# Effects of chronic psychosocial stress on the development of a PTSD- and SAD-like phenotype in male mice

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**“All you need [for your PhD] is faith, trust and a little bit of pixie dust”**

(J.M. Barrie, Peter Pan; revisited by Katharina Gryksa-Zotz)

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# Abstract

# Abstract

Chronic psychosocial stress is a burden of modern society and risk factor for numerous somatic and affective disorders, including anxiety and posttraumatic stress disorders (PTSD). Both, human and animal studies suggest that an over-reactive immune system accompanies stress-associated disorders and is involved in their pathogenesis. The mouse model of chronic subordinate colony housing (CSC) mimics this type of health-compromising stress by exposing male mice to 19 consecutive days of subordination by a larger dominant aggressor mouse (resident). CSC exposure induces behavioral, physiological, and immunological alterations described in PTSD patients. However, developing PTSD requires a traumatic event that is actively avoided and PTSD is highly comorbid with other psychiatric disorders like social anxiety disorder (SAD). The mouse social fear conditioning (SFC) paradigm resembles a social traumatic event and induces social avoidance, a core symptom of SAD, by pairing social investigation with punishments. Furthermore, social fear extinction training resembles exposure therapy, commonly used to treat PTSD and SAD patients. Nevertheless, treatment options for these diseases are highly limited, and many patients do not respond.

Thus, the present thesis addresses the impact of exposure to chronic psychosocial stress (CSC) on the susceptibility to develop social fear, induced by a social trauma (SFC). I hypothesized that CSC exposure prior to SFC accelerates the onset and strengthens symptoms of a PTSD- and SAD-like phenotype via an activation of the immune system. Therefore, I studied underlying effects, including social behavior following CSC exposure and changes of the peripheral and central immune system following CSC exposure, SFC acquisition and the combination of both. Hereby, I focused on central inflammation, i.e., microglia and pro- and anti-inflammatory mediators, in the amygdala, septum, and ventral hippocampus (vHC), which are critically involved in stress, fear, SFC, and stress-related psychiatric diseases including anxiety disorders and PTSD. Moreover, I investigated, how specific the effect of chronic psychosocial stress exposure on subsequent fear expression is, depending on the nature (social versus non-social) of the fear conditioning model, the nature and composition of the chronic stressor paradigm, and the mouse strain used. Thus, CSC mice and single housed controls (SHC) were exposed to non-social, i.e., cued fear conditioning (CFC). Next, I analyzed the effects of non-social, i.e., chronic unpredictable stress (CUS) exposure on CFC and SFC. Moreover, I examined potential strain differences in C57BL/6N (BL6) and CD1 mice, since BL6 mice are commonly used in the CSC paradigm, while the SFC paradigm was established in CD1 mice. Therefore, BL6 and CD1 mice were tested for their innate anxiety- and fear-related behavior, social behavior, and stress coping behavior during CSC exposure. Moreover, I investigated the impact of CSC exposure on SFC in CD1 mice. Finally, I

studied whether acute central oxytocin (OXT) treatment can improve the social fear extinction success in fear-susceptible BL6 mice and protect BL6 and CD1 mice against chronic stress-induced impaired SFC extinction, due to its manifold properties and SFC protective effects.

In the present thesis, I could show that during SFC, CSC mice showed facilitated acquisition and impaired extinction of social fear, compared to SHC. This was not accompanied by alterations in social preference behavior or social memory abilities following CSC exposure, but with a context and brain-dependent dysregulation of the central immune system. Here, 2 hours following CSC exposure, central inflammation was decreased, indicated by reduced mRNA expression of microglia marker, addressing microglia density and pro-inflammatory morphological changes, in the amygdala, and reduced inflammasome mRNA expression in the septum compared to SHC. Moreover, this seemed to be mediated by increased mRNA expression of the anti-inflammatory agent glucocorticoid-induced leucine zipper (GILZ), an early transcriptional target of glucocorticoids. In the vHC, CSC mice showed increased microglia density, which might contribute to the increased anxiety-like behavior, a robust effect following CSC exposure. Following SFC acquisition, peripheral stress and immune parameters were not affected, while in the brain, induction of social fear ($SFC^+$) was associated with increased GILZ mRNA expression in the amygdala. Foot shock exposure *per se* (shock controls), however, led to microglia inhibition in the vHC, indicating distinct inflammatory pathways in $SFC^+$ and shock controls. Combined exposure to CSC and SFC acquisition did not affect peripheral inflammation, but increased central inflammation. In contrast to CSC exposure only, unconditioned CSC mice ($CSC/SFC^-$) and CSC mice independent of the conditioning status, showed increased mRNA and / or protein levels of microglia density and pro-inflammatory microglia, as well as of the pro- and anti-inflammatory cytokines interleukin (IL)-6 and IL-4, respectively, in the amygdala and septum, compared to respective SHC. CSC exposure followed by an acute social trauma ($CSC/SFC^+$) seemed to exaggerate the central immune response in the amygdala via increased activation of the inflammatory nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) signaling pathway, compared to $SHC/SFC^+$ and $CSC/SFC^-$. This might be mediated by dysregulated GILZ expression, which was increased following CSC exposure and induction of social fear. Furthermore, CSC exposure did not affect freezing behavior during CFC, while CUS exposure affected fear-related behavior neither during CFC, nor during SFC. However, CUS exposure increased social investigation in $CUS/SFC^-$ compared to $SHC/SFC^-$ during SFC extinction training. Nevertheless, these results have to be interpreted with caution, as CUS mice exposed to CFC did barely show an induction of chronic stress, while during SFC, neither CUS mice nor SHC showed extinction of the social fear. Analyzing strain differences in BL6 and CD1 mice revealed reduced anxiety-like behavior, and CFC- and SFC-related fear behavior, as well as increased social behavior

in CD1 compared to BL6 mice. In addition, when both strains were exposed to the CSC paradigm, they differed in stress-coping behavior and stress-vulnerability. Here, during day 1 of CSC exposure, CD1 mice showed higher active stress coping behavior compared to BL6 mice, whereas during day 8 of CSC exposure, CD1 and BL6 mice differed in reactive emotional coping behavior. Thus, CD1 mice showed more submissive upright and less flight behavior compared to BL6, which was accompanied with less attacks received by the resident. Nevertheless, CSC exposure impaired SFC-related behavior also in CD1 mice. Finally, acute central OXT infusion did not affect social fear extinction in BL6 mice, as previous surgery seemed to prevent social fear extinction in BL6 mice. Nevertheless, in CD1 mice, acute icv OXT infusion 10 minutes prior to SFC extinction training rescued CSC-induced impairment of SFC extinction.

In sum, these data demonstrate that CSC exposure prior to SFC facilitated traumatic memory encoding and increased the risk to develop a PTSD- and SAD-like phenotype. This effect was not associated with altered social behavior, but brain region-dependent central inflammation following CSC exposure and SFC acquisition, shifting the central immune system towards an inflammatory state. Thus, an exaggerated immune response might mediate the impaired extinction of social fear after CSC exposure. Moreover, the effects of chronic psychosocial stress-induced impaired SFC-related behavior seemed to be specific to the social nature of the fear conditioning model and the chronic stressor paradigm, as CSC exposure did not influence freezing behavior during CFC, while CUS exposure affected neither CFC, nor SFC. Furthermore, even though BL6 and CD1 mice strongly differed in trait anxiety-, fear-, and stress-related behavior, the impact of CSC exposure on social fear extinction was comparable between strains. I could further demonstrate that OXT is effective in reversing CSC-induced impaired SFC extinction in CD1 mice. Thus, OXT seems to represent a potent treatment option during the development of a PTSD- and SAD-like phenotype and might serve as an add-on pharmacotherapy during exposure therapy in PTSD and SAD patients.

# Zusammenfassung

# Zusammenfassung

Chronischer psychosozialer Stress ist eine Belastung der modernen Gesellschaft und stellt einen hohen Risikofaktor für die Entstehung von somatischen und affektiven Krankheiten, wie beispielsweise Angststörungen und posttraumatischen Belastungsstörungen (PTBS), dar. Sowohl in Human-, als auch in Tierversuchsstudien wurde gezeigt, dass eine Überreaktion des Immunsystems mit stressbedingten Krankheiten einhergeht und an deren Entstehungen maßgeblich beteiligt ist. Das Maus-Modell der chronischen subordinierten Koloniehaltung (chronic subordinate colony housing, CSC) imitiert diesen gesundheitsgefährdenden Stress, indem Mäuse (die CSC Mäuse) für 19 Tage einer größeren, aggressiveren Maus (dem Resident) unterworfen werden. Es wurde gezeigt, dass CSC Mäuse Veränderungen bezüglich ihres Verhaltens, der Physiologie und der Immunologie zeigen, die auch in PTBS Patienten beschrieben wurden. Die Entstehung von PTBS benötigt es ein traumatisches Ereignis, das aktiv von den Patienten vermieden wird, wobei PTBS im höchsten Maße komorbid mit anderen affektiven Krankheiten ist, wie der sozialen Angststörung (social anxiety disorder, SAD). Das Maus- Modell der sozialen Furchtkonditionierung (social fear conditioning, SFC) stellt ein soziales Trauma dar und induziert ein soziales Meide-Verhalten, dem Hauptsymptom bei SAD, indem es soziale Interaktionen bestraft. Zudem ist die Auslöschungsphase des SFC ähnlich einer Expositionstherapie, die häufig verwendet wird um Patienten mit PTBS und SAD zu behandeln. Dennoch sind die Behandlungen dieser Krankheiten stark eingeschränkt, da unter anderem viele Patienten nicht auf die Therapien ansprechen.

Aus diesem Grund wurde die vorliegende Doktorarbeit mit dem Ziel verfasst, den Einfluss von chronischem psychosozialen Stress (mittels des CSC Modells) auf die Entwicklung sozialer Furcht zu untersuchen, welche durch ein soziales Trauma (mittels des SFC Modells) induziert wird. Ich nehme an, dass Mäuse, die dem CSC Modell ausgesetzt waren, bevor sie im SFC konditioniert wurden, schneller einen PTBS- und SAD- ähnlichen Phänotyp entwickeln und stärkere Symptome dieser Krankheiten aufzeigen, als Kontrolltiere (SHC), was auf eine starke Aktivierung des Immunsystems zurückzuführen ist. Daher habe ich auch zugrundeliegende Veränderungen in CSC Mäusen untersucht, einschließlich ihres Sozialverhaltens und Veränderungen des peripheren und zentralen Immunsystems nach einer CSC Exposition, SFC Akquisition sowie der Kombination beider Modelle. Hierbei habe ich mich vor allem auf Veränderungen des zentrale Immunsystems fokussiert, also auf Veränderungen der Mikroglia und pro- und anti-inflammatorischen Vermittlern des Immunsystems, in den Gehirnregionen Amygdala, Septum und ventralen Hippocampus (vHC), welche eine wichtige Rolle bei Stress, Furcht, SFC und stressbedingten Krankheiten wie Angststörungen und PTBS spielen. Zudem habe ich mich mit der Spezifität der

Effekte des chronischen psychosozialen Stressors auf eine darauffolgende Furcht befasst, also ob die Auswirkungen abhängig von der Art (sozial oder nicht-sozial) des Furchtkonditionierungs-Modells, der Art und Beschaffenheit des chronischen Stress Modells sowie die verwendete Mauslinie sind. Daher wurden CSC Mäuse und SHC einer nicht-sozialen, Ton-induzierten Furchtkonditionierung (cued fear conditioning, CFC), ausgesetzt. Darüber hinaus habe ich den Einfluss eines nicht-sozialen, also einem chronischen unvorhersehbaren Stress (CUS) Modell auf CFC und SFC untersucht. Zusätzlich habe ich mögliche Verhaltensunterschiede in den Mauslinien C57BL/6N (BL6) und CD1 untersucht. BL6 Mäuse werden üblicherweise für das CSC Modell verwendet, während in CD1 Mäusen das SFC Modell etabliert wurde. Hierbei wurden BL6 und CD1 Mäuse auf ihr inneres Angst- und Furchtverhalten, Sozialverhalten und Stress-Bewältigungsverhalten während des CSC Modells getestet. Außerdem habe ich den Einfluss einer CSC Exposition auf das Verhalten im SFC in CD1 Mäusen getestet. Zuletzt habe ich noch geprüft, inwiefern eine akute Verabreichung von Oxytocin (OXT) in das Gehirn die Auslöschung der sozialen Furcht in den anfälligeren BL6 Mäusen beschleunigen, sowie in BL6 und in CD1 Mäusen eine durch chronischen Stress verschlechterte Auslöschung der sozialen Furcht verhindert werden kann. Diese Effekte könnte OXT aufgrund der vielseitigen Wirkungsweisen und des schützenden Effekts während des SFC erzielen.

Ich konnte in dieser Doktorarbeit zeigen, dass während des SFC CSC Mäuse im Vergleich zu SHC schneller eine soziale Furcht generierten, wobei diese nur verzögert wieder ausgelöscht werden konnte. Dies ging nicht mit Veränderungen in der sozialen Präferenz oder dem sozialen Gedächtnis nach einer CSC Exposition einher, sondern mit einer Kontext- und Gehirnregion-abhängigen Veränderung des zentralen Immunsystems. Zwei Stunden, nachdem die Mäuse dem CSC ausgesetzt waren, kam es zu einer im Vergleich zu SHC herunterregulierten zentralen Immunantwort. Diese Beobachtung war auf eine verringerte Expression der mRNA verschiedener Mikroglia-Marker, sowohl die Anzahl als auch die pro-inflammatorische Morphologie von Mikroglia betreffend, in der Amygdala und die mRNA des Inflammasoms im Septum zurückzuführen. Diese Effekte schienen durch ein erhöhtes Aufkommen des anti-inflammatorisch wirkenden Glucocorticoid-induziertem leucine zipper (GILZ), einem Gen, das durch Glucocoticoide unmittelbar induziert wird, entstanden zu sein. Im vHC wiesen CSC Mäuse eine erhöhte Anzahl von Mikroglia auf, was zu dem erhöhten Angstverhalten in CSC Mäusen beitragen könnte, einer robusten Auswirkung des CSC. Nach einer SFC Akquisition waren weder periphere Stress noch Immun Parameter verändert, während im Gehirn die Induktion sozialer Furcht ($SFC^+$) zu einer Erhöhung der mRNA von GILZ in der Amygdala führte. Im Gegenteil dazu führte nur die Verabreichung eines elektrischen Fuß-Schocks (Schock Kontrolle) zu einer Inhibierung von Mikroglia in dem vHC. Diese Daten weisen darauf hin, dass $SFC^+$ und Schock Kontrollen einen

unterschiedlichen inflammatorischen Signalweg zugrunde liegen haben. Eine Kombination der Modelle des CSC und SFC hatte zwar keinen Einfluss auf das periphere Immunsystem, führte aber zu einer erhöhten Entzündungsreaktion im Gehirn. Im Gegenteil zu nur einer CSC Aussetzung, zeigen unkonditionierte CSC Mäuse (CSC/SFC$^{-}$) und CSC Mäuse unabhängig von ihrem Konditionierungs-Status im Vergleich zu den jeweiligen SHC eine erhöhte Expression der mRNA und / oder Proteine bezüglich der Anzahl und pro-inflammatorischen Morphologie von Mikroglia, als auch pro- und anti-inflammatorischer Zytokine wie Interleukin (IL)-6, beziehungsweise IL-4 in der Amygdala und dem Septum auf. Mäuse, die dem CSC ausgesetzt wurden und ein soziales Trauma erfuhren (CSC/SFC$^{+}$), schienen eine starke zentrale Immunantwort in der Amygdala zu haben, da sie, im Vergleich zu SHC/SFC$^{+}$ und CSC/SFC$^{-}$, eine stärkere Aktivierung des inflammatorischen „nuclear factor 'kappa-light-chain-enhancer' of activated B-cells" (NFκB) Signalwegs aufwiesen. Dies könnte durch eine erhöhte Expression von GILZ begünstigt werden, was sowohl nach einer CSC Exposition, als auch einer Induktion von sozialer Furcht aufgewiesen wurde. Des Weiteren hatte eine CSC Exposition keinen Einfluss auf das Furchtverhalten während des CFC, wobei auch Mäuse, die dem CUS Modell ausgesetzt waren, weder Verhaltensänderungen im CFC noch im SFC zeigten. CUS/SFC$^{-}$ aber zeigten während der Auslöschungsphase des SFC mehr Kontakt den Artgenossen gegenüber als SHC/SFC$^{-}$. Nichtsdestotrotz sollten diese Versuche vorsichtig interpretiert werden, da CUS Mäuse die anschließend im CFC getestet wurden, kaum Anzeichen chronischen Stresses zeigten, während beim SFC weder CUS Mäuse noch SHC eine erfolgreiche Auslöschung der sozialen Furcht zeigten. Bezüglich der Verhaltensunterschiede zwischen den beiden Mauslinien zeigten CD1 im Vergleich zu BL6 Mäusen weniger Angst-, CFC- und SFC-bezogenes Furchtverhalten, sowie ein höheres Sozialverhalten. Zudem unterschieden sich die beiden Mauslinien maßgeblich in ihrem Stress-Bewältigungsverhalten und der Stress-Anfälligkeit während des CSC. Am Tag 1 des CSC zeigten CD1 im Gegensatz zu BL6 Mäusen vermehrt eine aktive Stress-Bewältigung, wohingegen an Tag 8 CD1 und BL6 Mäuse ein unterschiedliches Verhaltensrepertoire der passiven Stress-Bewältigung aufwiesen. Folglich zeigten CD1 Mäuse vermehrt ihre empfindliche Bauchseite, eine unterwürfige Geste, und ergriffen seltener die Flucht als BL6 Mäuse, was zu weniger Angriffen seitens des Residents führte. Dennoch beeinträchtigte eine vorangegangene CSC Exposition das Verhalten während des SFC auch in CD1 Mäusen. Schlussendlich konnte ich zeigen, dass eine akute Verabreichung von OXT ins Gehirn das soziale Furchtverhalten in BL6 Mäusen nicht beeinflusste, da eine vorangegangene Operation generell die Auslöschung der erlernten sozialen Furcht in BL6 Mäusen zu verhindern schien. In CD1 Mäusen konnte jedoch eine akute Verabreichung von OXT in den lateralen Ventrikel die durch CSC beeinträchtigte Auslöschung der sozialen Angst verhindern.

Zusammengefasst zeigen diese Daten, dass eine CSC Exposition vor dem SFC die Kodierung von traumatischen Erinnerungen erleichtert und das Risiko erhöht, einen PTBS oder SAD ähnlichen Phänotypen zu entwickeln. Dieser Effekt ging nicht mit einem veränderten Sozialverhalten einher, jedoch mit einer erhöhten Entzündungsreaktion im Gehirn nach einer CSC und anschließender SFC Exposition, was dazu führte, dass die Balance des zentralen Immunsystems zu einem pro-inflammatorischen Status verschoben wurde. Demnach könnte eine Überreaktion des Immunsystems die verschlechterte Auslöschung der sozialen Furcht nach dem CSC begünstigen. Des Weiteren schien der Effekt einer beeinträchtigen Auslöschung der sozialen Furcht nach chronischem psychosozialen Stress spezifisch bezüglich der sozialen Art der Modelle der Konditionierung und des chronischen Stressors zu sein. Grund für diese Annahme ist, dass eine CSC Exposition Furchtverhalten im CFC nicht beeinflusste, und eine CUS Exposition das Furchtverhalten weder im CFC, noch im SFC beeinflusste. Obwohl BL6 und CD1 Mäuse sich stark im angebotenen Angst-, Furcht- und Stress-bezogenem Verhalten unterschieden, war der Einfluss des CSC auf die Auslöschung der sozialen Furcht in beiden Mauslinien gleich hoch. Ich konnte außerdem zeigen, dass OXT erfolgreich die durch CSC Exposition beeinträchtigte Auslöschung der sozialen Angst in CD1 Mäusen verhinderte. Demzufolge scheint OXT eine wirksame Behandlungsmöglichkeit bei der Entstehung eines PTBS- und SAD-ähnlichen Phänotyps zu sein und könnte demzufolge eine zusätzliche Pharmakotherapie im Zuge einer Expositionstherapie in PTBS und SAD Patienten darstellen.

# Abbreviations

| Abbreviations | Definitions |
| --- | --- |
| ACTH | Adrenocorticotropic hormone |
| ANOVA | Analysis of variance |
| Arg | Arginase |
| AVP | Arginine vasopressin |
| BL6 | C57BL/6N |
| BSA | Bovine serum albumin |
| Casp | Caspase |
| CBT | Cognitive behavioral therapy |
| CD | Cluster of Differentiation |
| cDNA | Complementary deoxyribonucleic acid |
| CFC | Cued fear conditioning |
| CNS | Central nervous system |
| CORT | Corticosterone |
| COVID-19 | Coronavirus disease 2019 |
| CRH | Corticotropin-releasing hormone |
| CRP | C-reactive protein |
| CS | Conditioned stimulus |
| CSC | Chronic subordinate colony housing |
| CSDS | Chronic social defeat stress |
| CUS | Chronic unpredictable stress |
| CV | Coefficients of variation |
| DEX | Dexamethasone |
| DSM | Diagnostic and statistical manual of mental disorders |
| DSS | Dextran sulfate sodium |
| ELISA | Enzyme linked immunosorbent assay |
| EPM | Elevated plusmaze |
| FBS | Fetal bovine serum |
| FS | Forced swim |
| GCs | Glucocorticoids |
| GILZ | Glucocorticoid-induced leucine zipper |
| GR | Glucocorticoid receptor |
| gSAD | Generalized social anxiety disorder |
| HC | Hippocampus |

| | |
|---|---|
| HPA | Hypothalamus-pituitary-adrenal |
| Iba-1 | Ionized calcium binding adaptor molecule 1 |
| Icv | Intracerebroventricular |
| IFN | Interferon |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| kDa | Kilodalton |
| LB | Light box |
| LDB | Light dark box |
| LPS | Lipopolysaccharide |
| LS | Lateral septum |
| *M. vaccae* | *Mycobacterium vaccae* |
| mesLNCs | Mesenteric lymph node cells |
| mesLNs | Mesenteric lymph nodes |
| MP | Milk powder |
| mRNA | Messenger Ribonucleic acid |
| mSDT | Modified social discrimination test |
| NFκB | Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |
| Nlrp | Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing |
| NOR | Novel object recognition |
| OC | Overcrowding |
| OF | Open field |
| OXT | Oxytocin |
| OXTR | Oxytocin receptor |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PFC | Prefrontal cortex |
| pNFκB | Phosphorylation of the NFκB subunit |
| PTSD | Posttraumatic stress disorder |
| PVN | Paraventricular nucleus |
| qPCR | Quantitative polymerase chain reaction |
| RM | Repeated measures |
| RPL | Ribosomal Protein L13a |
| RS | Restraint stress |

| | |
|---|---|
| RT | Room temperature |
| SAD | Social anxiety disorder |
| SARS-CoV | Severe acute respiratory syndrome coronavirus |
| SD | Social defeat |
| SEM | Standard error of the mean |
| Ser276 | Serine 276 |
| SFC | Social fear conditioning |
| SHC | Single housed controls |
| SNS | sympathetic nervous system |
| SPT | Social preference test |
| SSRIs | Selective serotonin reuptake inhibitors |
| TBS-T | Tris-buffered saline containing Tween-20 |
| TGF | Transforming growth factor |
| Tmem119 | Transmembrane Protein 119 |
| TNF | Tumor necrosis factor |
| Tregs | Regulatory T cells |
| US | Unconditioned stimulus |
| VEH | Vehicle |
| vHC | Ventral Hippocampus |
| WHO | World health organization |

# Introduction

# 1 Introduction

*"Everybody knows what stress is and nobody knows what it is"* (Selye, 1973).

Even though this statement has been issued by Hans Selye in 1973, it still holds true. Many people experience stress almost daily, while its aftereffects, underlying mechanisms and the impact of different types of stressors (i.e., stressor specificity) are still not entirely understood. Especially during the current "Corona crisis" the life of billions of people has changed, and the level of psychosocial stress has dramatically increased. The novel virus, named "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2), caused coronavirus disease 2019 (COVID-19), which has been characterized as pandemic by the world health organization (WHO) in March 2020 (WHO, 2020). Lockdowns, social distancing, and quarantine urged people to exclusively work and teach from home and to restrict social contacts to a minimum; lonely people got into further isolation, while families` nerves were strained to the extreme. Overall, this resulted in a dramatic and chronic increase in the level of psychosocial stress over several months and even years, mainly caused by social isolation on the one side and psychosocial stress associated with overcrowding (OC), social tension in families, and domestic violence on the other (Gryksa and Neumann, 2021). Even though the interest in studying the effects of chronic psychosocial stress on mental and general health is increasing, in-depth knowledge is still required to not only understand the term "stress" and its short- and long-term aftereffects, but also to develop potent treatment options.

## 1.1 Definition and physiological response to stress

Hans Selye was the first, who defined "stressor" as a specific challenge that causes a physiological response, and "stress" as the physiological response of the body to restore internal balance, in 1975 (Selye, 1975). However, the theory of stress is even older. In 1865, the French physiologist Claude Bernard described "homeostasis" as a stable internal equilibrium of an organism within the fluctuating external environment (Cannon, 1939). Thereby, the network of interacting factors, by which homeostasis was achieved, has been called "allostasis" (McEwen, 2004).

In response to a physical or psychological threat, the brain coordinates multiple physiological responses involving autonomic, neuroendocrine, metabolic, and immune systems. In mammals, the two main systems of the physiological stress response are the sympathetic nervous system (SNS) as part of the autonomous nervous system, and the hypothalamus-pituitary-adrenal (HPA) axis (Figure 1). These systems do not only differ in the time course of their response, but also in the nature of pathways and effector hormones involved to deal with the threat and to restore homeostasis. Activation of the SNS occurs rapidly within seconds to minutes via neuronal connections that originate in the thoracolumbar region of the spinal cord. These

preganglionergic neurons project to pre- or paravertebral ganglia, where they connect to postganglionergic neurons, which in turn project to the target organs, such as liver, lung, and heart. In this context, the adrenal glands are an exception, as preganglionergic, i.e., acetylcholinergic neurons, project directly to the chromaffin cells of the adrenal medulla to rapidly stimulate adrenalin and noradrenalin release. The SNS controls cardiovascular, gastrointestinal, respiratory and other somatic systems (Chrousos, 1998; Ulrich-Lai and Herman, 2009). In contrast, activation of the HPA axis occurs with a slight temporal delay of minutes to hours via exclusive hormonal release. Activation of the HPA axis is initiated by neurons in the medial parvocellular region of the hypothalamic paraventricular nucleus (PVN), which release corticotropin-releasing hormone (CRH) – and under some circumstances arginine vasopressin (AVP) (Aguilera, 1994; Keeney et al., 2006; Lupien et al., 2009) – into the median eminence, a circumventricular organ located in the infundibulum, where CRH is transported via the portal blood system to the anterior pituitary. Binding to the CRH receptor triggers the secretion of adrenocorticotropic hormone (ACTH) into the peripheral blood system. In the zona fasciculata of the cortex of the adrenal gland, ACTH stimulates the production and secretion of glucocorticoids (GCs; cortisol in humans, corticosterone (CORT) in rodents). After activation of the HPA axis an efficient negative feedback loop, mediated by GCs that act on glucocorticoid (GR) and mineralocorticoid receptors at several levels within the brain, like hippocampus (HC), hypothalamus and anterior pituitary, suppresses the HPA axis activity and, thus, reinstates homeostasis (reviewed in Herbert et al., 2006; Langgartner et al., 2015; Lupien et al., 2009).

The intensity and duration of the stress response towards a stressor is dependent on many factors, including the type and duration of the stressor, the way in which the organism perceives it and the individual ability to cope with it (Goldstein and Kopin, 2007). When the stress response is not adequately terminated due to prolonged stressor exposure, called chronic stress, or overused by frequent and repeated stressors over weeks or months, the body is engaged in prolonged or repeated attempts to reinstate homeostasis. This “allosteric overload” induces maladaptation of cardiovascular, metabolic and immune systems, and of brain functioning, resulting in a disease state (McEwen, 2004). Therefore, chronic stress is a common risk factor for developing somatic disorders like chronic inflammatory disorder (Bernstein et al., 2010) and cancer (Hou et al., 2013), but also mental diseases including depression, anxiety disorders (Chiba et al., 2012), and posttraumatic stress disorder (PTSD) (Davidson and Baum, 1986; reviewed in Langgartner et al., 2015; McEwen, 2004).

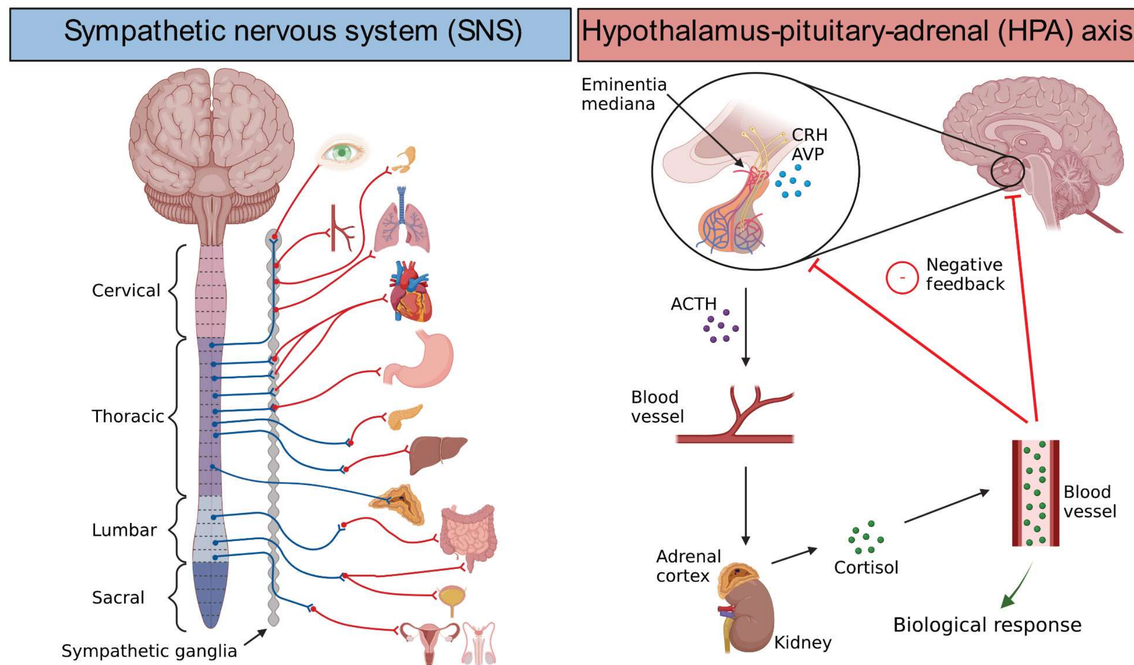



**Figure 1: Schematic illustration of the sympathetic nervous system (SNS) and the hypothalamus-pituitary-adrenal (HPA) axis.** Neuronal pathways of the SNS (left) are originating from the thoracolumbar region of the spinal cord innervating sympathetic ganglia and finally the end organs. The adrenal glands are directly innervated and stimulated to release adrenalin and noradrenalin from chromaffin cells of the medulla. The HPA axis (right) is activated via hormonal release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus into the eminentia mediana, from where they are transported via the portal blood to the anterior pituitary. Here, adrenocorticotropic hormone (ACTH) is released into the blood and transported to the adrenal cortex, where cortisol is released. An efficient negative feedback loop terminates HPA axis activity to restore homeostasis. The illustration has been created using BioRender.com.

## 1.2 Different types of stress

Stress is mostly afflicted with negative effects and disease state. However, the acute stress response is very important and even healthy for an organism, and represents one of the major survival mechanisms. In 1929, Walter Cannon named this important mechanism, mainly induced by activation of the SNS, the “fight-or-flight response”. Without this, a lion would have no chance to catch a gazelle, while a gazelle would have no chance to escape from a lion (Dhabhar, 2009). In accordance, activation of the SNS results in an instant behavioral response, such as increased arousal and alertness, as well as anxiety / fear or aggression, with numerous different brain regions being involved (Arble et al., 2019; Charmandari et al., 2005; Clement et al., 2005; Grillon et al., 2007; Hendley et al., 1988; Katz et al., 1981; Tonelli et al., 2008). However, as an opossum, for example, shows tonic immobility to escape from predators, researchers had to rethink and rephrase this response into the “freeze, flight, fight or fright” response (Bracha, 2004), which has originally been described by Gray in 1987 (Gray, 1987). In line, acute stress may also induce

freezing behavior (hypervigilance) and fright (tonic immobility) in vertebrates (Bracha et al., 2004; Bracha, 2004; Gray, 1987; Rupia et al., 2016).

In contrast to acute challenges, prolonged exposure to stressors can lead to a dysregulation of the stress systems and, thus, induce a disease state (Charmandari et al., 2005). Therefore, it is important to distinguish between acute and chronic stress. While acute stress was defined as the response to a short-lasting stressor that lasts for a period of minutes to hours, chronic stress lasts for days to months (Dhabhar, 2000), depending on the species. Acute stressor exposure in male mice raised blood concentrations of ACTH and CORT to peak level after 15 and 30 minutes, respectively, and declined slowly to basal levels, via the negative feedback loop, after 60 to 90 minutes (Keeney et al., 2006). In female rats, plasma ACTH and CORT levels raised to a peak after 5 and 15 minutes, respectively, and declined to basal levels after 60 minutes (Johnstone et al., 2000; Neumann et al., 1998). In contrast, following chronic social defeat stress (CSDS), GCs were still increased after 24 h (Keeney et al., 2006). In line, sustained increased basal plasma CORT levels were found in rats after 14 days of chronic subordination compared to unstressed controls (Albeck et al., 1997). Interestingly, it has been repeatedly hypothesized that the acute stress response is mediated by increased CRH levels, while the chronic stress response is additionally mediated via increased AVP activation from parvocellular PVN neurons (Aguilera, 1994; Füchsl et al., 2013a; Keeney et al., 2006).

Clinical studies confirmed that chronic activation of the SNS and the HPA axis increases the risk to develop not only various somatic disorders, like cardiovascular diseases (Dimsdale, 2008; Golbidi et al., 2015), inflammatory bowel disease (Duffy et al., 1991), infections (Cohen et al., 1991), and impaired wound healing (Kiecolt-Glaser et al., 1995), but also affective disorders like stress-induced anxiety (Maes et al., 1998), depression (Lopez-Duran et al., 2015; Steinhardt et al., 2011), PTSD (Davidson and Baum, 1986), alcohol and drug abuse disorder (Brown et al., 1990), Alzheimer's Disease (Dong and Csernansky, 2019), bipolar disorder, and schizophrenia (Agid et al., 1999). Thereby, it is hypothesized that stress triggers diseases via an altered output of cortisol, which can result of decreased hormone bioavailability (hypocorticism), attenuated GC sensitivity, and enhanced GC resistance of target cells, or a combination of all (Raison and Miller, 2003). This might lead to tissue damage and subsequent dysregulation of biological systems (reviewed in Miller et al., 2007).

Lastly, the nature of the stressors needs clear differentiation, as different stressors vary in their intensity, which can be measured via heart rate, blood pressure and stress hormone levels, like GCs and catecholamines (Dhabhar, 2000). Several pre-clinical studies support the hypothesis that the risk to develop "allosteric overload" is more likely, when the stressors are not only chronic, but especially unpredictable and of social nature (Bartolomucci et al., 2005).Today´s challenges

include in particular social and psychological stressors (Bartolomucci et al., 2005). In line, humans mostly encounter work-related stress like job insecurity and a poor work-life balance (Ferrie, 1999; Kivimäki and Kawachi, 2015), as well as bullying (Hoel et al., 2002; Hollins Martin and Martin, 2010; Wolke et al., 2015), social interactions (Sgoifo and Meerlo, 2002), and OC (Gove et al., 1979). This results in chronic psychosocial stress, which is a combination of prolonged psychological and social stressors and represents a major risk factor for numerous stress-related psychiatric diseases in humans (Backé et al., 2012; Sgoifo and Meerlo, 2002).

## 1.3 Psychiatric diseases linked to chronic psychosocial stress

Psychiatric diseases, or mental disorders, are defined as clinically significant disturbances of cognitive and emotional behaviors that reflect an impairment in developmental, biological or mental functioning (American Psychiatric Association, 2013). Moreover, this is often accompanied by distress or disability in social or occupational activities, and detraction of daily life (American Psychiatric Association, 2013). As an example, PTSD and anxiety disorders include a high level of fear and anxiety that are related to behavioral disturbances (American Psychiatric Association, 2013). Here, fear and anxiety need to be separated due to distinct definitions. Thus, fear is defined as the emotional response to a real or perceived imminent threat, while anxiety is the emotional response to a potential anticipated future threat. In line, fear is mostly associated with immediate reactions of the SNS including arousal, escape behavior, and immediate thoughts of danger, which are important for the flight or fight response. Anxiety, in contrast, is mostly associated with muscle tension and vigilance in preparation of future danger, as well as cautious or avoidant behaviors (American Psychiatric Association, 2013; Craske et al., 2011).

The global lifetime prevalence to develop a mental, disorder has been pooled out of 39 countries to an estimate of almost 30 % (Steel et al., 2014). Additionally, psychiatric diseases show a high degree of comorbidity, as 35 to 45 % of patients that suffer from one psychiatric disease fulfil the criteria for a second or even more comorbid disorders (van Loo et al., 2013). According to the WHO, anxiety disorders (including PTSD) and depression are the most common mental disorders, which are associated with enormous health care costs and a high burden of disease (Bandelow and Michaelis, 2015; World Health Organization, 2017). Within the USA, PTSD and social anxiety disorder (SAD) are the second and third most commonly diagnosed disorders, respectively (Bandelow and Michaelis, 2015; Kessler et al., 2009; McMillan et al., 2017).

### 1.3.1 Posttraumatic stress disorder (PTSD)

PTSD, which has been classified as an anxiety-disorder until 2013 (American Psychiatric Association, 1998), and is now classified as a trauma- and stressor- related disorder, has a lifetime prevalence of 8.7%. Other disorders that belong to this classification are reactive attachment disorder, disinhibited social engagement disorder, acute stress disorder and adjustment disorder. To develop PTSD requires a traumatic event and induces core symptoms like traumatic nightmares and dissociative flashbacks, which patients experience even when the real threat is not present. Moreover, PTSD patients show an active avoidance of everything related to the traumatic event, as well as alterations in behavior including hyperarousal, anxiety, anger, and impulsive aggression (Albucher and Liberzon, 2002; American Psychiatric Association, 2013). PTSD is common in active military personnel or veterans with a prevalence of about 14 to 16 %. For this reason, PTSD is also characterized as “shell shock” and “battle fatigue” (Shiromani et al., 2009). Nevertheless, not only war resembles a traumatic experience. Other severe and stressful threats that can cause PTSD are sexual abuse, bullying and violence (Carleton et al., 2011; Feerick and Snow, 2005), as well as the experience of natural disasters, like hurricane Katrina (Galea et al., 2007).

PTSD has been associated with a variety of alterations in biological systems including the SNS and HPA axis. In line, PTSD patients showed a hyperactivation of the SNS, which seems to underlie behavioral changes, including hyperarousal. In support, noradrenalin and adrenalin levels were found to be increased during a 24-hour urinary sampling in PTSD patients under basal conditions (Kosten et al., 1987) as well as during exposure to trauma-related stimuli (Liberzon, 1999). Furthermore, urinary catecholamines, like dopamine and noradrenalin, were found to correlate with the severity of PTSD symptoms in veterans of the Vietnam war (Yehuda et al., 1992). In response to awakening, Bosnian war refugees who suffer from PTSD revealed increased salivary alpha-amylase, a non-invasive marker to measure SNS activation (Nater and Rohleder, 2009). Moreover, this marker has been associated with psychiatric symptoms of PTSD and, interestingly, with GC sensitivity on inflammatory cytokine producing cells (Thoma et al., 2012). Moreover, many studies have focused on a dysregulation of HPA axis functioning and activation, when addressing the consequences and underlying mechanisms of PTSD (reviewed in Kloet et al., 2006). Fittingly, humans with low cortisol levels following acute traumatic experiences were more vulnerable to develop subsequent PTSD (Delahanty et al., 2000). In accordance, PTSD patients showed basal hypocortisol in plasma, saliva, and urine (Boscarino, 1996; Heim et al., 2000; Rohleder et al., 2004), as well as a stronger suppression of cortisol release after dexamethasone (DEX) supply, a synthetic GC mimicking the effects of cortisol and, thus, activating the negative feedback of the HPA axis at adenohypophysial level (Newport et al., 2004; Yehuda et al., 2002). In

contrast to a basally rather suppressed HPA axis functioning, PTSD patients show higher basal CRH concentrations within the cerebrospinal fluid (Baker et al., 1999; Bremner et al., 1997; Sautter et al., 2003) and a hyperreactive response of the HPA axis following a stressful challenge or traumatic reminder, indicated by increased cortisol levels (Bremner et al., 2003; Elzinga et al., 2003).

Unfortunately, pharmacological therapy of PTSD is challenging due to its complexity and comorbidity with other psychiatric disorders, e.g. anxiety disorders, depression, substance abuse, suicide and panic disorders (Albucher and Liberzon, 2002; van Minnen et al., 2012). This is particularly problematic, as PTSD is a chronic disorder. Common treatment options that are at least partly effective are tricyclic antidepressants, monoamine oxidase inhibitors, and selective serotonin reuptake inhibitors (SSRIs) (Albucher and Liberzon, 2002). In addition, PTSD patients are often treated with cognitive-behavioral therapies (CBT), especially (prolonged) exposure therapy, which has been shown to be more effective to treat PTSD, but also comorbid symptoms like anxiety and depression (Bradley et al., 2005; Rauch et al., 2012; van Minnen et al., 2012). In line, virtual reality has been established for exposure therapy to confront the patients with the trauma-relevant environment, thereby improving PTSD symptom reduction (Deng et al., 2019b; Shiromani et al., 2009). Nevertheless, despite advantages in therapeutic options, there is still a high number of patients that do not respond to treatments (about 30 to 40 %) (Albucher and Liberzon, 2002; Bradley et al., 2005). This might be based on the complexity of PTSD, as it includes interactions between biology and individual experience, and involves various neurobiological systems as well as different brain regions (Albucher and Liberzon, 2002).

Thus, studying the neuroanatomy of PTSD revealed an involvement of mainly three specific brain regions: the prefrontal cortex (PFC), the amygdala and the HC (Liberzon and Sripada, 2007; Nutt and Malizia, 2004; Shin and Liberzon, 2010). These regions were associated with symptom provocation, cognitive activation, and functional connectivity in PTSD patients. In detail, patients suffering from PTSD show a reduced activation of the medial PFC, leading to an inadequate top-down control of the amygdala, which maintains amygdala hyperresponsivity mediating symptoms like hyperarousal and vigilance (Liberzon and Sripada, 2007). In agreement, PTSD patients revealed a hyperactivity of the amygdala, when they were confronted with trauma-related stimuli (Nutt and Malizia, 2004). In contrast, other core symptoms like flashbacks seem to be related to dysregulations in higher brain regions like HC and medial PFC (Nutt and Malizia, 2004). In line, deficient HC functioning can also lead to an overconsolidation of traumatic memories, the beginning of the development of PTSD (reviewed in Liberzon and Sripada, 2007). Fittingly, the ventral part of the HC (vHC) has also been studied in the context of stress, social behavior, as well as fear and anxiety (Fanselow and Dong, 2010; Felix-Ortiz and Tye, 2014).

### 1.3.2 Social anxiety disorder (SAD)

An anxiety disorder can be diagnosed, if an individual experiences anxiety frequently, at high intensities and / or in inappropriate situations (Cohen et al., 2015; Kessler et al., 2009). Among anxiety disorders, generalized anxiety disorder, panic disorder, obsessive compulsive disorder, as well as SAD are most common (American Psychiatric Association, 2013). Among them, SAD with a lifetime prevalence of 12.1 %, represents one of the most prevalent anxiety disorders (American Psychiatric Association, 2013; Kessler et al., 2009; Kessler et al., 2005). It is often referred to social phobia and is defined as persistent or chronic fear and avoidance of social situations. In addition, it is strongly comorbid with other psychiatric disorders as 52 % of patients suffering from SAD reported at least one, while 27 % reported three or more, additional lifetime mental disorders (Chartier et al., 2003; Kessler et al., 2005), like generalized anxiety disorder, substance abuse, major depression, and PTSD (Chartier et al., 2003; Collimore et al., 2010; Schneier et al., 2010). Interestingly, SAD can be divided into the specific and the generalized subtype (gSAD) (Toth et al., 2012b). The specific form refers to the fear and avoidance of a particular social situation, like performance anxiety (e.g. fear of giving a public speech), interaction anxiety (fear of social interaction and observation), and the fear of showing physiological symptoms of anxiety. In contrast, gSAD is even more severe, as those patients fear and avoid a wide range of social situations (Bögels et al., 2010; Brunello et al., 2000; Kessler et al., 1998). Unsurprisingly, patients suffering from SAD reported an elevated level of anxiety before, during, immediately after, and one week after a stressful social situation, such as exposure to the Trier social stress test, compared to healthy controls. However, this was neither accompanied by changes in plasma or salivary cortisol, nor in salivary alpha-amylase (Klumbies et al., 2014). In line, urinary cortisol, measured during 24 hours, did not differ between SAD patients and healthy controls (Potts et al., 1991; Uhde et al., 1994). Another study showed that patients suffering from gSAD did not differ in cortisol levels, but had increased basal alpha-amylase levels compared to healthy controls. This indicates a hyperactivation of the SNS, not of the HPA axis, in patients suffering from gSAD, which is in line with hyperarousal, seen in these patients (van Veen et al., 2008). In agreement, Stein and colleagues could show increased levels of noradrenalin in gSAD patients (Stein et al., 1992), but they could not replicate this finding (Stein et al., 1994).

Similar to PTSD, effective treatment options for SAD are limited, while the best outcomes were warranted using CBT, especially exposure therapy (Hofmann et al., 2006), that can be combined with pharmacotherapy, e.g. antidepressants like SSRIs, beta-blockers, benzodiazepines, or D-cycloserine (Clark et al., 2003; Davidson et al., 2004; Gould et al., 1997; Hofmann et al., 2006; Stein et al., 1998). Also for SAD treatment, virtual reality and augmented reality have been employed to improve exposure therapy (Ben-Moussa et al., 2017; Horigome et al., 2020; Zainal et al., 2021).

For pharmacotherapy, SSRIs are most commonly used and provide the best response rate among medications, even though the amount of non-responder is still very high (approximately 30 to 50 % of the patients) (Davidson, 2003; Kamaradova et al., 2014; van Ameringen et al., 2004). Interestingly, SSRIs have been shown to induce anxiolytic effects by attenuating amygdala responsiveness. This affirms that the amygdala is studied as a key structure in the pathophysiology of anxiety disorders (Faria et al., 2012). In line, patients suffering from gSAD exhibited greater amygdala activation in response to angry and harsh, but not happy faces compared to healthy controls (Labuschagne et al., 2010; Phan et al., 2006; Stein et al., 2002).

Even though anxiety disorders are commonly linked to stressor exposure, there is only a limited amount of clinical studies addressing the impact of stressful situations on the development of SAD. However, researchers suggested that negative social stressors, which might resemble an acute social trauma, especially during childhood, display a powerful environmental factor, facilitating the development of SAD symptoms (Erwin et al., 2006; Rigby, 2003; Storch et al., 2004). In line, people that have experienced negative and distressing social events, like public humiliation or teasing, scored higher measures of social anxiety, which was accompanied by PTSD symptoms in 30 % of the participants (Carleton et al., 2011). Moreover, women with a history of childhood abuse reported significantly more anxiety and distress in social situations, which was again accompanied by PTSD symptoms (Feerick and Snow, 2005) and increased cortisol reactivity, indicating also a sensitization of the HPA axis in SAD patients (Elzinga et al., 2010).

Noticeably, these data indicate not only a high burden of PTSD or SAD itself, but also a particular high comorbidity of both. In line, patients suffering from PTSD have been diagnosed with SAD in a range of 12.5 % up to even 72 % in veterans (Kashdan et al., 2006; Orsillo et al., 1996b; Orsillo et al., 1996a; Roszell et al., 1991) and 4 % up to 46 % in civilian samples (Collimore et al., 2010; Green et al., 1992; Hubbard et al., 1995; Zayfert et al., 2005; Zayfert et al., 2002). In general, PTSD highly co-occurs with SAD, since traumatic events increase the risk of developing emotional disorders (Magee, 1999) and might also potentiate the development of socially related fears (Collimore et al., 2009). Remarkably, stress-related psychiatric diseases including PTSD, anxiety, and to a lesser extent SAD have been linked to a dysregulation of the immune system, while especially PTSD is described as a disease with a strong immunological component (reviewed in Wang and Young, 2016).

## 1.4 The immune system in the context of stress and behavior

In the last 40 years, the interest in studying alterations of the immune system following chronic stress as well as in stress-related pathologies has grown. While in earlier times the immune system has been regarded as autonomous, which is separated from the protected brain, there is increasing evidence showing a distinct cross-talk between the brain and the immune system (Nutma et al., 2019). Particularly, besides the peripheral immune system, the brain has its "own", the central immune system, and both communicate.

### 1.4.1 The peripheral and central immune system

The immune system in the periphery consists, in general, of cells and molecules that act together to defend the body against infections. There are two fundamentally different types of response against invading microbes: the innate (natural) response, which occurs always to the same extent, even if antigen structures are encountered many times, and the acquired (adaptive) response, which improves by repeated exposure to the same kind of antigen. The innate immune system consists of myeloid cells, i.e., (i) phagocytotic cells, such as neutrophils, monocytes, macrophages, (ii) cells that release inflammatory mediators, like basophils, mast cells and natural killer cells, (iii) and acute-phase proteins and cytokines (e.g. interferons; IFN). In contrast, the acquired immune system involves B and T cells, antigen-specific lymphocytes that possess surface receptors to bind antibodies, leading to their proliferation. In detail, in response to a foreign signal or pathogen, antibodies, which are produced by B cells, can bind to molecules of the antigen, neutralize it and elicit the immune response, and recruit other cells and molecules to destroy the antigen. When the same immunological challenge encounters the body, the respective specific antibodies can directly recognize the antigens, thus leading to a rapid detection and therefore fast response of the immune system (Delves and Roitt, 2000; Janeway et al., 2005). T cells can divide into cytotoxic T cells that kill infected cells, antigen presenting T cells that can activate other cells including B cells, and regulatory T cells (Tregs) that can suppress immune responses and maintain immunological homeostasis (Kondělková et al., 2010). Organs of the immune system are central (primary) lymphoid organs, where leucocytes and lymphocytes are produced, i.e., bone marrow and thymus, as well as peripheral (secondary) lymphoid organs, where adaptive immune responses are initiated and lymphocytes maintained, e.g. spleen and lymph nodes (Janeway et al., 2005; Mebius and Kraal, 2005; Pruett, 2003; Randall et al., 2008). Notably, inflammation is initiated and regulated by the release of second messenger molecules, i.e., cytokines and chemokines, which are important relators and communicators of the immune system. While chemokines are necessary to attract other immune cells, cytokines are defined as small molecules that are released by cells to affect the behavior of other cells possessing respective receptors.

Thus, cytokines are crucial in the regulation and surveillance of cell and tissue growth, migration, development, differentiation and communication (Foster, 2001). They are categorized in two main groups: those that promote inflammation, i.e., pro-inflammatory cytokines, and those that reduce inflammation and promote healing, i.e., anti-inflammatory cytokines (Dinarello, 2000).

The immune system within the central nervous system (CNS) is mainly mediated by microglia, which are resident cells of the CNS (Kreutzberg, 1996), representing about 10 % of cells in the brain, as well as - to a minor extent - infiltrating myeloid cells. These cells regulate the innate and adaptive immune responses in CNS tissue (Gundersen et al., 2015). Resting microglia can adopt a number of phenotypes following activation, e.g. by cytokines and chemokines, that release inflammatory mediators like cytokines and hence affect each other's activity (see Figure 2). One phenotype is the pro-inflammatory M1 microglia state, following "classical activation" by, e.g. lipopolysaccharide (LPS) or pro-inflammatory cytokines including IFN-γ, that can, in turn, produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, leading to increased inflammation and neuronal damage. The second phenotype are M2 microglia, induced via "alternative activation", that dampen inflammation, facilitate phagocytosis of, e.g. misfolded proteins, support tissue repair and neuronal survival, as well as regulate anti-inflammatory cytokines like IL-4 and IL-10, and transforming growth factor (TGF)-β (Crain et al., 2013). To analyze microglia number and morphology, different genes, which are expressed in the respective cells, were studied as specific markers (Kettenmann et al., 2011; Korzhevskii and Kirik, 2016; Lisi et al., 2017; Walker et al., 2020). Here, the ionized calcium binding adaptor molecule 1 (Iba-1), which increases following microglia activation, is most commonly used to verify the number and density of microglia. However, as microglia share many features with other immune cells, Iba-1 is also expressed in macrophages (Nakamura et al., 2013). Thus, a specific marker has been established that is exclusively expressed in microglia, but not macrophages, i.e., the transmembrane protein 119 (Tmem119) (Bennett et al., 2016). Analyzing microglia morphology, resting microglia express the purinergic receptor P2ry12 (Gómez Morillas et al., 2021; Walker et al., 2020), while M1 microglia can also be identified via inducible nitric oxide synthase (iNOS), as well as the cluster of differentiation (CD) molecules 86 (CD86), and CD11b, which are also expressed in macrophages (Kettenmann et al., 2011; Korzhevskii and Kirik, 2016; Lisi et al., 2017; Roy et al., 2006; Schmid et al., 2018). On the other hand, M2 microglia express, for instance, CD68 and Arginase (Arg)-1, which can be analyzed to determine anti-inflammatory microglia state (Kettenmann et al., 2011; Lisi et al., 2017). Cell contact-dependent mechanisms of microglia inhibition can also be analyzed by specific markers including the receptor CD200R1, which decreases in response to pro-inflammatory stimuli (Dentesano et al., 2012). In general, microglia were shown to play a central role in, stroke (Danton and Dietrich, 2003; Wood, 1995), multiple

sclerosis (Carson, 2002), Alzheimer´s disease (Eikelenboom et al., 2002; Li et al., 2014), schizophrenia, bipolar disorder, depression (Bayer et al., 1999), stress-induced depression and anxiety (Ramirez et al., 2016) as well as PTSD (Bhatt et al., 2020). In contrast to peripheral immune cells, the brain is lacking antigen-presenting cells like T cells. However, peripheral T cells can traffic to the CNS, where they respond to disease and present the antigen to central immune cells, while also B cells can produce antibodies into the CNS (Ransohoff et al., 2015).

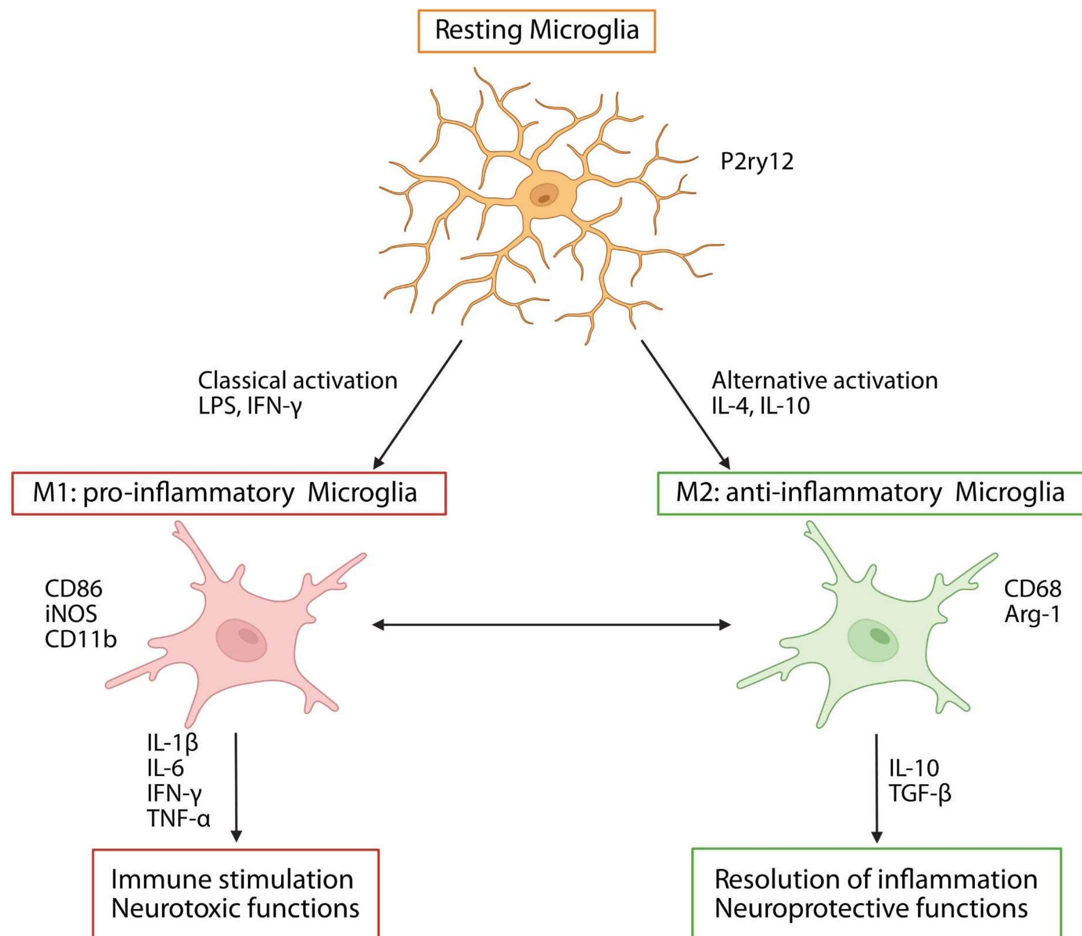



**Figure 2: Schematic illustration of microglia phenotypes and activation states.** Resting microglia, which express the purinergic receptor P2ry12, can adopt various phenotypes following activation, which can interact and affect each other. Classical activation, stimulated by lipopolysaccharide (LPS) or pro-inflammatory cytokines including interferon (IFN)-γ, induces the pro-inflammatory M1 microglia phenotype, which express the cluster of differentiation (CD) 86, inducible nitric oxide synthase (iNOS), or CD11b. M1 microglia release pro-inflammatory mediators, like interleukin (IL)-1β, IL-6, IFN-γ, and tumor necrosis factor (TNF)-α, leading to an increased immune response and neuronal damage. In contrast, alternative activation of resting microglia by, e.g. the anti-inflammatory cytokines IL-4 and IL-10 induces the M2 microglia phenotype, which express CD68 or Arginase (Arg)-1. M2 microglia are anti-inflammatory and release anti-inflammatory mediators like IL-10 and transforming growth factor (TGF)-β, leading to a dampening of the immune response and neuronal survival and protection. The illustration has been created using BioRender.com.

### 1.4.2 Crosstalk between the immune system and the HPA axis

Already in the 1940ies, a direct crosstalk of the immune and the stress systems has been described. Based on studies on "hormones of the adrenal cortex", by Kendall, Reichstein and Hench, which received the Nobel Prize of Medicine in 1950, the inherent and strong anti-inflammatory characteristics of GCs, as well as their regulatory role on a wide variety of immune cell functions, have been repeatedly described. Thus, GCs modulate cytokine and chemokine expression, as well as immune-cell trafficking, proliferation, differentiation, and functioning (Ashwell et al., 2000; Bellavance and Rivest, 2014; Di Rosa et al., 1990; Elenkov and CHROUSOS, 2002; Padgett and Glaser, 2003; Russo-Marie, 1992). In accordance, acute deficiency of GCs, following removal of adrenal cortical tissue, led to elevated levels of pro-inflammatory cytokines, especially IL-6 (Papanicolaou et al., 1996). Interestingly, many of the known anti-inflammatory properties of GCs are mediated by GC-induced leucine zipper (GILZ) (Ayroldi and Riccardi, 2009; Bereshchenko et al., 2019; Bereshchenko et al., 2014; Berrebi et al., 2003; Cannarile et al., 2019; Hoppstädter et al., 2016; Ronchetti et al., 2015), an ubiquitously expressed gene, which was found to be induced after DEX-treatment (Cannarile et al., 2001; D'Adamio et al., 1997), IL-10 stimulation (Godot et al., 2006) and even the natural product curcumin (Hoppstädter et al., 2016). Fittingly, GILZ is one of the earliest transcriptional targets of GCs (Bereshchenko et al., 2019; Ronchetti et al., 2015), but is also produced by immune cells like macrophages (Berrebi et al., 2003; Hoppstädter et al., 2018; Hoppstädter et al., 2016). When GILZ expression is induced by GCs, it mediates the anti-inflammatory actions of GCs on a broad variety of immune cells including the control of B and T cell activity, as well as the induction of Tregs (Bereshchenko et al., 2014; Cannarile et al., 2019; Ronchetti et al., 2015), the regulation of macrophage activity (Hoppstädter et al., 2018), cytokine release (Nataraja et al., 2021), and regulation of cell death and cell differentiation (Bereshchenko et al., 2014; Bruscoli et al., 2010; D'Adamio et al., 1997; Yosef et al., 2013). Moreover, GILZ interacts with several transcription factors to inhibit important inflammatory signaling pathways, including the Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) pathway (Auphan et al., 1995; Ayroldi et al., 2001; Di Marco et al., 2007) and Mitogen-Activated Protein Kinase (Bereshchenko et al., 2019). In this context, the NFκB transcription factor is of special interest, as it controls cytokine and chemokine production (Baldwin, 1996; Tak and Firestein, 2001). Interestingly, NFκB activity and functioning is regulated via phosphorylation of the NFκB subunits (pNFκB) like of the p65 subunit at Serine 276 (Ser276), involved in the cross-repression of NFκB and GC activity (Christian et al., 2016). In line, downregulation of GILZ in primary human and murine macrophages increased the production of pro-inflammatory cytokines and phagocytic activity, at least partly mediated through NFκB activity (Hoppstädter et al., 2018).

Besides, lymphocytes and myeloid cells including monocytes, macrophages, and granulocytes regulate or express receptors for several neuroendocrine signals of the HPA axis and SNS. As an example, monocytes possess CRH receptors (Wilbert-Lampen et al., 2006), while lymphocytes can even produce messenger ribonucleic acid (mRNA) of the ACTH precursor proopiomelanocortin and its derived peptides, which can be upregulated by CRH (Maddila et al., 2017; Weigent and Blalock, 1995), and suppress the activation of granulocytes (Smith et al., 1992). Moreover, CRH was shown to not only induce the production of IL-1, but also IL-1 can mediate some effects of CRH and AVP (Kavelaars et al., 1989). In support of the clinical studies, rodent studies revealed mRNA expression of the IL-1 receptor 1 throughout the components of the HPA axis including pituitary and adrenal glands (Cunningham et al., 1992). Thus, IL-1 can directly affect the HPA axis on various stages (Besedovsky and Del Rey, 1996; Sundar et al., 1990), such as CRH secretion from the PVN (Berkenbosch et al., 1987; Wong et al., 1997; Wong and Licinio, 1994), ACTH release from the anterior pituitary (Uehara et al., 1987), as well as a direct stimulation of the adrenal cortex (Roh et al., 1987). In support, various cytokines were shown to modulate HPA axis activity and functioning (Dunn, 2000; Lyson and McCann, 1991; Maes et al., 1998; Mastorakos et al., 1993; Smith et al., 1999; Tu et al., 2007). Here, especially pro-inflammatory cytokines like IL-1, IL-6 and TNF-α (Dunn, 2000; Lyson and McCann, 1991; Mastorakos et al., 1993), but also the anti-inflammatory cytokine IL-10 are potent activators of the HPA axis (Smith et al., 1999). Interestingly, cytokine-induced activation of the HPA axis can even induce sickness behavior and anxiety (Maes et al., 1998). In line, psychological stress triggered cytokine-induced activation of the HPA axis, which was accompanied by an increased risk, and partly even prediction (Baune et al., 2012), to develop stress-induced disorders, like anxiety (Maes et al., 1998), depression (Quinn et al., 2020), and PTSD (Wang and Young, 2016; reviewed in Gryksa and Neumann, 2021).

### 1.4.3 Inflammation in the development of stress-related psychiatric diseases

Inflammation has been repeatedly connected to behavioral changes and the development of neuropsychiatric disorders, and vice versa. Especially depression, anxiety and PTSD, but also schizophrenia are closely linked to inflammation. As a bidirectional phenomenon, inflammation increases the risk to develop psychiatric diseases, whereas psychiatric diseases facilitate inflammatory responses and impairs both the peripheral and central immune system (for review see Bauer and Teixeira, 2019). In accordance, patients suffering from mood disorders, like bipolar disorder, depression or schizophrenia, showed higher levels of peripheral pro-inflammatory cytokines, peripheral and central C-reactive protein (CRP), a marker for systemic inflammation, increase number of cells of the innate immune system, i.e., monocytes and neutrophils, as well as higher activation of T cells but reduction of Tregs (Barbosa et al., 2014; Bauer and Teixeira, 2019;

Dargél et al., 2015; Do Prado et al., 2013; Goldsmith et al., 2016; Inoshita et al., 2016; Köhler et al., 2017; Modabbernia et al., 2013). Interestingly, increased inflammation even correlated with the progression and clinical severity of mood disorders. In line, higher levels of CRP and IL-6 were shown to predict the development of depressive symptoms (Valkanova et al., 2013). This is of special interest as social stress exposure, such as being bullied during childhood and adolescence, increased the levels of CRP, which even correlated with the number of times being bullied (Copeland et al., 2014). In another study, anxiety was related to increased level of IL-6, while CRP levels were equal in anxious compared to non-anxiety participants, indicating an anxiety-specific effect on inflammatory IL-6 activity (O'Donovan et al., 2010). These data further confirm the interdependence of stress and inflammation in the development of psychiatric diseases.

In this context, the development of PTSD is of special interest, as it is highly associated with chronic low-grade inflammation (Gola et al., 2013; Hori and Kim, 2019; Kim et al., 2020; Michopoulos et al., 2017; Sumner et al., 2020; Wang and Young, 2016). Summarizing various publications, the development of PTSD shifts the immune system towards an inflammatory state, thereby increasing CRP and pro-inflammatory, e.g. IL-1 and IL-6, but also anti-inflammatory cytokines, e.g. IL-10, while downregulating other anti-inflammatory cytokines, e.g. IL-4 (Gola et al., 2013; Känel et al., 2007; Maes et al., 1999; Newton et al., 2014; Oliveira et al., 2018; Tursich et al., 2014; for review see Hori and Kim, 2019; Wang and Young, 2016). Additionally, PTSD patients revealed increased spontaneous production of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α in peripheral blood mononuclear cells (Gola et al., 2013), as well as increased pro-inflammatory T helper cells, but reduced number of the anti-inflammatory, immune balancing Tregs (Sommershof et al., 2009; Zhou et al., 2014). Moreover, the level of systemic inflammation, indicated by CRP expression, correlated positively with the severity of PTSD symptoms (Michopoulos et al., 2015). In line with systemic inflammation in PTSD patients, traumatic events have been shown to increase the risk to develop autoimmune disorders including thyroiditis, rheumatoid arthritis, inflammatory bowel disorders, multiple sclerosis, and lupus erythematosus (O'Donovan et al., 2015). Whether inflammation increases the risk to develop PTSD after trauma exposure, or if PTSD leads to an altered inflammatory response has been studied in both directions, indicating again a bidirectional phenomenon (Sumner et al., 2020). Interestingly, increased levels of the pro-inflammatory cytokine IL-6, a potent activator of the HPA axis (Dunn, 2000; Wang and Dunn, 1998), as well as altered levels of the anti-inflammatory GILZ, are hypothesized to act as biomarkers to predict the development of PTSD following a traumatic experience (Cohen et al., 2011; Lebow et al., 2019; Pervanidou et al., 2007). Moreover, high levels of GILZ expression were correlated with high level of PTSD symptoms (van Zuiden et al., 2012). These data indicate an involvement of the GC pathway in the development and severity of PTSD.

In agreement, hypocortisolism and increased GC sensitivity of leucocytes and lymphocytes to suppress the production of pro-inflammatory cytokines after DEX treatment, were found in PTSD patients (Rohleder et al., 2004).

As demonstrated, the knowledge about the interpendence of the immune system and the development of stress-related diseases, particularly of PTSD, has been intensively studied. Given the high comorbidity of PTSD and SAD, the role of the immune system in SAD as well as in SAD combined with PTSD seems noteworthy. Unfortunately, there is only limited literature on the interaction of SAD and immunological changes. As an example, LPS-induced production of the pro-inflammatory cytokine IL-6 was lower in patients suffering from obsessive-compulsive disorder, whereas patients suffering from gSAD showed no difference in cytokine production compared to healthy controls (Fluitman et al., 2010). Nevertheless, SAD patients showed alterations in the immune-kynurenine pathway, that is affected by stress and immune alterations due to cortisol and cytokine sensitivity. Here, SAD patients revealed a shift towards increased synthesis of the endogenous neuromodulator kynurenic acid, which was even elevated in SAD patients with suicide attempts (Butler et al., 2022), and also reported in patients suffering from schizophrenia (Chiappelli et al., 2014). In addition, pro-inflammatory cytokines were unchanged in SAD men and women, whereas, under basal conditions, the anti-inflammatory cytokine IL-10 was reduced in SAD men, but not women, compared to healthy controls (Butler et al., 2022). In line, childhood maltreatment and the level of IL-6 was found to be mediated by symptoms of SAD (Carlton et al., 2021).

Moreover, within the CNS, especially microglia and cytokines were studied in the development of psychiatric diseases (Bayer et al., 1999; Bhatt et al., 2020; Wang and Young, 2016) including depression (Lotrich, 2015; Ramirez et al., 2016; Wang et al., 2017), anxiety (McKim et al., 2018a; O'Donovan et al., 2010; Ramirez et al., 2016; Wohleb et al., 2011), social deficits (Kim et al., 2017; Zhan et al., 2014) and PTSD (Enomoto and Kato, 2021; Li et al., 2021; Loane et al., 2014; Smith et al., 2019; Wang and Young, 2016). With respect to cytokines in the CNS, they are among others important regulators of neuronal excitability, synaptic plasticity, synaptic refinement, neurogenesis, blood-brain-barrier integrity, as well as myelination of the neurons (Banks et al., 2009; Conti et al., 2008; Gottfried-Blackmore et al., 2008; Ignatowski and Spengler, 2008; Kim et al., 2017; Levin and Godukhin, 2017; Saija et al., 1995; Schmitz and Chew, 2008; Vallières et al., 2002), factors that control behavior and mood. Interestingly, the highest distribution of the IL-1 receptor 1 was found in the HC (Cunningham et al., 1992). Recent findings in mice revealed that HC IL-1 directly affected local glutamatergic neurons via actions at IL-1 receptors, thereby inducing stress-mediated social withdrawal and cognitive deficits (DiSabato et al., 2021).

Nevertheless, more detailed research is needed to gain a better understanding and unravel underlying mechanisms, including dysregulations in the immune response, in the development of mental disease like PTSD, SAD, and their comorbidity, especially following chronic stressor exposure. However, human resources, their access and research possibilities are highly restricted; therefore, appropriate and specific animal models are used for fundamental research.

## 1.5 Animal models to study chronic stress

To develop animal models for human diseases, they have to be validated by at least three criteria, elaborated by Paul Willner: predictive validity, face validity, and construct validity (Willner, 1991; Willner, 1984). According to Willner, predictive validity means that performance in the test predicts the performance in the condition that is modeled. For example, the drug that is specific and effective in humans can also manipulate the corresponding disease phenotype in the model. Face validity means that there are phenomenological similarities between the human condition and the animal model, so that the phenotype that is created by the model is highly similar to the characteristics of the human disease. Finally, construct validity means that the model is based on a theoretical and scientific rationale (Schmidt et al., 2008; Willner, 1991).

In agreement with human studies, preclinical studies confirmed long-term effects of chronic stress, as a result of allosteric overload, on behavioral, physiological, and neural systems. In mice and rats, chronic stress has repeatedly been shown to induce increased anxiety- as well as depressive-like behavior (D'Aquila et al., 1994; Grønli et al., 2005; Kloet et al., 2005; Langgartner et al., 2015; Nestler et al., 2002; Slattery et al., 2012; Tong et al., 2017), disturb social behavior (Kinley et al., 2021; Slattery et al., 2012), decrease sexual activity and induce hyperactivity (Grønli et al., 2005). Moreover, pre-clinical studies revealed that chronic stress has also an intense impact on neuronal structures of the brain, as it causes HC dendritic atrophy (Magarin˜os and McEwen, 1995), inhibits neurogenesis (Gould and Tanapat, 1999; Mirescu and Gould, 2006) and induces neuronal loss (McEwen, 2000). This can further reduce the negative feedback inhibition of the HPA axis, thus leading to a prolonged activation of the same (Warner-Schmidt and Duman, 2006).

In addition, rodent models of chronic stress confirmed a strong impact of the immune system on somatic and affective maladaptations. In response to various stressors including metabolic stress, the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing (Nlrp)3 gets activated and induces caspase (casp)-1, which leads to the processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18 (Leemans et al., 2011). In turn, casp-1 knockout mice revealed an attenuated stress response and decreased stress-induced depressive- and anxiety-like behavior following chronic stress (Wong et al., 2016). Moreover,

bioactive IL-18 plays a major role in the production of IFN-γ from T cells and natural killer cells, which were expressed at sites of chronic inflammation, like inflammatory bowel disease, autoimmune diseases or cancer (Fabbi et al., 2015; Novick et al., 2013). In line, chronic stress induced an overexpression of colonic and lymphatic IFN-γ, colonic inflammation, altered colonic mucosal barrier functions and bacterial translocation (Ferrier et al., 2003; Reber et al., 2008). Furthermore, central inflammatory mediators including Nlrp3, casp-1, NFκB, pro-inflammatory cytokines, and microglia were increased in response to chronic stress (Calcia et al., 2016; Kreisel et al., 2014; Tynan et al., 2010; Wohleb et al., 2012), mediating stress-induced affective dysregulations (Feng et al., 2019). Nevertheless, as mentioned in 1.2, not only the duration, also the nature of the stressor matters; therefore, different types of chronic stress paradigms have been developed.

### 1.5.1 Models of chronic non-social stress

Rodent models of chronic non-social stress are, for example, based on repeated exposure to physiological stressors like restraint stress (RS) (Goldwater et al., 2009; Jeong et al., 2013; McLaughlin et al., 2007). This animal model has been shown to induce behavioral and neuronal alterations including increased anxiety- and depressive-like behavior (Chiba et al., 2012; Yoon et al., 2014) and to impair neurogenesis (Yun et al., 2010), GC receptor expression (Chiba et al., 2012), as well as T-cell immunity, while promoting tumor progression (Frick et al., 2009). However, especially in mice, the effects of chronic RS were shown to be contradictory, as depressive-like behavior was increased when mice were exposed to RS for 3 weeks, 2 hours per day (Yoon et al., 2014), whereas Zhu and colleagues revealed no effect on depressive-like behavior when mice were exposed to RS for 4 weeks, 1 hour per day (Zhu et al., 2014). Interestingly, often repetition of the same stressor has been shown to induce stress habituation, indicated by an attenuated HPA axis response towards the stressor, which was accompanied by reduced neuronal activity in various brain regions including PVN and lateral septum (LS) (Girotti et al., 2006). In confirmation, microglia contributed to social behavioral adaptation following chronic RS (Piirainen et al., 2021).

To avoid habituation and predictability of the same repeated stressor, Willner and colleagues established the animal model of chronic unpredictable stress (CUS) (Willner, 2005; Willner et al., 1987). In this paradigm, rodents are exposed to a variety of physical stressors, e.g. cold, food deprivation, changes in light / dark cycle, wet bedding, cage shaking or RS, in an unpredictable and alternately manner (Kim and Han, 2006; Kreisel et al., 2014; Monteiro et al., 2015; Yoon et al., 2014; Yun et al., 2010). The CUS paradigm has been used as a model to induce depressive- and anxiety-like behavior (Kim and Han, 2006; Kreisel et al., 2014; Yoon et al., 2014), cognitive deficits (Bondi et al., 2008) and impaired spatial memory (Riaz et al., 2015). Moreover, CUS exposure

induced a time-independent increase in IL-6 expression, which contributed to a sustained CORT response (Girotti et al., 2013) and, GC dependently, exaggerated LPS-induced activation of NFκB in the frontal cortex and HC (Munhoz et al., 2006).

However, as already mentioned, several studies indicated that “allostatic overload” is more likely to develop when the stressor is not only chronic and unpredictable, but, especially, is of social nature (Bartolomucci et al., 2005).

### 1.5.2 Models of chronic psychosocial stress

Animal models of chronic psychosocial stress revealed a great impact on a broad spectrum of somatic disorders including hypertension, cardiovascular damage, HPA axis dysregulation and impairment of the peripheral and central immune system (reviewed in Bartolomucci et al., 2005; Langgartner et al., 2015; Masis-Calvo et al., 2018). In addition, animal models of the chronic psychosocial burden are promising tools to study the mechanisms underlying the development of psychiatric disorders like anxiety disorders and trauma and stressor-related disorders, as they mimic the current human situation more precisely. Commonly used animal models of chronic psychosocial stress in rats and mice (reviewed in Beery and Kaufer, 2015; Masis-Calvo et al., 2018) are (i) long-term social isolation, especially during the post-weaning period (Dunphy-Doherty et al., 2018; Guo et al., 2004; Lapiz et al., 2003), (ii) OC (BROWN, 1995; Reiss et al., 2007), (iii) social instability, i.e., alternation of social isolation and OC (Baranyi et al., 2005; Goñi-Balentziaga et al., 2018; Herzog et al., 2009), (iv) CSDS (Golden et al., 2011; Hollis et al., 2010), (v) social defeat (SD) / OC (Reber et al., 2006; Slattery et al., 2012), or (vi) chronic subordinate colony housing (CSC) (Langgartner et al., 2015; Nyuyki et al., 2012; Reber et al., 2007). These animal models induce increased anxiety-like behavior and social deficits (Baranyi et al., 2005; Bartolomucci et al., 2005; Golden et al., 2011; Goñi-Balentziaga et al., 2018; Kumari et al., 2016; Lukkes et al., 2009; Reiss et al., 2007; Seffer et al., 2015; Venzala et al., 2012; Walker et al., 2019). Depressive-like behavior was only induced by social isolation, OC, social instability, and CSDS (Goñi-Balentziaga et al., 2018; Herzog et al., 2009; Hollis et al., 2010; Venzala et al., 2012; Walker et al., 2019), whereas SD / OC and CSC did not show altered depressive-like behavior (Langgartner et al., 2015; Slattery et al., 2012). Interestingly, social isolation and social instability affect particularly females, while male rodents, especially male mice, are more vulnerable to group housing stressors like OC, CSDS, SD / OC and the CSC paradigm, as it induces hierarchical fights due to innate territorial behavior (Baranyi et al., 2005; Bartolomucci et al., 2005; Beery and Kaufer, 2015; BROWN, 1995; Goñi-Balentziaga et al., 2018; Langgartner et al., 2015; Palanza, 2001; Singewald et al., 2009). When males are exposed to one of these models, chronic psychosocial stress is induced by a prolonged or repeated experience of aggressive behavior. Thereby, the dominant rodent is continuously

showing offensive behavior such as attacks, to subordinate its opponents, which show defensive behavior like flight and submissive upright to avoid attacks (Blanchard et al., 1995; Reber and Neumann, 2008).

In the brain, microglia activity and morphology, as well as cytokine release were linked to the development of chronic psychosocial stress-related disorders like depression (Ramirez et al., 2016; Wang et al., 2017), anxiety (McKim et al., 2018a; Ramirez et al., 2016; Wohleb et al., 2011), and PTSD (Enomoto and Kato, 2021; Li et al., 2021; Smith et al., 2019). In line, male rats socially isolated for 7 weeks, showed an increased expression of microglia in the PFC and nucleus accumbens (Schiavone et al., 2009). In addition, early-life social isolation enhanced microglia activation and pro-inflammatory cytokine expression in the rat HC, which was accompanied by increased depressive-like behavior (Wang et al., 2017). Treatment with minocycline, an antibiotic with anti-depressant and anti-inflammatory properties, rescued microglial density in the PFC and HC of high trait anxiety rats (Schmidtner et al., 2019) and following early-life social isolation (Wang et al., 2017). Moreover, CSDS enhanced the secretion of pro-inflammatory cytokines and activation of microglia, and promoted amygdala hyperactivity, which resulted in an anxiogenic phenotype in male rats (Munshi et al., 2019). In female adolescent rats, social instability increased NFkB1 expression in the HC, but attenuated its rise when an additional immune challenge followed. However, opposite effects were found on HC IL-6 synthesis (McCormick et al., 2020). Furthermore, male mice that were exposed to chronic social isolation, revealed increased microglia cell density in the dentate gyrus of the HC as well as increased pro-inflammatory TNF-α and decreased anti-inflammatory IL-10 plasma levels (Du Preez et al., 2020). In female mice, chronic social instability stress reduced the expression of the anti-inflammatory cytokine IL-10, while increasing the ratio of pro- and anti-inflammatory cytokines, i.e., IL-1β/IL-10, IL-6/IL-10 and TNF-α/IL-10, in the HC, which was accompanied by increased anxiety- and depressive-like behavior (Labaka et al., 2017; reviewed in Gryksa and Neumann, 2021). Moreover, CSDS increased microglial recruitment, activity and reactivity in the amygdala, prelimbic cortex and HC. Additionally, CSDS enhanced microglial expression of IL-1β, while reducing GILZ expression, and increased the trafficking of CD11b macrophages and monocytes to the brain, thereby mediating the development of stress-induced anxiety (McKim et al., 2018a; Reader et al., 2015; Wohleb et al., 2011).

Notably, the CSC model is of special interest, not only due to its profound impact on the HPA axis and the immune system, but also as it induces a long-lasting increase in anxiety-like behavior and social dysregulations. Moreover, it has also been described as an animal model to study PTSD (Reber et al., 2016a).

### 1.5.3 Chronic subordinate colony housing (CSC)

The CSC model has been established in 2007 by Reber and colleagues and is based on the natural behavior of male mice to acquire and defend their territory, and to live in social hierarchies, thereby inducing long-term psychosocial stress in the subordinates. During the CSC procedure, 4 mice are housed together with a larger male aggressor mouse (resident) for 19 consecutive days, which results in an immediate and permanent subordination of the CSC mice (Reber et al., 2007). Although mainly C57BL/6N (BL6) mice were used for the CSC paradigm (Reber et al., 2008; Reber et al., 2007; Reber and Neumann, 2008; Slattery et al., 2012), it was shown that this model is also effective in other mouse strains, namely, BALB/c (Forkwa et al., 2014) and CD1 mice (Füchsl et al., 2014), as well as partially in rats (Nyuyki et al., 2012). In confirmation of its clinical relevance, the CSC model induces multiple chronic stress-related diseases including reduced GC signaling as well as somatic and affective dysregulations (see Figure 3).

Decreased GC signaling in CSC mice is caused by increased pituitary weight and number of corticotroph pituitary cells. This leads to an elevated capability of the pituitary gland to produce and secrete ACTH, and thus increased plasma ACTH concentrations (Füchsl et al., 2013a). In addition, CSC exposure increased adrenal weight through hyperplasia, resulting in an overall increased number of functional adrenal cells (Füchsl et al., 2013b). Contradictory, adrenal glands show a strong *in vitro* insufficiency to respond to ACTH stimulation with the production of CORT (Reber et al., 2007; Uschold-Schmidt et al., 2013). This alteration is thought to be an adaptive mechanism of the HPA axis to protect the body against prolonged CORT secretion leading to basal evening hypocorticism (Langgartner et al., 2020; Reber et al., 2007). In contrast, exposure to acute heterotypic stressors like forced swim (FS) or elevated platform following CSC led to an exaggerated CORT release, indicating a simultaneous hyperreactivity of the HPA axis (Füchsl et al., 2013a; Uschold-Schmidt et al., 2012). This seems to be an adaptive mechanism after chronic stress exposure in response to an acute challenge, as confirmed by other studies addressing chronic stress paradigms (Aguilera, 1994; Bhatnagar and Vining, 2003; Uschold-Schmidt et al., 2012). Furthermore, CSC mice display GC resistance of immune-related cells, including a decreased *in vitro* sensitivity of anti-CD3-stimulated mesenteric lymph node cells (mesLNCs) and LPS-stimulated splenocytes (Langgartner et al., 2015; Reber et al., 2007).

In line, *in vitro* cell viability of splenocytes showed a higher viability under basal conditions and following LPS-stimulation in CSC mice compared to single housed controls (SHC). Moreover, CSC mice revealed a splenic GC resistance, which correlated with the number and severity of bite wounds (Foertsch et al., 2017). Furthermore, analyzing peripheral lymph nodes of CSC mice revealed a reduction of Tregs and increased T cell effector functions, as activation of the T cells led to an increase in cytokine production (Schmidt et al., 2010). In line, stimulation of mesLNCs

showed increased levels of pro-inflammatory cytokines, such as IL-6, TNF-α and IFN−γ, in CSC mice (Reber et al., 2008). Moreover, chronic low-grade inflammation in CSC mice was confirmed by splenomegaly and thymus atrophy, organs that play important roles in the immune system as they are lymphatic organs (Langgartner et al., 2015; Reber et al., 2007), as well as increased inflammatory state of the liver and colon, as well as an enhanced risk of developing colorectal cancer and dextran sodium sulfate (DSS) induced colitis (Langgartner et al., 2015; Peters et al., 2012; Reber et al., 2008; Reber et al., 2007; Reichel et al., 2018). Interestingly, the inflammatory state of the colon as well as CSC-induced colitis were dependent on Helicobacter spp. transmission from resident to CSC mice (Langgartner et al., 2017).

Regarding affective dysregulations, CSC mice show a permanent and long-lasting increase in anxiety-related behavior (Reber et al., 2007; Slattery et al., 2012), long-lasting lack of social preference, hyperactivity (Amoroso et al., 2020; Foertsch et al., 2019; Slattery et al., 2012), and increased alcohol consumption (Peters et al., 2013). The latter also fits to clinical studies, demonstrating that chronic stress increases the risk of developing drug and alcohol dependence, at least partly, to reduce anxiety symptoms (reviewed in Pohorecky, 1991). Notably, in agreement with the three criteria of Paul Willner (Willner, 1991), the CSC paradigm is also described as an animal model for PTSD (Reber et al., 2016a). Here, (i) face validity was demonstrated as repeated exposure to trauma-inducing events, i.e., SD in CSC mice, mimics the type of health compromising traumatization in humans (Reber et al., 2016a). Moreover, avoidance of trauma-related stimuli, which is indicated by lack of social preference after CSC exposure (Foertsch et al., 2019; Slattery et al., 2012), as well as hyperarousal, i.e., hyperactivity following CSC exposure (Slattery et al., 2012), fulfil additional core symptoms of PTSD according to the Diagnostic and statistical manual of mental disorders (DSM)-V (American Psychiatric Association, 2013). Furthermore, (ii) construct validity has been clearly proven as CSC mice, similar to patients that suffer from PTSD, show a hyperactivity of the SNS indicated by increased plasma noradrenalin levels (Kosten et al., 1987; Reber et al., 2007), as well as a dysregulation of the HPA axis indicated by increased DEX suppression (Füchsl et al., 2013a; Newport et al., 2004; Yehuda et al., 2002), basal hypocorticism (Reber et al., 2007; Rohleder et al., 2004), and hyperactivity of the HPA axis towards novel heterotypic stressors (Bremner et al., 2003; Füchsl et al., 2013a; Uschold-Schmidt et al., 2012). Moreover, human studies revealed that PTSD patients show a strong dysregulation of the immune system, which has also been shown in CSC mice (Langgartner et al., 2019; Langgartner et al., 2015; Reber et al., 2016b; Schmidt et al., 2010; Wang and Young, 2016). Unfortunately, (iii) predictive validity could not be proven, as drugs that are normally applied to PTSD patients, like SSRIs or exposure therapy have not been assessed yet.

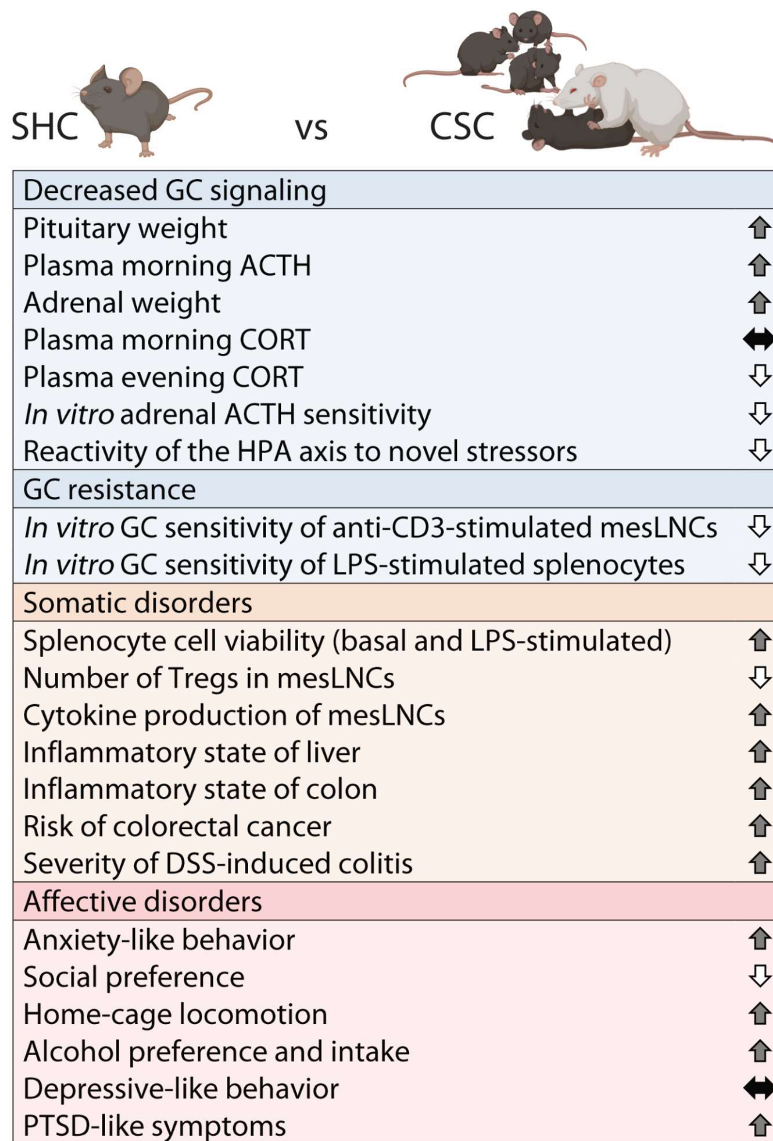



**Figure 3: Effects of chronic subordinate colony housing (CSC) on physiological, immunological and behavioral parameters in male mice.** In comparison to the single housed controls (SHC), CSC mice displayed various maladaptations including decreased glucocorticoid (GC) signaling as well as somatic and affective disorders. Therefore, the CSC paradigm represents a promising animal model to mimic stress-related pathologies in humans. Abbreviations: ACTH, adrenocorticotropic hormone; CORT, corticosterone; DSS, dextran sodium sulfate; HPA, hypothalamus-pituitary-adrenal; LPS, lipopolysaccharide; mesLNCs, mesenteric lymph node cells; PTSD, posttraumatic stress disorder. The illustration is adapted from Langgartner et al., 2015 and has been created using BioRender.com.

Regarding neuronal alterations, less is known about the impact of CSC exposure on brain region-dependent changes in neuronal functioning or activity. Nevertheless, chronic psychosocial stress predisposes an individual´s emotionality also by affecting different neuronal patterns and its activation in various brain regions that are involved in the processing of emotions and stress-response. In line, CSC mice revealed altered neuronal activation patterns, measured by c-FOS expression levels, an immediate early gene, 2 hours after acute open arm exposure in the amygdala, LS, HC, PVN and periaqueductal grey (Singewald et al., 2009). Those are areas that play a crucial role in the development of mood disorders like anxiety and PTSD (Graeff, 1994; Liberzon and Sripada, 2007; Nutt and Malizia, 2004). In the amygdala, open arm, but not CSC exposure increased c-FOS expression. However, a previous study showed that exposure to chronic social stressors induces neuronal adaptations in the amygdala, thus downregulating neuronal activity to the level of unstressed controls (Martinez et al., 1998). In the LS, c-FOS expression revealed a

decrease in neuronal activation in CSC mice following open arm exposure, compared to respective SHC. Nevertheless, analyzing the LS Zif-268 expression, which, in contrast to c-FOS, is also highly expressed under basal conditions, showed no differences in CSC mice compared to SHC under basal conditions, indicating adaptation of these neurons to chronic stressor exposure, but not following a heterotypic stressor (Singewald et al., 2009). Interestingly, neuronal adaptation in the LS seems to be dependent on the nature of the chronic stressor, as CSDS induced neuronal adaptation (Martinez et al., 1998), whereas repeated RS did not (Chen and Herbert, 1995). Moreover, the LS has been described to play a key role in anxiety-related responses, as increased neuronal activity in the same region reduced fear and anxiety, whereas septal lesions induced anxiogenic effects (Degroot et al., 2001; Menon et al., 2022; Sheehan et al., 2004; Yadin et al., 1993). Fittingly, reduced c-FOS expression in this region might contribute to the well-known increase in anxiety-like behavior in CSC mice (Singewald et al., 2009). Additionally, in the dorsal HC, c-FOS expression was increased following open arm exposure, which was dampened due to neuronal adaptation in CSC mice. However, this effect could not be observed in the vHC, despite its role in anxiety-related behavior (Bannerman et al., 2004).

Due to the high negative impact of CSC exposure on the immune system, positive immune modulation might have beneficial effects. In confirmation, CSC mice repeatedly treated with a heat-killed preparation of *Mycobacterium vaccae* (*M. vaccae*), an environmental microorganism with immunoregulatory properties (Amoroso et al., 2020; Amoroso et al., 2019; Reber et al., 2016b), exerted beneficial effects. In detail, subcutaneous pre-immunization promoted stress resilience in CSC mice, shown by increased proactive, i.e., offensive, but less re-active, i.e., defensive behavior, as well as reduced anxiety-like behavior, and partly rescued social preference behavior (Amoroso et al., 2020; Reber et al., 2016b). Moreover, *M. vaccae* pre-immunization prevented stress-induced spontaneous colitis (Reber et al., 2016b) and DSS induced colitis (Amoroso et al., 2019; Reber et al., 2016b), while reducing the production of pro-inflammatory cytokines by mesLNCs (Reber et al., 2016b). In the brain, *M. vaccae* treatment reduced CSC-induced increase in microglia density in the prelimbic cortex (Reber et al., 2016b), a brain region crucial for fear expression (Sierra-Mercado et al., 2011). Interestingly, the beneficial effects of *M. vaccae* were shown to be dependent on the presence of Tregs, as depletion of the same negated the positive effects on colitis and anxiety-like behavior (Reber et al., 2016b). These data demonstrate a strong influence of the immune system on CSC-induced alterations, which further demonstrated the CSC model as a potent paradigm to study stress-related psychopathologies that have a strong inflammatory character like PTSD.

## 1.6 Animal models to study stress-related affective dysregulations

Animal models to study stress-related affective disorders including fear, anxiety and PTSD are divided in unconditioned and conditioned tests. Unconditioned / ethological responses mimic natural stressful conditions to study innate fear / avoidance and can be exploration-based, i.e., elevated plusmaze (EPM), elevated Y-maze, light dark box (LDB), novelty-suppressed feeding, social interaction tests, or they can be predator-based. While exploration-based tests are used to analyze anxiety-like behavior in rodents, predator-based tests are used to identify the impact of threatening situations on defensive behavior, neurological alterations and the development of fear-related disorders like PTSD (Campos et al., 2013).

Regarding the regulation of anxiety-like behavior, two brain regions appeared to play a fundamental role, i.e., the amygdala and the vHC. The amygdala is implicated in some anxiolytic actions of benzodiazepines (Kang, 2000; Pesold and Treit, 1995), whereas lesions in the amygdala induced anxiolytic behavior (Korn et al., 2017) and reduced aggressive behavior, but did not affect social interaction (McHugh et al., 2004). In line, lesions in the vHC also induced anxiolytic behavior (Fanselow and Dong, 2010; Kjelstrup et al., 2002; McHugh et al., 2004), but increased social interaction without affecting aggressive behavior (McHugh et al., 2004). Interestingly, the social interaction test was the first to study anxiety-related behavior, as it is of high ethological relevance (Campos et al., 2013; File and Hyde, 1978).

Establishing animal models of PTSD is particularly challenging, as core symptoms like nightmares, flashbacks and invasive thoughts cannot be evaluated in rodents. Besides the mentioned CSC model (see 1.5.3), predator exposure has been widely used as a model to induce PTSD-like symptoms including hyperarousal and long-lasting increase in anxiety-like behavior (Adamec and Shallow, 1993; Campos et al., 2013). Commonly, animal models of fear conditioning paradigms that induce an acute non-social or social trauma are mostly used to study stress-related psychiatric diseases like PTSD and SAD. These animal models present a high validity as they involve encoding of a traumatic memory during acquisition, as well as extinction training following trauma induction, resembling exposure therapies that are commonly used to treat PTSD and SAD patients (Campos et al., 2013; Toth et al., 2012a, 2012b).

### 1.6.1 Models of non-social fear conditioning

Fear conditioning models include operant or classical, i.e., Pavlovian, fear conditioning tests. Operant conditioning involves spontaneous responses of an animal to an environmental stimulus that can be positively or negatively reinforced. Positive reinforcement, i.e., reward, can be obtained using pleasurable food when the animal shows the favored behavior, whereas negative reinforcement is seen when the animal is trying to avoid an unpleasant stimulus, which can be an electric shock (Campos et al., 2013). Classical, Pavlovian fear conditioning, in contrast, involves associative learning, where a neutral conditioned stimulus (CS), like an odor or a tone, is repeatedly paired with an unconditioned stimulus (US), like food or electric shock. Thus, the animals learn that CS predicts US, leading to a conditioned response, i.e., salivation or freezing behavior, respectively (Campos et al., 2013; Toth et al., 2012a). There are two commonly used paradigms for classical fear conditioning: the contextual and the cued fear conditioning (CFC). During contextual fear conditioning, an animal is placed in a novel environment, where it receives electric foot shocks, thus inducing aversive memories towards this environment. When the animal is placed again in this environment, it will show freezing behavior, as it predicts punishment. Brain regions that are involved during contextual fear conditioning are HC, frontal and cingulate cortex, and, to a lesser extent, the amygdala (Curzon et al., 2009; Phillips and LeDoux, 1992). During CFC, the CS is represented by an odor or tone that is co-terminated with an aversive stimulus (US), such as electric foot shock. This leads to aversive behavior, i.e., freezing, when the animal is exposed to the CS, involving learning processes. During fear extinction training, this traumatic memory is extinguished by exposing the animals to numerous repetitions of the CS without the US. Thereby the animals re-learn that the CS does not predict a threat anymore. To avoid negative associations with the environment, like during contextual fear conditioning, the environment during extinction can be changed (Cammarota et al., 2007; Curzon et al., 2009; Quirk et al., 2010; Toth et al., 2012a).

An inability to extinguish a traumatic memory is a core symptom of several psychiatric disorders, including PTSD and SAD, and has been shown to be accompanied by a hyperactivity of the amygdala (Liberzon and Sripada, 2007; Phan et al., 2006; Rauch et al., 2000; Stein et al., 2002). In confirmation, chronic stress impairs fear extinction and increases neuronal activity in the basolateral and central amygdala, as well as HC (Hoffman et al., 2014). In the context of CFC, the HC is differentially involved depending on the dorsal and ventral part. Here, pharmacological inhibition or lesions of the ventral, but not dorsal HC impaired cued fear acquisition (Chen et al., 2016; Phillips and LeDoux, 1992). An involvement of the amygdala and vHC in fear conditioning could be confirmed by another study that broke neuronal signaling down to fear expression and extinction. Here, fear expression was dependent on amygdala-signaling that receives inputs from

the prelimbic cortex and the vHC, whereas extinction processes required plasticity in infralimbic cortex, basolateral amygdala and vHC (Sierra-Mercado et al., 2011).

Furthermore, neuro-inflammation has been referred to fear conditioning and extinction processes. In line, the inflammasome Nlrp3 was found to be activated 3 hours after fear conditioning, which was accompanied with decreased postsynaptic density related proteins, 72 hours after the shocks. Depletion of Nlrp3 prevented the loss of postsynaptic density related proteins and facilitated extinction of the learned fear, as well as attenuated anxiety-like behavior. Promoting neuro-inflammation by intracerebroventricular (icv) infusion of IL-1β inhibited extinction learning, which could be prevented using an IL-1 receptor antagonist (Dong et al., 2020). In line, pre-immunization or immunization after fear conditioning exposure with *M. vaccae* was able to facilitate fear extinction in a fear-potentiated startle paradigm (Fox et al., 2017; Hassell et al., 2019). This might be mediated, as *M. vaccae* reduced CRH mRNA expression in the amygdala (Loupy et al., 2019) and induced an anti-inflammatory status in the HC, by increasing the anti-inflammatory cytokine IL-4 and reducing the pro-inflammatory mediators NFκB and Nlrp3 (Frank et al., 2018b). Moreover, *M. vaccae* pre-immunization prevented a stress-induced rise in IL-1β and downregulation of CD200R1, a receptor that inhibits microglia function (Frank et al., 2018b). In contrast, knockout of the inflammasome Nlrp3 induced an anxiogenic phenotype and impaired fear memory. Interestingly, this was not restricted to fear memory, but also included social memory, as Nlrp3 knockout mice showed no preference towards a novel conspecific in the social novelty test, whereas social behavior *per se* was not affected (Komleva et al., 2021).

### 1.6.2 Model of social fear conditioning

Not only non-social traumatic events, but also those of social nature are able to insert long-lasting fears that can be also socially related and increase the risk to develop PTSD or SAD (Collimore et al., 2009). The social fear conditioning (SFC) paradigm was established in our laboratory as a mouse model that induces a SAD-like phenotype. In line, SFC is based on operant conditioning and induces socially related fears and social avoidance in naïve mice by punishing them using an electric foot shock (US) when they investigate an unfamiliar conspecific (CS; social stimulus) (Toth et al., 2012b; Toth and Neumann, 2013). Similar to CFC, during extinction training animals do not receive a foot shock anymore and thus re-learn that the CS is not punished anymore. Of note, the SFC paradigm induces long-lasting - up to at least two weeks - generalized social fear, without affecting fear of novelty, generalized anxiety-like behavior, depressive-like behavior or locomotor activity (Toth et al., 2012b), which is in contrast to other animal models of social avoidance like SD (Lukas et al., 2011b; Toth and Neumann, 2013). Moreover, even though this paradigm was

originally reported to induce social fear in CD1 mice (Toth et al., 2012b), it is not restricted to a specific strain as it was also successfully performed in BL6 mice (Toth et al., 2013).

During SFC, the glutamatergic system seems to be involved in the process of social fear extinction, as injections of an allosteric antagonist or agonist of the metabotropic receptor subtypes 5 or 7, respectively, impaired mice' ability to extinguish social fear when administered prior to extinction, but not acquisition. This indicated that the glutamatergic system might provide treatment strategies for CBT like exposure therapies rather that preventing the encoding of traumatic memories (Slattery et al., 2017). It is important to mention, that during SFC the LS is decisive, which receives glutamatergic and sends GABAergic projections to hypothalamic and midbrain regions (Deng et al., 2019a; Menon et al., 2022; Zoicas et al., 2014). In the LS, oxytocin (OXT; for details see 1.8) signaling played a key role, as conditioned animals showed reduced release of OXT, which normalized after extinction training, while endogenous or exogenous increase in OXT abolished social fear expression (Menon et al., 2022; Menon et al., 2018; Zoicas et al., 2014). In addition, Zoicas and colleagues have performed OXT receptor (OXTR) autoradiography to unravel brain regions that are involved in SFC. Here, OXTR was increased following SFC in the LS, the dentate gyrus of the HC, and in the central amygdala (Zoicas et al., 2014). Interestingly, these brain regions were shown to be involved in PTSD, SAD and social behavior, too (Amaral, 2002; Felix-Ortiz and Tye, 2014; Liberzon and Sripada, 2007; Nutt and Malizia, 2004; Phan et al., 2006; Rauch et al., 2000; Stein et al., 2002). Moreover, icv infusion of Neuropeptide S reversed SFC-induced social fear and was even able to reinstate social preference in SD mice (Zoicas et al., 2016). In non-social fear conditioning, the effects of Neuropeptide S on anxiety- and fear-related behavior were shown to be mediated by GABAergic neurons in the amygdala (Jüngling et al., 2008). Additionally, successful versus unsuccessful extinction of social fear in male mice was associated with differently regulated levels of a long non-coding RNA, indicating a substantial role of non-coding RNAs in treatment resistance (Royer et al., 2022).

Nevertheless, there is only little evidence about the impact of previous stress on SFC outcome. Surprisingly, early-life stress, induced by maternal separation, facilitated extinction of social, but not non-social, fear in male mice. Thus, early-life stress seems to improve the abilities to cope with and / or recover from a traumatic social experience in adulthood (Zoicas and Neumann, 2016). However, the impact of chronic psychosocial stress on SFC needs to be further investigated. Thus, combining the CSC paradigm, a model of chronic psychosocial stress with the SFC paradigm, a model that induces an acute traumatic social event, allows me to study the development of a stress-induced PTSD- and SAD- like phenotype in male mice.

## 1.7 Strain differences in stress response and behavior

Even though there is superficial knowledge about the impact of stress, the role of the immune system, emotional processing, and on the development of stress-related disorders in humans and rodents, it is unneglectable that each individual acts and reacts differently towards stress (Albucher and Liberzon, 2002; Bartolomucci et al., 2005; Hamann and Canli, 2004; Hodes et al., 2014; Langgartner et al., 2017; McEwen, 1993; Sapolsky, 1994). In line, experiencing severe negative life events results in the development of stress-related pathologies in stress-vulnerable, (Duffy et al., 1991; Kiecolt-Glaser and Glaser, 1995; Langgartner et al., 2015; McEwen, 2004, 2003), but not in stress-resilient individuals (Bartolomucci et al., 2004; Castro et al., 2012; Hodes et al., 2014; Krishnan et al., 2007; Stiller et al., 2011). However, underlying mechanisms that create individual differences are not fully understood as they include genetic as well as environmental factors (DeRijk and Kloet, 2005; Eiland and McEwen, 2012; Ising et al., 2008; Stiller et al., 2011; reviewed in Langgartner et al., 2017). For instance, individual differences in HPA axis reactivity and a hyperactive immune system, in particular high plasma IL-6 levels, were associated with increased vulnerability towards stress (Hodes et al., 2014; Stiller et al., 2011). These observations were also seen in treatment-resistant depressed patients (Hodes et al., 2014). Interestingly, even behavioral differences that indicate diverse stress-coping styles, i.e. low attack latency in low aggressive mice showing passive stress coping behavior, and short attack latency in high aggressive mice showing active stress coping behavior, affected HPA axis responsiveness. These data suggest that individual differences in stress-coping also impact the susceptibility to develop stress-related affective dysregulations (Veenema et al., 2004).

To study innate differences in the vulnerability in rodents, distinct strains are commonly used. In rats, comparing Sprague-Dawley and Long-Evans males and females, nicotine administrations increased stress hormone release in Sprague-Dawley females only, whereas stress exposure, i.e., acute RS, increased stress hormone levels in all groups, except Long-Evans females (Faraday et al., 2005). These data indicate strain and sex specific differences in HPA axis activity and functioning (Chisari et al., 1995; Faraday et al., 2005). In mice, ethanol withdrawal severity, indicated by plasma ACTH and CORT levels, was greater in DBA/2 compared to C57BL/6 mice (Roberts et al., 1992). Moreover, acute and repeated SD stress induced behavioral changes like social avoidance in BALB/c, but not C57BL/6J mice (Razzoli et al., 2011a; Razzoli et al., 2011b). Interestingly, within the inbred C57BL/6 strain, the substrains C57BL/6J and C57BL/6N (here BL6) were shown to differ not only genetically (Simon et al., 2013), but also in behavior, physical fitness, and stress-vulnerability. In line, BL6 mice were more susceptible to hyperthermia-induced seizures (Kang et al., 2019), chronic CORT-induced depressive-like behavior (Sturm et al., 2015),

and showed lower basal locomotion and social interaction as well as higher basal anxiety-like behavior (Matsuo et al., 2010) compared to C57BL/6J mice.

In the present study, two mouse strains were used: (i) the inbred BL6, used in the CSC paradigm (Reber et al., 2007), and (ii) the outbred CD1, used in the SFC paradigm (Toth et al., 2012b). During CSC exposure, the impact of chronic psychosocial stress on CD1 mice differed from BL6 mice. Even though in both mouse strains the HPA axis seemed to be responsive to CSC exposure, CD1 mice were less vulnerable to CSC-induced immunological consequences as pro-inflammatory cytokines and the inflammatory state of the colon were unchanged in CD1 mice, but increased in chronically stressed BL6 mice (Füchsl et al., 2014; reviewed in Langgartner et al., 2015). This was confirmed by other studies showing that CD1 mice revealed a higher immune tolerance in response to an immune challenge during placental and fetal limb development (Prater et al., 2006) as well as pyometra (Kendziorski et al., 2012), compared to the immune-sensitive BL6 mice. However, these results are in contrast to another study showing that *in vitro* LPS stimulation resulted in a higher pro-inflammatory state in the outbred ICR/CD1-derived, compared to inbred C57BL/6 derived microglia cells, which confirms the theory that inbreeding reduces resistance to pathogens and parasites (Nikodemova and Watters, 2011). Thus, CD1 and BL6 mice might differ in stress- and pathogen-induced immune responses, which might be caused by distinct stress vulnerabilities. During SFC, BL6 mice showed lower levels of social investigation, less social motivation following repeated social exposure and less aggressive behavior towards the social stimuli compared to CD1 (Toth et al., 2013). Thus, CD1 and BL6 mice are highly social species, but differ in their social behavior. In line, C57BL/6J females were shown to prefer food over social interactions, which was in contrast to female CD1 mice, which showed also robust seeking for social support following social isolation (Ramsey et al., 2021). Moreover, CD1 mice are, in contrast to other strains, highly aggressive and territorial, which is accompanied by basally higher testosterone but lower CORT levels (van Loo et al., 2003b; van Loo et al., 2003a). In confirmation, lactating CD1 mice showed higher aggressive behavior towards male intruder compared to BL6 dams (Gryksa et al., 2020). Moreover, CD1 mice were shown to be less anxious in the novel open space test (Michalikova et al., 2010) and rat exposure test (Yang et al., 2004), compared to C57BL/6. Here, CD1 mice showed less freezing behavior in response to predator and more contact compared to C57BL/6, which spent more time in the home cage. Nevertheless, analyzing defensive strategies, CD1 mice displayed high defensive burying, whereas C57BL/6 mice showed higher risk assessment behavior, i.e. stretch attend posture (Yang et al., 2004). In sum, CD1 mice are, in comparison to BL6 mice, less immune sensitive, more social, more aggressive, and show a lower anxiety-like behavior.

These data indicate that the individual, genetically determined predisposition to coping behavior and trait anxiety can influence the vulnerability towards stress and, thus, the development of stress-related psychiatric diseases. This must be considered, when appropriate treatment options are developed, as it might play a crucial role in treatment resistance.

## 1.8 Oxytocin as a potential treatment option for stress-related psychiatric diseases

Due to its various pro-social, stress-buffering, anxiolytic, anti-nociceptive and anti-inflammatory properties, the impact of the OXT system on mental and physical health, including stress-vulnerability and immune resilience is of special interest to develop appropriate treatment options (see review Gryksa and Neumann, 2021). OXT is a nonapeptide, primarily synthesized in the PVN and supraoptic nucleus of the hypothalamus and released into the periphery as well as into different brain regions in response to various stimuli (Grinevich and Neumann, 2021; Jong et al., 2015; Jurek and Neumann, 2018; Landgraf and Neumann, 2004; Neumann, 2009; Neumann and Landgraf, 1989). Interestingly, the OXT system is closely related to stress regulation. Here, several stressors stimulate peripheral and/or central OXT release (Bernhard et al., 2018; Ebner et al., 2000; Gryksa and Neumann, 2021; Hew-Butler et al., 2008; Jurek and Neumann, 2018; Pierrehumbert et al., 2010), thereby exerting stress-protective, anxiolytic and pain-reducing effects (Bale et al., 2001; Bernhard et al., 2018; Blume et al., 2008; Eliava et al., 2016; Jurek et al., 2015; Neumann et al., 2000; Neumann et al., 1999; Waldherr and Neumann, 2007). In line, OXT is released during or after stressor exposure, depending on the nature of the stressor, and attenuates the response of the HPA-axis (Neumann, 2002; Neumann et al., 2000; Winter and Jurek, 2019). Additionally, OXT has been involved in the regulation of stress- and trauma-induced social vigilance and social avoidance (Duque-Wilckens et al., 2020; Zoicas et al., 2014). This is in line to rodent studies, where exogenous and endogenous OXT was found to play a pivotal role in social fear extinction (Menon et al., 2018; Zoicas et al., 2014), while acute social trauma induction by SFC reduced central OXT release, which was reversed following extinction of social fear (Zoicas et al., 2014). Moreover, chronic psychosocial stress, social trauma exposure in adulthood or early in life, as well as pair separation (Bosch et al., 2016; Frijling et al., 2015; Heim et al., 2009; Lukas et al., 2011a; Peters et al., 2014) resulted in mal-adaptations of the OXT system in rodent and human studies (Gryksa and Neumann, 2021; Jurek and Neumann, 2018; Neumann et al., 2000; Olff et al., 2013).

Besides, OXT has potent anti-nociceptive (Eliava et al., 2016; Lundeberg et al., 1994), anti-oxidant (Moosmann and Behl, 2002), and anti-inflammatory (Wang et al., 2015) properties (for review see

Gryksa and Neumann, 2021). In line, OXT alleviated tissue and organ damage in models of renal (Tuğtepe et al., 2007) and hepatic (Düşünceli et al., 2008) ischemia, sepsis induced multiple organ failure (Işeri et al., 2005a), skin injury (Işeri et al., 2008), and colitis (Işeri et al., 2005b; Peters et al., 2014). The involvement of OXT in inflammation is not surprising, since macrophages, monocytes and endothelial cells possess OXTRs, while OXT signaling can reduce the secretion of inflammatory cytokines from these cells (Szeto et al., 2008). Therefore, the anti-inflammatory properties of OXT might be mediated via downregulation of pro-inflammatory TNF-α and IL-6, as well as immune cell trafficking, e.g. neutrophil infiltration to the site of injury (Düşünceli et al., 2008; Işeri et al., 2005b; Işeri et al., 2005a; Tuğtepe et al., 2007). Consistently, high plasma OXT levels dampened pro-inflammatory cytokine production during early infection (Soumier and Sirigu, 2020; Wang et al., 2015). Of note, double-labeling studies revealed that many IL-1β positive neurons in the human hypothalamus showed a co-localization with OXT, which were reduced in patients suffering from multiple scleroses (Huitinga et al., 2000). In line, central administration of IL-1β stimulated the release of OXT (Landgraf et al., 1995).

Matching the close link of the immune system and the development of PTSD, numerous human studies proposed OXT as an effective treatment option for PTSD, either alone early after trauma exposure (Frijling, 2017; Frijling et al., 2014), or in combination with psychotherapies including exposure therapy (Flanagan et al., 2018; Koch et al., 2014). Hereby, OXT has been shown to dampen amygdala hyperreactivity in PTSD patients (Frijling, 2017; Koch et al., 2016). Interestingly, this could also be shown in patients suffering from gSAD, when they were exposed to fearful faces (Labuschagne et al., 2010). In line, intranasal OXT treatment improved positive evaluation of appearance and speech performance during a public speaking task in SAD patients, however, it did not enhance overall treatment outcome during exposure therapy (Guastella et al., 2009). Thus, elevated endogenous OXT levels might be beneficial to prevent the development of stress-induced psychiatric diseases. This can be achieved by, e.g. exercise or sexual stimulation (Jong et al., 2015), but also by probiotics, such as Lactobacillus bacteria that were described to increase OXT levels and thus to affect the stress and immune systems (Andersson et al., 2016; Bharwani et al., 2017). In rodent studies, acute OXT administration rescued psychosocial stress-induced social avoidance (Lukas et al., 2011b), abolished social fear following acute social trauma induction (Zoicas et al., 2014), and reduced alcohol intake in unstressed animals (Peters et al., 2013). Chronic OXT dose-dependently prevented CSC-induced mal-adaptations on anxiety-like behavior, thymus and adrenal weight, as well as adrenal *in vitro* ACTH insensitivity (Peters et al., 2014). Moreover, chronic OXT treatment rescued CSC-induced reduction of OXTR-binding in the median raphe nucleus (Peters et al., 2014), a brain region that is thought to mediate the anxiolytic effects of OXT (Yoshida et al., 2009), whereas OXT mRNA levels in the PVN were not altered (Peters et al.,

2014; Reber and Neumann, 2008). In line, chronic OXT administration, via subcutaneous injections, rescued long-term social isolation-induced increase in basal heart rate and depressive-like behavior in female prairie voles (Grippo et al., 2009).

Nevertheless, chronic use of OXT may exert negative outcomes and may affect the endogenous OXT system, resulting in downregulation of OXTR (Bale et al., 2001; Peters et al., 2014) and in alternative OXTR-mediated signaling pathways (Winter et al., 2021).

## 1.9 Aims of the thesis

In sum, the actual knowledge about the development of stress-related disorders, their comorbidity, risk factors, underlying mechanisms, as well as available efficient treatment options, is not sufficient. Especially the underlying mechanism of the comorbidity of PTSD and SAD remains deficient. Moreover, the impact of chronic psychosocial stress on the development of the disease phenotype and its comorbidity needs further investigation. The combination of CSC and SFC models represents a promising tool to answer these questions, as it induces a variety of core symptoms of PTSD and SAD, respectively. Thereby, unraveling behavioral and immunological changes, the importance of the nature of the fear conditioning paradigm and the chronic stressor model, as well as the innate state of anxiety and stress-susceptibility, can help to identify underlying mechanisms and to discover potential treatment options. In this connection, the oxytocinergic system is a promising target to develop potent and effective preventative or curative medications, which might be an add-on pharmacotherapy for CBT, like exposure therapy.

Therefore, in the present thesis I hypothesized that:

I. CSC exposure followed by an acute traumatic social experience accelerates the onset and strengthens the severity of PTSD- and SAD-like symptoms.

II. The chronic psychosocial stress-induced PTSD- and SAD- like phenotype might underlie CSC-induced dysregulations in social behavior, and / or a dysregulation of the peripheral and, in particular, the central immune system following CSC, SFC acquisition, and / or the combination of both.

III. The chronic psychosocial stress-induced PTSD- and SAD- like phenotype is dependent on i) the nature of the fear conditioning model, ii) the nature and composition of the chronic stressor paradigm, and iii) the mouse strain, as it requires a high innate state of anxiety and stress-vulnerability.

IV. Treatment with the pro-social, stress-buffering and anti-inflammatory nonapeptide OXT represents an effective add-on treatment in combination with exposure therapy. Thus,

OXT treatment might rescue the CSC-induced development of a PTSD- and SAD- like phenotype, when administered prior to SFC extinction.

In order to proof my hypotheses, I aimed to investigate:

I. The impact of CSC exposure on SFC acquisition, extinction and recall.

II. The impact of CSC exposure on social preference in the SPT, as well as short-term (2 hours after stimuli presentation) and long-term (24 hours after stimuli presentation) social discrimination abilities in the mSDT. Moreover, I aimed to analyze the peripheral and, especially, the central immune system 2 hours following CSC exposure, SFC acquisition, and the combination of both. In this context, I focused on neuro-inflammatory alterations in three brain regions, which are highly involved in stress, fear, social behavior, and SFC, as well as in the development of stress-related psychiatric disorders including anxiety, PTSD and SAD, i.e., amygdala, septum, and vHC.

III. The impact of CSC exposure on CFC, as well as the effects of CUS exposure on CFC and SFC. Moreover, BL6 and CD1 mice were tested for their innate anxiety- and fear-related behavior, social behavior, and stress coping behavior during CSC exposure. Additionally, I aimed to investigate the impact of CSC exposure on SFC in CD1 mice.

IV. The impact of acute OXT infusion (icv, 0.1 µg / 2 µl) 10 minutes prior to SFC extinction under basal conditions in BL6 mice, as well as following CSC exposure in BL6 and CD1 mice.

# Material and Methods

# 2 Material and Methods

## 2.1 Animals

For all experiments, male BL6 or CD1 mice were purchased from Charles River (Sulzfeld, Germany) with a weight of 19 to 22 g (BL6) or 27 to 30 g (CD1). On arrival day, mice were either directly single housed for 1 week (all CD1 mice and Experiment 1 to 3 for BL6 mice) or kept group housed for 1 week prior to single housing for another week (BL6 in Experiment 4). Male CD1 mice, either purchased from Charles River or kindly provided by the University Hospital Regensburg, were used as male dominant aggressor mice (residents). At a weight of 35 to 40 g, residents were individually housed in big observation cages (38 cm x 22 cm x 35 cm) 3 days prior to the experiments to develop territorial behavior. For all tests involving social interactions, mice were placed in small observation cages (30 cm x 25 cm x 35 cm), and weight and strain matched mice (purchased from Charles River) were used as social stimuli.

All mice were kept under standard laboratory conditions (22 ± 2°C, 50 % humidity, 12-hours light / dark cycle, lights on at 7 am) with free access to water and food. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health, Bethesda, MD, USA, approved by the government of Oberpfalz and performed according to international guidelines on the ethical use of animals and ARRIVE guidelines (Kilkenny et al., 2010). All efforts were made to minimize the number of animals used and their suffering.

## 2.2 Chronic stress models

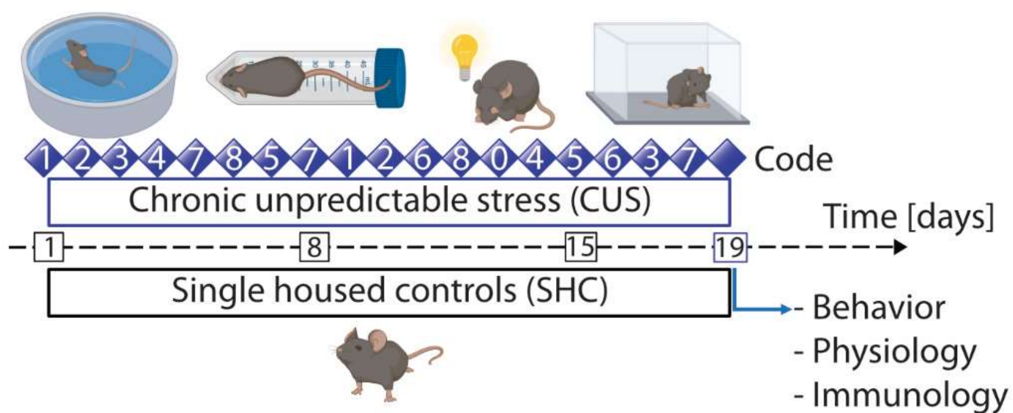
To investigate underlying mechanisms, as well as the impact of chronic stress on different types of behavior, I used two different chronic stressor paradigms, namely the CUS and the CSC paradigm.

### 2.2.1 Chronic unpredictable stress (CUS)

In order to induce chronic non-social stress, mice were exposed to the CUS paradigm. This model is based on published models (Kim and Han, 2006; Kreisel et al., 2014; Monteiro et al., 2015; Yoon et al., 2014; Yun et al., 2010) and has been adjusted to our laboratory conditions.

The CUS paradigm (Figure 4) lasts for 19 days and consists of a variety of stressors, i.e., RS, FS, no bedding and lights on overnight, which mice are exposed to once or twice a day at different durations in an unpredictable manner. For RS, mice were put in a well-ventilated 50-ml glass

falcon for 1 to 4 hours, where they were able to only move back and forth. For FS, mice were placed into a swim container (Plexiglas, 12 cm diameter, 40 cm in height) filled with water (23 to 24°C) up to 30 cm and were forced to swim for 10 to 30 minutes. For no bedding stress, mice stayed without bedding in their homecage for 24 hours. For lights on overnight, the sleep-wake cycle of mice were disturbed by exposing them to light for 24 hours. Stressors in the morning started at 8 am, while stressors in the evening started at 4.30 pm. On day 1 of the CUS model, mice were weighted-matched assigned to CUS mice or SHC. SHC were kept in a separate room and remained undisturbed except for changing the bedding and weighing once a week, while CUS mice were exposed to the CUS paradigm.




| Code | Morning (8 am) | Afternoon (4.30 pm) |
|---|---|---|
| 0 | | |
| 1 | RS for 2 hours | FS for 15 minutes |
| 2 | | Lights on overnight |
| 3 | RS for 4 hours | |
| 4 | FS for 20 minutes | RS for 1 hour |
| 5 | No bedding for 24 hours | |
| 6 | FS for 15 minutes | FS for 10 minutes |
| 7 | | FS for 30 minutes |
| 8 | RS for 3 hours | RS for 1 hour |

**Figure 4: Schematic illustration of the chronic unpredictable stress (CUS) paradigm.** On day 1, animals were weight-matched assigned to CUS mice or single housed controls (SHC). CUS mice were exposed to various stressors including restraint stress (RS), forced swim (FS), no bedding, and lights on overnight at different time points and durations. Type, duration and time point of the stressors are indicated as a code and listed in the table. SHC were left undisturbed in a separate room. At day 19, either behavioral, physiological and / or immunological parameter were measured.

### 2.2.2 Chronic subordinate colony housing (CSC)

To induce chronic psychosocial stress in male mice, 4 male mice (CSC mice) were housed together with a larger dominant male aggressor mouse (resident) in an observation cage for 19 consecutive days. The CSC paradigm (Figure 5) was performed as described earlier (Füchsl et al., 2013a; Langgartner et al., 2015; Reber et al., 2016a; Reber et al., 2008; Reber et al., 2007; Slattery et al., 2012). In detail, prior to the CSC procedure, residents were tested for their aggressive behavior. Dominant males that started to injure conspecifics by excessive aggression or harmful bites, as well as residents that did not show any aggression, were excluded from all experiments. On day 1 of CSC, mice were weighted-matched assigned to CSC mice or SHC. SHC were kept single housed in a separate room and remained undisturbed except for changing the bedding and weighing once a week (days 8 and 15). 4 CSC mice were put into the homecage of a resident and the 30 minutes, when CSC mice encountered a novel resident, was videotaped and further analyzed. To prevent habituation, the same procedure was repeated on days 8 and 15 of the protocol, whereat CSC mice were placed into the homecage of a novel, unknown resident. The residents seemed to generally obtain the dominant position, as they displayed offensive behavior such as chasing, mounting, lateral threat, keep down, aggressive grooming and attacks. CSC mice, in contrast, displayed mostly defensive behavior like grooming the resident, flight and submissive upright (Reber and Neumann, 2008).

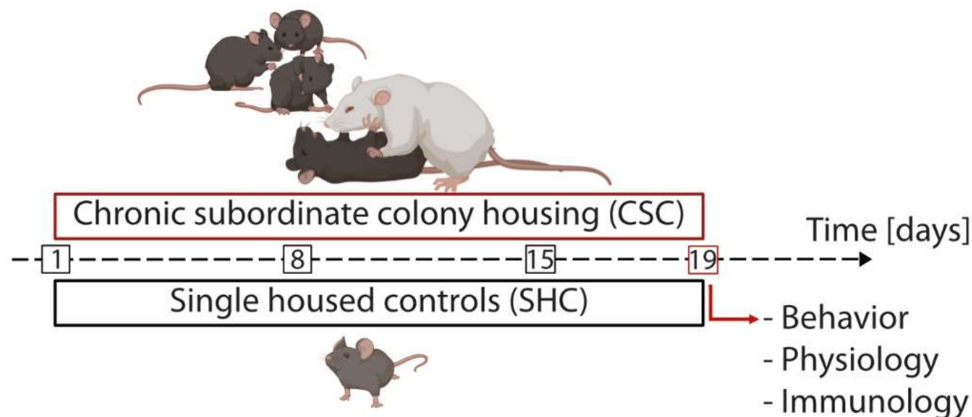



**Figure 5: Schematic illustration of the chronic subordinate colony (CSC) paradigm.** At day 1 of CSC, animals were weight-matched assigned to CSC or single housed controls (SHC). CSC mice were placed into the homecage of a dominant aggressor mouse (resident), resulting in subordination of the CSC mice, while SHC were left undisturbed. This paradigm lasts for 19 consecutive days, while CSC mice were put in the homecage of a novel resident on days 8 and 15 to prevent habituation. At day 19, either behavioral, physiological and / or immunological parameter were measured.

## 2.1 Fear conditioning paradigms

Using fear conditioning paradigms, I investigated non-social and social fear response of male mice under basal conditions as well as following chronic stress exposure.

### 2.1.1 Cued fear conditioning (CFC)

To investigate non-social fear responses, mice were exposed to the CFC paradigm (Figure 6), which was performed as described earlier (Toth et al., 2012a), in a computerized fear conditioning system (TSE System GmbH, Bad Homburg, Germany) with slight adaptations. The conditioning chamber consists of a transparent Perspex box (45 cm x 22 cm x 40 cm) enclosed in a chamber to reduce external noise and visual stimulation. The floor was made-up of a removable stainless steel grid, which was connected to a shock delivery unit to allow manual application of foot shocks. To perform CFC in distinct environments, different surroundings, floors, odors and light conditions were used for acquisition, or extinction and retention. An overhead camera recorded freezing behavior, which was measured by an automatic video analyzing system, as an index of fear expression.

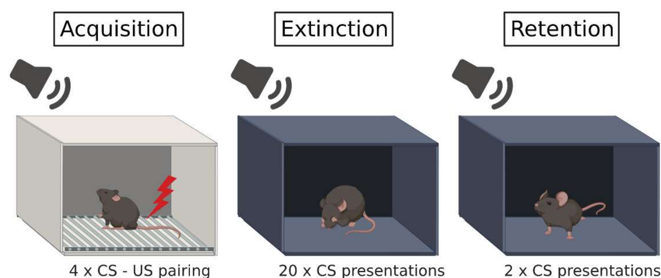



**Figure 6: Schematic illustration of the cued fear conditioning (CFC) paradigm.** On day 1 of CFC, mice were exposed to the acquisition, receiving 4 x a tone (conditioned stimulus, CS) for 30 seconds, which is co-terminated with an electric foot shock (unconditioned stimulus, US) during the last 2 seconds. During extinction, CS were presented 20 times and during retention 2 times in a different environment without US.

**Acquisition**

During acquisition, chamber A (23 cm x 23 cm x 36 cm, transparent surrounding, 300 lux, electric grid floor) was used and cleaned after each mouse with a lemon-scented detergent. All mice were placed individually in the middle of the chamber and subjected to the following acquisition protocol: 5 minutes adaptation, where mice were allowed to freely explore the box, followed by 4 (Experiment 3.1 and 3.3) or 3 (Experiment 3.2) times a 30 second tone (CS; 80 dB, 8 kHz) with a 2-minutes pause between them. The tones were co-terminated with an electric foot shock (US; 0.5 mA Experiment 3.2 or 0.7 mA Experiment 3.1 and 3.3, pulsed current) during the last 2 seconds. 5 minutes after the last CS-US pairing, mice were returned to their homecage.

**Extinction**

On the next day, extinction was performed in the same conditioning system, but in another chamber to provide a different environment (chamber B; 23 cm x 23 cm x 36 cm, dark surrounding, 20 lux, smooth floor) and a neutral smell (neutral smelling detergent). After 5 minutes of adaptation, each mouse was exposed to 20 repetitions of the 30-seconds tone (CS), without the US, with 5 seconds pause in-between. Thereafter, mice were habituated for 5 minutes in the chamber before returning them into their homecage. After each trial, the box was cleaned thoroughly with water containing a low concentration of neural detergent, to ensure the same criteria for every mouse. For analysis, freezing behavior during two CS-presentations were summarized to one data point, leading to 10 data points.

**Retention**

During retention the same chamber (chamber B) and environment was used as during extinction training. Each mouse was habituated for 5 minutes in the chamber, followed by 2 times 30 seconds tone with a 2-minute break in-between. After each trial, the box was cleaned thoroughly with water containing a low concentration of neural detergent, to ensure the same criteria for every mouse. For analysis, the time spent freezing during both CS presentations were summed in one data point.

### 2.1.2 Social fear conditioning (SFC)

To induce social avoidance behavior, a SAD-like symptom, mice were punished, using an electric foot shock, each time they investigate an unfamiliar conspecific in the SFC paradigm (Figure 7), which was performed as described earlier (Toth et al., 2013, 2012b; Toth and Neumann, 2013) using the same TSE System described above (see 2.1.1).

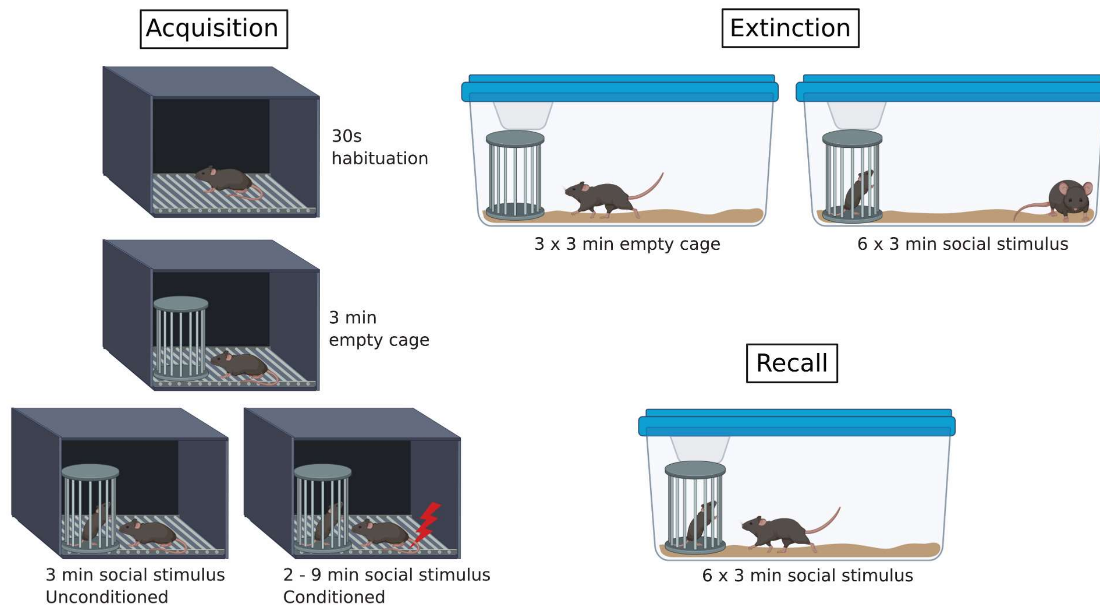



**Figure 7: Schematic illustration of the social fear conditioning (SFC) paradigm.** At day 1 of SFC, mice were placed in the acquisition chamber and allowed to habituate for 30 seconds before an empty cage (non-social stimulus) was put in the chamber for 3 minutes. Thereafter, an identical cage containing a conspecific (social stimulus) replaced the empty cage. Unconditioned mice (SFC⁻) were allowed to freely explore the conspecific for 3 minutes, while conditioned mice (SFC⁺) received an electric foot shock each time they had direct contact. After 2-9 minutes, depending on the number of shocks received, animals were returned to their homecage, where on the next day extinction training was performed. Here, SFC⁻ and SFC⁺ were presented 3 times a non-social stimulus, followed by 6 times a social stimulus, which they could freely explore for 3 minutes each with a 3-minute inter-exposure break. The same procedure was applied during recall on day 3 of SFC, presenting 6 times a conspecific for 3 minutes each with a 3-minute inter-exposure break.

## Acquisition

During acquisition, on day 1 of SFC, mice were individually placed in the conditioning chamber and after 30 seconds of habituation an empty wire mesh cage (non-social stimulus, 7 cm x 7 cm x 6 cm) was placed in the right corner of the door. Mice were able to investigate the non-social stimulus freely for 3 minutes before an identical one containing an unfamiliar, weight-matched male mouse (social stimulus) replaced the empty cage. Unconditioned mice (SFC⁻) were allowed to investigate the social stimulus freely for 3 minutes, while conditioned mice (SFC⁺) received an electric foot shock (0.7 mA, pulsed current), each time they had direct contact with the social stimulus, until they reached the opposite corner (about 0.8 to 1 second). SFC⁺ mice received between 1 to 3 foot shocks and were returned to their homecage when no further social contact was made for 6 minutes (in case of only 1 social contact) or 2 minutes (when they investigated the unfamiliar mouse more than once). For Experiment 2.2b, an additional shock control was added, which received one electric foot shock directly after the social stimulus was presented and a second one after additional 2 minutes, independent of social contact. Shock controls were returned into their homecage 2 minutes after the last shock. Therefore, mice spent between 2 to

6 minutes in the conditioning chamber in presence of a conspecific. An empty cage was placed over night in the homecage of all mice to extinguish the fear of an empty cage.

**Extinction**

On day 2 of SFC, extinction was performed in the homecage to test if SFC$^+$ mice displayed social fear and if the fear is extinguishable. Mice were exposed to 3 non-social stimuli (identical to the cages used during acquisition) placed in the middle of the homecage for 3 minutes per stimulus with 3-minute inter-exposure intervals, to assess non-social investigation. To analyze social investigation, each mouse was exposed to 6 different unfamiliar male conspecifics enclosed in a wire mesh cage, as used on day 1 of SFC, placed at the door of the homecage. Each stimulus was presented for 3 minutes with a 3-minute inter-exposure interval. Extinction was videotaped by a camera placed in front of the observation cages and analyzed by a trained observer blind to treatment using the JWatcher program (V 1.0, Macquarie University and UCLA). Reduced or no social investigation indicated social fear and, thus, successful conditioning, whereas, increasing investigation time along the social stimuli indicated successful extinction of the learned social fear.

**Recall**

To investigate, whether extinction of the learned social fear was successful, recall was performed on day 3 of SFC. Here, mice were exposed again to 6 unfamiliar social stimuli for 3 minutes with a 3-minute inter-exposure interval, as performed during extinction. Recall was videotaped by a camera placed in front of the observation cages and analyzed by a trained observer blind to treatment using the JWatcher program.

## 2.2 Behavioral testing for anxiety-like behavior

Anxiety-like behavior in mice was analyzed using the following tests that mimic the natural conflict of mice between their natural tendency to explore new environments and their innate avoidance of open, bright and / or elevated areas (Campos et al., 2013).

### 2.2.1 Light dark box (LDB)

The LDB was performed as described earlier (Bourin and Hascoët, 2003) and consists of two compartments: The light box (LB; 40 cm x 50 cm; 300 Lux) and the dark box (40 cm x 30 cm; 50 Lux). Those are separated by a partition wall that had a small opening (7.5 cm x 7.5 cm) at floor level to allow transitions. At the beginning of the test, mice were placed individually in the dark box of the LDB and, to allow habituation, the opening of the partition wall was closed for 30

seconds. Afterwards, mice were allowed to freely explore the complete arena for 5 minutes. Time spent in the LB and the latency to enter the LB were used as an indicator for anxiety-like behavior, which was analyzed using the tracking software EthoVision XT7 (Noldus Information Technology, Wageningen, Netherlands). After each trial, the LDB was cleaned thoroughly with water containing a low concentration of detergent, to ensure same criteria for every mouse.

### 2.2.2 Elevated plusmaze (EPM)

The EPM is a plus-shaped elevated maze (70 cm height) consisting of two closed arms (50 cm x 10 cm, 10 lux) and two open arms (50 cm x 10 cm, 50 lux) with a neutral zone in the middle, and was performed as described earlier (Veenema et al., 2007). Briefly, each mouse was placed into the neutral zone facing a closed arm and allowed to explore the EPM freely for 5 minutes. The following parameters were measured by an trained observer blind to treatment using the lab-owned plusmaze DOS program: time spent on the open arms, open arm entries (i.e. front paws and shoulder enter the open arm), number of full open arm entries (i.e. the full body enters the open arm), time spent on the full open arm, and closed arm entries (indicator of locomotion). After each trial, the box was cleaned thoroughly with water containing a low concentration of detergent, to ensure same criteria for every mouse.

### 2.2.3 Open field (OF) and novel object recognition (NOR)

The open field (OF) test with novel object recognition (NOR) was performed as described earlier (Veenema et al., 2007) with slight adaptations. In brief, the OF box (80 cm x 80 cm x 38.5 cm) is separated into four arenas (each 40 cm x 40 cm x 38.5 cm; 300 Lux) with a defined outer and inner zone (20 cm x 20 cm each). Experimental mice were placed individually in the center of one arena and allowed to explore it freely for 5 minutes. Afterwards, a novel object (i.e. round ion cylinder; 3.5 cm diameter x 1.5 cm height) was placed in the middle of the inner zone and mice were allowed to explore for further 5 minutes. Behavior was videotaped by an overhead camera and analyzed using the tracking software EthoVision XT7. Time spent in the inner zone and time and occurrence spent sniffing the novel object were used as parameters indicative for anxiety-like behavior, while total distance moved was used as an indicator of locomotion. After each trial, the OF box and the novel objects were cleaned thoroughly with water containing a low concentration of detergent, to ensure same criteria for every mouse.

## 2.3 Behavioral testing for depressive-like behavior

To determine the effect of chronic stress exposure on depressive-like behavior, the splash test was performed in the homecage as described earlier (Machado et al., 2012; Yalcin et al., 2005). In brief, one pump of a 10 % sucrose solution diluted in tap water was splashed on the dorsal coat of the mouse using a manual spray bottle. Directly after spraying the sucrose solution, the time spent grooming as well as the latency to groom were recorded for 5 minutes and analyzed by a trained observer blind to treatment using the JWatcher program.

## 2.4 Behavioral testing for social preference

To analyze natural social behavior of male mice under basal conditions, as well as following chronic stress exposure, the social preference test (SPT) was performed in the homecage as described earlier (Lukas et al., 2011b) in two different sets: (i) either, mice were exposed to a non-social stimulus (empty wire mesh cage) for 2.5 minutes, which was replaced by a social stimulus (wire mesh cage containing a weight matched conspecific) for addition 2.5 minutes (Experiment 3), or (ii) mice were exposed to both stimuli in parallel, placed at the opposite sides of the homecage, for 10 minutes (Experiment 2). The behavior was recorded and the time spend investigating the respective stimuli was analyzed by a trained observer blind to treatment using the JWatcher program.

## 2.5 Behavioral testing for social discrimination

Directly after the SPT in Experiment 2, a modified social discrimination test (mSDT) was performed as described earlier with slight adaptations (Lukas et al., 2013), to analyze mice social memory abilities after CSC exposure. Therefore, the social stimulus (enclosed in a wire mesh cage) used in the SPT, was re-used as known social stimulus, while a unknown conspecific was used as novel social stimulus. For each trial, the stimuli were presented in opposite sides of the homecage for 10 minutes, while the positions of the stimuli were changed after each test. To measure short-term memory, the mSDT was performed 2 hours after the SPT, for long-term social memory after additional 24 hours. Tests were recorded and time spend investigating the stimuli was analyzed by a trained observer blind to treatment using the JWatcher program.

## 2.6 Stereotactic implantation

To investigate the effects of OXT infusion into the lateral ventricle on social fear extinction, mice were implanted an icv cannula on day 1 of Experiment 4.1 or directly after CSC exposure (day 19, Experiment 4.2). Here, guide cannulas (21G, 8 mm length; Injecta GmbH, Germany) were implanted (from Bregma +0.2 mm, lateral +1.0 mm, depth -1.4 mm; coordinates based on the mouse brain atlas by Paxinos and Franklin, 2019) under anesthesia (Isoflurane, 4 %, Forene; Abbott GmbH, Wiesbaden, Germany) and semi-sterile conditions (Menon et al., 2018; Toth et al., 2012a). The cannula was fixed to the scull using two jeweler´s screws and dental cement (Kallocryl; Speiko-Dr. Speier GmbH, Münster, Germany). A stainless steel stylet (26 G, 8 mm) was inserted into the guide cannula to avoid infections by contact with the external environment. To avoid postsurgical infections and pain, mice received a subcutaneous injection of the antibiotic Baytril (Bayer GmbH, Leverkusen, Germany, 10 mg / kg) and of the analgesic Buprenovet (0.1 mg/kg Buprenorphine; Bayer), as well as the local anesthetic Lidocaine (Lidocainhydrochlorid 2 %; Bela-pharm). Following surgery, mice were handled daily for 10 days (Experiment 4.1) or 4 days (Experiment 4.2; twice a day in Experiment 4.2a) to clean the stylet and to allow the mice to habituate to the infusion procedure.

## 2.7 Intracerebral infusion

10 minutes prior to SFC extinction training (Experiment 4), mice received either vehicle (VEH, 2 µl sterile Ringer solution, NaCl) or 0.1 µg / 2 µl OXT (solved in sterile Ringer solution; Sigma Aldrich Chemie GmbH, Schnelldorf, Germany). Therefore, a stainless steel needle (26G, 10 mm) was inserted into the guide cannula, which was connected to a polyethylene tube and a Hamilton syringe (Hamilton Company, Bonaduz, Switzerland) to allow precise infusion of the appropriate volume (2 µl).

## 2.8 Organ collection

To determine the effects of stress exposure or fear conditioning on plasma stress hormones and / or cytokine levels as well as on organ weight and *in vitro* response, after the last behavioral experiment, mice were deeply anesthetized using $CO_2$ followed by rapid decapitation. Trunk blood was collected in EDTA-tubes (Sarstedt, Nürnbrecht, Germany) and centrifuged at 4°C (5 minutes at 5000 rpm) before storing it at -20°C until assayed. Moreover, brains were carefully removed from the cranium, snap frozen in -32°C cold n-methylbutane (Sigma Aldrich, Deisenhofen, Germany) and stored at -80°C until analyzed. Adrenal glands, thymus, spleen and mesenteric lymph nodes (mesLNs) were collected and stored on ice in 1 x phosphate buffered

saline (1 x PBS; 0.01 M) or cell culture medium (mesLNs; RPMI-1640 + Medium, i.e., RPMI-1640 medium with L-glutamine and sodium bicarbonate containing 10 % heat-inactivated fetal bovine serum (FBS), 100 U / ml Penicillin, 100 µg / ml Streptomycin, 3 x $10^{-5}$ M β-mercaptoethanol; all from Sigma Aldrich). Adrenal glands, thymus and spleen were pruned from fat, weighed separately and stored in 1 x PBS until further processed.

## 2.9 *In vitro* stimulations of adrenal glands, mesLNCs and splenocytes

***In vitro* ACTH stimulation of adrenal glands**

The *in vitro* stimulation of adrenal explants with ACTH (100 nM; Sigma Aldrich) or 0.9 % saline (B. Braun Melsungen AG, Melsungen, Germany) was performed as described earlier (Reber et al., 2007; Uschold-Schmidt et al., 2012). In brief, each left and right adrenal gland was bisected with each half containing cortical and medullary tissue, weighted and stored in Dulbecco´s Modified Eagle Medium-F12 (Life Technologies, Darmstadt, Germany) containing 0.1 % bovine serum albumin (BSA; Sigma Aldrich). Subsequently, adrenal glands were pre-incubated for 4 hours (Experiment 1 and 3.2) or until the beginning of the light cycle, i.e., 6.30 pm (Experiment 2.2a and 3.3) at 37°C (5 % $CO_2$). Afterwards, the culture medium was replaced with 100 µl fresh medium and each half of one adrenal gland was incubated with 25 µl medium containing either 0.9 % saline or ACTH (100 nM) for additional 6 hours (Experiment 1 and 3.2) or 1 hour (Experiment 2.2a and 3.3) at 37°C (5 % $CO_2$). Finally, supernatants were carefully collected and stored at -20°C until analyzed.

***In vitro* stimulation of mesLNCs**

Isolation and stimulation of the mesLNCs was performed as previously described (Füchsl et al., 2014; Reber et al., 2007; Veenema et al., 2008). Thus, mesLNs were mechanically homogenized using the back of a syringe, filtered through a 40-µm cell strainer (BB Falcon, Erembodegem, Belgium) and centrifuged at 4°C for 15 minutes (1600 rpm). The supernatant was removed and the cell pellet re-suspended in fresh medium. Following a second filtration through a 40-µm cell strainer, cell quantity was determined by mixing trypan blue (Life Technologies) with the cell solution at a ratio of 1:5 when counted in a Neubauer chamber or at a ratio of 1:1 when counted by an automated Dual Fluorescence Cell Counter Luna™ fl (Logos Biosystems, Villeneuve-d'Ascq, France; Experiment 3.3c). $10^6$ cells were transferred into a new falcon tube and filled up with fresh medium to a volume of 0.5 ml. After an additional centrifugation step, the medium was refreshed and 100 µl containing 500.000 cells were transferred per well (4 wells per animal) of a 96-well tissue plate pre-coated with anti-CD3 antibody (2.5 µg / ml; BD Biosciences, Heidelberg,

Germany). After 48 hours of incubation (37°C, 5 % $CO_2$), the supernatant was stored at -20°C until assayed.

***In vitro* stimulation of splenocytes**

Isolation and stimulation of the splenocytes has been performed as described earlier (Foertsch et al., 2017). Therefore, after pruning from fat, spleens were stored in 50-ml falcon tubes containing 1 ml cell culture medium (Hank´s Balanced Salt Solution; Sigma Aldrich), mechanically homogenized using the back of a syringe, and filtered through a 70-µm cell strainer (BB Falcon). Erythrocytes were removed by adding Ammonium-Chloride-Potassium lysis buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 10 mM EDTA) for 2 minutes, which was terminated by adding 15 ml Hank´s Balanced Salt Solution containing 10 % FBS. Following a second filtration through a 70-µm cell strainer, cell quantity was determined by mixing trypan blue (Life Technologies) with the cell solution at a ratio of 1:5 and counted in a Neubauer chamber. $2.5 \times 10^6$ cells were transferred into a new falcon tube and filled up with RPMI-1640 + medium. Cell suspensions were either treated with LPS (final concentration of 1 µg / ml, Escherichia coli O111:B4; Sigma Aldrich) or remained untreated (basal). Moreover, treated and untreated cells were stimulated in 96-well plates with ascending CORT concentrations (0.005, 0.05, 0.1, 0.5, 5 µM CORT final concentrations; Sigma Aldrich), diluted in 95 % ethanol at a final volume of 100 µl per well for 48 hours (37°C, 5 % $CO_2$). After incubation, 20 µl of a colometric assay (CellTiter 96® $AQ_{ueous}$ One Solution Cell Proliferation Assay, MTS; Promega, Madison, WI) was added to each well to determine cell viability. Living cells convert the containing formazan to a red dye, which can be measured using a plate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany) at 450 nm. In Experiment 2.2b, ascending CORT dilutions were not analyzed due to technical issues.

## 2.10 ELISA for IFN-γ, ACTH and CORT

Supernatant of mesLNCs were analyzed using the mouse IFN-γ enzyme linked immunosorbent assay (ELISA) MAX Deluxe Set (analytical sensitivity ≤ 4 pg / ml; BioLegend, San Diego, USA) of two wells per animal, which were averaged per animal.

Plasma and adrenal supernatant samples were analyzed using a commercial available ELISA for ACTH (plasma samples only; analytical sensitivity 0.22 pg / ml, intra-assay and inter-assay coefficients of variation (CV) ≤ 7.1 %; IBL International, Hamburg, Germany) and CORT (Corticosterone Human, Rat, Mouse ELISA, analytical sensitivity < 1.631 nmol / l, intra-assay and inter-assay CV ≤ 6.35 %; IBL International). Adrenal CORT concentrations were calculated in relation to the weight of the respective adrenal explants (i.e. relative CORT secretion) and expressed in ng / ml / mg. To illustrate possible treatment effects and *in vitro* CORT secretion in

relation to the whole organism, relative CORT secretion from the left and right adrenal gland of each mouse was summed up.

All ELISAs were measured using the plate reader FLUOstar Optima at 450 nm (BMG Labtech).

## 2.11 Multiplex analysis of peripheral cytokine level

Plasma cytokine levels were measured by microBIOMix GmbH (Regensburg, Germany) using a Multiplex Testing Service with home-brew murine Cytokine Panel 5-plex for monocyte chemoattractant protein-1, IL-1β, IL-6, IL-4, IL-10. Due to low concentrations below the detection limit of the multiplex, only IL-6, IL-4 and IL-10 levels could be analyzed in the plasma.

## 2.12 Brain mRNA and Protein quantification

To quantify brain immune parameters following CSC and / or SFC exposure, frozen brains were cut in 200-µm cryo-sections and micropunches from the amygdala, septum and vHC were obtained. Micropunches were flash frozen in liquid nitrogen and stored at -80°C until mRNA and protein isolation. As the LS is too small to obtain micropunch-tissue only from this specific area of the brain, the complete septum has been analyzed. In Experiment 2.2a and 2.2b, mRNA isolation was performed using isopropanol precipitation, while in Experiment 2.2c mRNA and protein were isolated simultaneously from the same sample using the NucleoSpin RNA/Protein kit (#740933; Macherey-Nagel GmbH & Co KG, Düren, Germany).

**mRNA isolation using isopropanol precipitation**

Total RNA was isolated in Experiment 2.2a and 2.2b using isopropanol precipitation. Therefore, tissue samples were homogenized in 1 ml TriFast (VWR Life Science, Darmstadt, Germany) and incubated for 5 minutes (room temperature; RT) before adding 200 µl chloroform for phase separation. After thoroughly mixing the samples, they were centrifuged (17,000 g, 20 minutes, 4°C) and the upper aqueous phase was carefully removed and mixed with isopropanol (45 % of the total volume) and 1 µl Glycogen. After incubating the samples over night at -20°C, they were centrifuged for 30 minutes (17,000 g, 4°C) and washed 3 times by adding 1 ml of 80 % EtOH and centrifuging for 15 minutes (17,000 g, 4°C) for each wash. After the last washing step, the samples were dried to remove all EtOH residues and resuspended in 1 µl 10 x Reaction Buffer (+ $MgCl_2$, FIRMA), 2 µl DNase I (Thermo Fisher Scientific, Schwerte, Germany) and 7 µl RNase-free $H_2O$ for DNA digestion. Samples were incubated at 37°C for 30 minutes (1000 rpm), the reaction was stopped by adding 1 µl 50 mM EDTA (Thermo Fisher Scientific) and further incubation at 65°C (10 minutes). mRNA was quantified with the Nanodrop (NanoDrop Technologies Inc., Wilmington, Delaware, USA). mRNA samples were stored at -80°C and if 260/280 and 260/230 ratios were

between 1.8 to 2, samples were used for reverse transcription and polymerase chain reaction (PCR).

**mRNA and protein isolation using the NucleoSpin RNA/Protein kit**

For Experiment 2.2c, mRNA and protein were isolated using the NucleoSpin RNA/Protein kit (Macherey-Nagel GmbH & Co KG) according to the manufacturer´s protocol. Total mRNA was eluted in 15 µl RNase-free $H_2O$ and stored at -80°C until processed.

The precipitated protein was solved in 40 µl Protein Solving buffer with reduced agent (PSB-TCEP), incubated at 95°C for 3 minutes until completely dissolved and denaturized, cooled down to RT and centrifuged at 11,000 g for 1 minute before stored at -20°C until processed.

**Reverse transcription**

To quantify mRNA levels in the quantitative PCR (qPCR) or PCR Array, total mRNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) using two different protocols.

For the reverse transcription of RNA samples of the amygdala, 500 ng RNA, adjusted to 13 µl total volume with RNase-free $H_2O$, was mixed with 1 µl Random Primers (Invitrogen, Carlsbad, California, USA), 1 µl 10 µM dNTP mix (Invitrogen) and 1 µl RNase-free $H_2O$. After an incubation time of 5 minutes at 65°C (primary annealing), 4 µl 5 x first strand buffer as well as 1 µl 0.1 M DTT and 1 µl RNaseOUT (all Invitrogen) were added, whereof 2 µl per sample were removed as negative control of reverse transcription for genomic DNA contamination of the RNA samples. 1 µl Superscript IV (Invitrogen) was added to the remaining samples and all samples were incubated at 50°C for 60 minutes and 70°C for 15 minutes before diluting the samples 10-fold and storing them at -20°C until analyzed.

For reverse transcription of RNA samples of the septum and vHC the $RT^2$ HT First Strand Kit 96 (#330411; Qiagen, Hilden, Germany) was used according to manufacturer´s protocol using 800 ng RNA. cDNA was stored at -20°C until analyzed.

**Quantification of mRNA expression using qPCR and PCR Array**

qPCR, also called real-time PCR, or a Custom $RT^2$ PCR Array, purchased from Qiagen (#330171; 384-well plates, Qiagen), were used to quantify mRNA expression levels. This was carried out by using the QuantiFast SYBR® Green PCR Kit (Qiagen) or RT2 SYBR Green ROX qPCR Mastermix (Qiagen), respectively, and the QuantStudio 5 Real-time PCR System (Thermo Fischer Scientific) for both. SYBR Green intercalates in double-stranded DNA and emits green light at 522 nm reflecting the amount of double-stranded DNA of the gene of interest.

qPCR was performed using samples of the amygdala as well as when analyzing IL-6 and GILZ mRNA expression in all samples. Custom RT$^2$ PCR Array was performed using samples of the septum and vHC as well as analyzing IL-10 mRNA expression levels in all samples.

For qPCR, 5 µl SYBR Green, 1 µl of the respective forward primer as well as 1 µl of the respective reverse primer of the gene of interest (see Table 1) and 1 µl RNase-free $H_2O$ per sample were added per well of a 384-well plate, while 5 µl SYBR Green, 2 µl forward and 2 µl reverse primer, as well as 5 µl RNase-free $H_2O$ per sample were added per well of a or 96-well plate. Subsequently, 2 µl cDNA of the respective sample was added in duplicates. To exclude contamination, RNase-free $H_2O$ was added as a negative control instead of cDNA. Moreover, the negative controls for reverse transcription were added to exclude contamination of genomic DNA. Detecting only one peak of the melt curves, measured by slowly heating up from 60°C (20 seconds) to 95°C (1 second), while constantly measuring the green fluorescence of the SYBR green, indicated the specificity of the primer and amplification of one specific product. Gene expression was quantified relative to the expression of the housekeeping Ribosomal Protein L13a (RPL) gene. Primer efficiency for each primer pair was determined beforehand by serial dilution of cDNA using the Pfaffl method (Bustin et al., 2009; Pfaffl, 2001). All primers were self-designed using publications or PubMed, except for Arg-1, purchased from Biomol (Biomol GmbH, Hamburg, Germany).

**Table 1:** Primer sequences for the detection and quantification of target genes (mus musculus) via qPCR.

| Gene | Forward | Reverse | Product Length [bp] |
|---|---|---|---|
| Tmem119 | CACCCAGAGCTGGTTCCATA | GTGACACAGAGTAGGCCACC | 100 |
| P2ry12 | AGGGGTTCAGCCAAAGTTCC | TGGCTCAGGGTGTAGGGAAT | 119 |
| Arg-1 | GTGAAGAACCCACGGTCTGT | CTGGTTGTCAGGGGAGTGTT | 209 |
| iNOS | CGAAACGCTTCACTTCCAA | TGAGCCTATATTGCTGTGGCT | 51 |
| CD11b | TCGCTACGTAATTGGGGTGG | TAGATGCGATGGTGTCGAGC | 74 |
| Casp-1 | GCATGCCGTGGAGAGAAACA | GTCACCCTATCAGCAGTGGG | 326 |
| IL-6 | CTACCCCAATTTCCAATGCT | ACCACAGTGAGGAATGTCCA | 187 |
| IL-1β | GCCACCTTTTGACAGTGATGAG | AAGGTCCACGGGAAAGACAC | 219 |
| GILZ | CAGCCTACTCCTTGCTTCAGGGC | TTCATGGTTCGGTTGCCGGGG | 88 |
| NFκB | TTGCAACTATGTGGGGCCT | CTGTCATCCGTGCTTCCAGT | 218 |
| RPL | CACTCTGGAGGAGAAACGGAAGG | GCAGGCATGAGGCAAACAGTC | 182 |

For the Custom RT$^2$ PCR Array (Qiagen), 5 µl SYBR Green, 1 µl cDNA and 4 µl RNase-free $H_2O$ were added in one well of a 384-well plate. For IL-10 mRNA expression level, 96-well plates were used, were 12 µl SYBR Green, 1 µl cDNA and 12 µl RNase-free $H_2O$ were added per well. All plates were pre-coated by the company with the respective primer pair. Gene expression was quantified relative to the expression of the estimated marginal mean of 4 housekeeping genes: TATA-Box Binding Protein, Phosphoglyceratkinase 1, Peptidylprolyl Isomerase H, and Succinat-Dehydrogenase Complex Flavoprotein Subunit A.

All plates were closed using a clear foil and centrifuged for 1 minute at 1000 g. Samples were run (in duplets for qPCR) using the respective protocol below (Table 2).

**Table 2:** Cycler program for qPCR and Custom $RT^2$ PCR Array.

| | qPCR | | | | Custom $RT^2$ PCR Array | | |
|---|---|---|---|---|---|---|---|
| **Step** | **°C** | **time** | **cycles** | **description** | **°C** | **time** | **cycles** |
| 1 | 95°C | 2 min | | denaturation | 95°C | 10 min | |
| 2 | 95°C | 3 sec | x 60 | amplification | 95°C | 15 sec | x 40 |
| | 60°C | 30 sec | | | 60°C | 1 min | |
| | 72°C | 1 min | | | | | |
| 3 | 72°C | 10 min | | elongation | 72°C | 10 min | |
| 4 | 4°C | ∞ | | cooling | 4°C | ∞ | |

**Protein quantification**

To quantify the amount of protein in the respective brain regions, the frozen protein samples dissolved in PBS-TCEB were processed according to manufacturer´s protocol using the protein quantification assay (#740967; Macherey-Nagel GmbH & Co KG). Therefore, a BSA dilution series (1.0, 0.75, 0.5, 0.3, 0.2, 0 µg / µl) was prepared and 20 µl per well pipetted in a 96-well plate. After adding 5 µl of the protein sample, PSB was added to a final volume of 60 µl each, and 40 µl Quantification Reagent was supplied before incubating the plate for 30 minutes at RT while gently shaking. Using the plate reader FLUOstar Optima (BMG Labtech) absorption was photometrically measured at 570 nm.

**Semi-quantitative analysis of protein levels using western blot (WB)**

20-30 µg protein were loaded onto Criterion™ TGX Strain-Free™ Precast or Mini PROTEAN TGX Strain-Free™ gels (all Bio-Rad, München, Germany) for electrophoretic separation at 140 V (1.5 – 2 hours). A pre-stained protein ladder (Fermentas Inc., Glen Bumie, USA) was used as a marker to measure protein size (kilodalton; kDa). After gel electrophoresis, the gels were activated with 2.5 minutes ultraviolet light exposure using the ChemiDoc XRS+ System (Bio-Rad). To transfer the proteins from the gel onto a nitrocellulose membrane, Trans-Blot@ Turbo™ Midi or Mini Nitrocellulose Transfer Packs (all Bio-Rad) were used. For better detection of the small proteins, the gels were cut into halves at 35 kDa (measured based on the visible protein ladder). The gels and the membranes (sizes adjusted to the cut gels) were stacked between layers of blotting paper and placed into the Semi-dry Trans-Blot® Turbo™ Transfer System (Bio-Rad). The upper parts of the gels, containing the larger proteins (>35 kDa), were blotted for 30 minutes (25 V, 1 A), the lower parts, containing the smaller proteins (<35 kDa), were blotted for 3-5 minutes (25 V, 2.5 A). After blotting and measuring total protein using the ChemiDoc XRS+ System, membranes were blocked for 1-2 hours (see Table 3) in blocking solution (5 % milk powder, MP, or 5 % BSA) at RT. Subsequently, membranes were washed 3 times for 5 minutes with Tris-buffered saline

containing 0.1 % Tween-20 (TBS-T, pH 7.6) and incubated with the primary antibody at 4°C overnight or for 2 days (see Table 3), while gently shaking. Thereafter, membranes were again washed 3 times in TBS-T for 5 minutes and incubated for 2 hours (RT, gently shaking) with the secondary antibody (Goat Anti-Rabbit #7074 or Goat Anti-Rat #7077, Cell Signaling, diluted in TBS-T, see Table 3), before the membranes were washed 3 times 5 minutes each. Protein bands were detected via chemiluminescent reaction with ECL western blot reagents (GE Healthcare, UK) or Super Signal West Dura Extended Duration Substrate (for IL-6 and IL-4, Thermo Fisher Scientific). Images were obtained with the ChemiDoc XRS+ System (Bio-Rad).

Images were analyzed using the Image Lab software (Bio-Rad) and abundance of the target protein was normalized to total protein of the lane. For the detection of additional proteins, primary and secondary antibodies were removed by incubating the membrane in Restore Western Blot Stripping Buffer (#21059; Thermo Fisher Scientific) for 15 minutes at RT. After washing 3 times 5 minutes in TBS-T, the membrane was blocked in blocking solution for 1 hour or 30 minutes (half of the respective first blocking time; see Table 3) and the protocol for another protein of interest was conducted.

**Table 3:** List of antibodies and protocols used in Western blot.

| Protein | Company | Size | Blocking | Primary Antibody | Secondary Antibody |
|---|---|---|---|---|---|
| Tmem119 | Proteintech #27585 | 45 kDa | 2 h / 1 h in 5 % MP | 1:1000 in 5 % MP for 1 day | Goat Anti-Rabbit 1:5000 |
| NFκB | Cell Signaling #8242 | 65 kDa | 2 h / 1 h in 5 % MP | 1:2000 in 5 % BSA for 1 day | Goat Anti-Rabbit 1:5000 |
| pNFκB (Ser276) | Absci #AB11011 | 65 kDa | 2 h / 1 h in 5 % MP | 1:2000 in 5 % MP for 1 day | Goat Anti-Rabbit 1:5000 |
| GILZ | NovusBio #NBP2-41210 | 23 kDa | 1 h / 30 min in 5 % BSA | 1:500 in 5 % MP for 1 day | Goat Anti-Rabbit 1:5000 |
| IL-6 | Cell Signaling #12912 | 24 kDa | 2 h / 1 h in 5 % MP | 1:300 in 5 % BSA for 2 days | Goat Anti-Rabbit 1:2000 |
| IL-4 | R&D Systems #AF-404 | 18 kDa | 1 h / 30 min in 5 % BSA | 1 µg / ml in 5 % BSA for 2 days | Goat Anti-Rat 1:3000 |

## 2.13 Statistics and figures

Behavioral, physiological and immunological data were analyzed using SPSS (IBM SPSS Statistics 26). All data were depicted using GraphPad Prism (Version 8, GraphPad Software), while all illustrations were created using BioRender.com.

For data analysis, independent T-Test if normally distributed, Mann-Whitney-U Test if not normally distributed, has been performed when comparting one factor for two groups (factor CSC, factor CUS, or factor strain in anxiety- and depressive-like behavior, physiological stress parameters, immune parameters, SFC acquisition, CFC retention, or stress coping behavior). One-way analysis of variance (ANOVA) has been performed when comparing one factor for three or more groups (factor SFC in delta cell viability, as well as in immune and physiological stress parameters). One-way ANOVA for repeated measures (RM) has been performed when comparing one factor for three or more time points (factor CSC, factor CUS, or factor strain in body weight, SPT, mSDT, or CFC acquisition and extinction). Two-way ANOVA has been performed when comparing two factors for two or more groups (factors CSC x SFC, or factors CUS x SFC in physiological stress parameters and in immune parameters). Two-way ANOVA for RM has been performed when comparing two factors for two or more time points (factors CSC x SFC x time, factors CUS x SFC x time, factors strain x SFC x time, or factors OXT x SFC x time in SFC extinction and recall). Three-way ANOVA has been performed when comparing three factors for two or more groups (factors CSC x SFC x ACTH in adrenal stimulation).

All tests were followed by a Bonferroni *post hoc* test (except for Experiment 2.2b, where LSD *post hoc* analysis has been performed), when appropriate, i.e., if ANOVA revealed significant differences for all factors that have been analyzed, except if mentioned otherwise. Values of the *post hoc* tests are given in the text, while statistical values of the T-Test, Mann-Whitney-U Test and ANOVAs are given in separate tables. Statistical significance was accepted at $p \leq 0.05$, a trend was accepted at $p \leq 0.07$. Values were specified in three panels and all data represent the mean +/± standard error of the mean (SEM). Statistical outliers were calculated with the formula “mean ± 2 x standard deviation”.

## 2.14 Experimental design

To investigate the effects of chronic psychosocial stress on the development of a PTSD- and SAD-like phenotype, as well as underlying behavioral and immunological changes, specificity of the fear conditioning and chronic stressor paradigm, as well as possible treatment options, the following experiments were performed. Trunk blood as well as brains and organs were collected, weighted and / or stimulated.

### Experiment 1: Effects of CSC exposure on SFC behavior

To investigate the effects of chronic psychosocial stress on subsequent SFC, mice were exposed to 19 days of CSC. On day 19, mice were tested for anxiety-like behavior in the OF test and NOR, or in the LDB, and after 2 to 3 days of habituation, mice were tested in the SFC paradigm to induce social fear (Figure 8). Due to the high animal numbers, the SFC paradigm was performed in two groups with a shift of one day in between. Therefore, group 1 started acquisition after 2 days of habituation (day 21), while group 2 started acquisition after 3 days of habituation (day 22).

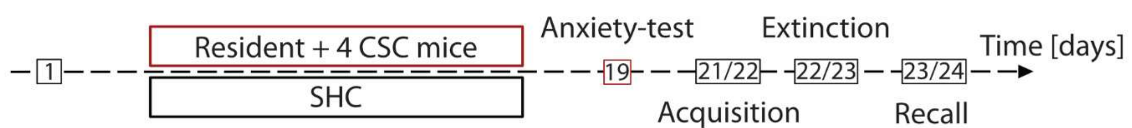



**Figure 8: Schematic timeline of Experiment 1.** Mice were either exposed to the chronic subordinate colony housing (CSC) paradigm or kept as single housed controls (SHC), before they were tested for anxiety-like behavior on day 19, social fear conditioning (SFC) acquisition on day 21 (group 1) or day 22 (group 2) and, subsequently, to SFC extinction (day 22/23) and recall (day 23/24).

### Experiment 2: Effects of CSC exposure on social behavior and the immune system

To determine behavioral and immunological changes underlying the impact of CSC exposure on SFC, mice were tested for social behavior after CSC exposure as well as for changes of the peripheral and central immune system.

**Experiment 2.1: Effects of CSC on social preference and social discrimination**

To test the impact of chronic psychosocial stress exposure on social behavior including social preference and social memory, mice were exposed to the CSC procedure and subsequently tested for anxiety-like behavior in the LDB on day 19. After 2 days of habituation, mice were tested for social preference in the SPT and subsequently for short- (2 hours after SPT) and long-term (24 hours after short-term mSDT) social memory in the mSDT (Figure 9).

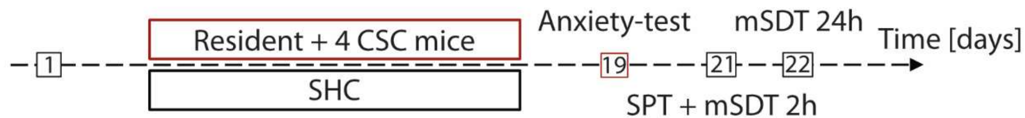



**Figure 9: Schematic timeline of Experiment 2.1.** Mice were either exposed to the chronic subordinate colony housing (CSC) paradigm or kept as single housed controls (SHC), before were tested for anxiety-like behavior on day 19. To analyze social behavior, mice were exposed to the social preference test (SPT) and to the modified social discrimination test (mSDT) for short-term social memory (2 hours after SPT) on day 21 and 24 hours later in the mSDT for long-term social memory on day 22.

**Experiment 2.2: Effects of CSC and SFC exposure on the immune system**

To unravel the impact of chronic psychosocial stress and SFC on the peripheral and central immune system, mice were exposed to either the CSC paradigm (Experiment 2.2a), SFC acquisition (Experiment 2.2b), or the combination of both. Here, CSC mice and SHC were tested for anxiety-like behavior on the EPM after CSC exposure (days 19) and following a habituation time of 2 to 3 days (see Experiment 1), exposed to SFC acquisition (Experiment 2.2c). For all experiment, brains were removed 2 hours after stressor termination.

### Experiment 3: Effects of CSC exposure on CFC and CUS exposure on non-social vs social fear conditioning

To determine the specificity of the effects of CSC expose on SFC, depending on the fear conditioning model, nature and composition of the chronic stressor paradigm and mouse strain, mice were firstly exposed to CSC followed by non-social fear conditioning, i.e., CFC (Experiment 3.1). Next, mice were exposed to CUS followed by CFC or SFC (Experiment 3.2), and BL6 and CD1 were tested for their innate anxiety- and fear-related behavior, social behavior, and stress coping behavior during CSC exposure. Additionally, I exposed CD1 mice to the CSC paradigm, followed by SFC (Experiment 3.3).

**Experiment 3.1: Effects of CSC exposure on CFC**

To investigate the effects of chronic psychosocial stress on non-social fear conditioning, mice were exposed to 19 days of CSC. On day 19, mice were tested for anxiety-like behavior in the LDB and, after 2 to 3 days of habituation, tested in the CFC paradigm to induce non-social fear (see Figure 8).

**Experiment 3.2: Effects of CUS exposure on CFC and SFC**

To investigate the importance of the nature and composition of the chronic stressor paradigm on non-social versus social fear expression and extinction, the CUS model was established in our laboratory. To verify the induction of robust chronic non-social stress following CUS exposure, mice were tested subsequently for anxiety-like behavior in the OF test and NOR on day 19, for

depressive-like behavior using the Splash test on day 20, and for social behavior using the SPT on day 21. Moreover, I investigated the effects of CUS exposure on physiological stress parameters 3 days after stressor termination. In a second set of animals, I analyzed short-term changes of CUS exposure primarily on physiology. Here, CUS mice and SHC were tested for anxiety-like behavior in the LDB followed by organ collection on day 19 (Experiment 3.2a). After a successful validation of the CUS model, another batch of mice were exposed to the CUS paradigm, tested for anxiety-like behavior on the EPM on day 19, and after 2 days of habituation exposed to the CFC (Experiment 3.2b) or SFC paradigm (Experiment 3.2c; Figure 10).

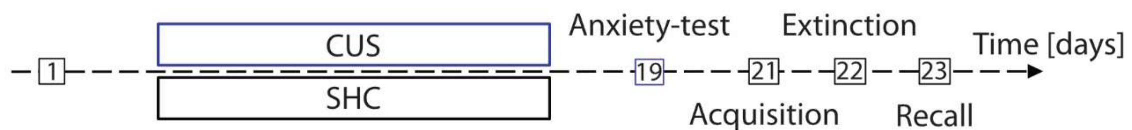



**Figure 10: Schematic timeline of Experiment 3.** Mice were either exposed to the chronic unpredictable stress (CUS) paradigm or kept as single housed controls (SHC), before they were tested for anxiety-like behavior on day 19 and cued or social fear conditioning on days 21 to 23.

**Experiment 3.3: Strain differences in behavior, fear- and stress-response**

As the CSC paradigm has originally been established in BL6 mice and SFC in CD1 mice, I further investigated strain differences in anxiety- and fear-related behaviors, as well as in their stress response during CSC exposure. Thus, CD1 and BL6 mice were tested for anxiety-like behavior in the EPM and three days later in the LDB. After 3 days of habituation they were tested for non-social fear in the CFC paradigm (Experiment 3.3a). Secondly, I analyzed social behavior in the SPT and after 2 days of habituation, I exposed them to SFC (Experiment 3.3b). For SPT and SFC same treated animals from separate cohorts, performed with Dr. Melanie Royer (Molecular and Behavioural Neurobiology, University of Regensburg, Germany) were added.

Lastly, I evaluated whether CSC exposure has the same effect on SFC in the less anxious and fear-susceptible CD1 mice, compared to the stress-vulnerable BL6 mice. Therefore, like during Experiment 1, CD1 mice were exposed to 19 days of CSC. On day 19, mice were tested for anxiety-like behavior and after 2 to 3 days of habituation, mice were tested in the SFC paradigm to induce social fear (Experiment 3.3c; see Figure 8).

### Experiment 4: Effects of acute OXT on social fear extinction behavior

Due to its stress-buffering, pro-social and anti-inflammatory effects, OXT has been tested as a potential treatment option to potentially improve extinction success in the generally stress-vulnerable BL6 mice. Moreover, I aimed to proof whether acute icv OXT treatment can rescue or counteract the negative effects of chronic psychosocial stress on SFC in BL6 and CD1 mice.

**Experiment 4.1: Effects of acute OXT on SFC extinction training in BL6 mice**

Therefore, I first investigated whether acute icv OXT treatment 10 minutes prior to SFC extinction was able to improve extinction success in fear- and stress-susceptible BL6 mice. Therefore, BL6 mice underwent surgery and a cannula was implanted icv. After 10 days of recovery, during which mice were handled daily, they underwent the SFC paradigm. 10 minutes prior to extinction training, 0.1 µg / 2 µl OXT or VEH was infused icv (Experiment 4.1).

**Experiment 4.2 Effects of acute OXT on SFC extinction following CSC exposure**

To further investigate a potential stress-buffering effect of OXT following chronic psychosocial stress, BL6 (Experiment 4.2a) and CD1 (Experiment 4.2b) mice were exposed to 19 days of CSC. On day 19, a subset of mice was tested for anxiety-like behavior on the EPM and all mice underwent icv surgery. After 4 days of recovery with daily handling (BL6 mice were handled twice a day), mice were tested in the SFC paradigm (day 23 to day 25). 10 minutes prior to extinction training (day 24), mice received icv infusion of either VEH or OXT (0.1 µg / 2 µl; see Figure 11).

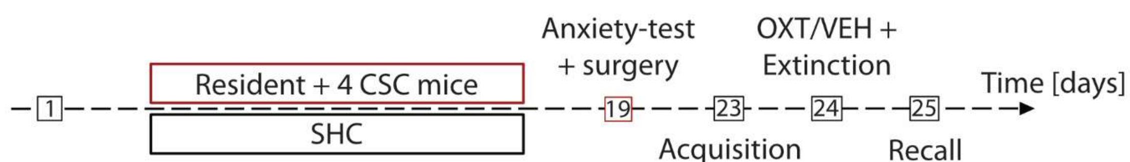



**Figure 11: Schematic timeline of Experiment 4.** Mice were either exposed to the chronic subordinate colony housing (CSC) paradigm or kept as single housed controls (SHC), before they were tested for anxiety-like behavior on the elevated plusmaze (EPM) and subsequently underwent surgery for cannula implantation on day 19. After 4 days of habitation and daily handling, mice were exposed to social fear conditioning (SFC) acquisition on day 23 and extinction and recall on days 24 and 25, respectively. 10 minutes prior to extinction, mice received either oxytocin (OXT) or vehicle (VEH) via cannula infusion.

# Results

# 3 Results

For all experiments using chronic stress models, the parameters for the validation of successful induction of chronic stress in CSC or CUS mice are presented only in statistical tables (see Appendix), without additional graphical illustration. Following stress parameters were analyzed: anxiety-like behavior, body weight, adrenal weight, thymus weight, spleen weight, plasma ACTH and CORT levels, *in vitro* adrenal ACTH responsiveness, number of mesLNCs, cytokine production by mesLNCs, and spleen cell viability.

## 3.1 Effects of CSC exposure on SFC behavior

To investigate the effects of chronic psychosocial stress on social fear acquisition and extinction, male mice were exposed to the CSC housing and, subsequently, tested in the SFC paradigm. Firstly, stress parameter including anxiety-like behavior, body weight, and other physiological parameters were analyzed to validate successful induction of chronic stress in CSC mice (see Table A1). Anxiety-like behavior was tested directly after CSC exposure in the OF and NOR, or - in a separate cohort- in the LDB. In the OF, CSC mice did not differ from SHC in the time spent in the inner zone of the box, indicating no difference in anxiety-related behavior, but they had reduced locomotion. In contrast, in the NOR, CSC mice displayed less object sniffing compared to SHC indicating increased anxiety-related behavior. In the LDB, CSC mice spent similar time in the LB, but had a reduced latency to enter it compared to SHC, indicating rather reduced anxiety levels. Moreover, body weight gain over the 19 days of CSC did not differ between CSC mice and SHC, whereas it was more pronounced in CSC mice over 4 to 5 days after stressor termination, i.e., on the day of SFC recall compared to SHC (post hoc analysis: $p < 0.001$). With respect to physiological parameters assessed after termination of the experiment, i.e., after exposure to SFC, I found an increased relative adrenal weight in stressed unconditioned, i.e., CSC/SFC$^{-}$ ($p < 0.050$) and conditioned, i.e., CSC/SFC$^{+}$ ($p < 0.050$) compared to unstressed unconditioned, i.e., SHC/SFC$^{-}$ and conditioned, i.e., SHC/SFC$^{+}$, mice, respectively, while SFC itself did not affect adrenal weight. In addition, relative spleen weight as well as IFN-γ production of the mesLNCs were increased in CSC mice compared to SHC, which was independent of conditioning, whereas relative thymus weight was not affected by CSC or SFC exposure. Moreover, plasma ACTH concentrations in blood sampled after SFC recall were also not affected by CSC or SFC exposure, while plasma CORT levels were reduced in CSC mice compared to SHC in dependence on conditioning. In detail, CSC/SFC$^{-}$ and CSC/SFC$^{+}$ showed reduced CORT levels compared to respective SHC ($p < 0.050$), while plasma

CORT level seemed to be lower in $CSC/SFC^+$ than in $CSC/SFC^-$, however, this did not reach significance ($p = 0.091$). *In vitro* stimulation of the adrenal glands with ACTH resulted in increased CORT release in all groups, which was, however, less pronounced in all CSC mice compared to SHC independent of conditioning. In sum, these data indicate a successful induction of chronic stress in CSC mice.

After validation of CSC effects on major stress parameters, I analyzed the effects of CSC exposure on behavioral responses in the SFC paradigm (for statistics see Table 4). I could find that CSC exposure prior to SFC affected both social fear acquisition as well as extinction. Thus, during acquisition, less shocks were needed in CSC mice compared to SHC to induce social avoidance behavior (Figure 12A). During social fear extinction, $SHC/SFC^+$ showed reduced social investigation at the beginning of extinction training (social stimuli 1 to 3: $p \leq 0.001$; social stimulus 4: $p = 0.053$), but equal social investigation at the last two social stimuli compared to $SHC/SFC^-$. These data indicate a successful induction of social fear, which was extinguished along stimuli presentations in SHC. Importantly, prior CSC exposure significantly impaired social fear extinction, reflected by reduced social investigation along all social stimuli ($p < 0.05$) in $CSC/SFC^+$ compared to $CSC/SFC^-$ and of the second ($p = 0.064$), fourth ($p = 0.053$) and sixth ($p < 0.050$) social stimulus compared to $SHC/SFC^+$. Interestingly, $CSC/SFC^-$ showed reduced social investigation at the first social stimulus ($p < 0.050$) compared to $SHC/SFC^-$, reflecting reduced social preference behavior following CSC exposure (Figure 12B). Moreover, during extinction training, $CSC/SFC^+$ showed less investigation time of the first ($p < 0.010$) and third ($p < 0.050$) non-social stimulus compared to $SHC/SFC^+$ as well as at the third non-social stimulus compared to $CSC/SFC^-$, indicating increased anxiety-related behavior in $CSC/SFC^+$ compared to $SHC/SFC^+$ and $CSC/SFC^-$. Furthermore, during recall $SHC/SFC^+$ showed reduced investigation time at the first social stimulus ($p < 0.050$) compared to $SHC/SFC^-$, whereas $CSC/SFC^+$ showed reduced investigation at social stimuli 1 to 3 compared to $CSC/SFC^-$ ($p < 0.050$) and towards social stimuli 1 to 4 compared to $SHC/SFC^+$ ($p < 0.050$; Figure 12C).

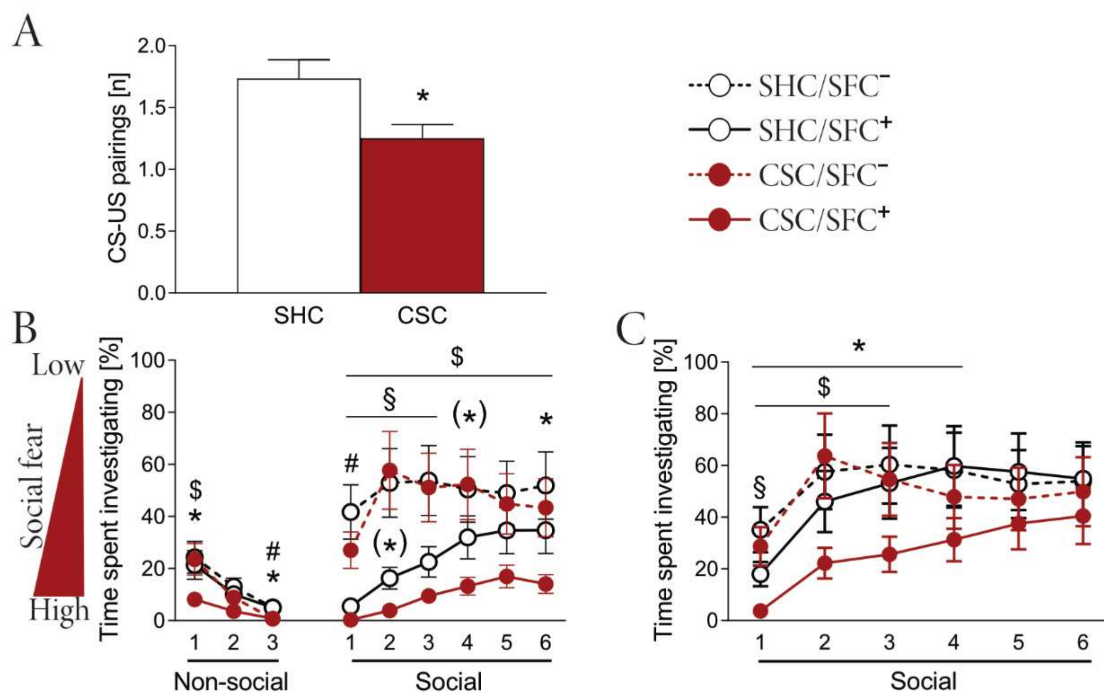



**Figure 12: Chronic subordinate colony housing (CSC) exposure facilitated social fear conditioning (SFC) acquisition and impaired its extinction.** CSC mice needed a lower number of shocks during acquisition to induce social avoidance behavior compared to single housed controls (SHC; A). Moreover, CSC/SFC⁺ showed reduced social investigation compared to CSC/SFC⁻ and SHC/SFC⁺ during extinction (B) and recall (C). Data represent mean +/± SEM. (*) p = 0.064 for s2 and p = 0.053 for s4, * p ≤ 0.050 CSC/SFC⁺ vs SHC/SFC⁺; # p ≤ 0.050 CSC/SFC⁻ vs SHC/SFC⁻; $ p ≤ 0.050 CSC/SFC⁺ vs CSC/SFC⁻; § p ≤ 0.05 SHC/SFC⁺ vs SHC/SFC⁻; n = 15-16 per group; for detailed statistics see Table 4.

**Table 4: Statistics of the effects of CSC exposure on SFC (Experiment 1).** Factor time represents stimulus presentations during SFC extinction and recall; factor CSC represents SHC vs CSC effects; factor SFC represents SFC⁻ vs SFC⁺ effects.

| Acquisition | Independent T-Test | Figure 12A |
|---|---|---|
| CS-US pairings | (CSC) $T_{29} = 2.57$ | P = 0.016 |
| **Extinction** | **Two-way ANOVA for RM** | **Figure 12B** |
| Time spent investigating | (Time) $F_{8,456} = 35.5$ | P < 0.001 |
| | (CSC) $F_{1,57} = 5.80$ | P = 0.019 |
| | (SFC) $F_{1,57} = 44.5$ | P < 0.001 |
| | (Time x CSC) $F_{8,456} = 0.625$ | P = 0.757 |
| | (Time x SFC) $F_{8,456} = 12.1$ | P < 0.001 |
| | (CSC x SFC) $F_{1,57} = 1.75$ | P = 0.192 |
| | (Time x CSC x SFC) $F_{8,456} = 1.18$ | P = 0.312 |
| **Recall** | **Two-way ANOVA for RM** | **Figure 12C** |
| Time spent investigating | (Time) $F_{5,275} = 26.9$ | P < 0.001 |
| | (CSC) $F_{1,55} = 6.29$ | P = 0.015 |
| | (SFC) $F_{1,55} = 5.36$ | P = 0.024 |
| | (Time x CSC) $F_{5,275} = 0.934$ | P = 0.459 |
| | (Time x SFC) $F_{5,275} = 4.66$ | P < 0.001 |
| | (CSC x SFC) $F_{1,55} = 2.05$ | P = 0.157 |
| | (Time x CSC x SFC) $F_{5,275} = 0.888$ | P = 0.447 |

## 3.2 Effects of CSC exposure on social behavior and the immune system

Secondly, I investigated behavioral and immunological changes underlying the observed impact of CSC exposure on SFC, including social behavior and changes of the immune system 2 hours after CSC exposure, SFC acquisition or a combination of both.

### 3.2.1 Effects of CSC on social preference and social discrimination

To investigate the effects of chronic psychosocial stress on social behavior, mice were exposed to the CSC paradigm before they were tested in the SPT and mSDT. In this experiment, the effects of CSC exposure (see 3.1) could be confirmed in the following parameters: increased body weight gain, relative spleen weight and by trend relative adrenal weight (see Table A2). Additionally, *in vitro* spleen cell stimulation showed increased cell viability following LPS stimulation compared to basal conditions (SHC: $p < 0.050$, CSC: $p < 0.001$). Interestingly, cell viability was increased in CSC mice compared to SHC by trend under basal conditions ($p = 0.055$), as well as significantly after LPS stimulation ($p < 0.001$), and when comparing delta cell viability. Following cell treatment with ascending CORT concentrations, delta cell viability was increased in CSC mice compared to SHC, as SHC showed a dose-dependent decline, which was absent in CSC mice, indicating splenic GC resistance in CSC mice. These data confirm again a successful induction of chronic stress in CSC mice.

For the analysis of the effects of chronic psychosocial stress on social behavior (for statistics see Table 5), mice were tested in the SPT 2 days following CSC exposure. Here, both CSC mice and SHC showed preference towards the conspecific, thus investigating the social stimulus more than the non-social stimulus (Figure 13A). Moreover, in the mSDT 2 hours after SPT, all mice investigated the novel social stimulus more than the known conspecific demonstrating short-term social memory abilities in both CSC mice and SHC (Figure 13B). However, 24 hours after presentation of the known conspecific, CSC mice did not show any preference whereas SHC showed reduced investigation towards the novel compared to the known conspecific ($p < 0.050$; Figure 13C). These data indicate that neither CSC mice nor SHC can remember the known social stimulus after 24 hours.

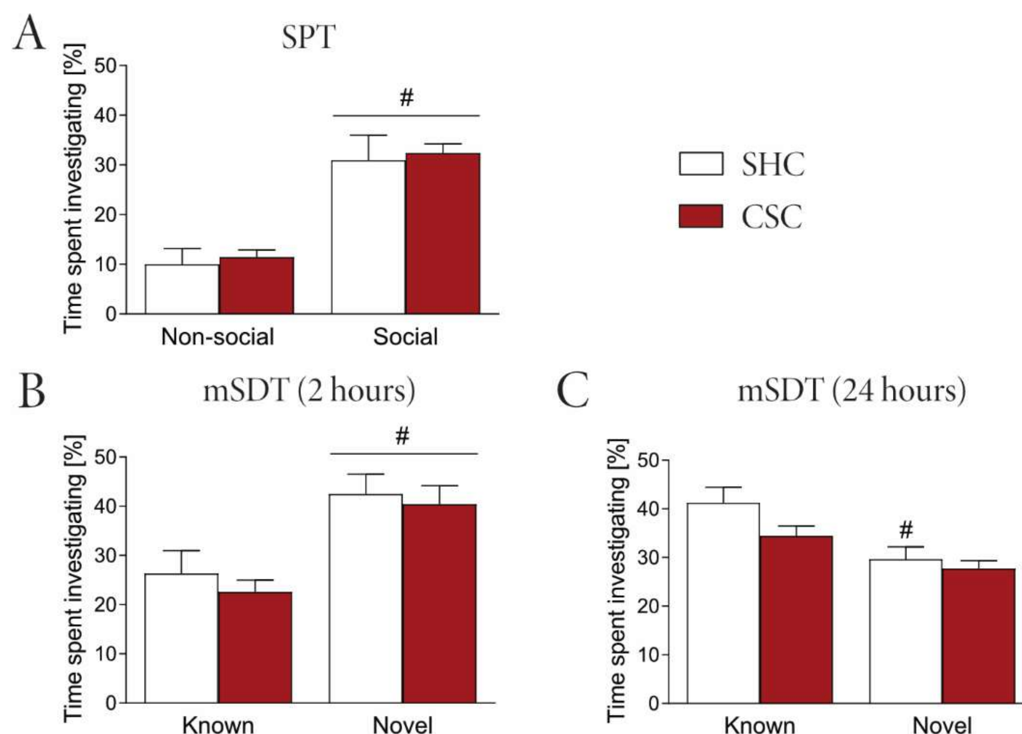



**Figure 13: Chronic subordinate colony housing (CSC) exposure did not affect social preference or social discrimination abilities.** In the social preference test (SPT), CSC mice and single housed controls (SHC) showed increased investigation of the social over the non-social stimulus (A) and in the modified social discrimination test (mSDT), increased investigation of the novel over the known social stimulus 2-hour after investigation of the known stimulus for the first time (B). However, 24-hour after presentation of the known conspecific, long-term social memory, measured in the mSDT, was absent in both CSC mice and SHC (C). Data represent mean + SEM. # $p \leq 0.050$ social vs non-social stimulus and novel vs known stimulus; n = 7-8 per group; for detailed statistics see Table 5.

**Table 5: Statistics of the effects of CSC exposure on social preference and social discrimination (Experiment 2.1).** Factor time represents stimulus presentations during SPT and mSDT; factor CSC represents SHC vs CSC effects.

| SPT | One-way ANOVA for RM | Figure 13A |
|---|---|---|
| Time spent investigating | (Time) $F_{1,13} = 48.8$<br>(CSC) $F_{1,13} = 0.146$<br>(Time x CSC) $F_{1,13} = 0.000$ | $P < 0.001$<br>$P = 0.708$<br>$P = 0.998$ |
| **mSDT (2 hours)** | **One-way ANOVA for RM** | **Figure 13B** |
| Time spent investigating | (Time) $F_{1,14} = 11.3$<br>(CSC) $F_{1,14} = 2.35$<br>(Time x CSC) $F_{1,14} = 0.028$ | $P = 0.005$<br>$P = 0.148$<br>$P = 0.871$ |
| **mSDT (24 hours)** | **One-way ANOVA for RM** | **Figure 13C** |
| Time spent investigating | (Time) $F_{1,14} = 10.8$<br>(CSC) $F_{1,14} = 4.62$<br>(Time x CSC) $F_{1,14} = 0.774$ | $P = 0.005$<br>$P = 0.050$<br>$P = 0.394$ |

### 3.2.2 Effects of CSC and SFC exposure on the immune system

To investigate whether a dysregulation of the immune system might underlie the effects of CSC exposure on SFC, mice were subjected to CSC or SFC acquisition or a combination of both. 2 hours after stressor exposure, parameters of the peripheral immune system, e.g. spleen cell viability, plasma cytokine level, and IFN-y production of the mesLNCs, as well as of the central immune system, e.g. expression of microglia marker and cytokines, were analyzed. To reveal changes of the central immune system, three brain regions, i.e., amygdala, septum and vHC, were selected based on their fundamental role in stress, emotion, fear, social behavior, and SFC as well as in the development of stress-related psychiatric disorders including anxiety, PTSD and SAD.

**Effects of CSC exposure on the peripheral and central immune system**

Firstly, immunological changes following CSC exposure were analyzed. In this experiment, the successful induction of chronic stress in CSC mice was reflected by increased relative adrenal and spleen weight, reduced thymus weight and elevated *in vitro* adrenal CORT response to ACTH compared to SHC (see Table A3).

To unravel the effects of CSC exposure on the peripheral immune system (for statistics see Table 6), I analyzed a known stress and immune parameter (see 3.2.1) as a representative of the peripheral immune status, i.e., *in vitro* cell viability of spleen cells. Here, cell viability was increased in both CSC mice and SHC after LPS stimulation compared to basal levels ($p < 0.010$). However, the cell viability was higher in CSC mice compared to SHC both under basal conditions ($p < 0.050$) and following LPS stimulation ($p < 0.001$; Figure 14A). Increased cell viability of spleen cells isolated from CSC mice was further reflected by respective delta cell viability (Figure 14B). In line, following cell treatment with ascending CORT concentrations, delta cell viability (Figure 14C) as well as percent delta cell viability (Figure 14D) were higher in CSC mice compared to SHC.

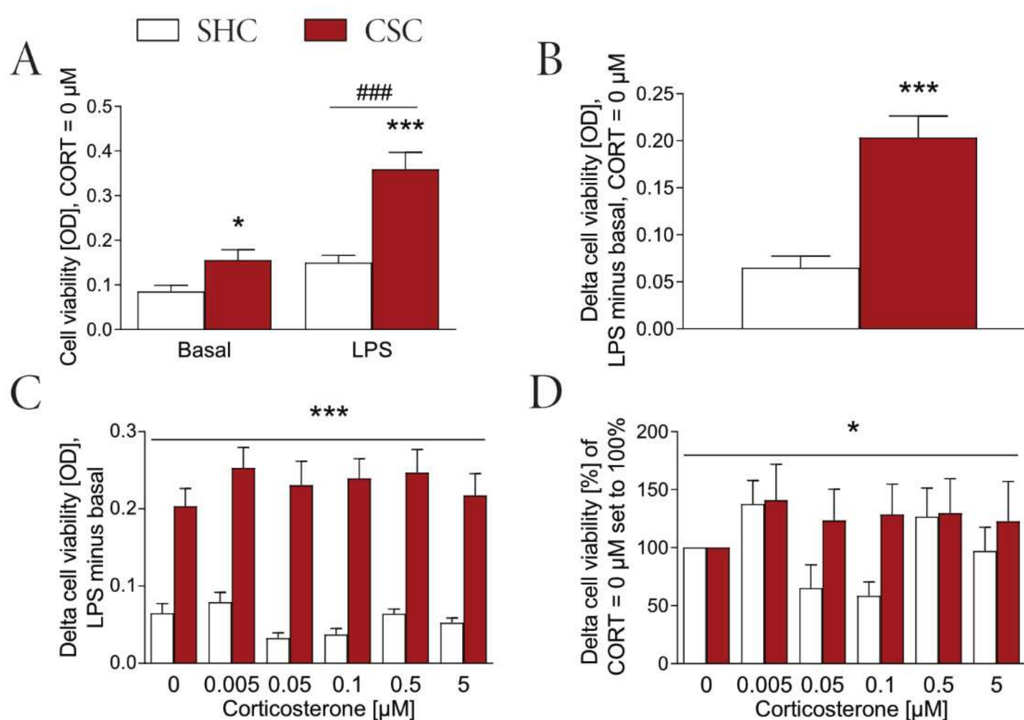



**Figure 14: Chronic subordinate colony housing (CSC) exposure increased splenocyte cell viability.** CSC mice showed increased cell viability of the splenocytes at basal conditions and after lipopolysaccharide (LPS) stimulation (A), as well as when analyzing delta cell viability (B), compared to single housed controls (SHC). The same effect was observed after corticosterone (CORT) stimulation with ascending concentrations (C - D). Data represent mean + SEM. * $p \leq 0.050$, *** $p \leq 0.001$ CSC vs SHC; ### $p \leq 0.001$ LPS vs basal stimulation; n = 7-8 per group; for detailed statistics see Table 6.

**Table 6: Statistics of the effects of CSC exposure on the peripheral immune system (Experiment 2.2a).** Factor LPS represents basal vs LPS effects; factor CORT represents ascending CORT concentrations; factor CSC represents SHC vs CSC effects.

| Spleen cell stimulation | Two-way ANOVA, Independent T-Test (delta CORT = 0 µM) | Figure 14 |
|---|---|---|
| Cell viability CORT = 0 µM | (LPS) $F_{1,28} = 29.3$<br>(CSC) $F_{1,28} = 31.7$<br>(LPS x CSC) $F_{1,28} = 7.81$ | $P < 0.001$<br>$P < 0.001$<br>$P = 0.009$ |
| Delta cell viability CORT = 0 µM | (CSC) $T_{14} = -5.27$ | $P < 0.001$ |
| Delta cell viability | (CORT) $F_{5,83} = 0.953$<br>(CSC) $F_{5,83} = 0.636$<br>(CORT x CSC) $F_{5,50} = 1.43$ | $P = 0.451$<br>$P < 0.001$<br>$P = 0.672$ |
| Delta cell viability CORT = 0 µM set to 100 % | (CORT) $F_{5,83} = 1.35$<br>(CSC) $F_{5,83} = 3.95$<br>(CORT x CSC) $F_{5,83} = 0.883$ | $P = 0.251$<br>$P = 0.050$<br>$P = 0.496$ |

For the analysis of central immune parameters, the expression levels of relevant immune factors were assessed and compared between CSC mice and SHC (for statistics see Table 7). In the amygdala, I found a reduced expression of microglia marker including the resting-state marker P2ry12, iNOS a marker for pro-inflammatory microglia, and the adhesion protein CD11b, a marker

for macrophages and more active microglia. Moreover, I found an increased expression of GILZ, an early transcriptional gene of GCs, mediating its immunosuppressive and anti-inflammatory properties (Figure 15A). Furthermore, in the septum the inflammasome Nlrp3 was reduced, while GILZ was again increased (Figure 15B), whereas in the vHC, mRNA expression of Tmem119, a specific microglia marker, was increased (Figure 15C) in CSC mice compared to SHC. However, among the cytokines, neither pro-inflammatory, i.e., IL-6 and IL-1β, nor anti-inflammatory cytokines, i.e., IL-4, were changed in any of these brain regions. These data indicate rather reduced inflammation in the amygdala and septum, caused by reduced microglia and increased GILZ expression. In contrast, in the vHC, which is highly involved in anxiety, increased number of microglia might promote stress-induced anxiety-like behavior in CSC mice, however, microglia phenotypes seemed not affected.

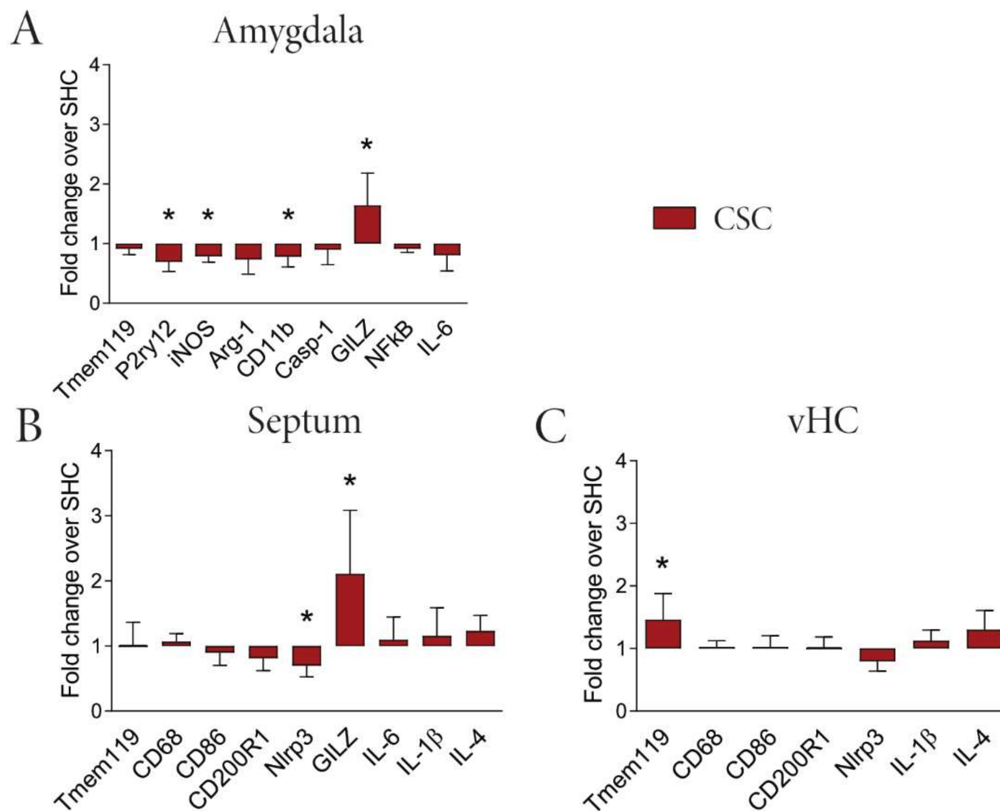



**Figure 15: Chronic subordinate colony housing (CSC) exposure differentially affected immune parameters in the brain.** CSC mice showed reduced mRNA of genes associated with microglia and other immune cells but increased glucocorticoid-induced leucine zipper (GILZ) in the amygdala (A), reduced inflammasome and increased GILZ expression in the septum (B), and increased microglia number in the ventral hippocampus (vHC; C), compared to single housed controls (SHC). Data represent mean + SEM. Group values were adapted to SHC (value 1). * $p \leq 0.050$ CSC vs SHC; n = 5-7 per group; for detailed statistics see Table 7.

**Table 7: Statistics of the effects of CSC exposure on the central immune system (Experiment 2.2a).** Factor represents SHC vs CSC effects.

| Amygdala | Independent T-Test, Mann-Whitney-U Test (Casp-1, GILZ) | Figure 15A |
|---|---|---|
| Tmem119 | $T_7 = 1.09$ | $P = 0.310$ |
| P2ry12 | $T_8 = 2.68$ | $P = 0.028$ |
| iNOS | $T_9 = 2.38$ | $P = 0.041$ |
| Arg-1 | $T_8 = 0.954$ | $P = 0.368$ |
| CD11b | $T_{10} = 2.45$ | $P = 0.034$ |
| Casp-1 | $T_{5.06} = 0.307$ | $P = 0.771$ |
| GILZ | $T_{6.73} = -3.06$ | $P = 0.019$ |
| NFκB | $T_7 = 1.04$ | $P = 0.334$ |
| IL-6 | $T_9 = 1.23$ | $P = 0.250$ |
| **Septum** | **Independent T-Test, Mann-Whitney-U Test (GILZ)** | **Figure 15B** |
| Tmem119 | $T_9 = -0.100$ | $P = 0.992$ |
| CD68 | $T_{10} = -0.869$ | $P = 0.405$ |
| CD86 | $T_{10} = 0.874$ | $P = 0.402$ |
| CD200R1 | $T_{10} = 1.22$ | $P = 0.252$ |
| Nlrp3 | $T_{10} = 2.89$ | $P = 0.016$ |
| GILZ | $T_{5.44} = -2.73$ | $P = 0.038$ |
| IL-6 | $T_{10} = 1.97$ | $P = 0.077$ |
| IL-1β | $T_{10} = -0.616$ | $P = 0.552$ |
| IL-4 | $T_{10} = -1.61$ | $P = 0.139$ |
| **vHC** | **Independent T-Test** | **Figure 15C** |
| Tmem119 | $T_{10} = -2.26$ | $P = 0.047$ |
| CD68 | $T_{10} = -0.392$ | $P = 0.703$ |
| CD86 | $T_{10} = -0.280$ | $P = 0.785$ |
| CD200R1 | $T_{10} = -0.087$ | $P = 0.933$ |
| Nlrp3 | $T_{10} = 1.70$ | $P = 0.121$ |
| IL-1β | $T_{10} = -1.30$ | $P = 0.223$ |
| IL-4 | $T_{10} = -1.95$ | $P = 0.080$ |

## Effects of SFC acquisition on the peripheral and central immune system

Next, I aimed to evaluate the acute effects of SFC acquisition on physiological stress and immune parameters, including organ weight, as well as plasma CORT and cytokine levels, respectively (for statistics see Table 8). To investigate the effect of an electric foot shock *per se*, shock controls were added. Though, neither relative adrenal nor spleen weight, nor plasma CORT level were altered 2 hours after SFC acquisition (Figure 16A-C). Additionally, plasma cytokine levels remained unchanged in all groups (Figure 16D-F). However, performing separate statistics on plasma IL-10 levels comparing $SFC^-$ and $SFC^+$, revealed a trend towards decreased IL-10 levels in $SFC^+$

(independent T-Test $T_{11} = 2.12$, $p = 0.058$; Figure 16F). Moreover, splenocyte stimulation revealed no differences between $SFC^{-}$, $SFC^{+}$ and shock controls (Figure 16G-H). Due to technical problems, the effect of ascending CORT concentrations on spleen cell viability could not be analyzed in this experiment.

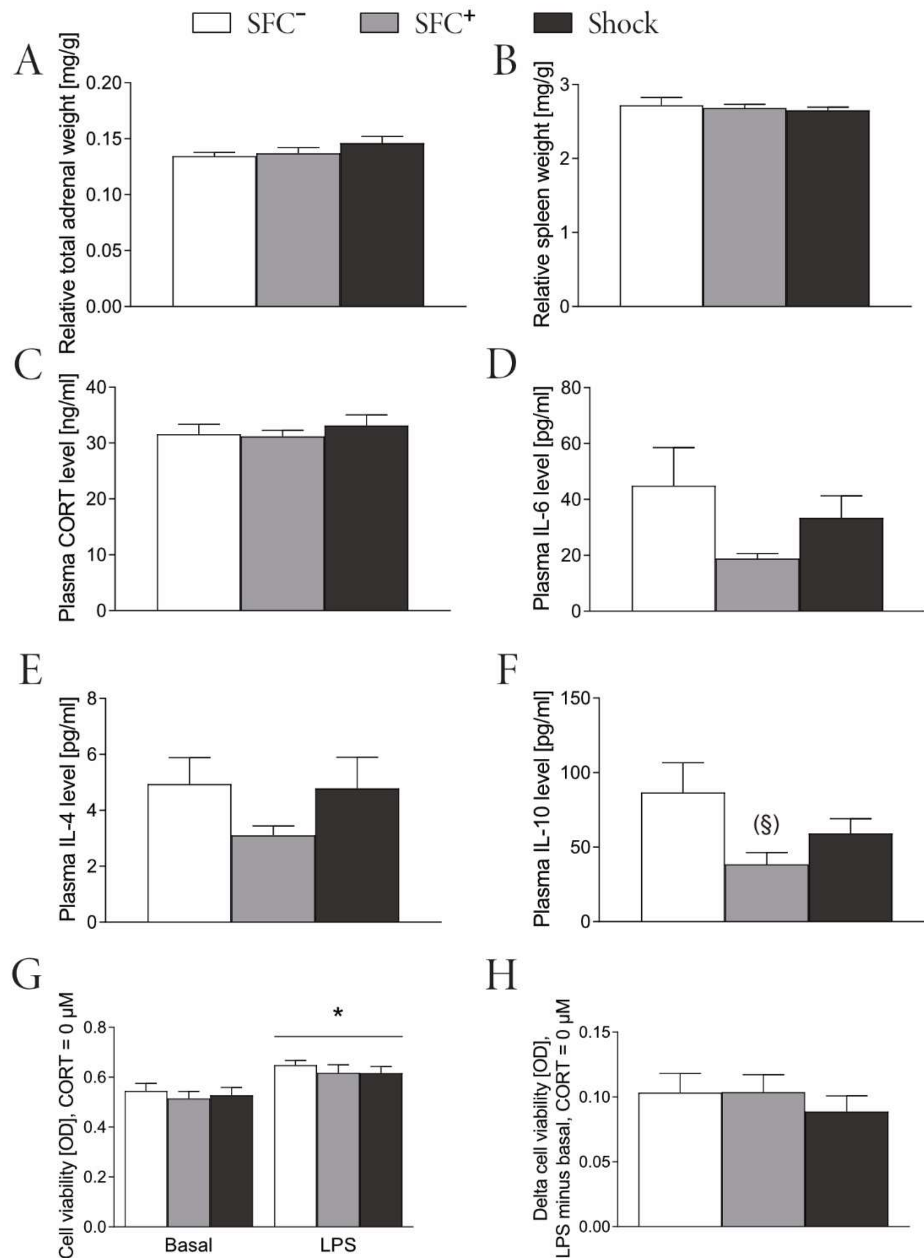



**Figure 16: Social fear conditioning (SFC) acquisition did not affect peripheral stress or immune parameters.** SFC acquisition did not affect organ weight (A-B), plasma corticosterone (CORT; C) or cytokine levels (D-F)Moreover, splenocyte cell viability remained unchanged under basal conditions and after lipopolysaccharide (LPS) stimulation (G), as well as when comparing delta cell viability (H). Using separate statistics, an independent T-Test revealed a trend towards reduced plasma interleukin (IL)-10 in $SFC^{+}$ compared to $SFC^{-}$ animals (F). Data represent mean + SEM. * $p \leq 0.050$ LPS vs basal; (§) separate statistics $p = 0.058$ $SFC^{+}$ vs $SFC^{-}$; n = 6-8 per group; for detailed statistics see Table 8.

**Table 8: Statistics of the effects of SFC acquisition on peripheral stress and immune parameters (Experiment 2.2b).** Factor LPS represents basal vs LPS effects; factor SFC represents SFC⁻ vs SFC⁺ vs shock effects.

| Physiological parameters | One-way ANOVA | Figure 16A-C |
|---|---|---|
| Relative total adrenal weight | (SFC) $F_{2,20} = 1.48$ | P = 0.252 |
| Relative spleen weight | (SFC) $F_{2,20} = 0.228$ | P = 0.799 |
| Plasma CORT | (SFC) $F_{2,19} = 0.428$ | P = 0.658 |
| **Plasma Cytokines** | **One-way ANOVA** | **Figure 16D-F** |
| Plasma IL-6 | (SFC) $F_{2,11} = 2.08$ | P = 0.171 |
| Plasma IL-4 | (SFC) $F_{2,14} = 1.06$ | P = 0.374 |
| Plasma IL-10 | (SFC) $F_{2,18} = 2.90$ | P = 0.082 |
| **Spleen cell stimulation** | **Two-way ANOVA, One-way ANOVA (delta CORT = 0 µM)** | **Figure 16G-H** |
| Cell viability CORT = 0 µM | (LPS) $F_{5,42} = 18.3$<br>(SFC) $F_{5,42} = 0.666$<br>(LPS x SFC) $F_{5,42} = 0.046$ | P < 0.001<br>P = 0.519<br>P = 0.955 |
| Delta cell viability CORT = 0 µM | (SFC) $F_{2,21} = 0.401$ | P = 0.675 |

Analyzing the effects of SFC acquisition on central immune parameters using mRNA expression levels (for statistics see Table 9), minor changes could be revealed in the three brain regions that have been studied. However, in the Amygdala, increased GILZ mRNA expression in $SFC^+$ compared to $SFC^-$ animals (LSD *post hoc* $p < 0.050$) and shock controls (LSD *post hoc* analysis $p < 0.050$; Figure 17A) could be shown. Nevertheless, no changes in immune parameters were found in the septum (Figure 17B), whereas in the vHC, the expression of CD200R1, a microglia inhibitory receptor, was reduced in shock animals compared to $SFC^-$ (LSD *post hoc* analysis $p < 0.050$; Figure 17C).

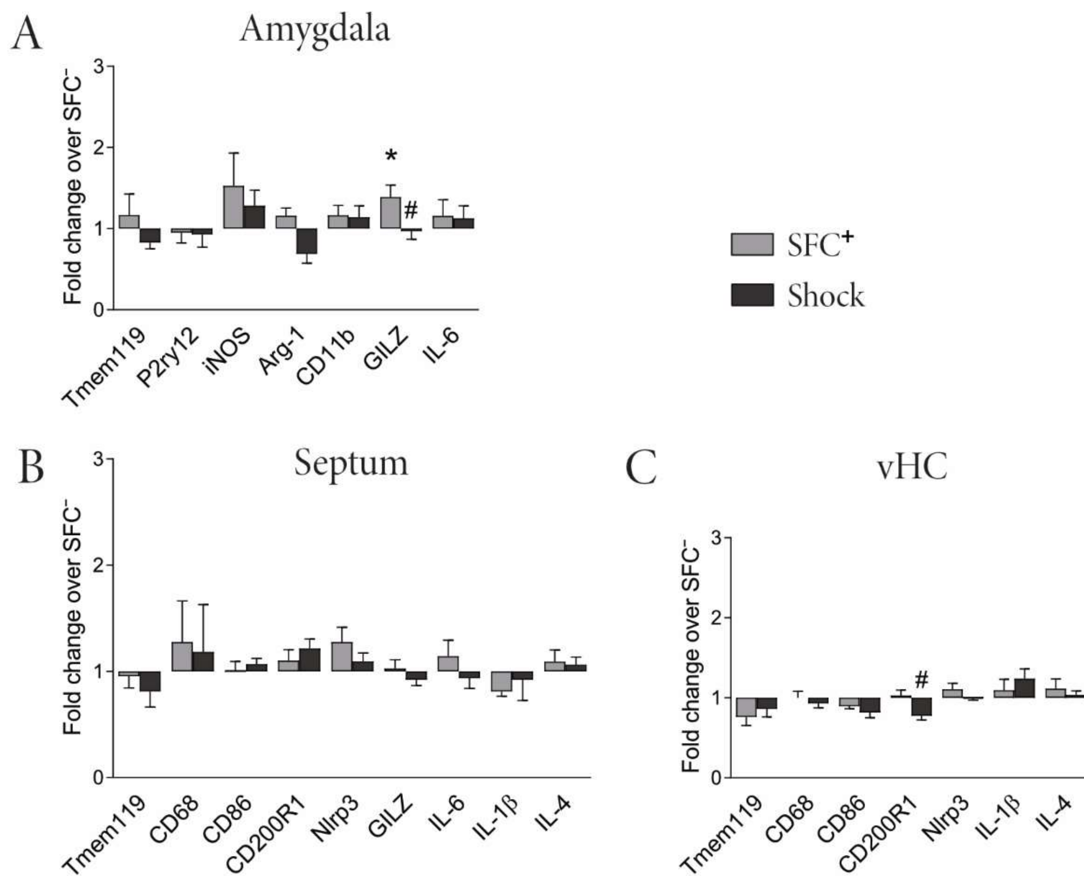



**Figure 17: Social fear conditioning (SFC) acquisition selectively affected central immune parameters.** In the amygdala, $SFC^+$ animals showed higher glucocorticoid-induced leucine zipper (GILZ) mRNA levels compared to $SFC^-$ and shock animals (A). Moreover, in the septum mRNA expression levels were not affected by SFC acquisition (B), while in the ventral hippocampus (vHC) shock controls showed reduced expression of the CD200 receptor 1 (CD200R1) compared to $SFC^-$ (C). Data represent mean + SEM. Group values were adapted to $SFC^-$ control mice (value 1). LSD *post hoc* analysis. * $p \leq 0.050$ $SFC^+$ vs $SFC^-$; # $p \leq 0.050$ shock control vs $SFC^+$ in amygdala and vs $SFC^-$ in vHC; n = 6-8 per group; for detailed statistics see Table 9.

**Table 9: Statistics of the effects of SFC acquisition on the central immune system (Experiment 2.2b).** Factor represents SFC⁻ vs SFC⁺ vs shock effects.

| Amygdala | One-way ANOVA | Figure 17A |
|---|---|---|
| Tmem119 | $F_{2,12} = 0.900$ | P = 0.432 |
| P2ry12 | $F_{2,14} = 0.046$ | P = 0.955 |
| iNOS | $F_{2,9} = 1.21$ | P = 0.341 |
| Arg-1 | $F_{2,10} = 3.40$ | P = 0.075 |
| CD11b | $F_{2,12} = 0.432$ | P = 0.659 |
| GILZ | $F_{2,15} = 4.09$ | P = 0.038 |
| IL-6 | $F_{2,11} = 0.237$ | P = 0.793 |
| **Septum** | **One-way ANOVA** | **Figure 17B** |
| Tmem119 | $F_{2,7} = 1.11$ | P = 0.992 |
| CD68 | $F_{2,9} = 2.59$ | P = 0.129 |
| CD86 | $F_{2,9} = 0.042$ | P = 0.959 |
| CD200R1 | $F_{2,9} = 0.562$ | P = 0.589 |
| Nlrp3 | $F_{2,9} = 2.11$ | P = 0.177 |
| GILZ | $F_{2,10} = 0.834$ | P = 0.462 |
| IL-6 | $F_{2,10} = 0.561$ | P = 0.588 |
| IL-1β | $F_{2,9} = 0.200$ | P = 0.822 |
| IL-4 | $F_{2,9} = 1.76$ | P = 0.227 |
| **vHC** | **One-way ANOVA** | **Figure 17C** |
| Tmem119 | $F_{2,10} = 0.133$ | P = 0.878 |
| CD68 | $F_{2,8} = 0.329$ | P = 0.729 |
| CD86 | $F_{2,8} = 0.671$ | P = 0.538 |
| CD200R1 | $F_{2,8} = 4.57$ | P = 0.047 |
| Nlrp3 | $F_{2,8} = 0.077$ | P = 0.926 |
| IL-1β | $F_{2,8} = 0.432$ | P = 0.663 |
| IL-4 | $F_{2,8} = 1.36$ | P = 0.310 |

### Effects of CSC and SFC acquisition on the peripheral and central immune system

Subsequently, I investigated the effects of the combination of CSC exposure and SFC acquisition on the peripheral and central immune system. Here, CSC mice showed increased anxiety-like behavior on the EPM, indicated by reduced time spent on the open arms and full open arm entries, as well as reduced open arm entries by trend, compared to SHC. Additionally, successful induction of chronic stress in CSC mice could be confirmed by the following physiological stress parameters: increased body weight gain after stressor termination, increased relative adrenal and spleen weight, and reduced relative thymus weight. Analyzing the number of shocks during SFC acquisition revealed no differences between CSC mice and SHC (see Table A4).

To unravel the effects of the combination of CSC exposure and SFC acquisition on the peripheral immune system (for statistics see Table 10), I analyzed a known stress and immune parameter (see 3.1) as a representative of the peripheral immune status, i.e., the number (Figure 18A) and IFN-γ release (Figure 18B) of mesLNCs. Here, both were increased in CSC mice compared to SHC, which was, however, independent of conditioning.

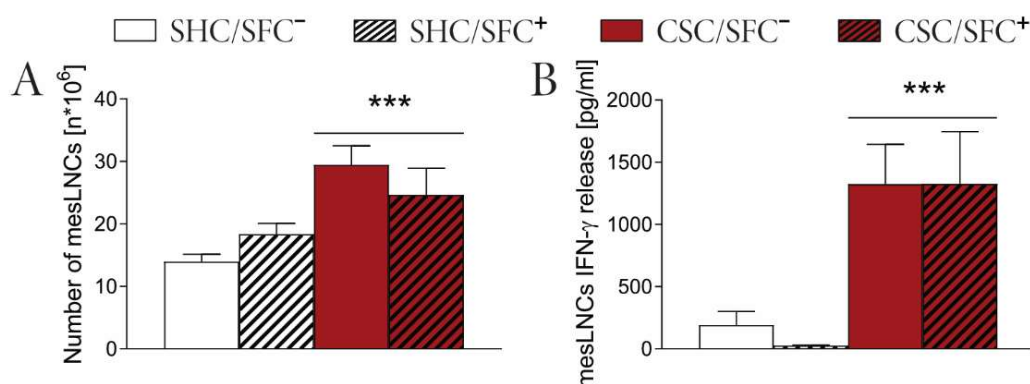



**Figure 18: Chronic subordinate colony housing (CSC) exposure increased peripheral inflammation independent of conditioning.** Independent of conditioning, CSC mice showed an increased number of mesenteric lymph node cells (mesLNCs; A) and interferon (IFN)-γ release (B) by them compared to single housed controls (SHC). Data represent mean + SEM. *** $p \leq 0.001$ CSC vs SHC; n = 7-8 per group; for detailed statistics see Table 10.

**Table 10: Statistics of the effects of CSC and subsequent SFC acquisition exposure on the peripheral immune system (Experiment 2.2c).** Factor CSC represents SHC vs CSC effects; factor SFC represents $SFC^-$ vs $SFC^+$ effects.

| mesLNCs | Two-way ANOVA | Figure 18 |
|---|---|---|
| Number of mesLNCs | (CSC) $F_{1,28} = 14.8$ | $P = 0.001$ |
| | (SFC) $F_{1,28} = 0.005$ | $P = 0.944$ |
| | (CSC x SFC) $F_{1,28} = 2.64$ | $P = 0.116$ |
| mesLNCs IFN-γ release | (CSC) $F_{1,28} = 20.4$ | $P < 0.001$ |
| | (SFC) $F_{1,28} = 0.090$ | $P = 0.766$ |
| | (CSC x SFC) $F_{1,28} = 0.095$ | $P = 0.760$ |

Analyzing central immune parameters after CSC exposure and subsequent SFC acquisition, qPCR analysis indicated systemic immune activation in the amygdala and septum, seen by molecular changes in microglia marker, pro- and anti-inflammatory cytokines, but also the immunosuppressant agent GILZ (for statistics see Table 11). In detail, in the amygdala, CSC mice revealed increased mRNA expression of the resting microglia marker P2ry12 compared to SHC, which was independent of conditioning, and there was an ANOVA trend for the interaction of factor CSC and factor SFC in iNOS, a marker for the pro-inflammatory microglia phenotype. Analyzing Bonferroni *post hoc* analysis, revealed increased mRNA expression of iNOS in CSC/SFC⁻

compared to SHC/SFC$^-$ ($p < 0.050$). Moreover, GILZ mRNA was increased in SFC$^+$ compared to SFC$^-$, which was independent of chronic stressor exposure (Figure 19A). In the septum, microglia number or phenotypes were not altered, while GILZ mRNA ($p < 0.050$) was increased in CSC/SFC$^-$ compared to SHC/SFC$^-$, and the pro-inflammatory cytokine IL-6 was increased in CSC/SFC$^-$ compared to SHC/SFC$^-$ ($p < 0.050$) and compared to CSC/SFC$^+$ ($p < 0.050$). Besides, the anti-inflammatory cytokine IL-4 was increased in CSC mice compared to SHC, independent of conditioning (Figure 19B). In the vHC, SFC$^+$ showed a trend towards reduced expression of CD68 mRNA expression, a marker of M2 microglia, compared to SFC$^-$, which was found to be independent of chronic stressor exposure (Figure 19C).

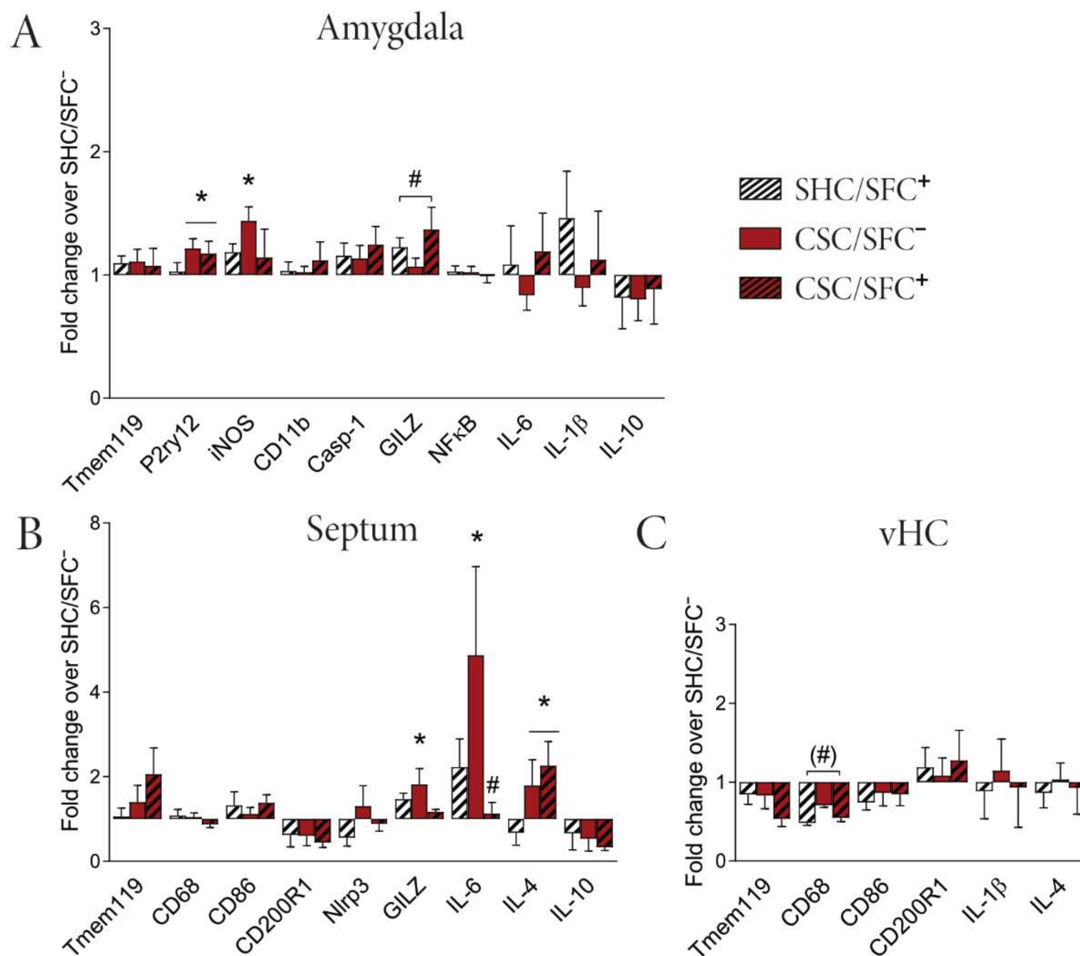



**Figure 19: Effects of exposure to either chronic subordinate colony housing (CSC/SFC⁻), or to social fear conditioning acquisition (SHC/SFC⁺), or to the combination of both (CSC/SFC⁺), increased central inflammation brain-region dependently, when measuring mRNA expression.** In the amygdala, CSC exposure increased microglia parameters independent of conditioning, while conditioning increased glucocorticoid-induced leucine zipper (GILZ) expression, independent of chronic stressor exposure (A). Moreover, in the septum, CSC exposure increased microglia number and anti-inflammatory cytokine expression, independent of conditioning, while CSC alone (CSC/SFC⁻) increased GILZ and pro-inflammatory cytokine expression. The combination of both, i.e., CSC/SFC⁺, reduced GILZ expression in the septum (B). In the ventral hippocampus (vHC), a M2 microglia marker tended to be reduced in SFC⁺ vs SFC⁻ mice, independent of chronic stress exposure (C). In the amygdala, iNOS reached only a trend for factor CSC and SFC. Data represent mean + SEM. Group values were adapted to SHC/SFC⁻ (value 1). * $p \leq 0.050$ CSC vs SHC; (#) $p = 0.058$, # $p \leq 0.050$ SFC⁺ vs SFC⁻; n = 6-8 per group; for detailed statistics see Table 11.

**Table 11: Statistics of the effects of CSC and subsequent SFC acquisition exposure on the central immune system using mRNA expression analysis (Experiment 2.2c).** Factor CSC represents SHC vs CSC effects; factor SFC represents $SFC^-$ vs $SFC^+$ effects.

| Amygdala | Two-way ANOVA | Figure 19A |
|---|---|---|
| Tmem119 | (CSC) $F_{1,25} = 0.197$<br>(SFC) $F_{1,25} = 0.764$<br>(CSC x SFC) $F_{1,25} = 0.516$ | $P = 0.661$<br>$P = 0.764$<br>$P = 0.516$ |
| P2ry12 | (CSC) $F_{1,26} = 5.63$<br>(SFC) $F_{1,26} = 0.011$<br>(CSC x SFC) $F_{1,26} = 0.201$ | $P = 0.025$<br>$P = 0.917$<br>$P = 0.658$ |
| iNOS | (CSC) $F_{1,21} = 2.57$<br>(SFC) $F_{1,21} = 0.211$<br>(CSC x SFC) $F_{1,21} = 3.81$ | $P = 0.124$<br>$P = 0.651$<br>$P = 0.064$ |
| CD11b | (CSC) $F_{1,24} = 0.400$<br>(SFC) $F_{1,23} = 0.562$<br>(CSC x SFC) $F_{1,23} = 0.135$ | $P = 0.533$<br>$P = 0.461$<br>$P = 0.761$ |
| Casp-1 | (CSC) $F_{1,23} = 0.839$<br>(SFC) $F_{1,23} = 1.20$<br>(CSC x SFC) $F_{1,23} = 0.031$ | $P = 0.369$<br>$P = 0.285$<br>$P = 0.861$ |
| GILZ | (CSC) $F_{1,24} = 0.916$<br>(SFC) $F_{1,24} = 5.65$<br>(CSC x SFC) $F_{1,24} = 0.115$ | $P = 0.348$<br>$P = 0.026$<br>$P = 0.738$ |
| NFκB | (CSC) $F_{1,25} = 0.005$<br>(SFC) $F_{1,25} = 0.001$<br>(CSC x SFC) $F_{1,25} = 0.243$ | $P = 0.943$<br>$P = 0.979$<br>$P = 0.626$ |
| IL-6 | (CSC) $F_{1,22} = 0.016$<br>(SFC) $F_{1,22} = 0.915$<br>(CSC x SFC) $F_{1,22} = 0353$ | $P = 0.901$<br>$P = 0.349$<br>$P = 0.558$ |
| IL-1β | (CSC) $F_{1,20} = 0.517$<br>(SFC) $F_{1,20} = 1.26$<br>(CSC x SFC) $F_{1,20} = 0.142$ | $P = 0.481$<br>$P = 0.274$<br>$P = 0.710$ |
| IL-10 | (CSC) $F_{1,19} = 0.070$<br>(SFC) $F_{1,19} = 0.046$<br>(CSC x SFC) $F_{1,19} = 0.313$ | $P = 0.794$<br>$P = 0.832$<br>$P = 0.582$ |

| Septum | Two-way ANOVA | Figure 19B |
|---|---|---|
| Tmem119 | (CSC) $F_{1,20} = 3.20$<br>(SFC) $F_{1,20} = 0.84$<br>(CSC x SFC) $F_{1,20} = 0.592$ | P = 0.089<br>P = 0.372<br>P = 0.450 |
| CD68 | (CSC) $F_{1,20} = 0.601$<br>(SFC) $F_{1,20} = 0.204$<br>(CSC x SFC) $F_{1,20} = 1.57$ | P = 0.447<br>P = 0.656<br>P = 0.224 |
| CD86 | (CSC) $F_{1,20} = 0.136$<br>(SFC) $F_{1,20} = 1.457$<br>(CSC x SFC) $F_{1,20} = 0.010$ | P = 0.716<br>P = 0.241<br>P = 0.923 |
| CD200R1 | (CSC) $F_{1,20} = 1.39$<br>(SFC) $F_{1,20} = 1.15$<br>(CSC x SFC) $F_{1,20} = 0.194$ | P = 0.252<br>P = 0.297<br>P = 0.664 |
| Nlrp3 | (CSC) $F_{1,19} = 0.698$<br>(SFC) $F_{1,19} = 1.30$<br>(CSC x SFC) $F_{1,19} = 0.001$ | P = 0.414<br>P = 0.269<br>P = 0.972 |
| GILZ | (CSC) $F_{1,20} = 1.44$<br>(SFC) $F_{1,20} = 0.185$<br>(CSC x SFC) $F_{1,20} = 6.75$ | P = 0.245<br>P = 0.672<br>P = 0.017 |
| IL-6 | (CSC) $F_{1,15} = 2.03$<br>(SFC) $F_{1,15} = 1.68$<br>(CSC x SFC) $F_{1,15} = 6.58$ | P = 0.175<br>P = 0.214<br>P = 0.022 |
| IL-4 | (CSC) $F_{1,20} = 6.01$<br>(SFC) $F_{1,20} = 0.023$<br>(CSC x SFC) $F_{1,20} = 0.671$ | P = 0.024<br>P = 0.882<br>P = 0.422 |
| IL-10 | (CSC) $F_{1,19} = 1.72$<br>(SFC) $F_{1,19} = 0.767$<br>(CSC x SFC) $F_{1,19} = 0.052$ | P = 0.206<br>P = 0.392<br>P = 0.822 |
| **vHC** | **Two-way ANOVA** | **Figure 19C** |
| Tmem119 | (CSC) $F_{1,20} = 2.57$<br>(SFC) $F_{1,20} = 2.32$<br>(CSC x SFC) $F_{1,20} = 0.230$ | P = 0.124<br>P = 0.143<br>P = 0.637 |
| CD68 | (CSC) $F_{1,20} = 0.461$<br>(SFC) $F_{1,20} = 4.06$<br>(CSC x SFC) $F_{1,20} = 1.12$ | P = 0.505<br>P = 0.058<br>P = 0.303 |
| CD86 | (CSC) $F_{1,20} = 0.007$<br>(SFC) $F_{1,20} = 0.647$<br>(CSC x SFC) $F_{1,20} = 0.497$ | P = 0.932<br>P = 0.431<br>P = 0.489 |
| CD200R1 | (CSC) $F_{1,20} = 0.074$<br>(SFC) $F_{1,20} = 0.376$<br>(CSC x SFC) $F_{1,20} = 0.000$ | P = 0.788<br>P = 0.547<br>P = 0.991 |
| IL-1β | (CSC) $F_{1,13} = 0,053$<br>(SFC) $F_{1,13} = 0.150$<br>(CSC x SFC) $F_{1,13} = 0.012$ | P = 0.821<br>P = 0.705<br>P = 0.913 |
| IL-4 | (CSC) $F_{1,12} = 0.038$<br>(SFC) $F_{1,12} = 0.232$<br>(CSC x SFC) $F_{1,12} = 0.004$ | P = 0.850<br>P = 0.639<br>P = 0.949 |

To analyze protein levels of each group, representative blots of every protein and brain region are depicted (Figure 20A). In line to the findings of mRNA expression levels, protein analysis (for statistics see Table 12) revealed increased inflammation in the amygdala, as the microglia marker Tmem119 and activation of the inflammatory NFκB signaling pathway were increased in CSC mice compared to SHC. In detail, CSC mice showed a trend towards increased pNFκB (Ser276) protein level and a significant increase in the ratio of pNFκB (Ser276) / NFκB, compared to SHC. Interestingly, analyzing *post hoc* Bonferroni without an ANOVA effect for factor SFC, revealed even a significant increase in the ratio of pNFκB (Ser276) / NFκB in CSC/SFC$^+$ compared to SHC/SFC$^+$ and CSC/SFC$^-$ (no ANOVA effect; $p \leq 0.05$). Moreover, the anti-inflammatory mediators GILZ and IL-4 were increased in CSC mice compared to SHC (Figure 20B). However, neither in the septum (Figure 20C), nor in the vHC (Figure 20D), protein levels differed between the groups. These data indicate that CSC-induced central inflammation, which seems to be exaggerated in CSC/SFC$^+$, in the amygdala, a brain region highly involved in PTSD and SAD, is mainly mediated via activation of the NFκB signaling pathway, maybe by a dysregulation of GILZ.

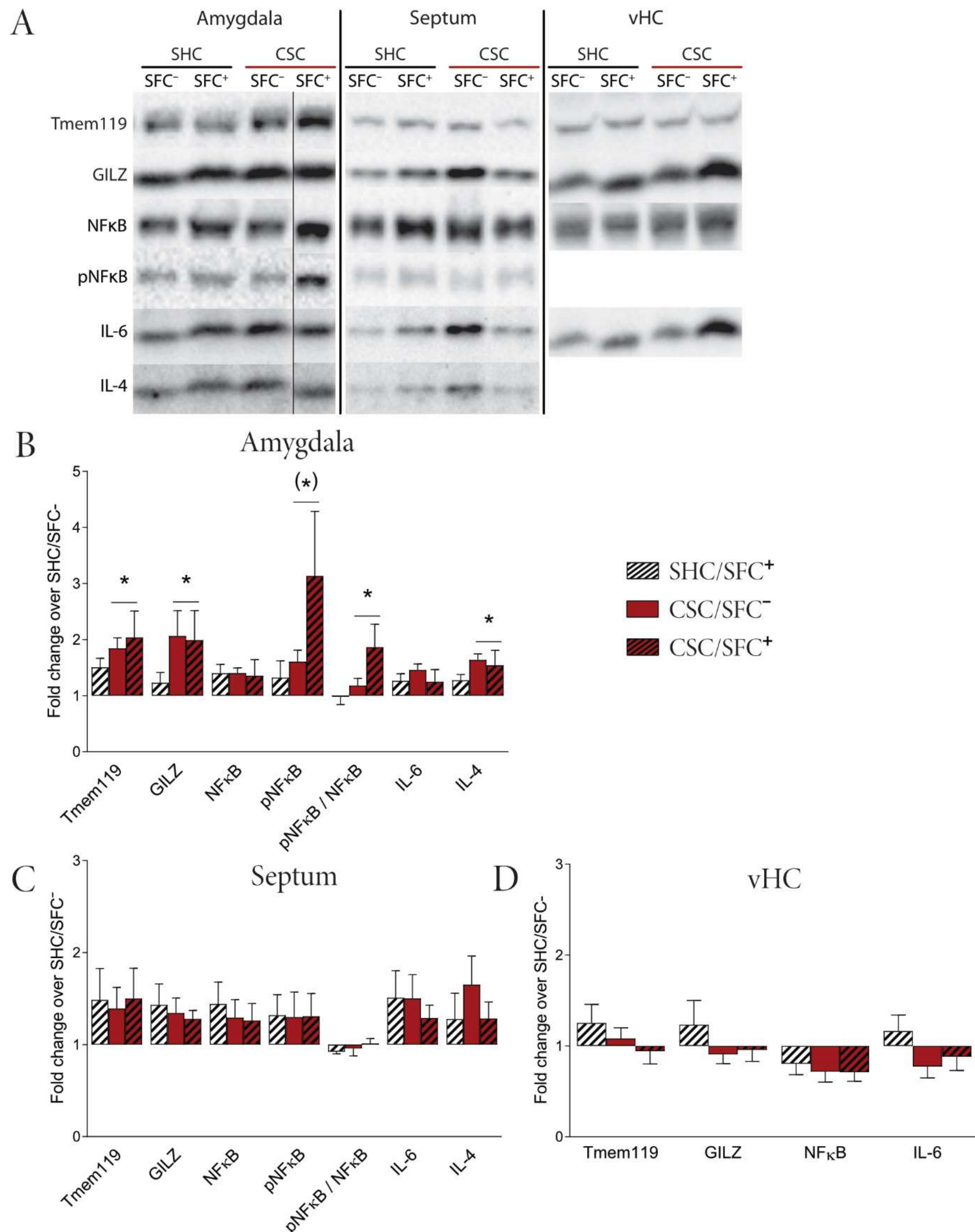



**Figure 20: Effects of exposure to either chronic subordinate colony housing (CSC/SFC⁻), or to social fear conditioning acquisition (SHC/SFC⁺), or to the combination of both (CSC/SFC⁺) increased central inflammation in the amygdala, when measuring protein level.** Representative blots of each protein and brain region are depicted in the first panel (A). In the amygdala, CSC mice showed increased protein levels of microglia, the anti-inflammatory glucocorticoid-induced leucine zipper (GILZ) and interleukin (IL)-4, as well as increased phospho-nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (pNFκB) and pNFκB / NFκB ratio compared to single housed controls (SHC; B), while the septum (C) and vHC (D) showed no alterations (C). Data represent mean + SEM. Group values were adapted to SHC/SFC⁻ (value 1). (*) $p = 0.059$, * $p \leq 0.050$ CSC vs SHC; n = 6-8 per group; for detailed statistics see Table 12.

**Table 12: Statistics of the effects of CSC and subsequent SFC acquisition exposure on the central immune system using protein analysis (Experiment 2.2c).** Factor CSC represents SHC vs CSC effects; factor SFC represents SFC⁻ vs SFC⁺ effects.

| Amygdala | Two-way ANOVA | Figure 20B |
|---|---|---|
| Tmem119 | (CSC) $F_{1,25} = 6.50$<br>(SFC) $F_{1,25} = 1.69$<br>(CSC x SFC) $F_{1,25} = 0.324$ | P = 0.017<br>P = 0.206<br>P = 0.574 |
| GILZ | (CSC) $F_{1,25} = 5.947$<br>(SFC) $F_{1,25} = 0.043$<br>(CSC x SFC) $F_{1,25} = 0.164$ | P = 0.022<br>P = 0.838<br>P = 0.689 |
| NFκB | (CSC) $F_{1,26} = 1.12$<br>(SFC) $F_{1,26} = 1.08$<br>(CSC x SFC) $F_{1,26} = 1.74$ | P = 0.300<br>P = 0.309<br>P = 0.199 |
| pNFκB | (CSC) $F_{1,24} = 3.95$<br>(SFC) $F_{1,24} = 2.32$<br>(CSC x SFC) $F_{1,24} = 0.987$ | P = 0.059<br>P = 0.141<br>P = 0.330 |
| pNFκB/ NFκB | (CSC) $F_{1,24} = 5.12$<br>(SFC) $F_{1,24} = 2.04$<br>(CSC x SFC) $F_{1,24} = 2.23$ | P = 0.033<br>P = 0.166<br>P = 0.148 |
| IL-6 | (CSC) $F_{1,26} = 2,33$<br>(SFC) $F_{1,26} = 0.030$<br>(CSC x SFC) $F_{1,26} = 2.70$ | P = 0.139<br>P = 0.863<br>P = 0.113 |
| IL-4 | (CSC) $F_{1,26} = 8.74$<br>(SFC) $F_{1,26} = 0.337$<br>(CSC x SFC) $F_{1,26} = 1.44$ | P = 0.007<br>P = 0.567<br>P = 0.241 |
| **Septum** | **Two-way ANOVA** | **Figure 20C** |
| Tmem119 | (CSC) $F_{1,28} = 0.525$<br>(SFC) $F_{1,28} = 1.13$<br>(CSC x SFC) $F_{1,28} = 0.458$ | P = 0.475<br>P = 0.296<br>P = 0.504 |
| GILZ | (CSC) $F_{1,28} = 0.355$<br>(SFC) $F_{1,28} = 1.28$<br>(CSC x SFC) $F_{1,28} = 2.37$ | P = 0.556<br>P = 0.267<br>P = 0.135 |
| NFκB | (CSC) $F_{1,28} = 0.085$<br>(SFC) $F_{1,28} = 1.16$<br>(CSC x SFC) $F_{1,28} = 1.51$ | P = 0.772<br>P = 0.290<br>P = 0.229 |
| pNFκB | (CSC) $F_{1,28} = 0.390$<br>(SFC) $F_{1,28} = 0.516$<br>(CSC x SFC) $F_{1,28} = 0.452$ | P = 0.537<br>P = 0.478<br>P = 0.507 |
| pNFκB/ NFκB | (CSC) $F_{1,28} = 0.204$<br>(SFC) $F_{1,28} = 0.042$<br>(CSC x SFC) $F_{1,28} = 1.57$ | P = 0.655<br>P = 0.838<br>P = 0.221 |
| IL-6 | (CSC) $F_{1,28} = 0.383$<br>(SFC) $F_{1,28} = 0.422$<br>(CSC x SFC) $F_{1,28} = 2.53$ | P = 0.541<br>P = 0.521<br>P = 0.123 |
| IL-4 | (CSC) $F_{1,20} = 1.65$<br>(SFC) $F_{1,20} = 0.031$<br>(CSC x SFC) $F_{1,20} = 1.58$ | P = 0.213<br>P = 0.862<br>P = 0.224 |

| vHC | Two-way ANOVA | Figure 20D |
|---|---|---|
| Tmem119 | (CSC) $F_{1,27} = 0.609$<br>(SFC) $F_{1,27} = 0.152$<br>(CSC x SFC) $F_{1,27} = 1.77$ | P = 0.442<br>P = 0.699<br>P = 0.195 |
| GILZ | (CSC) $F_{1,28} = 1.28$<br>(SFC) $F_{1,28} = 0.752$<br>(CSC x SFC) $F_{1,28} = 0.317$ | P = 0.267<br>P = 0.393<br>P = 0.578 |
| NFκB | (CSC) $F_{1,28} = 1.97$<br>(SFC) $F_{1,28} = 0.347$<br>(CSC x SFC) $F_{1,28} = 1.45$ | P = 0.172<br>P = 0.560<br>P = 0.239 |
| IL-6 | (CSC) $F_{1,27} = 3.03$<br>(SFC) $F_{1,27} = 0.858$<br>(CSC x SFC) $F_{1,27} = 0.035$ | P = 0.093<br>P = 0.363<br>P = 0.854 |

## 3.3 Effects of CSC exposure on CFC and CUS exposure on non-social vs social fear conditioning

Next, I aimed to investigate how specific the effects of chronic social stress on subsequent fear expression is, depending on the fear conditioning model, the nature and composition of the chronic stressor paradigm, and the mouse strain used. Thus, in the first experiment, CSC mice and SHC were exposed to CFC. Next, I analyzed the effects of chronic non-social stress, i.e., CUS, on CFC and SFC. Finally, I analyzed potential strain differences in BL6 and CD1 mice in anxiety-like behavior, social behavior, CFC, SFC and the effects of CSC exposure on SFC.

### 3.3.1 Effects of CSC exposure on CFC

First, CSC mice were exposed to CFC to unravel the effect of chronic psychosocial stress on non-social fear conditioning. A successful induction of chronic stress in CSC mice was confirmed by increased relative adrenal and spleen weight, as well as spleen cell viability under basal conditions, LPS stimulation, and ascending CORT concentrations (see Table A5).

Studying the effects of CFC showed increased freezing behavior of all mice along the number of CS-US pairings during acquisition and slightly reduced freezing behavior along CS presentations during extinction, indicating successful induction and partly extinction of cued fear. However, as the level of freezing stayed high even during retention in all mice (about 60 to 70 % time spent freezing), extinction success was very low. Nevertheless, CFC was found to be independent of CSC exposure as CSC mice and SHC did not differ in freezing behavior neither during acquisition (Figure 21A), nor extinction (Figure 21B), nor retention (Figure 21C; for statistics see Table 13).

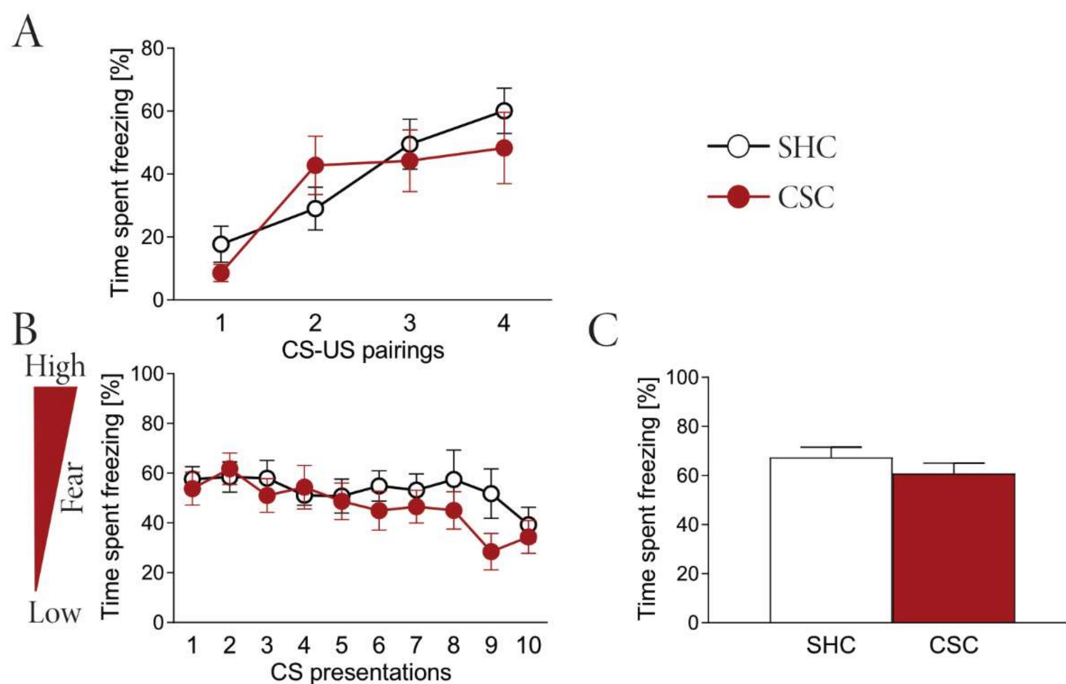



**Figure 21: Chronic subordinate colony housing (CSC) exposure did not affect freezing behavior during cued fear conditioning (CFC).** CSC mice and single housed controls (SHC) did not differ in their freezing behavior neither during acquisition (A), nor extinction (B), nor retention (C). CS: conditioned stimulus, US: unconditioned stimulus. Data represent mean +/± SEM; n = 7-8 per group; for detailed statistics see Table 13.

**Table 13: Statistics of the effects of CSC exposure on CFC (Experiment 3.1).** Factor time represents CS-US pairings during acquisition and CS presentations during extinction; factor CSC represents SHC vs CSC effects.

| Acquisition | One-way ANOVA for RM | Figure 21A |
|---|---|---|
| Time spent freezing | (Time) $F_{3,39} = 9.07$<br>(CSC) $F_{1,13} = 0.344$<br>(Time x CSC) $F_{3,39} = 0.944$ | $P < 0.001$<br>$P = 0.567$<br>$P = 0.429$ |
| **Extinction** | **One-way ANOVA for RM** | **Figure 21B** |
| Time spent freezing | (Time) $F_{9,108} = 3.71$<br>(CSC) $F_{1,12} = 0.677$<br>(Time x CSC) $F_{9,108} = 1.17$ | $P < 0.001$<br>$P = 0.427$<br>$P = 0.322$ |
| **Retention** | **Independent T-Test** | **Figure 21C** |
| Time spent freezing | (CSC) $T_{13} = 1.10$ | $P = 0.291$ |

### 3.3.2 Effects of CUS exposure on CFC and SFC

**Establishment and validation of the CUS paradigm**

To investigate the effects of chronic non-social stressor exposure on CFC and SFC, the CUS paradigm was established and evaluated with respect to behavioral and physiological stress parameters (see Table 14). Here, anxiety-like behavior in the OF was increased in CUS mice reflected by less time spent in the inner zone of the OF box compared to SHC (Figure 22A), without affecting locomotion (Figure 22B). In contrast, the time spent object sniffing in the NOR was significantly increased in CUS mice (Figure 22D), indicating reduced anxiety-like behavior, whereas the occurrence of object sniffing was not affected (Figure 22C). In addition, depressive-like behavior in the splash test indicated by time spent grooming (Figure 22E) and latency to groom (Figure 22F), as well as social preference in the SPT were not affected by CUS exposure, whereas both CUS mice and SHC preferred the social over the non-social stimulus (Figure 22G).

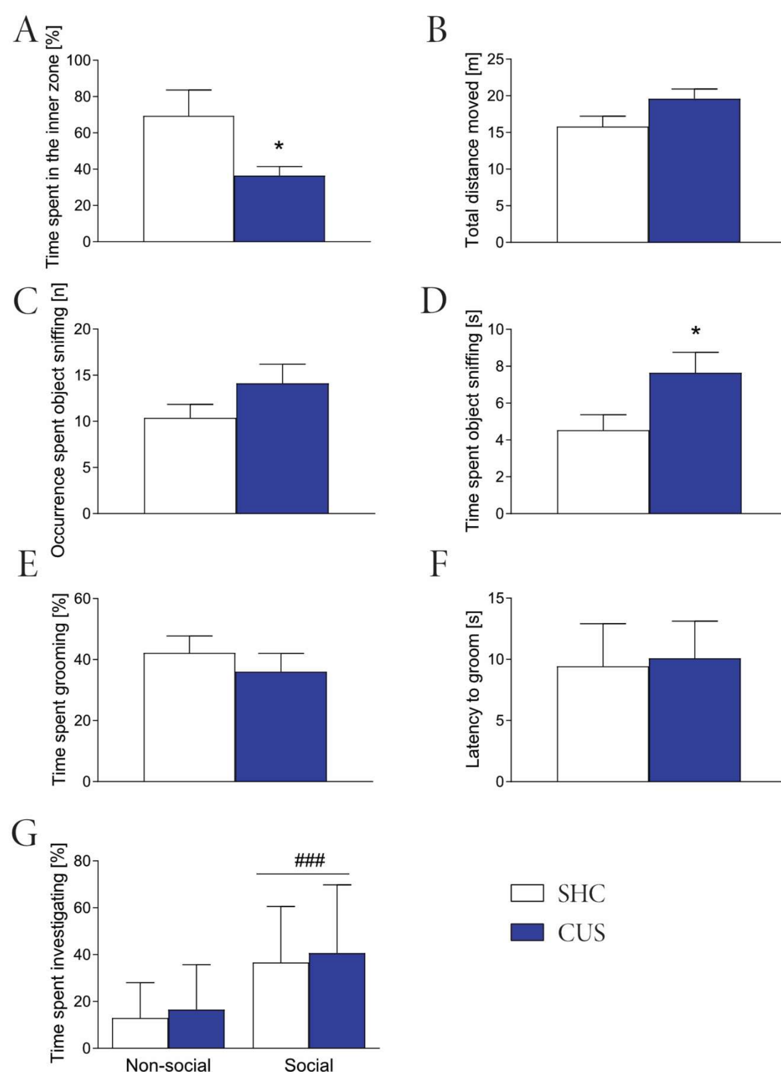



**Figure 22: Chronic unpredictable stress (CUS) exposure increased anxiety-like behavior without affecting depressive-like behavior and social preference.** In the open field (OF) box, CUS mice showed reduced time spent in the inner zone compared to single housed controls (SHC; A), without affecting locomotion (B). In contrast, in the novel object recognition (NOR) test, CUS mice showed increased time (D) but not occurrence (C) spent object sniffing, compared to SHC. In the splash test, CUS mice and SHC showed equal time (E) and latency (F) to groom. Furthermore, social preference was present in both CUS mice and SHC (G). Data represent mean + SEM. * $p \leq 0.050$ CUS vs SHC; ### $p \leq 0.001$ social vs non-social; n = 7-8 per group; for detailed statistics see Table 14.

**Table 14: Statistics of the effects of CUS exposure on anxiety-, depressive-like and social behavior (Experiment 3.2a).** Factor time represents stimulus presentations during SPT; factor CUS represents SHC vs CUS effects.

| Anxiety-like behavior | Independent T-Test | Figure 22A-D |
|---|---|---|
| Time spent in the inner zone | (CUS) $T_{14} = 2.18$ | P = 0.324 |
| Total distance moved | (CUS) $T_{14} = -1.96$ | P = 0.454 |
| Occurrence spent object sniffing | (CUS) $T_{14} = -1.48$ | P = 0.161 |
| Time spent object sniffing | (CUS) $T_{14} = -2.25$ | P = 0.041 |
| **Splash test** | **Independent T-Test** | **Figure 22E-F** |
| Time spent grooming | (CUS) $T_{14} = 0.756$ | P = 0.462 |
| Latency to groom | (CUS) $T_{14} = -0.140$ | P = 0.890 |
| **SPT** | **One-way ANOVA for RM** | **Figure 22G** |
| Time spent investigating | (Time) $F_{1,14} = 28.2$<br>(CUS) $F_{1,14} = 0.138$<br>(Time x CUS) $F_{1,14} = 0.002$ | P < 0.001<br>P = 0.716<br>P = 0.966 |

Analyzing physiological parameters (for statistics see Table 15), body weight gain was reduced in CUS mice compared to SHC during 19 days of stressor exposure and until 3 days after its termination (Figure 23A). Moreover, mice exposed to the CUS paradigm revealed unchanged relative adrenal (Figure 23B) and spleen weight (Figure 23D), while relative thymus weight was reduced compared to SHC (Figure 23C). However, neither number of mesLNCs (Figure 23E), nor IFN-γ release (Figure 23F) by mesLNCs, nor plasma CORT levels (Figure 23G) and adrenal CORT release (Figure 23H) were affected by CUS exposure. Even though, adrenal CORT release was increased in ACTH compared to saline stimulated adrenal glands, this effect was independent of chronic stressor exposure.

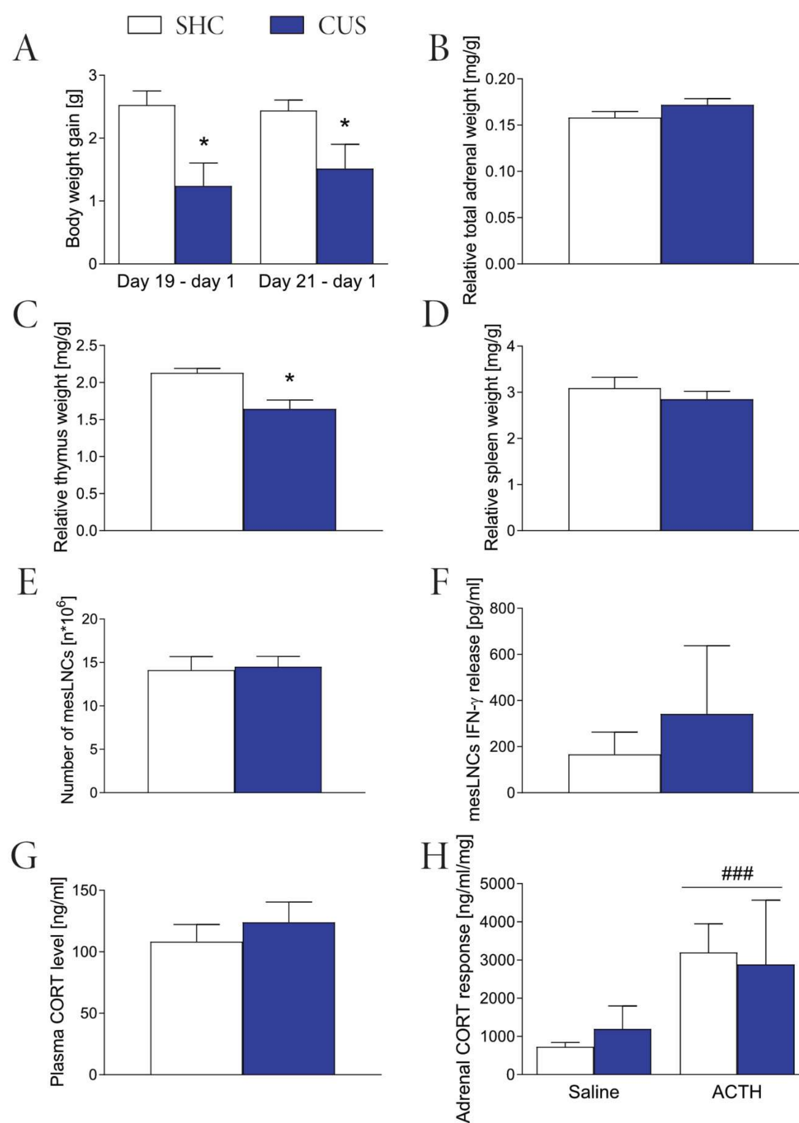



**Figure 23: Chronic unpredictable stress (CUS) exposure decreased body and thymus weight without affecting other physiological stress parameters.** Analyzing physiological stress parameters showed reduced body weight gain (A) and relative thymus weight (C) in CUS compared to single housed controls (SHC), without affecting relative adrenal (B) and spleen (D) weight, number of mesenteric lymph node cells (mesLNCs; E) and interferon (IFN)-γ release by mesLNCs (F), nor plasma (G) and adrenal corticosterone (CORT) levels (H). Data represent mean + SEM. * $p \leq 0.050$ CUS vs SHC; ### $p \leq 0.001$ adrenocorticotropic hormone (ACTH) vs saline; n = 7-8 per group; for detailed statistics see Table 15.

**Table 15: Statistics of the effects of CUS exposure on physiological stress parameters (Experiment 3.2a).** Factor time represents time effects over days; factor ACTH represents saline vs ACTH; factor CUS represents SHC vs CUS vs effects.

| Body weight | One way ANOVA for RM | Figure 23A |
|---|---|---|
| Body weight gain | (Time) $F_{1,14} = 0.464$<br>(CUS) $F_{1,14} = 7.48$<br>(Time x CUS) $F_{1,14} = 1.73$ | $P = 0.507$<br>$P = 0.016$<br>$P = 0.209$ |
| **Physiological parameters** | **Independent T-Test** | **Figure 23B-G** |
| Relative total adrenal weight | (CUS) $T_{12} = -1.48$ | $P = 0.166$ |
| Relative thymus weight | (CUS) $T_{14} = 2.70$ | $P = 0.017$ |
| Relative spleen weight | (CUS) $T_{14} = 0.810$ | $P = 0.432$ |
| Number of mesLNCs | (CUS) $T_{14} = -0.202$ | $P = 0.843$ |
| mesLNCs IFN-γ release | (CUS) $T_{12} = -0.633$ | $P = 0.538$ |
| Plasma CORT | (CUS) $T_{14} = -0.730$ | $P = 0.477$ |
| **Adrenal stimulation** | **Two-way ANOVA** | **Figure 23H** |
| Adrenal CORT release | (ACTH) $F_{1,24} = 31.0$<br>(CUS) $F_{1,24} = 0.043$<br>(ACTH x CUS) $F_{1,24} = 1.09$ | $P < 0.001$<br>$P = 0.837$<br>$P = 0.308$ |

Examining the short-term effects of CUS exposure on behavior and physiology directly after termination of stressor exposure on day 19 (for statistics see Table 16), CUS mice showed surprisingly reduced anxiety-like behavior measured in the LDB, indicated by increased time spent in the LB (Figure 24A) and reduced latency to enter the LB (Figure 24B), compared to SHC. However, physiological stress parameters could confirm induction of stress by CUS exposure, as CUS mice showed reduced body weight gain (Figure 24C), increased relative adrenal weight (Figure 24D), and reduced relative thymus weight (Figure 24E), compared to SHC, without affecting relative spleen weight (Figure 24F).

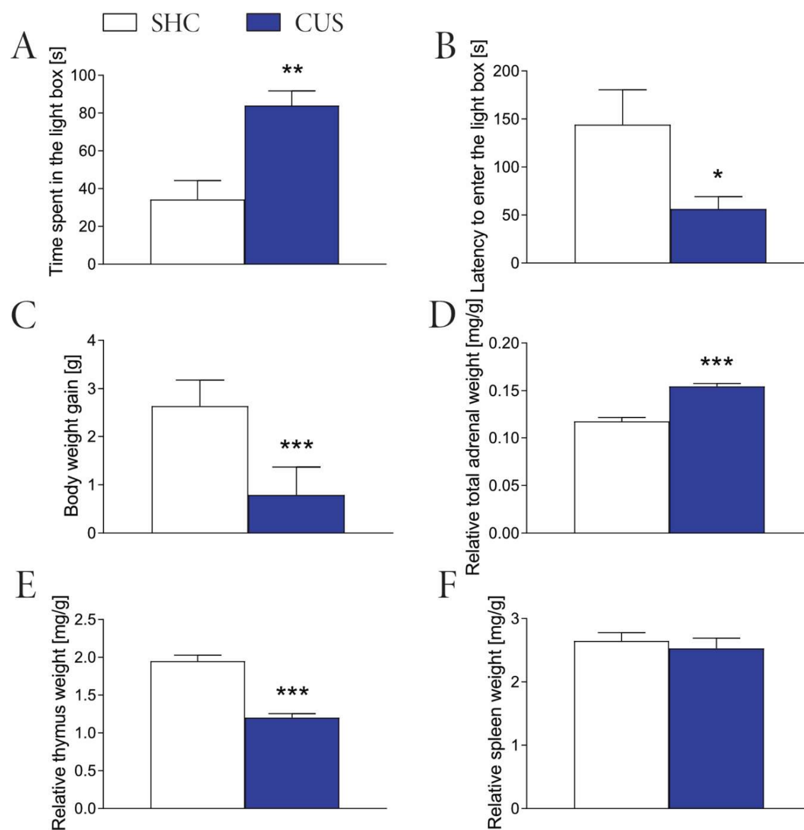



**Figure 24: Analyzing short-term effects, chronic unpredictable stress (CUS) exposure decreased anxiety-like behavior, but affected physiological stress parameters.** CUS animals showed reduced anxiety-like behavior in the light dark box (LDB; A-B), but decreased body weight gain (C) and relative thymus weight (E) while increasing relative adrenal weight (D), compared to single housed controls (SHC), without affecting spleen weight (F). Data represent mean + SEM. * p ≤ 0.050, ** p ≤ 0.010, *** p ≤ 0.001 CUS vs SHC; n = 7-8 per group; for detailed statistics see Table 16.

**Table 16: Statistics of short-term effects of CUS exposure on anxiety-like behavior and physiological stress parameters (Experiment 3.2a).** Factor represents SHC vs CUS effects.

| Anxiety-like behavior | Independent T-Test, Mann-Whitney-U Test (latency) | Figure 24A-B |
|---|---|---|
| Time spent in the light box | $T_{14} = -3.91$ | P = 0.002 |
| Latency to enter the light box | $T_{8.70} = 2.28$ | P = 0.050 |
| **Body weight** | **One way ANOVA for RM** | **Figure 24C** |
| Body weight gain | $T_{14} = 6.59$ | P < 0.001 |
| **Physiological parameters** | **Independent T-Test** | **Figure 24D-F** |
| Relative total adrenal weight | $T_{12} = -6.94$ | P < 0.001 |
| Relative thymus weight | $T_{14} = 7.79$ | P < 0.001 |
| Relative spleen weight | $T_{14} = 0.550$ | P = 0.591 |

### Effects of CUS exposure on CFC

In order to compare the effects of a social (CSC) versus a non-social (CUS) chronic stress paradigm on CFC outcome, mice were exposed to 19 days of CUS and after 2 days of habituation to the CFC paradigm. Successful induction of chronic stress was only validated by decreased body weight gain directly after CUS exposure, but not until 5 days after stressor termination on the day of CFC retention. In addition, CUS did not affect anxiety-like behavior (EPM) or organ weight, indicating insufficient induction of chronic stress in CUS mice of this specific experiment (see Table A6).

Studying the effects of CFC showed increased freezing behavior of all mice along the number of CS-US pairings during acquisition and slightly reduced freezing behavior along CS presentations during extinction, indicating successful induction and partly extinction of cued fear in mice. However, as the level of freezing stayed high even during retention in all mice (50 to 60 % time spent freezing), extinction success was low. Nevertheless, CFC was found to be independent of CUS exposure, as CUS mice and SHC did not differ in freezing behavior neither during acquisition (Figure 25A), nor extinction (Figure 25B), nor retention (Figure 25C; for statistics see Table 17). However, as this might be based on insufficient induction of chronic stress in CUS mice, this result has to be interpreted with caution.

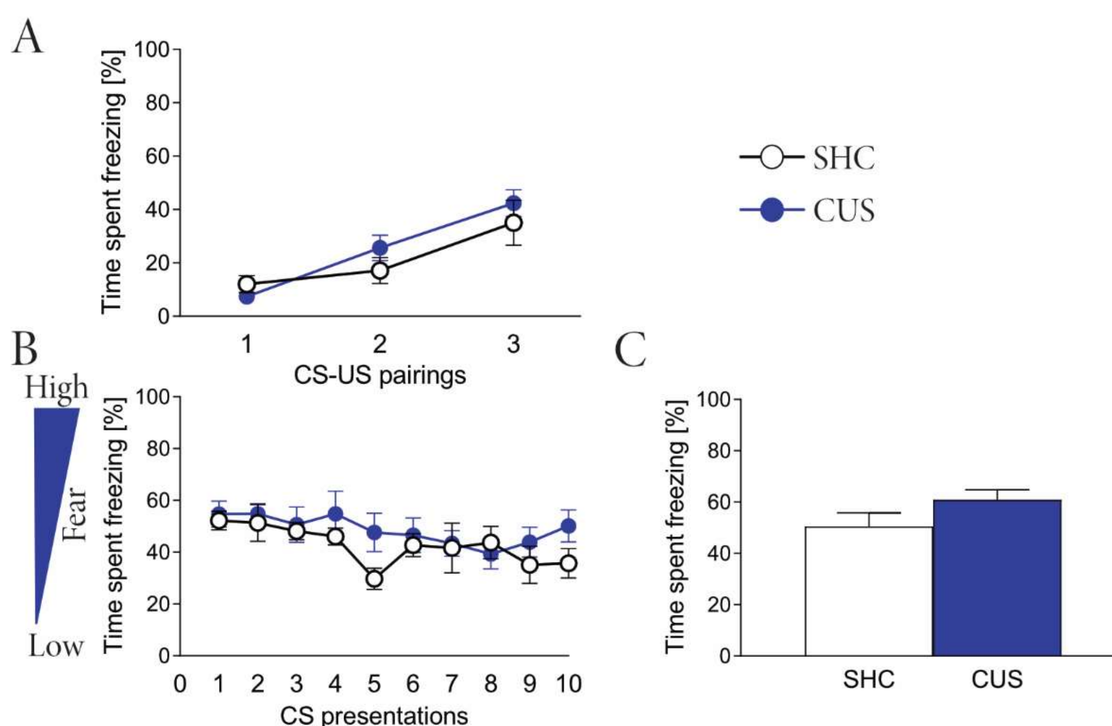



**Figure 25: Chronic unpredictable stress (CUS) exposure did not affect freezing behavior during cued fear conditioning (CFC).** CUS mice and single housed controls (SHC) did not differ in their freezing behavior neither during acquisition (A), nor extinction (B), nor retention (C). CS: conditioned stimulus, US: unconditioned stimulus. Data represent mean +/± SEM; n = 8 per group; for detailed statistics see Table 17.

**Table 17: Statistics of the effects of CUS exposure on CFC (Experiment 3.2b).** Factor time represents CS-US pairings during acquisition and CS presentations during extinction; factor CUS represents SHC vs CUS effects.

| Acquisition | One-way ANOVA for RM | Figure 25A |
|---|---|---|
| Time spent freezing | (Time) $F_{2,28} = 25.4$<br>(CUS) $F_{1,14} = 0.464$<br>(Time x CUS) $F_{2,28} = 1.59$ | $P < 0.001$<br>$P = 0.507$<br>$P = 0.221$ |
| **Extinction** | **One-way ANOVA for RM** | **Figure 25B** |
| Time spent freezing | (Time) $F_{9,126} = 2.46$<br>(CUS) $F_{1,14} = 1.24$<br>(Time x CUS) $F_{9,126} = 0.871$ | $P = 0.013$<br>$P = 0.284$<br>$P = 0.553$ |
| **Retention** | **Independent T-Test** | **Figure 25C** |
| Time spent freezing | (CUS) $T_{14} = -1.56$ | $P = 0.141$ |

### Effects of CUS exposure on SFC

Next, I compared the effects of social (CSC) versus non-social (CUS) chronic stress on social fear expression, thus, mice were exposed to 19 days of CUS and after 2 days of habituation, underwent the SFC paradigm. In this experiment, successful induction of chronic stress was validated by increased anxiety-like behavior on the EPM, decreased body weight gain directly ($p < 0.001$) and 5 days after stressor termination (SFC recall; $p < 0.010$), as well as increased relative adrenal and reduced absolute thymus weight in CUS mice compared to SHC (see Table A7).

After successful validation of CUS on major stress parameters, I analyzed the effects of CUS exposure on behavioral responses in the SFC paradigm (for statistics see Table 18). During social fear acquisition, CUS mice and SHC did not differ in the number of foot shocks, necessary to induce social avoidance (Figure 26A). Nevertheless, neither SHC nor CUS mice showed successful extinction of social fear as SHC/SFC$^+$ and CUS/SFC$^+$ showed lower social investigation at all social stimuli (1 to 6: $p < 0.001$) compared to respective SFC$^-$ during both extinction (Figure 26B) and recall (Figure 26C). Interestingly, during extinction training, CUS/SFC$^-$ showed more investigation at the second to sixth social stimulus compared to SHC/SFC$^-$ ($p < 0.05$), which might indicate increased seeking for social support in CUS mice, whereas social fear expression and extinction was not affected by CUS exposure.

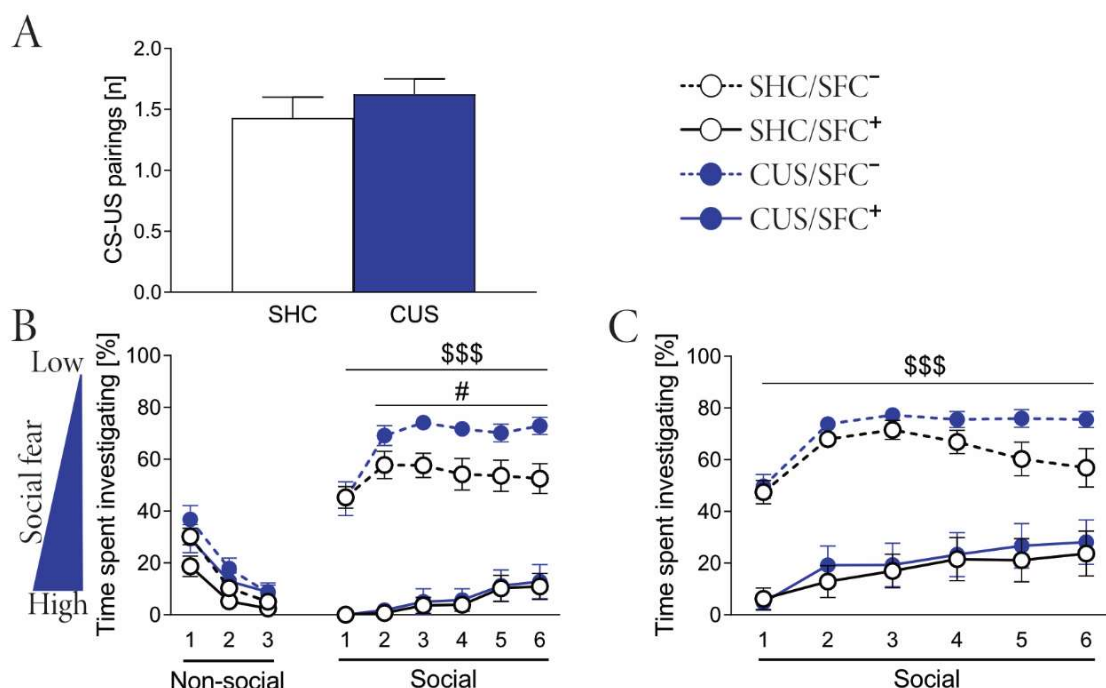



**Figure 26: During social fear conditioning (SFC), chronic unpredictable stress (CUS) exposure increased investigation time in SFC⁻ mice, without affecting social fear extinction behavior in SFC⁺.** During acquisition, CUS mice and single housed controls (SHC) needed the same amount of shocks to induce social avoidance (A). During extinction, CUS/SFC⁻ showed more social investigation compared to SHC/SFC⁻, but there was no difference between CUS/SFC⁺ and SHC/ SFC⁺ (B). However, recall was found to be independent of stress exposure (C). Data represent mean + SEM. # $p \leq 0.050$ CUS/SFC⁻ vs SHC/SFC⁻; $$$ $p \leq 0.001$ SFC⁺ vs SFC⁻; n = 16 per group; for detailed statistics see Table 18.

**Table 18: Statistics of the effects of CUS exposure on SFC (Experiment 3.2c).** Factor time represents stimulus presentations during SFC extinction and recall; factor CUS represents SHC vs CUS effects; factor SFC represents SFC⁻ vs SFC⁺ effects.

| Acquisition | Independent T-Test | Figure 26A |
|---|---|---|
| CS-US pairings | (CUS) $T_{29} = -1.10$ | $P = 0.279$ |
| **Extinction** | **Two-way ANOVA for RM** | **Figure 26B** |
| Time spent investigating | (Time) $F_{8,472} = 48.1$ | $P < 0.001$ |
| | (CUS) $F_{1,59} = 7.23$ | $P = 0.009$ |
| | (SFC) $F_{1,59} = 194$ | $P < 0.001$ |
| | (Time x CUS) $F_{8,472} = 1.09$ | $P = 0.367$ |
| | (Time x SFC) $F_{8,472} = 61.0$ | $P < 0.001$ |
| | (CUS x SFC) $F_{1,59} = 1.96$ | $P = 0.167$ |
| | (Time x CUS x SFC) $F_{8,472} = 2.19$ | $P = 0.027$ |
| **Recall** | **Two-way ANOVA for RM** | **Figure 26C** |
| Time spent investigating | (Time) $F_{5,295} = 19.5$ | $P < 0.001$ |
| | (CUS) $F_{1,59} = 1.08$ | $P = 0.302$ |
| | (SFC) $F_{1,59} = 87.3$ | $P < 0.001$ |
| | (Time x CUS) $F_{5,295} = 1.16$ | $P = 0.331$ |
| | (Time x SFC) $F_{5,295} = 3.68$ | $P = 0.003$ |
| | (CUS x SFC) $F_{1,59} = 0.602$ | $P = 0.441$ |
| | (Time x CUS x SFC) $F_{5,295} = 0.783$ | $P = 0.562$ |

### 3.3.3 Strain differences in behavior, fear- and stress-response

As the CSC paradigm was originally established in BL6 mice and SFC in CD1 mice, I further investigated differences between these two mouse strains with respect to anxiety-related and social behavior, as well as CFC and SFC. Moreover, I studied the effects of CSC exposure on social fear in CD1 mice.

**Strain differences in anxiety-like behavior and CFC**

First, I analyzed strain differences in anxiety-like behavior in the EPM and LDB (for statistics see Table 19). CD1 mice showed reduced anxiety-like behavior in both EPM and LDB compared to BL6 mice. In detail, CD1 mice spent more time on the open arms (Figure 27A) and displayed more open arm (Figure 27B) and full open arm entries in the EPM (Figure 27C), which was accompanied by increased locomotion (closed arm entries; Figure 27D). In line, in the LDB, CD1 mice spent more time in the LB compared to BL6 mice (Figure 27E), without affecting the latency to enter the LB (Figure 27F).

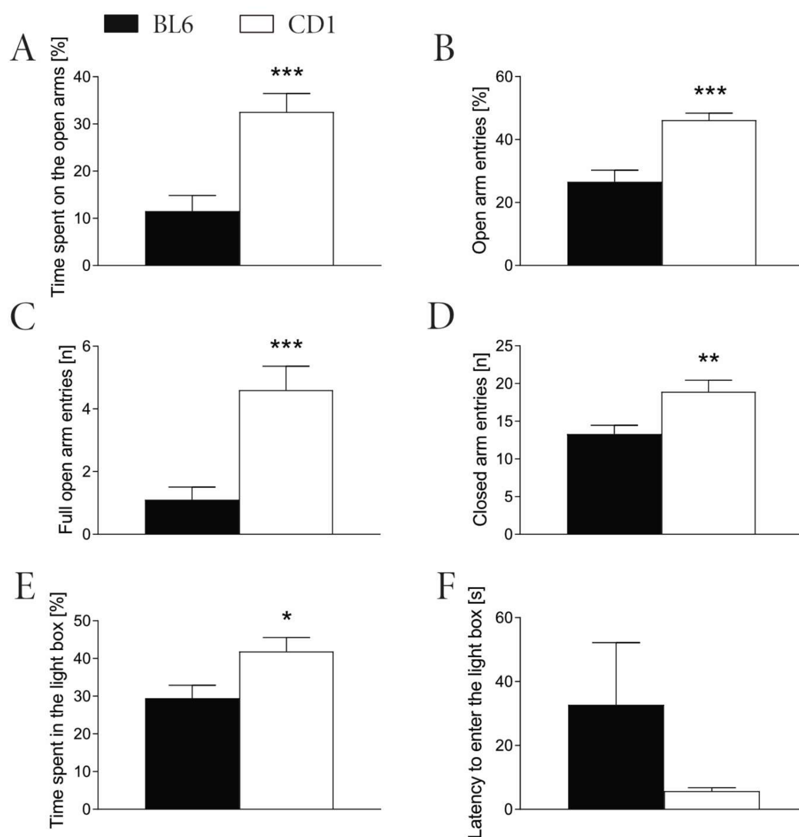



**Figure 27: CD1 mice showed reduced anxiety-like behavior compared to C57BL/6N (BL6) mice.** CD1 mice show reduced anxiety-like behavior on the elevated plusmaze (EPM; A-D) and light dark box (LDB; E-F). In detail, CD1 mice spent more time on the open arms (A) and had more open arm entries (B), full open arm entries (C) and latency to enter the open arm (D), compared to BL6. In the LDB, CD1 mice spent more time in the light box (LB; E), without affecting the latency to enter the LB (F), compared to BL6. Data represent mean + SEM. * $p \leq 0.050$, ** $p \leq 0.010$, *** $p \leq 0.001$ CD1 vs BL6; n = 10 per group; for detailed statistics see Table 19.

**Table 19: Statistics of strain differences on anxiety-like behavior (Experiment 3.3a).** Factor represents BL6 vs CD1 effects.

| Anxiety-like behavior | Independent T-Test, Mann-Whitney-U Test (latency) | Figure 27A-F |
|---|---|---|
| EPM: Time spent on the open arms | $T_{18} = -4.08$ | $P = 0.001$ |
| EPM: Open arm entries | $T_{18} = -4.57$ | $P < 0.001$ |
| EPM: Full open arm entries | $T_{18} = -4.05$ | $P = 0.001$ |
| EPM: Closed arm entries | $T_{18} = -2.89$ | $P = 0.010$ |
| LDB: Time spent in the light box | $T_{18} = -2.45$ | $P = 0.025$ |
| LDB: Latency to enter the light box | $T_{9.05} = 1.39$ | $P = 0.182$ |

I also found substantial differences between the mouse lines in the CFC paradigm (for statistics see Table 20). Specifically, during cued fear acquisition, CD1 mice showed less freezing behavior at the second to the last CS-US pairing compared to BL6 mice ($p < 0.010$; Figure 28A). In line, freezing behavior stayed lower in CD1 compared to the high freezing behavior of BL6 mice at all CS presentations during extinction ($p < 0.001$; Figure 28B) and retention (Figure 28C).

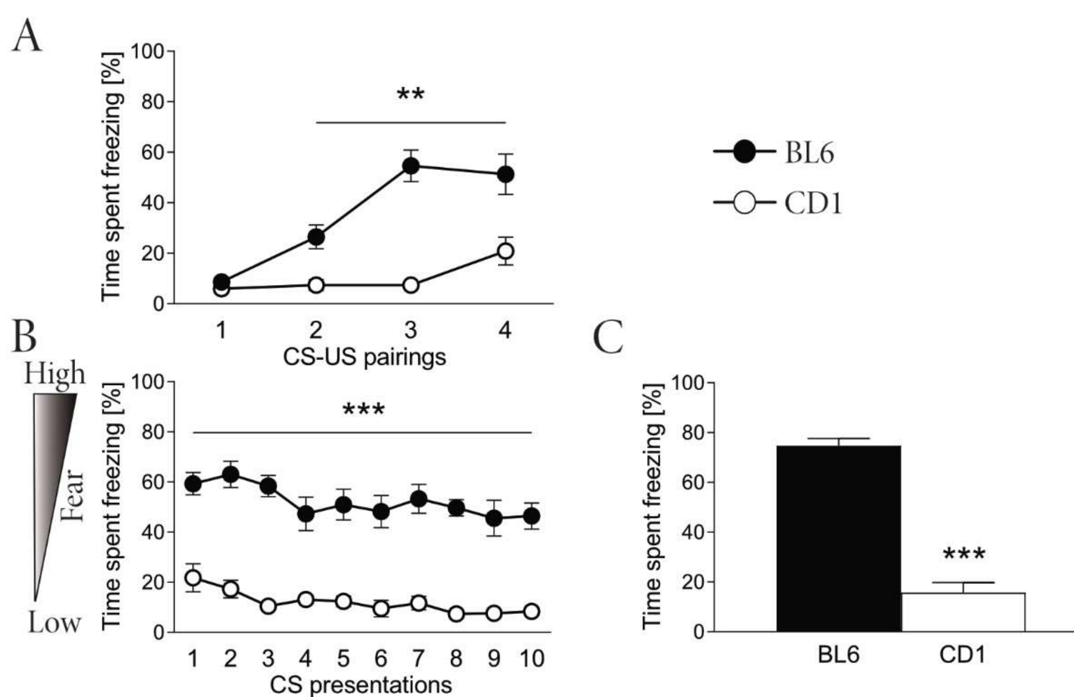



**Figure 28: During cued fear conditioning (CFC), CD1 mice showed lower freezing behavior compared to C57BL/6N (BL6) mice.** During acquisition (A), as well as extinction (B) and retention (C), CD1 mice showed lower freezing behavior compared to BL6 mice. CS: conditioned stimulus, US: unconditioned stimulus. Data represent mean +/± SEM. ** $p \leq 0.010$, *** $p \leq 0.001$ CD1 vs BL6; n = 10 per group; for detailed statistics see Table 20.

**Table 20: Statistics of strain differences on CFC (Experiment 3.3a).** Factor time represents CS-US pairings during acquisition and CS presentations during extinction; factor strain represents BL6 vs CD1 effects.

| Acquisition | One-way ANOVA for RM | Figure 28A |
|---|---|---|
| Time spent freezing | (Time) $F_{3,54} = 20.1$<br>(Strain) $F_{1,18} = 125$<br>(Time x strain) $F_{3,54} = 10.2$ | $P < 0.001$<br>$P < 0.001$<br>$P < 0.001$ |
| Extinction | One-way ANOVA for RM | Figure 28B |
| Time spent freezing | (Time) $F_{9,162} = 3.52$<br>(Strain) $F_{1,18} = 116$<br>(Time x strain) $F_{9,162} = 0.616$ | $P = 0.001$<br>$P < 0.001$<br>$P = 0.782$ |
| Retention | Independent T-Test | Figure 28C |
| Time spent freezing | (Strain) $T_{18} = 11.7$ | $P < 0.001$ |

**Strain differences in social preference and SFC**

Next, I revealed strain differences in social behavior, analyzing social preference and SFC outcome in CD1 and BL6 mice (for statistics see Table 21). In the SPT, both strains showed preference towards the social over the non-social stimulus ($p \leq 0.001$), however, CD1 mice spent generally more time investigating both stimuli compared to BL6 ($p < 0.001$; Figure 29A).

In agreement with the general lower stress-sensitivity of CD1 mice, they needed more shocks to induce social avoidance behavior during social fear acquisition compared to BL6 mice (Figure 29B). During social fear extinction training, performed on the next day, they expressed less social fear and showed proper extinction of the same, which was in contrast to the non-responding BL6 mice. In detail, CD1/SFC$^+$ showed reduced investigation towards social stimuli 1 and 2 ($p < 0.050$) compared to CD1/SFC$^-$, while BL6/SFC$^+$ avoided all social stimuli compared to BL6/SFC$^-$ ($p < 0.010$). Moreover, CD1/SFC$^+$ investigated all social stimuli, except the first one, longer (social stimuli 2 to 6: $p < 0.001$) than BL6/SFC$^+$ (Figure 29C). These finding were confirmed during recall, as BL6/SFC$^+$ still showed reduced investigation time at all social stimuli compared to BL6/SFC$^-$ and CD1/SFC$^+$ ($p < 0.001$; Figure 29D).

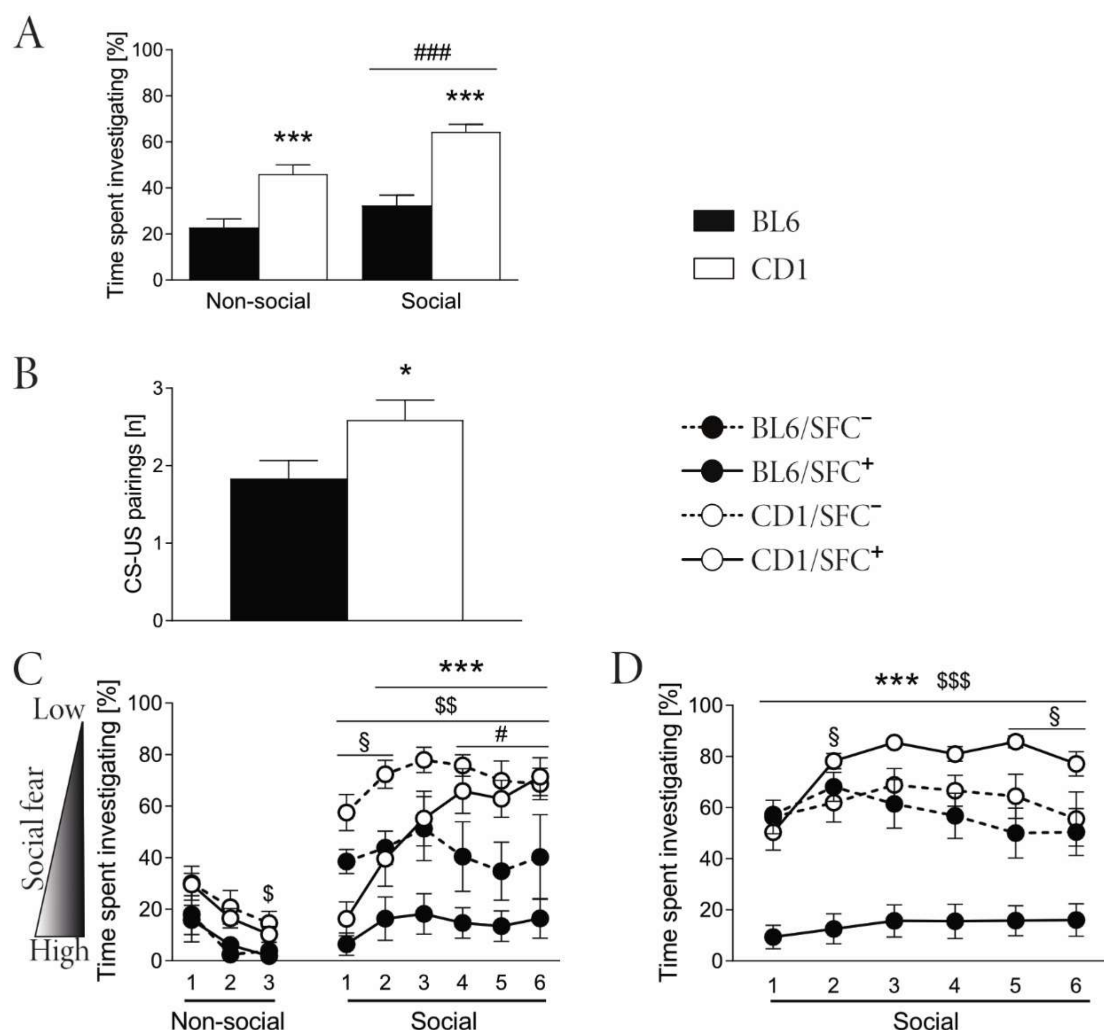



**Figure 29: Both CD1 and C57BL/6N (BL6) mice showed social preference abilities, while CD1 mice investigated both stimuli more. During social fear conditioning (SFC) acquisition CD1 mice needed less shocks, but showed a faster extinction of social fear compared to BL6 mice.** During the social preference test (SPT), CD1 and BL6 mice showed social preference abilities, while CD1 mice investigated both stimuli more (A). During SFC acquisition, CD1 mice needed less shocks to induce social avoidance behavior (B) and showed more social investigation time during extinction (C) and recall (D) compared to BL6 mice, which displayed an impaired extinction of the learned social fear. Data represent mean +/± SEM. * $p \leq 0.050$, *** $p \leq 0.001$ CD1 vs BL6 and CD1/SFC$^+$ vs BL6/SFC$^+$; # $p \leq 0.050$, ### $p \leq 0.001$ social vs non-social stimulus in the SPT and CD1/ SFC$^-$ vs BL6/ SFC$^-$; \$ $p \leq 0.050$, \$\$ $p \leq 0.010$, \$\$\$ $p \leq 0.001$ BL6/SFC$^+$ vs BL6/ SFC$^-$; § $p \leq 0.050$ CD1/SFC$^+$ vs CD1/ SFC$^-$; n = 31 – 33 per group for SPT, n = 11 – 18 for SFC; for detailed statistics see Table 21.

**Table 21: Statistics of strain differences on social preference and SFC (Experiment 3.3b).** Factor time represents time represents stimulus presentations during SPT and SFC extinction and recall; factor strain represents BL6 vs CD1 effects; factor SFC represents $SFC^-$ vs $SFC^+$ effects.

| SPT | One-way ANOVA for RM | Figure 29A |
|---|---|---|
| Time spent sniffing | (Time) $F_{1,62} = 45.8$<br>(Strain) $F_{1,62} = 29.6$<br>(Time x strain) $F_{1,62} = 4.43$ | $P < 0.001$<br>$P < 0.001$<br>$P = 0.039$ |
| **Acquisition** | **Independent T-Test** | **Figure 29B** |
| CS-US pairings | (Strain) $T_{33} = -2.179$ | $P = 0.037$ |
| **Extinction** | **Two-way ANOVA for RM** | **Figure 29C** |
| Time spent investigating | (Time) $F_{8,432} = 46.8$<br>(Strain) $F_{1,54} = 33.8$<br>(SFC) $F_{1,54} = 29.2$<br>(Time x strain) $F_{8,432} = 14.0$<br>(Time x SFC) $F_{8,432} = 12.3$<br>(Strain x SFC) $F_{1,54} = 8.1$<br>(Time x strain x SFC) $F_{8,432} = 2.48$ | $P < 0.001$<br>$P < 0.001$<br>$P < 0.001$<br>$P < 0.001$<br>$P < 0.001$<br>$P = 0.006$<br>$P = 0.012$ |
| **Recall** | **Two-way ANOVA for RM** | **Figure 29D** |
| Time spent investigating | (Time) $F_{5,265} = 5.18$<br>(Strain) $F_{1,53} = 40.5$<br>(SFC) $F_{1,53} = 7.24$<br>(Time x strain) $F_{5,265} = 3.00$<br>(Time x SFC) $F_{5,265} = 3.32$<br>(Strain x SFC) $F_{1,53} = 32.4$<br>(Time x strain x SFC) $F_{5,265} = 1.37$ | $P < 0.001$<br>$P < 0.001$<br>$P = 0.010$<br>$P = 0.012$<br>$P = 0.006$<br>$P < 0.001$<br>$P = 0.238$ |

**Strain differences in the CSC paradigm and consequences on SFC**

To reveal strain differences in the context of chronic psychosocial stress on social fear expression, CD1 mice were exposed to the CSC paradigm, too. Here, I compared stress coping strategies in CD1 and BL6 mice during the first 30 minutes, when the CSC mice encountered a novel resident on day 1 and day 8 of CSC housing. The analyzed behaviors included offensive, i.e., active stress coping, and defensive behaviors, i.e., passive stress coping. Moreover, during passive stress coping strategies, the reactive emotional coping behavior, i.e., submissive upright or flight behavior, was analyzed. On day 1 (for statistics see Table 22), CD1 mice showed a higher number of attacks (Figure 30A) and general more offensive behavior towards the larger resident and the other CSC mice compared to BL6 mice (Figure 30B). In turn, CD1 mice received less attacks by the opponents (Figure 30C) and showed less flight behavior (Figure 30D) compared to BL6 mice. However, submissive upright did not differ between CD1 and BL6 mice (Figure 30E). In sum, CD1 mice showed less total defensive behavior (Figure 30F) and a lower ratio of offensive / defensive behavior compared to BL6 (Figure 30G). Thus, during the initial phase of colony formation on day 1, CD1 mice showed more active stress coping behavior, whereas BL6 mice displayed mostly passive stress coping behavior, thus, receiving more attacks.

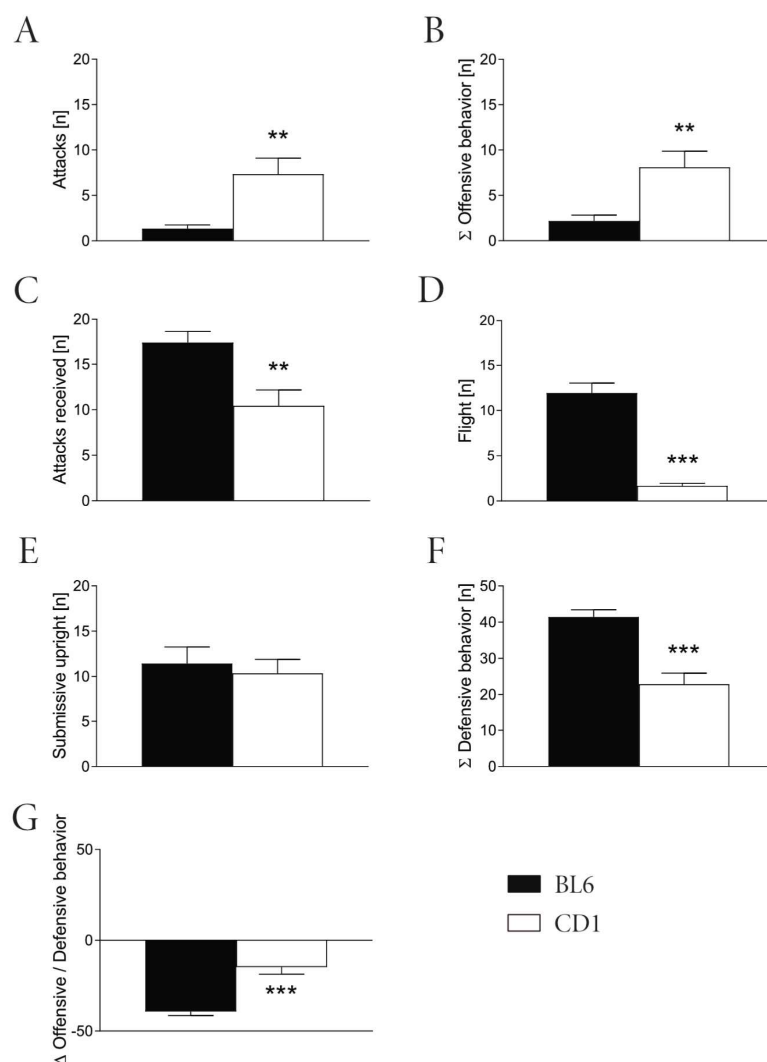



**Figure 30: On day 1 of chronic subordinate colony housing (CSC) exposure, CD1 mice showed more offensive, but less defensive behavior compared to C57BL/6N (BL6) mice.** During CSC on day 1, CD1 mice showed a higher number of attacks (A) and total offensive behavior (B) compared to BL6 mice. Moreover, CD1 mice received less attacks (C), showed less flight behavior (D) but equal submissive upright (E) compared to BL6 mice. Total defensive behavior (F) and the ratio offensive / defensive behavior (G) was lower in CD1 compared to BL6 mice. Data represent mean + SEM. ** $p \leq 0.010$, *** $p \leq 0.001$ BL6 vs CD1; n = 12 per group; for detailed statistics see Table 22.

**Table 22: Statistics of strain differences on coping behavior during CSC on Day 1 (Experiment 3.3c).** Factor represents BL6 vs CD1 mice.

| Behavior CSC day 1 | Independent T-Test, Mann-Whitney-U Test (attacks, offensive, flight) | Figure 30 |
|---|---|---|
| Attacks | $T_{12.2} = -3.29$ | P = 0.006 |
| Σ Offensive behavior | $T_{13.9} = -3.09$ | P = 0.008 |
| Attacks received | $T_{22} = 3.25$ | P = 0.004 |
| Flight | $T_{12.4} = 8.84$ | P < 0.001 |
| Submissive upright | $T_{22} = 0.450$ | P = 0.657 |
| Σ Defensive behavior | $T_{22} = 5.08$ | P < 0.001 |
| Δ Offensive / Defensive behavior | $T_{22} = -5.42$ | P < 0.001 |

In contrast to the behavioral differences seen at the beginning of CSC housing, on day 8 of CSC exposure (for statistics see Table 23), CD1 and BL6 mice did not differ in active or passive stress coping strategies. Here, CD1 and BL6 mice showed equal number of attacks (Figure 31A) and offensive behavior (Figure 31B). However, CD1 mice received less attacks (Figure 31C), and showed less flight behavior (Figure 31D), but increased submissive upright compared to BL6 mice (Figure 31E). Summed up, on day 8 of CSC, CD1 and BL6 mice did not differ in total defensive behavior (Figure 31F) or the ratio of offensive / defensive behavior (Figure 31G), but they differed in their reactive emotional coping behavior strategy.

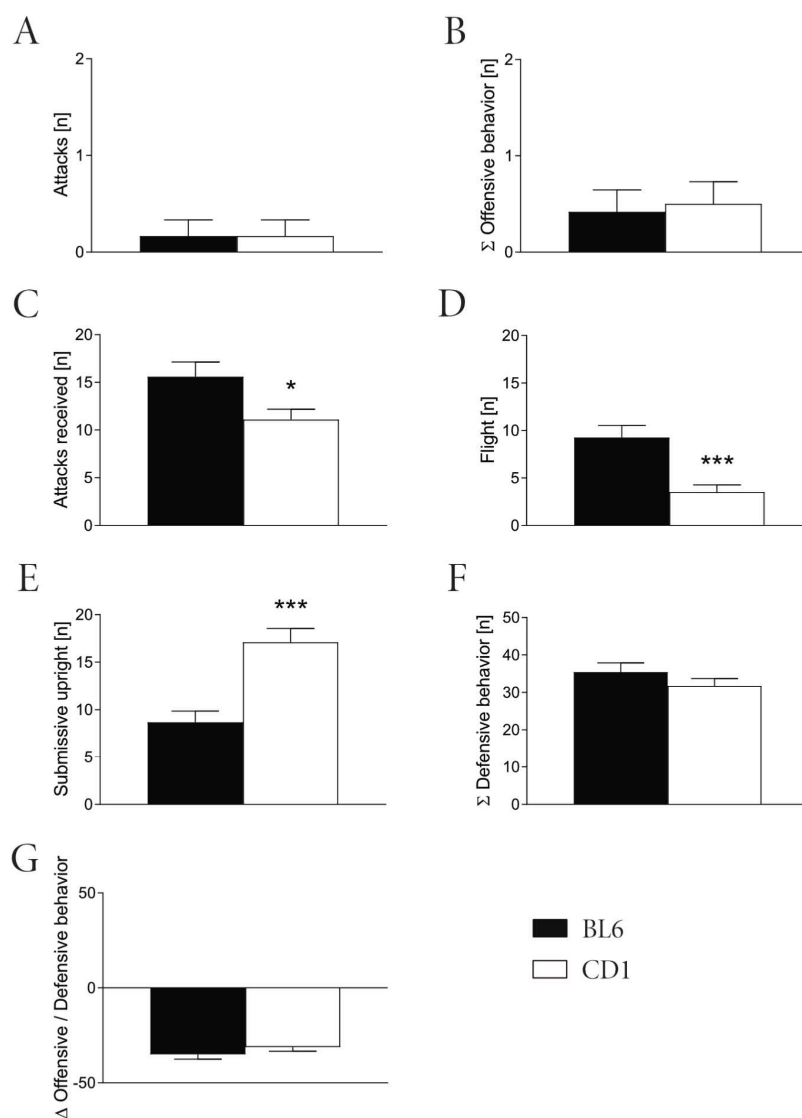



**Figure 31: On day 8 of chronic subordinate colony housing (CSC) exposure, CD1 and C57BL/6N (BL6) mice showed equal offensive and defensive behavior, but differed in reactive coping strategies.** During CSC on day 8, CD1 and BL6 mice showed equal number of attacks (A) and total offensive behavior (B). However, analyzing defensive behavior, CD1 mice received less attacks (C) and showed less flight behavior (D), but increased submissive upright (E) compared to BL6 mice. Total defensive behavior (F) and the ratio offensive / defensive behavior (G) were equal in CD1 and BL6 mice. Data represent mean + SEM. * $p \leq 0.050$, *** $p \leq 0.001$ BL6 vs CD1; $n = 12$ per group; for detailed statistics see Table 23.

**Table 23: Statistics of strain differences on coping behavior during CSC on Day 8 (Experiment 3.3c).** Factor represents BL6 vs CD1 mice.

| Behavior CSC day 8 | Independent T-Test | Figure 31 |
|---|---|---|
| Attacks | $T_{22} = 0.000$ | $P = 1.000$ |
| Σ Offensive behavior | $T_{22} = -0.257$ | $P = 0.800$ |
| Attacks received | $T_{22} = 2.36$ | $P = 0.028$ |
| Flight | $T_{22} = 3.87$ | $P = 0.001$ |
| Submissive upright | $T_{22} = -4.43$ | $P < 0.001$ |
| Σ Defensive behavior | $T_{22} = 1.16$ | $P = 0.260$ |
| Δ Offensive / Defensive behavior | $T_{22} = -1.13$ | $P = 0.269$ |

To analyze the effects of CSC exposure on SFC in CD1 mice, I firstly validated a successful induction of chronic stress in CSC mice, by confirming CSC effects in relative adrenal weight in CSC/SFC$^+$ compared to SHC/SFC$^+$ and in spleen weight in CSC mice compared to SHC, independent of conditioning, while body weight tended to be increased (see Table A8). However, as CD1 mice are lacking CSC-induced effects on anxiety-like behavior, number and IFN-γ release by mesLNCs, plasma CORT level, and adrenal *in vitro* ACTH sensitivity, as seen in BL6 mice (see 3.1), CD1 mice seem to be less stress-vulnerable compared to BL6 mice.

Next, CD1 CSC mice and SHC were tested in the SFC paradigm (for statistics see Table 24). Here, the number of shocks necessary to induce social avoidance during social fear acquisition did not differ between CSC mice and SHC (Figure 32A), which was in contrast to the findings in BL6 mice (see 3.1). During extinction training, all SFC$^+$ mice expressed social fear, indicated by reduced investigation time at the beginning. Here, SHC/SFC$^+$ showed less social investigation time at the first social stimulus compared to SHC/SFC$^-$ ($p < 0.05$), while CSC/SFC$^+$ investigated the social stimuli 1 to 5 less than CSC/SFC$^-$ ($p < 0.05$). Moreover, CSC/SFC$^+$ investigated all social stimuli, except the first one, less than SHC/SFC$^+$ ($p < 0.05$), indicating impaired extinction of social fear in CSC mice compared to SHC (Figure 32B), which confirms my findings in BL6 mice (see 3.1). During recall, only factor time and SFC showed an ANOVA effect, while CSC interactions showed only a tendency. Despite this, analyzing Bonferroni *post hoc* analysis of all factors revealed equal investigation time in SHC/SFC$^+$ and SHC/SFC$^-$ at all social stimuli, indicating successful extinction of social fear. In contrast, CSC/SFC$^+$ showed reduced investigation of social stimuli 1 to 3 compared to CSC/SFC$^-$. Moreover, CSC/SFC$^+$ showed reduced contact at social stimuli 1 and 2, and by trend at social stimulus 3 ($p = 0.061$), compared to SHC/SFC$^+$ ($p < 0.050$; Figure 32C), which is again in confirmation of my findings in BL6 mice (see 3.1).

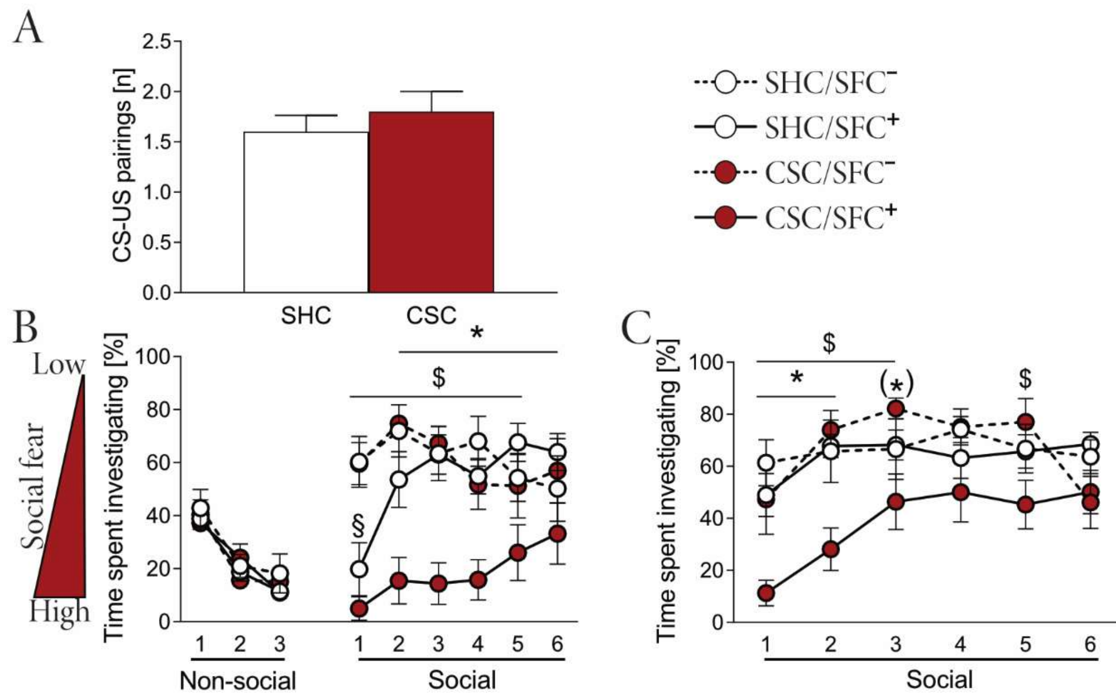



**Figure 32: Chronic subordinate colony housing (CSC) exposure impaired extinction of social fear in CD1 mice.** Even though CSC mice and single housed controls (SHC) show equal number of shocks during social fear conditioning (SFC) acquisition (A), CSC/SFC⁺ showed impaired extinction of learned social fear during extinction (B) and recall (C), compared to SHC/SFC⁺. During recall, factor CSC did only reach a trend. Data represent mean +/± SEM. (*) p = 0.061, * p ≤ 0.050 CSC/SFC⁺ vs SHC/SFC⁺; $ p ≤ 0.050 CSC/SFC⁺ vs CSC/SFC⁻; § p ≤ 0.050 SHC/SFC⁺ vs SHC/SFC⁻; unconditioned animals n = 6 per group; conditioned animals n = 10 per group; for detailed statistics see Table 24.

**Table 24: Statistics of the effects of CSC exposure on SFC in CD1 mice (Experiment 3.3c).** Factor time represents stimulus presentations during SFC extinction and recall; factor CSC represents SHC vs CSC effects; factor SFC represents SFC⁻ vs SFC⁺ effects.

| Acquisition | Independent T-Test | Figure 32A |
|---|---|---|
| CS-US pairings | (CSC) $T_{18}$ = -0.775 | P = 0.449 |
| **Extinction** | **Two-way ANOVA for RM** | **Figure 32B** |
| Time spent investigating | (Time) $F_{8,208}$ = 16.7 | P < 0.001 |
| | (CSC) $F_{1,26}$ = 5.80 | P = 0.023 |
| | (SFC) $F_{1,26}$ = 13.3 | P = 0.001 |
| | (Time x CSC) $F_{8,208}$ = 1.96 | P = 0.052 |
| | (Time x SFC) $F_{8,208}$ = 6.45 | P < 0.001 |
| | (CSC x SFC) $F_{1,26}$ = 5.51 | P = 0.027 |
| | (Time x CSC x SFC) $F_{8,208}$ = 2.82 | P = 0.005 |
| **Recall** | **Two-way ANOVA for RM** | **Figure 32C** |
| Time spent investigating | (Time) $F_{5,135}$ = 7.27 | P < 0.001 |
| | (CSC) $F_{1,27}$ = 2.87 | P = 0.102 |
| | (SFC) $F_{1,27}$ = 4.65 | P = 0.040 |
| | (Time x CSC) $F_{5,135}$ = 2.07 | P = 0.073 |
| | (Time x SFC) $F_{5,135}$ = 2.42 | P = 0.039 |
| | (CSC x SFC) $F_{1,27}$ = 3.70 | P = 0.065 |
| | (Time x CSC x SFC) $F_{5,135}$ = 2.00 | P = 0.083 |

## 3.4 Effects of acute OXT on social fear extinction behavior

In the previous experiments, I have revealed that CD1 mice have an enhanced social fear extinction compared to the impaired extinction in BL6 mice. In the next chapter, I will analyze OXT as a potential treatment option in fear-susceptible mice as well as in chronic stress-induced impaired SFC extinction.

### 3.4.1 Effects of acute OXT on SFC extinction training in BL6 mice

In order to investigate OXT as a potential treatment option in fear-susceptible mice, I firstly analyzed the effects of acute icv OXT (0.1 µg / 2 µl) treatment on social fear extinction in BL6 mice.

However, as during extinction only factor time and SFC, and during recall only factor SFC showed an ANOVA effect, whereas OXT interactions showed no significance (for detailed statistics see Table 25), *post hoc* analysis has not been performed in this experiment. Nevertheless, visual interpretation of extinction behavior revealed, that all $SFC^+$ mice expressed social fear as $SFC^+$/VEH and $SFC^+$/OXT showed reduced social investigation at social stimuli 1 to 6 compared to respective $SFC^-$/VEH and $SFC^-$/OXT (Figure 33A), which was still the case during recall (Figure 33B). However, neither $SFC^+$/VEH nor $SFC^+$/OXT showed extinction of the learned social fear throughout extinction and recall, as social investigated time stayed very low in all $SFC^+$ animals.

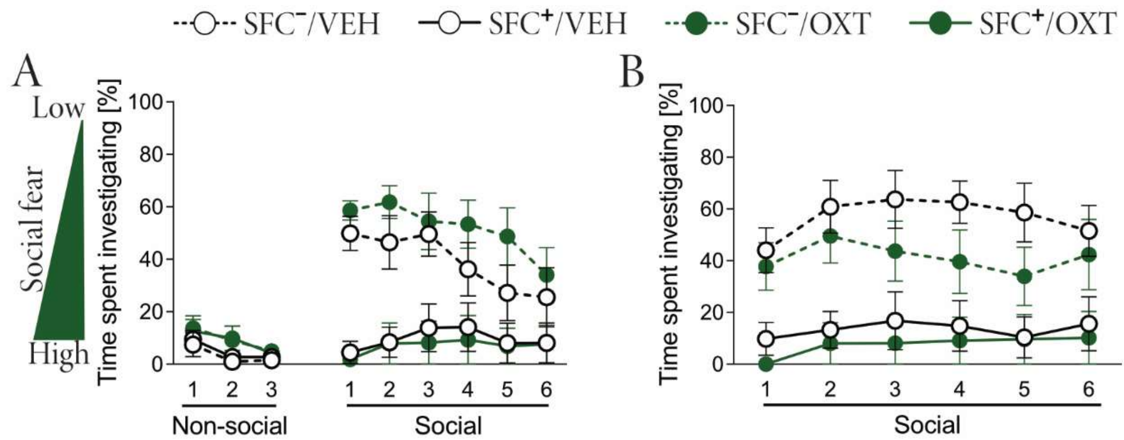



**Figure 33: Oxytocin (OXT; 0.1 µg / 2 µl) treatment did not affect behavior during social fear conditioning (SFC) in C57BL/6N (BL6) mice.** During SFC extinction, all $SFC^+$ animals showed social fear, revealed by reduced contact compared to $SFC^-$. However, neither OXT/$SFC^+$, nor VEH/$SFC^+$ showed extinction of the learned social fear during extinction (A) or recall (B). No *post hoc* analysis, as factor OXT did not reach significance. Data represent mean +/± SEM; n = 8-9 per group; for detailed statistics see Table 25.

**Table 25: Statistics of the effects of OXT on SFC extinction training in BL6 mice (Experiment 4.1).** Factor time represents stimulus presentations during SFC extinction and recall; factor OXT represents VEH vs OXT effects; factor SFC represents $SFC^-$ vs $SFC^+$ effects.

| Extinction | Two-way ANOVA for RM | Figure 33A |
|---|---|---|
| Time spent investigating | (Time) $F_{8,232} = 16.2$ | $P < 0.001$ |
| | (OXT) $F_{1,29} = 1.08$ | $P = 0.307$ |
| | (SFC) $F_{1,29} = 26.1$ | $P < 0.001$ |
| | (Time x OXT) $F_{8,232} = 0.342$ | $P = 0.949$ |
| | (Time x SFC) $F_{8,232} = 14.4$ | $P < 0.001$ |
| | (OXT x SFC) $F_{1,29} = 1.25$ | $P = 0.272$ |
| | (Time x OXT x SFC) $F_{8,232} = 0.550$ | $P = 0.818$ |
| **Recall** | **Two-way ANOVA for RM** | **Figure 33B** |
| Time spent investigating | (Time) $F_{5,145} = 1.91$ | $P = 0.096$ |
| | (OXT) $F_{1,29} = 1.49$ | $P = 0.232$ |
| | (SFC) $F_{1,29} = 18.2$ | $P < 0.001$ |
| | (Time x OXT) $F_{5,145} = 0.733$ | $P = 0.600$ |
| | (Time x SFC) $F_{5,145} = 0.701$ | $P = 0.624$ |
| | (OXT x SFC) $F_{1,29} = 0.316$ | $P = 0.578$ |
| | (Time x OXT x SFC) $F_{5,145} = 1.25$ | $P = 0.296$ |

### 3.4.2 Effects of acute OXT on SFC extinction following CSC exposure

Subsequently, I analyzed the effects of acute icv infusion of OXT (0.1 µg / 2 µl) 10 minutes prior to SFC extinction in both BL6 and CD1 mice, which underwent the CSC procedure beforehand (CSC/SFC$^{+}$) to see, if OXT can counteract the chronic stress-induced impairment of SFC extinction and whether there are strain differential effects.

**Effects of acute OXT on SFC extinction following CSC in BL6 mice**

First, I analyzed the effect of acute icv OXT on SFC extinction following CSC exposure in BL6 mice. Validation of successful induction of chronic stress in CSC mice was confirmed by increased anxiety-like behavior (tested on the EPM), decreased body weight gain directly after CSC exposure (day 19), increased body weight gain 5 to 6 days after CSC termination (SFC recall), and increased relative spleen weight in both VEH and OXT treated CSC mice compared to respective SHC (see Table A9).

After exposure to either CSC or SHC and surgical implantation of the icv cannula, mice were tested in the SFC paradigm (for statistics see Table 26). During SFC acquisition, CSC mice and SHC did not differ in the number of shocks necessary to induce social avoidance behavior (Figure 34A). During SFC extinction and after infusion of either VEH or OXT, all SHC/SFC$^{+}$, irrespective of treatment and in confirmation of the results in 3.4.1, showed induction of social fear, but did not extinguish it. In detail, SHC/SFC$^{+}$ investigated all social stimuli less compared to SHC/SFC$^{-}$ during both extinction ($p < 0.010$; Figure 34B), and recall ($p \leq 0.001$; Figure 34C), which was irrespective of treatment. Nevertheless, SFC$^{-}$/OXT showed increased investigation time at social stimuli 1, 3 ($p < 0.050$), and by trend 4 ($p = 0.058$) compared to SFC$^{-}$/VEH. Moreover, OXT treatment increased investigation time at non-social stimulus presentations in comparison to respective VEH treated mice (SFC$^{+}$/OXT vs SFC$^{+}$/VEH non-social 1 and 2: $p < 0.05$; SFC$^{-}$/OXT vs SFC$^{-}$/VEH non-social 1 to 3: $p < 0.050$).

Similar to the findings in SHC, also in CSC mice, all CSC/SFC$^{+}$, irrespective of treatment, showed induction of social fear, but did not extinguish it, as they investigated all social stimuli less compared to CSC/SFC$^{-}$ during both extinction ($p < 0.01$; Figure 34D), and recall ($p \leq 0.001$; Figure 34E). Unfortunately, in CSC mice, only factor time and SFC showed an ANOVA effect during extinction and recall, while OXT interactions showed no significance. Regardless, *post hoc* Bonferroni has been analyzed for all factors including factor OXT during extinction training. Here, OXT treatment did not affect extinction success, but again increased investigation time at social stimulus 1 ($p < 0.050$) in SFC$^{-}$/OXT compared to SFC$^{-}$/VEH. Moreover, OXT increased the contact towards non-social stimulus presentations in SFC$^{+}$ and SFC$^{-}$ compared to respective VEH treated

mice ($SFC^+$/OXT vs $SFC^+$/VEH non-social 2: $p < 0.050$; $SFC^-$/OXT vs $SFC^-$/VEH non-social 1 and 2: $p < 0.050$). These data indicate a pro-social effect of OXT in $SFC^-$ mice in both CSC mice and SHC, however, surgery prior to SFC in BL6 mice seems to prevent social fear extinction, which is in line to my findings in 3.4.1.

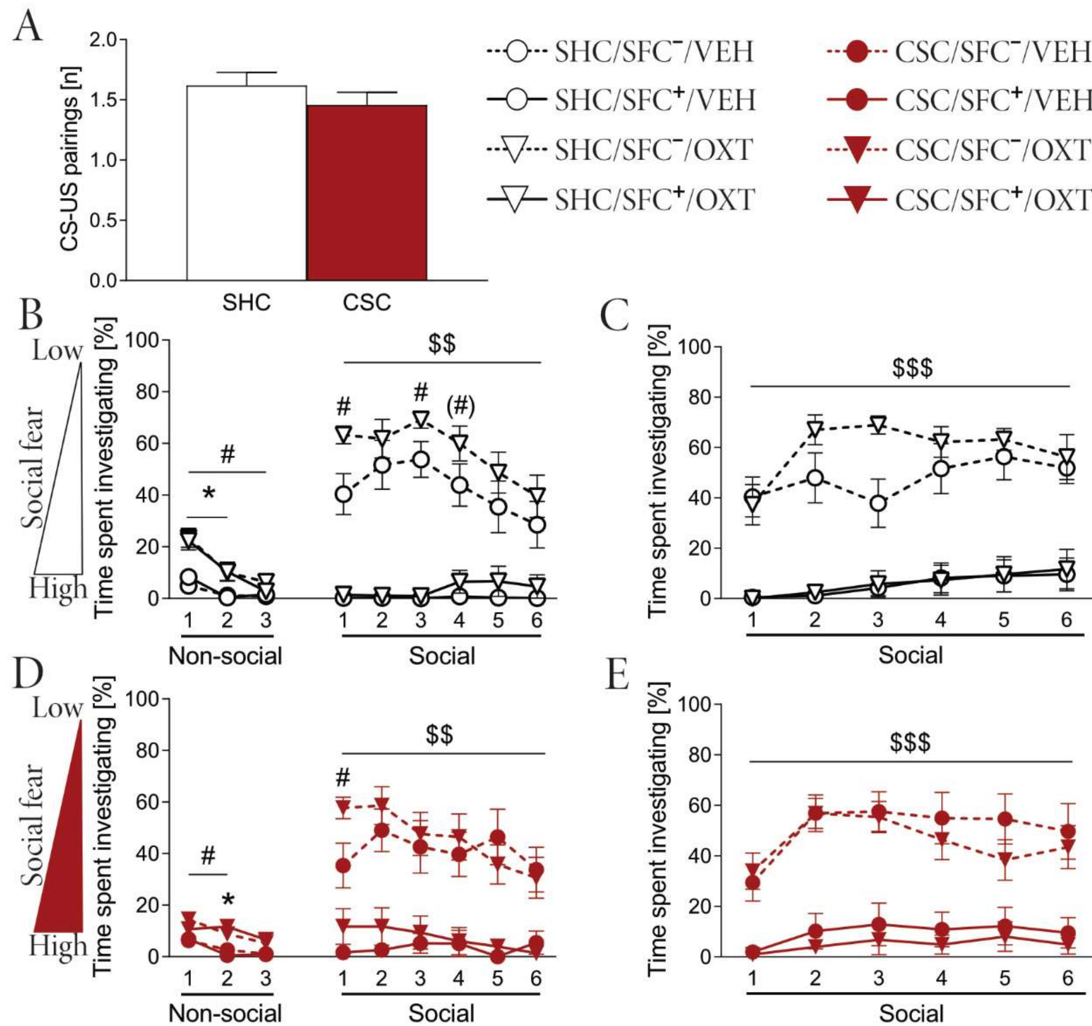



**Figure 34: Oxytocin (OXT; 0.1 µg / 2 µl) treatment did not affect social fear extinction training, neither in single housed controls (SHC), nor in chronic subordinate colony housing (CSC) C57BL/6N (BL6) mice, but increased social investigation in $SFC^-$.** During acquisition of the social fear conditioning (SFC) paradigm, CSC mice and SHC did not differ in the number of shocks (A). Moreover, in SHC, OXT treatment increased social investigation in SHC/$SFC^-$, while SHC/$SFC^+$ did not show extinction, independent of treatment (B), neither during recall (C). In CSC mice, OXT treatment increased non-social and social investigation in CSC/$SFC^-$, compared to respective vehicle (VEH), but did not affect social fear extinction in CSC/$SFC^+$. Independent of treatment, CSC/$SFC^+$ did not extinguish learned social fear neither during extinction (D), nor during recall (E). Factor OXT did not reach significance in CSC mice. Data represent mean +/± SEM. * $p \leq 0.050$ $SFC^+$/OXT vs $SFC^+$/VEH; (#) $p = 0.058$, # $p \leq 0.050$ $SFC^-$/OXT vs $SFC^-$/VEH; $$ $p \leq 0.010$, $$$ $p \leq 0.001$ $SFC^+$ vs $SFC^-$; acquisition n = 21-24 per group; extinction and recall n = 10-12 per group; for detailed statistics see Table 26.

**Table 26: Statistics of the effects of OXT on SFC extinction following CSC in BL6 mice (Experiment 4.2a).** Factor time represents stimulus presentations during SFC extinction and recall; factor OXT represents VEH vs OXT effects; factor SFC represents SFC$^-$ vs SFC$^+$ effects.

| Acquisition | Independent T-Test | Figure 34A |
|---|---|---|
| CS-US pairings | (CSC) $T_{43} = 1.07$ | $P = 0.292$ |
| **SHC** | | |
| Extinction | Two-way ANOVA for RM | Figure 34B |
| Time spent investigating | (Time) $F_{8,304} = 24.4$<br>(OXT) $F_{1,38} = 8.15$<br>(SFC) $F_{1,38} = 98.3$<br>(Time x OXT) $F_{8,304} = 0.789$<br>(Time x SFC) $F_{8,304} = 37.7$<br>(OXT x SFC) $F_{1,38} = 1.84$<br>(Time x OXT x SFC) $F_{8,304} = 0.617$ | $P < 0.001$<br>$P = 0.007$<br>$P < 0.001$<br>$P = 0.613$<br>$P < 0.001$<br>$P = 0.183$<br>$P = 0.764$ |
| Recall | Two-way ANOVA for RM | Figure 34C |
| Time spent investigating | (Time) $F_{5,195} = 4.15$<br>(OXT) $F_{1,39} = 1.77$<br>(SFC) $F_{1,39} = 108$<br>(Time x OXT) $F_{5,195} = 1.30$<br>(Time x SFC) $F_{5,195} = 1.46$<br>(OXT x SFC) $F_{1,39} = 1.38$<br>(Time x OXT x SFC) $F_{5,195} = 1.24$ | $P = 0.001$<br>$P = 0.192$<br>$P < 0.001$<br>$P = 0.266$<br>$P = 0.205$<br>$P = 0.247$<br>$P = 0.291$ |
| **CSC** | | |
| Extinction | Two-way ANOVA for RM | Figure 34D |
| Time spent investigating | (Time) $F_{8,336} = 17.5$<br>(OXT) $F_{1,42} = 2.53$<br>(SFC) $F_{1,42} = 61.6$<br>(Time x OXT) $F_{8,336} = 1.72$<br>(Time x SFC) $F_{8,336} = 17.2$<br>(OXT x SFC) $F_{1,42} = 0.004$<br>(Time x OXT x SFC) $F_{8,336} = 0.626$ | $P < 0.001$<br>$P = 0.119$<br>$P < 0.001$<br>$P = 0.094$<br>$P < 0.001$<br>$P = 0.950$<br>$P = 0.756$ |
| Recall | Two-way ANOVA for RM | Figure 34E |
| Time spent investigating | (Time) $F_{5,205} = 6.50$<br>(OXT) $F_{1,41} = 0.792$<br>(SFC) $F_{1,41} = 58.7$<br>(Time x OXT) $F_{5,205} = 0.761$<br>(Time x SFC) $F_{5,205} = 2.32$<br>(OXT x SFC) $F_{1,41} = 0.000$<br>(Time x OXT x SFC) $F_{5,205} = 0.557$ | $P < 0.001$<br>$P = 0.379$<br>$P < 0.001$<br>$P = 0.579$<br>$P = 0.044$<br>$P = 0.993$<br>$P = 0.733$ |

### Effects of acute OXT on SFC extinction following CSC in CD1 mice

Lastly, I investigated the effects of acute icv OXT treatment on SFC extinction following CSC exposure in CD1 mice. Induction of chronic stress in CSC mice was validated by increased anxiety-like behavior on the EPM, increased body weight gain, as well as by trend increased relative spleen weight in VEH treated CSC mice compared to respective SHC (see Table A10).

Next, mice were tested in the SFC paradigm (for statistics see Table 27). Here, they did not differ in the number of shocks, necessary to induce social avoidance behavior, during SFC acquisition (Figure 35A). As the effect of acute icv OXT treatment (0.1 µg / 2 µl)10 min prior to SFC extinction in CD1 males (without an additional stressor, mirroring SHC in this experiment) is already known

(Zoicas et al., 2014), the group size of SHC in this experiment is very low (n = 3-4 per group). On this account, analyzing SHC did not reach a statistical significance in factor OXT during extinction and recall. Thus, post hoc analysis has not been performed for SFC extinction and recall in SHC. However, social fear visually seemed to be successfully induced in all $SFC^+$ mice, which was at least party extinguished along social stimuli presentations with a higher investigation time in $SFC^+$/OXT mice compared to $SFC^+$/VEH during extinction (Figure 35B) and recall (Figure 35C).

In CSC mice, thru extinction, the negative effects of CSC exposure on social fear extinction seen in 3.3.3 could be confirmed, as $SFC^+$/VEH showed reduced investigation time at social stimuli 1 to 4 ($p < 0.050$) compared to $SFC^-$/VEH. Regarding OXT treatment, I could demonstrate that OXT rescued the impaired social fear extinction in CSC mice, as $SFC^+$/OXT, in comparison to $SFC^-$/OXT, showed reduced investigation time only at social stimulus 1 ($p < 0.050$). Moreover, $SFC^+$/OXT showed higher investigation at social stimuli 2 to 4 compared to $SFC^+$/VEH, indicating reduced social fear when CSC mice were treated with OXT prior to extinction training (Figure 35D). Interestingly, $SFC^+$/OXT showed also higher investigation at non-social stimulus 3 compared to $SFC^+$/VEH, indicating also reduced general anxiety-like behavior following OXT treatment. During recall, only factor time and SFC showed an ANOVA effect, while CSC interactions showed only a tendency. Despite this, analyzing post hoc Bonferroni for all factors revealed reduced social investigation at the first social stimulus in $SFC^+$/VEH compared to $SFC^-$/VEH. Regarding OXT treatment, I revealed more social investigation in $SFC^+$/OXT compared to $SFC^+$/VEH at social stimulus 1 ($p < 0.050$) and by trend social stimulus 2 ($p = 0.056$; Figure 35E). These data indicate that, at least in CD1 mice, CSC-induced impaired social fear extinction can be rescued by acute icv OXT treatment 10 min prior to extinction training, which even lasts until recall.

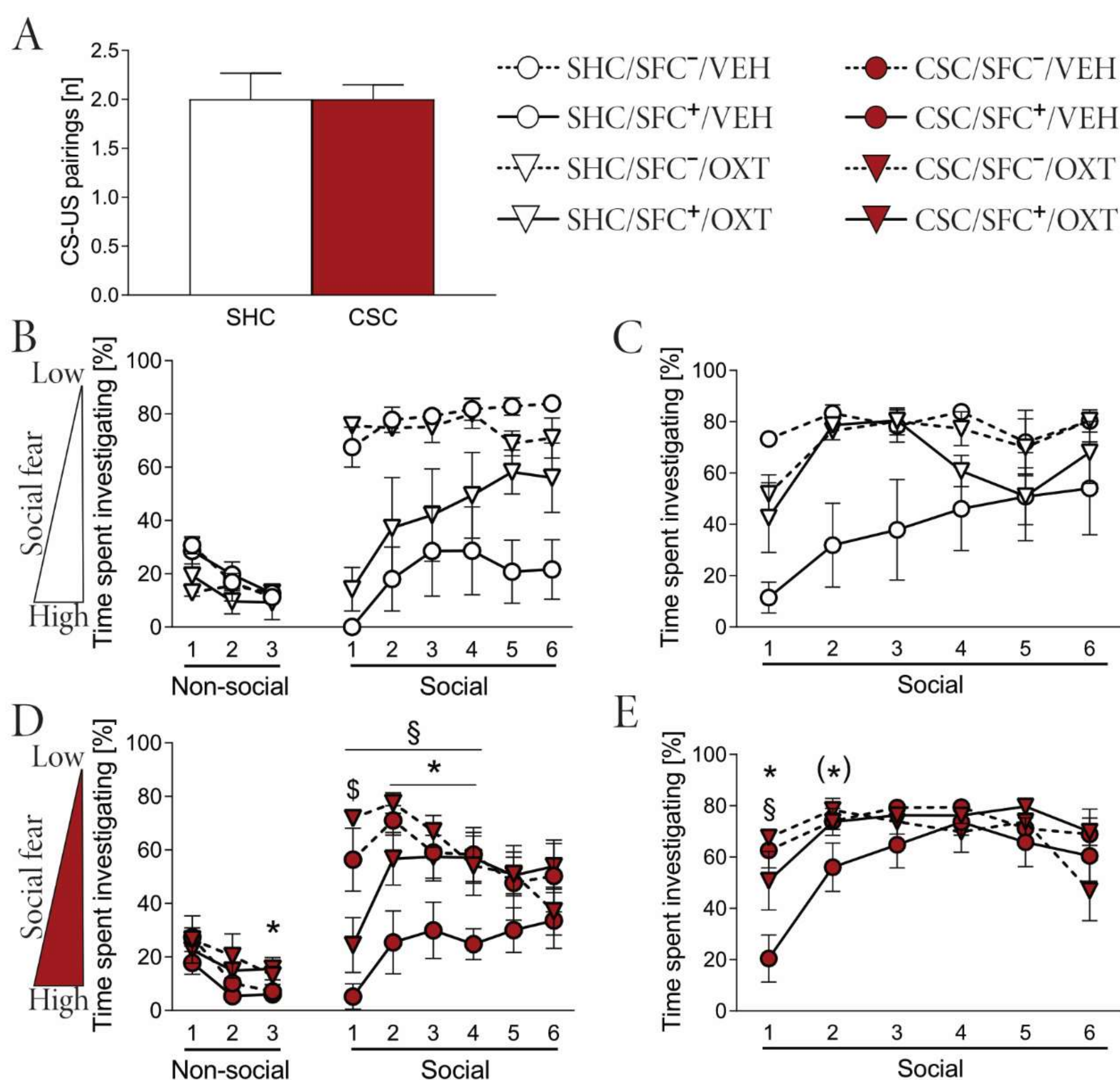



**Figure 35: Oxytocin (OXT; 0.1 µg / 2 µl) treatment facilitated social fear extinction in chronic subordinate colony housing (CSC) CD1 mice.** During acquisition of the social fear conditioning (SFC) paradigm, CSC mice and single housed controls (SHC) did not differ in the number of shocks (A). Moreover, in SHC, OXT treatment did not affect social fear extinction (B), or recall (C), which might be based on low animal numbers. In CSC mice, OXT treatment facilitated social fear extinction, indicated by increased social investigation in $SFC^+$/OXT compared to $SFC^+$/VEH. Moreover, CSC/$SFC^+$/VEH showed impaired extinction of social fear, as they revealed lower investigation levels throughout extinction training (D). These effects are even present during recall (E). Factor OXT did not reach significance in SHC, and reached only a trend during recall in CSC mice. Data represent mean +/± SEM. (*) $p = 0.056$, * $p \leq 0.050$ $SFC^+$/OXT vs $SFC^+$/VEH; $ $p \leq 0.050$ $SFC^+$/OXT vs $SFC^-$/OXT; § $p \leq 0.05$ $SFC^+$/VEH vs $SFC^-$/VEH; Acquisition: SHC n = 8 per group, CSC n = 17 per group; Extinction and recall: SHC n = 3-4 per group, CSC n = 6-8 per group; for detailed statistics see Table 27.

**Table 27: Statistics of the effects of OXT on SFC extinction following CSC in CD1 mice (Experiment 4.2b).** Factor time represents stimulus presentations during SFC extinction and recall; factor OXT represents VEH vs OXT effects; factor SFC represents $SFC^-$ vs $SFC^+$ effects.

| Acquisition | Independent T-Test | Figure 35A |
|---|---|---|
| CS-US pairings | (CSC) $T_{23} = 0.000$ | $P = 1.000$ |
| **SHC** | | |
| Extinction | Two-way ANOVA for RM | Figure 35B |
| Time spent investigating | (Time) $F_{5,88} = 23.5$<br>(OXT) $F_{1,11} = 0.582$<br>(SFC) $F_{1,11} = 33.6$<br>(Time x OXT) $F_{8,88} = 1.18$<br>(Time x SFC) $F_{8,88} = 8.63$<br>(OXT x SFC) $F_{1,11} = 2.95$<br>(Time x OXT x SFC) $F_{8,88} = 1.64$ | $P < 0.001$<br>$P = 0.461$<br>$P < 0.001$<br>$P = 0.320$<br>$P < 0.001$<br>$P = 0.114$<br>$P = 0.126$ |
| Recall | Two-way ANOVA for RM | Figure 35C |
| Time spent investigating | (Time) $F_{5,55} = 6.12$<br>(OXT) $F_{1,11} = 1.29$<br>(SFC) $F_{1,11} = 8.49$<br>(Time x OXT) $F_{5,55} = 1.42$<br>(Time x SFC) $F_{5,55} = 0.631$<br>(OXT x SFC) $F_{1,11} = 3.35$<br>(Time x OXT x SFC) $F_{5,55} = 1.87$ | $P < 0.001$<br>$P = 0.280$<br>$P = 0.014$<br>$P = 0.232$<br>$P = 0.676$<br>$P = 0.094$<br>$P = 0.114$ |
| **CSC** | | |
| Extinction | Two-way ANOVA for RM | Figure 35D |
| Time spent investigating | (Time) $F_{8,200} = 29.0$<br>(OXT) $F_{1,25} = 4.99$<br>(SFC) $F_{1,25} = 8.89$<br>(Time x OXT) $F_{8,200} = 0.918$<br>(Time x SFC) $F_{8,200} = 6.60$<br>(OXT x SFC) $F_{1,25} = 2.43$<br>(Time x OXT x SFC) $F_{8,200} = 1.126$ | $P < 0.001$<br>$P = 0.035$<br>$P = 0.006$<br>$P = 0.502$<br>$P < 0.001$<br>$P = 0.131$<br>$P = 0.347$ |
| Recall | Two-way ANOVA for RM | Figure 35E |
| Time spent investigating | (Time) $F_{5,125} = 9.75$<br>(OXT) $F_{1,25} = 1.123$<br>(SFC) $F_{1,25} = 1.87$<br>(Time x OXT) $F_{5,125} = 2.21$<br>(Time x SFC) $F_{5,125} = 4.48$<br>(OXT x SFC) $F_{1,25} = 3.761$<br>(Time x OXT x SFC) $F_{5,125} = 0.449$ | $P < 0.001$<br>$P = 0.299$<br>$P = 0.184$<br>$P = 0.057$<br>$P = 0.001$<br>$P = 0.064$<br>$P = 0.814$ |

# Discussion

# 4 Discussion

In the present thesis, I could demonstrate for the first time that chronic psychosocial stress prior to acute social trauma exposure facilitated traumatic memory encoding and increased the risk to develop a PTSD- and SAD-like phenotype in mice. Hence, CSC exposure prior to SFC exaggerated social fear acquisition and impaired its extinction. Moreover, this effect seemed to be not associated with social deficits in CSC mice, but with a brain region-dependent increase of the immune status. Especially dysregulated GILZ expression in the amygdala might play a critical role in this context. Nevertheless, the nature of the traumatic event, i.e., whether the fear conditioning procedure includes a social component, and the nature and composition of the chronic stressor paradigm, seem to be essential for the impact of chronic stress-induced impaired fear extinction, as CSC exposure did not affect CFC outcome. Moreover, CUS exposure did not affect fear-related behavior during CFC or SFC. Nevertheless, it has to be mentioned that CUS mice tested in the CFC paradigm did not show successful induction of chronic stress, despite temporally reduced body weight gain, while during SFC, neither CUS mice nor SHC extinguished the learned social fear. Furthermore, BL6 and CD1 mice were found to strongly differ in trait anxiety-, fear-, and stress-related behavior. Unexpectedly, these mouse strains did not differ in the impact of CSC exposure on social fear extinction, indicating a severe and general high influence of chronic psychosocial stress on social trauma extinction. Finally, treating mice with the pro-social, stress-buffering, and anti-inflammatory nonapeptide OXT did not show any effects in BL6 mice, since BL6/SFC$^{+}$ did not show social fear extinction following surgery, neither under basal conditions 10 days after recovery, nor in CSC mice or SHC after 4 days of recovery. However, in CD1 mice acute OXT infusion (icv, 0.1 µg / 2 µl) facilitated extinction in SFC$^{+}$ and prevented CSC-induced impairment of social fear extinction.

## 4.1 Effects of chronic psychosocial stress on the consequences of social fear conditioning

Chronic psychosocial stress is a well-known risk factor for the development of somatic and affective disorders in humans and rodents (Backé et al., 2012; Langgartner et al., 2015; Nestler et al., 2002; Sgoifo and Meerlo, 2002; Slattery et al., 2012). The CSC model closely mimics this type of health-compromising stress and has been repeatedly described to induce numerous stress-related dysregulations (for details see introduction 1.5.3), e.g. a dysregulation of the HPA-axis, systemic inflammation, and increased anxiety-like behavior (Langgartner et al., 2015; Reber et al., 2016b; Reber et al., 2008; Reber et al., 2007). In confirmation, in all experiments using the CSC

paradigm in BL6 mice, successful induction of chronic stress in CSC mice was validated, by revealing at least three of the following known parameters: increased anxiety-like behavior, changes in body weight gain, increased relative adrenal and spleen weight, reduced thymus weight, elevated number of mesLNCs and production of IFN-γ by mesLNCs, increased spleen cell viability and splenic GC resistance, reduced *in vitro* ACTH sensitivity of the adrenal glands, and hypocorticism compared to SHC. In combination with SFC, relative adrenal weight and plasma CORT concentrations were the only parameters that were altered depending on both CSC and SFC exposure. However, neither in BL6 nor in CD1 mice, SFC exposure *per se* affected adrenal weight or plasma CORT level significantly, while visually adrenal glands and plasma CORT level seemed to be reduced in $SFC^+$ compared to $SFC^-$. Adrenal weight has been shown to increase during in the initial phase of stressor exposure (Uschold-Schmidt et al., 2013), indicating that also acute stress has an impact on adrenal weight. However, to my knowledge, there are no studies reporting reduced adrenal weight following severe acute traumatic experience. Since $SFC^+$ animals seemed to have also lower plasma CORT levels, especially in CSC mice, smaller adrenal glands might be a compensating mechanism following continuous low CORT release. Fittingly, BL6 CSC mice showed reduced CORT levels compared to SHC, reflecting hypocorticism, which seemed even lower in $CSC/SFC^+$ compared to $CSC/SFC^-$, although this did not reach statistical significance (see Table A1). As hypocorticism is a common PTSD symptom (Boscarino, 1996; Heim et al., 2000; Rohleder et al., 2004), these data confirm my hypothesis that combined exposure to chronic psychosocial stress and an acute traumatic event even elevates the risk to develop a PTSD-, but also SAD-like phenotype. In line, CSC mice show a hyperactivity of the HPA axis following an acute heterotypic stressor (Füchsl et al., 2013a; Uschold-Schmidt et al., 2012), which is commonly known in PTSD patients re-experiencing the traumatic event (Bremner et al., 2003; Elzinga et al., 2003). In agreement, social fear acquisition resembles such an acute stressor inducing long-lasting socially related fears (Toth et al., 2012b). Nevertheless, 2 hours after SFC acquisition, CSC mice did neither show hyperactivity of the HPA axis (see Table A4), nor hypocorticism. This might be based on the assessed time point of plasma sampling, as CORT levels drop to basal levels 60 to 90 minutes after stressor termination (Keeney et al., 2006; Neumann et al., 1998), and, therefore, the initial rise following SFC acquisition might have declined to the level of SHC. In line, hyperactivity of the HPA axis following acute stressor exposure in CSC mice has been studied 5 to 10 minutes following stressor termination (Füchsl et al., 2013a; Uschold-Schmidt et al., 2012), while in PTSD patients hyperactivity of the HPA axis following trauma re-exposure was present until 20 to 25 minutes, declining to the level of healthy controls thereafter (Bremner et al., 2003; Elzinga et al., 2003). Nevertheless, as hypocorticism is also absent, plasma CORT level might still be higher in $CSC/SFC^+$ than prior to SFC acquisition.

Moreover, as social stimuli presentations resemble traumatic memory re-exposure, the HPA axis might be hyperactive in CSC mice following the first social stimulus during extinction training. However, this needs to be further analyzed.

In addition, successful induction of chronic stress in CSC mice was confirmed by increased anxiety-like behavior, tested directly following CSC exposure in the NOR, indicated by lower occurrence spent object sniffing. However, CSC mice did not show altered anxiety-like behavior in the OF test, as the time spent in the inner zone did not differ compared to SHC, which was accompanied with reduced locomotion (see Table A1). Nevertheless, reduced locomotion is in line to changes of homecage locomotion in CSC mice during the dark phase directly after CSC exposure, whereas hyperactivity was measured one week later (Slattery et al., 2012). Fittingly, CSDS reduced locomotion in the OF test (Sterlemann et al., 2008), and has been described as a characteristic of anxious animals (Landgraf and Wigger, 2002). In contrast, in the LDB, CSC mice showed reduced latency to enter the light compartment, indicating reduced anxiety-like behavior. However, as the time spent in the LB was unchanged between CSC mice and SHC (see Table A1), CSC exposure decreased the initial aversion against the light compartment, without affecting anxiety-like behavior in the LDB. But, unaffected anxiety-like behavior in the LDB might also be a methodical issue, as increased anxiety-like behavior following CSC exposure is a robust and long-lasting effect (Reber et al., 2007; Slattery et al., 2012). In confirmation, CSC mice revealed increased anxiety-like behavior when tested on the EPM (see Table A4, Table A9, Table A10), but repeatedly failed to show increased anxiety-like behavior when measured in the LDB (see Table A1, Table A2, Table A5). Accordingly, during SFC extinction training, CSC mice showed reduced investigation time towards non-social stimuli presentations compared to SHC, which was even reduced in CSC/SFC$^+$ compared to SHC/SFC$^+$ and CSC/SFC$^-$ (see Figure 12), indicating an increased anxiety-like behavior in CSC mice, which is exaggerated when CSC mice were exposed to an acute traumatic and stressful event. These data again confirm my hypothesis of an increased risk to develop stress-related psychiatric dysregulations including anxiety and PTSD, when mice are exposure to chronic psychosocial stress followed by a traumatic event. Moreover, during SFC extinction, CSC/SFC$^-$ showed reduced social investigation compared to SHC/SFC$^-$ at the first social stimulus, indicating reduced social interest or social motivation, which is in line to a lack of social preference in CSC mice (Slattery et al., 2012).

It is of special interest that CSC exposure facilitated the onset of social fear during acquisition, as CSC mice needed less shocks to show social avoidance behavior (see Figure 12), a core symptom of SAD, compared to SHC. This indicates that chronic psychosocial stressor exposure, probably by inducing PTSD-like symptoms (see 1.5.3), facilitates the encoding of a socially related traumatic

memory. In accordance, CSC/SFC$^+$ showed impaired extinction of social fear, as they had lower social investigation compared to CSC/SFC$^-$ and SHC/SFC$^+$, which even lasted until the last two social stimuli during recall. These data are in line to previous studies, showing that chronic psychosocial stress increased non-social fear acquisition and impaired its extinction (Azzinnari et al., 2014; Fuertig et al., 2016). Moreover, early-life stress in combination with stressor exposure during adulthood prevented extinction of non-social fear during the CFC paradigm (Mancini et al., 2021; Remmes et al., 2016). In contrast, early-life stress facilitated social fear extinction during adulthood, without affecting non-social fear expression in the CFC paradigm. Thus, early-life stress might improve (Zoicas and Neumann, 2016), whereas chronic psychosocial stress during adulthood seems to impair coping with and / or recovery from a traumatic social experience. Additionally, early-life stress spanning the early postnatal through peri-adolescent periods differentially affected the susceptibility towards chronic stressor exposure during adulthood (Peña et al., 2019). Thus, the timing and duration experiencing stressful events, as well as the composition and nature of the chronic stressor and the fear conditioning paradigms seem to influence fear-related behavior and memory. In rats, chronic RS exposure induced a hyperactivity of the amygdala, leading to an exaggerated and long-lasting fear memory following CFC (Hoffman et al., 2015). Moreover, increased amygdala activity has been associated with PTSD and social phobia (Liberzon and Sripada, 2007; Nutt and Malizia, 2004; Shin and Liberzon, 2010) and was found in CSC mice following acute stressor, i.e., open arm, exposure, but not following CSC exposure *per se*, which is thought to be caused by neuronal adaptation (Martinez et al., 1998; Singewald et al., 2009). Thus, analyzing the impact of the combination of CSC and SFC exposure on amygdala activity might reveal neuronal hyperactivity, especially in response to the trauma-reminding stimuli, i.e., the conspecifics, which might be an underlying mechanism in enhanced social fear memory and impaired extinction in CSC mice. Additionally, chronic psychosocial stress increased neuronal activity among others in the LS (Laine et al., 2017) a key region in SFC (Zoicas et al., 2014), as well as in the vHC (Laine et al., 2017), crucial in the regulation of fear and anxiety (Bannerman et al., 2004; Chen et al., 2016; Kjelstrup et al., 2002). Thus, impaired extinction of social fear following CSC exposure might be based on central mechanisms in the amygdala, LS and vHC.

These data confirm CSC as a robust model of chronic psychosocial stress in mice, leading to numerous long-lasting changes including PTSD-like symptoms, while SFC resembles an acute traumatic social event inducing social avoidance, a core symptom of the highly comorbid SAD. Thus, CSC exposure is hypothesized to sensitize an individual, leading to the manifestation of a PTSD- and SAD-like phenotype, when experiencing a traumatic social event. In confirmation, CSC/SFC$^+$ showed even a strengthening of PTSD- and SAD-like symptoms, as well as an impaired ability to extinguish the learned social fear. Interestingly, SFC extinction and recall training

resemble exposure therapies that are commonly performed to treat PTSD and SAD patients (Bradley et al., 2005; Hofmann et al., 2006; Rauch et al., 2012). Therefore, exposure to chronic psychosocial stress prior to the traumatic memory encoding might contribute to treatment-resistance, which is very common in patients suffering from PTSD (30 – 40 %) and SAD (30 – 50 %; see 1.3).

## 4.2 Behavioral and immunological changes underlying the consequences of chronic psychosocial stress on social fear conditioning

In the next part of the present thesis, I aimed to unravel possible behavioral and immunological changes underlying facilitated traumatic memory encoding and impaired extinction of social fear in CSC mice. As CSC exposure is associated with both impaired social behavior (Amoroso et al., 2020; Foertsch et al., 2019; Slattery et al., 2012) and systemic inflammation (Foertsch et al., 2019; Foertsch et al., 2017; Langgartner et al., 2019; Langgartner et al., 2015), which are highly involved in the development of a PTSD- and SAD-like phenotype (American Psychiatric Association, 2013; Carleton et al., 2011; Hofmann et al., 2003; Wang and Young, 2016), these two parameters will be discussed in this section.

### 4.2.1 Impact of chronic psychosocial stress on social behavior

Regarding social behavior, CSC mice were described to display lack of social preference (Slattery et al., 2012), however, nothing is known about the impact of CSC exposure on social memory abilities. In the present thesis, I could show that CSC mice showed normal social behavior including social preference in the SPT and intact social memory abilities in the mSDT (see Figure 13). The first result is in contrast to recent findings of long-term lack of social preference (Amoroso et al., 2020; Foertsch et al., 2019; Slattery et al., 2012). One reason might be that I performed the SPT in the homecage, which is in contrast to literature (Amoroso et al., 2020; Foertsch et al., 2019; Slattery et al., 2012). Performing the SPT in a novel environment represents an additional challenge for the mice that might influence social interaction. Moreover, performing the SPT in a novel environment, another animal model of chronic psychosocial stress, i.e., CSDS, induced social avoidance in stress-susceptible, but not resilient mice, which correlated with increased circulating IL-6 levels, a potent activator of the HPA axis (Lyson and McCann, 1991; Mastorakos et al., 1993). These effects were even independent of physical injury, i.e., amount of bite wounds (Hodes et al., 2014). Thus, CSC mice that are exposed to an additional challenge, like novel environment, might reveal a susceptible phenotype, probably caused by a hyperactivation of the HPA axis (Füchsl et al., 2013a; Uschold-Schmidt et al., 2012), thereby inducing social avoidance or

lack of social preference. In line, during SFC extinction training, $CSC/SFC^-$ showed reduced investigation time towards the first social stimulus compared to $SHC/SFC^-$ (see Figure 12), which strengthens my hypothesis, as extinction training is performed following an acute challenge, i.e., SFC acquisition.

Even though CSC mice and SHC showed intact short-term social memory abilities, neither CSC mice nor SHC could distinguish the conspecifics after 24 hours, indicating lack of long-term social memory in both. Nevertheless, mice are highly social species that can discriminate conspecifics for 24 hours (Noack et al., 2010; Richter et al., 2005) up to 7 days (Kogan et al., 2000). However, social memory formation requires olfactory information (Lukas et al., 2013; Richter et al., 2005) that is provided by close contact and anogenital sniffing. Thus, during social memory tests, juvenile conspecifics and freely moving animals are used (Lukas et al., 2013), which was not given in the present protocol, as conspecifics were enclosed in a wire mesh cage. Nevertheless, CUS exposure has been shown to impair short-term social discrimination abilities (van Boxelaere et al., 2017), while chronic social isolation in mice prevented long-term, but not short-term social discrimination (Kogan et al., 2000). Additionally, early-life stress, induced by maternal separation, impaired social memory without affecting social preference, while facilitating social fear extinction (Zoicas and Neumann, 2016). These data indicate that different types and timing of chronic stress exposure can affect social memory, however, cognition and fear memory seem to be differently regulated within the brain. In line, learning and memory tasks, including social memory, depend on HC signaling and activity (Fanselow and Dong, 2010; Jarrard, 1993; Kogan et al., 2000; Tzakis and Holahan, 2019). Moreover, stress-induced dendritic atrophy in HC neurons results in cognitive impairments (McEwen and Gianaros, 2010), which is in line to impaired memory formation following chronic stressor exposure (Ohl et al., 2000; Park et al., 2001). In contrast, I could show that chronic psychosocial stress enhanced social fear memory, which is in agreement to other studies demonstrating chronic stress-enhanced non-social fear memory and impaired extinction (Hoffman et al., 2015; Hoffman et al., 2014; Miracle et al., 2006). In fear-related memory, distinct brain regions were found to be involved including amygdala (Hoffman et al., 2015; Hoffman et al., 2014) and LS (Zoicas et al., 2014). Moreover, various studies revealed that the dorsal and ventral part of the HC have to be strictly distinguished, as the dorsal HC performs primarily cognitive functions, while the vHC regulates stress, emotion and affect (Fanselow and Dong, 2010). Thus, it is important to differentiate between dorsal HC-mediated cognitive memory (Ohl et al., 2000; Park et al., 2001) and vHC, amygdala and LS-mediated fear memory (Chen et al., 2016; Hoffman et al., 2014; Phillips and LeDoux, 1992; Zoicas et al., 2014). The latter being involved in CSC-induced enhanced social fear consolidation and impaired extinction.

### 4.2.2 Impact of chronic psychosocial stress, social fear acquisition and the combination of both on the immune system

The immune system is highly involved in stress and stress-related psychiatric disorders including PTSD and anxiety (Bauer and Teixeira, 2019; Dunn, 2000; Pruett, 2003; Wang and Young, 2016), while also the CSC paradigm has been shown to induce systemic peripheral inflammation (Langgartner et al., 2019; Langgartner et al., 2015; Reber et al., 2016a; Reber et al., 2016b). As discussed above, the amygdala, LS, and vHC are of special interest, due to its involvement in stress-related non-social and social fear memory, as well as social behavior, anxiety, PTSD, and SAD (Chen et al., 2016; Fanselow and Dong, 2010; Felix-Ortiz and Tye, 2014; Hoffman et al., 2015; Hoffman et al., 2014; Labuschagne et al., 2010; Liberzon and Sripada, 2007; Nutt and Malizia, 2004; Phillips and LeDoux, 1992; Shin and Liberzon, 2010; Stein et al., 2002; Zoicas et al., 2014). Thus, I studied inflammatory alterations in the periphery and, especially, the central immune system, focusing on the amygdala, septum, and vHC, following CSC exposure, SFC acquisition and the combination of both. Tissue samples were analyzed 2 hours after stressor termination, i.e., 2 hours after CSC exposure or SFC acquisition, as cytokine levels were shown to be increased 20 minutes to 48 hours after SD stress (Hodes et al., 2014). However, as the LS is too small to obtain micropunch-tissue only from this specific area, the complete septum has been analyzed.

**Impact of chronic psychosocial stress on the immune system**

Following CSC exposure, I could confirm systemic inflammation in the periphery by analyzing one of the known parameters (Foertsch et al., 2017), i.e., *in vitro* splenocyte cell viability of CSC mice and SHC following basal and LPS stimulation, as well as ascending CORT concentrations. In line to literature (Avitsur et al., 2003; Foertsch et al., 2017), CSC mice showed increased cell viability during all conditions, as well as a GC resistance of the splenocytes, since spleen cell viability stayed high when the cells were treated with ascending CORT concentrations. In contrast, spleen cell viability decreased along ascending concentrations in SHC until 0.1 µM CORT (see Figure 14). Increased cell viability at higher CORT concentrations (0.5 µM and 5 µM CORT) in SHC seems to be a technical or methodical issue and is in contrast to literature (Foertsch et al., 2017). Interestingly, splenocyte GC resistance as well as spleen weight were shown to be dependent on bite wounds that mice receive during social stressor exposure (Avitsur et al., 2001; Foertsch et al., 2017). However, bite wounds have not been scored in the present thesis, but CSC mice did freely interact with the residents, thus I cannot exclude physical harm. However, visually only few CSC mice had bite wounds in all experiments using BL6 CSC mice. Nevertheless, also other factors might contribute to splenic GC resistance and increased spleen weight, the latter being present following all CSC experiments in the present thesis (see Appendix). In line, CSDS has been shown

to increase the expression of non-coding micro RNAs that are involved in GC signaling and correlated with GC mRNA expression in splenic macrophages. In cell culture studies, two of these micro RNAs showed direct effects on mRNA expression of GRs, thereby significantly reducing LPS-induced overexpression of GRs (Jung et al., 2015). However, to my knowledge, there are no studies showing GC resistance in animal models using non-social stressors (Sheridan et al., 2000), except for one study of Mahanti and colleagues, demonstrating GC resistance following RS (Mahanti et al., 2018). Thus, it seems to be substantial that the chronic stressor paradigm involves a social situation, enabling the appearance of physical harm by bite wounds in the development of GC resistance (Foertsch et al., 2017; Sheridan et al., 2000).

In contrast to the periphery, analyzing central inflammation in the amygdala, septum, and vHC following CSC exposure, revealed rather decreased inflammation. Here, CSC exposure reduced mRNA expression of various microglia marker in the amygdala, including the resting microglia marker P2ry12, iNOS, a marker for pro-inflammatory microglia, and CD11b, which is expressed by microglia and macrophages with increased activity. In addition, CSC exposure increased expression of the anti-inflammatory mediator GILZ in the amygdala, as well as in the septum, where also the mRNA of the inflammasome Nlrp3 was reduced (see Figure 15). Reduced central inflammation is in line to literature showing reduced IL-1β, TNF-α and GRs in the striatum and HC following CSDS (Bartolomucci et al., 2003). In accordance, serotonin-induced activation of the HPA-axis, indicated by increased plasma CORT levels, reduced IL-1α expression in the HC and hypothalamus, as well as time-dependently IL-1β expression, indicating that central inflammation is dependent on CORT release (Gemma et al., 2003). However, this is in contrast to my findings, demonstrating hypocorticism in CSC mice, even though CSC exposure initially increases CORT release, which dampens already 48 hours after CSC exposure (Uschold-Schmidt et al., 2013). Another explanation might be the simultaneous release of anti-inflammatory mediators following stress that protect the brain against systemic inflammation (García-Bueno et al., 2008). In line, GILZ, which was increased in the amygdala and septum in CSC mice, is known to mediate the anti-inflammatory properties of GC (Ayroldi and Riccardi, 2009; Cannarile et al., 2019; Ronchetti et al., 2015). Following RS, GILZ was increased in the PFC and HC, which was absent in adrenalectomized mice (Yachi et al., 2007). Additionally, GILZ downregulated the pro-inflammatory and HPA axis stimulating cytokine IL-6 via the NFκB pathway (Thiagarajah et al., 2014), which has been shown to be increased in the periphery of CSC mice (Reber et al., 2016b). Interestingly, increased GILZ expression has been correlated with increased risk for a high symptom severity of PTSD (van Zuiden et al., 2012), while the amygdala is highly associated with the development of PTSD and SAD (Hoffman et al., 2015; Hoffman et al., 2014; Labuschagne et al., 2010; Phan et al., 2006). Nonetheless, another study showed reduced GILZ expression in male

PTSD patients (Lebow et al., 2019). Thus, dysregulated GILZ expression seems to be involved in PTSD, but might predict the risk of symptom development as well as the ongoing disorder differently. However, most human studies addressed peripheral inflammation, whereas only little is known about central inflammation in PTSD patients. Nevertheless, increased GILZ expression in the amygdala strengthens the findings of the CSC model as an animal model to study PTSD-like behavior (Reber et al., 2016a). In confirmation, PTSD has been associated with deficient microglia activation (Bhatt et al., 2020). Accordingly, amygdala mRNA analysis revealed increased GILZ expression accompanied by reduced microglia number and activity. Fittingly, GILZ is also expressed in microglia, which has been shown to be reduced following repeated SD stress, accompanied by increased pro-inflammatory mediators like IL-1β (Wohleb et al., 2011). Even though little is known about the interaction of GILZ and microglia activity, increased GILZ expression is highly likely to downregulate microglia activity to protect the brain against continuously high inflammation, as microglia are commonly increased following chronic stress (Tynan et al., 2010). Moreover, in the septum increased GILZ expression was accompanied by reduced mRNA expression of the inflammasome Nlrp3 in CSC mice compared to SHC. Interestingly, Nlrp3 is highly involved in the development of depression (Alcocer-Gómez and Cordero, 2014; Kaufmann et al., 2017), while stress-induced depressive-like behavior even requires Nlrp3 signaling (Alcocer-Gómez et al., 2016; Zhang et al., 2015). Additionally, neurons in the septum regulate and manifest depressive-like behaviors (Sheehan et al., 2004; Wang et al., 2021). This is of special interest, as CSC exposure does not affect depressive-like behavior, while CSDS and other chronic psychosocial and non-social stressor paradigms robustly induce depressive-like behavior (Goñi-Balentziaga et al., 2018; Herzog et al., 2009; Hollis et al., 2010; Kim and Han, 2006; Kreisel et al., 2014; Venzala et al., 2012; Walker et al., 2019; Yoon et al., 2014). Thus, reduced Nlrp3 expression in the septum might contribute to the lack of depressive-like behavior in CSC mice. Interestingly, reduced Nlrp3 and IL-1β expression in the PFC and HC were linked to increased stress-susceptibility following CUS exposure. However, stress-susceptibility was measured based on anxiety-like behavior, social behavior and depressive-like behavior (Yang et al., 2021). Therefore, Nlrp3 might have contributed specifically to an increase in depressive-like behavior, whereas IL-1β was not altered following CSC exposure. Nevertheless, decreased inflammation following CSC exposure in the amygdala and septum might be a protective mechanism of the brain to prevent systemic inflammation, which is seen in the periphery. In contrast, in the vHC, microglia number was increased, indicated by elevated Tmem119 mRNA expression, without affecting microglia phenotype or other inflammatory mediators (see Figure 15). In line, the vHC has been repeatedly described as a brain region involved in fear and anxiety (Fanselow and Dong, 2010), while stress-induced increased microglia number and activation

underlie the development of anxiety-like behavior (McKim et al., 2018a; Ramirez et al., 2016). Thus, increased microglia number in the vHC might contribute to the long-lasting and robust increase in anxiety-like behavior in CSC mice (Langgartner et al., 2015; Reber et al., 2007; Slattery et al., 2012).

In sum, CSC exposure rather decreased inflammation in the brain, probably mediated via increased GILZ expression, which might be a protective mechanism to prevent the brain from sustained inflammation. For a critical discussion, other relevant factors need to be included to prove reduced inflammation, which were not assessed, e.g. NFκB signaling pathways, by which GILZ exerts its anti-inflammatory properties (Auphan et al., 1995; Di Marco et al., 2007) and CRP, a marker for systemic inflammation (Copeland et al., 2014; Känel et al., 2007). Moreover, other studies suggest that reduced inflammation following chronic stress is mediated via sustained CORT release (Gemma et al., 2003). Even though, CSC mice show increased CORT expression during the initial time of CSC exposure, this declines already following 48 hours, resulting in hypocorticism (Uschold-Schmidt et al., 2013). Thus, central immune system might initially increase following reduced CORT levels, as seen in the periphery, leading to systemic inflammation also in the brain. However, to protect the brain, similar to the observations in HPA axis activity (see 1.5.3) (Langgartner et al., 2020; Reber et al., 2007), an adaptive mechanisms might downregulate inflammation, leading to decreased inflammation. Thus, measuring plasma CORT and central GC levels, as well as simultaneously central inflammatory mediators at different time points following CSC exposure might provide a better knowledge.

**Impact of social fear acquisition on the immune system**

Analyzing peripheral stress and immune parameters following SFC acquisition showed barely an effect (see Figure 16). In this experiment, an additional group, i.e., shock controls, has been added to unravel, whether electric foot shock exposure induces alterations in the stress and / or immune system, or if the induction of social fear ($SFC^+$) is required. Analyzing stress parameters, neither adrenal gland nor spleen weight or plasma CORT level were affected in $SFC^+$ or shock controls compared to $SFC^-$ 2 hours after stressor exposure. This is not surprising, because, e.g. an increase in adrenal glands weight has only been described after 10 hours or longer of social stressor exposure (Uschold-Schmidt et al., 2013). In addition, plasma CORT levels following acute stress were described to return to basal levels after 60 to 90 minutes (Johnstone et al., 2000; Keeney et al., 2006; Neumann et al., 1998). Moreover, social fear / social anxiety has been shown to affect rather the SNS than the HPA axis (Potts et al., 1991; Uhde et al., 1994; van Veen et al., 2008). Thus, follow up studies need to analyze the level of alpha-amylase, adrenalin, or noradrenalin in this context. Furthermore, plasma cytokine level were analyzed following SFC acquisition. Here,

neither IL-6, nor IL-4 were affected, while the anti-inflammatory cytokine IL-10 seemed to be downregulated in SFC⁺ compared to SFC⁻, as separate statistics revealed a trend. Fittingly, male SAD patients showed reduced plasma IL-10 concentrations compared to healthy men (Butler et al., 2022), while increased level of IL-1β, which is known to enhance fear learning, also decreases IL-10 level (Song et al., 2003). Unfortunately, due to low concentrations below the detection limit of the Luminex, plasma IL-1β levels could not be measured. However, as also spleen cell viability was not affected by SFC exposure, peripheral inflammation seemed to be not altered 2 hours after SFC acquisition.

Regarding the central immune system, the amygdala revealed increased GILZ expression in SFC⁺ compared to the other groups, whereas in the vHC, CD200R1 was reduced in shock controls compared to SFC⁻ (see Figure 17). Interestingly, the amygdala has been found to be involved in SFC processes (Zoicas et al., 2014), and is a key structure in anxiety disorders (Faria et al., 2012; Korn et al., 2017), PTSD (Hoffman et al., 2015; Hoffman et al., 2014) and SAD (Amaral, 2002; Labuschagne et al., 2010; Phan et al., 2006; Stein et al., 2002). Moreover, GILZ is one of the earliest genes following GC signaling (Bereshchenko et al., 2019) and rises following HPA axis stimulation (Cannarile et al., 2001; D'Adamio et al., 1997), while it regulates anti-inflammatory and pro-inflammatory pathways (Auphan et al., 1995; Di Marco et al., 2007; Nataraja et al., 2021). Thus, acute stress increases GILZ expression, which is in line to my findings. Nevertheless, shock controls did not show this rise in GILZ mRNA, even though they received two electric foot shocks. Thus, GILZ might have been high immediately following SFC exposure in both SFC⁺ and shock controls, but stayed high in SFC⁺ mice, developing social fear thereafter. Due to the fact that socially related fears can promote the development of PTSD (Collimore et al., 2009), my results are in line to the findings of van Zuiden and colleagues showing a correlation of increased GILZ expression with the development of PTSD symptoms (van Zuiden et al., 2012). Nevertheless, neither microglia nor cytokine expression were affected in the amygdala, while also no changes were found in the septum. The latter is surprising, as the LS plays a key role in SFC (Menon et al., 2018; Zoicas et al., 2014), while central inflammatory pathways, especially IL-1β signaling, are involved in social processes (DiSabato et al., 2021; Moieni and Eisenberger, 2018), fear memory (Song et al., 2003) and learning (Dong et al., 2020). Additionally, I analyzed the vHC, where SFC⁺ showed no differences, but shock controls revealed reduced expression of the microglia inhibitor CD200R1 compared to SFC⁻. CD200R1 binds to CD200, which inhibits microglia activity and the expression of pro-inflammatory mediators including TNF-α, IFNs, and iNOS (Vaine and Soberman, 2014). Thus, reduced expression of CD200R1 increases inflammation in the vHC, a brain region involved in fear and anxiety (Fanselow and Dong, 2010). In line, high doses of DEX treatment (Park et al., 2019), as well as inescapable tail shock, reduced CD200R1 in total HC tissue and more

specific in microglia isolated from the HC (Frank et al., 2018b; Frank et al., 2018a), while no other inflammatory marker was changed (Frank et al., 2018a). Thus, inescapable shock exposure might disinhibit microglia via reduction of CD200R1 leading to neuro-inflammatory priming (Frank et al., 2018a). Nevertheless, $SFC^+$ animals, which were able to escape the punishment by avoiding the conspecifics, which is in contrast to the shock controls, did not show changes in CD200R1 expression, indicating different inflammatory pathways in the development of social fear and following inescapable shock exposure.

Similar to PTSD, increased GILZ expression in the amygdala seems to be involved in the development of a SAD-like phenotype, which might be a possible mechanism of the high comorbidity of PTSD and SAD. Nevertheless, nothing is known so far about the impact of SFC on inflammation. Thus, also other brain regions might be involved like the dorsal HC, which is important for learning processes (Fanselow and Dong, 2010) and has the highest density of IL-1 receptor 1 (Cunningham et al., 1992).

**Impact of the combination of chronic psychosocial stress and social fear acquisition on the immune system**

When combining the CSC and the SFC paradigms, I first analyzed the peripheral immune system. Here, CSC mice showed increased number of mesLNCs, as well as an increased release of IFN-γ from mesLNCs, which is in confirmation of literature (Langgartner et al., 2015; Reber et al., 2016b; Reber et al., 2007), indicating systemic inflammation. However, this was not affected by SFC acquisition (see Figure 18), which is in line to my findings above. Nevertheless, re-exposing conditioned mice to the traumatic stimulus has been shown to rapidly increase plasma IFN-γ and IL-6 levels (Young et al., 2018), indicating systemic inflammation during extinction, but not acquisition of social fear. However, this has not been analyzed.

Examining changes of the central immune system following CSC exposure and SFC acquisition, revealed increased inflammation in all brain regions (see Figure 19), especially in the amygdala, where also protein levels were affected (see Figure 20). In detail, in the amygdala, CSC mice showed increased P2ry12 mRNA expression compared to SHC, while the M1 microglia marker iNOS was increased in CSC/$SFC^-$ compared to SHC/$SFC^-$. Moreover, protein levels revealed increased Tmem119, GILZ, and IL-4 expression, as well as increased phosphorylation at Ser276, i.e., activation, of the NFκB signaling pathway in CSC mice compared to SHC. These results are in contrast to my findings above analyzing central inflammation 2 hours following CSC exposure, where CSC mice showed reduced levels of P2ry12 and iNOS compared to SHC. However, the procedure of placing CSC mice in the SFC acquisition chamber, which is a novel environment, represents a stressful situation, even when they did not receive a foot shock ($SFC^-$), since mice are

also exposed to an unknown conspecific. As discussed in 4.2.1, exposing CSC mice to conspecifics in a novel environment resulted in a lack of social preference / social avoidance (Amoroso et al., 2020; Foertsch et al., 2019; Slattery et al., 2012). Accordingly, central inflammation might be decreased following chronic psychosocial stress exposure, but exaggerated when an acute challenge follows chronic psychosocial stress, which might be the reason for different outcomes in CSC mice and CSC/SFC$^-$. Nevertheless, increased expression of the resting microglia marker P2ry12 has been found to mediate cell migration following injury, and to play a critical role in synaptic plasticity (Bollinger and Wohleb, 2019; Sipe et al., 2016), which is essential for learning processes and memory (Abbott and Nelson, 2000). Moreover, in the amygdala, synaptic plasticity underlies fear memory and fear conditioning (Blair et al., 2001), thus, contributing to the develop of stress-related psychiatric diseases like PTSD (Mahan and Ressler, 2012). Even though, Tmem119 and P2ry12 are specific microglia marker, present in the homeostatic state of microglia, P2RY12 expression has been shown to increase following IL-4 stimulation (Moore et al., 2015), while Tmem119 is highly stable expressed in microglia, despite reduced mRNA levels following LPS stimulation (Bennett et al., 2016). These data indicate differentially regulated expression of both markers in microglia, depending on inflammatory mediators (van Wageningen et al., 2019). Fittingly, IL-4 is also highly associated to learning and memory processes (Gadani et al., 2012), thus, probably contributing to increased P2ry12 expressing microglia that mediate synaptic plasticity. In line, IL-4 protein levels, even though not mRNA levels, were increased in the amygdala in CSC mice compared to SHC. Hence, increased P2ry12 and IL-4 levels might contribute to a facilitated traumatic memory encoding in CSC mice and subsequently the development of the observed phenotype. Moreover, microglia density, indicated by Tmem119, was also increased on a protein level in CSC mice compared to SHC, which was independent from conditioning. In line, chronic psychosocial stress has been repeatedly shown to induce microglia activation, mediating the development of stress-induced psychiatric dysregulations like anxiety- and PTSD-like symptoms, as well as social deficits (Enomoto and Kato, 2021; McKim et al., 2018a; Ramirez et al., 2016; Reader et al., 2015; Wohleb et al., 2011). Interestingly, microglia are thought to be involved in fear memory, leading to a dysregulated fear network within the brain, which might contribute to an increased risk to develop PTSD (Enomoto and Kato, 2021), but probably also socially related fears, due to the involvement of the amygdala in SFC (Zoicas et al., 2014). Moreover, iNOS was increased in CSC/SFC$^-$ compared to SHC/SFC$^-$, which was not seen in CSC/SFC$^+$ animals. In line, iNOS increased following chronic non-social and social stress (Gądek-Michalska et al., 2016; Olivenza et al., 2000; Zlatković and Filipović, 2013), however, previous long-term psychosocial stress, but not RS, inhibited increased iNOS expression in the brain following an additional acute stressor (Gądek-Michalska et al., 2016), probably mediated by increased CORT

release following acute stress in CSC mice (Uschold-Schmidt et al., 2012). Additionally, iNOS knockout mice showed enhanced fear memory and affirmed a critical involvement of iNOS in stress-related psychiatric diseases like anxiety disorders and PTSD, as well as their comorbidity (Lisboa et al., 2015; Pitsikas, 2018). Thus, increased microglia number and M1 phenotype in the amygdala is highly likely to underlie facilitated traumatic memory encoding in CSC mice, as well as an impaired extinction of the learned fear. Interestingly, CSC/SFC$^+$ and SHC/SFC$^+$ showed increased GILZ mRNA expression in the amygdala compared to respective SFC$^-$, which is in line to my previous result in SFC$^+$ described above (see Experiment 2.2b), while protein analysis showed increased GILZ expression in CSC mice compared to SHC, which is also in line to the results described above (see Experiment 2.2a). These data further indicate a specific time course of GILZ mRNA translation to protein following CSC exposure and SFC acquisition. However, GILZ mRNA expression seemed to be higher in CSC/SFC$^+$ compared to SHC/SFC$^+$, even though this did not reach significance, which is in line to an increased risk to develop a PTSD-like phenotype (van Zuiden et al., 2012) in CSC/SFC$^+$. Interestingly, analyzing one of the major signaling pathways of GILZ on a protein level, revealed increased activation of the NFκB signaling pathway in CSC mice compared to SHC. In detail, CSC mice showed a trend towards increased pNFκB (Ser276) protein level and significantly increased pNFκB (Ser276) / NFκB protein ratio, which was even higher in CSC/SFC$^+$ compared to CSC/SFC$^-$. The NFκB transcription factor regulates multiple inflammatory functions, including the production of pro-inflammatory mediators, thus promoting inflammatory responses. For this reason, NFκB activation and functioning has been associated with various inflammatory diseases (Liu et al., 2017). In line, NFκB has been found to be increased early after trauma exposure (Stegmaier et al., 2008), which might induce the known increased inflammatory state in PTSD patients. Nevertheless, activation of NFκB is mediated via phosphorylation of NFκB subunits. Among them, the pNFκB (Ser276), a Protein Kinase A phosphorylation site, is of special interest as it is involved in the interaction of NFκB and GC (Christian et al., 2016), which suggests that also GILZ is interacting with NFκB on this site-specific phosphorylation. Additionally, NFκB-induced repression of GR activity requires Protein Kinase A phosphorylation at Ser276, while GR-mediated inhibition of NFκB activity was reported to be dependent on the same (Christian et al., 2016). Interestingly, increased activation of the NFκB signaling pathway via phosphorylation of the p65 subunit at Ser276 was even higher in CSC/SFC$^+$, which is likely to exaggerate the inflammatory response that which might underlie facilitated social fear memory encoding and impaired extinction. However, increased GILZ expression along with increased NFκB signaling seems contradictive, as GILZ inhibits the NFκB signaling pathway (Auphan et al., 1995; Ayroldi et al., 2001; Di Marco et al., 2007). However, this might be based on a disordered functioning of GILZ in stress-related diseases (Lebow et al., 2019; van Zuiden et al.,

2012), or GILZ is interacting with another site- or subunit- specific phosphorylation of NFκB, which is not known so far.

In the septum, CSC/SFC⁻ showed increased GILZ and IL-6 expression compared to respective SHC, which was absent in CSC/SFC⁺, as well as increased IL-4 in CSC mice compared to SHC. Importantly, the LS is highly associated with SFC (Menon et al., 2022; Menon et al., 2018; Zoicas et al., 2014), as well as emotion and stress responses (Singewald et al., 2011). Moreover, GILZ and IL-6 were studied as marker for PTSD (Cohen et al., 2011), while increased expression of IL-6 and GILZ predicted the development of PTSD (Cohen et al., 2011; van Zuiden et al., 2012), whereas reduced level of GILZ were found to increase the susceptibility to PTSD in men (Lebow et al., 2019). Moreover, increased IL-6 levels are associated with increased anxiety-like behavior, learning deficits, synaptic functioning, and decreased social behavior (Gruol, 2015; Wei et al., 2012). Interestingly, the effects of IL-6 on neuronal development are partly mediated via the kynurenine pathway (Brown et al., 2014), which was affected in SAD patients (Butler et al., 2022). Nevertheless, IL-6 seems to be regulated sex-dependently, since in male rats central IL-6 expression was increased following chronic psychosocial stress exposure, but reduced to basal levels following an additional challenge, i.e., LPS injection, which was the opposite in females (McCormick et al., 2020). However, these date are in line to my findings showing increased IL-6 in CSC/SFC⁻, which went back to basal levels in CSC/SFC⁺. Moreover, as acute heterotypic stressor exposure highly increases CORT release in CSC mice (Uschold-Schmidt et al., 2012), lower IL-6 expression in CSC/SFC⁺ might be based on a rise of GCs following SFC acquisition. Additionally, the anti-inflammatory cytokine IL-4 was increased in CSC compared to SHC mice, independent of conditioning. Due to its substantial role in learning and memory (Gadani et al., 2012), increased IL-4 expression might contribute to a facilitated traumatic memory encoding in CSC mice compared to SHC mice, as discussed above (see Figure 12). Moreover, IL-4, as well as IL-6, IL-10 and TNF-α were found to be increased following stress exposure (Himmerich et al., 2013), indicating increased release of pro- and anti-inflammatory cytokines during systemic inflammation.

In the vHC, less parameters were measured due to methodical issues, however, CSC exposure did not affect the expression of inflammatory mediators. This is in contrast to my expectations, as the vHC is highly involved in fear and anxiety (Fanselow and Dong, 2010), and revealed increased microglia expression in CSC mice compared to SHC without an additional challenge (described above). In addition, CSC/SFC⁺ seemed to be more anxious than CSC/SFC⁻ by showing reduced contact towards the non-social stimuli (see Figure 12). However, anti-inflammatory M2 microglia seemed to be reduced in SFC⁺ compared to SFC⁻, which is in line to microglia inhibition following

SFC acquisition in shock controls, even though SFC$^+$ were not affected here (see Experiment 2.2b). In line, M2 microglia were reduced in mice that received an elective foot shock compared to unshocked controls, which returned to basal levels following fear extinction (Yu et al., 2017). Thus, microglia seemed to be inhibited in the vHC during electric shock exposure.

In sum, combining CSC and SFC acquisition, peripheral inflammation was not altered, however, central inflammation was increased in all brain regions, while in the amygdala even protein levels were affected. Thus, CSC exposure and SFC acquisition shifted the immune system towards a pro-inflammatory state, not only in CSC/SFC$^+$, but also in CSC/SFC$^-$. Here, CSC exposure following a mild stressor, i.e., novel environment during SFC acquisition, (CSC/SFC$^-$ or CSC independent of conditioning) induced increased microglia density and pro-inflammatory morphological changes, as well as cytokine production, whereas CSC exposure followed by induction of social fear (CSC/SFC$^+$) increased inflammation via activation of the NFκB signaling pathway, probably via dysregulated GILZ expression. This indicates that CSC exposure followed by an acute stressor, which can be mild like exposure to novel environments, or strong like the induction of social fear, differentially affect central inflammation, probably mediating either increased susceptibility in CSC mice (CSC/SFC$^-$) or the development of a PTSD- and SAD-like phenotype (CSC/SFC$^+$). Nevertheless, it would be interesting to measure central inflammation not only after trauma induction but also after re-exposure to the traumatic event. Accordingly, re-exposing mice to the traumatic event exaggerated peripheral inflammation, mediating enhanced fear memory (Young et al., 2018). Furthermore, positive modulation of the immune system, especially of the brain, via pre-immunization of CSC mice with the stress-protective M. vaccae (Reber et al., 2016b), will intense the understanding of the role of the immune system in CSC-induced impaired SFC.

## 4.3 Specificity of the effects of chronic psychosocial stress on the consequences of social fear conditioning

After unraveling behavioral and immunological alterations following CSC and SFC exposure, I aimed to analyze the specificity of CSC-induced facilitated traumatic memory encoding and impaired extinction of social fear. Therefore, I exposed CSC mice to a non-social traumatic event, namely CFC, to investigate acute trauma specificity. Next, I established a chronic non-social stressor paradigm, the CUS model, to investigate, if chronic non-social stress has similar effects on CFC and SFC compared to CSC. Finally, I tested two strains for their innate anxiety-like behavior, as well as non-social and social fear-related behavior, stress-vulnerability and the impact of CSC exposure on SFC.

### 4.3.1 Effects of chronic psychosocial stress on the consequences of non-social fear conditioning

Analyzing the importance of the nature of the traumatic event following chronic psychosocial stress, CSC mice were exposed to CFC. Here, CSC exposure did not affect CFC acquisition or extinction behavior, neither retention (see Figure 21). These results are in contrast to literature (for review see Farrell et al., 2013), showing that in rats and mice, chronic RS or CUS facilitates acquisition of cued and contextual fear conditioning (Conrad et al., 1999; Farrell et al., 2010; Sanders et al., 2010), and impaired its extinction (Farrell et al., 2010; Hoffman et al., 2015; Miracle et al., 2006). In addition, chronic psychosocial stress increased non-social fear acquisition and impaired its extinction (Azzinnari et al., 2014; Fuertig et al., 2016). Moreover, CSDS during adolescence followed by a single exposure to SD during adulthood impaired cued fear extinction (Mancini et al., 2021). Interestingly, stress-susceptible and stress-resilient mice following CSDS, assigned by the presence or absence of social preference behavior, respectively, reacted differently to CFC or contextual fear conditioning. Here, stress-susceptible mice showed increased freezing behavior during contextual, but not CFC, whereas resistant mice showed increased freezing during CFC (Dulka et al., 2015). Therefore, mice that were exposed to the CSC paradigm, which represents a strong and long-lasting stressor inducing long-lasting lack of social preference in novel environments (Amoroso et al., 2020; Foertsch et al., 2019; Slattery et al., 2012), might represent stress-susceptible mice, thus CFC was not affected. However, contextual fear conditioning was not performed in the present study. Nevertheless, most studies analyzing the impact of chronic stress on CFC used chronic non-social stressor paradigms, indicating that the nature and composition of the chronic stressor and the fear-conditioning paradigms may need to coincide to affect fear-related behavior. In line, I could show that chronic psychosocial stress facilitates acquisition and impairs extinction of SFC, while social stress exposure early in life facilitated social fear extinction, but did not affect non-social fear extinction (Zoicas and Neumann, 2016). To test my hypothesis, I established the CUS model and subsequently exposed CUS mice to the CFC and SFC paradigm. Nonetheless, in the current experiment, cued fear extinction was very low in both groups, as CSC mice and SHC show high freezing behavior during extinction. Even though freezing behavior marginally reduced, it was still high during retention, indicating a high susceptibility in BL6 mice, which might have been improved using less shocks (e.g. only three CS during acquisition) and a lower intensity (e.g. 0.5 mA).

### 4.3.2 Effects of chronic non-social stress on the consequences of non-social and social fear conditioning

To study the effects of CUS exposure on CFC and SFC, the CUS model has been adapted to our laboratory conditions and was subsequently evaluated to successfully induce chronic stress in CUS mice. Therefore, I studied first the impact of CUS exposure on anxiety- and depressive-like behavior, as well as social behavior (see Figure 22). Here, CUS animals showed increased anxiety-like behavior in the OF test, indicated by reduced time spent in the inner zone, while locomotion was not affected. Moreover, CUS mice showed reduced anxiety-like behavior in the NOR, shown by increased time spent object sniffing, as well as in the LDB, indicated by increased time spent in the light and reduced latency to enter the light. In line, in rats and mice different behavioral tests revealed that CUS exposure either increases anxiety-like behavior (Bondi et al., 2008; Chaby et al., 2015; Ge et al., 2020; Griebel et al., 2002; Matuszewich et al., 2007; Monteiro et al., 2015), to not alter anxiety-like behavior (Yoon et al., 2014), and to reduce anxiety-like behavior, termed anomalous anxiolytic-like behavior or blunted emotionality (Cancela et al., 1995; Costa-Nunes et al., 2014; D'Aquila et al., 1994; Ducottet et al., 2003; Kompagne et al., 2008; Schweizer et al., 2009). On which factors different outcomes in anxiety-like behavior might underlie is not clear yet, but might involve differences in stressor protocols or strains (Ducottet et al., 2003), and assessed time points (Matuszewich et al., 2007). However, as in the present thesis the CUS paradigm has been performed identical with the same mouse strain, i.e., BL6 mice, while also the time point of measuring anxiety-like behavior was equal (day 19 of CUS exposure), methodical differences or delayed effects of CUS exposure on anxiety-like behavior (Matuszewich et al., 2007) can be excluded. Nevertheless, as already mentioned in 4.1, the LDB seemed to be afflicted with methodical issues, as also CSC exposure did not reveal differences in anxiety-like behavior using this anxiety test. Moreover, as the NOR test was performed directly following the OF test, these contradictory results might underlie the habituation period during the OF test prior to NOR. Here, mice explored the OF box already for 5 minutes before the novel object was placed in the middle of the box. Furthermore, I studied depressive-like behavior in CUS mice compared to SHC using the splash test, a common behavioral test to measure depressive-like behavior (Machado et al., 2012; Yalcin et al., 2005). Here, I could not show differences in depressive-like behavior, since CUS mice and SHC showed equal time and latency to groom. This is in contrast to previous studies, repeatedly describing the CUS paradigm as a model for depressive-like behavior measured in the FST, sucrose preference test, or tail suspension test (Griebel et al., 2002; Kreisel et al., 2014; Yoon et al., 2014). However, these tests have not been performed in the present study, which might have revealed different outcomes. Nevertheless, no changes in depressive-like behavior are in line to the findings following CSC exposure (Langgartner et al., 2015; Slattery et al., 2012),

suggesting that the CUS model is a suitable model to compare CUS and CSC outcomes during fear conditioning. Moreover, analyzing social behavior following CUS exposure revealed normal social preference in the SPT in CUS mice and SHC. This is again in line to my findings in the CSC model, as following both chronic stress models, the SPT has been performed in the homecage. However, previous studied reported both normal social interaction (D'Aquila et al., 1994) or social avoidance (Kompagne et al., 2008) following CUS exposure, measured in novel environments. Similar to the CSC paradigm (discussed above, see 4.2), performing the SPT in a novel environment might have shaped to outcome, however, this has not been tested in the present thesis.

Analyzing stress-related physiological parameters following CUS exposure (see Figure 23 and Figure 24), revealed a robust reduction of body weight gain directly after CUS exposure, as well as following 2 days after stressor termination. In line, as a measure of chronic stress, chronic social and non-social stressor paradigms have been repeatedly shown to reduce body weight gain in rats and mice (Cox et al., 2011; Monteiro et al., 2015; Schmidt et al., 2010; Solomon et al., 2010; Tamashiro et al., 2007; Willner et al., 1996). Moreover, body weight further decreased in stressed animals following lesions of the medial amygdala (Solomon et al., 2010), whose activity and CRH expression were altered following CUS exposure (Sandi et al., 2008; Wang et al., 2010), indicating a substantial role of the amygdala in stress-induced physical alterations. Moreover, CUS exposure temporally increased relative adrenal weight in CUS mice, which declined 2 days following stressor termination. Increased adrenal weight, mediated by cell hyperplasia, leads to an increased bioavailability and mobilization capacity of cholesterol, the precursor of GC (Füchsl et al., 2013b; Reber et al., 2007; Uschold-Schmidt et al., 2013). This indicates prolonged HPA axis activity, since plasma CORT levels were shown to correlate with adrenal weight in rats (Baranyi et al., 2005; Schwartz et al., 1997) and mice (Uschold-Schmidt et al., 2013). This is in line to my results, showing equal plasma CORT level and adrenal weight in CUS mice and SHC 2 days after the CUS paradigm, while directly after stressor termination, adrenal weight was increased, whereas plasma CORT levels were not measured. Nevertheless, increased adrenal weight provides a reliable chronic stress parameter (Forkwa et al., 2014; Füchsl et al., 2014; Füchsl et al., 2013b; Reber et al., 2007; Slattery et al., 2012; Uschold-Schmidt et al., 2013; Uschold-Schmidt et al., 2012). However, as adrenal weight declined 2 days after stressor termination, the stress effects of CUS exposure on the HPA axis seemed to be not long-lasting, which is in contrast to the CSC paradigm (Slattery et al., 2012). In line, neither plasma CORT level, not adrenal CORT release were altered 2 days after stressor termination, however these parameters have not been measured directly after CUS exposure.

Analyzing the lymphatic organs, CUS mice showed reduced relative thymus weight directly after CUS exposure, as well as 2 days after stressor termination, while relative spleen weight was unchanged. In line, thymus atrophy or involution is a common consequence following CUS exposure (Kioukia-Fougia, 2002; Monteiro et al., 2015), chronic RS (Domínguez-Gerpe and Rey-Méndez, 1997), and CSC exposure (Langgartner et al., 2015; Reber et al., 2007). Reduced thymus weight is probably caused by an acute loss of cortical thymocytes and reduced output of naïve T cells (Wang et al., 1994), as well as GC-induced immune cell apoptosis and prevented proliferation (McEwen et al., 1997; Reber et al., 2007; Schwartzman and Cidlowski, 1994). Moreover, also the spleen has been described to increase following chronic stressor exposure (Langgartner et al., 2015; Reber et al., 2007). In confirmation, chronic psychosocial stress induces a rise in immature myeloid cells and Tregs, as well as increased germinal center formation in the spleen, thus enhancing the activation of B cells (Schmidt et al., 2016; Schmidt et al., 2010). Fittingly, chronic psychosocial stress exposure robustly induced splenomegaly (DiSabato et al., 2021; Langgartner et al., 2015; McKim et al., 2018a), which has been shown to correlate with bite wounds during social interactions (Foertsch et al., 2017). Interestingly, increased spleen weight has been described as a biomarker of psychosocial stress (DiSabato et al., 2021; McKim et al., 2018b; Wohleb et al., 2014), which might explain the lack of splenomegaly following CUS exposure. In line, most studies fail to demonstrate increased spleen weight following chronic non-social stressor paradigms, such as chronic RS (Dhabhar et al., 1997), CUS (Konkle et al., 2003), repeated shock exposure (Pitman et al., 1995), or chronic noise exposure (van Raaij et al., 1997). Moreover, 2 days following CUS exposure, neither number of mesLNC, nor IFN-γ production of mesLNCs were affected. However, increased number and cytokine production of mesLNCs is a common companion of chronic psychosocial stress (Langgartner et al., 2015; Reber et al., 2016b). CUS exposure, however, seems to have a lower impact on the immune system, because physical injury, e.g. bite wounds during social interactions, was shown to be required for stress-induced immune activation (Foertsch and Reber, 2020). Another explanation might be the assessed time point, since CUS exposure seems to have rather short-term effects, as discussed above.

Nevertheless, I could demonstrate that CUS exposure successfully induced chronic stress in CUS mice, even though this seems to be temporally restricted, which is in contrast to the long-term effects after CSC exposure (Langgartner et al., 2015; Slattery et al., 2012), indicating that the CSC model provides a more stressful condition to male mice. This is in line to the statement that "allosteric overload" is more likely to develop, when the stressor is of chronic, but especially social nature (Bartolomucci et al., 2005). The short-term effects of the CUS paradigm on stress parameters might be particularly problematic, as fear conditioning paradigms were performed after 2 days of habitation following CUS exposure. In line, CUS mice that were subsequently tested

in the CFC, showed only reduced body weight gain directly after CUS exposure (day 19), but not after CFC retention (day 23), while anxiety-like behavior, as well as organ weight were not affected, indicating insufficient induction of chronic stress in CUS mice. Nevertheless, CUS mice that were tested in the SFC paradigm showed increased anxiety-like behavior on the EPM, measured by reduced open arm entries, as well as reduced body weight gain, increased adrenal and reduced thymus weight, indicating successful induction of chronic stress in CUS mice, in this experiment.

Analyzing the effects of CUS exposure on CFC revealed no differences in freezing behavior in CUS mice compared to SHC, neither during acquisition, nor extinction, nor retention (see Figure 25). This is in contrast to literature, as discussed above (see 4.3.1), as chronic non-social stress has been repeatedly shown to facilitate CFC acquisition and impair its extinction (Conrad et al., 1999; Farrell et al., 2013; Hoffman et al., 2015; Hoffman et al., 2014). However, in this experiment, CUS mice did not show successful induction of chronic stress, despite reduced body weight gain, which might be the underlying reason. Visually, CUS exposure seemed to impair CFC extinction, as during the last CS presentation CUS mice showed higher freezing behavior compared to SHC, as well as during retention, however, this did not reach significance. Nevertheless, to make a clear statement, this experiment needs to be repeated in another set of animals, where a successful induction of chronic stress in CUS mice is revealed. Moreover, neither CUS mice, nor SHC showed proper extinction of the cued fear, even though the protocol of the CFC paradigm has been adapted from Experiment 3.1 (mentioned in 4.3.1) using less shocks (e.g. only three CS during acquisition) and a lower intensity (e.g. 0.5 mA of the shocks).

When CUS mice and SHC were exposed to the SFC paradigm, CUS exposure had no impact on social fear acquisition or extinction, while CUS/SFC$^-$ showed higher investigation time towards the conspecifics during extinction training compared to SHC/SFC$^-$ (see Figure 26). Thus, chronically stressed mice seem to be in higher "needs" for social interactions, seeking for social support, as rewarding social interactions and social support have beneficial effects on physical and emotional fitness, protecting against psychopathologies (for review see Gryksa and Neumann, 2021). Therefore, social support can counteract stress-induced epigenetic modifications, decrease plasma CORT levels and reduce the reactions to stress in different animal models (Beery and Kaufer, 2015; Kikusui et al., 2006; Viana Borges et al., 2019). Moreover, increased seeking for social support is seen in highly anxious rats (Ohl et al., 2001b; Ohl et al., 2001a) and in female mice following chronic social isolation (Ramsey et al., 2021). Nevertheless, the impact of chronic non-social stress on SFC is unknown yet, however, psychosocial early-life stress has been shown to facilitate social fear extinction (Zoicas and Neumann, 2016), whereas

CSC exposure facilitated SFC acquisition and impaired extinction (see Figure 12). However, as already mentioned, the composition of the stressor and the acute trauma might have to coincide or the social component in the stressor and the subsequent trauma might be crucial for the development of a PTSD- and SAD-like phenotype. Nonetheless, it has to be taken into account that neither CUS mice nor SHC showed successful extinction of social fear, which might have been a matter of the animal cohort or the use of highly fear-susceptible BL6 mice (discussed below), thus this experiment needs to be interpreted with caution.

### 4.3.3 Strain differences of BL6 and CD1 mice in anxiety- and fear-related behavior, social behavior, and stress- response

Next, I studied behavioral differences between the two mouse strains, which are commonly used during CSC, i.e., BL6, and SFC, i.e., CD1. Therefore, BL6 and CD1 mice were tested for innate anxiety- and fear- related behavior, stress coping styles and CSC-induced social fear-related behavior. Here, CD1 mice were less anxious and less susceptible to fear conditioning paradigms, while displaying more social behavior. In detail, CD1 mice showed reduced anxiety-like behavior on the EPM and LDB, and showed higher locomotion on the EPM (see Figure 27). This is in confirmation with previous studies, reporting reduced anxiety in CD1 mice compared to C57BL/6 and BALB/c mice in the novel open space test (Michalikova et al., 2010) and rat exposure test (Yang et al., 2004). Additionally, I tested CD1 and BL6 mice for their social behavior during the SPT (see Figure 29), where both strains showed social preference, which is in line to literature (Lukas et al., 2011b; Toth et al., 2013; Zoicas et al., 2016), even though CD1 mice explored the non-social and social cages more than BL6 mice. This seems to be based on their lower anxiety-like behavior, as both stimuli are unknown, while social interaction tests are one of the earliest anxiety tests in rodents (Campos et al., 2013; File and Hyde, 1978).

During CFC, CD1 mice showed less freezing behavior during acquisition, extinction and retention compared to BL6 mice (see Figure 28). This is in line to literature, showing highly reduced freezing behavior in CD1 mice compared to the fear-susceptible BL6 mice during contextual fear conditioning (Gerlai et al., 2002), as well as compared to ICR mice during CFC (Adams et al., 2002). Interestingly, these effects were independent of learning tasks (Adams et al., 2002; Gerlai et al., 2002), and seemed to be not based on differences in pain sensitivity (Kovacsics and Gould, 2010). Thus, either reduced anxiety-like behavior might result in reduced fear expression (Sartori et al., 2011), or CD1 mice might show a more active avoidance behavior rather than freezing during fear conditioning (Adams et al., 2002), or CD1 mice have an increased stress-tolerance (discussed below). However, to compare freezing behavior between the anxious BL6 and more resilient CD1 mice, the CFC protocol had to be adjusted to induce fear in CD1, but to not “overwhelm” BL6

mice. Therefore, mice received four shocks at 0.7 mA, which seemed insufficient to induce a high level of freezing behavior in CD1 mice, but seemed too high for BL6 mice, as they did not extinguish cued fear, revealing high freezing behavior even during retention (about 75 %). In accordance, testing CD1 and BL6 mice in the SFC paradigm revealed that CD1 mice needed more shocks to successfully induce social avoidance, while CD1/SFC$^{+}$ mice showed faster extinction of social fear compared to BL6/SFC$^{+}$ (see Figure 29). Moreover, CD1/SFC$^{-}$ showed more social exploration compared to BL6/SFC$^{-}$, which even lost social interest / motivation, as social investigation dropped along social stimuli presentations during extinction and recall in BL6/SFC$^{-}$. This indicates higher sociality in CD1 mice compared to BL6, which is again in line with literature (Ramsey et al., 2021; Toth et al., 2013). Interestingly, females tested in a social self-administration paradigm, CD1 mice preferred social interactions over foot pellets, whereas C57BL/6J mice preferred the foot pellet (Ramsey et al., 2021). These data indicate, that inbreed C57BL/6 mice have reduced social motivation or social interest, thus BL6 mice show an impaired extinction of social fear, as their internal drive might not be strong enough to overcome the fear. Furthermore, during recall CD1/SFC$^{+}$ showed even increased social investigation compared to CD1/SFC$^{-}$, which might be mediated by increased seeking for social support in stressed CD1 mice (Ramsey et al., 2021), or a side effect of initially lower investigation time during extinction.

Finally, CD1 and BL6 mice were compared in their stress coping behavior and stress susceptibility during CSC exposure. On day 1 of CSC exposure, when the colonies are build, CD1 mice showed more active stress coping strategies, i.e., offensive behavior, which was accompanied by lower passive stress coping behavior, i.e., defensive behavior, which was higher in BL6 mice (see Figure 30). Hence, CD1 mice attacked the resident and the other CSC mice more and showed less flight behavior, therefore receiving less attacks, which has been shown to correlate (Foertsch et al., 2017). Interestingly, increased passive stress coping behavior increases the vulnerability to develop stress-related behavioral dysregulations including anxiety- and depressive-like behavior (Koolhaas et al., 1999; Wood et al., 2010; Wood and Bhatnagar, 2015), which is at least partly mediated via increased inflammation in passive stress coping rats (Wood et al., 2015). In line, positive modulation of the immune system induced increased stress-resilience in CSC mice, and increased active stress coping behavior, while reducing passive strategies (Reber et al., 2016b). Moreover, passive stress coping has been associated with lower HC cell proliferation under basal conditions, as well as a hyperreactive HPA axis and almost 50 % reduction in HC cell proliferation following acute stress (Veenema et al., 2004). One underlying mechanism might be reduced signaling of the LS in BL6 compared to CD1 mice, which is also in agreement with impaired social fear extinction, described above. In line, lesions of the LS, a brain region critically involved in SFC (Menon et al., 2018; Zoicas et al., 2014), increased the response of the HPA axis towards acute

stress, which was accompanied with increased passive stress coping behavior (Singewald et al., 2011). However, if reduced signaling in the LS underlies stress- and SFC-susceptibility in BL6 mice needs to be investigated. On day 8 of CSC exposure, when CSC mice were already subordinated for one week, CD1 and BL6 mice did not differ in active or passive stress coping strategies, but in reactive emotional coping behavior, i.e., flight and submissive upright behavior (Reber et al., 2016b; Reber and Neumann, 2008). Here, CD1 mice showed less flight behavior but increased submissive upright compared to BL6, which was accompanied by less attacks received (see Figure 31), indicating that especially flight behavior triggers the resident to attack and therefore might drive susceptibility during passive stress coping behavior. In line, CD1 mice seemed less susceptible towards CSC-induces peripheral alterations (see 1.7), as they revealed only increased relative adrenal and spleen weight (see Table A8). Thus, CD1 CSC mice showed successful induction of chronic stress, but reduced stress- and immune-susceptibility, because neither anxiety-like behavior, nor body weight gain, nor plasma CORT or immunological parameters including thymus weight, number of mesLNCs, and IFN-γ production of the mesLNCs were affected (see Table A8). These results are in line to previous findings, demonstrating reduced immune-susceptibility in CD1 mice following CSC exposure (Füchsl et al., 2014). These data suggest that CD1 mice are, at least compared to BL6 mice, less susceptible to develop stress-related affective disorders like PTSD and SAD. Nevertheless, underlying factors causing these strong strain differences in anxiety- and fear-related behavior, as well as stress susceptibility are unknown. However, differences in the immune system, LS signaling and the OXT system, possessing anxiolytic, pro-social, stress-buffering and anti-inflammatory properties (reviewed in Gryksa and Neumann, 2021; Jurek and Neumann, 2018), are likely.

Unexpectedly, when CD1 CSC mice were exposed to SFC, they showed the same phenotype like BL6 mice. Even though CD1 CSC mice and SHC needed the same amount of shocks during acquisition to successfully induce social avoidance, CSC/SFC$^+$ showed impaired extinction of social fear compared to SHC/SFC$^+$, which lasted until recall (see Figure 32), to a comparable extent seen in BL6 mice (see Figure 12, discussed in 4.1). This result was surprising, as CD1 mice were less anxious, more social, showed less fear-related behavior during both CFC and SFC, and showed even a higher immune- and stress-tolerance compared to BL6 mice. Thus, CSC-induced impaired extinction of social fear seems to be a robust and powerful effect, strengthening my hypothesis of a severe and serious impact of chronic psychosocial stress on the development of a PTSD- and SAD-like phenotype, where not even innate susceptibility or tolerance seem to impact the outcome.

## 4.4 Oxytocin as a potential treatment option to prevent stress-related symptoms

Finally, I analyzed, if acute icv OXT (0.1 µg / 2 µl) treatment 10 minutes prior to SFC extinction training can improve extinction success in the highly anxious, stress-vulnerable and fear-susceptible BL6 mice, and whether it can prevent or counteract CSC-induced impaired SFC extinction in BL6 and CD1 mice.

Under basal conditions, 10 days after recovery, acute OXT treatment failed to affect behavior during SFC extinction in BL6/SFC$^-$ and BL6/SFC$^+$ (see Figure 33). Furthermore, SFC$^-$/VEH and SFC$^-$/OXT lost social motivation during the extinction process, as they showed reduced social investigation along the social stimuli presentations, which is in line to my previous results (see Figure 29). Nevertheless, neither SFC$^+$/VEH nor SFC$^+$/OXT showed extinction of the learned social fear, which was also seen following CSC exposure in both SHC and CSC mice (see Figure 34), indicating that previous surgery prevents social fear extinction in BL6 mice. In line, minor abdominal surgery increased HC IL-1β expression, without cognitive impairments in aged BALB/c mice (Rosczyk et al., 2008), while anesthesia and surgery impaired preference for social novelty in C57BL/6 mice, when performed during the neonatal period (Jin et al., 2020). Moreover, anesthesia and surgery age-dependently increased BBB permeability and IL-6 expression, which was associated with cognitive impairments in C57BL/6J mice (Yang et al., 2017). However, in the present study, mice that underwent surgery seemed to have intact cognitive fear memory, as they showed normal behavior during acquisition. Interestingly, chronic psychosocial stress-induced immune activation has been shown to be mediated by physical wounding, i.e., bite wounds or surgery, rather than the social stressor itself, mediating stress-related psychopathologies (Foertsch et al., 2017; Foertsch and Reber, 2020). This might contribute to the impaired extinction in the highly susceptible BL6 mice, when they underwent surgery, even though this is in contrast to my findings in CD1 mice (discussed below). Nevertheless, as Foertsch and colleagues also performed their studies in BL6 mice (Foertsch et al., 2017; Foertsch and Reber, 2020), a high trait anxiety, as well as immune- and stress- susceptible phenotype of the mouse strain might be deciding in surgery-induced increased inflammation and behavioral impairment.

As described above, following CSC exposure, surgery also impaired social fear extinction in BL6 SHC/SFC$^+$ and CSC/SFC$^+$ mice, but OXT treatment increased investigation towards non-social stimuli in SFC$^-$ and SFC$^+$, as well as social investigation in SFC$^-$ (see Figure 34). This effect is mainly seen in SHC, reaching the investigation level of the more social CD1 mice (about 70 % social investigation). These data are in line with the anxiolytic and pro-social properties of OXT (Bale et al., 2001; Jurek and Neumann, 2018; Lukas et al., 2011b; Ring et al., 2006). Interestingly, in

CSC/SFC$^{-}$, OXT increased social investigation at the first social stimulus compared to respective VEH treated mice, indicating a rescuing effect of OXT on CSC-induced lack of social preference, which was seen during Experiment 1 (discussed in 4.1 and 4.2.1). However, OXT did not increase social investigation in CSC/SFC$^{-}$ thereafter compared to respective VEH treated mice, indicating again an inhibited action of OXT following surgery combined with stressor exposure.

Finally, CD1 mice were exposed to the CSC paradigm, underwent surgery, and OXT effects on social fear extinction were measured. Here, CSC mice showed successful induction of chronic stress, indicated by increased anxiety-like behavior on the EPM, as well as increased body weight gain and relative spleen weight (see Table A10). During SFC acquisition, CSC exposure did not influence the number of shocks, necessary to induce social avoidance behavior. As the effect of acute icv OXT infusion in male CD1 mice 10 minutes prior to social fear extinction is already known (Zoicas et al., 2014), the group size of SHC was kept very low (n = 3-4 per group). Therefore, interpreting the results is difficult, however, OXT treatment seemed to facilitate social fear extinction, which is in line to literature (Zoicas et al., 2014). In CSC mice, I could repeat my findings demonstrating impaired social fear extinction in CSC/SFC$^{+}$/VEH, since they showed reduced investigation time towards the conspecifics compared to CSC/SFC$^{-}$/VEH until social stimulus 5, which was also seen in CSC/SFC$^{+}$ in Experiment 3.3c (see Figure 32). However, CSC/SFC$^{-}$ seemed to lose social interest and / or social motivation during the extinction process, independent of treatment. Thus, CSC/SFC$^{-}$ showed lower investigation time towards the conspecifics along stimuli presentations, which has been reported in BL6 mice (Toth et al., 2013) and might be a consequence of surgery.

Analyzing the effects of OXT treatment on CSC-induced stress parameters, acute OXT infusion seemed to rescue CSC-induced splenomegaly, as VEH treated CSC mice showed increased spleen weight compared to SHC, which was absent in OXT treated CSC mice (see Table A10). This is in line to the stress-protective effects of chronic OXT treatment during CSC exposure (Peters et al., 2014). Moreover, acute icv OXT infusion prevented CSC-induced impaired extinction of social fear (see Figure 35). In detail, in CSC mice SFC$^{+}$/OXT showed reduced social investigation at the first social stimulus, but revealed a fast extinction by demonstrating equal investigation time towards the remaining social stimuli compared to SFC$^{-}$/OXT. Moreover, CSC/SFC$^{+}$/OXT showed more social investigation compared to CSC/SFC$^{+}$/VEH. These data are in line with the anxiolytic, stress-buffering and pro-social effects of OXT (Bale et al., 2001; Bernhard et al., 2018; Jurek and Neumann, 2018; Lukas et al., 2011b) and its protective effects on social fear extinction (Zoicas et al., 2014). Additionally, chronic OXT treatment was able to prevented CSC-induced mal-adaptations on various stress parameters including anxiety-like behavior, thymus and adrenal

weight, and adrenal *in vitro* ACTH insensitivity, as well as CSC-induced impaired OXTR signaling in the median raphe nucleus (Peters et al., 2014). Moreover, as CSC-induced impaired SFC extinction seemed to be associated with increased central inflammation (see 4.2.2), the anti-inflammatory properties of OXT (Eliava et al., 2016; Jurek and Neumann, 2018; Wang et al., 2015) might underlie the protective effects on the observed phenotype. In line, in an animal model of PTSD, OXT prevented single prolonged stress-induced impaired fear extinction, and the stress-induced increase in central and peripheral pro-inflammatory cytokine expression including IL-1β in both, IFN-γ in the brain, and TNF-α in the periphery (Wang et al., 2018). However, central inflammation following OXT treatment has not been measured in the present thesis. Another hypothesis is that CSC-induced hyperactivity of the HPA axis might contribute to the impaired extinction in CSC mice. Acute central infusion of OXT might prevent HPA hyperactivity and, thus improve extinction of social fear to the level of unstressed controls (SHC). In line, increased CORT release is known to increase fear memory (Dos Santos Corrêa et al., 2019), while patients suffering from social phobia reveal also a hyperresponsive cortisol release (Condren, 2002). OXT, in contrast has stress-buffering effects, regulating HPA axis activity and dampening the stress response (Neumann, 2002; Winter and Jurek, 2019). Another explanation might be the dampening effect of OXT on amygdala hyperreactivity (Koch et al., 2016; Labuschagne et al., 2010), which might occur following CSC and SFC combination, since it is a common symptom in PTSD and SAD patients (Frijling, 2017; Koch et al., 2016; Labuschagne et al., 2010; Phan et al., 2006). However, this needs to be further analyzed, as amygdala activity has not been studied in the present thesis.

Nevertheless, OXT seems to represent an effective treatment option in the development of a PTSD- and SAD-like phenotype, which is strengthened by chronic psychosocial stress exposure. Here, OXT seems to be a highly potent add-on pharmacotherapy during exposure therapy in PTSD and SAD patients, which is in line to previous studies (Flanagan et al., 2018; Frijling, 2017; Frijling et al., 2014; Guastella et al., 2009; Koch et al., 2016; Koch et al., 2014). However, OXT has to be applied advisedly, as high doses of OXT affect the endogenous OXT system, resulting in OXTR downregulation (Bale et al., 2001; Peters et al., 2014), activation of alternative OXTR-mediated pathways (Winter et al., 2021), and different behavioral outcome reaching from sedation to anxiogenesis (Peters et al., 2014; Uvnäs-Moberg et al., 1994).

## 4.5 Conclusion

In conclusion, I could show that chronic psychosocial stress prior to acute social trauma exposure facilitated traumatic memory encoding and increased the risk to develop a PTSD- and SAD-like phenotype in mice. This effect was robustly seen on a behavioral level in two mouse strains strongly differing in innate anxiety and susceptibility. Moreover, this effect was associated with a context and brain region-dependent alteration of the immune status. While CSC exposure decreased central inflammation, mainly mediated by reduced microglia number and pro-inflammatory morphology, reduced Nlrp3 inflammasome, as well as increased GILZ expression, whereas SFC acquisition barely affected central inflammation. However, developing a SAD-like phenotype ($SFC^+$) seemed to be associated with increased GILZ expression. Combining CSC and SFC acquisition shifted the immune status towards a pro-inflammatory state. Here, CSC exposure following a mild stressor, i.e., novel environment during SFC acquisition, induced increased microglia density and pro-inflammatory morphological changes, as well as cytokine production, while CSC exposure followed by an acute social trauma seemed to exaggerate the central immune response in the amygdala via activation of the inflammatory NFκB signaling pathway. Interestingly, GILZ seemed to be involved in the development of both a PTSD- and a SAD-like phenotype, since it was increased following CSC exposure and induction of social fear, which might underlie the high comorbidity. However, in general, less is known about the impact of SAD on the immune system. Moreover, CSC-induced impaired fear extinction seemed to be specific for chronic psychosocial stress and social fear paradigms, since CSC did not affect CFC-related behavior, while CUS exposure did neither affect CFC, nor SFC -related fear behavior. Additionally, acute OXT treatment, used to treat PTSD patients either alone or as an add-on pharmacotherapy during exposure therapy, prior to extinction training rescued stress-induced impaired extinction of social fear in CD1 mice (see Figure 36).

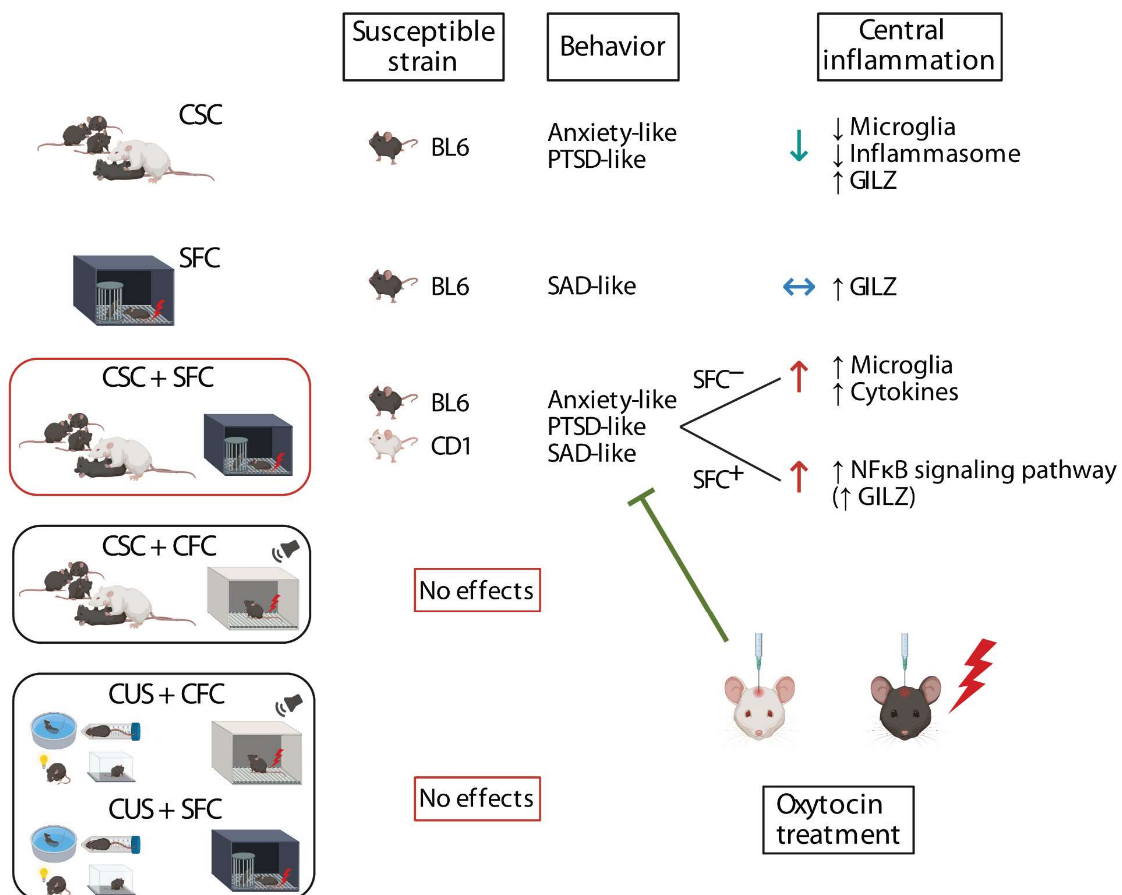



**Figure 36: Schematic summary of the present thesis.** Chronic subordinate colony housing (CSC) exposure induced anxiety- and PTSD-like behavior, where especially C57BL/6N (BL6) mice were most susceptible, but reduced central inflammation. Social fear conditioning (SFC), induced a social anxiety disorder (SAD)-like behavior in mice, whereas BL6 mice were more susceptible compared to CD1, but did barely affect central inflammation, despite increased glucocorticoid-induced leucine zipper (GILZ) expression in $SFC^+$. The combination of CSC and SFC acquisition induced an anxiety- PTSD- and SAD-like phenotype in BL6 and CD1 mice and increased central inflammation. Here, $CSC/SFC^-$ showed increase in microglia and cytokines, whereas $CSC/SFC^+$ showed increased activation of the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) signaling pathway, probably mediated by dysregulated GILZ expression. However, CSC exposure did not affect cued fear conditioning (CFC), while chronic unpredictable stress (CUS) affected neither CFC, nor SFC. Interestingly, oxytocin treatment did not affect social fear extinction in BL6 mice, but rescued stress-induced impaired extinction of social fear in CD1 mice. The illustration has been created using BioRender.com.

Interestingly, the combination of CSC exposure and SFC even fulfils the three criteria evaluating an animal model to be suitable to study human diseases, i.e., predictive validity, face validity, and construct validity (Willner, 1991; Willner, 1984). In confirmation, the combination of CSC exposure followed by SFC seems to be a suitable model to study the comorbidity of PTSD and SAD, as it induces trauma and socially related fears, i.e., facilitated social fear acquisition leading to robust social avoidance and impaired extinction of the learned fear, demonstrating face validity. Moreover, CSC and SFC exposure induces symptoms seen in PTSD and SAD patients including

inflammatory mediators in brain regions, both highly involved in PTSD and SAD, demonstrating construct validity. Finally, predictive validity could be proven as OXT administration prior to extinction training could rescue CSC-induced impaired extinction of social fear, which has been shown to be also an effective add-on pharmacotherapy in PTSD and partially SAD patients in combination with exposure therapy (Flanagan et al., 2018; Guastella et al., 2009; Koch et al., 2014; Labuschagne et al., 2010).

The present data are of special importance in the current situation, as the ongoing corona crisis increased the level of chronic psychosocial stress in the population (Gryksa and Neumann, 2021). In addition, the Ukraine war, which started February the 24$^{th}$ in 2022, is a highly traumatic event for young and adults all over the world. Experiencing both highly stressful conditions will certainly heavily impact humans' mental health and increase the level of stress-related psychiatric disorders including PTSD and comorbid diseases like SAD. Thus, further research is needed and outstanding questions need to be addressed to understand the development of the disease state and to generate effective and potent treatment options.

## 4.6 Future perspectives

CSC exposure, SFC acquisition and the combination of both, context and brain region dependently affected central inflammation. In line, CSC exposure decreased inflammation in the amygdala and septum, which is thought to be a protective mechanism. However, to prove this hypothesis, it would be interesting to measure central inflammatory mediators simultaneously with central GC and plasma CORT levels, which might contribute to reduced central inflammation, at different time points following CSC exposure. Furthermore, SFC acquisition barely affected peripheral and central inflammation. When combining CSC and SFC acquisition, central inflammation was increased in all brain regions, indicating systemic inflammation. Notably, GILZ, a key mediator in the development of PTSD, was increased following CSC exposure, SFC acquisition and the combination of both, especially in the amygdala, a brain region critically involved in PTSD and SAD. Thus, dysregulated GILZ expression in the amygdala might play a critical role in mediating the effects of chronic psychosocial stress on the development of a PTSD- and SAD-like phenotype, leading to an exaggerated central immune response, which might induce impaired social fear extinction after CSC exposure. To further prove this hypothesis, it would be of special interest to pre-immunize CSC mice with *M. vaccae*, or related derivatives, to positively modulate the immune system, which has been shown to be stress-protective (Reber et al., 2016b). Subsequently, exposing pre-immunized CSC mice to the SFC paradigm, will demonstrate if positive modulation of the immune system can prevent traumatic memory

encoding and / or stress-induced impaired social fear extinction. Furthermore, analyzing central inflammatory parameters, in CSC mice treated with *M. vaccae* compared to SHC, as well as following SFC acquisition, might help to unravel underlying inflammatory changes within the brain. Hereby, it seems crucial to focus on GILZ expression and activation of the NFκB signaling pathway in the amygdala. In addition, central inflammation in CSC mice needs to be studied not only after trauma induction, but also after re-exposure to the traumatic event, i.e., social stimulus presentation, as this might additionally exaggerate the immune response, mediating enhanced fear memory (Young et al., 2018). Moreover, some immunological parameters have to be added to analyze peripheral and central inflammation following CSC exposure, SFC acquisition and the combination of both, such as CRP, a marker for systemic inflammation, which is highly involved in the development of stress-related disorders including depression (Valkanova et al., 2013) and PTSD (O'Donovan et al., 2015). In the brain, TNF-α production from pro-inflammatory microglia mediated sustained fear memory, contributing to posttraumatic reactions by persistent fear memory (Yu et al., 2017). Thus, these parameters might be involved in CSC-induced impaired social fear extinction, contributing to the increased risk to develop a PTSD- and SAD-like phenotype. Moreover, these results will provide a broader knowledge about the impact of SAD on the immune system, which is lacking so far. Additionally, SAD has been shown to affect rather the SNS than HPA axis (Stein et al., 1992; van Veen et al., 2008). Thus measuring alpha-amylase, adrenalin, or noradrenalin following SFC acquisition and extinction, as well as following the combination of CSC exposure and SFC acquisition are of special interest, to see whether CSC-induced development of social avoidance, a SAD-like symptom, is accompanied by an altered reactivity of the SNS. In addition, amygdala reactivity has been repeatedly shown to play a fundamental role in the development of PTSD (Liberzon and Sripada, 2007; Nutt and Malizia, 2004; Rauch et al., 2000) and SAD (Phan et al., 2006; Stein et al., 2002; van Veen et al., 2008), as well as stress-induced enhanced fear memory (Hoffman et al., 2015), and acute stress following CSC exposure (Singewald et al., 2009). Thus, measuring amygdala activity following CSC exposure, SFC acquisition, and the combination of both might unravel another potential central mechanism, mediating chronic stress-induced impaired social fear extinction. Unraveling these mechanisms will help to understand the development of stress-related diseases and to generate effective and potent treatment options in humans.

Moreover, CUS exposure affected neither CFC, nor SFC. However, CUS mice exposed to CFC did barely show an induction of chronic stress, while during SFC, neither CUS mice nor SHC showed extinction of the social fear. Thus, to make a clear statement these experiments have to be repeated, probably using less susceptible mice, i.e., CD1 mice.

When testing BL6 and CD1 mice in various behavioral tests, CD1 mice showed lower trait anxiety, fear-related behavior, immune-sensitivity and stress-vulnerability, accompanied with higher active stress coping behavior, compared to BL6 mice. However, the underlying neurological and molecular differences between the mouse strains, mediating these extreme behavioral differences need to be identified, as innate stress-susceptibility or -tolerance may influence the probability to develop a disease state and to develop treatment resistance. I hypothesize that these differences are mediated via distinct responses of the central immune system, the SNS, HPA axis, endogenous OXT system, and / or distinct neuronal activity in the amygdala and LS. Nevertheless, both mouse strains showed an equally high impact of CSC exposure on social fear extinction. Thus, CSC-induced impaired extinction of social fear seems to be a robust and powerful effect, strengthening my hypothesis of a severe and serious impact of chronic psychosocial stress on the development of a PTSD- and SAD-like phenotype, where not even innate susceptibility or tolerance seem to impact the outcome.

Additionally, I could demonstrate that OXT represents a potent treatment option in the development of a PTSD- and SAD-like phenotype and might serve as an add-on pharmacotherapy during exposure therapy in PTSD and SAD patients. Nonetheless, unraveling underlying mechanisms of acute central OXT on the protective effects during CSC-induced impaired social fear extinction will help to improve further treatment options. These mechanisms are highly likely to include anti-inflammatory or stress-buffering effects, as well as dampening effects on neuronal activity in the amygdala and LS. In line, OXT treatment has been shown to dampen amygdala reactivity (Frijling, 2017; Koch et al., 2016), thus, preventing PTSD symptoms.

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# Appendix

# 6 Appendix

**Table A1: Statistics of the stress parameters in Experiment 1.** Factor time represents time effects over days; factor ACTH represents saline vs ACTH effects; factor CSC represents SHC vs CSC effects; factor SFC represents $SFC^-$ vs $SFC^+$ effects; body weight n = 30 – 31 per group; plasma ACTH n = 4-8 per group; mesLNCs, plasma and adrenal CORT n = 7-8 per group; other parameter n = 13-16 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CSC effects and the direction in which the parameter changed in CSC mice compared to SHC.

| Anxiety-like behavior | Independent T-Test, Mann-Whitney-U Test (latency) | *CSC effect |
|---|---|---|
| OF: Time spent in the inner zone [%]: SHC 12.6±1.19; CSC 10.1±1.30 | (CSC) $T_{28} = 1.41$ | $P = 0.169$ |
| OF: Total distance moved [m]: SHC 18.2±0.645 CSC 15.8±0.586*↓ | (CSC) $T_{29} = 2.74$ | $P = 0.010$ |
| NOR: Occurrence sniffing [n]: SHC 7.60±0.815; CSC 4.93±0.802*↓ | (CSC) $T_{27} = 2.33$ | $P = 0.027$ |
| LDB: Time spent in the LB [%): SHC 40.0±2.02; CSC 40.7±2.23 | (CSC) $T_{27} = -0.209$ | $P = 0.836$ |
| LDB: Latency to enter the LB [s]: SHC 62.5±14.7; CSC 24.7±5.59*↓ | (CSC) $T_{17.9} = 2.40$ | $P = 0.027$ |
| **Body weight** | **One way ANOVA for RM** | ***CSC effect** |
| Body weight gain [g]*↑<br>Δ Day 19 – day 1: SHC 2.57±0.160; CSC 2.63±0.213<br>Δ Recall – day 1: SHC 2.55±0.170; CSC 4.19±0.206 | (Time) $F_{1,59} = 52.4$<br>(CSC) $F_{1,59} = 12.2$<br>(Time x CSC) $F_{1,59} = 55.5$ | $P < 0.001$<br>$P = 0.001$<br>$P < 0.001$ |
| **Physiological parameters** | **Two-way ANOVA** | ***CSC effect** |
| Relative total adrenal weight [mg/g]*↑<br>SHC: $SFC^-$ 0.131±0.003; $SFC^+$ 0.123±0.006<br>CSC: $SFC^-$ 0.143±0.003; $SFC^+$ 0.135±0.003 | (CSC) $F_{1,56} = 8.76$<br>(SFC) $F_{1,56} = 4.45$<br>(CSC x SFC) $F_{1,56} = 0.013$ | $P = 0.005$<br>$P = 0.039$<br>$P = 0.908$ |
| Relative thymus weight [mg/g]<br>SHC: $SFC^-$ 1.61±0.048; $SFC^+$ 1.62±0.046<br>CSC: $SFC^-$ 1.56±0.064; $SFC^+$ 1.55±0.070 | (CSC) $F_{1,55} = 1.06$<br>(SFC) $F_{1,55} = 0.012$<br>(CSC x SFC) $F_{1,55} = 0.034$ | $P = 0.307$<br>$P = 0.912$<br>$P = 0.854$ |
| Relative spleen weight [mg/g]*↑<br>SHC: $SFC^-$ 2.72±0.081; $SFC^+$ 2.60±0.052<br>CSC: $SFC^-$ 3.12±0.092; $SFC^+$ 2.99±0.079 | (CSC) $F_{1,51} = 25.8$<br>(SFC) $F_{1,51} = 2.63$<br>(CSC x SFC) $F_{1,51} = 0.005$ | $P < 0.001$<br>$P = 0.111$<br>$P = 0.945$ |
| mesLNCs IFN-γ release [pg/ml]*↑<br>SHC: $SFC^-$ 18.2±1.06; $SFC^+$ 123±101<br>CSC: $SFC^-$ 532±158; $SFC^+$ 667±253 | (CSC) $F_{1,25} = 7.39$<br>(SFC) $F_{1,25} = 0.381$<br>(CSC x SFC) $F_{1,25} = 0.006$ | $P = 0.012$<br>$P = 0.543$<br>$P = 0.941$ |
| Plasma ACTH [pg/ml]<br>SHC: $SFC^-$ 169±56.2; $SFC^+$ 96.4±23.8<br>CSC: $SFC^-$ 142±42.0; $SFC^+$ 94.5±50.9 | (CSC) $F_{1,20} = 0.103$<br>(SFC) $F_{1,20} = 1.76$<br>(CSC x SFC) $F_{1,20} = 0.077$ | $P = 0.752$<br>$P = 0.199$<br>$P = 0.784$ |
| Plasma CORT [ng/ml]*↓<br>SHC: $SFC^-$ 289±46.6; $SFC^+$ 207±28.0<br>CSC: $SFC^-$ 175±49.6; $SFC^+$ 80.8±20.7 | (CSC) $F_{1,20} = 9.95$<br>(SFC) $F_{1,20} = 5.30$<br>(CSC x SFC) $F_{1,20} = 0.029$ | $P = 0.004$<br>$P = 0.029$<br>$P = 0.866$ |
| **Adrenal stimulation** | **Three-way ANOVA** | ***CSC effect** |
| Adrenal CORT release [ng/ml/mg]*↓<br>SHC saline: $SFC^-$ 839±127; $SFC^+$ 627±72.0<br>SHC ACTH: $SFC^-$ 1817±298; $SFC^+$ 2158±250<br>CSC saline: $SFC^-$ 594±58.6; $SFC^+$ 553±52.0<br>CSC ACTH: $SFC^-$ 1312±116; $SFC^+$ 1365±170 | (ACTH) $F_{1,51} = 73.1$<br>(CSC) $F_{1,51} = 11.7$<br>(SFC) $F_{1,51} = 0.088$<br>(ACTH x CSC) $F_{1,51} = 4.30$<br>(ACTH x SFC) $F_{1,51} = 1.88$<br>(CSC x SFC) $F_{1,51} = 0.063$<br>(ACTH x CSC x SFC)<br>$F_{1,51} = 0.947$ | $P < 0.001$<br>$P < 0.001$<br>$P = 0.767$<br>$P = 0.043$<br>$P = 0.176$<br>$P = 0.803$<br>$P = 0.335$ |

**Table A2: Statistics of the stress parameters in Experiment 2.1.** Factor time represents time effects over days; factor LPS represents basal vs LPS effects; factor CORT represents ascending CORT concentrations; factor CSC represents SHC vs CSC effects; n = 7-8 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CSC effects and the direction in which the parameter changed in CSC mice compared to SHC.

| Anxiety-like behavior | Independent T-Test | |
|---|---|---|
| Time spent in the LB [%]: SHC 62.9±9.89; CSC 52.1±9.88 | (CSC) $T_{12} = 0.761$ | $P = 0.462$ |
| Latency to enter the LB [s]: SHC 34.4±11.2; CSC 14.0±5.02 | (CSC) $T_{12} = -0.302$ | $P = 0.768$ |
| **Body weight** | **One way ANOVA for RM** | ***CSC effect** |
| Body weight gain [g]<br>Δ Day 19 – day 1: SHC 2.75±0.253; CSC 3.49±0.385<br>Δ Recall – day 1: SHC 2.66±0.366; CSC 4.35±0.525*↑ | (Time) $F_{1,14} = 5.58$<br>(CSC) $F_{1,14} = 5.18$<br>(Time x CSC) $F_{1,14} = 8.39$ | $P = 0.033$<br>$P = 0.039$<br>$P = 0.012$ |
| **Physiological parameters** | **Independent T-Test, Mann-Whitney-U Test (spleen)** | ***CSC effect** |
| Relative total adrenal weight [mg/g]*(↑)<br>SHC 0.125±0.004; CSC 0.133±0.002 | (CSC) $T_{14} = -2.07$ | $P = 0.058$ |
| Relative thymus weight [mg/g]: SHC 1.82±0.100; CSC 1.62±0.063 | (CSC) $T_{13} = 1.73$ | $P = 0.107$ |
| Relative spleen weight [mg/g]: SHC 2.39±0.069; CSC 3.01±0.260*↑ | (CSC) $T_{7.97} = -2.31$ | $P = 0.050$ |
| **Spleen cell stimulation** | **Two-way ANOVA, Mann-Whitney-U Test (delta CORT = 0 µM)** | ***CSC effect** |
| Cell viability CORT = 0 µM [OD]*↑<br>Basal: SHC 0.100±0.017; CSC 0.188±0.030<br>LPS: SHC 0.211±0.022; CSC 0.394±0.046 | (LPS) $F_{1,20} = 27.0$<br>(CSC) $F_{1,20} = 19.7$<br>(LPS x CSC) $F_{1,20} = 2.41$ | $P < 0.001$<br>$P < 0.001$<br>$P = 0.136$ |
| Delta cell viability CORT = 0 µM [OD]<br>SHC 0.112±0.011; CSC 0.207±0.026*↑ | (CSC) $T_{6.705} = -3.31$ | $P = 0.014$ |
| Delta cell viability [OD]*↑<br>SHC: 0µM 0.112±0.011; 0.005µM 0.110±0.011; 0.05µM 0.083±0.009; 0.1µM 0.071±0.007; 0.5µM 0.072±0.016; 5µM 0.078±0.011<br>CSC: 0µM 0.207±0.026; 0.005µM 0.245±0.048; 0.05µM 0.245±0.047; 0.1µM 0.203±0.063; 0.5µM 0.215±0.057; 5µM 0.208±0.050 | (CORT) $F_{5,60} = 0.364$<br>(CSC) $F_{1,60} = 40.6$<br>(CORT x CSC) $F_{5,60} = 0.183$ | $P = 0.871$<br>$P < 0.001$<br>$P = 0.968$ |
| Delta cell viability CORT = 0 µM set to 100 % [%]*↑<br>SHC: 0µM 100±0; 0.005µM 101±9.34; 0.05µM 78.1±11.1; 0.1µM 65.0±5.62; 0.5µM 65.3±11.6; 5µM 73.0±11.5<br>CSC: 0µM 100±0; 0.005µM 116±15.0; 0.05µM 114±8.01; 0.1µM 88.7±16.6; 0.5µM 95.6±14.4; 5µM 96.1±14.4 | (CORT) $F_{5,60} = 2.52$<br>(CSC) $F_{1,60} = 11.2$<br>(CORT x CSC) $F_{5,60} = 0.642$ | $P = 0.039$<br>$P = 0.001$<br>$P = 0.668$ |

**Table A3: Statistics of the stress parameters in Experiment 2.2a.** Factor ACTH represents saline vs ACTH effects; factor CSC represents SHC vs CSC effects; n = 7-8 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CSC effects and the direction in which the parameter changed in CSC mice compared to SHC.

| Body weight | Mann-Whitney-U Test | |
|---|---|---|
| Body weight gain [g]: SHC 1.51±0.182; CSC 1.91±0.341 | (CSC) $T_{10.7} = -1.03$ | $P = 0.324$ |
| **Physiological parameters** | **Independent T-Test, Mann-Whitney-U Test (spleen)** | ***CSC effect** |
| Relative adrenal weight [mg/g]: SHC 0.122±0.003; CSC 0.159±0.003*↑ | (CSC) $T_{14} = -9.20$ | $P < 0.001$ |
| Relative thymus weight [mg/g]: SHC 1.83±0.043; CSC 1.09±0.065*↓ | (CSC) $T_{13} = 9.27$ | $P < 0.001$ |
| Relative spleen weight [mg/g]: SHC 2.59±0.053; CSC 4.18±0.224*↑ | (CSC) $T_{6.681} = -6.91$ | $P < 0.001$ |
| **Adrenal stimulation** | **Two-way ANOVA** | ***CSC effect** |
| Adrenal CORT release [ng/ml/mg]*↓<br>SHC: saline 1063±168; ACTH 9289±2844<br>CSC: saline 698±64.0; ACTH 2148±247 | (ACTH) $F_{1,28} = 11.4$<br>(CSC) $F_{1,28} = 6.86$<br>(ACTH x CSC)<br>$F_{1,28} = 5.59$ | $P = 0.002$<br>$P = 0.014$<br>$P = 0.025$ |

**Table A4: Statistics of the stress parameters in Experiment 2.2c.** Factor time represents time effects over days; factor CSC represents SHC vs CSC effects; factor SFC represents $SFC^-$ vs $SFC^+$ effects; body weight and anxiety-like behavior n = 16 per group; other parameters n = 7-8 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CSC effects and the direction in which the parameter changed in CSC mice compared to SHC.

| Anxiety-like behavior | Independent T-Test, Mann-Whitney-U Test (time OA, OA entries, full OA) | *CSC effect |
|---|---|---|
| Time spent on the open arms [%]: SHC 29.8±9.33; CSC 6.8±1.66*↓ | (CSC) $T_{16.4} = 2.74$ | P = 0.014 |
| Open arm entries [%]: SHC 39.5±6.82; CSC 24.5±2.52*(↓) | (CSC) $T_{30} = 1.99$ | P = 0.059 |
| Full open arm entries [n]: SHC 4.89±1.60; CSC 1.33±0.408*↓ | (CSC) $T_{17.6} = 2.52$ | P = 0.022 |
| Closed arm entries [n]: SHC 12.6±2.01; CSC 12.8±1.47 | (CSC) $T_{30} = -0.169$ | P = 0.867 |
| **Body weight** | **One way ANOVA for RM** | ***CSC effect** |
| Body weight gain [g]*↑<br>Δ Day 19 – day 1: SHC 2.23±0.162; CSC 2.89±0.373<br>Δ Acquisition – day 1: SHC 2.42±0.096; CSC 3.84±0.316 | (Time) $F_{1,30} = 28.6$<br>(CSC) $F_{1,30} = 8.68$<br>(Time x CSC) $F_{1,30} = 12.6$ | P < 0.001<br>P = 0.006<br>P = 0.001 |
| **Physiological parameters** | **Two-way ANOVA** | ***CSC effect** |
| Relative total adrenal weight [mg/g]*↑<br>SHC: $SFC^-$ 0.131±0.002; $SFC^+$ 0.139±0.003<br>CSC: $SFC^-$ 0.151±0.006; $SFC^+$ 0.151±0.003 | (CSC) $F_{1,27} = 17.0$<br>(SFC) $F_{1,27} = 0.829$<br>(CSC x SFC) $F_{1,27} = 1.26$ | P < 0.001<br>P = 0.371<br>P = 0.272 |
| Relative thymus weight [mg/g]*↓<br>SHC: $SFC^-$ 2.02±0.081; $SFC^+$ 1.90±0.062<br>CSC: $SFC^-$ 1.60±0.116; $SFC^+$ 1.75±0.079 | (CSC) $F_{1,28} = 10.3$<br>(SFC) $F_{1,28} = 0.034$<br>(CSC x SFC) $F_{1,28} = 2.31$ | P = 0.003<br>P = 0.855<br>P = 0.140 |
| Relative spleen weight [mg/g]*↑<br>SHC: $SFC^-$ 3.00±0.221; $SFC^+$ 2.63±0.076<br>CSC: $SFC^-$ 3.58±0.281; $SFC^+$ 3.28±0.119 | (CSC) $F_{1,28} = 10.3$<br>(SFC) $F_{1,28} = 3.07$<br>(CSC x SFC) $F_{1,28} = 0.044$ | P = 0.003<br>P = 0.091<br>P = 0.836 |
| Plasma CORT [ng/ml]<br>SHC: $SFC^-$ 43.4±3.07; $SFC^+$ 45.7±8.04<br>CSC: $SFC^-$ 46.3±2.30; $SFC^+$ 52.0±4.89 | (CSC) $F_{1,24} = 0.818$<br>(SFC) $F_{1,24} = 0.623$<br>(CSC x SFC) $F_{1,24} = 0.117$ | P = 0.375<br>P = 0.438<br>P = 0.735 |
| **SFC acquisition** | **Independent T-Test** | |
| Number of shocks [n]: SHC 1.50±0.267; CSC 1.38±0.183 | (CSC) $T_{14} = 0.386$ | P = 0.705 |

**Table A5: Statistics of the stress parameters in Experiment 3.1.** Factor time represents time effects over days; factor LPS represents basal vs LPS effects; factor CORT represents ascending CORT concentrations; factor CSC represents SHC vs CSC effects; n = 7-8 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CSC effects and the direction in which the parameter changed in CSC mice compared to SHC.

| Anxiety-like behavior | Independent T-Test, Mann-Whitney-U Test (latency) | |
|---|---|---|
| Time spent in the light box [%]: SHC 43.7±2.85; CSC 40.7±2.59 | (CSC) $T_{13}$ = 0.771 | P = 0.454 |
| Latency to enter the light box [s]: SHC 14.4±3.96; CSC 21.9±6.05 | (CSC) $T_{11.8}$ = -1.03 | P = 0.324 |
| **Body weight** | **One way ANOVA for RM** | |
| Body weight gain [g]<br>Δ Day 19 – day 1: SHC 2.11±0.261; CSC 2.70±0.357<br>Δ Retention – day 1: SHC 2.56±0.376; CSC 3.83±0.602 | (Time) $F_{1,13}$ = 11.2<br>(CSC) $F_{1,13}$ = 2.69<br>(Time x CSC) $F_{1,13}$ = 2.13 | P = 0.005<br>P = 0.125<br>P = 0.168 |
| **Physiological parameters** | **Independent T-Test, Mann-Whitney-U Test (spleen)** | ***CSC effect** |
| Relative total adrenal weight [mg/g]:<br>SHC 0.124±0.002; CSC 0.129±0.004 | (CSC) $T_{12}$ = -1.08 | P = 0.302 |
| Absolute total adrenal weight [mg]: SHC 3.17±0.087; CSC 3.49±0.106*↑ | (CSC) $T_{12}$ = -2.30 | P = 0.040 |
| Relative thymus weight [mg/g]: SHC 1.90±0.101; CSC 1.68±0.103 | (CSC) $T_{13}$ = 1.53 | P = 0.151 |
| Relative spleen weight [mg/g]: SHC 2.26±0.061; CSC 2.94±0.210*↑ | (CSC) $T_{8.17}$ = -3.10 | P = 0.014 |
| **Spleen cell stimulation** | **Two-way ANOVA, Independent T-Test (delta CORT = 0 µM)** | ***CSC effect** |
| Cell viability CORT = 0 µM [OD]*↑<br>Basal: SHC 0.212±0.021; CSC 0.435±0.082<br>LPS: SHC 0.487±0.043; CSC 0.759±0.078 | (LPS) $F_{1,26}$ = 21.5<br>(CSC) $F_{1,26}$ = 14.6<br>(LPS x CSC) $F_{1,26}$ = 0.145 | P < 0.001<br>P = 0.001<br>P = 0.706 |
| Delta cell viability CORT = 0 µM [OD]: SHC 0.275±0.034; CSC 0.324±0.038 | (CSC) $T_{13}$ = -0.959 | P = 0.355 |
| Delta cell viability [OD]*↑<br>SHC: 0µM 0.275±0.034; 0.005µM 0.256±0.034; 0.05µM 0.119±0.014; 0.1µM 0.104±0.026; 0.5µM 0.130±0.017; 5µM 0.086±0.012<br>CSC: 0µM 0.324±0.038; 0.005µM 0.302±0.026; 0.05µM 0.261±0.055; 0.1µM 0.257±0.042; 0.5µM 0.340±0.067; 5µM 0.291±0.059 | (CORT) $F_{5,77}$ = 3.27<br>(CSC) $F_{5,77}$ = 33.5<br>(CORT x CSC) $F_{5,77}$ = 1.62 | P = 0.010<br>P < 0.001<br>P = 0.165 |
| Delta cell viability CORT = 0 µM set to 100 % [%]*↑<br>SHC: 0µM 100±0; 0.005µM 94.9±9.05; 0.05µM 48.1±7.89; 0.1µM 36.4±6.49; 0.5µM 40.0±4.86; 5µM 34.8±6.65<br>CSC: 0µM 100±0; 0.005µM 99.7±9.67; 0.05µM 94.0±30.8; 0.1µM 86.0±14.9; 0.5µM 113±36.3; 5µM 99.6±20.3 | (CORT) $F_{5,76}$ = 1.85<br>(CSC) $F_{5,76}$ = 16.5<br>(CORT x CSC) $F_{5,76}$ = 1.62 | P = 0.114<br>P < 0.001<br>P = 0.165 |

**Table A6: Statistics of the stress parameters in Experiment 3.2b.** Factor time represents time effects over days; factor CUS represents SHC vs CUS effects; n = 6-8 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CUS effects and the direction in which the parameter changed in CUS mice compared to SHC.

| Anxiety-like behavior | Independent T-Test | |
|---|---|---|
| Time spent on the open arms [%]: SHC 31.1±8.62; CUS 31.1±7.91 | (CUS) $T_{14}$ = 0.004 | P = 0.997 |
| Open arm entries [%]: SHC 38.7±6.62; CUS 40.2±5.88 | (CUS) $T_{14}$ = -0.161 | P = 0.874 |
| Full open arm entries [n]: SHC 7.13±1.41; CUS 7.38±0.75 | (CUS) $T_{14}$ = -0.157 | P = 0.878 |
| Closed arm entries [n]: SHC 10.6±1.38; CUS 11.1±1.06 | (CUS) $T_{14}$ = -0.288 | P = 0.778 |
| **Body weight** | **One way ANOVA for RM** | ***CUS effect** |
| Body weight gain [g]*↓<br>Δ Day 19 – day 1: SHC 1.59±0.282; CUS 0.46±0.233<br>Δ Retention – day 1: SHC 1.50±0.224; CUS 0.91±0.272 | (Time) $F_{1,14}$ = 2.98<br>(CUS) $F_{1,14}$ = 6.23<br>(Time x CUS) $F_{1,14}$ = 6.55 | P = 0.106<br>P = 0.026<br>P = 0.023 |
| **Physiological parameters** | **Independent T-Test, Mann-Whitney-U Test (spleen)** | |
| Relative total adrenal weight [mg/g]: SHC 0.162±0.01; CUS 0.182±0.01 | (CUS) $T_{12}$ = -1.32 | P = 0.211 |
| Relative thymus weight [mg/g]: SHC 1.83±0.109; CUS 1.67±0.080 | (CUS) $T_{14}$ = 1.15 | P = 0.271 |
| Relative spleen weight [mg/g]: SHC 2.44±0.075; CUS 2.60±0.184 | (CUS) $T_{9.25}$ = -0.794 | P = 0.441 |

**Table A7: Statistics of the stress parameters in Experiment 3.2c.** Factor time represents time effects over days; factor CUS represents SHC vs CUS effects; factor SFC represents $SFC^-$ vs $SFC^+$; body weight n = 32 per group; anxiety n = 24 per group; other parameters n = 14-16 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CUS effects and the direction in which the parameter changed in CUS mice compared to SHC.

| Anxiety-like behavior | Independent T-Test, Mann-Whitney-U Test (latency) | ***CUS effect** |
|---|---|---|
| Time spent on the open arms [%]: SHC 20.2±2.65; CUS 18.0±1.74 | (CUS) $T_{46}$ = 0.706 | P = 0.484 |
| Open arm entries [%]: SHC 34.1±2.13; CUS 28.5±1.24*↓ | (CUS) $T_{46}$ = 2.28 | P = 0.028 |
| Full open arm entries [n]: SHC 4.71±0.569; CUS 4.17±0.428 | (CUS) $T_{46}$ = 0.760 | P = 0.451 |
| Closed arm entries [n]: SHC 13.2±1.29; CUS 14.8±1.15 | (CUS) $T_{46}$ = -0.915 | P = 0.365 |
| **Body weight** | **One way ANOVA for RM** | ***CUS effect** |
| Body weight gain [g]*↓<br>Δ Day 19 – day 1: SHC 2.66±0.111; CUS 0.76±0.086<br>Δ Recall – day 1: SHC 2.18±0.169; CUS 1.57±0.117 | (Time) $F_{1,62}$ = 4.52<br>(CUS) $F_{1,62}$ = 64.3<br>(Time x CUS) $F_{1,62}$ = 65.7 | P = 0.038<br>P < 0.001<br>P < 0.001 |
| **Physiological parameters** | **Two-way ANOVA** | ***CUS effect** |
| Relative total adrenal weight [mg/g]*↑<br>SHC: $SFC^-$ 0.141±0.004; $SFC^+$ 0.143±0.003<br>CUS: $SFC^-$ 0.162±0.005; $SFC^+$ 0.156±0.002 | (CUS)$F_{1,56}$ = 20.9<br>(SFC) $F_{1,56}$ = 0.335<br>(CUS x SFC) $F_{1,56}$ = 0.969 | P < 0.001<br>P = 0.565<br>P = 0.329 |
| Relative thymus weight [mg/g]<br>SHC: $SFC^-$ 2.10±0.078; $SFC^+$ 2.00±0.096<br>CUS: $SFC^-$ 1.93±0.070; $SFC^+$ 1.90±0.092 | (CUS) $F_{1,60}$ = 2.74<br>(SFC) $F_{1,60}$ = 0.566<br>(CUS x SFC) $F_{1,60}$ = 0.191 | P = 0.103<br>P = 0.455<br>P = 0.664 |
| Absolute thymus weight [mg]*↓<br>SHC: $SFC^-$ 52.4±2.54; $SFC^+$ 48.4±2.28<br>CUS: $SFC^-$ 47.7±2.06; $SFC^+$ 47.0±1.90 | (CUS) $F_{1,60}$ = 5.81<br>(SFC) $F_{1,60}$ = 0.389<br>(CUS x SFC) $F_{1,60}$ = 0.164 | P = 0.019<br>P = 0.535<br>P = 0.687 |
| Relative spleen weight [mg/g]<br>SHC: $SFC^-$ 2.66±0.084; $SFC^+$ 2.57±0.089<br>CUS: $SFC^-$ 2.48±0.040; $SFC^+$ 2.57±0.073 | (CUS) $F_{1,60}$ = 1.16<br>(SFC) $F_{1,60}$ = 0.001<br>(CUS x SFC) $F_{1,60}$ = 1.24 | P = 0.287<br>P = 0.981<br>P = 0.270 |

**Table A8: Statistics of the stress parameters in Experiment 3.3c.** Factor time represents time effects over days; factor ACTH represents saline vs ACTH effects; factor CSC represents SHC vs CSC effects; factor SFC represents SFC⁻ vs SFC⁺ effects; body weight and anxiety-like behavior n = 16 per group; unconditioned animals n = 6 per group; conditioned animals n = 10 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CSC effects and the direction in which the parameter changed in CSC mice compared to SHC.

| Anxiety-like behavior | Independent T-Test, Mann-Whitney-U Test (full OA) | |
|---|---|---|
| Time spent on the open arms [%]: SHC 26.2±2.35; CSC 20.9±2.25 | (CSC) $T_{28} = 1.62$ | $P = 0.117$ |
| Open arm entries [%]: SHC 47.5±1.41; CSC 47.2±2.12 | (CSC) $T_{28} = 0.116$ | $P = 0.909$ |
| Full open arm entries [n]: SHC 1.9±0.530; CSC 1.1±0.363 | (CSC) $T_{24.8} = 1.25$ | $P = 0.225$ |
| Closed arm entries [n]: SHC 18.0±1.21; CSC 17.0±1.21 | (CSC) $T_{28} = 0.583$ | $P = 0.565$ |
| **Body weight** | **One way ANOVA for RM** | ***(CSC trend)** |
| Body weight gain [g] ***(↑)**<br>Δ Day 19 – day 1: SHC 1.78±0.302; CSC 1.77±0.276<br>Δ Recall – day 1: SHC 2.13±0.254; CSC 2.60±0.278 | (Time) $F_{1,30} = 22.4$<br>(CSC) $F_{1,30} = 0.385$<br>(Time x CSC) $F_{1,30} = 3.85$ | $P < 0.001$<br>$P = 0.540$<br>$P = 0.059$ |
| **Physiological parameters** | **Two-way ANOVA** | ***CSC effect** |
| Relative total adrenal weight [mg/g]*↑<br>SHC: SFC⁻ 0.114±0.008; SFC⁺ 0.101±0.003<br>CSC: SFC⁻ 0.126±0.004; SFC⁺ 0.114±0.004 | (CSC) $F_{1,26} = 7.07$<br>(SFC) $F_{1,26} = 6.21$<br>(CSC x SFC) $F_{1,26} = 0.009$ | $P = 0.013$<br>$P = 0.019$<br>$P = 0.925$ |
| Relative thymus weight [mg/g]<br>SHC: SFC⁻ 0.955±0.164; SFC⁺ 0.823±0.063<br>CSC: SFC⁻ 0.749±0.045; SFC⁺ 0.966±0.072 | (CSC) $F_{1,27} = 0.112$<br>(SFC) $F_{1,27} = 0.206$<br>(CSC x SFC) $F_{1,27} = 3.45$ | $P = 0.741$<br>$P = 0.654$<br>$P = 0.074$ |
| Relative spleen weight [mg/g]*↑<br>SHC: SFC⁻ 3.04±0.181; SFC⁺ 3.01±0.131<br>CSC: SFC⁻ 4.06±0.283; SFC⁺ 3.86±0.217 | (CSC) $F_{1,28} = 20.1$<br>(SFC) $F_{1,28} = 0.283$<br>(CSC x SFC) $F_{1,28} = 0.177$ | $P < 0.001$<br>$P = 0.599$<br>$P = 0.677$ |
| Number of mesLNCs [n*10⁶]<br>SHC: SFC⁻ 2.26±0.073; SFC⁺ 2.47±0.242<br>CSC: SFC⁻ 2.83±0.517; SFC⁺ 2.40±0.318 | (CSC) $F_{1,28} = 0.596$<br>(SFC) $F_{1,28} = 0.112$<br>(CSC x SFC) $F_{1,28} = 0.945$ | $P = 0.447$<br>$P = 0.740$<br>$P = 0.339$ |
| mesLNCs IFN-γ release [pg/ml]<br>SHC: SFC⁻ 4.00±0.239; SFC⁺ 17.6±8.06<br>CSC: SFC⁻17.7±4.56; SFC⁺ 28.8±14.0 | (CSC) $F_{1,25} = 1.48$<br>(SFC) $F_{1,25} = 1.45$<br>(CSC x SFC) $F_{1,25} = 0.015$ | $P = 0.235$<br>$P = 0.239$<br>$P = 0.903$ |
| Plasma CORT [ng/ml]<br>SHC: SFC⁻ 76.2±13.7; SFC⁺ 124±24.9<br>CSC: SFC⁻ 86.0±8.21; SFC⁺ 112±17.1 | (CSC) $F_{1,28} = 0.502$<br>(SFC) $F_{1,28} = 2.33$<br>(CSC x SFC) $F_{1,28} = 0.267$ | $P = 0.485$<br>$P = 0.138$<br>$P = 0.609$ |
| **Adrenal stimulation** | **Three-way ANOVA** | |
| Adrenal CORT release [ng/ml/mg]<br>SHC saline: SFC⁻ 494±58.4; SFC⁺ 581±66.0<br>SHC ACTH: SFC⁻ 954±161; SFC⁺ 1018±92.1<br>CSC saline: SFC⁻ 500±69.4; SFC⁺ 561±73.9<br>CSC ACTH: SFC⁻ 1200±217; SFC⁺ 1195±151 | (ACTH) $F_{1,50} = 33.8$<br>(CSC) $F_{1,50} = 1.96$<br>(SFC) $F_{1,50} = 0.025$<br>(ACTH x CSC) $F_{1,50} = 2.61$<br>(ACTH x SFC) $F_{1,50} = 0.308$<br>(CSC x SFC) $F_{1,50} = 0.300$<br>(ACTH x CSC x SFC) $F_{1,50} = 0.201$ | $P < 0.001$<br>$P = 0.168$<br>$P = 0.876$<br>$P = 0.113$<br>$P = 0.581$<br>$P = 0.587$<br>$P = 0.656$ |

**Table A9: Statistics of the stress parameters in Experiment 4.2a.** Factor time represents time effects over days; factor CSC represents SHC vs CSC effects; factor SFC represents $SFC^-$ vs $SFC^+$ effects; body weight n = 40-47 per group; anxiety-like behavior n = 12 per group; other parameters n = 10-12 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CSC effects and the direction in which the parameter changed in CSC mice compared to SHC.

| Anxiety-like behavior | Independent T-Test, Mann-Whitney-U Test (time OA) | *CSC effect |
|---|---|---|
| Time spent on the open arms [%]: SHC 51.5±8.35; CSC 23.1±2.49*↓ | (CSC) $T_{12.9} = 3.26$ | P = 0.006 |
| Open arm entries [%]: SHC 48.8±4.75; CSC 39.3±3.09 | (CSC) $T_{22} = 1.67$ | P = 0.109 |
| Full open arm entries [n]: SHC 4.75±0.509; CSC 3.92±0.596 | (CSC) $T_{22} = 1.06$ | P = 0.299 |
| Closed arm entries [n]: SHC 8.42±1.32; CSC 11.7±0.899*(↑) | (CSC) $T_{22} = -2.04$ | P = 0.054 |
| **Body weight** | **One way ANOVA for RM** | ***CSC effect** |
| Body weight gain [g]<br>Δ Day 19 – day 1: SHC 1.99±0.079; CSC 1.42±0.181*↓<br>Δ Recall – day 1: SHC 1.15±0.213; CSC 1.95±0.265*↑ | (Time) $F_{1,77} = 1.93$<br>(CSC) $F_{1,77} = 0.150$<br>(Time x CSC) $F_{1,77} = 21.1$ | P = 0.168<br>P = 0.700<br>P < 0.001 |
| **Physiological parameters VEH** | **Two-way ANOVA** | ***CSC effect** |
| Relative total adrenal weight [mg/g]<br>SHC: $SFC^-$ 0.146±0.005; $SFC^+$ 0.144±0.005<br>CSC: $SFC^-$ 0.155±0.007; $SFC^+$ 0.144±0.004 | (CSC) $F_{1,32} = 0.755$<br>(SFC) $F_{1,32} = 1.39$<br>(CSC x SFC) $F_{1,32} = 0.492$ | P = 0.391<br>P = 0.247<br>P = 0.488 |
| Relative thymus weight [mg/g]<br>SHC: $SFC^-$ 1.63±0.099; $SFC^+$ 1.54±0.080<br>CSC: $SFC^-$ 1.59±0.084; $SFC^+$ 1.46±0.058 | (CSC) $F_{1,33} = 0.565$<br>(SFC) $F_{1,33} = 2.00$<br>(CSC x SFC) $F_{1,33} = 0.063$ | P = 0.457<br>P = 0.167<br>P = 0.803 |
| Relative spleen weight [mg/g]*↑<br>SHC: $SFC^-$ 2.68±0.150; $SFC^+$ 2.60±0.095<br>CSC: $SFC^-$ 3.38±0.312; $SFC^+$ 3.22±0.287 | (CSC) $F_{1,34} = 10.0$<br>(SFC) $F_{1,34} = 0.601$<br>(CSC x SFC) $F_{1,34} = 0.018$ | P = 0.003<br>P = 0.444<br>P = 0.894 |
| **Physiological parameters OXT** | **Two-way ANOVA** | ***CSC effect** |
| Relative total adrenal weight<br>SHC: $SFC^-$ 0.139±0.004; $SFC^+$ 0.066±0.003<br>CSC: $SFC^-$ 0.155±0.007; $SFC^+$ 0.147±0.009 | (CSC) $F_{1,34} = 2.08$<br>(SFC) $F_{1,34} = 0.084$<br>(CSC x SFC) $F_{1,34} = 0.823$ | P = 0.158<br>P = 0.774<br>P = 0.371 |
| Relative thymus weight<br>SHC: $SFC^-$ 1.55±0.068; $SFC^+$ 1.53±0.097<br>CSC: $SFC^-$ 1.63±0.090; $SFC^+$ 1.48±0.116 | (CSC) $F_{1,36} = 0.033$<br>(SFC) $F_{1,36} = 0.841$<br>(CSC x SFC) $F_{1,36} = 0.460$ | P = 0.856<br>P = 0.365<br>P = 0.502 |
| Relative spleen weight*↑<br>SHC: $SFC^-$ 2.58±0.061; $SFC^+$ 2.47±0.105<br>CSC: $SFC^-$ 3.29±0.221; $SFC^+$ 3.15±0.206 | (CSC) $F_{1,36} = 20.0$<br>(SFC) $F_{1,36} = 1.34$<br>(CSC x SFC) $F_{1,36} = 0.199$ | P < 0.001<br>P = 0.254<br>P = 0.658 |

**Table A10: Statistics of the stress parameters in Experiment 4.2b.** Factor time represents time effects over days; factor CSC represents SHC vs CSC effects; factor SFC represents $SFC^-$ vs $SFC^+$ effects; body weight n = 14-32 per group; anxiety-like behavior n = 12-14 per group; other parameters n = 3-9 per group. Data are given as mean ± SEM. * and ↑/↓ indicate CSC effects and the direction in which the parameter changed in CSC mice compared to SHC.

| Anxiety-like behavior | Mann-Whitney-U Test, Independent T-Test (CA entries) | ***CSC effect** |
|---|---|---|
| Time spent on the open arms [%]: SHC 20.7±2.82; CSC 16.6±1.41 | (CSC) $T_{16.3}$ = 1.30 | P = 0.211 |
| Open arm entries [%]: SHC 43.3±1.91; CSC 48.7±0.878*↑ | (CSC) $T_{15.6}$ = -2.60 | P = 0.020 |
| Full open arm entries [n]: SHC 2.33±0.655; CSC 0.714±0.286*↓ | (CSC) $T_{15.1}$ = 2.27 | P = 0.039 |
| Time spent on the full open arms [s]: SHC 20.3±5.92; CSC 3.86±1.37*↓ | (CSC) $T_{12.2}$ = 2.70 | P = 0.019 |
| Closed arm entries [n]: SHC 18.1±1.16; CSC 16.8±1.23 | (CSC) $T_{24}$ = 0.759 | P = 0.455 |
| **Body weight** | **One way ANOVA for RM** | ***CSC effect** |
| Body weight gain [g]<br>Δ Day 19 – day 1: SHC 0.550±0.276; CSC 1.51±0.202*↑<br>Δ Recall – day 1: SHC -0.486±0.361; CSC 1.29±0.261*↑ | (Time) $F_{1,43}$ = 13.8<br>(CSC) $F_{1,43}$ = 12.5<br>(Time x CSC) $F_{1,43}$ = 6.43 | P = 0.001<br>P = 0.001<br>P = 0.015 |
| **Physiological parameters VEH** | **Two-way ANOVA** | ***(CSC trend)** |
| Relative total adrenal weight [mg/g]<br>SHC: $SFC^-$ 0.114±0.012; $SFC^+$ 0.116±0.020<br>CSC: $SFC^-$ 0.124±0.009; $SFC^+$ 0.132±0.007 | (CSC) $F_{1,16}$ = 1.34<br>(SFC) $F_{1,16}$ = 0.218<br>(CSC x SFC) $F_{1,16}$ = 0.041 | P = 0.264<br>P = 0.647<br>P = 0.842 |
| Relative thymus weight [mg/g]<br>SHC: $SFC^-$ 0.820±0.068; $SFC^+$ 0.942±0.145<br>CSC: $SFC^-$ 0.839±0.088; $SFC^+$ 0.813±0.056 | (CSC) $F_{1,17}$ = 0.353<br>(SFC) $F_{1,17}$ = 0.270<br>(CSC x SFC) $F_{1,17}$ = 0.633 | P = 0.560<br>P = 0.610<br>P = 0.437 |
| Relative spleen weight [mg/g] *(↑)<br>SHC: $SFC^-$ 2.79±0.125; $SFC^+$ 3.09±0.317<br>CSC: $SFC^-$ 3.64±0.247; $SFC^+$ 3.40±0.205 | (CSC) $F_{1,17}$ = 4.39<br>(SFC) $F_{1,17}$ = 0.012<br>(CSC x SFC) $F_{1,17}$ = 0.923 | P = 0.051<br>P = 0.913<br>P = 0.350 |
| **Physiological parameters OXT** | **Two-way ANOVA** | |
| Relative total adrenal weight<br>SHC: $SFC^-$ 0.114±0.011; $SFC^+$ 0.117±0.012<br>CSC: $SFC^-$ 0.124±0.006; $SFC^+$ 0.121±0.006 | (CSC) $F_{1,20}$ = 0.748<br>(SFC) $F_{1,20}$ = 0.001<br>(CSC x SFC) $F_{1,20}$ = 0.190 | P = 0.397<br>P = 0.972<br>P = 0.668 |
| Relative thymus weight<br>SHC: $SFC^-$ 0.801±0.088; $SFC^+$ 0.854±0.071<br>CSC: $SFC^-$ 0.881±0.051; $SFC^+$ 0.793±0.065 | (CSC) $F_{1,17}$ = 0.015<br>(SFC) $F_{1,17}$ = 0.049<br>(CSC x SFC) $F_{1,17}$ = 0.815 | P = 0.905<br>P = 0.827<br>P = 0.379 |
| Relative spleen weight<br>SHC: $SFC^-$ 2.96±0.206; $SFC^+$ 2.66±0.145<br>CSC: $SFC^-$ 3.16±0.230; $SFC^+$ 3.20±0.164 | (CSC) $F_{1,19}$ = 3.00<br>(SFC) $F_{1,19}$ = 0.358<br>(CSC x SFC) $F_{1,19}$ = 0.643 | P = 0.099<br>P = 0.557<br>P = 0.432 |

# Curriculum vitae

| **Education** | |
|---|---|
| Since 04/2018 | Dissertation at the University of Regensburg<br>Department of Behavioural and Molecular Neurobiology<br>PhD thesis: “Effects of chronic psychosocial stress on the development of a PTSD- and SAD-like phenotype in male mice” |
| 04/2015 – 09/2017 | Master of Science Biology<br>Master thesis: University of Regensburg, Department of Behavioral and Molecular Neurobiology |
| 10/2011 – 10/2014 | Bachelor of Science Biology<br>Bachelor thesis: University of Regensburg, Molecular and Cellular Neurobiology |
| 09/2002 – 03/2011 | General higher education entrance qualification (Abitur)<br>Gymnasium Markt Indersdorf, Germany<br>Examinations in Mathematics, Geography, Biology and English |
| **Professional experience** | |
| 03/2016 – 05/2016,<br>09/2017 – 03/2018 | Research Assistant at the University of Regensburg, Department of Behavioral and Molecular Neurobiology |
| 03/2014 – 01/2018 | Commercial Department at G-EaC |
| 2006 – 2018 | Private tutoring in Mathematics, Biology, English and Chemistry |
| **Grants** | |
| 04/2018 – 09/2018 | Grant for the start of the promotion for young scientists<br>(Stipendium zur Promotionsanschubfinanzierung für Nachwuchswissenschaftlerinnen)<br>University of Regensburg, Koordinationsstelle Chancengleichheit & Familie |
| 03/2019 | Travel grant for the 70$^{th}$ annual meeting of DKPM and DGPM – Deutscher Kongress für Psychosomatische Medizin und Psychotherape |
| **Skills** | |
| Certificate | FELASA Recommendations for the Education and Training of Persons Carrying out Animal Experiments (Category B) |
| EDV | Basic knowledge in MS-Office, SPSS |
| Others | Writing applications for animal licenses |
| | PhD Student representative of the section neurobiology |

**References**

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# Publications

- Anna K. Schmidtner, David A. Slattery, Joachim Gläsner, Andreas Hiergeist, **Katharina Gryksa**, Victoria A. Malik, Julian Hellmann-Regen, Isabella Heuser, Thomas C. Baghai, André Gessner, Rainer Rupprecht, Barbara Di Benedetto, Inga D. Neumann, 2019. „Minocycline alters behavior, microglia and the gut microbiome in a trait-anxiety-dependent manner“. *Translational Psychiatry*. https://doi.org/10.1038/s41398-019-0556-9

- **Katharina Gryksa**, Laura Mittmann, Angelika Bauer, Daniel Peterlik, Peter J. Flor, Nicole Uschold-Schmidt, Oliver J. Bosch, 2019. „Metabotropic glutamate receptor subtype 7 controls maternal care, maternal motivation and maternal aggression in mice“. *Genes, Brain and Behavior*. https://doi.org/10.1111/gbb.12627

- **Katharina Gryksa**, Inga D. Neumann, 2021. "Consequences of pandemic-associated social restrictions: Role of social support and the oxytocin system". *Psychoneuroendocrinology*. https://doi.org/10.1016/j.psyneuen.2021.105601

- Melanie Royer, Balagopal Pai, Rohit Menon, Anna Bludau, **Katharina Gryksa**, Rotem Ben-Tov Perry, Igor Ulitsky, Gunter Meister, Inga D. Neumann, 2022. "Transcriptome and chromatin alterations in social fear indicate association of MEG3 with successful extinction of fear". *Molecular Psychiatry*. https://doi.org/10.1038/s41380-022-01481-2

- **Katharina Gryksa**, Anna K. Schmidtner, Marianella Masis-Calvo, Odir Antonio Rodríguez-Villagra, Andrea Havasi, Gwendolyn Wirobski, Rodrigue Maloumby, Herbert Jägele, Oliver J. Bosch, David Slattery, Inga D. Neumann, 2022. "30 years of selective breeding of rats for high (HAB) and low (LAB) anxiety-related behaviour". *In preparation*.

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