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

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

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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 extend 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.

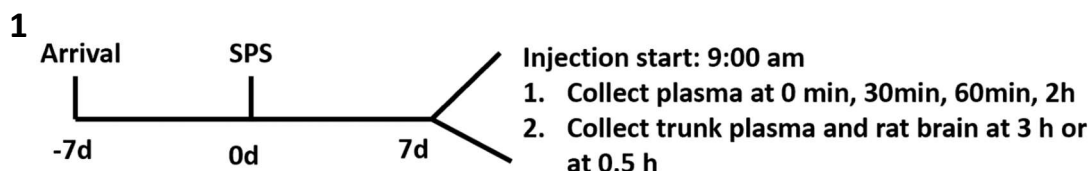


Figure 1. Schematic diagram of the study. One week after arrival in the facility, rats were exposed to SPS (day 0). 7 days after SPS, rats were injected with corticosterone or vehicle. In experiment 1 plasma was collected via a tail cut at 0 min, 30 min, 60 min, and 2 h. Rats were sacrificed at 3 h (experiment 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 μ m 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 $2^{-\Delta\Delta C_t}$ 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	AGTGCAGGTTCTCTCGTCAAC
Ntf3	CAAGTCCTCAGCCATTGACA	CTGGCCTGGCTTCTTTACAC
Drd1a	AGATCCATCGAGTCCCCTCT	TGTTGCAACTGCTTCCAAAG

2.7 Statistical analysis

Data are expressed as mean \pm 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).

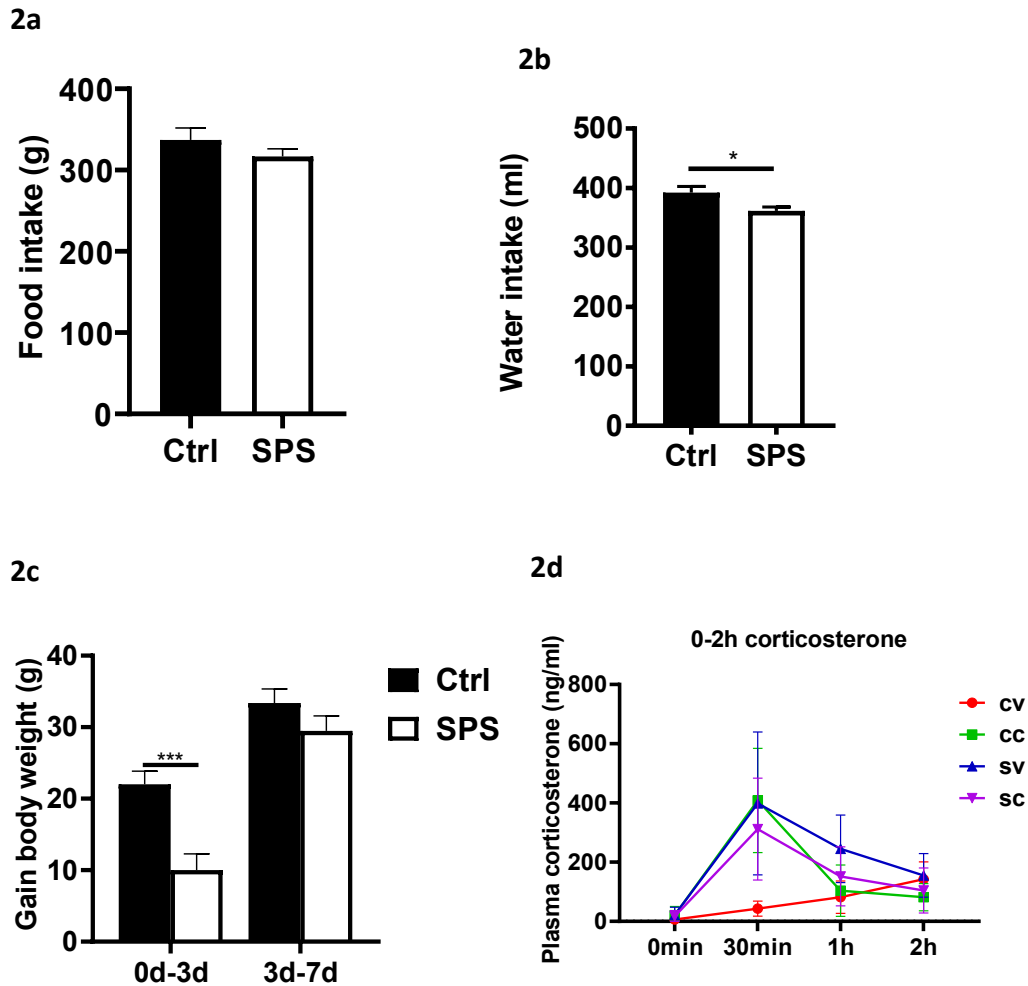


Figure 2. Effects of SPS on food and water intake, body parameters and corticosterone levels after injections. a: Food consumption. b: Water consumption. c: Gain in body weight. d: Corticosterone levels at 0 min, 0.5 h, 1 h and 2 h after injection. CV: control + vehicle group; CC: control + CORT group;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

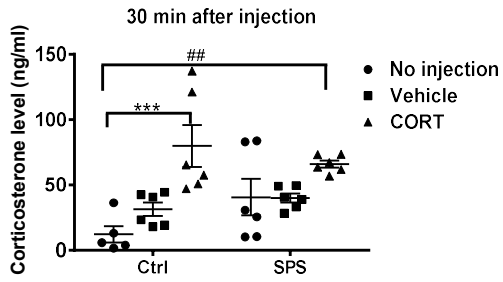
The elevated corticosterone in SPS-vehicle rats in our first experiment could have been caused by the injection, the tail blood sampling or both. To control for the acute effects of the injection itself, in our next experiment we included SPS and control groups that did not receive an injection, and 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.

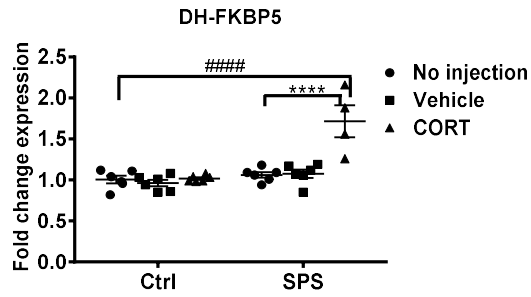
In the amygdala, *FKBP5* mRNA levels showed a main effect of stress (stress: $F_{(1, 30)} = 16.11$, $p < 0.001$, figure 3f). Post-hoc tests showed higher mRNA level of the SPS-CORT group higher compared to the control without injection group. There was no significant upregulation after corticosterone 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.

3a



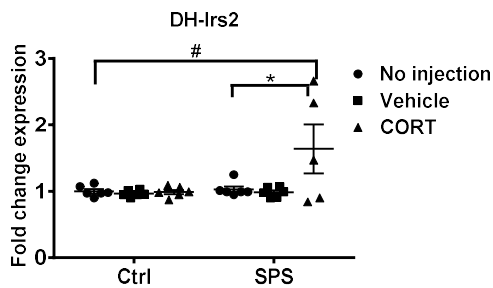
S: $p = 0.33$ C: $p < 0.001$ SxC: $p = 0.11$

3b



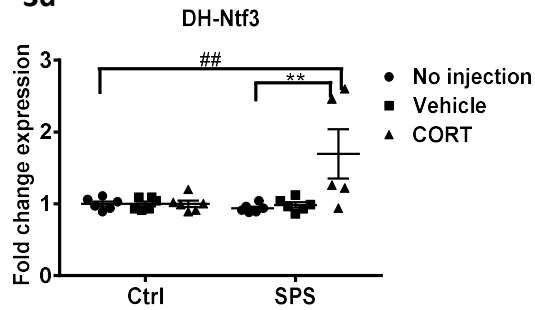
S: $p < 0.001$ C: $p < 0.001$ SxC: $p < 0.001$

3c



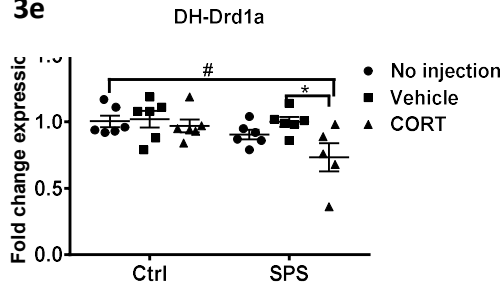
S: $p = 0.04$ C: $p = 0.03$ SxC: $p = 0.04$

3d



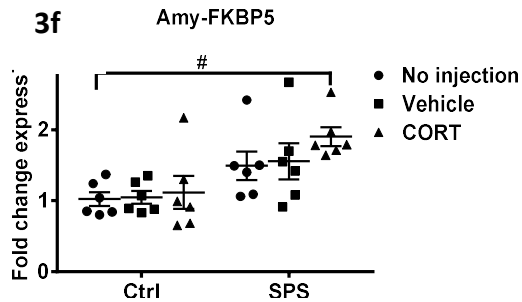
S: $p = 0.05$ C: $p = 0.008$ SxC: $p = 0.008$

3e



S: $p = 0.02$ C: $p = 0.03$ SxC: $p = 0.19$

3f



S: $p < 0.001$ C: $p = 0.34$ SxC: $p = 0.63$

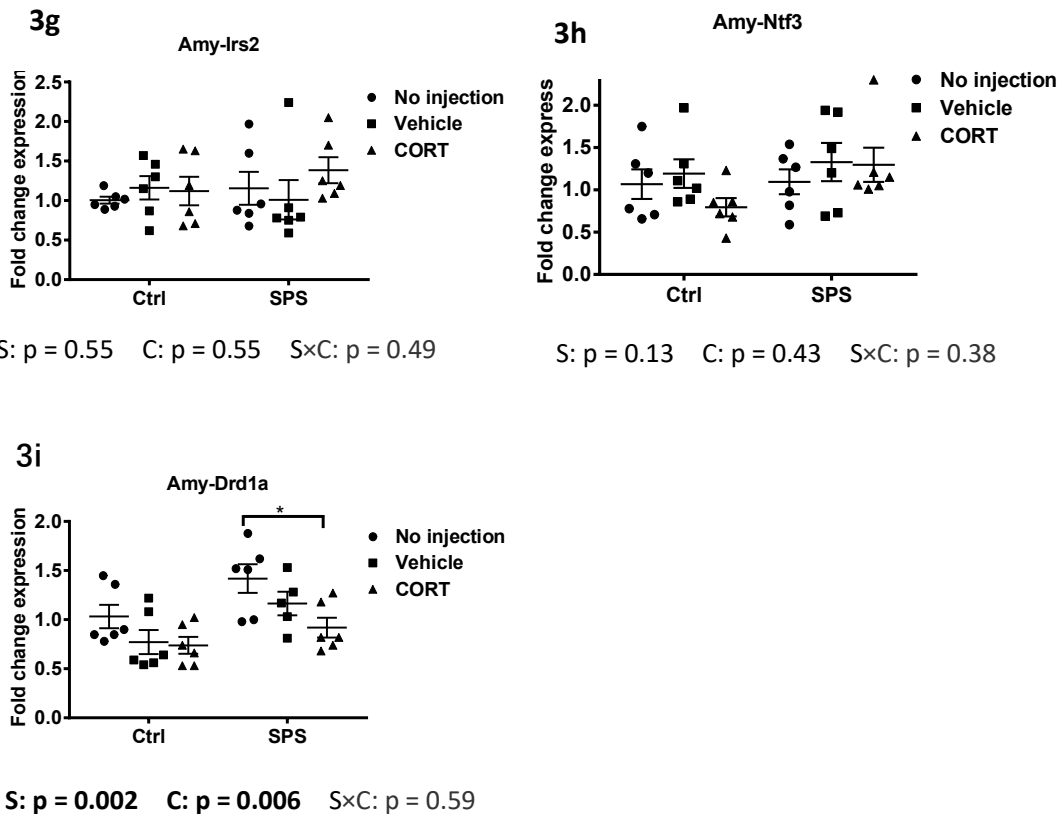


Figure 3. Plasma corticosterone levels, and gene expression in the dorsal hippocampus and in the amygdala at 0.5 h after injection. a: Corticosterone levels b: FKBP5 mRNA expression in the dorsal hippocampus. c: Irs2 mRNA expression in the dorsal hippocampus. d: Ntf3 mRNA expression in the dorsal hippocampus. 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 \pm 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 studied 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.

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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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