Glucocorticoid signaling in a rat model of post-traumatic stress disorder

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Late glucocorticoid receptor antagonism changes the outcome of adult life stress

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Abstract

Background: Stressors activate a wide spectrum of interacting hormonal and neuronal systems resulting in behavioral and physiological responses, with consequences for the development of psychopathology. Several recent studies demonstrated that treatment with the glucocorticoid receptor (GR) antagonist RU486 during adulthood normalized effects of early life stress. We aimed to evaluate the potential of RU486 to reverse stress-induced changes in an animal model of adult stress.

Method: We employed the single-prolonged stress (SPS) model as a multimodal stress exposure protocol in male rats. SPS rats and unstressed controls were treated with RU486 on days 8, 9, 10 after stress exposure and the effects of treatment were evaluated after another 4 days. We determined body weight gain, corticosterone levels, behavioral reactivity in anxiety tests, and brain gene expression of c-fos, corticosteroid receptors, drivers of the stress response and genes (epi-)genitally linked to PTSD.

Results: RU486 affected body weight gain, corticosterone levels and open field behavior only in SPS rats. RU486 had history-independent effects in reducing fear in the elevated plus maze and fear conditioning behavior. Gene expression analysis showed a diversity of in- and interdependent effects of stress and RU486.

Conclusion: The effects of RU486 applied 1 week after stress and measured 4 days after treatment demonstrate that in the state of post-SPS the GR-dependence of homeostatic processes has changed. This suggests that GR-mediated processes are part of allostatic regulation after adult stress. The normalization of a number of SPS-effects after RU486 treatment reinforces the potential of targeting GR for treatment of stress-related psychopathologies.

1. Introduction

Acute responses to stress are aimed to restore homeostatic balance, but chronic or severe stressors may involve a change in homeostatic set points, in a process that has been called allostasis [1]. In such situations the organism will structurally require more, or other resources maintain homeostasis [2-4]. Moreover, when a stress response is, for any reason, too strong or lasts too long the outcome can become maladaptive, increasing the risk for disease in many systems, including psychopathologies [5, 6].

Diverse stressors activate a wide spectrum of interacting hormonal and neuronal systems resulting in behavioral and physiological responses [7], such as adrenal corticosteroid hormone release. In the brain corticosteroids affect neuronal excitability and structure via binding to high affinity mineralocorticoid receptors (MR) and lower affinity glucocorticoid receptors (GR) [2]. The GR in particular is considered as the mediator of maladaptive effects of excessive corticosteroid exposure, including vulnerability to psychiatric disease [8]. This may be the case in early life stress and adult traumatic experience, which both can increase vulnerability and/or lead to posttraumatic stress disorder (PTSD) in some individuals.

Disorders like PTSD are characterized by impaired abilities to use contextual information (safety cues) in a situation of potential threat [9, 10], or impaired abilities to acquire and express inhibitory memory [11-13]. This then may result in enhanced expression of fear. Several studies have shown that administration of the GR antagonist RU38486 (RU486/Mifepristone) can block the acute effects of stress on memory and impairs formation of aversive memory such as contextual fear conditioning when administered shortly after training [14, 15]. However, in psychopathological settings, reversal of established maladaptive responses would be needed. Strikingly, recent studies demonstrated that RU486 treatment during adulthood normalized effects of early life stress in male rats, including deficits in contextual memory, changed neuronal activity and enhanced freezing behavior [16, 17]. Similar findings were obtained after stress in adolescence [18]. Although RU486 also is a potent antagonist of the progesterone receptor, and a weak antagonist for the androgen receptor [19], all these effects are generally assumed to reflect interference with GR signaling.

Here we aimed to evaluate the potential of RU486 to reverse stress-induced changes in an animal model of adult stress. We employed the single-prolonged stress (SPS) model as a multimodal stress exposure protocol for traumatic memories in rats. SPS induces changed behavioral reactivity [20] and has been proposed to model aspects of PTSD [21]. Using a factorial design, we evaluated the effects of RU486 on the behavioral and neuroendocrine consequences of SPS. To underpin these observations, we examined gene expression in the paraventricular nucleus (PVN) of hypothalamus, hippocampus and amygdala. We measured expression of *c-fos* as a marker for neuronal activity, corticosteroid receptors, drivers of the stress response (*(Crh, Avp*) and genes that have been (epi-)genitally linked to PTSD (e.g. *Pacap, Fkbp5*).

2. Materials and methods

2.1 Animals

32 adult male Wistar rats (200-220 g, 7 weeks old) were obtained from China Medical University Animal Centre to make four experimental groups of n = 8. Rats were housed (two per cage) under controlled conditions of temperature and fixed light-dark cycle (22 ± 1 °C, 12 h light/dark cycle, lights on at 7:00-19:00) with free access to standard food and tap water. All experiments were approved by the China Medical University Animal Care and were performed in accordance with the National Guideline on Animal Care.

2.2 Single prolonged stress (SPS) model

SPS was performed as previously described [22]. The protocol consisted a 2 h immobilization period, in an acrylic animal holder, which was immediately followed by a 20 min forced swim in a plexiglass cylinder (50 cm height, 24 cm diameter) filled with 24 °C fresh water (water depth: 40 cm). Rats were allowed to recuperate for 15 min and then were exposed to ether vapors until loss of consciousness. After recovery, the animals were then returned to their home cage and left undisturbed for 7 days (to allow PTSD symptoms to develop). Control animals remained in their home cage with no handling and were injected at the same time as the stressed groups.

2.3 Drugs

Mifepristone (RU486, Sigma, USA) was dissolved in DMSO (Beyotime, China) 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 performed by previously study [23, 24].

2.4 Experimental design

The design is depicted in figure 1. Animals were given 1 week of habituation after arrival in the vivarium. Body weight was first determined 3 days before SPS using electronic weighing scale. The rats were then randomly assigned into two groups: SPS or control (16 animals per group). On day 0, rats received SPS exposure, or remained in their home cage. The SPS procedure took place in a different room, and was not witnessed by control rats. On days 8, 9 and 10 the animals from both groups received intraperitoneal injection of RU486 (30 mg/Kg), or vehicle leading to 4 groups of 8 animals. One animal from the SPS plus RU486 group died during the forced swim experiment, probably from cardiac arrest. After the injections the animals were left undisturbed until day 14, when behavioral experiments were performed, with the exception of a tail bleeding for corticosterone and body weight measurements on day 11. Animals we sacrificed one day after behavioral testing in the morning.

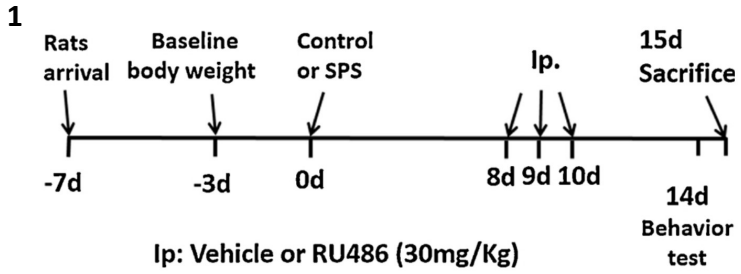


Figure 1. Schematic representation of the experimental design. On day -7, 32 rats were began to habituate 1 week after arrival in the vivarium. On day -3, determined the body weight using electronic scale as the baseline body weight. On day 0, 16 rats were exposed to the SPS stressor. The control group (another 16 rats) remained undisturbed. On days 8, 9, and 10, the animals from each group received intraperitoneal injection of RU486, or vehicle (4 groups; n = 8 rats per group). Behavioral tests include open field, elevate plus maze and freezing conditioning

were applied on day 14. Rats were sacrificed on day 15.

2.5 Plasma corticosterone measurement and body weight measurement

Blood was collected from tail in Lithium Heparinized micro tubes (#20.1282, Saestedt, Germany) on day 1, 7, 11 and centrifuged at 2000 rpm for 5 min at 20 °C to obtain the plasma and then stored at -70 °C. Tail blood samples were collected between 9:00-10:00 and, between 19:00-20:00. At sacrifice, we collected trunk blood between 10:00-11:00. Corticosterone levels were determined with an ELISA assay kit (AC-15F1, Immunodiagnostic Systems, UK) according to the manufacturer's instructions. Body weight was determined using weighing scale on day -3, 1, 3, 7, 11. Body weight on day -3 as the baseline was 222 ± 10 g on average. We expressed as the percentage weight of the increase relative to baseline.

2.6 Open-field (OF) test

The open-field test was used to study anxiety/fear-related behavior. The procedure was done as previous described [25]. The 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 corner 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 distance and time in the centre compartment were recorded. Total distance was used as locomotor activity. Percentage of time in the central compartment was used as parameter to assess anxiety-related behavior. The apparatus was cleaned with 10% ethanol before the introduction of each rat.

2.7 Elevated plus maze (EPM) test

The EPM apparatus consists of a plus-shaped maze elevated (80 cm) from the floor with two oppositely positioned closed arms (50 cm×10 cm, the walls are 30 cm high), two oppositely positioned open arms (50 cm×10 cm), and a center area (10×10 cm). Rats were placed in the central area of the maze, facing an enclosed arm, and permitted to explore freely for 5 min. Each trial was recorded by an automatic analysis system (Smart 3.0, Panlab, Barcelona, Spain). Total distance, number of center crossings, percentage time spent in the open arms and closed arms were determined. Anxiety- like behavior was assessed as decreased percentage time in

the open arm and increased percentage time in the closed arms. The maze was cleaned with 10 % ethanol solution between the trials.

2.8 Fear- condition test

Training test was performed as described previously [20]. Rats were placed in the conditioning chamber (23 × 23 × 35 cm) for 2 min with white noise (background, 60 dB). After a 2 min habituation period, an auditory cue (conditioned stimulus (CS), 2000 Hz, 80 dB) was presented for 30 s and an electrical foot shock (unconditioned stimulus (US), 2 s 1.5 mA) stimulation was delivered continuously during the last 2 s of the auditory cue. This presentation of CS-US repeated five times per session with a 30 s interval during each repeat. 30 s after the last shock the rats were returned to home-cage (figure 4g).

We measured the short-term fear memory. Two hours after training, animals were placed in this chamber and tested for freezing [26, 27]. After 2 min exploration (pre-CS) with white noise (background, 60 dB), the tone (CS, 2000 Hz, 80 dB) was presented for 30 s without a foot shock. The behavior was recorded for another 90 s, after which the rat was put back in its home-cage (figure 4i). The freezing activity was recorded and measured using Packwin 2.0 software (Panlab, Barcelona, Spain). Freezing time was used as an index of fear conditioning. Freezing was defined as immobility, excluding respiratory movements with a freezing posture more than 2 s. The chamber was cleaned using 10% ethanol after each animal.

2.9 RNA extraction, cDNA synthesis and real time quantitative PCR

Frozen brains were sliced into 60 µm coronal sections. To collect the PVN, amygdala and dorsal hippocampus, punches were made using a 1.00 mm sample corer (Fine Science Tools, Foster City, CA, USA). RNA isolation, cDNA synthesis and qPCR were performed as described previously [28]. Tested genes and their primers are described in Table 1. The relative expression of the target gene was calculated based on the threshold cycle (Ct). The ΔΔCt method was used to determine differences between groups.

Table 1. Primer sequences and size of expected product of target genes.

Gene	Primer	Sequence	Size product (bp)
GAPDH	Forward	5'-ACGGCAAGTTCAACGGCACAG-3'	148
	Reverse	5'-AAGACGCCAGTAGACTCCACGACA-3'	
Nr3c1 (GR)	Forward	5'-GCATTACCACAGCTCACCCCTAC-3'	149
	Reverse	5'-GCAATCACTTGACGCCCACC-3'	
Adcyap1 (PACAP)	Forward	5'- AACTCTTTCCTAGCCGCGAA-3'	158
	Reverse	5'-TTCCGTCCTGATCGTAAGCC-3'	
c-fos	Forward	5'-CCAAGCGGAGACAGATCAAC-3'	174
	Reverse	5'-AAGTCCAGGGAGGTCACAGA-3'	
AVP	Forward	5'-TGCCTGCTACTTCCAGAACTGC-3'	77
	Reverse	5'-AGGGGAGACACTGTCTCAGCTC-3'	
Adcyap1r1 (PAC1)	Forward	5'-GGTGAGATGGTCCTTGTAAGC-3'	198
	Reverse	5'-CCCACAAGCATCGAAGTAGT-3'	
CRH	Forward	5'- CAGAACAACAGTGCGGGCTCA-3'	119
	Reverse	5'- AAGGCAGACAGGGCGACAGAG-3'	
MR	Forward	5'-TCCAAGATCTGCTTGGTGTG-3'	239
	Reverse	5'-CCCAGCTTCTTTGACTTTCG-3'	
FKBP5	Forward	5'-AAGCATTGAGCAAGAAGGCAGTA-3'	139
	Reverse	5'-GAGGAGGGCCGAGTTCATTAG-3'	

2.10 Statistical analysis

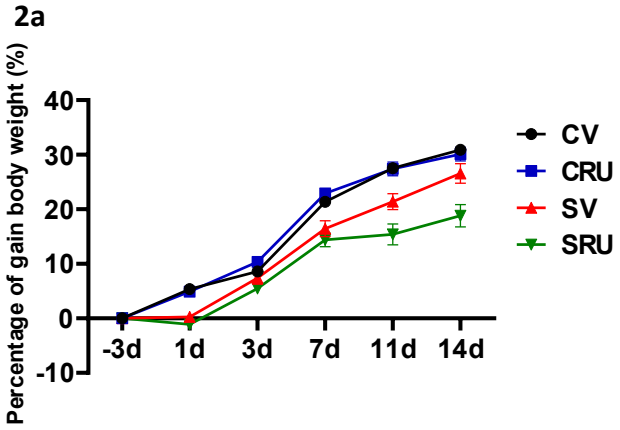
The results were expressed as Mean ± SEM. Two-way ANOVA analysis of the data was performed with SPSS 23.0 to determine main effects of treatment. Turkey's post-hoc test was used to assess significant post-hoc differences between individual groups. Unpaired t test was performed during only two group data. Differences with P-values below 0.05 were considered statistically significant.

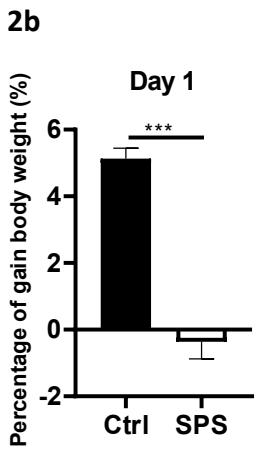
3. Results

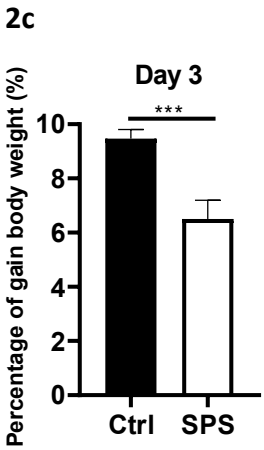
3.1 A stress x RU486 interaction in reduction of body weight

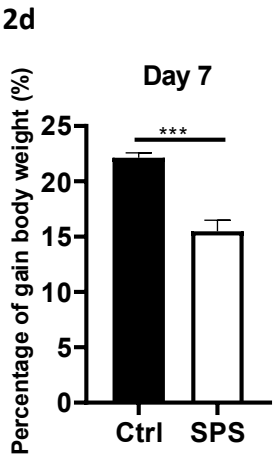
On day 1, 3 and 7, after stress and before injection of RU486, the SPS rats gained less weight than the control animals ($t = 9.54$, $p < 0.05$; $t = 4.09$, $p < 0.05$; $t = 6.50$, $p < 0.05$; Figure 2a-d). After drug treatment, the percentage body weight gain showed an effect of stress and an

interaction between stress and RU486 on day 11 ($F_{(1,25)} = 44.10$, $p < 0.05$; $F_{(1,25)} = 4.69$, $p < 0.05$, Figure 2e) and on day 14 ($F_{(1,25)} = 28.50$, $p < 0.05$; $F_{(1,25)} = 5.65$, $p < 0.05$, Figure 2f). Post hoc analysis showed that in vehicle-treated SPS rats the percentage body weight gain increased and normalized towards unstressed rats on day 14. In contrast, RU486 treated rats still had a decreased percentage of body weight gain on day 11 and on day 14. These findings indicate that stress had a transient effect on body weight and that RU486 can attenuate body weight gain, but only did so in the context of prior stress exposure.









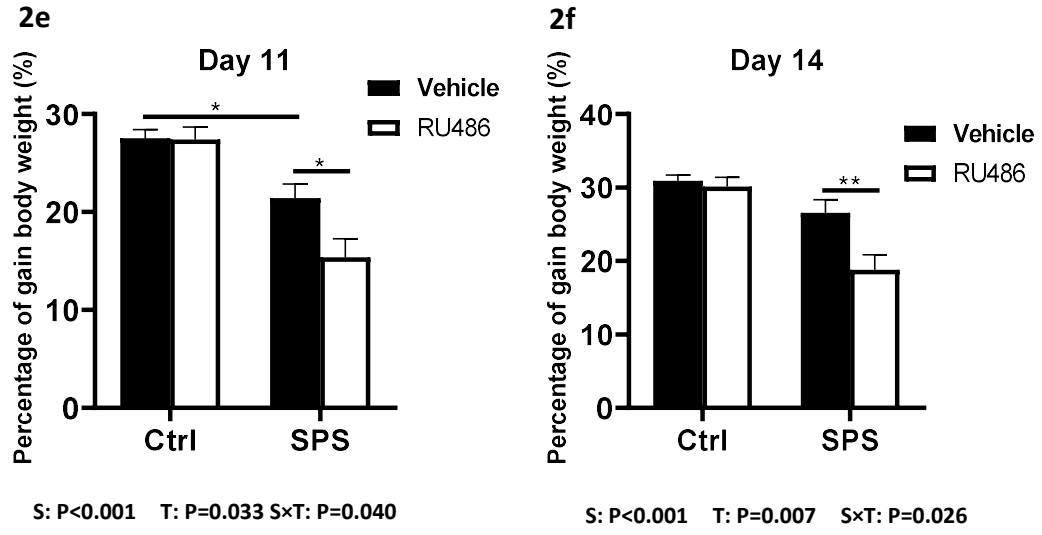
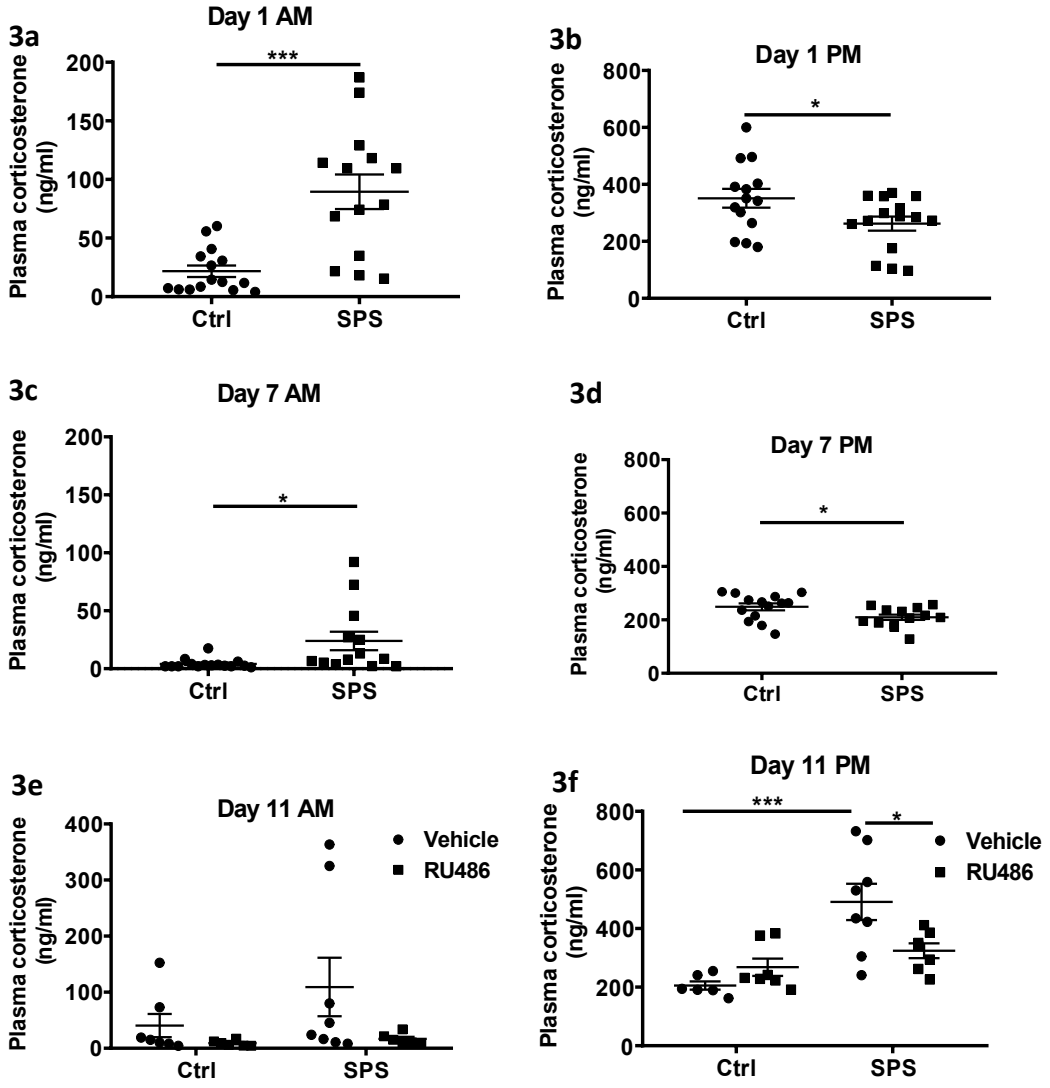


Figure 2. SPS and RU486 affect gain in body weight. 2a: The percentage gain in body weight over the experimental period. RU = RU486, V = vehicle, S = SPS, C = control. 2b: SPS attenuated percentage body weight gain on day 1, SPS vs control. 2c: SPS attenuated body weight gain percentage on day 3, SPS vs control. 2d: SPS attenuated body weight gain percentage on day 7, SPS vs control. 2e: SPS attenuated body weight gain percentage on day 11, while RU486 selectively did so only in SPS rats. 2f: RU486 selectively reduced body weight gain percentage on day 14 only in SPS rats. 2-way ANOVA outcomes are indicated by S: effect of stress; T: effect of RU486 treatment; S × T: interaction effect. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2 Plasma corticosterone level

Trough or AM corticosterone levels of the control rats were in the normal range (< 50 ng/ml). Control rats PM levels were high relative to the normal range on day 1 and lowered over time to reach 200 ng/ml on day 11 [29]. On day 1 and 7 the SPS rats had elevated AM corticosterone levels compared to the control rats ($t = 4.38$, $p < 0.05$; $t = 2.44$, $p < 0.05$; Figure 3a and Figure 3c). In contrast, evening corticosterone (PM) levels were significantly decreased in SPS rats compared to control rats ($t = 2.17$, $p < 0.05$; $t = 2.35$, $p < 0.05$) (Figure 3b and Figure 3d). Therefore, SPS led to an apparent flattening of corticosterone rhythm. After drug treatment, morning corticosterone levels on day 11 tended to be suppressed by RU486, irrespective of stress history ($F_{(1,23)} = 3.439$, $p = 0.077$) (Figure 3e). On day 11, the afternoon corticosterone levels showed a significant effect of stress ($F_{(1,24)} = 17.14$, $p < 0.05$) and a significant interaction

between stress and RU treatment (Interaction, $F_{(1,24)} = 7.668$ $p < 0.05$). Specifically, 11 days after the stress, PM corticosterone levels were clearly elevated in vehicle-treated SPS rats, while prior RU486 treatment normalized these values towards control levels (Figure 3f). On day 15, the trunk plasma corticosterone levels were consistent with the results on day 11 as RU486 treatment lead to normalization of corticosterone levels towards control levels (treatment, $F_{(1, 26)} = 19.25$, $p < 0.05$) (Figure 3g). These results indicate that in vehicle treated SPS rats, there was a trajectory from an initially blunted circadian HPA axis activity towards an overall elevated activity (with the caveat that the animals received three injections on days 8-10), and that RU486 had both intrinsic and history-dependent effects that led to normalization of the axis towards control animals.



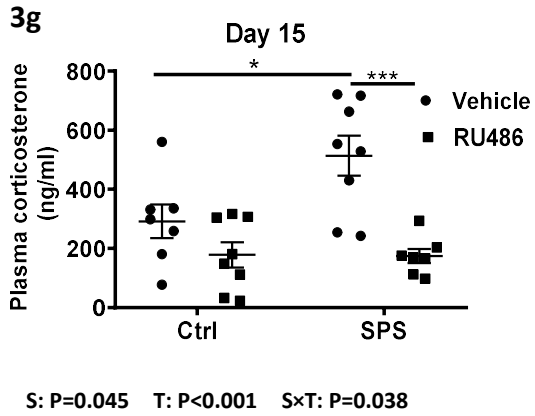


Figure 3. Plasma corticosterone level changes after SPS and RU486 treatment. 3a and 3c: Increased AM corticosterone levels in SPS rats compared with control rats on day 1 and day 7 morning. 3b and 3d: Decreased PM corticosterone level after SPS rats compared with control rats on day 1 and day 7 evening. 3e: AM corticosterone levels on day 11. The levels tended to be suppressed by RU486, irrespective of treatment. 3f: On day 11 PM corticosterone levels were increased after SPS (SPS Vehicle vs Ctrl Vehicle) and reduced after RU486 treatment (SPS Vehicle vs SPS RU486). 3 g: For trunk blood, the post-hoc data showed that corticosterone levels elevated in SPS Vehicle group compared with Ctrl Vehicle group; RU486 reversed the corticosterone levels in SPS RU486 towards to normal (SPS Vehicle vs SPS RU486). 2-way ANOVA outcomes are indicated by S: effect of stress; T: effect of RU486 treatment; S × T: interaction effect. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3 Behavioral reactivity in anxiety and fear freezing

3.3.1 Open-field test: partial reversal of stress effects by RU486

In the Open Field test there were no differences between the four groups for total distance walked, *i.e.* locomotor activity was very similar (Figure 4a). Data for time spent in the central area showed main effects for stress and RU486 (stress, $F_{(1, 27)} = 14.578$, $p < 0.05$; RU486 treatment, $F_{(1, 27)} = 5.089$, $p < 0.05$; Figure 4b). SPS led to reduced time in the central area, while RU486 lead to increased time in the central area. Although there was no formal interaction effect, post-hoc analysis showed that animals from the SPS Vehicle group spent significantly less time in the central area in comparison with Ctrl Vehicle group, but that RU486

treated SPS rats did not differ from non-stressed animals. These data indicate that RU486 was able to overcome some of the SPS-induced changes in behavioral reactivity.

3.3.2 Elevated Plus Maze test: independent effects of stress and RU486

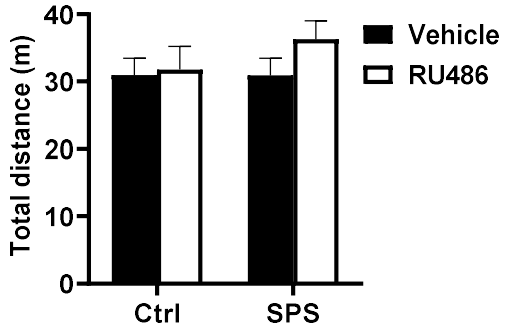
In the elevated plus maze test there were no differences for total distance and the number of center crossings (figure 4c - 4d). This indicates that the locomotor activity was similar for all four groups. The percentage time spent in open or closed arms was not affected by SPS, but RU486-treatment resulted in a lower percentage of time spent in the closed arms (RU treatment $F_{(1, 27)} = 4.992$, $P < 0.05$; Figure 4e - 4f). This was mirrored in the time spent in open arms, but this effect was not significant, likely because of interference with the central compartment. There was no interaction between SPS and RU486. These latter data indicate that RU486 had a long lasting (days) effect on behavioral reactivity irrespective of stress-history.

3.3.3 Fear conditioning test: effects of RU486 on acquisition

During the acquisition phase (Figure 4g), animals consistently froze following the shock. Freezing time during acquisition was lower after RU486 treatment for all phases following the first shock (during shock: $F_{(1, 27)} = 7.327$, $p< 0.05$; during intervals ($F_{(1, 27)} = 14.01$, $p< 0.05$, Figure 4h) and during the whole training time ($F_{(1, 27)} = 11.47$, $p< 0.05$ figure 4h). These data indicate that RU486 affected the acquisition phase of the fear conditioning, irrespective of prior stress history.

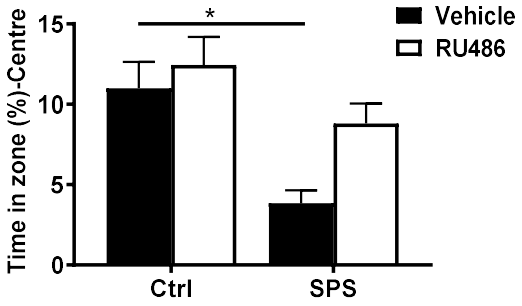
During re-exposure two hours after training (Figure 4i), the percentage of freezing time in the 120 s exploration, the data showed that RU486-treated groups had a significantly decreased percentage freezing time compare vehicle groups (treatment, $F_{(1, 27)} = 5.08$, $p < 0.05$, Figure 4j). This is in line with a reduced freezing during the acquisition phase. In the total time of the re-exposure period, the percentage time spent also was significantly lower in RU-treated animals compared to vehicle treated groups ($F_{(1, 27)} = 4.22$, $p < 0.05$, Figure 4k). These data indicate that RU486 treatment effect the fear memory acquisition and that this effect likely underlies decreased responses to re-exposure in the short term of fear memory setup.

4a



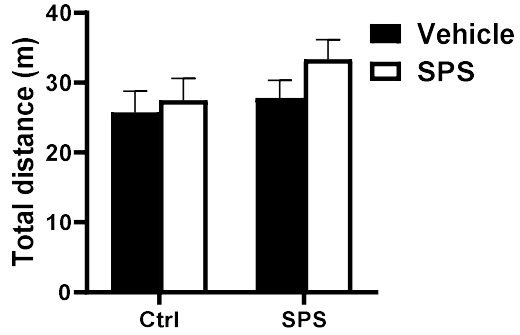
S: P=0.442 T: P=0.287 S×T: P=0.431

4b



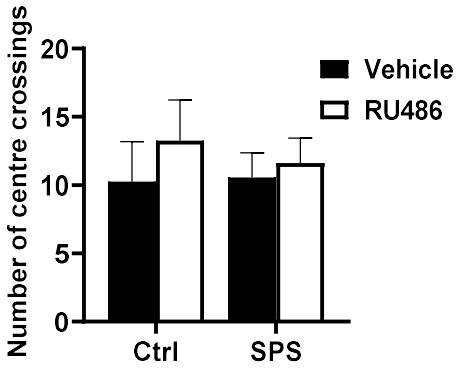
S: P<0.001 T: P=0.032 S×T: P=0.222

4c



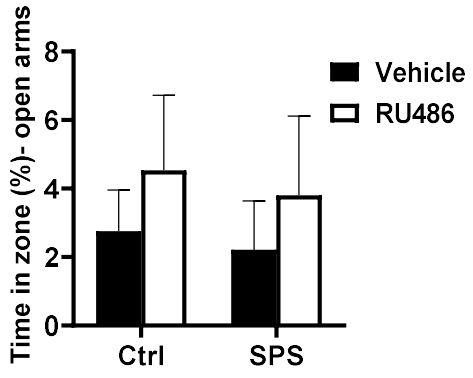
S: P=0.188 T: P=0.224 S×T: P=0.520

4d



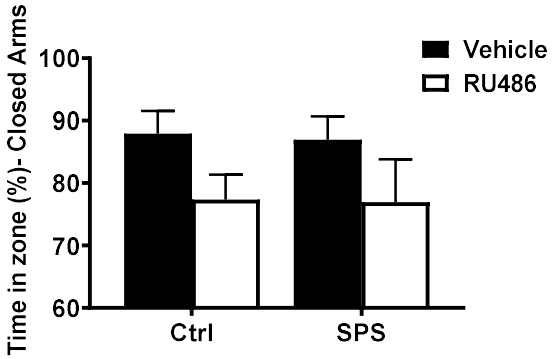
S: P=0.797 T: P=0.426 S×T: P=0.700

4e



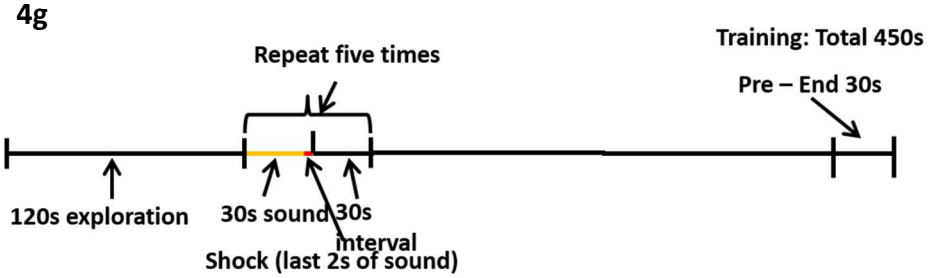
S: P=0.737 T: P=0.377 S×T: P=0.962

4f

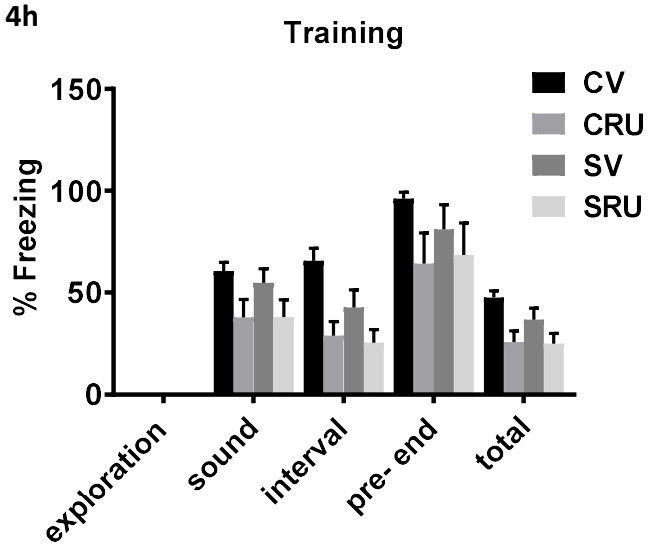


S: P=0.876 T: P=0.034 S×T: P=0.952

4g

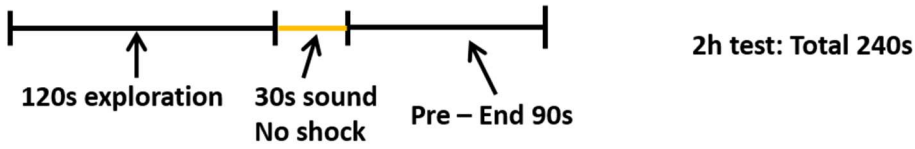


4h



Sound, T: P=0.01 Relax, T: P<0.001 Total, T: P=0.002

4i



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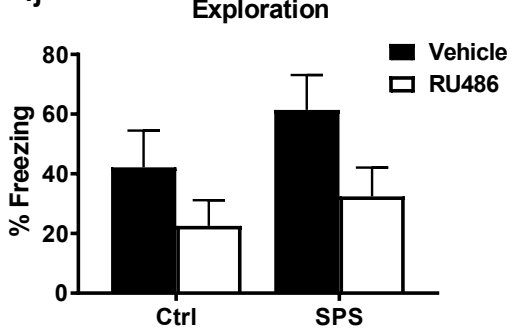
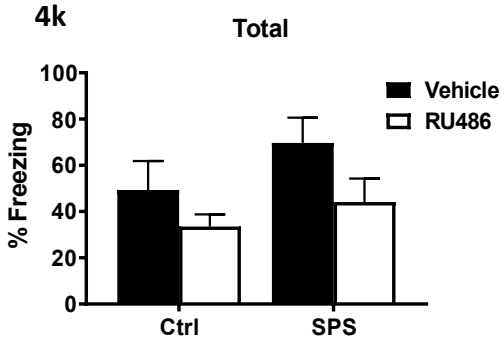


S: P=0.19 T: P=0.03 S×T: P=0.66

4k



S: P=0.14 T: P=0.049 S×T: P=0.64

Figure 4. Effects of single prolonged stress (SPS) and treatment on anxiety/fear-like behavior in the Open Field Test (4a - b), elevate plus maze test (4c – f) and freezing test (4g – k). 4a: Rats run the total distance was no difference of four groups. 4b: SPS significantly decreased the time that rats spent in centre zone, RU486 treatment led to increased time in the centre zone (main effects, Control vs SPS: $F_{(1, 27)} = 14.578$, $P < 0.05$, Vehicle vs RU486: $F_{(1, 27)} = 5.089$, $P < 0.05$). 4c–d: Total distance and the number of central crossings almost the same. 4e-f: There were no significant differences between SPS rats and control rats of percentage time in the open arms and closed arms. RU486 treatment resulted in less time spent in the closed arms (main effects, Treatment: $F_{(1, 27)} = 4.992$, $P < 0.05$). 4 g: Schematic representation of the training process design. 4 h: the percentage of freezing in all various phase during training. RU486 treatment led to decreased freezing during all stages of the acquisition phase. 4i: The re-exposure protocol 2 h after training. 4 j-k: the percentage of freezing time during exploration, re-exposure and total showed reduced freezing in RU486 treated rats irrespective of stress history. 2-way ANOVA outcomes are indicated by S: effect of stress; T: effect of RU486 treatment; S × T: interaction effect. * $p < 0.05$.

3.4 qPCR results

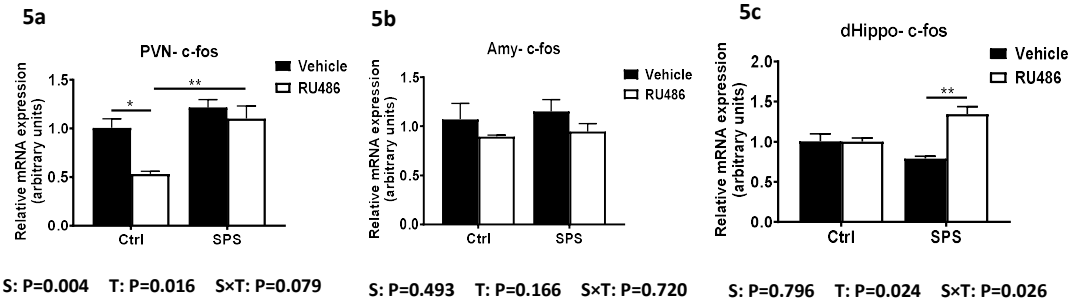
In order to find correlates for endocrine and behavioral changes, we determined gene expression in punches from the PVN (Figure 5k-5l), the dorsal hippocampus and the amygdala. C-fos mRNA was determined as a proxy for neuronal (re-)activity. Of note, these were basal c-fos mRNA levels, in the morning one day after behavioral testing. We determined expression of MR, GR as potential mediators of corticosterone effects. *Crh* and *Avp* expression was measured, given their role in driving the HPA axis. *Fkbp5, Pacap* and the gene coding for the PACAP receptor (*Pac1*) were included based on human genetic studies implicating these genes in the pathogenesis of PTSD [30, 31]. In no areas did we observe changes in PAC1, FKBP5, MR, AVP and CRH mRNA two weeks after SPS (not shown in figures; AVP and CRH mRNA (figure 5j) was only measured in PVN).

In the PVN, c-fos mRNA levels were increased in the SPS group ($F_{(1, 27)} = 10.239$, $p < 0.05$) and decreased in the RU486 group ($F_{(1, 27)} = 6.786$, $p < 0.05$, Figure 5a). There was a trend towards an interaction, in that RU486 clearly suppressed basal c-fos mRNA expression in control rats, but not in SPS animals. In the amygdala we observed no changes in c-fos mRNA (Figure 5b), while in the dorsal hippocampus there was an effect of RU486, and an interaction between stress and RU486 ($F_{(1, 27)} = 5.837$, $p < 0.05$). Here, c-fos mRNA level in SPS/RU486 group was higher than SPS/Vehicle group. This indicates that RU486 selectively led to increased basal c-fos mRNA levels in the hippocampus stressed rats (figure 5c).

In the PVN, PACAP mRNA levels were suppressed after RU486, but only in control rats (Figure 5d), mirroring the picture of c-fos mRNA. In the amygdala PACAP mRNA was decreased after stress, irrespective of RU486 treatment (Figure 5e). In the dorsal hippocampus, PACAP mRNA was higher after SPS, without an effect of RU486 (Figure 5f).

For GR mRNA changes tended to be modest in effect size. In the PVN GR mRNA was lower after SPS (Stress, $F_{(1, 27)} = 7.137$, $p < 0.05$, Figure 5g). In the amygdala, there was an interaction between stress and RU486, in that RU486 modestly suppressed GR expression only in the SPS rats (Figure 5h). In the hippocampus no changes in GR mRNA were observed (Figure 5i).

The mRNA expression indicates that two weeks after SPS and 5 days after RU486 treatment (and after behavioral testing), there are substantial changes in basal c-fos and PACAP mRNA expression, and modest changes in GR expression. These changes vary strongly by brain area and may occur either independently for SPS and RU486, or in interaction.



S: P=0.004 T: P=0.016 S×T: P=0.079 **S: P=0.493 T: P=0.166 S×T: P=0.720** **S: P=0.796 T: P=0.024 S×T: P=0.026**

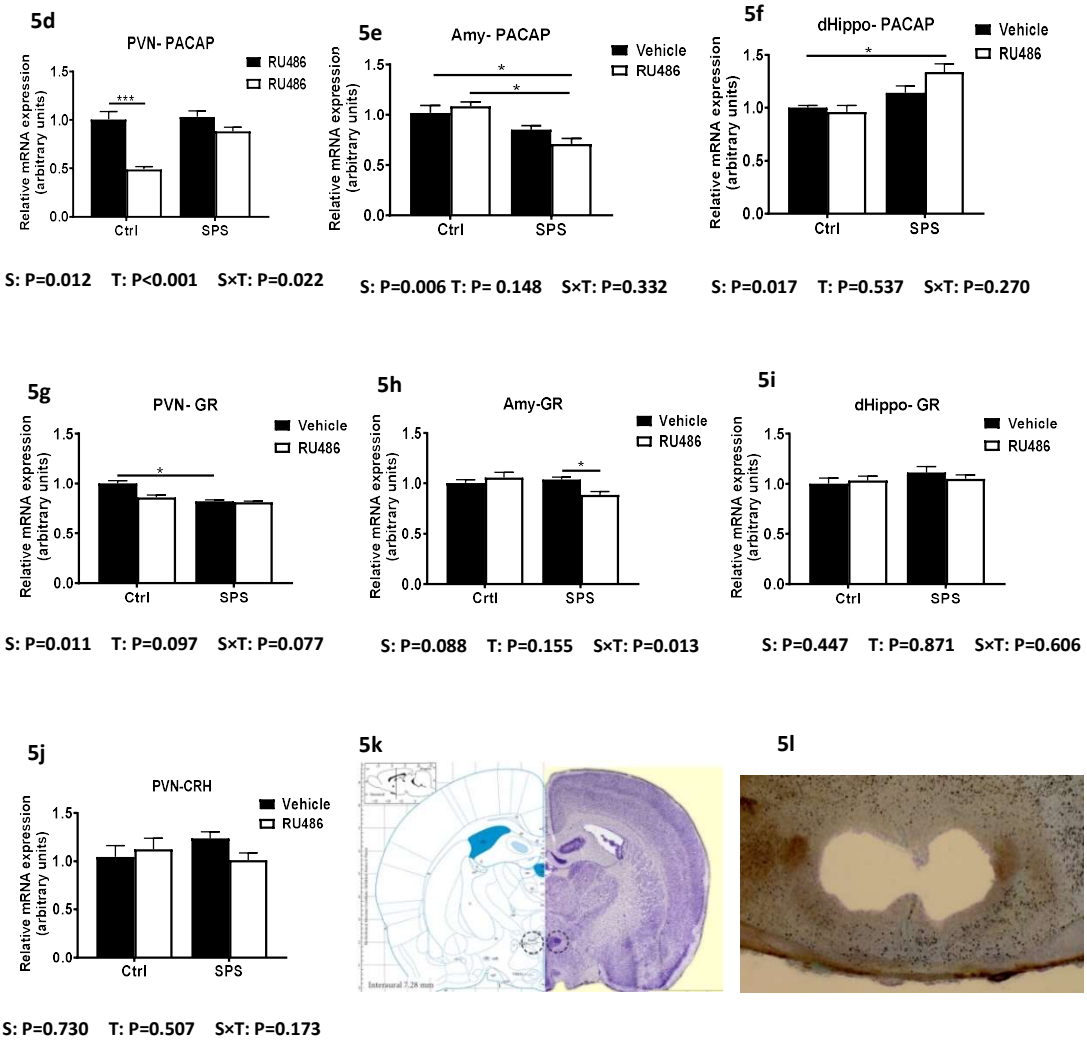


Figure 5. The results of gene mRNA expression in different brain areas. 5a: In the PVN, c-fos decreased after mifepristone treatment (treatment, $F_{(1, 27)} = 6.786$, $p < 0.05$). 5b: No change of each group. 5c: In the dorsal hippocampus, c-fos gene mRNA up-regulated between SPS versus Vehicle and SPS versus RU486 group (interaction, $F_{(1, 27)} = 5.837$, $p < 0.05$). 5d: PACAP mRNA was suppressed by RU486 compared with Vehicle in control rats in the PVN. 5e: In the amygdala, PACAP gene mRNA was decreased after stress (stress, $F_{(1, 26)} = 8.786$, $p < 0.05$). 5f: In the dorsal hippocampus, PACAP gene mRNA was increased after stress (SPS vs Ctrl, $F_{(1, 27)} = 6.909$, $p < 0.05$), post-hoc showed PACAP up-regulate in SPS RU486 rats compared with Ctrl Vehicle rats. 5 g: In the PVN, GR down regulate after SPS stress (SPS vs Ctrl, $F_{(1, 27)} = 7.137$, $p < 0.05$). 5 h: In the amygdala, The GR mRNA expression in SPS RU486 group decreased compared

with SPS Vehicle group. 5i: GR expression was no significant difference between each group in the dorsal hippocampus. 5 j: CRH mRNA expression was no significant difference between each group in the PVN. 5k: Schematic punch place in PVN in Watson and Paxinos rat atlas brain. 5l: Example of PVN punch out. 2-way ANOVA outcomes are indicated by S: effect of stress; T: effect of RU486 treatment; S × T: interaction effect. * $p < 0.05$, ** $p < 0.01$.

4. Discussion

In this study we evaluated the effects of RU486 treatment one week after rats were exposed to the three consecutive stressors of the SPS model. We timed this intervention based on the many effects one week after the SPS procedure that have been reported in the literature [32, 33]. The effects of treatment were evaluated again several days later. We found that GR antagonism had intrinsic effects on fear behavior, the HPA axis and gene expression in the brain. These effects reveal a role of GR in normal (or naïve) homeostatic processes. Moreover, RU486 interacted with stress history, in that it was able to reverse a number of stress induced changes. These effects reveal a role of GR in stress adaption over days – weeks, or allostatic processes.

It is clear from the clinic and many animal models that GR can contribute to disease processes in many different body systems [34-37]. The effects of RU486 in interaction with earlier stress experiences actually show that GR is part of maintaining an altered state of homeostatic control for days or weeks after stress. Corticosteroid signaling has been considered a cornerstone of such allostatic adaptation [38, 39], but to which extent this is the case can only be revealed by blocking GR signaling. The most basic example of such 'acquired GR dependence' in our data is perhaps the effect of RU486 on body weight gain. While SPS caused the expected reduction in body weight, this normalized after two weeks. RU486 blocked this normalization, which suggests that the restoring / maintaining normal body weight after stress depended on GR signaling, while in control rats GR signaling apparently had no role in maintaining body weight. This is reminiscent of the role of glucocorticoids during adolescence and puberty to promote ponderal growth [40].

RU486 was previously shown to partially normalize effects induced by early life stress (ELS),

in particularly enhanced fear learning [16, 17]. ELS also can act as a 'second hit' in neurodegenerative mice models, and also here RU486 can have beneficial effects [41]. GR targeting also proved effective in reinstating hippocampus neurogenesis when given in the last days of a chronic stress paradigm [42, 43]. Here, we extend these data to (reversal) effects by RU486 treatment one week after a single stress experience in adulthood. Of note, these reversal effects of RU486 occurred without reinstatement of the stressful context (save handling and injection), which is in contrast to the use of GR *agonists* in treatment of trauma or phobia [44].

4.1 HPA axis

Corticosterone levels in SPS animals revealed a trajectory from an initially blunted circadian HPA axis activity towards an overall elevated activity. In particular on day 11, some rats in the two vehicle groups showed high corticosterone plasma levels. This likely reflects stress that was induced by the sampling procedure. These elevations did not occur in RU486 treated rats – and the effect of RU486-treatment may therefore reflect stress reactivity rather than true basal levels. Initially, SPS was reported to enhance glucocorticoid feedback sensitivity 7 days after stress, which was attributed to changed MR and GR expression [45]. The lower PM peak levels are in line with a GR-dependent increased feedback sensitivity, while the increased basal trough levels would classically suggest lower MR-mediated feedback [46, 47]. In addition, the changed circadian rhythm may well reflect changed central drive to the axis. Disrupted circadian patterns of CORT may result in a 'sluggish' HPA axis response [48], Rhythmicity of the HPA axis is essential for normal homeostatic control [49], and has been linked to psychopathology in the clinic [50, 51].

Of note, our data suggest that the HPA axis is still in the process of regaining a new set point, because after day 7, PM levels became elevated. We cannot exclude that this change in trajectory may be caused by the injection paradigm. Regardless, RU486 suppressed PM corticosterone levels in SPS rats, without affecting levels in control rats. In contrast to the effect of RU486 on body weight, corticosterone levels were reversed to normal by RU486. Acute and single RU486 exposure disinhibits the HPA axis in rodents [46], and in humans this remains the case for at least 7 days [52]. In rodents, the effects of several days of RU486 treatment vary,

and can lead to suppression rather than disinhibition of the axis [53]. The mechanism for suppression is unknown, but may involve pharmacokinetic aspects (shorter half-life in rodents and 'rebound' effects after RU486 clearance while corticosterone is still elevated, changes in brain penetration), or differences in partial agonism of RU486 [54]. The present data suggest that indeed, in SPS rats, RU486 treatment can lead a normalization of basal corticosterone levels.

4.2 Behavior

In all three behavioral tests, RU486 had effects that were independent of SPS exposure. SPS 14 days earlier only affected behavior in the open field test, and – although there was no formal interaction - the combined stress and RU486 effects led to behavior of SPS-RU486 animals that was similar with control rats. The SPS procedure has previously been shown to have behavioral consequences after 7 days, including the open field test [55], elevated plus maze [56] and strength of fear conditioning [57]. One study reported that normalization of effects 14 days after SPS [58]. We found that some of the presumed changes in stress induced behavioral reactivity had normalized after 14 days, but that open field behavior still indicated increased anxiety. We used sequential analysis with three behavioral setups, which may have resulted in carry over effects between tasks and may have masked differential reactivity in for example the elevated plus maze. The effects of RU486 may be mediated via changed activity of the HPA axis. However, they also occurred in control animals where there were few changes in corticosterone level. RU486 effects may therefore also reflect changes in the brain regions important for appraisal and fear processing, including hippocampus and amygdala [59-61]. The effect on acquisition in the fear conditioning paradigm precludes strong conclusions about fear related memory formation (that is strongly affected by acute post training RU486 treatment [14, 62-65].

4.3 Gene expression in the brain

Given that the GR is a transcription factor, it seems reasonable to assume that the effects of RU486 on endocrine and behavioral (re)activity depend on changes in gene expression. We evaluated expression of a limited number of genes in three brain regions that may be involved in these effects [66-70]. C-fos was taken as a measure for neuronal activity [71]. The other

genes are either known regulators of the HPA axis and behavior (MR, GR, CRH, AVP, FKBP5), or have been implicated in pathogenesis of PTSD (PACAP, PAC1, FKBP5) [72, 73]. The punch-based mRNA quantification has limited spatial resolution, but nevertheless the results are informative. Interestingly, there is a number of clear interactions between stress and the effect of RU486, but in no instance there was an outspoken or specific normalization of SPS-induced gene expression by RU486.

C-fos mRNA expression showed a clear interaction between SPS and RU486 treatment. In the PVN, c-fos expression was dependent on (systemic) GR activation under basal conditions, but not after SPS exposure (combined with behavioral testing on day 14). Suppression of PVN neuronal activity by GR antagonism would not *a priori* be expected to depend on GR blockade in the parvocellular neurons of the HPA axis, and may rather reflect inhibition of excitatory inputs into the PVN. Such inputs would have become independent of GR activation in SPS rats. In contrast, in the hippocampus c-fos had become dependent on GR after stress, as RU486 treatment led to increased c-fos expression only in SPS rats. These stress history dependent effects of RU486 on neuronal activity may point to activation of the hippocampus (or under-activation of the PVN) in normalizing open field behavior in the SPS/RU486 group. The weak trend towards decreased basal activity of the amygdala is the only (but consistent) parallel to the dominant history-independent behavioral effects of RU486 effects that we observed.

PACAP has emerged as a key regulator of the stress response [74-76]. The PACAP expression in PVN mirrored c-fos expression, but directionality of this association remains unknown. Amygdala PACAP mRNA expression was lower after SPS and remained so after RU486 treatment, indicating changes in the brain even 14 days after stress exposure. In contrast, in the hippocampus PACAP expression was increased. RU486 was without effects in amygdala and hippocampus. The PACAP receptor gene, PAC1 did not show differences between any of the groups. We conclude that PACAP gene expression shows substantial plasticity, but that also outspoken regional specificity.

GR mRNA expression showed small history-dependent changes in PVN and amygdala, while other genes did not show differences, (PVN AVP/CRH, FKBP5, MR). Therefore, many of the

previously reported changes – mainly after 7 days – are likely to be transient. However, it is also clear from our data that at 14 days after SPS behavioral and endocrine responses and brain gene expression have not fully normalized. It will be interesting to further study the trajectory of adaptive changes during the first two weeks after SPS and beyond. It will also be of interest to vary frequency and timing or RU486 treatment. Given that RU486 had effects in naïve rats, treatment before the stressor may also change the trajectory of stressor-induced changes. Moreover, it will be of interest to see whether newer more selective antagonists and GR modulators will have similar effects [43, 77, 78].

In conclusion, the GR antagonist RU486 led to history-independent and history-dependent effects when applied one week after the single SPS procedure and tested several days after treatment. The latter demonstrate that in the state of post-SPS the GR-dependence of homeostatic processes has changed and in this way suggest that GR is part of allostatic regulation after adult stress. The fact that a number of SPS-induced changes were normalized after RU486 treatment reinforces the potential of targeting GR for treatment of stress-related psychopathologies.

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Conflict of interest

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

Author Contributions

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

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