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

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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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