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Molecular and Cellular Neuroscience 108 (2020) 103541

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Effects of RU486 treatment after single prolonged stress depend on the post-stress interval

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ARTICLE INFO

Keywords:
Single prolonged stress
Behaviour
Mifepristone
Sgk1
FKBP5

ABSTRACT

The Single Prolonged Stress protocol is considered a model for PTSD, as it induces long lasting changes in rat behaviour and endocrine regulation. Previous work demonstrated that some of these changes can be prevented by treatment with the glucocorticoid receptor antagonist RU486, administered a week after the stressor. The current study evaluated the effects of an earlier intervention with RU486, as evaluated 1 week after SPS-exposure. Most RU486 effects occurred independent of prior stress, except for the reversal of a stress-induced increase in locomotor behaviour. The accompanying changes in gene expression depended on gene, brain region, and time. DNA methylation of the robustly down-regulated Fkbp5 gene was dissociated of changes in mRNA expression. The findings reinforce the long term effects of GR antagonist treatment, but also emphasize the need to evaluate changes over time to allow the identification of robust correlates between gene expression and behavioural/endocrine outcome of stressful experiences.

1. Introduction

Stress leads to many neuronal and endocrine responses that promote homeostatic and behavioural adaptations. However, when stress is excessive it can lead to pathogenic maladaptive responses within brain stress-integrative systems and to the development of stress-related psychiatric disorders, such as post-traumatic stress disorder (PTSD) (Sabban et al., 2015). PTSD is a difficult-to-treat psychiatric disorder. Patients with PTSD have altered hypothalamus-pituitary-adrenal (HPA) axis reactivity and increased glucocorticoid receptor (GR) sensitivity (Bergen-Cico et al., 2014; Szeszko et al., 2018). In PTSD animal models altered (re)activity of the HPA axis is also observed, in association with altered expression of corticosteroid receptors, particularly the GR (Danan et al., 2018; Han et al., 2014; Whitaker et al., 2016).

Unlike for other psychiatric disorders, PTSD is generally associated with a specific triggering stressor. This may allow for early pharmacological intervention with the goal to increase resilience and thereby prevent PTSD development (Glaspey et al., 2017; Kearns et al., 2012; Laukova et al., 2014; Rothbaum et al., 2014). Understanding both the nature and timing of potential interventions is critical to develop such a pharmacotherapeutic approach (Rasmusson et al., 2017). GR may contribute to the disease process, either through excessive activation by stress-induced cortisol during the traumatizing event, or through its ensuing dysfunction. Regardless, the receptor may form a target for intervention. Strikingly, GR antagonists can ameliorate stress-induced changes even when administered weeks after a stressor. For example, the GR antagonist mifepristone (RU486) administered at adolescent age prevented fear responses and contextual memory deficits after early life stress(Arp et al., 2016; Loi et al., 2017a; Loi et al., 2017b), although such reversal effects are not always found (Kentrop et al., 2016). GR antagonist treatment therefore is a potential strategy for PTSD and other stress-related disease (Daskalakis et al., 2018; Girgenti and Duman, 2018; Nees et al., 2018).

Previously, we demonstrated that treatment with GR antagonist RU486/mifepristone changes the outcome of adult rodent stress of PTSD model, when administered a week after the Single Prolonged Stress paradigm and evaluated after two weeks (Ding et al., 2019). Because in many studies the effects of SPS are evaluated one week after the stressor, in the current study we treated with RU486 at an earlier timepoint to be able to evaluate the effects after one week. We measured behaviour, the expression of several candidate genes in the hypothalamic paraventricular nucleus (PVN) and limbic brain regions,

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https://doi.org/10.1016/j.mcn.2020.103541
Received 19 March 2020; Received in revised form 17 August 2020; Accepted 20 August 2020
Available online 25 August 2020

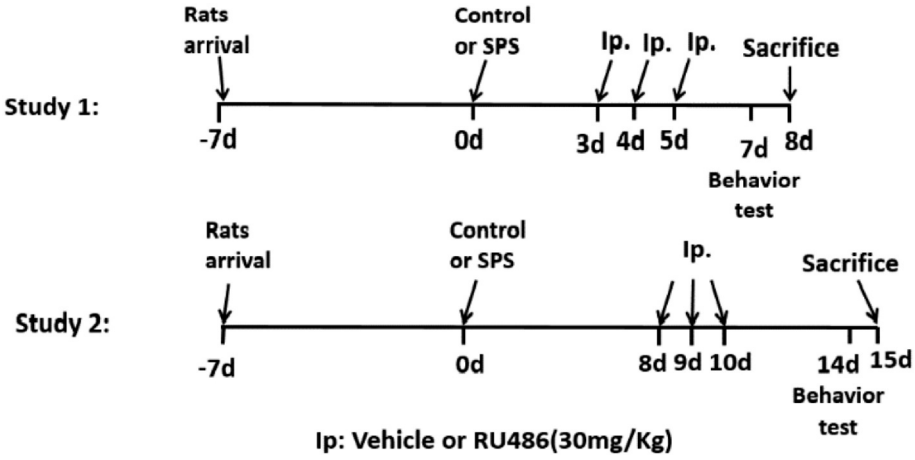


Fig. 1. Schematic diagram of experiment timeline. Animals habituated 1 week after arrival in the vivarium. On day 0, the stress paradigm was performed. From day 3 (study 1) or 8 (study 2), the animals from control or SPS group received three consecutive days intraperitoneal injection of RU486, or vehicle (n = 8 rats per group). Behavioural tests were applied on day 7 or 14. Rats were sacrificed on day 8 or 15.

and a potential epigenetic mechanism underlying a consistent effect on the *Fkbp5* expression.

2. Materials and methods

2.1. Subjects

All experiments were performed in accordance with the Chinese National Guideline on Animal Care. Animals were obtained from the China Medical University Animal center. A total of 32 male Wistar rats of 7 weeks old, weighing 200–220 g at arrival, were housed (two per cage) on a 12-hour light/dark cycle (lights on at 7:00–19:00) at 22 ± 1 °C, with ad libitum access to food and water. After 7 days of acclimatization, animals were randomly assigned to experimental (n = 16) or control groups (n = 16).

2.2. Experimental design

We conducted two studies assessing the effect of RU486 treatment intervention at different times after stress. The experimental design is depicted in Fig. 1. The second experiment was published previously (Ding et al., 2019), here we include new measurements on some target genes.

2.2.1. Single-prolonged stress (SPS) paradigm

The single session of prolonged stress was performed as previously described (Liberzon and Young, 1997). SPS consisted of restraint for 2 h in an acrylic animal holder followed by forced swim for 20 min in a plexiglass cylinder (50 cm height, 24 cm diameter) filled with 24 °C fresh water. Rats were allowed recuperate for 15 min and then subjected to ether anesthesia. Control animals remained in their home cage with no handling and were injected and sacrificed at the same time as the stressed groups.

2.2.2. Drugs

Mifepristone (RU486, Sigma, USA) was dissolved in DMSO and diluted into 0.9% saline/20% DMSO immediately before intraperitoneal injection (30 mg/kg). Vehicle injections were saline containing 20% DMSO. The dose and DMSO concentration were chosen based on previous studies (Bohacek et al., 2015; Taubenfeld et al., 2009).

2.2.3. Treatment & testing

Starting on the third day after SPS, half the animals from both control (n = 16) and SPS (n = 15, one rat died during the forced swim) groups received on three consecutive days an intraperitoneal injection of RU486, or vehicle, leading to 4 groups of animals. On day 7, the behavioural experiments were performed and animals were sacrificed on the morning of the next day, 8 days after SPS. Gene expression data from this study ('study 1') were compared with a longer experiment in which RU486 treatment was administered at days 8–10 after SPS, tested for behaviour at 14 days, and killed on the morning of day 15 ('study 2') (Ding et al., 2019).

2.3. Plasma corticosterone measurement

Blood was collected via the caudal vein in microtubes (Lithium-Heparin, #20.1282, Saerstedt, Germany) on the third day after SPS between 9:00–10:00 for the measurement of basal corticosterone. At sacrifice, trunk blood was collected between 10:00–11:00 am. Blood was centrifuged at 2000*g* for 5 min at 4 °C to obtain the plasma and then stored at −70 °C. Corticosterone levels were determined with the ELISA assay kit (AC-15F1, Immunodiagnostic Systems, UK) according to the manufacturer's instructions. Some animals (1 in control vs vehicle group, 1 in control vs RU486 group and 1 in SPS vs vehicle group) were removed from the endocrine analyses due to insufficient sample collection. For study 2, corticosterone levels were published previously (Ding et al., 2019).

2.4. Locomotor activity and anxiety in open-field (OF) test and elevated plus maze (EPM) test

Locomotor activity and anxiety were measured using the OF and EPM test. The OF apparatus was surrounded by black walls 40 cm in height, and the floor was 90 cm × 90 cm, subdivided into central (18 cm far from the wall) and peripheral compartments. During the experiment, each rat was put in the center of apparatus, and permitted to explore freely for 5 min. Each trial was recorded by an automatic analysis system (Smart 3.0, Panlab, Barcelona, Spain). Total and center distance, times crossing and time in the centre compartment were recorded. The maze was cleaned with 10% ethanol solution between the trials. The EPM apparatus consisted of a plus-shaped maze elevated 80 cm above the floor with two oppositely positioned closed arms, two oppositely positioned open arms, and a central area. Rats were placed in the central area of the maze facing an open arm and allowed to explore freely for 5 min. Movement was monitored and quantified by an automatic analysis system (Smart 3.0, Panlab, Barcelona, Spain). Distance in total and closed arms, percentage time spent in the open arms were determined.

2.5. Determination of changes in mRNA levels for candidate genes in the PVN, amygdala and dorsal hippocampus

Following sacrifice, brains were immediately removed and frozen on dry ice (−80 °C). Coronal sections (80 μm) were sectioned using a

Table 1
Primer sequences for qPCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	ACGGCAAGTTCAACGGCACAG	AAGACGCCAGTAGACTCCACGACA
FKBP5	AAGCATTGAGCAAGAAGGCAGTA	GAGGAGGGCCGAGTTCATTAG
sgk1	GAAGATCACGCCCCCATTTA	TGTGACAAGGATGCTGTCAGG
COMT	CTGGAGGCCATCGACACCTA	AGTAAGCTCCCAGCTCCAGCA
c-fos	CCAAGCGGAGACAGATCAAC	AAGTCCAGGGAGGTCACAGA
PACAP	AACTCTTTCCTAGCCGCGAA	TTCCGTCCTGATCGTAAGCC
GR	GCATTACCACAGCTCACCCCTAC	GCAATCACTTGACGCCCACC

cryostat and regions of interest were punched out as described previously (Ding et al., 2019): the PVN, amygdala and dorsal hippocampus. Tested genes and their primers are described in Table 1. RNA isolation, cDNA synthesis and qPCR were performed as the manufacturer's instructions. The $2^{-\Delta\Delta Ct}$ method was used to determine differences between groups, using GAPDH as a housekeeping gene.

2.6. FKBP5 DNA methylation analysis

DNA was isolated from tissue punches of the dorsal hippocampus using the QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. For methylation assays, 400 ng DNA was bisulfite - converted using the EpiTect bisulfite Qiagen kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. Illumina - sequencing PCR was used to measure methylation status directly at 7 CG sites in FKBP5 intron V upstream from a conserved glucocorticoid-response element (GRE) as previously reported (Kitraki et al., 2015; St-Cyr et al., 2017, Table 2, Fig. 7a).

2.7. Statistical analysis

The results were expressed as Mean ± SEM. Comparisons between two groups were evaluated using unpaired *t*-tests. For all 2 × 2 designs, two-way ANOVA analysis of the data was performed with GraphPad Prism 8.0. Turkey's post-hoc test was used to assess significant post-hoc differences between individual groups. Differences with $p < 0.05$ were considered statistically significant. Pearson correlation analyses were performed using GraphPad Prism 8.0. Given that we determined potential correlations in total 54 parameters, we only report on correlations that were consistent in the data as a whole, as well as in subgroups, or that had a $p < 0.01$.

3. Results

3.1. Plasma corticosterone level of study 1

On day 3 after SPS, the morning basal corticosterone concentration was higher in stressed animals compared to controls (Fig. 2a, $p < 0.05$). On day 8 after SPS, there were main effects of stress ($F_{(1,25)} = 6.056$, $p < 0.05$, Fig. 2b) and treatment ($F_{(1,25)} = 8.13$, $p < 0.05$): stressor exposed rats had higher plasma corticosterone levels, while RU486 treatment suppressed plasma corticosterone. Of note, values were substantially higher than at day three, indicating that the conditions before sampling were not stress free, perhaps in part due to the behavioural testing the day before.

3.2. Anxiety and locomotion activity of OF and EPM test at SPS day 7

In the Open Field test, the percentage time in the central zone showed an interaction effect ($F_{(1,26)} = 8.965$, $p < 0.05$, Fig. 3a). Post-hoc analysis showed that animals from the SPS Vehicle group, surprisingly, spent significantly more time in the central area in comparison with Ctrl Vehicle group, but that RU486 treated SPS rats did not differ from non-stressed animals. RU486 treated control rats also spent more time in the central zone compared to vehicle-treated controls. There was a significant interaction effect of total distance ($F_{(1,27)} = 10.94$, $p < 0.05$, Fig. 3b), and post-hoc analysis showed that RU486 increased locomotor activity only in the control group. There was a significant interaction effect for distance in the central zone ($F_{(1,26)} = 9.725$, $p < 0.05$, Fig. 3c), with more locomotor activity only in SPS vehicle group compared to controls. Data for entries in the central area showed main effects for stress and interaction (stress: $F_{(1,26)} = 6.878$, $p < 0.05$, interaction: $F_{(1,26)} = 18.22$, $p < 0.05$, Fig. 3d). Post hoc tests revealed that SPS led to increased times in the central area, while RU486 led to reduced times in the central area for the stress group.

As shown in Fig. 3e–h, analysis of the behaviour in the elevated plus maze identified several significant effects of stress and treatment. A significant main effect of RU486 treatment indicated more time spent in the open arms ($F_{(1,24)} = 5.021$, $p < 0.05$, Fig. 3e). For total distance moved, there was a significant main effect of stress and an interaction effect (stress: $F_{(1,27)} = 5.858$, $p < 0.05$, Interaction: $F_{(1,27)} = 5.427$, $p < 0.05$, Fig. 3f). Post hoc tests revealed that SPS vehicle rats had moved more total distance than non-stressed vehicle rats. For distance moved in the open arms, there was a significant main effect of RU486 treatment ($F_{(1,26)} = 6.197$, $p < 0.05$, Fig. 3g). Post hoc tests indicated a higher distance in the open arms in RU486-treated control animals compared to vehicle. There were main effects of both stress and treatment for distance moved in the closed arms (stress: $F_{(1,27)} = 7.267$, $p < 0.05$, RU486 treatment, $F_{(1,27)} = 5.911$, $p < 0.05$, Fig. 3h).

In summary, SPS led to overall higher locomotor activity in the OF and the EPM. Indeed, we observed that some animals seemed agitated, perhaps pointing to a panic-like state. These effects were in interaction with RU486 treatment.

3.3. Gene expression

Gene expression was determined in punches from the PVN, the amygdala and the dorsal hippocampus in the animals 8 days after SPS. Data were compared with those previously reported (Table 3) as well as newly determined expression levels from the previous 15 days experiment, in order to delineate the time trajectory of stress-induced

Table 2
Primer sequences for DNA methylation.

FKBP5-1 forward	5′-GATGTGTATAAGAGACAGATGATTTAGTTATTGTTTGGGGATAG-3′
FKBP5-1 reverse	5′ CGTGTGCTCTTCCGATCTCCAAACTATACAACTTATATTTCAAAAAAC-3′
FKBP5-2 forward	5′-GATGTGTATAAGAGACAGGAAATATAAGTTGTATAGTTTGGGGTTTTT-3′
FKBP5-2 reverse	5′-CGTGTGCTCTTCCGATCT AACACCCTATTCTAAATATAACTAACAC-3′

FKBP5-1: FKBP5 methylation pair 1 (CpG 1-5), FKBP5-2: FKBP5 methylation pair 2 (CpG 6-7).

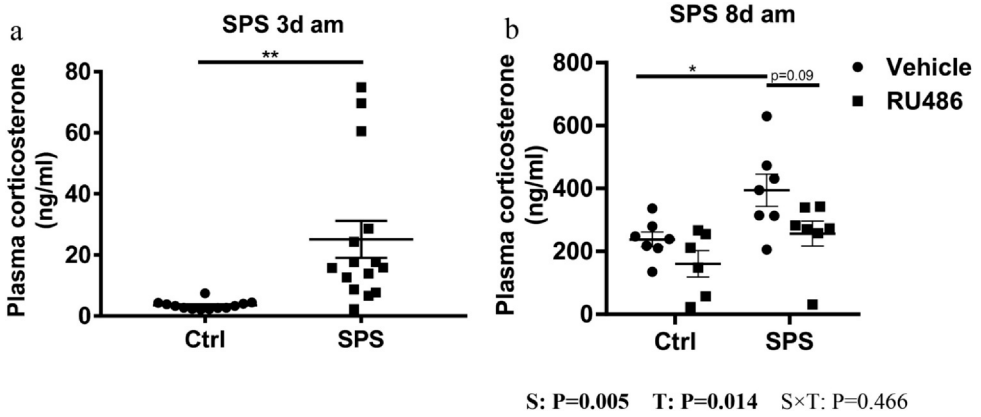


Fig. 2. Corticosterone neuroendocrine responses on stress and RU486 treatment. 2a: Stress significantly increased AM corticosterone plasma levels three days after SPS. 2b: Corticosterone levels at sacrifice day 8 were higher after SPS and reduced by prior RU486 treatment. *p < 0.05, **p < 0.01.

changes, and the importance of timing of RU486 treatment.

3.3.1. Dynamic gene expression in the PVN on day 8 and day 15

In the PVN, c-fos mRNA, a proxy for neuronal (re-)activity, at 8 days showed a significant main treatment of RU486 and an interaction effect (RU486 treatment: $F_{(1,26)} = 21.26$, $p < 0.0001$, interaction: $F_{(1,26)} = 15.36$, $p < 0.001$, Fig. 4a, Table 3). Post hoc tests revealed that c-fos mRNA expression was reduced after RU486, but only in non-stressed rats. This is similar to previous data found at 15 days after SPS (Table 3). In addition, c-fos mRNA was lower in vehicle treated SPS rats, compared to non-stressed controls.

Sgk1 mRNA in the PVN was measured in tissue from animals both 8 and 15 days after SPS, as it is a direct GR target gene (Mifsud and Reul, 2016; Webster et al., 1993) for which transcriptional regulation in the brain has been implicated in adaptation to stress (Licznerski et al., 2015). At 8 days there was a significant interaction effect between stress and RU486 and a trend towards a main effect of stress (interaction: $F_{(1,26)} = 13.91$, $p < 0.0001$, stress: $F_{(1,26)} = 3.226$, $p = 0.084$, Fig. 4b, Table 3). Post hoc tests revealed that RU486 supressed Sgk1 mRNA in controls, and this effect was absent in SPS rats. Sgk1 expression was lower in SPS-vehicle rats than in control vehicle rats. In the material from study 2, at 15 days after SPS there was a weak trend for an effect of stress, which tended to be slightly higher is SPS rats ($F_{(1,25)} = 3.02$, $p = 0.095$, Fig. 4e, Table 3). The relatively low expression in the control-RU486 group seemed to drive this trend, although there was no interaction effect.

PVN FKBP5 mRNA expression at the day 8 time point showed a significant main effect for stress ($F_{(1,26)} = 16.8$, $p < 0.001$, Fig. 4c, Table 3), indicating lower expression after stress. This was significant in post hoc tests for control rats. At the day 15 time point, 2-way ANOVA showed a main effect of stress ($F_{(1,24)} = 5.84$, $p = 0.024$, Fig. 4f, Table 3), but now indicating (slightly) higher expression after stress. There were no significant differences between the groups in pairwise comparisons. Of the genes reported earlier to be differentially expressed 15 days after SPS, PACAP mRNA expression in the PVN 8 days after stress had a significant main effect for RU486 treatment ($F_{(1,26)} = 5.032$, $p < 0.05$, Fig. 4d, Table 3).

In sum, in the PVN there were effects of stress at mRNA expression at 8 days after SPS, but these were mostly absent at 15 days after SPS. However, the suppressive effect of RU486 on c-fos mRNA that occurred selectively in control rats is similar to what we observed earlier on day 15 (Ding et al., 2019).

3.3.2. Gene expression in the amygdala on day 8 and day 15

In the amygdala at 8 days after SPS, c-fos mRNA levels were suppressed after RU486 but, similarly to the PVN, only in control rats (RU486 treatment: $F_{(1,26)} = 7.156$, $p < 0.05$, Fig. 5a, Table 3). The expression of Sgk1 mRNA of day 8 was overall similar to that in the PVN (interaction: $F_{(1,24)} = 8.82$, $p < 0.01$, Fig. 5b, Table 3). Post hoc tests showed a trend towards upregulation of Sgk1 mRNA by RU486 treatment in stressed rats. For study 2 at day 15, stress upregulated the sgk1 mRNA expression independent of RU486 treatment (stress: $F_{(1,26)} = 7.63$, $p = 0.01$, Fig. 5f, Table 3).

At day 8, stress had significant main effect on FKBP5 mRNA expression within the amygdala ($F_{(1,25)} = 26.04$, $p < 0.001$, Fig. 5c, Table 3). In post-hoc tests, the downregulation was significant only for vehicle treated rats, but there was no significant main effect of RU486. In study 2, FKBP5 expression showed a trend towards an opposite main effect of stress (increased expression: $F_{(1,26)} = 3.46$, $p = 0.074$) and of RU486 treatment (increased expression; $F_{(1,26)} = 3.95$, $p = 0.058$, Fig. 5g, Table 3). The expression of PACAP mRNA of day 8 showed a significant main effect of stress and RU486 (stress: $F_{(1,26)} = 4.34$, $p < 0.05$, RU486 treatment: $F_{(1,26)} = 4.49$, $p < 0.05$, Fig. 5d, Table 3).

Based on behaviour test results where the behaviour of the SPS rats suggested a possible panic-like state, we measured expression of the panic related gene COMT in the amygdala. At day 8, COMT mRNA expression showed a significant interaction effect ($F_{(1,25)} = 11.92$, $p = 0.002$, Fig. 5e, Table 3). Post-hoc tests showed lower COMT mRNA levels in the SPS vehicle group compare with the control vehicle group. RU486 treatment seemed to recover to the level observed in the control group. COMT expression was not different between groups of study 2 on day 15 (Fig. 5h, Table 3).

3.3.3. Gene expression in the dorsal hippocampus on day 8 and day 15

In the dorsal hippocampus of day 8, RU486 treatment had a significant main effect on c-fos mRNA expression ($F_{(1,25)} = 5.34$, $p < 0.05$, Fig. 6a, Table 3) within the dorsal hippocampus, indicating a slightly lower expression. This contrasts with our prior day 15 data, where RU486 led to increased c-fos mRNA in the hippocampus of stressed animals (Table 3).

The expression of Sgk1 mRNA of day 8 showed a significant interaction between stress and RU486 treatment ($F_{(1,27)} = 7.80$, $p < 0.01$, Fig. 6b, Table 3). Post hoc tests showed that RU486 decreased the Sgk1 mRNA expression only in the control group. Sgk1 mRNA expression in the stress-vehicle group was lower than in the control vehicle group, mirroring the PVN effect. In the dorsal hippocampus of day 15, Sgk1 mRNA expression showed a significant main effect of RU486 treatment and an interaction (treatment: $F_{(1,17)} = 7.765$, $p < 0.05$, interaction: $F_{(1,17)} = 22.32$, $p < 0.001$, Fig. 6e, Table 3). Post-hoc analysis indicated that RU486 increased Sgk1 mRNA expression only in the SPS group, similar to the amygdala data on day 8.

At day 8, stress had significant main effect for FKBP5 mRNA expression within the dorsal hippocampus ($F_{(1,27)} = 28.74$, $p < 0.001$,

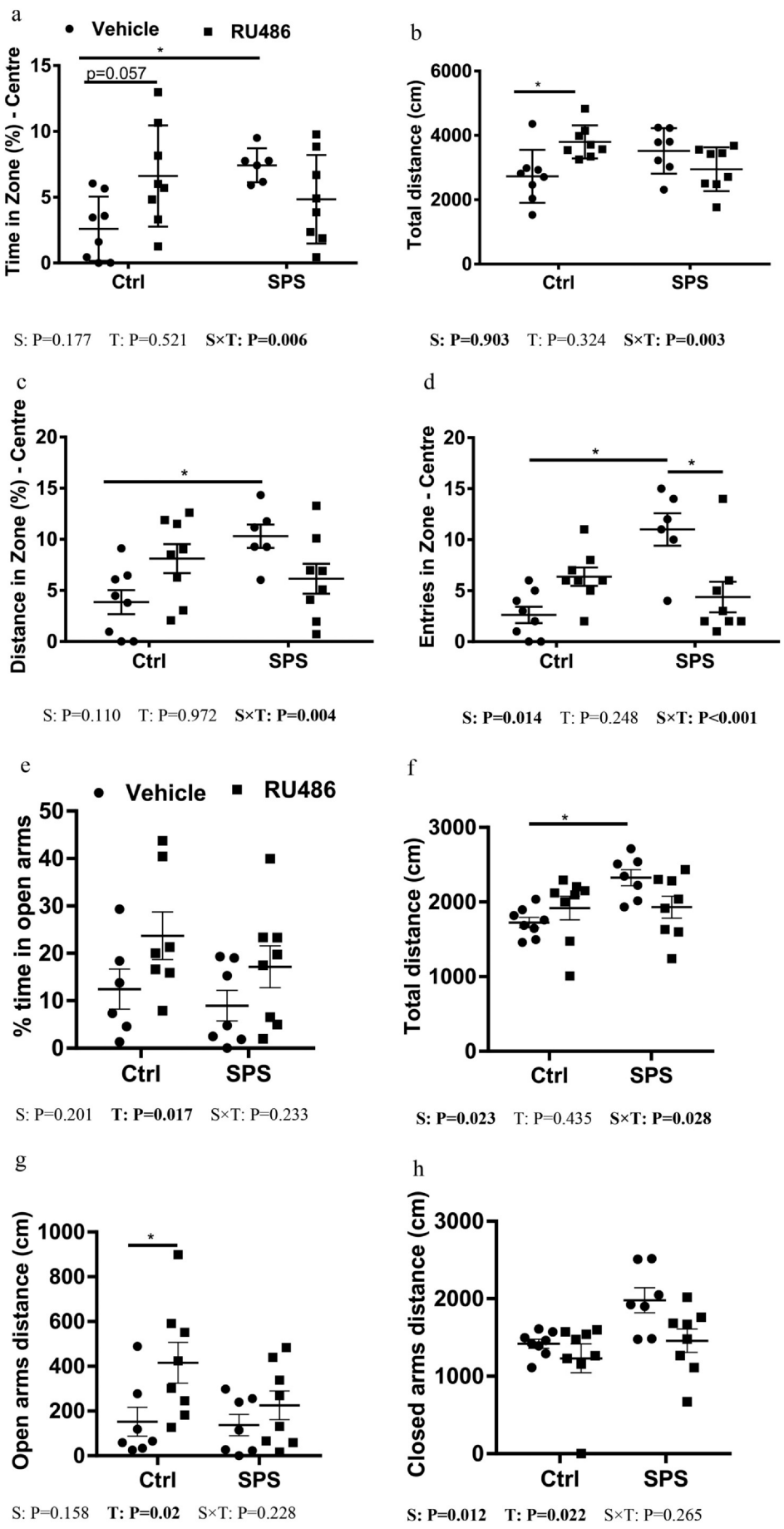


Fig. 3. Effects of stress and RU486 in the OF (a–d) test and EPM (e–h). 3a–b: Strong interaction effects between SPS and RU486 in the open field, for the measure Percentage time in central zone (a), Total distance (b), Percentage distance in the central zone (c) and Entries in the central zone (d). SPS led to an unexpected increase in Distance in central zone (c) and Entries in central zone (d). 3e: RU486 treatment led to increased time in the open arms of the EPM. 3f: SPS led to high total distance moved in the EPM, and RU486 normalized this. 3g: RU486 increased the distance moved in the open arms. 3h: Distance moved in the closed arms was increased by stress and decreased by RU486.

Table 3
RT-qPCR validation of genes regulated by SPS stressor and RU486 treatment in the PVN, amygdala and dorsal hippocampus.

		8d			15d		
		RU486	SPS	Interaction	RU486	SPS	Interaction
cfos	PVN	↓	−	+	↓	↑	~+
	Amydala	↓	−	~	−	−	−
	Dorsal hippocampus	↓	−	−	↑	−	+
FKBP5	PVN	−	↓	−	−	↑	−
	Amydala	−	↓	−	~↑	↑	−
	Dorsal hippocampus	−	↓	−	−	−	+
Sgk1	PVN	−	~↓	+	−	~↑	−
	Amydala	−	−	+	−	↑	−
	Dorsal hippocampus	−	−	+	↑	−	+
PACAP	PVN	↓	−	−	↓	↑	+
	Amygdala	↓	↑	−	−	↓	−
	Dorsal hippocampus	↓	−	~+	−	↑	−
COMT	Amygdala	−	−	+	−	−	−

Arrows indicate whether the gene is up-regulated (↑) or down-regulated (↓) by stress or treatment. (+) indicate interaction has statistically significant. (−) indicate the $p > 0.05$ of the factor. (~) indicate has the tendency of factor, $0.05 < p < 0.1$.

Fig. 6c, Table 3). In post-hoc tests, the downregulation was significant only for vehicle treated rats, but there was no significant main effect of RU486, similar to the situation in the amygdala. At day 15, FKBP5 expression showed a significant interaction between stress and RU486 treatment ($F_{(1,18)} = 6.82$, $p = 0.018$, Fig. 6f, Table 3), in absence of significantly different pairwise comparisons. At day 8, RU486 treatment had a significant main effect on PACAP mRNA expression ($F_{(1,26)} = 6.31$, $p < 0.05$) and interaction had a trend significant on PACAP mRNA expression ($F_{(1,26)} = 3.56$, $p = 0.071$, Fig. 6d, Table 3). Post hoc comparison showed that RU486 treatment downregulated the PACAP mRNA expression only in the stress group. The data differ from previously observed effects at day 15, where stressed animals showed overall higher PACAP mRNA levels in the SPS rats.

3.4. FKBP5 DNA methylation

FKBP5 expression has been linked to epigenetic regulation via CpG methylation. In view of the observed decrease in FKBP5 mRNA expression in all three brain regions 8 days after SPS, we analyzed in the dorsal hippocampus on day 8 DNA methylation levels for 7 CpG sites in the FKBP5 intron V (St-Cyr et al., 2017) (Fig. 7a). We observed changes at CpG site 5 and 7 (Fig. 7b). At CpG site 5 there was a significant main effect of RU486 treatment ($F_{(1,15)} = 5.492$, $p < 0.05$) and an interaction effect ($F_{(1,16)} = 13.48$, $p < 0.05$, Fig. 7b). The post hoc results showed that the levels of DNA methylation decreased after RU486 and with stress after vehicle treatment, but that RU486 had no effect in stressed rats. CpG site 7 showed a significant main effect of stress and an interaction effect (stress, $F_{(1,15)} = 5.336$, $p < 0.05$, interaction, $F_{(1,15)} = 12.09$, $p < 0.05$). The post hoc data showed that RU486 reversed the decreased methylation level only in the stress group. Thus, the CpG methylation levels did not match the observed mRNA expression levels.

3.5. Correlations between outcomes

The data showed substantial variation in corticosterone levels, which may indicate individual differences in stress responsiveness. In order to further understand relationships between corticosterone responses and outcomes at the level of behaviour and gene expression we performed correlation analyses. For corticosterone values at day 3 we found no significant correlations. Because the variation in corticosterone levels in the control group was minimal, we also analyzed these data for SPS rats only, but again found no correlations. The three rats with very high corticosterone levels at day 3 (1 veh, 2 RU486) showed low distance in the open arms of the EPM but did not otherwise stand out.

Corticosterone levels at day 8 correlated positively with c-fos mRNA expression in the dorsal hippocampus for the group as a whole ($r^2 = 0.17$, $p = 0.03$; Fig. 8a), as well as for all vehicle rats (control and SPS; $r^2 = 0.77$; $p < 0.0001$, Fig. 8b), all control rats (vehicle & RU486; $r^2 = 0.359$; $p = 0.04$) the SPS-vehicle rats ($r^2 = 0.789$; $p = 0.008$).

PACAP mRNA in the dorsal hippocampus was positively correlated with corticosterone in the vehicle group as a whole ($r^2 = 0.476$; $p = 0.009$; Fig. 8c).

4. Discussion

In this study we administered RU486 starting three days after SPS exposure and evaluated the effects 8 days after SPS. We compared the treatment with the previously performed intervention at 7 days after SPS and testing after 2 weeks. Our rationale for reducing the time course of the experiment to one week was that most effects of SPS exposure have been reported at 7 days after stress (Souza et al., 2017). We found that treatment with RU486 starting 3 days after the stressor lowered plasma corticosterone concentrations. RU486 also normalized the overall increased locomotor activity that we observed in stressed rats in the EPM and the OF test. Although some of the effects also occurred in control rats, they led to a de facto normalization towards unstressed, vehicle treated control rats. Overall, it is clear that RU486 treatment in rat acted in interaction with stress, and can normalize stress-induced parameters. There are also intrinsic effects of treatment in control animals that last for days or (in our 15 days experiment) weeks. These may or may not be of benefit to the stress-responsiveness of the individual.

The behavioural effects that we observed were atypical, in that we did not see a clear anxiety effect of SPS. We found increased locomotor activity in the anxiogenic areas of the tests (open arms of the EPM and central arena of the OF). We have no clear explanation for the fact that we did not replicate earlier effects on anxiety at one week after SPS (Han et al., 2014; Lee et al., 2016; Serova et al., 2019). We can be positive that the SPS protocol worked, given effects on corticosterone and gene expression. We also have earlier observed the anxiety provoking effects of SPS in our own facility (Han et al., 2017). We speculate that the daily injections per se may have altered the time course of brain reorganization that is normally occurring after SPS exposure (and this is something we have observed in preliminary experiments in our lab). Our vehicle for RU486 was 20% DMSO, and this may additionally have caused neurotoxic or behavioural effects (Cavas et al., 2005). Of course, a form of drug delivery is inevitable to address effects of RU486 on the development of emotional reactivity, and the vehicle-controlled data do show clear effects of the antagonist. However, we cannot straightforwardly compare the effects with data from non-treated SPS exposed rats.

For gene expression, we selected some additional genes compared to our previous analyses (Ding et al., 2019). Our choice was based on potential relevance for PTSD and (COMT) panic disorder. The latter was motivated by the hyperactive behaviour of the SPS rats in the EPM and OF, although this behaviour constitutes only a hint towards such a state. Sgk1 and Fkbp5 are stress responsive genes that are under direct transcriptional control of GR (Cattaneo and Riva, 2016; Mifsud and Reul, 2016; Webster et al., 1993). Both have been implicated in the pathophysiology psychiatric disease (Binder, 2009; Iurato et al., 2017; Licznerski et al., 2015). In addition, COMT was identified as risk gene for PTSD (Boscarino et al., 2011; Kolassa et al., 2010; Zhang et al., 2018) and panic disorder (Howe et al., 2016; Iurato et al., 2017).

Gene expression changes in PVN, amygdala and hippocampus

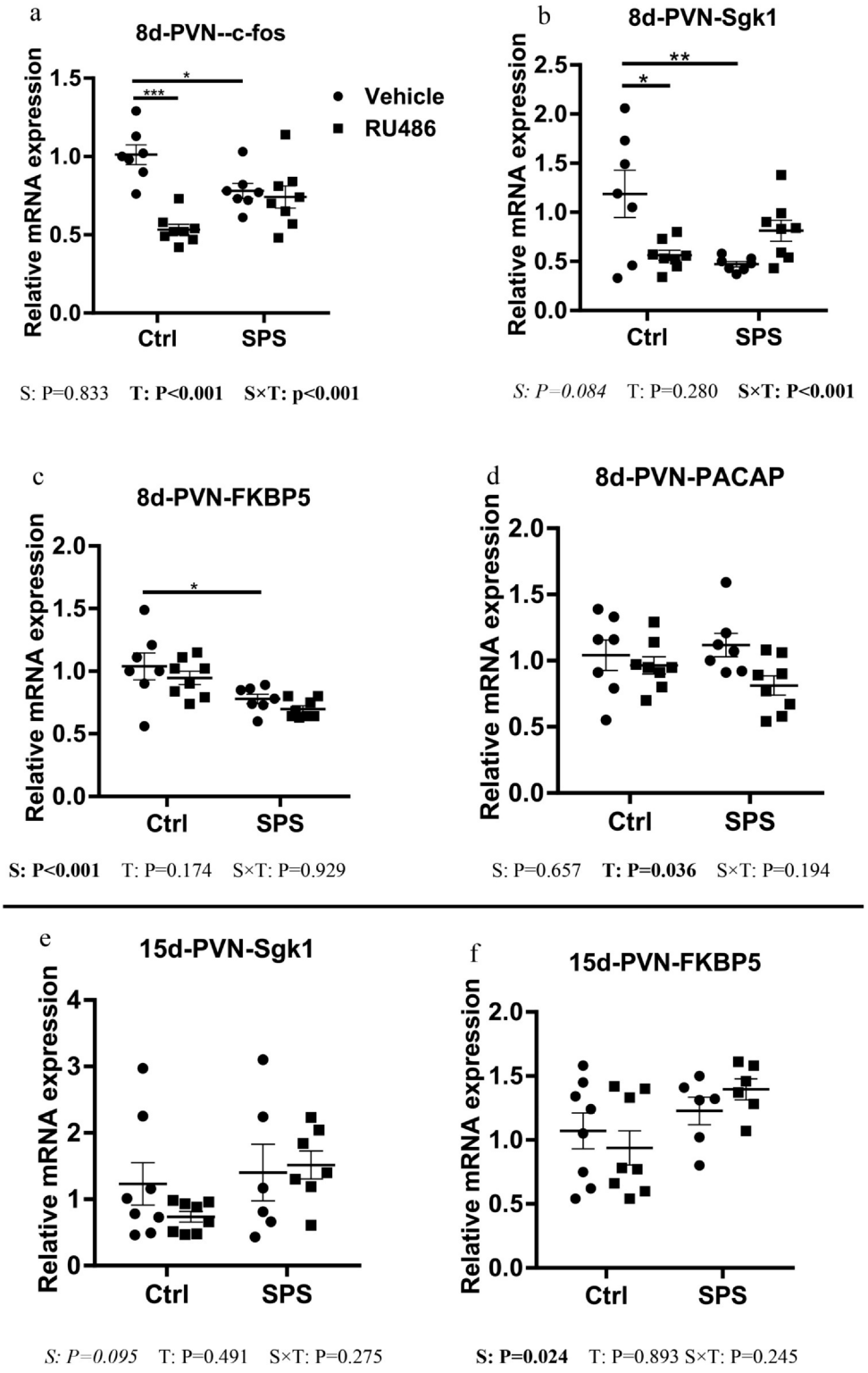


Fig 4. Effect of stress and RU486 treatment on gene expression in the hypothalamus. a: C-fos mRNA expression at day 8 was lower after RU486 only in control rats. b: Sgk1 mRNA expression at day 8 showed a strong interaction effect between SPS and RU486. c: FKBP5 mRNA expression at the day 8 was suppressed. d: PACAP mRNA expression at the day 8. e: At 15 days after SPS Sgk1 mRNA was not different between the groups. f: At day 15, FKBP5 mRNA was higher in stressed animals, irrespective of RU486 treatment. *p < 0.05, **p < 0.05, ***p < 0.001.

revealed complex interactions between brain region, stress, RU486 and time. Notwithstanding this complexity the data do yield insights in consistent or, rather, transient changes after stress and the RU486 intervention. The comparison between the effects of stress after 8 and 15 days shows that adaptations to a single day of stress are dynamic and certainly are not complete after one week. For example, the expression of Sgk1 and FKBP5 mRNA in PVN and amygdala was initially reduced, but after 15 days actually higher in SPS rats compared to non-stressed controls. This observation alone begs the question of what happens upon longer term follow up after SPS. This notion of longer term follow

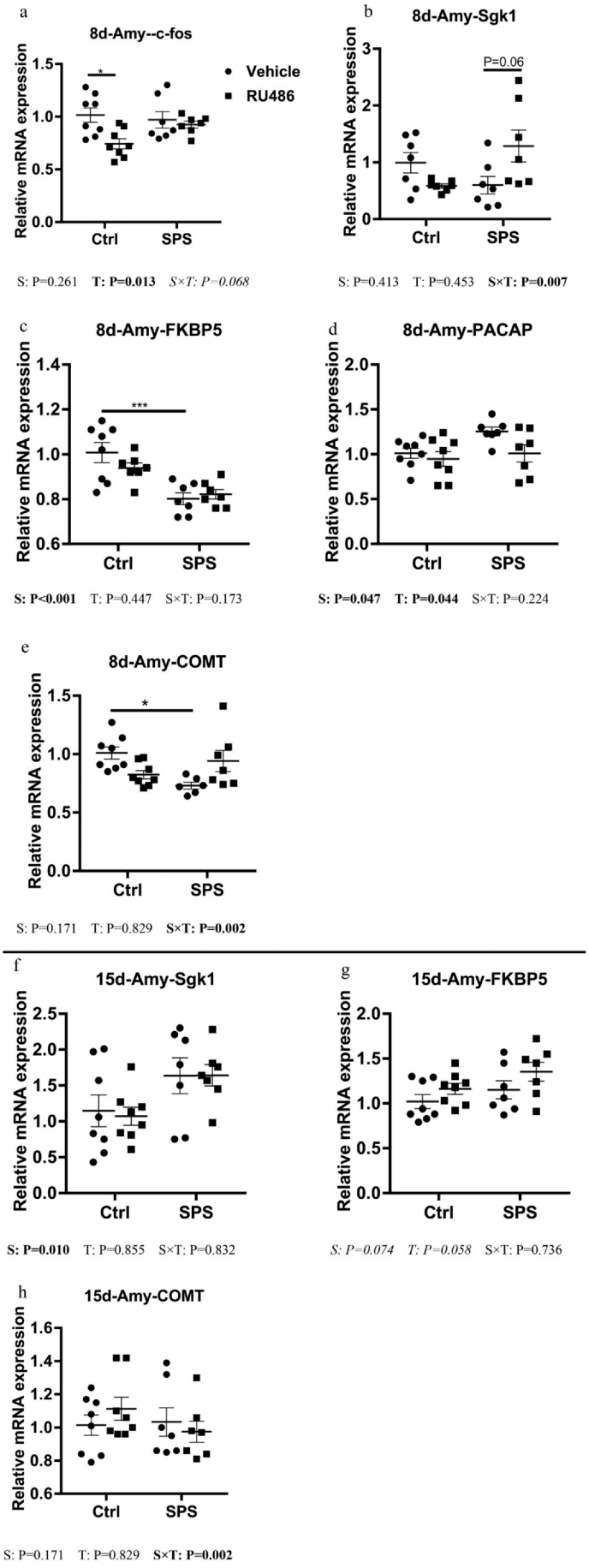


Fig. 5. Effect of stress and RU486 treatment on gene expression in the amygdala. a: C-fos mRNA expression at day 8was overall suppressed by RU486 treatment, and this effect was more pronounced in control rats. b: Sgk1 mRNA at day 8 showed a strong interaction between SPS and RU486, similar to the PVN data. c: At day 8, stress suppressed FKBP5 mRNA. d: PACAP mRNA at day 8 showed significant main effect of stress and treatment. e: At day 8, COMT mRNA expression showed a significant interaction between stress and RU486, similar to Sgk1 mRNA. f: At day 15, stress upregulated the sgk1 mRNA expression. g: At day 15 FKBP5 mRNA expression was not different between the groups, with a tendency for upregulation by both stress and RU486. h: At day 15 COMT mRNA expression was not different between groups. *$p < 0.05$, ***$p < 0.001$.

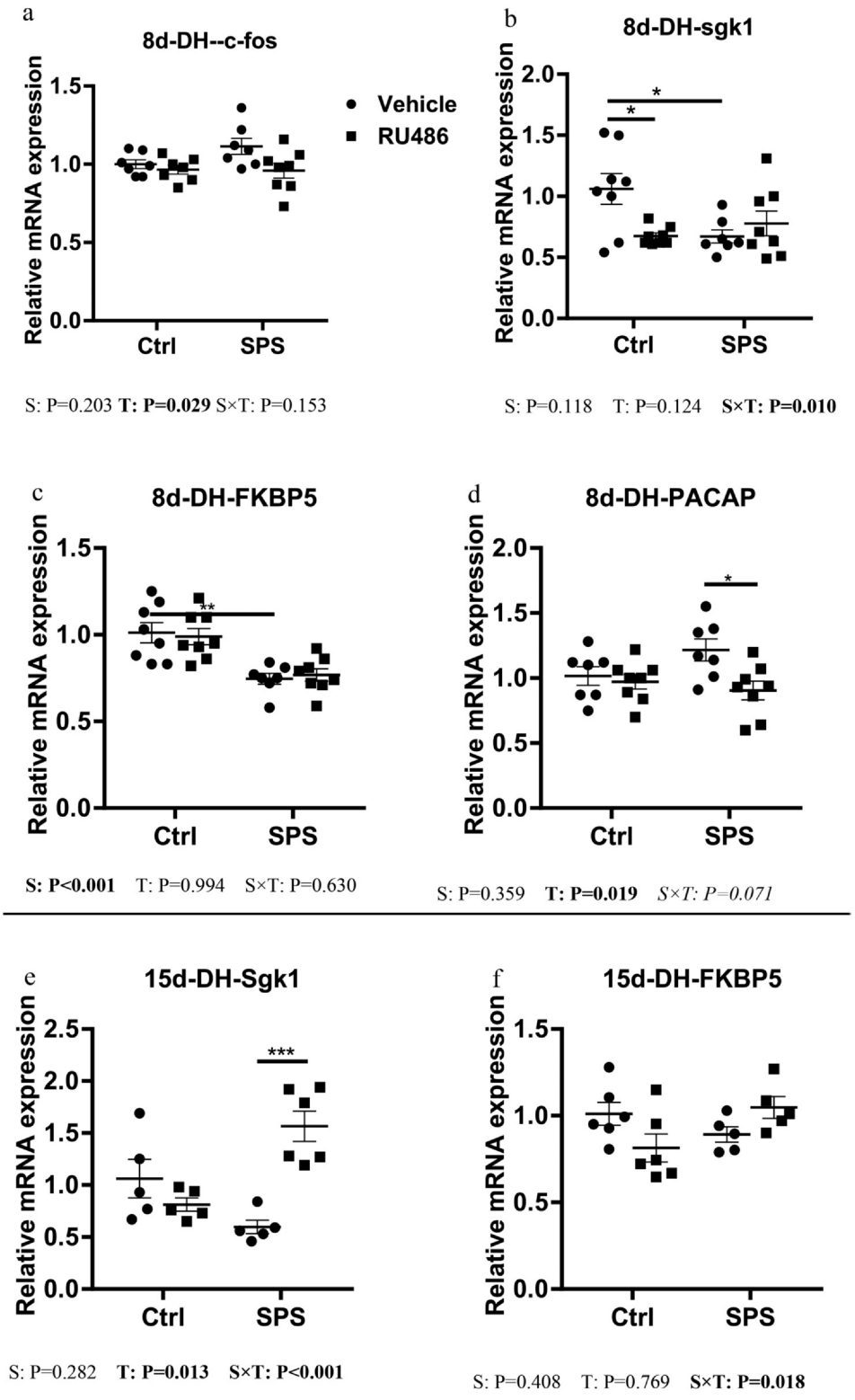


Fig. 6. Effect of stress and RU486 treatment on gene expression in the dorsal hippocampus. a: At day 8, c-fos expression was significantly, but very modestly higher after RU486 treatment. b: At day 8, Sgk1 mRNA of day 8 showed a significant interaction between stress and RU486 treatment, with reduced levels after stress and after RU486, but no further reduction by the combination. c: At day 8, stress suppressed FKBP5 mRNA expression. d: At day 8, RU486 treatment significantly suppressed PACAP mRNA expression, and this effect was stronger in stressed rats. e: At day 15, Sgk1 mRNA expression was significantly upregulated after RU486 only in stressed rats. f: At day 15, FKBP5 mRNA showed a significant interaction between stress and RU486 treatment, but no subtantial intergroup differences. *p < 0.05, **p < 0.01, ***p < 0.001.

up is supported by earlier studies that demonstrated behavioural and endocrine effects as late as 1 month after stress in adulthood (van Dijken et al., 1993; van Dijken et al., 1992). Bidirectional changes over time also were also observed for GR and FKBP5 mRNA levels in the locus coeruleus, but in an opposite direction (Serova et al., 2019). The transition from decreased to increased expression in our work and that of others also suggests that the term 'normalization' should be used with caution, as by definition levels would have momentarily 'normalized' during the transition from low to high.

C-fos mRNA expression was consistently suppressed after RU486 treatment in PVN and amygdala, but this only occurred in non-stressed rats. In addition, in the PVN c-fos mRNA showed a transient suppression one week after the stressor. Given the fact that corticosterone levels after sacrifice were in the stress-range, we cannot say whether the expression of c-fos was basal or stimulated. Regardless, RU486 treatment had long term consequences on (basal) neuronal activity in stress-

a

FKBP5 intron V

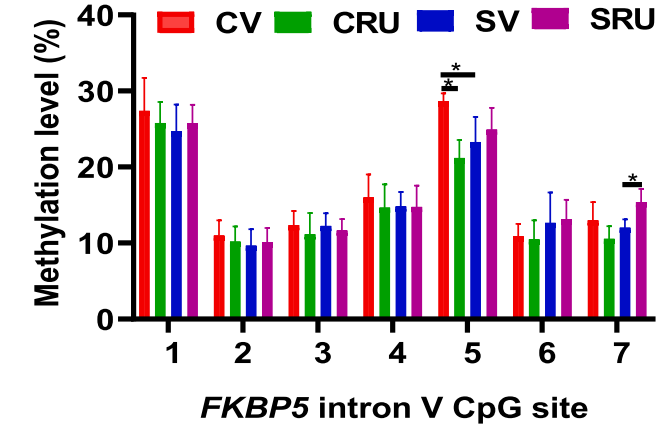
TAG**CG$_1$**TAAAGTTATTAGA**CG$_2$**TTAGTTGTTATAATTAGAGAAGAGAAAGTA

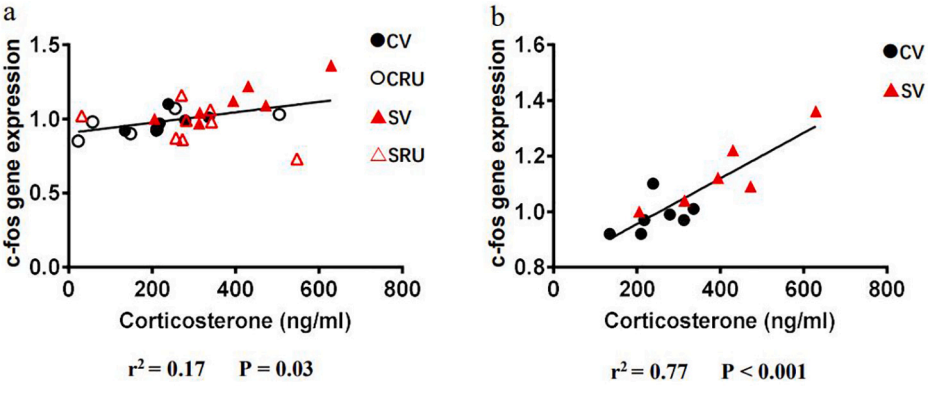
GATATTTAT**CG$_3$**AGTTAA**CG$_4$**TTTTAGGTTTTGG**CG$_5$**GTTATAGTATTAAAAA

GTTTTATAGTTTTTGTTTTTAGTTTTGTTTTTTGAAATATAAGTTGTATAGTT

Fig. 7. FKBP5 DNA methylation is affected by SPS and RU486. 7a: CpG sites in intron V of the rat FKBP5 gene. 7b: DNA methylation level (%) of the 7 sequenced CpG sites within the Fkbp5 intron V in the dorsal hippocampus. At CpG site 5 there was a significant main effect of RU486 treatment ($F_{(1,15)} = 5.492$, $p < 0.05$) and an interaction effect ($F_{(1,16)} = 13.48$, $p < 0.05$). CpG site 7 showed a significant main effect of stress and an interaction effect (stress, $F_{(1,15)} = 5.336$, $p < 0.05$, interaction, $F_{(1,15)} = 12.09$, $p < 0.05$). CV: Control + Vehicle; CRU: Control + RU486; SV: SPS + Vehicle; SRU: SPS + RU486 *$p < 0.05$.

b





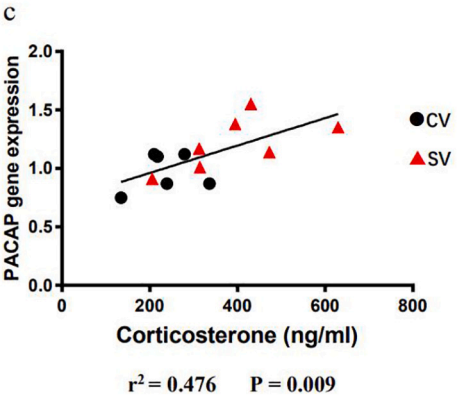


Fig. 8. Correlations between corticosterone levels at day 8 with gene expression. a: Correlations between corticosterone and dorsal hippocampus c-fos expression for all rats. b: Correlations between corticosterone and dorsal hippocampus c-fos expression only for vehicle-treated rats. c: Correlations between corticosterone and dorsal hippocampus PACAP expression for vehicle-treated rats. CV: Control + Vehicle; CRU: Control + RU486; SV: SPS + Vehicle; SRU: SPS + RU486.

related brain areas. This might well change behavioural reactivity, but it is also true that c-fos mRNA expression across all four treatment groups did not consistently correspond with behavioural readouts. The lack of efficacy of RU486 in stressed rats may reflect competition with elevated corticosterone levels, but given the high dose of RU486 used this does not seem probable. The alternative interpretation is that after stress, processes underlying neuronal reactivity had become independent of GR signalling. Interestingly, also Sgk-1 mRNA expression ceased to respond to the RU486 intervention after SPS exposure.

The c-fos mRNA expression in the dorsal hippocampus correlated with the corticosterone values on day 8. Given that corticosterone levels in all likelihood reflected an activated HPA-axis, we interpret these findings as two connected measures for stress reactivity that likely indicate the state of the animal at the moment of sacrifice. The RU486 treatment seemed to interfere with this correlation in SPS rats.

Because RU486 is a potent antagonist of the GR, we evaluated the expression of two direct GR target genes, Sgk1 and Fkbp5. Both genes showed major changes, but their being GR targets did not predict responsiveness to RU486 treatment. For example, in the 8 days experiment, Fkbp5 mRNA was reduced in all stress groups without any effect of RU486 treatment. Also in the 15 days protocol, there were only borderline significant (interaction) effects of RU486 on the expression of Fkbp5 mRNA. High FKBP5 expression is thought to suppress GR signalling (Touma et al., 2011), and low FKBP5 expression after SPS would therefore be supportive of the previously reported hyper-sensitivity of GR at 7 days after SPS that was originally reported (Liberzon et al., 1999). It is unclear whether and how the low FKBP5 expression in the SPS rats relates to higher corticosterone concentrations at sacrifice; this would be in line with one study that found that hippocampal GR actually *stimulates* HPA axis activity (van Haarst et al., 1997).

In contrast to FKBP5, Sgk1 mRNA showed pronounced interaction effects between stress and RU486 treatment in the 8 days protocol, and for the hippocampus also in the 15 days protocol. Because the genomic binding site for Sgk1 is known, it may be of interest to study dynamics of GR binding at this locus with ChIP (Mifsud and Reul, 2016). COMT mRNA expression in the amygdala was low in the 8 days SPS rats. However, given that expression is also low in RU486-treated control rats, low COMT mRNA is certainly not sufficient to explain the behavioural data.

Perhaps the most robust change in gene expression that we observed was the lowered expression of Fkbp5 mRNA in all brain evaluated brain regions at 8 days after SPS, irrespective of RU486 treatment. As methylation of the Fkbp5 regulatory regions in the DNA has received much attention (Zhu et al., 2016), we evaluated CpG methylation at this timepoint for the hippocampus. We observed a lowered methylation of CpG 7 in SPS rats, but also in RU486-treated control rats. The fact that a lower methylation degree is coupled to higher expression is counter-intuitive but not by definition impossible (Sawamura et al., 2016). However, the dissociation between mRNA expression and methylation suggests that the demethylation is at best necessary, but not sufficient for changes in gene expression of FKBP5.

Overall RU486 treatment affects the outcome of SPS both in the 8 days and 15 days protocol, in that behaviour and corticosterone levels moved towards normalization. However, brain correlates tended to be specific to either protocol. Unfortunately, we had to change more than one variable going from the 15 days to the 8 days protocol: not only time after stress, but also time of RU486 treatment (given that treatment for the 15 day protocol coincides with termination of the 8 day experiment). This for now precludes conclusions on the exact cause of the different effects of RU486 between the two experiments, that is: total time after stress at the moment of testing, or timing of RU486 treatment after stress. The data however do allow to define a trajectory of SPS-induced changes over time, in line with a recent paper studying the noradrenergic system (Serova et al., 2019). The data also show which correlates between gene expression and behavioural/ endocrine reactivity hold over time, and this may be of use to identify factors that are involved in the effects of stress and RU486 treatment. The current data also can help to decide on time points and brain areas that should be subject to future genome wide mRNA expression studies.

After early life stress, RU486 treatment during adolescence seems to actually reverse some of the consequences of stress (Arp et al., 2016; Loi et al., 2017b; Papilloud et al., 2019). However, these studies did not extensively evaluate gene expression. Our data suggest that RU486 treatment may also be of benefit after adult life stress, although it will also have intrinsic effects (which may have gone unnoticed in previous studies). Whether changed behavioural responsiveness depends on direct effects in emotion-regulating brain regions or on endocrine reorganization (Dalm et al., 2019) remains to be determined. Moreover, it is important to realize that RU486 also best known as an antiprogestin and an abortifacient, but it has broad medical applicability, it could counteract the stress-related disease (Baulieu, 1991; Regelson, 1992). The effects of pure glucocorticoid antagonists that act on the brain (Meyer et al., 2018) will be important to evaluate in the future.

Earlier RU486 has been studied in clinical trials for treatment of depression and stress disorders (Flores et al., 2006; Taubenfeld et al., 2009). However, the changed emotional reactivity and HPA axis (re) activity that are observed suggest that its effects may be permissive rather than curative. Therefore, GR antagonism should be perhaps be considered as add-on therapy rather than monotherapy, and only in patients with a clear history of stress. In sum, our data support GR targeting as a potential treatment in stress-related psychiatric disease, but the precise mechanistic underpinning remains as yet unresolved.

Declaration of competing interest

Onno C Meijer receives research funding from Concept Therapeutics that develops GR antagonists. All other authors declare that they have no conflicts of interest.

Acknowledgements

The authors thank Trea Streefland and Rob Buurstede at the department of endocrinology, LUMC, for help with the measurement of corticosterone. This work was supported by China Scholarship Council grant 201608210229 to JD. Animal experiments were supported a grant to FH from the National Natural Science Foundation of China (NO. 81571324).

Author contributions

JD, FH and OM designed the experiments; JD, XC, MS and JL performed the animal experiments; JD, MS and OM performed the statistical analysis; JD and OM wrote the paper.

Author statement

All other authors agreed with the revised manuscript.

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