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Characterization of mouse stress models on allostatic overload of the HPA axis and depressive-like behavior

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Abstract

Introduction: Depression is one of the most prevalent mental disorders worldwide and it negatively affects mood, sleep, sexual desire and pleasure [1, 2]. The current treatment options do not have an effect in 35% of patients [3] and there are no objective diagnostic tools such as biomarkers in depression [4, 5]. Therefore, there is a need to develop and optimize animal stress models for further investigations into this mental disorder. This thesis examine the ability of three different stress models to induce allostatic overload of the HPA axis and a depressive-like phenotype in mice.

Methods: Three different stress paradigms in male mice were examined in this thesis; a Chronic stress (CS) model based on Chronic mild stress (CMS) [6, 7, 8, 9] together with jetlag models [10, 11], a 14-day Repeated restraint stress (RRS) model (RRS14) and interrupted RRS (iRRS) consisting of 3 days of stress followed by 48h of rest which continues for 20 days. After the stress, the behavioral assays Elevated plus maze (EPM), diurnal locomotor activity (72h), locomotor activity (3h), burrowing and nest building were used to determine behavioral changes indicative of a depressive-like phenotype.

Results: CS and iRRS induced significant increase in basal Corticosterone (CORT) levels while RRS14 demonstrated acutely increased CORT in response to RS. Moreover, CS induced changes in motivated behavior and sleep rhythm in the stress group with diurnal disruption during the whole study. CS also show significant upregulation of clock genes in the brain. CS and RRS14 demonstrate a tendency to increase locomotor activity in the stress groups while iRRS show decreased locomotor activity in the stress group. Finally, gene expressions measures in the brain reveal significant upregulation of Il-1β acutely in RRS14 while iRRS show significant upregulation of Il-1β 48h after last stress exposure.

Conclusion: in conclusion, CS and iRRS seem to induce allostatic overload of the Hypothalamic-pituitary-adrenal (HPA) axis. Moreover, CS induce a depressive-like phenotype in mice and may lead to disturbances in the circadian rhythm by altering the gene expression of clock genes in the brain. In addition, both RRS paradigms induce neuroinflammation.

Abbreviations

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ANS	Autonomous nervous system
BDNF	Brain-derived neurotrophic factor
BMAL1	Brain and Muscle Arnt-like protein-1
cDNA	complementary DNA
CK1	Casein kinase 1
CLOCK	Circadian Locomotor Output Cycles Kaput
CMS	Chronic mild stress
CNS	Central nervous system
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
Cry	Cryptochrome
CS	Chronic stress
CUS	Chronic unpredictable stress
DNA	Deoxyribonucleic acid
DSM-V	Diagnostic and Statistical Manual of Mental Disorders - Fifth Edition
E-box	Enhancer box
ELISA	Enzyme-Linked Immunosorbent Assay
EPM	Elevated plus maze
GC	Glucocorticoid
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor
Ha	Handling
HPA	Hypothalamic-pituitary-adrenal
Iba-1	Ionized calcium-binding adapter molecule 1
ICD-10	International Classification of Diseases - 10th version
IL	Interleukin
iRRS	Interrupted RRS
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
MAOI	Monoamine oxidase inhibitor
mRNA	messenger RNA
NEST	Nest building assay
NFκB	Nuclear Factor κ-Light-Chain-Enhancer of Activated B Cells
NSAID	Nonsteroidal anti-inflammatory drugs

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Per	Period
PFC	Prefrontal cortex
PVN	Paraventricular nucleus
QNP	Quiescent neural progenitor cells
Rev-erb	Retinoic Acid-related Orphan Receptor
RRS	Repeated restraint stress
RS	Restraint stress
RT	Reverse transcriptase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SNRI	Selective serotonin and noradrenalin reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic and tetracyclic antidepressant
TNF	Tumor necrosis factor
TTFL	Transcriptional/translational feedback loop
UNG	Uracil-DNA glycosylase
WHO	The World Health Organization

1 Introduction

Depression is a mental disorder which negatively affects mood, sleep, sexual desire and pleasure [1, 2] and it is one of the most prevalent mental disorders with approximately 1 in 20 people reported to have a depressive episode in 2011 in 17 different countries [1]. Furthermore, The World Health Organization (WHO) states that depression is the leading cause of disability worldwide and greatly contributes to the overall global disease burden [12, 13]. Depression is characterized by the following symptoms; low mood, loss of interest (apathy), sleep disturbances, changes in appetite, slower actions among others [14, 1]. These symptoms may become chronic or recurrent, which will cause impairment of the daily living of the individual [1].

To be diagnosed with depression there is two major classification systems, namely International Classification of Diseases - 10th version (ICD-10) and Diagnostic and Statistical Manual of Mental Disorders - Fifth Edition (DSM-V). The two systems overlap considerably in their criteria for diagnosing depressive episodes. The symptoms included in the ICD-10 and DSM-V criteria are listed in table 1.1. ICD-10 requires the presence of at least two of the first three core symptoms together with at least two of the remaining seven symptoms. On the other hand, DSM-V requires that five out of the nine symptoms are present where one of these must be one of the two core symptoms [15, 16]. Furthermore, these symptoms must persist for at least two weeks and have a substantial impact on everyday life for the patient to be diagnosed with depression [14]. The two classification systems can assess the severity of depressive episodes by categorizing them as either mild, moderate or severe based on the type, number and severity of symptoms together with the degree of functional impairment [15].

Another category of symptoms in depression is vegetative symptoms, which include fatigue, dyspnea, cardiac arrythmias as well as changes in sleep, body temperature, weight and appetite. These somatic symptoms are often

Table 1.1.: Comparison of depression symptoms in ICD–10 and DSM–V [15, 16]
Abbreviations: International Classification of Diseases - 10th version (ICD-10), Diagnostic and Statistical Manual of Mental Disorders - Fifth Edition (DSM-V).

ICD-10	DSM–V major/minor depressive disorder
Depressed mood*	Depressed mood by subjective report or observation made by others*
Loss of interest*	Loss of interest or pleasure in almost all activities*
Reduction in energy*	Fatigue/loss of energy
Loss of confidence or self-esteem	Worthlessness/excessive or inappropriate guilt
Unreasonable feelings of self-reproach or inappropriate guilt	
Recurrent thoughts of death or suicide	Recurrent thoughts of death, suicidal thoughts or actual suicide attempts
Diminished ability to think/concentrate or indecisiveness	Diminished ability to think/concentrate or indecisiveness
Change in psychomotor activity with agitation or retardation	Psychomotor agitation or retardation
Sleep disturbance	Insomnia/hypersomnia
Change in appetite with weight change	Significant appetite and/or weight loss

**Core symptoms*

presented in depressive patients together with the affective, behavioral and cognitive symptoms of depression [17].

The current treatments for depression is based on the role of monoamines in depression [18, 19]. Several antidepressants work by blocking the reuptake of monoamines such as norepinephrine and serotonin by the presynaptic neuron (Selective serotonin reuptake inhibitor (SSRI), Selective serotonin and noradrenalin reuptake inhibitor (SNRI), Tricyclic and tetracyclic antidepressant (TCA)) or inhibiting the degradation of these monoamines (Monoamine oxidase inhibitor (MAOI)) in order to increase the number of available neurotransmitters in the brain [4, 18]. These monoamines have many different functions that contribute to the functional benefit of these antidepressants in reducing depressive symptoms in patients. For instance, they affect the Hypothalamic-pituitary-adrenal (HPA) axis, stimulate the expression of Brain-derived neurotrophic factor (BDNF) and cause proliferation of neural precursor cells in the hippocampal dentate gyrus [14, 20, 21]. However, the current antidepressants do not have an effect in 35% of patients and it takes between

three to six weeks before the patients feel an effect [3]. This long delay for efficacy of the treatment may be due to the monoamine function causing proliferation of neural precursor cells in the hippocampus, which is a time-consuming process [21]. Therefore, there is a need for animal stress models in order to fully cover all the aspects of depression and hereby create more treatment options, discover biomarkers and expand the knowledge about depression.

1.1 Causes of depression

It is suggested that the heritability of depression is between 28-46% [22, 23, 24, 25]. This is much lower than for other mental disorders indicating a considerable influence of environmental factors and lifestyle in depression. Therefore, depression is not a consequence of a single gene or risk factor but instead a complex interplay between biological, psychological and social factors [14, 2].

1.1.1 Chronic stress

Brain regions known to be involved in depression includes the hippocampus, hypothalamus, amygdala, the Prefrontal cortex (PFC) among others [2, 20, 14, 26, 27, 28]. Moreover, the HPA axis also plays a crucial role in depression [14, 2, 20, 29], since one of the primary causes of depression is chronic stress [21]. Chronic or repeated environmental changes in which the individual find particularly stressful lead to increased neuroendocrine responses and is defined as allostatic load. The term allostatic load derives from allostasis, which is the organism's ability to mediate changes in order to achieve balance (homeostasis), but also the idea that healthy functioning requires ongoing adjustments of the internal physiological milieu. Allostatic overload is the cumulative impact of stressful daily life experiences and environmental challenges that exceed the individual's ability to cope, which leads to repeated activation of the stress response systems. Exposure to chronic stress, lack of adaption to repeated stressors or ability to inhibit the stress response after the termination of a stressor and inefficient allostatic response to handle the stressor are situations that lead to allostatic overload [30]. Chronic

stress may be the reason why depression is so prevalent in the modern society because the modern lifestyle is full of mild stressful events every day, especially at work [31].

Stressful events are perceived by the cortex and amygdala in the brain, which will transmit signals to the hypothalamus. In response, the hypothalamus will release Corticotropin-releasing hormone (CRH) to the pituitary gland. Pituitary receptors are stimulated by CRH, which leads to secretion of Adrenocorticotropic hormone (ACTH) into the blood transporting it to the adrenal cortex. Here, the corticotropin receptors will bind ACTH causing release of Glucocorticoid (GC) into the bloodstream (figure 1.1). This system is called the HPA axis [14, 32]. Another system which is activated in response to stress along with the HPA axis is the Autonomous nervous system (ANS). Exposure to stress leads to activation of the sympathic preganglionic neurons in the spinal cord (the sympathetic nervous system). This signal will cause secretion of catecholamines via splanchnic nerves to the adrenal medulla as well as continue through postganglionic neurons projecting to peripheral organs creating the fight-or-flight response known as increased heart rate and blood pressure, stimulation of sweat glands, energy mobilization etc. [33].

The mechanism by which GCs mediate negative feedback is through the Glucocorticoid receptor (GR) at the level of the hippocampus, hypothalamus and the pituitary leading to decreased activity of the HPA axis in order to maintain homeostasis [34, 14, 32]. This feedback-loop is lacking in almost half of the severe cases of depression [14, 32]. Hippocampus also exerts negative feedback on the hypothalamus, which decreases the activity of the HPA axis [21]. The hippocampus expresses a high number of GRs allowing it to detect circulating GC levels and thereby adjust the negative feedback on HPA axis to the number of GCs [2, 35]. GCs stimulate the hippocampus to inhibit the HPA axis [35] while blocking the GRs in hippocampus diminishes the inhibitory effect of hippocampus on the HPA axis [36]. Opposite to the hippocampus, amygdala activity promotes activation of the HPA axis [37].

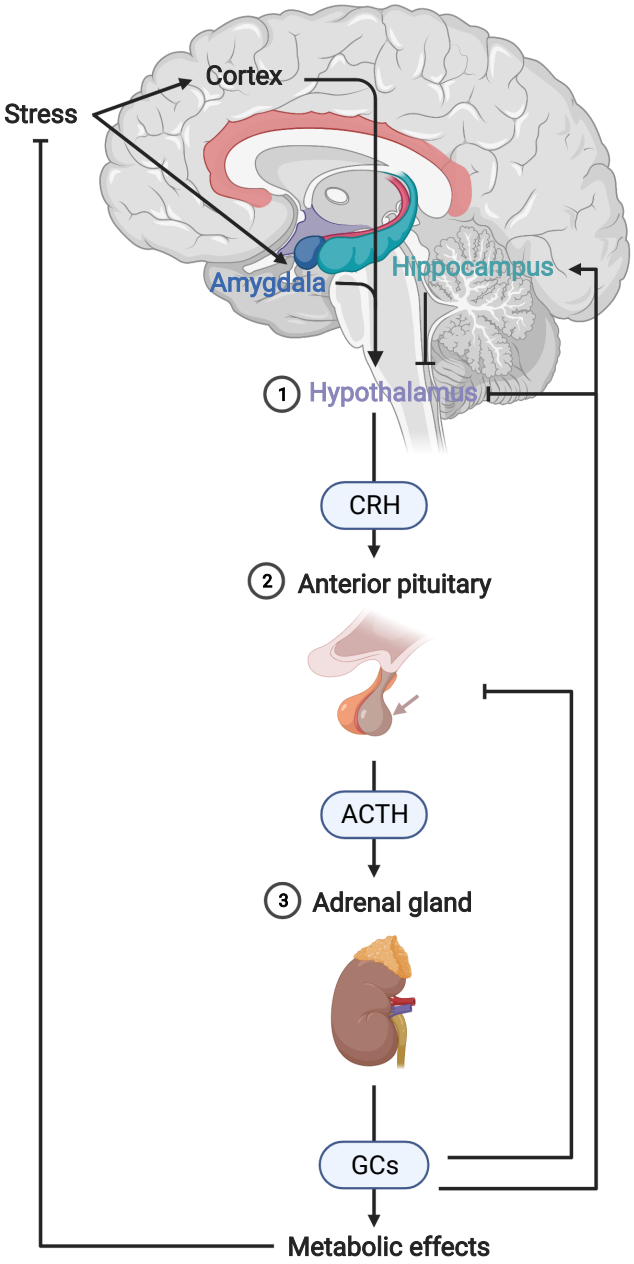


Figure 1.1.: Illustration of the Hypothalamic-pituitary-adrenal (HPA) axis. The cortex and amygdala is stimulated by stress and will activate the hypothalamus leading to secretion of CRH. CRH stimulate receptors in the anterior pituitary causing ACTH secretion which will travel by the bloodstream to the adrenal glands. ACTH stimulate synthesis and secretion of GCs which have several metabolic effects in the body. GCs exerts negative feedback on three levels of the HPA axis; the pituitary, hypothalamus and hippocampus which maintains homeostasis.
Abbreviations: Adrenocorticotropic hormone (ACTH), Glucocorticoid (GC), Corticotropin-releasing hormone (CRH). Made in BioRender by Freja Pretzmann.

Lack of negative feedback on the HPA axis may lead to allostatic overload of the HPA axis and thereby abnormal high GC levels, which are often seen in patients with depression. Therefore, the dysfunction of the HPA axis can be evaluated in patients with depression with the dexamethasone suppression test or the dexamethasone/CRH test [38].

Allostatic overload have unfavorable effects in the body such as cognitive impairment, decreased BDNF and decreased neurogenesis in hippocampus leading to reduced volume in patients with depression [2, 14, 38, 39, 40]. While low concentrations of GCs are immune stimulatory, high concentrations are known to be immunosuppressive (Schiepers, Wichers and Maes, 2004). Nevertheless, increased inflammation is often seen in patients with depression [41, 42, 43, 44]. An explanation may be GR resistance both in the brain and the periphery resulting in lack of suppression of the immune system [45]. Psychosocial stress is known to reduce the activity of areas in the limbic system such as hypothalamus and hippocampus [46, 47] and increase the activity of amygdala and PFC [28, 47]. The hippocampus is sensitive to harmful effects of chronic stress that leads to elevated GCs due to the high expression of GRs in this structure [2]. On the other hand, hippocampus is important in the stress response since it exerts negative feedback on the Paraventricular nucleus (PVN) of the hypothalamus, which will decrease the activity of the HPA axis. Without this feedback loop, there will be less negative regulation of the HPA axis, which may lead to allostatic overload. This means, that chronic stress may initiate a negative spiral with elevated GC causing decreased volume and activity of hippocampus through neuronal death. The neuronal loss in hippocampus together with GC resistence leads to failure of the negative feedback on the HPA axis, and consequently, there will be further elevation of GC due to allostatic overload of the HPA axis, resulting in increased risk of depression [21].

1.1.2 Neuroinflammation

Signaling molecules of the immune system, called cytokines, are thought to play a role in the pathophysiology of depression since they are important for the stress response [5]. Pro-inflammatory cytokines such as Interleukin (IL)-6, Tumor necrosis factor (TNF)-α and IL-1β, are known to be potent activators of the HPA axis directly, or indirectly by cytokine-induced GC resistance [48, 42,

49, 50, 51, 52]. Hereby, these cytokines can cause dysregulation of the HPA axis by either hyperactivation or counteracting the negative feedback of GCs on the HPA axis. The latter is based on a study in rats where the potency of dexamethasone to suppress the secretion of GCs is reduced by systemically administered Lipopolysaccharide (LPS), which implies that it may be cytokines interfering with the negative feedback mechanism of GCs [53].

TNF-α and IL-6 have also been shown to exert a direct suppressive effect on the neurogenesis in hippocampus [54, 55]. Furthermore, the cytokine IL-1 can cause decreased proliferation of neural progenitor cells of hippocampus through the Nuclear Factor κ-Light-Chain-Enhancer of Activated B Cells (NFκB) signaling pathway by binding to type 1 IL-1 receptors expressed on the progenitor cells [56]. Thus, stress induced neuroinflammation may also explain the hippocampal reduction and hyperactivity of the HPA axis in depression. Blood samples from patients with depression have shown significantly elevated levels of pro-inflammatory cytokines in their circulation, such as IL-6, IL-1β and TNF-α [41, 42, 43, 44], but also significantly decreased anti-inflammatory IL-10 [57]. IL-10 is important for the HPA axis homeostasis due to the suppressive effect on the ACTH-induced cortisol production and decreased IL-10 may therefore lead to allostatic overload of the HPA axis and GC resistance [5]. Furthermore, an in vivo study has shown that IL-10 knockout mice display depressive behavior and overexpression of IL-10 decreases depressive behavior in mice [58].

Chronic mild stress (CMS) in rats have also resulted in microglial activation in the hippocampus as well as upregulation of pro-inflammatory cytokines such as IL-1β and IL-6 [56, 59]. Even though several studies find an increase in pro-inflammatory cytokines in blood from patients with depression, a significant decrease in IL-1β has also been observed in serum of depressive patients [60] as well as no significant difference for IL-6 [61]. Therefore, there is a need for further investigation into these cytokines in depression. Still, anti-inflammatory treatment such as Nonsteroidal anti-inflammatory drugs (NSAID) and cytokine inhibitors have been shown to reduce depressive symptoms in patients compared to placebo [62]. These results support the idea that inflammation may be an important factor in depression.

Pro-inflammatory cytokines and their receptors that are present in the Central nervous system (CNS) are primarily produced within the CNS by astrocytes

and microglia, which are cells of the immune system that primarily exert their functions toward neurons [51, 63]. Astrocytes are important for maintaining homeostasis of ions and neurotransmitters, refine synaptic connections and support neurons with metabolic substrates, while microglia monitor synaptic networks and molecules through responses to dyshomeostasis by promoting or removing synaptic molecules and modulating activity of neurons [63]. Therefore, these two cell types may be important in the pathophysiology of depression. For instance, it has been shown that the density and number of astrocytes were reduced in the PFC, hippocampus, anterior cingulate cortex and amygdala in patients with depression shown by a decrease in Glial fibrillary acidic protein (GFAP) [64, 65, 66], which is highly expressed on astrocytes [3]. Astrocytes are also thought to promote neurogenesis in hippocampus since co-culturing of hippocampus-derived neural precursor cells from rats with adult astrocytes from rat hippocampus increase the proliferation of the precursor cells [67]. Therefore, these brain regions as well as astrocytes are thought to be involved in depression.

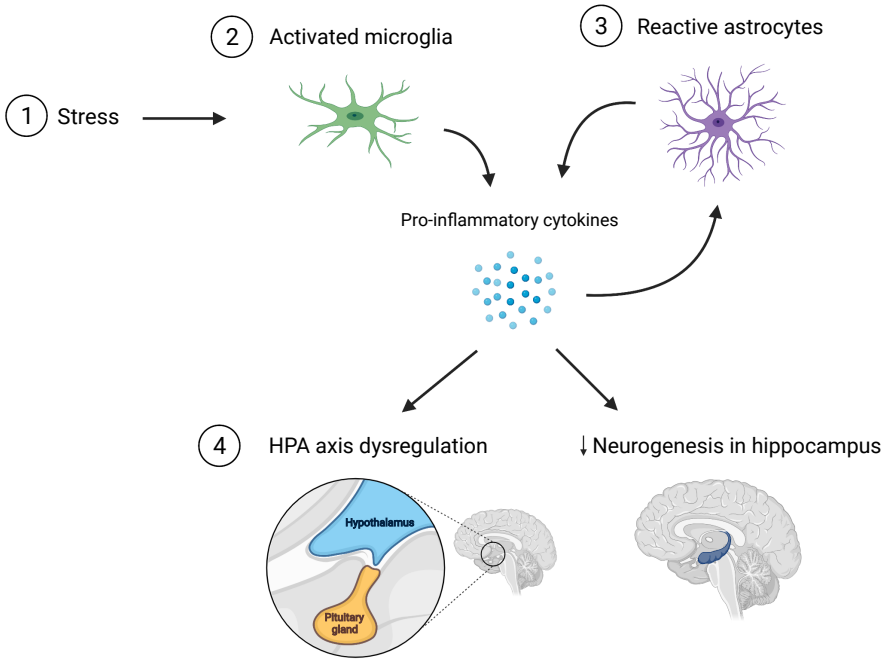


Figure 1.2.: Illustration of how stress activates the immune system. Stress activates the immune cells microglia and astrocytes resulting in pro-inflammatory cytokines which may cause HPA axis dysregulation and decreased neurogenesis in hippocampus.
Abbreviations: Hypothalamic-pituitary-adrenal (HPA). Made in BioRender by Freja Pretzmann.

Microglia are activated upon pathological conditions such as infections, neurodegeneration [59, 56] and stress [56] and a commonly used marker for microglial activation is Ionized calcium-binding adapter molecule 1 (Iba-1) [56, 68, 69]. Iba-1 gene expression has been shown to be downregulated in the hippocampus of rodents exposed to different types of chronic stress [70, 71], however, the protein expression has been shown to be increased [72]. Microglia seem to play an important role in the response to stress and inflammation but also induce a negative influence on mood and behavior in both human and animal models [73, 74, 75, 76, 77]. Furthermore, changed morphology of microglia or loss of these cells due to stress exposure can lead to depressive-like behavior in rodent models together with associated pathological findings such as impaired neuroplasticity and neurogenesis [71, 56, 70]. This correlates with the fact that the hippocampus volume is reduced in depression and there is a high density of microglia in this brain area [68]. In vitro studies have shown that conditioned medium from microglia stimulated with LPS induce apoptosis of hippocampal neuroblasts, which is caused by IL-6 and TNF-α in the medium [55, 78]. Medium from non-activated microglia, however, increased the survival of hippocampal neuroblasts [78]. In summary, chronic stress continuously activate microglia and astrocytes resulting in altered function and structure of these cells as well as secretion of cytokines, which in turn may dysregulate the HPA axis and reduce neurogenesis in the hippocampus (figure 1.2). Furthermore, current antidepressant treatments with SSRI as well as cognitive therapy recover astrocyte and microglia functioning [79]. Altogether, these studies provide evidence that these two cell types together with their cytokine secretion may be involved in the pathophysiology of depression.

1.1.3 Circadian rhythm disturbances

The circadian rhythm is thought to play an important role in depression since patients with depression often experience disruption of the sleep-wake rhythm, temperature, reward and mood, which are all modulated by the circadian rhythm [80, 81, 82]. Moreover, 40-60% of patients with depression improve in symptoms with alternative therapies such as sleep deprivation therapy and bright light therapy indicating that maybe about 50% of depressed patients have circadian abnormalities [81]. In addition, it has been shown that

circadian alterations precede depressive behavior in rodents and, therefore, these alterations may be the cause or predict the onset of depression [83].

The SCN of the hypothalamus is known as the circadian rhythm pacemaker and the internal clock in this pacemaker is controlled by clock genes [80]. The clock genes are present not only in the SCN but in cells throughout the body in order to maintain the circadian rhythm in the whole body [82]. Non-SCN cellular clocks are more vulnerable than the SCN clock to genetic or environmental insults, and this may lead to impairments in processes normally regulated by the clock in non-SCN cells [82]. Throughout the day, different clock genes maintain the body in synchrony with the 24h solar day through an autoregulatory Transcriptional/translational feedback loop (TTFL) [84, 80].

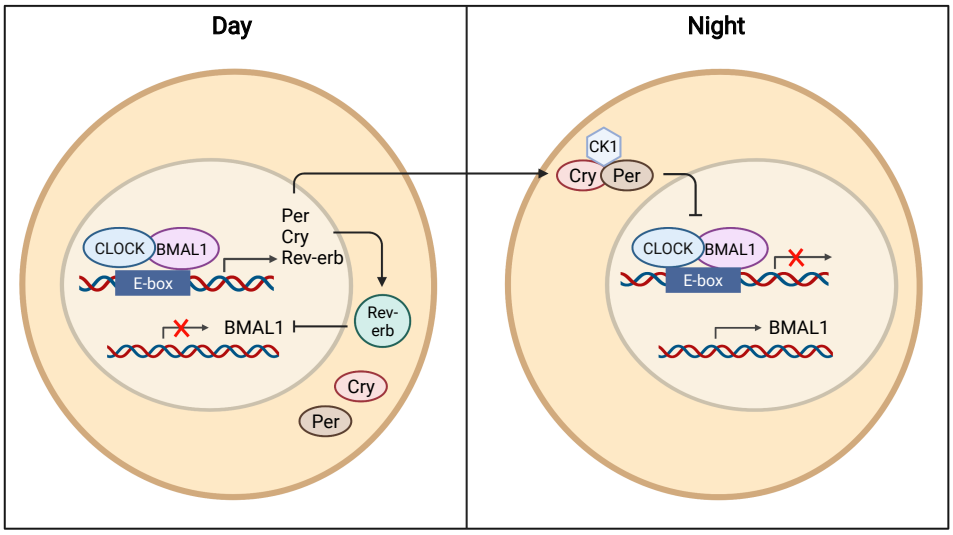


Figure 1.3.: Circadian rhythm of clock genes (inspired by [85]). During the day, CLOCK and BMAL1 forms a heterodimer in the nucleus that bind to an E-box with promoters for Per and Cry respectively. The binding initiates the transcription of Per and Cry which afterwards will be translated into proteins in the cytoplasm. After formation of Per and Cry protein during the day, Per and Cry proteins form heterodimers that are translocated back to the nucleus to interact with the BMAL1/CLOCK heterodimer inhibiting the transcription of Per and Cry during the evening and night. *Abbreviations: Period (Per), Cryptochrome (Cry), Circadian Locomotor Output Cycles Kaput (CLOCK), Brain and Muscle Arnt-like protein-1 (BMAL1), Casein kinase 1 (CK1), Retinoic Acid-related Orphan Receptor (Rev-erb), Enhancer box (E-box)*. Made in BioRender by Freja Pretzmann.

The main clock genes regulating the circadian rhythm are Period (Per) and Cryptochrome (Cry) and their expression is controlled by Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle Arnt-like protein-1 (BMAL1) together with their own protein products. The transcription of CLOCK and BMAL1 are regulated by Retinoic Acid-related Orphan Receptor (Rev-erb) [80, 86]. BMAL1 and CLOCK form a heterodimer in the nucleus, which permanently bind to Enhancer box (E-box) promotor elements in promoters for Per or Cry. This initiates the transcription of Per and Cry DNAs into mRNAs, which move into the cytoplasm where they are translated into proteins. The Per and Cry proteins form heterodimers, which create a complex with Casein kinase 1 (CK1) leading to translocation of the Per/Cry heterodimer back to the nucleus to interact with the BMAL1/CLOCK heterodimer causing inhibition of their own transcription (figure 1.3). An imbalance between these circadian clock genes can disrupt the circadian rhythm, which may lead to changes in metabolism, mood and sleep [80, 82, 81, 87, 88].

The SCN provides the circadian regulation through major outputs to the PVN of the hypothalamus. The PVN converts signals from the SCN into autonomic and hormonal signals through the ANS and the HPA axis [89]. Thereby, GC secretion throughout the day is adjusted to the light/dark cycle by the SCN. When the active phase begins (light phase for humans, dark phase for mice), GC levels (cortisol in humans, Corticosterone (CORT) in mice) are high in order to increase metabolism and prepare the body for activities. Throughout the active phase, the GC levels fall and reach a minimum at the end of the active phase, when it is time for the body to relax and fall asleep. During the inactive phase, the GC levels start to rise again to reach a maximum at the beginning of the active phase (figure 1.4 and 1.5) [83, 33].

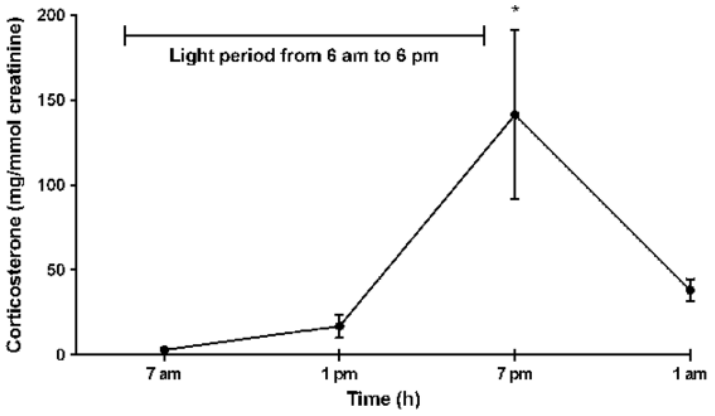


Figure 1.4.: Diurnal urine corticosterone levels in mice [90].

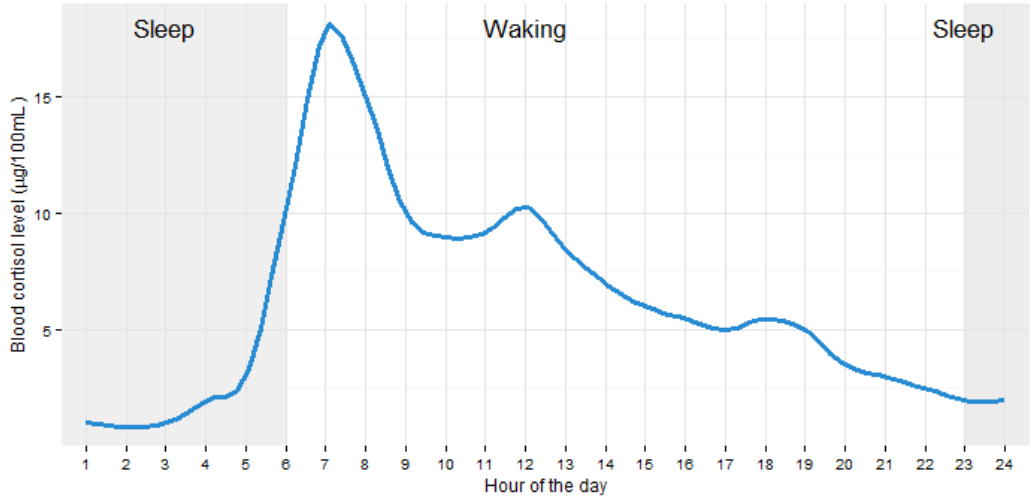


Figure 1.5.: Diurnal blood cortisol [91].

Clock genes regulate sleep-wake rhythms and has shown to be associated with mood symptoms. The relation to mood symptoms may be through circadian regulation of target neuronal firing rate, cellular metabolism rate or number of released neurotransmitters and neuromodulators [89]. In addition, the clock is phase delayed in depression and the extent of delay correlated with the severity of depression [92]. This phase delay of the clock is in line with the tendency for patients with depression to prefer later schedules [93]. A study on patients with major depression also showed disruption of the diurnal rhythm of Per1, Per2, Cry1 and BMAL1 gene expressions in the blood [94]. Thus, it may be interesting to investigate these clock genes in animal models of depression to further discover their impact on this disorder.

Studies in mice have shown that clock gene mutations in BMAL1, CLOCK or Per2 lead to hypocortisolism [95, 96, 97], while mutations in Cry result in hypercortisolism [98, 99]. In addition, a study has found Cry2 gene expression to be associated with depression [100]. Moreover, Cry1 and Cry2 can regulate the activation of GR through direct binding to the C-terminal domain, which is where the ligand-binding also happens. Thereby, Cry repress GR-mediated activation of target genes but also participate in the GC-dependent suppression of the HPA axis [33, 101, 84]. However, the binding does not affect the ability of GRs to suppress the expression of several inflammatory genes [101]. Moreover, deletion of both Cry genes resulted in elevated GC levels without diurnal rhythm due to the lack of inhibiting feedback [99]. Rodents exposed

to acute stress during the inactive phase had increased Per1 mRNA in non-SCN cells in the PVN and PFC but not the SCN itself [102, 103] since this structure lacks GRs [104]. No change was seen for Per2 (Takahashi et al. 2001). In addition, chronic stress or repeated stress changes the rhythmicity of Per1 and Per2 mRNA in non-SCN tissue [105, 106]. These results suggest that stress affects the circadian activity of the HPA axis through changes in the gene expression of Per1 and Per2. On the other hand, Per2-deficient mice show reduced depression- and anxiety-like behaviors [107] and the gene expression of Per2 in the PFC of rats exposed to CMS were significantly decreased [108]. A study showed that a mouse line mutant in the Per2 gene have a higher density of proliferating neural progenitor cells as well as increased number of new neurons in the dentate gyrus. However, lack of the PER2 gene does not change the total generated number of adult neurons since it also causes increase in neuronal cell death [109]. These results are in line with the fact that clock genes control the timing of cell-cycle entry and exit of Quiescent neural progenitor cells (QNP). Particularly, lack of Per2 eliminates gating of cell cycle entry for QNPs while absence of BMAL1 lead to increased proliferation and delayed exit of the cell cycle [110]. The role of Cry in regulating GR activation and Per in adjusting the circadian rhythm in response to environmental stimuli such as stress, makes these genes relevant to investigate further in animal stress models.

1.2 Stress-induced depression models in rodents

In general, animal models are crucial in biomedical research [111], and regarding stress response, animal models are optimal since the HPA axis is preserved in many species, including rodents [112]. Animal models can be used to determine essential biological factors in depression such as inflammation, clock dysfunction of circadian related genes and allostatic overload of the HPA axis as well as discovering new drug targets and biomarkers. Therefore, it is important to keep developing and optimizing animal models for mood disorders. The critical challenge with animal models for investigation of mood disorders is that these models do not fully recapitulate the spectrum of these disorders [82]. Furthermore, there is no objective diagnostic tools such as biomarkers, neuroimaging, biopsies etc. for depression. Right now, clinical examination

together with a subjective evaluation of depressive symptoms is the only way to diagnose depression [4, 5], which makes it more difficult to develop animal models of depression. It is complicated to define a depressive-like state for an animal compared to humans. We can ask a human how one feels, but with animals, we must define a change in behavior, which can be qualified to reflect a depressive-like state since there is a lack of more objective diagnostics [4]. While humans can describe the feeling of anhedonia and apathy, in rodents, nest building and burrowing can be evaluated to determine spontaneous motivation [113, 114]. Both nest building and burrowing are natural mouse phenotypic behaviors corresponding to activities of daily living [115, 116], which are disrupted in depression and is indicative of symptoms like reduction of energy and loss of interest as described in patients with depression (table 1.1). As anxiety and depression are closely associated [117] the Elevated plus maze (EPM), which is the gold standard assay to measure anxiety-like behavior, is commonly used in animal stress models [118]. Furthermore, movement patterns in mice throughout hours or days may be interesting in stress models since increased locomotor activity has been observed in mice exposed to chronic stress [119, 120] as well as disturbed diurnal activity patterns indicative of sleep disturbances in mice exposed to repeated stress [121]. Moreover, another physical sign of chronic stress in rodents, which can easily be measured is body weight change [122], which is also commonly seen in patients with depression. In addition, we can combine these behavioral results with biochemical assays such as Enzyme-Linked Immunosorbent Assay (ELISA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR) that measure the same changes as previously seen in patients with depression.

Many of the current stress models have been predominantly developed in rats for several reasons such as larger amount of tissue for downstream analysis and better circuit understanding, however, in neuroscience research the use of mice has gone from 20% in the 1970s and 1980s to about 50% the past 10 years [123]. Still, it is easier to handle rats as they are not as easily stressed by human contact as mice, and therefore can be repeatedly handled prior to behavioral assays as a routine practice [123], whereas mice often would be stressed by this procedure [124]. However, mice have several advantages over rats such as less inter-strain variability, more economical and they can be made transgenic [122]. Chronic stress models in mice show similar changes as those observed in depressed patients such as allostatic overload of the HPA axis, glial abnormalities and depressive-like behavior [27, 122, 79, 125, 126, 127] but

the reproducibility of these chronic stress models to induce a depressive-like state in rodents remains disappointing [120, 128]. There can be variability in resistance and vulnerability to stress between mice strains [129] and it is therefore important to consider the strain of mice for a chronic stress model but also when comparing different studies. One of the most used mouse strains in neuroscience is C57BL/6 [123], which was also used in this thesis, more specifically C57BL/6 J. This strain is more resilient to stress than others [130, 131, 132].

Several different chronic stress models such as Chronic unpredictable stress (CUS) [133, 122], CMS [108, 134], Repeated restraint stress (RRS) [133, 127, 75], social defeat stress [135] among others [120], have been made and studied in rodents. Furthermore, there is many different assays to assess depressive-like and anxiety-like behavior [128]. Consequently, the protocol variability is huge, which makes it hard to compare the results of different studies and the behavioral results are inconsistent and not reproducible [120]. Therefore, this thesis seeks to characterize three different stress models, a Chronic stress (CS) model developed by Lundbeck A/S with multiple milder stressors, a RRS model with just a single severe stressor for 14 days (RRS14) and an Interrupted RRS (iRRS) model with 3 days of stress followed by 48 hours of rest continuing for 20 days. The purpose is to find a mouse model that creates allostatic overload of the HPA axis and a depressive-like phenotype, which can be used to study stress biology and depression with the ambition of discovering potential biomarkers.

1.3 CS

The CS model seeks to mimic stress-induced depression in humans with daily mild stressors as previous CMS models in rodents have been shown to do by causing long-lasting changes in behavior and in the brain, similar to those changes observed in patients with depression [6, 7, 8, 9]. However, the CS protocol combine CMS with jetlag stress, inspired by jetlag models (10h/10h or 8h/8h light/dark cycle) which have previously induced depressive-like behavior in rodents [10, 11] as well as impaired cognition and decreased neuronal complexity in mice [10]. Furthermore, rats exposed to CUS for 4 weeks displayed depressive-like behavior and changes in clock gene expression in the

hippocampus, which persisted for at least two weeks after the termination of stressors [136, 137]. This evidence from previous studies were used as the basis of the CS model described in this thesis.

1.4 RRS14

The RRS14 model is shorter and simpler to perform with only one stressor compared to the CS model. However, it does not mimic stress-induced depression, as known in humans, to the same extent as CS. Still, RRS in rats has been shown to induce atrophy of neurons and shortening the lengths of apical dendrites as well as decrease the density of spines in hippocampal neurons [138, 139]. Additionally, mice exposed to RRS show depressive-like behavior [140]. These studies, however, performed RRS for 21 days and for several hours each day, while in this thesis the animals were only stressed for 30 minutes each day for a maximum of 14 days. Still, studies have found that 2h of Restraint stress (RS) each day for 14 days induced depressive-like behavior in mice [141, 142, 143], which may persist up to two months [141].

1.5 iRRS

The last model is iRRS, which seeks to investigate whether there is a change in the response to stress when interrupted by rest periods. The model was inspired by Benini et al. 2019 [144] that investigates cardiovascular responses to RS of different lengths, frequencies and number of stress sessions in male rats where the combination of 3 days of stress and 48h rest seemed to be most stressful [144].

2 Aim

Examine the ability of three different stress models to induce allostatic overload of the HPA axis and a depressive-like phenotype in male mice.

2.1 Hypotheses

1. CS with multiple stressors induces sustained elevated basal plasma CORT levels, changes in phenotypic behavior in mice and changes in gene expression of clock genes and markers of neuroinflammation in the brain.

2. RRS14 induces an increase in acute and basal plasma CORT, changes in phenotypic mouse behavior and changes in gene expression of neuroinflammation markers.

3. iRRS induces sustained elevated plasma CORT, changes in phenotypic behavior in mice and changes in gene expression of neuroinflammation markers.

3 Method

All in-vivo and ex-vivo studies were performed at Lundbeck A/S by the author, sometimes with assistance from Lundbeck employees.

3.1 General housing of mice

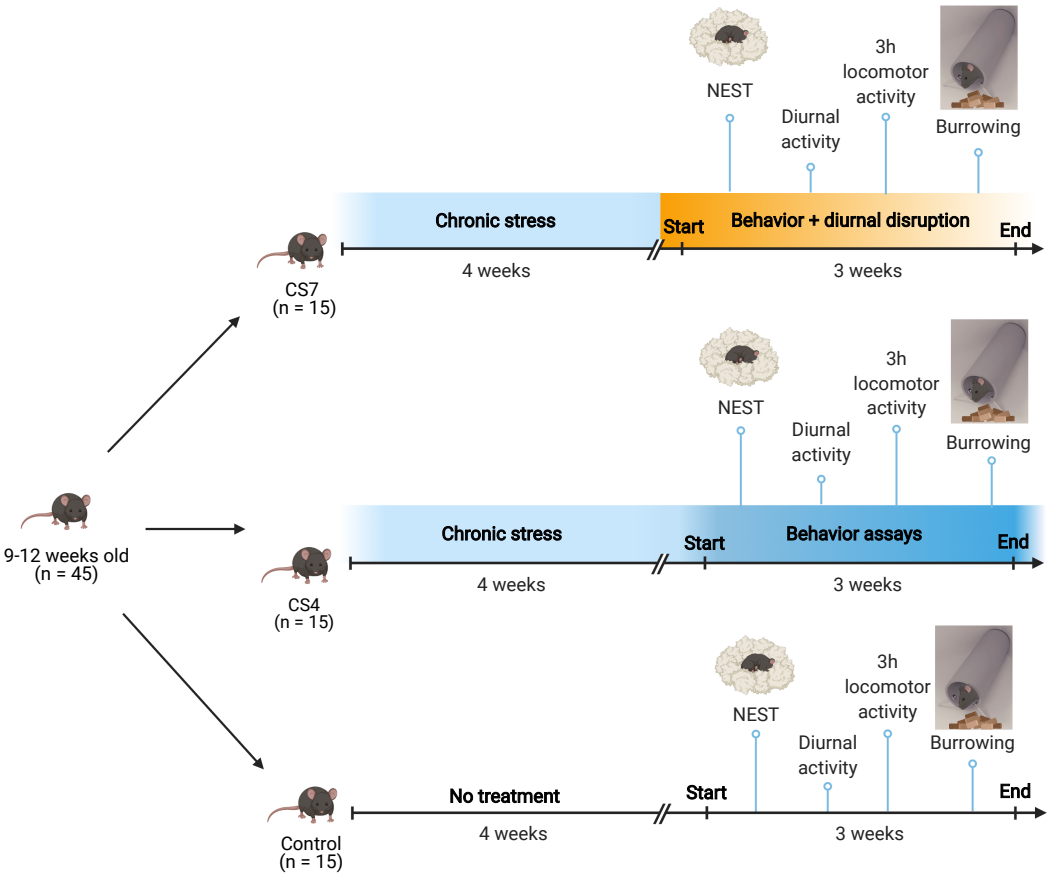
All experiments in this thesis was performed using male C57BL/6JBomTac mice from Taconic Biosciences; age: 9-12 weeks, weight: 20-30g. All mice were single housed in a Macrolon type III low cage (20 $\times$ 35 $\times$ 18 cm) with food and water ad libitum together with plastic igloos (Bio-Serv Mouse Igloo), aspen bricks, nesting material and food enrichment. Furthermore, the room temperature and humidity were 21$\pm$2°C and 55%$\pm$5%, respectively. The mice had a 12h/12h light/dark cycle with lights on at 06:00 am. The mice were allowed to acclimatize for at least one week after arrival before experiments were initiated. As per standard in the Lundbeck animal facilities, radio noise was provided during the light period and turned off during the dark period. All animal procedures were carried out in compliance with the Danish legislation regulating animal experiments; Law and Order on Animal experiments; Act No. 474 of 15/05/2014 and Order No. 2028 of 14/12/2020, and with the specific license for this experiment issued by the National Authority.

3.2 Experimental designs

3.2.1 Chronic stress (CS)

The CS protocol was inspired by previous CMS protocols in mice [120, 128, 145, 6] in combination with jetlag models [10, 11]. In the CS model, a total of 45 mice were used, which were randomly assigned to one of three groups (CS4, CS7 or Control, n=15/group). CS4 and CS7 were exposed to diurnal

disruption by a 10h/10h light/dark cycle for four weeks together with different stressors each weekday in the following order: Monday; food deprivation overnight, Tuesday; cage tilt at 45°C overnight, Wednesday; water deprivation overnight, Thursday; food deprivation overnight, Friday; confinement to small container for 1h (4,5 cm $\times$ 8 cm $\times$ 12 cm) (figure 3.1). Every Monday morning the mice were weighed. After the 4 weeks of stress, CS7 animals continued with a 10h/10h light/dark cycle while the other stress group, CS4, returned to a 12h/12h light/dark cycle during the three-week period of behavioral assays. The behavioral assays for this study included Nest building assay (NEST), diurnal locomotor activity (72hrs), locomotor activity (3hrs) and burrowing assay.



Days	Stressors	Diurnal disruption
Monday	Food deprivation overnight	10h light/10h dark
Tuesday	Cage tilt (30-45°) overnight	10h light/10h dark
Wednesday	Water deprivation overnight	10h light/10h dark
Thursday	Food deprivation overnight	10h light/10h dark
Friday	Confinement to small container (1h)	10h light/10h dark
Saturday	-	10h light/10h dark
Sunday	-	10h light/10h dark

Figure 3.1.: The experimental setup for the CS protocol. Two stress groups, CS4 and CS7, were exposed to the same 4-week CS protocol while only one continued with diurnal disruption during the behavioral assays (CS7). The CS protocol consists of different stressors throughout the week together with a 10h light/10h dark cycle during the 4 weeks of stress, which is displayed in the table.
Abbreviations: Nest building assay (NEST). Made in BioRender by Freja Pretzmann.

3.2.2 Repeated restraint stress (RRS)

The mice were restrained for 30 minutes in a clear plastic syringe equipped with air holes (15 cm in length and 3.1 cm in diameter) with a plunger to adjust the space to the size of the mouse, consequently restricting movement in all directions (figure 3.2). The mouse tail was sticking out from the cylinder and the tip of the syringe had been cut open, allowing the mice to breathe through this hole. The restrained mice were placed on an Abri Soft 60 × 60 (Abena) mat to prevent the mice from freezing.

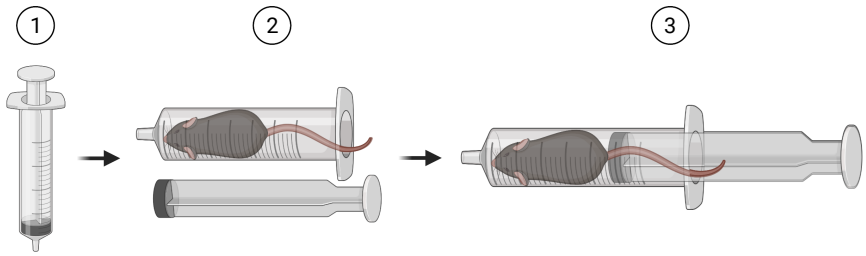


Figure 3.2.: The RRS procedure. 1) Illustration of the restrainer used for RS. 2) The mouse is placed into the syringe tube headfirst 3) the plunger is pushed into the syringe making the mouse unable to move with careful consideration not to trap toes or tail. The tail is taken out of the tube through a hole in the plunger.
Abbreviations: Restraint stress (RS) Repeated restraint stress (RRS). Made in BioRender by Freja Pretzmann.

3.2.3 RRS14

In the RRS14 model, a total of 48 mice were used, which were randomly assigned to control (n=15) or RRS (n=33). On day 1, five control mice were euthanized while the remaining 10 were euthanized at the end of the study. Moreover, five of the RRS mice were euthanized on day 1, 7 and 14 right after stress exposure and another four mice were euthanized on day 15 which is 24h after the last stress exposure to evaluate on both the acute and sustained effect of RS on plasma CORT. The remaining stress mice were euthanized at the end of the study. RS were performed each day between 07:00-11:00 for 14 consecutive days (figure 3.3). All mice were weighed daily during the 14-day stress period and the stress mice were always weighed right before restraint

stress. After the stress period, the behavior of the mice was evaluated with the NEST and the EPM.

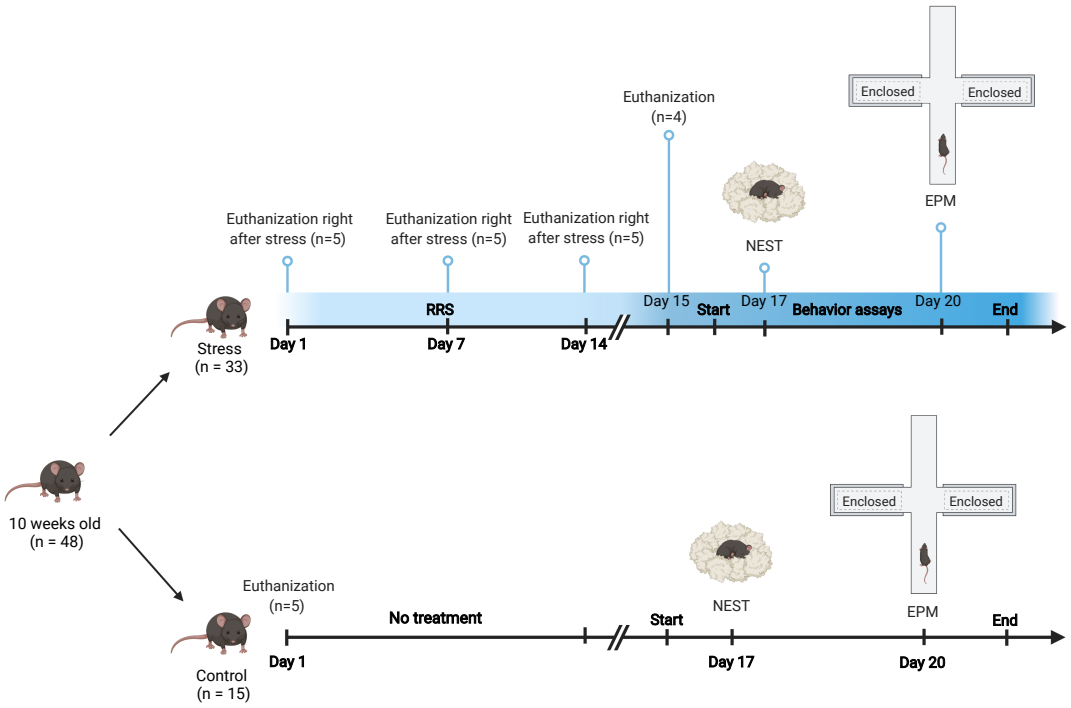


Figure 3.3.: Timeline of the RRS14 study protocol. The study had a total of 48 mice, 33 RRS and 15 Control. The stress group was exposed to 30 minutes of RS every day for 14 consecutive days. On day 1, 7 and 14 a group of five stress mice were euthanized right after stress exposure and another four mice were euthanized on day 15. Five control mice were also euthanized at day 1. After the stress period, the behavioral assays EPM and NEST were performed on all remaining mice.
Abbreviations: Nest building assay (NEST), Elevated plus maze (EPM).
Made in BioRender by Freja Pretzmann.

3.2.4 Interrupted RRS (iRRS)

The iRRS study used a total of 43 mice which were randomly assigned to a control group (n=20) or a RRS group (n=23). A group of 10 control mice were euthanized on day 1 and the last 10 at the end of the study, in order to evaluate on the effect of the study on the control group. On day 10 and 20 respectively, six and seven RRS mice were euthanized (48 hours after last stress exposure) in order to investigate the ongoing effect of the stress paradigm on plasma CORT and inflammatory markers in the brain. A timeline of the study can be seen in figure 3.4. The control mice were handled for 1 minute each

day for 7 days prior to the study in order to achieve lower steady-state basal CORT. The handling procedure include gentle placement of the mouse in the hand (cubbing) and allowing it to explore the handler by walking along the hands and arms. To maintain the effect of handling, the control mice were also handled for 1 minute each day on stress days. The RRS group were exposed to RS each day for 3 days followed by 48h of rest, which continued for a total of 20 days. The stress paradigm was performed before 12 am. The body weight was measured on stress days before exposure to RS. Moreover, control animals were also weighed on stress days. In addition, all the mice were given 200g of food at day 1 to monitor food intake throughout the stress period. Burrowing and locomotor activity were assessed on day 21 and day 23, respectively, to understand potential stress-induced behavioral abnormalities.

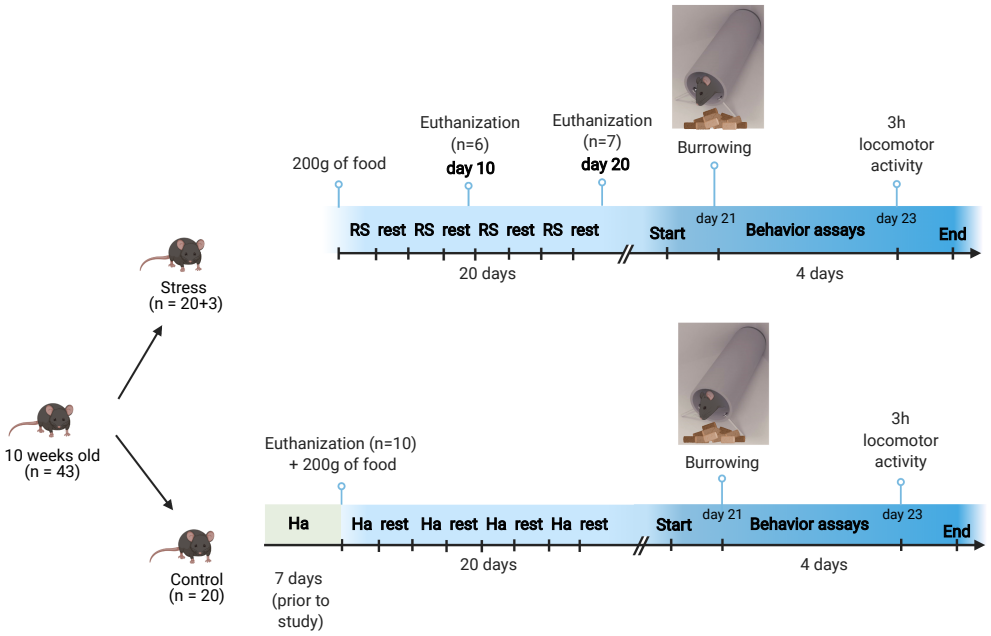


Figure 3.4.: Timeline of the iRRS study protocol. The stress mice were exposed to 30 minutes of RS each day for 3 days followed by 48h of rest. Control mice were handled for 1 minute each day 7 days prior to the study initiation and on all stress days. This process continued for 20 days followed by behavioral assays. On day 1, 10 control mice were euthanized and on day 10 and 20 a subcohort of stress mice were euthanized.
Abbreviations: Restraint stress (RS), Handling (Ha). Made in BioRender by Freja Pretzmann.

3.3 Behavioral assays

3.3.1 Locomotor activity

The locomotor activity of RRS14 and CS mice were investigated by a 3h locomotor activity assay in the UMOT system (figure 3.5).

Figure 3.5.: The UMOT system. The system is used for determining locomotor activity in mice. It consists of a large cabinet (C) with 16 empty Macrolon type III low cages (A). The cabinet is equipped with infrared light sources and photocells to detect photo beam crossings

The UMOT system consist of a large cabinet with 16 empty Macrolon type III low cages with a plastic lid with holes for ventilation. The cabinet is equipped with 4 $\times$ 8 infrared light sources and photocells; two placed 2.5 cm above the floor of the cage on each side (activity measure) and two placed 7.5 cm above the floor of the cage on each side (rearing measure). The mice were transferred to the experimental room at least 2h prior to the test. Mice were placed individually in the activity box and allowed to freely explore for 3h with no disturbances and afterwards returned to their respective home cages. Photo beam crossings were automatically scored and collected in 5-minute bins using UMOTWin (Ellegaard Systems A/S, Denmark). The resulting data output was locomotor activity count (lower light beam source) and rearing count (higher light beam source).

3.3.2 Diurnal locomotor activity

CS mice were placed individually in Macrolon type III low cages with minimal bedding and food enrichment as well as food and water ad libitum. The same equipment as described in section 3.3.1 was used for the measurement of diurnal activity (figure 3.5). The mice were monitored for 72h. The light/dark cycle was 12h/12h for control and CS4 while CS7 was assayed under 10h/10h light/dark conditions. Activity counts were pooled in 1h intervals. Data were collected using UMOTWin (Ellegaard systems, Denmark).

3.3.3 Nest building assay (NEST)

NEST was performed in the CS and RRS14 studies according to the previous protocol by Pedersen et al. 2014 [114] with a few adjustments. The equipment for NEST included Igloos and nestlets (5 $\times$ 5 cm squares of cotton batting). NEST was performed in the respective home cages and initiated at 7 am by removing any prior nesting material, placing the igloo in the left back corner and placing one nestlet in the right front corner of the cage. Quality of nests were scored every hour for 8h and after 24h, starting 1h after nestlet placement. NEST scores were given for two criteria; the degree to which the nestlets were shredded (on a scale from 0 to 5 points) and the coverage of the three igloo-openings (one point per coverage). The two scores were summed up for each mouse per time point, giving a maximum score of 8 (figure 3.6).

Furthermore, the score cannot be lower than the previous time points. The room temperature is of great importance for nest building activity of the mice, since making nests serves the purpose of controlling temperature [146]. Thus, the temperature in the test rooms was measured at all time points using a medical precision thermometer DM 852 (Ellab Copenhagen). The optimal room temperature for NEST has previously been reported to be 21°C ± 1°C [114].

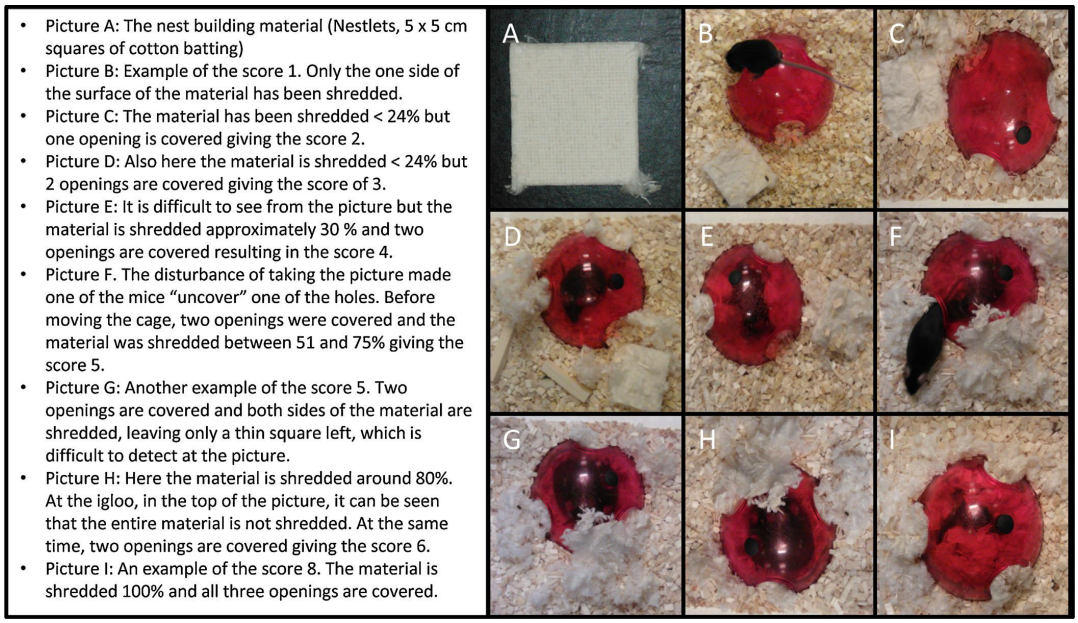


Figure 3.6.: **Nest score system.** Illustration of how to score nesting behavior in the nest building assay [114]. Up to 5 point can be given for shredding of the nestlet while 1 point is given for each opening of the igloo that has been covered. This gives a maximum score of 8 points.

3.3.4 Burrowing

The burrowing assay was used for CS and iRRS and involves tubes with a 45-degree slope filled with 270g of food pellets (figure 3.7). One tube filled with food was added to each of the mouse home cages containing only bedding and food enrichment. The tubes were added to the mouse home cages at 8 am and the weight of the food remaining in the tube was measured after 2h, 6h and 24h and deducted from the starting amount of 270g to calculate the amount of food pellets removed from the tube. Acclimatization to the burrowing tube overnight in the home cage prior to the study was performed for iRRS but not CS, which means the mice in iRRS also performed burrowing in a new clean cage, and not their respective home cage.

Figure 3.7.: Burrowing tube. Placement of the burrowing tube in the cage for the burrowing assay. After 2h, 6h and 24h respectively, the number of pellets displaced from the tube was calculated in grams. At the end of the assay, the mice were returned to a clean cage

3.3.5 Elevated plus maze (EPM)

The EPM assay was used to measure anxiety-like behavior in mice. It consists of two closed arms (25 $\times$ 5 $\times$ 30 cm) with high dark walls, two open arms (25 $\times$ 5 cm) with 1 cm tall transparent walls to avoid falls and a center area of 5 $\times$ 5 cm (figure 3.8). The maze is elevated 50 cm from the ground and placed in the center of the test room to ensure an "open space" feeling in the open arms. An overhead camera records all mouse movements, which is automatically scored using EthoVision XT Noldus. The mice were brought to the experimental room at least 20 minutes before testing. A mouse was placed in the center of the EPM and allowed to explore freely for 5 minutes and afterwards returned to its home cage. The EPM was cleaned with 70% ethanol before initiation of a new trial.

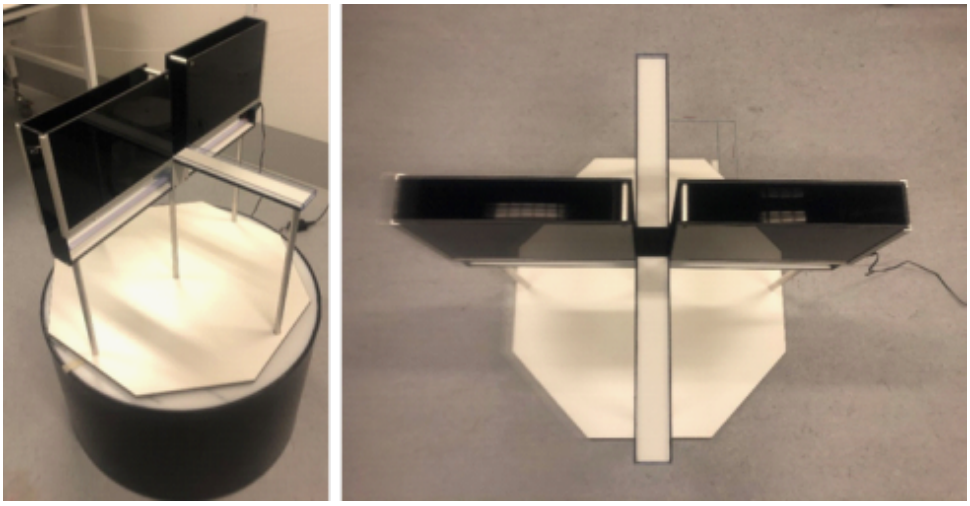


Figure 3.8.: The EPM setup for mice. A mouse was placed in the center of the EPM at the beginning of the test and was allowed to explore freely for 5 minutes. Afterwards, the mouse was returned to the home-cage and the maze was cleaned with 70% ethanol.
Abbreviations: Elevated plus maze (EPM)

3.4 Euthanization and tissue collection

All mice were euthanized by decapitation with help from trained staff at Lundbeck A/S. Thorax blood was quickly collected in 500 μl Microvette® EDTA K3E tubes (Sarstedt, Germany) and centrifuged for 15 minutes at 3500 x g at 4°C. Plasma was collected in 1.40 ml Micronic tubes (Micronic, MP32022) and stored at -80°C. Brains were removed and either divided sagittally into right and left hemisphere (CS) or portioned into cortex, middle brain and hippocampus (RRS14 and iRRS) as shown in figure 3.9. All tissues were snap-frozen on dry ice and stored at -80°C until further use. Later, the left hemispheres from the CS study were divided into forebrain and center brain, as illustrated in figure 3.9, for further analysis.

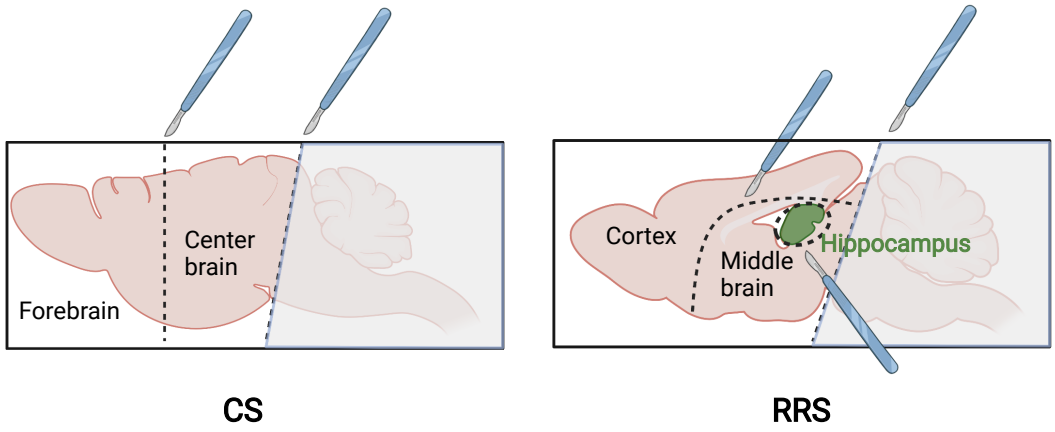


Figure 3.9.: Tissue collection. illustration of how forebrain and center brain sections from the CS model were cut as well as hippocampus, middle brain and cortex for the RRS studies.
Abbreviations: Chronic stress (CS), Repeated restraint stress (RRS). Made in BioRender by Freja Pretzmann.

3.5 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Brain samples from all studies were weighed and homogenized in Precellys tubes of 0.5 ml (Bertin Corp. Precellys lysing kit, cat. no. P000933-LYSK0-A), 2 ml (Bertin Corp. Precellys lysing kit, cat. no. P000973-LYSK0-A.0) or 7 ml (Bertin Corp. Precellys lysing kit, cat. no. P000935-LYSK0-A). Prior to homogenization, the samples were allowed to thaw for 5 minutes. Samples were homogenized in 10 volumes of Phosphate buffered saline (PBS) containing 0.11% diethyl pyrocarbonate (Aldrich, CAS no. 1609-47-8) and cOmplete protease inhibitor (Roche, cat no. 11697498001/50-100-3301) using a Precellys Evolution Homogenizer (Bertin Technologies) at 5000 RPM at 4°C for 2 $\times$ 50s cycle with 10s interval between cycles. Each sample was split into two aliquots; 300 μl for RNA extraction and the remaining was saved for potential protein analyses. The aliquots were stored at -80°C until use. RNA purification of homogenized brain tissue was accomplished by following the manufacturer's protocol; NucleoSpin RNA Mini (Machery-Nagel, cat. no 740955.50). A NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific™) was used to quantify the resulting RNA concentration and purity. All RNA concentrations were used to calculate the amount of RNA needed for comple-

mentary DNA (cDNA) synthesis. Calculation were made for 500ng RNA pr. sample. In order to synthesize cDNA from the purified RNA samples, iScript cDNA Synthesis Kit (Bio-Rad, cat. no 1708891) was used and performed according to the manufacturer's protocol. The samples were run in a PTC-200 Peltier Thermal Cycler (Bio-Rad/MJ Research) with a profile of 5 minutes of priming at 25°C, 20 minutes of reverse transcription at 46°C and 1 minute of Reverse transcriptase (RT) inactivation at 95°C followed by hold at 4°C. Afterwards, cDNA was diluted 1:4 in distilled RNAse/DNAse free water. PCR analyses were performed on the synthesized cDNA to understand potential stress-effects on target genes normalized to the reference gene GAPDH (table 3.1).

Table 3.1.: TaqMan Gene Expression assay primer probes used for RT-PCR.

Target gene	# Catalog	Assay-ID
GAPDH	4331182	Mm99999915_g1
Per1	4331182	Mm00501813_m1
Per2	4331182	Mm00478099_m1
Cry1	4331182	Mm00514392_m1
Cry2	4331182	Mm01331539_m1
IL-6	4331182	Mm00446190_m1
IL-1β	4331182	Mm00434228_m1
TNF-α	4331182	Mm00443258_m1
IL-10	4331182	Mm01288386_m1
Nr3c1 (GR)	4331182	Mm00433832_m1
GFAP	4331182	Mm01253033_m1
Aif-1 (Iba-1)	4331182	Mm00479862_g1

TaqMan® Gene Expression Assay primer probes were purchased from ThermoFisher. These contain forward and reverse primers and a minor-groove-binding (MGB) Taqman probe labelled with the fluorescent dye FAM. The PCR was carried out according to TaqMan® Fast Advanced Master Mix User Guide (Applied Biosystems). Each sample was analyzed in duplicates with 4 μl cDNA pr. well followed by 6 μl mastermix containing TaqMan Fast Advanced Mastermix (5 μl), RNAse/DNAse free water (0.5 μl) and Taqman primer probe (0.5 μl). A no template control (NTC) was included in all assays. Each well had a final volume of 10 μl. The PCR reactions were carried out using CFX384

Touch Real-Time System (Bio-Rad) with the thermal profile presented in figure 3.10. CFX Maestro Software (Bio-Rad) was used to obtain and withdraw data for further analyses. The results were calculated by the $2^{-\Delta\Delta CT}$ method relative to the control group. For iRRS and RRS14, expression of target genes was related to control groups euthanized on day 1.

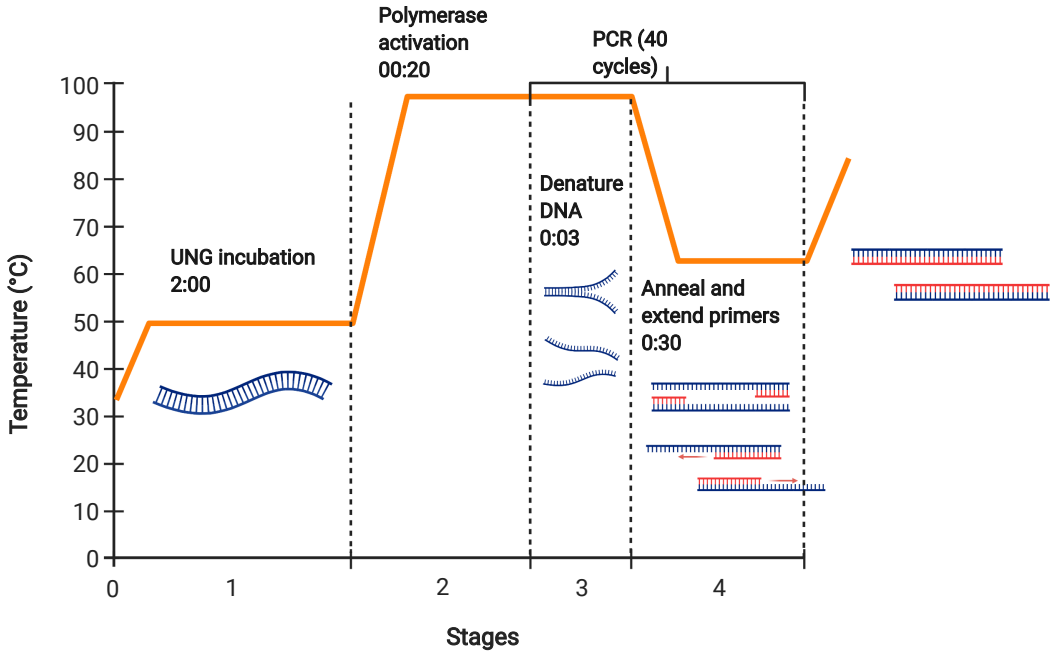


Figure 3.10.: PCR thermal profile. Thermal profile of the TaqMan RT-PCR run in this thesis.
Abbreviations: Polymerase Chain Reaction (PCR), Deoxyribonucleic acid (DNA), Uracil-DNA glycosylase (UNG)

3.6 Enzyme-linked Immunosorbant Assay (ELISA)

Plasma samples was assayed for CORT levels with a competitive ELISA according to the manufacturer's protocol; DetectX Corticosterone Enzyme Immunoassay Kit (Cat. No. K014-H1) from Arbor Assays. The ELISA plate was read with a Multiskan™ FC Microplate Photometer (Thermo Scientific™) at a 450 nm wavelength.

3.7 Statistical analysis

All data and graphs were analysed using GraphPad Prism V9. Values are shown as means ± Standard error of the mean (SEM). The statistical analyses used to discover significant differences were unpaired Student's t tests (with or without Welch's correction) when comparing two groups, and one-way or two-way Analysis of variance (ANOVA) with Bonferroni correction for more than two groups or two independent variables. ANOVA was also used for making multiple comparisons which allows pairwise comparisons (Bonferroni) or comparison with a control (Dunnett's method) to find more specific significant differences. A statistically significant difference was defined as a p-value $\leq$ 0.05.

4 Results

4.1 CS

4.1.1 CS lead to changes in motivated behavior but not in body weight

To monitor potential metabolic effects of the CS protocol and general health status of the mice, body weights were measured weekly. Changes in body weight is presented as percentage of change from baseline for each animal (figure 4.1 A). All mice increased in body weight from baseline showing a significant effect of time on body weight ($F(2.262, 95.02) = 65.89$, $P<0.0001$). However, there was no significant difference in body weight gain between Control, CS4 and CS7 during the test period ($F(8, 168) = 1.664$, $P=0.1106$), thus CS did not affect body weight in mice ($F(2, 42) = 0.1524$, $P=0.8592$).

Burrowing and nest building are phenotypic rodent behavior and are used for measuring goal-directed motivated behavior (Roof et a. 2010, Deacon et al. 2001, Jirkof 2014, Pedersen et al. 2014). Therefore, these assays were used to investigate changes in phenotypic mouse behavior indicative of an apathy-like phenotype. Only the burrowing results after 24h is shown in figure 4.1 C, while 2h and 6h are not shown, since none of the mice showed any burrowing behavior at these timepoints (Appendix A). The burrowing results after 24h show two populations within each group, the mice that perform burrowing (>200g removed from the tube) and the mice that poorly perform burrowing (<100g removed from the tube). Therefore, the results are illustrated as how many of the mice within each group that performs burrowing or not in figure 4.1, D. It is clear that about 50% of the mice in the stress groups do not burrow while only 2/15 mice in the control group do not burrow. Thus, this indicate that the CS paradigm results in reduced goal-directed motivated behavior. Next, mice were subjected to the nest building assay. However, there

were no significant difference in nest score between any of the groups at any timepoint (F (16, 336) = 0.6176, P=0.8698) and thereby no effect of CS on nest building (F (2, 42) = 0.1686, P=0.8454) (figure 4.1 B).

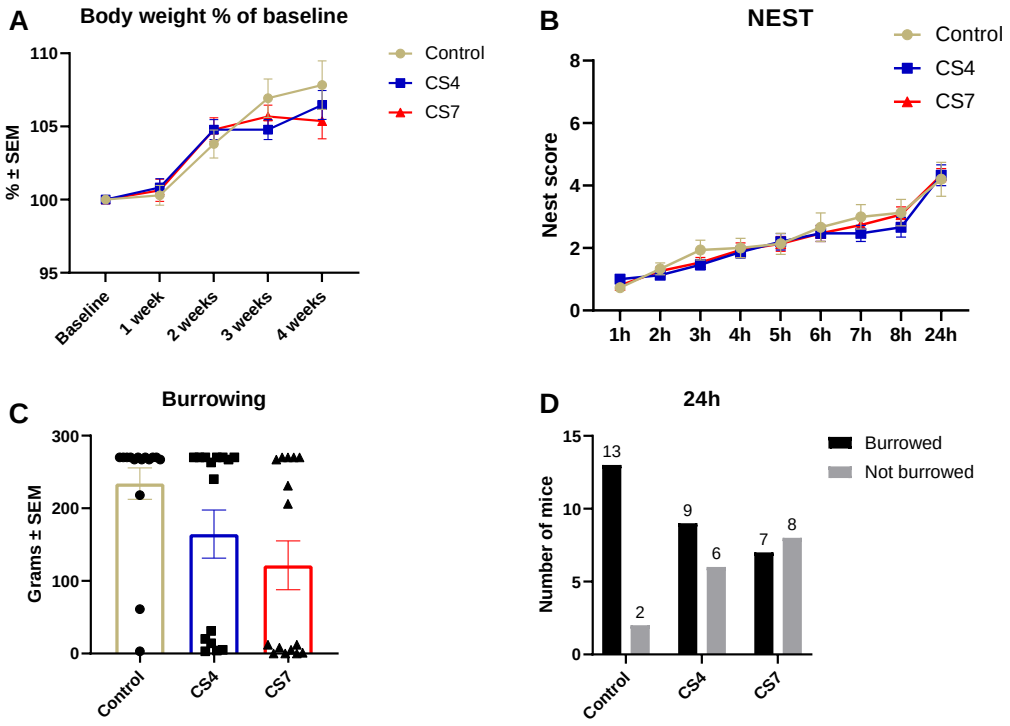


Figure 4.1.: Body weight and behavior results for CS. A) Body weight shown as percent of baseline weight. B) Nest building illustrated over 24h. C) Burrowing shown as the amount of food pellets in grams removed from the burrowing tube after 24h. These data clearly shows two populations. D) The figure shows the number of mice within each group that performed burrowing or did not perform burrowing. Two-way ANOVA with repeated measures was used to measure significant differences between groups.
Abbreviations: Nest building assay (NEST)

4.1.2 Diurnal disruption causes disturbed locomotor activity pattern and clock gene expression in the brain

Diurnal locomotor activity measurement for 72h was performed to evaluate the locomotor activity patterns in light and dark periods, which may also be reflective of disturbed sleep patterns. There is a clear pattern in locomotor activity for control and CS4 which show peaks in activity at the beginning of the dark phase and low activity levels close to or below the light phase mean activity of the control group (335.08, green line figure 4.3) during the light phase. On the other hand, CS7 show no clear activity pattern with several smaller peaks during the dark phase and less rest below the green line during the light phase, indicative of disturbed activity and sleep rhythm. When dividing total activity into light and dark phases, there is a significant difference between groups in the dark phases ($F(2, 42) = 3.484$, $P=0.0398$) but no difference in the light phases ($F(2, 42) = 1.582$, $P=0.2175$) (4.2. Specifically, there is a tendency for CS7 to have decreased activity in the dark phase compared to control ($P=0.0766$) and CS4 ($P=0.0882$). Control and CS4 on the other hand are almost identical ($P = >0.9999$). These data indicate a disturbed locomotor activity pattern in the active phase in CS7 together with a changed activity pattern during the inactive phase suggesting disturbed sleep rhythm. Thus, the ongoing diurnal disruption in CS7 seem to cause the disturbed activity in these mice, since we do not see a changed activity pattern for CS4.

The gene expression of different clock genes in the forebrain and center brain was measured by RT-PCR to evaluate on stress induced disturbances in clock gene expression in the brain. The results are shown in figure 4.5. No significant difference in gene expressions between groups were found in the forebrain and center brain for Cry2 (forebrain: $F(2, 42) = 0.8367$, $P=0.4402$, center brain: $F(2, 42) = 0.1657$, $P=0.8478$). In addition, Per1 gene expression is significantly different between groups in the forebrain ($F(2, 42) = 5.013$, $P=0.0112$) but not the center brain ($F(2, 42) = 1.825$, $P=0.1737$). Moreover, Per2 (forebrain: $F(2, 42) = 10.75$, $P=0.0002$, center brain: $F(2, 41) = 10.86$, $P=0.0002$) and Cry1 (forebrain: $F(2, 42) = 7.731$, $P=0.0014$, center brain: $F(2, 42) = 5.579$, $P=0.0071$) show significant difference in gene expression

between groups in both the forebrain and center brain. The post hoc multiple comparisons analyses show significantly upregulated Per1 (P=0.0075), Per2 (P=0.005) and Cry1 (P=0.0017) in the forebrain as well as Per2 (P=0.0002) and Cry1 (P=0.0068) in the center brain for CS7 compared to control. On the other hand, CS4 only show a tendency to upregulated Per1 in the forebrain (P=0.0648). Therefore, the dysregulated expression of Per1 were more closely associated with the CS protocol, while the expression of Per2 and Cry1 were more affected by continued diurnal disruption.

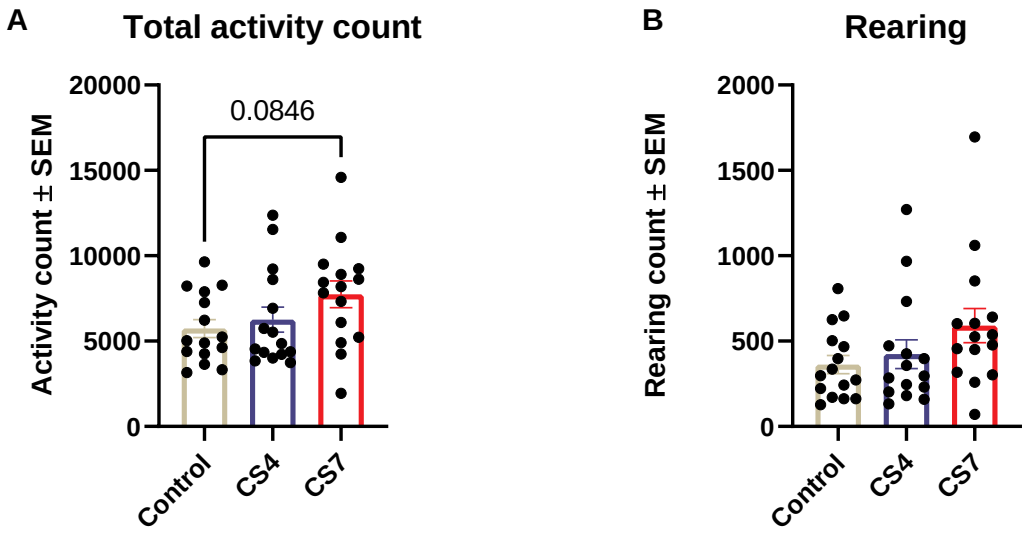


Figure 4.2.: Locomotor activity. Locomotor activity for 3h shown as total activity count and total rearing count measured by the UMOT system. There is a tendency towards increased activity (P=0.0846) in the CS7 group compared to the control group. One-way ANOVA was used to measure significant differences.

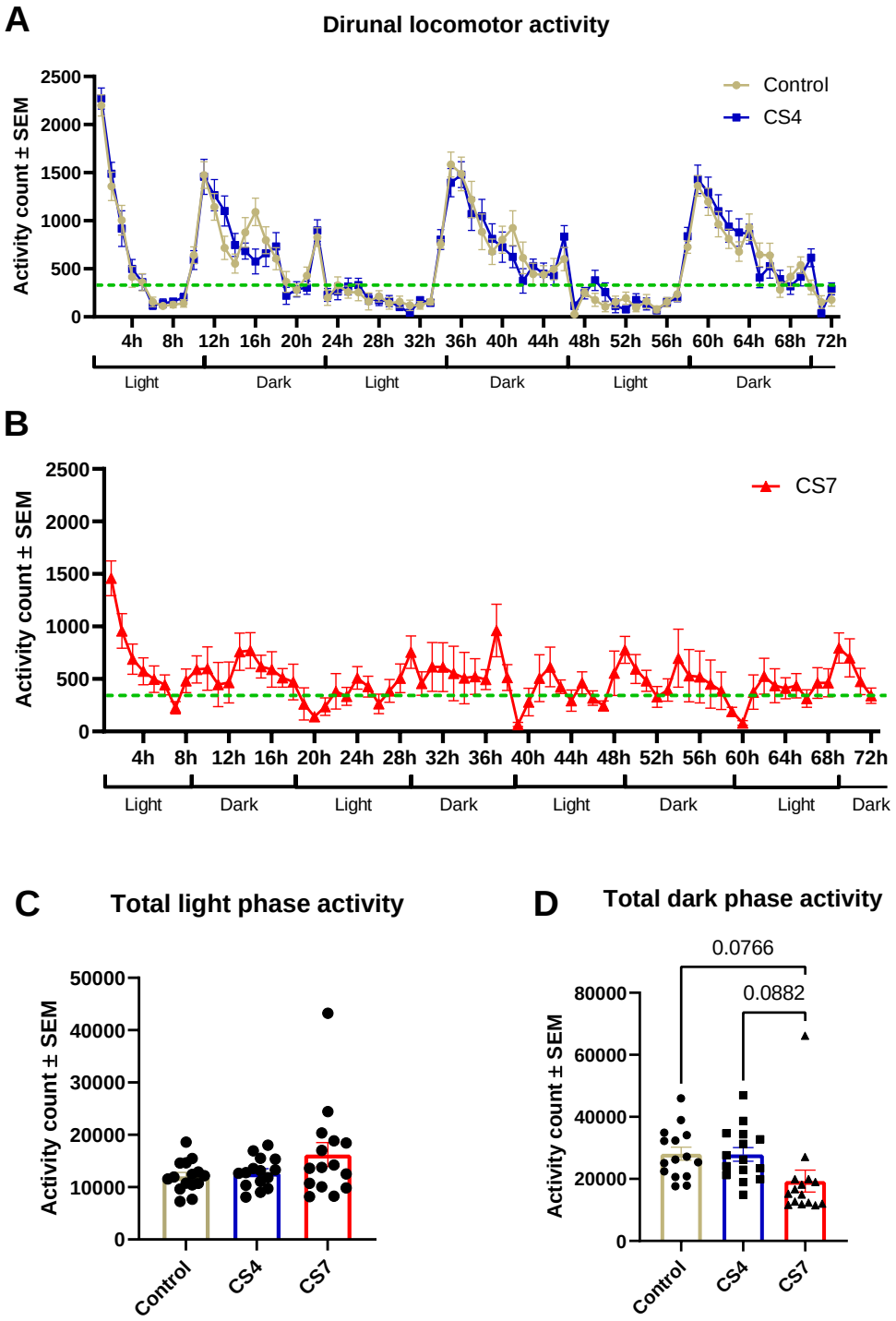


Figure 4.3.: Diurnal locomotor activity. Diurnal locomotor activity pattern over 72h measured with the UMOT system (A and B). A clear change in activity pattern can be observed for the CS7 compared to the two other groups. The green line is the mean light-phase activity of the control group (335.08). C and D shows the total diurnal activity divided into light and dark phase measured for 72h with the UMOT system. One-way ANOVA was used to measure significant differences.

Previous studies have demonstrated increased locomotor activity [119, 121, 120] and decreased rearing in stressed mice [72, 147]. Therefore, the locomotion and rearing were measured for 3h with the UMOT system. There is no significant difference between the groups ($F(2, 42) = 2.259$, $P=0.1170$), however the multiple comparisons results indicate a tendency to increase locomotor activity ($P=0.0846$) in CS7 compared to control. The same tendency seems to be present for rearing, however, the results varies a lot within each group leading to no statistical trend. Since this effect was unique to CS7 and not CS4, it can be postulated that this effect is driven by diurnal disruption.

4.1.3 CS induce allostatic overload of the HPA axis but only in CS7

In order to determine the impact of CS on the HPA axis, plasma CORT was measured, which is a well-established blood-based biomarker of HPA axis activity [148, 5]. The CS paradigm was hypothesized to induce elevated basal CORT levels which would stay elevated throughout the 3-week behavioral period, and thereby indicating allostatic overload of the HPA axis. There is a significant difference between groups for both the tail blood ($F(2, 34) = 4.568$, $P=0.0175$; figure 4.4, A) and euthanization blood ($F(2, 40) = 8.957$, $P=0.0006$; figure 4.4, B). The post hoc multiple comparisons analyses revealed a significant increase in tail blood CORT for CS7 compared to control ($P=0.0115$). However, the tail blood sample were taken after the 4 weeks of stress where CS4 and CS7 had been exposed to the same CS protocol, and thus CS4 and CS7 mice were expected to have comparable plasma CORT levels. This data therefore indicates a variability between groups or variation in the method leading to inconsistencies in the model in its ability to hyperactivation the HPA axis. Furthermore, there is a clear drop in CORT from day 26 to day 51 for both stress groups even though CS7 basal CORT levels are still significantly different from control ($P=0.0007$). However, the difference is still significantly stronger at day 51 ($P=0.0007$) than at day 26 ($P=0.0115$).

Gene expression of GR was measured to evaluate on the effect of stress on this gene. No significant difference was found between groups in GR gene expressions in the forebrain or center brain (forebrain: $F(2, 42) = 1.780$, $P=0.1812$, center brain: $F(2, 41) = 1.086$, $P=0.3470$) (figure 4.5).

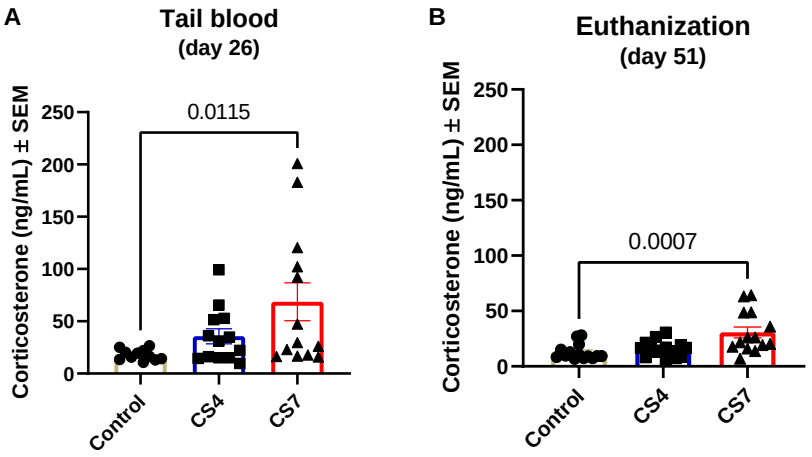
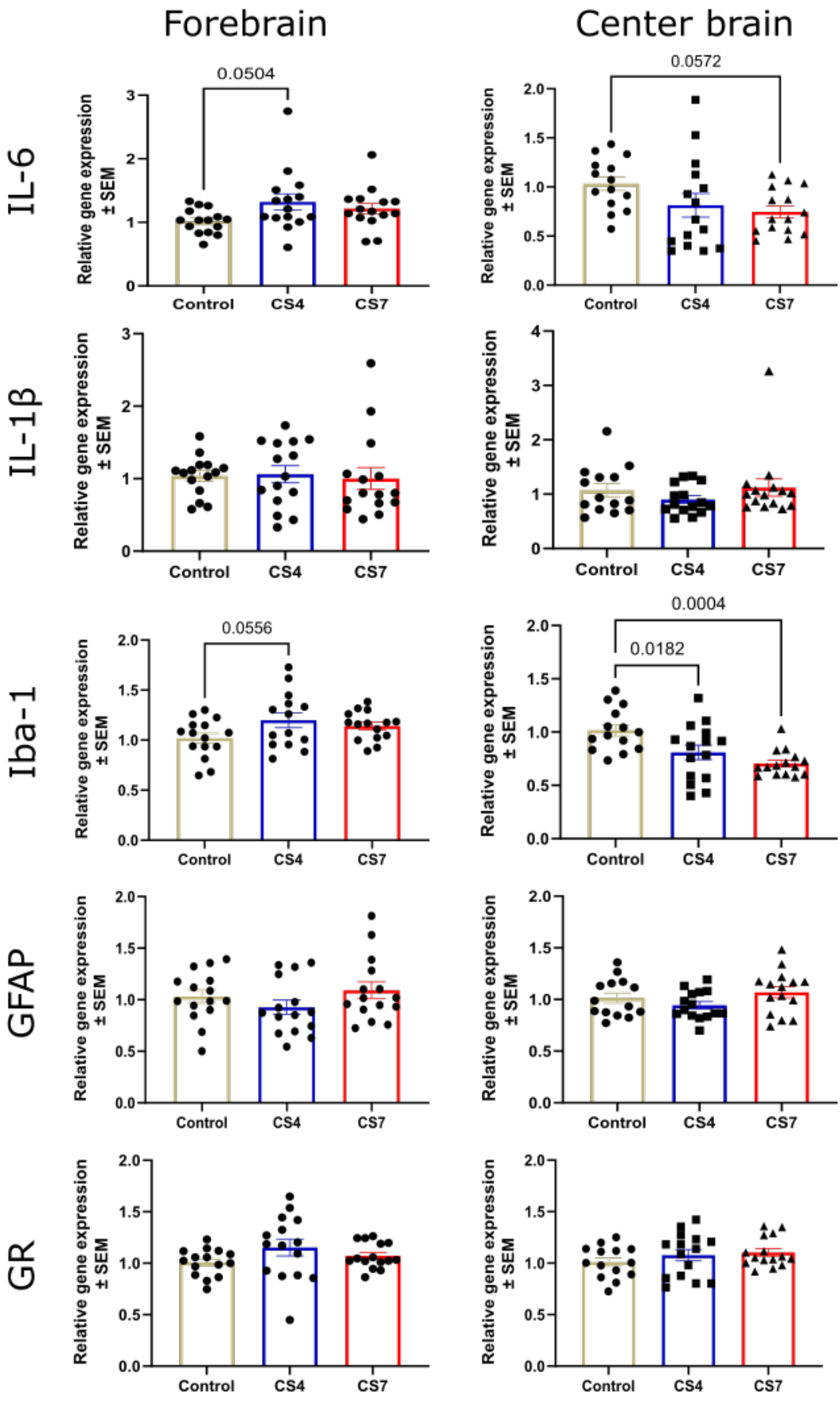


Figure 4.4.: Plasma CORT levels measured with competitive ELISA. CS7 have significantly increased plasma CORT compared to the control group at day 26 (P=0.0115) and day 51 (P=0.0007). However, there is a clear drop in CORT levels from tail blood samples to euthanization samples in the stress groups. One-way ANOVA was used to find statistically significant differences.

4.1.4 CS does not increase neuroinflammation

The gene expression of different inflammatory cytokines in the forebrain and center brain (figure 4.5) was measured by RT-PCR to evaluate on stress induced inflammation in the brain. The data of IL-10 and TNF-α are not shown due to low expression levels in the tissue resulting in high CT values(>38). No significant difference in gene expressions between groups were found in the forebrain and center brain for IL-6 (forebrain: F (2, 42) = 2.778, P=0.0736, center brain: F (2, 41) = 2.793, P=0.0729), IL-1β (forebrain: F (2, 42) = 0.06347, P=0.9386, center brain: F (2, 41) = 0.9214, P=0.4060) and GFAP (forebrain: F (2, 42) = 1.358, P=0.2682, center brain: F (2, 41) = 1.872, P=0.1667). However, Iba-1 gene expression is significantly different between groups in the center brain (F (2, 41) = 8.621, P=0.0007) but not in the forebrain (F (2, 41) = 2.739, P=0.0764). Looking at multiple comparisons, CS7 show a trend towards downregulated IL-6 in the center brain (P=0.0572). Moreover Iba-1 in the center brain show to be significantly downregulated for both CS4 (P=0.0182) and CS7 (P=0.0004) compared to control. It can be postulated that the reduced Iba-1 expression indicates a decrease in microglia.


Forebrain
Center brain
IL-6
IL-1β
Iba-1
GFAP
GR
Relative gene expression ± SEM
Control
CS4
CS7
0.0504
0.0572
0.0556
0.0004
0.0182

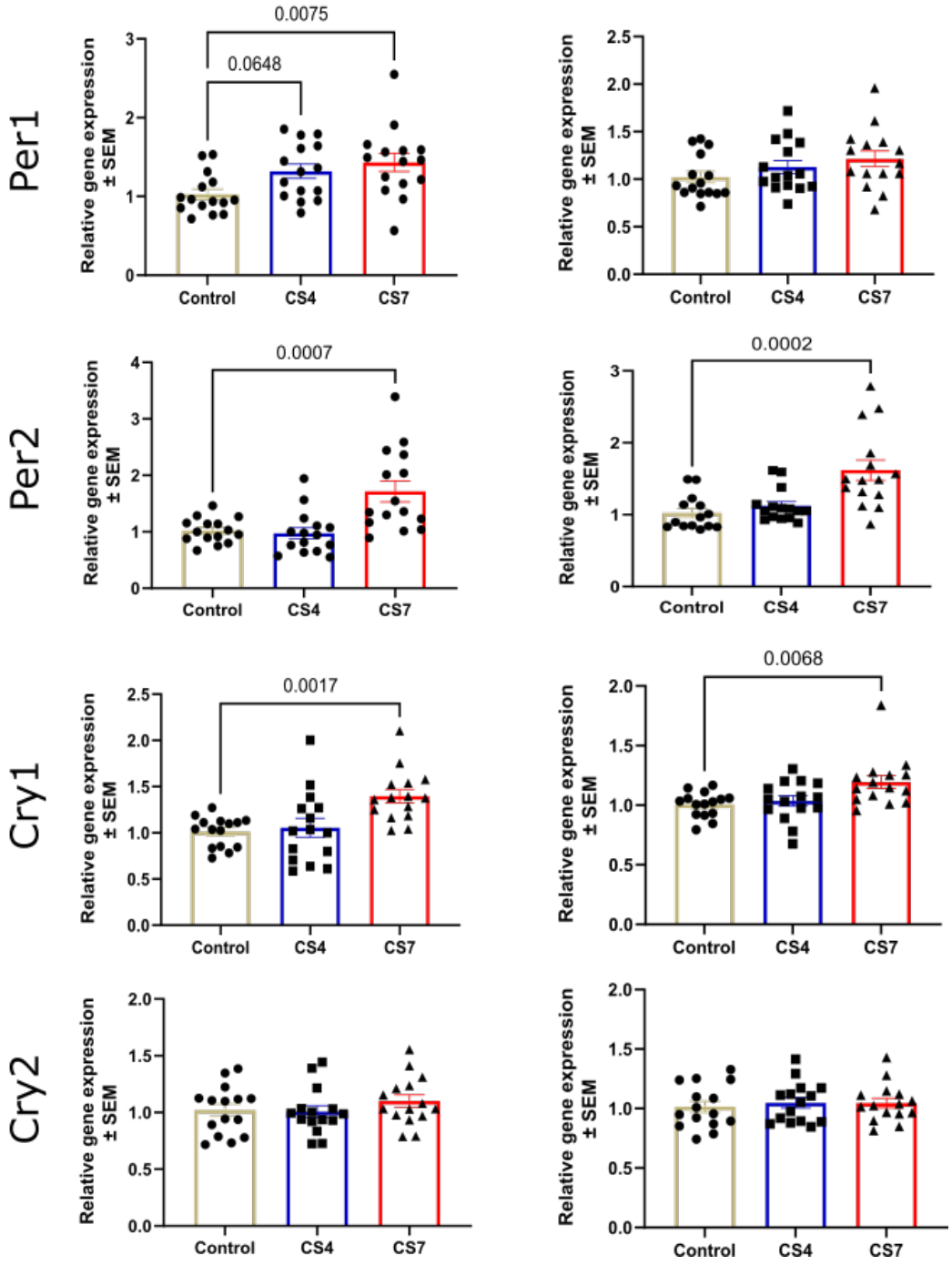


Figure 4.5.: RT-PCR results from the CS study. Gene expression of Per1, Per2, Cry1, Cry2, IL-1β, IL-6, Iba-1, GR and GFAP in the forebrain and center brain shown as gene expression relative to GAPDH measured by RT-PCR. One-way ANOVA was used to find significant differences.

4.2 RRS14

4.2.1 RRS14 lead to significant weight loss

Body weight was measured every morning for 14 days to evaluate on the health status of the mice. Changes in body weight is presented as percentage of change from baseline for each animal (figure 4.6 A). ANOVA showed a significant main effect of time ($F(4.173, 126.2) = 2.874$, $P=0.0239$), stress ($F(1, 34) = 97.64$, $P=<0.0001$) and corresponding interaction ($F(13, 393) = 6.999$, $P=<0.0001$). The control mice have a slight weight gain during the study while the stress group show significant weight loss. Multiple comparisons analyses showed that the stressed mice have lost weight significantly from day 3 ($P= 0.0004$) and forward compared to control. This indicates that RRS induce significant weight loss in mice.

4.2.2 RRS14 does not induce a depressive-like phenotype but increased locomotor activity in stressed mice

The NEST results are shown in figure 4.6 B and demonstrate no significant difference in nest scores ($F(1, 17) = 0.6343$, $P=0.4368$) between RRS and control at any timepoint ($F(8, 136) = 1.162$, $P=0.3264$). Mice perform nest building to stay warm among other things (Deacon 2006), therefore, it was investigated if RRS14 affected body temperature in mice. However, rectal body temperature was comparable between the groups ($P = 0.1417$; figure 4.6 C).

The EPM is the gold standard for measuring anxiety-like behavior [118], which RS has previously shown to induce in mice [133]. However, there was no significant difference in time spent in open ($P= 0.5938$) or closed arms ($P= 0.9241$) between control and RRS (figure 4.7 A and B). RRS show a trend towards increased number of head dips ($P=0.0546$) and total distanced moved ($P=0.0529$) compared to the control (figure 4.7 C and D). This may be an indication of increased locomotor activity as seen in the previous studies (CS and RRS pilot study, appendix B.3) rather than anxiety-like behavior.

Nevertheless, heatmaps of EPM data indicate that control mice moved around the maze and spent more time in the open arms, while the stress group spent more time in the closed arms and center area than in the open arms.

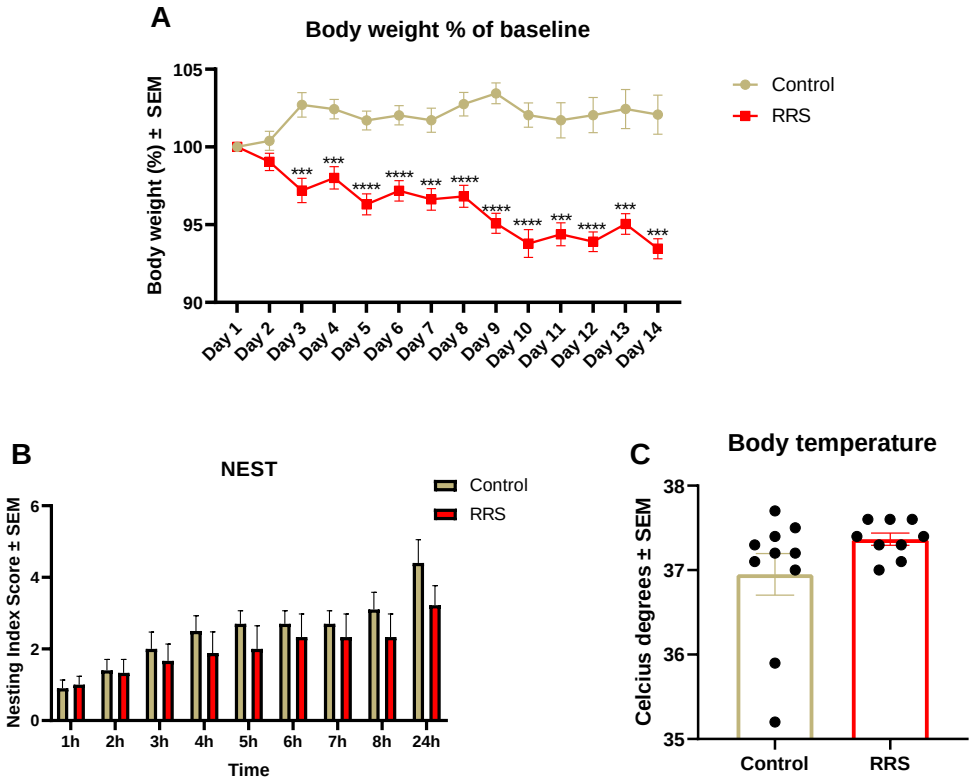


Figure 4.6.: Body weight and behavior results for RRS14. A) Body weight shown as percent of baseline weight. Significant differences in body weight between the control group and RRS group was identified from day 3 to 14 of the stress period. B) Nest building was scored on a scale from 0-8, where 8 is a perfect nest and 0 is no nest building at all. C) Rectal body temperature was measured within a few hours after the end of NEST. There is no significant difference in body temperature between control and RRS (P=0.1417). Student's T-test and Two-way ANOVA with repeated measures was used to calculate significant differences. *Abbreviations: Nest building assay (NEST), Repeated restraint stress (RRS)*

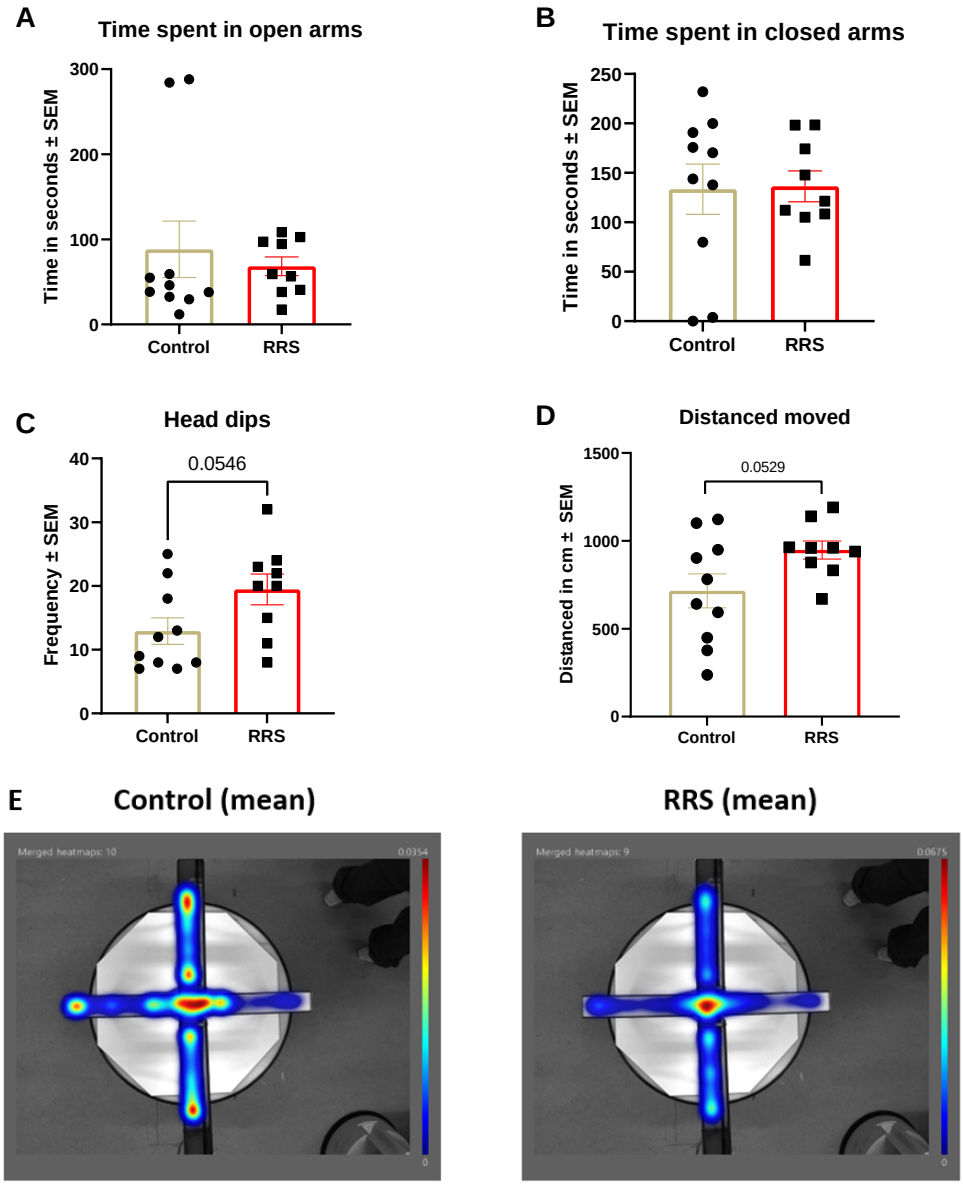


Figure 4.7.: EPM results from RRS14. The EPM data is displayed as time spent in open and closed arms, number of head dips and total distanced moved as well as a heatmap for each group indicating the mean movement pattern of the groups. Student's T test was used to measure significant differences.
Abbreviations: Repeated restraint stress (RRS)

4.2.3 RRS14 significantly increases acute plasma CORT levels and handling of control mice might lower basal plasma CORT

Plasma CORT was measured at several timepoints in order to determine if RRS14 would induce allostatic overload of the HPA axis and increase acute plasma CORT (figure 4.8). One-way ANOVA showed a significant difference in plasma CORT ($F(4, 19) = 140$, $P<0.0001$). Multiple comparisons demonstrated significantly increased plasma CORT in mice euthanized immediately after stress at both day 1 ($P=<0.0001$), day 7 ($P=<0.0001$) and day 14 ($P=<0.0001$) compared to control on day 1. Furthmore, there is a significant increase in CORT levels from RRS day 1 to day 7 ($P=0.0105$) and RRS day 7 to day 14 ($P=0.0036$). This illustrates acute activation of the HPA axis in response to RS, and that the mice do not habituate to the stressor over time. Furthermore, the plasma CORT levels on day 15 (24h after last stress exposure) and day 21 (7 days after last stress exposure) drops notably (day 15: $P=0.5146$, day 21: $P=0.1032$). Zooming in on the two control groups, there is a significant difference in CORT levels between the control group on day 1 and day 21 ($P=0.0257$). Control mice were weighted on the same days as RS was performed, thus it is likely that this handling lowered the plasma CORT levels to a statistically significant degree.

The gene expression of GR was also measured (figure 4.9) but no significant differences could be observed in any of the brain tissues (hippocampus: $F(6, 28) = 1.169$, $P=0.3506$, middle brain: $F(6, 36) = 1.036$, $P=0.4185$, cortex: $F(6, 36) = 1.642$, $P=0.1641$).

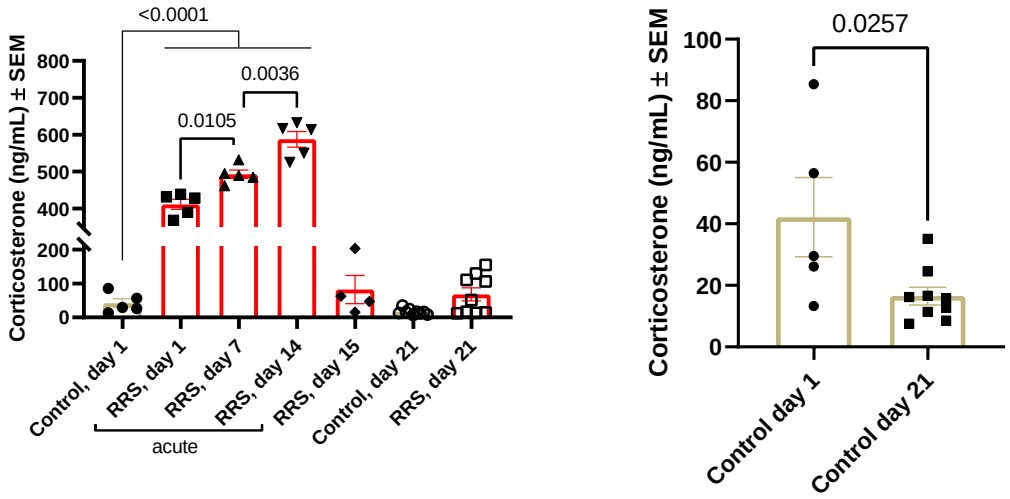


Figure 4.8.: Plasma CORT levels measured with competitive ELISA. There is a significant increase in acute CORT levels is response to RS and a significant difference between the two control groups. One-way ANOVA and Student's T-test was used to find significant differences
Abbreviations: Repeated restraint stress (RRS)

4.2.4 Acute upregulated IL-1β gene expression indicates stress-induced neuroinflammation

To evaluate on neuroinflammation, the gene expression of different inflammatory markers was investigated in cortex, middle brain and hippocampus (figure 4.9). One-way ANOVA showed that there is significant difference between groups in the hippocampus for the gene expression of IL-1β ($F(5, 22) = 7.225$, $P=0.0004$) and no significant difference for IL-6 ($F(6, 23) = 0.9529$, $P=0.4778$) and Iba-1 ($F(6, 30) = 1.340$, $P=0.2708$). Gene expressions in the middle brain showed significant difference between groups for IL-6 ($F(6, 36) = 3.254$, $P=0.0117$), IL-1β ($F(5, 31) = 4.403$, $P=0.0038$) and Iba-1 ($F(6, 36) = 2.361$, $P=0.05$). Cortex gene expressions showed a trend of difference between groups for Iba-1 ($F(6, 36) = 2.266$, $P=0.0587$) and no significant difference for IL-6 ($F(6, 36) = 1.621$, $P=0.1698$) and IL-1β ($F(5, 32) = 1.865$, $P=0.1284$). Multiple comparisons reveal that RS significantly upregulated IL-1β acutely on day 1 and day 7 in the cortex (day 1: $P=<0.0404$, day 7: $P=0.0374$) and middle brain (day 1: $P=<0.0001$, day 7: $P=0.0094$) of stressed mice compared to control. The effect is mostly pronounced in the middle brain where the acute effect also decreases along the study compared

to control on day 1. Microglia is a major source of Il-1β in the CNS during immune activation (Jesudasan et al. 2014), thus it was investigated whether the upregulation of Il-1β was due to activated microglia, by examining the gene expression of the microglial activation marker Iba-1. However, there was no significant difference of Iba-1 gene expression between control and RRS. Suprisingly, a significant increase of iba-1 gene expression was observed in the control group on day 21 compared to control on day 1 in middle brain (P= 0.0324) and cortex (P= 0.0352). In addition, control day 21 had significantly increased gene expression of IL-6 in the middle brain (P=0.005) and Iba-1 in cortex (P=0.0352) compared to control day 1. These observed increases in the control group on day 21 are puzzling. Still, the acutely increased IL-1β levels indicate immune activation in the brain in response to RS.

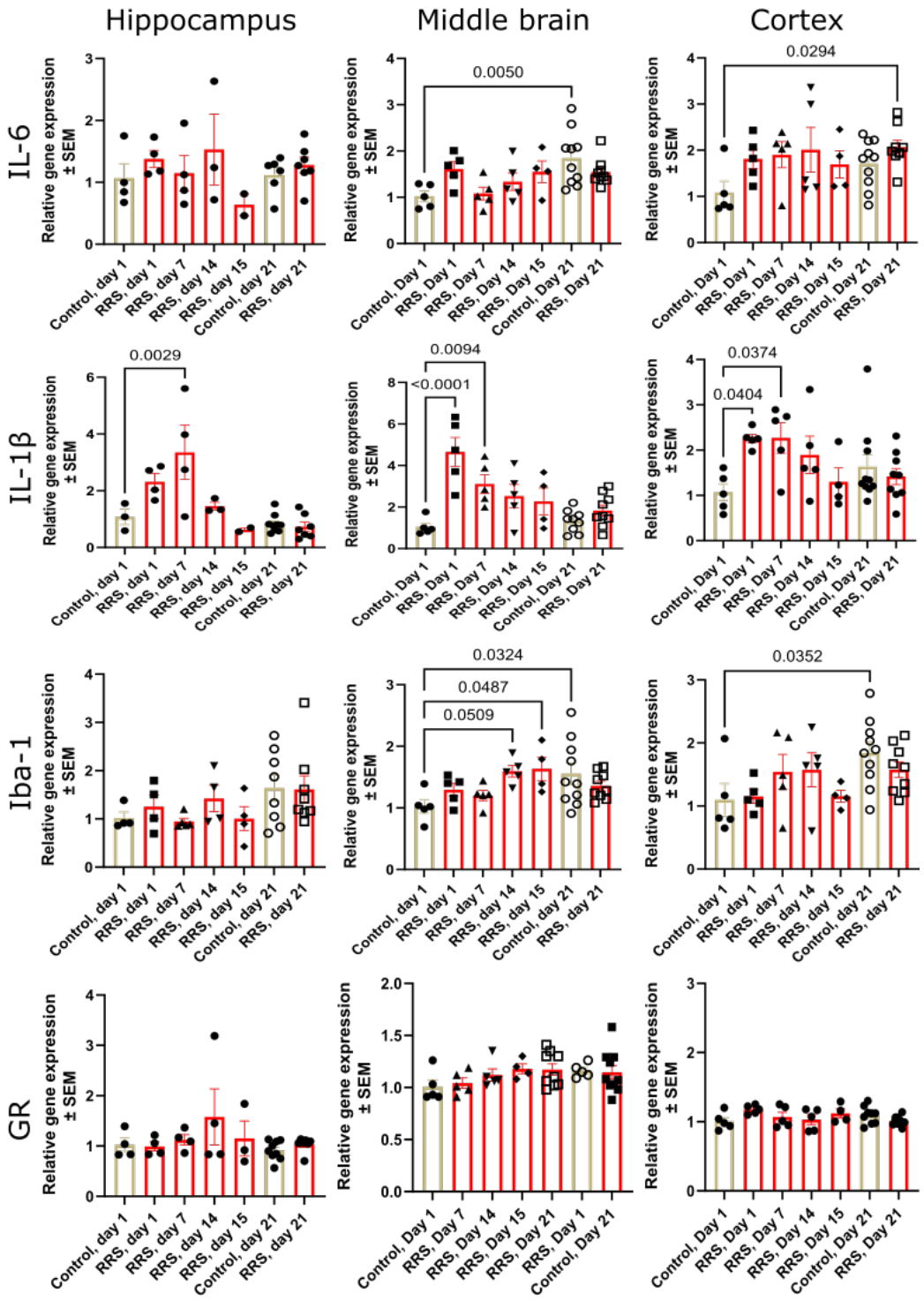


Figure 4.9.: RT-PCR results from RRS14. Gene expression of IL-1β, IL-6, Iba-1 and GR in the hippocampus, middle brain and cortex shown as gene expression normalized to GAPDH and relative to Control on day 1. One-way ANOVA was used to find significant differences.
Abbreviations: Repeated restraint stress (RRS), Glucocorticoid receptor (GR), Ionized calcium-binding adapter molecule 1 (Iba-1), Interleukin (IL)

4.3 iRRS

4.3.1 iRRS resulted in significant weight loss and it is not due to loss of appetite

Potential metabolic effects of the iRRS protocol and general health status of the mice were monitored by measurements of body weight and food intake during the study period. Changes in body weight is presented as percentage of change from baseline for each animal (figure 4.10 A). Two-way ANOVA showed a significant main effect of time ($F(5.855, 157.6) = 8.448$, $P=<0.0001$), stress ($F(1, 31) = 36.71$, $P=<0.0001$) and the corresponding interaction ($F(12, 323) = 9.555$, $P=<0.0001$) on body weight. The control mice have a slight weight gain during the study, while the stressed mice lost weight significantly on day 3 ($P=<0.0001$), which is reversed during the 48h rest period (day 6: $P= >0.9999$). From day 7 onward the weight difference is significant between RRS and control. In the stress-free period from day 18-22, iRRS mice increase drastically in body weight, although this do not reach control levels. This indicates that the stressed mice compensate for the lost weight during stress as soon as the stress stops. Two-way ANOVA showed a significant main effect of time ($F(2.794, 75.43) = 25.87$, $P=<0.0001$), stress ($F(1, 31) = 11.47$, $P= 0.0019$) and the corresponding interaction ($F(6, 162) = 4.864$, $P= 0.0001$) on food intake between iRRS and control mice. Overall, the results displayed in figure 4.10 C likewise show that the iRRS mice tend to eat less than the control mice during the stress periods and a significant difference was found on day 18 ($P=0.014$). However, the total food intake during the stress period was comparable between iRRS and control mice (figure 4.10 D), thus food intake cannot alone explain the body weight loss in iRRS mice, and it can be speculated that metabolic mechanisms might explain this.

4.3.2 iRRS lead to lower activity but no change in motivated behavior

The burrowing assay was chosen to understand the effect of iRRS on goal-directed motivated behavior. However, the results illustrated in figure 4.10 show that there is no noticeable difference in burrowing behavior between

iRRS and control. Locomotor activity was measured for 3h in order to evaluate the effect of iRRS on locomotor activity in mice (figure 4.10 B). There is a trend of lower locomotor activity in iRRS mice compared to control mice (P=0.069).

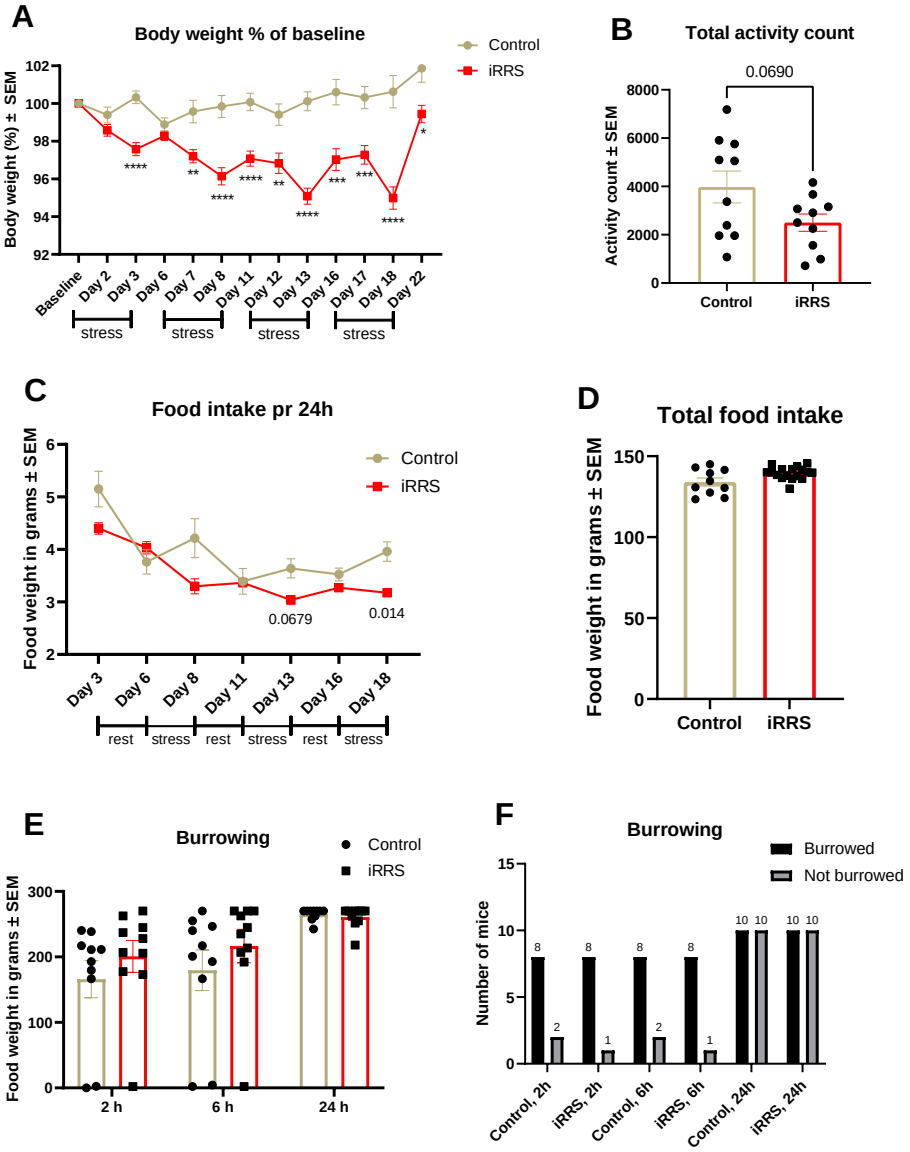


Figure 4.10.: Body weight and behavior from iRRS. A) Body weight shown as percent of baseline weight. B) 3h locomotor activity shown as total activity count. C) Food intake shown as the amount in grams eaten in average each 24h. D) The total food intake during the first 18 days of the study. E) Burrowing shown as the amount of food pellets in grams removed from the burrowing tube. F) The figure shows the number of mice within each group that performed burrowing or did not perform burrowing. Two-way ANOVA with repeated measures and Student's T-test was used for statistical analysis.

4.3.3 iRRS causes allostatic overload of the HPA axis

To understand if the iRRS protocol could induce allostatic overload of the HPA axis the blood-based biomarker CORT was measured. Based on the learnings from the RRS14 study, the protocol was modified to include handling of the control mice 7 days prior to the iRRS period and at the same days as RS to reduce basal plasma CORT in control mice and thus allow a bigger window between stressed and control mice. One-way ANOVA showed a significant difference between groups ($F(4, 36) = 5.038$, $P=0.0025$). Additional multiple comparisons analyses showed a significant increase at day 20 ($P=0.0069$) compared to control while a slight increase in CORT also can be observed on day 10 (figure 4.11). Furthermore, a trend towards increased plasma CORT in iRRS mice is still present at day 24 ($P=0.0744$) when comparing to the control on day 24, even though the CORT levels have dropped since day 20.

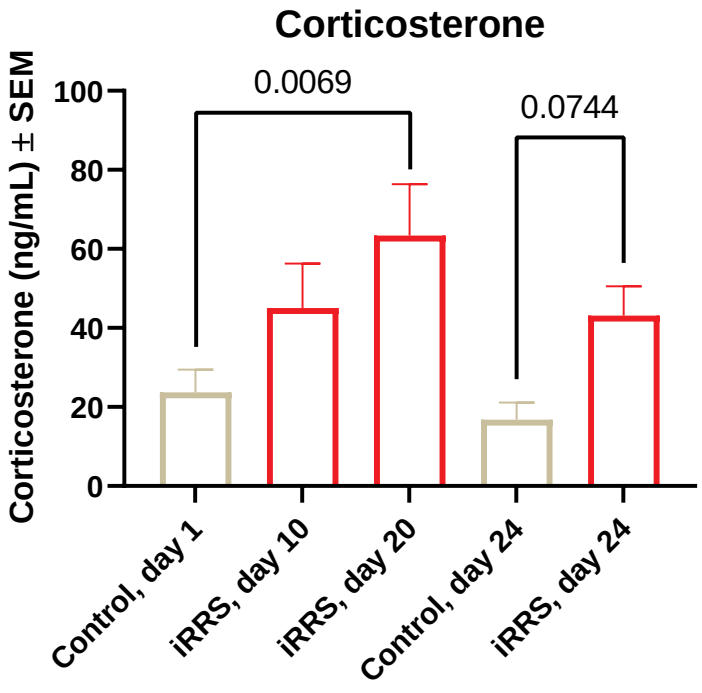


Figure 4.11.: Plasma CORT levels measured with competitive ELISA. One-way ANOVA was used to identify significant differences.

4.3.4 Upregulated IL-1β gene expression in the brain indicates stress-induced neuroinflammation

Others have demonstrated increased inflammatory markers in the brain of patients with depression (Pandey et al. 2011) as well as in animal stress models (Wang et al. 2018 and Yirmiya et al. 2015). Thus, the gene expression of Il-1β, IL-6 and GFAP was measured in cortex, middle brain and hippocampus. One-way ANOVA showed that there is significant difference between groups in the hippocampus for the gene expression of IL-1β ($F(4, 31) = 13.26$, $P<0.0001$) and no significant difference for IL-6 ($F(4, 31) = 1.703$, $P=0.1745$) and GFAP ($F(4, 32) = 0.4226$, $P=0.7911$). Gene expressions in the middle brain showed significant difference between groups for IL-1β ($F(4, 30) = 8.808$, $P<0.0001$) and no significant difference for IL-6 ($F(4, 36) = 0.2280$, $P=0.9209$) and GFAP ($F(4, 36) = 1.882$, $P=0.1349$). Cortex gene expressions showed significant difference between groups for GFAP ($F(4, 32) = 2.743$, $P=0.0455$), IL-6 ($F(4, 32) = 3.818$, $P=0.0120$) and IL-1β ($F(4, 32) = 7.207$, $P=0.0003$). Multiple comparisons reveal a significant upregulation of IL-1β in the hippocampus ($P=<0.0001$), middle brain ($P=<0.0001$) and cortex ($P=0.0005$) in iRRS mice at day 20 compared to the control at day 1. Furthermore, GFAP is significantly downregulated in cortex ($P=0.0219$) and middle brain ($P=0.0454$) at day 24 in iRRS mice compared to control on day 1, while IL-6 tend to be downregulated on day 20 in cortex (figure 4.12). These results suggests immune activation in the brain in response to iRRS leading to secretion of IL-1β. The decreased GFAP expression may be a result of astrocytic loss.

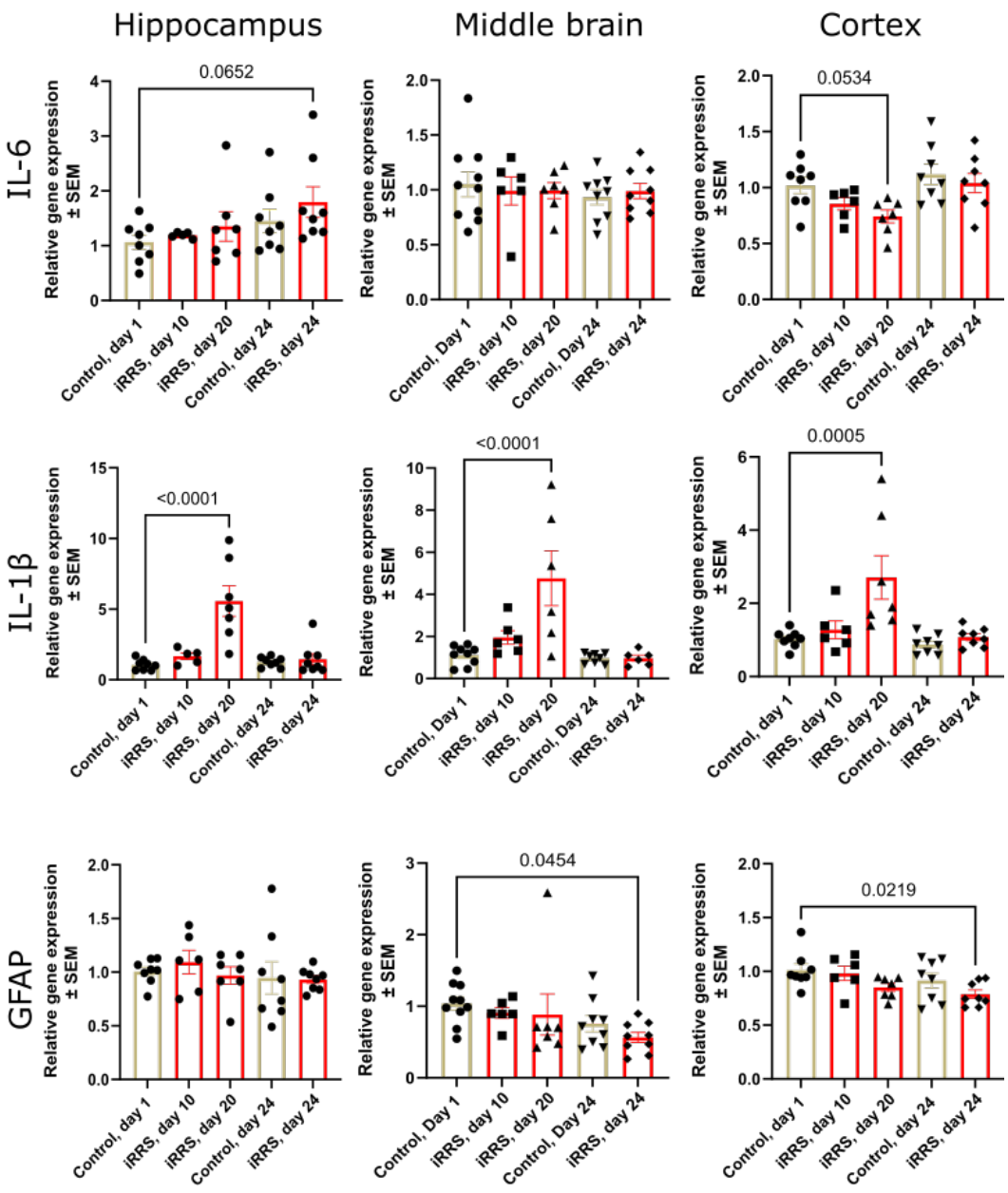


Figure 4.12.: **RT-PCR results from iRRS.** gene expression of IL-6, IL-1β and GFAP in the hippocampus, middle brain and cortex shown as gene expression normalized to GAPDH and relative to the control on day 1. One-way ANOVA was used to find significant differences.

4.3.5 RRS14 and iRRS may be equally stressful; handling of the control mice makes the difference

Comparing the plasma CORT results from the controls of RRS14 and iRRS, one-way ANOVA reveal a significant difference between groups ($F(3, 28) = 3.150$, $P=0.0405$). Specifically, the control group on day 1 in RRS14 show increased CORT compared to the remaining control groups (figure 4.13). These results indicate an effect of handling mice to lower basal CORT levels. Plasma CORT levels for the stress groups of iRRS and RRS14 show no significant difference between groups ($F(4, 31) = 0.8103$, $P=0.5282$). This suggests that both the RRS models are equally stressful. Thus, the baseline CORT level of the control group on day 1 highly influences whether there is a significant difference between stress groups and the control group.

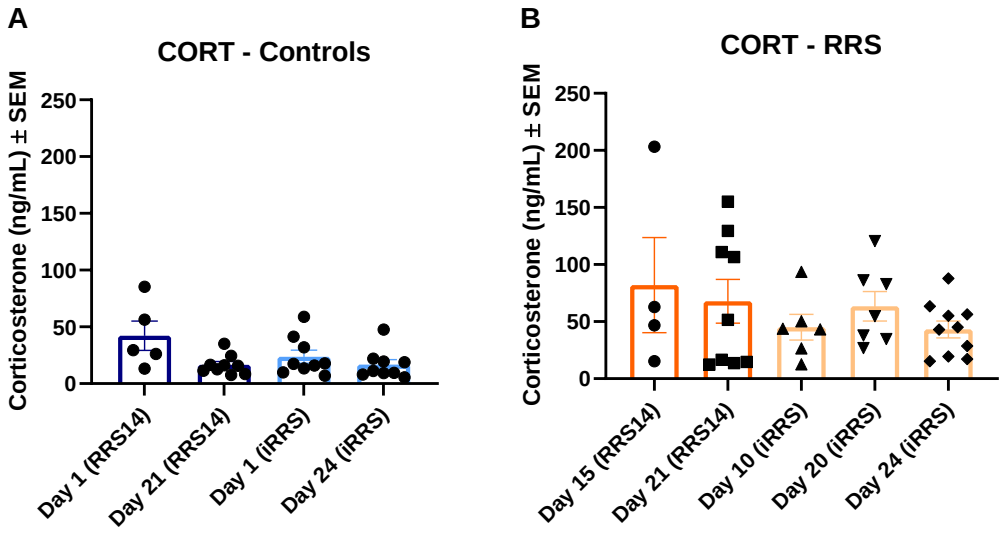


Figure 4.13.: CORT results from RRS14 and iRRS. comparison of plasma CORT levels from RRS14 and iRRS. A) Plasma CORT levels of the control groups from the two studies. B) Plasma CORT levels of the RRS groups from the two studies. Statistical analyses were performed using One-way ANOVA.

5 Discussion

5.1 CS

One of the most pronounced findings from the CS study is disturbed diurnal locomotor activity pattern together with the upregulation of Per1, Per2 and Cry1 in the forebrain and center brain of CS7 mice. Because these observations were not found in the CS4 group, it suggests that this effect is driven by the contingency of the diurnal disruption. However, studies in rodents have shown changes in the gene expression of Per1 and Per2 due to CUS [136, 105] and sub-acute stress [106]. The CUS studies used a different protocol with two stressors daily, while the CS in this thesis only had one stressor each day together with the diurnal disruption. Moreover, they focus on very specific brain areas while in the CS study the brains were divided into forebrain and center brain. Jiang et al. 2011 [136], Logan et al. 2015 [105] and Tahara et al. 2015 [106] all measured the rhythmic expression of the clock genes while this thesis analyzed a single timepoint. Since clock gene expression varies during the day, it is possible that dysregulation on the cohort of animals in this thesis might be more pronounced at other timepoints.

The same issue is relevant with the CORT measures since CORT also have a rhythmic secretion during the day. However, the single timepoint of CS4 and CS7 after the 4 weeks of CS are not comparable even though the two groups had been exposed to the same stress protocol at this timepoint. Working with living animals there is a chance of individual variability and likewise, not all humans exposed to stressful environments will develop allostatic overload of the HPA axis. A study by Kim et al. 2013 [149] found that the basal steady-state CORT level may predict a susceptible or resilient phenotype in male C57BL/6 N mice in reaction to stress. Therefore, it may be relevant to investigate whether these phenotypes also can be identified in C57BL/6 J mice which were used in this thesis. Thus, the mice may be divided into a

susceptible and resilient group when performing stress protocols to investigate the variability in HPA axis responses in mice.

Previous studies have shown increased neuroinflammation in response to stress such as increased Iba-1 and IL-6 [59, 56, 72]. The CS study, however, revealed significant downregulation of the inflammatory markers IL-6 and Iba-1 in the center brain of CS7 mice and significant downregulation of Iba-1 in the center brain of CS4 three weeks after the end of the CS protocol. Similarly, a study has shown decreased Iba-1 gene expression in the brain of rats in response to CMS even though an increase in Iba-1 gene expression was seen 24h after initiation of the stress [71]. A study by Tong et al. 2017 [70] demonstrate significantly decreased Iba-1 protein expression as well as decreased microglial number in hippocampus of mice exposed to CUS, chronic restraint stress or chronic social defeat stress. Furthermore, this study concludes that the decrease in Iba-1 reflects the loss of hippocampal microglia and that loss of microglia in hippocampus plays a role in the development of depression. This was based on behavioral results where restoration of microglial loss with Macrophage colony stimulating factor (M-CSF) reverse the behavioral abnormalities caused by the three different stress protocols. In order to discover if the downregulation of Iba-1 in this thesis is due to microglial loss, immunostaining of brain sections together with Western Blot analysis for protein expression could be useful. Since cytokines present in the CNS is mainly secreted by microglia and astrocytes, there may be a link between decreased Iba-1 and decreased Il-6 in the center brain of the cohort of animals in this thesis. However, both IL-6 and Iba-1 tend to be upregulated in CS4 in the forebrain demonstrating the opposite effect than what is seen in the center brain. This may suggest varying responses to stress dependent on the brain area as seen in other studies [2, 127, 150].

5.2 RRS14 and iRRS

One of the most significant findings from the RRS studies was the weight loss in the stress groups. The iRRS study indicate that the stressed mice eat less during stress periods and about the same during rest periods compared to the control group. This is consistent with the weight loss during stress periods and weight gain during the a rest periods. However, the results also

show that the total food consumption during the study is comparable for both groups, thus food intake cannot explain the significant weight loss. In addition, increased locomotor activity has been seen in several stress models presented in this thesis, namely CS, RRS14 and RRS pilot study (Appendix B.3) as well as in published research [119, 120]. Therefore, the significant weight loss may be a result of increased locomotor activity in stressed mice. However, in the iRRS study there was a trend of decreased locomotor activity in the stress group compared to control. A study by Miyazaki et al. 2013 [121] also found that stress significantly reduced body weight of mice even though the stressed mice had a higher food intake than the control group. However, Miyazaki and colleagues used a different stress paradigm while Son et al. 2019 [151] showed that restraint stress lead to significant weight loss and less food intake at some timepoints during stress. Altogether, stress-induced metabolic pathways may be responsible for the observed significant weight loss, thus it would be interesting to investigate this hypothesis further.

The gene expression results of IL-6 and Iba-1 show an unexpected increase from control day 1 to control day 21 in both the middle brain and cortex of the cohort from the study RRS14. This may indicate an influence of the study on the control mice leading to immune activation in the brain. Moreover, this change was not seen for iRRS, where the control mice were handled during the study. A possible explanation may be that the single housing leads to social isolation. One study has shown that 6 weeks of social isolation of male mice resulted in a depressive-like phenotype as well as increased TNF-α in plasma and decreased microglial cell density in the dentate gyrus of hippocampus [152]. Therefore, it might be that the 21 days of single housing of the control mice lead to alterations in the brain as a result of social isolation. Moreover, the RRS group at day 21 also show similar tendencies to increased IL-6 and Iba-1. A study has shown that regular and gentle handling of mice reduce anxious- and depressive-like behavior [153] and regular handling of rats reduced the effects of social isolation [154, 155]. If this is also applicable to mice, it correlates with the fact that the control on day 1 and on day 21 in iRRS show similar gene expressions for IL-6, while control mice in RRS14 show significantly increased IL-6 on day 21 compared to day 1. However, the lower plasma CORT of the control on day 21 is in conflict with this hypothesis. Moreover, a study by Prevot et al. 2019 [128] chose to single-house the mice exposed to unpredictable CMS as a part of the procedure while control mice were group-housed to eliminate the stress of single-housing. This could

be considered in the optimization of the RRS14 as well as the other stress models to clarify the results. The effect of social isolation in mice should be investigated further in order to solidly conclude on this matter.

There was a significant difference in plasma CORT from the control on day 1 compared to day 21 in the RRS14 study. The lower CORT levels on day 21 may be due to larger sample size compared to day 1 and/or the daily routine of weighing the control group during the study which may habituate the mice to the handler and thereby lower basal steady-state plasma CORT levels. The same trend was observed in a similar study at Lundbeck A/S performed by Amalie Clement, where the control groups demonstrate decreasing plasma CORT levels during the study period (Appendix D), supporting the handling hypothesis. Thus, in the iRRS study the control mice were handled 7 days prior to the study and during the study. The clear difference in baseline CORT between control day 1 in RRS14 and iRRS indicate an effect of handling on steady-state baseline CORT. However, the study would have to be repeated several times to ensure that this effect of handling is reproducible. A study by Ghosal et al. [156] practised cup/massage handling which involves "cupping" the mice with two hands followed by a massage of 5–10 strokes with the second and third fingers of one hand, starting at the level of the ears extending down the head/neck. This study discovered that cup/massage handling of mice results in reduced acute CORT response to a stressor such as a behavioral assay.

5.2.1 RRS14

The EPM results reveal that two mice in the control group only spent time in the open arms during the 5-minute testing which deviates from the rest of the group. This may highly influence the heatmap of the control group. In addition, the RRS group spend more time in the center and the beginning of the open arms while the control group move all the way to the end of the open arms. Dividing the open arms into sections may provide a more realistic picture of the anxiety-like state of the cohort of animals in the RRS14 study. Furthermore, it may be relevant to look at the head dips which were increased in RRS. Whether the dip is performed from the center area or in the open arms may also tell more about the state of the mice. Conclusively, dividing the open arms of the maze into more sections and evaluating the way of head dipping

would give us more information about the nature of the animals and should be considered for the next EPM assay.

5.2.2 iRRS

The RT-PCR results from iRRS showed significantly increased IL-1β at day 20 in hippocampus, middle brain and cortex of the stress group. In addition, there was a significant increase in CORT at day 20 in stressed mice. Microglia is one of main sources of cytokines such as IL-1β in the CNS and also express GRs. In response to chronic stress, immune cells will over time compensate for the increased CORT levels with downregulated GR activity. These changes are speculated to be an adaptive mechanism induced by cytokines in order to increase the immune response during stress [157]. Furthermore, cytokines can activate the HPA axis leading to further increase in CORT [48, 42, 49, 50, 51, 52]. GR gene expression was measured for RRS14 and CS where no difference was found, however, it was not evaluated in iRRS. Since IL-1β is significantly upregulated on day 20 in iRRS mice, it may activate the HPA axis further and thereby increased CORT levels. On the other hand, when looking at Il-1β gene expression for the other timepoints there is no difference compared to control while CORT tend to be increased on day 10 and day 24 compared to control. This indicates that an increase in plasma CORT happens prior to increased IL-1β expression in the brain. This hypothesis is supported by the fact that microglial activation markers, microglial proliferation and IL-1β are induced by GCs [158, 159, 72].

There is a significant downregulation of GFAP gene expression in the middle brain and cortex on day 24 in iRRS mice compared to control mice on day 1, which may indicate loss of astrocytes. Previous studies have demonstrated decreased GFAP in the brain of patients with depression [64, 65, 66] and in rodents exposed to different stress models [160, 161, 66, 162]. These results may therefore be indicative of a depressive-like state in the brain of mice exposed to iRRS. To further understand this data, measurement of the protein levels of Il-1β and GFAP in the brain might be beneficial. It may also be interesting to know if Iba-1 is upregulated in the same manner as IL-1β indicating that microglia are the main source of this cytokine secretion.

5.3 Comparison across studies

The NEST was one of the behavioral assays used for CS and RRS14. The results showed no difference between control and stress animals in the two studies. Moreover, the control animals scored lower than expected based on prior experience. A Lundbeck employee specialized in NEST, Christian S. Pedersen, conducted a study to evaluate the influence of single housing on nest building behavior (Appendix C). The study showed no difference between single housed mice and two-hosed mice in nest building behavior which attenuates the social aspect of NEST.

The other assay of motivated behavior was burrowing, which showed a marked difference between stress groups and control in the CS study but no difference in iRRS. In the CS study, the mice do not burrow during the first 6h of the assay. This may be due to the lack of acclimation to the burrowing tube prior to the assay which is normally a part of the burrowing protocol and was also done for iRRS. This difference alone may influence the interpretation of the two studies. Furthermore, the stress protocols are very different, and it might be that burrowing behavior in mice is not affected by the short iRRS protocol but instead require a longer period of stress to alter burrowing behavior, such as the CS protocol. The motivated behavior results of the RRS studies are not indicative of any changes. Therefore, it is necessary to either extent the stress period or increase the daily stressors and reevaluate the motivated behavior or look towards other behavioral assays to fully cover the effect of the specific stress model on mouse behavior.

Lever and colleagues [163] have shown a connection between rearing behavior and the hippocampus, which is highly involved in stress and depression. Rearing is when the mouse temporarily stands on its hind legs to explore the environment, and it can be either supported (touches the wall) or unsupported. The supported rearing is thought to be related to locomotion alone, while unsupported is more related to emotional behavior [147]. For instance, Sturman and colleagues [147] showed that male mice only had a change in unsupported rearing and fecal boli in the open field test when environmental factors such as brightness and noise changed. Especially removal of background noise led to reduced anxiety showed by reduction in fecal boli and increased unsupported rearing. Moreover, the male mice performed significantly more unsupported

rears during the last 5 minutes of testing compared to the first 5 minutes, indicating increased unsupported rearing after familiarizing to the arena [147]. The study also showed that acute RS lead to significantly less supported and unsupported rears in male mice [147]. However, the rearing measures in this thesis do not distinguish between supported and unsupported rearing, thus it may be difficult to completely understand these results. Dividing rears into supported and unsupported may clarify the rearing results and expand on the findings by Sturman and colleagues.

As previously mentioned, most studies performed in this thesis as well as publications demonstrate increased locomotor activity in the stress group compared to the control group [119, 121, 120]. However, iRRS mice showed decreased locomotor activity. This may be a result of the specific protocol with interrupted stress periods. Strekalova and Steinbusch report that hyperlocomotion triggered by the stressful experience of behavioral tests is a consequence of chronic stress [120]. Furthermore, this hyperlocomotion interferes with the assessment of anxiety-like behavior in stressed mice, as suspected in the RRS14 study. In addition, the previous mentioned study by Kim et al. 2013 [149] on susceptible and resilient mice also show that resilient mice have increased locomotor activity after stress compared susceptible mice. Based on this, the decreased locomotor activity in the iRRS group may be indicative of an anxiety-like phenotype in stressed mice susceptible to stress while the other studies may have more resilient mice resulting in increased locomotor activity. Although it may be unlikely that only stress susceptible mice were incidentally assigned to the iRRS group and stress resilient mice to the stress groups of the other studies. Therefore, these deviating locomotor activity results in the iRRS study is probably due to the stress paradigm.

When comparing the three models presented in this thesis there are advantages and disadvantages for all models (Table 5.1). First, the CS protocol is twice as long as the two RRS protocols and the changed light/dark rhythm makes it more difficult to organize the behavioral assays. Second, weight changes were only observed in the RRS studies and not the CS model. Third, RRS14 showed increased locomotor activity in the stress group compared to control, while iRRS showed the opposite. This may indicate a different response in mice to the iRRS protocol compared to RRS14 meaning that even though it is the same type of stressor the reaction may change with the number and frequencies of stress sessions. Fourth, an increase in plasma CORT was found

Table 5.1.: Comparison of the results from the three studies CS, RRS14 and iRRS.

Model	**CS**	**RRS14**	**iRRS**
Duration	8 weeks	3 weeks	3.5 weeks
Weight	No change	Significiant change	Significant change
Food intake	-	-	Significant change during stress
Burrowing	Changed behavior	-	No change
NEST	No change	No change	-
Locomotor activity	increase and disturbed diurnal activity	Increased	Decreased
CORT	Significant increase but inconsistent	Significant increase acutely	Significant increase
Gene expression	Significant upregulated clock genes and downregulated Iba-1	Significant upregulated IL-1β acutely	Significant upregulated IL-1β on day 20

in all three models. However, in the CS study only CS7 showed the significant increase even at the timepoint where CS4 and CS7 had been exposed to the same stress protocol. This may indicate variations in the performance of the CS protocol by the executioner or that there is variability between groups which could be investigated further e.g. by screening for resilient and susceptible mice to stress as demonstrated by Kim and colleagues [149]. On the other hand, RRS14 showed a significant increase in acute plasma CORT, while iRRS mice showed significantly increased CORT 48h after the last stress exposure indicating allostatic overload of the HPA axis. Handling of the control groups in RRS14 might have resulted in lower basal steady-state CORT leading to a significant difference between RRS day 15 (24h after the last stress exposure) and control day 1. This should be investigated to conclude on whether both RRS14 and iRRS can cause allostatic overload of the HPA axis in male mice. RRS14 does not show a significant increase in IL-1β on day 15 in stressed mice which is almost corresponding to day 20 in the iRRS study. This further strengthen the idea that the number and frequencies of stress sessions changes the reaction to the stressor in mice. Since RRS leads to significant upregulation of IL-1β 48h after the last stress exposure, it is possible that the CS paradigm

led to increased IL-1β earlier in the study. To investigate this further, one would need to sample brain from subcohorts during the CS period.

5.4 Recommendation

1. The iRRS model seem to be the most interesting model to move forward with.

2. The iRRS model is the most interesting due to protocol length, it induce allostatic overload of the HPA axis, changed behavior compared to previous discoveries and strong IL-1β upregulation in the brain.

3. To optimize and improve the iRRS protocol more behavioral assays is needed to describe the state of the mice. Moreover, the acute plasma CORT response during the study and the following hours after stress is relevant to investigate the stress recovery. Long-term effects of the stress paradigm is also interesting. Furthermore, it is necessary to make sure that the results are reproducible.

6 Conclusion

In conclusion, the CS model induced allostatic overload of the HPA axis together with changes in motivated behavior and sleep rhythm which may indicate a depressive-like phenotype. A significant upregulation of clock genes in the brain which may be related to the disturbed sleep rhythm was also found in stressed mice. Thus, the hypotheses for CS can not be rejected. However, all these changes were only found in CS7 and not CS4. Further investigations are needed to substantiate the hypothesis on the involvement of clock genes in disturbed sleep rhythm. RRS14 demonstrated acute increased CORT in response to RS during the whole study but no significant increase in basal CORT levels. In addition, RRS14 demonstrate a trend of increased locomotor activity in the stress group but not a depressive-like phenotype. Significant upregulation of Il-1β gene expression acutely suggests that RS induce neuroinflammation in mice. Thus, the hypotheses about acute plasma CORT and neuroinflammation can not be rejected. Finally, iRRS induced a significant increase in basal CORT levels indicative of allostatic overload of the HPA axis. Behavior assays of iRRS showed decreased locomotor activity but no change in motivated behavior, which is not sufficient to define a depressive-like phenotype. At last, iRRS had significantly upregulated Il-1β 48h after the last stress exposure indicating that RS induce neuroinflammation. Thus, the hypotheses for allostatic overload of the HPA axis and neuroinflammation can not be rejected.

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Appendix

CS: Burrowing results

A

The full data set from the burrowing assay in the CS study is displayed in figure A.1.

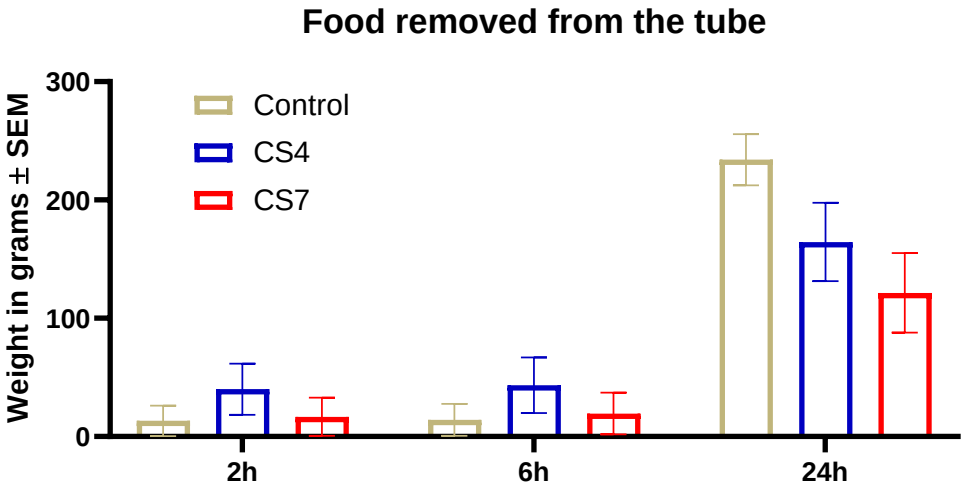

Figure A.1.: All burrowing data from the CS study. The mice does not seem to perform burrowing during the first 6h, however, a notable difference is seen between groups after 24h.

B

RRS pilot study

The aim of the study was to investigate if 5 days of RRS was sufficient to induce a sustained significant increase in plasma CORT of C57Bl6 J male mice. Furthermore, body weight was measured during the study and locomotor activity was measured 3 days after the last stress day.

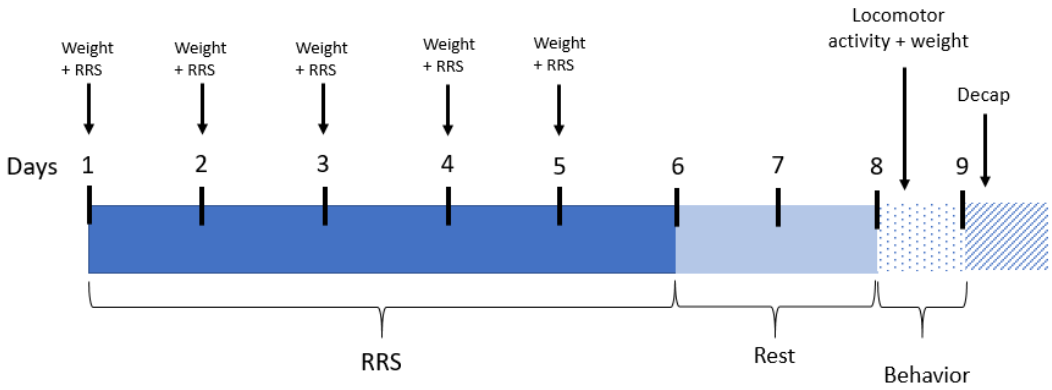

Figure B.1.: Timeline of the RRS pilot study. Five days of 30 minutes RS was performed. Body weight was measured on all stress days and on day 8 after behavior. Locomotor activity was measured before noon on day 8.

The results show no significant increase in basal CORT but a tendency to increased locomotor activity in stressed mice. Moreover, ANOVA showed a significant effect of time ($F(3.904, 62.47) = 7.519$, $P<0.0001$), stress ($F(1, 16) = 10.89$, $P=0.0045$) and corresponding interaction ($F(5, 80) = 4.338$, $P=0.0015$) on body weight. Multiple comparisons showed a significant difference between RRS and control on day 4 ($P=0.0223$) and 5 ($P=0.0283$).

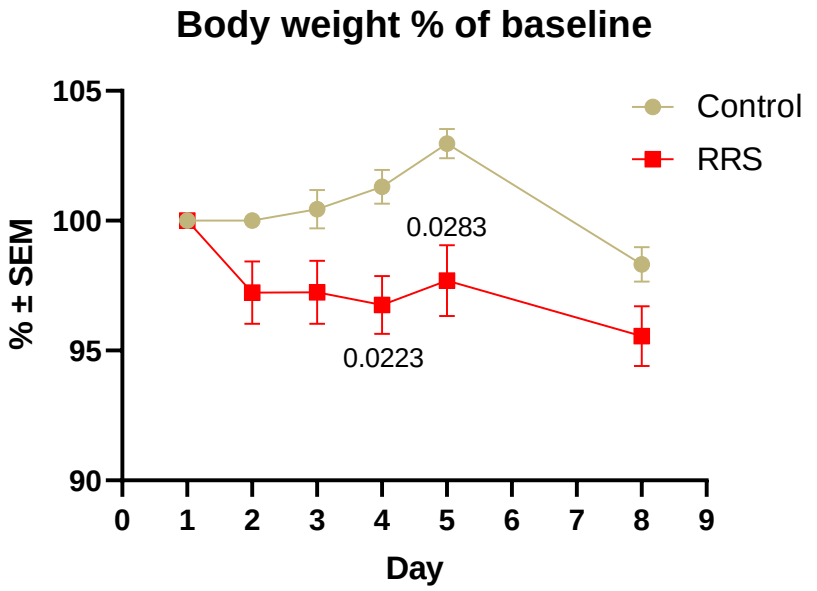

Figure B.2.: Body weight results of the RRS pilot study. Five days of 30 minutes RS was performed. Body weight was measured on all stress days and on day 8 after behavior. Significant difference between RRS and control was found on day 4 (P=0.0223) and 5 (P=0.0283)

Locomotor activity and rearing was measured for 3h hours and plasma was taken at euthanization to measure CORT levels. There was no significant difference between control and RRS in locomotor activity and plasma CORT.

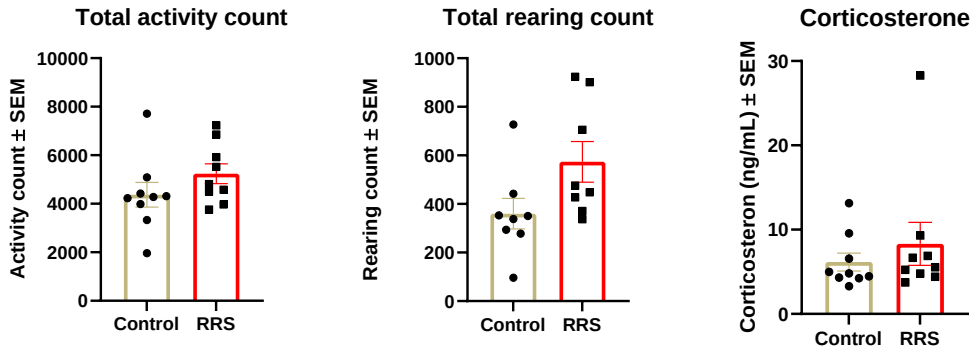

Figure B.3.: Locomotor activity and plasma CORT levels from RRS pilot study. No significant difference was found between control and RRS for locomotor activity, rearing and plasma CORT.

C

NEST data by Christian Spang Pedersen

Investigation of the social aspect of nest building in C57Bl6 J male mice (single-housing vs. 2 mice pr. cage) performed by Christian Spang Pedersen from Lundbeck A/S. The results presented in figure C.1 show there there is no difference in nest building between single-housed mice and 2 mice pr. cage.

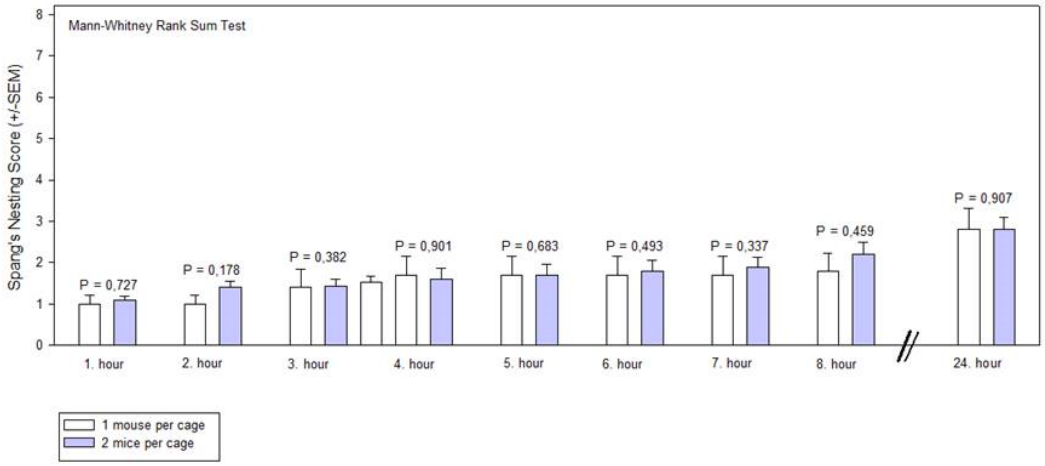

Figure C.1.: Results from NEST by Christian Spang Pedersen from Lundbeck A/S. The social aspect of nest building was investigated by comparing single-housed mice with 2 mice pr. cage.

D

Plasma CORT of control groups from a 14-day RRS study by Amalie Clement

Investigation of plasma CORT levels in control groups during a 14-day RRS study. The study was performed by Amalie Clement from Lundbeck A/S and is almost identical with RRS14 in this thesis. The results show decreasing plasma CORT levels in the control group along the study.

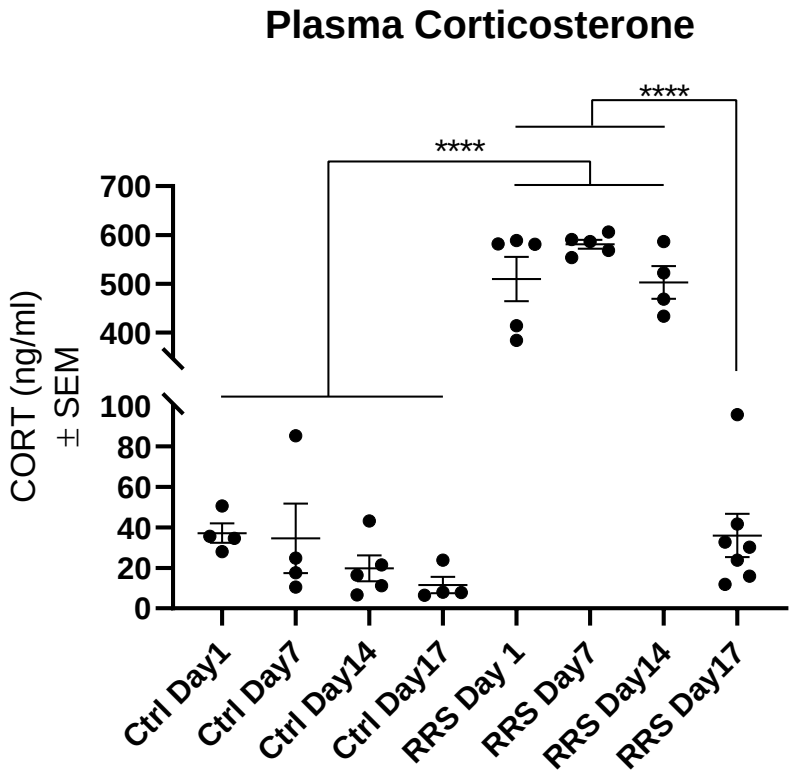

Figure D.1.: Plasma CORT results from a 14-day RRS study by Amalie Clement from Lundbeck A/S. Investigation of plasma CORT levels in the control groups during the study in C57Bl6 J male mice. The results show that the controls show decreasing levels in plasma CORT towards the end of the study.