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Stressors affect urocortin 1 and urocortin 2 gene expression in rat spleen: The role of glucocorticoids

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ABSTRACT

The mechanisms underlying stress-related modulation of immune function via urocortin 1 and urocortin 2 have been only vaguely described. Therefore, we investigated the effect of LPS injection or immobilization stress on gene expression of urocortin 1 and urocortin 2 in the rat spleen, along with the potential involvement of glucocorticoids. Our data showed: a) different regulation of urocortin 1 and urocortin 2 gene expression in the rat spleen under different stressful conditions (LPS vs. immobilization stress) and b) diverse effects of stress-induced adrenal glucocorticoids on this process. Our findings indicate a specific, rather than general regulation of splenic immune function by urocortins during stressful conditions.

1. Introduction

Immune system activity is at least partially regulated by the principal systems of the neuroendocrine stress response, particularly the hypothalamic-pituitary-adrenocortical (HPA) axis and sympathoadrenal system (SAS) (Cain and Cidlowski, 2017; Elenkov et al., 2000). The mechanisms and pathways participating in the immunomodulatory effects of effector molecules of the HPA axis, particularly glucocorticoids, are relatively well described (Cain and Cidlowski, 2017; Webster Marketon and Glaser, 2008).

The neuroendocrine stress response is also activated by immune challenges that disturb the homeostasis of the organism and therefore represent a stressor. It is well known that immune challenges such as bacterial lipopolysaccharide (LPS) exposure stimulate hypothalamic structures underlying the stress response (Elenkov et al., 2000; Chrousos, 1995). Administration of LPS into the circulation induces a cascade of events, including increased plasma levels of prostaglandin E_2 within minutes after administration, followed by increased ACTH and corticosterone levels 20–30 min later. Finally, 30–40 min after LPS administration, there is an increase in plasma levels of the pro-inflammatory cytokines TNF-α and IL-6 (Soto-Tinoco et al., 2016).

Corticotropin releasing hormone (CRH) is a crucial factor in the regulation of HPA axis activity. This peptide was discovered in 1980's by the Vale group (Vale et al., 1981) and from that time on, more CRH-like peptides were identified and are known as urocortins 1, 2, and 3. Together with their specific receptors, CRHR1 and CRHR2, they are widely distributed throughout the central nervous system as well as in the periphery (Fekete and Zorrilla, 2007; Yamauchi et al., 2005). The CRH family of peptides and their receptors also are involved in many other physiological processes, including the regulation of immune function (Deussing and Chen, 2018; Fekete and Zorrilla, 2007; Zhu et al., 2011).

Urocortins (Ucn1 and Ucn2) and their specific receptors (CRHR1 and CRHR2) are expressed in immune tissues, including spleen, thymus, and skin (Fekete and Zorrilla, 2007). Expression of Ucn1 has been observed in various types of immune cells, including T and B cells, macrophages, monocytes, and mast cells. CRH receptors are expressed in T cells, macrophages, monocytes, and dendritic cells (Varela et al., 2007). However, the role of urocortins in regulating immune function has been only vaguely described. Some data indicate a pro-inflammatory role while other suggest an anti-inflammatory role for these peptides. These ambiguous observations may result from different effects induced by activation of specific receptor subtypes, as well as types of activated cells and tissues. The available data indicate that activation of CRHR1 has pro-inflammatory effects, whereas activation of CRHR2 has anti-inflammatory effects on peripheral inflammation processes (Zhu et al., 2011). These observations are analogous to the effects of the CRH family of peptides in the stress response, where different CRH family members have different effects depending on type of the receptor activated. These actions are usually also tissue specific, showing a very

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complex role for the CRH system in regulating the stress response that goes beyond simple dualism where CRHR1 induced signaling participates in the stress response and anxiety-like behavior, while activation of CRHR2 is responsible for stress recovery and the restoration of homeostasis (Henckens et al., 2016; Janssen and Kozicz, 2013).

To determine the involvement of urocortins in stress-related immune regulation, we investigated the gene expression of Ucn1 and Ucn2 in the spleen of animals exposed to either an immune stressor (injection of LPS) or immobilization (IMO), a combined physical-psychological stressor. A pharmacological adrenalectomy (PhADX) was performed to investigate any potential interconnection between the effect of urocortinon immune function and the neuroendocrine stress response. In addition, we also investigated the effect of the used stressors on splenic catecholaminergic system and immune function.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats, 8–9 weeks old (250 - 300 g, Charles River, Germany), were used in our experiments. Prior to experiments, the animals were housed for at least 1 week, four animals per cage, in a controlled environment (22 ± 2 °C, 12 h light/dark cycle, lights on at 6 a.m.). Food and water were available ad libitum. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and in accordance with the Council Directive 2010/63EU of the European Parliament and the Council of 22nd September 2010 on the protection of animals used for scientific purposes.

2.2. Pharmacological adrenalectomy

On the day of the experiment animals were divided into four groups (8 animals in each group). Out of these, two groups of animals underwent pharmacological adrenalectomy (PhADX). For PhADX, rats were administered *s.c.* metyrapone, a 11-β hydroxylase inhibitor (100 mg/kg, Enzo Life Sciences) and aminoglutethimide, a 20-α hydroxylase inhibitor (100 mg/kg, Alfa Aesar) both dissolved in vehicle (40% propyleneglycol in saline). Animals were injected 4.5 h and 3.5 h before decapitation, respectively, to suppress the synthesis of endogenous corticosterone, as previously described (Tillinger et al., 2018). Sham-injected rats were administered *s.c.* vehicle using the same time schedule. Afterwards, all animals were kept in their home cages. One group of PhADX animals and one group of Sham animals were assigned to stressed groups that underwent immobilization protocol 2.5 h after the beginning of the experiment.

2.3. Immobilization procedure

Immobilization (IMO) was performed as originally described by Kvetnansky and Mikulaj (Kvetnansky and Mikulaj, 1970) by taping the limbs of animals with surgical tape and restricting the motion of the head on an immobilization board. Rats were immobilized once for 2 h and sacrificed immediately after termination of IMO. Control animals were not exposed to stress and were sacrificed immediately after removal from their home cages. The spleen was dissected from each individual animal, frozen in liquid nitrogen, and then stored at −80 °C for further analysis.

2.4. Lipopolysaccharide administration

On the day of the experiment animals were divided into two groups (6 animals in each group). Animals in first group were administered a single *i.p.* injection of a lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5, Sigma-Aldrich) at a dose of 50 μg/kg of body weight. Control animals received single *i.p.* injection of saline (SAL; 0.9% NaCl).Three hours after LPS injection, the animals were sacrificed by decapitation. The spleen was quickly removed, frozen in liquid nitrogen, and then stored at −80 °C.

2.5. Measurement of plasma corticosterone

Immediately after decapitation, trunk blood was collected in polyethylene tubes containing EDTA (1 mg/mL blood) and centrifuged for 20 min at 1000 ×*g* and 4 °C. Separated plasma was stored at −20 °C until later analysis. Plasma corticosterone (CORT) levels were measured by RIA kit (MP Biochemicals), according to manufacturer's instructions.

2.6. RNA isolation and real time PCR

Total RNA was isolated using the TRI Reagent®RT (MRC, Inc.) according to the manufacturers' protocol and concentrations were quantified using the NanoDrop 2000 (Thermo Fisher Scientific). Reverse transcription of RNA (1000 ng) was performed with the RevertAid H minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's protocol, using an oligo dT primer. Semi-quantitative Real Time PCR was set up in total volume of 25 μL containing: ~30 ng of template cDNA mixed with 12.5 μLof FastStart Universal SYBR Green Master Rox (Roche Diagnostics), 1 μLof specific primer pair set, and water to achieve the final volume. Sequences of specific primers used are as follows: **Ucn1** For. 5′-TCCGAATCTGCGATGGAACC-3′, Rev. 5′-CCGCTAACAGCAAGAGGAGT-3′; **Ucn2** For. 5′-CCTGGACACCCGTGTCATAC-3′, Rev. 5′-ACACGGGCTAGTATTTGGGC-3′; tyrosine hydroxylase (**TH**) For. 5′-CCGGTCTACTGTCCGCCCGT-3′, Rev. 5′-TCATGGCAGCAGTCCGGCTC-3′; phenylethanolamine *N*-methyltransferase (**PNMT**) For. 5′-GGTCCTCATTGACATCGGCT-3′, Rev. 5′-GGACTCTCCCTTGTCCTCGAT-3′; **IL-1β** For. 5′-CAGCTTTCGACAGTGAGGAGA-3′, Rev. 5′-TGTCGAGATGCTGCTGTGAG-3′; **IL-6** For. 5′-ATACCACCCACAACAGACCAGT-3′, Rev. 5′-GATGAGTTGGATGGTCTTGGT-3′; **TNF-α** For. 5′-GATCGGTCCCAACAAGGAGG-3′, Rev. 5′-GTTTGCTACGACGTGGGC TA-3′; **GAPDH** For. 5′-TGGACCACCCAGCCCAGCAAG-3′, Rev. 5′-GGCCCCTCCTGTTGTTATGGGGT-3′. Each sample was analyzed on a ABI7900HT Fast Real-Time PCR instrument (Applied Biosystems) under the following conditions: 1 cycle of 2 min at 50 °C, followed by 1 cycle of 10 min at 95 °C and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were normalized to GAPDH levels and expressed as the relative fold change, as calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). A melting curve analysis was performed to confirm the specificity of the amplified products.

2.7. Statistical analysis

All data are presented as mean ± SEM with 6–8 animals per group. Differences were analyzed by *t*-test when two experimental groups were compared (SAL vs. LPS) and with a two-way analysis of variance (Two-Way ANOVA) for more than two groups, using the factors treatment (Sham/PhADX) and stress (Control/IMO), followed by a Tukey post-hoc analysis using statistical software SigmaPlotv.11.0 (Systat Software, Inc.). A value of $p \leq .05$ was considered significant.

3. Results

3.1. Effect of stressors on plasma corticosterone levels

Plasma corticosterone (CORT) levels were significantly increased in animals exposed to acute IMO as well as in animals injected with LPS ($p < .001$) (Fig. 1A-B). These findings confirmed the effectiveness of both stressors. We also validated the effect of pharmacological adrenalectomy (PhADX) as this intervention completely abolished the IMO-induced increase of plasma corticosterone levels in stressed animals (Fig. 1A).

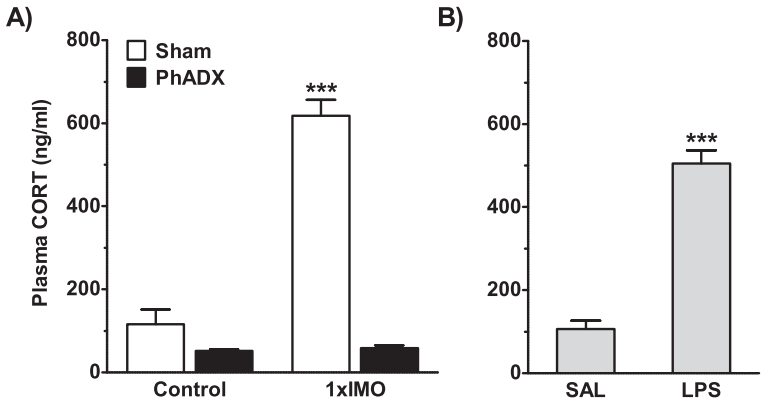

Fig. 1. Effect of stressors on plasma levels of corticosterone in rat. A) Animals underwent pharmacological adrenalectomy (PhADX) or were injected *s.c.* with vehicle (Sham). Afterwards, some of the animals were exposed to acute immobilization stress for 2 h (1xIMO) and sacrificed immediately after the IMO. Control animals were not exposed to stress. B) Animals were administered a single *i.p.* injection of lipopolysaccharide (LPS) at a dose of 50 µg/kg of body weight or received a single *i.p.* injection of saline (SAL) and were sacrificed 3 h later. Data are presented as the fold change relative to control, taken as 1 (mean ± SEM, $n = 6–8$). *t*-Test: ***$p < .001$.

3.2. Effect of stressors on gene expression of urocortins

To investigate the effect of stress on the gene expression of urocortins in the spleen, rats were exposed to an immune stressor (LPS) or to a combined physical-psychological stressor (immobilization). Whereas *i.p.* injection of LPS significantly reduced Ucn1 mRNA levels in the spleen ($p < .001$; Fig. 2A), gene expression of Ucn2 was not affected (Fig. 2B). In contrast, IMO led to a significant, 6-fold increase of Ucn2 mRNA levels in rat spleen (1xIMO/Sham; $p < .001$, Fig. 3B) and had no effect on Ucn1 mRNA levels in naive animals (Fig. 3A) These data indicate that gene expression of urocortins in rat spleen is regulated in a stressor specific manner.

Based on our previous findings that showed a modulatory effect of glucocorticoids on the gene expression of urocortins in rat adrenal medulla and brain, we investigated the effect of pharmacological adrenalectomy on the gene expression of splenic urocortins. We found that IMO-induced increase of Ucn2 mRNA levels in rat spleen was blocked in adrenalectomized animals (1xIMO/PhADX; $p < .001$, Fig. 3B), but there was no effect of PhADX on basal Ucn2 gene expression in the spleen. However, glucocorticoid withdrawal affected IMO-induced changes of Ucn1 gene expression. While IMO did not affect splenic Ucn1 gene expression in Sham animals, it did induce a significant increase of Ucn1 mRNA levels in PhADX animals (1xIMO/PhADX; $p = .009$, Fig. 3A). This increase was also significant when compared to stressed Sham animals ($p = .013$, Fig. 3A).

3.3. Effect of stressors on gene expression of catecholamine-synthesizing enzymes in the spleen

Both LPS and IMO-induced significant changes in the gene expression of catecholamine-synthesizing enzymes. While TH was significantly decreased only after LPS administration ($p < .001$, Fig. 4A), PNMT was significantly decreased not only after LPS administration ($p = .005$, Fig. 4B),but also after exposure to a single IMO (1xIMO/Sham; $p < .001$, Fig. 5B). Pharmacological adrenalectomy did not affect basal nor IMO-induced changes of TH and PNMT mRNA levels.

3.4. Effect of stressors on gene expression of inflammation markers in the spleen

To confirm the efficiency of our immune stressor, we determined the gene expression of splenic pro-inflammatory cytokines of rats injected with LPS. In addition, LPS administration allowed us to compare the efficiency of stressors in stimulating animmune response in the spleen. We found that LPS administration induced a profound increase in the gene expression ofIL-1β, IL-6, and TNF-α in the spleen ($p < .001$; Fig. 6A-C). In contrast, acute immobilization only induced a significant increase of IL-1β (1xIMO/Sham; $p = .04$; Fig. 7A), whereas mRNA levels of IL-6 (1xIMO/Sham; $p = .004$; Fig. 7B) and TNF-α (1xIMO/Sham; $p = .003$; Fig. 7C) were significantly reduced.

Withdrawal of glucocorticoids had a diverse effect on the gene expression of investigated cytokines in immobilized rats. IMO induced an increase of IL-1β in andrenalectomized animals (1xIMO/PhADX; $p = .013$; Fig. 7A) that was significantly greater when compared to Sham animals (IL-1β, $p = .021$; Fig. 7A). IMO-induced decrease of TNF-α observed in Sham animals was blocked in the spleens of adrenalectomized animals (1xIMO/PhADX; $p = .003$; Fig. 7C) and finally there was no effect of IMO in PhADX rats on IL-6 gene expression in spleen, when compared to the decreased levels observed in Sham animals.

4. Discussion

Previously, we have found that exposure of rats to immobilization stress affects urocortin2 expression in both the adrenal medulla and brain areas involved in the stress response. In addition, we have found that these effects are influenced by glucocorticoids (Tillinger et al., 2018; Tillinger et al., 2013). In the present paper we summarized data

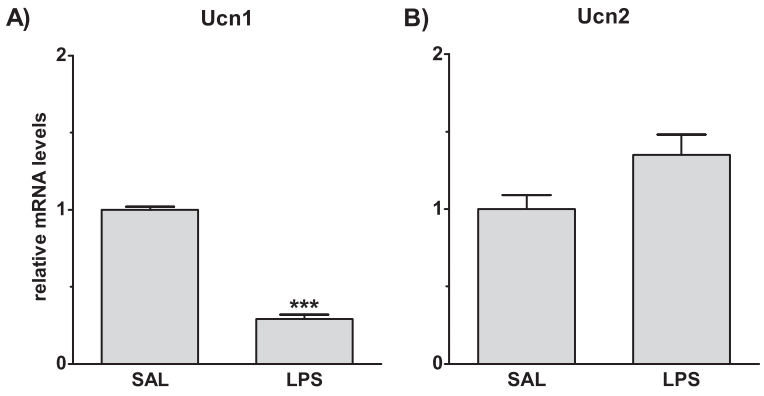

Fig. 2. Effect of immune challenge (LPS administration) on Ucn1 and Ucn2 gene expression in rat spleen. Animals were administered a single *i.p.* injection of lipopolysaccharide (LPS) at a dose of 50 µg/kg of body weight or received a single *i.p.* injection of saline (SAL) and were sacrificed 3 h later. Data are presented as fold change relative to control, taken as 1 (mean ± SEM, n = 6–8 animals per group). *t*-Test: *** $p < .001$.

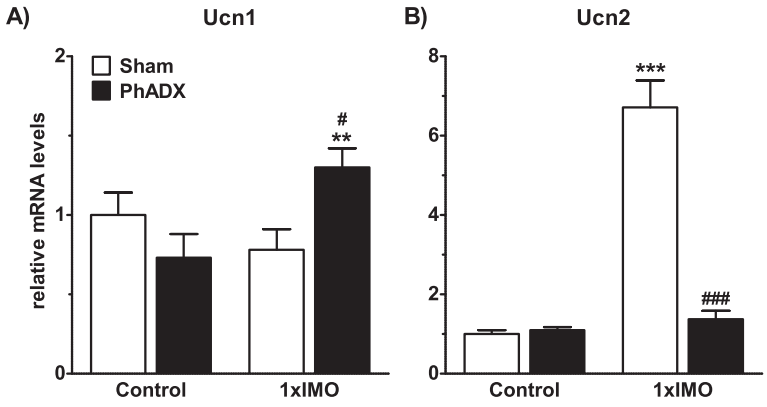

Fig. 3. Effects of immobilization stress and glucocorticoid withdrawal on Ucn1 and Ucn2 gene expression in rat spleen. Animals underwent pharmacological adrenalectomy (PhADX) or were injected *s.c.* with vehicle (Sham). Afterwards, some of the animals were exposed to acute immobilization stress for 2 h (1xIMO) and sacrificed immediately after the IMO. Control animals were not exposed to stress. Data are presented as the fold change relative to control, taken as 1 (mean ± SEM, n = 6–8 animals per group). Tukey post hoc analysis: $^{**}p < .01$, $^{***}\ p < .001$ 1xIMO vs. Control; $\#p < .05$, $\#\#\#\ p < .001$ PhADX vs. Sham.

related to experiments focused on describing of the role of urocortins in stress-related regulation of immune function. We investigated the effect of two different stressors on the gene expression of urocortins, catecholamine synthesizing enzymes, and cytokines in rat spleen. In addition, we determined the effect of pharmacological adrenalectomy on the response to these stressors.

We have shown that immobilization stress leads to a significant, several fold increase in Ucn2 mRNA levels in the rat spleen, which is very similar to what we have previously seen in the adrenal medulla and selected brain regions related to neuroendocrine components of the stress response, in particular the HPA axis and SAS (Tillinger et al., 2018; Tillinger et al., 2013). In the same experimental setup, we found that IMO did not affect the gene expression of Ucn1 in rat spleen, while a different effect on the gene expression of splenic urocortins was induced by immune challenge (LPS administration). While there was no change of Ucn2 mRNA levels in spleen of rats injected by LPS, we observed a significant decrease in Ucn1 mRNA levels in these rats that was in agreement with the findings of Kageyama (Kageyama et al., 1999). The different responses of urocortins gene expression induced by different stressful challenges indicate that LPS, compared to immobilization, activated different mechanisms related to urocortins synthesis in the spleen.

Glucocorticoids play a crucial role in the stress response as well as in the regulation of inflammation. We and others have shown that glucocorticoids are also involved in regulating gene expression of Ucn2 (Chen et al., 2004; Chen et al., 2003; Tillinger et al., 2018; Tillinger et al., 2013). Therefore, we investigated the effect of adrenalectomy (PhADX) on IMO-induced changes of urocortins mRNA levels. The IMO-induced increase of Ucn2 mRNA levels was complete blocked by pharmacological adrenalectomy. However, the adrenalectomy didn't affect basal levels of Ucn2 in control animals. When comparing adrenalectomy-induced effects in the spleen with its effects on the gene expression of urocortins in the adrenal medulla, a tissue specific regulatory effect of glucocorticoids on gene expression of urocortins might be proposed. These findings support our previously published hypothesis, along with observations from other research groups, that gene expression of Ucn2 is regulated in a tissue specific manner, and in some of them like in the spleen glucocorticoids play a crucial role in this process. Interestingly PhADX led to an increase in Ucn1 mRNA levels after IMO compared to non-stressed controls, indicating an inhibitory role of glucocorticoids in stress-related regulation of Ucn1 gene expression. In the thymus, another immunological organ, endogenous glucocorticoids increased the gene expression of Ucn1 whereas LPS administration also led to increases in thymic Ucn1 mRNA levels (Kageyama et al., 1999). Based on our findings, we hypothesize both Ucn2 and Ucn1 expression is tissue specific and regulated by different mechanisms when the organism is exposed to different stressors (LPS vs. IMO).

Immune functions are also affected by catecholamines. Therefore, to determine the role of the catecholaminergic system in splenic immune function and to elucidate any possible interconnection between changes in local catecholaminergic and the CRH system, we also measured gene expression of two catecholamine synthesizing enzymes in the spleen. We investigated gene expression of tyrosine hydroxylase (TH) which represents the rate limiting enzyme and phenylethanolamine *N*-methyltransferase (PNMT) which is responsible for converting norepinephrine to epinephrine (Kvetnansky et al., 2009). Immobilization induced a significant decrease in gene expression of PNMT but did not affect TH mRNA levels in spleen, while adrenalectomy did not affect IMO induced changes of neither TH nor PNMT expression in spleen. In contrast, LPS administration led to a significant decrease in splenic TH and PNMT mRNA levels. Similar findings were also described by Laukova et al. (2018). Moreover, they have shown significantly elevated levels of splenic catecholamines, suggesting increased input from the sympathoadrenal system rather than from local catecholaminergic system (Laukova et al., 2018). It was also shown that chronic stress leads to decreased gene expression of catecholamine biosynthetic enzymes in rat spleen (Gavrilovic et al., 2010; Gavrilovic et al., 2012).

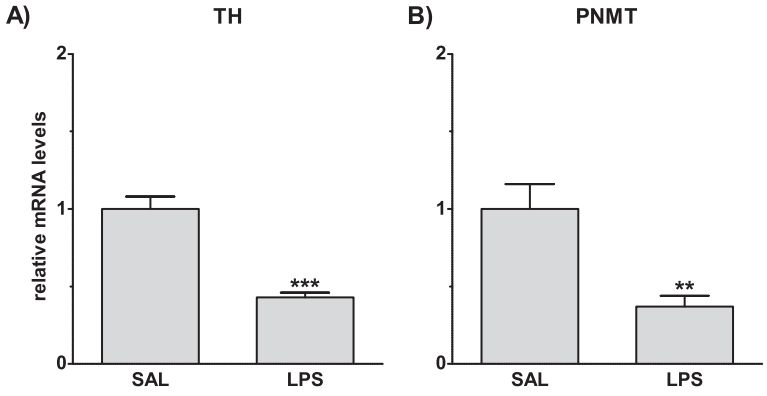

Fig. 4. Effect of immune challenge (LPS administration) on catecholamine synthesizing enzymes (TH and PNMT) gene expression in rat spleen. Animals were administered a single *i.p.* injection of lipopolysaccharide (LPS) at a dose of 50 µg/kg of body weight or received a single *i.p.* injection of saline (SAL) and were sacrificed 3 h after. Data are presented as the fold change relative to control, taken as 1 (mean ± SEM, n = 6–8 animals per group). *t*-Test: $^{**}p < .01$, $^{***}\ p < .001$.

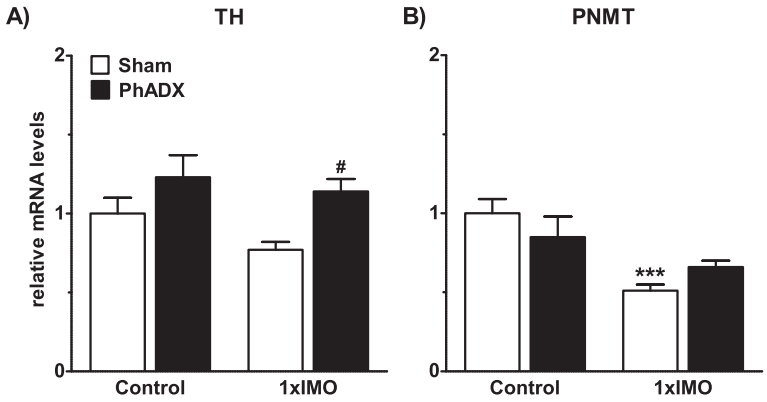

Fig. 5. Effects of immobilization stress and glucocorticoid withdrawal on catecholamine synthesizing enzymes (TH and PNMT) gene expression in rat spleen. Animals underwent pharmacological adrenalectomy (PhADX) or were injected *s.c.* with vehicle (Sham). Afterwards some of the animals were exposed to acute immobilization stress for 2 h (1xIMO) and sacrificed immediately after IMO. Control animals were not exposed to stress. Data are presented as fold change relative to control, taken as 1 (mean ± SEM, n = 6–8 animals per group). Tukey post hoc analysis: *** $p < .001$ 1xIMO vs. Control; #$p < .05$ PhADX vs. Sham.

This would suggest a primary role of the HPA axis and its components (CRH system and glucocorticoids) over the local catecholaminergic system in regulating splenic immune responses under acute stress conditions.

The spleen represents a crucial organ triggering a systemic response to immune-related stimuli via synthesis and release of pro-inflammatory cytokines. Therefore, we also investigated the effect of pharmacological adrenalectomy on the gene expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in the spleen of immobilized rats and compared them to those exposed to the LPS model of immune challenge. As expected, LPS-induced activation of immune cells led to a robust increase in the gene expression of splenic IL-1β, IL-6, and TNF-α, in accordance with previously published data (Li et al., 2005; Meltzer et al., 2003; Turrin et al., 2001). Interestingly, we saw a very different response of splenic cytokines in immobilized animals compared to those exposed to LPS. Whereas IMO induced a slight, but significant increase of IL-1β mRNA levels, mRNA levels of IL-6 and TNF-α were significantly reduced. Similar results have been observed in the spleen of rats exposed to acute physical stressors like tail- or foot-shock (Goujon et al., 1995; Meltzer et al., 2004; O'Connor et al., 2003). We have confirmed the involvement of glucocorticoids in the regulation of cytokine expression as shown in stressed adrenalectomized animals (PhADX). Withdrawal of glucocorticoids led to a significant increase of IL-1β and TNF-α mRNA levels and increase of IL-6 mRNA levels after immobilization when compared to stressed animals with intact adrenals (Sham). These results are in accordance with data obtained from experiments in which animals that underwent surgical adrenalectomy were exposed to either a stressor (foot-shock) or inflammatory stimulus (LPS) and suggests an inhibitory role of acute stress on cytokine production in the spleen, with a role of glucocorticoids as anti-inflammatory factor (Goujon et al., 1996; Meltzer et al., 2003, 2004).

Our results have shown that acute physical-psychological stress (IMO) is not a very potent activator of the splenic immune response when compared to a classical immune challenge induced by LPS. Based on our findings we can suggest a suppressive effect of acute stress on splenic immune function. However, we cannot generalize these observations, because the effect of stress on immune function is complex and depends on the type of stressor and exposure duration (Dhabhar, 2014). In addition to decreased levels of cytokine gene expression in the spleen, we also found inhibition of the splenic catecholaminergic system in stressed animals. In contrast, we found a several fold increase in the expression of Ucn2, a CRH family member, in rat spleen. The CRH family of peptides and their receptors are involved in regulating the stress response at a central level and also at the periphery. In addition, they are also involved in regulating immune functions at different levels (Deussing and Chen, 2018; Zhu et al., 2011). The increased mRNA levels of Ucn2, without changes in splenic Ucnl, in animals exposed to acute immobilization indicates an anti-inflammatory role of the Ucn2/CRHR2 pathway. The most common view of the role of Ucn2/CRHR2 in stress responses is that it plays a role in restoring homeostasis after exposure to stressors. In the spleen of stressed adrenalectomized animals we have also observed a significant increase in cytokine mRNA levels compared to stressed animals with preserved adrenal function, confirming the well-known anti-inflammatory role of glucocorticoids. Moreover, the Ucn2 mRNA rise was completely abolished in the spleen of adrenalectomized animals, but there was an increase of Ucn1 mRNA levels, which would suggest a pro-inflammatory role of the Ucn1/CRHR1 pathway and a negative regulatory role of glucocorticoids in Ucn1 mRNA gene expression. Increased Ucn1 immunoreactivity and mRNA levels have been observed in the synovium of rheumatoid arthritis patients (Uzuki et al., 2001) and in the lung-airway epithelial cells of rats with experimental allergic asthma, which was reversed by glucocorticoid treatment (Wu et al., 2006). Zhu et al. (2011) in his review also proposed similar mechanisms of CRHR1/pro-inflammatory

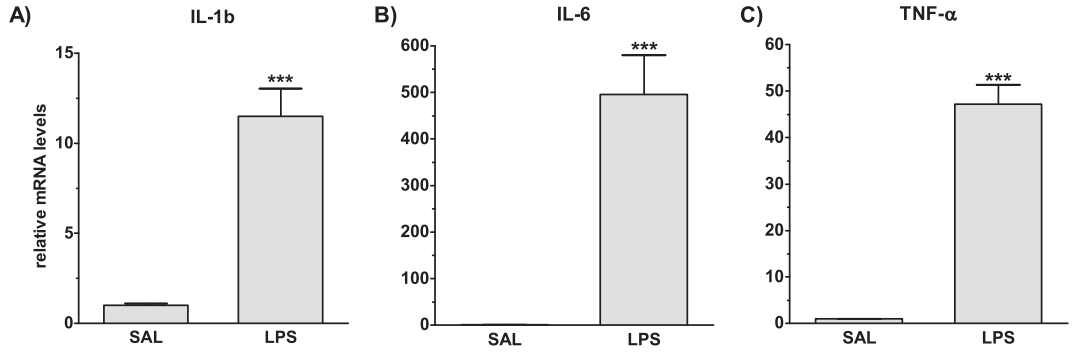

Fig. 6. Effect of immune challenge (LPS administration) on gene expression of selected cytokines (IL-1β, IL-6, and TNF-α) in rat spleen. Animals were administered a single *i.p.* injection of a lipopolysaccharide (LPS) at a dose of 50 μg/kg of body weight or received a single *i.p.* injection of saline (SAL) and were sacrificed 3 h after. Data are presented as fold change relative to control, taken as 1 (mean ± SEM, n = 6–8 animals per group). *t*-Test: *** $p < .001$.

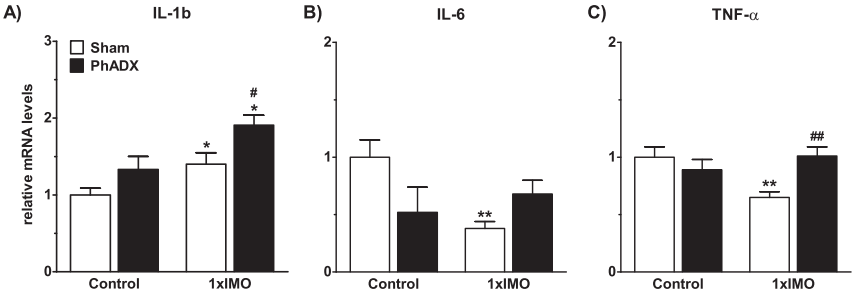

Fig. 7. Effects of immobilization stress and glucocorticoid withdrawal on gene expression of selected cytokines (IL-1β, IL-6, and TNF-α) in rat spleen. Animals underwent pharmacological adrenalectomy (PhADX) or were injected *s.c.* with vehicle (Sham). Afterwards, some of the animals were exposed to acute immobilization stress for 2 h (1xIMO) and sacrificed immediately after the IMO. Control animals were not exposed to stress. Data are presented as fold change relative to control, taken as 1 (mean ± SEM, n = 6–8 animals per group). Tukey post hoc analysis: $*p < .05$, $**p < .01$ 1xIMO vs. Control; $\#p < .05$, $\#\# p < .01$ PhADX vs. Sham.

and CRHR2/anti-inflammatory effects. Nevertheless, tissue and stressor specific immune responses cannot be ruled out and the exact mechanisms of how CRH family members and their receptors participate in regulating immune system activity needs to be elucidated in more detail.

In our previous paper, and also in the work of others, there is a strong suggestion of glucocorticoids regulatory effect of Ucn2 gene expression (Chen et al., 2004; Chen et al., 2003; Tillinger et al., 2018; Tillinger et al., 2013). While there is an increase of Ucn2 mRNA levels in the adrenal medulla and spleen induced by IMO, this increase was completely abolished after glucocorticoid withdrawal induced by PhADX, in spleen but it was preserved in the adrenal medulla (Tillinger et al., 2018). Therefore, our findings confirm previous observations showing an important role of glucocorticoids in the regulation of Ucn2 gene expression. However, it is necessary to note that this effect is tissue and stressor specific. Based on published data and our findings it can be concluded that Ucn2 is a promising marker of stressor disturbed homeostasis of the organism.

5. Limitations of the study

We are aware that our study has some limitations. We did not determine changes at the protein level of Ucn1 and Ucn2, which would give us a more complex picture. Partially, this was caused due to the lack of reliable and commercially available antibodies to Ucn2. In addition, to better characterize the role of the urocortin system in regulating immune function, it will be necessary to determine the dynamics of changes in Ucn1 and Ucn2 gene expression/protein levels by employing more time intervals both during, as well as after exposure of animals to stressors.

6. Conclusion

Our data have shown different effects of immobilization stress on Ucn1 and Ucn2 gene expression in the spleen, finding a significant rise in Ucn2 mRNA levels. We have also shown that the gene expression of splenic urocortins is regulated by glucocorticoids, and we confirmed previous observations that their effect is tissue and stressor specific. Based on our findings, we suggest the existence of a local paracrine effect of urocortins in the spleen, with Ucn2/CRHR2 having an anti-inflammatory effect that would be in line with its suggested role in restoring homeostasis after exposure of the organism to stressors. However, further studies will be necessary to elucidate the role of urocortins and other CRH family members in regulating splenic immune function under various physiological and pathological conditions in more detail.

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Declaration of Competing Interest

The authors of the manuscript have no conflicts of interest to declare.

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