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Research paper

Maternal deprivation causes CaMKII downregulation and modulates glutamate, norepinephrine and serotonin in limbic brain areas in a rat model of single prolonged stress

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ABSTRACT

Background: Early life stress is a major risk factor for later development of psychiatric disorders, including post-traumatic stress disorder (PTSD). An intricate relationship exists between various neurotransmitters (such as glutamate, norepinephrine or serotonin), calcium/calmodulin-dependent protein kinase II (CaMKII), as an important regulator of glutamatergic synaptic function, and PTSD. Here, we developed a double-hit model to investigate the interaction of maternal deprivation (MD) as an early life stress model and single prolonged stress (SPS) as a PTSD model at the behavioral and molecular levels.

Methods: Male Wistar rats exposed to these stress paradigms were subjected to a comprehensive behavioral analysis. In hippocampal synaptosomes we investigated neurotransmitter release and glutamate concentration. The expression of CaMKII and the content of monoamines were determined in selected brain regions. Brain-derived neurotrophic factor (BDNF) mRNA was quantified by radioactive in situ hybridization.

Results: We report a distinct behavioral phenotype in the double-hit group. Double-hit and SPS groups had decreased hippocampal presynaptic glutamatergic function. In hippocampus, double-hit stress caused a decrease in autophosphorylation of CaMKII. In prefrontal cortex, both SPS and double-hit stress had a similar effect on CaMKII autophosphorylation. Double-hit stress, rather than SPS, affected the norepinephrine and serotonin levels in prefrontal cortex, and suppressed BDNF gene expression in prefrontal cortex and hippocampus.

Limitations: The study was conducted in male rats only. The affected brain regions cannot be restricted to hippocampus, prefrontal cortex and amygdala.

Conclusion: Double-hit stress caused more pronounced and distinct behavioral, molecular and functional changes, compared to MD or SPS alone.

1. Introduction

Diathesis stress model is a concept of pathogenesis of psychiatric disorders, which states that factors interfering with normal brain development during childhood, such as adverse life events, create an evolving phenotype, whereupon exposure to later-life environmental factors may serve as a trigger for the development of psychiatric disorders (Monroe and Cummins, 2015). In this vein, people who have suffered early life adversity have a higher risk coefficient for post-traumatic stress disorder (PTSD) (McGowan, 2013). Also, veterans exposed to combat in early adulthood are more susceptible to develop PTSD following exposure to later stressors (Sachs-Ericsson et al., 2016).

Maternal deprivation (MD) is one of the most widely used models of early life stress. MD represents a single episode of 24 h separation of rat pups from their mother on postnatal day nine (PN 9) (Marco et al., 2015). The effects are profound because the period of brain

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development between PN9 and PN10 is crucial for the establishment of normative control of behavior, metabolism, and energy homeostasis (Marco et al., 2015).

Single prolonged stress (SPS) is currently a prevalent animal model of PTSD (Lisieski et al., 2018). It shows a good validity in producing PTSD-like behavioral and pathophysiological changes in animals, which are similar to those seen in patients (Daskalakis et al., 2013). It is a multimodal traumatic stress exposure protocol, including sequential exposure to three stressors during a single continuous session.

Several converging lines of evidence suggest that glutamate, as the most abundant excitatory neurotransmitter, plays a leading role in the pathophysiology of PTSD. This cumulative evidence has led to a shift in the field from monoaminergic hypothesis of PTSD, which for a long time was the dominant hypothesis, to a more integrative neurochemistry and neuroplasticity hypothesis (Averill et al., 2017). Chronic excess glutamate is one of the pathogenic mechanisms associated with a loss of resilience to stress (Nasca et al., 2015) that leads to development of stress-related mental disorders (Popoli et al., 2011). Rats subjected to acute footshock-stress in a paradigm that induces learned helplessness showed a marked, rapid change in the depolarization-evoked release of glutamate in isolated nerve terminals (synaptosomes) from the prefrontal cortex (PFC) (Musazzi et al., 2010). Interestingly, the effect of acute stress on depolarization-evoked glutamate release in the PFC could be prevented by treating the rats with various classes of antidepressant drugs for two weeks prior to the stress exposure (Musazzi et al., 2010). Footshock-stress also increases the readily releasable pool of glutamate vesicles in synaptic terminals of prefrontal and frontal cortex (Wegener et al., 2014). Chronic stress provokes dysregulation of glutamate release. Rats showing anhedonic behavior after chronic mild stress have a decreased glutamate release in the hippocampus, which is normalized by ketamine (Tornese et al., 2019). Sustained alterations of glutamate transmission may play a key role in the long-term structural/functional changes associated with mood disorders in patients (Musazzi et al., 2015).

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a critical modulator of glutamatergic synaptic function. It is a multifunctional serine/threonine protein kinase, which comprises >1 % of total protein in the brain (especially in hippocampus and neocortex). It is ubiquitously expressed throughout the brain, and in all subcellular neuronal compartments (Bayer and Schulman, 2019). It is suggested that CaMKII dysfunction may underlie multiple psychiatric disorders, possibly by causing maladaptation in glutamate signaling and neuroplasticity (Robison, 2014). CaMKII is involved in the consolidation of traumatic memories as part of a signaling cascade, which plays a major role in triggering PTSD-like memory impairment during severe stress (Chen et al., 2012; Finsterwald and Alberini, 2014; Kaouane et al., 2012). In view of this and its crucial role as a regulator of glutamatergic synaptic function (Bayer and Schulman, 2019), our aim was to investigate whether CaMKII is affected by the combination of the two stress paradigms mentioned above.

Both MD and SPS stress paradigms affect the glutamatergic system. MD at PN9 reduces the expression of two main NMDA receptor subunits in the hippocampus of adult rats (Roceri et al., 2002). SPS (7 days after the stressing procedure) decreases medial prefrontal cortex (mPFC) glutamate levels (Knox et al., 2010). 14 days after SPS exposure, there is a significant increase in glutamate levels in the rat cerebrospinal fluid, suggesting a brain wide alteration of the glutamatergic system (Feng et al., 2015). The interaction of 24 h-MD at PN9 (neonatal period) and SPS at PN60 (early adulthood), especially its effects on the presynaptic compartment of the glutamatergic synapse, has not been described in the literature so far.

In our study we examined how double-hit stress affects animal behavior, in terms of assessing locomotor activity, emotional reactivity, social interaction and hedonic behavior, as well as the function and molecular composition of glutamatergic synapses in key brain regions involved in stress response (prefrontal cortex, hippocampus and amygdala). We also examined the concentrations of monoamines and BDNF mRNA, which play a role in mediating stress responses (Popoli et al., 2011). Although both serotonin (5-HT) and norepinephrine (NE) have been implicated in the pathobiological processes in PTSD, there are many more consistent reports linking PTSD and NE than those linking 5-HT and PTSD (Krystal and Neumeister, 2009), with a general consensus that PTSD is associated with increased NE function (Krystal and Neumeister, 2009). The relationship between 5-HT and PTSD is less straightforward. The only two drugs approved for PTSD, with limited efficacy, are 5-HT reuptake inhibitors, while administration of a non-selective 5-HT agonist causes transient PTSD symptoms (Krystal and Neumeister, 2009).

2. Materials and methods

2.1. Animals and treatments

In the experiments we used male and nulliparous female Wistar rats obtained from the breeding colony at the Department of Biochemistry, Belgrade University School of Medi-cine, which originated from Charles River (Charles River Laboratories, Erkrath, Germany). All experiments were carried out according to the NIH Guide for Care and Use of Laboratory Animals and were approved by the Ethical Council for the Protection of Experimental Animals of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia.

Animals were kept in male-female pairs in standard Plexiglass cages with sawdust (26 × 42 × 15 cm), in a temperature-controlled room (23 ± 1°C), on a standard 12 h light/dark cycle with lights on from 7:00 to 19:00 h, with water and food available ad libitum. After two weeks, the males were removed, and the dams were checked twice daily for delivery. The day of delivery was denoted as postnatal day (PN) 0. As presented in Fig. 1, rat pups were subjected to maternal deprivation (MD) procedure on PN9. Dams were removed from the litter at 10:00 am, after which the pups were weighed and returned to their home cage. They remained in their home cage at room temperature for 24 h. On P10, the pups were weighed again, and dams were returned to their cages. The dams of the control litters were removed from their home cages for 3 min, and the pups also weighed on both PN9 and PN10. All litters were after that left undisturbed except for the routine cleaning of the cages until PN21, when the rats were weaned and classified according to sex. Male rats were placed in new standard Plexiglass cages with sawdust (26 × 42 × 15 cm) and housed in pairs.

Single prolonged stress (SPS) procedure was implemented on PN 60. Both, animals exposed to MD and control animals were restrained for two hours by placing inside clear specially designed plastic restraint tubes. After immobilization, rats were individually placed in a clear acrylic cylinder (24 cm diameter and 50 cm height) filled two thirds with water (24 °C). Rats were forced to swim for 20 min. Following recuperation for 15 min, animals were exposed to diethyl ether until the loss of consciousness and then left undisturbed in their home cage for 7 days.

Animals were either used for proceeding behavioral testing or sacrificed by cervical dislocation and decapitation without anesthesia, with the heads immediately frozen in liquid nitrogen and kept at -80oC until further use for biochemical and functional assays. For schematic representation see supplementary fig. S1.

2.2. Behavioral testing

Behavioral testing started on PN68. In order to elucidate the effect of the above-mentioned experimental protocols on animal behavior we performed a battery of behavioral tests which included elevated plus maze, spontaneous locomotor activity test, sucrose preference test and social interaction test in that order. Animal tracking was done using AnyMaze software (Stoelting Co., USA). The equipment and detailed protocols are described in the Supplement.

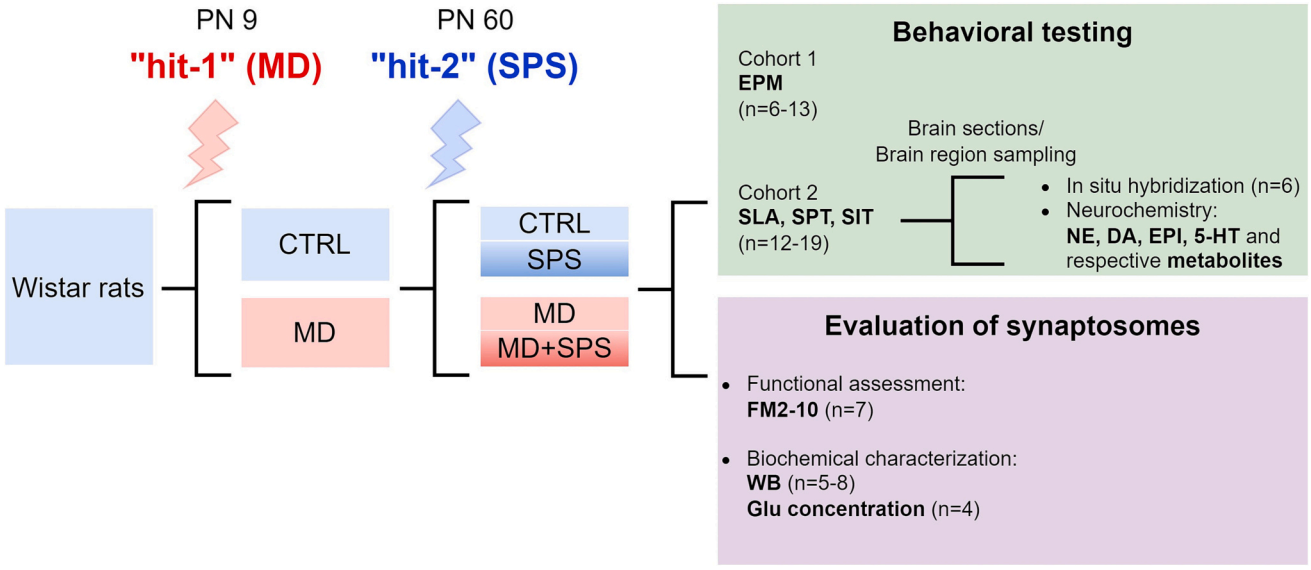

Fig. 1. Experimental design. Rats were subjected to two different stressors at different time points: first ("hit-1") in early postnatal period (PN9; early stress) and second ("hit-2") in early adulthood (PN60; later stress). Early stress represents maternal deprivation (MD). Later stress represents single prolonged stress (SPS). After the application of SPS, four groups of animals were formed: control group without stress exposure (CTRL), single prolonged stress group (SPS), maternal deprivation group (MD), double-hit group (MD + SPS). Animals were either used for proceeding behavioral testing, or sacrificed by cervical dislocation and decapitation without anesthesia, with the heads immediately frozen in liquid nitrogen and kept at -80oC until further use for biochemical and functional assays. Rat brains from cohort 2 were harvested after behavioral testing and used for in situ hybridization and neurochemistry experiments. Abbreviations: PN (postnatal day), WB (western blot), EPM (elevated plus maze), SLA (spontaneous locomotor activity), SPT (sucrose preference test), SIT (social interaction test), NE (norepinephrine), DA (dopamine), EPI (epinephrine), 5-HT (serotonin).

2.3. Synaptosome isolation

Synaptosomes were isolated from the hippocampus, prefrontal cortex and amygdala. Tissue samples were weighted and homogenized in Syn-PER Reagent (Thermo Fisher) in a ratio 10 ml per 1 g of tissue. Homogenates were centrifuged for 10 min, 1200 *g* at 4 °C, and the resulting supernatants centrifuged for 20 min, 15.000 g at 4 °C. Resulting pellets, which contain synaptosomes, were further resuspended in Hank's balanced salt solution (HBSS; for functional study using FM 2–10 dye) or in RIPA buffer (for biochemical analysis using WB).

2.4. FM2–10 dye uptake/release protocol

For the assessment of neurotransmitter release, we performed FM2–10 dye uptake/release protocol. FM dyes are lipophilic styryl compounds, widely used to image vesicle recycling. Synaptosomes were resuspended in 500 µl of HBSS buffer (with Ca2+/Mg2+) containing 100 µM FM2–10 at room temperature (RT). KCl was added to the suspension in order to stimulate dye uptake and incubated for 15 min at room temperature. Depolarization induced by KCl causes exocytosis, and subsequent compensatory endocytosis, which causes the uptake of FM2–10. After that synaptosomes are centrifuged for 5 min at 15.000 g and washed two times with HBSS to remove excess dye. Finally, synaptosomes are resuspended in HBSS. Stimulation with KCl causes exocytosis and release of the dye in the medium, which results in decreased fluorescence. Reduction in fluorescence was used to quantify exocytosis.

2.5. Western blot

Synaptosome pellets were resuspended in RIPA buffer (300 mM NaCl, 20 mM HEPES pH = 7.5, 0.2 % SDS, 2 % Na-deoxycholate, 2 % Triton X-100) which is supplemented with protease and phosphatase inhibitors. Protein concentration was determined by using BCA (bicinchoninic acid) Protein Assay (Pierce). Equal amounts of protein were loaded onto polyacrylamide gels, and the proteins were separated by SDS-PAGE and transferred to Immobilon®-FL Polyvinylidene Difluoride membranes (Sigma). Primary antibodies (anti-pCaMKII (T286) rabbit monoclonal, 1:500, Cell Signaling Technology, cat. no. 12716; anti-CaMKII-α, mouse monoclonal, 1:1000, Cell Signaling Technology, Cat. No. 50049, anti-β-actin, 1:1000, mouse monoclonal, Sigma-Aldrich, A5441) were applied overnight at 4 °C and fluorescently labelled secondary antibodies for 1 h at RT. Membranes were scanned using an Odyssey CLx Infra Red Imaging system from LI-COR Biosciences (Lincoln, NE, USA). Quantification of the signals was done using software Image Studio 3.1. From obtained intensity for each band the background subtraction was made, and the values were normalized to β-actin and, in the case of phospho-CaMKIIα, to total-CaMKII-α.

2.6. Glutamate concentration

Glutamate concentration was determined by using fluorometric glutamate assay (Abcam, USA, ab138883) in synaptosomes, according to the manufacturer's protocol.

2.7. Analysis of neurotransmitters by high-performance liquid chromatography (HPLC)

Concentration of different neurotransmitters was measured in brain tissue by high-performance liquid chromatography (HPLC). See Supplementary for full details.

2.8. In situ hybridization

In situ hybridization was performed in fresh frozen post-fixed brain sections that were hybridized with ^{35}S-labelled anti-sense and sense cRNA probes against brain-derived neurotrophic factor (BDNF) gene. Details are described in Supplemental Information.

2.9. Statistics

Data were processed using GraphPrism 9, using two-way ANOVA or two-way ANOVA with repeated measures followed by Fisher's least significance difference (LSD) post hoc test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Double-hit stress caused decreased locomotor response in a novel environment, and combined negative behavioral effects of MD and SPS

We performed a comprehensive behavioral analysis by using a battery of behavioral tests that consisted of elevated plus maze (EPM), spontaneous locomotor activity test (SLA), sucrose preference test (SPT)

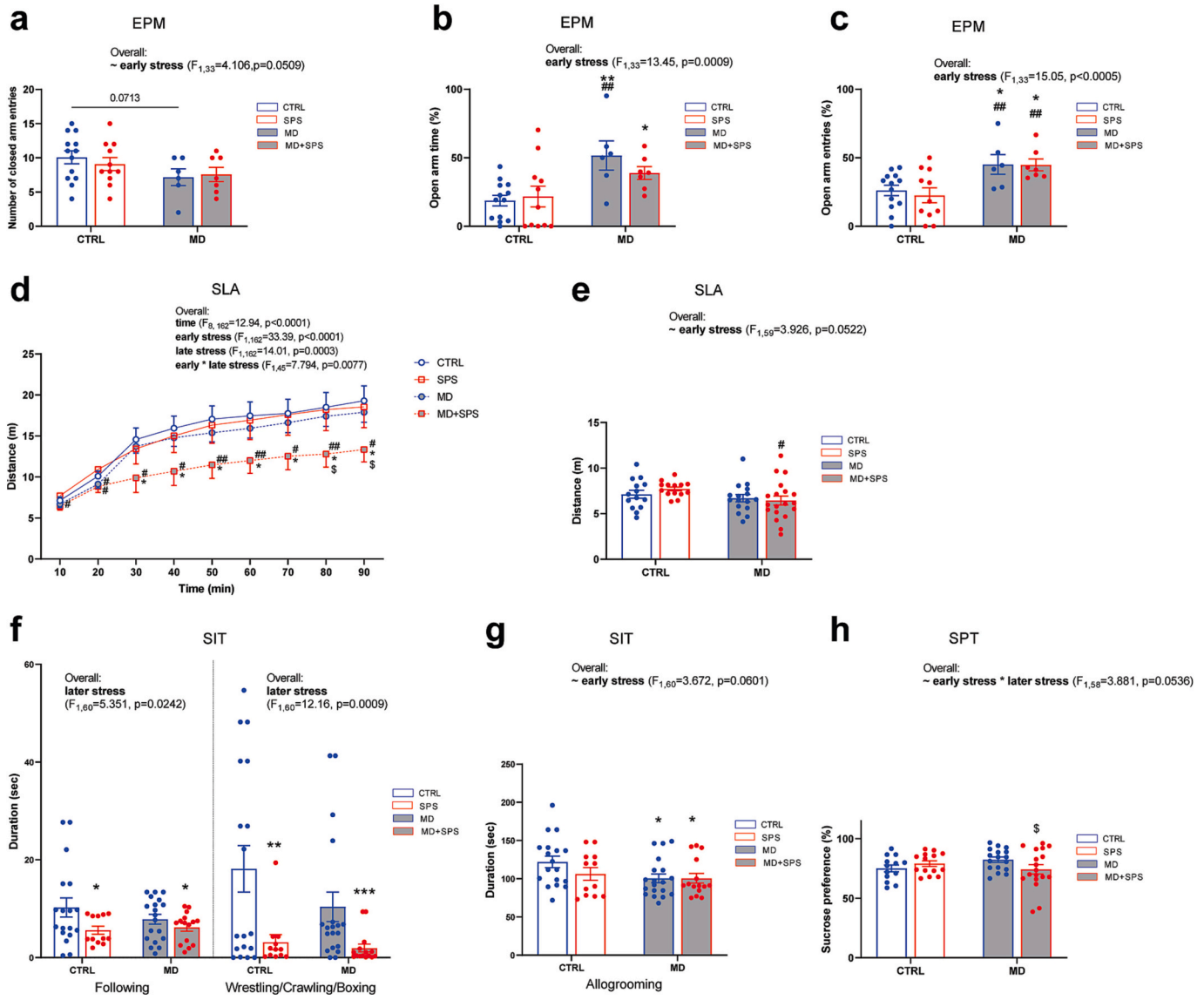

Fig. 2. Behavioral testing. Data was analyzed using two-way ANOVA or two-way ANOVA with repeated measures (SLA data) followed by Fisher's least significance difference (LSD) post hoc test. F-values and overall effect of early stress or later stress or early stress x later stress is depicted in the upper right corner of each graph if significant, or at trend level. Statistical difference from control: $0.01 < p < 0.05$, $0.001 < p < 0.01$, $p < 0.001$ is presented as *, **, ***, respectively. Statistical difference from SPS: $0.01 < p < 0.05$, $0.001 < p < 0.01$, is presented as #, ##, respectively. Statistical difference from MD: $0.01 < p < 0.05$ is presented as $. Individual data points are presented as dots in each figure, in addition to the group average and error bars representing SEM. Exact numerical values per group are given in Supplementary table 1. Trend effect ($0.05 < p < 0.1$) is labelled as ~ or a specific p value. a, b, c: EPM results. Three parameters are displayed: number of closed arm entries (a), percentage of open arm time (b), percentage of open arm entries (c). SPS had no effect on behavior in the EPM. MD had an overall trend effect on the number of closed arm entries ($p = 0{,}051$). MD and MD + SPS animals displayed disinhibited behavior in the EPM: percentage of open arm time and percentage of open arm entries was significantly higher for MD group compared to SPS and CTRL groups. Likewise, MD + SPS had significantly increased percentage of open arm time compared to CTRL group, and increased percentage of open arm entries compared to both SPS and CTRL groups. d, e: SLA results. Locomotor activity during 90 min of testing (d), represented in 10 min bins. MD + SPS animals consistently displayed decreased distance travelled compared to SPS and CTRL groups. MD and SPS groups did not differ significantly from CTRL. Locomotor activity during the first ten minutes of testing (habitutation period) (e). MD had a trend effect on distance travelled in the first ten minutes. f, g: SIT results. Three parameters of social interaction were quantified: following and social play (wrestling, crawling and boxing) (f), allogrooming (g). SPS had a significant overall effect on following and social play behavior. Animals subjected to SPS and MD + SPS spent significantly less time following and engaging in social play, compared to non-stressed controls. Early life stress had an overall tendency of reducing allogrooming ($p = 0{,}060$). h: SPT results. Strong trend for the interaction effect of early stress and later stress on sucrose preference ($p = 0{,}054$).

and social interaction test (SIT). First, we assessed anxiety related behavior in the EPM (Fig. 2a, b, c). In the EPM, early stress had a significant main effect on the percentage of open arm time (Fig. 2b) and open arm entries (Fig. 2c). Both maternally deprived and double-hit rats displayed disinhibited behavior in the EPM, evident by significantly increased percentage of open arm time (Fig. 2b) and open arm entries (Fig. 2c), compared to the control group. Early stress had a trend effect on decreasing the number of closed arm entries in the EMP (Fig. 2a), indicating a tendency to decrease locomotor activity (cf. Cruz et al., 1994; Rodgers and Johnson, 1996). Locomotor response in a novel environment was assessed via SLA test (Fig. 2d, e). Double-hit stress caused a significant reduction in locomotor activity compared to controls and experimental groups (Fig. 2d). During the first ten minutes (habituation period) early stress had a strong trend effect on locomotor activity (Fig. 2e). Social interaction was significantly impaired by later stress (Fig. 2f, g). This effect was most pronounced for following and social play (wrestling, crawling, boxing) behaviors (Fig. 2f). Early stress had a trend effect on allogrooming behavior (Fig. 2g). Stress did not have a significant effect on sucrose preference, although there was a strong trend for the overall interaction effect of early and later stress (Fig. 2h).

3.2. Double-hit stress caused a significant reduction in neurotransmitter release along with reduced glutamate concentration in hippocampal synaptosomes

Previous reports (Pitman et al., 2012; Lener et al., 2017) have linked impairments in hippocampal glutamatergic function to behavioral symptoms of mood disorders. Consistent with this, animals exposed to SPS and double-hit stress showed a reduced depolarization-evoked neurotransmitter release in hippocampal synaptosomes (Fig. 3a). Next, we wanted to investigate whether this impairment is associated with a change in glutamate concentration in hippocampal synaptosomes. Using FM2–10 dye uptake/release protocol, only double-hit stress caused a significant reduction in glutamate concentration in hippocampal synaptosomes (Fig. 3b).

3.3. Reduced T286 phosphorylation of CaMKII in key brain regions after SPS and double-hit stress

To identify the mechanism of the reduction in depolarization-evoked neurotransmitter release, we examined the levels of presynaptic proteins known to regulate glutamate release in hippocampal synaptosomes. The most striking finding was a reduction in T286 phosphorylation of CaMKII in MD + SPS group (Fig. 4a, b, c). We measured T286 phosphorylation of CaMKIIα and CaMKIIβ, the two dominant CaMKII isoforms in the brain (Schulman and Greengard, 1978; Bennett et al., 1983), and determined that both were similarly affected. We then tested P-T286 CaMKII in PFC and amygdala. It was found that both SPS and double-hit stress caused a significant reduction of P-T286 CaMKII in prefrontal cortex (Fig. 4d, e, f) and SPS had a significant effect in amygdala (Fig. 4g, h, i).

3.4. HPLC measures of monoamines revealed alterations in PFC

We performed a comprehensive analysis of monoamines and their key metabolites in PFC, amygdala and hippocampus (Fig. 5a-d; supplementary Fig. S1). A significant increase in the concentration of NE and EPI was found in PFC only in double-hit stressed animals (Fig. 5a, b). Early stress had a significant overall effect on MHPG concentration in amygdala (Fig. 5c), whereas VMA was not significantly affected by stress (Fig. 5c). Consistent with the changes in NE and EPI in PFC, 5-HT and 5-HIAA were similarly increased in PFC only in double-hit stressed animals (Fig. 5d). No change in the concentration of DA or its related metabolites was detected in any of the regions examined (supplementary Fig. S1).

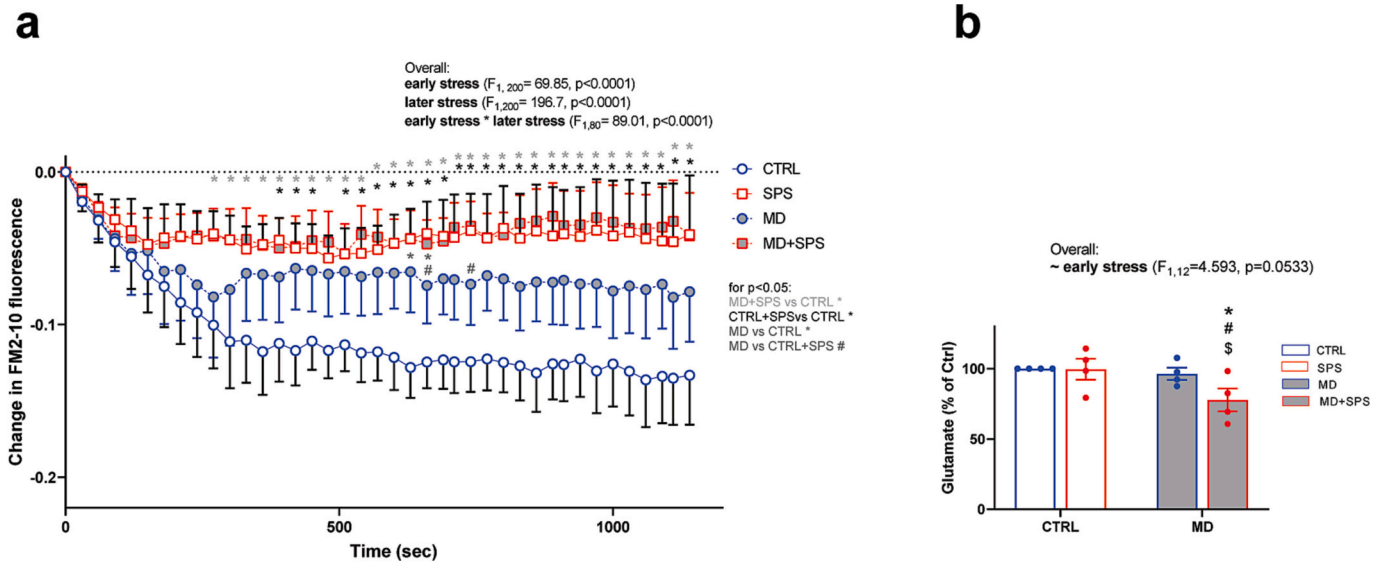

Fig. 3. Neurotransmitter release and glutamate concentration in hippocampal synaptosomes. Data was analyzed using two-way ANOVA (glutamate concentration) or two-way ANOVA with repeated measures (FM2–10 data) followed by Fisher's least significance difference (LSD) post hoc test. F-values and overall effect of early stress or later stress or early stress x later stress is depicted in the upper right corner of each graph if significant, or at trend level. Statistical difference from control: $0.01 < p < 0.05$ is presented as *. Statistical difference from SPS: $0.01 < p < 0.05$ is presented as #. Statistical difference from MD: $0.01 < p < 0.05$ is presented as $. Individual data points are presented as dots in each figure, in addition to the group average and error bars representing SEM. Exact numerical values per group are given in Supplementary table 1. Trend effect ($0.05 < p < 0.1$) is labelled as ~ or a specific p value.
a Results of the FM2–10 experiment. Change in FM2–10 florescence was quantified after KCL stimulation of synaptosomes. SPS and MD + SPS group demonstrated significantly reduced extinction of fluorescence compared to non-stressed controls, indicating reduced depolarization-evoked neurotransmitter release. b Quantification of glutamate concentration. Within each experimental setup data were normalized to the mean of the control. Significantly reduced glutamate concentration was detected in hippocampal synaptosomes of MD + SPS compared to MD, SPS and CTRL groups.

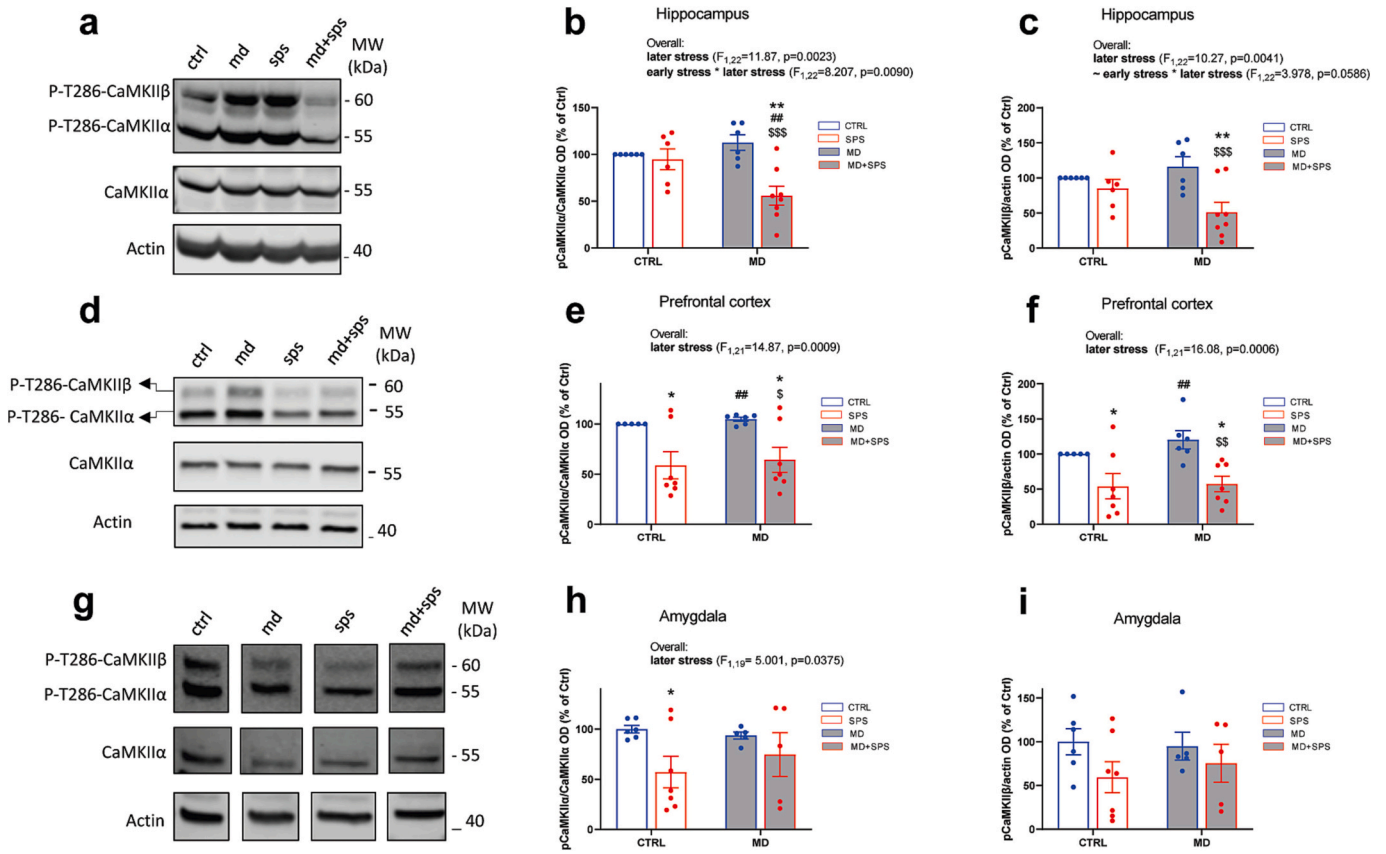

Fig. 4. Effects of stress on CaMKII-T286 phosphorylation. Data was analyzed using two-way ANOVA followed by Fisher's least significance difference (LSD) post hoc test. F-values and overall effect of early stress or later stress or early stress x later stress is depicted in the upper right corner of each graph if significant, or at trend level. Statistical difference from control: $0.01 < p < 0.05$, $0.001 < p < 0.01$ is presented as *, ** respectively. Statistical difference from SPS: $0.001 < p < 0.01$ is presented as ##. Statistical difference from MD: $0.01 < p < 0.05$, $0.001 < p < 0.01$, $p < 0.001$ is presented as $, $$, $$$ respectively. Individual data points are presented as dots in each figure, in addition to the group average and error bars representing SEM. Exact numerical values per group are given in Supplementary table 1. Trend effects ($0.05 < p < 0.1$) is labelled as ~ or a specific p value.
CTRL and MD designations below the X axes are related to the absence and presence, respectively, of early life stress. Representative immunoblots and corresponding quantification of pCaMKIIα/CaMKIIα and pCaMKIIβ/actin in synaptosomes from hippocampus (a, b, c), prefrontal cortex (d, e, f) and amygdala (g, h, i). All the images shown are from the same membrane. However, in some instances, the respective groups were not sequentially loaded onto the membrane, which is why the immunoreactive bands in Fig. 4g are separated to show all groups. All original Western blot images can be found in the supplementary fig. S2. SPS caused region specific effects in CTRL and MD animals.

3.5. Double-hit stress did not modulate BDNF mRNA

Finally, we wanted to assess possible effects of stress on BDNF mRNA in hippocampus and mPFC (Fig. 6a-h). Early stress caused a significant reduction in BDNF mRNA in CA1 region of dorsal hippocampus (Fig. 6b), while later stress had significant overall effect in DG of dorsal hippocampus (Fig. 6d). BDNF mRNA expression in ventral hippocampus was not affected by stress (Fig. 6e-g). Early stress also caused a significant reduction of BDNF mRNA in mPFC (Fig. 6h). However, there was no interaction effect of the two stressors in any of the investigated regions.

4. Discussion

We established a novel double-hit stress paradigm to test the effects of early life stress, in the form of maternal deprivation, on single prolonged stress in early adulthood, which is per se a common model of PTSD (Lisieski et al., 2018; Daskalakis et al., 2013). The combined results of the behavioral tests suggest synergistic negative effects of MD and SPS.

First, we measured spontaneous locomotor activity in a novel environment. Stressed animals showed characteristic behavioral phenotypes. MD or SPS alone had no effect on spontaneous locomotor activity; however, intriguingly, these two stressors in combination produced a significant reduction in spontaneous locomotor activity compared to controls. Activity in a novel environment is used as an indicator of emotional and motivational state (Katz et al., 1981). This finding could be linked to emotional deficits that are associated with PTSD (Litz et al., 2000). There is a report that showed an increased locomotor response in adult MD rats, which the authors attributed to increased dopaminergic activity within the mesolimbic system, as a translational consequence of "psychomotor agitation" in schizophrenia (Janetsian-Fritz et al., 2018). We did not detect any change in dopamine levels in the MD animals, which could explain the absence of hyperlocomotion in our study. Locomotor activity after SPS exposure has been used to differentiate between vulnerable and resilient rats, with vulnerable rats displaying locomotor depression (Le Dorze and Gisquet-Verrier, 2016a, 2016b).

Second, we examined anxiety-like behavior in the elevated plus maze. Increased open arm activity observed in MD and MD + SPS group is consistent with data from the literature on MD male rats in adulthood (Burke et al., 2013; Llorente-Berzal et al., 2011) and suggests an anxiolytic effect or increased risk-taking behavior. In our hands, SPS did not affect anxiety-related behavior in the EPM. The literature on the effect of SPS on anxiety is contradictory, which may be at least partly related to differences in the rat strain, the exact day of stress exposure, and other subtle differences in the protocol (e.g. substitution of ether for halothane). Some studies show an anxiety-like phenotype following SPS

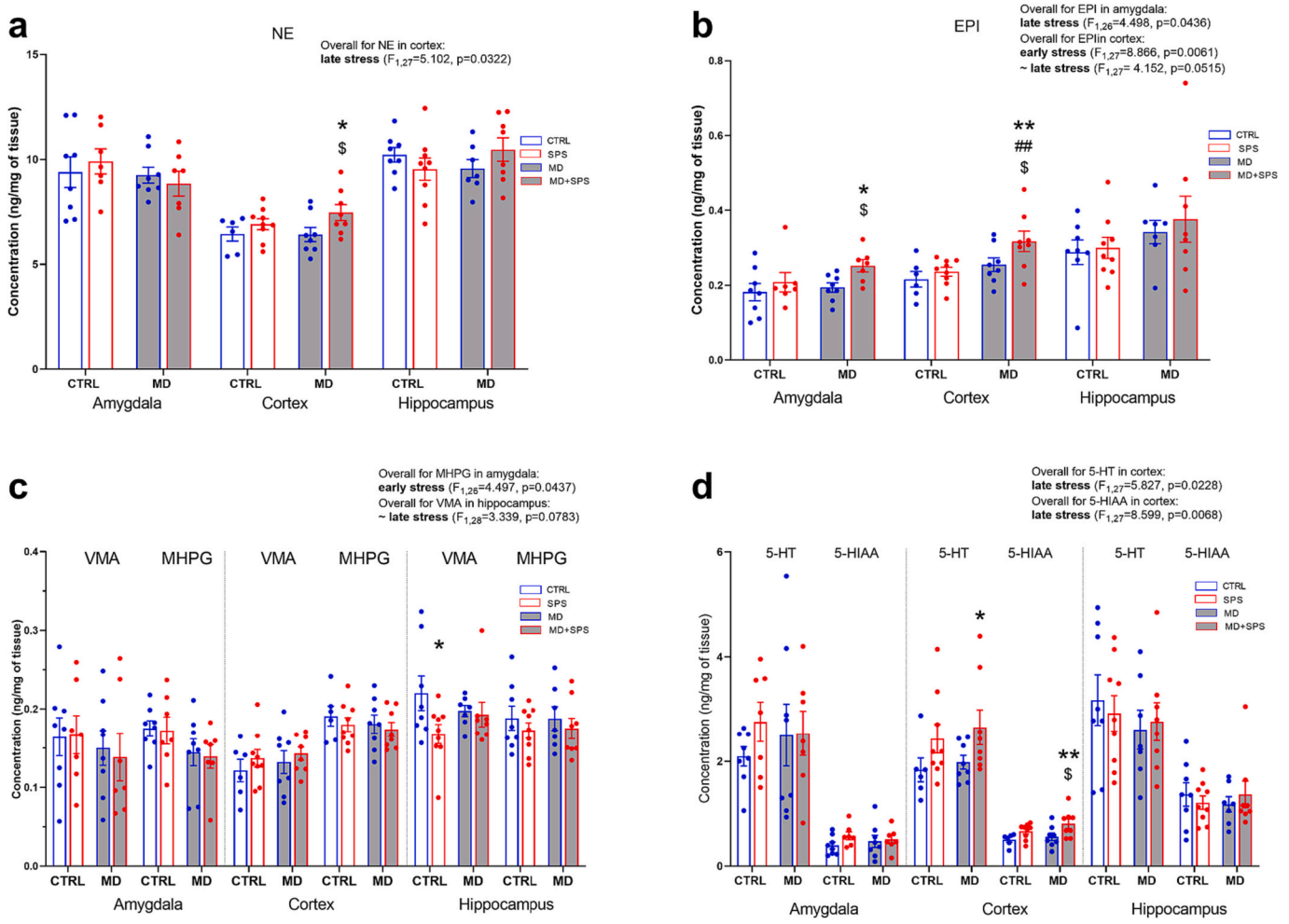

Fig. 5. Neurochemical parameters in adult rats exposed to MD, SPS or MD + SPS. Data was analyzed using two-way ANOVA followed by Fisher's least significance difference (LSD) post hoc test. F-values and overall effect of early stress or later stress or early stress x later stress is depicted in the upper right corner of each graph if significant, or at trend level. Statistical difference from control: $0.01 < p < 0.05$, $0.001 < p < 0.01$ is presented as *, ** respectively. Statistical difference from SPS: $0.001 < p < 0.01$ is presented as ##. Statistical difference from MD: $0.01 < p < 0.05$ is presented as $.
Individual data points are presented as dots in each figure, in addition to the group average and error bars representing SEM. Exact numerical values per group are given in Supplementary table 1. Trend effects ($0.05 < p < 0.1$) is labelled as ~ or a specific p value.
Different neurotransmitters and their metabolites were analyzed in amygdala, prefrontal cortex and hippocampus. Affected neurochemical parameters include NE (a), EPI (b), VMA/MHPG (c), 5-HT/5-HIAA (d). MD + SPS caused a significant increase of NE, EPI, 5-HT and 5-HIAA in PFC, as well as EPI in amygdala.

exposure (Imanaka et al., 2006; Feng et al., 2015), while others have failed to replicate this effect (Harvey et al., 2006; Eagle et al., 2013). While generalized anxiety is not a defining feature of PTSD (American Psychiatric Association, 2013), behavioral disinhibition is an important characteristic of this disorder (Sadeh et al., 2015; Sadeh et al., 2018) and corresponds to self-destructive or reckless behavior, which is a diagnostic criterion (American Psychiatric Association, 2013). It must still be noted that the decreased locomotor activity measured in MD + SPS group hinders the interpretation of EPM results, and the future addition of a less stressful and less locomotion-dependent test to measure risk-taking/exploration would help elucidate the emotional reactivity phenotype presented in this study.

Next, we assessed social interaction. Social avoidance is a diagnostic criterion for PTSD (American Psychiatric Association, 2013) and represents one of its hallmarks. Consistent with a previous report (Zamberletti et al., 2012), MD had no effect on social interaction. SPS significantly impaired social interaction compared to controls, and MD did not mitigate these effects in the MD + SPS group. A previous study reported decreased elements of active social interaction in rats 7 days after SPS exposure (Han et al., 2020). In rodents, reduction in social interaction is one of the indicators of vulnerability to stress (Krishnan, 2007). There was a highly significant reduction in social play behavior in both MD + SPS and SPS group compared to controls. Social play is very pleasurable and has a high hedonic impact (Trezza et al., 2011; Vanderschuren, 2010), and lack of motivation to engage in social play implies a state of stress-induced apathy (Trezza et al., 2011; Cathomas et al., 2015).

Finally, we examined hedonic behavior. No significant effect of either stressor on sucrose preference was found. There is a dual explanation for this result. A previous study showed a significant effect of sucrose concentration on sucrose preference in SPS subjected animals (they compared preference for three different sucrose solutions: 1 %, 0.5 % and 0.25 %), with larger differences in sucrose preference observed for lower sucrose concentrations (Enman et al., 2015). In our experiment, 1.5 % sucrose solution was used, so the difference in sucrose preference between the groups could be greater at a lower sucrose concentration. This result could also be expected, as in a PTSD rat model, a behavioral profiling approach showed that anhedonia only occurs in 5/18 stressed rats (Ritov et al., 2016). This is also consistent with clinical data on the rate of comorbid major depression in PTSD patients (Conner et al., 2014).

Dysfunction of the glutamatergic system in hippocampus and in

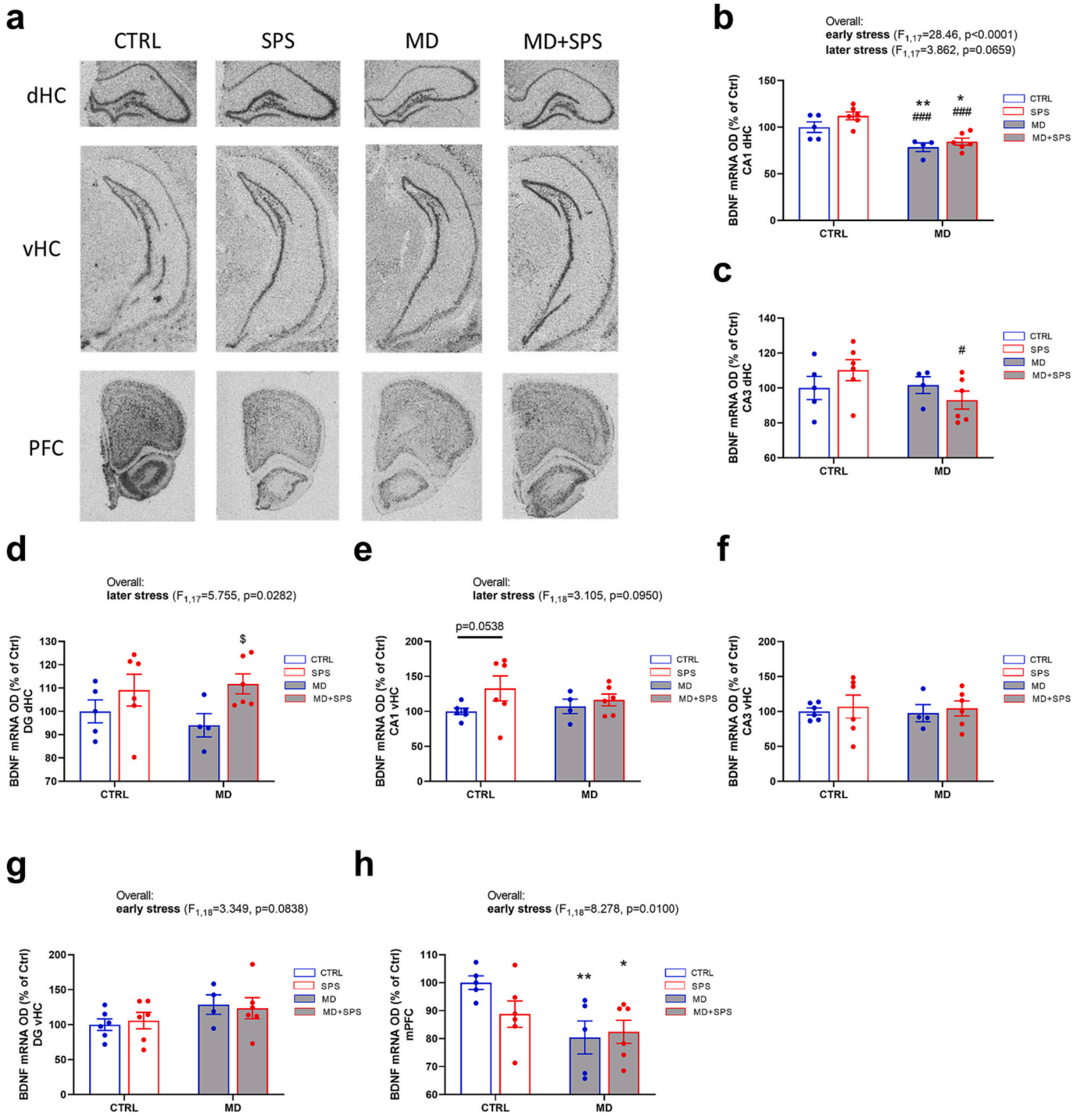

Fig. 6. Quantitative measurement of BDNF mRNA expression by radioactive in situ hybridization. Data was analyzed using two-way ANOVA followed by Fisher's least significance difference (LSD) post hoc test. F-values and overall effect of early stress or later stress or early stress x later stress is depicted in the upper right corner of each graph if significant, or at trend level. Statistical difference from control: $0.01 < p < 0.05$, $0.001 < p < 0.01$ is presented as *, ** respectively. Statistical difference from SPS: $0.01 < p < 0.05$, $p < 0.001$ is presented as #, ###, respectively. Statistical difference from MD: $0.01 < p < 0.05$ is presented as $. Individual data points are presented as dots in each figure, in addition to the group average and error bars representing SEM. Exact numerical values per group are given in Supplementary table 1. Trend effects ($0.05 < p < 0.1$) is labelled as ~ or a specific p value.

a Representative autoradiograms for dorsal hippocampus (dHC), ventral hippocampus (vHC) and prefrontal cortex (PFC). BDNF mRNA quantification in dHC: CA1 (b), CA3 (c), DG (d); vHC: CA1 (e), CA3 (f), DG (g) and medial PFC (h). Significant overall effect of MD was detected in dHC CA1 and mPFC. Significant overall effect of SPS was detected in dorsal DG. MD and MD + SPS groups had significantly decreased BDNF mRNA in dHC CA1 and mPFC compared to unstressed controls. MD + SPS group differed significantly from SPS in dHC CA3 and from MD in dHC DG.

cortical areas is considered the main feature of stress-related mental disorders (Pitman et al., 2012; Lener et al., 2017). In their review of synaptic loss and the pathophysiology of PTSD, Krystal et al. (2017) highlight a large cluster of PTSD symptoms, that cannot be explained as a consequence of dysregulated fear, but rather may be a direct result of the effects of stress on glutamatergic synaptic integrity. Some of these symptoms, such as behavioral disinhibition and apathy, overlap with diseases that impair brain functional connectivity (traumatic brain injury or cerebrovascular disease) (Laskowski et al., 2015; Starkstein and Pahissa, 2014; Baldassarre et al., 2016), with glutamatergic toxicity as an important pathogenic mechanism (Dirnagl et al., 1999; Yi and Hazell, 2006). Traumatic brain injury is a known risk factor for PTSD (Bryant, 2011).

Our second aim was therefore to investigate hippocampal glutamatergic synaptic function in stressed animals. Seven days after SPS exposure, we observed reduced depolarization-evoked neurotransmitter release in hippocampal synaptosomes from MD + SPS and SPS animals compared to controls. Since 80–90 % of hippocampal synapses are glutamatergic (Shinohara and Hirase, 2009), this result mainly reflects a reduced depolarization-evoked release of glutamate. To our knowledge, this is the first study to examine this type of effect in a PTSD animal model.

It is generally recognized that stress activates glutamatergic circuits (Popoli et al., 2011). Acute stress of low and moderate intensity enhances glutamate release. It leads to increased excitability and enhanced LTP. Chronic stress has an opposite effect (Tornese et al., 2019; Kallarackal et al., 2013; Marrocco et al., 2014; Yuen et al., 2012). Indeed, the relationship between intensity and duration of stress and glutamate release most closely resembles an inverted U-shaped dose response curve and has been associated with loss of resilience to stress (Tornese et al., 2019; Joëls, 2006; McEwen et al., 2016). Our results suggest that SPS induces a significant and persistent reduction in hippocampal glutamate release. The molecular mechanisms of these effects are largely unknown.

A recent study investigating the effects of SPS on the molecular composition of the presynaptic compartment in the hippocampus showed a reduced capacity for vesicle fusion (Guan et al., 2022). Such changes could explain the decreased hippocampal neurotransmitter release induced by SPS in our study. Impaired glutamatergic neurotransmission (primarily in the hippocampus) induced by stress leads to impaired top-down control of subcortical areas, such as reward circuitry and amygdala, which has long been recognized as a pathophysiological hallmark of dysregulated mood and emotion (Price and Drevets, 2010; Duman et al., 2016; Workman et al., 2018).

Another finding of our study that complements the observed changes in depolarization-evoked neurotransmitter release is the decreased glutamate concentration in hippocampal synaptosomes only in the MD + SPS group. The sample size is relatively small ($n = 4$) and the overall effect showed a strong trend ($p = 0.053$), so any conclusions from these data should be taken with caution. However, the effects reflecting reduced glutamate concentration in synaptic vesicles are potentially significant. This could be due to either depletion of vesicular glutamate reserves or reduced glutamate import into the vesicles. Reduced glutamate import into vesicles would imply impairment of the glutamate-glutamine cycle and could be a consequence of reduced astrocyte number or function, which has been found in animal models of stress-related disorders as well as in postmortem brain tissue from patients with stress-related major depression (Nagy et al., 2015; Banasr et al., 2010). Such impairment has been proposed as a model of pathogenesis of major depression (Walter et al., 2009), and a similar mechanism has been proposed for PTSD (Krystal et al., 2017).

These results further demonstrate that the combination of two stressors induced a complex disruption of glutamatergic synaptic function. It is important to note that these changes were detected after a 7-day quiescent period following SPS, and thus represent a delayed response likely due to dysfunctional adaptation to the stress. This complements a previous observation that MD in rats impedes the normal response to acute restrain stress in adulthood (Roceri et al., 2002) and points to a possible mechanism of how early life stress might contribute to disease pathogenesis. This interpretation is supported by a report of excitotoxicity in hippocampus of PTSD patients, where elevated glutamate levels were correlated with re-experiencing symptoms (Rosso et al., 2017).

The neurochemical HPLC profile suggested PFC as the focal point of change. Significant changes were detected only in the double-hit group. A significant difference in NE, 5-HT, 5-HIAA and EPI concentration was detected in PFC of MD + SPS animals compared to controls. There is extensive literature on the changes in the NE system in PTSD, with a general consensus that PTSD is associated with increased NE function (Krystal and Neumeister, 2009). Additionally, increased catecholamine availability, due to genetic dysfunction of the catecholamine catabolic enzyme – catechol-*O*-methyltransferase, has been shown to increase the incidence of PTSD in people exposed to trauma (Kolassa et al., 2010). Interestingly, in veterans with PTSD and a history of trauma exposure, there is significantly more positive correlation between NE concentration in CSF and the expression of PTSD symptoms than in individuals without history of trauma exposure, suggesting that past trauma increases brain reactivity to NE and predisposes to the expression of PTSD symptoms (Hendrickson et al., 2018). Increased NE activity in the PFC has been causally linked to stress-induced PFC dysfunction (Arnsten et al., 2015). Increased NE activity in a PTSD animal model led to increased aggression and impaired social interaction (Olson et al., 2011).

The role of 5-HT in PTSD is far from clear. There is evidence that stress upregulates the serotonergic system (Chaouloff et al., 1999) and some 5-HT agonists transiently induce PTSD symptoms (Krystal et al., 1996). However, it should be noted that 5-HT reuptake inhibitors are approved for the treatment of PTSD (Krystal and Neumeister, 2009).

When we examined presynaptic protein expression, the most striking finding was a substantial decrease in T286 phosphorylation of both CaMKIIα and CaMKIIβ in cortical synaptosomes from SPS and MD + SPS groups and in hippocampal synaptosomes only from MD + SPS group, compared to controls. Total CaMKII expression was normal in all groups. The decrease in T286 phosphorylation reflects a loss of autonomous CaMKII activity. CaMKII autonomy is essential for synaptic plasticity and learning (Bayer and Schulman, 2019). Also, autonomous CaMKII activity has been identified as a drug target (Vest et al., 2010; Ashpole and Hudmon, 2011; Deng et al., 2017). To our knowledge, this is the first evidence of disruption of autonomous CaMKII activity in these regions in the context of PTSD.

Upregulation of CaMKII expression in lateral amygdala and autophosphorylation 4 weeks after stress was recently revealed in susceptible mice exposed to different stress paradigms (underwater trauma and four conditioned stimulus/unconditioned stimulus paradigm) (An et al., 2021). On the other hand, we have shown that SPS decreases P-T286 CaMKII in amygdala synaptosomes, whereas total CaMKII levels remained unchanged. A common explanation for these seemingly contradictory results requires further investigation.

CaMKII autonomous activity is crucial for neuronal survival, through its influence on glutamate homeostasis (Ashpole et al., 2012; Ashpole et al., 2013; Chawla et al., 2017). Prolonged pharmacological inhibition of CaMKII exacerbates excitotoxicity following a submaximal glutamate challenge (Ashpole and Hudmon, 2011). Pharmacological inhibition of CaMKII immediately after an excitotoxic insult prevents the aggregation and prolonged inactivation of the kinase, which protects cultured neurons from injury (Ashpole and Hudmon, 2011). In cerebral ischemia, administration of CaMKII inhibitors immediately after ischemic insult results in reduced neuronal death, improved functional plasticity, and improved behavioral outcome in rats (Vest et al., 2010; Deng et al., 2017; Coultrap et al., 2011). CaMKII pharmacologic modulation in PTSD is an avenue we plan to explore with our model in future studies.

Finally, we examined the extent of these stress paradigms on BDNF

mRNA. MD decreased BDNF mRNA in dorsal CA1 and mPFC, and there was no modulation of this effect by SPS. This led us to conclude that effects on BDNF mRNA are of relatively minor importance in these models, at least at the level of gene transcription.

4.1. Limitations

The study was conducted in male rats only, so the conclusions cannot be generalized to the female population. Also, the affected brain regions cannot be restricted to hippocampus, prefrontal cortex and amygdala. The second stressor was applied in early adulthood, when cortical circuits are still maturing, thus the possibility that developing and adult brains respond differently to trauma should be considered. Accordingly, our results may be translationally more relevant to military populations, for example, as veterans are typically exposed to combat in late adolescence or early adulthood. The results were obtained at a single time point only - seven days after SPS. Subsequent studies should analyze changes during the consolidation phase (the first week after SPS) – before the full manifestation of behavioral changes – as well as long-term changes after SPS.

5. Conclusions

Our findings suggest that MD has a significant impact on SPS in early adulthood at the behavioral, neurochemical, and molecular levels, and indicate how early life stress enhances the effects of severe stress in early adulthood, which, given that SPS is a validated model of PTSD, may be pathophysiologically related to the increased risk for PTSD in individuals exposed to early life stress. The results point out that the reduction of glutamate release, as a presynaptic effect is particularly influenced by double-hit stress. Additionally, the observed changes in pCaMKII may indicate a pathophysiological mechanism that could be a potential pharmacological target. As an important translational implication, the existence of a time window between the second hit and the full manifestation of behavioral and molecular/cellular changes may enable protocols that exploit both preventive and therapeutic interventions in parallel in this rapidly evolving area of psychiatry.

CRediT authorship contribution statement

Đorđe Đorović: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Vesna Lazarevic:** Methodology, Investigation, Conceptualization. **Jovana Aranđelović:** Formal analysis. **Vladimir Stevanović:** Methodology, Investigation. **Wojciech Paslawski:** Methodology, Investigation. **Xiaoqun Zhang:** Methodology, Investigation. **Milica Velimirović:** Investigation. **Nataša Petronijević:** Resources, Funding acquisition. **Laslo Puškaš:** Resources. **Miroslav M. Savić:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Per Svenningsson:** Writing – review & editing, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jad.2024.01.087.

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