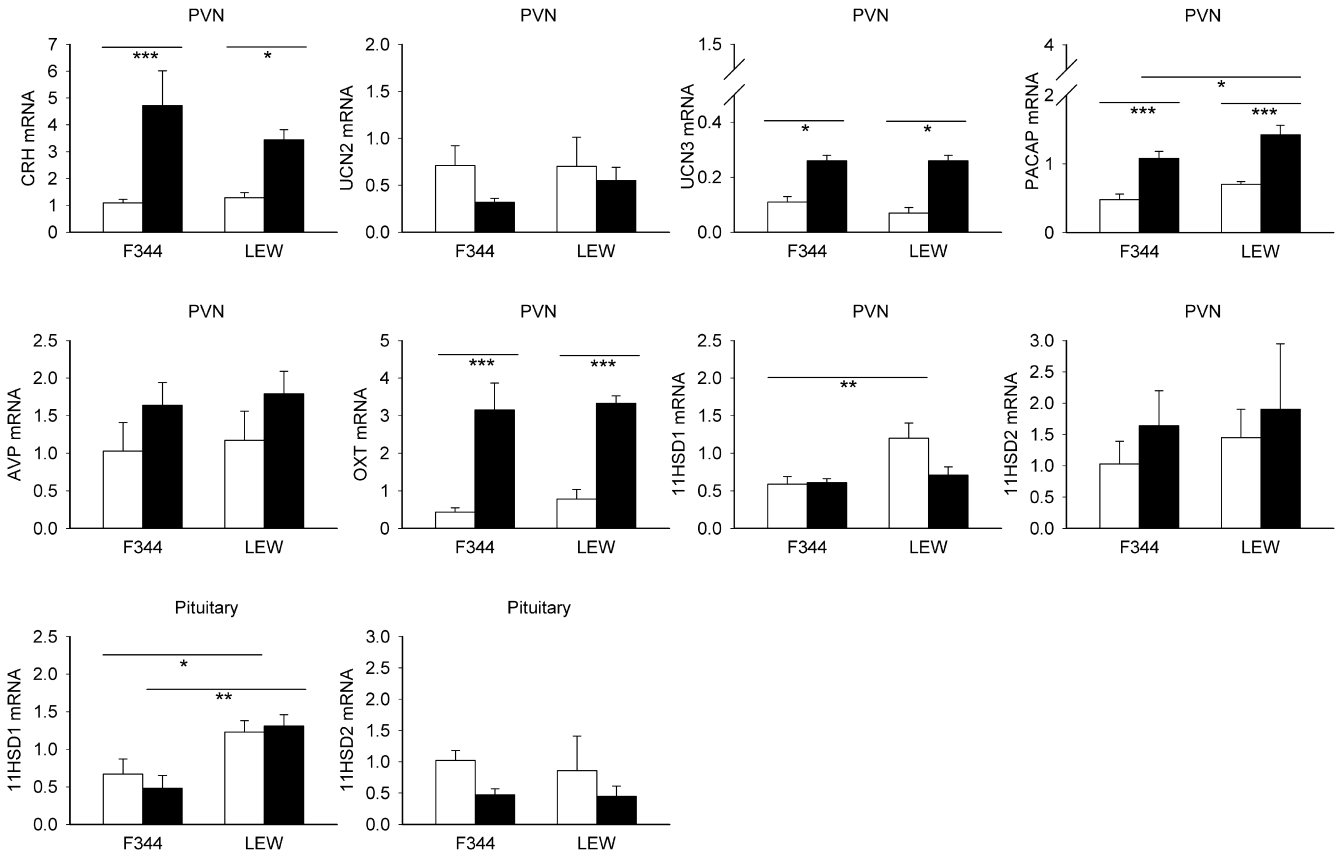

Figure 1 Stress-induced expression of neuropeptides and enzymes of glucocorticoid metabolism in hypothalamic paraventricular nucleus (PVN) and pituitary of Fisher 344 (F344) and Lewis (LEW) rats. *Open bars*, unstressed rats, *full bars*, rats exposed for three days to variable stressors; CRH, corticotropin-releasing hormone; UCN2, urocortin 2; UCN3, urocortin 3; PACAP, adenylate cyclase-activating polypeptide; AVP, arginine vasopressin; OXT, oxytocin, 11HSD1 and 11HSD2, 11β-hydroxysteroid dehydrogenase type 1 and type 2. The results are expressed as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 2 Comparison of expression of genes encoding receptors of CRH and VIP families in specific brain areas and pituitary gland of stressed and unstressed Fisher 344 and Lewis rats.

	Fisher 344		Lewis		Two-way ANOVA		
	CTRL	Stress	CTRL	Stress	Strain	Stress	Strain × stress
CRHR1							
PVN	0.14 ± 0.03	0.17 ± 0.01	0.13 ± 0.02	0.15 ± 0.02	ns	ns	ns
Pituitary	0.10 ± 0.03	0.02 ± 0.03*	0.20 ± 0.03^{++}	0.10 ± 0.01$^{**,+}$	<0.001 (16.16)	<0.001 (14.80)	ns
CeA	0.24 ± 0.04	0.25 ± 0.04	0.31 ± 0.05	0.23 ± 0.08	ns	ns	ns
LaA	0.74 ± 0.09	0.83 ± 0.07	1.01 ± 0.12	0.92 ± 0.10	ns	ns	ns
CRHR2							
PVN	0.07 ± 0.01	0.38 ± 0.10***	0.05 ± 0.01	0.27 ± 0.03**	ns	<0.001 (29.53)	ns
Pituitary	0.81 ± 0.21	0.74 ± 0.10	0.41 ± 0.08	0.64 ± 0.10	ns	ns	ns
PAC1							
PVN	3.41 ± 0.57	5.58 ± 0.81*	3.65 ± 0.25	4.82 ± 0.38	ns	<0.01 (10.58)	ns
Pituitary	0.09 ± 0.03	0.02 ± 0.00	0.33 ± 0.03^{+++}	0.32 ± 0.06^{+++}	<0.001 (46.90)	ns	ns
CeA	0.76 ± 0.05	1.32 ± 0.10*	0.57 ± 0.02	1.32 ± 0.25**	ns	<0.001 (19.66)	ns
LaA	0.62 ± 0.04	1.34 ± 0.17**	0.52 ± 0.11	1.28 ± 0.17**	ns	<0.001 (33.20)	ns

CeA, central nucleus of amygdala; LaA, lateral nucleus of amygdala; PVN, hypothalamic paraventricular nucleus; results are expressed as means ± SEM. 5—7 animals in each group. *F* values are given in parentheses; $^{*}P < 0.05$ or $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. control group; $^{+}P < 0.05$ or $^{++}P < 0.01$ or $^{+++}P < 0.001$ vs. Fisher 344 rats.

Table 3 Changes in expression of the gene encoding glucocorticoid receptor followed by a 3-day stress in brain areas and pituitary gland of Fisher 344 and Lewis rats.

	Fisher 344		Lewis		Two-way ANOVA		
	CTRL	Stress	CTRL	Stress	Strain	Stress	Strain × stress
PVN	0.22 ± 0.05	0.30 ± 0.03	0.25 ± 0.01	0.33 ± 0.03	ns	ns	ns
Pituitary	0.02 ± 0.00	0.03 ± 0.01	0.12 ± 0.01^{+++}	0.15 ± 0.01$^{*,+++}$	<0.001 (66.72)	<0.05 (6.41)	ns
plPFC	0.13 ± 0.03	0.16 ± 0.01	0.19 ± 0.02	0.18 ± 0.01	ns	ns	ns
ilPFC	0.17 ± 0.02	0.18 ± 0.02	0.16 ± 0.04	0.18 ± 0.02	ns	ns	ns
CeA	0.26 ± 0.03	0.30 ± 0.01	0.34 ± 0.02	0.29 ± 0.05	ns	ns	ns
LaA	0.11 ± 0.02	0.12 ± 0.01	0.13 ± 0.01	0.15 ± 0.02	ns	ns	ns
dCA1	0.78 ± 0.08	0.61 ± 0.04*	0.82 ± 0.08	0.51 ± 0.04**	ns	<0.001 (15.60)	ns
vCA1	0.26 ± 0.04	0.25 ± 0.02	0.22 ± 0.06	0.26 ± 0.02	ns	ns	ns

plPFC, prelimbic prefrontal cortex; ilPFC, infralimbic prefrontal cortex; CeA, central nucleus of amygdala; LaA, lateral nucleus of amygdala; PVN, hypothalamic paraventricular nucleus; dCA1, dorsal part of CA1 hippocampus; vCA1, ventral part of CA1 hippocampus; CA2 and CA3, hippocampal subfields. Results are expressed as means ± SEM. 5—7 animals in each group. *F* values are given in parentheses; $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. control group; $^{+++}P < 0.001$ vs. Fisher 344 rats.

without any effect on 11HSD1 and 11HSD2 in both strains (Fig. 1). The inter-strain differences were found in both 11HSD1 (Fig. 1; $F = 16.97$, $P < 0.001$) and GR (Table 2) and the expression of GR and 11HSD1 was significantly higher in LEW compared to F344 rats. As CRH represents not only an autocrine—paracrine factor in specific brain areas but also a hypothalamic—pituitary releasing factor, and PACAP seems to modulate biosynthesis and secretion of some pituitary hormones, we further examined the expression of CRHR1, CRHR2 and PAC1 receptors in the pituitary gland. As shown in Table 2, effects of stress as well as strain were observed on CRHR1, where F344 rats had lower expression of this receptor than LEW rats and stress downregulated CRHR1 in both strains. In contrast, CRHR2 expression was independent of strain and stress and PAC1 expression was stress-independent but was significantly lower in F344 that LEW.

As shown in Fig. 2, F344 and LEW rats exhibited significant differences in the expression of adrenal 11HSD1 and 11HSD2. The two-way ANOVA revealed inter-strain differences in the expression of 11HSD1 in adrenal cortex ($F = 21.88$, $P < 0.001$) and 11HSD2 in both adrenal cortex ($F = 5.95$, $P < 0.05$) and medulla ($F = 7.21$, $P < 0.01$) in addition to the effect of stress on adrenocortical 11HSD2 ($F = 16.08$, $P < 0.001$). Post hoc analysis revealed a considerably higher 11HSD1 level in the adrenal cortex of F344 than LEW rats and significantly higher stress-induced upregulation of 11HSD2 in F344 than LEW rats.

As LEW rats show a blunted stress-induced epinephrine response compared to the F344 strain and stress alters the synthesis of catecholamines within the adrenal medulla through the activity of some catecholamine-synthesizing enzymes (Elenkov et al., 2008), we studied further the effect of our stress paradigm on expression of genes of the epinephrine biosynthesis. As expected, the main effect of stress and strain was observed on TH (stress: $F = 41.19$, $P < 0.001$; strain: $F = 6.08$, $P < 0.05$) and PNMT (stress: $F = 32.00$, $P < 0.001$; strain: $F = 7.23$, $P < 0.01$), whereas strain x stress interaction was evident only for TH ($F = 5.42$, $P < 0.05$). In contrast to TH and PNMT, the expression of DDC and DBH was not dependent on strain and stress. Post hoc tests revealed stress-dependent upregulation of TH and PNMT expression in both strains and the response was higher in F344 than LEW rats (Fig. 2).

3.3. Effect of stress on the limbic structures involved in the HPA axis integration

To examine the impact of stress on activation of glucocorticoid and neuropeptide signaling in structures that are responsible for modulation of HPA axis activity, we studied the expression of 11HSD1, CRH, UCN2, UCN3, PACAP and their receptors in prefrontal cortex, amygdala and hippocampus. As shown in Fig. 3, exposure to stress significantly modulated 11HSD1 expression in prefrontal cortex (plPFC: $F = 8.91$, $P < 0.01$; ilPFC: $F = 9.05$, $P < 0.01$), amygdala (CeA: $F = 6.79$, $P < 0.05$; LaA: $F = 8.19$, $P < 0.05$) and in vCA1 and CA2 regions of hippocampus (vCA1: $F = 10.18$, $P < 0.01$; CA2: $F = 5.96$, $P < 0.05$), whereas no effect was observed in dCA1 and CA3. In contrast, the effect of strain was found in prefrontal cortex (plPFC: $F = 8.72$, $P < 0.01$; ilPFC: $F = 9.11$, $P < 0.01$) and vCA1 and CA3 hippocampus (vCA1: $F = 10.95$, $P < 0.01$; CA3: $F = 23.81$, $P < 0.001$). The analysis did not find any significant strain x stress interaction. Post hoc comparisons revealed that naïve F344 rats had higher expression of 11HSD1 in plPFC, ilPFC and in CA3 hippocampus than LEW rats. The stressors upregulated 11HSD1 in plPFC, ilPFC and LaA of LEW rats and in CeA, vCA1 and CA2 of F344 rats (Fig. 3). To further assess the effect of stressors on glucocorticoid signaling in the brain, we measured the expression of GR. As summarized in Table 3, no strain differences were found in expression of GR. Similarly, stress did not change the expression of GR in any investigated brain areas with the exception of the dorsal CA1 hippocampus.

To clarify whether F344 and LEW rats might differ in signaling via CRF and PACAP pathways, we assayed mRNA levels of CRH, UCN2, UCN3, PACAP and their receptors in the amygdala, a crucial complex of nuclei involved in the control of emotional and autonomic response to stress. As shown in Fig. 4, the expression of CRH in amygdala differed significantly between F344 and LEW strain and CRH reached 40 times lower level in LaA than in CeA. In amygdalar nuclei, the statistical analysis revealed an effect of stress (CeA; $F = 7.51$, $P < 0.05$) and strain (CeA: $F = 7.83$, $P < 0.01$; LaA: $F = 8.18$, $P < 0.01$) as well as strain x stress interaction (CeA: $F = 6.15$, $P < 0.05$; LaA: $F = 5.75$, $P < 0.05$). Post hoc comparisons to the LEW rats showed that the unstressed F344 rats have higher expression of CRH in the amygdala and the stressors stimulated the expression of amygdalar CRH only

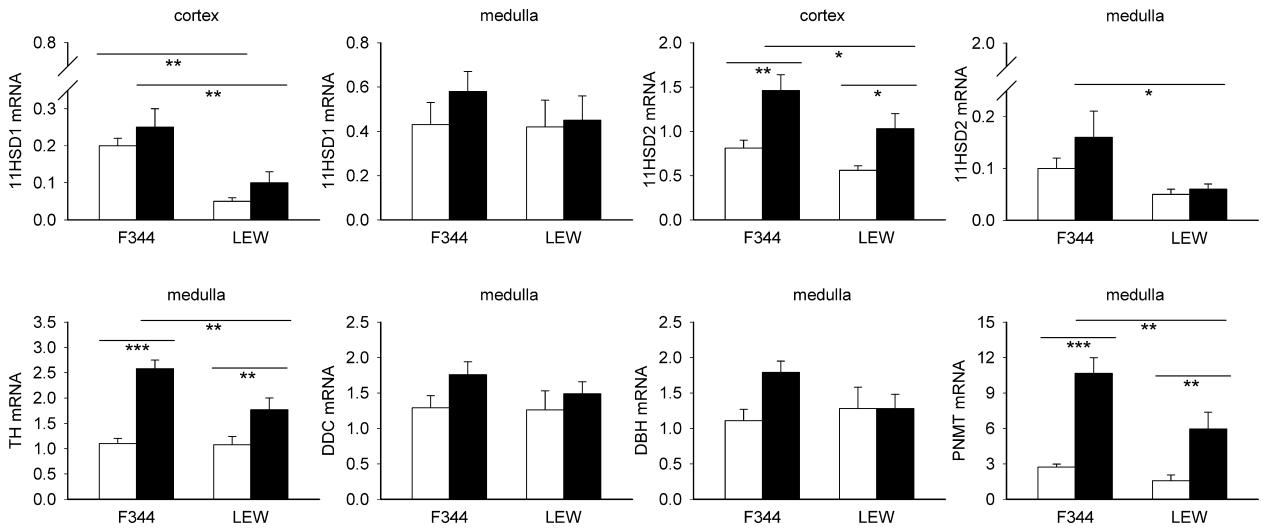

Figure 2 Differential response of adrenal enzymes of glucocorticoid metabolism and catecholamine biosynthesis following stress. Expression of 11β-hydroxysteroid dehydrogenase type 1 (11HSD1), type 2 (11HSD2) and enzymes of epinephrine biosynthesis: tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (DDC), dopamine β-hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT), was analyzed in unstressed (*open bars*) and stressed (*full bars*) Fisher 344 (F344) and Lewis (LEW) rats. The results are expressed as the means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

in LEW but not F344 rats. The distribution of urocortins UCN2 and UCN3 differed considerably from the expression of CRH. Two-way ANOVA revealed stimulatory effect of stress on amygdalar UCN2 (CeA: $F = 9.74$, $P < 0.01$; LaA: $F = 25.57$, $P < 0.001$) and UCN3 (CeA: $F = 5.31$, $P < 0.05$; LaA: $F = 7.95$ $P < 0.05$). In addition, the effect of strain and interaction between strain and stress was found in CeA (UCN3: strain: $F = 4.62$, $P < 0.05$, interaction: $F = 5.33$, $P < 0.05$) and LaA (UCN2: strain: $F = 36.98$, $P < 0.001$, interaction: $F = 11.54$, $P < 0.01$). Post hoc comparisons demonstrated that the stressors preferentially upregulated UCN2 and UCN3 in amygdala of F344 rats (Fig. 4). The expression of CRHR1 receptor was not different between both strains and was not influenced by stress (Table 2); the expression of CRHR2 was below the detection limit of our method.

Expression of PACAP was much higher in LaA than CeA and stress significantly upregulated the expression of this neuropeptide (LaA: $F = 7.85$, $P < 0.05$; Fig. 4) and its receptor PAC1 (Table 2) in both strains.

4. Discussion

Stress has been shown to be associated with activation of the HPA and sympathoadrenal medullary axes. Consistent

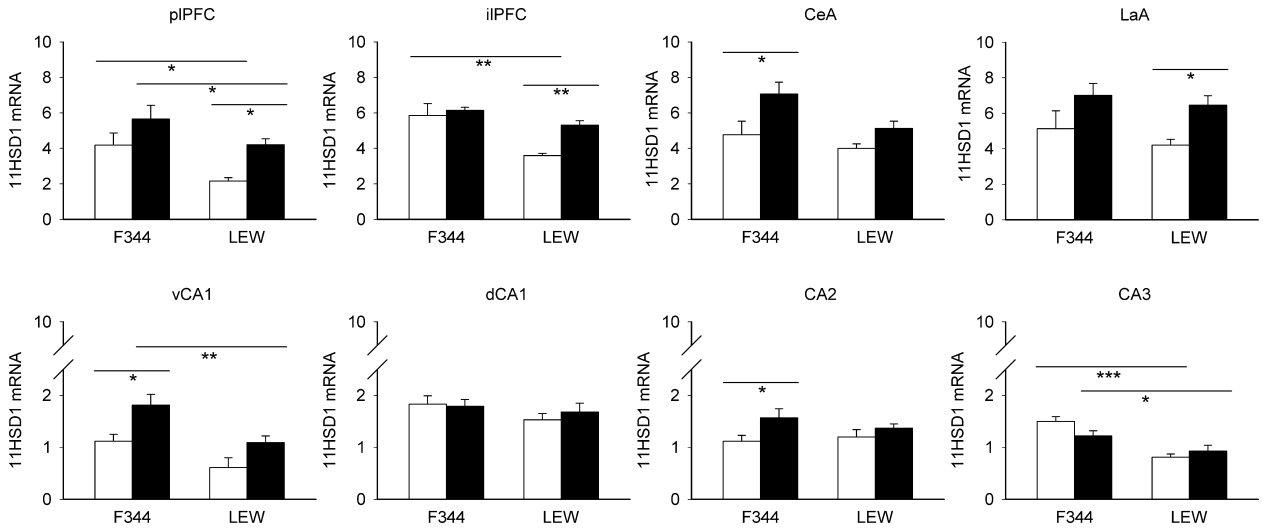

Figure 3 Effect of stress on expression of 11β-hydroxysteroid dehydrogenase type 1 (11HSD1) in brain structures associated with the HPA axis in Fisher 344 (F344) and Lewis (LEW) rats. *Open bars*, unstressed rats, *full bars*, rats exposed for three days to variable stressors; plPFC, prelimbic prefrontal cortex; ilPFC, infralimbic prefrontal cortex; CeA, central nucleus of amygdala; LaA, lateral nucleus of amygdala; vCA1 and dCA1, ventral and dorsal part of CA1 hippocampus; CA2 and CA3, hippocampal CA2 and CA3 subfields. The results are expressed as the means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

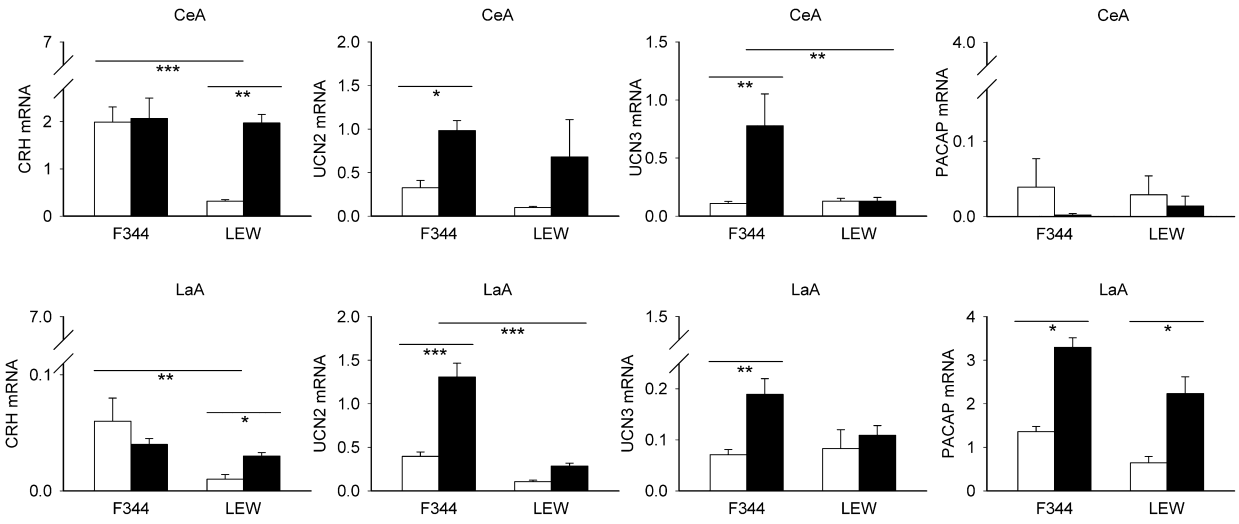

Figure 4 Stress-induced expression of neuropeptides in central (CeA) and lateral (LaA) nucleus of amygdala in unstressed (*open bars*) and stressed (*full bars*) Fisher 344 (F344) and Lewis (LEW) rats. CRH, corticotropin-releasing hormone; UCN2, urocortin 2; UCN3, urocortin 3; PACAP, adenylate cyclase-activating polypeptide. The results are expressed as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

with previous studies (Sternberg et al., 1989; Dhabhar et al., 1993; Moncek et al., 2001; Elenkov et al., 2008), we found an exaggerated stress response in F344 rats compared to those of LEW strain as indicated by higher plasma corticosterone and higher upregulation of TH and PNMT, the key regulatory enzymes of catecholamine biosynthesis in the adrenal medulla. Although the original differences between LEW and F344 rats were found in females and the sexual dimorphism of HPA axis is well-known (Sternberg et al., 1989), our finding in males shows that that the lower stress response of LEW rats in comparison to F344 strain is not limited to one gender and is in line with previous reports (Dhabhar et al., 1993; Grota et al., 1997; Stöhr et al., 2000; Elenkov et al., 2008). The results indicate that short-term variable stress modulates in brain structures associated with HPA axis the expression of some genes encoding 11HSD1, glucocorticoid-sensitive neuropeptides and their receptors and that these changes are not only stress- but also strain-dependent.

4.1. Strain differences in 11HSD1 and GR and their stress responsiveness in specific components of the HPA axis

The present data reveal clear inter-strain differences in the response to stress using the paradigm based on presentation of the three different stressors within three days. The combination of emotional and physical stressors significantly upregulated 11HSD1 in the prefrontal cortex and amygdala and some regions of the hippocampus (vCA1, CA2) but not in the PVN and pituitary and adrenal gland; i.e., stress upregulated 11HSD1 in the limbic structures that are associated with the regulation of the HPA axis (de Kloet et al., 2005; Ulrich-Lai and Herman, 2009) but not in the canonical components of this axis. This is concordant with our earlier report in which we showed that chronic psychosocial stress (resident-intruder paradigm) is also able to upregulate 11HSD1 in some limbic structures without any effects on the principal components of the HPA axis (Vodička et al., 2014). When subjected to stress, the response of LEW rats was higher in plPFC, ilPFC and LaA, whereas F344 rats were more sensitive to stressors in CeA, vCA1 and CA2. The regionally distinct effects may be related to several factors, including (i) ceiling effects in highly stress-responsive strain or (ii) the role of specific structures in glucocorticoid feedback regulation. First, LEW rats had constitutively lower 11HSD1 expression in the prefrontal cortex, vCA1 and CA3 than the F344 strains and stress never stimulated this expression in the brain of LEW rats to a higher level than in the F344 strain. Second, the prelimbic cortex inhibits the HPA axis (Herman et al., 2003), and corticosterone implantation to this region reduces glucocorticoid secretion after stress (Diorio et al., 1993). Similarly, the hippocampus and amygdala and their GR have been repeatedly shown to modulate the HPA response (Herman et al., 2003), and sustained GR deletion in the hippocampus, prefrontal cortex and amygdala showed delayed inhibition of the HPA axis response to stressors (Furay et al., 2008). One possible interpretation for this pattern of changes might be that upregulation of 11HSD1 expression amplifies the negative regulatory feedback of glucocorticoids to control the intensity and duration of the stress response and that this amplification shows inter-strain and regional differences. Indeed, it was shown recently (Cobice et al., 2013) that the ratio of corticosterone/11-dehydrocorticosterone is significantly different among the brain cortex, amygdala and hippocampus and hippocampal 11HSD1 reactivates inactive 11-dehydrocorticosterone to active corticosterone (Rajan et al., 1996). The regional differences in response to stress-induced hippocampal 11HSD1 expression are difficult to reconcile with the hippocampal functions; however, it was recently shown that the ventral part of the hippocampus responds to glucocorticoids much differently than does the dorsal hippocampus (Maggio and Segal, 2009). In addition, the prominent role of the ventral hippocampus in inhibiting the HPA axis stress response, its importance in the processing of anxiety and existence of more connections of ventral than dorsal hippocampus with amygdala and prefrontal cortex (Fanselow and Dong, 2010) raises the possibility that increased hippocampal 11HSD1 expression may be relevant to stress regulation. On the basis of the absence of changes in expression of GR combined with the upregulation of 11HSD1, we can hypothesize that

our stress paradigm might intensify the glucocorticoid signal in limbic structures predominantly due to the conversion of local corticosterone to 11-dehydrocorticosterone but not via upregulation of GR.

In contrast to the limbic structures, no effect of stress on 11HSD1 expression was observed in the PVN and stress-relevant peripheral organs, such as the pituitary and adrenal gland, even if pronounced strain differences were evident. Similarly, no effect of stress was observed in case of 11HSD2 expression. Thus, stress does not seem to modulate the intracrine/paracrine glucocorticoid signaling in canonical components of the HPA axis. The lower expression of GR and 11HSD1 in the pituitary gland of F344 than in LEW rats suggests that these changes could contribute to aggravated efficacy of the pituitary glucocorticoid negative feedback of HPA in F344 (Simar et al., 1997). With regard to the adrenal gland, strain-dependent differences of 11HSD1 and 11HSD2 were found both in the adrenal cortex and medulla. The evidence that adrenal 11HSDs may play a physiological role has been demonstrated in experiments with nonspecific inhibitors of 11HSDs, which reduced the release of 11-dehydrocorticosterone from adrenal gland and the expression of the glucocorticoid-dependent enzyme PNMT in adrenal medulla (Musajo et al., 1996; Shimojo et al., 1996). However, the absence of any changes in medullary 11HSD1 and 11HSD2 under stressful conditions and concomitant stress-dependent upregulation of glucocorticoid-dependent PNMT and glucocorticoid-independent TH in both strains does not indicate any role for intracrine regulation of glucocorticoids in upregulation of genes of the epinephrine biosynthetic pathway. The surprising stress-dependent upregulation of adrenal 11HSD2 that was more obvious in F344 than in LEW rats agrees with the previous findings of higher adrenal corticosterone level in stressed LEW than F344 rats (Moncek et al., 2001).

4.2. Strain-dependent neuropeptide responses to stress

The importance of maintaining stress-induced activation of the HPA system within tolerable limits requires the efficient regulation of CRH signaling pathway, which has been extensively characterized both in vivo and in vitro. However, only limited data are available concerning the central regulation of this pathway in F344 and LEW rats. Whereas the 3-day stress upregulated CRH and OXT expression in the PVN of both strains, the upregulation of CRH in the amygdala was observed only in LEW rats, in particular in CeA, which is involved in long term modulation of HPA activity (Prewitt and Herman, 1997). The increased expression of amygdalar CRH and absence of further stimulation by stress in hyper-responsive F344 rats in comparison with LEW strain is in accord with the recent findings of Flandreau et al. (2012) who have shown that overexpression of CRH in CeA induces HPA axis hyperactivity. Similar to CRH, we have shown that the stressors not only stimulate the expression of UCN2 and UCN3, but that this expression is region-selective and strain-dependent. Whereas in the amygdala UCN2 and UCN3 were upregulated in F344 but not LEW rats, the expression of paraventricular UCN2 was not changed and UCN3 increased in both strains. Considering that UCN2 is increased by glucocorticoids (Chen et al., 2003) and the rat UCN2 gene promoter has several putative glucocorticoid responsive elements (Tillinger et al., 2013), the upregulation of 11HSD1 in CeA of stressed F344 rats might intensify the stress-associated increase of UCN2 in this strain. In contrast, the expression of PACAP, a neuropeptide, which has been shown to modulate CRH expression in the amygdala (Stroth et al., 2011), was significantly stimulated by stress in both strains, a finding similar to Hammack et al. (2009) in Sprague-Dawley rats.

We next investigated whether the differences in stress-response mediators described above are associated with changes of their receptors. To address this, we analyzed the mRNA levels of PACAP receptor PAC1 and CRH receptors CRHR1 and CRHR2, which have preferential specificity for CRH and UCN2 and UCN3, respectively. In both strains, the stressors upregulated the expression of PAC1 in CeA, LaA and PVN, downregulated the expression of CRHR1 in pituitary gland and upregulated CRHR2 in PVN. Interestingly, the effect of strain was found only in pituitary CRHR1 and PAC1; no effect of strain x stress interaction was found in any investigated structure. In amygdala, the expression of CRH receptors was unchanged (CRHR1) or was under the detection limit of our technique (CRHR2). Similar findings were observed by Zohar and Weinstock (2011), who observed in stressed animals the upregulation of paraventricular CRHR2 without any changes of paraventricular and amygdalar CRHR1 and by Ochedalski et al. (1998), who found downregulation of pituitary CRHR1 by glucocorticoids. Moreover, stress-dependent upregulation of PAC1 was described recently in bed nucleus of the stria terminalis (Hammack et al., 2009).

In summary, the analysis of CRH(UCN2,3)/CRHR1,2 system in amygdala, PVN and pituitary revealed the effect of stress, strain and their interactions predominantly on the amygdalar CRH pathway at the level of neuropeptides CRH, UCN2 and UCN3 but not their receptors CRHR1 and CRHR2. As stress response of amygdalar UCN2/UCN3 are higher in F344 than in LEW rats, whereas the expression of amygdalar CRH is stimulated predominantly in LEW rats, it is reasonable to assume that the blunted response to stress challenges in LEW rats might depend partly on the observed differences. Stress-induced glucocorticoids stimulate the expression of amygdalar CRH, which is in contrast to the PVN (Watts and Sanchez-Watts, 1995), and thus the upregulation of 11HSD1 in CeA of F344 rats might intensify the glucocorticoid signal for the stimulation of amygdalar CRH expression and result it modulation of stress response projections to the PVN and fear- and anxiety-like behavior (Ulrich-Lai and Herman, 2009). However, the different patterns of the effect of stress on the expression of 11HSD1 and the studied neuropeptides in CeA and LaA suggest that stress-induced 11HSD1 elevation may not be the primary driver of CRH, UCN2 and UCN3 in amygdala.

4.3. Conclusions

The present results show that (i) there are significant inter-strain differences in basal level of 11HSD1 expression in some components of the HPA axis, specifically the stress hyper-responsive F344 rats have higher 11HSD1 expression in the prefrontal cortex, vCA1, CA3 and adrenal cortex and lower expression in the PVN and pituitary gland than their stress hypo-responsive LEW counterparts, (ii) stress does not modulate the expression of 11HSD1 in canonical components of the HPA axis (PVN, pituitary gland, adrenal cortex) but selectively upregulates 11HSD1 in brain structures associated with regulation of HPA axis (prefrontal cortex, amygdala, some regions of hippocampus), (iii) inter-strain differences are also found in neuropeptides that participate in activation of HPA axis, namely amygdalar CRH/UCN2,3 but not in their receptors CRHR1 and CRHR2, and (iv) the effect of stress on the expression of

the neuropeptides and their receptors is site-specific. Taken together, the results from the current experiments combined with the observations by others suggest that the local glucocorticoid metabolic system and CRH pathway in the brain structures associated with HPA axis is not identical in both strains and might be involved in the differences of the HPA axis responsiveness between F344 and LEW rats.

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Conflict of interest statement

None declared.

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References

Bisschop, P.H., Dekker, M.J., Osterthun, W., Kwakkel, J., Anink, J.J., Boelen, A., Unmehopa, U.A., Koper, J.W., Lamberts, S.W., Stewart, P.M., Swaab, D.F., Fliers, E., 2013. Expression of 11β-hydroxysteroid dehydrogenase type 1 in the human hypothalamus. J. Neuroendocrinol. 25, 425—432.

Carter, R.N., Paterson, J.M., Tworowska, U., Stenvers, D.J., Mullins, J.J., Seckl, J.R., Holmes, M.C., 2009. Hypothalamic—pituitary—adrenal axis abnormalities in response to deletion of 11β-HSD1 is strain-dependent. J. Neuroendocrinol. 21, 879—887.

Chen, A., Vaughan, J., Vale, W.W., 2003. Glucocorticoids regulate the expression of the mouse urocortin II gene: a putative connection between the corticotropin-releasing factor receptor pathways. Mol. Endocrinol. 17, 1622—1639.

Cobice, D.F., Mackay, C.L., Goodwin, R.J., McBride, A., Langridge-Smith, P.R., Webster, S.P., Walker, B.R., Andrew, R., 2013. Mass spectrometry imaging for dissecting steroid intracrinology within target tissues. Anal. Chem. 85, 11576—11584.

de Kloet, E.R., Joëls, M., Holsboer, F., 2005. Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6, 463—475.

Dhabhar, F.S., McEwen, B.S., Spencer, R.L., 1993. Stress response, adrenal steroid receptor levels and corticosteroid-binding globulin levels — a comparison between Sprague-Dawley, Fischer 344 and Lewis rats. Brain Res. 616, 89—98.

Diorio, D., Viau, V., Meaney, M.J., 1993. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic—pituitary—adrenal responses to stress. J. Neurosci. 13, 3839—3847.

Elenkov, I.J., Kvetnansky, R., Hashiramoto, A., Bakalov, V.K., Link, A.A., Zachman, K., Crane, M., Jezova, D., Rovensky, J., Dimitrov, M.A., Gold, P.W., Bonini, S., Fleisher, T., Chrousos, G.P., Wilder, R.L., 2008. Low- versus high-baseline epinephrine output shapes opposite innate cytokine profiles: presence of Lewis- and Fischer-like neurohormonal immune phenotypes in humans? J. Immunol. 181, 1737—1745.

Fanselow, M.S., Dong, H.W., 2010. Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 65, 7—19.

Flandreau, E.I., Ressler, K.J., Owens, M.J., Nemeroff, C.B., 2012. Chronic overexpression of corticotropin-releasing factor from the central amygdala produces HPA axis hyperactivity and behavioral anxiety associated with gene-expression changes in the hippocampus and paraventricular nucleus of the hypothalamus. Psychoneuroendocrinology 37, 27—38.

Furay, A.R., Bruestle, A.E., Herman, J.P., 2008. The role of the forebrain glucocorticoid receptor in acute and chronic stress. Endocrinology 149, 5482—5490.

Grota, L.J., Bienen, T., Felten, D.L., 1997. Corticosterone responses of adult Lewis and Fischer rats. J. Neuroimmunol. 74, 95—101.

Halvorson, L.M., 2014. PACAP modulates GnRH signaling in gonadotropes. Mol. Cell. Endocrinol. 385, 45—55.

Hammack, S.E., Cheung, J., Rhodes, K.M., Schutz, K.C., Falls, W.A., Braas, K.M., May, V.V., 2009. Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): roles for PACAP in anxiety-like behavior. Psychoneuroendocrinology 34, 833—843.

Hanafusa, J., Mune, T., Tanahashi, T., Isomura, Y., Suwa, T., Isaji, M., Daido, H., Morita, H., Murayama, M., Yasuda, K., 2002. Altered corticosteroid metabolism differentially affects pituitary corticotropin response. Am. J. Physiol. Endocrinol. Metab. 282, E466—E473.

Harris, H.J., Kotelevtsev, Y., Mullins, J.J., Seckl, J.R., Holmes, M.C., 2001. Intracellular regeneration of glucocorticoids by 11β-hydroxysteroid dehydrogenase (11β-HSD)-1 plays a key role in regulation of the hypothalamic—pituitary—adrenal axis: analysis of 11β-HSD-1-deficient mice. Endocrinology 142, 114—120.

Herman, J.P., Figueiredo, H., Mueller, N.K., Ulrich-Lai, Y., Ostrander, M.M., Choi, D.C., Cullinan, W.E., 2003. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front. Neuroendocrinol. 24, 151—180.

Ilin, Y., Richter-Levin, G., 2009. Enriched environment experience overcomes learning deficits and depressive-like behavior induced by juvenile stress. PLoS ONE 4, e4329.

Jamieson, P.M., Fuchs, E., Flugge, G., Seckl, J.R., 1997. Attenuation of hippocampal 11β-hydroxysteroid dehydrogenase type 1 by chronic psychosocial stress in the tree shrew. Stress 2, 123—132.

Jamieson, P.M., Li, C., Kukura, C., Vaughan, J., Vale, W., 2006. Urocortin 3 modulates the neuroendocrine stress response and is regulated in rat amygdala and hypothalamus by stress and glucocorticoids. Endocrinology 147, 4578—4588.

Joëls, M., Baram, T.Z., 2009. The neuro-symphony of stress. Nat. Rev. Neurosci. 10, 459—466.

Kageyama, K., Suda, T., 2010. Transcriptional regulation of hypothalamic corticotropin-releasing factor gene. Vitam. Horm. 82, 301—317.

Kim, J.K., Summer, S.N., Wood, W.M., Schrier, R.W., 2001. Role of glucocorticoid hormones in arginine vasopressin gene regulation. Biochem. Biophys. Res. Commun. 289, 1252—1256.

Lee, H.J., Macbeth, A.H., Pagani, J.H., Zouny, W.S., 2009. Oxytocin: the great facilitator of life. Prog. Neurobiol. 88, 127—151.

Lezak, K.R., Roman, C.W., Braas, K.M., Schutz, K.C., Falls, W.A., Schulkin, J., May, V., Hammack, S.E., 2014. Regulation of bed nucleus of the stria terminalis PACAP expression by stress and corticosterone. J. Mol. Neurosci. 54, 477—484.

Low, S.C., Moisan, M.P., Noble, J.M., Edwards, C.R., Seckl, J.R., 1994. Glucocorticoids regulate hippocampal 11β-hydroxysteroid dehydrogenase activity and gene expression in vivo in the rat. J. Neuroendocrinol. 6, 285—290.

Maggio, N., Segal, M., 2009. Differential corticosteroid modulation of inhibitory synaptic currents in the dorsal and ventral hippocampus. J. Neurosci. 29, 2857—2866.

Moncek, F., Kvetnansky, R., Jezova, D., 2001. Differential responses to stress stimuli of Lewis and Fischer rats at the pituitary and adrenocortical level. Endocr. Regul. 35, 35—41.

Monder, C., Sakai, R.R., Miroff, Y., Blanchard, D.C., Blanchard, R.J., 1994. Reciprocal changes in plasma corticosterone and testosterone in stressed male rats maintained in a visible burrow system: evidence for a mediating role of testicular 11β-hydroxysteroid dehydrogenase. Endocrinology 134, 1193—1198.

Musajo, F., Neri, G., Tortorella, C., Mazzocchi, G., Nussdorfer, G.G., 1996. Intra-adrenal 11β-hydroxysteroid dehydrogenase plays a role in the regulation of corticosteroid secretion: an in vitro study in the rat. Life Sci. 59, 1401—1406.

Ochedalski, T., Rabadan-Diehl, C., Aguilera, G., 1998. Interaction between glucocorticoids and corticotropin releasing hormone (CRH) in the regulation of the pituitary CRH receptor in vivo in the rat. J. Neuroendocrinol. 10, 363—369.

Pan, J.Z., Wei, H., Hecker, J.G., Tobias, J.W., Eckenhoff, R.G., Eckenhoff, M.F., 2006. Rat brain DNA transcript profile of halothane and isoflurane exposure. Pharmacogenet. Genom. 16, 171—182.

Paxinos, G., Watson, C., 2007. The Rat Brain in Stereotaxic Coordinates. Elsevier, Amsterdam.

Prewitt, C.M., Herman, J.P., 1997. Hypothalamo-pituitary-adrenocortical regulation following lesions of the central nucleus of the amygdala. Stress 1, 263—280.

Porterfield, V.M., Zimomra, Z.R., Caldwell, E.A., Camp, R.M., Gabella, K.M., Johnson, J.D., 2011. Rat strain differences in restraint stress-induced brain cytokines. Neuroscience 188, 48—54.

Rajan, V., Edwards, C.R., Seckl, J.R., 1996. 11β-Hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. J. Neurosci. 16, 65—70.

Rogoff, D., Ryder, J.W., Black, K., Yan, Z., Burgess, S.C., McMillan, D.R., White, P.C., 2007. Abnormalities of glucose homeostasis and the hypothalamic—pituitary—adrenal axis in mice lacking hexose-6-phosphate dehydrogenase. Endocrinology 148, 5072—5080.

Sesti-Costa, R., Ignacchiti, M.D., Chedraoui-Silva, S., Marchi, L.F., Mantovani, B., 2012. Chronic cold stress in mice induces a regulatory phenotype in macrophages: correlation with increased 11β-hydroxysteroid dehydrogenase expression. Brain Behav. Immun. 26, 50—60.

Shimojo, M., Whorwood, C.B., Stewart, P.M., 1996. 11β-Hydroxysteroid dehydrogenase in the rat adrenal. J. Mol. Endocrinol. 17, 121—130.

Simar, M.R., Saphier, D., Goeders, N.E., 1997. Dexamethasone suppression of the effects of cocaine on adrenocortical secretion in Lewis and Fischer rats. Psychoneuroendocrinology 22, 141—153.

Sternberg, E.M., Young, W.S., Bernardini, R., Calogero, A.E., Chrousos, G.P., Gold, P.W., Wilder, R.L., 1989. A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. Proc. Natl. Acad. Sci. U. S. A. 86, 4771—4775.

Stöhr, T., Szuran, T., Welzl, H., Pliska, V., Feldon, J., Pryce, C.R., 2000. Lewis/Fischer rat strain differences in endocrine and behavioural responses to environmental challenge. Pharmacol. Biochem. Behav. 67, 809—819.

Stroth, N., Liu, Y., Aguilera, G., Eiden, L.E., 2011. Pituitary adenylate cyclase-activating polypeptide controls stimulus-transcription coupling in the hypothalamic—pituitary—adrenal axis to mediate sustained hormone secretion during stress. J. Neuroendocrinol. 23, 944—955.

Tanaka, Y., Makino, S., Noguchi, T., Tamura, K., Kaneda, T., Hashimoto, K., 2003. Effect of stress and adrenalectomy on urocortin II mRNA expression in the hypothalamic paraventricular nucleus of the rat. Neuroendocrinology 78, 1—11.

Tillinger, A., Nostramo, R., Kvetnansky, R., Serova, L., Sabban, E.L., 2013. Stress-induced changes in gene expression of urocortin 2 and other CRH peptides in rat adrenal medulla: involvement of glucocorticoids. J. Neurochem. 125, 185—192.

Tomlinson, J.W., Walker, E.A., Bujalska, I.J., Draper, N., Lavery, G.G., Cooper, M.S., Hewison, M., Stewart, P.M., 2004. 11β-Hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. Endocr. Rev. 25, 831—866.

Uchoa, E.T., Mendes da Silva, L.E., de Castro, M., Antunes-Rodrigues, J., Elias, L.L., 2009. Hypothalamic oxytocin neurons modulate hypophagic effect induced by adrenalectomy. Horm. Behav. 56, 532—538.

Ulrich-Lai, Y.M., Herman, J.P., 2009. Neural regulation of endocrine and autonomic stress responses. Nat. Rev. Neurosci. 10, 397—409.

Vodička, M., Ergang, P., Mikulecká, A., Řeháková, L., Klusoňová, P., Makal, J., Soták, M., Musílková, J., Zach, P., Pácha, J., 2014. Regulation of 11β-hydroxysteroid dehydrogenase type 1 and 7α-hydroxylase CYP7B1 during social stress. PLOS ONE 9, e89421.

Watts, A.G., Sanchez-Watts, G., 1995. Region-specific regulation of neuropeptide mRNAs in rat limbic forebrain neurones by aldosterone and corticosterone. J. Physiol. 484, 721—736.

Wyrwoll, C.S., Holmes, M.C., Seckl, J.R., 2011. 11β-Hydroxysteroid dehydrogenases and the brain: from zero to hero, a decade of progress. Front. Neuroendocrinol. 32, 265—286.

Zohar, I., Weinstock, M., 2011. Differential effect of prenatal stress on the expression of corticotrophin-releasing hormone and its receptors in the hypothalamus and amygdala in male and female rats. J. Neuroendocrinol. 23, 320—328.

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Full-length Article

Microbiota affects the expression of genes involved in HPA axis regulation and local metabolism of glucocorticoids in chronic psychosocial stress

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ABSTRACT

The commensal microbiota affects brain functioning, emotional behavior and ACTH and corticosterone responses to acute stress. However, little is known about the role of the microbiota in shaping the chronic stress response in the peripheral components of the hypothalamus-pituitary-adrenocortical (HPA) axis and in the colon. Here, we studied the effects of the chronic stress-microbiota interaction on HPA axis activity and on the expression of colonic corticotropin-releasing hormone (CRH) system, cytokines and 11β-hydroxysteroid dehydrogenase type 1 (11HSD1), an enzyme that determines locally produced glucocorticoids. Using specific pathogen-free (SPF) and germ-free (GF) BALB/c mice, we showed that the microbiota modulates emotional behavior in social conflicts and the response of the HPA axis, colon and mesenteric lymph nodes (MLN) to chronic psychosocial stress. In the pituitary gland, microbiota attenuated the expression of Fkbp5, a gene regulating glucocorticoid receptor sensitivity, while in the adrenal gland, it attenuated the expression of genes encoding steroidogenesis (MC2R, StaR, Cyp11a1) and catecholamine synthesis (TH, PNMT). The pituitary expression of CRH receptor type 1 (CRHR1) and of proopiomelanocortin was not influenced by microbiota. In the colon, the microbiota attenuated the expression of 11HSD1, CRH, urocortin UCN2 and its receptor, CRHR2, but potentiated the expression of cytokines TNFα, IFNγ, IL-4, IL-5, IL-6, IL-10, IL-13 and IL-17, with the exception of IL-1β. Compared to GF mice, chronic stress upregulated in SPF animals the expression of pituitary Fkbp5 and colonic CRH and UCN2 and downregulated the expression of colonic cytokines. Differences in the stress responses of both GF and SPF animals were also observed when immunophenotype of MLN cells and their secretion of cytokines were analyzed. The data suggest that the presence of microbiota/intestinal commensals plays an important role in shaping the response of peripheral tissues to stress and indicates possible pathways by which the environment can interact with glucocorticoid signaling.

1. Introduction

Stress is a common aspect of the life experience of all living creatures and generally serves as an adaptation mechanism to meet various challenges to survive and benefit from potentially threatening environments. The principal endocrine components of the stress response are the sympathetic adrenomedullary (SAM) and hypothalamus-pituitary-adrenocortical (HPA) axes, which represent neuroendocrine cascades culminating in the synthesis and secretion of catecholamines and glucocorticoids, respectively (McEwen, 2007). The HPA axis is triggered by the activation of parvocellular neurons located in the paraventricular nucleus of the hypothalamus (PVN) that produce corticotropin-releasing hormone (CRH); CRH binds to CRH receptors type 1 (CRHR1) in the pituitary, causing the activation of proopiomelanocortin (POMC), a precursor of adrenocorticotropic hormone (ACTH) (Herman et al., 2016). Similar to CRH, stress regulates the expression of two other members of the CRH family, the urocortins UCN2 and UCN3, which have a much higher affinity for CRHR2 than CRHR1 receptors and have been implicated, particularly during stress, in several functions of the brain and peripheral organs, including the gastrointestinal tract (Larauche et al., 2009).

The response of target cells to glucocorticoids depends not only on the level of the hormone in blood but also on the prereceptor metabolism in the target cells that controls the intracellular concentration of

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the hormone. This metabolism is determined by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11HSD1), which catalyzes the conversion of inactive cortisone and 11-dehydrocorticosterone to the active glucocorticoid hormones cortisol and corticosterone. In contrast, the analog 11β-hydroxysteroid dehydrogenase type 2 (11HSD2) is an enzyme that inactivates cortisol and corticosterone to their 11-oxo derivatives (Chapman et al., 2013). 11HSD1 is expressed in many tissues, including the pituitary and adrenal glands (Ergang et al., 2015), which suggests that 11HSD1 might modulate the activity of the HPA axis. Some data also indicate that stress modulates the expression of 11HSD1, but results are contradictory (Jamieson et al., 1997; Monder et al., 1994; Vodička et al., 2014). Data from our lab and others have revealed that some cytokines play a significant role in the regulation of 11HSDs (Chapman et al., 2013; Ergang et al., 2011; Jun et al., 2014).

A series of studies has demonstrated that the gastrointestinal microbiota has a large effect on HPA axis activity and emotional behavior. Mice or rats raised under germ-free (GF) conditions display exaggerated HPA axis activity in response to acute stressors (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Sudo et al., 2004) and decreased anxiety-like behavior (Clarke et al., 2013; de Palma et al., 2015; Diaz Heijtz et al., 2011; Neufeld et al., 2011). The changes observed in GF animals can be partially normalized after colonization with commensal bacteria from control mice (Clarke et al., 2013; de Palma et al., 2015; Sudo et al., 2004), and probiotic intervention can modulate stress-induced corticosterone and anxiety-related behavior (Ait-Belgnaoui et al., 2012; Moya-Pérez et al., 2017).

The above studies link stress to changes in the microbiota; however, the key question remaining is how does the microbiota of the gut modulate glucocorticoid response to stress. Several studies have linked the microbiota to plasma corticosterone and plasma ACTH levels in stressed animals, yet only a few studies have investigated the mechanisms that might be responsible for the modulatory effect of the microbiota on the activation of the HPA axis. For example, it has been shown that GF mice display an increased rate of norepinephrine, dopamine, and serotonin turnover in the brain striatum (Diaz Heijtz et al., 2011) and reduced expression of cortical and hippocampal BDNF (Sudo et al., 2004); however, these findings have not been supported by other studies (Crumeyrolle-Arias et al., 2014; Neufeld et al., 2011; Nishino et al., 2013). As these studies were limited to the brain and only acute stress, we focused on the impact of the microbiome on the peripheral components of the HPA axis and assessed whether the microbiome modified the pituitary and adrenal glands under basal conditions and in response to a chronic stress challenge. The second objective of this study was to analyze the effects of the microbiome and stress on the expression of 11HSDs in peripheral tissues and to assess whether immune activation could differentially influence the expression of cytokines as a function of stressor impact.

2. Materials and methods

2.1. Animals

Two-month-old germ-free (GF) and specific pathogen-free (SPF) male BALB/c mice (Institute of Microbiology, Nový Hrádek, Czech Republic) were used. The animals were split into four groups: unstressed GF (n = 7), stressed GF (n = 7), unstressed SPF (n = 6) and stressed SPF (n = 7) animals. GF animals were kept under sterile conditions in Trexler-type isolators since birth. One month before the beginning of the experiments, the SPF mice were transferred to similar isolators to ensure identical conditions for all groups during the experiments. Animals were housed in groups of 4–5 per cage with free access to autoclaved tap water and fed 50 kGy irradiated sterile pellet diet Altromin 1410 (Altromin, Lage, Germany) ad libitum under a 12-h light/dark cycle. The GF mice were monitored weekly for fecal microbial contamination. Retired male breeders (7-months to 1-year-old) of the BALB/c strain were used as residents, GF-BALB/c residents in experiments with GF-BALB/c intruders and SPF-BALB/c residents in experiments with SPF-BALB/c intruders. The experiments were approved by the Committee for the Protection and Use of Experimental Animals of the Institute of Microbiology v.v.i., Academy of Sciences of the Czech Republic.

2.2. Social defeat stress and tissue sampling

The social defeat procedure used in this study is a modified version of the resident-intruder paradigm used in our previous work (Vodička et al., 2014) and adapted for mice (Golden et al., 2011). This procedure relies on the concept of ethological analysis of rodent behavior and represents an unpredictable allostatic load, which is associated with the activation of both HPA and SAM axes. The procedure is based on the fact that a male mouse will defend its territory against an unfamiliar male intruder. Male mice designated as residents (older, sexually experienced males) were housed individually for 7 days before the experiment without a change of bedding (a manipulation often used to enhance territoriality and aggression). Intruders were housed in groups of 4–5. On the days of testing, each intruder was removed from his home cage and placed into the home cage of a resident; GF intruders were exposed to GF residents and SPF intruders to SPF residents. The cages of the residents and intruders were placed in the same isolator where social interactions were repeatedly done. The data summarized in Supplementary data (Fig. S1) show that both GF and SPF intruders were exposed to similar aggressive behavior of residents irrespective of their different origin. During social interaction, the behavior of the animals was videotaped for off-line evaluation. Following the 10 min interaction, the mice were divided by a steel mesh to preserve sensory contact between the mice for the next 50 min. Thus, the intruder was subjected to continuous psychological stress due to sensory interaction with the resident. Then, the intruders were examined for wounds and returned to their home cage until the next exposure. No wounded animals were found, indicating that no pathological form of offensive aggression occurred during the experiment. The procedure was repeated for 5 consecutive days with different residents to prevent any habituation to the resident. All stress experiments were carried out between 9 and 11 am. Unstressed (control) mice were undisturbed in their home cage, moreover control mice never witnessed stress procedure because resident-intruder interaction was never done in isolators containing unstressed animals.

Following the last stress session, the animals were removed from the isolator and anesthetized with isoflurane vapor. Isoflurane was used because it does not interfere with gene transcriptional responses and leaves the stress response intact (Wu et al., 2015). Anesthetized mice were decapitated, and the colon, mesenteric lymph nodes (MLN), pituitary and adrenal glands were harvested and either snap-frozen in liquid nitrogen or used for the preparation of single-cell suspensions of MLN cells. For technical reasons (to keep sterility of isolators) all mice from one cage (n = 4) were taken out of the isolator simultaneously and placed individually into clean cages located in a quiet room. Then the mice were sacrificed serially. This procedure was performed equally for all groups.

2.3. Behavioral analysis

A detailed behavioral analysis was performed using the commercial software Observer Video-Pro (Noldus Information Technology, Wageningen, The Netherland) to analyze behavioral responses during social defeat. For each resident-intruder interaction, the number and the time spent in offensive or defensive behaviors were evaluated. The following patterns of behavior were classified as offensive: chasing/following (rapid pursuit of the fleeing opponent), clinch/fight (mice roll around floor wrestling, their bodies clasped tightly together) and genital investigation (sniffing of the intruder's anogenital region). The following patterns of behavior were classified as defensive: defensive

upright (the intruder rears on his hind paws and extends the forepaws while facing the resident), escape/flight (rapid running or jumping away from the resident), and freezing posture (the intruder assumes a completely immobile crouched posture with all four limbs on the ground and usually no activity except for the movement associated with respiration).

2.4. Tissue sampling and gene expression analysis

Total RNA was isolated using a GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions and quantified by spectrophotometry using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was removed during RNA purification using on-column digestion of DNA (RNase-free DNase set, Qiagen, Hilden Germany). First-strand cDNA was prepared from total RNA using random hexamers and a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Quantitative RT-PCR was carried out using a Viia 7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), 5x Hot Firepol Probe QPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) and TaqMan Assays (Life Technologies) specific for the studied transcript. The following assays were used: 11HSD1 (cat. no. Mm00476182_m1), 11HSD2 (Mm01251104_m1), POMC (Mm00435874_m1), CRH (Mm01293920_s1), urocortin 2 (UCN2, Mm01227928_s1), urocortin 3 (UCN3, Mm00453206_s1), CRH receptor type 1 (CRHR1, Mm00432670_m1), CRH receptor type 2 (CRHR2, Mm00438308_m1), MC2R (Mm01262510_m1), steroidogenic acute regulatory protein (Star, Mm00441558_m1), cholesterol side-chain cleavage enzyme (Cyp11a1, Mm00490735_m1), tyrosine hydroxylase (TH, Mm00447557_m1), phenylethanolamine N-methyltransferase (PNMT, Mm00476993_m1), interferon γ(IFNγ, Mm00801778_m1), interleukin 1β (IL-1β, Mm01336189_m1), interleukin 4 (IL-4, Mm00445259-m1), interleukin 5 (IL-5, Mm00439646_m1), interleukin 6 (IL-6, Mm00446190_m1), interleukin 10 (IL-10, Mm00439614_m1), interleukin 13 (IL-13, Mm00434204_m1), interleukin 17 (IL-17, Mm00439618_m1), tumor necrosis factor α (TNFα, cat. no. Mm00443258_m1), and immunophilin Fkbp5 (Mm00487401_m1). The quantity of the PCR product was determined using the standard curve method with 3-fold dilutions of the mixed cDNA sample. To identify the stability of the reference genes, a panel of 12 potential reference genes was compared using geNorm analysis, and the genes HPRT1 (Mm01545399-m1) and TBP (Mm00446973_m1) were identified as the optimal combination to provide reliable normalization in the colon; HPRT1 and GAPDH (cat. no. 4351309), in the adrenal gland; and PPIB and SDHA (Mm01352366_m1), in the pituitary. The expressions of the genes of interest were calculated relative to the geometric mean of the reference genes in each sample. Due to the low expression levels of several genes in colon (Ct of the gene of interest higher than 35), we applied the specific preamplification step of gene assays using the TaqMan PreAmp Master Mix (Life Technologies) according to the manufacturer's protocol.

2.5. Detection of cytokine secretion in MLN cells

To determine whether stress modulates cytokine production, MLNs were harvested, mashed into a cell suspension, washed in complete RPMI-1640 medium, and filtered through a 70 μm cell strainer. The single cell suspension in RPMI containing 10% fetal bovine serum and 1% penicillin-streptomycin was plated in 96-well flat bottom plates (1×10^5 cells/well) and incubated for 72 h at 37 °C prior to collecting supernatants. Supernatants were kept frozen at −40 °C until IL-1β, IL-6, IL-10, IFNγ and TNFα were measured by ELISA R&D Systems® (Minneapolis, MN, USA) according to the manufacturer's instruction. The values are reported in pg/ml.

2.6. Immunophenotyping by flow cytometry

Primary MLN cells were prepared as mentioned above on the day of sacrifice and stained for regulatory T cell and intracellular cytokine production. To detect the regulatory CD4 + FoxP3 + T cells, the cell suspensions were washed, labeled with Fixable Viability Dye (eBioscience, San Diego, CA, USA), blocked with anti-CD16/CD32 antibody, stained for surface CD4, fixed, permeabilized overnight with fixation/permeabilization buffer (eBioscience), and stained for intracellular FoxP3. To analyze intracellular cytokine production, cells (2×10^6 cells/ml in complete RPMI) were incubated for 5 h with 50 ng/ml PMA, 500 ng/ml ionomycin (both from Sigma-Aldrich, Prague, Czech Republic), and 2 μM monensin (eBioscience). After incubation, the cells were washed, labeled with a viability dye, blocked, stained for surface CD4, fixed, and permeabilized as described above. Next, the cells were stained for intracellular cytokines with antibodies against IFNγ, IL-17, and TNFα. The data were acquired on a FACSCalibur flow cytometer and analyzed with FlowJo software. The cytokines were analyzed while gating in viable CD4 + cells. All monoclonal antibodies were purchased from eBioscience.

2.7. Statistical analysis

The results are expressed as the mean ± SEM. The data were analyzed by two-way ANOVA (microbial status and stress treatment) followed by *post hoc* test when the results of ANOVA were significant. Behavioral data were analyzed by two-way repeated-measure ANOVA with one between-group factor (microbial status) and one within-subject factor (time; 5 days of repeated social defeat). Statistical analyses yielded highly similar results for both the frequency and duration of behavioral patterns; therefore, to simplify the text, only the duration of patterns is reported. Pearson's correlation analysis was used to determine the relationship between the levels of 11HSD1 mRNA expression and those of various cytokines. The threshold for statistical significance was set as $P \leq 0.05$.

3. Results

3.1. Impact of microbiota on behavior of intruder and resident mice

Both the resident and intruder mouse behaviors were assessed over a 5-day period. All intruder mice were defeated by residents, showing upright posture. ANOVA showed significant effects of both microbiome ($F_{1,52} = 7.10$, $P = 0.019$) and time ($F_{4.52} = 3.27$, $P < 0.018$) on the defensive behavior of intruders. *Post hoc* analysis revealed that GF mice spent less time in defensive behavior than SPF animals on the 3rd and 4th day of confrontation (Fig. 1A) and analysis of individual patterns of defensive behavior showed an increase in the escape/flight of SPF intruders (Fig. 1B). Because GF intruders were exposed to GF residents and SPF intruders to SPF residents, we analyzed also behavior of residents to reveal whether changes in behavior of intruders could be provoked by different offensive behavior of residents. ANOVA did not show any significant overall effect of microbiome (Supplementary Fig. S1; however, this analysis indicated a significant effect of time ($F_{4,52} = 3.43$, $P < 0.014$). *Post hoc* analysis revealed that SPF mice spent less time in offensive behavior than GF animals only on the second day of confrontation (Supplementary Fig. S1). Collectively, these results show that exposure to repeated social defeat leads to a significant decrease in the time that GF mice spent in defensive behaviors compared to their SPF counterparts.

3.2. Effect of stress and microbiota on pituitary and adrenal glands

To test whether the gut microbiota modulates the periphery of the HPA axis, we examined the expression of CRHR1 and POMC in the pituitary and the expression of selected genes encoding proteins of

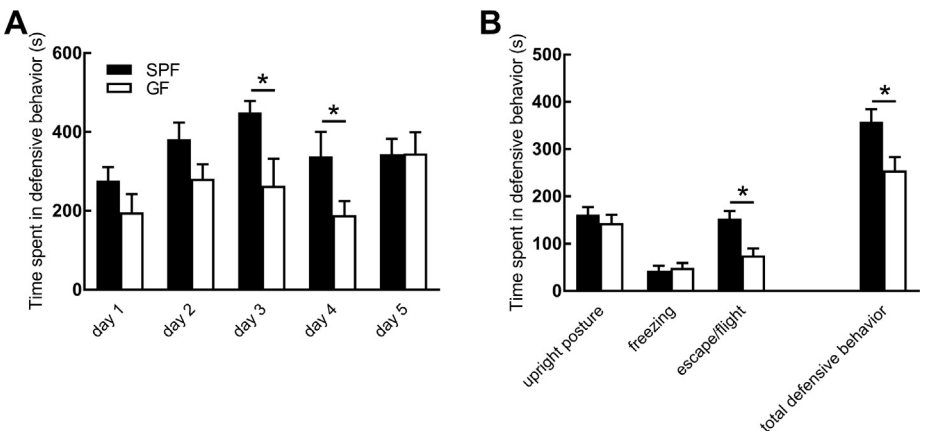

Fig. 1. Time spent in defensive behavior in each day of social interaction (A) and time spent in individual defensive behavioral patterns (upright posture, freezing and escape/flight) and in total defensive behavior (B) of specific pathogen-free (SPF) and germ-free (GF) mice. Data are expressed as the means ± SEM; Only the statistical differences determined by two-way ANOVA followed by post hoc test are shown; the P-value of the post hoc test: $^{*}P < 0.05$.

steroidogenesis and synthesis of catecholamines in the adrenal gland. Social defeat had a general effect on POMC expression in the pituitary in both the SPF and GF mice ($F_{1,22} = 63.85$, $P < 0.001$); however, there was no significant effect of microbiota. *Post hoc* analysis demonstrated a significant increase in POMC transcript levels in stressed mice from both the SPF and GF groups (Fig. 2A). The expression of CRHR1 was not influenced either by stress or by the microbiome (Fig. 2B). To further characterize the effect of the microbiome on the HPA axis in the pituitary, we measured the expression of the Fkbp5 gene, which encodes the co-chaperone that participates in the regulation of glucocorticoid receptor sensitivity and efficiency of the negative feedback process of the HPA axis (Bekhbat et al., 2017). Strikingly, the expression of Fkbp5 was affected not only by stress ($F_{1,24} = 11.03$, $P = 0.002$) but also by microbiota ($F_{1,24} = 19.62$, $P < 0.001$). SPF unstressed mice exhibited significantly lower levels of Fkbp5 transcripts than all other groups of animals (Fig. 2C).

We next examined the impact of microbiota and stress on the activation of the adrenal gland. First, we analyzed the mRNA level of MC2R, the adrenal receptor for ACTH hormone, and the transcripts of Star and Cyp11a1, which are genes encoding the key proteins of cholesterol mobilization and conversion to steroid hormones. Social defeat did not influence the expression of any of these three genes; however, a significant effect of microbiota was observed on MC2R ($F_{1,22} = 12.76$, $P = 0.002$) as well as on Star ($F_{1,22} = 8.69$, $P = 0.008$) and Cyp11a1 ($F_{1,22} = 8.20$, $P = 0.009$). The absence of microbiota led to the upregulation of MC2R, Star and Cyp11a1 in the adrenal gland (Fig. 3A–C). In contrast to steroidogenesis, the expression of the genes encoding the key enzymes of epinephrine biosynthesis TH and PNMT was significantly affected both by stress (TH: $F_{1,22} = 5.71$, $P = 0.026$; PNMT: $F_{1,22} = 12.23$, $P = 0.002$) and by the microbiota (TH: $F_{1,22} = 23.30$, $P < 0.001$; PNMT: $F_{1,22} = 18.03$, $P < 0.001$). A comparison of group means demonstrated significantly higher TH and PNMT transcripts in unstressed and stressed GF compared to SPF animals and significantly higher levels of both transcripts after social defeat in GF mice. Although the effect of social defeat on SPF mice did not reach statistical significance, the results showed a trend very similar to that seen in the GF group (Fig. 3D, E).

As PNMT is a glucocorticoid-dependent enzyme and as glucocorticoids released by stress are known to regulate biosynthesis and the secretion of epinephrine in the adrenal medulla (Kvetnansky et al., 2009), we further examined the impact of microbial colonization on the expression of adrenal 11HSD1 and 11HSD2. Stress affected 11HSD2 ($F_{1,22} = 14.45$, $P < 0.001$), and the microbiota affected 11HSD1 ($F_{1,22} = 10.43$, $P = 0.003$). Stress significantly downregulated 11HSD2 transcript in both GF and SPF mice (Fig. 3F), and microbial colonization decreased the expression of 11HSD1 in both groups, although statistical significance was seen between only stressed SPF and stressed GF mice (Fig. 3G).

3.3. Effect of stress and microbiota on colonic CRH signaling system

There is well-established evidence that the CRH system is highly organized not only in the brain but also in some peripheral tissues, including the colon, where it seems to generate functions analogous to the HPA axis, i.e., the coordination and execution of local responses to stress (Larauche et al., 2009). To investigate whether stress and gut microbiota affect the expression of genes encoding peripheral stress-associated peptides, we analyzed the transcripts of CRH, its related peptides UCN2 and UCN3, and their receptors, CRHR1 and CRHR2. There was no significant effect of stress on all analyzed transcripts; however, ANOVA revealed a significant effect of gut microbiota on UCN2 ($F_{1,33} = 24.03$, $P < 0.001$) and CRHR2 ($F_{1,34} = 17.15$, $P < 0.001$) as well as a significant interaction between stress and microbiota in the cases of UCN2 ($F_{1,33} = 9.47$, $P = 0.004$) and CRH ($F_{1,34} = 12.45$, $P = 0.001$). As shown in Fig. 4, CRH, UCN2 and CRHR2 transcripts were higher in control GF than control SPF animals, and stress significantly upregulated the expression of UCN2 and CRH in SPF, but not GF, mice.

3.4. Effect of stress and microbiota on 11HSD1 and cytokine expression in colon

The local effect of glucocorticoids is modulated by 11HSD1, whose expression is regulated by cytokines. To identify whether immune activation/microbiota could differentially influence local glucocorticoid metabolism, we compared the expression of colonic 11HSD1 and cytokines that are known to modulate 11HSD1 expression. Both stress ($F_{1,22} = 29.43$, $P < 0.001$) and microbiota ($F_{1,22} = 9.74$, $P = 0.005$) significantly affected colonic 11HSD1. The presence of microbiota, similar to stress, was associated with downregulation of 11HSD1 (Fig. 4F).

Having established that the commensal microbiota could modify local glucocorticoid regulation in the colon, we then analyzed cytokine gene profiling in stressed and unstressed SPF and GF mice. As shown in Supplementary Table S1 and Fig. 5A, the microbiota upregulated the expression of all studied cytokines (IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, TNFα and IFNγ) except for IL-1β, and stress downregulated the expression of all cytokines except for IL-5. The effect of stress was significant predominantly in the SPF animals, as the GF animals showed a much lower level of cytokine transcripts in naive unstressed mice than their SPF counterparts (Fig. 5A). These data indicate that cytokine milieu in stressed SPF mice is similar to stressed and unstressed GF mice.

In vitro experiments demonstrated that various cytokines stimulate 11HSD1 (Ergang et al., 2011; Jun et al., 2014). To identify which of the cytokines might contribute to the regulation of 11HSD1 in GF and SPF mice, we performed correlation analysis. As shown in Supplementary Table S2, we found a statistically positive correlation between 11HSD1

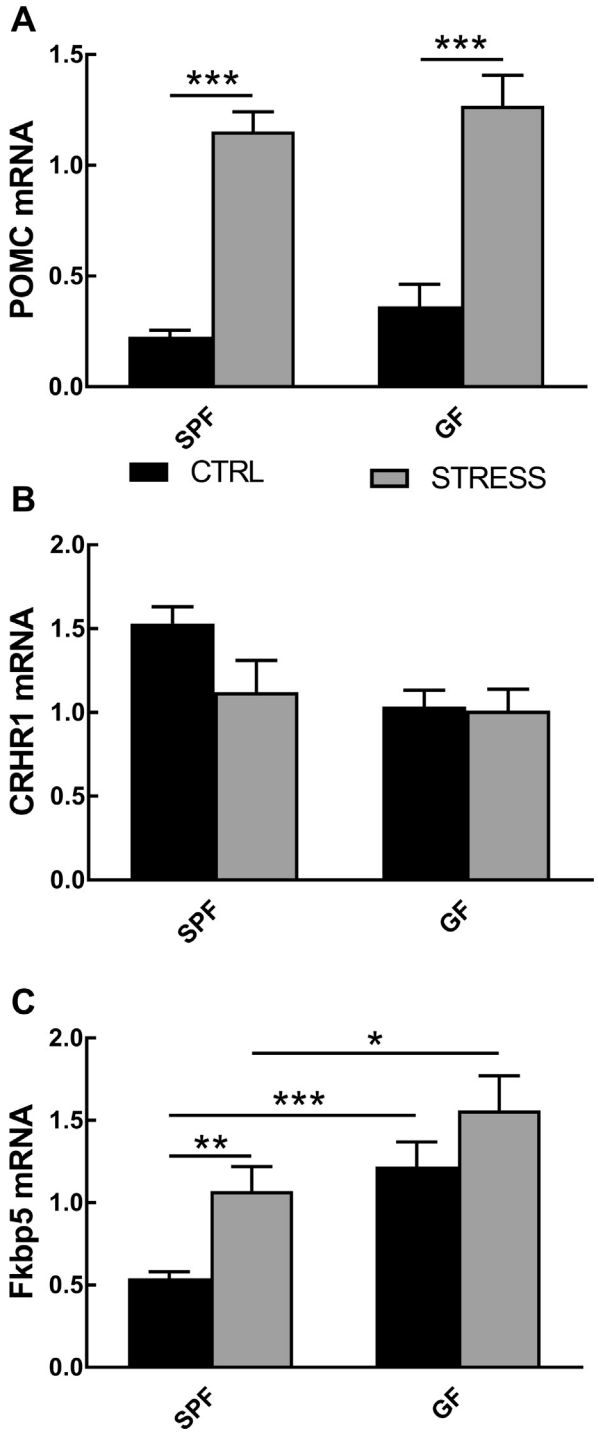

Fig. 2. Effect of social defeat stress on proopiomelanocortin (POMC), CRH receptor type 1 (CRHR1) and immunophilin Fkbp5 mRNA levels in the pituitary of specific pathogen-free (SPF) and germ-free (GF) mice. The mRNA levels were determined in isolated tissues collected from unstressed mice and mice exposed to a social defeat procedure. The data are expressed as the means ± SEM; Only the statistical differences determined by two-way ANOVA followed by post hoc test are shown; the P-value of the post hoc test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and IL-1β, IL-4, IL-6, IL-10, IL-13 and TNFα in SPF mice but only between 11HSD1 and IL-6, IL-13 and TNFα in GF mice. Fig. 5B–D show the statistically significant correlations between 11HSD and cytokines, which were found in both SPF and GF mice.

3.5. Effect of stress and microbiota on the immunophenotype of mesenteric lymph nodes

To better understand the effect of stress on cytokine response, we evaluated in vitro secretion of different pro- (IFNγ, TNFα, IL-1β) and anti-inflammatory cytokines (IL-10). Analysis of MLN cells culture supernatant identified spontaneous production of TNFα, IL-6 and IL-10 but not IFNγ and IL-1β. As shown in Fig. 6, secretion of TNFα, IL-6 and IL-10 varied as a function of microbiome (TNFα: $F_{1,21} = 6.31$, $P = 0.020$; IL-6: $F_{1,21} = 8.87$, $P = 0.007$; IL-10: $F_{1,20} = 4.99$, $P = 0.038$) and stress x microbiome interaction (TNFα: $F_{1,21} = 5.14$, $P = 0.034$; IL-10: $F_{1,20} = 6.36$, $P = 0.021$). Social defeat provoked a significant decrease of IL-10 and TNFα secretion in SPF but not GF animals. Although the statistical analysis of the effect of stress on production of IL-6 did not reached statistical significance, the data indicate that stress might also decrease the production of IL-6. Secretion of cytokines from isolated MLN cells of GF mice approximates the state in stressed SPF mice.

To analyze further the effect of gut microbiota on the immune system of stressed mice, we evaluated the phenotypes of MLN cells by flow cytometry. As shown in Fig. 7, we observed a decreased percentage of $Foxp3^+$ Treg cell in unstressed GF mice compared to unstressed SPF animals and the sensitivity of these cells in SPF mice to be down-regulated by social defeat stress. Similar to the $Foxp3^+$ Treg cells, the TNFα-producing $CD4^+$ cells of unstressed GF mice were significantly reduced compared to unstressed SPF animals and stress significantly decreased the percentage of these cells in SPF mice. In addition, the gut microbiota was essential for the expansion of pro-inflammatory IFNγ-producing $CD4^+$ T cells, which was observed in stressed SPF mice. In the absence of microbiota, stress did not change the percentage of IFNγ-producing $CD4^+$ T cells. The frequency of IL-17A-producing $CD4^+$ T cells remained unchanged in stressed GF mice compared to stressed SPF mice. To summarize, the analysis shows that stress has a modulatory effect on MLN and that the gut microbiota is a necessary mediator of this effect. In addition, the milieu of MLNs of stressed SPF mice was more pro-inflammatory compared to stressed GF mice, as seen by the higher percentage of IFNγ-producing CD4 + cells.

4. Discussion

The present study aimed to determine the effect of commensal microbiota on behavioral phenotypes and the HPA axis during repeated exposure to social defeat stress. The results show that stress-induced behavior and the expression of genes encoding proteins that participate in the HPA and SAM axes and in the peripheral metabolism of glucocorticoids are largely affected by the microbiota. Our study extends the findings of others that show changes in the behavioral and HPA axis phenotypes of GF animals by demonstrating, for the first time, that the microbiota acts as a profound modulator of pituitary and adrenal gland function and the genes involved in the metabolism of glucocorticoids in peripheral tissues.

The present study revealed differences in the behavioral phenotypes of GF and SPF mice during chronic resident-intruder stress. In contrast to SPF mice, GF animals displayed less defensive behavior, which was caused mainly by escape/flight behavior. Since we found no differences in total offensive behavior between GF and SPF residents, we can assume that this behavior was not induced as a reaction to the resident's aggression. There are no studies that have yet addressed behavioral phenotypes during social conflict in GF and SPF mice; however, it has been shown that microbiota can alter social behavior in mice (Arentsen et al., 2015; Desbonnet et al., 2014). In contrast, more studies on anxiety-like behavior have been conducted and most but not all reported decreased anxiety-like behavior in GF mice (Arentsen et al., 2015; Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; De Palma et al., 2015; Diaz Heijtz et al., 2011; Neufeld et al., 2011; Nishino et al., 2013). Since the escape/flight pertains to anxiety-related behavior, our

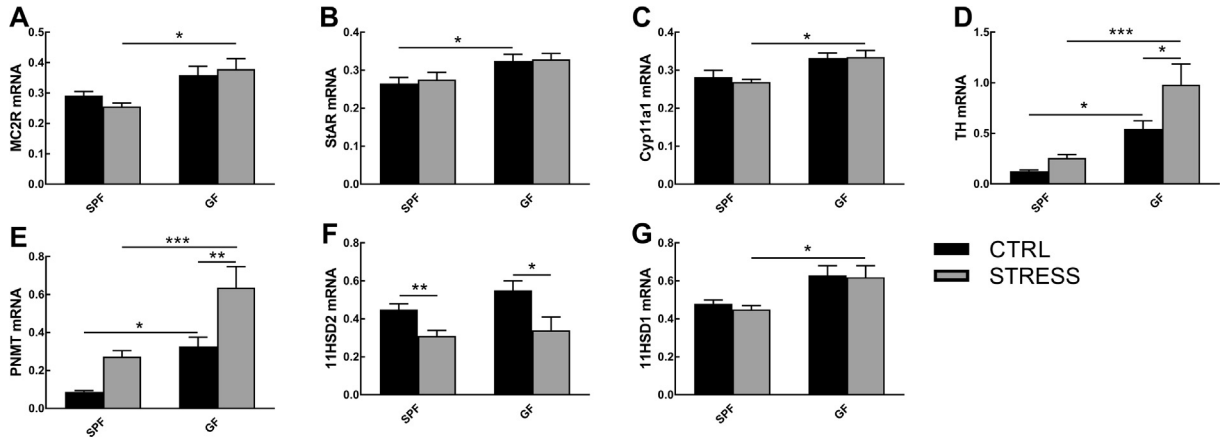

Fig. 3. Responses of genes encoding adrenal steroidogenesis, catecholamines biogenesis and glucocorticoid metabolism following social defeat in specific pathogen-free (SPF) and germ-free (GF) mice. MC2R, melanocortin 2 receptor; StAR, steroidogenic acute regulatory protein; Cyp11a1, cholesterol side-chain cleavage enzyme; TH, tyrosine hydroxylase; PNMT, phenylethanolamine N-methyltransferase; 11HSD1 and 11HSD2, 11β-hydroxysteroid dehydrogenase type 1 and type 2. For further details, see the description of Fig. 2.

results indicate that GF mice are less prone to anxiety-like behavior in repeated social stress.

The present study identified multiple changes in the HPA axis induced by stress and/or microbiota. While the microbiota did not elicit any effect on the expression of POMC or the CRHR1 receptor in the pituitary, stress upregulated the expression of POMC without any significant effect on CRHR1. This finding is in line with our previous observation in Lewis rats exposed to repeated social defeat, where we also

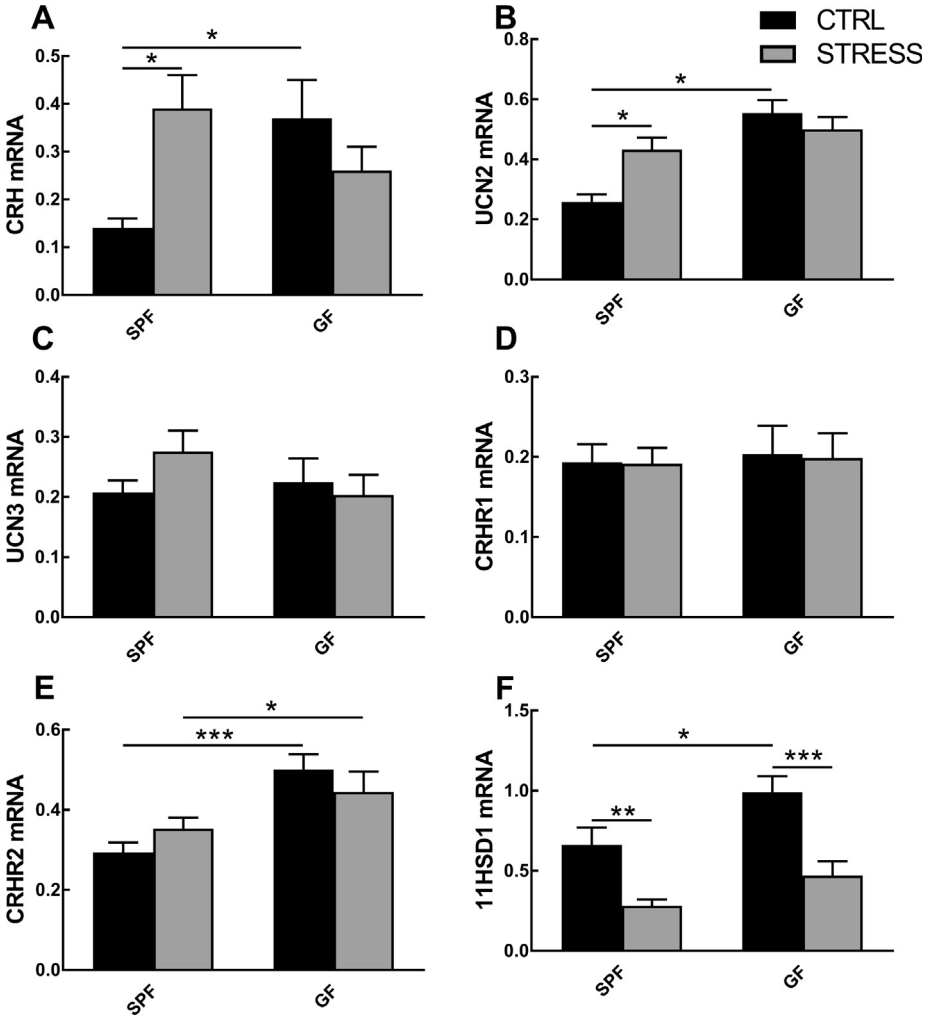

Fig. 4. Comparison of the gene expression of corticotropin-releasing hormone (CRH), urocortin 2 (UCN2), urocortin 3 (UCN3), their receptors, CRHR1 and CRHR2, and 11β-hydroxysteroid dehydrogenase type 1 (11HSD1) within the colon of stressed and unstressed specific pathogen-free (SPF) and germ-free (GF) mice. For further details, see the description of Fig. 2.

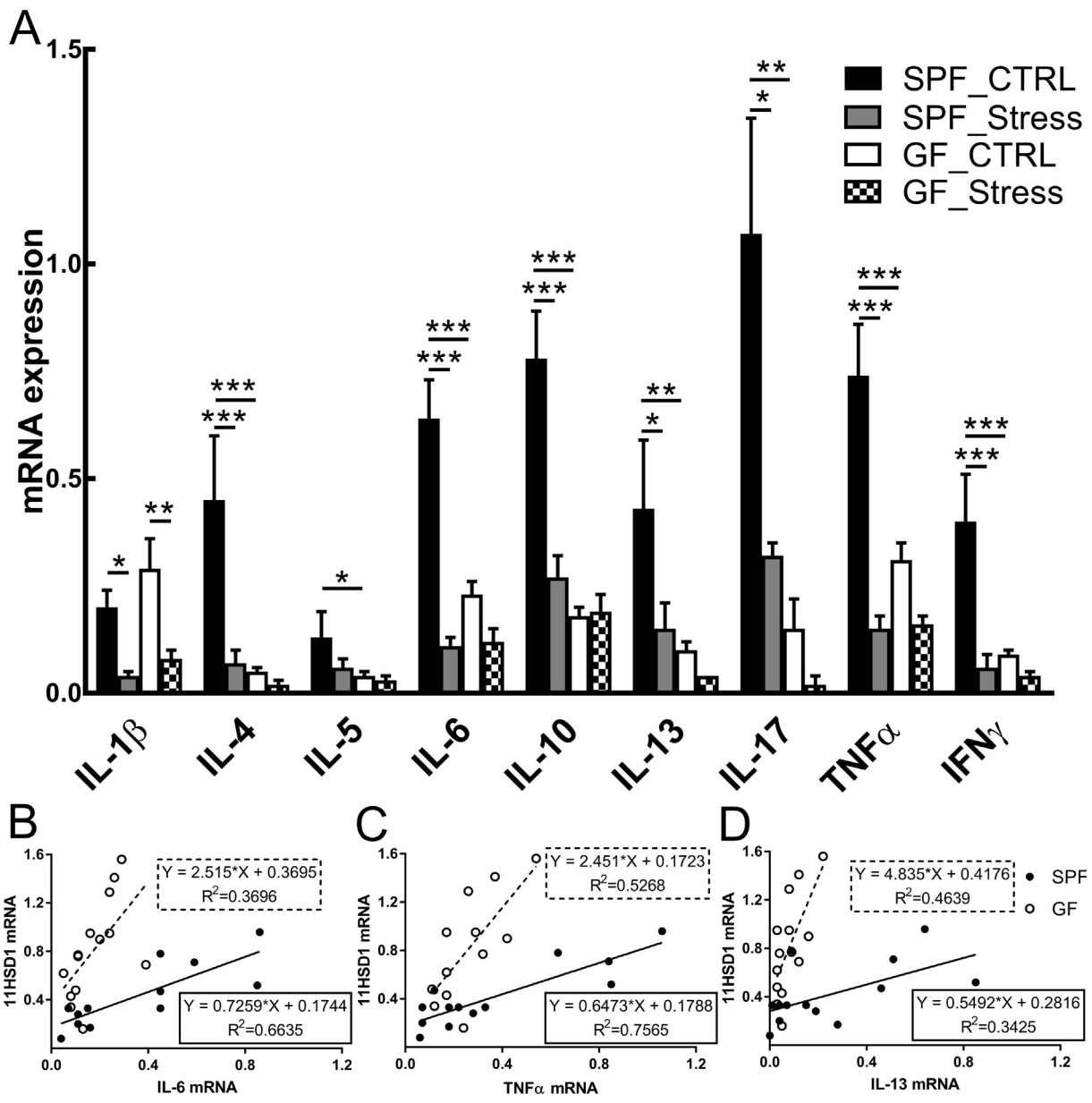

Fig. 5. Effect of social defeat stress on cytokine expression in colon of specific pathogen-free (SPF) and germ-free (GF) mice. (A) Comparison of mRNA expression levels in stressed and unstressed animals. (B–D) Correlation between the expression of 11HSD1 and cytokines with significant correlation coefficients both in SPF and in GF mice. IL, interleukin; TNF, tumor necrosis factor, IFN, interferon. For further details, see the description of Fig. 2.

observed an upregulation of POMC expression without any changes in the expression of CRHR1 (Vodička et al., 2014). Similarly, no changes were observed in pituitary CRHR1 protein after chronic stress based on a shock-escape paradigm (Raone et al., 2007). As the absence of a microbiota upregulates the HPA stress response (Ait-Belgnaoui et al., 2012; Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Sudo et al., 2004), the absence of any significant difference in the expression of POMC and CRHR1 indicates that the exaggerated HPA stress response in GF mice must be localized to either brain structures or the negative feedback regulation of the pituitary gland. First, some studies support the view that the mechanisms behind this change operate in brain structures associated with the HPA axis, as alterations of brain neurochemistry have been observed in GF mice (Clarke et al., 2013; Neufeld et al., 2011; Sudo et al., 2004); these results, however, are contradictory. Second, microbiota downregulated Fkbp5 in the pituitary, and it is possible that higher Fkbp5 expression in the pituitary gland of GF mice might induce decreased efficiency of the negative feedback via GR. The Fkbp5 gene encodes a protein that regulates the glucocorticoid signaling negative feedback loop through a reduction in the affinity of GR for corticosterone and its trafficking to the nucleus (Bekhbat et al., 2017). Thus, increased expression of Fkbp5 in GF mice might partially explain the exaggerated HPA response observed in GF mice by others. In line with this possibility, chronic mild stress in rats has been shown to increase the expression of Fkbp5 and the cytoplasmic level of GR (Guidotti et al., 2013), and the chronic treatment of mice with corticosterone upregulated Fkbp5 expression (Lee et al., 2010).

While it is well established that the absence of a microbiota modulates emotional-related behavior and brain neurochemistry and upregulates HPA-axis activity, the effect of microbiota on the stress-induced response of the adrenal gland is unknown, despite this gland participating in both the HPA and SAM axes. Using SPF and GF mice, we showed that exposing mice to microbiota downregulated the expression of key genes encoding proteins involved in steroidogenesis and catecholamine biosynthesis in the adrenal gland. However, the impact

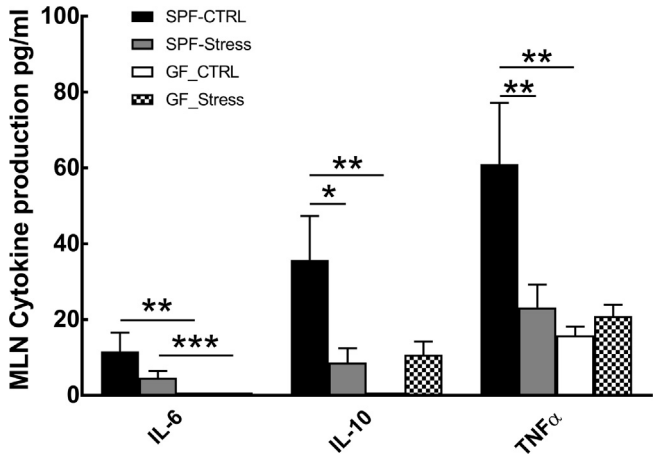

Fig. 6. Effect of social defeat stress on in vitro cytokine secretion from cells of mesenteric lymph nodes (MLN) of specific pathogen-free (SPF) and germ-free (GF) mice. For further details, see the description of Fig. 2.

of stress on only the genes encoding the synthesis of epinephrine and not corticosteroids was observed. These data support previous findings showing the absence of changes in Star and CYP11a1 mRNA levels in the adrenal gland of mice exposed to chronic subordinate colonic housing compared with single-housed controls (Uschold-Schmidt et al., 2012). Previous studies have also demonstrated dysregulated brain synthesis and degradation of catecholamines in GF mice (Crumeyrolle-Arias et al., 2014; De Palma et al., 2015; Diaz Heijtz et al., 2011; Nishino et al., 2013); however, the inconsistencies in the direction of changes in brain induced by microbiota make comparisons between the brain and the adrenal medulla difficult. Nevertheless, our study demonstrates that the absence of microbiota can strongly affect catecholamine biosynthesis and, to a lesser degree, steroidogenesis. The mechanism of the effects of microbiota is unknown, but it may stem from (1) the indirect activation via immune cells or enterocytes, which release pro-inflammatory cytokines and prostaglandins influencing the corticosteroid production cells (Bornstein et al., 2014) or (2) the effect mediated by the activation of Toll-like receptors expressed by adrenocortical cells (Kanczkowski et al., 2011). Although glucocorticoids are critical regulators of PNMT, the final enzyme in epinephrine biosynthesis (Kvetnansky et al., 2009), the increased expression of 11HSD1 in the adrenal gland of GF mice may not participate in the upregulation of PNMT expression, as the expression profile of both glucocorticoid-independent TH and glucocorticoid-dependent PNMT genes in GF animals was similar. Future studies will have to be conducted to reveal the detailed mechanisms underlying the effect of microbiota on adrenal gland functions.

Beyond their central actions, microbiota and stressors may also influence peripheral actions (Allen et al., 2012). The present study indicates that local glucocorticoid synthesis via colonic 11HSD1 may be modulated by microbiota and stress. The mechanisms that underlie the microbiota and stress effects on 11HSD1 are currently unknown. However, one explanation is likely. Cytokines are important modulators of 11HSD1 expression (Ergang et al., 2011; Jun et al., 2014), and their expression is modulated by the microbiome (Steinberg et al., 2014) and by stress (Ait-Belgnaoui et al., 2012; Audet et al., 2011; Gibb et al., 2011). Here, we showed that the expression of cytokines in the colon is upregulated in the presence of gut microbiota and downregulated by stress irrespective of whether the cytokine belongs to the Th1, Th2 or Th17 pathway. The reduced colonic expression of cytokines in stressed animals, which partially depends on the microbiota, provides further evidence of the stress-induced immune suppression described by others (Reber et al., 2011). Correlation analysis of the relationship between 11HSD1 and cytokine expression suggests that cytokine milieu modulates 11HSD1 expression in colon and that the response of GF mice to

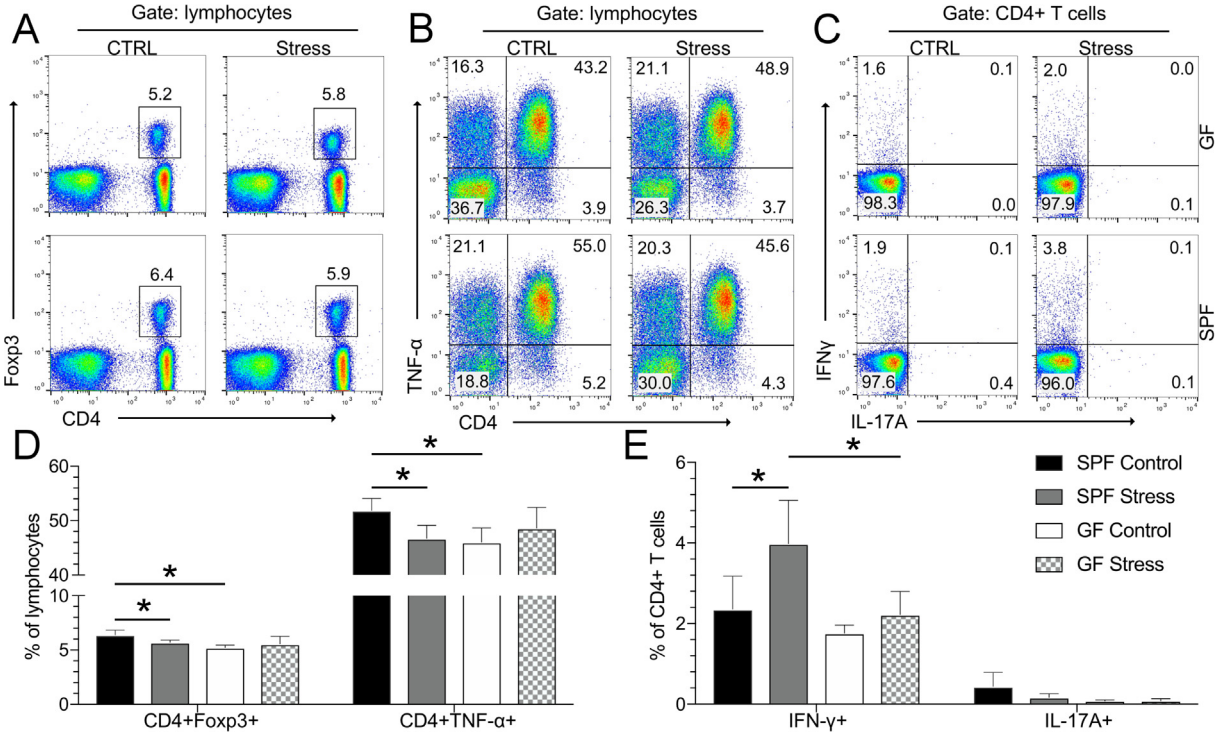

Fig. 7. Flow cytometric analysis of lymphocyte populations in mesenteric lymph nodes of specific-pathogen free (SPF) and germ-free mice (GF); (6A) the percentage of regulatory Foxp3- expressing CD4 + T cells, (6B) the percentage of TNFα-producing CD4 + T cells, (6C) the percentage of IFNγ- and IL-17-producing CD4 + T. All dotplots are representative of at least two independent experiments. Column graphs (6D) and (6E) summarize the frequency of lymphocyte subpopulations in mesenteric lymph nodes. Each graph represents data from three independent experiments. Only the statistical differences determined by two-way ANOVA followed by post hoc test are shown; the P-value of the post hoc test: $^{*}P < 0.05$.

cytokines might be stronger than in the case of SPF mice. This conclusion is in accordance with the significantly higher expression of colonic 11HSD1 in control unstressed GF mice than in their SPF counterpart. Similarly, our previous work demonstrated that TNFα and IL-1β upregulated colonic 11HSD1 in vitro (Ergang et al., 2011) and that the expression of 11HSD1 in vivo was positively correlated with TNFα in MLN (Ergang et al., 2017). In contrast, the decrease of colonic 11HSD1 in stressed animals might be associated with the downregulated expression of colonic cytokines in stressed animals. This downregulation can be at least partially attributed to glucocorticoids that are secreted in response to stress and that suppress cytokine expression. In addition, signals other than cytokines might participate in the regulation of 11HSD1. Previous studies have shown that the stimulation of CRHR1 downregulates 11HSD1 in pancreatic islets (Schmid et al., 2011) and have demonstrated the physiological relevance of the CRH signaling system in the colon during stress (Larauche et al., 2009) associated with the modulation of urocortins and CRH receptors (Arase et al., 2016; O'Malley et al., 2010; Yuan et al., 2016). Similarly, the present study found stress-induced upregulation of UCN2 and CRH in the colon and extended these findings by demonstrating the downregulatory effects of the gut microbiota on the expression of CRH and UCN2.

In summary, using GF and SPF mice, we provide evidence for the role of microbiota in the regulation of the peripheral components of the HPA axis and the local metabolism of glucocorticoids as well as in the alteration of social behaviors during chronic psychosocial stress. Our study reveals the importance of the microbiota/intestinal commensals in shaping the response of the peripheral tissue to stress and indicates possible pathways by which the environment can interact with glucocorticoid signaling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbi.2018.07.007.

References

Ait-Belgnaoui, A., Durand, H., Cartier, C., Chaumaz, G., Eutamene, H., Ferrier, L., Houdeau, E., Fioramonti, J., Bueno, L., Theodorou, V., 2012. Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. Psychoneuroendocrinology 37, 1885–1895.

Allen, R.G., Lafuse, W.P., Galley, J.D., Ali, M.M., Ahmer, B.M., Bailey, M.T., 2012. The intestinal microbiota are necessary for stressor-induced enhancement of splenic macrophage microbicidal activity. Brain Behav. Immun. 26, 371–382.

Arase, S., Watanabe, Y., Setoyama, H., Nagaoka, N., Kawai, M., Matsumoto, S., 2016. Disturbance in the mucosa-associated commensal bacteria is associated with the exacerbation of chronic colitis by repeated psychological stress; is that the new target of probiotics? PLoS One 11, e0160736.

Arentsen, T., Raith, H., Qian, Y., Forssberg, H., Diaz Heijtz, R., 2015. Host microbiota modulates development of social preference in mice. Microb. Ecol. Health Dis. 26, 29719.

Audet, M.C., Jacobson-Pick, S., Wann, B.P., Anisman, H., 2011. Social defeat promotes specific cytokine variations within the prefrontal cortex upon subsequent aggressive or endotoxin challenges. Brain Behav. Immun. 25, 1197–1205.

Bekhbat, M., Rowson, S.A., Neigh, G.N., 2017. Checks and balances: the glucocorticoid receptor and NFκB in good times and bad. Front. Neuroendocrinol. 46, 15–31.

Bornstein, S.R., Rutkowski, H., Vrezas, I., 2004. Cytokines and steroidogenesis. Mol. Cell Endocrinol. 215, 135–141.

Chapman, K., Holmes, M., Seckl, J., 2013. 11β-hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action. Physiol. Rev. 93, 1139–1206.

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R.D., Shanahan, F., Dinan, T.G., Cryan, J.F., 2013. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol. Psychiatry 18, 666–673.

Crumeyrolle-Arias, M., Jaglin, M., Bruneau, A., Vancassel, S., Cardona, A., Daugé, V., Naudon, L., Rabot, S., 2014. Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. Psychoneuroendocrinology 42, 207–217.

De Palma, G., Blennerhassett, P., Lu, J., Deng, Y., Park, A.J., Green, W., Denou, E., Silva, M.A., Santacruz, A., Sanz, Y., Surette, M.G., Verdu, E.F., Collins, S.M., Bercik, P., 2015. Microbiota and host determinants of behavioural phenotype in maternally separated mice. Nat. Commun. 6, 7735.

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T.G., Cryan, J.F., 2014. Microbiota is essential for social development in the mouse. Mol. Psychiatry 19, 146–148.

Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H., Pettersson, S., 2011. Normal gut microbiota modulates brain development and behavior. Proc. Natl. Acad. Sci. U.S.A. 108, 3047–3052.

Ergang, P., Vodička, M., Vagnerová, K., Moravec, M., Kvapilová, P., Kment, M., Pácha, J., 2017. Inflammation regulates 11β-hydroxysteroid dehydrogenase type 1 differentially in specific compartments of the gut mucosal immune system. Steroids 126, 66–73.

Ergang, P., Vodička, M., Soták, M., Klusoňová, P., Behuliak, M., Řeháková, L., Zach, P., Pácha, J., 2015. Differential impact of stress on hypothalamic-pituitary-adrenal axis: gene expression changes in Lewis and Fisher rats. Psychoneuroendocrinology 53, 49–59.

Ergang, P., Vytáčková, K., Švec, J., Bryndová, J., Mikšík, I., Pácha, J., 2011. Upregulation of 11β-hydroxysteroid dehydrogenase 1 in lymphoid organs during inflammation in the rat. J. Steroid Biochem. Mol. Biol. 126, 19–25.

Gibb, J., Hayley, S., Poulter, M.O., Anisman, H., 2011. Effects of stressors and immune activating agents on peripheral and central cytokines in mouse strains that differ in stressor responsivity. Brain Behav. Immun. 25, 468–482.

Golden, S.A., Covington, H.E., Berton, O., Russo, S.J., 2011. A standardized protocol for repeated social defeat stress in mice. Nat. Protoc. 6, 1183–1191.

Guidotti, G., Calabrese, F., Anacker, C., Racagni, G., Pariante, C.M., Riva, M.A., 2013. Glucocorticoid receptor and FKBP5 expression is altered following exposure to chronic stress: modulation by antidepressant treatment. Neuropsychopharmacology 38, 616–627.

Herman, J.P., McKlveen, J.M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J., Myers, B., 2016. Regulation of the hypothalamic-pituitary-adrenocortical stress response. Compr. Physiol. 6, 603–621.

Jamieson, P.M., Fuchs, E., Flugge, G., Seckl, J.R., 1997. Attenuation of hippocampal 11β-hydroxysteroid dehydrogenase type 1 by chronic psychosocial stress in the tree shrew. Stress 2, 123–132.

Jun, Y.J., Park, S.J., Kim, T.H., Lee, S.H., Lee, K.J., Hwang, S.M., Lee, S.H., 2014. Expression of 11β-hydroxysteroid dehydrogenase 1 and 2 in patients with chronic rhinosinusitis and their possible contribution to local glucocorticoid activation in sinus mucosa. J. Allergy Clin. Immunol. 134, 926–934.

Kanczkowski, W., Tymoszuk, P., Chavakis, T., Janitzky, V., Weirich, T., Zacharowski, K., Ehrhart-Bornstein, M., Bornstein, S.R., 2011. Upregulation of TLR2 and TLR4 in the human adrenocortical cells differentially modulates adrenal steroidogenesis. Mol. Cell Endocrinol. 336, 41–46.

Kvetnansky, R., Sabban, E.L., Palkovits, M., 2009. Catecholaminergic systems in stress: structural and molecular genetic approaches. Physiol. Rev. 89, 535–606.

Larauche, M., Kiank, C., Tache, Y., 2009. Corticotropin releasing factor signaling in colon and ileum: regulation by stress and pathophysiological implications. J. Physiol. Pharmacol. 60 (Suppl. 7), 33–46.

Lee, R.S., Tamashiro, K.L., Yang, X., Purcell, R.H., Harvey, A., Willour, V.L., Huo, Y., Rongione, M., Wand, G.S., Potash, J.B., 2010. Chronic corticosterone exposure increases expression and decreases deoxyribonucleic acid methylation of Fkbp5 in mice. Endocrinology 151, 4332–4343.

McEwen, B.S., 2007. Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol. Rev. 87, 873–904.

Monder, C., Sakai, R.R., Miroff, Y., Blanchard, D.C., Blanchard, R.J., 1994. Reciprocal changes in plasma corticosterone and testosterone in stressed male rats maintained in a visible burrow system: evidence for a mediating role of testicular 11β-hydroxysteroid dehydrogenase. Endocrinology 134, 1193–1198.

Moya-Pérez, A., Perez-Villalba, A., Benítez-Páez, A., Campillo, I., Sanz, Y., 2017. Bifidobacterium CECT 7765 modulates early stress-induced immune, neuroendocrine and behavioral alterations in mice. Brain Behav. Immun. 65, 43–56.

Neufeld, K.M., Kang, N., Bienenstock, J., Foster, J.A., 2011. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol. Motil. 23, 255–264.

Nishino, R., Mikami, K., Takahashi, H., Tomonaga, S., Furuse, M., Hiramoto, T., Aiba, Y., Koga, Y., Sudo, N., 2013. Commensal microbiota modulate murine behaviors in a strictly contamination-free environment confirmed by culture-based methods. Neurogastroenterol. Motil. 25, 521–528.

O'Malley, D., Dinan, T.G., Cryan, J.F., 2010. Alterations in colonic corticotropin-releasing factor receptors in the maternally separated rat model of irritable bowel syndrome: differential effects of acute psychological and physical stressors. Peptides 31, 662–670.

Raone, A., Cassanelli, A., Scheggi, S., Rauggi, R., Danielli, B., De Montis, M.G., 2007. Hypothalamus-pituitary-adrenal modifications consequent to chronic stress exposure in an experimental model of depression in rats. Neuroscience 146, 1734–1742.

Reber, S.O., Peters, S., Slattery, D.A., Hofmann, C., Schölmerich, J., Neumann, I.D., Obermeier, F., 2011. Mucosal immunosuppression and epithelial barrier defects are key events in murine psychosocial stress-induced colitis. Brain Behav. Immun. 25, 1153–1161.

Schmid, J., Ludwig, B., Schally, A.V., Steffen, A., Ziegler, C.G., Block, N.L., Koutmani, Y., Brendel, M.D., Karalis, K.P., Simeonovic, C.J., Licinio, J., Ehrhart-Bornstein, M., Bornstein, S.R., 2011. Modulation of pancreatic islets-stress axis by hypothalamic releasing hormones and 11β-hydroxysteroid dehydrogenase. Proc. Natl. Acad. Sci.

U.S.A. 108, 13722–13727.
Steinberg, R.S., Lima, M., Gomes de Oliveira, N.L., Miyoshi, A., Nicoli, J.R., Neumann, E., Nunes, A.C., 2014. Effect of intestinal colonisation by two Lactobacillus strains on the immune response of gnotobiotic mice. Benef. Microbes 5, 409–419.
Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.N., Kubo, C., Koga, Y., 2004. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J. Physiol. 558 (Pt 1), 263–275.
Uschold-Schmidt, N., Nyuyki, K.D., Füchsl, A.M., Neumann, I.D., Reber, S.O., 2012. Chronic psychosocial stress results in sensitization of the HPA axis to acute heterotypic stressors despite a reduction of adrenal in vitro ACTH responsiveness. Psychoneuroendocrinology 2012 (37), 1676–1687.
Vodička, M., Ergang, P., Mikulecká, A., Řeháková, L., Klusoňová, P., Makal, J., Soták, M., Musílková, J., Zach, P., Pácha, J., 2014. Regulation of 11β-hydroxysteroid dehydrogenase type 1 and 7α-hydroxylase CYP7B1 during social stress. PLoS One 9, e89421.
Wu, X.Y., Hu, Y.T., Guo, L., Lu, J., Zhu, Q.B., Yu, E., Wu, J.L., Shi, L.G., Huang, M.L., Bao, A.M., 2015. Effect of pentobarbital and isoflurane on acute stress response in rat. Physiol. Behav. 145, 118–121.
Yuan, P.Q., Wu, S.V., Pothoulakis, C., Taché, Y., 2016. Urocortins and CRF receptor type 2 variants in the male rat colon: gene expression and regulation by endotoxin and anti-inflammatory effect. Am. J. Physiol.Gastrointest. Liver Physiol. 310, G387–G398.

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Interactions Between Gut Microbiota and Acute Restraint Stress in Peripheral Structures of the Hypothalamic–Pituitary–Adrenal Axis and the Intestine of Male Mice

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The gut microbiota play an important role in shaping brain functions and behavior, including the activity of the hypothalamus-pituitary-adrenocortical (HPA) axis. However, little is known about the effect of the microbiota on the distinct structures (hypothalamus, pituitary, and adrenals) of the HPA axis. In the present study, we analyzed the influence of the microbiota on acute restraint stress (ARS) response in the pituitary, adrenal gland, and intestine, an organ of extra-adrenal glucocorticoid synthesis. Using specific pathogen-free (SPF) and germ-free (GF) male BALB/c mice, we showed that the plasma corticosterone response to ARS was higher in GF than in SPF mice. In the pituitary, stress downregulated the expression of the gene encoding CRH receptor type 1 (*Crhr1*), upregulated the expression of the *Fkbp5* gene regulating glucocorticoid receptor sensitivity and did not affect the expression of the proopiomelanocortin (*Pomc*) and glucocorticoid receptor (*Gr*) genes. In contrast, the microbiota downregulated the expression of pituitary *Pomc* and *Crhr1* but had no effect on *Fkbp5* and *Gr*. In the adrenals, the steroidogenic pathway was strongly stimulated by ARS at the level of the steroidogenic transcriptional regulator *Sf-1*, cholesterol transporter *Star* and *Cyp11a1*, the first enzyme of steroidogenic pathway. In contrast, the effect of the microbiota was significantly detected at the level of genes encoding steroidogenic enzymes but not at the level of *Sf-1* and *Star*. Unlike adrenal *Sf-1,* the expression of the gene *Lrh-1*, which encodes the crucial transcriptional regulator of intestinal steroidogenesis, was modulated by the microbiota and ARS and this effect differed between the ileum and colon. The findings demonstrate that gut microbiota have an impact on the response of the pituitary, adrenals and intestine to ARS and that the interaction between stress and the microbiota during activation of glucocorticoid steroidogenesis differs between organs. The results suggest that downregulated expression of pituitary *Pomc* and *Crhr1* in SPF animals might be an important factor in the exaggerated HPA response of GF mice to stress.

Keywords: acute restraint stress, gut microbiota, germ-free, mice, HPA axis, intestine, extra-adrenal glucocorticoid synthesis

INTRODUCTION

Stressful stimuli induce a cascade of events in the hypothalamic–pituitary–adrenal (HPA) axis, which culminate in the secretion of glucocorticoids from the adrenal gland. The HPA axis is a self-regulatory network, utilizing its end-products, corticosterone in rats and mice and cortisol in humans, to regulate its own activity through a negative feedback mechanism at varying levels of the HPA axis (1). Changes in the HPA axis affect many physiological systems, including the immune system (2), and exposure to stressors modulates the pro-inflammatory cytokines and inflammatory pathways in the brain, endocrine glands, and plasma (3).

Studies performed on germ-free (GF) mice and rats showed that stress modifies not only gut microbiota but also vice versa; gut microbiota alter the stress response and brain neurochemistry (4, 5). GF mice exposed to acute restraint stress exhibited an exaggerated response of the HPA axis with elevated plasma adrenocorticotropic hormone (ACTH) and corticosterone levels, and this discrepancy was normalized after colonization of GF mice with commensal bacteria (6). A similar exaggerated response of the HPA axis was observed in response to acute novel-environment stress in GF mice and rats (7, 8). In contrast, treatment with prebiotics (9) or probiotics (10, 11) attenuated the HPA response to acute restraint or forced swim stress, even if this was not confirmed in all studies (12), probably due to strain-specific effects of the probiotic bacteria.

Taken together, these data strongly demonstrate that gut microbiota play a significant role in the activity of the HPA axis, including the plasma level of glucocorticoids. However, it is unknown what microbiota-induced changes underlie the exaggerated HPA axis activity. The signals originating from microbiota must be transmitted to the brain and/or the peripheral tissues that secrete glucocorticoids. These steroids are secreted primarily from the adrenal cortex, but they can also be generated in peripheral tissues such as the intestine via extra-adrenal glucocorticoid synthesis (13) or via regeneration of biologically active glucocorticoids, corticosterone, or cortisol from their inactive 11-oxo derivatives by enzyme 11β-hydroxysteroid dehydrogenase type 1 (11HSD1) (14). Numerous studies have also shown that neural, immune and endocrine pathways interact with each other at various levels, including the brain and adrenal glands, under normal and stress conditions and that a number of neuropeptides, cytokines, and even bacterial ligands are capable modulating glucocorticoid secretion independently of pituitary ACTH (15–17). Therefore, it is conceivable that gut microbiota might affect steroidogenesis of glucocorticoids. Enterocytes express a wide range of innate immune receptors, cytokines and chemokines (18), and cytokines influence the adrenal steroidogenesis (19), the regeneration of glucocorticoids via 11HSD1 (20) and the brain, including the activity of the HPA axis (21). Similarly, adrenal and intestinal extra-adrenal glucocorticoid synthesis is upregulated by systemic administration of endotoxins (22, 23), and activation of innate immune receptors stimulates steroidogenesis in adrenocortical cells (24).

The physiological response to acute stress related to gut bacteria has only sparsely been studied and focused only on upstream stress regulatory pathways in the brain (6–8). Therefore, in the present study, we aimed to reveal the relationship between gut microbiota and acute stress challenge downstream in the HPA axis, within the pituitary and adrenal gland, and in the intestine, which expresses the machinery of local glucocorticoid synthesis regulated by tumor necrosis factor α (TNFα) (13, 25).

MATERIALS AND METHODS

Animals

Nine-week-old germ-free (GF) and specific pathogen-free (SPF) male BALB/c mice (Institute of Microbiology of the Czech Academy of Sciences, Nový Hrádek, Czechia) were split into four groups: unstressed GF ($n = 10$), stressed GF ($n = 10$), unstressed SPF ($n = 10$), and stressed SPF ($n = 10$) mice. The animals were kept under a 12-h light/dark cycle and were given free access to autoclaved tap water and an irradiated (50 kGy) sterile pellet diet Altromin 1414 (Altromin, Lage, Germany). The GF animals were kept under sterile conditions in Trexler-type isolators since birth and their sterility was assessed every week by microbial cultivation and staining methods. The absence of bacteria, molds, and yeast was confirmed by aerobic and anaerobic cultivation of mouse feces and swabs from the isolators. Germfree status of the mice was further confirmed by the cecal size, weight, and bacterial DNA content when the GF mice were used in the experiments. Breeding of animals in isolators represents very specific environment in terms of handling, exposure to staff, noise level, air pressure etc. In order to ensure equal conditions for all groups during the experiment, the SPF mice were transferred to identical isolator as GF mice 1 month before the beginning of the experiments and were kept under the same conditions as GF mice, i.e., they were fed a sterile diet, drunk autoclaved water, were reared on the sterile bedding and were manipulated by the same staff as the GF mice. As the transfer of mice out of the isolator through a sterilized transfer port via an autoclave jar is a stressful procedure, control mice were transferred into sterile "individually ventilated cages" equipped with a filter system (IVC box; Tecniplast S.p.A., Buguggiate, Italy) 1 week before the end of the experiment. In preliminary experiments, we showed that the transfer of mice from the isolator through the transfer port increased the plasma level of corticosterone from 17.4 ± 4.7 to 103.3 ± 14.3 ng/ml. To minimize the effect of diurnal factors, the mice were stressed between 9:00 and 11:00 and sacrificed between 11:00 and 13:00. The experiments were approved by the Committee for the Protection and Use of Experimental Animals of the Institute of Microbiology of the Czech Academy of Sciences.

Acute Restraint Stress

The GF and SPF animals were subjected to a 2-h restraint stress in 50-ml conical centrifuge tubes equipped with multiple ventilation holes (26). First, the mice were inserted into the restrainer in the isolator and then during the restraint session,

they were transferred out of the isolator through the sterilized transfer port. Immediately after the stress period, the mice were anesthetized with isoflurane vapor, blood was collected by cardiac puncture in K_3EDTA coated tubes (Sarstedt, Nümbrecht, Germany), centrifuged and the plasma was stored at −80°C before being assayed. Anesthetized mice were decapitated, and the pituitary, adrenal gland, ileum and colon were harvested and snap-frozen in liquid nitrogen for assessment of mRNA expression. Isoflurane was used as an anesthetic because it does not interfere with gene transcriptional responses and leaves the stress response intact (27).

Sample Preparation and Gene Expression Analysis

Total RNA was extracted from the pituitary, adrenal gland, ileum, and colon using a commercially available kit (Quick-RNA Miniprep Plus, ZYMO Research, Irvine, CA, USA) according to the manufacturer's instructions and quantified by spectrophotometry using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was prepared from total RNA using random hexamers and a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Quantitative RT-PCR was carried out using the LightCycler 480 PCR System (Roche Diagnostic GmbH, Mannheim, Germany), 5x Hot Firepol Probe QPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) and the primers and probes specific for studied transcript (TaqMan Assays, Life Technologies; Generi Biotech, Hradec Králové, Czechia). The following assays were used: pro-opiomelanocortin (*Pomc*, Mm00435874_m1), co-chaperone FK506 binding protein 5 (*Fkbp5*, Mm00487401_m1), corticotropin-releasing hormone (CRH) receptor type 1 (*Crhr1*, Mm00432670_m1), corticotropin-releasing hormone receptor type 2 (*Crhr2*, Mm00438308_m1), glucocorticoid receptor (*Gr*, Mm00433832_m1), melanocortin-2 receptor (*Mc2r*, Mm01262510_m1), steroidogenic acute regulatory protein (*Star*, Mm00441558_m1), lymph node protein 64, a functional homolog of StAR (*Mln64*, Mm00445524_m1), cholesterol side-chain cleavage enzyme (*Cyp11a1*, Mm00490735_m1), 3β-hydroxysteroid dehydrogenase type 1, the major isoform expressed in adrenal gland (*Hsd3b1*, Mm01261921_mH), 3β-hydroxysteroid dehydrogenase type 2, the isoform predominantly expressed in extra-adrenal tissues (*Hsd3b2*, Mm00462685_m1), 11β-hydroxylase (*Cyp11b1*, Mm01204952_m1), steroidogenic factor-1 (*Sf-1*, Mm00446826-m1), liver receptor homolog-1 (*Lrh-1*, Mm00446088), and tumor necrosis factor α (*Tnfα*, Mm00443258_m1). For PCR amplification of 21-hydroxylase (*CYP21a1*) were used the following primers: sense TGGTGCTAAATTCTAACAGA and antisense CTTCCACATGAGAGAGTAATC; probe: ACAGGTCCAAGTCCATCTTTCCAT. To identify the stability of the reference genes, a panel of 12 potential reference genes was compared using geNorm analysis, and the genes *Hprt1* (hypoxanthine-guanine phosphoribosyltransferase 1, Mm01545399-m1) and *Tbp* (TATA-box binding protein, Mm00446973_m1) were identified as the optimal combination to provide reliable normalization in the ileum and colon and *Ppib* (peptidylprolyl isomerase B, cyclophilin B, Mm00478295_m1) and *Sdha* (succinate dehydrogenase subunit A, Mm01352366_m1) in the pituitary and adrenal glands. The expressions of the genes of interest were calculated relative to the geometric mean of the reference genes in each sample. The quantity of the PCR product was determined using the standard curve method with 3-fold dilutions of the mixed cDNA sample.

Corticosterone Assay

Plasma corticosterone levels were determined by a commercially available Corticosterone rat/mouse ELISA KIT (AR E-8100, LDN GmbH, Nordhorn, Germany). The samples for the assay were determined in a single run to prevent inter-assay variability according to the manufacturer's instructions. The sensitivity of the corticosterone assay was 6.1 ng/ml.

Statistical Analysis

For statistical comparison, the STATISTICA 9 software package (StatSoft Inc., Tulsa, OK, USA) was used. The data were analyzed by two-way analysis of variance (ANOVA; main factors: microbial status and stress treatment). If there was not a significant interaction effect between both factors, the interaction term was removed from the model and the main effects ANOVA was run. Follow-up comparisons of the means comprising main effects or simple effects of significant interactions were conducted using Tukey's test. The data were expressed as the mean ± SEM, and $p < 0.05$ were considered statistically significant.

RESULTS

Effect of Microbiota on Plasma Corticosterone Level in Response to Acute Restraint Stress

The two-way ANOVA revealed a significant effect of stress [$F_{1,34} = 283.45$, $p < 0.001$] and microbiota [$F_{1,34} = 4.82$, $p = 0.035$] but no significant interaction effect between the factors. *Post-hoc* analysis demonstrated that both stress and microbiota resulted in increased plasma level of corticosterone (**Figure 1**).

Effect of Microbiota on Pituitary Response to Acute Restraint Stress

To establish the impact of microbiota on glucocorticoid and neuropeptide signaling pathways in the pituitary, we examined the expression of the *Crhr1* gene encoding the CRHR1 receptor, whose activation enhances the transcription of *Pomc*, a gene encoding the ACTH precursor (**Figure 1**). Microbiota had a main effect on both *Crhr1* and *Pomc* expression [*Crhr1*: $F_{1,47} = 24.42$, $p < 0.001$; *Pomc*: $F_{1,47} = 17.26$, $p < 0.001$], but a significant effect of stress was revealed only for the expression of *Crhr1* [$F_{1,47} = 37.53$, $p < 0.001$] but not for *Pomc*. No significant interaction between stress and microbiota was observed in either case. The expression of *Crhr1*, which has been suggested together with the hormones CRH and ACTH to be critical for initiating the stress response, was significantly decreased in stressed mice. Similarly, microbiota downregulated the expression of *Crhr1* and *Pomc*.

To assess the potential differences in the pituitary glucocorticoid feedback between GF and SPF mice, we measured

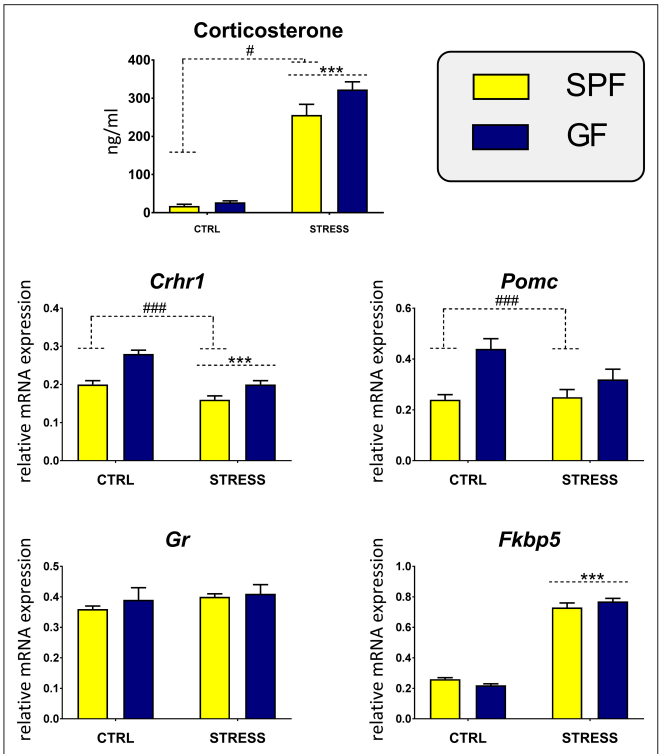

FIGURE 1 | Effect of acute restraint stress on plasma corticosterone and on genes participating in secretion of adrenocorticotropic hormone in the pituitary of specific pathogen-free (SPF) and germ-free (GF) mice. CTRL, unstressed mice; STRESS, mice exposed to restraint stress for 2 h; *Crhr1*, corticotropin-releasing hormone receptor type 1; *Pomc*, pro-opiomelanocortin; *Gr*, glucocorticoid receptor; *Fkbp5*, co-chaperone FK506 binding protein 5. Data are shown as mean ± SEM. A main effect of stress has been identified by placing a dashed horizontal line with an asterisk (***$p < 0.001$) above the bars for the stress-exposed groups, whereas a main effect of microbiota by placing a dashed horizontal line with a hash sign (###$p < 0.001$, #$p < 0.05$) above the bars for the SPF groups. No interaction effects between stress and microbiota were observed in any of the analyses.

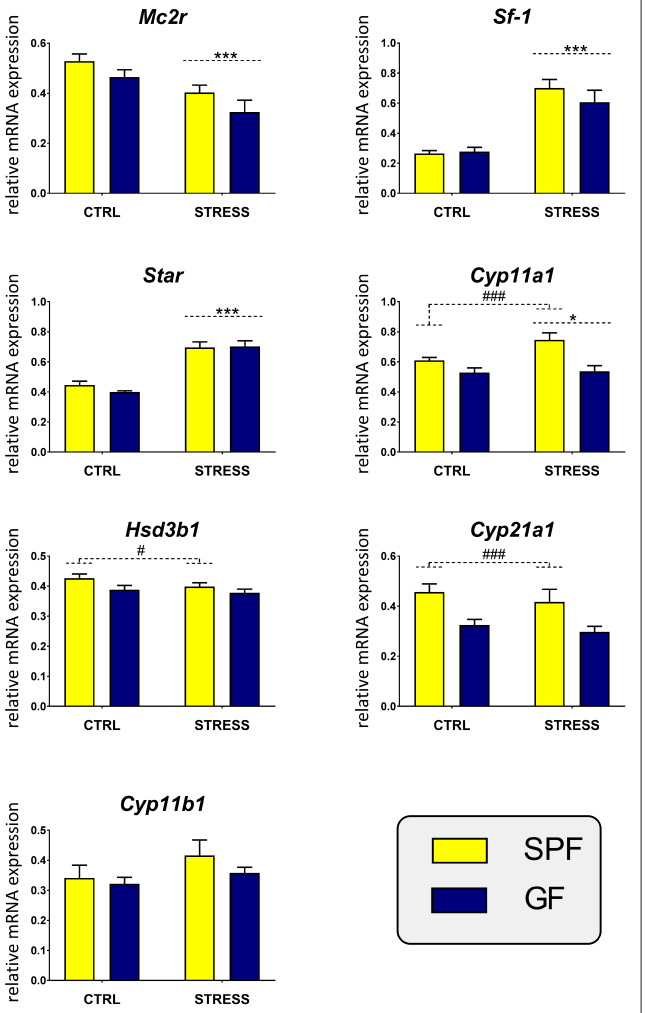

FIGURE 2 | Effect of microbiota and acute restraint stress on the expression of genes participating in adrenal steroidogenesis and its regulation. SPF, specific pathogen-free mice; GF, germ-free mice; CTRL, unstressed mice; STRESS, mice exposed to restraint stress for 2 h; *Mc2r*, melanocortin-2 receptor; *Sf-1*, steroidogenic factor 1; *Star*, steroidogenic acute regulatory protein, *Cyp11a1*, cholesterol side-chain cleavage enzyme, *Hsd3b1*, 3β-hydroxysteroid dehydrogenase type 1; *Cyp21a1*, 21-hydroxylase; *Cyp11b1*, 11β-hydroxylase. All data are expressed as mean ± SEM. A main effect of stress has been identified by placing a dashed horizontal line with an asterisk (***$p < 0.001$, *$p < 0.05$) above the bars for the stress-exposed groups, whereas a main effect of microbiota by placing a dashed horizontal line with a hash sign (###$p < 0.001$, #$p < 0.05$) above the bars for the SPF groups. No interaction effects between stress and microbiota were observed in any of the analyses.

the expression of *Gr* and *Fkbp5*, which encode the glucocorticoid receptor and the co-chaperone participating in the regulation of glucocorticoid receptor sensitivity and in the efficiency of the negative feedback pathway of the HPA axis (28) (**Figure 1**). Within the pituitary, two-way ANOVA revealed a significant effect of acute stress on the expression of *Fkbp5* [$F_{1,47} = 556.11$, $p < 0.001$], without any significant effect of microbiota or the stress × microbiota interaction. As shown in **Figure 1**, stress upregulated *Fkbp5* compared with unstressed counterparts. Neither stress nor microbiota modulated the expression of *Gr*.

Effect of Microbiota and Acute Restraint Stress on Expression of the ACTH Receptor and Steroidogenesis Enzymes in Adrenal Glands

To evaluate the effect of microbiota on the acute stress response in the adrenal glands, the expression of genes participating in adrenal steroidogenesis was quantified, namely, the genes encoding the ACTH receptor (*Mc2r*), a critical transcriptional factor regulating adrenal steroidogenesis (*Sf-1*), a protein that triggers the flow of cholesterol to the steroidogenic machinery (*Star*) and the steroidogenic enzymes (*Cyp11a1*, *Hsd3b1*, *Cyp21a1*, *Cyp11b1*) (29) (**Figure 2**). A two-way ANOVA of these transcripts did not indicate any statistically significant interaction effect of stress and microbiota. In contrast, the analysis proved the main effect of stress on the expression of genes encoding the first regulatory elements of the steroidogenic pathway, whereas the subsequent elements of this pathway

TABLE 1 | Results of two-way analysis of variance comparing the effects of microbiota and acute restraint stress in the intestine.

	Colon				Ileum			
	Df	**Microbiota**	**Stress**	**Interaction**	**Df**	**Microbiota**	**Stress**	**Interaction**
Lrh-1	1, 36	**<0.001** (69.64)	**<0.001** (68.29)	NS	1, 34	0.816 (0.05)	**<0.001** (16.56)	**0.003** (10.10)
Sf-1	1, 33	0.095 (2.95)	0.109 (2.71)	NS	1, 28	0.051 (4.15)	0.171 (1.98)	NS
Star	1, 32	0.388 (0.77)	0.603 (0.28)	**0.035** (4.84)	1, 33	0.784 (0.07)	0.704 (0.15)	NS
Mln64	1, 37	0.864 (0.03)	0.897 (0.02)	NS	1, 33	**0.045** (4.36)	**0.002** (11.49)	**0.039** (4.62)
Cyp11a1	1, 35	**<0.001** (20.61)	**<0.001** (32.14)	**<0.001** (14.79)	1, 33	0.161 (2.06)	0.298 (1.12)	NS
Hsd3b1	1, 33	0.164 (2.03)	0.960 (0.01)	**0.017** (6.27)	1, 20	0.140 (2.37)	0.182 (1.92)	NS
Hsd3b2	1, 37	**<0.001** (71.45)	**0.005** (8.88)	NS	1, 36	**0.003** (9.78)	0.931 (0.01)	NS
Cyp11b1	1, 28	0.082 (3.25)	**0.038** (3.00)	NS	1, 34	0.169 (1.97)	0.173 (1.94)	NS
Tnfα	1, 37	**0.046** (5.21)	**<0.001** (17.37)	NS	1, 33	**0.009** (7.74)	**0.035** (4.86)	**0.006** (8.71)
Crhr1	1, 34	0.517 (0.43)	0.616 (0.25)	NS	1, 25	0.147 (2.23)	0.358 (0.88)	NS
Crhr2	1, 36	0.143 (2.24)	**<0.001** (50.62)	NS	1, 32	0.801 (0.06)	0.157 (2.10)	**0.007** (8.37)
Mc2r	1, 26	0.108 (2.77)	0.107 (2.79)	**0.035** (4.63)	1, 27	0.095 (2.99)	0.907 (0.01)	NS

The data represent p-values with bolding indicating a statistically significant main effect or interaction effect; F values are given in parentheses; Df, degrees of freedom; NS, no significant interaction effect between microbiota and stress.

were modulated by microbial status but not by acute stress. Namely, the results showed that *Mc2r* was dependent on stress [$F_{1,34} = 14.47$, $p < 0.001$] but not on microbiota and stress significantly downregulated *Mc2r* expression. Similarly, the expression of adrenal genes, which are known to respond to acute stress, showed a main effect of stress [*Star*: $F_{1,34} = 79.30$, $p < 0.001$; *Sf-1*: $F_{1,34} = 52.23$, $p < 0.001$] accompanied by upregulation of the expression. No significant main effect of stress was found in the expression of genes encoding the enzymes of glucocorticoid synthesis excepting *Cyp11a1* [$F_{1,35} = 4.21$, $p = 0.048$], which was weakly increased by stress. In contrast, microbiota had a main effect on the expression of *Cyp11a1* [$F_{(1,35} = 14.96$, $p < 0.001$], *Hsd3b1* [$F_{1,35} = 5.08$, $p = 0.031$], and *Cyp21a1* [$F_{1,35} = 13.83$, $p < 0.001$] and *post hoc* tests revealed upregulation of all three genes by gut microbiota. No change due to microbiota was seen in *Cyp11b1*.

Effect of Microbiota and Acute Restraint Stress on Expression of Genes Encoding Intestinal Biogenesis

As acute inflammatory stress upregulates glucocorticoid synthesis in the intestine (13), we determined the effects of microbiome and stress on the expression of genes encoding selective enzymes and regulatory factors associated with steroidogenesis in GF and SPF mice. Two-way ANOVA revealed that both microbiota and stress modulate the expression of several genes associated with intestinal steroidogenesis. In the colon, the ANOVA indicated a significant interaction between the effect of stress and microbiota on the expression of *Star*, *Cyp11a1,* and *Hsd3b1* (**Table 1**). As shown in **Figure 3**, the *post hoc* analysis revealed a significant stress-dependent decrease of *Cyp11a1* in GF but not SPF mice and the stimulatory effect of germ-free status only in unstressed but not stressed animals. In contrast, germ-free status decreased the response of *Hsd3b1* to stress but did not modulate the expression in unstressed animals. There was no significant effect of a stress × microbiota interaction in other genes participating in intestinal steroidogenesis (**Table 1**). However, there were significant main effects of stress and microbiota on the expression of *Lrh-1,* encoding a functional homolog of adrenal *SF-1* in the intestine (30) and *Hsd3b2,* the second enzyme of the steroidogenic pathway (**Table 1**). The presence of microbiota led to the upregulation and stress to the downregulation of *Lrh-1* and *Hsd3b2* (**Figure 3**). In the case of *Cyp11b1,* only the effect of stress but not microbiota approached significance (**Table 1**).

Within the ileum, the main effect of microbiota approached significance only on the expression of *Hsd3b2* and the effect of stress × microbiota interaction on *Lrh-1* and *Mln64* (**Table 1**). As shown in **Figure 4**, microbiota downregulated the expression of *Hsd3b2* and stress upregulated *Lrh-1* and *Mln64,* but here the effect depended on the absence of microbiota.

Effect of Microbiota and Acute Restraint Stress on Expression of TNFα and Melanocortin and CRH Signaling in the Intestine

Both stress and microbiota affected the expression of *Tnfα* in the colon and ileum. Whereas, the interaction between both factors was not significant in the colon, the two-way ANOVA proved a robust interaction between stress and microbiota in the ileum (**Table 1**). As shown in **Figure 5**, stress significantly downregulated and microbiota upregulated the expression of *Tnfα* in the colon. However, in the ileum, stress downregulated *Tnfα* only in SPF but not GF mice, where the expression of the cytokine was very low.

To determine whether microbiota modulate peripheral CRH and melanocortin signaling in acute stress, the expression of

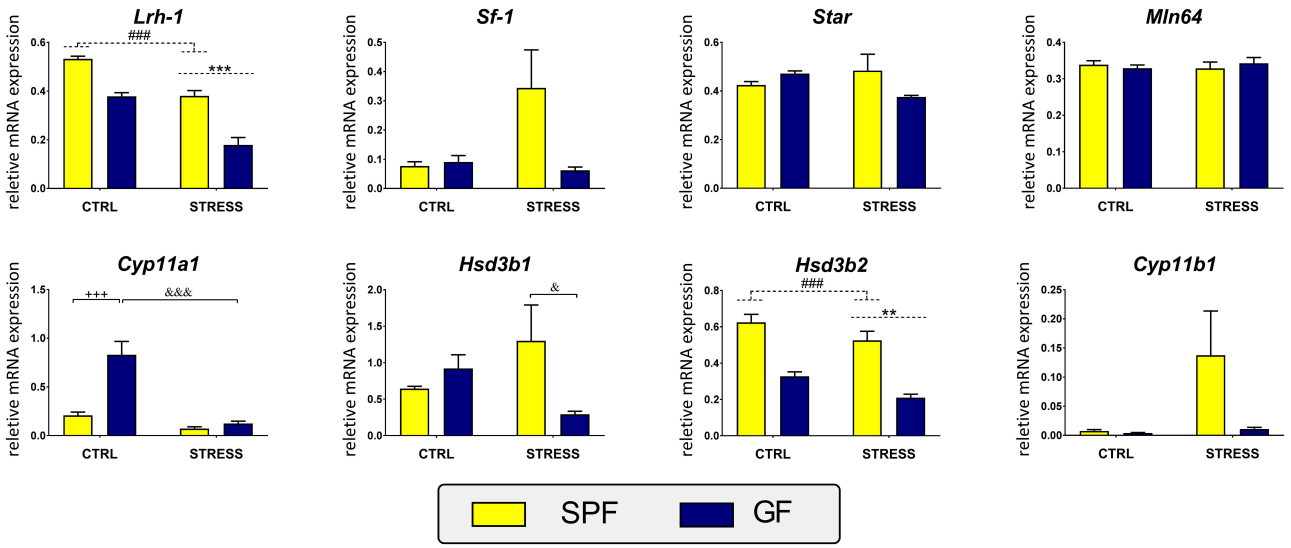

FIGURE 3 | Effect of microbiota and acute restraint stress on the expression of genes participating in colon steroidogenesis and its regulation. SPF, specific pathogen-free mice; GF, germ-free mice; CTRL, unstressed mice; STRESS, mice exposed to restraint stress for 2 h; *Lrh-1*, liver receptor homolog-1; *Sf-1*, steroidogenic factor 1; *Star*, steroidogenic acute regulatory protein; *Mln64,* lymph node protein 64; *Cyp11a1*, cholesterol side-chain cleavage enzyme; *Hsd3b1*, 3β-hydroxysteroid dehydrogenase type 1; *Hsd3b2*, 3β-hydroxysteroid dehydrogenase type 2; *Cyp11b1*, 11β-hydroxylase. All data are expressed as mean ± SEM. Where an interaction effect was observed, the ampersand sign ($^{\&\&\&}p < 0.001$, $^{\&}p < 0.05$) indicates a significant difference from the stressed GF mice and the plus sign ($^{+++}p < 0.001$) indicates a significant difference relative to the unstressed SPF animals. Where no interaction effect was observed, a main effect of stress has been identified by placing a horizontal dashed line with an asterisk ($^{***}p < 0.001$, $^{**}p < 0.01$) above the bars for the stress-exposed groups, whereas a main effect of microbiota by placing a dashed horizontal line with a hash sign ($^{\#\#\#}p < 0.001$) above the bars for the SPF groups.

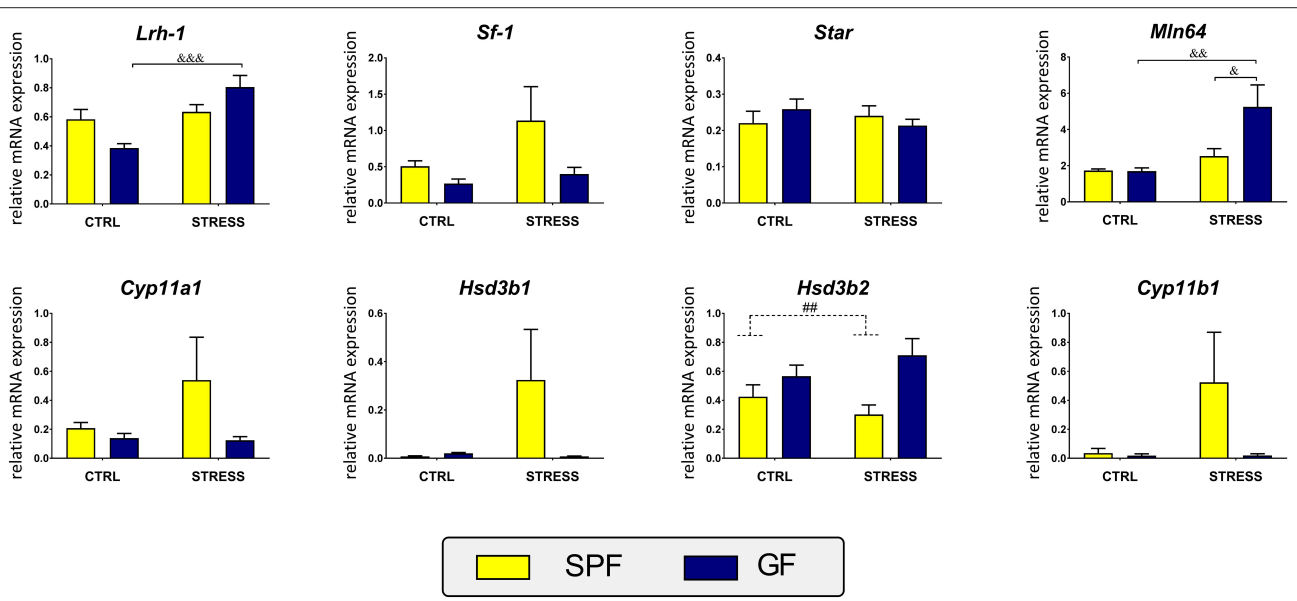

FIGURE 4 | Effect of microbiota and acute restraint stress on expression of genes participating in ileal steroidogenesis and its regulation. For further details, see the description of **Figure 3**. All data are expressed as mean ± SEM. Where an interaction effect was observed, the ampersand sign ($^{\&\&\&}p < 0.001$, $^{\&\&}p < 0.01$, $^{\&}p < 0.05$) indicates a significant difference from the stressed GF mice. Where no interaction effect was observed, a main effect of microbiome has been identified by placing a dashed horizontal line with a hash sign ($^{\#\#}p < 0.01$) above the bars for the SPF groups. No main effects of stress were found in any of the analyses.

the receptors *Crhr1*, *Crhr2,* and *Mc2r* were compared between stressed and unstressed SPF and GF mice. A significant effect of stress was found on *Crhr2* in the colon and a stress × microbiota interaction on *Crhr2* in the ileum and *Mc2r* in the colon. No effects of stress and microbiota were found on *Crhr1* expression either in the colon or ileum (**Table 1**). *Post-hoc* tests revealed

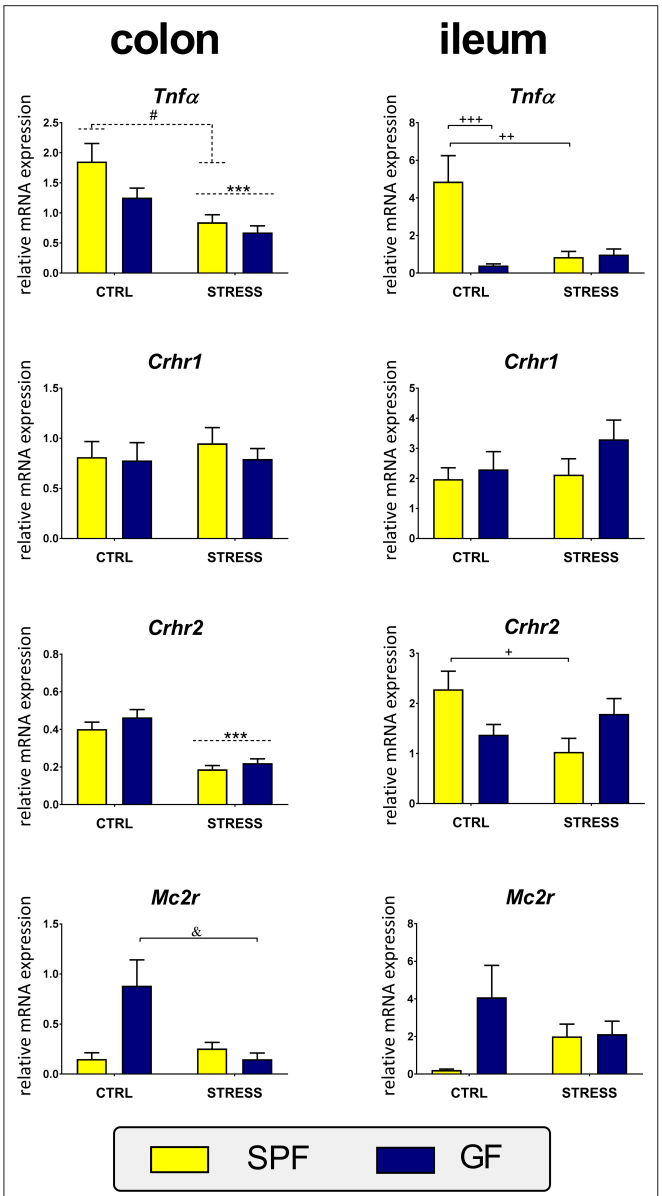

FIGURE 5 | Effect of stress on expression of the genes encoding tumor necrosis factor α and receptors for adrenocorticotropic and corticotropin-releasing hormones in the ileum and colon of specific pathogen-free (SPF) and germ-free (GF) mice. CTRL, unstressed mice; STRESS, mice exposed to restraint stress for 2 h; *Tnfα*, tumor necrosis factor α; *Crhr1*, corticotropin-releasing hormone receptor type 1; *Crhr2*, corticotropin-releasing hormone receptor type 2; *Mc2r*, melanocortin-2 receptor. All data are expressed as mean ± SEM. Where an interaction effect was observed, the ampersand sign ($^{\&}p < 0.05$) indicates a significant difference from the stressed GF mice and the plus sign ($^{+++}p < 0.001$, $^{++}p < 0.01$, $^{+}p < 0.05$) indicates a significant difference relative to the unstressed SPF animals. Where no interaction effect was observed, a main effect of stress has been identified by placing a dashed horizontal line with an asterisk ($^{***}p < 0.001$) above the bars for the stress-exposed groups, whereas a main effect of microbiota by placing a dashed horizontal line with a hash sign ($^{\#}p < 0.05$) above the bars for the SPF groups.

that stress significantly decreased the expression of *Crhr2* in the colon but in the ileum this effect was observed only in SPF mice (**Figure 5**). Expression of *Mc2r* showed a similar pattern in both intestinal segments, with a significant downregulation of *Mc2r* expression by stress only in the colon of GF mice (**Figure 5**).

DISCUSSION

There is growing evidence that microbiota regulate the responsiveness of the HPA axis to stress. Similar to Sudo et al. (6) and Clarke et al. (7), we showed an exaggerated response of the HPA axis to acute restraint stress in GF mice, but our study extends this finding by demonstrating that the microbiota have a profound modulatory effect not only on brain neurochemistry (6, 8, 31) but also on the pituitary and adrenal glands and extra-adrenal tissues.

Contrary to our expectations, we did not observe higher expression of *Pomc* in the pituitary after acute restraint stress, despite our previous report demonstrating upregulation of pituitary *Pomc* after chronic psychosocial stress (32) and the findings of Aguilera et al. showing increased expression of this gene after 14 days of repeated immobilization (33). This discrepancy may reflect either the different timelines of the experiments or different stressors used. In acute stress, the absence of increased pituitary *Pomc* expression after 45 min of restraint stress was observed in domestic chickens (34), whereas 15 min of restraint upregulated *Pomc* levels in the rat pituitary (35), but after 2 h of restraint, this level was already at the control value (36). The decreased *Crhr1* expression during acute stress is in line with previous findings in rat (37). Nevertheless, the expression of pituitary *Pomc* and *Crhr1* were upregulated in GF animals without any significant effect of microbiota on the expression of *Gr* and *Fkbp5*. This finding differs from our previous results (32), which showed an absence of any effect of microbiota on the expression of *Pomc* and *Crhr1* in the pituitary. This discrepancy seems to reflect differences in the treatment of control groups in both experiments. In our previous experiment, the GF and SPF mice were kept in groups of 4–5 per cage and were transferred from the isolator through a sterilized transfer port, where the animals had to spend some time in the transfer jar, whereas in the current experiment, the mice were kept in sterile IVC boxes and thus were not exposed to acute transfer and handling stress, which increased the instantaneous plasma level of corticosterone (see Materials and Methods). Regarding the effect of stress, the upregulation of pituitary *Fkbp5* and downregulation of *Crhr1* were also described in other studies (32, 38), and this downregulation was connected with the action of microRNA (36). To achieve homeostasis, glucocorticoids suppress the HPA axis through feedback inhibition of hypothalamic CRH and pituitary POMC synthesis and secretion (39). Therefore, the appropriate regulation of adrenal glucocorticoid synthesis is dependent not only on the adrenal responsiveness to ACTH but also on the synthesis and secretion of CRH in the hypothalamus and the degree of glucocorticoid-mediated feedback inhibition of the HPA axis. The absence of any effect of microbiota on *Gr* and *Fkbp5* expression indicates that the efficiency of the negative feedback loop via pituitary glucocorticoid receptors

is not modulated by the microbiota. The *Fkbp5* gene encodes a protein that regulates the glucocorticoid-mediated negative feedback loop through a decrease of the corticosterone affinity to glucocorticoid receptors and the trafficking of the receptor ligand complex to the nucleus (28). In contrast to the absence of any effect of microbiota on the glucocorticoid negative feedback loop, the downregulation of pituitary *Pomc* and *Crhr1* expression by microbiota suggests the possibility that the higher expression of *Pomc* and *Crhr1* in GF mice might contribute to the exaggerated HPA response to stress in these animals.

The ACTH-dependent regulation of glucocorticoid production requires the precisely coordinated transcription of a variety of genes involved in numerous aspects of steroidogenesis within the adrenal cortex where the nuclear receptor SF-1 represents the critical mediator, which transcriptionally regulates a variety of steroid biosynthetic enzymes (29). However, although the GF mice showed higher HPA axis reactivity to stress than SPF animals, the genes of the ACTH signaling pathway in the adrenal gland were independent of microbiota, particularly *Mc2r*, *Sf-1,* and *Star*, a gene whose transcription is rapidly stimulated by ACTH. Despite the effect of microbiota on the expression of genes encoding enzymes of adrenal steroidogenesis, acute restraint stress strongly upregulated *Sf-1* and *Star* but had no significant effect on the expression of steroidogenic enzyme genes with the exception of a weak effect of stress on *Cyp11a1*. Similar resistance of steroidogenic genes to acute restraint stress was shown recently by others (34, 40). These findings are in accordance with rapid stimulation of *Star* transcription during acute regulation of steroidogenesis and with less obvious effect on *Cyp11a1*, whose increased expression is associated predominantly with chronic maintenance of steroidogenesis (29).

The microbiota impact the systemic glucocorticoid response to stress (6–8), but whether the microbiota are involved in stressor-induced extra-adrenal glucocorticoid synthesis is not clear. It has been previously shown that acute inflammation stress increases local secretion of corticosterone from the intestine due to upregulation of *Cyp11a1* and *Cyp11b1* (13). As the design of our study allowed us to evaluate steroidogenesis not only in the adrenal gland but also in other tissues, we further studied whether acute restraint stress and the microbiota modulate intestinal glucocorticoid steroidogenesis. First, we provided evidence in favor of the effect of our stress paradigm in the intestine. Microbiota were shown to upregulate and chronic stress to downregulate *TNFα* mRNA and protein secretion (32). Second, the intestinal CRH system, a well-established regulatory system in the gastrointestinal tract, was shown to respond to various stressors (41). Consistent with this, microbiota upregulated intestinal *Tnfα* expression, and acute restraint stress downregulated *Crhr2* and *Tnfα*. Detailed analysis of steroidogenic genes in the colon showed a profound effect of stress and microbiota on the expression of several genes, particularly *Lrh-1*, whose gene product is a functional homolog of the transcription factor SF-1 and plays a crucial role in the regulation of intestinal steroidogenesis (42). Surprisingly, despite the upregulation of *Sf-1* transcript in the adrenals of stressed animals, we found downregulation of colonic *Lrh-1* by stress in both GF and SPF animals, and this downregulation was not followed by a corresponding decrease in *Star* expression. Stress-induced downregulation was identified only in the case of *Cyp11a1* in GF animals, and the transcripts of all other genes of glucocorticoid synthesis were not significantly downregulated by stress. In contrast to the colon, stress upregulated the expression of *Lrh-1* in the ileum but only in GF mice and the same pattern was observed in the case of *Mln64*, a gene encoding protein that has been implicated in cholesterol transport and steroidogenesis (43).These data are not in line with previous studies, which have shown that acute inflammatory stress upregulated the expression of *Lrh-1*, *Cyp11a1,* and *Cyp11b1* in the intestine (13, 42). The different responses of steroidogenic genes to acute inflammatory and restraint stress may be because the gene encoding TNFα, a master regulator of intestinal glucocorticoid synthesis during inflammation (25), was either downregulated or unchanged after restraint stress. Final proof regarding, whether acute restraint stress has a similar effect on intestinal synthesis of glucocorticoids as acute inflammatory stress will require further experiments. Nevertheless, the data indicate that acute restraint stress might influence intestinal steroidogenesis and that this effect depends on microbiota. First, the stimulatory effect of stress on the expression of ileal *Lrh-1* and *Mln64* was observed only in GF mice. Second, the expression of *Sf-1*, which activates the promoter of intestinal *Cyp11a1* and *Cyp11b1* similar to *Lrh-1* (30), showed a trend toward a significant increase by a stressor only in SPF animals. Third, the interaction between stress and microbiota determined the expression of *Cyp11a1*.

While our experiments show the impact of gut microbiota on the response of the pituitary, adrenal and intestine to stress, there are several limitations to these data. The current study used only males and thus the impact of sexual dimorphism of HPA axis cannot be excluded. First, compared to males, female mice and rats show a more robust HPA axis response, as a result of circulating estradiol, which elevates stress hormones levels during non-threatening situations and during stress (44). This sexual dimorphism reflects not only differences in the central components of the HPA axis but also in the adrenal responsiveness to ACTH (45–47). However, the mechanisms surrounding the stronger adrenal phenotype of females are not well understood (48–51). Second, recent data indicate that microbiome leads to alterations of sex-dimorphic gene expression (52) but no interaction between stress, sex, and GF status was observed in the release of corticosterone following a novel-environment stressor (7). Therefore, more studies will be necessary to assess whether the effect of stress and microbiota on activation of glucocorticoid steroidogenesis is a sex-specific process.

In conclusion, the findings reported here demonstrate that the microbiota have a significant impact on the response of the peripheral components of the HPA axis and extra-adrenal glucocorticoid steroidogenic pathway to acute restraint stress. In particular, we found that a lower expression of *Pomc* and *Crhr1* in the pituitary of SPF mice could partially explain the exaggerated HPA axis reactivity in GF animals. In contrast, the weak effect of microbiota on the expression of genes of

the adrenal glucocorticoid synthetic pathway indicates that the increased reactivity of the HPA axis in GF mice is not related to changes in the expression of adrenal steroidogenic enzymes. Finally, our study revealed that the response of the intestinal extra-adrenal glucocorticoid pathway to acute stressors depends on the microbiota. Although the precise mechanisms by which microbiota mediate these changes have yet to be elucidated, our findings show that the acute stress response is shaped by microbiota not only in the components of the HPA axis but also in peripheral organs and that the activation of intestinal steroidogenesis is controlled differently from that in the adrenals.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The experiments were approved by the Committee for the Protection and Use of Experimental Animals of the Institute of Microbiology of the Czech Academy of Sciences.

AUTHOR CONTRIBUTIONS

JP, MV, and TH conceived the study, designed the experiments, and led the project. KV, PH, PE, PK, and KB performed the experiments and analyses. DŠ prepared the germ-free animals. JP, KV, and MV wrote the manuscript. All of the authors read and approved the final manuscript.

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REFERENCES

1. Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, et al. Regulation of the hypothalamic–pituitary–adrenocortical stress response. *Compr Physiol*. (2016) 6:603–21. doi: 10.1002/cphy.c150015
2. Webster Marketon JI, Glaser R. Stress hormones and immune function. *Cell Immunol*. (2008) 252:16–26. doi: 10.1016/j.cellimm.2007.09.006
3. Hueston CM, Deak T. The inflamed axis: the interaction between stress, hormones, and the expression of inflammatory-related genes within key structures comprising the hypothalamic–pituitary–adrenal axis. *Physiol Behav*. (2014) 124:77–91. doi: 10.1016/j.physbeh.2013.10.035
4. Foster JA, Rinaman L, Cryan JF. Stress & the gut-brain axis: regulation by the microbiome. *Neurobiol Stress*. (2017) 7:124–36. doi: 10.1016/j.ynstr.2017.03.001
5. Mackos AR, Maltz R, Bailey MT. The role of the commensal microbiota in adaptive and maladaptive stressor-induced immunomodulation. *Horm Behav*. (2017) 88:70–8. doi: 10.1016/j.yhbeh.2016.10.006
6. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *J Physiol*. (2004) 558:263–75. doi: 10.1113/jphysiol.2004.063388
7. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, et al. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry*. (2013) 18:666–73. doi: 10.1038/mp.2012.77
8. Crumeyrolle-Arias M, Jaglin M, Bruneau A, Vancassel S, Cardona A, Daugé V, et al. Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. *Psychoneuroendocrinology*. (2014) 42:207–17. doi: 10.1016/j.psyneuen.2014.01.014
9. Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G, et al. Targeting the microbiota-gut-brain axis: prebiotics have anxiolytic and antidepressant-like effects and reverse the impact of chronic stress in mice. *Biol Psychiatry*. (2017) 82:472–87. doi: 10.1016/j.biopsych.2016.12.031
10. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA*. (2011) 108:16050–5. doi: 10.1073/pnas.1102999108
11. Ait-Belgnaoui A, Durand H, Cartier C, Chaumaz G, Eutamene H, Ferrier L, et al. Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology*. (2012) 37:1885–95. doi: 10.1016/j.psyneuen.2012.03.024
12. Savignac HM, Kiely B, Dinan TG, Cryan JF. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. *Neurogastroenterol Motil*. (2014) 26:1615–27. doi: 10.1111/nmo.12427
13. Cima I, Corazza N, Dick B, Fuhrer A, Herren S, Jakob S, et al. Intestinal epithelial cells synthesize glucocorticoids and regulate T cell activation. *J Exp Med*. (2004) 200:1635–46. doi: 10.1084/jem.20031958
14. Vagnerová K, Kverka M, Klusonová P, Ergang P, Mikšík I, Tlaskalová-Hogenová H, et al. Intestinal inflammation modulates expression of 11β-hydroxysteroid dehydrogenase in murine gut. *J Endocrinol*. (2006) 191:497–503. doi: 10.1677/joe.1.06732
15. Bornstein SR, Engeland WC, Ehrhart-Bornstein M, Herman JP. Dissociation of ACTH and glucocorticoids. *Trends Endocrinol Metab*. (2008) 19:175–80. doi: 10.1016/j.tem.2008.01.009
16. Chrousos GP. The hypothalamic–pituitary–adrenal axis and immune-mediated inflammation. *N Engl J Med*. (1995) 332:1351–62. doi: 10.1056/NEJM199505183322008
17. Nance DM, Sanders VM. Autonomic innervation and regulation of the immune system (1987–2007). *Brain Behav Immun*. (2007) 21:736–45. doi: 10.1016/j.bbi.2007.03.008
18. Lavelle EC, Murphy C, O'Neill LA, Creagh EM. The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. *Mucosal Immunol*. (2010) 3:17–28. doi: 10.1038/mi.2009.124
19. Bornstein SR, Zacharowski P, Schumann RR, Barthel A, Tran N, Papewalis C, et al. Impaired adrenal stress response in Toll-like receptor 2-deficient mice. *Proc Natl Acad Sci USA*. (2004) 101:16695–700. doi: 10.1073/pnas.0407550101
20. Ergang P, Vytáčková K, Švec J, Bryndová J, Mikšík I, Pácha J. Upregulation of 11β-hydroxysteroid dehydrogenase 1 in lymphoid organs during inflammation in the rat. *J Steroid Biochem Mol Biol*. (2011) 126:19–25. doi: 10.1016/j.jsbmb.2011.04.002
21. Turnbull AV, Rivier CL. Regulation of the hypothalamic–pituitary–adrenal axis by cytokines: actions and mechanisms of action. *Physiol Rev*. (1999) 79:1–71. doi: 10.1152/physrev.1999.79.1.1
22. Kanczkowski W, Chatzigeorgiou A, Samus M, Tran N, Zacharowski K, Chavakis T, et al. Characterization of the LPS-induced inflammation of the adrenal gland in mice. *Mol Cell Endocrinol*. (2013) 371:228–35. doi: 10.1016/j.mce.2012.12.020

23. Noti M, Corazza N, Tuffin G, Schoonjans K, Brunner T. Lipopolysaccharide induces intestinal glucocorticoid synthesis in a TNFα-dependent manner. *FASEB J*. (2010) 24:1340–6. doi: 10.1096/fj.09-140913
24. Kanczkowski W, Tymoszuk P, Chavakis T, Janitzky V, Weirich T, Zacharowski K, et al. Upregulation of TLR2 and TLR4 in the human adrenocortical cells differentially modulates adrenal steroidogenesis. *Mol Cell Endocrinol*. (2011) 336:41–6. doi: 10.1016/j.mce.2010.12.013
25. Noti M, Corazza N, Mueller C, Berger B, Brunner T. TNF suppresses acute intestinal inflammation by inducing local glucocorticoid synthesis. *J Exp Med*. (2010) 207:1057–66. doi: 10.1084/jem.20090849
26. Zimprich A, Garrett L, Deussing JM, Wotjak CT, Fuchs H, Gailus-Durner V, et al. A robust and reliable non-invasive test for stress responsivity in mice. *Front Behav Neurosci*. (2014) 8:125. doi: 10.3389/fnbeh.2014.00125
27. Wu XY, Hu YT, Guo L, Lu J, Zhu QB, Yu E, et al. Effect of pentobarbital and isoflurane on acute stress response in rat. *Physiol Behav*. (2015) 145:118–21. doi: 10.1016/j.physbeh.2015.04.003
28. Bekhbat M, Rowson SA, Neigh GN. Checks and balances: The glucocorticoid receptor and NFkB in good times and bad. *Front Neuroendocrinol*. (2017) 46:15–31. doi: 10.1016/j.yfrne.2017.05.001
29. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. (2011) 32:81–151. doi: 10.1210/er.2010-0013
30. Mueller M, Atanasov A, Cima I, Corazza N, Schoonjans K, Brunner T. Differential regulation of glucocorticoid synthesis in murine intestinal epithelial versus adrenocortical cell lines. *Endocrinology*. (2007) 148:1445–53. doi: 10.1210/en.2006-0591
31. Desbonnet L, Clarke G, Traplin A, O'Sullivan O, Crispie F, Moloney RD, et al. Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain Behav Immun*. (2015) 48:165–73. doi: 10.1016/j.bbi.2015.04.004
32. Vodička M, Ergang P, Hrnčír T, Mikulecká A, Kvapilová P, Vagnerová K, et al. Microbiota affects the expression of genes involved in HPA axis regulation and local metabolism of glucocorticoids in chronic psychosocial stress. *Brain Behav Immun*. (2018) 73:615–24. doi: 10.1016/j.bbi.2018.07.007
33. Aguilera G. Regulation of pituitary ACTH secretion during chronic stress. *Front Neuroendocrinol*. (1994) 15:321–50. doi: 10.1006/frne.1994.1013
34. Løtvedt P, Fallahshahroudi A, Bektic L, Altimiras J, Jensen P. Chicken domestication changes expression of stress-related genes in brain, pituitary and adrenals. *Neurobiol Stress*. (2017) 7:113–21. doi: 10.1016/j.ynstr.2017.08.002
35. Ginsberg AB, Frank MG, Francis AB, Rubin BA, O'Connor KA, Spencer RL. Specific and time-dependent effects of glucocorticoid receptor agonist RU28362 on stress-induced pro-opiomelanocortin hnRNA, c-fos mRNA and zif268 mRNA in the pituitary. *J Neuroendocrinol*. (2006) 18:129–38. doi: 10.1111/j.1365-2826.2005.01396.x
36. Nemoto T, Mano A, Shibasaki T. miR-449a contributes to glucocorticoid-induced CRF-R1 downregulation in the pituitary during stress. *Mol Endocrinol*. (2013) 27:1593–602. doi: 10.1210/me.2012-1357
37. Rabadan-Diehl C, Kiss A, Camacho C, Aguilera G. Regulation of messenger ribonucleic acid for corticotropin releasing hormone receptor in the pituitary during stress. *Endocrinology*. (1996) 137:3808–14. doi: 10.1210/endo.137.9.8756551
38. Klenerova V, Kvetnansky R, Hynie S. The effect of acute and repeated stress on CRH-R1 and CRH-R2 mRNA expression in pituitaries of wild type and CRH knock-out mice. *Cell Mol Neurobiol*. (2018) 38:163–9. doi: 10.1007/s10571-017-0556-3
39. Gagner JP, Drouin J. Opposite regulation of pro-opiomelanocortin gene transcription by glucocorticoids and CRH. *Mol Cell Endocrinol*. (1985) 40:25–32. doi: 10.1016/0303-7207(85)90154-6
40. Fallahsharoudi A, de Kock N, Johnsson M, Ubhayasekera SJ, Bergquist J, Wright D, et al. Domestication effects on stress induced steroid secretion and adrenal gene expression in chickens. *Sci Rep*. (2015) 5:15345. doi: 10.1038/srep15345
41. Larauche M, Kiank C, Tache Y. Corticotropin releasing factor signaling in colon and ileum: regulation by stress and pathophysiological implications. *J Physiol Pharmacol*. (2009) 60(Suppl. 7):33–46.
42. Mueller M, Cima I, Noti M, Fuhrer A, Jakob S, Dubuquoy L, et al. The nuclear receptor LRH-1 critically regulates extra-adrenal glucocorticoid synthesis in the intestine. *J Exp Med*. (2006) 203:2057–62. doi: 10.1084/jem.20060357
43. Watari H, Arakane F, Moog-Lutz C, Kallen CB, Tomasetto C, Gerton GL, et al. MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. *Proc Natl Acad Sci USA*. (1997) 94:8462–7. doi: 10.1073/pnas.94.16.8462
44. Oyola MG, Handa RJ. Hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes: sex differences in regulation of stress responsivity. *Stress*. (2017) 20:476–94. doi: 10.1080/10253890.2017.1369523
45. Atkinson HC, Waddell BJ. Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. *Endocrinology*. (1997) 138:3842–48. doi: 10.1210/en.138.9.3842
46. Figueiredo HF, Ulrich-Lai YM, Choi DC, Herman JP. Estrogen potentiates adrenocortical responses to stress in female rats. *Am J Physiol Endocrinol Metab*. (2007) 292:E1173–82. doi: 10.1152/ajpendo.00102.2006
47. Osborn JA, Yu C, Stelzl GE, Weinberg J. Effects of fetal ethanol exposure on pituitary-adrenal sensitivity to secretagogues. *Alcohol Clin Exp Res*. (2000) 24:1110–9. doi: 10.1111/j.1530-0277.2000.tb04657.x
48. Bastida CM, Cremades A, Castells MT, López-Contreras AJ, López-García C, Sánchez-Mas J, et al. Sexual dimorphism of ornithine decarboxylase in the mouse adrenal: influence of polyamine deprivation on catecholamine and corticoid levels. *Am J Physiol Endocrinol Metab*. (2007) 292:E1010–7. doi: 10.1152/ajpendo.00316.2006
49. Engeland WC, Massman L, Miller L, Leng S, Pignatti E, Pantano L, et al. Sex differences in adrenal Bmal1 deletion-induced augmentation of glucocorticoid responses to stress and ACTH in mice. *Endocrinology*. (2019) 160:2215–29. doi: 10.1210/en.2019-00357
50. Lo MJ, Chang LL, Wang PS. Effects of estradiol on corticosterone secretion in ovariectomized rats. *J Cell Biochem*. (2000) 77:560–8. doi: 10.1002/(SICI)1097-4644(20000615)77:4<560::AID-JCB4>3.0.CO;2-D
51. Nowak KW, Neri G, Nussdorfer GG, Malendowicz LK. Effects of sex hormones on the steroidogenic activity of dispersed adrenocortical cells of the rat adrenal cortex. *Life Sci*. (1995) 57:833–7. doi: 10.1016/0024-3205(95)02015-B
52. Weger BD, Gobet C, Yeung J, Martin E, Jimenez S, Betrisey B, et al. The mouse microbiome is required for sex-specific diurnal rhythms of gene expression and metabolism. *Cell Metab*. (2019) 29:362–82. doi: 10.1016/j.cmet.2018.09.023

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RESEARCH

Social defeat stimulates local glucocorticoid regeneration in lymphoid organs

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Abstract

Stress is an important risk factors for human diseases. It activates the hypothalamic–pituitary–adrenal (HPA) axis and increases plasma glucocorticoids, which are powerful regulators of immune system. The response of the target cells to glucocorticoids depends not only on the plasma concentrations of cortisol and corticosterone but also on their local metabolism. This metabolism is catalyzed by 11β-hydroxysteroid dehydrogenases type 1 and 2, which interconvert glucocorticoid hormones cortisol and corticosterone and their 11-oxo metabolites cortisone and 11-dehydrocorticosterone. The goal of this study was to determine whether stress modulates glucocorticoid metabolism within lymphoid organs – the structures where immune cells undergo development and activation. Using the resident-intruder paradigm, we studied the effect of social stress on glucocorticoid metabolism in primary and secondary lymphoid organs of Fisher 344 (F344) and Lewis (LEW) rats, which exhibit marked differences in their HPA axis response to social stressors and inflammation. We show that repeated social defeat increased the regeneration of corticosterone from 11-dehydrocorticosterone in the thymus, spleen and mesenteric lymphatic nodes (MLN). Compared with the F344 strain, LEW rats showed higher corticosterone regeneration in splenocytes of unstressed rats and in thymic and MLN mobile cells after stress but corticosterone regeneration in the stroma of all lymphoid organs was similar in both strains. Inactivation of corticosterone to 11-dehydrocorticosterone was found only in the stroma of lymphoid organs but not in mobile lymphoid cells and was not upregulated by stress. Together, our findings demonstrate the tissue- and strain-dependent regeneration of glucocorticoids following social stress.

Key Words

- glucocorticoid metabolism
- lymphoid organs
- Lewis rats
- Fisher 344 rats
- resident-intruder paradigm
- social stress

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Introduction

Stress is a ubiquitous condition that affects both people and animals. It initiates a series of events, culminating in the activation of the hypothalamic–pituitary–adrenal (HPA) axis and sympathetic nervous system (SNS), including the sympathetic–adrenal–medullary axis, which subsequently release glucocorticoids and catecholamines, respectively. The HPA and SNS axes are the two major pathways through which stress is able to modulate immune functions depending on the nature, intensity and duration of stress (1). Chronic stress can stimulate immunosuppression and increase susceptibility to diseases (2) or can enhance immune reactivity and induce insensitivity to glucocorticoids, which prevents glucocorticoid-induced suppression of inflammation (3). Acute stress increases transiently plasma glucocorticoids, whereas chronic stress is associated with a chronic elevation of circulating glucocorticoids (4).

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The response of the target cells to glucocorticoids does not merely depend on the level of the free hormone, activity of multidrug resistance efflux pumps and density of glucocorticoid receptors in target cells but also on the prereceptor metabolism of glucocorticoids, which is catalyzed by two enzymes, 11β-hydroxysteroid dehydrogenase type 1 (11HSD1) and type 2 (11HSD2). 11HSD2 is an enzyme that catalyzes the oxidations of cortisol and corticosterone to the inactive cortisone and 11-dehydrocorticosterone, reducing the local glucocorticoid signals. In contrast, 11HSD1 converts biologically inactive 11-oxo steroids (cortisone and 11-dehydrocorticosterone) to cortisol and corticosterone, amplifying the cellular glucocorticoid action (5). 11HSD1 is expressed in many organs and tissues, including lymphoid organs and immune cells (6, 7, 8, 9). 11HSD2 is predominantly expressed in mineralocorticoid target tissues, but moderate levels of 11HSD2 have also been found in lymphoid organs (10). Stressful situations have been shown to modulate the expression of 11HSD1 in the brain and some peripheral organs, but the results are contradictory (11, 12, 13, 14, 15, 16). For example, chronic social stress increased 11HSD1 expression in the rat hippocampus (12) but decreased it in the hippocampus of tree shrews (13) and in the rat testes (15). Similarly, chronic restraint stress upregulated 11HSD1 in the liver (11) but not in the thymus (14).

Given the fact that chronic stress is associated with an increased risk of many diseases including autoimmune disorders (17, 18), these effects are often attributed to the dysregulation of the HPA axis (1, 17) and the activity of lymphocytes and other leukocytes is potently modulated by glucocorticoids (19, 20, 21), the goal of this study was to determine (i) whether social stress influences the local metabolism of glucocorticoids in primary and secondary lymphoid organs, i.e. in the structures where immune cells undergo development and activation and (ii) whether there is any strain predisposition for the effect of stress on the lymphoid organs. To study these questions, we used two inbred rat strains, Fisher 344 (F344) and Lewis (LEW) rats, which represent two ends of a spectrum of HPA axis responsiveness to stress (22, 23) and vulnerability to immune diseases (24). The F344 strain has been classically used as a model of HPA axis hyperactivity and hyperreactivity to stress, whereas the LEW strain shows vulnerability to immune diseases due to a hypoactive and hyporeactive HPA axis (22). To induce stress, the animals were submitted to repeated social defeat, which is the result of intraspecific confrontation between male rats. This model provides a relevant tool to study stress response features, as well as differences in the vulnerability and resilience to stress and exposing the test animal to a dominant and aggressive counterpart for a known period of time is thought to mimic psychological stress in humans (25).

Materials and methods

Animals and social defeat paradigm

The animals used in the present study were male F344 and LEW rats, aged 65 days, that were purchased from Charles River, Germany. Animals were kept in groups of three to four in standard transparent cages in a temperature-controlled room (23 ± 1°C) on a 12/12-h light/darkness cycle with *ad libitum* access to food and water, and they were left for 3 weeks to acclimatize before any experimental procedure. Additionally, Long Evans retired male breeders (Institute of Physiology, Academy of Science, Prague) were chosen for consistent aggressive behavior. In contrast to the experimental animals, the Long Evans rats were housed individually. The F344 and LEW animals were randomly assigned to four groups, each consisted of eight rats, as follows: (1) control F344 rats, (2) defeated F344 rats, (3) control LEW rats and (4) defeated LEW rats. Control rats were placed in an adjacent room under the lighting conditions mention above and were then left undisturbed in their home cages.

To stress the animals, we used a slight modification of the resident-intruder paradigm validated by other authors (26). The resident-intruder test consisted of placing a smaller experimental rat (intruder) in the home cage of a larger and aggressive conspecific rat (resident), which defended its territory and defeated the intruder. The experimental rats were confronted with a resident male for 15 min each in the home cage of a Long Evans rat. The paradigm was repeated once daily for ten consecutive days, and each intruder was exposed to a novel resident to prevent habituation to the resident. No intruder was wounded by the residents during the repeated confrontations. The male rats were used for this study because they are more sensitive to social defeat than females (27).

The experiments were performed in the morning (between 09:00 and 12:00 h), and all animal procedures were performed in accordance with Institutional Animal Care and Use Committee regulations.

Tissue collection and processing

Control rats and the rats after the last social interaction session were immediately anesthetized with isoflurane

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and blood was collected by cardiac puncture. Then, the rats were decapitated, and the pituitary, thymus, spleen and mesenteric lymphatic nodes (MLNs) were quickly collected, cleaned of fat and connective tissues and weighted. The harvested thymus, spleen and MLN were used immediately for preparation of cell suspension of mobile cells and stroma, as described previously (6). Briefly, the lymphoid cell suspensions were prepared in RPMI 1640 medium by pressing the organs with a syringe plunger, filtering the suspension through nylon cell strainer (mesh size 45 µm) and washing the cell suspensions and remaining stroma twice in RPMI 1640 before measurement of the 11HSD activity. Erythrocytes were depleted from the spleen cell suspension by lysis in ACK lysis buffer.

Measurement of 11HSD activity

Isolated cells and stroma minced into fine pieces were used immediately to measure 11HSD1 and 11HSD2 activities. The 11-reductase activity assay for 11HSD1 was performed by measuring corticosterone produced from 11-dehydrocorticosterone and 11-oxidase assay for 11HSD2 was done by measuring the conversion of corticosterone to 11-dehydrocorticosterone as described previously (6). In brief, isolated cells and stroma were incubated in culture media consisting of RPMI 1640 supplemented with 5% charcoal-stripped fetal bovine serum (Biochrom GmbH), 100 IU/mL penicillin, 10 µg/mL streptomycin, 0.3 mg/L L-glutamine and 4.5 g/L glucose in the presence of 12.8 nM [^{3}H]11-dehydrocorticosterone or [^{3}H]corticosterone in an atmosphere of 5% CO_2 and 95% O_2 at 37°C. [^{3}H]Corticosterone was purchased from MP Biomedicals (Santa Anna, USA) and [^{3}H]11-dehydrocorticosterone was synthesized 'in house' from [^{3}H]corticosterone using kidney microsomes prepared from guinea pig. After 24 h of incubation, the samples were centrifuged, the pellets used for protein quantification using the BCA method and the steroids extracted from the supernatants using C18 reverse phase Sep-Pak cartridges (Phenomenex, USA). Pituitary 11HSD1 activity was measured in minced pituitary explants using the same tissue culture procedure as for lymphoid organs.

The extracted samples were evaporated to dryness under nitrogen at 40°C, reconstituted in methanol and analyzed using HPLC as previously described (28, 29). The elution of radioactive steroids was detected using Radiomatic 150TR Flow Scintillation Analyzer (Canberra Packard, USA) and the identification of radiolabeled corticosterone and 11-dehydrocorticosterone peaks (Supplementary Fig. 1, see section on supplementary data given at the end of this article) was performed by comparison with the elution profiles of the unlabeled steroid standards (Steraloids, Newport, RI, USA).

Plasma corticosterone and ACTH determination

The plasma corticosterone and ACTH concentrations were determined using commercially available radioimmunoassay (Corticosterone ^{125}I RIA, MP Biomedicals, Solon, OH, USA) and enzyme immunoassay (ACTH ELISA, MD Bioproducts, Eggs, Switzerland) kits, respectively. The samples for each assay were determined in a single run to prevent inter-assay variability according to the manufacturer's instructions. The sensitivity of the corticosterone and ACTH assays were 7.7 ng/mL and 0.22 pg/mL, respectively.

Statistical analysis

The results are expressed as the means±S.E.M. All calculations were conducted in Statistica 6.1. (StatSoft Inc., Tulsa, OK, USA) using a two-way ANOVA for comparisons involving the effects of stress and strain. The independent variables were the treatment and strain, consisting of two levels, which were unstressed or stressed and F344 or LEW, respectively. *Post hoc* analyses were performed using Fisher's LSD test. Differences were considered significant at $P<0.05$.

Results

Effect of stress on weights of thymus, spleen and adrenal gland and on plasma levels of ACTH and corticosterone

Statistical analysis revealed a decreased body weight in repeatedly stressed LEW rats but control and stressed F344 rats had similar body weight not only on Day 1 but also on Day 10 (Table 1). Stress significantly increased the relative adrenal weight in F344 rats but not in LEW rats. No differences were observed in the spleen and thymus weights of stressed and unstressed animals of both strains.

Figure 1 shows plasma ACTH and corticosterone levels in unstressed and stressed F344 and LEW rats. A two-way ANOVA revealed a significant effect of stress ($F_{1,24}=16.17$, $P<0.01$) and strain ($F_{1,24}=11.11$, $P<0.01$) on ACTH. As given in Fig. 1A, stress significantly increased ACTH level in F344 rats and had a nonsignificant tendency to increase ACTH level also in LEW rats ($P=0.067$). Whereas the plasma

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Table 1 Effect of repeated social defeat on the body and organ weights.

	F344		LEW	
	Control	Defeat	Control	Defeat
Body weight day 1	184±2	188±2	247±4	244±4
Body weight day 10	225±3	224±3	303±3	283±2***
Thymus	179±5	171±6	190±7	172±5
Spleen	241±3	239±2	202±2##	222±15
Adrenal gland	15±1	22±2*	15±1	17±0

Body weight is expressed in grams and organ weights in milligrams per 100 g of body weight (shown as mean ± S.E.M., n=6–7 rats per group). Statistically significant differences between control and social defeat group: *P<0.05 and ***P<0.001 and between the strains: ##P<0.01.

levels of ACTH in unstressed LEW and F344 rats were similar, ACTH levels in stressed F344 rats were significantly higher than those in stressed LEW rats. As shown in Fig. 1B, stress significantly affected the plasma corticosterone ($F_{1,26}$=37.08, P<0.001), and this effect depended on the strain ($F_{1,26}$=11.77, P<0.01) and showed a stress×strain interaction ($F_{1,26}$=12.58, P<0.01). *Post hoc* analysis revealed similar baseline levels of corticosterone with a significantly higher effect of stress on the plasma corticosterone in F344 rats compared to LEW rats.

Effect of stress on local metabolism of glucocorticoids in lymphoid organs

As shown in Fig. 2, exposure to stress significantly affected the 11-reductase activity of 11HSD1, in both stroma (thymus: $F_{1,21}$=33.72, P<0.001; spleen: $F_{1,20}$=126.58, P<0.001; MLN: $F_{1,20}$=52.16, P<0.001) and mobile cells (thymus: $F_{1,20}$=32.96, P<0.001; spleen: $F_{1,20}$=4.71, P<0.05; MLN: $F_{1,14}$=53.90, P<0.001), indicating stress-induced modulation of glucocorticoid regeneration in lymphoid tissues. Similarly, two-way ANOVA revealed a significant effect of the strain on 11-reductase activity in mobile cells of the thymus ($F_{1,20}$=14.80, P<0.01), spleen ($F_{1,20}$=5.46, P<0.05) and MLN ($F_{1,14}$=25.85, P<0.001), but no significant strain differences were found in the stroma of the three lymphoid organs. The stress×strain interactions were insignificant in all three tissues. *Post hoc* analysis showed a stimulatory effect of stress on the 11-reductase activity in studied lymphoid tissues of both strains, except for LEW splenocytes. In addition, a significantly higher effect of social defeat was found in thymocytes and MLN mobile cells of LEW rats than in their F344 counterparts. 11-Reductase activity in splenocytes of control unstressed LEW rats was significantly higher than that in the F344 rats and was not further upregulated in splenocytes of stressed LEW rats.

Expression of 11HSD2 measured as 11-oxidase activity was found in stroma, but it was not observed in mobile cells (Fig. 3), which agrees with previous findings of absent 11HSD2 in lymphoid cells (9). Expression of 11HSD2 in the MLN and spleen was not changed following social defeat or strain differences. In contrast, the thymic activity was influenced by stress ($F_{1,18}$=4.43, P<0.05) and strain ($F_{1,18}$=13.25, P<0.01), with significantly increased levels in LEW compared to F344 rats and a significant downregulation by stress (Fig. 3).

Effect of stress on local metabolism of glucocorticoids in pituitary

As release of ACTH in pituitary is controlled by glucocorticoid negative feedback (30), this gland expresses 11HSD1 (12) and ACTH and corticosterone response to stressogenic stimulus differs between F344 and LEW rats (Fig. 1), we

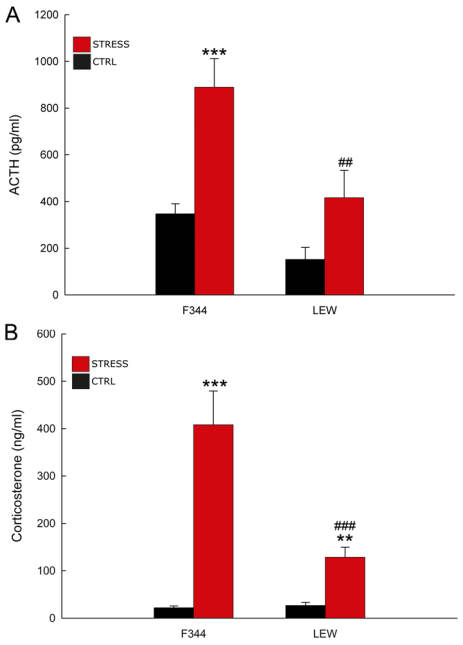

Figure 1
Plasma ACTH (A) and corticosterone (B) levels in rats exposed to repeated social defeat. The bars represent control unstressed (CTRL) and stressed rats. Data are given as the mean ± S.E.M. Significant differences between the stressed and unstressed animals of the same strain: **P<0.01 and ***P<0.001 and between the F344 and LEW rats of the same treatment: ##P<0.01 and ###P<0.001.

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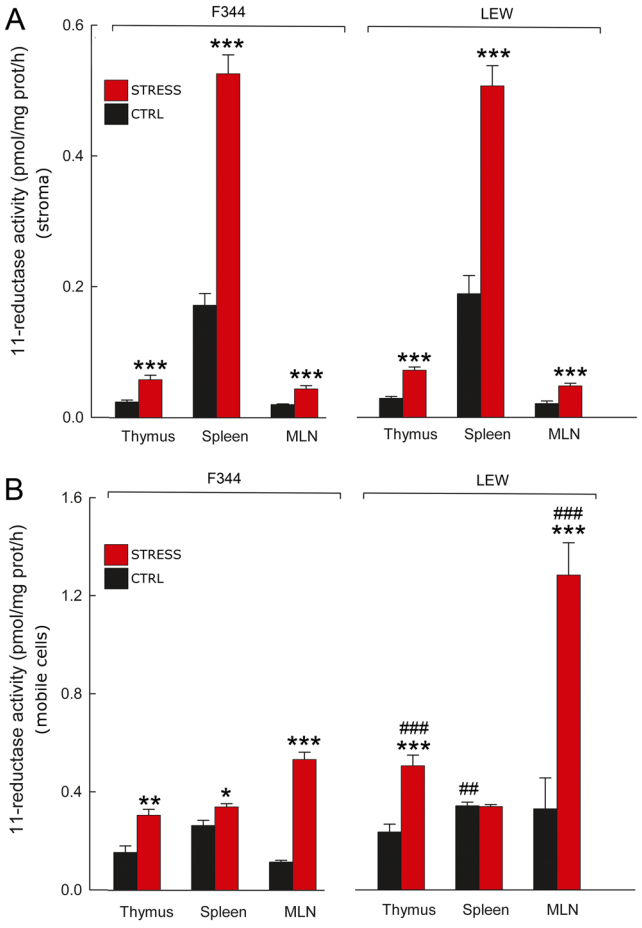

Figure 2
Effect of stress on 11-reductase activity of 11HSD1 in the stroma (A) and mobile cells (B) in lymphoid organs of control unstressed (CTLR) and stressed rats. Data are given as the mean ± S.E.M. Significant differences between the stressed and unstressed animals of the same strain: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ and between the F344 and LEW rats of the same treatment: $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$.

determined whether stress results in changes of pituitary corticosterone metabolism. In contrast to lymphoid organs, pituitary tissue incubated *in vitro* with radioactive steroids only resulted in conversion of 11-dehydrocorticosterone to corticosterone, while oxidation of corticosterone was not detected. This finding indicates the presence of 11HSD1 but not 11HSD2 in the pituitary. As shown in Fig. 4, the 11-reductase activity of 11HSD1 was higher in F344 rats than in LEW rats ($F_{1,21}=12.56$, $P<0.01$), but stress did not modulate the conversion.

Discussion

In the present study, we have characterized the consequences of social stress on glucocorticoid metabolism in the lymphoid organs and pituitary of two inbred rat strains with differing HPA axis responsiveness. The F344 strain has been classically used as a model of HPA axis hyperactivity and hyperreactivity to stress associated with inflammation resistance, whereas the LEW strain has a hypoactive and hyporeactive HPA axis, which has been associated with vulnerability to immune diseases (22, 23). Consistent with previous report (22), the plasma corticosterone and ACTH levels were elevated in both strains after the social defeat challenge, and the effect was greater in F344 rats. Although this result confirms the strain difference in the HPA axis, our findings demonstrate the tissue-dependent effect of stress on the local regeneration of biologically active glucocorticoids and its partial strain dependence.

Amplification of glucocorticoid metabolism in the immune system during social defeat indicates that the local effect of glucocorticoids follows both the systemic patterns of glucocorticoid synthesis by the adrenal gland and the local regeneration of corticosterone catalyzed by 11HSD1 via its 11-reductase activity. In contrast, the absence of any stress-dependent changes of pituitary 11-reductase activity excludes the possibility that stress might be associated with amplification of glucocorticoid signals, which are known to influence the secretion of pituitary ACTH via negative feedback (30). Considering the regulatory effects of glucocorticoids in immune cells (19, 20, 21) and the expression of 11HSD1 in lymphocytes and immune organs (6, 8, 9), the stress-induced upregulation of corticosterone regeneration in lymphoid organs might provide immune cells/organs with a novel

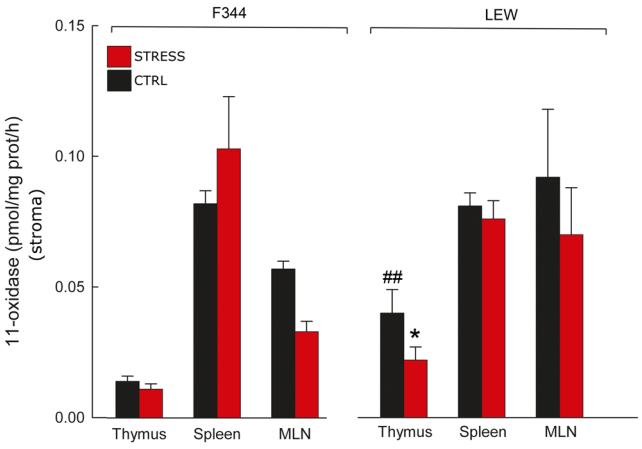

Figure 3
Effect of stress on 11-oxidase activity referring to 11HSD2 in the stroma of lymphoid organs of control unstressed (CTRL) and stressed rats. Data are given as the mean ± S.E.M. Significant differences between the stressed and unstressed animals of the same strain: $^{*}P<0.05$ and between the F344 and LEW rats of the same treatment: $^{\#\#}P<0.01$.

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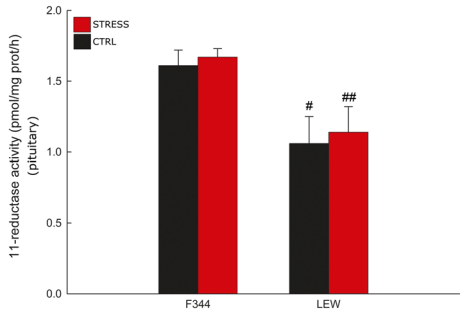

Figure 4
Strain difference in 11-reductase activity of 11HSD1 in the pituitary of F344 and LEW rats. The bars represent the mean ± SEM of control unstressed (CTRL) and stressed rats. Significant differences between the F344 and LEW rats of the same treatment: $^{\#}P<0.05$ and $^{\#\#}P<0.01$.

intracrine regulatory pathway. Similar stress-induced increase of 11HSD1 was recently shown in liver (11) and murine macrophages (16), respectively.

The presence of 11-oxidase and 11-reductase activities in the stroma tissue of lymphoid organs but the absence of 11-oxidase activity in the mobile cells of lymphoid organs may reflect the presence of different subsets of cells such lymphocytes, macrophages and thymocytes in the pool of mobile cells and fibroblast, thymic epithelial cells and vascular endothelial and smooth muscle cells in the stroma. Both isoforms of 11HSDs were identified in vascular cells, whereas epithelial cells express only 11HSD2 and fibroblasts, lymphocytes, thymocytes and macrophages only 11HSD1 (5, 7, 9). In addition, it has been shown recently that thymic 11HSD2 is located at the perivascular sites of capillaries and small vessels penetrating the thymus (31) and in the thymic epithelial cells (32). Our findings are in full concordance with these data. We identified 11-reductase and 11-oxidase activities in the stroma and 11-reductase activity in the mobile cells, which indicates operational 11HSD1 and 11HSD2 in lymphoid organ stroma but 11HSD1 only in the mobile cells.

The mechanism by which social stress modulates 11HSD1 remains unknown; however, several studies have demonstrated the regulation of 11HSD1 by transcription factors of the CCAT/enhancer-binding protein (C/EBP) family (33, 34) and their induction by catecholamines (35, 36) or glucocorticoid (37). Thus, it is likely that upregulation of 11HSD1 during stress might be associated with indirect regulation via C/EBP pathway activated either by increased plasma level of corticosterone or by increased activity of catecholaminergic pathway through norepinephrine released from adrenergic terminal or through local biogenesis of catecholamines in the target tissue (38).

Consistent with previous studies, F344 rats had an exaggerated stress response compared to the LEW strain, as measured by the plasma ACTH and corticosterone, but the present data also revealed clear inter-strain differences in the local regeneration of glucocorticoids both in the pituitary and lymphoid organs, particularly in mobile cells. Compared to the F344 strain, the LEW rats exhibited a lower resting regeneration of corticosterone in the pituitary, which was insensitive to repeated social challenge. In contrast, such challenge in LEW strain was associated with an increased regeneration of corticosterone in thymocytes and MLN mobile cells and with decreased corticosterone inactivation in thymic stroma. In addition, the splenocytes from untreated LEW vs F344 rats showed an increased corticosterone regeneration, which was not further upregulated by stress. These findings suggest that the hyporesponsiveness of the HPA axis in LEW rats compared to the F344 strain (22, 23) is not associated with the LEW vs F344 difference in glucocorticoid metabolism in the pituitary. However, the well-known increased vulnerability of LEW rats to immune/inflammatory challenge (24) might be associated with a higher regeneration of corticosterone from 11-dehydrocorticosterone in thymocytes and MLN mobile cells of LEW rats exposed to stress. Considering that glucocorticoids can antagonize the signal transduction delivered through T cell receptors in lymphocytes (21, 39), differences in corticosterone regeneration might distinctly modulate the activation and survival of T cells in the immune organs of both strains, even if further studies will be needed to evaluate this possibility. Analogous to our findings, immune tissues of stressed LEW rats exhibit reduced glucocorticoid receptor binding compared to F344 rats, even if there are no strain differences in the total glucocorticoid receptor levels in most immune tissues (22).

In summary, our findings indicate that social stress increases the local glucocorticoid production in lymphoid organs via corticosterone regeneration from a biologically inactive 11-oxo derivative, 11-dehydrocorticosterone, and this regeneration partially depends on the strain. As the stress-dependent increase of glucocorticoid production in mobile lymphoid organ cells is higher in LEW than in F344 rats, it is reasonable to assume that these strain-dependent differences might participate in the higher susceptibility of the LEW strain to inflammatory diseases.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/EC-18-0319.

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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References

1 McEwen BS, Biron CA, Brunson KW, Bulloch K, Chambers WH, Dhabhar FS, Goldfarb RH, Kitson RP, Miller AH, Spencer RL, *et al*. The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Research Reviews* 1997 **23** 79–133. (https://doi.org/10.1016/S0165-0173(96)00012-4)

2 Cohen S, Janicki-Deverts D, Doyle WJ, Miller GE, Frank E, Rabin BS & Turner RB. Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. *PNAS* 2012 **109** 5995–5999. (https://doi.org/10.1073/pnas.1118355109)

3 Silverman MN & Sternberg EM. Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. *Annals of the New York Academy of Sciences* 2012 **1261** 55–63. (https://doi.org/10.1111/j.1749-6632.2012.06633.x)

4 Bowers SL, Bilbo SD, Dhabhar FS & Nelson RJ. Stressor-specific alterations in corticosterone and immune responses in mice. *Brain Behavior and Immunity* 2008 **22** 105–113. (https://doi.org/10.1016/j.bbi.2007.07.012)

5 Chapman K, Holmes M & Seckl J. 11β-Hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action. *Physiological Reviews* 2013 **93** 1139–1206. (https://doi.org/10.1152/physrev.00020.2012)

6 Ergang P, Vytáčková K, Švec J, Bryndová J, Mikšík I & Pácha J. Upregulation of 11β-hydroxysteroid dehydrogenase 1 in lymphoid organs during inflammation in the rat. *Journal of Steroid Biochemistry and Molecular Biology* 2011 **126** 19–25. (https://doi.org/10.1016/j.jsbmb.2011.04.002)

7 Nuotio-Antar AM, Hasty AH & Kovacs WJ. Quantitation and cellular localization of 11β-HSD1 expression in murine thymus. *Journal of Steroid Biochemistry and Molecular Biology* 2006 **99** 93–99. (https://doi.org/10.1016/j.jsbmb.2006.01.011)

8 Taves MD, Plumb AW, Korol AM, Van Der Gugten JG, Holmes DT, Abraham N & Soma KK. Lymphoid organs of neonatal and adult mice preferentially produce active glucocorticoids from metabolites, not precursors. *Brain Behavior and Immunity* 2016 **57** 271–281. (https://doi.org/10.1016/j.bbi.2016.05.003)

9 Zhang TY, Ding X & Daynes RA. The expression of 11β-hydroxysteroid dehydrogenase type I by lymphocytes provides a novel means for intracrine regulation of glucocorticoid activities. *Journal of Immunology* 2005 **174** 879–889. (https://doi.org/10.4049/jimmunol.174.2.879)

10 Hennebold JD, Ryu SY, Mu HH, Galbraith A & Daynes RA. 11β-Hydroxysteroid dehydrogenase modulation of glucocorticoid activities in lymphoid organs. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* 1996 **270** R1296–R1306. (https://doi.org/10.1152/ajpregu.1996.270.6.R1296)

11 Corona-Pérez A, Díaz-Muñoz M, Rodríguez IS, Cuevas E, Martínez-Gómez M, Castelán F, Rodríguez-Antolín J & Nicolás-Toledo L. High sucrose intake ameliorates the accumulation of hepatic triacylglycerol promoted by restraint stress in young rats. *Lipids* 2015 **50** 1103–1113.

12 Ergang P, Vodička M, Soták M, Klusoňová P, Behuliak M, Řeháková L, Zach P & Pácha J. Differential impact of stress on hypothalamic-pituitary-adrenal axis: gene expression changes in Lewis and Fisher rats. *Psychoneuroendocrinology* 2015 **53** 49–59. (https://doi.org/10.1016/j.psyneuen.2014.12.013)

13 Jamieson PM, Fuchs E, Flugge G & Seckl JR. Attenuation of hippocampal 11β-hydroxysteroid dehydrogenase type 1 by chronic psychosocial stress in the tree shrew. *Stress* 1997 2 123–132. (https://doi.org/10.3109/10253899709014743)

14 Jellinck PH, Dhabhar FS, Sakai RR & McEwen BS. Long-term corticosteroid treatment but not chronic stress affects 11β-hydroxysteroid dehydrogenase type I activity in rat brain and peripheral tissues. *Journal of Steroid Biochemistry and Molecular Biology* 1997 **60** 319–323. (https://doi.org/10.1016/S0960-0760(96)00197-5)

15 Monder C, Sakai RR, Miroff Y, Blanchard DC & Blanchard RJ. Reciprocal changes in plasma corticosterone and testosterone in stressed male rats maintained in a visible burrow system: evidence for a mediating role of testicular 11β-hydroxysteroid dehydrogenase. *Endocrinology* 1994 **134** 1193–1198. (https://doi.org/10.1210/endo.134.3.8119159)

16 Sesti-Costa R, Ignacchiti MD, Chedraoui-Silva S, Marchi LF & Mantovani B. Chronic cold stress in mice induces a regulatory phenotype in macrophages: correlation with increased 11β-hydroxysteroid dehydrogenase expression. *Brain Behavior and Immunity* 2012 **26** 50–60. (https://doi.org/10.1016/j.bbi.2011.07.234)

17 Reber SO, Peters S, Slattery DA, Hofmann C, Schölmerich J, Neumann ID & Obermeier F. Mucosal immunosuppression and epithelial barrier defects are key events in murine psychosocial stress-induced colitis. *Brain Behavior and Immunity* 2011 **25** 1153–1161. (https://doi.org/10.1016/j.bbi.2011.03.004)

18 Cohen S, Janicki-Deverts D & Miller GE. Psychological stress and disease. *JAMA* 2007 **298** 1685–1687. (https://doi.org/10.1001/jama.298.14.1685)

19 Ashwell JD, Lu FW & Vacchio MS. Glucocorticoids in T cell development and function. *Annul Review of Immunology* 2000 **18** 309–345. (https://doi.org/10.1146/annurev.immunol.18.1.309)

20 Tuckermann JP, Kleiman A, Moriggl R, Spanbroek R, Neumann A, Illing A, Clausen BE, Stride B, Förster I & Habenicht AJ, *et al*. Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. *Journal of Clinical Investigation* 2007 **117** 1381–1390. (https://doi.org/10.1172/JCI28034)

21 Mittelstadt PR, Monteiro JP & Ashwell JD. Thymocyte responsiveness to endogenous glucocorticoids is required for immunological fitness. *Journal of Clinical Investigation* 2012 **122** 2384–2394. (https://doi.org/10.1172/JCI63067)

22 Dhabhar FS, Miller AH, McEwen BS & Spencer RL. Differential activation of adrenal steroid receptors in neural and immune tissues of Sprague Dawley, Fischer 344, and Lewis rats. *Journal of Neuroimmunology* 1995 **56** 77–90. (https://doi.org/10.1016/0165-5728(94)00135-B)

23 Dhabhar FS, McEwen BS & Spencer RL. Adaptation to prolonged or repeated stress – comparison between rat strains showing intrinsic differences in reactivity to acute stress. *Neuroendocrinology* 1997 **65** 360–368. (https://doi.org/10.1159/000127196)

24 Sternberg EM, Hill JM, Chrousos GP, Kamilaris T, Listwak SJ, Gold PW & Wilder RL. Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. *PNAS* 1989 **86** 2374–2378. (https://doi.org/10.1073/pnas.86.7.2374)

25 Chaouloff F. Social stress models in depression research: what do they tell us? *Cell and Tissue Research* 2013 **354** 179–190. (https://doi.org/10.1007/s00441-013-1606-x)

26 Buwalda B, Kole MH, Veenema AH, Huininga M, de Boer SF, Korte SM & Koolhaas JM. Long-term effects of social stress on brain and behavior: a focus on hippocampal functioning. *Neuroscience and Biobehavioral Reviews* 2005 **29** 83–97. (https://doi.org/10.1016/j.neubiorev.2004.05.005)

27 Haller J, Fuchs E, Halász J & Makara GB. Defeat is a major stressor in males while social instability is stressful mainly in females: towards the development of a social stress model in female rats. *Brain Research Bulletin* 1999 **50** 33–39. (https://doi.org/10.1016/S0361-9230(99)00087-8)

28 Pácha J, Mikšík I, Lisá V & Pohlová I. Hormonal regulation of intestinal 11β-hydroxysteroid dehydrogenase. *Life Sciences* 1997 **61** 2391–2396. (https://doi.org/10.1016/S0024-3205(97)00956-9)

29 Pácha J, Mikšík I, Mrnka L, Zemanová Z, Bryndová J, Mazancová K & Kučka M. Corticosteroid regulation of colonic ion transport during postnatal development: methods for corticosteroid analysis. *Physiological Research* 2004 **53** S63–S80.

30 Keller-Wood M. Hypothalamic-pituitary-adrenal axis-feedback control. *Comprehensive Physiology* 2015 **53** 1161–1182. (https://doi.org/10.1002/cphy.c140065)

31 Almanzar G, Mayerl C, Seitz JC, Höfner K, Brunner A, Wild V, Jahn D, Geier A, Fassnacht M & Prelog M. Expression of 11β-hydroxysteroid-dehydrogenase type 2 in human thymus. *Steroids* 2016 **110** 35–40. (https://doi.org/10.1016/j.steroids.2016.03.019)

32 Qiao S, Chen L, Okret S & Jondal M. Age-related synthesis of glucocorticoids in thymocytes. *Experimental Cell Research* 2008 **314** 3027–3035. (https://doi.org/10.1016/j.yexcr.2008.06.014)

33 Esteves CL, Kelly V, Breton A, Taylor AI, West CC, Donadeu FX, Péault B, Seckl JR & Chapman KE. Proinflammatory cytokine induction of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in human adipocytes is mediated by MEK, C/EBPβ, and NF-κB/RelA. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** E160–E168. (https://doi.org/10.1210/jc.2013-1708)

34 Ignatova ID, Kostadinova RM, Goldring CE, Nawrocki AR, Frey FJ & Frey BM. Tumor necrosis factor-α upregulates 11β-hydroxysteroid dehydrogenase type 1 expression by CCAAT/enhancer binding protein-β in HepG2 cells. *American Journal of Physiology: Endocrinology and Metabolism* 2009 **296** E367–E377. (https://doi.org/10.1152/ajpendo.90531.2008)

35 Briest W, Rassler B, Deten A, Leicht M, Morwinski R, Neichel D, Wallukat G, Ziegelhöffer T & Zimmer HG. Norepinephrine-induced interleukin-6 increase in rat hearts: differential signal transduction in myocytes and non-myocytes. *Pflügers Archiv: Europen Journal of Physiology* 2003 **446** 437–446. (https://doi.org/10.1007/s00424-003-1043-x)

36 Cardinaux JR & Magistretti PJ. Vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide, and noradrenaline induce the transcription factors CCAAT/enhancer binding protein (C/EBP)-β and C/EBP-δ in mouse cortical astrocytes: involvement in cAMP-regulated glycogen metabolism. *Journal of Neuroscience* 1996 **16** 919–929. (https://doi.org/10.1523/JNEUROSCI.16-03-00919.1996)

37 Sai S, Esteves CL, Kelly V, Michailidou Z, Anderson K, Coll AP, Nakagawa Y, Ohzeki T, Seckl JR & Chapman KE. Glucocorticoid regulation of the promoter of 11β-hydroxysteroid dehydrogenase type 1 is indirect and requires CCAAT/enhancer-binding protein-β. *Molecular Endocrinology* 2008 **22** 2049–2060. (https://doi.org/10.1210/me.2007-0489)

38 Laukova M, Vargovic P, Vlcek M, Lejavova K, Hudecova S, Krizanova O & Kvetnansky R. Catecholamine production is differently regulated in splenic T- and B-cells following stress exposure. *Immunobiology* 2013 **218** 780–789. (https://doi.org/10.1016/j.imbio.2012.08.279)

39 Jondal M, Pazirandeh A & Okret S. Different roles for glucocorticoids in thymocyte homeostasis? *Trends in Immunology* 2004 **25** 595–600. (https://doi.org/10.1016/j.it.2004.09.003)

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Inflammation regulates 11β-hydroxysteroid dehydrogenase type 1 differentially in specific compartments of the gut mucosal immune system

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ABSTRACT

The bioavailability of glucocorticoids is modulated by enzyme 11β-hydroxysteroid dehydrogenase type 1 (11HSD1), which catalyzes the conversion of inactive 11-oxo-glucocorticoids to active 11-hydroxy-glucocorticoids cortisol and corticosterone and is regulated by pro-inflammatory cytokines. Our aim was to assess the effect of colitis on the expression of 11HSD1 in specific microanatomical compartments of the mucosal immune system. Using qRT-PCR we quantified the expression of 11HSD1 and cytokines in the colon, mesenteric lymph nodes (MLN) and spleen of mice with colitis. Microsamples of the MLN cortex, paracortex and medulla, colonic crypt epithelium (CCE), lamina propria and isolated intestinal lymphoid follicles (ILF) were harvested by laser microdissection, whereas splenic and MLN lymphocytes by flow cytometry. Colitis increased 11HSD1 in the CCE, ILF, and MLN cortex but not in the lamina propria and the MLN paracortex and medulla. Expression of IL-4, IL-21 and TNFα was increased in both the cortex of MLN and ILF, whereas IL-1β and IL-10 were only increased in the follicles. No positive effect was observed in the case of IFNγ and TGFβ. 11HSD1 was positively correlated with TNFα and less strongly with IL-21, IL-1β, and IL-4. Colitis also upregulated the 11HSD1 expression of T cells in the spleen and MLN. The study demonstrates the stimulatory effect of inflammation on local glucocorticoid metabolism only in particular compartments of the mucosal immune system. The correlation between cytokines and 11HSD1 in the ILF and MLN cortex indicates that pro-inflammatory cytokines may amplify glucocorticoid signals in inductive compartments of the mucosal immune system.

1. Introduction

Inflammation is a first-line host defense mechanism that is controlled by many regulators. Some of the most effective regulators of inflammation are glucocorticoids, which modulate the differentiation, trafficking and distribution of immune cells, block the transcription of pro-inflammatory cytokines, and promote the expression of anti-inflammatory cytokines [1,2]. Glucocorticoid bioavailability in target tissues depends not only on the concentration of free, unbound glucocorticoids in the blood and the receptor density but also on the corticosteroid metabolism in target cells, which is catalyzed by 11 β-hydroxysteroid dehydrogenase (11HSD). This enzyme interconverts active glucocorticoids cortisol and corticosterone, with their inert 11-oxo metabolites cortisone and 11-dehydrocorticosterone, respectively. The isoform 11HSD1 activates *in vivo* cortisone and 11-dehydrocorticosterone into active cortisol and corticosterone, whereas the 11HSD2 isoform operates in the opposite direction and inactivates glucocorticoids. 11HSD1 is expressed in various tissues, including lymphoid organs and immune cells, such as lymphocytes, macrophages and dendritic cells (DC), where the level of 11HSD1 mRNA and enzyme activity depends on the activation of these cells. The obligatory cofactor for 11HSD1 oxo-reductase activity is NADPH, which is generated from $NADP^+$ by hexose-6-phosphate dehydrogenase (H6PDH) [3–7]. The augmentation of 11HSD1 was shown during lipopolysaccharide- and thioglycollate-induced immune cell activation and in TNBS-induced colitis [4,8,9]. By contrast, 11HSD1-deficiency in mice increased the severity of the inflammation [10]. In addition, accumulating evidence indicates that 11HSD1 expression is modulated by the pro-inflammatory cytokine milieu. *In vitro* experiments demonstrated that TNF-α and IL-1β stimulate 11HSD1 in a variety of immune and non-immune cells and the monocytes and the nasal mucosa are potently induced by IL-4 and IL-13 [6,11–13].

These findings led to the hypothesis that the immunomodulatory effects of glucocorticoids in immune reactions depend, at least in part,

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on 11HSD1. In particular, inflammatory diseases such as arthritis, atherosclerosis and colitis have been associated with changes in 11HSD1 expression [10,14–17]. In previous studies, we demonstrated that experimental colitis upregulates 11HSD1 expression in the colon, the colon-draining lymph nodes and the spleen [9,18,19]. However, intestinal inflammation is associated with specific dynamic changes in the numbers and functions of inflammatory cells in different anatomical compartments. To promote immune responses in the gut, complex networks of specialized cells operate within the lamina propria and the intestinal epithelium, which represent the effector compartments, and within the gut-associated lymphoid tissue (GALT), which functions as a sensory region of the antigen and an inductive site of T effector cells. Enteric antigens are endocytosed by macrophages and DCs. Antigen-loaded DCs then interact with T cells in the sub-epithelial structures of the GALT such as Peyer's patches and isolated lymphoid follicles (ILF) or migrate to the gut-draining mesenteric lymph nodes (MLN) to prime naive T cells to yield T effector cells, some of which provide help for B cells in the follicular structures. Immune cells from the MLN reenter the blood and are transported to the gut interstitium, where they interact with a variety of cells to initiate intestinal inflammation. In addition, some antigens may be transported to the spleen where they are presented to splenic DCs and initiate immune responses within the spleen [20–22].

Although the process of the colonic inflammatory response takes place in different microanatomical compartments of the colon and the secondary lymphoid organs, so far no data are available on 11HSD1 and the cytokine microenvironment. Most experiments concerning the regulation of 11HSD1 have been performed using the whole colon, MLN, and spleen [9,16,18] when information about the local compartment and its microenvironment was lost, even though it may be assumed that the local cytokine microenvironment differs in various compartments. Therefore, the aim of this study was to analyze the expression of 11HSD1 and the local cytokines in the specific microanatomical compartments of the immune system associated with colitis.

2. Experimental

2.1. Animals, treatment and tissue sampling

Acute colitis was induced in male Balb/c mice (six to seven weeks old; Institute of Physiology, Prague) by administering dextran sodium sulfate (DSS, M_w = 40,000–50,000; USB Corp., Cleveland, OH, USA) in drinking water [19]. Because initial studies revealed that mice exposed to 5% DSS (wt/vol) became too diseased, a lower concentration (2%) was used for a five-day period during the course of which the mice developed a spontaneous colitis featuring typical clinical parameters such as decreased weight gain, diarrhea and anal bleeding. Controls consisted of time-matched naive mice that received only water instead of DSS solution. The mice were sacrificed on day five, and the colon, draining MLNs and spleen were harvested. The colon, MLNs and liver (only for Western blot analysis) were either quickly frozen in liquid nitrogen for protein isolation or oriented in a cryomold, embedded in Tissue-Tek (Sakura, Tokyo, Japan) and frozen for immunohistochemistry and laser capture microdissection. All samples were stored at −80 °C. The spleen and remaining MLNs were used immediately for the isolation of immune cells. All experiments were approved by the Animal Care Committee of the Institute of Physiology and performed in accordance with the guidelines of the European Community Council Directive 86/609EEC.

2.2. Western blot analysis

The colonic, hepatic and MLN tissues were homogenized in RIPA buffer (approximately 1:9 w/v, Sigma-Aldrich, St. Louis, MO, USA) containing protease inhibitor cocktail (1×; Roche Diagnostics GmbH, Mannheim, Germany) using MagNA Lyser instrument and MagNA Lyser green beads. The tissue homogenates were filtered through filtration columns (Sigma-Aldrich) by 10,000 × g for 10 min, subsequently sonicated and centrifuged at 12,000 × g for 10 min. Protein concentrations in the resulting supernatant were determined by the BCA method. The supernatant samples were suspended in 4x Laemmli Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA), heated to 70 °C for 10 min and stored at −20 °C. Proteins (41 μg of the colon, 29 μg of the MLN and 1.9 μg of the liver) were run in 10% Mini-PROTEAN TGX gels (Bio-Rad Laboratories), transferred to PVDF-low fluorescent membranes using semi-dry transfer technique and blocked in 10% SEA block buffer (TBS buffer containing 10% SEA and 0.1% Tween-20) for 30 min (Termo Fisher Scientific, Rockford, IL, USA). The membranes were treated with secondary antibody (rabbit anti-goat IRDye800 conjugated, 1:7500; Rockland, Gilbertsville, PA, USA) diluted in the SignalBoost Immunoreaction Enhancer Kit (Millipore, Temecula, CA, USA) for 60 min and washed 5 times with TBS buffer containing 0.1% Tween-20 (TBST) and 2 times with TBS buffer. Nonspecific interaction was detected by ChemiDoc-MP Imaging System (Bio-Rad Laboratories). The membranes were then incubated overnight at 4 °C with the goat anti-11HSD1 primary antibody (R & D System, McKinley Place, MN, USA) diluted 1:7500 in the SignalBoost Immunoreaction Enhancer Kit. The blots were washed with TBST buffer (5 times) and incubated with secondary antibody as described above. After capturing the signal for 11HSD1, the blots were analyzed for β-actin as the loading standard using the anti-β-actin Clone AC-15 primary antibody (1:5000, Sigma-Aldrich) in 10% SEA and goat anti-rabbit IRDye700 conjugated secondary antibody (1:10,000, Rockland). Liver was used as positive control tissue.

2.3. Immunohistochemistry

Sections 20-μm thick were cut on Cryostat Leica 1850, mounted on SuperFrost Plus slides (Thermo Fisher Scientific), dried at room temperature for 1 h and fixed in 4% paraformaldehyde (pH = 7.5) for 24 h followed by washing with TBS (3 × 5 min). The sections were then subjected to heat-induced antigen retrieval using citrate buffer (pH = 6) at 92–96 °C for 30 min and permeabilized with 0.2% Triton-100 for 15 min. Following these steps, the sections were rinsed in TBS and blocked with 0.2% normal goat serum in TBST for 1 h (room temperature, humidified chamber) prior to incubation overnight (4 °C) with the rabbit anti-11HSD1 primary antibody (ab39364, Abcam, Cambridge, UK) diluted 1:200 in blocking buffer. After being washed with TBST (5 × 5 min) and incubated with peroxidase blocking solution Bloxall (Vector Laboratories, Burlingame, CA, USA) for 10 min, the sections were incubated with secondary antibody. Tissue-bound primary antibody was detected using biotinylated goat anti-rabbit secondary antibody (1:400, 90 min) and the avidin-biotin peroxidase complex method (Vectastain Elite ABC HRP Kit, Vector Laboratories). The sections were then washed with TBST and finally incubated with 3,3′-diaminobenzidine (Sigma-Aldrich) as the chromogen. Negative control sections were incubated with each immunohistochemistry run by omission of the primary antibody. The samples were embedded in Mowiol medium (Sigma-Aldrich).

2.4. Laser capture microdissection of the colon and the MLN and real-time RT-PCR

For microdissection, 20-μm tissue sections were cut from frozen blocks of the colon and MLN, using a cryostat Leica CM 1850 (Leica Microsystems, Wetzlar, Germany), and transferred to polyethylene-naphtalate membrane slides. The tissues were dehydrated and stained with cresyl violet acetate and eosin B. Immediately after staining, the tissues were dissected using the Leica LMD 6000 Laser Microdissection System. Staining allowed for the identification of functionally different compartments in the gut (ILF, lamina propria, colonic crypts) and MLN (cortex, paracortex, medulla). To yield enough RNA for the analyses,

we dissected an area measuring 25–75 × 10^3 μm^2 per compartment, which was usually harvested and combined from 7 (colon) or 5 (MLN) sections prepared from one animal.

The harvested tissues were placed directly into RLT buffer and stored at −80 °C until RNA isolation. RNA was extracted using Micro RNeasy kit (Qiagen) and evaluated with a NanoDrop spectrophotometer (Nanodrop Products, Wilmington, DE, USA). The total RNA was used to prepare cDNA using random hexamers and Enhanced Avian Reverse Transcriptase (Sigma-Aldrich) and the cDNA samples were analyzed by real-time PCR on a Viia 7 (Applied Biosystems) using 5x Hot Firepol Probe QPCR Master Mix Plus ROX (Solis Biodyne) and TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays (Life Technologies) specific for mouse 11HSD1 (cat. No. Mm00476182_m1), hexose-6-phosphate dehydrogenase (H6PDH, cat. No. Mm00557617_m1), interferon γ (IFNγ, cat. No. Mm00801778_m1), interleukin 1β (IL-1β, cat. No. Mm01336189_m1), interleukin 4 (IL-4, cat. No. Mm00445259-m1), interleukin 10 (IL-10, cat. No. Mm00439614_m1), interleukin 17 (IL-17, cat. No. Mm00439618_m1), interleukin 21 (IL-21, cat. No. Mm00517640_m1), transforming growth factor β (TGFβ, cat. No. Mm01178820_m1), tumor necrosis factor α (TNFα, cat. No. Mm00443258_m1), the T cell marker CD3ε coding a part of TCR complex (cat. No. Mm00599683_m1), and the B cell marker CD19 (cat. No. Mm00515420_m1). To identify the stability of reference genes, the panel of 12 potential reference genes was compared using the geNorm analysis and the genes HPRT1 (cat. No. Mm01545399-m1), GUSB (cat. No. Mm01197698_m1), GAPDH (cat. No. 4351309) and PPIB (cat. No. Mm00478295_m1) were identified as the optimal combination to provide reliable normalization and the expressions of genes of interest were expressed relative to the mean of the mentioned four reference genes in each sample. The quantity of the PCR product was determined using the standard curve method with 10-fold dilutions of the mixed cDNA sample. Due to the limited RNA amounts and low expression levels of 11HSD2 and H6PDH in some tissues (Ct of gene of interest higher than 35), we applied in cDNA aliquots the specific pre-amplification step of gene assay using the TaqMan PreAmp Master Mix (Life Technologies) according to the manufacturer's protocol.

2.5. Isolation of splenocytes and mobile MLN cells, flow cytometric analysis, sorting and real-time RT-PCR

Single cell suspensions from the spleen and MLNs of individual mice were prepared as previously described [9]. Briefly, mononuclear cells were released by the mechanical disruption of the spleen capsule in cold RPMI 1640, the cell suspension was filtered through nylon mesh (mesh size 45 μm), red blood cells were lysed with ACK lysis buffer, and mononuclear cell populations were collected by centrifugation and re-suspended in RPMI 1640 buffer. After five minutes at room temperature, the cells were washed three times with HBSS buffer and re-suspended at a concentration of 5 × 10^6 cells/ml. Lymphocyte populations were prepared for cell sorting by staining aliquots of the cell suspension (0.5 × 10^6 cells) using anti-mouse CD3ε-FITC, CD4-Alexa Fluor 488, CD8a-PE, CD49b-PE, and CD19-APC (all purchased from Affymetrix/eBioscience). Cells were incubated with antibodies at 4 °C for 30 min, washed three times with HBSS and sorted on a BD FACSJazz (BD Biosciences). Sorted cells were collected into tubes containing lysis buffer and stored at −80 °C until needed. RNA was purified using GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich) according to the manufacturer's protocol. After RNA isolation, the RNA was reverse-transcribed into cDNA using random hexamers and the High Capacity cDNA Reverse Transcription kit (Life Technologies). PCR amplification of cDNA was performed in a final volume of 10 μl.

2.6. Statistical analysis

All data are expressed as the mean ± SEM. Data were analyzed by two-way ANOVA (main effects: compartments/cell subsets and treatment) or Student's *t* test, as appropriate. When the results of ANOVA were significant, *post hoc* analysis was performed using the Fisher LSD test. Pearson's correlation analysis was used to determine the relationship between the levels of 11HSD1 mRNA expression (dependent variable) and those of various cytokines (independent variables). All calculations were performed using Statistica v.6 software (StatSoft Inc., Tulsa, OK). Differences between groups were considered statistically significant at $P < 0.05$.

3. Results

3.1. Colitis and expression of 11HSD1 in specific immune-cell compartments

Because colon and secondary lymphoid organs convert inactive 11-oxo derivatives of glucocorticoids to active hormones [9,23], show non-homogenous distribution of 11HSD1 immunoreactivity [9,24] and have upregulated 11HSD1 mRNA and enzyme activity during colitis [16–19], we initially asked whether colitis per se increases expression of 11HSD1 in colon and MLN. Western blot analysis demonstrated different expression of 11HSD1 in liver (control tissue), colon and MLN and the stimulatory effect of colitis on its expression. As shown in Fig. 1, the band for 1.9 μg liver protein of untreated mice was greater intensity than the bands for 41 μg colonic and 29 μg MLN protein isolated from DSS-treated mice. In contrast, the bands for colon and MLN of control untreated mice were faint.

Immunohistochemistry revealed 11HSD1 immunoreactivity both in MLN and colon, where the staining was more intense (Fig. 2). In MLN, the moderate 11HSD1 staining was present predominantly in the subcapsular sinuses and cortex containing follicles (Fig. 2B). In follicles, the staining was observed mainly in the periphery of the follicle, together with some stronger staining in its central area. In the longitudinal sections of the colon, 11HSD1 immunoreactivity was found mainly in the crypt epithelium, cells lining the crypts and in lamina propria (Fig. 2E).

To determine whether the upregulation of 11HSD1 differs in various immune-cell compartments of the gut and MLN, the levels of 11HSD1 transcripts were quantified in the laser microdissection samples of colonic crypt epithelium (CCE), intestinal lymphoid follicles (ILF) and lamina propria of the gut and in the cortex, paracortex and medulla of the MLN (Fig. 3). The expression of 11HSD1 was found in all the investigated compartments both in the colon and the MLN, even though this expression differed among the compartments (both colon and MLN, $P < 0.001$). Constitutively higher expression was found in the medulla of the MLN and in the lamina propria, which represent an immune cell effector compartment (Fig. 4). Colitis stimulated expression of 11HSD1 both in the colon ($P < 0.01$) and in the MLN ($P < 0.05$), but *post hoc* analysis revealed significant upregulation only in the colonic ILF and CCE and in the cortex of the MLN but not in the lamina propria, paracortex or medulla (Fig. 4). These data suggest that animals challenged with inflammation increase 11HSD1 expression, not only in immune-inductive sites (ILF, MLN cortex), but also in the CCE, an

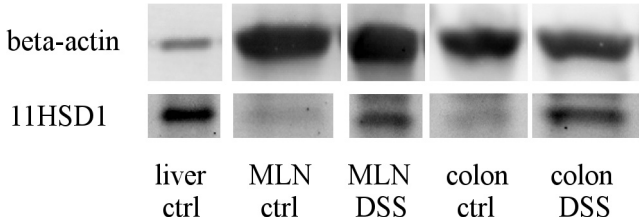

Fig. 1. Western blot analysis for 11HSD1 expression in liver, colon, and MLN of control (ctrl) and DSS-treated mice detected by the antibody against 11HSD1 at 32 kDa and actin at 42 kDa (liver, 1.9 μg prot.; colon, 41 μg prot.; MLN, 29 μg prot.).

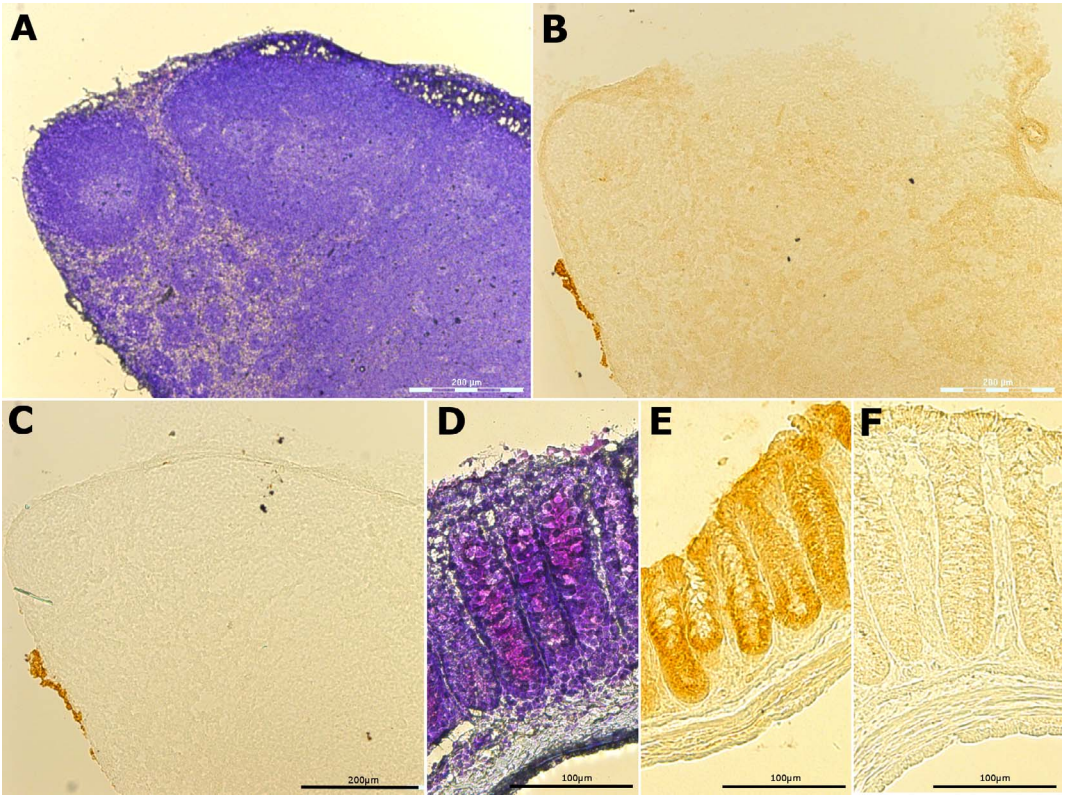

Fig. 2. Immunolocalization of 11HSD1 in mouse mesenteric lymphatic node (A–C) and colon (D–E). (A, D) Sections stained with cresyl violet. (B, E) Sections stained with anti-11HSD1 antibody. (C, F) Respective negative controls.

immune-effector compartment, which contains not only enterocytes but also many immune cells such as $CD8^+$ intraepithelial lymphocytes. In contrast to 11HSD1, H6PDH was found only in MLN and was below the detection limit in gut compartments. In MLN, the H6PDH expression was significantly higher in medulla than in cortex and paracortex ($P < 0.01$) and was not influenced by colitis (Fig. 4). 11HSD2, which inactivates glucocorticoids, was expressed in CCE and ILF but not in lamina propria and immune compartments of MLN. Colitis downregulated 11HSD2 in CCE (control mice: 2.94 ± 0.54, $n = 7$; DSS-mice: 1.51 ± 0.34, $n = 7$; $P < 0.05$), whereas in ILF the detectable 11HSD2 mRNA was found only in control (0.11 ± 0.04, $n = 6$) but not DSS-mice.

To characterize and confirm that the isolated zones were accurately identified, we further examined the expression of CD3ε and CD19, T and B cell markers, respectively. As shown in Fig. 5, the distribution of CD3ε and CD19 mRNA was not homogenous in either the MLN (CD3ε, $P < 0.01$; CD19, $P < 0.05$) or the colon (CD3ε, CD19, $P < 0.001$). In the MLN, CD3ε mRNA was detected predominantly in the paracortex and medulla whereas CD19 was detected predominantly in the cortex and the medulla. In addition, colitis downregulated CD3ε and CD19 in the cortex and medulla, but this effect reached significance only in the case of CD3ε in medulla and CD19 in cortex. (Fig. 5A). In contrast to the MLN, colitis was associated with upregulation of colonic CD3ε ($P < 0.001$), and this effect depended on the compartment (Fig. 5C).

After establishing the upregulation of 11HSD1 in the colonic ILF, CCE and the cortex of the MLN, further analysis was focused only on ILF and the cortex of the MLN.

3.2. Cytokine expression in specific compartments during colitis

As 11HSD1 is upregulated by cytokines *in vitro* [13], we focused further on determining how the expression of 11HSD1 changes in the context of inflammation *in vivo*. In particular, we studied the cytokines that are known to play a role in the pathogenesis of inflammatory bowel disease: the Th1/Th2 effector cytokines, IFNγ and IL-4; the Th17 cytokines IL-17 and IL-21; and the homeostatic cytokines IL-10 and TGFβ [25,26]. In addition, we assessed the expression of TNFα and IL-1β, the cytokines that generally arise secondary to the earlier Th1/Th2 or Th17-like response as a result of the stimulation of epithelial cells, macrophages and other innate immune cells [25].

As shown in Fig. 6, colitis induced changes in the local cytokine microenvironment that differed between the ILF and the MLN cortex.

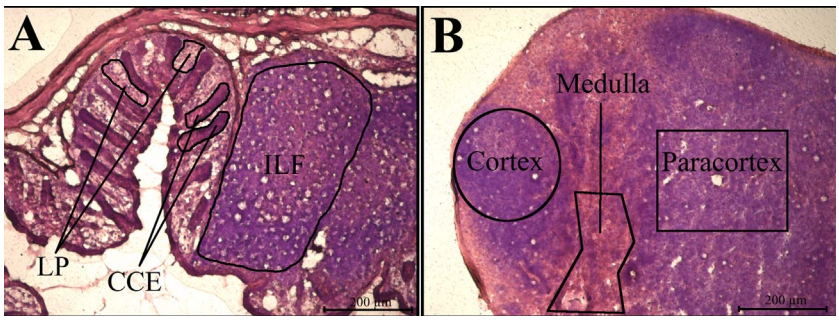

Fig. 3. Laser-capture microdissection of the functionally different lymphoid compartments in the colon (A) and mesenteric lymph nodes (B). Representative 20-µm thick cryosections of colon and mesenteric lymph nodes were rapidly stained with cresyl violet and eosin B just before dissection and then three colonic (CCE, epithelial crypts; LP, lamina propria; ILF, isolated lymphoid follicles) and three nodular areas (cortex, paracortex, medulla) were dissected. Scale: 200 µm.

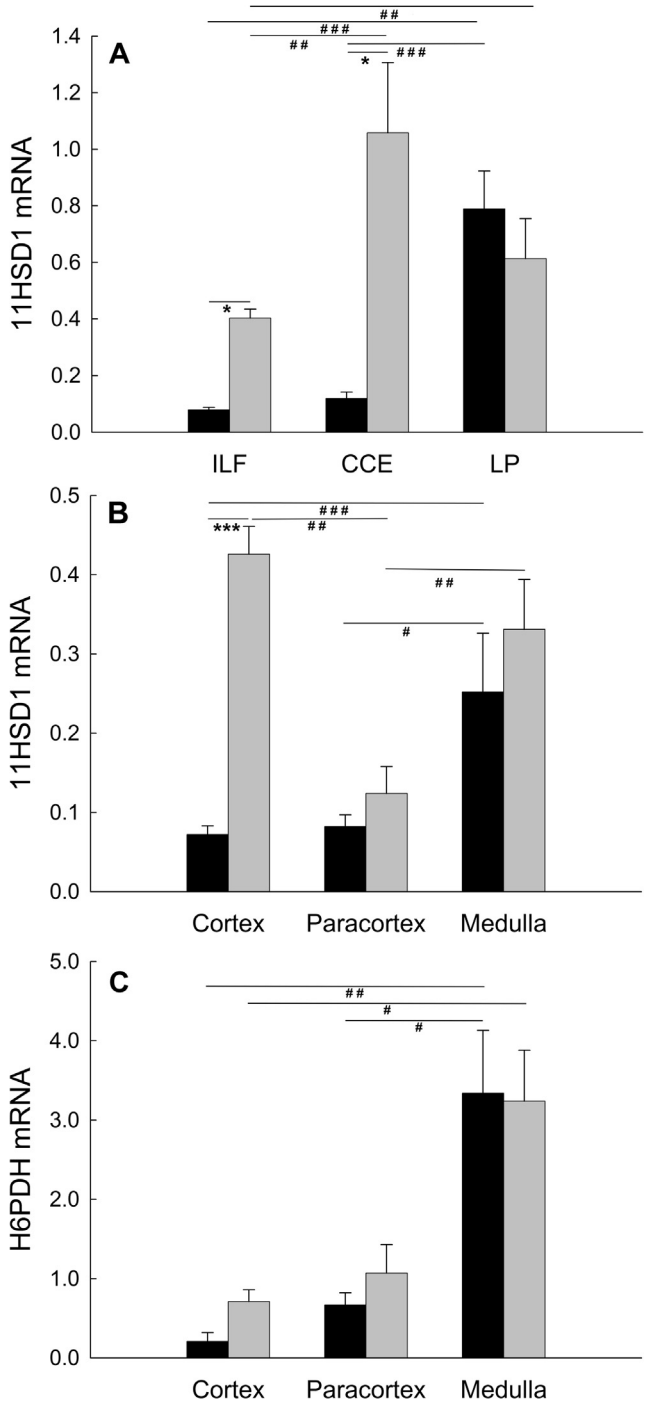

Fig. 4. Effect of colitis on the expression of 11β-hydroxysteroid dehydrogenase type 1 (11HSD1) and hexose-6-phosphate dehydrogenase (H6PDH) in different anatomical compartments of the colon (A) and mesenteric lymph nodes (B, C). The bars represent control healthy mice (*black bars*) and mice with DSS-colitis (*grey bars*). ILF, isolated lymphoid follicles; CCE, colonic crypt epithelium; LP, lamina propria. Data are given as the mean ± SEM (n = 7–9); comparison between the identical anatomical compartments of controls and animals treated with DSS: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; comparison between anatomical compartments: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

Colitis significantly affected the expression of genes encoding IL-1β ($P < 0.01$), IL-4 ($P < 0.001$), IL-10 ($P < 0.05$), IL-21 ($P < 0.001$) and TNFα ($P < 0.001$) but not IFNγ and TGFβ, and this effect was more obvious in the colonic ILF than in the cortex of the MLN. Quantitative differences in the cytokine expression between the ILF and MLN cortex were found in the case of IL-1β ($P < 0.01$), IL-4 ($P < 0.05$), IL-10 ($P < 0.01$), TNFα ($P < 0.001$) and IFNγ ($P < 0.01$), but not IL-21 and TGFβ. In contrast to other cytokines, IL-1β and IL-10 had preferential distribution in the ILF, whereas their expression was very low in the MLN cortex and was not upregulated during colitis. Similarly, the expression of IL-17 was not detected in any of the studied compartments either in the controls or in animals with colitis (data not shown).

Collectively, these data indicate that the cytokine milieu is different among the lymphoid compartments in which 11HSD1 is upregulated during colitis. To identify which of the cytokines might contribute most to the upregulation of 11HSD1, Pearson's correlation coefficient analysis was performed. As shown in Table 1, we found a statistically significant positive correlation between the levels of 11HSD1 and IL-1β, IL-4, IL-21, and TNFα, but there was no correlation between 11HSD1 and IFNγ, IL-10 or TGFβ. In addition, the determination of R^2 contributed by individual variables suggested that TNFα mRNA is the most important variable in predicting the 11HSD1 mRNA level, and that the increase of 11HSD1 expression during colitis is related predominantly to the increased expression of TNFα. Fig. 7 summarizes the experimental data of 11HSD1 and TNFα obtained in ILF and MLN cortex of control and DSS-mice.

3.2.1. 11HSD1 expression in purified T cells

Given that colitis significantly increased 11HSD1 expression in the ILF and the MLN cortex, which contain not only B cells and stroma cells, but also DCs and various subsets of T cells [21,27,28], we also analyzed FACS-sorted lymphocyte cell subsets from the spleen and MLN of control and DSS-treated mice. Five phenotypes of lymphocytes were isolated: $CD19^+$ (B cells), $CD3^+$ (T cells) and their subsets $CD8^+$, $CD4^+$, and natural killer T cells. As shown in Fig. 8A and B, the freshly isolated splenic and MLN T cells had significantly upregulated 11HSD1 during colitis ($P < 0.01$), whereas 11HSD1 expression in B cells had only a tendency to increase ($P = 0.074$). Detailed analysis of splenic T cells proved that colitis stimulates 11HSD1 expression in all three subsets of these cells (Fig. 8C).

4. Discussion

In this study, we found a topographically specific effect of colitis on 11HSD1 expression. First, we showed that 11HSD1 expression was upregulated in the ILF and colonic crypts but not in the lamina propria. In parallel with this finding, we observed upregulation of 11HSD1 expression in the cortex but not in the paracortex or medulla of the MLN. Collectively, these results indicate that inflammation stimulates 11HSD1 expression both in the effector and inductive compartments of the colonic lymphoid tissue and in the secondary lymphoid organs. All of the microanatomical compartments analyzed encompass different types of cells. The follicular compartments of the ILF and MLN cortex contain mainly B cells and stromal cells, but also populations of other cell types such as DCs, follicle-associated epithelium and subsets of T cells. These subsets include T follicular regulatory cells and T helper cells, a class of activated T lymphocytes that migrate to the cortex to assist B cells [21,28]. Our data do not allow to determine definitely what types of cells have upregulated 11HSD1 expression. However, a comparison of our findings with other data indicates that 11HSD1 was not increased in stromal or DC cells. Even if the resident stromal cells that comprise the backbone of the nodes are important in shaping a unique microenvironment [27], the absence of the stimulatory effect in the paracortex and medulla supports the hypothesis that glucocorticoid metabolism is not upregulated in stromal cells. Similarly, Soulier et al. [5] demonstrated that glucocorticoid amplification operates in murine DCs at a maximal rate, and is unaffected by additional innate or adaptive immune stimuli. In contrast, we have found that T cells respond to colitis with an increase in 11HSD1 expression and Zhang et al. [7] showed that *in vitro* activation of the splenic and lymph node T cells is accompanied by upregulation of 11HSD1 activity. The hypothesis about upregulation of 11HSD1 expression in follicular T cells during

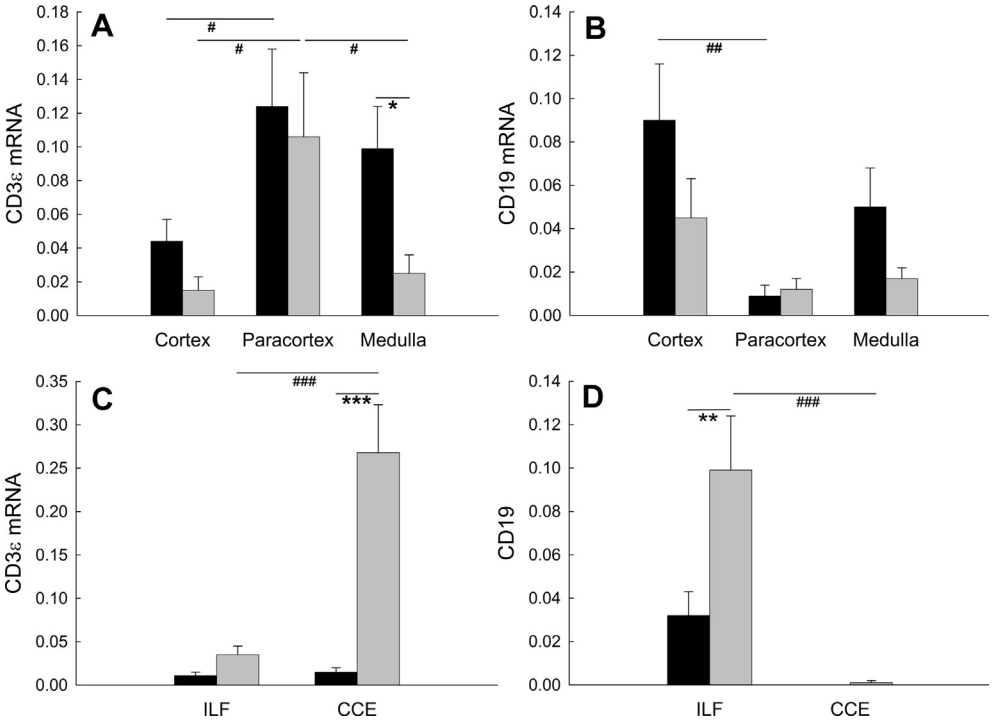

Fig. 5. Expression of the T and B cell markers CD3ε and CD19 in different compartments of the mesenteric lymph nodes (A, B) and the colon (C, D). *Black bars* represent control healthy mice and *grey bars* the mice with DSS-colitis, (n = 7–9). For further details, see Fig. 2.

inflammation is concordant with the finding of the T cell marker CD3ε in the ILF and the cortex of the MLN and with previous papers showing that multiple types of T cells are interspersed within and at the boundary of immune follicles [20,21]. Consistent with this notion is also the recently described role of glucocorticoids in regulatory T cells whose differentiation initiated by Treg-cell-activating cytokines is modulated by glucocorticoid-induced protein GILZ [29].

Our data suggest that inflammation induces changes in glucocorticoid metabolism not only in inductive sites of the lymphoid tissue but also in crypts, which represent a very specific microanatomical

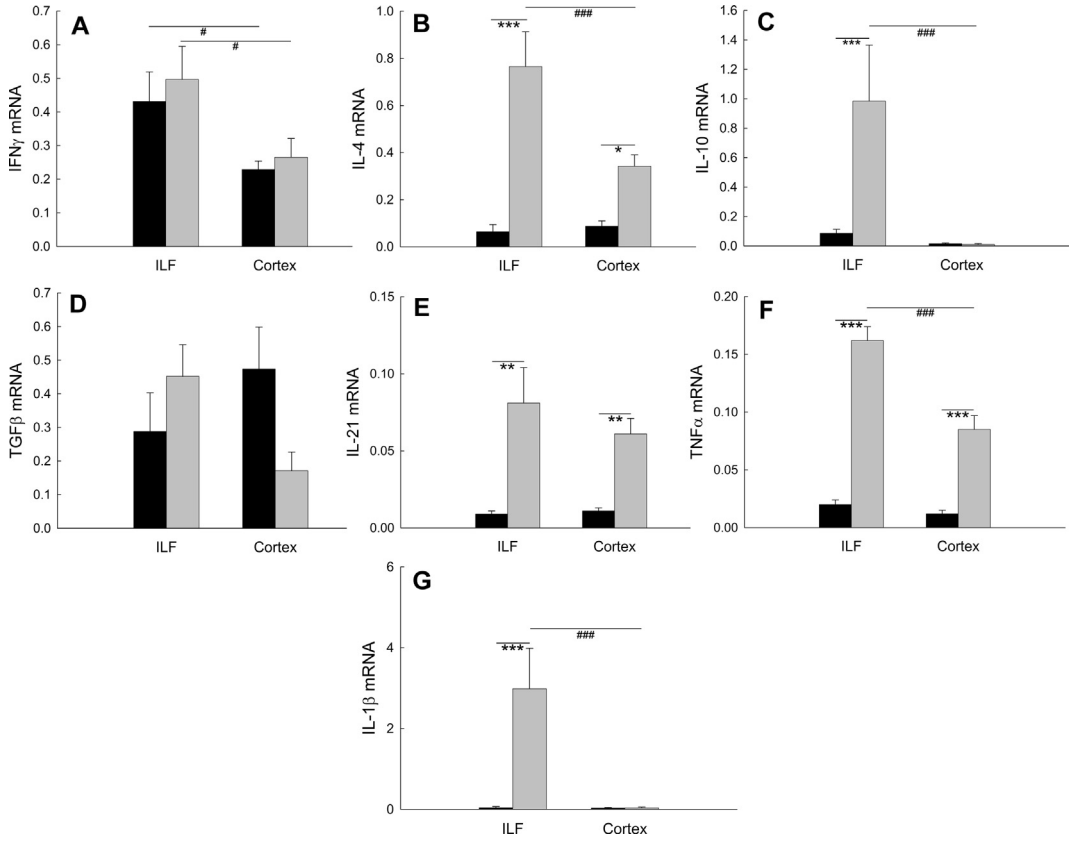

Fig. 6. Effect of colitis on the expression of cytokine mRNAs in isolated lymphoid follicles (ILF) and the cortex of mesenteric lymph nodes. *Black bars* indicate mRNA levels in the control, healthy mice and *grey bars* represent mice with DSS-colitis, (n = 6–8). For further details, see Fig. 2.

Table 1
Regression analysis for expression of 11HSD1 mRNA and mRNA of cytokines in intestinal lymphoid follicles (ILF) and cortex of mesenteric lymph nodes.

	Beta	P	R^2
IFNγ	0.293	ns	0.010
IL-1β	0.622	< 0.05	0.340
IL-4	0.440	< 0.05	0.157
IL-10	0.295	ns	0.022
IL-21	0.588	< 0.01	0.318
TGFβ	0.282	ns	0.047
TNFα	0.751	< 0.0001	0.562

Beta, regression coefficient; P, statistical significance; R^2, coefficient of determination; ns, not significant.

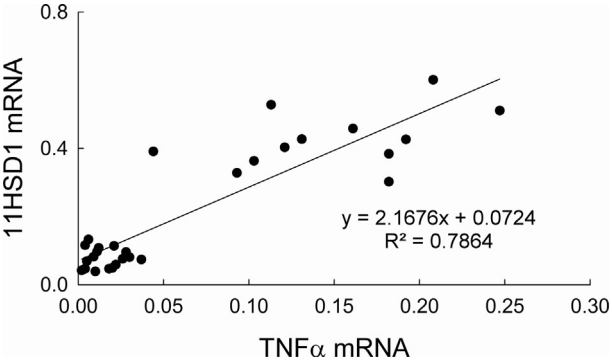

Fig. 7. Regression analysis plot showing the correlation between the levels of 11HSD1 and TNFα mRNA in healthy controls and mice with DSS-colitis, (n = 30).

compartment containing not only epithelial cells, but also a unique niche of intraepithelial lymphocytes, a heterogeneous population of T cells composed mainly of cytotoxic $CD8^+$ T cells displaying features that distinguish them from T cells organized in lymphoid tissues and in the lamina propria [30]. As 11HSD1 enzyme activity was identified in isolated intraepithelial leukocytes but not in colonocytes [9,31], it is reasonable to consider intraepithelial lymphocytes to be a principal subset of cells whose 11HSD1 expression is upregulated by colitis. Concerning macrophages whose 11HSD1 expression is upregulated by Th2 cytokines during the process of differentiation from monocytes into macrophages [6], we do not believe that they are responsible for the upregulation of 11HSD1 expression because intestinal macrophages are rare in the epithelium itself and upregulation of 11HSD1 was not observed in the lamina propria, where macrophages are mostly located [32].

Although 11HSD1 expression was significantly increased during colitis, we did not find any parallel upregulation of H6PDH, an enzyme which mediates glucocorticoid generation via 11HSD1 oxo-reductase activity [33]. This means that H6PDH is resistant to inflammation or operates maximally already in control animals. The negative finding of H6PDH in colon may be due to the levels of the transcript too low for detection. Despite the absence of H6PDH transcript signal in microsamples of colonic compartments, H6PDH transcript and protein were identified in macrosamples of not only secondary lymphoid organs but also colon and small intestine [24,34]. It seems that the H6PDH copy number relative to 11HSD1 copies is much lower in ILF, LP and CCE than in MLN. Similar to other studies, the isozyme 11HSD2, which inactivates glucocorticoids, was found in colonic epithelium but not in lymphoid organs and immune cells [7,9,31] and was decreased during inflammation [17,18]. Identification of 11HSD2 transcript in ILF seems to reflect the epithelium overlying ILF and containing microfold (M) cells [28]. The undetectable levels of 11HSD2 mRNA in MLN compartments and the lack of enzyme activity in immune cells [7] indicate that the deactivation of glucocorticoids is not catalyzed in secondary and most likely also in tertiary lymphoid organs.

The initiation and evolution of intestinal inflammation is governed by cytokines, and cytokines have been shown to regulate 11HSD1 expression and the local metabolism of glucocorticoids [13,25,35]. To further explore the possibility that changes in the cytokine milieu in microanatomical compartments might provide signaling to upregulate 11HSD1, we compared the relationship between 11HSD1 expression and the cytokines characteristic of the Th1/Th2/Th17 and Treg pathways: IFNγ, IL-4, IL-10, IL-17A, and TGFβ, respectively. In addition, we studied the cytokine IL-21, which amplifies Th17 differentiation, and the pro-inflammatory cytokines TNFα and IL-1β, which are more promiscuous in their function. Whereas colitis upregulated the expression of TNFα, IL-1β, IL-4, IL-10 and IL-21 in ILF and partially in the MLN cortex, no changes were observed in the case of IFNγ and TGFβ. On the basis of evidence obtained from *in vitro* experiments, one possible explanation for the upregulation of 11HSD1 expression is that the increased level of cytokines in specific microanatomical compartments might stimulate 11HSD1 expression. In our experiments, the strongest statistically significant positive correlation between 11HSD1 expression and cytokines was found above all in the case of TNFα. A weaker correlation was found for IL-21 and IL-1β (only in ILF) and especially for IL-4. These data suggest that TNFα is the most crucial determinant of 11HSD1 upregulation even if the effects of other cytokines cannot be excluded. This conclusion is in accordance with the previously described stimulatory effects of cytokines on 11HSD1 expression in various *in vitro* experiments. 11HSD1 expression was shown to be upregulated by Th2/Th17 but not Th1 cytokines in airways [11,12,36], fibroblasts [37] and monocytes [6] and by the pleiotropic cytokines TNFα and IL-1β in a large variety of cell cultures of various origin [13,37]. Similarly, the absence of any significant correlation between IFNγ or TGFβ and 11HSD1 corresponds with the inability of these two cytokines to modulate the expression of 11HSD1 in cell culture [12]. The exact mechanism by which TNFα carries out the stimulatory effect on 11HSD1 expression during inflammation is unknown. Nevertheless, findings in hepatocytes, adipocytes and synovial fibroblasts show that TNFα and IL-1β upregulate 11HSD1 expression by MAPK-mediated

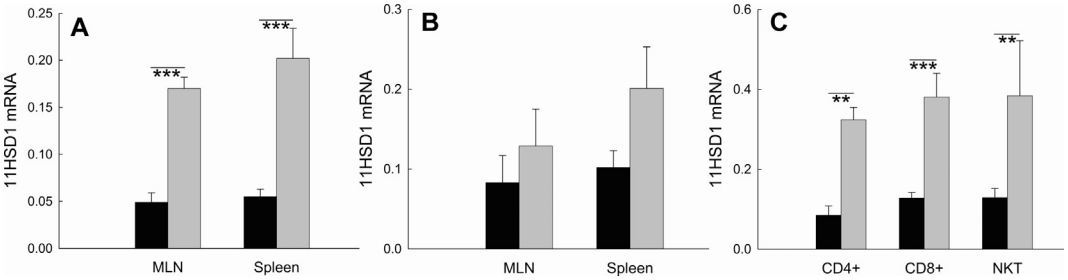

Fig. 8. Effect of colitis on 11HSD1 expression in subpopulations of lymphocytes isolated from the spleen and mesenteric lymph nodes. (A) T cells, (B) B cells, (C) subpopulations of splenic T cells ($CD4^+$; $CD8^+$; NKT, natural killer T cells). The *black bars* represent the control healthy mice and the *grey bars* the mice with DSS-colitis. The results are shown as the mean ± SEM of data obtained from 6 control and 6 DSS-treated mice; the analyzed samples of lymphocyte subsets were always prepared from one animal. Significant difference between controls and colitis: $^{**}P < 0.01$, $^{***}P < 0.001$.

increased binding of transcription factor C/EBPβ to the 11HSD1 promoter or activate NF-κB/ReIA pathway [38–40].

In conclusion, we have identified topographically distinct changes in the regulation of 11HSD1 expression in specific anatomical compartments of the colon and the MLN during colitis, which suggests differences in the bioavailability of endogenous glucocorticoids and in local glucocorticoid signaling amplification. These changes may facilitate a regulatory and/or anti-inflammatory role of glucocorticoids in immune processes generated in response to activation of the GALT system. We postulate, that the upregulation of 11HSD1 expression in inductive sites of the immune system during inflammation is associated with the activation of Th2 but not Th1 and Th17 regulatory pathways. The strongest regulatory effect appears to be TNFα, a pleiotropic pro-inflammatory cytokine, produced in a broad range of cell types.

Acknowledgments

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References

[1] B.C. McEwen, C.A. Biron, K.W. Brunson, K. Bulloch, W.H. Chambers, F.S. Dhabhar, et al., The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions, Brain Res. Rev. 23 (1997) 79–133.
[2] F.S. Dhabhar, B.S. McEwen, Enhancing versus suppressive effects of stress hormones on skin immune function, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 1059–1064.
[3] L. Freeman, M. Hewison, S.V. Hughes, K.N. Evans, D. Hardie, T.K. Means, et al., Expression of 11β-hydroxysteroid dehydrogenase type 1 permits regulation of glucocorticoid bioavailability by human dendritic cells, Blood 106 (2005) 2042–2049.
[4] J.S. Gilmour, A.E. Coutinho, J.F. Cailhier, T.Y. Man, M. Clay, G. Thomas, et al., Local amplification of glucocorticoids by 11β -hydroxysteroid dehydrogenase type 1 promotes macrophage phagocytosis of apoptotic leukocytes, J. Immunol. 176 (2006) 7605–7611.
[5] A. Soulier, S.M. Blois, S. Sivakumaran, F. Fallah-Arani, S. Henderson, B. Flutter, et al., Cell-intrinsic regulation of murine dendritic cell function and survival by prereceptor amplification of glucocorticoid, Blood 122 (2013) 3288–3297.
[6] R. Thieringer, C.B. Le Grand, L. Carbin, T.Q. Cai, B. Wong, S.D. Wright, et al., 11β-hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages, J. Immunol. 167 (2001) 30–35.
[7] T.Y. Zhang, X. Ding, R.A. Daynes, The expression of 11β-hydroxysteroid dehydrogenase type I by lymphocytes provides a novel means for intracrine regulation of glucocorticoid activities, J. Immunol. 174 (2005) 879–889.
[8] T. Ishii, H. Masuzaki, T. Tanaka, N. Arai, S. Yasue, N. Koayashi, et al., Augmentation of 11β -hydroxysteroid dehydrogenase type 1 in LPS-activated J774.1 macrophages – role of 11β -HSD1 in pro-inflammatory properties in macrophages, FEBS Lett. 581 (2007) 349–354.
[9] P. Ergang, K. Vytáčková, J. Švec, J. Bryndová, I. Mikšík, J. Pácha, Upregulation of 11β-hydroxysteroid dehydrogenase 1 in lymphoid organs during inflammation in the rat, J. Steroid Biochem. Mol. Biol. 126 (2011) 19–25.
[10] A.E. Coutinho, M. Gray, D.G. Brownstein, D.M. Salter, D.A. Sawatzky, S. Clay, et al., 11β-Hydroxysteroid dehydrogenase type 1, but not type 2, deficiency worsens acute inflammation and experimental arthritis in mice, Endocrinology 153 (2012) 234–240.
[11] Y.J. Jun, S.J. Park, J.W. Hwang, K.J. Jung, J.Y. Jung, G.H. Hwang, et al., Differential expression of 11β-hydroxysteroid dehydrogenase type 1 and 2 in mild and moderate/severe persistent allergic nasal mucosa and regulation of their expression by Th2 cytokines: asthma and rhinitis, Clin. Exp. Allergy 44 (2014) 197–211.
[12] Y.J. Jun, S.J. Park, T.H. Kim, S.H. Lee, K.J. Lee, S.M. Hwang, et al., Expression of 11β-hydroxysteroid dehydrogenase 1 and 2 in patients with chronic rhinosinusitis and their possible contribution to local glucocorticoid activation in sinus mucosa, J. Allergy Clin. Immunol. 134 (2014) 926–934.
[13] C.A. Staab, E. Maser, 11β -Hydroxysteroid dehydrogenase type 1 is an important regulator at the interface of obesity and inflammation, J. Steroid Biochem. Mol. Biol. 119 (2010) 56–72.
[14] P. Ergang, P. Leden, K. Vagnerová, P. Klusoňová, I. Mikšík, J. Jurčovičová, et al., Local metabolism of glucocorticoids and its role in rat adjuvant arthritis, Mol. Cell. Endocrinol. 323 (2010) 155–160.
[15] A. Hermanowski-Vosatka, J.M. Balkovec, K. Cheng, H.Y. Chen, M. Hernandez, G.C. Koo, et al., 11β-hydroxysteroid inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice, J. Exp. Med. 202 (2005) 517–527.
[16] J.P. Stegk, B. Ebert, H.J. Martin, E. Maser, Expression profiles of human 11β -hydroxysteroid dehydrogenases type 1 and type 2 in inflammatory bowel diseases, Mol. Cell. Endocrinol. 301 (2009) 104–108.
[17] S. Žbánková, J. Bryndová, P. Leden, M. Kment, A. Švec, J. Pácha, 11β-Hydroxysteroid dehydrogenase 1 and 2 expression in colon from patients with ulcerative colitis, J. Gastroenterol. Hepatol. 22 (2007) 1019–1023.
[18] J. Bryndová, S. Žbánková, M. Kment, J. Pácha, Colitis up-regulates local glucocorticoid activation and down-regulates inactivation in colonic tissue, Scand. J. Gastroenterol. 39 (2004) 549–553.
[19] K. Vagnerová, M. Kverka, P. Klusoňová, P. Ergang, I. Mikšík, H. Tlaskalová-Hogenová, et al., Intestinal inflammation modulates expression of 11β-hydroxysteroid dehydrogenase in murine gut, J. Endocrinol. 191 (2006) 497–503.
[20] J.H. Chang, Y. Chung, Regulatory T cells in B cell follicles, Immune Netw. 14 (2014) 227–236.
[21] D. Yu, C.G. Vinuesa, The elusive identity of T follicular helper cells, Trends Immunol. 31 (2010) 377–383.
[22] I. Koboziev, F. Karlsson, M.B. Grisham, Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation, Ann. N.Y. Acad. Sci. 1207 (Suppl. 1) (2010) E86–E93.
[23] M.D. Taves, A.W. Plumb, A.M. Korol, J.G. van Der Gugten, D.T. Holmes, N. Abraham, et al., Lymphoid organs of neonatal and adult mice preferentially produce active glucocorticoids from metabolites, not precursors, Brain Behav. Immun. 57 (2016) 271–281.
[24] E.P. Gomez-Sanchez, D.G. Romero, A.F. de Rodriguez, M.P. Warden, Z. Krozowski, C.E. Gomez-Sanchez, Hexose-6-phosphate dehydrogenase and 11β-hydroxysteroid dehydrogenase-1 tissue distribution in the rat, Endocrinology 149 (2008) 525–533.
[25] W. Strober, I.J. Fuss, Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases, Gastroenterology 140 (2011) 1756–1767.
[26] L.A. Zenewicz, A. Antov, R.A. Flavell, CD4T-cell differentiation and inflammatory bowel disease, Trends Mol. Med. 15 (2009) 199–207.
[27] M. Ahrendt, S.I. Hammerschmidt, O. Pabst, R. Pabst, U. Bode, Stromal cells confer lymph node-specific properties by shaping a unique microenvironment influencing local immune responses, J. Immunol. 181 (2008) 1898–1907.
[28] M. Buettner, M. Lochner, Development and function of secondary and tertiary lymphoid organs in the small intestine and the colon, Front. Immunol. 7 (2016) 342.
[29] O. Bereshchenko, M. Coppo, S. Bruscoli, M. Biagioli, M. Cimino, T. Frammartino, et al., GILZ promotes production of peripherally induced Treg cells and mediates the crosstalk between glucocorticoids and TGF-β signaling, Cell Rep. 7 (2014) 464–475.
[30] D.L. Gibbons, J. Spencer, Mouse and human intestinal immunity: same ballpark, different players; different rules, same score, Mucosal Immunol. 4 (2011) 148–157.
[31] K.E. Sheppard, K.X. Li, D.J. Autelitano, Corticosteroid receptors and 11β-hydroxysteroid dehydrogenase isoforms in rat intestinal epithelia, Am. J. Physiol. 277 (1999) G541–G547.
[32] C.C. Bain, A.M. Mowat, Macrophages in intestinal homeostasis and in inflammation, Immunol. Rev. 206 (2014) 102–117.
[33] G.G. Lavery, E.A. Walker, N. Draper, P. Jeyasuria, J. Marcos, C.H. Shackleton, et al., Hexose-6-phosphate dehydrogenase knock-out mice lack 11β-hydroxysteroid dehydrogenase type 1-mediated glucocorticoid generation, J. Biol. Chem. 281 (2006) 6546–6551.
[34] S. Ohno, M. Ohta, S. Nakajin, Quantitative determination and tissue distribution of human 11β-hydroxysteroid dehydrogenase, hexose-6-phosphate dehydrogenase, glucose-6-phosphate transporter, glucocorticoid receptor and mineralocorticoid receptor mRNAs, Horm. Mol. Biol. Clin. Investig. 2 (2010) 219–226.
[35] K.E. Chapman, A.E. Coutinho, Z. Zhang, T. Kipari, J.S. Savill, J.R. Seckl, Changing glucocorticoid action: 11β-hydroxysteroid dehydrogenase type 1 in acute and chronic inflammation, J. Steroid Biochem. Mol. Biol. 137 (2013) 82–92.
[36] A. Hu, S. Fatma, J. Cao, J.S. Grunstein, G. Nino, Y. Grumbach, et al., Th2 cytokine-induced upregulation of 11β-hydroxysteroid dehydrogenase-1 facilitates glucocorticoid suppression of proasthmatic airway smooth muscle function, Am. J. Physiol. 296 (2009) L790–L803.
[37] R.S. Hardy, A. Filer, M.S. Cooper, G. Parsonage, K. Raza, D.L. Hardie, et al., Differential expression, function and response to inflammatory stimuli of 11β-hydroxysteroid dehydrogenase type 1 in human fibroblasts: a mechanism for tissue-specific regulation of inflammation, Arthritis Res. Ther. 8 (2006) R108.
[38] M.M. Ahasan, R. Hardy, C. Jones, K. Kaur, D. Nanus, M. Juarez, et al., Inflammatory regulation of glucocorticoid metabolism in mesenchymal stromal cells, Arthritis Rheum. 64 (2012) 2404–2413.
[39] I.D. Ignatova, R.M. Kostadinova, C.E. Goldring, A.R. Nawrocki, F.J. Frey, B.M. Frey, Tumor necrosis factor-α upregulates 11β-hydroxysteroid dehydrogenase type 1 expression by CCAAT/enhancer binding protein-β in HepG2 cells, Am. J. Physiol. Endocrinol. Metab. 296 (2009) E367–E377.
[40] C.L. Esteves, V. Kelly, A. Breton, A.I. Taylor, C.C. West, F.X. Donadeu, et al., Proinflammatory cytokine induction of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in human adipocytes is mediated by MEK, C/EBPβ, and NF-κB/RelA, J. Clin. Endocrinol. Metab. 88 (2014) E160–E168.