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PRESYNAPTIC FUNCTION OF GLUTAMATERGIC SYNAPSES IN A “TWO-HIT” STRESS MODEL

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PRESINAPTIČKA FUNKCIJA GLUTAMATERGIČKE SINAPSE U MODELU DVOSTRUKOG STRESA

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PRESYNAPTIC FUNCTION OF GLUTAMATERGIC SYNAPSES IN A "TWO-HIT" STRESS MODEL

Abstract

Background: Post-traumatic stress disorder (PTSD) is a common psychiatric disorder that requires a deeper pathophysiologic understanding and improved therapies. Early adversity significantly increases the risk of developing psychiatric disorders, including PTSD. Therefore, it is crucial to understand how such experiences influence the pathophysiology of PTSD. With this aim, we created a two-hit stress model to explore the interplay between two commonly used stressors: maternal deprivation (MD) and single prolonged stress (SPS), each administered at two key stages of development: the neonatal period and early adulthood.

Methods: We conducted a thorough analysis of animal behavior after exposure to these stress paradigms. We examined neurotransmitter release by utilizing FM2-10 dyes and measured glutamate concentration using a fluorometric assay in synaptosomes isolated from the hippocampus. In addition, we analyzed the expression of calcium/calmodulin-dependent protein kinase II (CaMKII) in different brain regions by Western blot analysis. Furthermore, we examined the levels of monoamines and brain-derived neurotrophic factor (BDNF) mRNA.

Results: We observed distinct behavioral changes in the two-hit group. Both the two-hit and SPS groups showed reduced depolarization evoked neurotransmitter release in the hippocampus, which was related to glutamate. Two-hit stress decreased p-T286 CaMKII levels in the hippocampus, while in the prefrontal cortex, both two-hit stress and SPS decreased p-T286 CaMKII levels. Regarding monoamine concentration, the most significant change was found in the prefrontal cortex, where two-hit stress caused an increase in norepinephrine and serotonin concentrations. Similarly, two-hit stress led to a decrease in the level of BDNF mRNA in the prefrontal cortex and hippocampus.

Conclusion: The combination of the two stressors had a more profound effect on animal behavior as well as on molecular and functional changes compared to single stressors.

Keywords: post-traumatic stress disorder, two-hit stress, maternal deprivation, single prolonged stress, glutamate release, CaMKII

Scientific field: Medicine

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PRESINAPTIČKA FUNKCIJA GLUTAMATERGIČKE SINAPSE U MODELU DVOSTRUKOG STRESA

SAŽETAK

Uvod: Posttraumatski stresni poremećaj (PTSP) je težak psihijatrijski poremećaj, koga karakteriše visoka učestalost komorbiditeta, kao i povećana stopa mortaliteta. Rani stres predstavlja važan faktor rizika za nastanak psihijatrijskih poremećaja, uključujući PTSP-a. Razumevanje kako rani stres utiče na patofiziologiju PTSP-a ima veliki translacioni značaj. Imajući to u vidu, razvili smo model "dvostrukog stresa", kako bi ispitali interakciju dva visoko korišćena stresora: maternalne deprivacije (MD) kao modela ranog stresa i pojedinačnog produženog stresa (PPS), koji predstavlja model PTSP-a.

Metode: Izvršili smo analizu ponašanja životinja izloženih ovim stresorima. U sinaptozomima hipokampusa ispitali smo egzocitozu neurotransmitera korišćenjem protokola preuzimanja/ekstinkcije FM2-10 boje i koncentraciju glutamata fluorimetrijskim testom. Takođe, korišćenjem Western blot metode, odredili smo ekspresiju Ca2+/kalmodulin zavisne protein kinaze II (CaMKII) u različitim regijama mozga. Dodatno, ispitivali smo koncentraciju monoamina metodom tečne hromatografije visokih performansi i mRNA neurotrofnog moždanog faktora korišćenjem radioaktivne *in situ* hibridizacije.

Rezultati: Grupa životinja izložena dvostrukom stresu razvila je karakteristične promene ponašanje, koje nisu registrovane kod životinja izloženih pojedinačnim stresorima. Kod dvostruko stresiranih pacova kao i pacova izloženih PPS-u utvrđena je smanjena egzocitoza glutamata u hipokampusu. Ovaj nalaz praćen je smanjenjem autofosforilacije CaMKII, enzima koji predstavlja jedan od glavnih regulatora glutamatergičke sinaptičke funkcije. U prefrontalnom korteksu, kako PPS tako i dvostruki stres " imali su sličan efekat na autofosforilaciju CaMKII. Dvostruki stres, za razliku od PPS-a, uzrokovao je povećanje koncentracije noradrenalina i serotonina u prefrontalnom korteksu kao i smanjenje ekspresije neurotrofnog moždanog faktora -a u prefrontalnom korteksu i hipokampusu.

Zaključak: Dvostruki stres uzrokovao je izraženije i specifične promene ponašanja, kao i molekularne i funkcionalne promene, u poređenju sa MD ili PPS-om.

Ključne reči: posttraumatski stresni poremećaj; dvostruki stres; maternalna deprivacija, pojedinačni produženi stres, egzocitoza glutamata, CaMKII

Naučna oblast: Medicina

Uža naučna oblast: Neuronauke

UDK broj: ……

Contents

1. Introduction

1.1 Two-hit stress

The diathesis-stress model is a psychological theory that suggests that mental disorders result from the interaction of a predispositional vulnerability (diathesis) and stressors (stress) (Monroe and Cummins, 2015). According to this model, individuals inherit certain genetic or biological predispositions that make them more susceptible to developing a mental disorder. These predispositions may include genetic vulnerabilities, personality traits, or early life experiences.

However, the development of a mental disorder is not solely determined by these predispositions. The diathesis-stress model posits that the interaction between these predispositions and environmental stressors is critical. Stressors can be any external or internal factors that disrupt a person's psychological equilibrium, such as traumatic events, chronic stress, or major life changes. The presence of these stressors can trigger or exacerbate the underlying vulnerability, leading to the onset of a mental disorder (Monroe and Cummins, 2015).

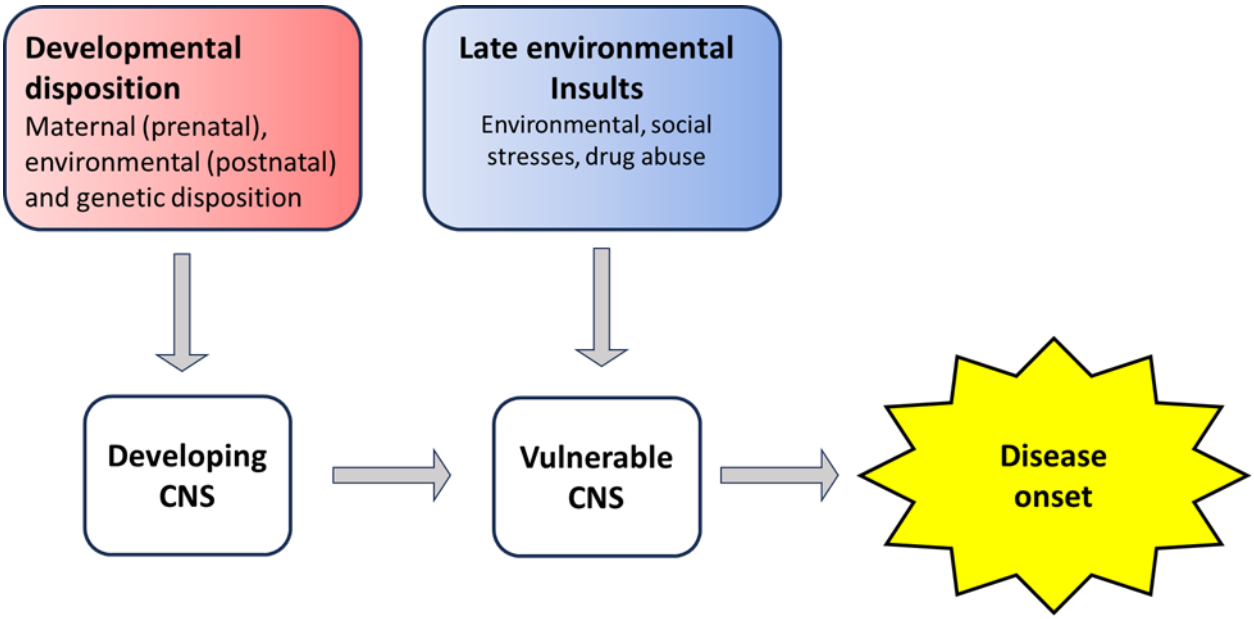


Figure 1. Diathesis stress model. Modified from Maynard et al (2001).

Allostatic load is a concept that describes the physiological consequences of chronic exposure to stressors and the body's efforts to adapt to them. It reflects the cumulative wear and tear on the body's systems, including the neuroendocrine, immune, and cardiovascular systems, as they strive to maintain stability in the face of stress (McEwen, 2005).

The term "allostatic" refers to the body's adaptive responses to stress, which involve changes in various physiological processes to maintain stability, or homeostasis, in the face of challenges. However, when stressors are chronic or severe, the body's adaptive mechanisms can become dysregulated, leading to a state of high allostatic load (McEwen, 2005).

Allostatic load is often measured using a combination of biomarkers that reflect the functioning of different physiological systems, such as cortisol levels, blood pressure, heart rate variability, and markers of inflammation. High allostatic load is associated with an increased risk of developing various health problems, including cardiovascular disease, metabolic disorders, and mental health conditions (McEwen, 2005).

One of the main drawbacks on PTSD animal models is that they don't include exposure to risk factors in addition to exposure to trauma (Richter-Levin et al., 2019). Thus, we decided to combine two highly replicated animal models: maternal deprivation (MD) at postnatal day (PN) 9, which is a model of early life stress, and single prolonged stress (SPS), which is a PTSD model. Maternal deprivation one of the most prevalent models of early life stress. It was developed as a model of schizophrenia, since MD at PN9 caused a more profound deficit in paired pulse inhibition compared to other timepoints (Ellenbroek et al., 1998). It has evolved overtime as a suitable model for the study of mental disorders with a developmental component (depression, schizophrenia, PTSD) (Marco et al., 2015). SPS is one of the most replicated models of PTSD (Lisieski et al., 2018). It faithfully recapitulates key aspects of PTSD symptomatology seen in patients (Daskalakis et al., 2013). We administered SPS at PN60, which corresponds to early adulthood in rats. Early adulthood is a period of heightened vulnerability for the development of PTSD, which is especially evident in the military population (Sachs-Ericsson et al., 2016). PTSD is a very heterogenous disease which probably consists of a variety of endophenotypes (Ressler et al., 2022). We believe that our model is translationally most relevant for the military population.

1.2 CaMKII structure and regulation

Ca2+/calmodulin (CaM)-dependent protein kinase II (CaMKII) is a serin/threonine protein kinase. It is very abundant and represents more than 1% of brain proteins (Bayer and Schulman, 2019). It is comprised of four domains: the N-terminal kinase domain (catalytic domain), which has an ATP and a substrate binding site, regulatory domain, that is divided into an autoinhibitory and CaM-binding domain which partially overlap, a variable domain and a C-terminal association domain (Bayer and Schulman, 2019). Under basal conditions CaMKII is inhibited by the interaction of the autoinhibitory and the catalytic domain, which blocks substrate binding. Ca2+/CaM displaces the autoinhibitory domain and lifts the autoinhibition. The CaM-biding domain is under regulatory control by inhibitory autophosphorylation at T305/306 which prevents Ca2+/CaM binding (Bayer and Schulman, 2019). CaMKII has a tendency to form a 12-subunit holoenzyme by linking through C-terminal association domains. A very important feature of the function of CaMKII is its ability to generate autonomous activity (Ca2+ independent activity) by autophosphorylation at T286 (Bayer and Schulman, 2019).

Autonomous activity is generated upon Ca2+/CaM binding and is organized so that the neighboring components in the holoenzyme autophosphorylate each other. In this scenario, one subunit functions as a kinase and the other as a substrate. CaM binding to both subunits is necessary for the autophosphorylation to occur. CaM has a dual role in this process (Hanson et al., 1994). On the one hand it will activate one kinase by lifting the autoinhibition, however its binding on the neighboring kinase in the holoenzyme will uncover T286, which is normally concealed by the regulatory domain which will make it accessible for autophosphorylation. This mechanism is sensitive to the frequency of Ca2+ spikes and is considered to be important for the molecular memory aspect of CaMKII function (Bayer and Schulman, 2019). CaMKII response to high frequency Ca2+ spikes represents the basis for its role in long-term potentiation (LTP) induction. Low frequency Ca2+ stimulation can also activate the kinase, only this process lasts longer and that represents the basis of CaMKII activation in induction of long-term depression (LTD). In a bound state, CaM covers the T305/306 sites, which prevents the autoinhibitory phosphorylation while CaM is bound. Dissociation of CaM from the kinase automatically triggers T305/306 phosphorylation, thus this occurs only in the autonomous subunit (Bayer and Schulman, 2019). This phosphorylation prevents subsequent Ca2+/CaM binding, and thus can't be considered a true inhibition, but rather a mechanism that limits the activation of the kinase.

Phosphorylation at any of the mentioned threonine residues in the target of phosphatases. There are three phosphatases that regulate CaMKII function: phosphatase 1 (PP1), phosphatase 2A (PP2A) and phosphatase 2C (PP2C), and it is believed that their function is restricted to different neuronal compartments (Stark et al., 1997). PP2A is active primarily in the cytoplasm, whereas PP1 is responsible for dephosphorylation of CaMKII in the postsynaptic density.

Another important CaMKII regulatory mechanism is the interaction between CaMKII and GluN2B (a subunit of NMDA receptors) which is dependent on binding of Ca2+/CaM or autophosphorylation (Bayer and Schulman, 2019). It causes accumulation of CaMKII at the postsynaptic membrane. This process is favored during LTP induction, while during LTD this interaction is prevented by the competitive binding of another kinase: DAPK1 to GluN2B. This seems to be a key molecular determinant that regulates the role of CaMKII in these opposing processes. It is enabled by opposing regulation, whereby factors that favor CaMKII/GluN2B interaction (which include Ca2+/CaM binding and GluN2B phosphorylation at S1303) inhibit DAPK1/GluN2B interaction and vice versa (Bayer and Schulman, 2019).

1.3. CaMKII and glutamatergic neurotransmission as a target of antidepressants

It is well established that presynaptic CaMKII represents a target of antidepressants. Chronic treatment with fluoxetine (serotonin reuptake inhibitor), reboxetine (norepinephrine reuptake inhibitor) and desipramine (tricyclic antidepressant) reduced depolarization evoked glutamate release in superfused hippocampal synaptosomes (Bonanno et al., 2005). In this experiment synaptosomes were placed on a filter paper inside a superfusion chamber, depolarization was evoked by KCl, and the amount of neurotransmitter released in the medium was measured using high-performance liquid chromatography (HPLC). Chronic antidepressant treatment affected the structure of the presynaptic compartment by altering the expression of three key SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex proteins (synaptobrevin, syntaxin and SNAP-25) in the fraction containing synaptic membranes, but not the one containing synaptic vesicles, which could signify that this effect was restricted to the readily releasable pool of vesicles (vesicles which are primed for exocytosis upon stimulation). From a follow up experiment, it was determined that this change did not affect the number of SNARE complexes and thus was not the cause of the reduced glutamate release. Chronic antidepressant treatment had an effect on the level of p-T286 CaMKII in hippocampal synaptosomes. Their results showed a substantial decrease of autophosphorylated CaMKIIα in synaptosomes as well as the synaptic membrane fraction, accompanied by a reduction in CaMKII expression level in the membrane fraction. Reduced CaMKII autophosphorylation can affect glutamate release, by altering CaMKII protein-protein interactions in the presynaptic compartment. Indeed, there was a reduction in the interaction between syntaxin 1 and CaMKIIα, which in known to stimulate exocytosis (Ohyama et al., 2022). Furthermore, the treatment induced increased interaction of syntaxin 1 and Munc-18 which is known to have an opposite effect on neurotransmitter release (Yang et al 2000). Thus, apparently antidepressants with different primary mechanisms of action converge to cause reduced glutamate release though altering CaMKII autophosphorylation in a specific compartment of the presynaptic terminal in the hippocampus. A subsequent study concluded that there was a redistribution of CaMKII between synaptic membranes (corresponding to the readily releasable pool of vesicles) and synaptic vesicles (corresponding to the reserve pool) in the hippocampus after chronic antidepressant treatment (Barbiero et al., 2007).

1.4. Neuroanatomical basis of PTSD symptoms

Amygdala, hippocampus, insular cortex, parts of the prefrontal cortex including the subgenual area (area 25) and the dorsal anterior cingulate cortex (area 32) (Fenster et al., 2018) have been implicated in the pathophysiology of PTSD. Additional structures that likely play a role in PTSD are the dorsolateral prefrontal cortex, striatum, thalamus, and sensory areas (Fenster et al., 2018).

Fear dysregulation is an important feature of PTSD. Exposure to trauma that leads to PTSD is considered an episode of natural fear conditioning (Jovanovic and Ressler, 2010). Fear conditioning is a form of classical conditioning that involves the association of a neutral stimulus (conditioned stimulus, CS) with an aversive or fear-inducing stimulus (unconditioned stimulus, US). Through this association, the neutral stimulus acquires the ability to elicit a fear response, even if the aversive stimulus is not present. Fear acquisition in fear conditioning refers to the process by which an individual learns to associate the CS and the US. The lateral amygdala (LA) receives sensory information about the CS and US from the thalamus and other sensory regions (Tovote et al., 2015). The convergence of these inputs in the LA allows the association between the two stimuli to be established. Synaptic plasticity mechanisms, such as long-term potentiation (LTP), play a crucial role in strengthening the connections between neurons representing the CS and the US (Eichenbaum, 1996). In addition to the amygdala, other brain regions, such as the hippocampus and the prefrontal cortex, also contribute to fear acquisition (Tovote et al., 2015). The hippocampus is involved in contextual fear conditioning, in which a particular context (in which the fear conditioning takes please) is associated with fear. During fear acquisition, the basal amygdala (BA) is activated in conjunction with the dorsal anterior cingulate cortex (area 32, analogous to the prelimbic cortex in rats) (Tovote et al., 2015). The most important output structure of the amygdala is the central amygdala (CA). It controls distal structures involved in the expression of fear, and CA microcircuits play an important role in fear acquisition (Tovote et al., 2015).

PTSD patients generally do not exhibit altered fear acquisition (Garfinkel et al., 2014; Milad et al., 2009). Fear extinction is a process of learning that involves the gradual reduction or elimination of a conditioned fear response. It occurs when neutral CS that was associated with an US is presented repeatedly without the US. Through this process, the association between the CS and the fear response weakens, leading to a decrease in fear behavior. Extinction is not the same as forgetting, as the original fear memory remains intact but is inhibited by new learning that the CS no longer predicts the US (Bouton, 1993). Extinction learning is context-dependent and can be influenced by various factors, including the timing and frequency of the CS presentations (Bouton, 1993; Rescorla and Heth, 1975). During extinction, the basolateral complex (BLC) is thought to undergo plasticity that weakens the association between the conditioned stimulus and the fear response (Quirk and Mueller, 2008). The prefrontal cortex, especially the subgenual area (area 25, which is analogous with the infralimbic cortex in rats), plays a key role in the inhibition of fear responses (Quirk and Mueller, 2008). This regulates the amygdala's response to fear-inducing stimuli, thereby facilitating extinction. A specific group of BLC neurons interacts with the infralimbic cortex and the disorganization of its output can cause extinction deficits (Jasnow et al., 2013). The hippocampus is involved in contextualizing fear memories and is essential for the formation of new, safety-related memories during extinction (Corcoran and Quirk, 2007). It is hypothesized that PTSD may result from a dysfunction in this process, leading to persistent fear (Jovanovic and Ressler, 2010). Indeed, individuals with PTSD often exhibit deficits in fear extinction (Norrholm et al., 2011).

One group of studies concludes that PTSD is not a disorder of fear extinction per se, but rather a deficit in extinction retention (Garfinkel et al., 2014; Milad et al., 2009). Garfinkel et al. (2014) conducted fear conditioning followed by extinction in veterans with PTSD and combat controls in different contexts to create a danger context (fear conditioning) and a safety context (extinction). After 24 hours, they tested extinction retention and fear renewal by exposing subjects to the extinguished CS in these two contexts. Interestingly, PTSD patients showed normal fear acquisition and extinction, but abnormal extinction retention and fear renewal, as evidenced by increased fear responses in the safety context and reduced fear responses in the danger context. These findings were accompanied by a specific activation pattern of brain regions, and they speculate that the main deficit lies in the inability of PTSD patients to process contextual information. Both the hippocampus and the prefrontal cortex play a role in the generation of contextual information (Garfinkel et al., 2014). A deficit in the processing of contextual information has been documented in PTSD patients and it is clear that this plays an important role in the extinction retention deficit (Corcoran and Quirk, 2007).

Reduced volume and activity of the ventromedial prefrontal cortex have been recorded in patients with PTSD and are increasingly considered crucial in the neurobiology of PTSD (Santhanam et al., 2019). Its reduced function is also associated with a deficit in fear inhibition (Jovanovic et al., 2012). Reduced volume of the hippocampus is a highly replicated finding in PTSD (Fenster et al., 2018). Impaired function of these regions has also been associated with fear generalization (Duvarci and Pare, 2014; Likhtik et al., 2014; Xu and Sudhof, 2013). This is a tendency of individuals with PTSD to generalize their fear responses to stimuli that are similar, but not identical, to the original traumatic event. This means that stimuli that are similar to the original traumatic trigger can elicit a fear response, even if they are not inherently threatening. Generalization of fear can occur along a spectrum ranging from stimuli that are very similar to the original trigger to those that are less similar but still evoke fear (Lissek and Meurs, 2015). Interestingly, with increasing similarity to the conditioned stimulus, a decrease in activity of the insula and the dorsomedial prefrontal cortex was found, while the opposite was observed for the ventromedial prefrontal cortex and the hippocampus (Lissek et al., 2013).

The salience network, which includes the amygdala, dorsal anterior cingulate cortex, and insula/operculum, plays a crucial role in filtering and identifying salient stimuli (Seeley et al., 2007). In PTSD, a hypersensitive salience network may lead to core symptoms such as hypervigilance and increased physiological reactivity due to inadequate threat detection (Sripada et al., 2012). Altered activity in all nodes of this network has been detected in PTSD (Sripada et al., 2012), and increased activity in this network is associated with a higher risk of developing PTSD as well as a negative prognosis for PTSD therapy (van Rooij et al., 2016; Admon et al., 2009). The dorsolateral prefrontal cortex, part of the central executive network, undergoes changes in PTSD that can lead to cognitive impairments, including deficits in attention, memory, and top-down control of emotions (Dunkley et al., 2015; Powers et al., 2015; Niendam et al., 2012; Ochsner et al., 2012).

2. Aims

The aims of this doctoral thesis were to:

1. Establish the ”two-hit” stress model and perform its behavioural characterization
2. Assess the changes in weight gain and the level of corticosterone caused by “two-hit” stress and single stressors (MD and SPS)
3. Assess the underlying molecular mechanism of the effects of stress on the presynaptic compartment, by measuring glutamate release and glutamate concentration in hippocampal synaptosomes as well as the expression of Ca2+/calmodulin protein kinase (CaMKII) in synaptosomes isolated from the rat hippocampus, prefrontal cortex and amygdala.
4. Assess the effects of “two-hit” stress and single stressors on the concentration of norepinephrine, epinephrine, serotonin, and dopamine and their respective metabolites in the hippocampus, prefrontal cortex and amygdala.
5. Assess the possible postsynaptic effects of stress by measuring dendritic BDNF transcript in hippocampus and prefrontal cortex.

3. Materials and methods

3.1 Animals and treatments

Wistar rats were used in the study, which were sourced from a breeding colony at the Department of Biochemistry, Belgrade University School of Medicine, provided by Charles River Laboratories. The study was conducted in accordance with NIH guidelines for animal experimentation and was approved by the Animal Ethics Committee.

The animals were housed in pairs in standard Plexiglas cages with sawdust (26 × 42 × 15 cm) in a temperature-controlled room (23 ± 1°C), on a standard 12-hour light/dark cycle (lights on from 7:00 am to 7:00 pm), with ad libitum access to food and water. After a two-week acclimatization period, male rats were removed and dams were monitored twice daily for parturition. The day of parturition was designated as postnatal day (PN) 0.

On PN9, the rat pups were subjected to maternal deprivation (MD), in which the dams were removed from the litter at 10:00 am and placed in another room to prevent vocalizations between mother and pups. The weight of the pups was measured and they were returned to their home cage where they remained at room temperature for 24 hours. On PN10, the weight of the pups was measured again and the mothers were returned to their cages. For the control litters, the mothers were removed for 3 minutes, while the pups were weighted, at PN9 and PN10. All litters were then left undisturbed, apart from routine cage cleaning, until weaning at PN21, when they were separated by sex. Only male rats were used in the study, which were kept in groups of two under standard conditions.

The single prolonged stress treatment (SPS) was performed at PN60. Both MD and control animals were restrained for two hours in clear, specially designed plastic tubes. After restraint, the rats were individually placed in a clear acrylic cylinder (24 cm diameter and 50 cm height) two-thirds filled with water (24°C) and forced to swim for 20 minutes. After a 15-minute recovery period, the animals were exposed to diethyl ether until loss of consciousness and then left undisturbed in their home cage for seven days.

The animals were thereafter either subjected to behavioral testing or euthanized by cervical dislocation and decapitation without anesthesia. The heads were snap frozen in liquid nitrogen and stored at -80°C until further use.

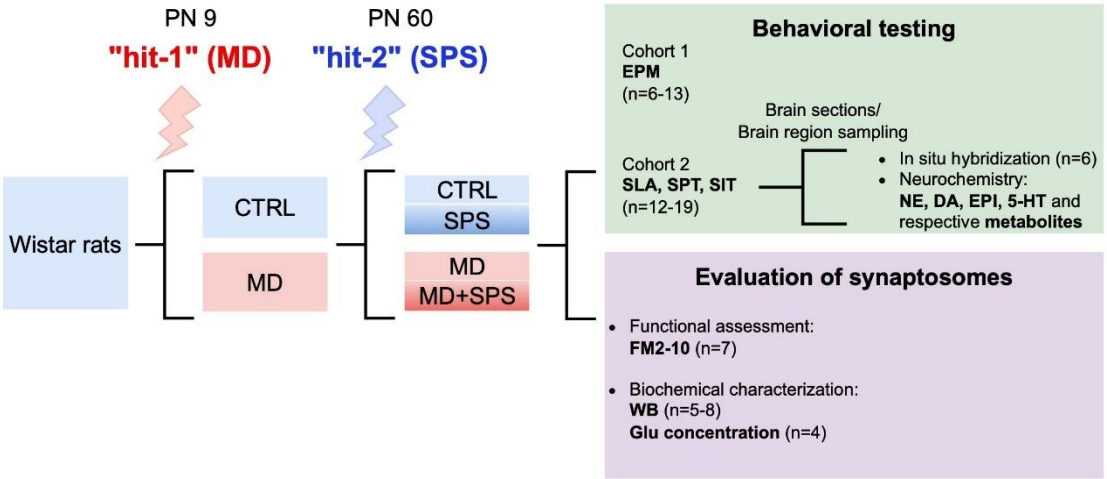


Figure 2. Experimental design

3.2 Behavioral testing

3.2.1. Elevated plus maze (EPM)

The Elevated Plus Maze (EPM) apparatus utilized in our study was crafted from robust sheet metal and had a resilient black rubber base. It comprised two open arms (50 cm × 10 cm) with slight ledges (0.3 cm high) and two closed arms (50 cm × 10 cm × 40 cm), interconnected by a central junction area (10 cm × 10 cm). The entire structure was elevated to a height of 50 cm above the floor. Illumination within the experimental room was provided by a red neon tube placed on the ceiling directly above the maze, maintaining a consistent light intensity of 10 lx on the surface of the closed arms. Individual rats were positioned in the junction area, oriented towards one of the closed arms, and were allowed to freely move around the maze for a period of five minutes. Quantification was done using ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA). Primary indicators of anxiety-like behavior encompassed the percentage of entries and time spent in the open arms. Motor activity was assessed by quantifying the number of entries into the closed arms. An entry into an area of the maze was registered when 90% of the animal traversed the virtual demarcation line separating the adjacent zones, while an exit was recorded when more than 90% of the animal departed from the respective zone.

Figure 3. Elevated plus maze apparatus. Picture taken from Ari et al (2019).

3.2.2. Spontaneous locomotor activity (SLA)

Locomotor activity testing was conducted in four blurred Plexiglas chambers (40 × 25 × 35 cm), under indirect white light (80 lx), without prior habituation. In each trial testing lasted for 90 min.

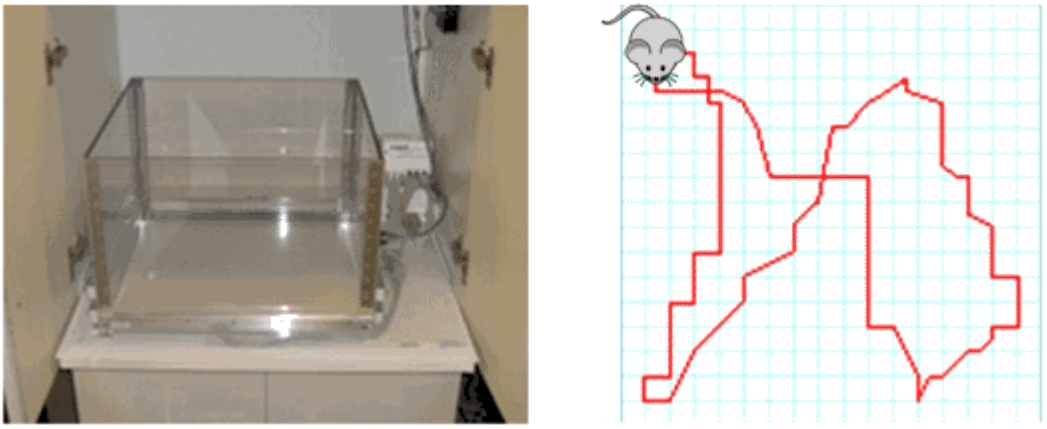


Figure 4. Spontaneous locomotor activity chamber. Picture taken from the internet (https://med.stanford.edu/sbfnl/services/bm/sm/activity-chamber.html)

3.2.3. Social interaction test (SIT)

The assessment of social behavior was conducted in an open wooden arena (100 × 100 × 40 cm) with a gray Plexiglas floor that allows easy removal of olfactory traces. The tests were carried out under dim red light (20 lx). 24 hours before the test, the rats were brought into the test room in individual cages, where they were allowed to acclimatize for 10 minutes. On the test day, two male rats of approximately equal weight (±10%), which had never been placed together before, were simultaneously placed in opposite corners of the arena facing the wall. Behavior was recorded for 10 minutes and manually scored by the researcher using AnyMaze software (Stoelting Co.). Any contact between the animals and their proximity in the tail-length area was generally considered as social interaction, while the researcher scoring the behavior was blind to the treatment. The experiment was conducted during the dark cycle.

3.2.4. Sucrose preference test (SPT)

Rats were individually housed. The first step was a 24h acclimation period to two bottles of 1.5% sucrose solution. After this period, one of the bottles was replaced with water over the next 24 hours. Subsequently the rats were deprived of food and water for 18h, and then presented with the two manually alternated preweighed bottles containing eighter 1.5% sucrose solution or plain water for 1 hour. The weight of the bottles was measured after 1h. Sucrose preference was calculated as the ratio of the weight of sucrose consumption to the weight of total fluid intake.

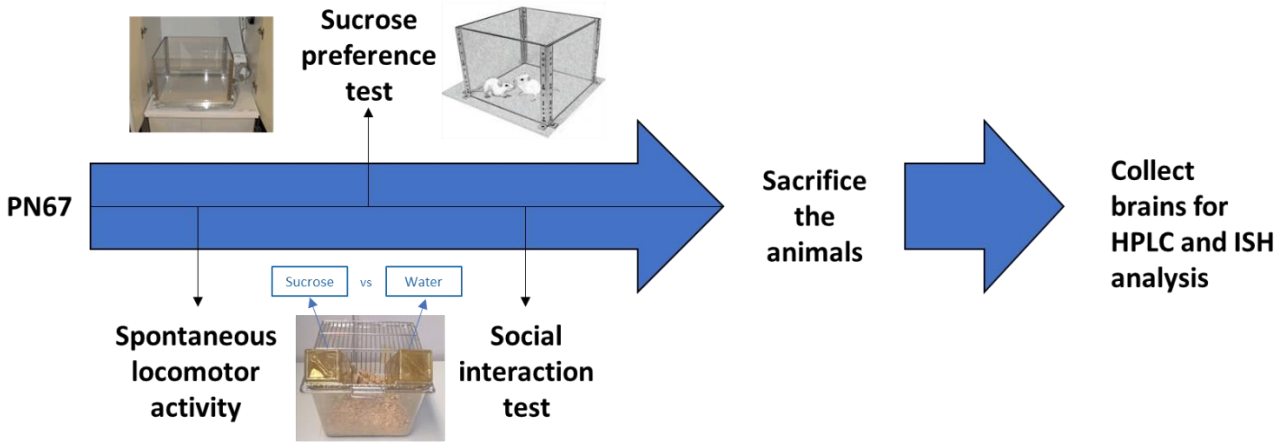


Figure 5. The workflow of behavioral testing in the second cohort

3.3 Synaptosome isolation

To isolate synaptosomes, we first dissected the hippocampus, prefrontal cortex, and amygdala from rat brains. We then measured the weight of each sample and homogenized the tissue in Syn-PER reagent (Thermo Fisher) by adding 10 ml of the reagent per 1 g of tissue. Following homogenization, the samples were centrifuged at 1200 g for 10 minutes, and the supernatants were collected and centrifuged again at 15,000 g for 20 minutes, to isolate the pellets representing the P2 fraction containing synaptosomes. All the manipulations with the tissue were performed at 4°C. The final pellets were resuspended in either Hank's balanced salt solution (HBSS) for quantification of neurotransmitter release or in RIPA buffer for Western blot analysis.

3.4. FM2–10 dye uptake/release protocol

To examine neurotransmitter release, we employed the FM2–10 dye uptake/release protocol, a standard method for investigating vesicle recycling. Initially, synaptosomes were suspended in 500 µl of HBSS buffer containing 100 µM FM2–10 at room temperature. Following this, KCl was added to induce dye uptake, and the synaptosomes were incubated for 15 minutes. Subsequently, the synaptosomes were centrifuged at 15,000 g for 5 minutes and washed twice with HBSS to remove excess dye. The synaptosomes were then resuspended in HBSS. Stimulation with KCl induced exocytosis, causing the release of the dye into the medium and a subsequent decrease in fluorescence, which was quantified to assess exocytosis.

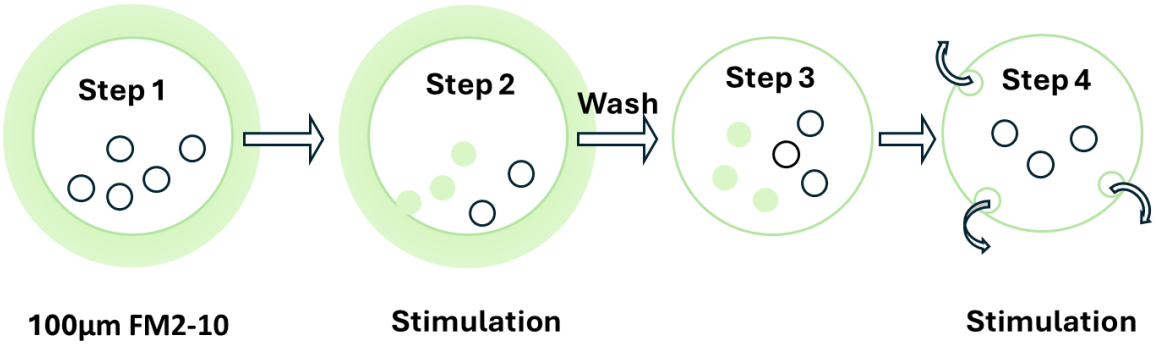


Figure 6. Schematic representation of the steps in the FM2-10 dye uptake/release protocol. Modified from Marks and Mahon, 1998.

3.5. Western blot

The 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) with protease and phosphatase inhibitors. The protein concentration was ascertained via BCA protein assay kit (Pierce). Protein concentration in the samples was normalized, and they were separated by SDS-PAGE on polyacrylamide gels, followed by transfer to Immobilon®-FL polyvinylidene difluoride membranes (Sigma). Primary antibodies, including anti-phospho-CaMKII (T286) rabbit monoclonal (1:500, Cell Signaling Technology, Cat. No. 12716), anti-CaMKII-α mouse monoclonal (1:1000, Cell Signaling Technology, Cat. No. 50049), and anti-β-actin mouse monoclonal (1:1000, Sigma-Aldrich, A5441), were incubated overnight at 4°C. After washing, membranes were incubated with fluorescently labeled secondary antibodies for 1 hour at room temperature. The blots were captured using Odyssey CLx Infra Red Imaging System from LI-COR Biosciences (Lincoln, NE, USA), and band intensity was quantified via Image Studio 3.1. Background intensity was deducted from the band intensity, and normalization was performed to β-actin and, for phospho-CaMKIIα, to total CaMKIIα.

3.6. Glutamate concentration

Glutamate concentration was assessed via a commercial kit (Abcam, USA, ab138883), based on instructions provided by the manufacturer.

3.7. Corticosterone quantification

Corticosterone was quantified in rat serum by using the corticosterone EIA kit, IDS, based on instructions provided by the manufacturer.

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

For this purpose, HPLC with electrochemical detection (ECD) was utilized. Tissue samples were treated with ice-cold 0.1 M perchloric acid (PCA) at a ratio of 50 µL PCA to 10 mg tissue, then incubated on ice for 10 minutes, vortexed, and centrifuged at 16000 x g at 4°C for 10 minutes.

Supernatants were filtered through 0.2 µm nylon membrane inserts and then centrifuged at 4000 x g for 5 minutes. The eluents were stored at -80°C and analysed within 1 week. Standard solutions (L-norepinephrine hydrochloride (NE), (+/-)-epinephrine hydrochloride (EPI), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxy-L-phenylalanine (DOPA), dopamine hydrochloride (DA), 5-hydroxyindole-3-acetic acid (5-HIAA), homovanillic acid (HVA), serotonin hydrochloride (5-HT), 4-hydroxy-3-methoxyphenylglycol hemipiperazinium salt (MHPG), DL-4-hydroxy-3-methoxymandelic acid (VMA) and 3-methoxytyramine hydrochloride (3-MT)) in 0.1 M PCA, had a final concentration of 200, 100, 50, 10, 5, 2, and 1 ng/mL. Calibration curves were constructed using Chromeleon software by performing linear regression of peak area against concentration. The HPLC-ECD system utilized was a Dionex Ultimate 3000 series with a Dionex C18 reversed-phase column (3 µm particle size), maintained at 30°C. The mobile phase consisted of 75 mM monobasic sodium phosphate, 2.2 mM 1-octanesulfonic acid (OSA) sodium salt, 100 µL/L triethylamine (TEA), 25 µM ethylenediaminetetraacetic acid (EDTA) disodium salt, and 10% acetonitrile (pH 3.0 adjusted with 85% phosphoric acid) at a flow rate of 0.4 mL/min. Detection of neurotransmitters and metabolites was achieved at -100 mV and +300 mV, respectively. Tissue samples were thawed on ice in the dark for approximately 1 hour, placed in the autosampler, and kept at 5°C before injection. Chromatograms were acquired using Dionex Chromeleon 7 software over a 55-minute period, and analyte concentrations in tissue samples were expressed in ng/mg tissue.

3.9. In situ hybridization

In situ hybridization was conducted on 12 µm thick cryostat fresh-frozen sections using a Leica CM 3050 S cryostat. 35S-labeled anti-sense and sense cRNA probes were generated by in vitro transcription from cDNA clones corresponding to fragments of brain-derived neurotrophic factor (BDNF). Transcription was carried out using [35S] UTP (1250 Ci/mmol) and T7 RNA polymerase with 50–100 ng of linearized plasmid. The fresh-frozen sections were post-fixed in 4% PFA for 5 minutes at room temperature, washed twice in 4× sodium chloride–sodium citrate buffer (SSC), and then treated with 0.25% acetic anhydride in 0.1 M triethanolamine/4×SSC (pH 8) for 10 minutes at room temperature. Following dehydration in a series of alcohols, the sections were incubated overnight at 55°C with a 35S-labeled probe in 50 µl of hybridization solution containing 20 mM Tris–HCl, 1 mM EDTA, 300 mM NaCl, 50% formamide, 10% dextran sulfate, 1×Denhardt's solution, 250 µg/ml yeast tRNA, 100 µg/ml salmon sperm DNA, 0.1% SDS, and 0.1% sodium thiosulfate. Then a series of washes was performed in 4×SSC (5 min, four times), followed by a treatment with RNAse A (20 µg/ml) for 20 minutes at 37°C, and another series of washes, first two times in 2×SSC (5 min each), 1×SSC (5 min), and 0.5×SSC (5 min) at room temperature, followed by a final two washes in 0.1×SSC at 65°C for 30 minutes (all washes contained 1 mM DTT) before dehydration in a series of alcohols. Finally, the slides were exposed to X-ray film for 4 to 28 days.

3.10. Statistics

Data were processed with GraphPrism 9, using two-way ANOVA or two-way ANOVA with repeated measures, followed by Fisher's Least Significant Difference (LSD) post hoc test. $p < 0.05$ was designated as statistically significant.

3.10.1. Sample sizes

	Number of animals per group			
Behavioral parameter/Group	**CTRL**	**SPS**	**MD**	**MD+SPS**
EPM parameters (fig. 2a-c)	13	11	6	7
SLA parameters (90min) (fig. 2d)	14	14	16	19
SLA parameters (10min) (fig. 2e)	14	14	16	19
SIT parameters (fig. 2f, g)	18	12	19	15
SPT parameters (fig. 2h)	13	14	17	18

	Number of animals per group			
Biomarker/Group	**CTRL**	**SPS**	**MD**	**MD+SPS**
FM2-10 experiment (fig. 3a)	7	7	7	7
Glutamate concentration (fig. 3b)	4	4	4	4
CaMKIIa/b HC (fig. 4b, c)	6	6	6	8
CaMKIIa/b mPFC (fig. 4e, f)	5	7	6	7
CaMKIIa/b AMY (fig. 4 h, i)	6	7	5	5
NTS/Metabolites HC (fig. 5)	8	9	7	8
NTS/Metabolites PFC (fig. 5)	6	9	8	8
NTS/Metabolites AMY (fig. 5)	8	7	8	7
BDNF HC (fig. 6b-g)	5	6	5	6
BDNF mPFC (fig. 6h)	5	6	6	6

Table 1: number of animals per group. HC (hippocampus), PFC (prefrontal cortex), AMY (amygdala)

4. Results

4.1. Behavior

4.1.1. Elevated plus maze (EPM)

For the analysis of the behavior in the EPM two-way ANOVA was used followed by by Fisher's Least Significant Difference (LSD) post hoc test. Regarding the behavior in the closed arms of the EPM there was an overall trend effect of early stress (p=0,0509) on decreasing the number of entries in the closed arms. Post hoc analysis revealed that MD animals had a lower number of entries in the closed arms, although the effect was at a trend level (p=0,0509). Regarding the behavior in the open arms of the EPM early stress had a significant effect on both the percentage of entries in the open arms and the percentage of time spent in the open arms. Post hoc analysis revealed that there was a statistically significant increase in both parameters in the MD and MD+SPS group compared to controls. There was also a statistically significant increase in both parameters in the MD group compared to SPS group, while MD+SPS displayed a significant increase only in the percentage of entries in the open arms compared to SPS group. SPS group displayed no change in these behaviors compared to the control group.

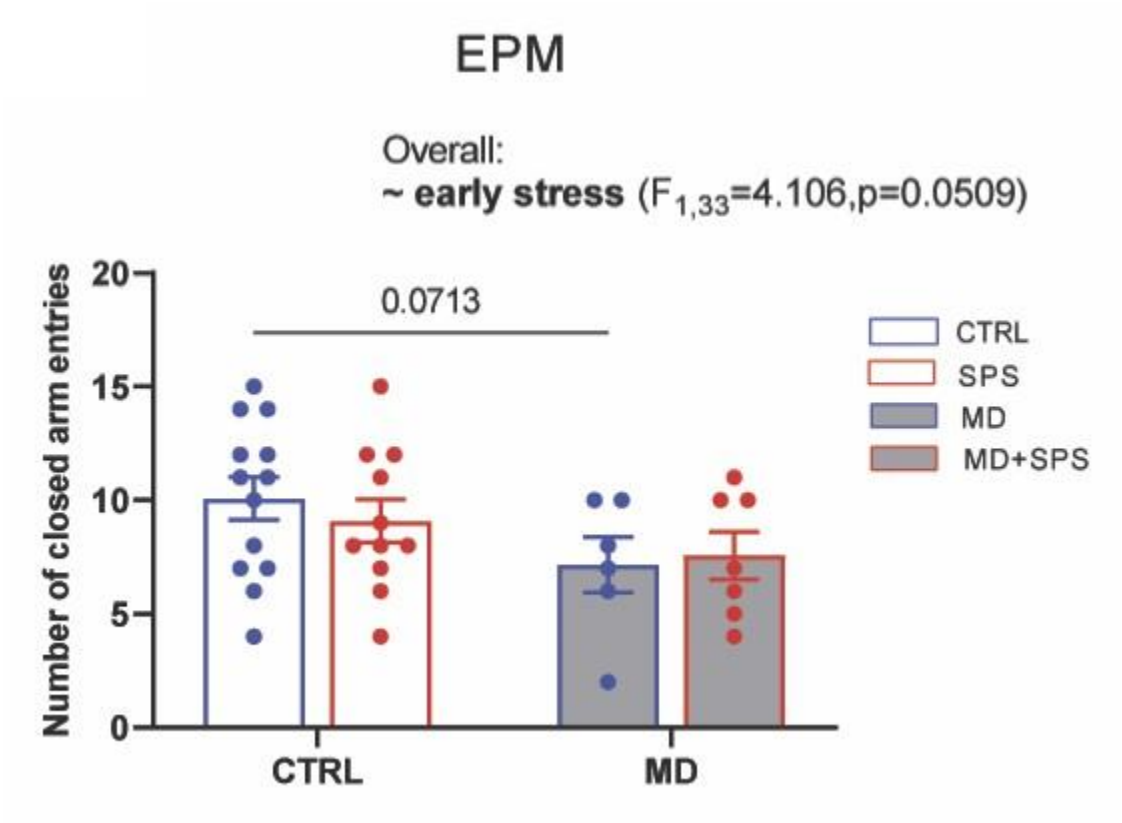


Figure 7. Number of closed arm entries. F-values and overall effects are presented if they are above the threshold of significance or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$).

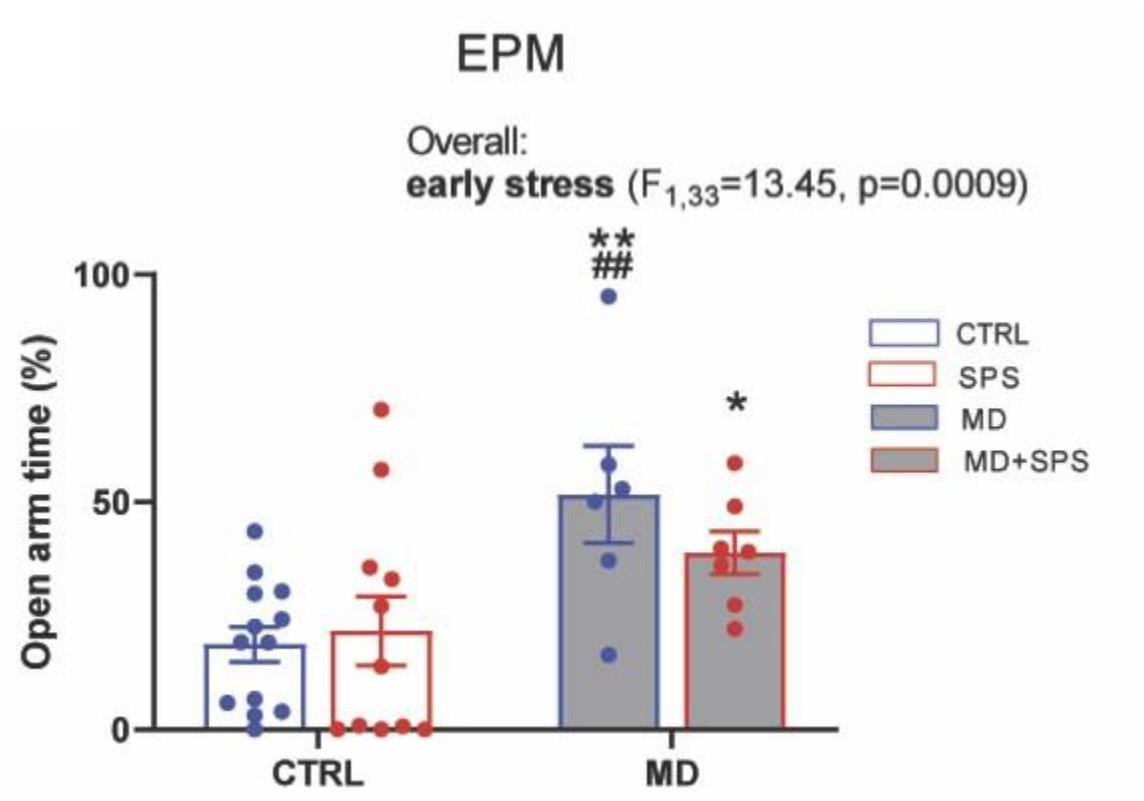


Figure 8. Percentage of open arm time. F-values and overall effects are presented if they are above the threshold of significance or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). * $0.01 < p < 0.05$ vs ctrl; ** $0.001 < p < 0.01$ vs ctrl; ## $0.001 < p < 0.01$ vs SPS

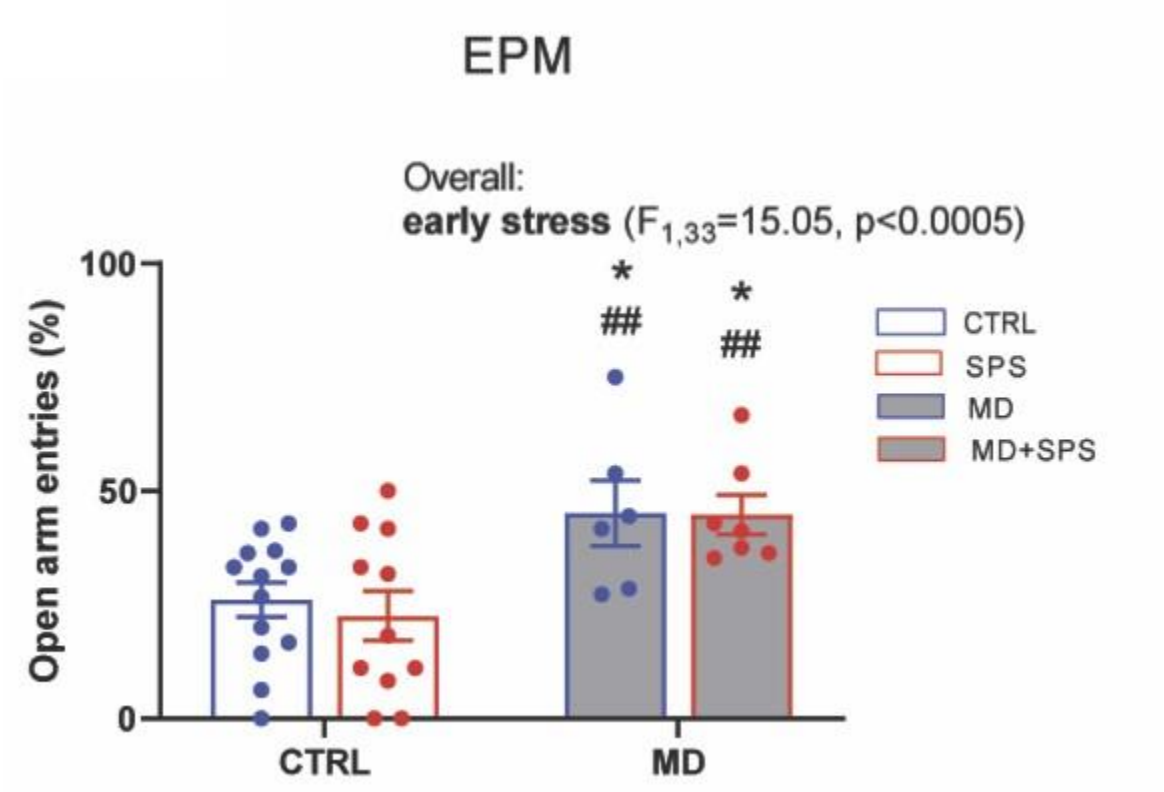


Figure 9. Percentage of open arm entries. F-values and overall effects are presented if they are above the threshold of significance or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). * $0.01 < p < 0.05$ vs ctrl; ## $0.001 < p < 0.01$ vs SPS.

4.1.2. Spontaneous locomotor activity (SLA)

Spontaneous locomotor activity was assessed during 90 minutes and analysed in 10-minute bins. The analysis was performed using two-way ANOVA with repeated measures followed by Fisher's Least Significant Difference (LSD) post hoc test. Post hoc analysis revealed that MD+SPS animals consistently displayed decreased distance travelled compared with control and SPS group respectively. Animals from MD and SPS groups did not display any alterations in this behavior compared to controls. We separately presented the distance travelled during the first ten minutes of testing, which is considered a habituation period. In that timeline early stress had an overall trend effect and MD+SPS group had a significantly lower locomotor activity compared to SPS group but not controls.

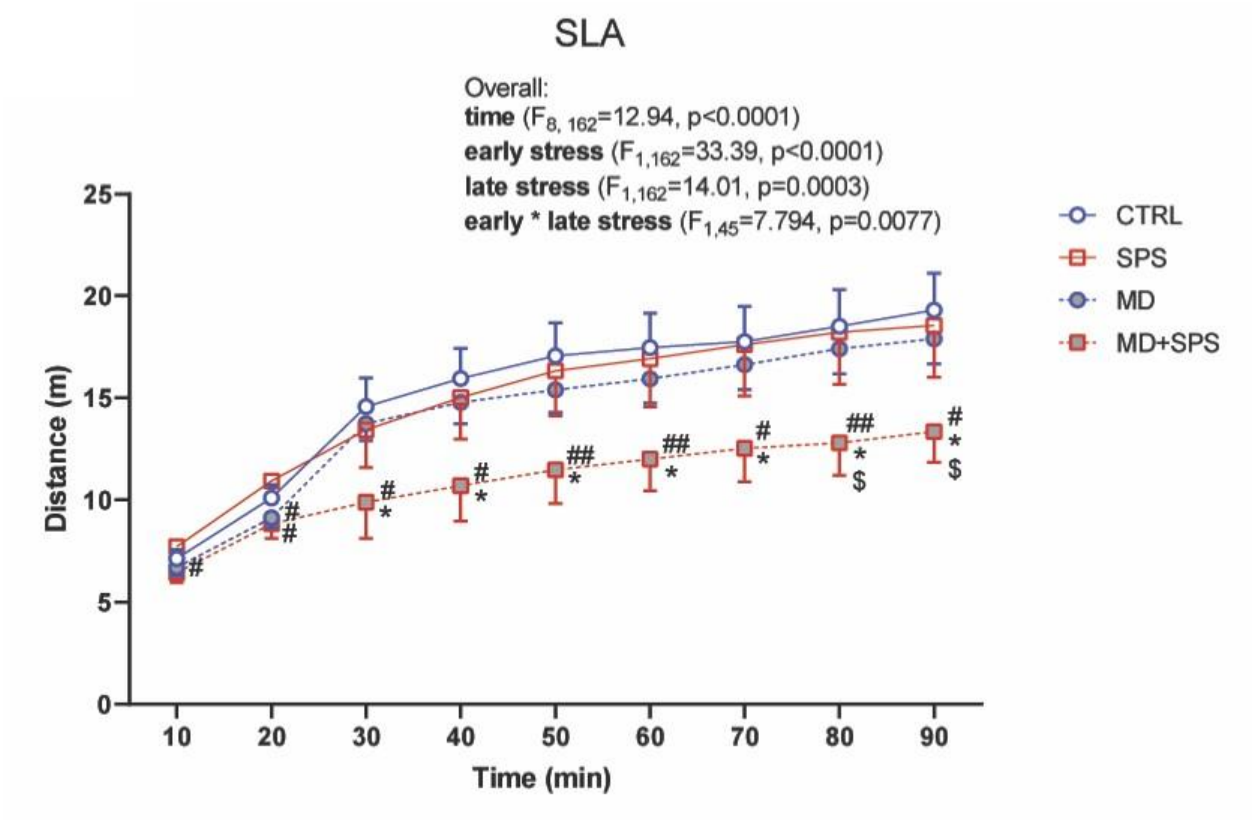


Figure 10. Spontaneous locomotor activity. F-values and overall effects are presented if they are above the threshold of significance or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl; $^{\#}$ $0.01 < p < 0.05$ vs SPS; $^{\#\#}$ $0.001 < p < 0.01$ vs SPS; $^{\$}$ $0.01 < p < 0.05$ vs MD.

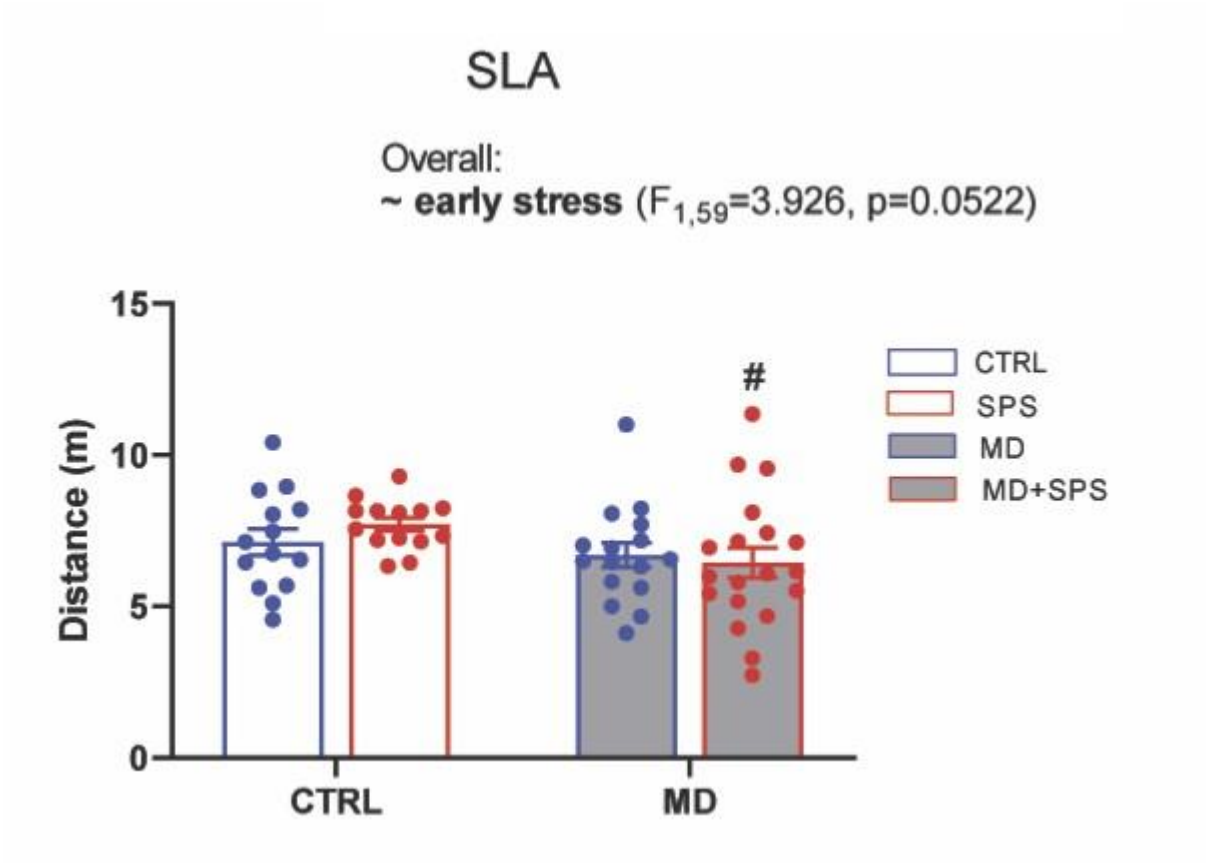


Figure 11. Distance travelled during the first ten minutes of testing. F-values and overall effects are presented if they are above the significance threshold or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq$ 0,1). $^{\#}$ $0.01 < p < 0.05$ vs SPS.

4.1.3. Social interaction test (SIT)

Social interaction data were analysed using two-way ANOVA followed by by Fisher's Least Significant Difference (LSD) post hoc test. Three parameters were analysed: following, social play (wrestling, crawling, boxing), and allogrooming. Later stress had an overall effect on following and social play. Post hoc analysis revealed that MD+SPS and SPS groups had a statistically significant decrease in these parameters compared to the control group. Later stress on the other hand had a trend effect on allogrooming ($p=0,0601$). Post hoc analyses showed that MD+SPS and MD group displayed decreased allogrooming compared to the control group.

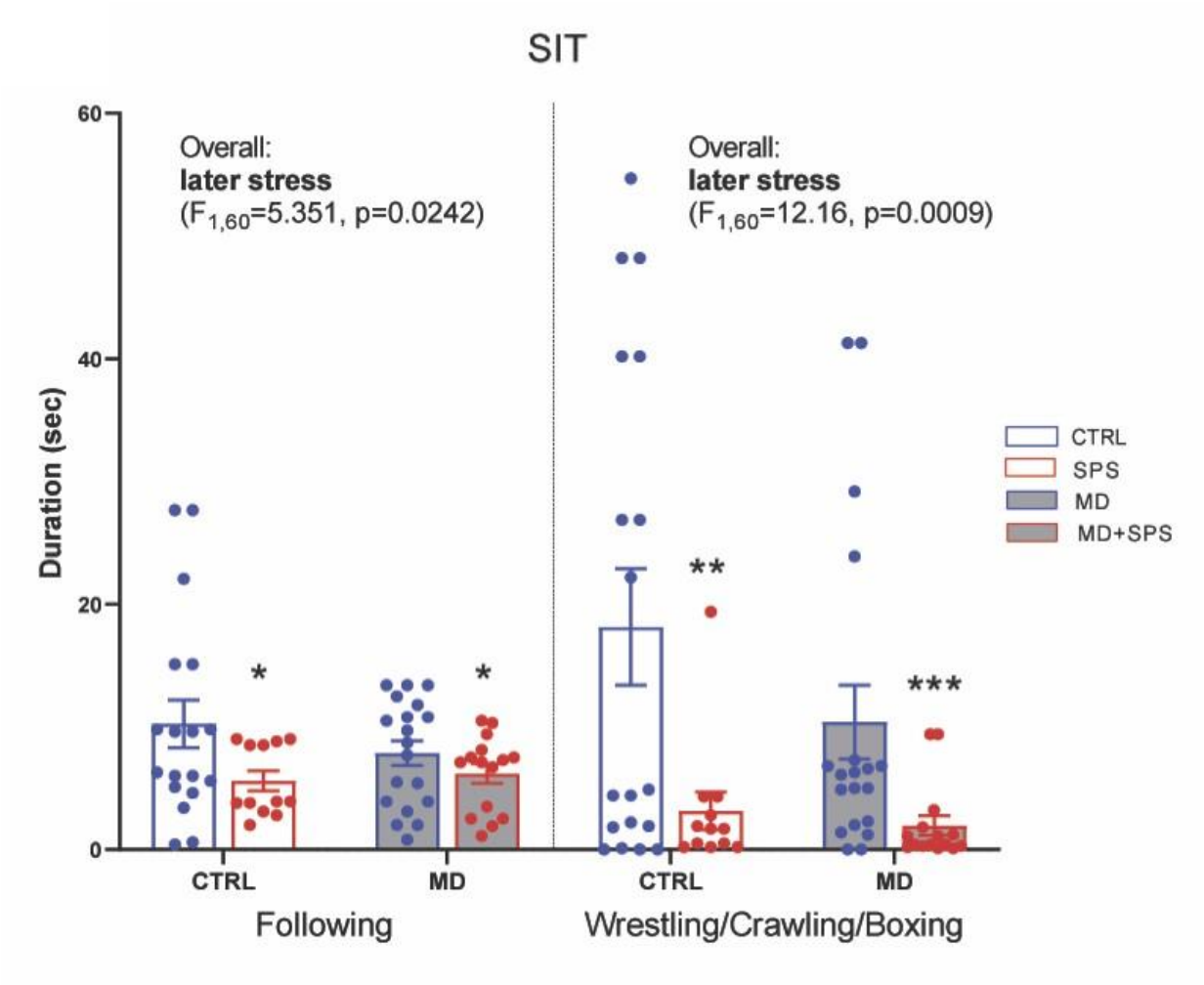


Figure 12. Following and social play (wrestling/crawling/boxing) F-values and overall effects are presented if they are above the significance threshold or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl; ** $0.001 < p < 0.01$ vs ctrl; *** $p<0{,}001$ vs ctrl.

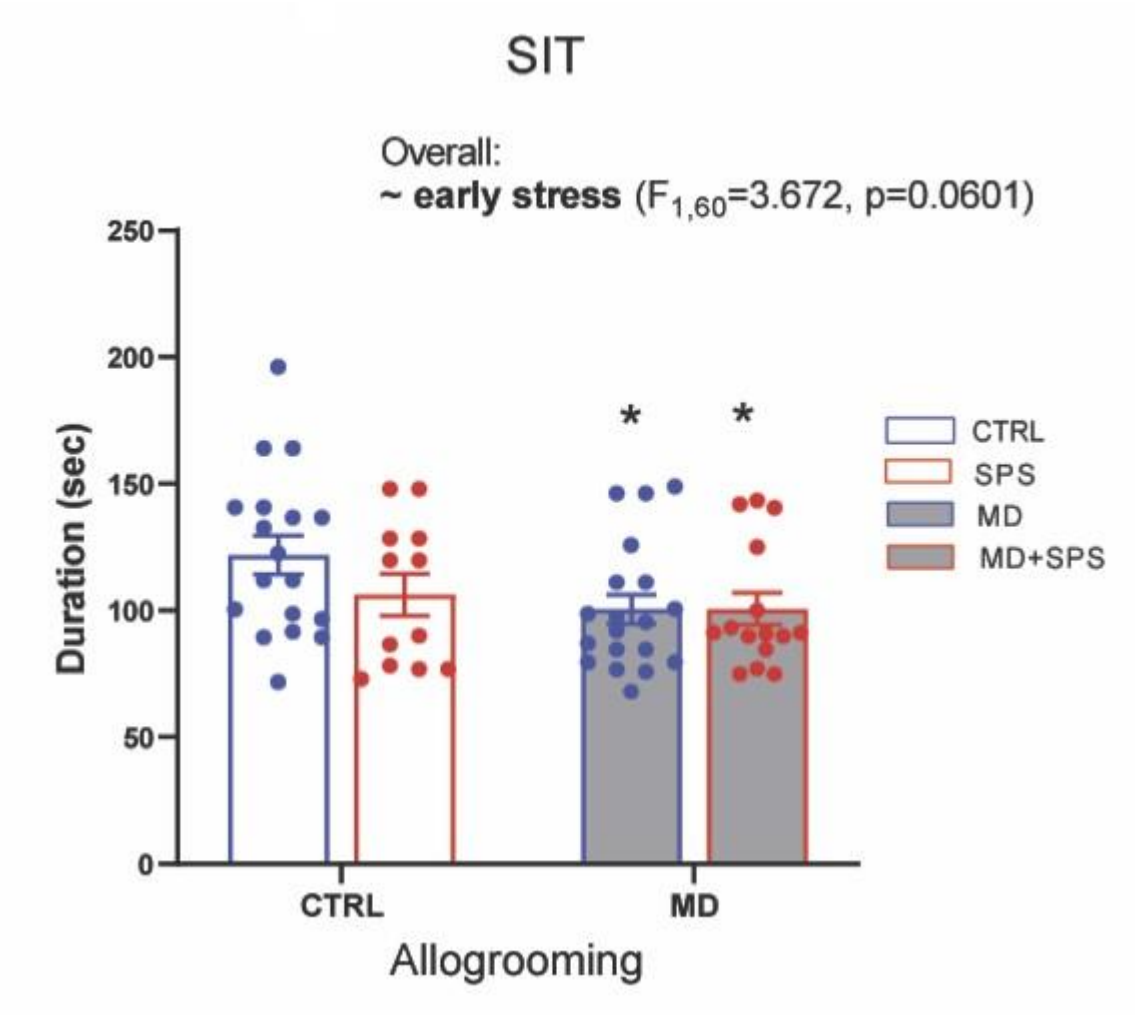


Figure 13. Allogrooming. F-values and overall effects are presented if they are above the significance threshold or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl.

4.1.4. Sucrose preference test (SPT)

Sucrose preference was analysed using two-way ANOVA followed by by Fisher's Least Significant Difference (LSD) post hoc test. There was an interaction effects of early stress * late stress, however it was at a trend level (p=0,053). The only statistically significant difference between groups involved MD+SPS and MD, where MD+SPS had a significantly lower sucrose preference. There was no significant difference between any of the experimental groups compared to controls.

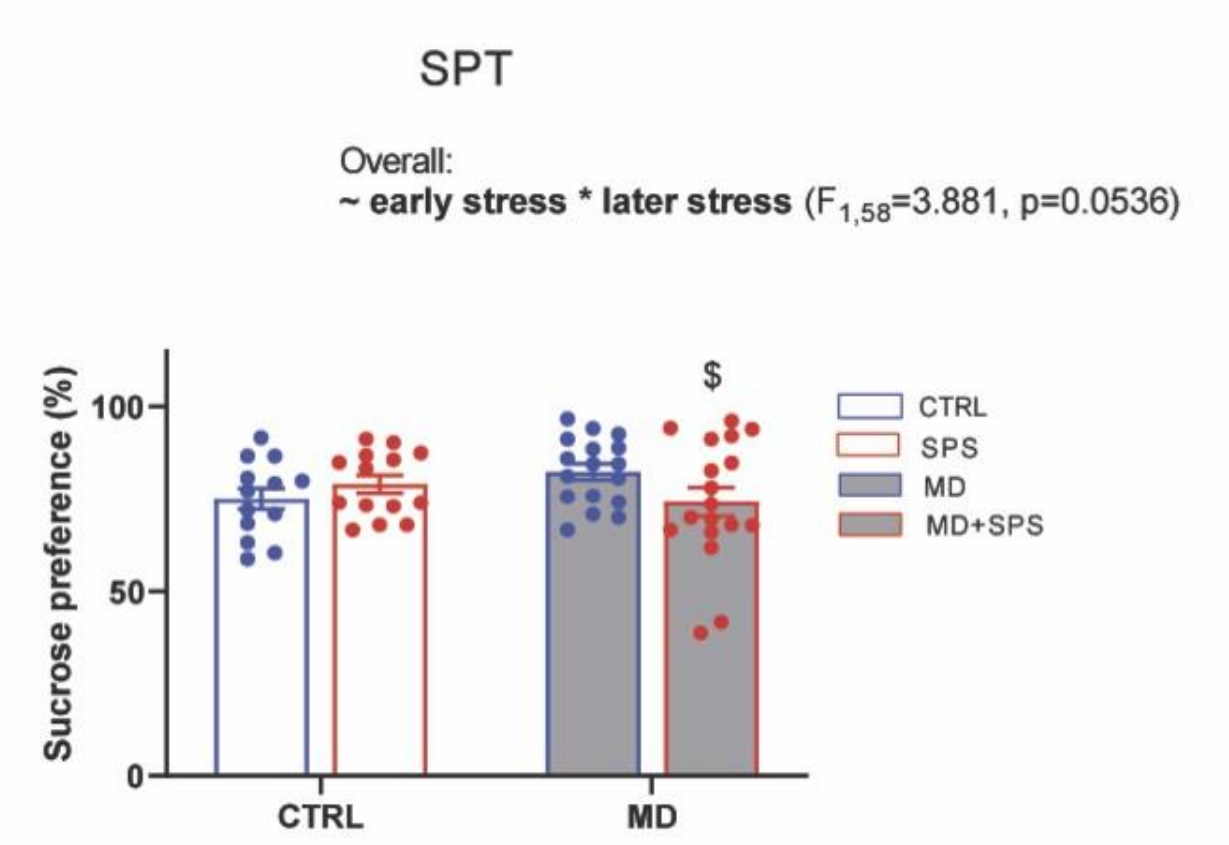


Figure 14. Sucrose preference. F-values and overall effects are presented if they are above the significance threshold or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). $^{\$}$ $0.01 < p < 0.05$ vs MD.

4.1.5. Animal weight

To assess the effects of the different stress paradigms on the body weight, measurements were performed at six time points. The first was before and after maternal deprivation (on PN 9 and 10), at weaning (PN21) during puberty (PN35), at early adulthood (PN60 – before SPS administration) and after the SPS consolidation period (at PN70), when the behavioral symptoms of SPS are expressed. The data was analysed using two-way ANOVA followed by Tuckey post hoc test (for PN9/10 and PN21-60 data) and Dunnett test (for PN70 data). There was a significant overall effect of early stress on body weight in all the time points. On PN9 MD group had a significantly lower body weight compared to CTRL group, however as expected only the control group showed a significant increase in body weight on PN10, while MD group body weight remained unchanged. MD group body weight remained significantly reduced compared to the CTRL group in all the subsequent measurements (at PN21, PN35 and PN60). At PN70 significant overall interaction effect was detected between early and late stress, and post hoc analysis detected a that MD+SPS group had a significantly lower body weight compared to CTRL group, while SPS group had a comparable body weight to the unstressed rats.

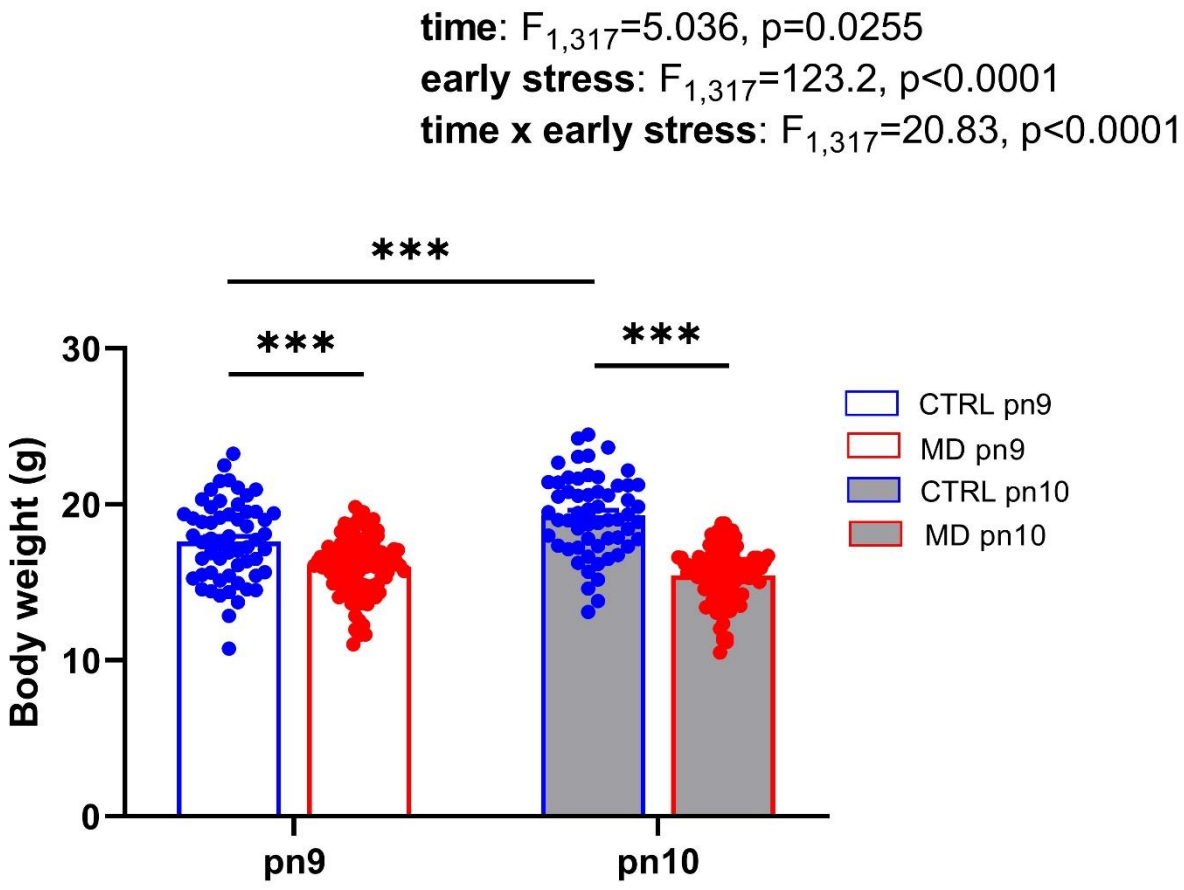


Figure 15. Body weight on PN9 and PN10. F-values and overall effects are presented if they are above the significance threshold or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). *** p<0,001.

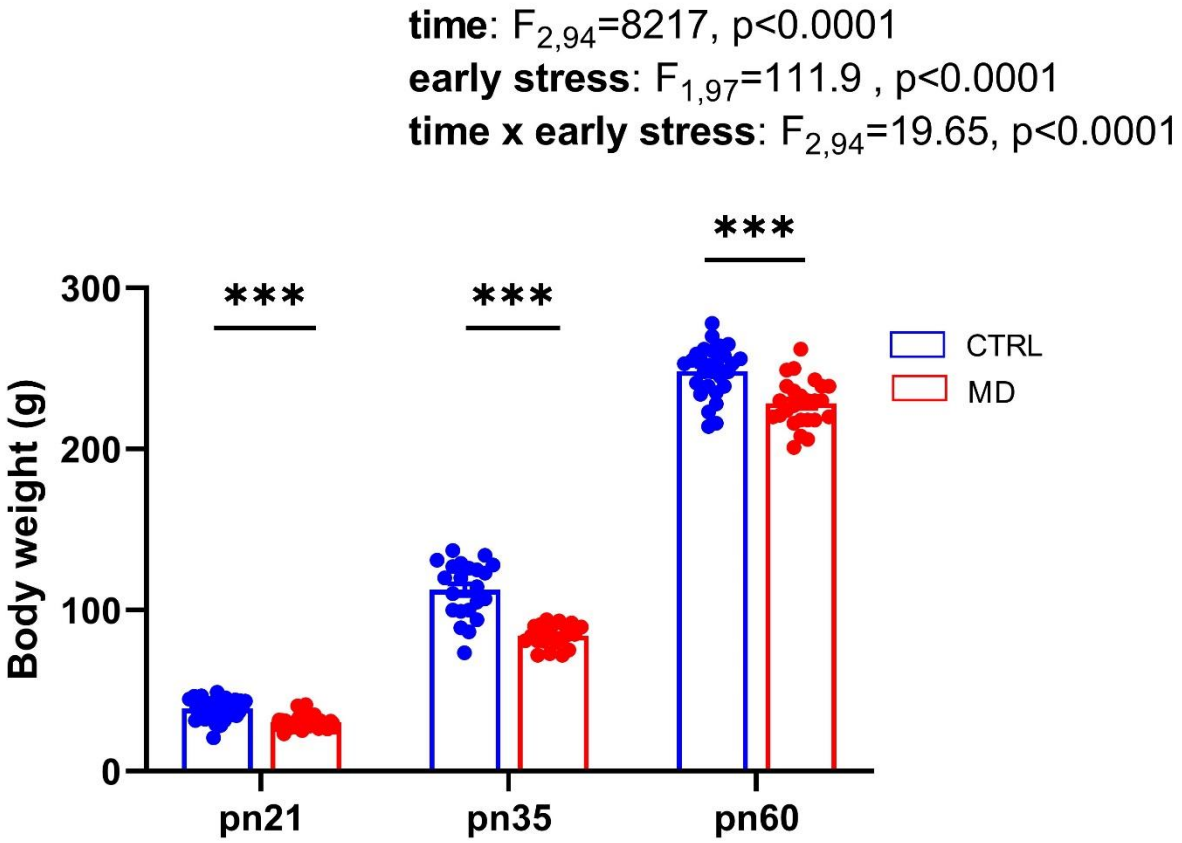


Figure 16. Body weight on PN21, PN35 and PN60. F-values and overall effects are presented if they are above the significance threshold or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). *** p<0,001.

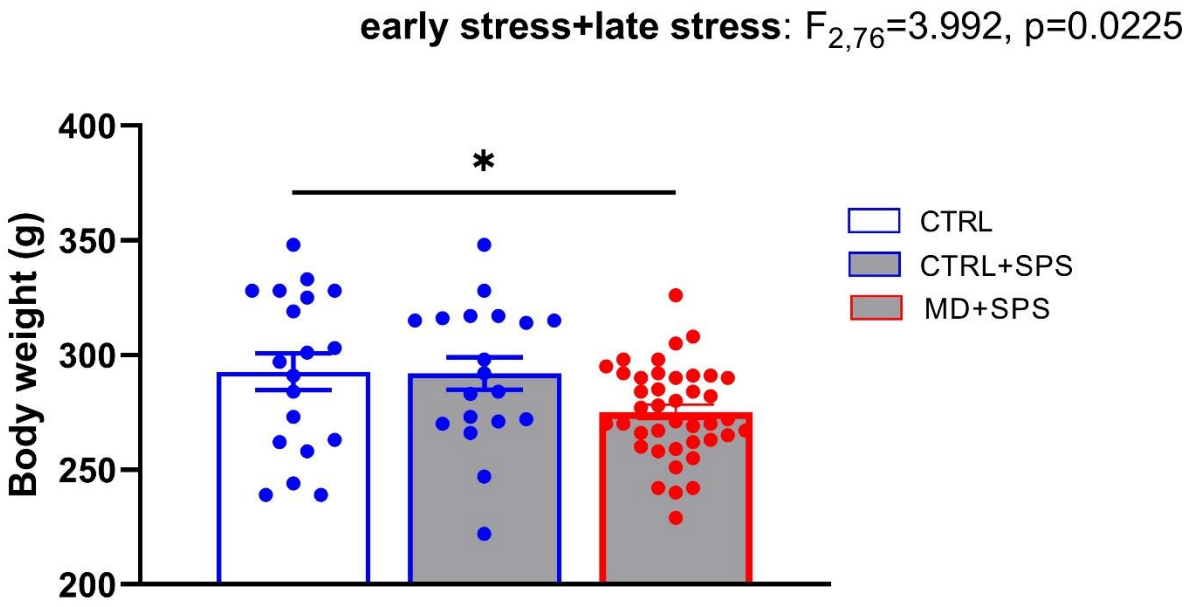


Figure 17. Body weight on PN70. F-values and overall effects are presented if they are above the significance threshold or almost significant. Each dot represents a data value. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). * $0.01 < p < 0.05$.

4.2. Corticosterone

Neither stress paradigm had a significant effect on the level of corticosterone.

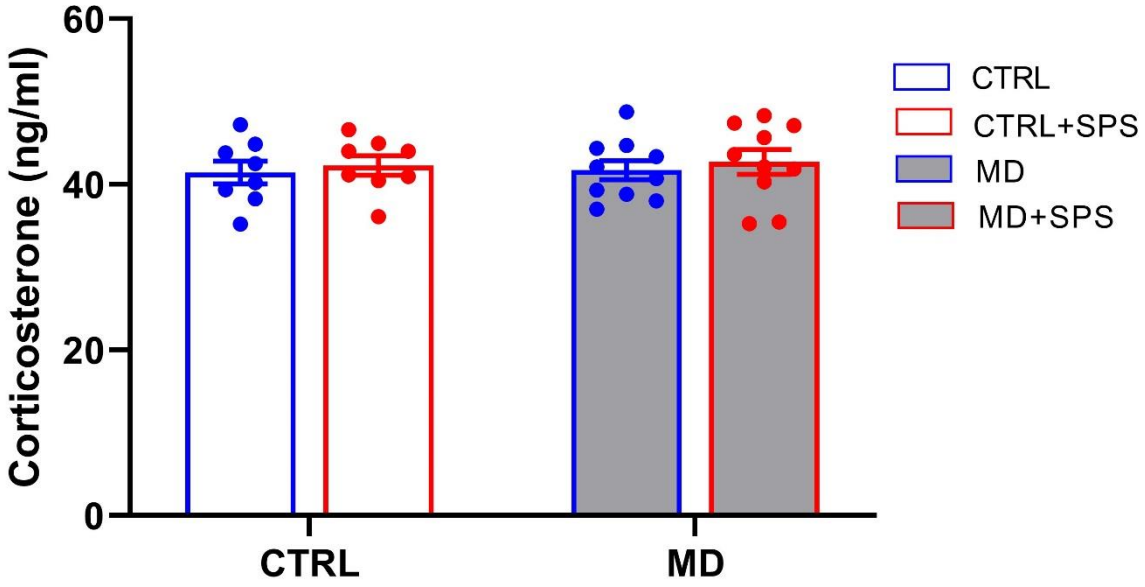


Figure 18. Concentration of serum corticosterone. No significant overall effect was detected. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). Each dot represents a data value.

4.3. Glutamate

FM2-10 data was analysed using two-way ANOVA with repeated measures followed by Fisher's Least Significant Difference (LSD) post hoc test. To assess neurotransmitter release a reduction of fluorescence was quantified following depolarization of synaptosomes with KCL. MD+SPS and SPS groups displayed reduced extinction of fluorescence compared to the control group.

Glutamate concentration was quantified in synaptosomes, and the data was analysed by two-way ANOVA followed by Fisher's Least Significant Difference (LSD) post hoc test. There was an overall effect of early stress on glutamate concentration at a trend level ($p=0,0533$). Post hoc analysis revealed that MD+SPS group displayed significantly reduced vesicular glutamate concentration compared to all other groups.

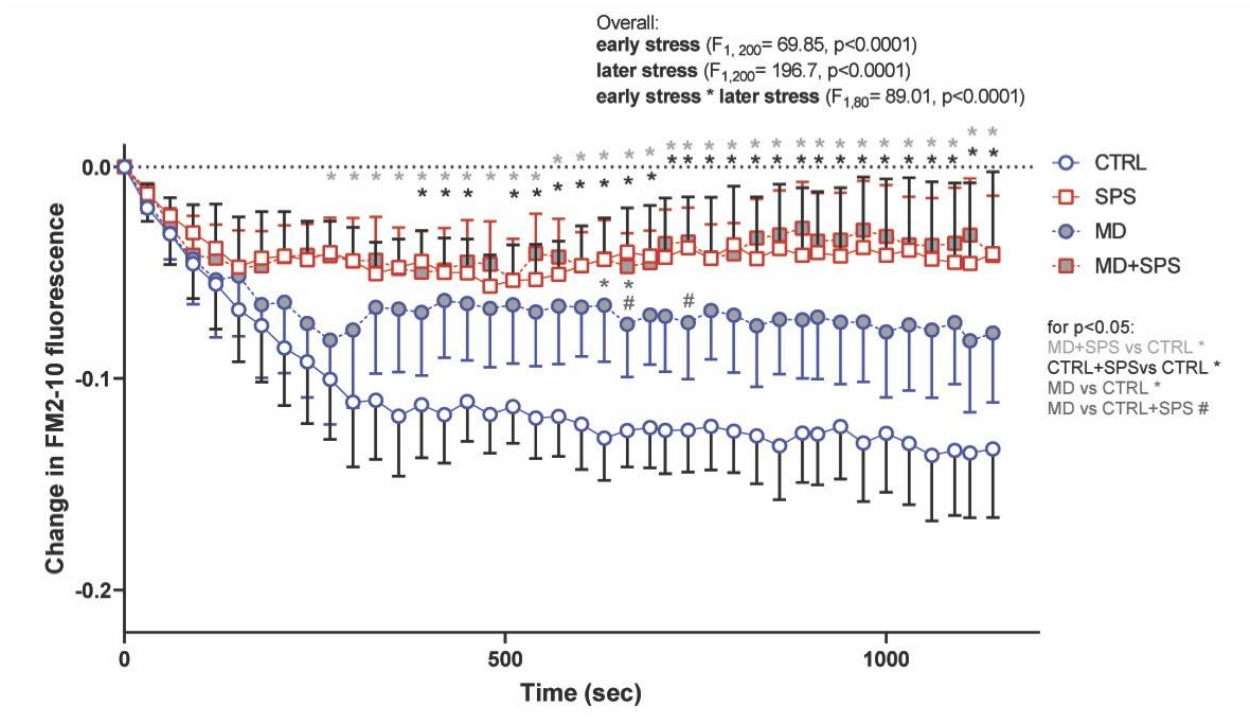


Figure 19. Neurotransmitter release in hippocampal synaptosomes. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl; $^{\#}$ $0.01 < p < 0.05$ vs SPS.

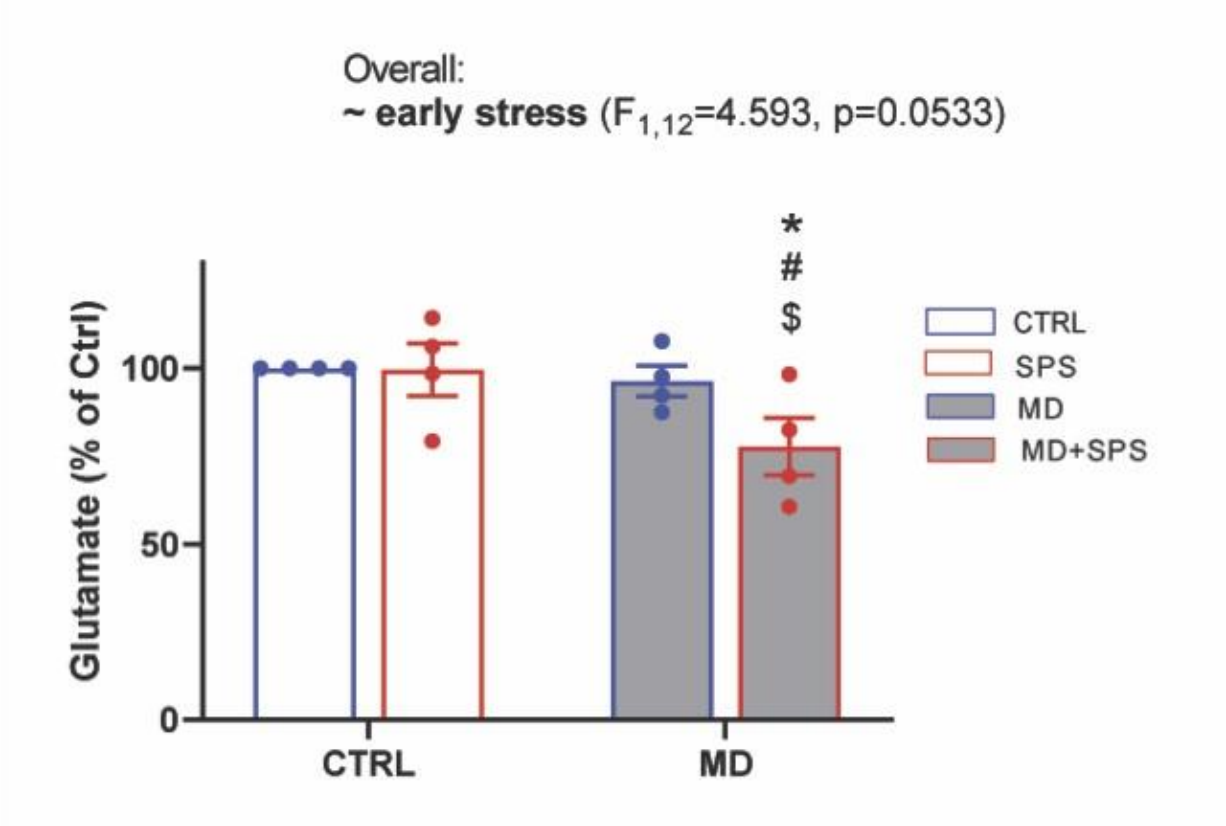
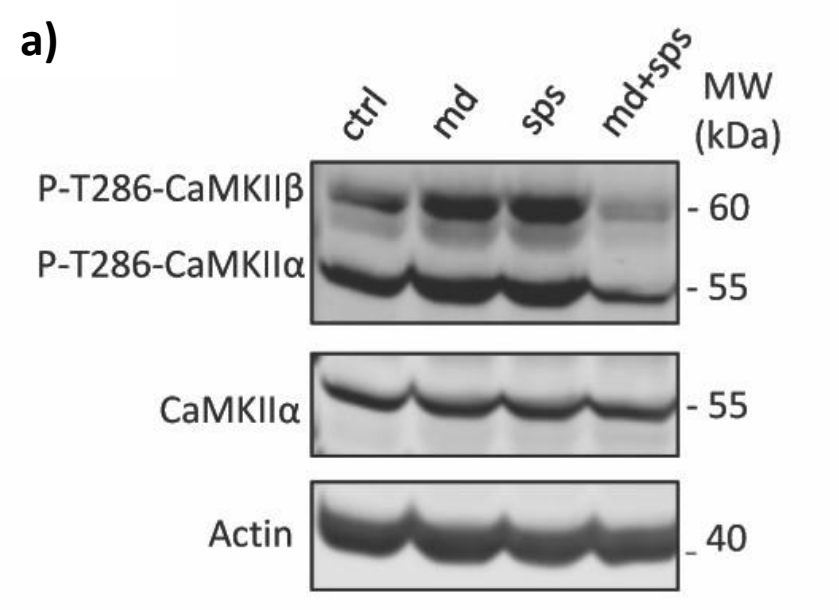


Figure 20. Glutamate concentration in hippocampal synaptosomes. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl; $^{\#}$ $0.01 < p < 0.05$ vs SPS; $^{\$}$ $0.01 < p < 0.05$ vs MD

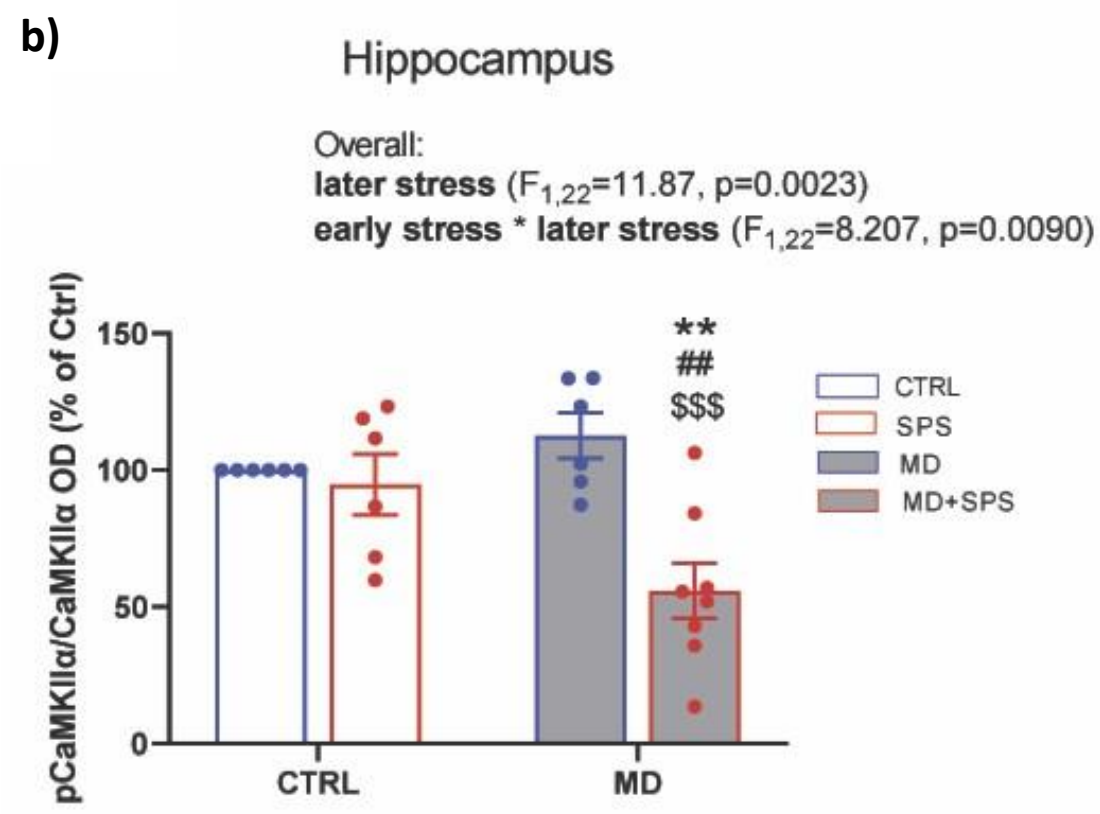
4.4 Ca2+/calmodulin (CaM)- dependent protein kinase II (CaMKII)

In the hippocampus there was a significant overall effect of late stress as well as an interaction effect of early stress and late stress. Post hoc analysis showed a significant reduction of p-T286 CaMKII in MD+SPS group compared to all other groups. Effects were similar for CaMKIIα and CaMKIIβ. In the prefrontal cortex there was a significant overall effect of late stress. MD+SPS and SPS group had a similar decrease of p-T286 CaMKII which was significantly lower compared to the control group, and both CaMKIIα and CaMKIIβ were similarly affected. In the amygdala only p-T286 CaMKIIα was significantly affected by later stress. Decrease was detected in both MD+SPS and SPS groups compared to controls, however the difference reached significance only in the SPS group.

a)


ctrl
md
sps
md+sps
MW (kDa)
P-T286-CaMKIIβ
P-T286-CaMKIIα
- 60
- 55
CaMKIIα
- 55
Actin
- 40

b)


Hippocampus
Overall:
later stress ($F_{1,22}$=11.87, p=0.0023)
early stress * later stress ($F_{1,22}$=8.207, p=0.0090)
pCaMKIIα/CaMKIIα OD (% of Ctrl)
150
100
50
0
**
##
$$$
CTRL
SPS
MD
MD+SPS
CTRL
MD

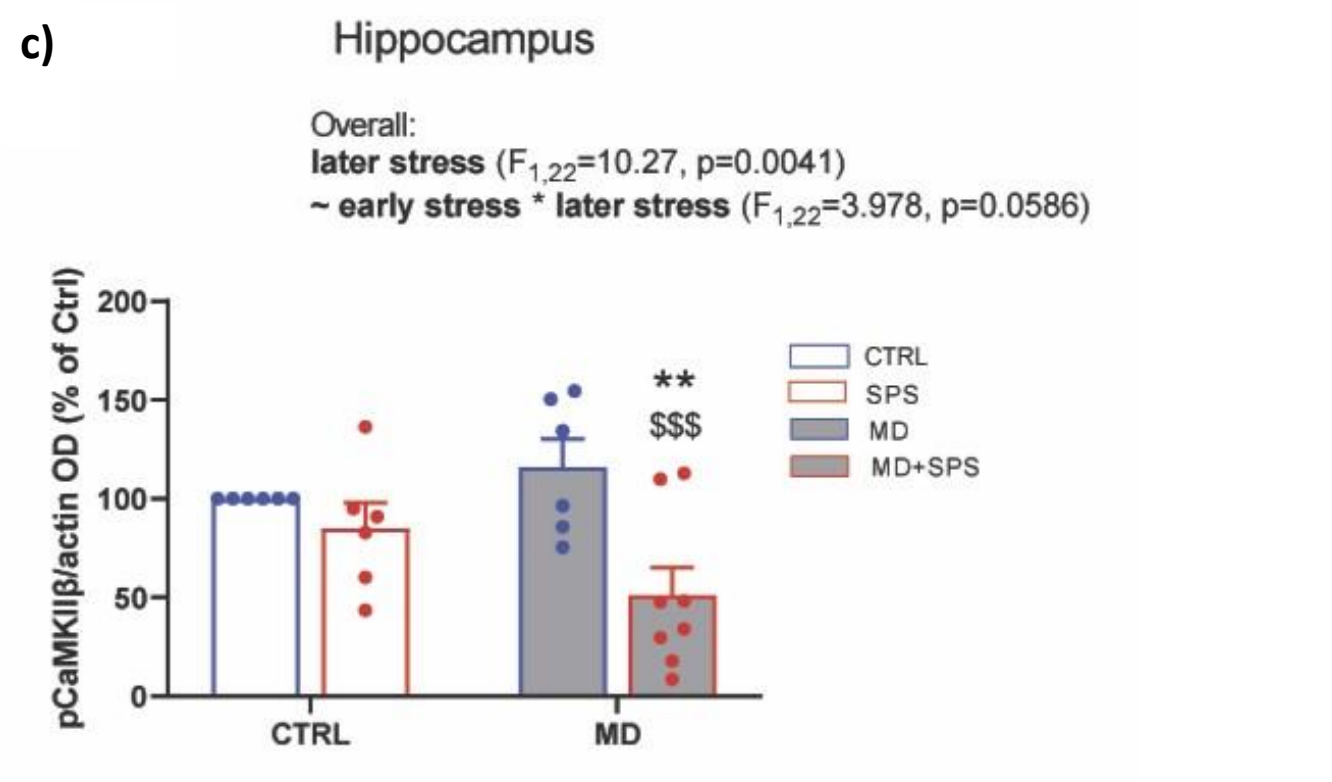
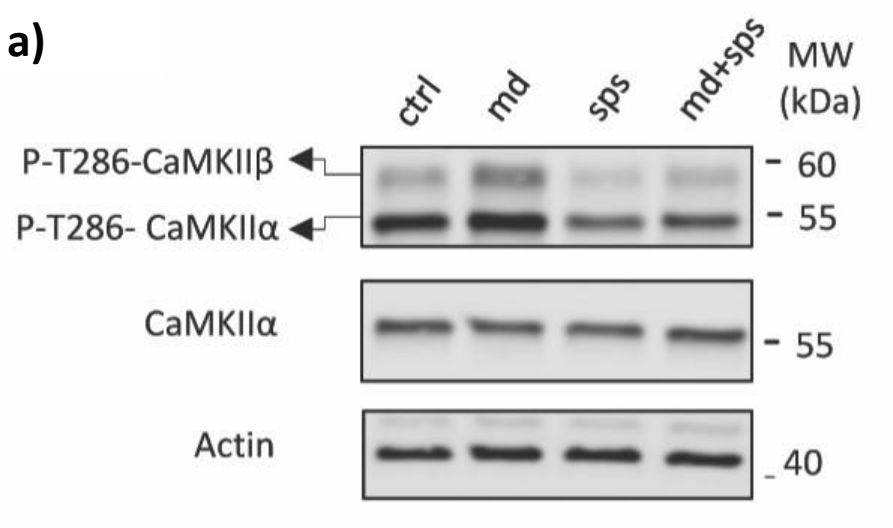
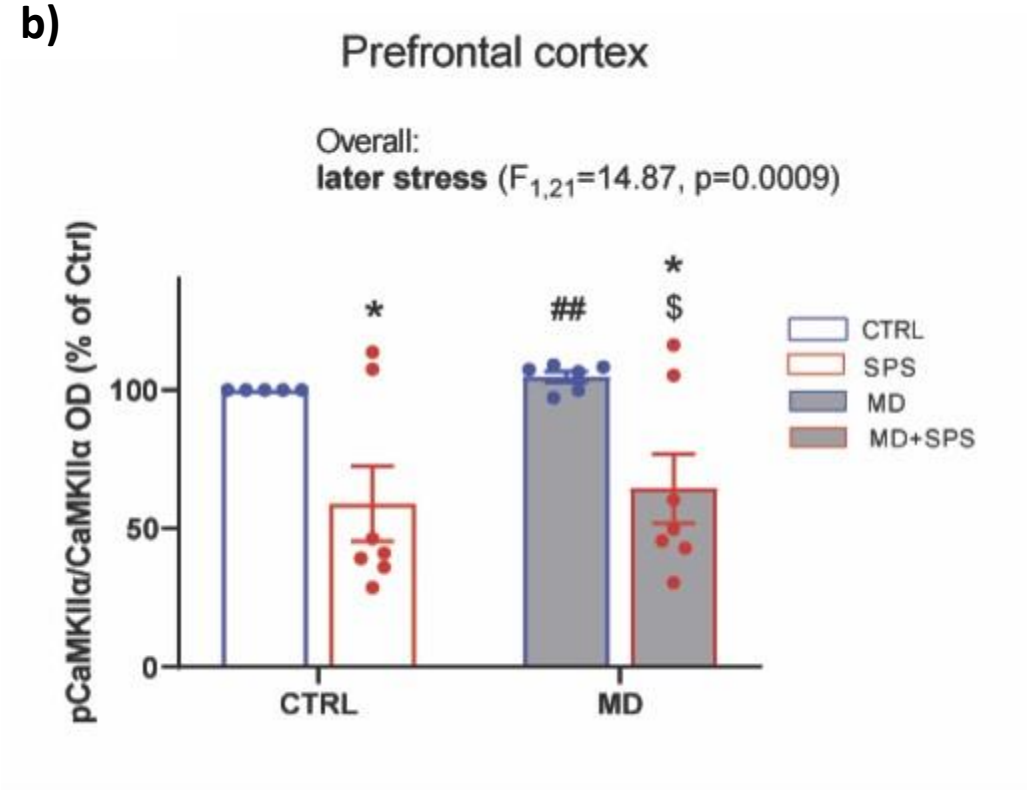


Figure 21. CaMKII-T286 phosphorylation in the hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). ** $0.001 < p < 0.01$ vs ctrl; $^{\$\$\$}$ p<0,001 vs MD. Representative blots (a) and quantitative analysis of p-T286 CaMKIIα (b) and p-T286 CaMKIIβ (c).

a)


ctrl
md
sps
md+sps
MW (kDa)
P-T286-CaMKIIβ
P-T286- CaMKIIα
CaMKIIα
Actin
60
55
55
40

b)


Prefrontal cortex
Overall:
later stress ($F_{1,21}$=14.87, p=0.0009)
pCaMKIIα/CaMKIIα OD (% of Ctrl)
100
50
0
CTRL
MD
*
##
*
$
CTRL
SPS
MD
MD+SPS

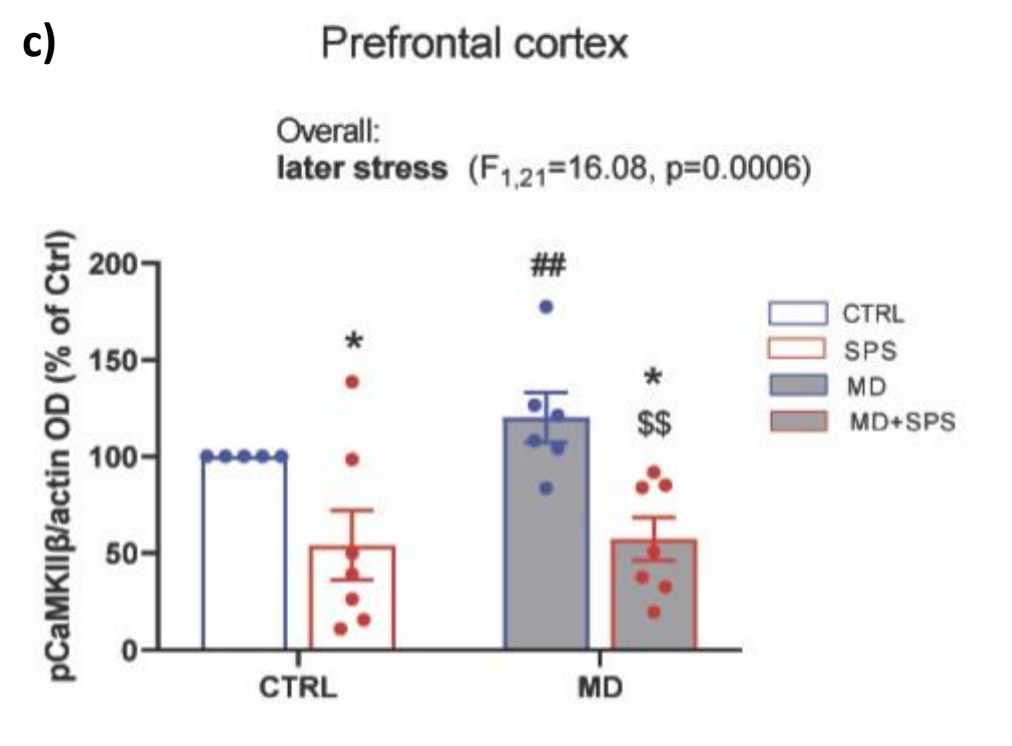
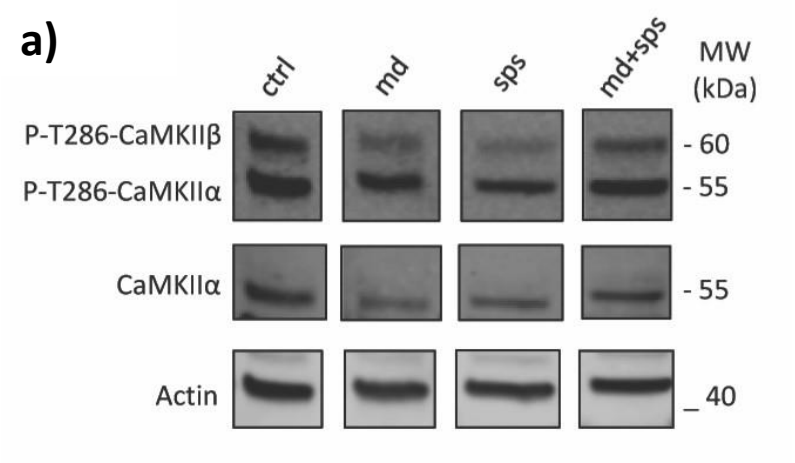
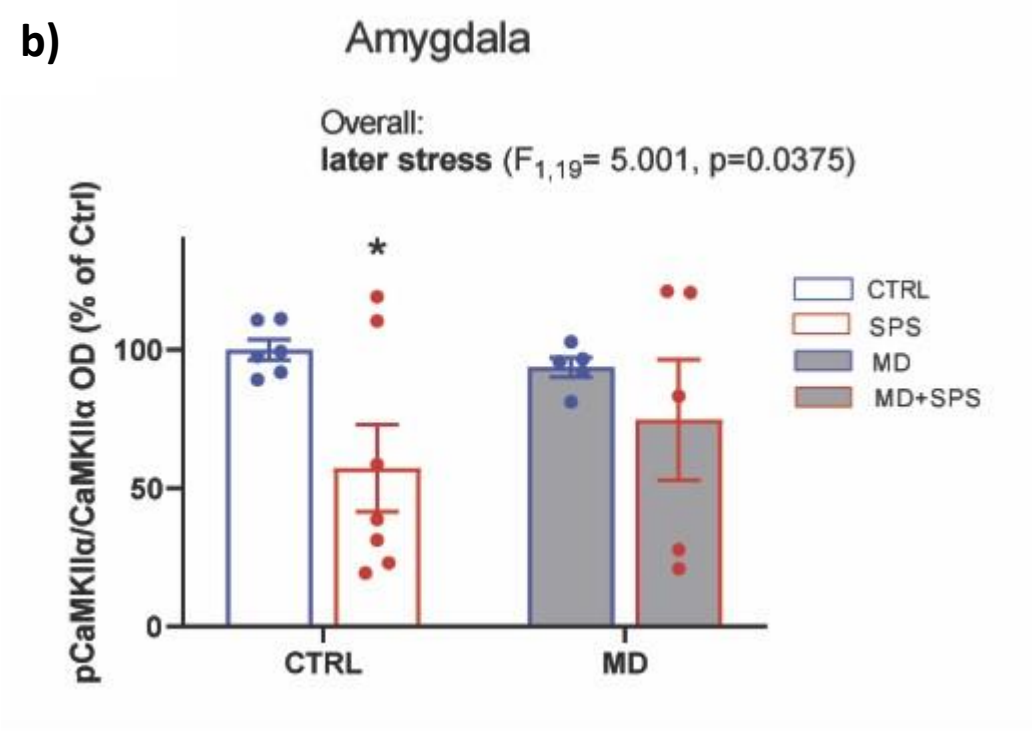


Figure 22. CaMKII-T286 phosphorylation in the prefrontal cortex. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl; $^{\#\#}$ $0.001 < p < 0.01$ vs SPS; $^{\$\$}$ $0.001 < p < 0.01$ vs MD. Representative blots (a) and quantitative analysis of p-T286 CaMKIIα (b) and p-T286 CaMKIIβ (c).

a)


ctrl
md
sps
md+sps
MW (kDa)
P-T286-CaMKIIβ
P-T286-CaMKIIα
- 60
- 55
CaMKIIα
- 55
Actin
_ 40

b)


Amygdala
Overall:
later stress ($F_{1,19}$= 5.001, p=0.0375)
pCaMKIIα/CaMKIIα OD (% of Ctrl)
100
50
0
*
CTRL
MD
CTRL
SPS
MD
MD+SPS

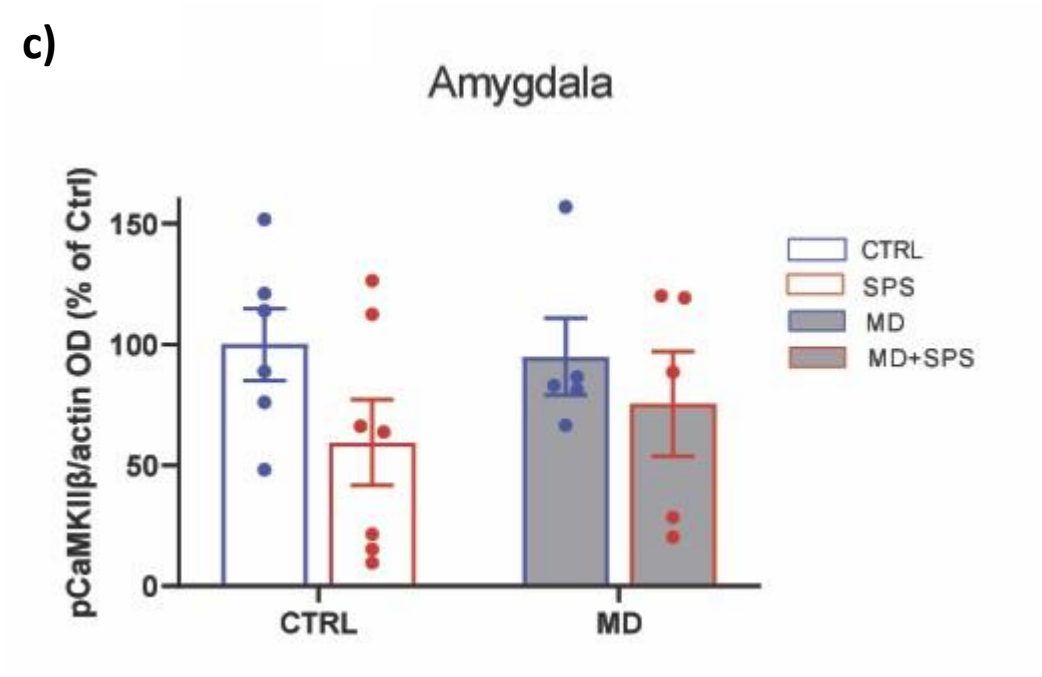


Figure 23. CaMKII-T286 phosphorylation in the amygdala. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl. Representative blots (a) and quantitative analysis of p-T286 CaMKIIα (b) and p-T286 CaMKIIβ (c).

4.5 Monoamines

Significant effect of late stress on the concentration of norepinephrine (NE) was detected in the prefrontal cortex. Post hoc analysis revealed statistically significant increase that parameter in the MD+SPS group compared to MD and CTRL group. Early and late stress had a significant overall effect on epinephrine (EPI) concentration in prefrontal cortex and amygdala respectively. There was a significant increase in the concentration of EPI in the MD+SPS group compared to controls. NA metabolite: 3-Methoxy-4-hydroxyphenylglycol (MHPG) was significantly affected by early stress in amygdala. Both serotonin (5-HT) and its metabolite (5-Hydroxyindoleacetic acid – 5-HIAA) were significantly affected in the prefrontal cortex by late stress, with post hoc analyses revealing a significant increase in the concentration of both chemicals in the MD+SPS group compared to controls. Dopamine and related chemicals (3,4-dihydroxyphenylalanine – DOPA; 3,4-Dihydroxyphenylacetic acid – DOPAC; 3-Methoxytyramine - 3-MT; homovanillic acid - HVA) were not significantly affected by the stress protocols.

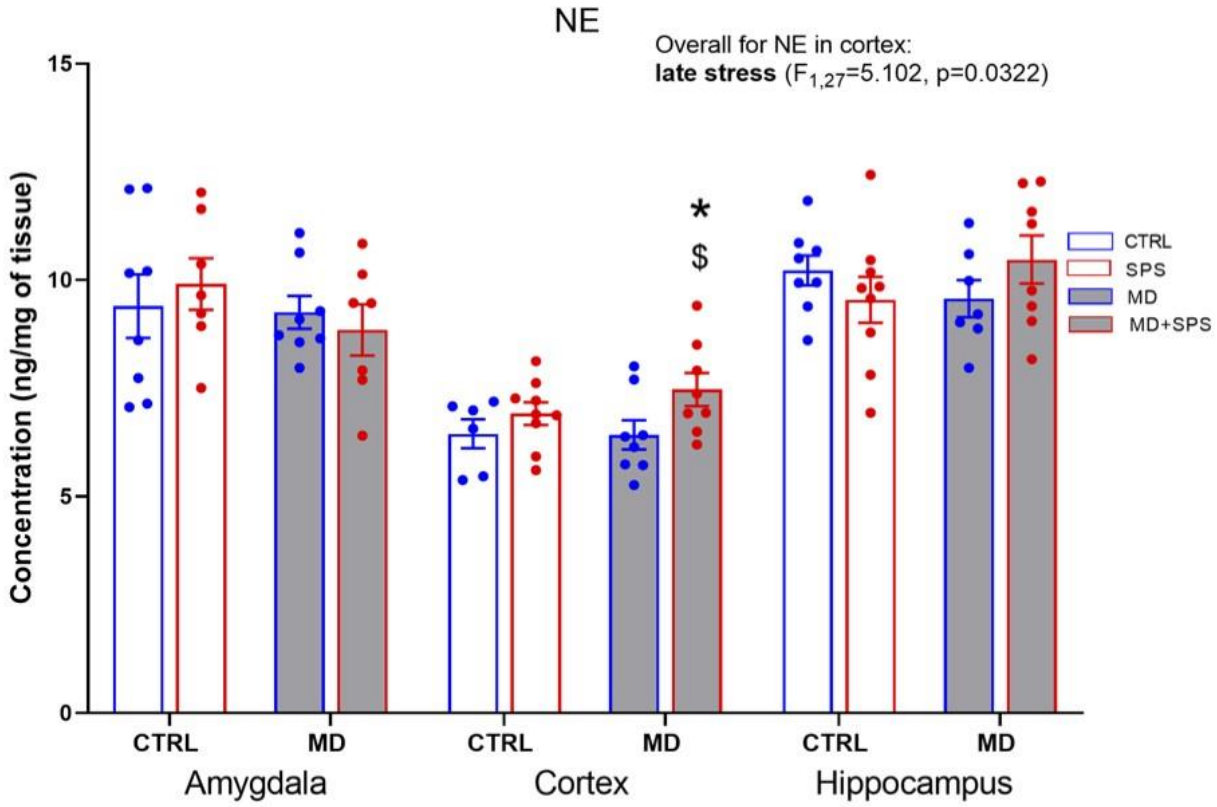


Figure 24. The effects of MD, SPS and MD + SPS on the concentration of NE in amygdala, prefrontal cortex and hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). * $0.01 < p < 0.05$ vs ctrl.

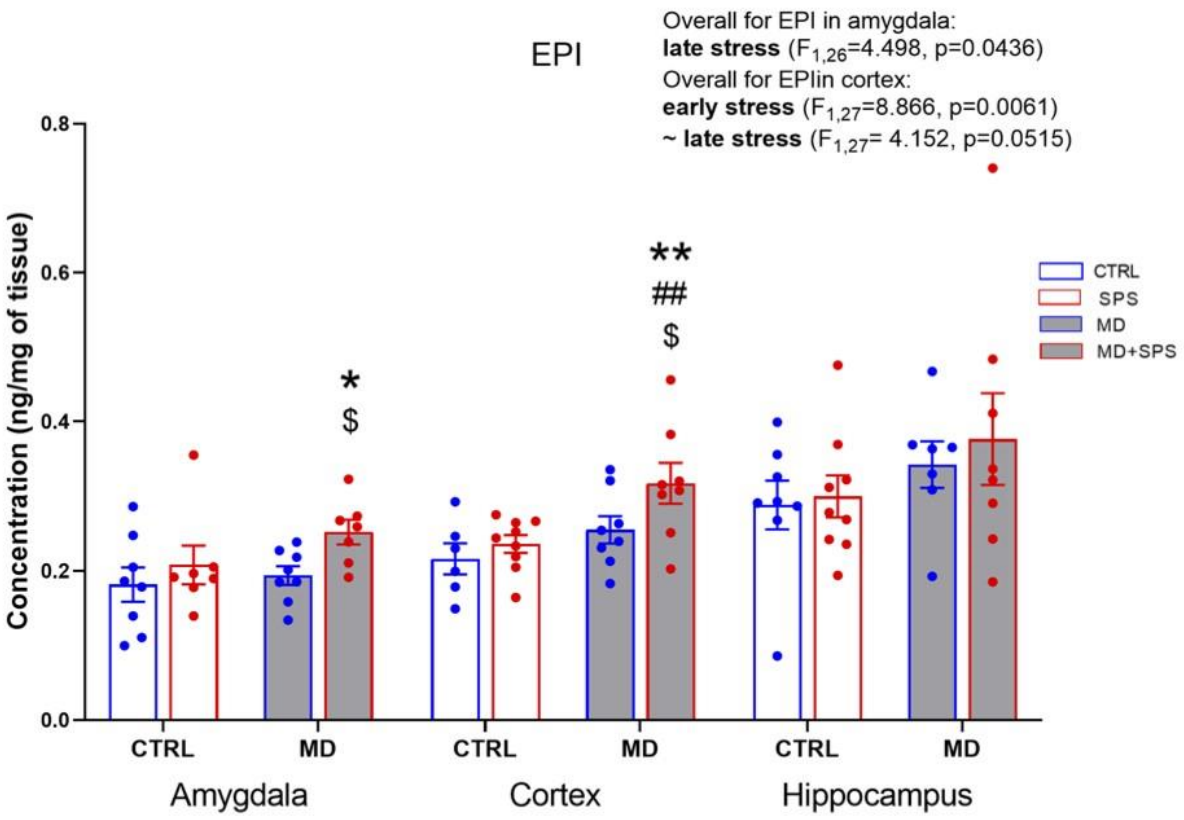


Figure 25. The effects of MD, SPS and MD + SPS on the concentration of EPI in amygdala, prefrontal cortex and hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl; ** $0.001 < p < 0.01$ vs ctrl; $^{\#\#}$ $0.001 < p < 0.01$ vs SPS; $^{\$}$ $0.01 < p < 0.05$ vs MD

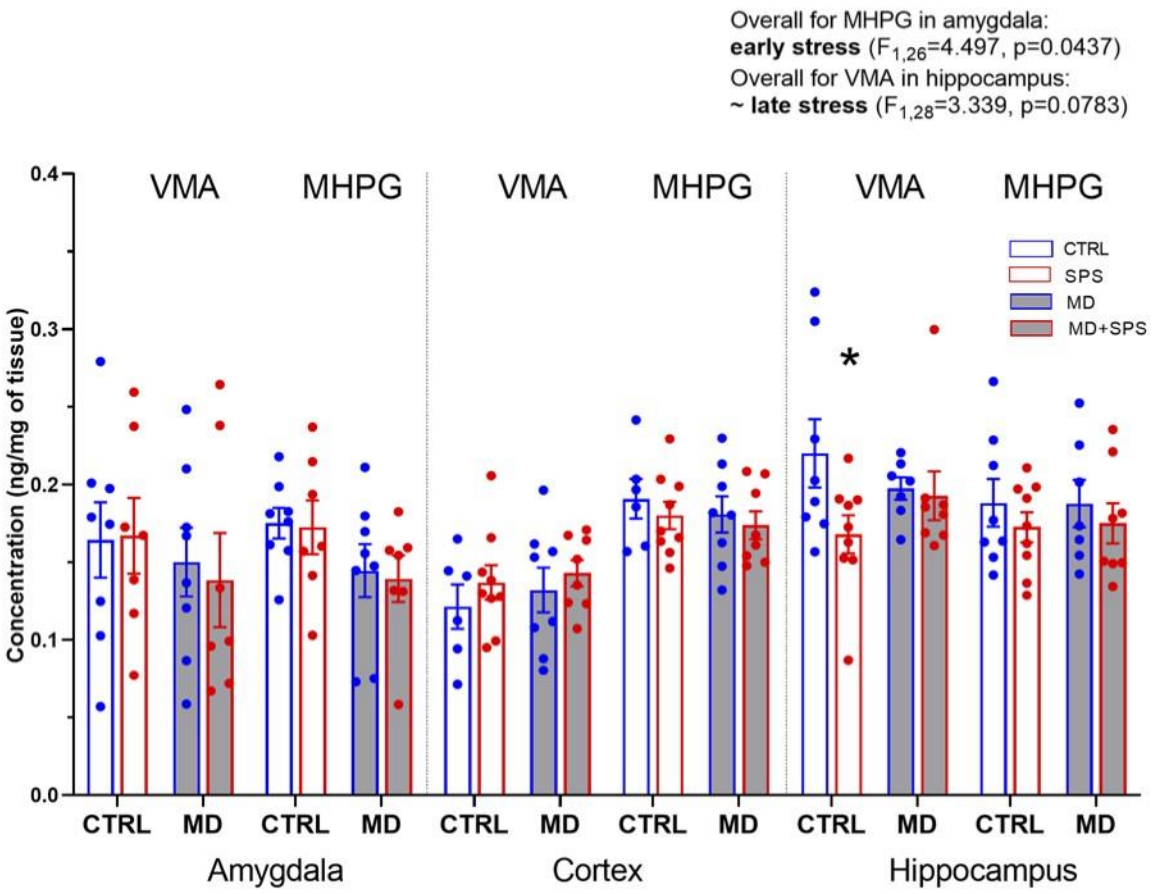


Figure 26. The effects of MD, SPS and MD + SPS on the concentration of VMA and MHPG in amygdala, prefrontal cortex and hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl.

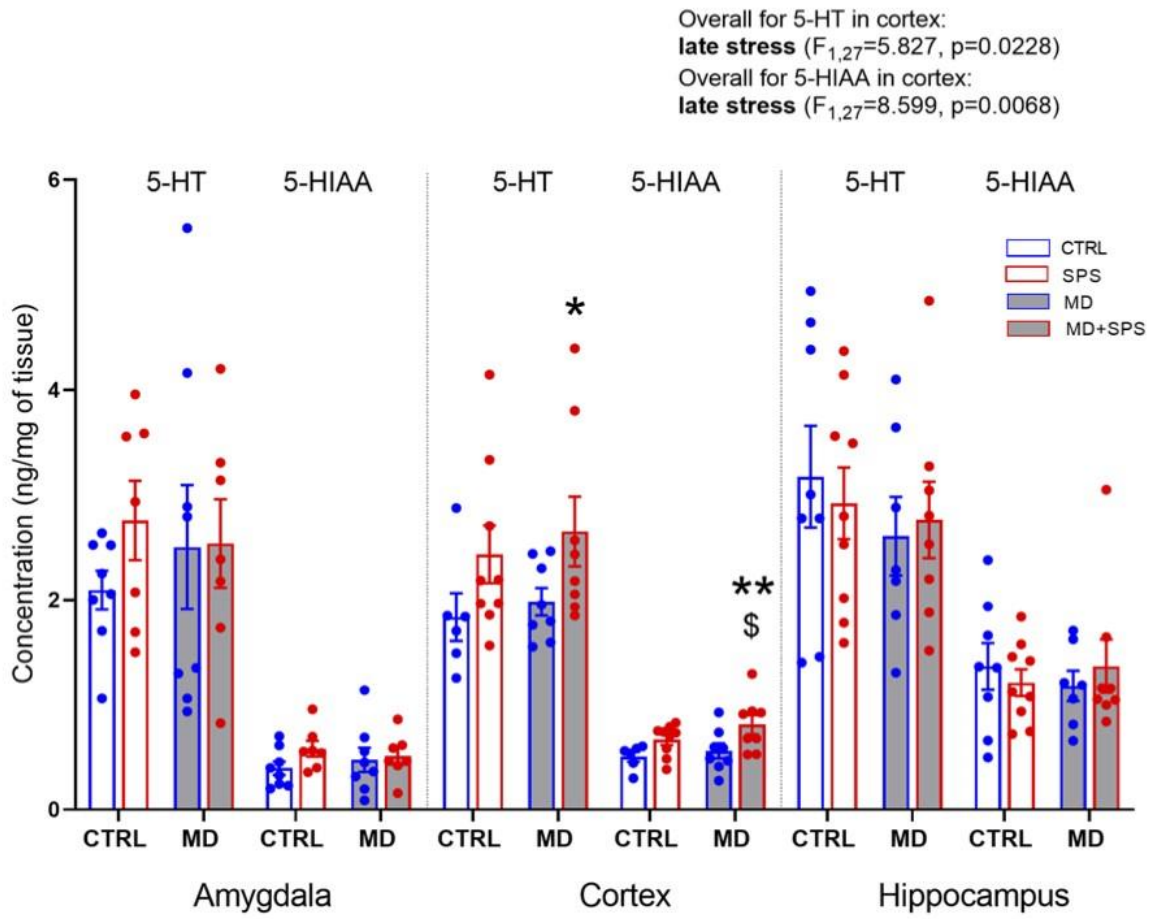
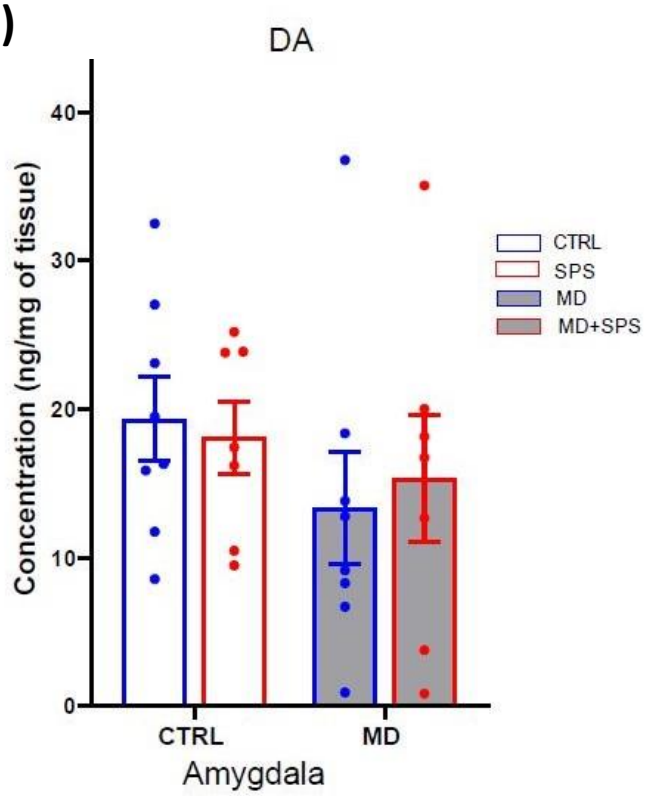


Figure 27. The effects of MD, SPS and MD + SPS on the concentration of 5-HT and 5-HIAA in amygdala, prefrontal cortex and hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). * $0.01 < p < 0.05$ vs ctrl; ** $0.001 < p < 0.01$ vs ctrl; $^{\$}$ $0.01 < p < 0.05$ vs MD.

a)


DA
Concentration (ng/mg of tissue)
40
30
20
10
0
CTRL
SPS
MD
MD+SPS
CTRL
MD
Amygdala

b)

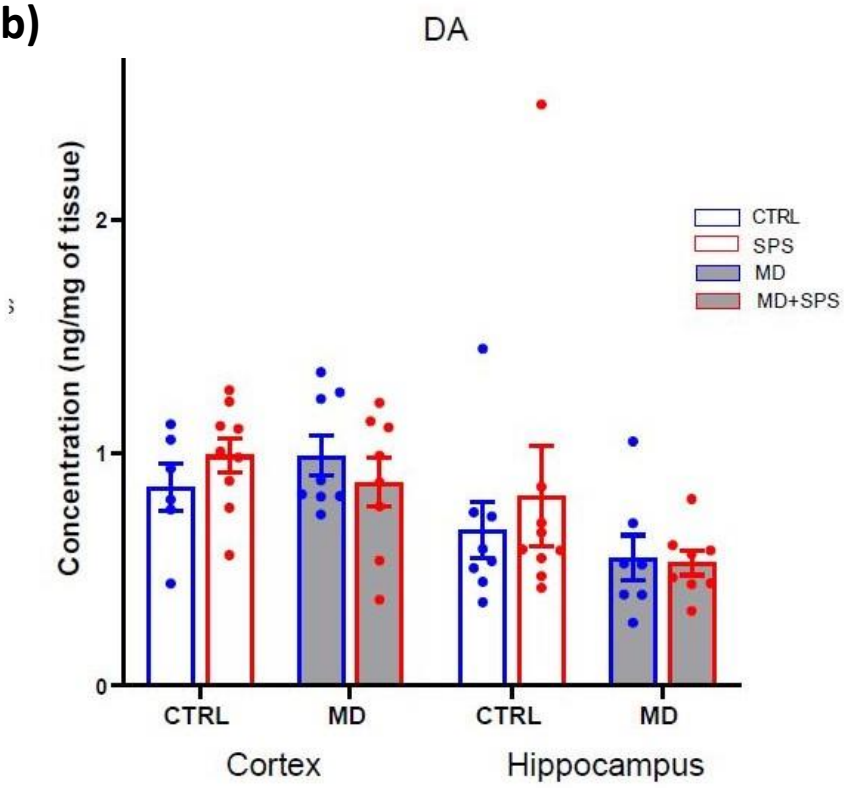

DA
Concentration (ng/mg of tissue)
2
1
0
CTRL
SPS
MD
MD+SPS
CTRL
MD
CTRL
MD
Cortex
Hippocampus

Figure 28. The effects of MD, SPS and MD + SPS on the concentration of DA in amygdala (a), prefrontal cortex and hippocampus (b). F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$).

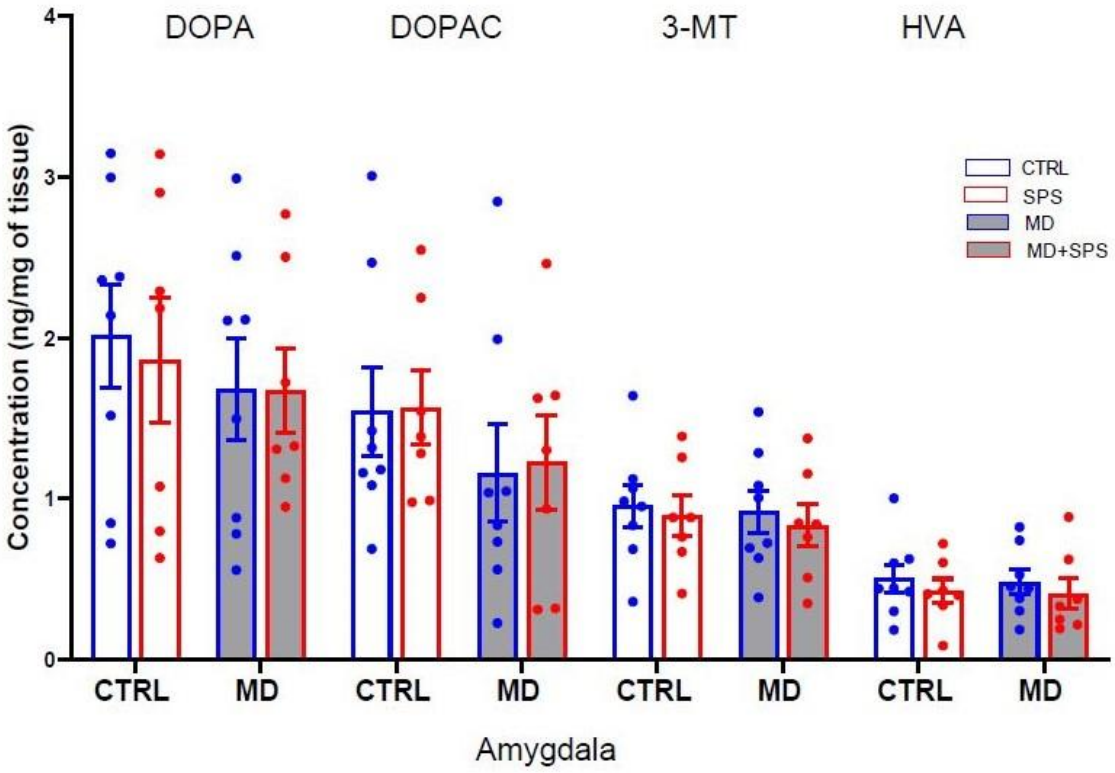


Figure 29. The effects of MD, SPS and MD + SPS on the concentration of DOPA, DOPAC, 3-MT and HVA in amygdala. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$).

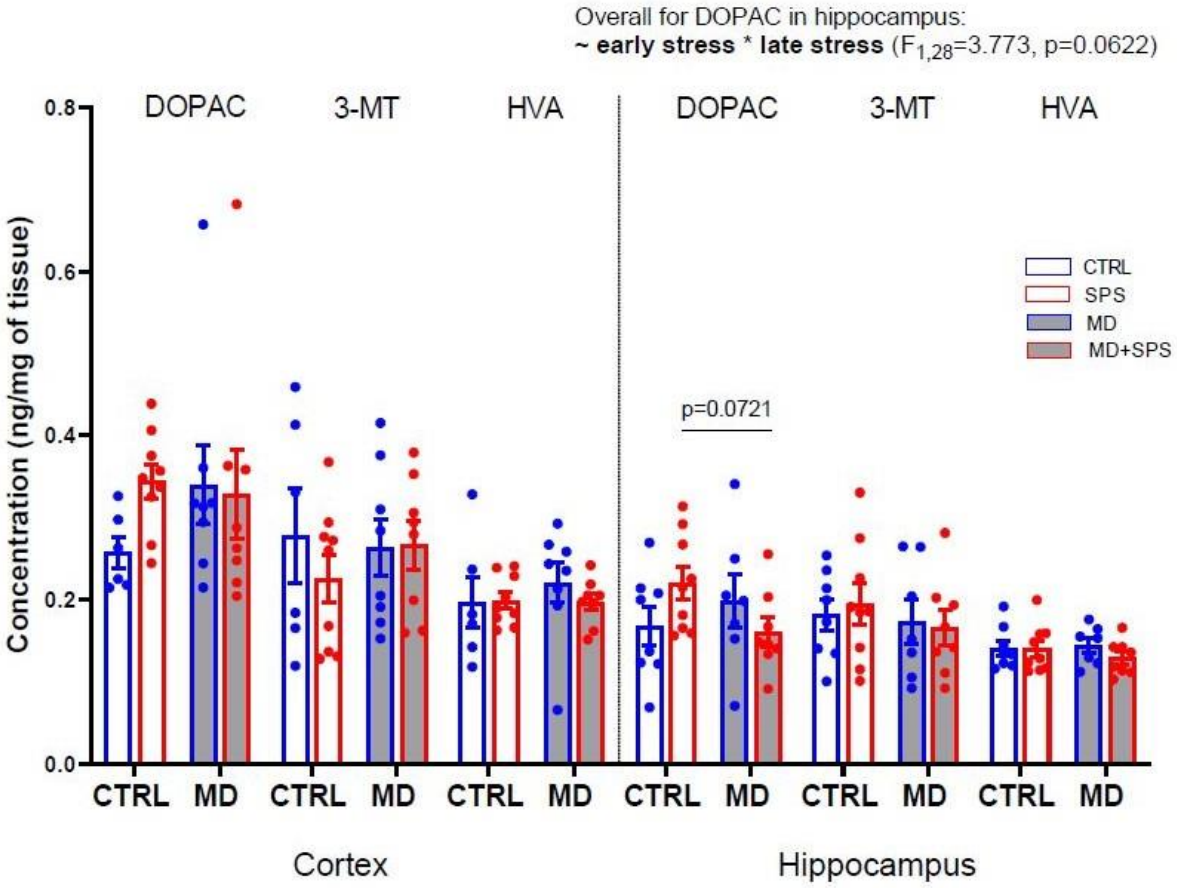


Figure 30. The effects of MD, SPS and MD + SPS on the concentration of DOPAC, 3-MT and HVA in prefrontal cortex and hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$).

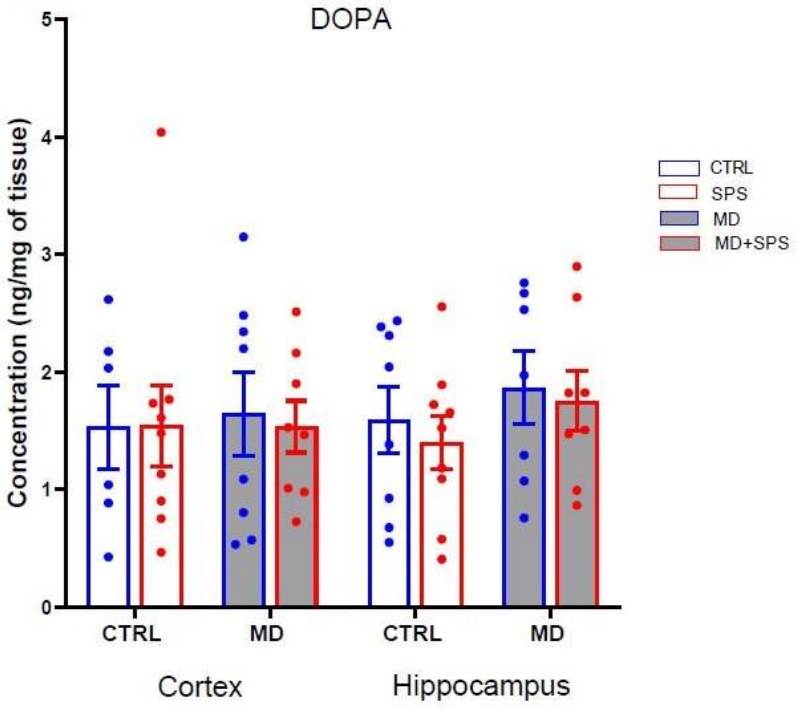


Figure 31. The effects of MD, SPS and MD + SPS on the concentration of DOPA in prefrontal cortex and hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$).

4.6 Brain-derived neurotrophic factor (BDNF)

Significant overall effect of early stress was observed in CA1 region of the dorsal hippocampus. Post hoc test revealed a statistically significant decrease in the MD+SPS and MD groups compared to CTRL and SPS groups. Late stress had a significant overall effect on increasing the BDNF mRNA in the dentate gyrus of the dorsal hippocampus which was apparent in the MD+SPS and SPS groups, however post hoc test revealed a significant difference only between MD+SPS and MD group and no significant difference between any of the experimental groups and controls. Concentration of BDNF mRNA in the ventral hippocampus was not affected by the stress paradigms. Late stress had a significant overall effect also in the medial prefrontal cortex, where similarly to the dCA1 there was a significant decrease in the MD+SPS and MD groups compared to SPS and CTRL groups.

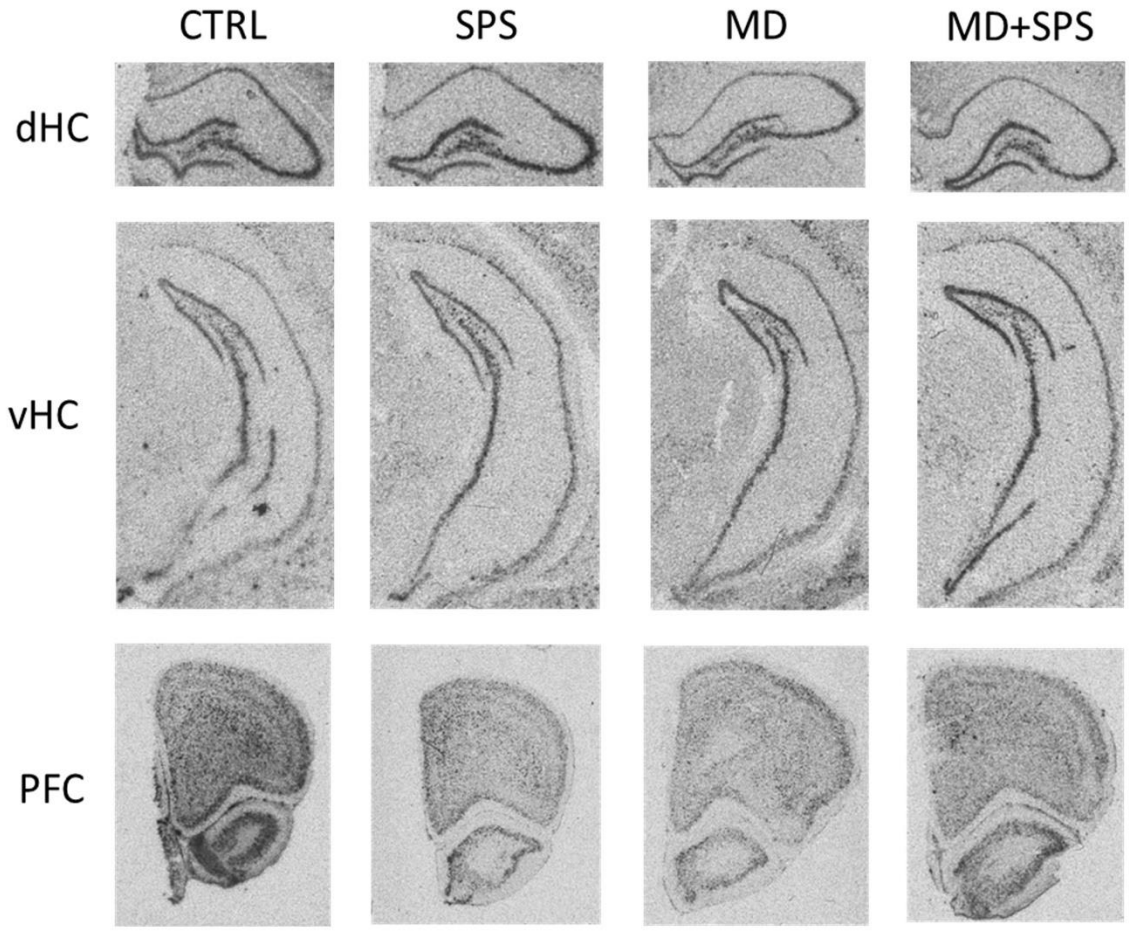


Figure 32. Autoradiograms representing BNDF mRNA in dorsal hippocampus (dHC), ventral hippocampus (vHC) and prefrontal cortex (PFC).

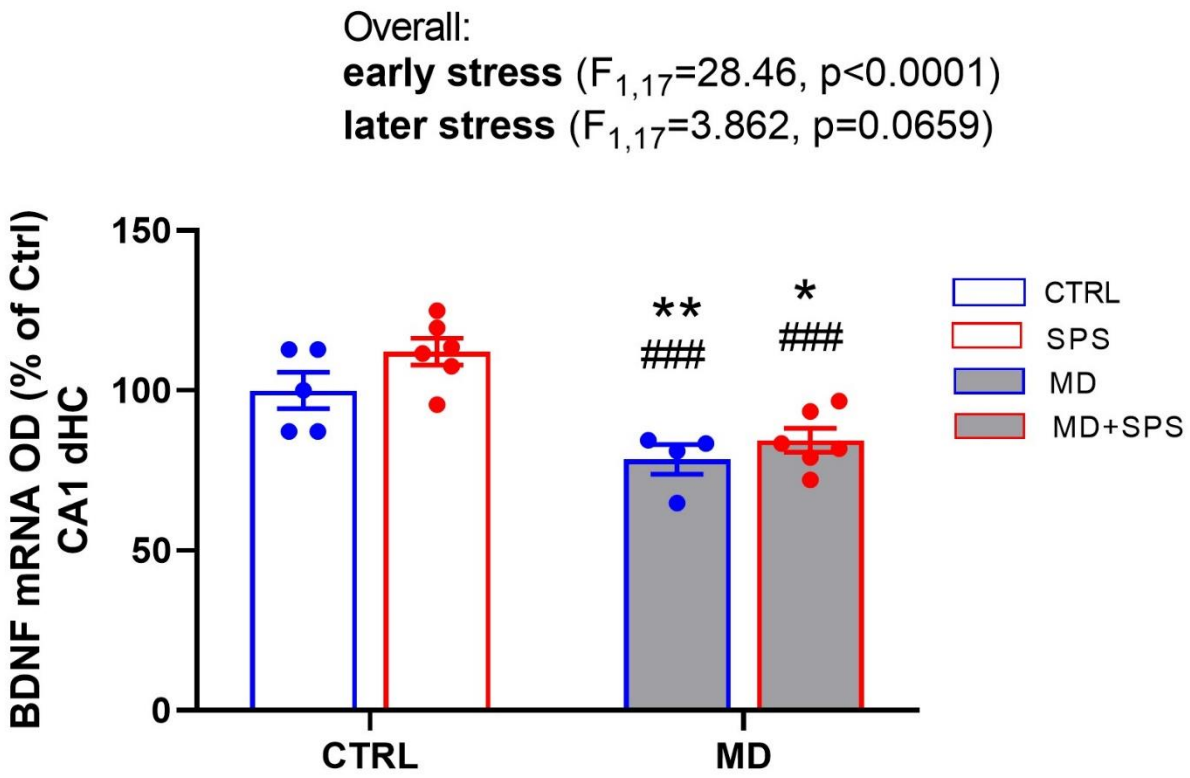


Figure 33. BDNF mRNA in the CA1 region of dorsal hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). * $0.01 < p < 0.05$ vs ctrl; ** $0.001 < p < 0.01$ vs ctrl; $^{\#\#\#}$ p<0,001 vs SPS

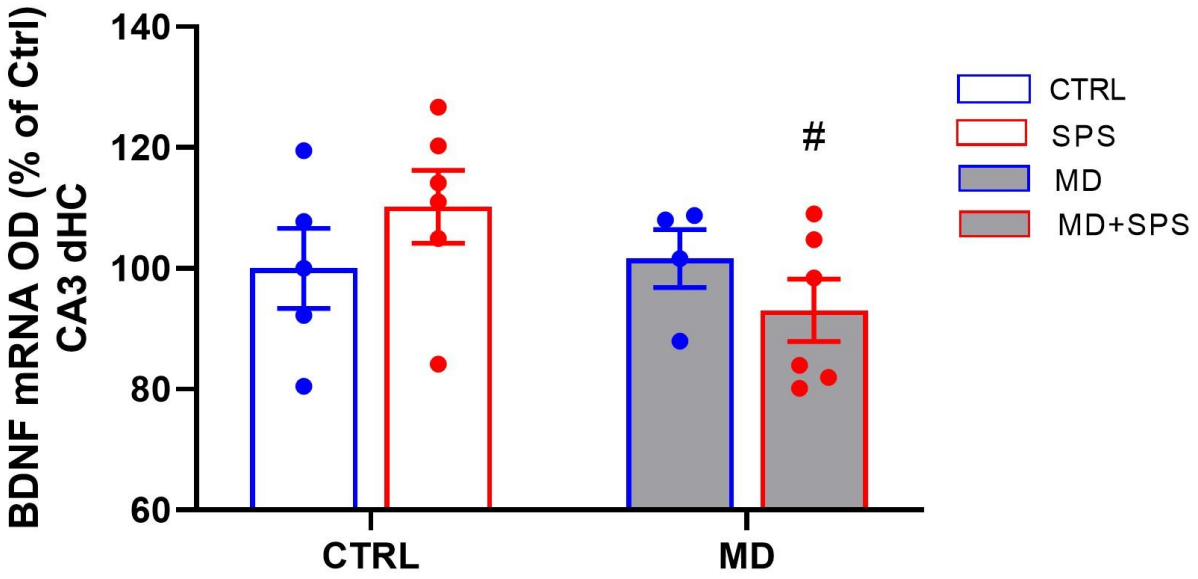


Figure 34. BDNF mRNA in the CA3 region of dorsal hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). $^{\#}$ $0.01 < p < 0.05$ vs SPS.

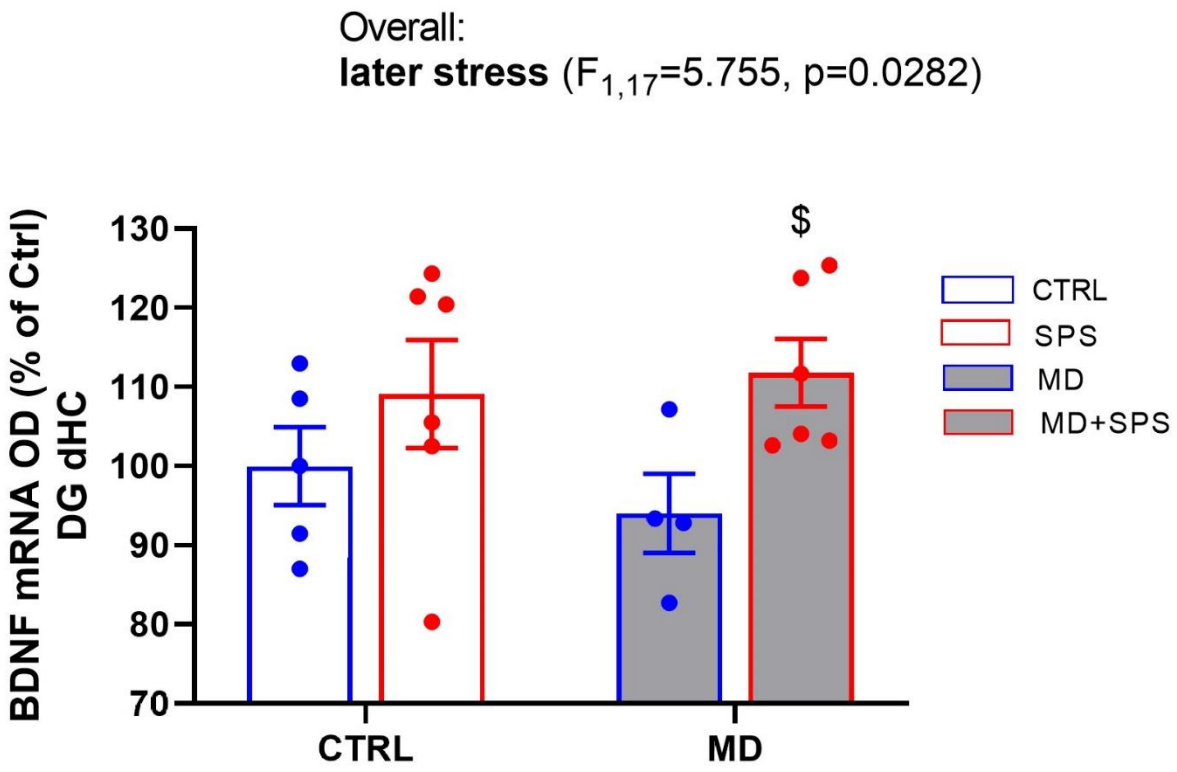


Figure 35. BDNF mRNA in the dentate gyrus (DG) of dorsal hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$). $^{\$}$ $0.01 < p < 0.05$ vs MD.

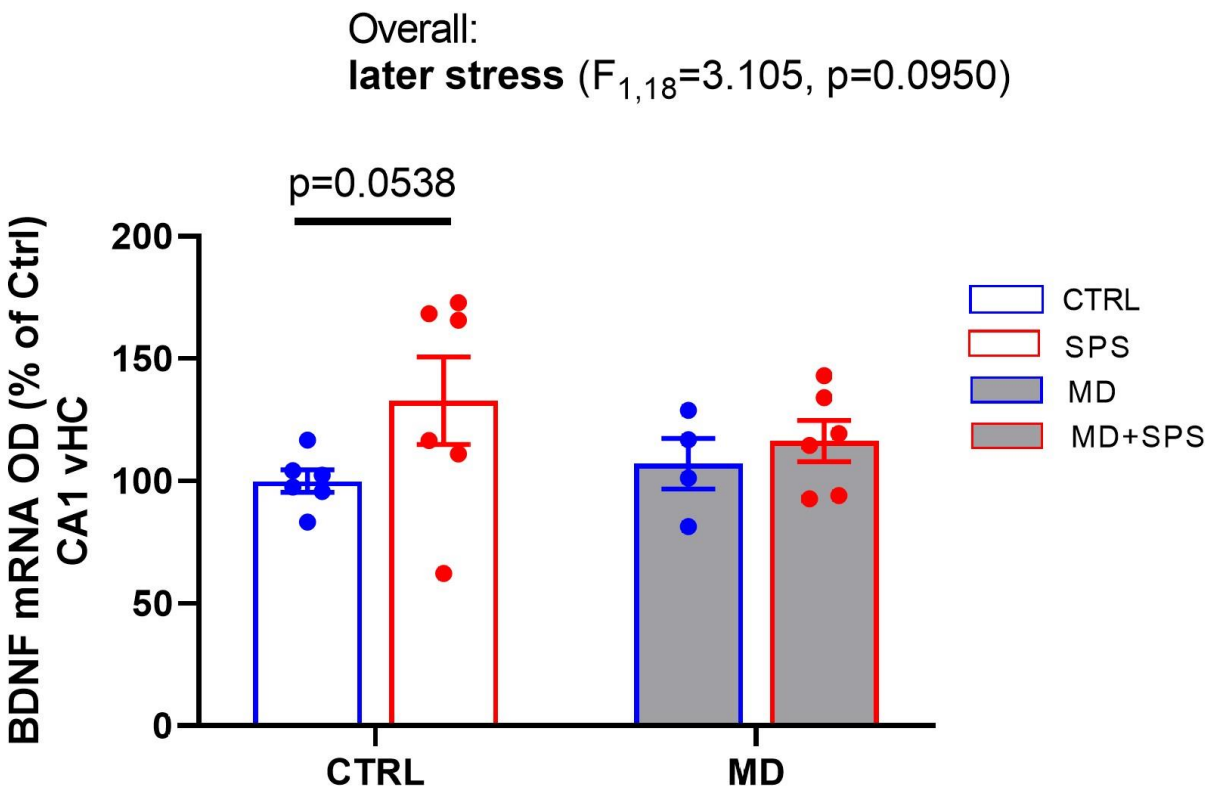


Figure 36. BDNF mRNA in the CA1 region of the ventral hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level (0.05 ≤ 0,1).

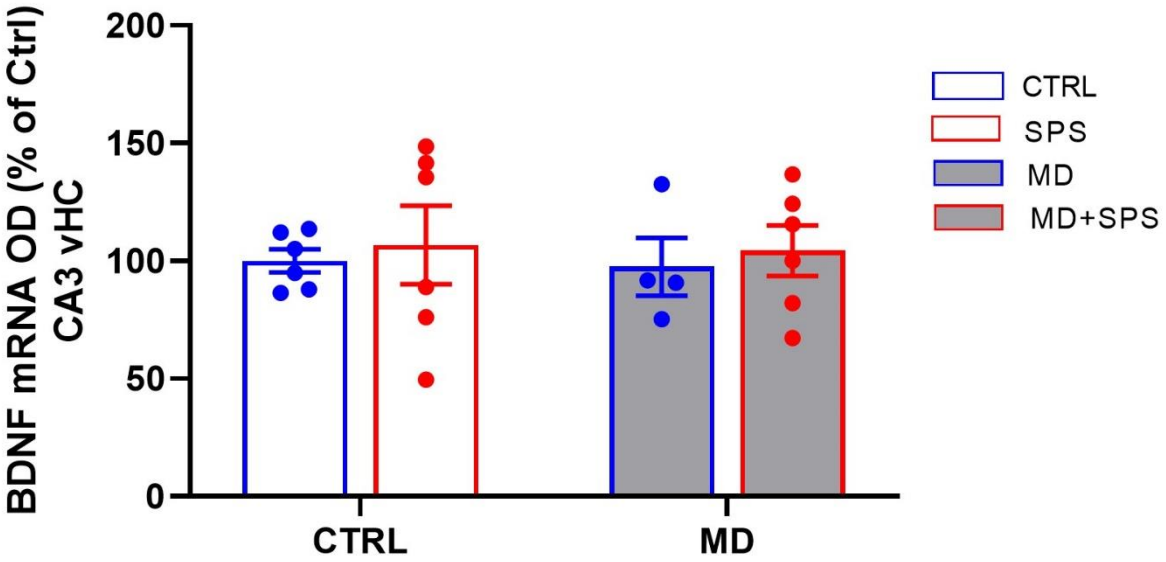


Figure 37. BDNF mRNA in the CA3 region of ventral hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$).

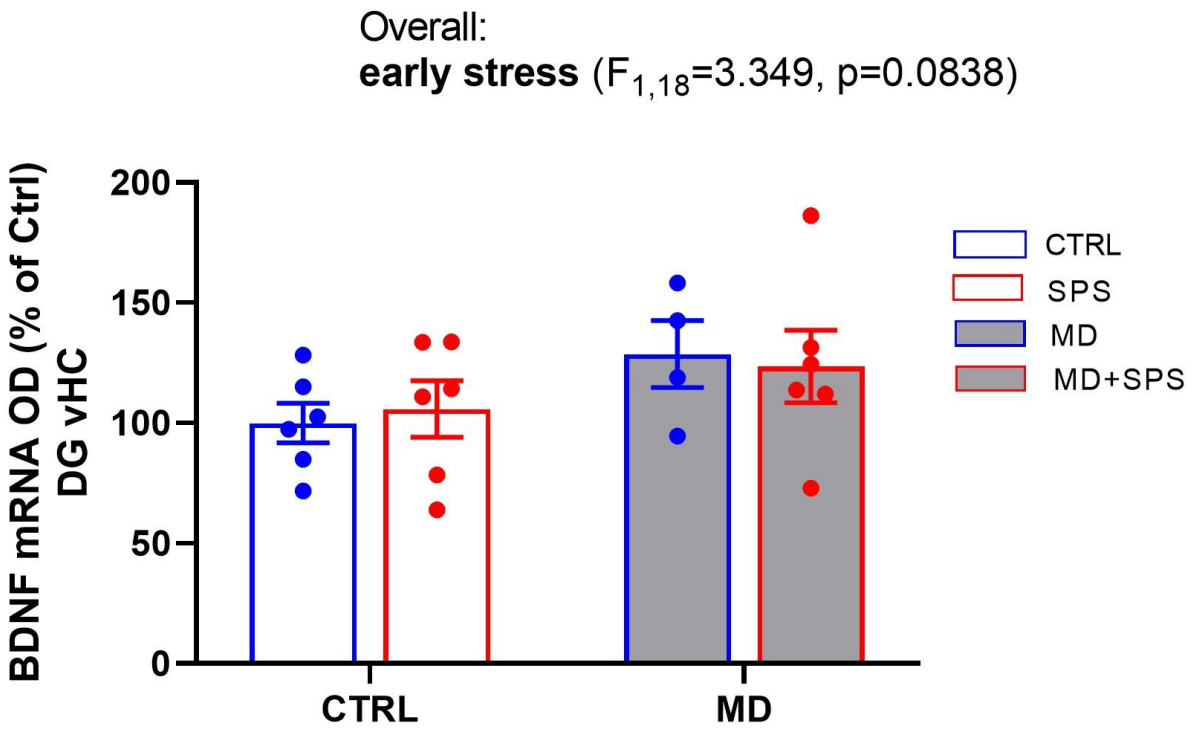


Figure 38. BDNF mRNA in the DG region of ventral hippocampus. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0,1$).

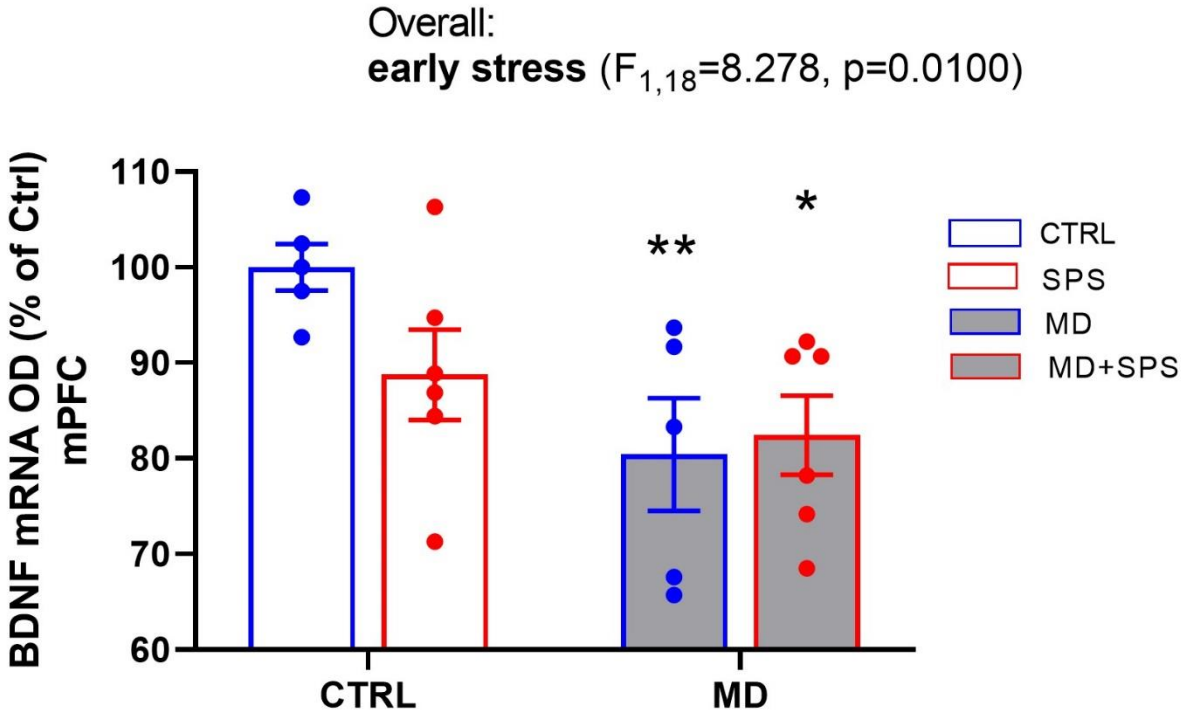


Figure 39. mRNA in the medial prefrontal cortex. F-values and overall effects are presented if they are above the significance threshold or almost significant. Dots represent data values. Error bars represent standard error of the mean. ~ designates p value at a trend level ($0.05 \leq 0{,}1$). * $0.01 < p < 0.05$ vs ctrl; ** $0.001 < p < 0.01$ vs ctrl.

5. Discussion

Animals exposed to two-hit stress displayed decreased spontaneous locomotor activity (SLA) compared to all other groups. Rodents have a natural tendency to explore a novel environment (Denenberg, 1969). Changes in exploratory activity in a novel environment are related to altered emotional or motivational state in animals (Katz et al., 1981).
Interestingly a recent study on susceptibility and resilience in an animal model of PTSD highlighted exploratory activity in the open field arena early after trauma as a robust predictive variable for the later expression of PTSD related behaviors (increased consolidation of trauma memory, impaired fear extinction and impaired social interaction), thus highlighting its usefulness in screening for identifying PTSD susceptible rats. Importantly they analysed pretrauma locomotor activity in a novel environment and determined that it was not a significant predictor of PTSD susceptibility. The same study concluded that time spent in the centre of the open field arena as well as social interaction test early after trauma were also not predictive for PTSD susceptibility (Colucci et al., 2020). A lack of motivation to explore a novel environment could be a reflection of stress induced apathy (Cathomas et al., 2015). Five subdomains of apathy have been defined in humans which could be modelled in animals. These correspond to reduced self-care, social interaction, exploration (interest in novel experiences), recreation and interest in work/education (Cathomas et al., 2015).

The finding of reduced reaction to novelty in MD+SPS group could be related to emotional deficits seen in PTSD patients (Litz et al., 2000). Symptoms from the DSM-IV avoidance/numbing cluster can be attributed to motivational deficits (Simmen-Jenevska et al., 2012). It is hypothesized that emotional symptoms of PTSD and motivational deficits originate from the same source, which is a dysfunctional reward processing system. This dysfunction can be attributed to external reward seeking, where people with PTSD are tuned to respond to intense stimulation, whereas normal activities are not motivating or pleasurable to them (Seidemann et al., 2021)
It is thought that this is the cause of self-destructive or reckless behaviour, which according to the DSM-5 is a diagnostic criterion for PTSD (Seidemann et al., 2021).
Physical exercise has been recognized as a stress coping mechanism in humans (https://www.cdc.gov/mentalhealth/cope-with-stress/index.html). Reduced engagement in physical activity has been observed in PTSD patients (Rosenbaum, et al., 2016; Vancampfort, et al., 2016; de Assis et al., 2008), and it has been linked to anhedonia (Leventhal et al., 2012), however people with PTSD have a tendency to avoid any new experiences, because environmental cues (people, places, conversations, activities, objects and situations) can trigger intrusive symptoms, and result in re-experiencing of the trauma (Johannessen and Berntsen, 2010)
There are studies which indicate that physical activity is negatively associated with PTSD symptom severity (Rosenbaum, et al., 2016).
Regarding anxiety related behavior in the elevated plus maze (EPM), animals subjected to two-hit stress had a very similar readout as MD animals. Both groups displayed increased activity in the open arms of the EPM. A common interpretation of this behavior is either decreased anxiety or behavioral disinhibition (Burke et al., 2013; Llorente-Berzal et al., 2011). Interpretation of the EPM results is hindered by the SLA data. Nevertheless, behavioral disinhibition is an important characteristic of PTSD, and represents the foundation of self-destructive or reckless behaviour (Sadeh et al., 2018; Sadeh et al 2015). Increased open arm exploration in the EPM attributed to loss of behavioral inhibition was observed in animal models of global cerebral ischemia and traumatic brain injury (TBI) (Morin et al., 2021; Leconte et al., 2020; Lu et al., 2016; Washington et al 2012). TBI is a known risk factor for PTSD (Bryant, 2011). EPM has been used to assess risk-taking behavior in laboratory animals (Cortese et al., 2010; Löfgren et al., 2009; Mikics et al., 2005; Griebel et al., 1997). Utilizing this methodology, risk-taking behavior was linked to region specific glutamate alterations (Cortese et al., 2010). In this study sleep deprivation, which is a very common comorbidity of PTSD, was used to model risk-taking behavior in rats. Still in order to better parse out these changes future experiments should more

carefully investigate behavioral disinhibition/impulsivity is this model, by utilizing a less locomotion dependent test than EPM.
Deficits in behavioral inhibition have been identified both as a pretrauma vulnerability for the development of PTSD, and as a contributing factor to PTSD chronicity, indicating its role in both aetiology and progression of PTSD (Lusk et al., 2017).
Interestingly it has been suggested by Krystal et al (2017) that a large group of symptoms which includes apathy and behavioral disinhibition might stem from a common pathophysiological process, and that this might be a throughline between PTSD and diseases that impair functional connectivity of the brain, like cerebral ischemia and TBI. Furthermore, self-destructive and reckless behavior has been linked to retraumatization induced by constantly putting oneself in dangerous situations, which could explain its role in progression and chronicity of PTSD (Lusk et al., 2017).
MD+SPS and SPS groups displayed social avoidance in the social interaction test. Parameters of social interaction were particularly decreased with regards to social play. Social avoidance is a diagnostic criterion of PTSD (American Psychiatric Association, 2013). Social play is a highly pleasurable and rewarding activity for rodents (Trezza et al., 2011; Vanderschuren, 2010).

Social interaction has a hedonic and a motivational component (Trezza et al., 2011), and the interpretation of diminished engagement in social interaction is comparable with the interpretation of SLA data in a sense that it could reflect a decrement in both emotional and motivational wellbeing of the animal.

Both parameters are indicative of loss of resilience to stress (Krishnan et al, 2007). Like physical activity social interaction after trauma is recognized as stress coping mechanisms in humans (https://www.cdc.gov/mentalhealth/cope-with-stress/index.html).

Lastly, we determined sucrose preference, as an indicator of consummatory anhedonia. A reduction of preference for a sweetened solution, was not apparent in any of the experimental groups. Research suggests that a small percentage of rats in a PTSD model develop decreased sucrose preference, which is interpreted as comorbid depression (Ritov et al., 2016), a common finding in PTSD patients (Conner et al., 2014).

Apathy and anhedonia represent disorders of reward functioning (Husain and Roiser, 2018). Reward related behavior can be broken down into three components: reward anticipation (motivational component), reward consumption (hedonic component) and reward-based learning (defined as a natural bias toward reward generating behavior) (Berridge and Kringelbach, 2008).
It has been suggested that anhedonia in trauma exposed individuals stems from deficits in reward anticipation, rather than reward consumption, further highlighting the significance of the motivational component of behavior (Eskelund et al., 2018).
Although, in the same study it was determined that emotional numbing, which is a PTSD symptom, was linked to consummatory anhedonia (Eskelund et al., 2018). Importantly it has been determined that reduced reward functioning in PTSD patients is more clearly present in response to social rather than non-social stimuli (Nawijn et al., 2015).
In fact it has been proposed that social and non-social anhedonia are linked to different affective, behavioral and biological phenotypes (Nawijn et al., 2015). Anhedonia was causatively linked to substance use disorder in PTSD patients, which could represent a link between the reward system disfunction and thrill-seeking behavior in PTSD (Fani et al., 2020).

Decreased neurotransmitter release was apparent in the hippocampus of rats from both MD+SPS and SPS group. This reflects decreased glutamate release, since 80-90% of synapses in the hippocampus are glutamatergic (Shinohara and Hirase, 2009).

This was coupled with a finding of reduced glutamate concentration in hippocampal synaptosomes from MD+SPS animals. Importantly these findings were observed seven days after SPS administration, thus they represent a postponed reaction, probably reflecting dysfunctional adaptation to stress. Together they reflect a profound dysregulation of the glutamatergic system in the hippocampus induced by stress in these animals.

The finding of decreased glutamate release in the hippocampus can be explained by the inverted U-shaped dose response relationship between glutamate release and stress intensity (Popoli et al., 2011). It is well established that acute stress of low or moderate intensity produces enhanced glutamate release, whereas chronic or intense stress causes decreased glutamate release. This has been related to loss of resilience to stress (Popoli et al., 2011).

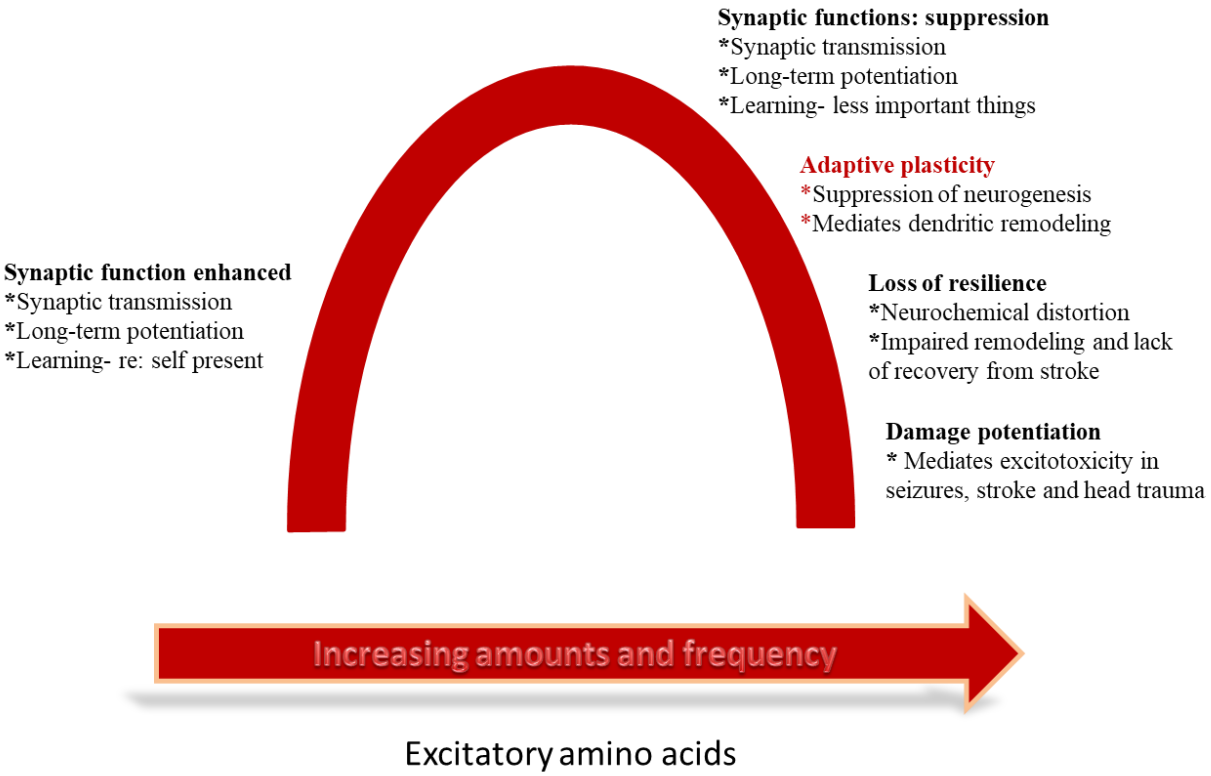


Figure 40. Inverted U-shape dose response curve. Adapted from McEwen et al (2016)

A 2022 study investigated the molecular changes in hippocampal synaptosomes caused by SPS (Guan et al., 2022). It showed that it induced an increase in the expression of VAMP, STX1A, Munc18-1, but caused a decrease in the expression of synaptotagmin-1, which suggests increased SNARE complex formation but decreased ability of vesicle fusion. This could explain our finding of decreased glutamate release caused by SPS.

Impairment in the presynaptic glutamatergic regulatory mechanisms in the hippocampus have been implicated in stress vulnerability (Nasca et al., 2015). mGlu2 is an inhibitory presynaptic glutamate receptor, which has an important role in regulating glutamate homeostasis by inhibiting glutamate release and preventing overactivation of the glutamatergic system. Genetic deletion of these receptors increases susceptibility to chronic unpredictable stress. Glucocorticoids decrease the expression of this receptor via epigenetic modification of mGlu2 promoter, an effect exerted through binding to high affinity mineralocorticoid receptors. Also, it has been shown that a natural compound acetyl-L-carnitine

which has rapid antidepressant properties acts through the epigenetic upregulation of mGlu2 receptors (Nasca et al., 2013).

The second regulatory mechanism that prevents excessive glutamatergic activation involves presynaptic CB1 receptors on glutamatergic neurons. The neuroprotective role of endocannabinoids is well established, and it is based on their regulatory function on glutamate release (Shohami et al., 2011). A 2014 study (Chiarlone et al., 2014) investigated which group of CB1 receptors is responsible for these effects in the striatum, by using knockout mice deficient for CB1 receptors on either GABAergic (CB1RGABA deficient) or glutamatergic neurons (CB1RGlu deficient), concluded that only the CB1RGlu deficient mice had a greater sensitivity to excitotoxicity induced by NMDA receptor agonist – quinolinic acid, whereas CB1RGABA or wild-type mice were protected from this effect. Additionally, it is well known that glucocorticoids recruit endocannabinoids, which are released from the postsynaptic neuron, and act on presynaptic CB1 receptors to reduce glutamate release (Balsevich et al., 2017).

Decreased excitatory glutamatergic function in cortex and hippocampus, resulting in a deficit of top-down control of the reward circuitry and amygdala, is considered a key mechanism in the pathogenesis of emotion and mood dysregulation (Price et al., 2010).

Interestingly short multimodal stress, compared to single stressors, caused more profound behavioral symptoms, region specific dendritic atrophy and altered functional connectivity patterns between regions, where it reduced functional connectivity of the hippocampus with septum and thalamus, but strengthened its connectivity with amygdala and bed nucleus of the stria terminalis (Maras et al., 2014). The second finding of reduced vesicular concentration of glutamate, could be related to depleted glutamate vesicular reserves due to overactivation or an impairment in glutamate/glutamine (Glu/Gln) cycle. A widely replicated finding in animal models of stress relates to decreased number and function of astrocytes, which mediate the Glu/Gln cycle (Banasr et al., 2010). This finding has also been replicated in postmortem samples from depressed patients (Nagy et al., 2015).

A model of pathogenesis of severe anhedonia in depression based on excitotoxicity was proposed, where they determined a correlation between negative blood oxygenation level-dependent (BOLD) responses induced by emotional stimulation in the pregenual anterior cingulate cortex in depressed patients, with markers of neuronal integrity, glutamate and N-acetyl aspartate (NAA), whereas in healthy controls a correlation with gamma aminobutyric acid (GABA) was observed (Walter et al., 2009).
It has been shown that chronic unpredictable stress alters glial metabolism, decreases the expression of astrocytic marker glial fibrillary acidic protein (GFAP) in the prefrontal cortex. These changes along with behavioral abnormalities caused by stress were prevented by glutamate-modifying drug riluzole (Banasr et al., 2010). Riluzole is anti-glutamatergic drug used for the treatment of amyotrophic lateral sclerosis, which was originally developed as an antiepileptic (Borowicz et al., 2004). It is thought that is acts by decreasing presynaptic release of glutamate and increasing the uptake of glutamate by glial cells, among other mechanisms (Lazarevic et al., 2018). There is evidence of the effectiveness of this drug for the treatment of mood and anxiety disorders (Salardini et al., 2016; Grant et al., 2010; Pittenger et al., 2008). Chronic restraint stress which causes dendritic remodelling in the hippocampus and supresses neurogenesis in the dentate gyrus, also affects the expression of glial glutamate transporter (GLT-1) in the hippocampus. Administration of antidepressant tianeptine reverses structural changes induced by stress in the hippocampus and also prevents the stress induced increase in the GLT-1 expression (Reagan et al., 2004).

A 2017 study found evidence of excitotoxicity in PTSD patients (Rosso et al., 2017). Using high-field proton magnetic resonance spectroscopy they detected decreased levels of neuronal marker NAA and high levels of glutamate in the hippocampus, which are considered biomarkers of glutamate neurotoxicity. Interestingly these alterations corelated with re-experiencing symptoms. Hippocampal atrophy, which could be at least partly a result of excitotoxicity is a common finding in PTSD patients, although the debate is still not settled on whether it is a consequence of the disease or a predisposing factor (Ressler et al., 2022).

A meta-analysis concluded that both people exposed to trauma without PTSD and PTSD patients have significantly lower hippocampal volume compared to trauma-unexposed subjects. These results further point to a possible causative role of stress induced excitotoxicity in PTSD. (Woon et al., 2010). Increased likelihood of developing PTSD was associated with single nucleotide polymorphism of the gene for excitatory amino acid transporter 3 (EAAT3) in combat-exposed veterans (Zhang et al., 2014).

A recent study (Bonifacino et al., 2023) proposed that rats can be deemed resilient or vulnerable based on anhedonic phenotype 24h after a single footshock stress. They determined differences between the two groups in basal and depolarization evoked glutamate release, astrocytic glutamate release, expression of GLT-1 and astroglial glutamate exchanger xCt, molecular changes which they designated as “early determinants of maladaptive response related to vulnerability”. An interesting mechanism of stress susceptibility has been identified which includes stress induced suppression of xCT gene expression in the ventral dentate gyrus by histone acetylation. A rescue experiment using GFAP+ -Cre dependent overexpression of xCT in the ventral dentate gyrus alleviated the effects of stress. Interestingly they found that acetyl-L-carnitine has pro-resilient effects which are mediated through increasing xCT expression, and regulating glutamate homeostasis (Nasca et al., 2017). Reduced astrocyte density and altered morphology was determined in the CA1 region of the hippocampus, but not amygdala in a PTSD animal model (Saur et al., 2016).

The glutamatergic abnormalities which we observed are complemented by the finding of decreased autohosphorylated CaMKII in prefrontal cortex and hippocampus of SPS and/or MD+SPS animals. CaMKII is a major regulator of the glutamatergic synapse and is heavily implicated in mediating learning and memory, which are functions that are central to the pathophysiology of PTSD (Bayer and Schulman, 2019). Both main CaMKII isoforms in the brain (CaMKIIα and CaMKIIβ) were similarly affected by the different stress paradigms. CaMKII autonomous activity is crucial for the survival of neurons due to its influence on glutamate homeostasis. Prolonged inhibition of CaMKII with small molecule and peptide inhibitors has been shown to induce apoptosis in cultured cortical neurons (Ashpole et al., 2012). This process disrupts calcium signaling and leads to the accumulation of extracellular glutamate. Furthermore, inhibition of CaMKII results in decreased glutamate uptake in cultured primary rodent astrocytes through EAAT1 (Ashpole et al., 2013; Chawla et al., 2017). Sustained pharmacological inhibition of CaMKII in primary cortical neuronal cultures increased neuronal death following a submaximal glutamate challenge (Ashpole and Hudmon, 2011). However, immediate pharmacologic inhibition of CaMKII after an excitotoxic insult prevents aggregation and prolonged inactivation of the kinase, which in turn has neuroprotective effects (Ashpole and Hudmon, 2011). Administration of CaMKII inhibitors in an animal model of global cerebral ischemia, 30 minutes after resuscitation has been shown to reduce neuronal death, improve functional plasticity, and improves behavioral symptoms (Vest et al., 2010; Coultrap et al., 2011; Deng et al., 2017).

A model which might provide a framework for a unified interpretation of our results could be the synaptic model of PTSD, proposed by Krystal et al (2017), which puts glutamatergic abnormalities at the center of PTSD pathophysiology. This is a speculative model based on clinical and preclinical evidence. First speculation which they assert is that trauma exposure combined with predisposing

vulnerability causes dysregulated glutamate transmission, proinflammatory processes and HPA axis abnormalities which result in a glutamate spillover that along with the disrupted Glu/Gln cycle causes accumulation of extracellular glutamate that triggers excitotoxicity in key brain areas involved in PTSD symptomatology. Second speculation is that PTSD represents a self-perpetuating paradigm of chronic stress, where PTSD symptoms (fear, depression, insomnia, guilt, demoralization, shame and numbing) themselves are a trigger of stress, which results in a progressive compromise of synaptic integrity through a vicious cycle that represents the basis for PTSD chronicity. The second statement is corroborated by the finding that certain behavioral symptoms of PTSD increase the likelihood of a chronic course of illness (Carper et al., 2015). CaMKII inhibition which we observed in our model could be a potential link between neuronal damage proposed by this model and PTSD symptomatology.

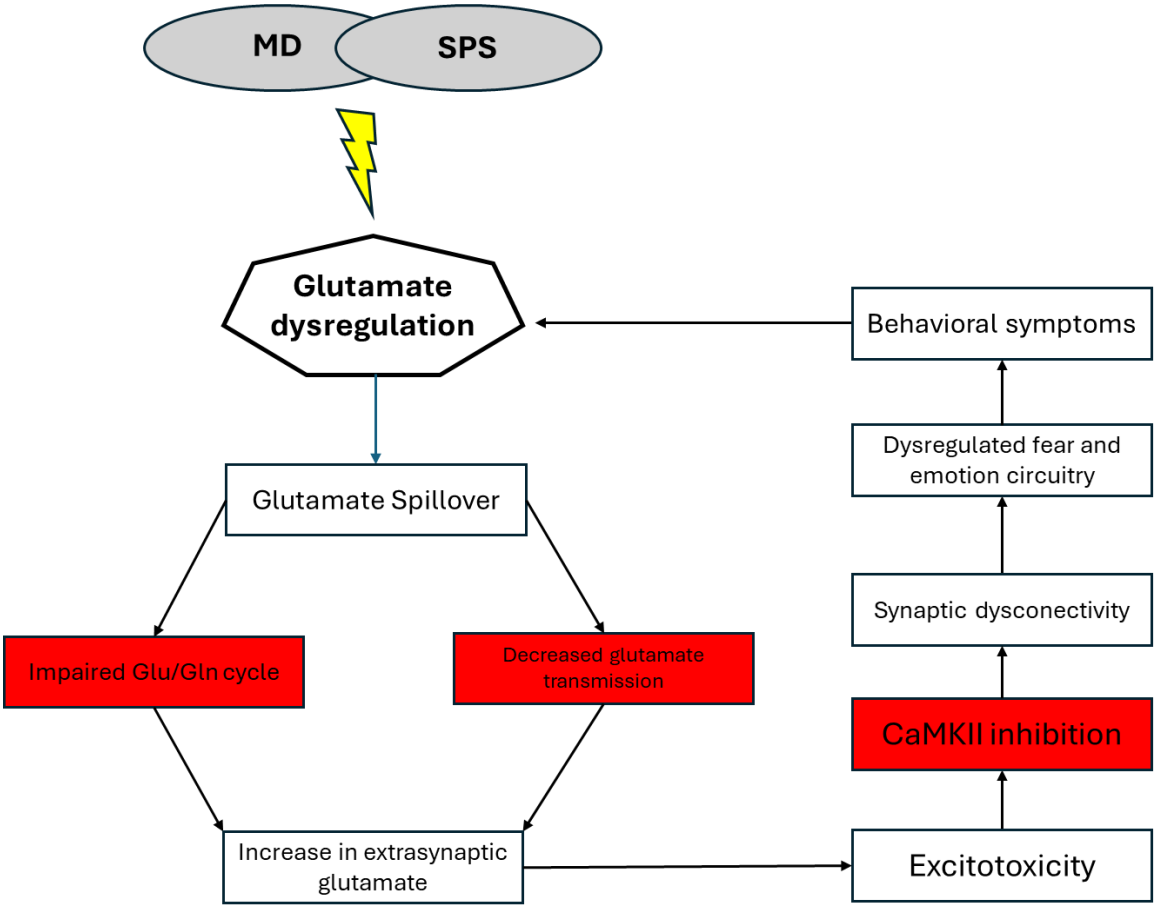


Figure 41. A hypothetical model of the unified interpretation of our glutamate data based on the synaptic model of PTSD proposed by Krystal et al. (2017)

Our results showed a statistically significant increase of norepinephrine (NE) and serotonin (5-HT) and their metabolites in the prefrontal cortex of MD+SPS animals compared to controls. The link between increased noradrenergic function and PTSD has been well established (Arnsten et al., 2015). Elevated NE in the prefrontal cortex represents an indication of prefrontal cortical disfunction (Arnsten et al., 2015).

Catechol-O-methyltransferase (COMT) a catecholamine catabolic enzyme, is associated with a G→A single nucleotide polymorphism in exon four of its gene (val158met polymorphism), which leads to a valine (val) to methionine (met) peptide change in the mature enzyme at amino acid 158, diminished functional capacity of the enzyme (Lachman et al., 1996) and increased catecholamine availability. Heterozygous Val/Met genotype produces an intermediary phenotype, while Met/Met genotype results in a 40% reduction of enzyme activity compared to Val/Val genotype (Malhotra et al., 2002). COMT has been one of the most studied genes for psychosis (Craddock et al., 2006). Careers of the low activity variants display decreased fear extinction (Lonsdorf et al., 2009) and increased startle reactivity (Montag et al., 2008), which has prompted research on a possible connection of these genotypes with PTSD. Indeed, it has been determined that Met/Met homozygotes have greater risk of PTSD independent of the number of previously experienced traumatic events (trauma load), whereas Val/Val or Val/Met careers displayed normal dose response relationship between trauma load and the incidence of PTSD (Kolassa et al., 2010)

A 2018 study (Hendrickson et al., 2018) had a goal of determining whether a previously experienced traumatic event had an influence on brain reactivity to NE, manifested as increased NE concentration in cerebrospinal fluid (CSF), and whether that had an effect on the expression of PTSD symptoms. They analyzed combat veterans, post deployment, who experienced a major trauma (sufficient to meet DSM-5 criterion A for the diagnosis of PTSD), that had or had not developed PTSD. Control group consisted of veterans who did not meet the criterion for trauma exposure. No difference in the concentration of NE in the CSF was detected between any of the groups. Trauma exposed individuals without a PTSD diagnosis had a significantly greater positive correlation between NE concentration in the CSF and PTSD symptom expression compared to the control group. In the control group a negative correlation between symptom expression and NE concentration in the CSF was observed. Their explanation for these results is that trauma changes the postsynaptic sensitivity to NE, and that higher postsynaptic sensitivity along with high NE release (corresponding to higher NE concentration in the CSF) in trauma exposed individuals causes higher symptom expression.

The effects of NE on prefrontal cortex have been detailly elucidated at the molecular level (Arnsten and Jin, 2014). The key aspect is its action is the modulation of dynamic network connectivity. This is a mechanism for coordinating cognitive state with arousal state, through modulation of network inputs and neuronal firing without inducing structural neuronal changes. This mechanism was described in layer III dorsolateral prefrontal cortex dendritic spines, and it involves the inhibition or activation of K+ channels, by controlling Ca2+ - cAMP (Arnsten et al., 2010). It is thought that this is the process that enables loosening or strengthening of top-down control of prefrontal cortex over subcortical structures. Concentration of Ca2+ inside the dendritic spines increases via NMDAR or IP3 mediated internal Ca2+ release through α1 AR or mGluR1α or mGluR5 activation. Ca2+ induces cAMP generation and cAMP further stimulates internal Ca2+ release. Ca2+ can activate SK channels, while cAMP can activate HCN and KCNQ channels. Activation of all of these receptors on dendritic spines causes rapid weakening of the synaptic activity and a disconnect of the dlPFC neuronal networks (Arnsten and Jin, 2014). Activation of postsynaptic α2 AR induces and opposite effect which is mediated through the inhibition of cAMP production (Arnsten and Jin, 2014).

The function of norepinephrine in the PFC has been characterized using delay ocular response task in monkeys (Arnsten, 2009). This is a spatial working memory task which consists of a series of trials in which monkeys are trained to move their eyes in a certain direction after an intertrial interval, where they receive a reward if they perform the task correctly. The correct position changes after every trial which is essential for the probing of working memory. PFC is the region of interest for the successful completion of this task. Single unit recordings have enabled the characterization of the neuronal

activity necessary to perform this task (Arnsten, 2009). Spatially tuned ensembles of PFC neurons selected by the input from the parietal cortex, are simultaneously activated during the intertrial interval via recurrent excitation, to encode a particular location in space, corresponding to the correct choice in the task. These neurons are called preferred direction neurons. Surrounding neurons are inhibited via recurrent inhibition. Those neurons are called non-preferred direction neurons. Catecholamines determine the strength of persistent firing and the degree of spatial tuning. An inverted U-shaped curve has been determined for the action of norepinephrine in this task, where under the optimal concentration of this neurotransmitter (which is in a non-stressed state) it binds to low affinity α2 AR receptors which results in excitation of preferred direction and inhibition of non-preferred direction neurons (Arnsten, 2009). Either a low or a high concentration of NE results in diminished firing of preferred direction neurons and a working memory impairment (Arnsten, 2009).

Activation of α2 AR has beneficial effects on working memory tasks, which has been proven in a number of experiments using α2 AR agonists such clonidine, guanfacine, or medetomidine (Arnsten, 2000). Also, α2 AR inhibitor yohimbine causes impairment in working memory (Li and Mei, 1994). Infusion of the α1 AR agonist phenylephrine on the other hand causes an impairment in working memory task, which is reversed by its antagonist urapidil (Arnsten et al., 1999).

Intraperitoneal injection of a pharmacological stressor FG7142 induced a deficit on delayed alternation task, which is a spatial working memory task (Birnbaum et al., 1999). It impaired accuracy on the task and induced perseverative responses which are indicative of PFC dysfunction. This deficit was completely reversed by injecting urapidil 15 minutes prior to the task, which suggest that activation of α1 AR during stress is the likely mechanism of stress induced impairment of PFC function.

In situations of acute and sudden danger PFC in naturally weakened to loosen top-down control over amygdala and enable reflexive, rapid action. It is thought that this mechanism of working memory impairment is relevant in situations of uncontrollable stress, where the subject perceives that he is not in control of the situation (e.g. unescapable shock). A 2006 study (Morgan et al., 2006) analyzed performance on a visuo-spatial working memory task in healthy cohort of Special Forces soldiers under conditions of acute stress. Nearly all of the subjects displayed a working memory deficit under acute stress, but previously experienced traumatic event that qualified under "fear for life" designation was determined to be a significant factor for working memory deficit in this visuo-spatial task.
Chronic stress strengthens the noradrenergic system. It influences the locus coeruleus neurons, by increasing their firing (Jedema and Grace, 2003) and it increases synthetic capacity of NA neurons (Fan et al., 2013).
The basic studies of NE changes in stress have resulted in the use of alpha-1 receptor antagonist, prazosin for the treatment of PTSD, and it has been particularly effective for the treatment of nightmares in PTSD patients (Arnsten et al., 2015). Guanfacine is used to treat various disorders which involve diminished PFC function (including attention deficit hyperactivity disorder, Tourette's Syndrome, autism spectrum disorder, traumatic brain injury) (Arnsten, 2020).
We have already mentioned that traumatic brain injury is a risk factor for PTSD. TBI in an animal model induced increased CREB mediated expression of α1-AR in the rat mPFC (Kobori et al., 2011). The working memory deficit observed in animals after TBI induction, was ameliorated by the administration of prazosin, which indicates that α1-AR are responsible for working memory deficits in TBI, similarly to PTSD.
MD and double hit stressed animals caused comparable changes in BDNF mRNA. Reduction of BDNF mRNA was detected in the medial prefrontal cortex and CA1 region of the dorsal hippocampus of both groups.

BDNF is a neurotrophic factor and one of the most studied molecules regarding stress induced psychopathology (Autry and Monteggia, 2012). Decrease in BDNF has been related to loss of resilience to stress (McEwen et al., 2016).
It is synthesized as preproBDNF which is cleaved to proBDNF in the Golgi apparatus. The proBDNF is further cleaved in the mature form (mBDNF) both extracellularly, by proteolytic cleavage via plasmin, or intracellularly. Neurons secret both proBDNF and mBDNF. Both forms are active, and have distinct and opposing roles, which they exert by binding to two different receptors: proBDNF binds to p75 neurotrophin receptor, whereas mBDNF binds to TrkB receptor. proBDNF is more prevalent during development, while mBDNF is the predominant form in adulthood, where it is involved in the regulation of synaptic function and plasticity, and the modulation of neuronal survival, cytoarchitecture, and function. (Colucci et al., 2020)
There are four different BDNF exons, and alternative splicing results in seven possible BDNF transcripts (Timmusk et al, 1993).
An interesting 2007 paper (Nair et al., 2007) analyzed the differential expression of BDNF exons in the hippocampus, during a wide variety of stress paradigms, administered both postnatally and during adulthood. A single episode of 3h maternal separation (MS) on postnatal day 7 resulted in an increase in BDNF III transcript, whereas a 6h MS on the same postnatal day resulted in a decrease in BDNF I transcript. Total BDNF (exon V) and CREB were unchanged. Interestingly chronic MS (3h per day between postnatal days 2 and 14) resulted in an increase in BDNF II and CREB at postnatal day 14 and increase in BDNF IV and total BDNF and CREB at postnatal day 21, with normal levels in adulthood. 24h MD at postnatal day 9 was shown to induce a decrease in total hippocampal BDNF in adulthood (Roceri et al, 2002), which is consistent with our results. This discrepancy indicates that different stress protocols have very different consequences with regards to BDNF expression as well as behavioral outcome. They provide an interpretation of this increase in BDNF expression induced by MS as a potential causal factor for the expression of behavioral changes that are characteristic for the model that they used, which include increased fear and anxiety. In adulthood chronic immobilization stress increased BDNF I/II transcript and decreased III/IV transcript while total BDNF transcript remained unchanged, whereas chronic unpredictable stress had a different effect on BDNF expression. Interestingly prior maternal separation altered the pattern of BDNF expression in response to stress in adulthood, which suggests that these changes could represent a mechanism of predisposition for the development of adult psychopathology.
Tornese et al. (2019) found a correlation between decreased BDNF expression and glutamate release in the hippocampus and anhedonia after chronic mild stress in rats. Interestingly ketamine which restored the behavioral deficit, had no effect on the total hippocampal BDNF transcript, however completely restored BDNF dendritic transcript assessed by in situ hybridization, which corroborates a general assessment that BDNF dendritic transcript is key for the beneficial effects of ketamine (Song et al., 2017).
Also, importantly only the dendritic BDNF transcript in the CA1 and CA2 was a significant discriminant between vulnerable and resilient rats.
In our study we also quantified the dendritic BDNF transcript, and our results indicate the significance of postsynaptic effects in the two-hit model, compared to SPS alone which displayed no effect in this regard.
Similarly, Hu et al. (2023) analyzed BDNF protein level and glutamate release after 14, 21 and 35 days of chronic mild stress (CMS) in adult rats in the prefrontal cortex. They found an increase in glutamate release coupled with a decrease in BDNF in susceptible rats after 14 days of CMS, whereas after a 35-day stress protocol glutamate release was decreased along with BDNF in the same group of rats. These results corroborate the interpretation of our findings and provide a strong indication of the key role of inverted U-shape effects on glutamate release in vulnerability to stress, which they speculate was

mediated through excitotoxicity. Caution and further clarification were however necessary because of the anhedonia detected after 14 days of CMS. They provide an explanation that this behavioral change could be caused by a different neural mechanism than after 35 days of CMS since the structure of the glutamatergic synapse after 14 days was unchanged, whereas after 35 days there was a reduction in the total levels of synapsin-1, PSD-95 and spine density.

6.Conclusions

1. Two-hit stress causes unique behavioral changes compared to MD and SPS.
2. Two-hit stress and SPS cause decreased depolarization evoked glutamate release in the hippocampus
3. Two-hit stress causes decreased glutamate concentration in hippocampal synaptosomes
4. Two-hit stress causes decreased p-T286 CaMKII in hippocampal synaptosomes
5. Two-hit stress and SPS cause decreased p-T286 CaMKII in prefrontal cortical synaptosomes
6. Two-hit stress increases the concentration of norepinephrine and serotonin in the prefrontal cortex
7. Two-hit stress and MD cause a decrease in BDNF mRNA in the CA1 region of dorsal hippocampus and the medial prefrontal cortex.

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Biography

Dr. Đorđe Đorović was born on November 3, 1990 in Belgrade, Serbia. He graduated from the elementary school "Vladimir Rolović" and the primary music school "Davorin Jenko" in Belgrade as well as the Fifteenth Belgrade High School through an accelerated programme for outstanding students as the best student of his generation. He graduated from the Faculty of Medicine at the University of Belgrade in 2015 with an average grade of 9.82. Since 2016, he has been working as a teaching assistant at the Institute of Anatomy “Niko Miljanić” at the Faculty of Medicine, University of Belgrade. He is a PhD student in Neuroscience at the Faculty of Medicine, University of Belgrade. During his doctoral studies, he received a number of prestigious European fellowships from various professional societies, including twice the IBRO PERC fellowship. In the period 2017-2021, he completed several research stays at Karolinska Institutet, Department of Clinical Neuroscience, where he completed part of his PhD thesis in the laboratory of Professor Per Svenningsson, who is the co-supervisor of his PhD thesis. He is the author of 5 articles on the JCR list.

Изјава о ауторству

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Изјављујем

да је докторска дисертација под насловом

„Presinaptička funkcija glutamatergičke sinapse u modelu dvostrukog stresa“

- резултат сопственог истраживачког рада;
- да дисертација у целини ни у деловима није била предложена за стицање друге дипломе према студијским програмима других високошколских установа;
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Име и презиме аутора: Ђорђе Ђоровић

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Студијски програм: Неуронауке

Наслов рада: "Presinaptička funkcija glutamatergičke sinapse u modelu dvostrukog stresa"

Ментор: Проф др Ласло Пушкаш

Коментор: Проф др Пер Свенингсон (Per Svenningsson)

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањивања у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, _____

_____

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

“Presinaptička funkcija glutamatergičke sinapse u modelu dvostrukog stresa”

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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Потпис аутора

У Београду, _____

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