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Ding, J.

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Jinlan Ding

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Jinlan Ding

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Promotor Prof. Dr. O. C. Meijer

Leden promotiecommissie

Prof. dr. H. G. J. M. Vermetten

Prof. dr. K. Gapp (ETHZ, Zürich)

Prof. dr. P. Campolongo (Sapienza University, Rome)

Prof. dr. C. Vinkers (AUMC, Amsterdam)

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General introduction and outline

Stress disrupts homeostasis

Stress may be defined as the state of the organism in response to a situation that (almost) exceeds our capacity to routinely adapt to it [1]. Diverse stressors activate a wide spectrum of interacting hormonal and neuronal systems to support an appropriate physiological and behavioral response. Behavior refers to the observable motor activities, that are however driven unobservable psychological and neurobiological processes. The behavioral response to stress includes the fight–flight–freeze system (related to fear) and the behavioral inhibition system (e.g., approach–avoidance conflicts, that related to anxiety) [2, 3]. The initial physiological response to stress is mediated in large measure by the neurotransmitter noradrenaline and the hormone adrenaline. The stress response also induces activation of the hypothalamus pituitary adrenal (HPA-axis) which leads to elevated concentrations of glucocorticoid hormones in the blood. These hormones are central to the work in this thesis. In the brain, increased noradrenergic activity, in concert with other mediators such as CRH, is responsible for both physiological and psychological aspects of the stress response [4].

In case of acute and transient stressors, the body's equilibrium quickly returns to normal once the threat is over. A successful acute response to stress is aimed to protect homeostatic balance. However, if the stressor continues over time, chronic stressors may involve a change in homeostatic setpoints, to a less optimal level of functioning, in a process that has been called allostasis [5]. The human body is capable of adapting its physiological processes when faced with repeated or severe stressors. Nevertheless, exposure to such stressors can through increased secretion of stress hormones ultimately result in increased allostatic load (AL) [6]. AL is a measure used to indicate the accumulated strain on physiological responses that surpasses the usual operating limits[7, 8]. This metric serves as an integrated measure of metabolic dysregulation, immune and neuroendocrine in response to stress [9]. AL is thought to cumulatively increase the risk for both physical and mental disease over the life span.

While transient acute stressors are often conceptualized as adaptive, they may contribute to disease if they are very strong. Exposure to such traumatic stressors can lead to (suppressed or overactive) deviant activities of physiological systems, and this can produce sufficient AL to disturb proper tissue- and organ functioning and ultimately lead to a disease state [5]. Post-traumatic stress disorder (PTSD) is the clearest example, and involves not only psychiatric symptoms, but also pervasive physiological impairments [10]. Several physiological disruptions commonly observed in individuals with PTSD have been documented in various systems which are associated with elevated AL [11-15]. The research discovered proof consistent with early or accelerated aging in individuals with PTSD, and the physiological consequences of aging are often linked to elevated AL [16]. However, the acute psychiatric symptoms of PTSD are the main concern in practice, and will be the focus of this thesis.

Stress and PTSD

Feeling scared is a normal response that can occur during and after experiencing traumatic stress. This instinctive "fight-or-flight" reaction is designed to safeguard individuals from potential danger. However, in PTSD the stress-induced changes act on a much longer time scale. PTSD develops only in a subset of people who have experienced an extremely traumatic event. In the most recent version of the DSM-5 (American Psychiatric Association, DSM-5), PTSD is classified into 20 symptoms in four clusters: active avoidance, intrusion, alterations in arousal and reactivity, and negative alterations in cognition and mood. The diagnostic criteria can be summarized as experiencing a stressor and having at least one intrusion symptom in association with it, one avoidance symptom, two negative changes in cognitions and mood-related symptoms, along with two symptoms related to heightened arousal and reactivity, enduring for a minimum period of one month, with functional impairment [17]. The PTSD patients display fear generalization, for example, it demonstrates how hypervigilance and exaggerated reactions towards potential dangers and even irrelevant signals [18]. Clearly military personnel and people with 'first responder' occupations (police, firefighter, medics) get regular exposure to various traumatic events frequently and are at high risk for PTSD [19-

21].

Several stress-related signaling molecules may be part of the development of PTSD. Central noradrenalin is related to arousal and vigilance [22]. CRH (corticotropin releasing hormone) is a coordinating factor of the stress response in the brain, which is activated within seconds after exposure to stress and play a central role in the adaptation of the organism to stress [23]. The high levels of glucocorticoid stress hormones secreted by the adrenal gland also may impact on the brain at different levels, and they have been hypothesized to be a major factor toward the development of PTSD [24].

Stress and HPA axis

The increased (nor-adrenalin) signaling upon stress is the consequence of activation of the sympathetic nervous system (SNS), which also includes (indirect) feedback to the brain [25]. The increased levels of glucocorticoid hormones are brought about by activation of the hypothalamic–pituitary–adrenal (HPA) axis [26]. The HPA axis is a slower system. Stress exposure stimulates parvocellular neurons in the hypothalamus produce CRH, which activates release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. This in turn stimulates cortisol secretion in humans or corticosterone release in rats from the adrenal cortex (Figure 1A). In response to acute stressors, these glucocorticoids peak at 10 to 15 minutes after the onset of the stress response.

Cortisol is a potent corticosteroid hormone and plays a key role in the body's response to stress. Corticosteroids bind to two receptor types in the brain: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Disruption of MR and GR signaling is proposed to underlie HPA axis dysregulation seen in stress-related psychiatric disorders [27]. Compared to the GR, corticosteroids have a 10-fold higher MR affinity, and this makes that MR and GR have different roles in the regulation of processes in the brain, including HPA-axis regulation [28, 29]. Its high affinity results in a high MR occupancy even under basal (non-stressful) conditions. This is thought to maintain the excitability of neuronal circuits [30] and helps maintaining low

basal corticosteroid levels through negative feedback on MR in the hippocampus. These effects involve the genomic effects, as MR and GR both act as ligand-dependent transcription factors. In contrast, full GR occupancy is increased when cortisol concentrations peak during the circadian peak or following stress [31]. The genomic effects mediated by GR take effect in the second phase of an acute stress response, typically starting around 30 minutes after the onset of stress. The peak stress concentrations also activate rapid MR- and GR-mediated non-genomic effects, presumably via membrane bound receptors [32]. Negative feedback mediated by GR involves both rapid and slow mechanisms.

The enhancement of memory consolidation for arousing experiences by glucocorticoid hormones is widely recognized [33-35]. Previous work revealed that enhanced corticosterone synthesis during fear learning strengthens the consolidation of fear memory [36, 37]. Effects mediated by GR have been associated with subsequent adaptive mechanisms, like negative feedback systems and the consolidation of recently acquired memories [38]. Corticosterone binding to GR is the principal mechanism for activation of GR to exert its memory-enhance effects [39, 40]. The administration of corticosterone or GR agonist administered into the basolateral amygdala (BLA) or hippocampus has been found to improve memory consolidation in inhibitory avoidance training or in any other training involving a significant contextual component [41, 42]. Of note, recent evidence suggests that while both noradrenalin and glucocorticoids can enhance memory strength, the effect of corticosterone is to also generalize the memories around stressful events [43]. As generalization of memories is highly relevant for PTSD, these findings emphasize the potential of glucocorticoids contribution to the pathogenesis of the disease [44].

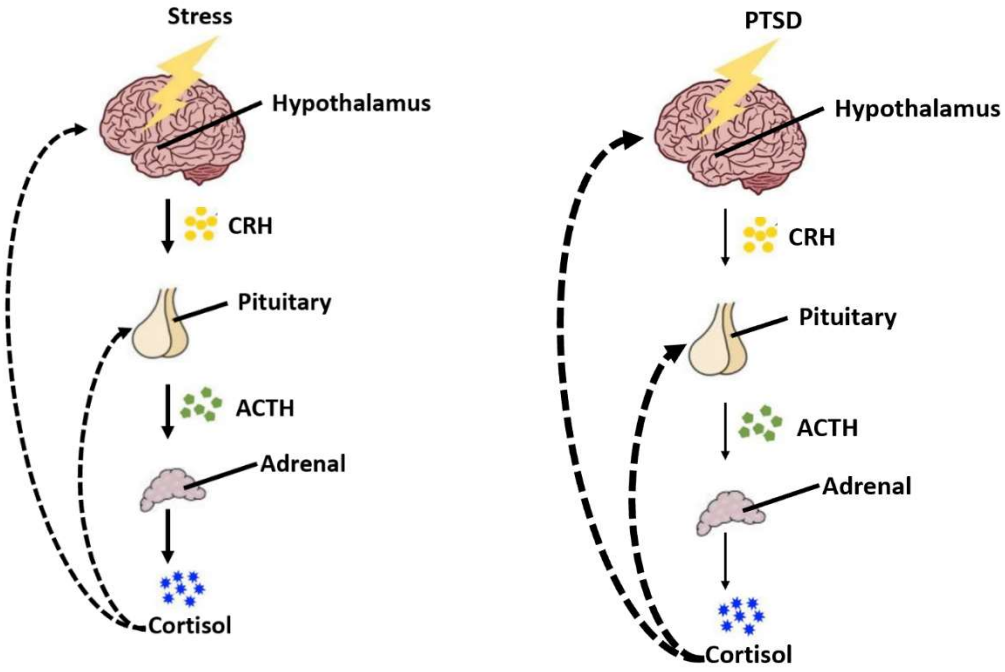

Figure 1. A: Exposure to stress and PTSD results in the release of corticosteroids via the hypothalamic-pituitary-adrenal (HPA)-axis. Cortisol exerts negative feedback on the HPA-axis and prevents a damaging overshoot. B: Most people with PTSD show a low secretion of cortisol and high secretion of CRH in hypothalamus, these suggest that enhanced negative feedback to inhibition of cortisol, itself likely due to an increased sensitivity of GR.

PTSD, the HPA axis: GR sensitivity

The initial implication of GR signaling in the pathogenesis of PTSD was based on the finding that people with PTSD display abnormally low levels of cortisol (and high concentrations of catecholamines) in urine, with (as a consequence) – a higher norepinephrine/cortisol ratio than in comparable healthy individuals [45]. This contrasts the typical acute stressor, in which both catecholamine and cortisol are elevated. With the dexamethasone suppression test, the sensitivity of GR-mediated negative feedback can be assessed. Hypersensitivity of the GR has consistently emerged as a prominent aspect in the impaired functioning of HPA axis in individuals with PTSD [46, 47]. The greater suppression of cortisol following dexamethasone administration demonstrates increased GR sensitivity at the level of the pituitary [48]. It is unknown whether this GR sensitivity generalizes to the brain. In PTSD individuals, increased

GR sensitivity may lead to negative feedback inhibition of cortisol at the pituitary, hypothalamus, or other brain regions comprising - and projecting to - the HPA axis (Figure 1B). Enhanced GR central sensitivity could possibly also be linked to changes in hippocampal volume and potentially impact various physiological systems regulated by glucocorticoids [49].

PTSD and GR genomic target genes

GR gets activated strongly by increases levels of cortisol that follow strong stressors. GR is a member of the nuclear receptor superfamily of ligand-dependent transcription factors. Upon ligand binding, GR translocates to the cell nucleus to enhance or repress transcription of target genes by a diversity of transcriptional mechanisms [50-52]. In the brain, the predominant mode of action seems to involve GR binding specific elements of DNA, termed GREs [53]. Although the genomic action of GRs has been well investigated, which of these actions play a role in the behavioral responses is still not yet very well understood at present. The expression of GR and possibly its downstream targets could serve as potential biomarkers for assessing vulnerability and treatment in (a subgroup of) PTSD patients. The FKBP5 gene is a prominent target gene of the GR. At the same time, FKBP5 protein serves as an inhibitory co-chaperone that prevents GR translocation to the nucleus. Interestingly, genetic variability in the GRE regulating FKBP5 expression was previously linked to vulnerability for negative consequences of childhood trauma – lending further credibility to a role of the GR in PTSD development [54]. Other GR target genes that can be used to assess the strength of glucocorticoid signaling include GILZ and SGK-1, which have been proposed as biomarkers of trauma-related vulnerabilities [55]. In addition, Sgk1 is reported to play a role in cellular and behavioral models of learning and memory [56]. Per1 is a GR-responsive period gene associated with the circadian rhythm, and may play a role in various cellular processes, e.g. the regulation of neuronal function [29].

β-arrestin-2 is glucocorticoid-responsive target gene that is suppressed by GR. This is accomplished at the transcriptional level by the binding of GR to intragenic glucocorticoid response elements (GREs) [57, 58]. β-arrestin-2 is of functional interest as it regulates fear/anxious memory formation, but functions in PTSD are remains unknown.

PTSD animal models

Animal models serve as a vital instrument in scientific research to investigate underlying diseases pathophysiology and neural mechanisms and for the development of novel treatments [59]. The goal of animal research in PTSD include a better comprehension of the intricate interactions among genetic, neuroendocrine and environmental aspects, to identify potential targets for innovative pharmaceutical therapies, and to evaluate drugs for their viability in treating PTSD in humans [60].

In view of the complexity of PTSD, there is no single widely accepted animal model of PTSD, but fear memory abnormalities and HPA axis dysfunction are central features of PTSD patients that should be incorporated in models. At present, numerous stress paradigms in rodents that mimic this behavioral symptom and/or neuroendocrinology alterations in PTSD. For example, fear conditioning (FC) is one of the predominant animal models of PTSD [61]. However, as the formation of fear memory is in principle adaptive, it is mainly the extent to which learned fear generalizes from 'cue' to 'context' or even further that may be considered as an aspect that is relevant to PTSD [43]. Utilizing foot shock models, researchers can effectively replicate several key symptoms of PTSD, including anxiety behavior and avoidance [62], re-experiencing, aggression and hyperarousal [63]. They have revealed that these induce substantial levels of extreme fear and stress in rodents, subsequently leading to enduring behavioral and endocrine stress responses [64, 65]. Other (components of) PTSD models include restraint stress, tail suspension, social isolation, underwater trauma, social defeat, early-life stress, chronic stress, and single-prolonged stress (SPS) [66, 67].

We have used the SPS paradigm in the work described in this thesis. SPS is the first experimental paradigm that could replicate changes in HPA axis similar to those observed in PTSD patients [68]. It reflects the core of PTSD the endocrine phenotype [69], namely negative feedback enhancement. SPS is a protocol that exposes individual rats to three stressors in a sequential and multimodal manner, as a means to mimic traumatic stress. Given that SPS rats mimic both the enhanced glucocorticoid negative feedback and anxiety-like behavior that are

observed in PTSD, the model provides a valuable means to investigate the involvement of the HPA-axis in PTSD [70]. In addition, SPS rats exhibit enhanced consolidation and impaired extinction of conditioned fear memory suggests that this model has additional value [71]. The validity of the SPS model in investigating PTSD are highlighted by its ability to replicate a range of behavioral, molecular, and physiological changes observed in PTSD patients [72].

PTSD and treatment

PTSD is not easy to treat, and treatment options do not suffice to help all patients. It is estimated that approximately 30% of individuals with PTSD do not respond to first-line treatments such as cognitive behavioral therapy (CBT) and antidepressant drugs such as SSRIs [73, 74]. This can be a frustrating and it can lead to a sense of hopelessness and a belief that the condition is untreatable [75]. At present, various therapeutic options have been recommended for patients suffering from PTSD, which mainly include pharmacotherapy and psychotherapy. The latter is often combined with eye movement desensitization and reprocessing (EMDR), many patients have good response to exposure therapy and EMDR. Since the precise mechanisms of PTSD remain unknown, rational (mechanism based) pharmacotherapeutic treatment interventions have not yet been established. The GR has been proposed as a potential factor in the neurobiological processes related to PTSD development or maintenance [76]. The reduced cortisol levels in patients with PTSD have been linked to heightened GR responsiveness or sensitivity, at least at the level of the pituitary [77]. An excessively active central GR may be a crucial factor in the development of PTSD, due to its disruption of adaptive fear memory regulation [78]. In one of the chapters of this thesis we address the question of central GR sensitivity.

If central GR overactivation contributes to the maintenance or development of PTSD, using GR antagonism could be beneficial in preventing the onset of PTSD [79]. Strikingly, the previous studies in rodents has shown that administering the GR antagonist RU486 to adult male rats can restore the negative effects of early life stress. These effects consisted of deficits in contextual memory, altered neuronal activity and increased freezing behavior [80, 81]. The

studies from Papilloud et al. [82] also showed that treatment with RU486 during adulthood successfully reversed the atypical aggressive behavior in rats that experienced stress in prepuberty. In this thesis, we evaluated reversibility of the effects of adult stress by GR antagonist RU486.

THESIS OUTLINE

In this thesis, we investigated the GR sensitivity and behavior in the PTSD. We evaluated the effect of RU486 treatment after rats were exposed to the three consecutive stressors of the SPS model (chapter 2-3). We aimed to identify a sensitization of brain GR signaling that extends beyond direct negative feedback regulation (chapter 4). Lastly, we provide evidence for a role of β-arrestin-2 as a modulator of regulating amygdala activity in response to fear/anxious memory of PTSD (chapter 5).

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2

Late glucocorticoid receptor antagonism changes the outcome of adult life stress

Jinlan Ding

Marcia Santos da Silvaa

Jolanthe Lingeman

Xinzhao Chen

Yuxiu Shi

Fang Han

Onno C. Meijer

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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.

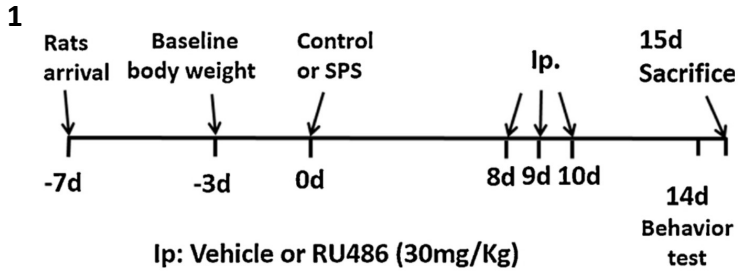


Figure 1. Schematic representation of the experimental design. On day -7, 32 rats were began to habituate 1 week after arrival in the vivarium. On day -3, determined the body weight using electronic scale as the baseline body weight. On day 0, 16 rats were exposed to the SPS stressor. The control group (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

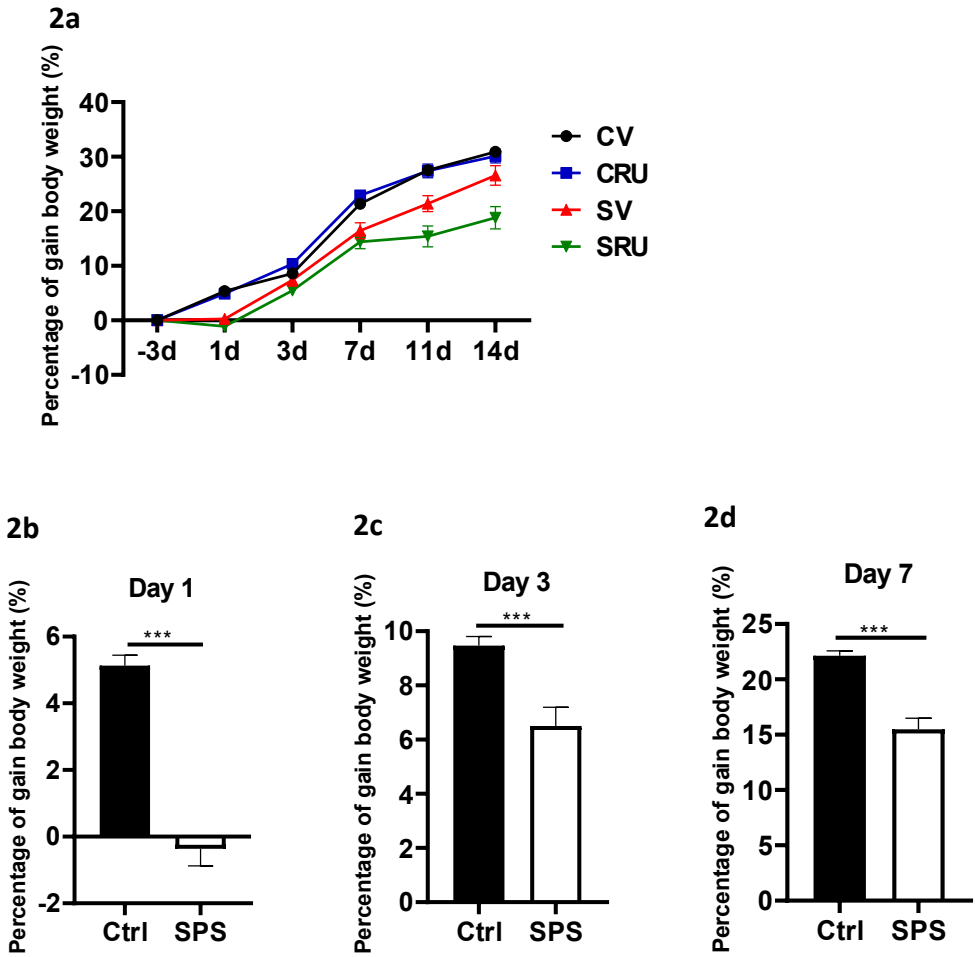
The results were expressed as Mean ± SEM. Two-way ANOVA analysis of the data was performed with SPSS 23.0 to determine main effects of treatment. Turkey's post-hoc test was used to assess significant post-hoc differences between individual groups. Unpaired t test was performed during only two group data.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



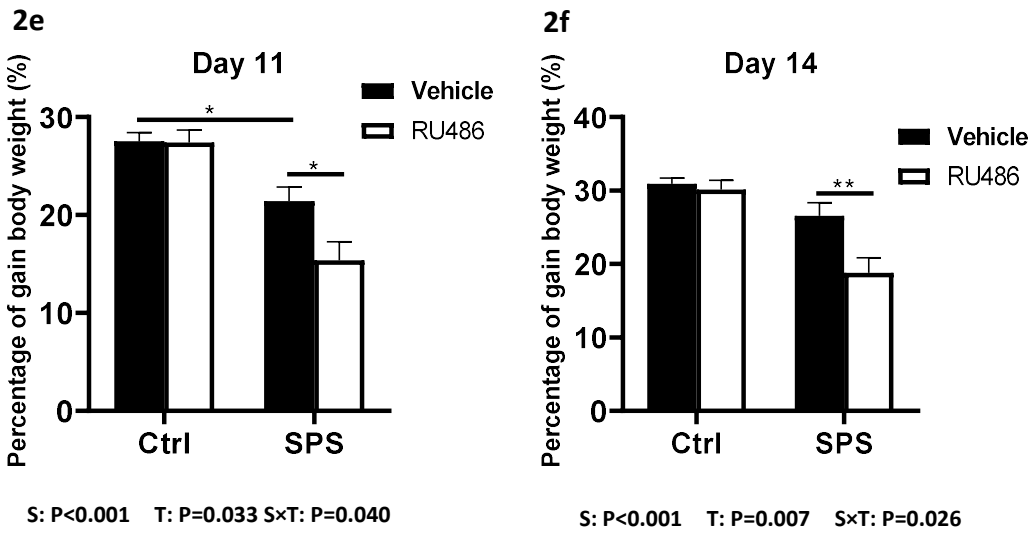
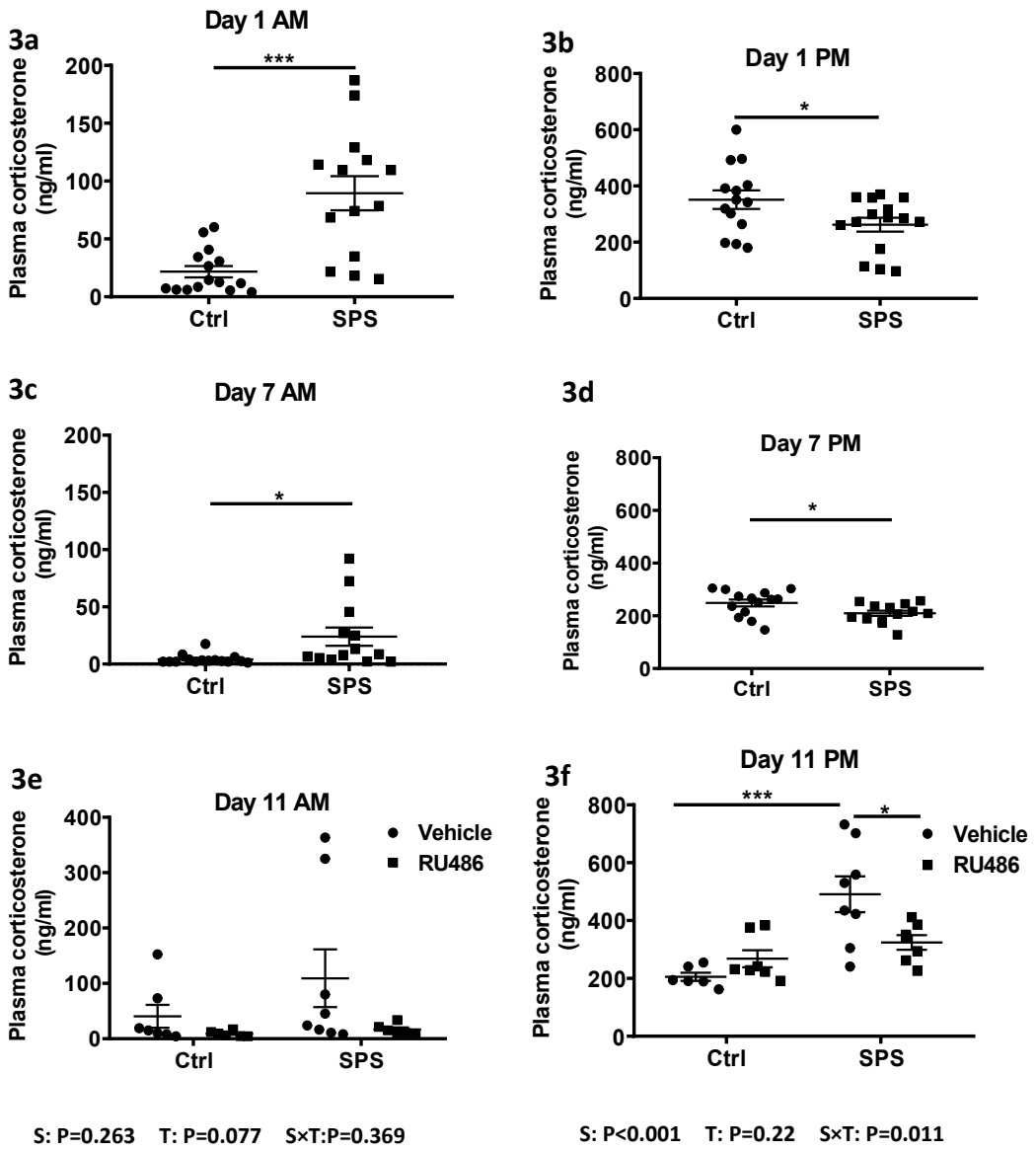


Figure 2. SPS and RU486 affect gain in body weight. 2a: The percentage gain in body weight over the experimental period. RU = RU486, V = vehicle, S = SPS, C = control. 2b: SPS attenuated percentage body weight gain on day 1, SPS vs control. 2c: SPS attenuated body weight gain percentage on day 3, SPS 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.



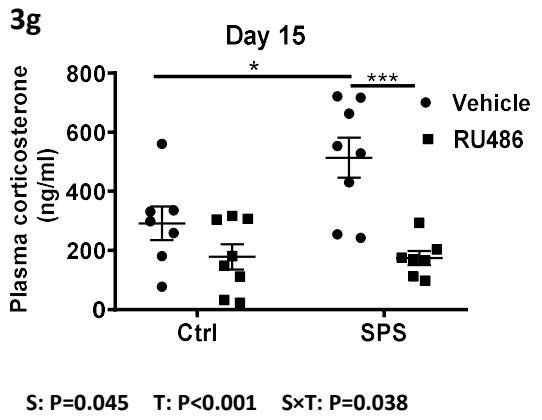


Figure 3. Plasma corticosterone level changes after SPS and RU486 treatment. 3a and 3c: Increased AM corticosterone levels in SPS rats compared with control rats on day 1 and day 7 morning. 3b and 3d: Decreased PM corticosterone level after SPS rats compared with control rats on day 1 and day 7 evening. 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.

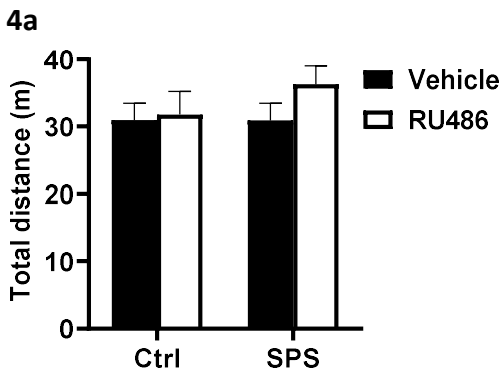
3.3.2 Elevated Plus Maze test: independent effects of stress and RU486

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.

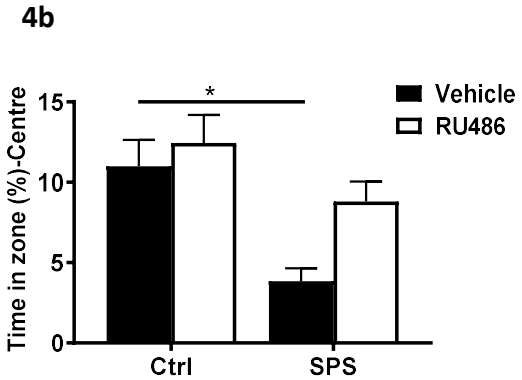
3.3.3 Fear conditioning test: effects of RU486 on acquisition

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.

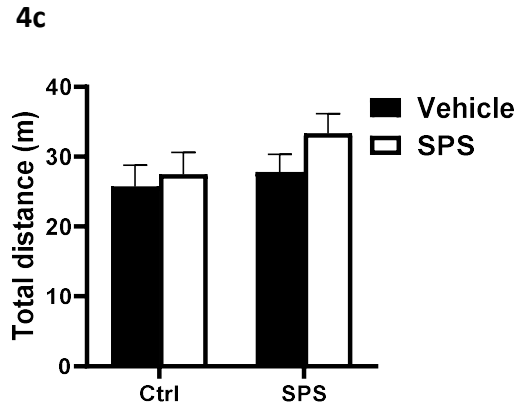
During re-exposure two hours after training (Figure 4i), the percentage of freezing time in the 120 s exploration, the data showed that RU486-treated groups had a significantly decreased percentage freezing time compare vehicle groups (treatment, $F_{(1, 27)} = 5.08$, $p < 0.05$, Figure 4j). This is 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.



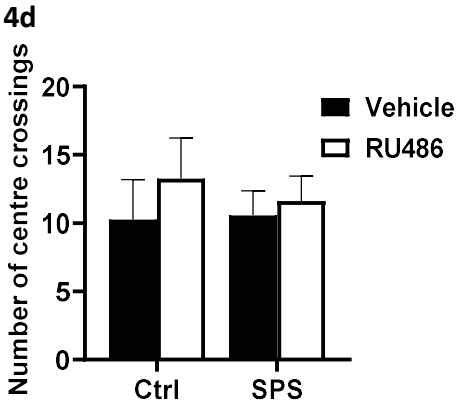
S: P=0.442 T: P=0.287 S×T: P=0.431



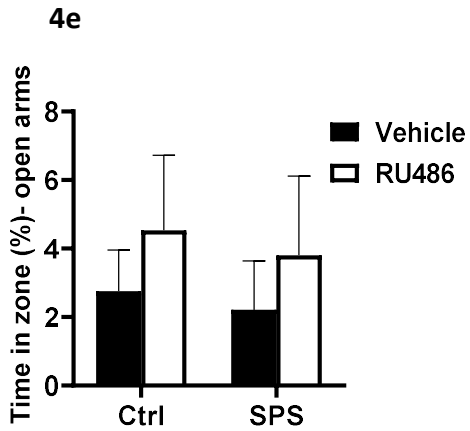
S: P<0.001 T: P=0.032 S×T: P=0.222



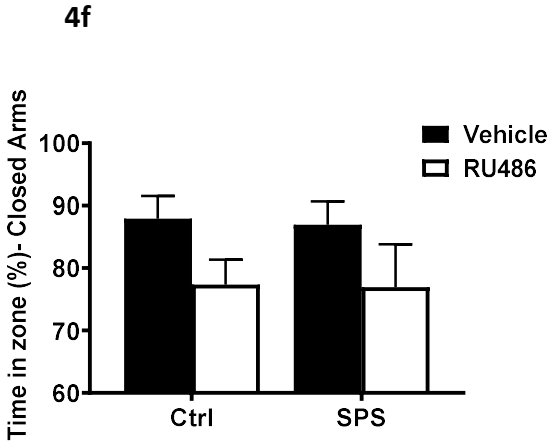
S: P=0.188 T: P=0.224 S×T: P=0.520



S: P=0.797 T: P=0.426 S×T: P=0.700

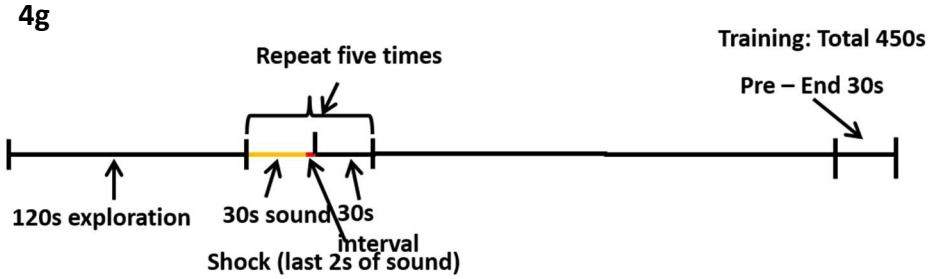


S: P=0.737 T: P=0.377 S×T: P=0.962

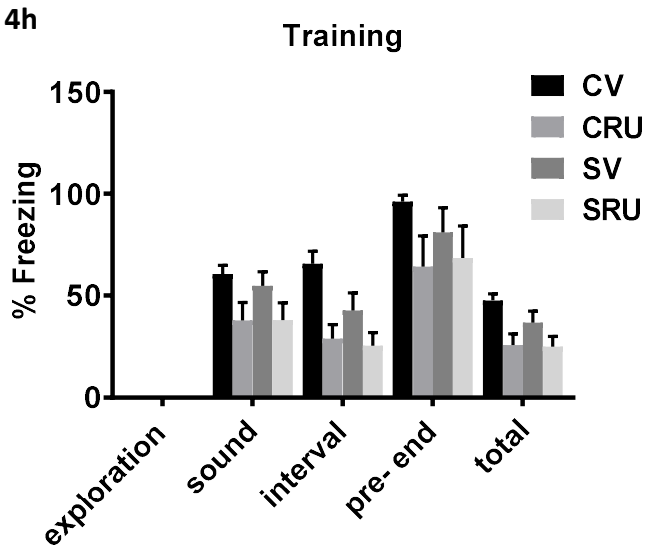


S: P=0.876 T: P=0.034 S×T: P=0.952

4g

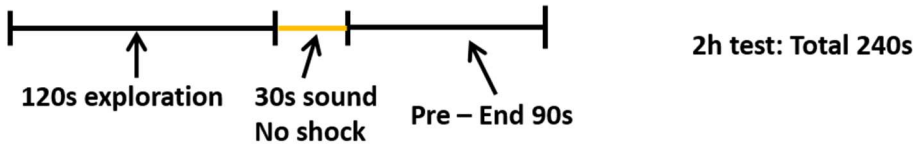


4h

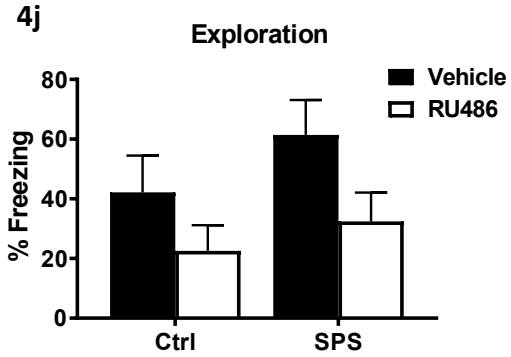


Sound, T: P=0.01 Relax, T: P<0.001 Total, T: P=0.002

4i

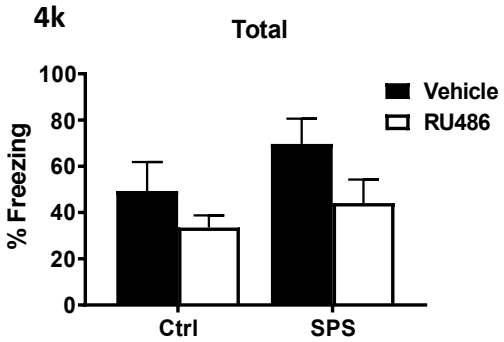


4j



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

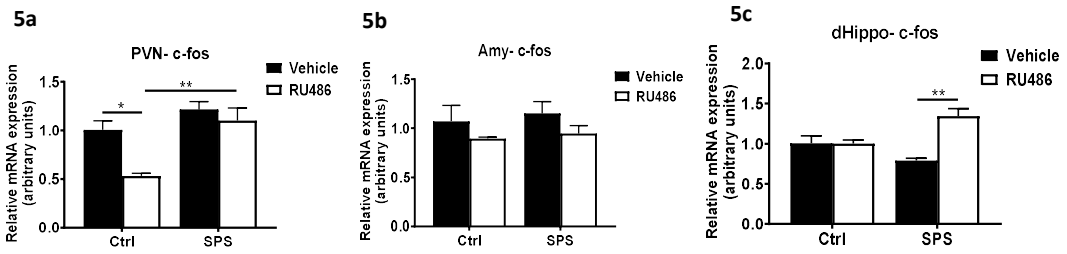
In order to find correlates for endocrine and behavioral changes, we determined gene expression in punches from the PVN (Figure 5k-5l), the dorsal hippocampus and the amygdala. C-fos mRNA was determined as a proxy for neuronal (re-)activity. Of note, these were basal c-fos mRNA levels, in the morning 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

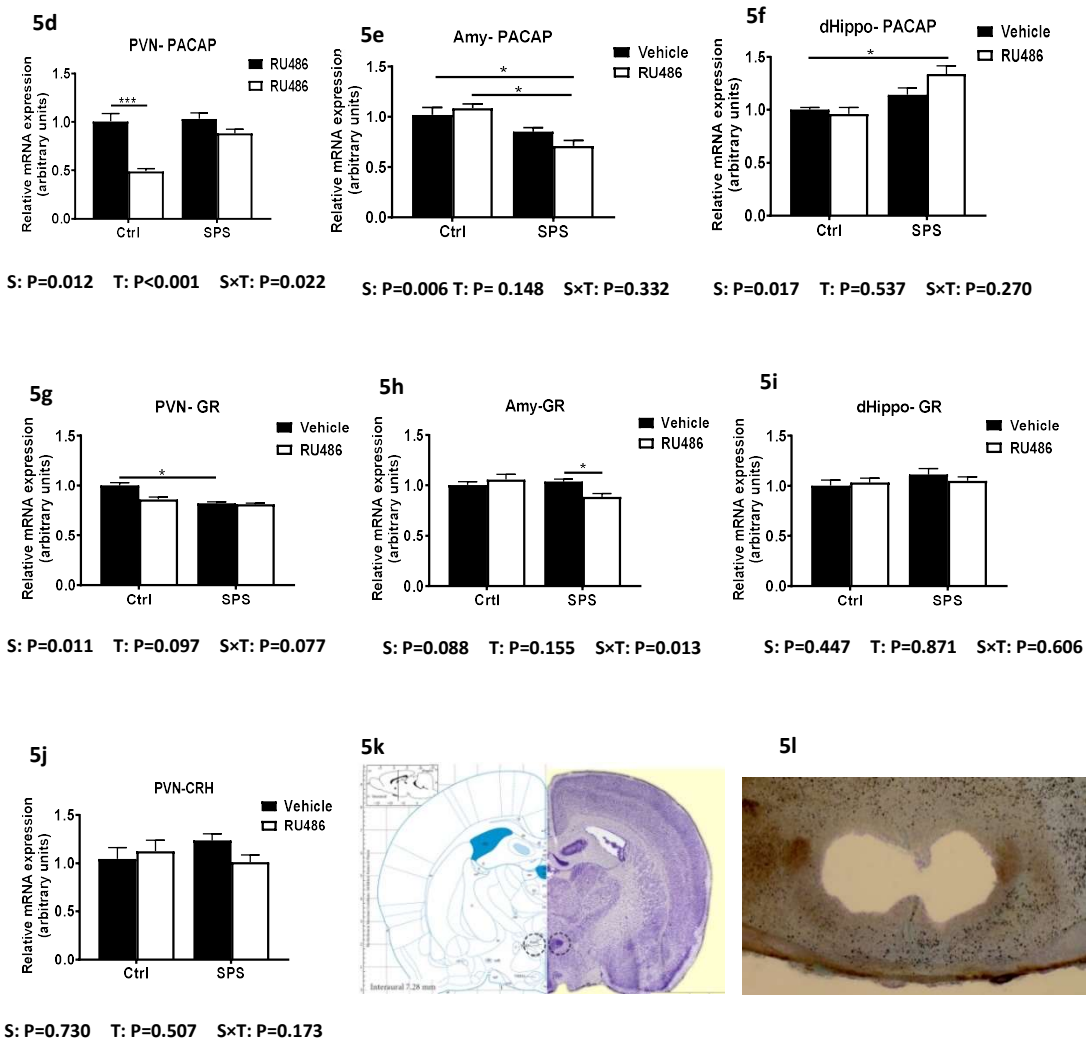


Figure 5. The results of gene mRNA expression in different brain areas. 5a: In the PVN, c-fos decreased after mifepristone treatment (treatment, $F_{(1, 27)} = 6.786$, $p < 0.05$). 5b: No change of each group. 5c: In the dorsal hippocampus, c-fos gene mRNA up-regulated between SPS versus Vehicle and 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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3

Effects of RU486 treatment after Single Prolonged Stress depend on the post-stress interval

Jinlan Ding

Xinzhao Chen

Marcia Santos da Silva

Jolanthe Lingeman

Fang Han

Onno C Meijer

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Abstract

The Single Prolonged Stress protocol is considered a model for PTSD, as it induces long lasting changes in rat behavior 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 behavior. 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 behavioral/ endocrine outcome of stressful experiences.

1. Introduction

Stress leads to many neuronal and endocrine responses that promote homeostatic and behavioral 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) [1]. 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 [2, 3]. 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 [4-6].

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 [7-10]. Understanding both the nature and timing of potential interventions is critical to develop such a pharmacotherapeutic approach [11]. 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[12-14], although such reversal effects are not always found [15]. GR antagonist treatment therefore is a potential strategy for PTSD and other stress-related disease [16-18].

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 [19]. 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 behavior, the expression of several candidate genes in the hypothalamic paraventricular

nucleus (PVN) and limbic brain regions, 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 figure 1. The second experiment was published previously [19], here we include new measurements on some target genes.

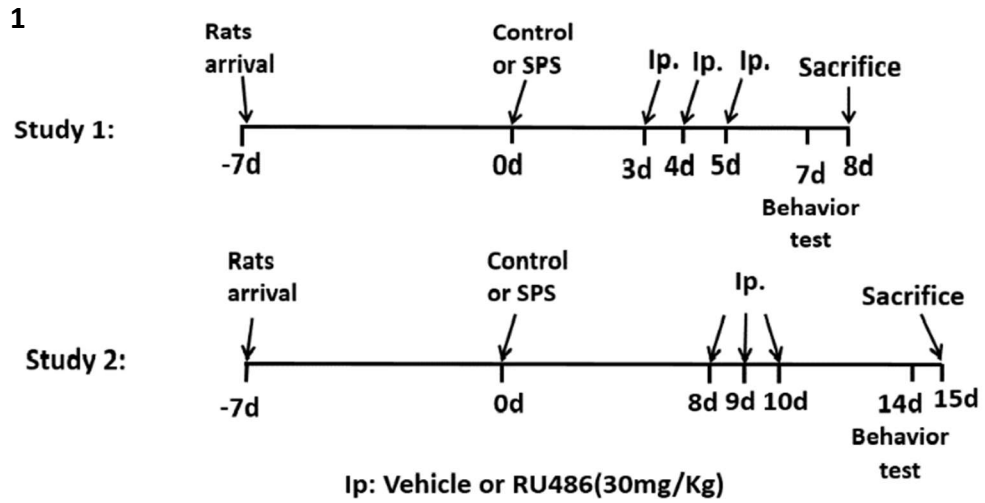


Figure 1. Schematic diagram of experiment timeline. Animals habituated 1 week after arrival in the vivarium. On day 0, the stress paradigm was performed. From day 3 (study 1) or 8 (study 2), the animals from control or SPS group received three consecutive days intraperitoneal

injection of RU486, or vehicle (n = 8 rats per group). Behavioral tests were applied on day 7 or 14. Rats were sacrificed on day 8 or 15.

2.2.1 Single-prolonged stress (SPS) paradigm.

The single session of prolonged stress was performed as previously described [20]. 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 [21, 22].

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 behavioral 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 behavior at 14 days, and killed on the morning of day 15 ('study 2') [19].

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 12000 rpm 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 [19].

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 5min. 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 cryostat and regions of interest were punched out as described previously [19]: 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.

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

3

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 ([23], table 2, Figure 7a).

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)

2.7 Statistical analysis

The results were expressed as Mean ± SEM. Comparisons between two groups were evaluated using unpaired t-tests. For all 2 x 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 (figure 2a, $p < 0.05$). On day 8 after SPS, there were main effects of stress ($F_{(1,25)} = 6.056$, $P<0.05$, figure 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 behavioral testing the day before.

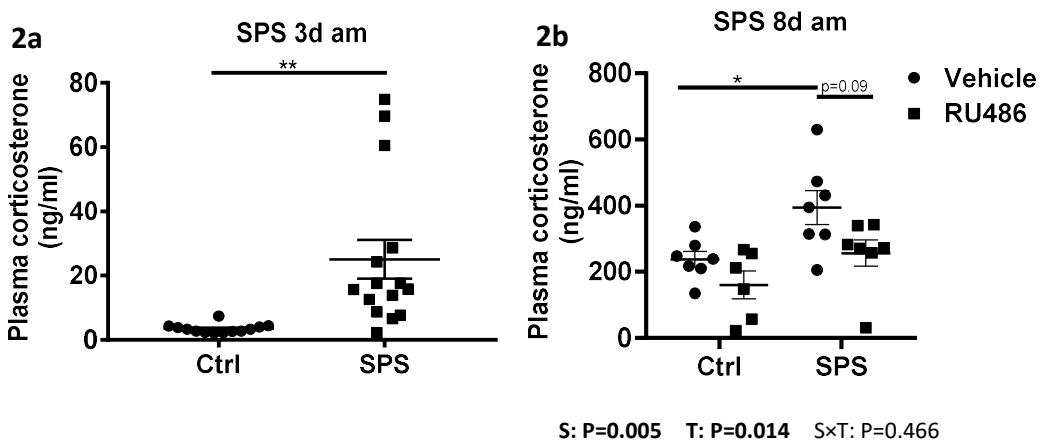


Figure 2. Corticosterone neuroendocrine responses on stress and RU486 treatment. 2a: Stress significantly increased AM corticosterone plasma levels three days after SPS.

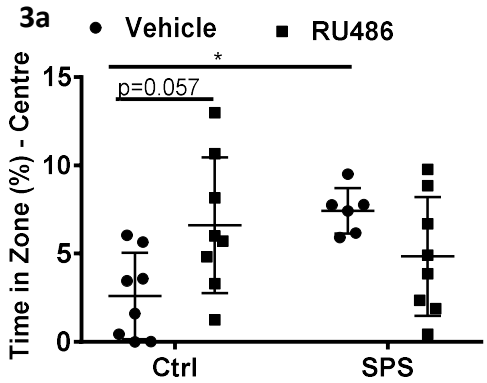
2b: Corticosterone levels at sacrifice day 8 were higher after SPS and reduced by prior RU486 treatment. * $p < 0.05$, **$p < 0.01$.

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

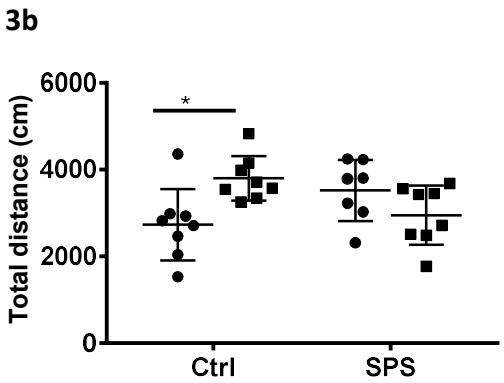
In the Open Field test, the percentage time in the central zone showed an interaction effect ($F_{(1,26)} = 8.965$, $p < 0.05$, figure 3a). Post-hoc analysis showed that animals from the SPS Vehicle group, surprisingly, spent significantly more time in the central area in comparison with Ctrl Vehicle 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 ($F_{(1,27)} = 10.94$, $p < 0.05$, figure 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 ($F_{(1,26)} = 9.725$, $p < 0.05$, figure 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: $F_{(1,26)} = 18.22$, $p < 0.05$, figure 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 figure 3 e-h, analysis of the behavior 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 ($F_{(1, 24)} = 5.021$, $p < 0.05$, figure 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: $F_{(1, 27)} = 5.427$, $p < 0.05$, figure 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 ($F_{(1, 26)} = 6.197$, $p < 0.05$, figure 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, $F_{(1, 27)} = 5.911$, $p < 0.05$, figure 3h).

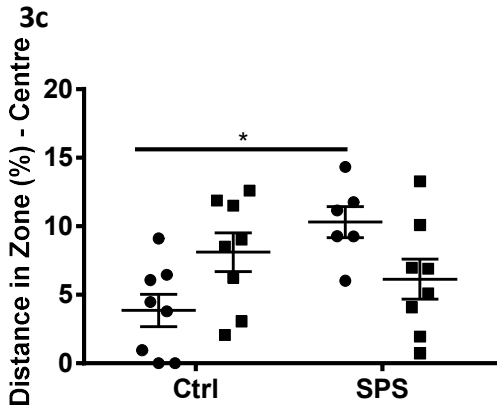
In summary, SPS led to overall higher locomotor activity in the OF and the EPM. Indeed, we observed that some animals seemed agitated, perhaps pointing to a panic-like state. These effects were in interaction with RU486 treatment.



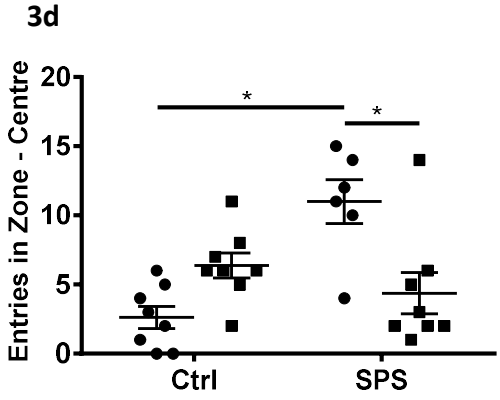
S: P=0.177 T: P=0.521 **S×T: P=0.006**



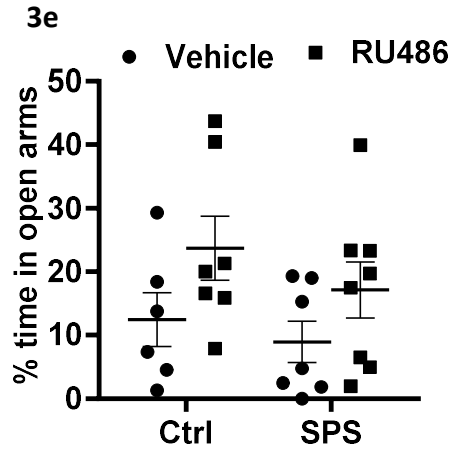
S: P=0.903 T: P=0.324 **S×T: P=0.003**



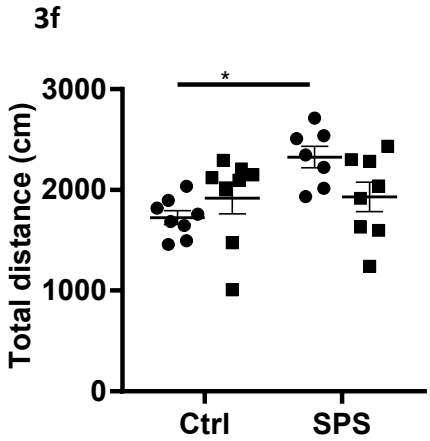
S: P=0.110 T: P=0.972 **S×T: P=0.004**



S: P=0.014 T: P=0.248 **S×T: P<0.001**



S: P=0.201 **T: P=0.017** S×T: P=0.233



S: P=0.023 T: P=0.435 **S×T: P=0.028**

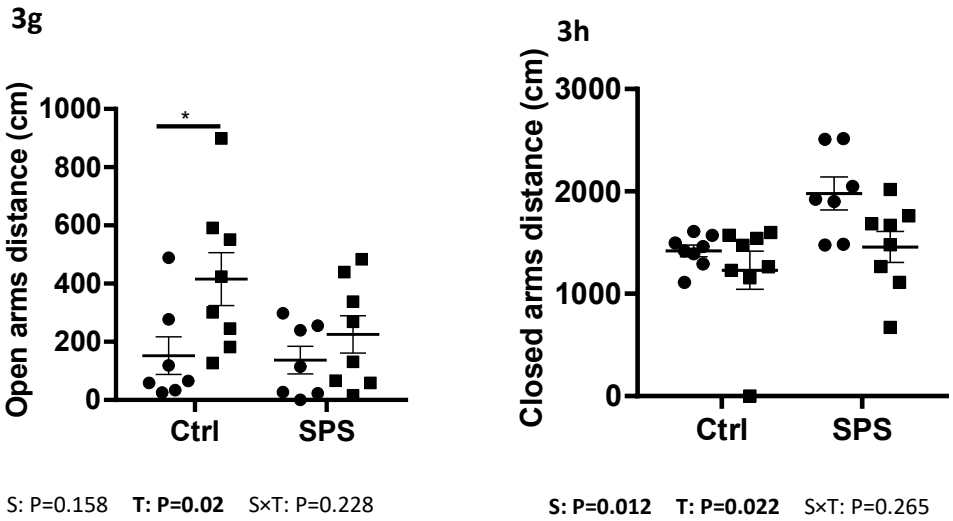


Figure 3. Effects of stress and RU486 in the OF (a - d) test and EPM (e - h). 3a-b: Strong interaction effects between SPS and RU486 in the open field, for the measure Percentage time in central zone (a), Total distance (b), Percentage distance in the central zone (c) and Entries in the central zone (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.

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 changes, and the importance of timing of RU486 treatment.

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
c-fos	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.

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

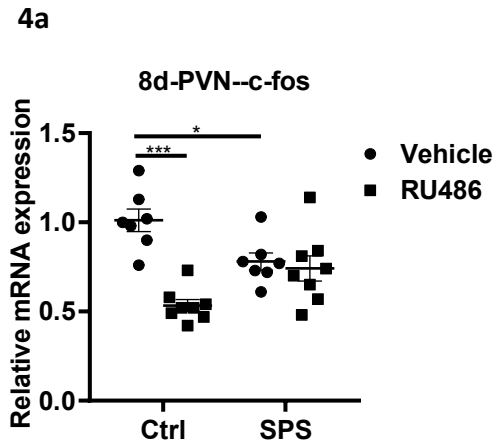
In the PVN, c-fos mRNA, a proxy for neuronal (re-)activity, at 8 days showed a significant main treatment of RU486 and an interaction effect (RU486 treatment: $F_{(1,26)} = 21.26$, $p < 0.0001$, interaction: $F_{(1,26)} = 15.36$, $p < 0.001$, figure 4a, table 3). Post hoc tests revealed that c-fos mRNA 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 [24, 25] for which transcriptional regulation in the brain has been implicated in adaptation to stress [26]. At 8 days there was a significant interaction effect between stress and RU486 and a trend towards a main effect of stress (interaction: $F_{(1,26)}$ =

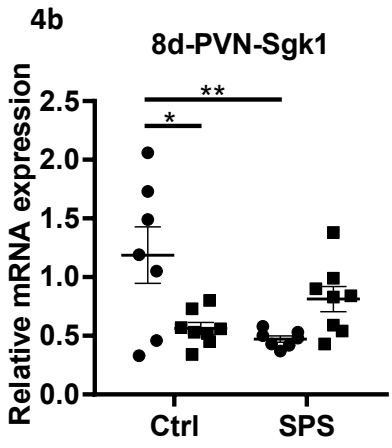
13.91, $p < 0.0001$, stress: $F_{(1,26)} = 3.226$, $p = 0.084$, figure 4b, table 3). Post hoc tests revealed that RU486 suppressed Sgk1 mRNA in controls, and this effect was absent in SPS rats. Sgk1 expression was lower in SPS-vehicle rats than in control vehicle rats. In the material from study 2, at 15 days after SPS there was a weak trend for an effect of stress, which tended to be slightly higher is SPS rats ($F_{(1,25)} = 3.02$, $p = 0.095$, figure 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 ($F_{(1,26)} = 16.8$, $p < 0.001$, figure 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 ($F_{(1,24)} = 5.84$, $p = 0.024$, figure 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 ($F_{(1,26)} = 5.032$, $p < 0.05$, figure 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 [19].



S: P=0.833 **T: P<0.001** **S×T: p<0.001**



S: P=0.084 T: P=0.280 **S×T: P<0.001**

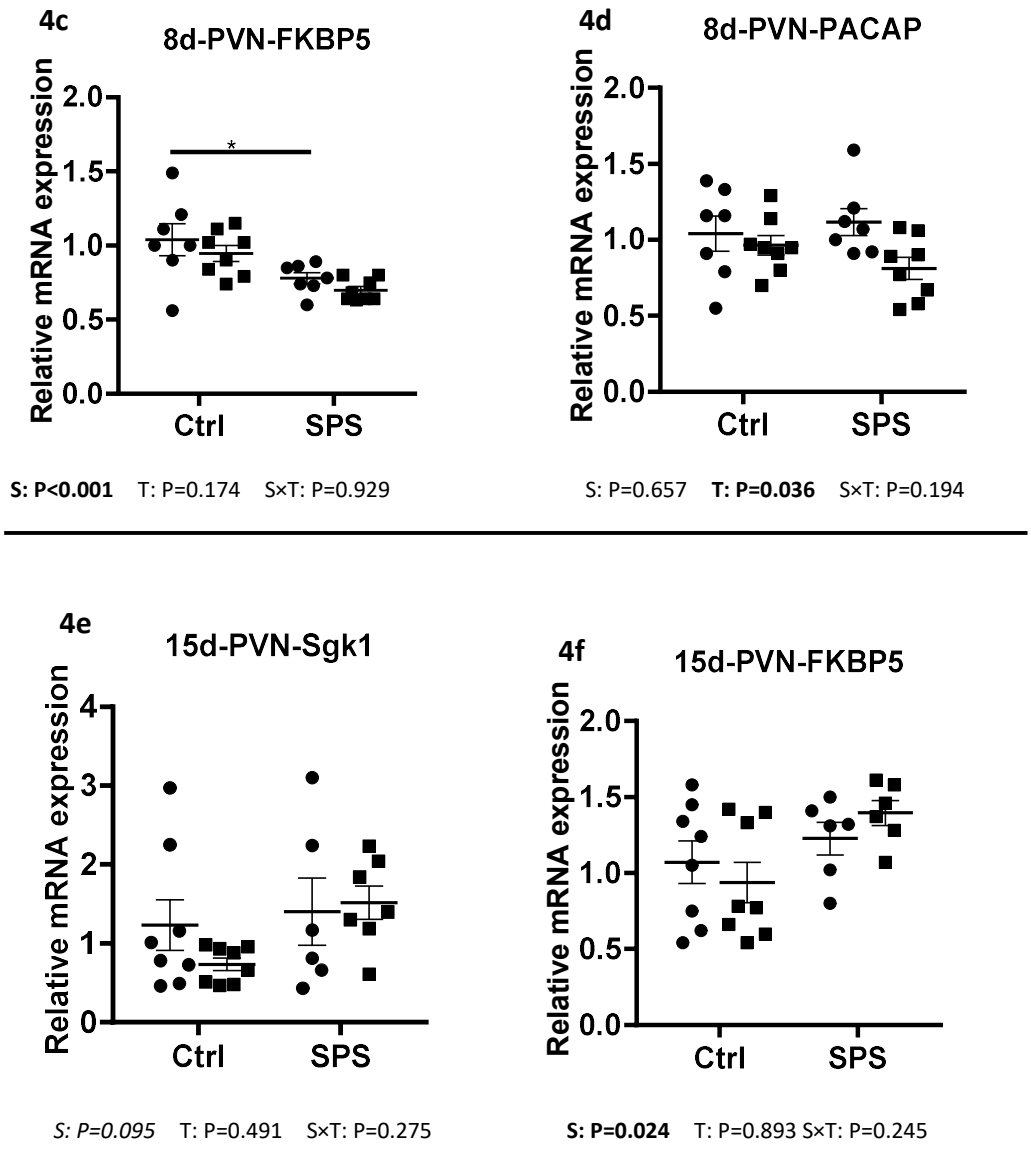


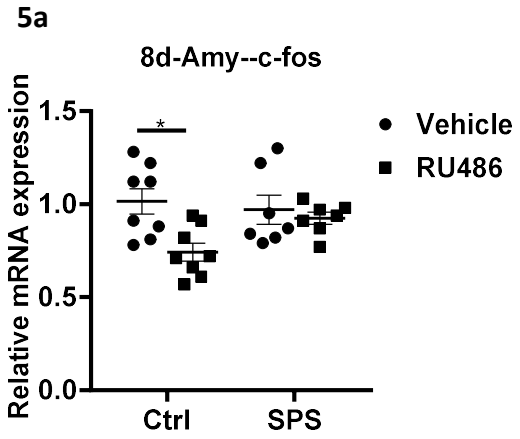
Figure 4. Effect of stress and RU486 treatment on gene expression in the hypothalamus. a: C-fos mRNA expression at day 8 was lower after RU486 only in control rats. b: Sgk1 mRNA expression at day 8 showed a strong interaction effect between SPS and RU486. c: FKBP5 mRNA expression at the day 8 was suppressed. 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. * $p < 0.05$, **$p < 0.05$) ***$p < 0.001$.

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

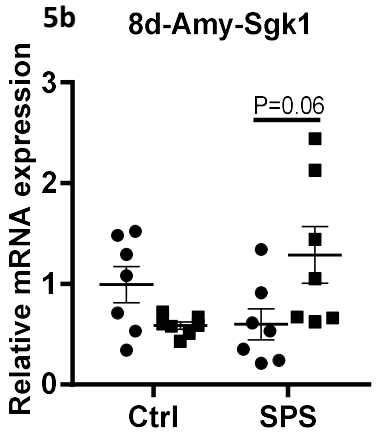
In the amygdala at 8 days after SPS, c-fos mRNA levels were suppressed after RU486 but, similarly to the PVN, only in control rats (RU486 treatment: $F_{(1,26)} = 7.156$, $p < 0.05$, figure 5a, table 3). The expression of Sgk1 mRNA of day 8 was overall similar to that in the PVN (interaction: $F_{(1,24)} = 8.82$, $p < 0.01$, figure 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: $F_{(1,26)} = 7.63$, $p = 0.01$, figure 5f, table 3).

At day 8, stress had significant main effect on FKBP5 mRNA expression within the amygdala ($F_{(1,25)} = 26.04$, $p < 0.001$, figure 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; $F_{(1,26)} = 3.95$, $p = 0.058$, figure 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: $F_{(1,26)} = 4.49$, $p < 0.05$, figure 5d, table 3).

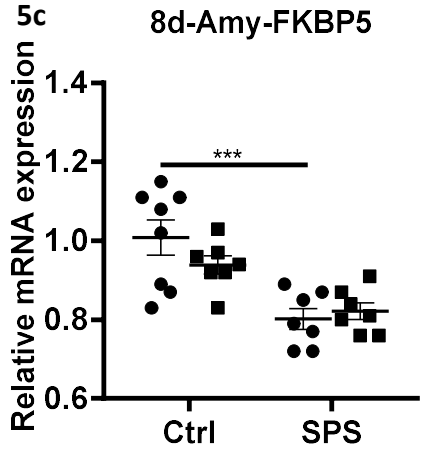
Based on behavior test results where the behavior of the SPS rats suggested a possible panic-like state, we measured expression of the panic related gene COMT in the amygdala. At day 8, COMT mRNA expression showed a significant interaction effect ($F_{(1,25)} = 11.92$, $p = 0.002$, figure 5e, table 3). 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 (figure 5h, table 3).



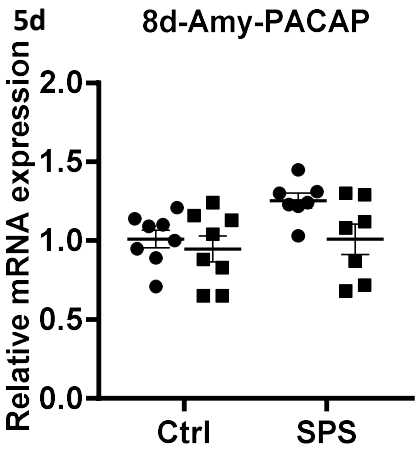
S: P=0.261 **T: P=0.013** *S×T: P=0.068*



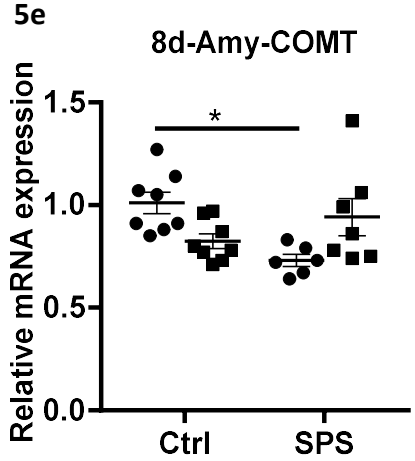
S: P=0.413 T: P=0.453 **S×T: P=0.007**



S: P<0.001 T: P=0.447 S×T: P=0.173



S: P=0.047 **T: P=0.044** S×T: P=0.224



S: P=0.171 T: P=0.829 **S×T: P=0.002**

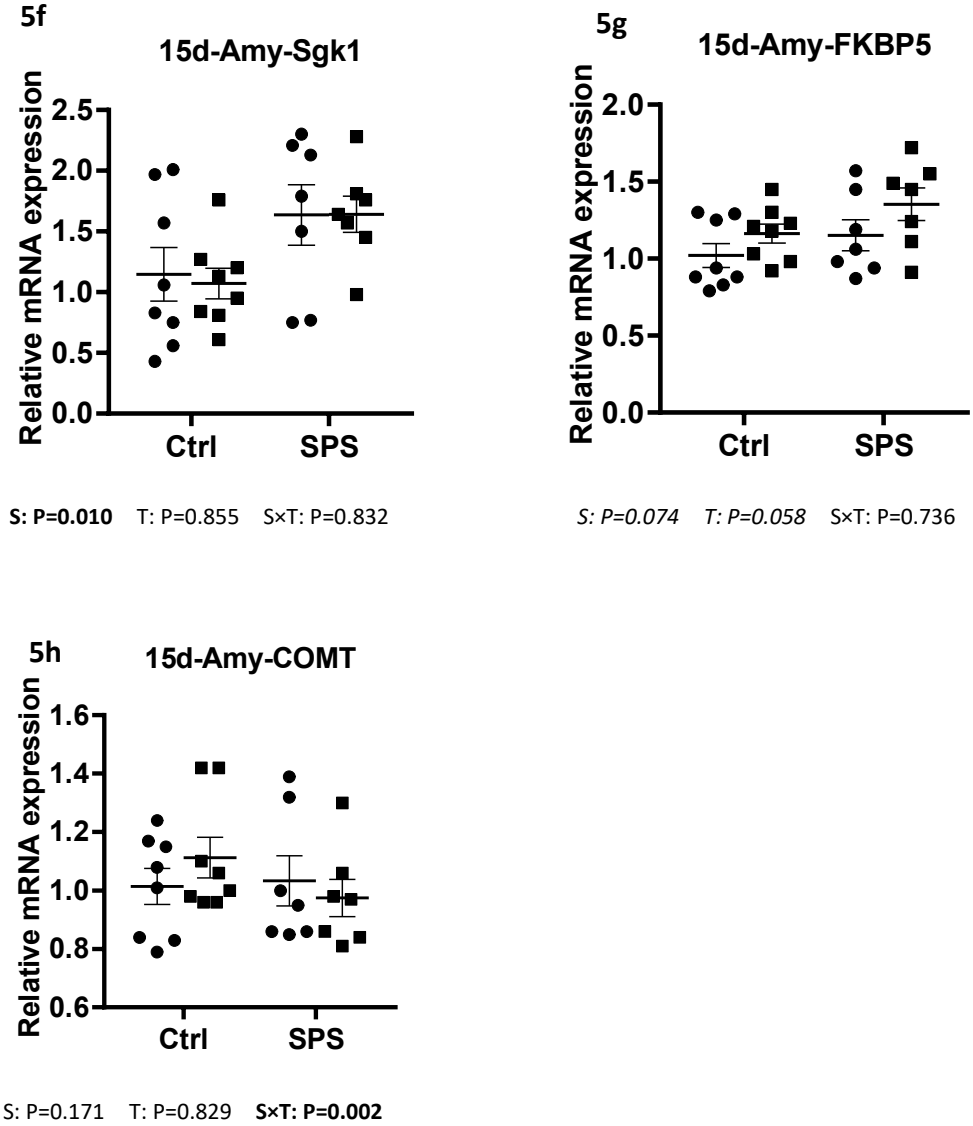


Figure 5. Effect of stress and RU486 treatment on gene expression in the amygdala. a: C-fos mRNA expression at day 8 was overall suppressed by RU486 treatment, and this effect was more pronounced in control rats. b: Sgk1 mRNA at day 8 showed a strong interaction between SPS and RU486, similar to the 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

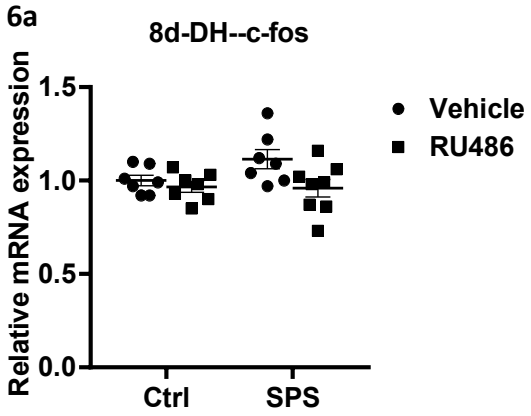
< 0.05, ***p < 0.001.

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

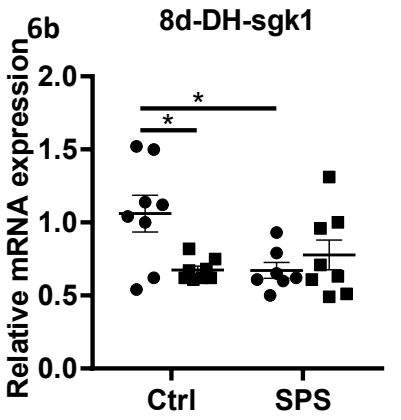
In the dorsal hippocampus of day 8, RU486 treatment had a significant main effect on c-fos mRNA expression ($F_{(1,25)} = 5.34$, $p < 0.05$, figure 6a, table 3) within the dorsal hippocampus, indicating a slightly lower expression. This contrasts with our prior day 15 data, where RU486 led to increased 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 ($F_{(1,27)} = 7.80$, $p < 0.01$, figure 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: $F_{(1,17)} = 22.32$, $p < 0.001$, figure 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.

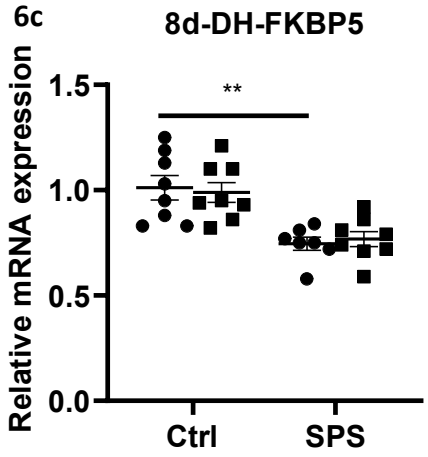
At day 8, stress had significant main effect for FKBP5 mRNA expression within the dorsal hippocampus ($F_{(1,27)} = 28.74$, $p < 0.001$, figure 6c, table 3). In post-hoc tests, the downregulation was significant only for vehicle treated rats, but there was no significant main effect of RU486, similar to the situation in the amygdala. At day 15, FKBP5 expression showed a significant interaction between stress and RU486 treatment ($F_{(1,18)} = 6.82$, $p = 0.018$, figure 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 ($F_{(1,26)} = 3.56$, $p = 0.071$, figure 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.



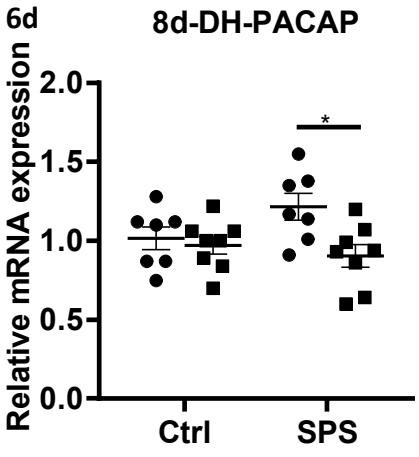
S: P=0.203 **T: P=0.029** S×T: P=0.153



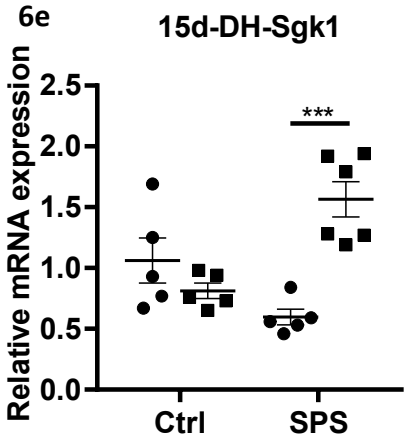
S: P=0.118 T: P=0.124 **S×T: P=0.010**



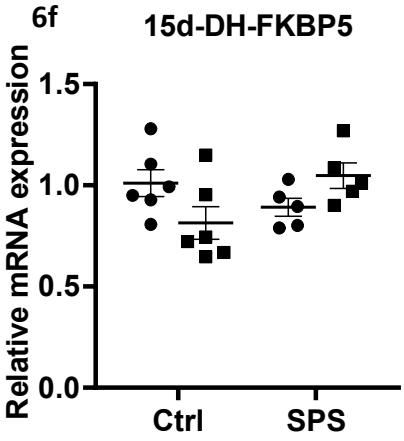
S: P<0.001 T: P=0.994 S×T: P=0.630



S: P=0.359 **T: P=0.019** *S×T: P=0.071*



S: P=0.282 **T: P=0.013** **S×T: P<0.001**



S: P=0.408 T: P=0.769 **S×T: P=0.018**

3

Figure 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 substantial intergroup differences. * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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 [27] (figure 7a). We observed changes at CpG site 5 and 7 (figure 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 ($F_{(1,16)} = 13.48$, $p < 0.05$, figure 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.

7a

FKBP5 intron V

TAG**CG**$_1$TAAAGTTATTAGA**CG**$_2$TTAGTTGTTATAATTAGAGAAGAGAAAGTAGATATT

TAT**CG**$_3$AGTTAA**CG**$_4$TTTTAGGTTTTGG**CG**$_5$GTTATAGTATTAAAAAGTTTTATAGTTTT

TGTTTTTAGTTTTGTTTTTTGAAATATAAGTTGTATAGTTTGGGGTTTTTTGTATTTTAG

7b

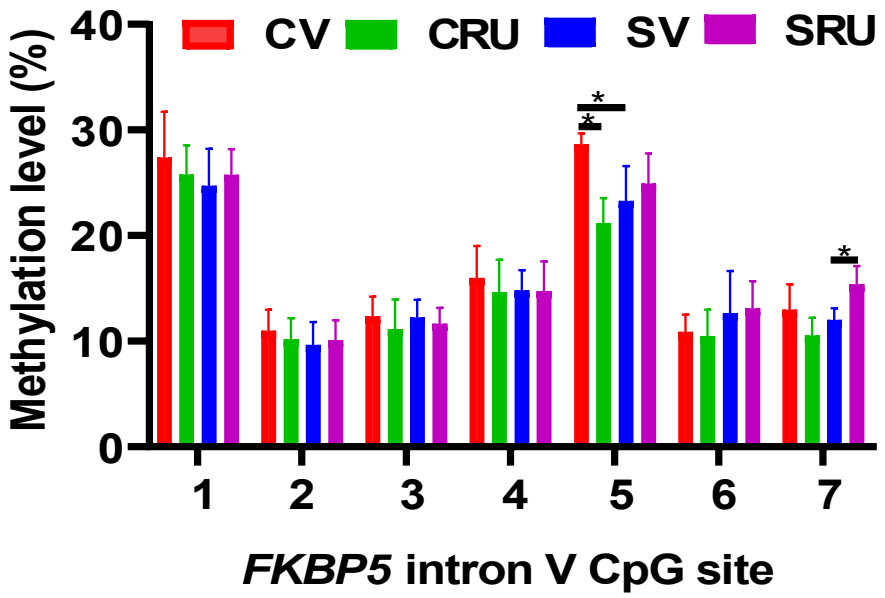


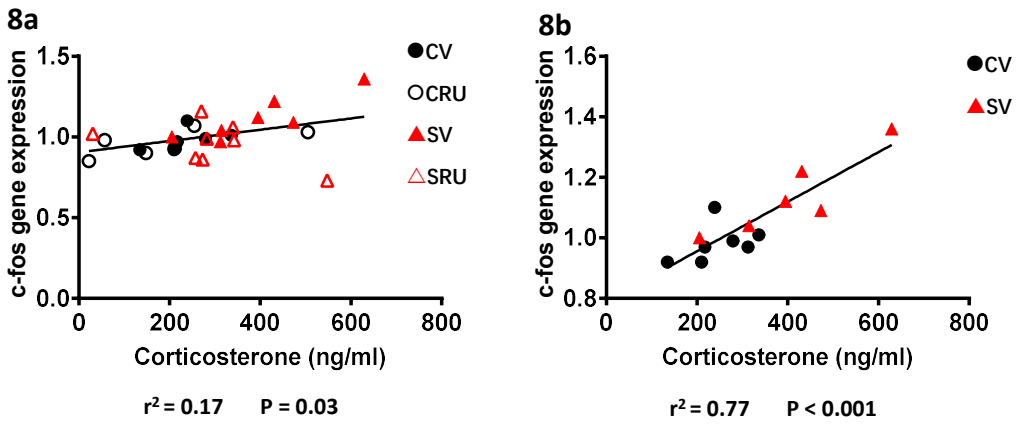
Figure 7. FKBP5 DNA methylation is affected by SPS and RU486. 7a: CpG sites in intron V of the rat FKBP5 gene. 7b: DNA methylation level (%) of the 7 sequenced CpG sites within the Fkbp5 intron V in the dorsal hippocampus. At CpG site 5 there was a significant main effect of RU486 treatment ($F_{(1,15)} = 5.492$, $p < 0.05$) 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 * $p < 0.05$.

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 behavior 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 ($r^2 = 0.17$, $p = 0.03$; Figure 8a), as well as for all vehicle rats (control and SPS; $r^2 = 0.77$; $p < 0.0001$, Figure 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 ($r^2 = 0.476$; $p = 0.009$; Figure 8c).



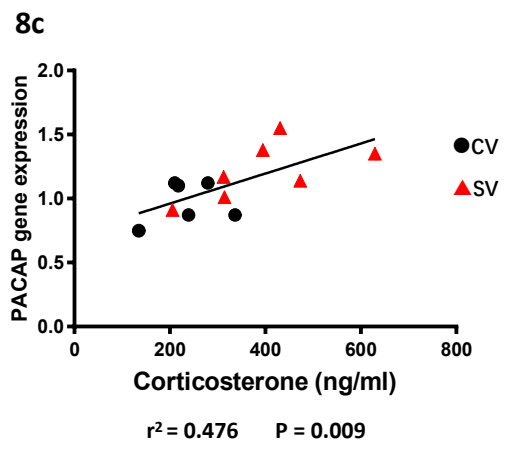


Figure 8. Correlations between corticosterone levels at day 8 with gene expression. a: Correlations between corticosterone and dorsal hippocampus c-fos expression for all rats. b: Correlations between corticosterone and dorsal hippocampus c-fos expression only for vehicle-treated rats. c: Correlations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

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 [28]. 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 behavioral 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 [6, 29, 30]. 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 [31]. 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 behavioral effects [32]. 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 [19]. Our choice was based on potential relevance for PTSD and (COMT) panic disorder. The latter was motivated by the hyperactive behavior of the SPS rats in the EPM and OF, although this behavior constitutes only a hint toward such a state. Sgk1 and Fkbp5 are stress responsive genes that are under direct transcriptional control of GR [24, 25, 33]. Both have been implicated in the pathophysiology psychiatric disease [26, 34, 35]. In addition, COMT was identified as risk gene for PTSD [36-38] and panic disorder [34, 39].

Gene expression changes in PVN, amygdala and hippocampus 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 up is supported by earlier studies that demonstrated behavioral and endocrine effects as late as 1 month after stress in adulthood [40, 41]. Bidirectional changes over time also were observed for GR and FKBP5 mRNA levels in the locus coeruleus, but in an opposite direction [42]. 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-related brain areas. This might well change behavioral reactivity, but it is also true that c-fos mRNA expression across all four treatment groups did not consistently correspond with behavioral 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 signaling. 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 signaling [43], 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 [44]. 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 [45].

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 [25]. 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 behavioral 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 [46], 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 counterintuitive but not by definition impossible [47]. 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 behavior 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 [42]. The data also show which correlates between gene expression and behavioral/ 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 [12, 14, 48]. 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 behavioral responsiveness depends on direct effects in emotion-regulating brain regions or on endocrine reorganization [49] 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 [50, 51]. The effects of pure glucocorticoid antagonists that act on the brain [52] will be important to evaluate in the future.

Earlier RU486 has been studied in clinical trials for treatment of depression and stress disorders [53, 54]. 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.

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Conflict of 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.

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.

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4

An advanced transcriptional response to corticosterone after single prolonged stress in male rats

Jinlan Ding

Xinzhao Chen

Fang Han

Onno C Meijer

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Abstract

Stress-related neuropsychiatric disorders are often accompanied by dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis. In patients suffering from posttraumatic stress disorder (PTSD), increased sensitivity of glucocorticoid negative feedback has regularly been observed. Here, we sought to investigate the overall GR responsiveness in the brains of rats exposed to Single Prolonged Stress (SPS), which was developed to model increased negative feedback and other aspects of PTSD. We injected corticosterone or vehicle in 7 days after SPS, and evaluated plasma corticosterone, as well as gene expression in the dorsal hippocampus and amygdala. We observed a strikingly rapid change in expression of established GR target genes (t = 30 minutes) only in the SPS group upon exogenous corticosterone injection. Our results extent the notion of increased GR sensitivity in PTSD to include transcriptional responses in the hippocampus.

1 Introduction

In physiological conditions, glucocorticoid hormone levels increase systemically in response to stress, as a consequence of activation of the hypothalamic-pituitary-adrenal (HPA) axis [1-4]. Stress-related neuropsychiatric disorders are often accompanied by dysfunction of the HPA axis. Specifically, patients suffering from posttraumatic stress disorder (PTSD) show alterations of the HPA system [5]. Prior studies reported inconsistent data on basal cortisol levels in individuals with PTSD [6, 7]. However, the general consensus is that these patients exhibit increased sensitivity of glucocorticoid negative feedback [8], based on e.g. the dexamethasone suppression test and the metyrapone stimulation test [9-11]. Glucocorticoid negative feedback is primarily mediated by the glucocorticoid receptor (GR) the anterior pituitary (outside the brain) and hypothalamus [12, 13].

The Single Prolonged Stress (SPS) paradigm in rats was developed to model PTSD, including enhanced negative feedback on the HPA axis [14]. However, GR is expressed widely in the brain and regulates the transcription of gene networks necessary for adaption to stressors [15]. Indeed, changes in expression and subcellular distribution of GR (and of the related mineralocorticoid receptor) have previously been found in hippocampus, amygdala and medial prefrontal cortex [16]. Recent evidence suggests that hippocampal GR signaling may also be affected in a different animal model for PTSD [17]. However, to our knowledge no study has directly tested GR functionality by evaluation of corticosterone-induced changes in gene expression in SPS rats. Here, we tested the hypothesis that SPS affects the overall GR responsiveness in the brains of male rats. We found that the mRNA induction of established GR target genes in the hippocampus and amygdala occurred as early as 30’ after corticosterone injection in SPS rats only.

2 Methods and materials

2.1 Animals

Adult male Wistar rats (200-220 g, 7 weeks old) were paired-housed on a 12 h light/dark cycle and controlled conditions of temperature (light on at 7:00-19:00 at 22 ± 1 °C) with standard

rat diet and *ad libitum* access to water. A total of 68 animals were used in this study (32 to make four experimental groups of n=8 for plasma collection at 3 h after injection and 36 to make six experimental groups of n = 6 for the gene response experiment at 0.5 h after injection). Animal procedures were approved by China Medical University Animal Care and were performed in accordance with the Chinese National Guideline on Animal Care.

2.2 Drugs

Rats were injected intraperitoneally with Vehicle (5% Ethanol in PBS) or corticosterone (3 mg/kg). Corticosterone (Sigma, USA) was dissolved in 100% ethanol and diluted to a final 5% ethanol solution in normal PBS, and injected in a volume of 5 ml/kg. The doses of corticosterone we used led to plasma corticosterone concentrations in the range of those observed after stress [18, 19].

2.3 Experimental design

Rats were allowed adapt for one week prior to initiating the experimental protocols. All experimental procedures were started at 9:00 AM. On day 0, rats were subjected to the single-prolonged stress (SPS) paradigm. The single session of prolonged stress was performed as previously described [20]. SPS consisted of restraint for 2 h in an acrylic animal holder followed immediately by a forced swim for 20 min in 24 °C fresh water (water depth: 40 cm). Animals were given 15 min to recuperate and then were exposed to the ether vapor until loss of consciousness. The animals were then returned to their home cage and left undisturbed for 7 days (to allow the behavioral phenotypes relevant to the PTSD symptomatology to develop). Control animals remained in their home cage with no handling and were injected and sacrificed at the same time with the stressed groups.

On day 7, animals were injected with corticosterone or vehicle according to the bodyweight, leading to control-vehicle (CV), control-corticosterone (CC), SPS-vehicle (SV) and SPS-corticosterone (SC) groups. In one experiment blood was collected from the caudal vein at 0 min, 30 min, 60 min and at 2 h, all rats were sacrificed to collect brains at 3 h after injection.

In a second experiment, we sacrificed the rats at 0.5 h after injection the trunk blood and brains were collected. In the second experiment we also included non-injected rats. The design of experiment is outlined in Figure 1.

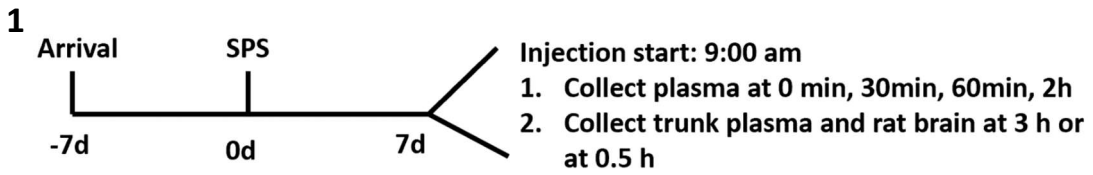


Figure 1. Schematic diagram of the study. One week after arrival in the facility, rats were exposed to SPS (day 0). 7 days after SPS, rats were injected with corticosterone or vehicle. In experiment 1 plasma was collected via a tail cut at 0 min, 30 min, 60 min, and 2 h. Rats were sacrificed at 3 h (experiment 1) or 0.5 h (experiment 2) after injection.

2.4 General body parameters of the second experiment

Body weight was determined using weighing scale on day 0, 3 and 7 after SPS. Baseline body weight at day 0 was 249 ± 17 g on average. We expressed the gain in body weight relative to the start of the SPS exposure. Food and water intake were recorded from day 0 to day 7.

2.5 Elisa analysis for corticosterone

The blood samples were collected in heparinized capillaries and centrifuged 12000 rpm for 5 min to remove blood cells and obtain plasma, and then stored at −80 °C till measurements were performed. The plasma concentration of corticosterone was quantified using enzyme-linked immunosorbent assay (ELISA, AC-15F1, Immunodiagnostic Systems, UK) according to the manufacturer's manual.

2.6 Determination of changes in mRNA levels for candidate genes in the dorsal hippocampus and amygdala

Following the sacrifice, brains were immediately removed, and frozen on dry ice. 80 µm sections were cut on a cryostat, and the dorsal hippocampus from Bregma-2.40 mm to Bregma

-4.36 mm, according to the (Paxinos and Watson, 2007) and the amygdala (the central amygdala and the basolateral complex and part of the medial nucleus), from Bregma -2.16 mm to Bregma -3.36 mm (Paxinos and Watson, 2007) were punched out using a 1.00 mm sample corer (Fine Science Tools, Foster City, CA, USA). Total mRNA was isolated, and concentrations were determined using a Nano Drop 2000 (Thermo Fisher Scientific, Pittsburgh, PA). cDNA synthesis and qPCR were performed per the manufacturer's instructions. Data were normalized to GAPDH mRNA and expressed as the relative fold change calculated using the $2^{-\Delta\Delta Ct}$ method. Tested genes and their primers are described in Table 1.

Table 1. primer sequences for qPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	ACGGCAAGTTCAACGGCACAG	AAGACGCCAGTAGACTCCACGACA
FKBP5	AAGCATTGAGCAAGAAGGCAGTA	GAGGAGGGCCGAGTTCATTAG
Irs2	GGAAGTCTGTTCGGGTGTGT	AGTGCAGGTTCCTCGTCAAC
Ntf3	CAAGTCCTCAGCCATTGACA	CTGGCCTGGCTTCTTTACAC
Drd1a	AGATCCATCGAGTCCCCTCT	TGTTGCAACTGCTTCCAAAG

2.7 Statistical analysis

Data are expressed as mean ± S.E.M. Statistical testing was done with unpaired Student's t-test, or two-way ANOVA followed by turkey's Multiple Comparisons post-hoc (as appropriate) using GraphPad Prism 8.0 software. Results were considered statistically significant at $p < 0.05$.

3 Results

3.1 Food/water intake and body weight parameters

Data on food/water intake and body weights were consistent in both experiments, here we only show the data of second experiment. Total food intake in the week after the SPS procedure did not significantly differ from the control group (figure 2a). However, water intake of SPS group was significantly reduced compared to the control group ($t = 2.416$, $p < 0.05$, figure 2b). The control rats gained more body weight than the SPS group during the first 3 days

after SPS (t = 4.097, p < 0.05). During the last 4 days the body weight gain did not differ between the groups (figure 2c).

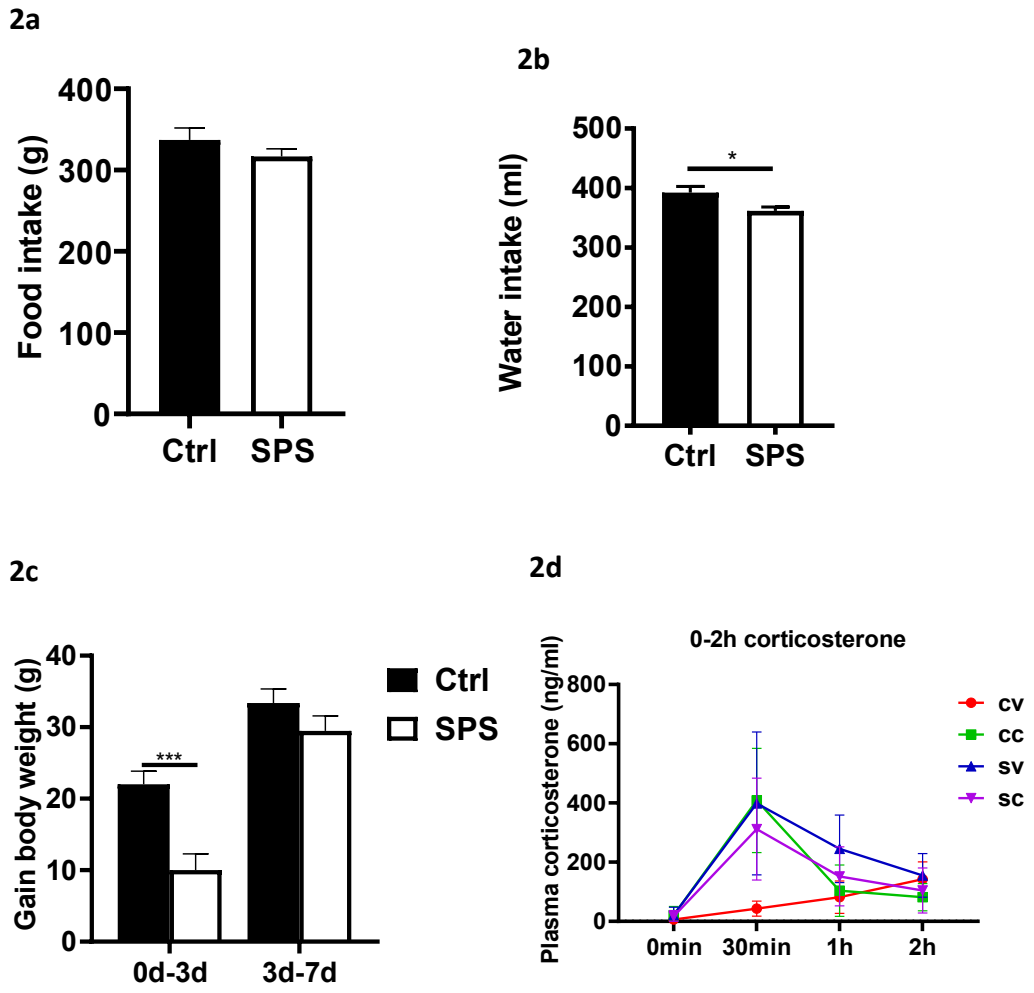


Figure 2. Effects of SPS on food and water intake, body parameters and corticosterone levels after injections. a: Food consumption. b: Water consumption. c: Gain in body weight. d: Corticosterone levels at 0 min, 0.5 h, 1 h and 2 h after injection. CV: control + vehicle group; CC: control + CORT group; SV: SPS + vehicle group; SC: SPS + CORT group * p < 0.05, *** P < 0.001.

3.2 A HPA axis response in SPS-control rats

Plasma corticosterone (figure 2d) levels were measured at different times points after injections in the first experiment to evaluate the response to vehicle injection. Corticosterone

levels showed an interaction effect and a trend toward a SPS main effect at 30 min after injection (interaction, $F_{(1, 11)} = 12.84$, $p < 0.05$, stress: $p = 0.078$), as well as an SPS effect at 60 min after injection (stress: $F_{(1, 22)} = 11.48$, $p < 0.01$). As expected, exogenous corticosterone injection led to similarly increased concentrations that returned to baseline after 60 minutes. The lack of an ANOVA corticosterone injection main effect (CORT: $p > 0.05$) *per se*, could be attributed by a strong increase of corticosterone levels in the vehicle-injected SPS rats at the 30 min time point. Corticosterone levels were still elevated 60 minutes after vehicle injection at 60 min in the SPS group. The high levels of corticosterone in vehicle treated SPS rats indicated enhanced stress reactivity in these animals, but precluded comparing the transcriptional response to corticosterone, for lack of a low-corticosterone SPS-group.

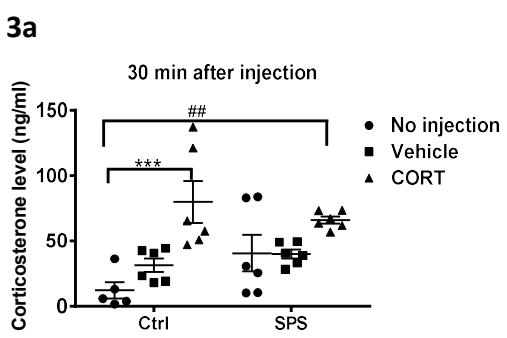
3.3 Gene expression effects of corticosterone half an hour after injection in the dorsal hippocampus and in the amygdala

The elevated corticosterone in SPS-vehicle rats in our first experiment could have been caused by the injection, the tail blood sampling or both. To control for the acute effects of the injection itself, in our next experiment we included SPS and control groups that did not receive an injection, and compared corticosterone levels half an hour after injection of vehicle or corticosterone. We did not apply tail cuts in these rats. Corticosterone results showed a significant exogenous CORT main effect ($F_{(2,29)} = 13.16$, $p < 0.001$, figure 3a). Post-hoc test confirmed a significant increase in plasma corticosterone only in the CORT-injected control and SPS animals, relative to untreated and vehicle-treated controls.

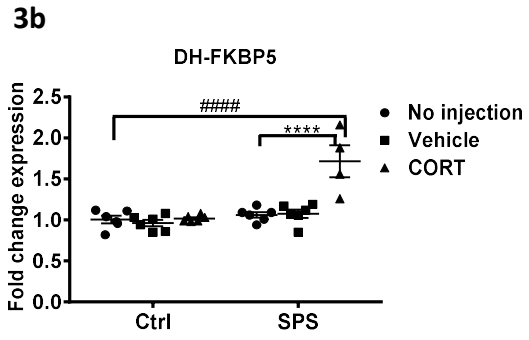
Because corticosterone levels were strongly induced only after injection of the hormone, we decided to evaluate mRNA responses in the brains of the animals in this experiment. Strikingly, in the dorsal hippocampus, the FKBP5 mRNA showed an interaction and two main effects between stress and exogenous corticosterone (interaction: $F_{(2,28)} = 13.3$, $p < 0.001$; stress: $F_{(1,28)} = 28,72$, $p < 0.001$; CORT: $F_{(2,28)} = 16.42$, $p < 0.001$, figure 3b). Post-hoc analysis showed FKBP5 mRNA levels were increased only in SPS rats after corticosterone injection. We also evaluated expression of additional target genes, based on some robust corticosterone-induced

target genes as identified in a previous study [21]. The Irs2 mRNA expression was similar to the FKBP5 mRNA expression. It showed a significant interaction and two main effects for stress and exogenous corticosterone (interaction: $F_{(2,29)} = 3.692$, $p < 0.05$; stress: $F_{(1,29)} = 4.71$, $p < 0.05$; CORT: $F_{(2,29)} = 3.879$, $p < 0.05$, figure 3c). In the post-hoc comparison, Irs2 mRNA expression upregulation after corticosterone only occurred in SPS rats. Ntf3 mRNA levels showed a very similar pattern. The 2-way ANOVA showed effect of exogenous corticosterone, and an interaction between stress and CORT (CORT: $F_{(2, 29)} = 5.772$, $p < 0.01$; interaction: $F_{(2, 29)} = 5.697$, $p < 0.01$, figure 3d). Post-hoc tests showed that Ntf3 mRNA expression was only upregulated by corticosterone in SPS rats. As a downregulated gene we selected Drd1a, which was earlier found to be downregulated irrespective of stress history [21, 22] . For this mRNA there were significant main effects for stress and CORT (stress: $F_{(1, 29)} = 6.555$, $p < 0.05$; CORT: $F_{(2, 29)} = 3.898$, $p < 0.05$, figure 3e). Post-hoc test revealed Drd1a mRNA levels were suppressed after exogenous corticosterone, but only in SPS rats. Thus, in the hippocampus, these 4 genes responded to corticosterone after 30 minutes in the SPS rats only.

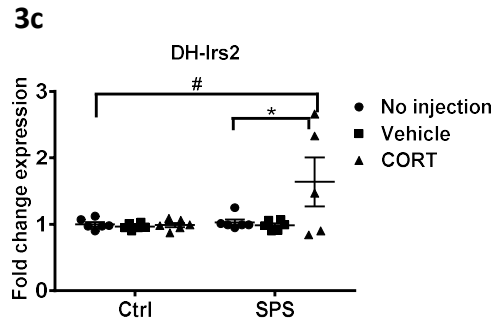
In the amygdala, FKBP5 mRNA levels showed a main effect of stress (stress: $F_{(1, 30)} = 16.11$, $p < 0.001$, figure 3f). Post-hoc tests showed higher mRNA level of the SPS-CORT group higher compared to the control without injection group. There was no significant upregulation after corticosterone within the stress or control groups. There was no difference of each group for Irs2 and Ntf3 mRNA expression (figure 3g and figure 3h). Drd1a mRNA showed main effects of stress and CORT (stress: $F_{(1, 29)} = 11.12$, $p < 0.01$; CORT: $F_{(2, 29)} = 6.058$, $p < 0.01$, figure 3i). In pairwise comparisons, Drd1a in the SPS with CORT injection group was lower than in the SPS without injection group. In sum, in the amygdala most genes identified previously as corticosterone targets in hippocampus did not differ between groups, but for those genes that were responsive to corticosterone, the effect was observed only in rats that had undergone SPS.



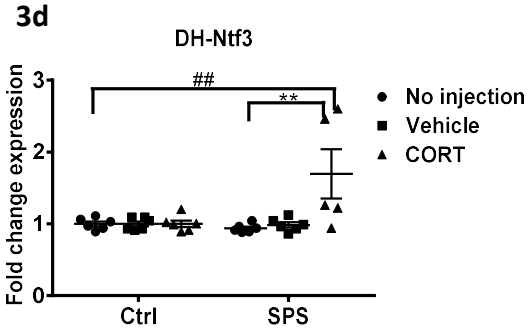
S: p= 0.33 **C: p<0.001** S×C: p=0.11



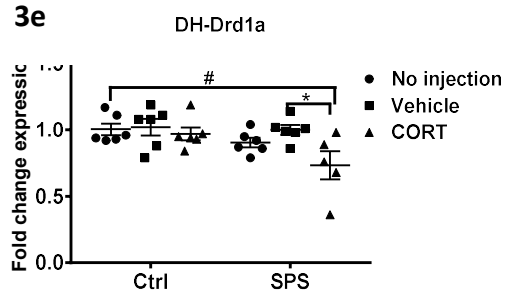
S: p < 0.001 C: p<0.001 S×C: p < 0.001



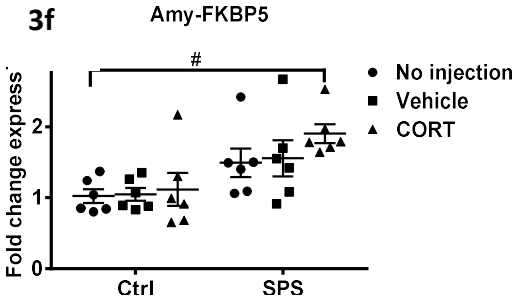
S: p = 0.04 C: p = 0.03 S×C: p = 0.04



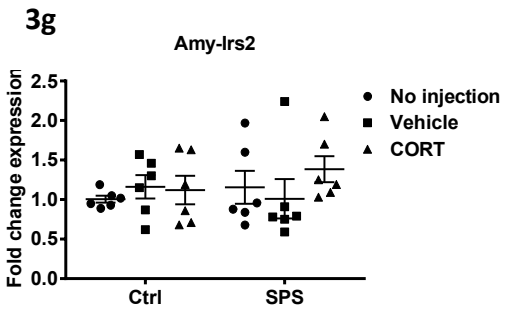
S: p = 0.05 C: p =0.008 S×C: p =0.008



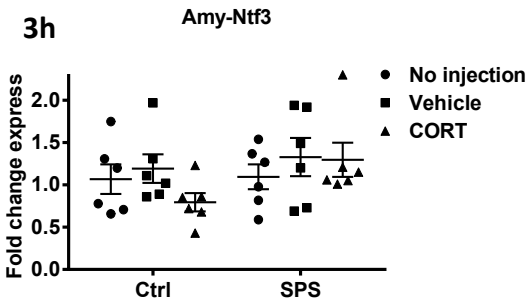
S: p= 0.02 C: p=0.03 S×C: p=0.19



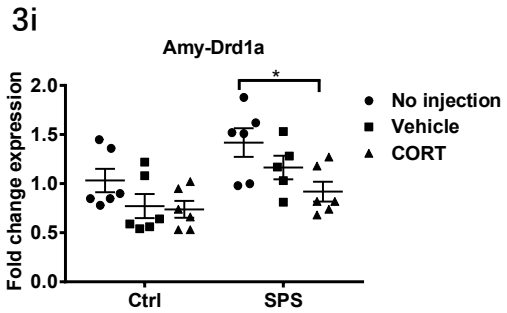
S: p < 0.001 C: p = 0.34 S×C: p = 0.63



S: p = 0.55 C: p = 0.55 S×C: p = 0.49



S: p = 0.13 C: p = 0.43 S×C: p = 0.38



S: p = 0.002 C: p = 0.006 S×C: p = 0.59

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Figure 3. Plasma corticosterone levels, and gene expression in the dorsal hippocampus and in the amygdala at 0.5 h after injection. a: Corticosterone levels b: FKBP5 mRNA expression in the dorsal hippocampus. c: Irs2 mRNA expression in the dorsal hippocampus. d: Ntf3 mRNA expression in the dorsal hippocampus. e: Drd1a mRNA expression in the dorsal hippocampus. f: FKBP5 mRNA expression in the amygdala. g: Irs2 mRNA expression in the amygdala. h: Ntf3 mRNA expression in the amygdala. i: Drd1a mRNA expression in the amygdala. The data are expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by post-hoc turkey's test. #: differences between Control and SPS groups; *: differences within control or SPS groups. $p < 0.05$, ## $p < 0.01$, #### $p < 0.001$, */# $P < 0.05$, **/## $P < 0.01$, *** $P < 0.001$, ****/#### $P < 0.001$.

4 Discussion

In this study we administered exogenous CORT to evaluate the GR sensitivity in hippocampus and amygdala one week after the SPS procedure. Our rationale was the documented feedback sensitivity of the HPA-axis in this model [20] and the likely importance of enhanced GR

sensitivity in limbic brain regions [23]. We found that the experimental procedure of injection and repeated blood sampling via the tail led to a pronounced adrenocortical activation in SPS rats, which precluded a properly controlled evaluation of GR target gene expression after three hours. In contrast, 30 minutes after a vehicle injection alone, SPS rats did not show a corticosterone elevation. We then observed in the corticosterone treated animals a striking mRNA response of up- and downregulated GR target genes, at this early time point in SPS rats. Our data suggest an enhanced stress responsiveness after SPS to moderate but not mild stressors, and a sensitization of brain GR signaling that extends beyond direct negative feedback regulation.

An enhanced GR activity in models of traumatic stressors has mainly been observed for negative feedback changes. This is a complex phenomenon in itself, with both non-genomic and genomic effects of primarily GR [24, 25]. It involves GR activation in the pituitary (the primary targeted of dexamethasone) and in the brain. The responsible brain GRs reside foremost in the hypothalamic paraventricular nucleus [24-27], and secondarily in higher brain centers project to the hypothalamus [28]. In higher brain centers, GR acts in concert with mineralocorticoid receptors [29, 30]. Our understanding of the nature of enhanced feedback has remained limited, although in patients both pituitary and central GRs have been implicated [31, 32], and probing MR functionality suggested no differences [33].

Our data do not allow further insights in negative feedback strength per se, because SPS rats reacted strongly to the initial protocol of injection followed by tail blood sampling. In control rats, this method may be used as a mild, essentially stress free way of collecting blood [34]. Enhanced stress reactivity one week after SPS is well established as evaluated by readouts such as the elevated plus maze [16, 35]. The clear stress response in SPS rats after vehicle injection followed by repeated handling confirms this, and unfortunately stood in the way of a meaningful comparison of gene expression changes in these animals. The lack of an adrenocortical response of rats in our second experiment, at 30’ after injection showed that likely the tail incision was the immediate cause of the response in the first experiment.

Our experimental setting was not suited to determine whether negative feedback sensitivity had changed. In our second experiment, the corticosterone treatment mimics the setting in which enhanced rapid negative feedback was initially observed, but this was defined at the level of ACTH, rather than corticosterone [14]. In other studies, dexamethasone was used, typically two hours before measuring plasma corticosterone. These studies consistently demonstrate enhanced suppression of the HPA axis in male rats [36-38]. While the later studies seem to indicate enhanced genomic effects of glucocorticoids, we do not know whether the SPS-exposed rats in our study actually showed enhanced feedback sensitivity.

Evaluation of gene expression at 30' after corticosterone could be performed, given the lack of strong injection effects. This showed the pronounced early effects on GR target genes. From a technical point, it is good to note that the strong response to corticosterone occurred not only for upregulated genes, but also for the previously established suppressed Drd1a mRNA [21, 22]. This argues against an effect on the housekeeping gene used in normalization, and for a bona fide difference in responsiveness.

Previous studies have evaluated the expression level of GR in this model. Soon after the development of the model, increased GR mRNA expression levels were reported in the hippocampus, 1 week after SPS [39]. Also other studies reported substantially higher (nuclear) GR immunoreactivity in the prefrontal cortex and amygdala 8 - 15 days after SPS [40-43]. The data are however not immediately intuitive in relation our previous work which did not find decreased receptor expression one week after SPS [44, 45]. However, it is clear from e.g. Cushing's Disease (mouse models) that there still may be enhanced GR activity in spite of homologous downregulation of the GR [46].

Rather than the number of receptors being different, the genomic GR signaling seems to be primed in SPS rats. This notion was previously explored, by looking at GR nuclear translocation 7 days after SPS, and these data suggested enhanced 'basal' nuclear GR presence in amygdala

and ventral (but not dorsal) hippocampus based on Western blot analysis [47]. Another study observed high nuclear GR signal in dorsal CA1 and dentate gyrus only in rats that were strongly affected by predator scent exposure [17]. While GR nuclear presence generally follows corticosterone levels, there are additional regulatory mechanisms governing nuclear translocation [48], and these may be relevant to the brain as evidenced by nuclear GR localization even in adrenalectomized rats [49]. FKBP5 is an often studies factor in this respect, that is both target and regulator of GR function [50-52]. In our current data, FKBP5 mRNA levels were affected by SPS in the amygdala, but do not explain the enhanced response to corticosterone at 30' after injection.

The idea that in PTSD and PTSD models the GR functionality is changed beyond negative feedback sensitivity goes back to human studies on lymphocyte GR expression [53], and in rodent models has logically been extended to higher brain centers which may be involved in the actual psychopathological symptoms of PTSD [54]. Our data add to the notion that GR is not only involved in the initiation of SPS-induced effects [55], but also in their maintenance. The changed GR signaling status might explain why treatment with the GR antagonist RU486/mifepristone can reverse the long term effects of stressors even when these are administered days to months later in the SPS [56, 57] and other stress paradigms [58, 59].

There is still a bias towards research in male experimental animals [60]. Enhanced negative feedback after SPS seems to be specific to male rats [38]. Given that our hypothesis directly derives from the enhanced feedback, the use of males makes sense. However, SPS does affect the female rat brain in different ways, and it will be interesting to also test our hypothesis in females in future studies, using the SPS as well as other models of PTSD.

In summary, we observed a strikingly rapid transcriptional response in the hippocampus and amygdala after corticosterone administration. It will be interesting to extend these findings to individual cell types [61], functional consequences, and, in the long run, to the PTSD patient population.

Acknowledgements

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Conflict of 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.

Author contributions

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

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5

The role of β-arrestin-2 on Fear/anxious-related memory in a rat model of post-traumatic stress disorder

Jinlan Ding

Fang Han

Lili Wen

Bing Xiao

Yuxiu Shia

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Abstract

Background: Post-traumatic stress disorder (PTSD) can be categorized as a disorder of dysregulated fear processing. In the formation and development of PTSD, whether fear/anxious-related memory is involves changes in β-arrestin-2, and its associated signal transduction pathways remains unknown.

Method: We used the single prolonged stress (SPS) as a rat model of PTSD. Next, the elevated plus maze test (EPM) was performed to examine fear/anxious memory- related behaviors. Then, we determined β-arrestin-2, PDE-4, and signal transduction pathways with immunofluorescence, co-immunoprecipitation, immune-histochemistry, Elisa, western blot, and real-time PCR in the basolateral amygdala.

Results: Our data indicated that SPS enhanced fear/anxious memory-related behaviors. This was associated with low expression of β-arrestin-2, PDE-4 and their complex, and high activity of signal transduction pathways 7 days after SPS.

Conclusions: The data indicate that β-arrestin-2 may be involved in the formation of abnormal fear/anxious memory in PTSD; through activation the signal transduction pathways. This may be relevant for the formation and development of PTSD.

1. Introduction

Post-traumatic stress disorder (PTSD) is an anxiety disorder arising as a certain severe psychological consequence of exposure to, or confrontation with, stressful events that a person experiences as highly traumatic. PTSD can be categorized as a disorder of dysregulated fear processing [1]. Fear/anxious memory is a form of emotional memory that recruits the amygdala [2-4] and is often disturbed in individuals suffering from PTSD [5-7].

The amygdala has been implicated in the storage and expression of fear/anxious memory in both animal [2, 3, 8] and human studies [9]. The amygdala can be divided into three distinct subgroups: central nucleus (CeA), corticomedial nucleus (MeA) and basolateral nucleus (BLA) [10]. BLA is the largest among these three and is the key region for the initiation of fear/anxiety.

Many signal molecules, such as protein kinase A (PKA), are involved in fear memory consolidation. Accumulating evidence revealed that the formation of associative fear/anxious memory involved multiple signal cascades, including cAMP- PKA and ERK- MAPK. It was revealed that perfusion of the PKA or ERK inhibitor into lateral amygdala (LA) before fear conditioning results in the impairment of fear memory [11, 12]. This PKA signal transduction pathway is necessary for the formation of long-term memory. Various activated signaling cascades converge upon transcription factors within the nucleus. cAMP response element binding protein (CREB), a key target of PKA, is one particular transcription factor that is responsible for regulating protein synthesis. Phosphorylation of CREB at Ser133 occurs when upstream signaling cascades get activated. CREB is also activated in the amygdala after fear conditioning [13]. Therefore, the cAMP- PKA- CREB signal transduction pathway is involved in the physiological processing of fear/anxious memory.

β-arrestins, including β-arrestin-1 and β-arrestin-2, play a critical role in a wide variety of physiological and pathophysiological cellular processes [14] and are found in high abundance in the immune and central nervous systems [15, 16]. Of the two types, β-arrestin-2 is widely

distributed but functions in PTSD are remains unknown. Emerging evidence implicates that β-arrestin-2 may play an important role in regulating basic brain functions, particularly fear/anxious memory formation and in the synaptic plasticity of the amygdala. β-arrestin-2 was reported to be a key molecule of feedback regulating cAMP signal transduction pathways [17]. In the mechanism regulating stress and anxiety responses, β-arrestin-2 recruitment also play an important role [18].

PDE-4 can interfere with the formation of long-term memory by its mechanism of degradation of specific enzymes of cAMP; this leads to a decrease in cAMP levels and alteration of the cAMP- PKA- CREB signaling pathways. Therefore, CREB-dependent gene expression and the synthesis of the associated proteins that involved in learning and memory are attenuated after PDE-4 activation. Therefore, an optimum PDE-4 activity is required for normal conditioning of fear memory [19]. β-arrestins are known to recruit PDE-4, thus controlling PKA activity at the membrane [20, 21]. Accordingly, PDE-4 a role in both memory and anxiety, and several lines of evidence suggest specific inhibition of PDE4B as a promising therapeutic approach for disorders of memory and anxiety [22].

PTSD likely involves changes in the amygdala, leading to enhanced fear/anxious memory. However, to date, in the process of formation of PTSD, the roles of β-arrestin-2 and PDE-4 in the regulation of fear/anxious memory remain unknown. It is also uncertain whether changes in signal transduction pathways are part of the PTSD pathophysiology.

Here we evaluate the activity of β-arrestin-2 and PDE-4 as essential modulators of regulating amygdala PKA activity, in response to fear/anxious memory formation in the SPS model of PTSD. Our results suggest that β-arrestin-2, PDE-4 and signal transduction pathways may be involved in the formation and development of PTSD.

2. Materials and methods

2.1 Animals

Male Wistar rats (China medical university, about 8 weeks old, weighing 150-180 g) were used

for all experiments. All rats were housed in the experimental animal facility for a week to let them acclimate to their new environment (temperature: (22±1 °C, humidity: 50~60%, lights on: 07:00~19:00). Standard food pellets and tap water were available ad libitum. All procedures followed the National Guidelines on Animal Care.

The SPS procedure is internationally recognized method for the preparation of an animal model of PTSD [23]. SPS is one of the animal models proposed for PTSD [24]. The SPS rats show enhanced inhibition of the HPA axis, which has been frequently demonstrated in patients with PTSD. In brief, the SPS model consisted of a 2 h whole body restraint in an acrylic animal holder, which was followed immediately by 20 min forced swimming (temperature: 25 °C, water depth: 40 cm). These rats were then allowed to recuperate for 15 min. Next, the rats were exposed to ether vapor until loss of consciousness [25, 26] and then placed to their home cages and left undisturbed until the behavioral testing. The rats were divided randomly into four groups (15/group), including three SPS groups (1d, 7d, and 14d) and the control group. For each group, three rats were used for histological analysis, three for Elisa, three for Western blotting, and three for Real-Time PCR.

2.2 Behavioral test -Elevated Plus Maze (EPM) test

All rats of each group underwent the behavioral test (EPM test) at two hours before being killed. The EPM apparatus consists of a plus-shaped maze elevated above the floor with 2 oppositely positioned closed arms (50 cm × 10 cm), 2 oppositely positioned open arms (50 cm × 10 cm), and a center area (10 × 10 cm). At the beginning, rats were placed in the central area of the maze, facing a closed arm. Behavior was recorded with a video camera during 5 min. The number of entries into open arms, into closed arms and the time spent in the open arms, in the closed arms were measured. The percentage of open arm entries (number of entries into the open arm/total number of entries in both arms), and the percentage of time in the open arms (time in the open arms /the time in both arms) were calculated. The measures of fear/ anxiety are the percentage (%) of open arm entries and the percentage (%) of time spent

on the open arms.

2.3 Fixation and sections making

Rats of each group were anaesthetized with 50 mg/kg body weight sodium pentobarbital and then infused with 500 ml of 0.01 M PBS (pH 7.4) including 4% paraformaldehyde. The brains were rapidly removed and put into the same fixative for 24 h at 4 °C. The brains were immersed in 30% sucrose in 0.1 M PB for 3 days for cryoprotection. The brain tissue was cut into slices of 14 um thickness using a cryostat (Leica CM 3050, Germany).

2.4 Double immunofluorescent labeling for β-arrestin-2 and PDE-4

The sections were incubated with mouse monoclonal antibody against β-arrestin-2 (Santa Cruz, USA; 1:200) plus rabbit polyclonal antibody against PDE-4 (Santa Cruz, USA; 1:200) overnight at 4 ℃. After three times washing, the sections were incubated with FITC anti-mouse IgG (Company of Zhongshan Goldenbridge, Beijing, China; 1:1000) plus CY3 anti-rabbit IgG (Company of Zhongshan Goldenbridge, Beijing, China; 1:1000) for 0.5 h at room temperature. After being washed in PBS and mounted. Confocal laser scanning microscope was applied for colocalization observation.

Six slides were randomly selected from each group. Each slide was randomly selected five visual fields in BLA (×40). The immunoreactivity of β-arrestin-2 and PDE-4- immuno-positive cells were collected using an EZ-C1 Thumbnailler morphology image analysis system.

2.5 Western blotting used to detect β-arrestin-2, PDE-4 and PKA

Rats were decapitated, and the brain were removed and immediately placed in an ice-cold dish. Then BLA was dissected according to the atlas (Paxinos and Watson, 1998) by use of a stereomicroscope. Fresh BLA tissue samples of control rats and SPS rats were respectively homogenized with a sample buffer and were denatured by boiling for 3 min. Samples were loaded on a 10%SDS- polyacrylamide gel, and electroblotted onto a PVDF membrane (Millipore Corp., Bedford, MA) from the gel by a semi-dry blotting apparatus (Bio-Rad Laboratories, Inc,

Hercules, CA). The blotted membrane was then blocked with 1.5% skim milk, 0.05% Tween-20 in TBS (TBST) at 4 ℃ overnight, and then incubated with 1:500 mouse monoclonal antibody against β-arrestin-2 (Santa Cruz, USA), 1:500 rabbit polyclonal antibody against PDE-4 (Santa Cruz, USA) and 1:500 rabbit polyclonal antibody against PKA (Santa Cruz, USA) at 4 ℃ for 24 h. Blots were washed three times with TBST, and then incubated with a second antibody (anti-mouse or anti-rabbit IgG-HRP from Santa Cruz, USA; 1:5000) for 2 h at room temperature. After incubation, blots were washed three times with TBST before visualization by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). To confirm equal protein loading, the same blots were incubated with antibodies specific for GAPDH (Abcam, British; 1:1,000). The protein levels of β-arrestin-2, PDE-4 and PKA were determined by calculating the OD ratio of β-arrestin-2 /GAPDH, PDE-4 /GAPDH and PKA/GAPDH. The OD of β-arrestin-2, PDE-4 and PKA were analyzed on the Gel Image Analysis System (Tanon 2500R, Shanghai, China). The procedures were repeated 3 times to obtain the average value.

2.6 Assessing the interaction of β-arrestin-2 and PDE-4 using Co-immuno- precipitation

The protein samples were extracted from the fresh BLA tissues, and then mixed with non-specific mouse or rabbit immunoglobulin G and the fully resuspended Protein A+G Agarose (Beyotime Institute of Biotechnology) and slowly shaken at 4 °C for 2 h. 2500 rpm for 5 min and the supernatant was used for subsequent immunoprecipitation. Mouse antibody against β-arrestin-2 or rabbit antibody against PDE-4 (Sata Ltd.) was added at 4 °C, the mixture was slowly agitated overnight and then fully resuspended in Protein A+G Agarose with 4 °C with gentle agitation for 2 h, 2500 rpm for 5 min, PDE-4 or β-arrestin-2 protein was immunoprecipitated from the whole cell lysates. Immuno-precipitates were washed, and subsequently subjected to western blot analysis using anti- PDE-4 or anti- β-arrestin-2 antibody.

2.7 Using Elisa to detect the concentration of cAMP

After the evaporation of liquid nitrogen, the frozen BLA tissue was weighed and homogenized

in 10 volumes of 0.1 M HCl. Pipet all the liquid in plate wells reference manual (cAMP Elisa kit, ewEast, China). The plate was incubated at room temperature for 2 h on a plate shaker at 250-500 rpm. The contents of the wells were emptied and each well was washed three times with 400 µL of solution; after the final wash, any remaining wash buffer was removed. Next, 200 µL of the Substrate solution was add in each well and then incubated at room temperature for 5-30 min without shaking. Finally, 50 µL of stop solution was added to every well to halt the reaction; the plates were read immediately with an optical density of 450 nm.

2.8 CREB immunohistochemistry

Cryosections sections were washed 3 times (5 min each) with 0.01 M PBS, and then treated with 2% BSA in PBS for 2 h at RT for blocking nonspecific reactions. The sections were treated with mouse monoclonal anti-CREB antibody (diluted to 1:200; Santa Cruz; CA, USA) in PBS solution for 24 h at 4 °C. The sections were washed 3 times with PBS, and then incubated with two-step IHC detection reagent (PV6001 and PV6002, Company of Zhongshan Golden bridge, Beijing, China) at 37 °C for 30 min. A brown color appeared in the slices after 3, 3'-diaminobenzidine colorization. Slices were then dehydrated and mounted with neutral gum. Five slides were randomly selected from each rat. For each slide, 5 randomly selected visual fields in the amygdala were chosen (×40 magnification). We recorded the optical density (OD) of positive cells in each field to evaluate the average OD. The OD of CREB immunopositive cells were analyzed using a Meta Morph/DPIO/BX41 morphology image analysis system.

2.9 Using real-time PCR to detect β-arrestin-2, PDE-4 and CREB

After decapitation, rat brains were dissected and BLA was removed. Total RNA was extracted using TRIzol (Invitrogen, Japan) according to the manufacturer's instructions. Reverse transcription of 1 µg of total RNA was into cDNA, and was performed with an RNA PCR Kit (AM Ver.3.0, TaKaRa bio, Otsu, Japan). The primers were designed and synthesized by Sangon Biotech Limited Company (Shanghai, China).

The primer sequences used for PCR amplification are shown in Table 1. The levels of β-arrestin-2, PDE-4 and CREB mRNA were determined from the ratio of β-arrestin-2/β-actin, PDE-4/β-

actin and CREB/β-actin.

Table 1. Primers respectively used for PCR.

Name		Primer	Product size
β-arrestin-2	Sense:	5'-CCA CAA AAG GAA CTC CGT GC-3'	185
	Antisense:	5'-GGA CGT TGA CAT TGA GGG GT-3'	
PDE-4	Sense:	5'-GAT GCG CTT GGA ACT TGA GC-3'	173
	Antisense:	5'-CCA CAT CAA AGC ATG TAT GAG CC-3'	
CREB	Sense:	5'-ATG CTG CGT CCA AAC ATA AAC AC-3'	121
	Antisense:	5'-CTG GCA CTC ACA TTG CCT ATC-3'	
β-actin	Sense:	5'-CGG AAA GAA GAT GAC GCA GAT A-3'	159
	Antisense:	5'-ACC AGA GTC CAA GAC AAT GC-3'	

2.10 Statistics

The results were expressed as Mean± S.D. The differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test using SPSS 17.0 software. A level of P <0.05 was considered to be statistically significant.

5

3. Results

3.1 Animal behavioral test

In the EPM Test (Table 2), the percentage of time in the open arms and the percentage of open arm entries were calculated. Rats showed a significant reduction in the percentage of time spent in the open arm ($F_{(3,8)} = 24.64$, $P < 0.05$) and percentage of the number of entries into open arms ($F_{(3,8)} = 23.65$, $P < 0.05$) on SPS 1d, SPS 7d and SPS 14d in comparison with control group. These results indicated SPS induced increased fear/anxiety-related behaviors.

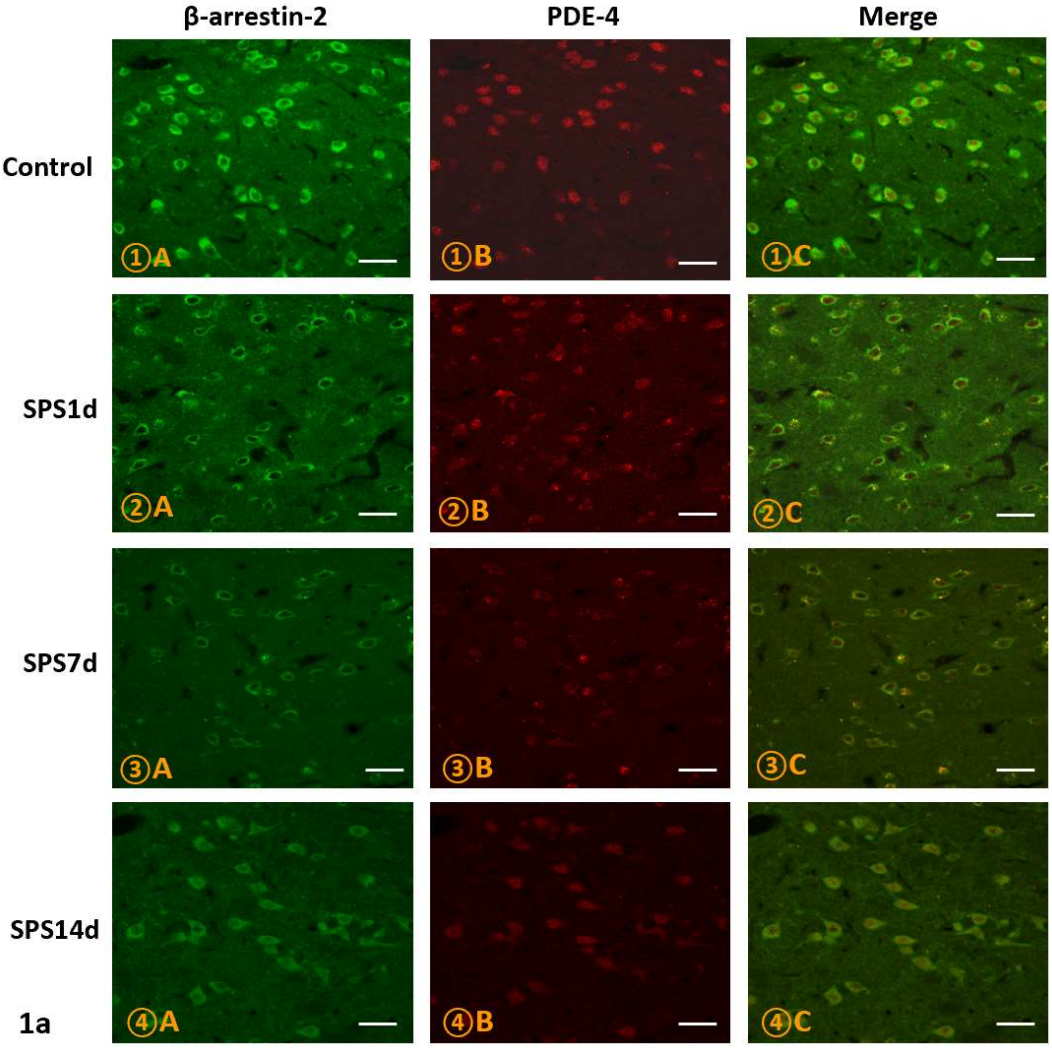
Table 2. The results of EMP test

Group	Time spent in open arm (%)	Number of open arm entries (%)
Control	30.92±2.17	45.69±3.48
SPS 1d	22.67±2.22*	38.04±2.57*
SPS 7d	16.99±2.08*	28.39±2.00*
SPS 14d	20.91±1.67*	35.52±1.77*

Statistical analysis was carried out by ANOVA test (*P < 0.05 compared with control group).

3.2 Immunofluorescent observation of β-arrestin-2 and PDE-4 expression

The concentrations of FITC- and CY3- labelled β-arrestin-2 and PDE-4 were measured in the BLA. The immunofluorescence staining results are shown in Figure 1. In the control group, immunoreactivity of β-arrestin-2 was mainly distributed in the cytoplasm of BLA neurons. At all the time points after SPS, the expression of β-arrestin-2 decreased significantly in comparison with the control group. In addition, at 7d after SPS, β-arrestin-2-signal was mainly distributed near the cell membrane (Fig. 1a) suggesting that after SPS, β-arrestin-2-positive products may transfer from the cytoplasm to the cell membrane. PDE-4 signal was mainly distributed in the nucleus and the cytoplasm of BLA neurons. BLA neurons showed strong positive reactions in the control group with relatively heavier staining. Like β-arrestin-2, PDE-4 was significantly decreased in the BLA region of SPS 7d rats compared with control rats, and then gradually increased to normal (Fig. 1b).



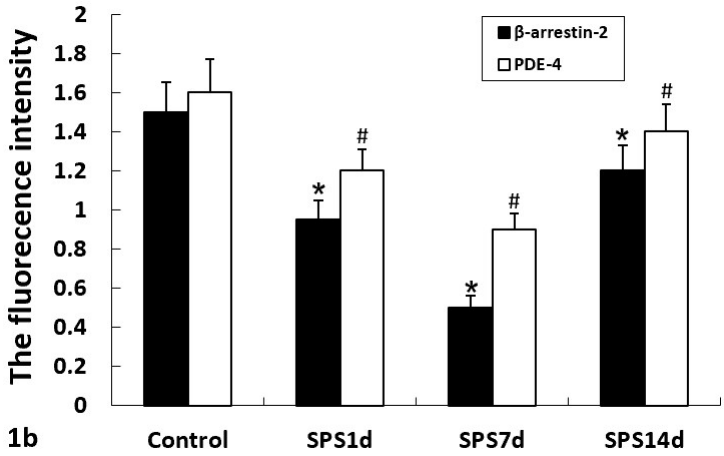


Fig. 1 Immunofluorescent positive result and quantitative analysis of β-arrestin-2 and PDE-

4 in the amygdala. 1a: Positive images (×400). The merged images show that β-arrestin-2 (green) and PDE-4 (red) were co-located. ①A →③A, showed that β-arrestin-2-positive products may transfer from the cytoplasm to the cell membrane. Bar = 50 μm. 1b. Quantitative analysis. The intensity of β-arrestin-2 and PDE-4 decreased after SPS, with a minimum at SPS 7d. *P < 0.05 compared with rats in the control group. # P < 0.05 compared with rats in the control group.

3.3 Western blot analysis protein expression levels for β-arrestin-2, PDE-4 and PKA

Similar findings were observed in the results of the western blot for β-arrestin-2 and PDE-4, as shown in Figure 2. Molecular weights of β-arrestin-2, PDE-4, PKA and GADPH were 55, 90, 42 and 36 kDa, respectively, showing clear bands (Fig. 2a). After SPS, the density of β-arrestin-2 ($F_{(3, 8)} = 93.82$) and PDE-4 ($F_{(3, 8)} = 37.55$) bands showed a significant decrease on SPS 1d and a further decrease on SPS 7d (Fig. 2b, $P < 0.05$). The levels of PKA significantly increased on SPS 1d and peaked on SPS 7d (Fig. 3), and then decreased on SPS 14d ($F_{(3, 8)} = 52.20$, $P < 0.05$).

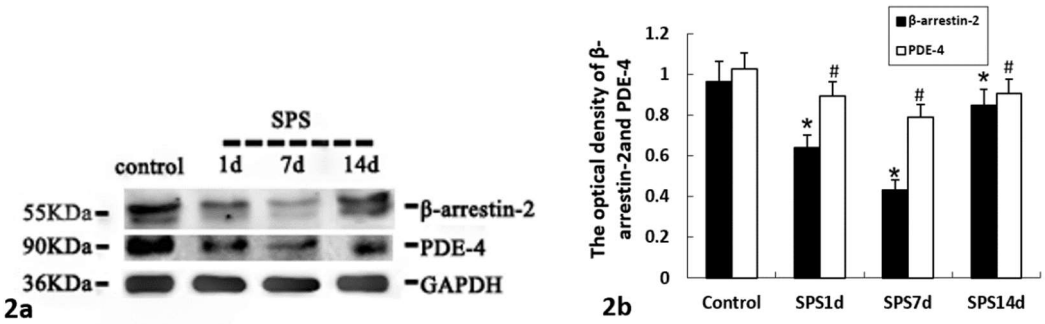


Fig. 2. Protein expression in the BLA detected by western blot. Fig 2a: Presentation of representative bands of β-arrestin-2 and PDE-4 protein levels. Fig. 2b. Quantitative results. A decrease in β-arrestin-2 and PDE-4 protein expression was observed in SPS rats. *P < 0.05 compared with rats in the control group. # P < 0.05 compared with rats in the control group.

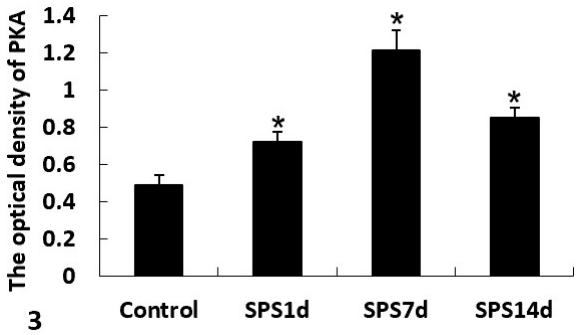


Fig. 3. Quantitative analysis for PKA based on western blot results. The level of PKA was peaked on SPS 7d. *P < 0.05 compared with the control group.

3.4 The results of co-immunoprecipitation for β-arrestin-2 and PDE-4

The results of co-immunoprecipitation showed that β-arrestin-2 and PDE-4 were present as a complex in the amygdala. The amount of the complex decreased in SPS7d (Fig. 4).

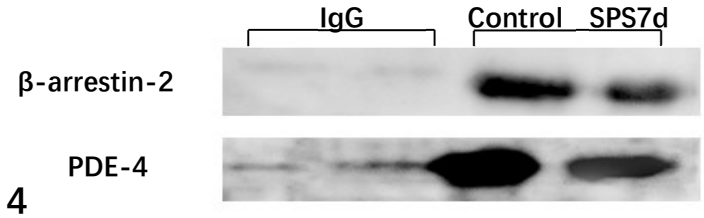
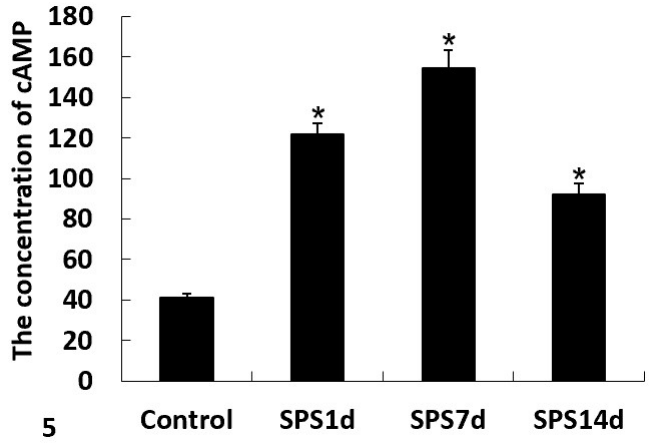


Fig. 4 Co- immunoprecipitation of β-arrestin-2 and PDE-4 in amygdala. Above bands β-arrestin-2: Homogenates were treated with antibody against β-arrestin-2, and presence of the partner protein PDE-4 was determined by Western for blot. Below band PDE-4: Homogenates were treated with antibody against PDE-4, and presence of the partner protein β-arrestin-2 was determined by Western for blot. Normal IgG as negative control which is non-specific interference.

3.5 cAMP levels were increased in SPS rats

A significant increase in cAMP levels in the amygdala was observed at 1 day, 7 days and 14 days after SPS exposure in comparison with the control group. The levels of cAMP began to increase on SPS 1d, and peaked on SPS 7d and then returned towards normal ($F_{(3, 8)} = 196.72$, $P < 0.05$, Fig. 5).

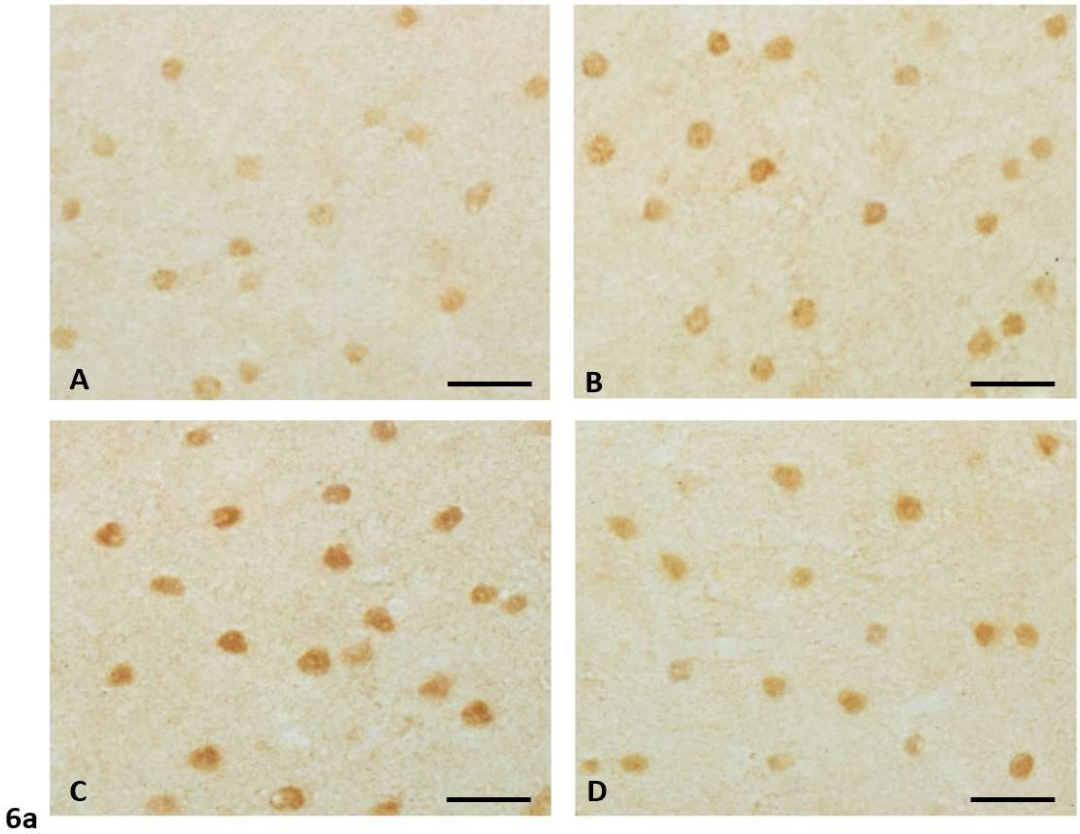


5

Fig.5. cAMP levels in the amygdala based on Elisa results. The concentration of cAMP began to increase on SPS 1d and peaked on SPS 7d. *P < 0.05 vs. the control group.

3.6 Increase of CREB in the BLA neurons after SPS exposure in immunohistochemical assay

Because CREB is downstream of cAMP signaling, we performed immunohistochemical staining in the BLA (Fig. 6). The immunoreactivity of CREB was localized in the nucleus (Fig. 6a). We observed an upregulation of the immunoreactivity of CREB on 1d after SPS. It peaked on 7d after SPS and then declined on 14d after SPS (Fig. 6b) ($F_{(3, 8)} = 41.83$, Fig. 7, $P < 0.05$).



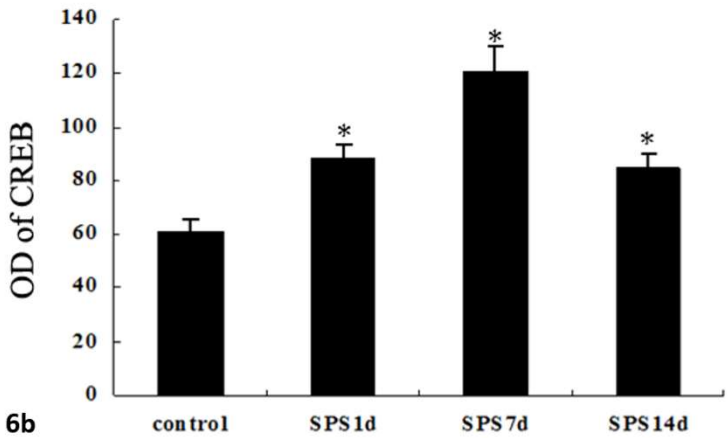


Fig.6. Immunoreactivity and quantitative analysis of CREB in the BLA (×400). 6a: is the expression of CREB in the SPS groups was increased compared to the control group. A: Control group; B: SPS 1d group; C: SPS 7d group; D: SPS 14d group. Bar=50μm. 6b: Quantitative analysis results. The intensity of CREB increased at the SPS 1d, and peaked at SPS 7d. *P< 0.05 compared with the control group.

3.7 Real-time PCR results of mRNA for β-arrestin-2, PDE-4 and CREB

The expressions of β-arrestin-2/β-actin ($F_{(3, 8)} = 16.51$, $P < 0.05$), PDE-4/β-actin ($F_{(3, 8)} = 21.37$, $P < 0.05$) decreased significantly after SPS stimulation and began to come towards normal on SPS 14d (Fig. 7a), which was consistent with the results of immunofluorescence and western blot.

The expression of CREB mRNA analyzed by real-time PCR showed a significant increase in the SPS group compared with that in the control group (Fig. 7b). The ratio of CREB/β-actin peaked on SPS 7d and then gradually decreased ($F_{(3, 8)} = 26.22$, $P < 0.05$).

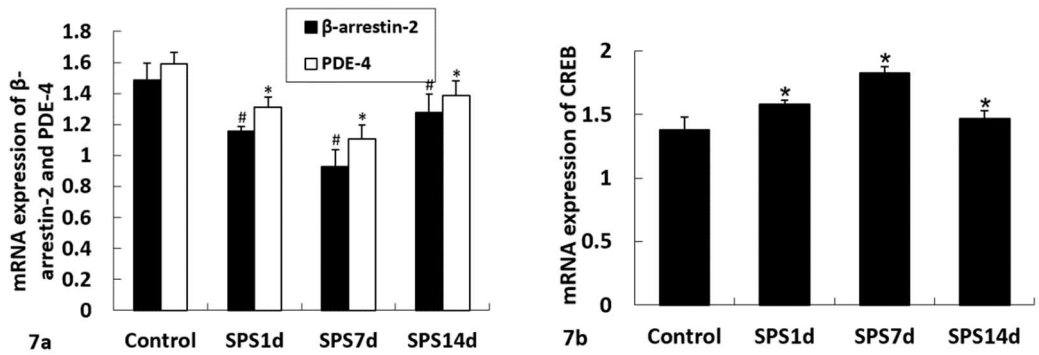


Fig. 7. Real-time PCR was used to detect changes in the mRNA expression of β-arrestin-2, PDE-4 and CREB. 7a: β-arrestin-2 and PDE-4 mRNA expression. 7b: CREB mRNA expression. *P < 0.05 and # P < 0.05 compared with the control group.

4. Discussion

PTSD is an anxiety disorder caused by a life-threatening traumatic experience, which affects a patient's quality of life and social stability. PTSD can be categorized as a disorder of dysregulated fear processing [1]. Aberrant fear learning is one of the central features of this disorder as demonstrated by cue-induced re-experiencing responses (e.g. flashback) that are slow to extinguish in humans [27]. Different types of memory depend on different parts of the

brain; for example, space location memory is associated with the hippocampus and fear/anxious emotional memories are associated with the amygdala. It is well-known that the amygdala and prefrontal cortex are key sites of synaptic plasticity that mediates aspects of fear learning and memory [28].

Many animal studies have suggested that molecular mechanisms of synaptic plasticity in the amygdala play a key role in fear extinction and ultimately in the PTSD symptoms. Recent studies have found that the morphology and arborization of dendritic spines (small protrusions that receive the majority of excitatory synapses) change as a result of fear conditioning and extinction in the cortical areas of the brain that are central to these learning processes [29-32]. On the basis of these findings, it is hypothesized that the amygdala, particularly BLA may be the key region of fear initiation.

The amygdala has also been directly implicated in PTSD. Evidence from clinical studies comparing individuals with PTSD to healthy controls showed that those with PTSD have increased amygdala activity to both negative stimuli and to trauma-specific stimuli [33]. The amygdala is a key brain structure in emotional processing and plays a critical role in the acquisition, consolidation and behavioral response to associative fear [34]. Thus, we aimed to detect changes in BLA.

In the present study, we used elevated plus maze tests to examine fear/anxious -related behaviors and then to confirm the main symptom of PTSD was abnormal fear memory. We found that SPS induced fear/anxious memory enhancement, and peaked on SPS 7d. After SPS, β-arrestin-2, PDE4 and the complex of β-arrestin-2/PDE4 were reduced. In line with a reduced containment of stimulatory G-protein signaling we found that the amygdala cAMP levels gradually increased after PTSD and peaked at SPS 7d. The enzyme immediately downstream of cAMP is PKA, and so is predicted to show higher activity. PKA levels were also increased after SPS stimulus and peaked on SPS 7d. Thus, our data suggest that response to fear conditioning, cAMP/PKA signaling is increased for 2 weeks, perhaps as a consequence of lower

activity of the β-arrestin-2/ PDE-4 pathway.

According to literature, the activity of PKA change is a necessary signal for fear memory consolidation [35]. Because it can bring about a change in the activity of nuclear transcription factors, such as CREB, to cause a new protein synthesis. CREB activation can lead to structural change of dendritic spines in BLA to promote and to maintain long-term fear/anxious memory. Experimental results showed that phosphorylation of CREB had a regulatory role in the synaptic plasticity of hippocampal neurons [36-38]. Thus, CREB can be considered molecular master switch of fear memory/anxious mechanism. Our results showed that CREB increased after SPS, peaked on SPS 7d, and then decreased to normal, and we expect that elevated CREB signaling leads to an abnormal amygdala-driven fear/anxious memory of PTSD. A caveat is that we quantified total CREB levels, and not the specific phosphorylated protein that is linked more directly to transcriptional activity.

Therefore, β-arrestin-2 and PDE-4 may act through the cAMP-PKA signaling pathways and further influence CREB phosphorylation, which further affect changes in neuron synaptic plasticity in BLA. Taken together, our data demonstrate that the reductions in β-arrestin-2, PDE-4 and the complex of β-arrestin-2/PDE-4 may lead to fear/anxious memory enhancement after SPS.

5. Conclusions

Our results suggest that β-arrestin-2 and PDE-4 may be involved in the formation of PTSD; low β-arrestin-2 and PDE-4 expression may cause or maintain high signal transduction pathway activity promote the formation and development of PTSD by influencing BLA in fear/anxious memory. β-arrestin-2 and PDE-4 may provide alternative intervention targets for more effective treatment for PTSD.

Acknowledgments

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6

Discussion and perspectives

In this thesis, we evaluated GR-related changes in the brain of rats that were exposed to the three consecutive stressors of the SPS model for PTSD. We tested the potential of RU486-treatment in reversing stress-induced effects, and evaluated the GR sensitivity after administered exogenous CORT. We found that GR antagonism had effects on fear behavior, the HPA axis and gene expression in the brain when administered one week after SPS and evaluated the effects 15 days after SPS (**chapter 2**). We also administered RU486 starting 3 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. We demonstrated that GR antagonist RU486 treatment in rat acted in interaction with stress, and can normalize some stress-induced parameters (**chapter 3**). However, varying the timing of RU486 administration and evaluation gave different behavioral results and dynamics of gene expression, that revealed complex interactions between stress and RU486 over time. In **chapter 4**, tested the hypothesis that after SPS GR sensitivity is enhanced not only in the HPA axis, but at multiple sites in the brain. Our data suggest the enhanced stress responsiveness after SPS to moderate but not mild stressors and a sensitization of brain GR signaling that extends beyond direct negative feedback regulation. Increased GR sensitivity may explain the effects of GR antagonists that occur relatively long after stressor exposure. In **chapter 5**, the changes of β-arrestin-2 and PDE-4 related to fear/anxious behavior one week after SPS. That showed that these factors may be involved in the formation and development of PTSD.

Overall, our findings support the notion that severe stressors induce a trajectory of changes in behavioral responsiveness and in the brain circuits that underlie this responsiveness. However, the adaptations that occur are broader than this, and include HPA axis reactivity. These adaptations may be considered as 'allostasis' – in that different internal setpoints are used to achieve homeostasis, or different mechanisms are employed to regulate a setpoint (leading to allostatic load). This is perhaps most clearly demonstrated by the (unanticipated) effects of GR antagonism on body weight in our experiments (chapter 2). In control conditions RU486-treatment did not affect body weight, but in SPS rats RU486 led to a reduced body weight gain

(away from normalization of the stress effect). Thus, a physiological parameter that had been GR-independent became GR-dependent after the SPS-procedure. As such, this effect confirms the central notion of the work in this thesis, namely that there is a substantial change in glucocorticoid signaling after SPS, analogous to existing hypotheses about PTSD development [1].

Glucocorticoid levels in PTSD

Clinically, lower baseline cortisol levels and enhanced negative feedback in the HPA axis have been often reported in PTSD [2-4]. Such enhanced negative feedback was indeed one of the reasons that the SPS model became widely adopted as a model for PTSD [5-7]. However, a previous systematic review reported no differences in basal cortisol levels between PTSD patients and controls [8]. Some studies even showed the AM cortisol levels increased in PTSD patients [9]. Such differences may be in part methodological. There may be differences in blood and saliva cortisol, and there may be differences in how stressed subjects were at the moment of sample collection. The time after the trauma may also be a factor – in SPS rats there are also change over time.

Under unstressed conditions, GC hormones have a characteristic circadian pattern of secretion. In addition, there is an ultradian rhythm with a period of 1-2 hours, which arises due to intrinsic activation and inhibition loops in the HPA axis [10, 11]. We used two therapeutic schedules of late RU486 administration (from 8-10 d after SPS, chapter 2) and early RU486 administration (from 3-5 d after SPS, chapter 3) to evaluated basal AM and PM hormone levels, and stress responses, but for lack of intravenous sampling can provide no information on the ultradian rhythm. We also used exogenous to test GR sensitivity, but our setup did not include validation of the enhanced negative feedback in our SPS rats (chapter 4).

Our results (chapter 2 and chapter 3) showed that the circadian corticosterone rhythm of the SPS rats was blunted in the first week after stress exposure, with elevated levels in the morning and decreased levels in the evening. This blunted basal corticosterone pulse amplitude is

consistent with a previous study, where the authors used the predator scent stress (PSS) exposure as the animal model of PTSD and evaluated in the acute aftermath of trauma at 6.5 hours [12]. However, in other studies, corticosterone was elevated within one day of SPS initiation but had returned to baseline levels at 7 days after SPS [13, 14]. The reduced PM levels that we observed are in line with a GR-dependent increased feedback sensitivity [15]. The increased basal AM levels may reflect lower MR-mediated feedback [16, 17], or rather an increased stimulation of the HPA axis as a consequence of continued stress. A model first suggested in a paper by Avital et al. [18] and again by Peters et al. [19] also implicates the binding of corticosterone to the high-affinity MR as a forward modulator of the HPA axis.

Two weeks after SPS we found the corticosterone level of stressed rats towards an overall elevated activity. In another study, serum CORT levels were evaluated on 9, 14 and 28 days after SPS. The corticosterone levels on day 9 and 14 showed a non-significant trend towards an increase, and then dropped below normal between 14 and 28 days after SPS [20]. Lin *et al* saw decreased PM levels of corticosterone at two weeks after SPS. They could reverse or rather prevent this, by continuous treatment with RU486 for a week, starting immediately after the SPS procedure but not when treatment started at day 8 after SPS [21]. In different animal models of PTSD, the results of corticosterone level are inconsistent. This may be due to the nature of the stressor, the time after the stressor, and the context. For example, a PTSD model involving both repeated maternal separation and adult exposure to inescapable foot shock reduced basal PM (between 13:00-16:00 h） corticosterone levels in plasma two months later [22].

In our studies, after SPS the corticosterone AM levels were more or less consistently higher than the normal. RU486 normalized these high values of SPS rats towards to the control levels. Late RU486 administration could adjust the SPS-induced GR overactivity and HPA axis dysfunction. Such a reversal effect of RU486 treatment reinforces the potential of targeting GR for treatment of PTSD. Interestingly, early RU486 administration reversed the SPS-induced

increase in plasma corticosterone concentrations, but did not completely normalize it (**chapter 3**). The lack of full 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. This lack of full efficacy of RU486 could be caused by the abnormally high elevation of corticosterone caused by stress, which might necessitate extended treatment durations [23]. Overall, both late and early of RU486 administration affects the outcome of SPS, in that corticosterone levels moved towards normalization.

In chapter 4, we designed two experiments to measure the corticosterone level at different time points. The first experiment showed that corticosterone levels were still elevated 60 min after vehicle injection at 60 min in the SPS group. This high levels of corticosterone in vehicle-treated SPS rats indicated enhanced stress reactivity in these animals. We hypothesize that this was caused by the combination of the injection and the tail blood sampling, as corticosterone level elevated only after the injection of the exogenous hormone (without blood sampling) in another experiment. Our data suggest the enhanced stress responsiveness to moderate stressors after SPS. Although enhanced negative feedback of the HPA axis in SPS rats was previously found, the stress response of the SPS rats did not allow us to observe that. As mentioned, enhanced negative feedback may be reflected in the lower PM corticosterone levels that we observed after SPS in chapter 2.

In PTSD patients, previous findings have not been fully consistent in cortisol levels before and after therapy [24]. This study showed higher average cortisol levels before and after therapy predicted greater PTSD symptom improvement. That preliminary evidence indicated that cortisol levels during therapy sessions could serve as a biomarker for assessing the response to exposure-based treatments for PTSD. The administration of RU486 can potentially modify the SPS-induced GR excessive activition and HPA axis dysfunction. Restoring the levels of plasma cortisol after inhibiting the GR may be beneficial for individuals diagnosed with PTSD, but it is not clear what this would mean for the use of cortisol as a biomarker for therapy response.

GR target genes

Disruption of GR and MR signaling is believed to be the cause of HPA axis dysregulation, which is observed in stress-related psychiatric disorders [25] such as PTSD. Particularly, heightened sensitivity to GR has been one of the most consistent discoveries in the field of altered HPA-axis function in PTSD [26, 27]. Corticosterone and cortisol promote GR activation, and GR as a transcription factor regulates a diverse set of genes upon activation [28]. Although there is substantial variation in GR target genes between cell and tissues, a number of direct target genes are shared between many cell types, such as the gene *FKBP5*. Others are expressed in fewer cell types, and may or may not be direct target genes, such as *PACAP.* In this thesis, we examined the expression of these several candidate genes and a potential epigenetic mechanism in the PVN and limbic brain regions. C-fos was used as a marker for neuronal activity, rather than a direct GR target gene. The gene expression changes in PVN, amygdala and hippocampus revealed complex interactions between brain region, stress, RU486 and time. Notwithstanding this complexity the data do yield insights in sustained or, rather, transient changes after stress and the RU486 intervention.

While ultimately all GR targets interact in a complex manner to shape the state of the brain, here our ambition was not to fully explain the diseased brain state. We rather chose to evaluate a number of relevant genes in different brain areas to prober their potential involvement in affecting behavior and endocrine responses. Below, we discuss the most prominent GR targets one by one.

The expression of GR is widespread in most cell types throughout the brain, and found in highest abundance in typical stress regulatory centers, like the PVN, amygdala and hippocampus [29], which is where we measured gene expression. GR in the medial parvocellular part of the PVN co-localize with CRH and play a key role in the regulation of the HPA axis [30]. The hippocampus is crucial in regulation of the stress response and memory formation. Lesion studies of the hippocampus suggest a critical role in the processing of

contextual information and retrieval of memory [31-33]. (Reversible) deactivation of the dorsal hippocampus disrupted the memory of a threat in a specific context [34, 35]. Our research group has showed that GR is relatively highly expressed in oligodendrocytes, microglia and endothelial cells [36], and that microglia GR may play a role in memory consolidation [37]. The amygdala is critical for the implicit, physiological expression of threat learning in humans. GR play a role in several subregions of the amygdala, e.g. the basolateral nucleus and the central nucleus of the amygdala [38, 39]. The hippocampus and amygdala, two vital components of the HPA axis, which play an key role in the regulation of the activation and negative feedback control of the HPA axis. Prior research indicated that PTSD is related to dysfunction of the neural circuitry that supports fear learning and memory processes. Both the hippocampus and amygdala seem to play an important role in the cognitive-affective dysfunction associated with PTSD [40]. Based on the above reasons, we chose these three brain regions to measure the GR target genes expression.

MR/GR expression

Because the expression of MR and GR forms the basis of transcriptional effects of corticosterone, we determined their mRNA expression as potential mediators of corticosterone effects. Our results showed no substantial differences in GR and MR mRNA after SPS. However, as we saw in chapter 4, there can be differences in GR signaling (chapter 4) in absence of changes in receptor expression. Next to ligand availability, mechanisms for these differences in corticosterone signaling can be many. GR can translocate into the nucleus and bind directly to GREs and then regulate the expression of target genes. GR can also have effects through non-genomic mechanisms, triggering fast cellular reactions that occur within a few seconds to minutes and do not require alterations in gene expression [41, 42]. All the processes involve many interactions with other proteins in the cytoplasm and/or the cell nucleus. Many types of post-translational modification of GR subtypes expands the diversity of glucocorticoid responses [43, 44]. The activity of other signaling pathways with which the GR interacts ('cross-talk') may differ. The MR and GR transcriptional activity will be influenced by the "state" of other active signaling pathways in addition to the set "trait" of cellular context

[45]. These include the β-arresting pathway, that is a downstream target of GR signaling and was studied in **chapter 5** [46]. β-arrestin2 is essential for termination and transduction of GPCR signals. Glucocorticoids modify the equilibrium between G-protein and β-arrestin-dependent signaling responses of GPCRs, and may play a role in the changes observed in SPS rats.

FKBP5

While there were no striking changes in MR and GR expression, their direct target genes responded to the SPS procedure. Fkbp5 is cochaperone of the GR-HSP70/90 heterocomplex, can lower GR affinity and thereby affects glucocorticoid binding [47]. This gene's expression depends on GREs located within introns 2, 5 and 7. The FKBP5 gene is also subject to epigenetic regulation. The DNA methylation of FKBP5 intronic regions is the primary epigenetic mark under examination [48]. FKBP5 DNA methylation has traditionally been considered a static process associated with transcriptional repression [49]. An influential study showed that the SNP rs1360780 in FKBP5 which confers risk to develop PTSD is located in intron 2, close to a functional GRE shown to mediate the transcriptional effects of the GR. Methylation of *FKBP5* could be considered as a marker of PTSD symptom alteration [50].

We determined the FKBP5 mRNA expression at 8 and 14 days after SPS (chapters 2/3). FKBP5 mRNA was consistently down-regulated 8 days after SPS. The lower expression after 8 days would reflect less GR drive on the Fkbp5 gene, but may also reflect enhanced GR activity, which should then be apparent for other genes. This is in line with enhanced feedback sensitivity, and with the enhanced response we observed in Chapter 4, but Fkbp5 protein levels should be determined to substantiate this notion. At day 15, FKBP5 mRNA expression showed a significant interaction between stress and RU486 treatment. The comparison between 8 and 15 days shows that adaptations to a single day of stress are dynamic and certainly are not complete after one week. This may be reflected in the human literature on PTSD (see below), and is consistent with early work that showed long lasting adaptive processes after a single stressor [51]. In chapter 4, we tested the hypothesis that SPS affects the GR responsiveness in the brains. Here we observed that basal Fkbp5 mRNA expression did not change in SPS rats,

and changed in SPS rats treated with RU486.

FKBP5 methylation was tested at 8 days after SPS in the hippocampus, we observed changes at CpG site 5 and 7. CpG site 5 showed that the levels of DNA methylation decreased after RU486 and with stress after vehicle treatment, CpG site 7 showed that RU486 reversed the decreased methylation level only in the stress group. However, the CpG methylation levels did not match the observed mRNA expression levels.

In chapter2 - 4 we found no difference or decreased in total FKBP5 expression between PTSD and control animals. Given that FKBP5 expression should increase after GR activation, this is somewhat surprising. However, these findings do show that prior-stress experience may impair levels of FKBP5 which may result in poor adaptation to future stress [52]. Another study discovered that the increased GR and FKBP5 complex in blood cells of PTSD patients could lead to decreased GR phosphorylation and nuclear translocation, which would be expected to affect gene transcription regulated by GR [53].

For humans, a study found that patients with PTSD showed a noticeable decrease in *FKBP5* mRNA expression in their whole blood [54]. Another study showed that the methylation levels of FKBP5 reduced significantly as CAPS score decreased in responders, while no changes occurred in non-responders [55]. Two other studies have tested whether FKBP5 methylation is related to treatment responses in veterans with PTSD. Yehuda et al. [56] found that positive outcomes corresponded to reductions in methylation of the FKBP5 exon 1 promoter region during the treatment period. Bishop et al. [57] reported that significant decreases in FKBP5 methylation in intron 7 region for those who responded to treatment whereas increases in methylation in non-responders.

Overall, our results indicated that FKBP5 had changed both at mRNA and DNA methylation level after stress and RU486. On the other hand, these results also have limitations, and overall the data are not consistent enough to consider FKBP5 expression as a substrate for disease

state or as biomarker for SPS effects.

Sgk1

The kinase Sgk1 is a downstream mediator of glucocorticoid effects on the brain and under direct transcriptional control of GR [58, 59]. Other evidence suggested that Sgk1 also directly enhances GR function and potentiates glucocorticoid effects [60]. So, Sgk1 may be a key enzyme involved in the downstream mechanisms and in the upstream potentiation and maintenance of GR function. Sgk1 expression was found to be down-regulated in the postmortem prefrontal cortex of six subjects with PTSD [61]. As with FKBP5, this may be interpreted either as a cause or a consequence of dysregulation of glucocorticoid signaling in the brain of patients.

Because of the reported highly significant reduced expression in PTSD subjects, we have tested the regulation and function of Sgk1 on both 8 and 15 days in SPS models. Sgk1 expression differed strongly between conditions of stress and RU486, but the effects depended on the brain region and time after SPS/treatment. In control animals, RU486 led to lower expression in PVN and hippocampus, in line with GR-dependence of Sgk1 gene expression. However, some of our findings are counterintuitive, if we consider Skg1 effects in stress to be purely GR-driven. In the amygdala, SPS induced Sgk1 mRNA levels, regardless of antagonist treatment. In animals that underwent SPS 15 days earlier, treatment with RU486 led to a strong *increase* in Sgk1 mRNA levels. This latter finding is – next to bodyweight – an example of some biological process that may become GR-dependent after stress.

The difficulty to interpret these findings in term of GR activity was one of the arguments to evaluate the response to an acute challenge with corticosterone, as described in chapter 4.

PACAP

The neuropeptide PACAP affects many cellular stress processes within hypothalamic and limbic systems in mammals [62]. A previous study found that a polymorphism of PAC1R in the PACAP-

PAC1R system is linked to increased risk of PTSD in women, and these women had higher blood PACAP levels [63]. In addition, following classical fear conditioning, mRNA levels of PACAP are increased in the extended amygdala of adult rodents [64].

Substantial changes in PACAP mRNA levels were only observed in the two weeks experiment (chapter 2). As with Sgk1, the effects differed greatly between brain regions. In the PVN PACAP mRNA levels were suppressed after RU486, but only in control rats. Amygdala PACAP mRNA expression was decreased 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 higher after SPS, and this would be the only area that matches the increase that was observed in the data by Ressler et al. We conclude that PACAP gene expression shows substantial plasticity, but that it does not consistently respond to trauma-like stressors across brain areas.

COMT

The COMT allele rs4633C may be causally related to PTSD symptoms [65]. The COMT val158met polymorphism has been associated with risk for PTSD and hippocampal volume [66] and impaired fear inhibition [67]. Based on genetic variation COMT also may be considered the most promising gene for panic disorder diagnostic to date [68]. Because of our behavioral test result in chapter 3 where the behavior 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 lower COMT mRNA levels in the SPS vehicle group compare with the control vehicle group on day 8. The limitation is that this low COMT mRNA is certainly not sufficient to explain the behavioral data.

β-arrestin2 signaling pathway

The work in chapters 2-4 was designed with a focus on GR signaling. Because GR is a transcription factor, effects at the mRNA level may be taken as a valid approach. The work in chapter 5 was performed earlier, and here we looked at factors that may be relevant for other

parts of the stress response. Here we looked at the protein level.

We evaluated the expression of β-arrestin2, PDE-4 and their regulated downstream signaling pathway in chapter 5. β-arrestin2 is important for stress adaptation through its regulatory role in Gs-coupled receptor signaling, including CRF-R1 [69-71]. PDE-4 affects learning and memory formation function from decrease cAMP levels and then led the expression alteration of the cAMP- PKA- CREB signaling pathways [72, 73]. Our data indicated the expression of β-arrestin-2, PDE-4 and their complex were decreased at 7 days after SPS, and these low expressions stimulated the high activity of signaling pathways at 7 days after SPS. It suggested that β-arrestin-2, PDE-4 and cAMP- PKA- CREB pathway may be influencing the fear/anxious memory.

GR-sensitivity

From our data it is clear that SPS and RU486 treatment led to changes in gene expression, and that these changes form trajectories over time. Even for well described GR target genes is very difficult to relate the changes to GR signaling, largely because of the time between treatments and measurements of gene expression. We therefore also directly tested GR-sensitivity by acute corticosterone treatment (chapter 4). we evaluated the mRNA responses on 30 min after corticosterone injection because the corticosterone levels were strongly changed on this time. next to FKBP5, we evaluated the expression of additional corticosterone-induced target genes. Our results showed that FKBP5 and Drd1a were responsive to corticosterone only in the SPS rats in the hippocampus and in the amygdala. Irs2 and Ntf3 responded to corticosterone only in the hippocampus of SPS rats. These data suggest the enhanced stress responsiveness after SPS to stressors.

We had hoped to evaluate the expression of target genes at more time points. However, the tail incision for repeated blood collection led to a strong corticosterone response only in SPS rats, and this stood in the way of a meaningful comparison of gene expression changes in these animals. It also prevented further evaluation of differences in negative feedback strength *per se*. Except for the uncertainty of whether SPS rats enhance negative feedback sensitivity, we

are data suggest that GR nuclear translocation and the genomic GR signaling seems to be primed in SPS rats. Previous studies suggesting that PTSD is associated with enhanced GR signaling [74]. There is also data supporting the notion that insufficient glucocorticoid signaling is present in PTSD [75]. GR nuclear translocation is also one of the molecular mechanisms of PTSD [53]. However, our data suggest overall more rapid GR-mediated responses, and if anything to enhanced nuclear translocation in the SPS-subjected rats.

Behavior in PTSD

PTSD is classically characterized by anxiety, avoidance and enhanced fear memory [76]. RU486 may be a promising pharmacological treatment for PTSD which can block reconsolidation of cue-conditioned fear in preclinical research [77]. The preliminary results of the first study to examine mifepristone in PTSD patients showed mifepristone was significantly more effective than placebo [78]. Other clinical evidence implied that a controlled amount of mifepristone might have circumscribed cognitive-enhancing effects in Gulf War veterans suffering from chronic multi-symptom illness [79]. It is very challenging to model the complex human psychiatry in animals. SPS is one of the animal models proposed for PTSD, as it more or less consistently causes a range of behavioral changes closely resembling those described in PTSD, which marks SPS as a potential PTSD model [80].

In this thesis, open field, elevated plus maze and fear condition test were used to evaluate behavioral changes of SPS rats. In chapter 2, The results indicated that anxiety behavior and fear conditioning were increased at 15 days after SPS. RU486 was able to overcome some of the SPS-induced changes in behavioral reactivity and affect the fear memory acquisition. These results suggested that RU486 has a good prospect as a treatment for PTSD. However, in chapter 3 we observed a different result, as SPS led to overall higher locomotor activity in the OF and the EPM one week after the SPS exposure. This may be due to the additional stimulus of the daily injection, which may have changed the formation of latent symptoms in incubation period. Indeed, we observed that some animals seemed agitated, perhaps pointing to a panic-like state. These effects were in interaction with RU486 treatment. In chapter 5, we observed

behavioral changes in SPS rats at 7 days, which is in line with other work [81]. Overall, our data showed that SPS-induced behavior changes over time, RU486 treatment affects the outcome of SPS both in the 3 days and 8 days intervention, in which behavior and corticosterone levels moved towards normalization. The data also showed which correlates between gene expression and behavioral/ 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. Thus, the optimal intervention timing should be considered. Lin et al. [21] examined the effects of early or late RU486 administration in SPS rats. They demonstrated that early RU486 administration could inhibit SPS-induced fear and anxiety abnormalities and glucocorticoid system dysregulation. Their results showed both early and late administration changed the gene expression. However, in clinical practice it may be difficult to start treatment immediately after trauma, given that it is not clear who will develop PTSD, and given that RU486 may also have intrinsic effects.

Short-term administration of RU486 could potentially counteract certain stress-related neurobiological changes and restore homeostasis to the HPA axis. Excessive levels of glucocorticoids may be an important cause of anxiety. In addition to their direct connection to anxiety [82], it also may affect the processing of information thereby influencing the behavioral reaction to particular forms of stress. After three days of repeated treatment, RU486 effectively lowered the levels of plasma corticosterone, reduced the excitability of the HPA axis and adjusted the HPA axis basic function to normalize abnormal behavior in rats.

GR antagonist RU486 treatment mechanism

The experiments described in this thesis have the explicit goal to model PTSD, and in part to test whether RU486 (mifepristone) may be used in pharmacotherapy. RU486 clearly had effects in the SPS model. It is important to mention that besides being a GR antagonist, RU486 also is a potent blocker of the progesterone receptor, and - with lower affinity – the androgen receptor. Even if we related its effects to GR antagonism, we cannot exclude that these other activities of RU486 played a role. For example, AR and PR are expressed at appreciable levels

in the rodent hippocampus [83].

If we interpret the RU486 effects as reflecting GR antagonism, its efficacy suggests ongoing GR-mediated signaling in the brains of SPS rats for many days after the stressor. Alternatively, RU486 may act as an 'inverse agonist': it is able to drive GR to the cell nucleus, and may cause recruitment of transcriptional repressor proteins by GR. Theoretically RU486-GR complexes may in this way silence transcriptional processes that were initiated earlier by GR [84]. This notion however remains unproven. In this respect it would be of interest to test other, selective GR antagonists for their capacity to reverse stress-induced changes in the rodent brain [85].

Future perspective

In our thesis, we studied the pathogenesis and potential treatment of PTSD, as modeled by the SPS procedure. The SPS procedure certainly led to changes, both in term of behavioral responsiveness, HPA axis function and gene/protein expression. However, these changes were dynamic over time, and brain region specific. Also, if treatment with GR antagonists is a viable treatment strategy, the optimal timing of such treatment is unclear. Immediate treatment may be optimal [21], but clinically this may not be always feasible. It is also interesting to consider the contrasting approach of treating PTSD patients (or SPS rats) with GR agonists. Clinical trials investigating the administration of low-dose cortisol have demonstrated a significant decrease in symptoms associated with PTSD [86, 87].

Future research may address such aspects. In order to better understand region-specific changes, (single-cell) whole transcriptome approaches may be used. In this way, we may capture a comprehensive overview of all the changes in e.g. transcription. Yet, it will be critical to first define the optimal time to do so, and the proper brain region. We may use c-fos staining to identify those brain regions that have a truly long-lasting change in reactivity after SPS, and focus on these. We can combine the GR signaling with the β-arrestin2 signaling, and to observe the β-arrestin2 signaling changes after RU486 administration. We may also use ongoing and future clinical studies as guidance to plan experiments in SPS rats that should explain and

support clinical findings.

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7

Summary

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Summary

Posttraumatic stress disorder (PTSD) is a psychological disorder that develops following exposure to perceived life-threatening trauma. Characteristic features include behavioral changes caused by heightened arousal, including fear and anxiety. PTSD also can cause functional changes in the HPA axis, and in brain regions such as the hippocampus, amygdala and so on. GR hypersensitivity, as defined by strong negative feedback, has been one of the most robust findings of altered HPA axis function in PTSD, but it is unknown whether this GR sensitivity generalizes to the brain. In this thesis, we evaluated GR-related changes in the rat brain that were exposed to the three consecutive stressors of the single prolonged stress (SPS) model for PTSD. We tested the potential of the GR antagonist RU486 treatment in reversing these stress-induced effects, and evaluated the GR sensitivity after administered exogenous corticosterone.

In **chapter 2**, we found that 3 days of GR antagonism had effects on fear behavior, the HPA axis and gene expression in the brain when the antagonist was administered one week after SPS and we subsequently evaluated the effects 15 days after SPS. RU486 had history-independent effects in reducing fear behavior. Gene expression analysis showed a diversity of in- and interdependent effects of stress and RU486. This normalization of a number of SPS effects after RU486 treatment reinforces the potential of targeting GR for treatment of stress-related psychopathologies.

In **chapter 3**, because many studies report behavioral changes one week after SPS, we administered RU486 starting 3 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. We demonstrated that the GR antagonist RU486 treatment in the rat acted in interaction with stress, and, again, that it can normalize some stress-induced parameters. However, varying the timing of RU486 administration and evaluation gave different behavioral results and dynamics of gene expression, which revealed complex

interactions between stress and RU486 over time.

In **chapter 4**, we hypothesized that after SPS GR sensitivity is enhanced not only in the HPA axis, but at multiple sites in the brain. We found that at an early time point gene expression of the GR target gene FKBP5 was induced in SPS rats, but not in control rats. Apparently, GR responses were exaggerated, or primed, as a consequence of SPS exposure. Next to sensitization of brain GR signaling that extends beyond direct negative feedback regulation, our data also suggest enhanced stress responsiveness after SPS to moderate but not mild stressors. Increased GR sensitivity may explain the effects of GR antagonists that occur relatively long after stressor exposure.

In **chapter 5**, we hypothesized that intracellular signaling that involves β-arrestin-2, PDE-4 and related signal transduction pathways relates to the fear memory regulation. We evaluated the activity of this pathway in the amygdala in relation to behavior using the SPS model. Our data indicated that SPS caused enhanced fear memory. The changes of β-arrestin-2 and PDE-4 related to fear behavior one week after SPS showed that these factors may be involved in the formation and development of PTSD. We conclude that the SPS lead to a decrease in β-arrestin-2 and a decrease in recruitment of PDE-4 which activates the cAMP-PKA-CREB pathway, and then leading to an enhancement of fear memory.

Samenvatting

Posttraumatische stress stoornis (PTSS) is een psychiatrische ziekte die kan optreden na blootstelling aan psychisch trauma. Kenmerkende aspecten van PTSS zijn gedragsveranderingen als gevolg van verstoorde emotieregulatie, inclusief verhoogde *arousal* en angst. PTSS kan ook gevolgen hebben voor de hypothalamus-hypofyse-bijnier (HBB) as, de niveaus van de stress-gerelateerde glucocorticoïde hormonen, en voor de activiteit van hersengebieden zoals de hippocampus en de amygdala. Een van de meest gerapporteerde biologische bevindingen in PTSS is het optreden van hypergevoeligheid van de glucocorticoïde receptor (GR) bij het proces van negatieve terugkoppeling binnen de HBB-as. Ofwel: blootstelling van het dier aan glucocorticoïde stresshormonen heeft meer effect in PTSS dan in gezonde mensen. Het is niet bekend of deze verhoogde gevoeligheid van de GR op meerdere plekken in het brein optreedt. In dit proefschrift bestudeerden we de GR in het brein van mannelijke ratten die blootgesteld werden aan het *single prolonged stress* model (SPS), dat aspecten van PTSS modelleert. Geïnspireerd op bevindingen in andere diermodellen voor stress, toetsten we in hoeverre een antagonist van de GR, RU486, in staat was om de gevolgen van blootstelling aan de SPS-procedure kon tegengaan. We toetsten ook de hypothese dat de gevoeligheid van de GR in meerdere hersengebieden veranderde na SPS. Daarnaast keken we in meer detail naar cellulaire veranderingen in de amygdala van ratten na SPS.

In **hoofdstuk 2** beschrijven we de effecten van behandeling met de GR antagonist, een week na SPS, gedurende 3 dagen. We evalueerden de effecten van deze behandeling twee weken na SPS. We zagen een heel aantal effecten van SPS op gedragsmaten voor angst, op de HBB-as, en op genexpressie in meerdere hersengebieden. RU486 verminderde angstgedrag, onafhankelijk van blootstelling aan SPS. RU486 had effecten op genexpressie in de hersenen, soms afhankelijk maar soms ook onafhankelijk van eerdere SPS. Een aantal veranderingen die optraden na SPS werden genormaliseerd door RU486. Dit geeft aan dat RU486 mogelijk nut kan hebben bij de behandeling van PTSS, of andere psychopathologieën die door stress veroorzaakt of verergerd worden.

In **hoofdstuk 3** beschrijven we een eendere, maar kortere studie. Omdat in veel van het eerdere onderzoek het effect van SPS al 1 week na de stressor bestudeerd werd, startten we de behandeling met RU486 3 dagen na de SPS-procedure, en keken we 8 dagen na SPS naar de effecten van de behandeling. We vergeleken de uitkomsten met het eerdere experiment, beschreven in hoofdstuk 2, waarbij de interventie na 7 dagen plaatsvond en we na 2 weken naar gedrag en genexpressie keken. We zagen ook hier dat de behandeling met de GR antagonist RU486 effecten had in interactie met blootstelling aan stress, en dat RU486 een aantal van de gevolgen van stress kon normaliseren. Echter, er waren behoorlijk veel verschillen tussen de kortere en langere proef, zowel wat betreft de effecten SPS en de effecten van RU486. Het is daarmee niet eenvoudig om de effecten dit diermodel te vertalen naar (fases van) de ziekte PTSS.

In het werk beschreven in **hoofdstuk 4**, toetsten we de hypothese dat er niet alleen hogere gevoeligheid van de GR is bij negatieve terugkoppeling binnen de HHB-as, maar ook op meerdere plekken in het brein. We zagen dat het bekende GR target gen FKBP5 op een heel vroeg moment na hormoonbehandeling reageerde in SPS ratten, maar niet in dieren uit de controlegroep. Blijkbaar leidde de SPS-procedure tot meer uitgesproken, of 'geprimede' effecten via GR. We zagen ook in deze studie dat de reactie van de HHB-as op matig sterke stressoren verhoogd was in dieren die eerder aan SPS blootgesteld waren. De verhoogde GR-gevoeligheid die we zagen, is mogelijk een van de redenen dat antagonisme van de GR werkzaam kan zijn, vele dagen na blootstelling aan het SPS-protocol.

In **hoofdstuk 5**, beschrijven we metingen aan de intracellulaire signaaltransductie van o.a. β-arrestin-2 en PDE-4 signaleringspaden, in relatie tot de sterkte van angstherinneringen bij de rat. Dit deden we in de amygdala van dieren die blootgesteld werden aan het SPS-protocol. We zagen dat SPS leidde tot een versterkt angstgeheugen bij een klassiek conditioneringsexperiment. We zagen veranderingen in β-arrestin-2 en PDE-4 die geassocieerd waren met deze versterkte angstconditionering. Via een uiteindelijke versterking

van het cAMP-PKA-CREB pathway, zouden deze effecten op geheugenvorming en angst relevant kunnen zijn voor de ontwikkeling van PTSS.

List of publications

Ding J, Chen X, Han F, Meijer OC. An Advanced Transcriptional Response to Corticosterone After Single Prolonged Stress in Male Rats. Front Behav Neurosci 2021, Nov 17.

Ding J, Chen X, da Silva MS, Lingeman J, Han F, Meijer OC. Effects of RU486 treatment after single prolonged stress depend on the post-stress interval. Mol Cell Neuroscin 2020, Oct; 108.

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Curriculum vitae

Jinlan Ding was born on 6 October 1981 in Lanxi, Heilongjiang province, China. In September 2001, she started her bachelor program 'Clinical medicine' and received her bachelor's degree in June 2006, at the Mudanjiang Medical college, Mudanjiang, Heilongjiang province, China. Later on, in September 2006, she was admitted as a postgraduate and majored in 'histology and Embryology' at the China Medical University, Shenyang, China. During this 3-year master program, she completed her thesis and obtained her master's degree in June 2009. Subsequently, she worked in the Teaching and Research Section of Histology and Embryology, China Medical University, Shenyang, China. In 2015, she studied as an on-the-job doctoral student at China Medical University and obtained her doctoral degree in June 2020.

In 2016, she was awarded financial support from the China Scholarship Council and started her another PhD program in January 2017 under the supervision of Prof. dr. Onno. C. Meijer, at the Division of Endocrinology of the Department of Internal Medicine, Leiden University Medical Center, Leiden, The Netherlands. Her research mainly focused on the sensitivity of GR and the treatment of GR antagonist in post-traumatic stress disorder. The results of her PhD studies are presented in this thesis.

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