FKBP51 in a dynamic environment

How FKBP51 shapes stress resilience in a sex- and cell type-specific manner

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Lotte van Doeselaar

Munich, Germany

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First examiner: PD. Dr. Mathias Schmidt

Second examiner: Prof. Dr. Laura Busse

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Abstract

Mental health disorders are a pressing global health-threat, affecting millions of people world-wide, which has already cost the world economy over 2.5 trillion dollars, making it a critical burden to society. These psychiatric disorders, including major depressive disorders, anxiety disorders and post-traumatic stress disorders, commonly arise as a combination of genetic and environmental factors. In the past decades, these gene by environment interactions (GxE) have increasingly been studied in both clinical and pre-clinical settings. Exposure to early life adversity has often been associated with negative outcomes on brain and behaviour and it has frequently been described as a risk factor for developing psychiatric disease. Nevertheless, there is also cumulative evidence that exposure to early life stress (ELS) in a milder form can result in adaptive responses that prepare an individual to cope with future life challenges. One gene that has repeatedly been implicated in the risk for psychiatric disease development is the FK506-binding protein 5 (*FKBP5*) gene, that encodes the glucocorticoid receptor (GR) co-chaperone FKBP51. FKBP51 plays an important role in regulating the sensitivity of the GR to the stress-hormone cortisol in humans or corticosterone in rodents. Interestingly, polymorphisms in the *FKBP5* gene were found to interact with traumatic early life events to increase the risk for developing psychiatric disorders. In recent years, FKBP51 has extensively been studied in relation to stress resilience and vulnerability, however the mechanisms by which it contributes to these processes, particularly in combination with ELS, are not yet fully understood. Apart from genetic factors and early life events, there are a number of additional environmental factors that can be of great influence on mental health, such as age or sex. In fact, research from the past decades has shed an increasing light on the pivotal role that sex plays in the resilience to (early life) stress. Nevertheless, since many studies historically only included males, there is unfortunately still a large gap in information on the female sex when it comes to stress resilience and vulnerability mechanisms. In this thesis, the importance of including both sexes in rodent stress research study designs is emphasized, by demonstrating sex-differential phenotypes of chronic social defeat stress using a recently developed hands-on protocol for chronic social defeat in females. Moreover, using genetic mouse models, this thesis demonstrates not only clear sex-dependent, but also cell-type specific functionality of FKBP51, either under baseline conditions in an older aged sample or in interaction with ELS stress exposure. Furthermore, it underlines the FKBP51-mediated beneficial effects of ELS exposure in female mice and proposes novel underlying pathways in this process. Ultimately, this thesis corroborates the notion that *FKBP5* is not per se a psychiatric risk factor, but rather a highly dynamic stress-responsive gene that interacts with the environment in shaping stress resilience.

Abbreviations

11β-HSD2	11β-hydroxysteroid dehydrogenase 2
ACTH	Adrenocorticotropic hormone
AR	Androgen receptor
AVP	Vasopressin
BDNF	Brain-derived neurotrophic factor
CeA	Central amygdala
CNS	Central nervous system
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
CSDS	Chronic social defeat stress
DG	Dentate gyrus
DSM	Diagnostic and statistical manual of mental disorders
EC	Entorhinal cortex
ER	Estrogen receptor
FKBP51	FK506-binding protein of 51-kDa
FKBP52	FK506-binding protein of 52-kDa
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element
HFS	High frequency stimulation
HPA	Hypothalamic-pituitary-adrenal
Hsp70	Heat-shock protein 70
Hsp90	Heat-shock protein 90
ICD	International classification of disease
LBN	Limited bedding and nesting material
LDP	Long-term depression
LTP	Long-term potentiation
MDD	Major depressive disorder
MR	Mineralocorticoid receptor
MRI	Magnetic Resonance Imaging
MS	Maternal separation

PHLPP	PH domain leucine-rich repeat phosphatase
PFC	Prefrontal cortex
POMC	Pro-opiomelanocortin
PR	Progesterone receptor
PTSD	Post- traumatic stress disorder
PVN	Paraventricular nucleus
SNP	Single Nucleotide Polymorphism
STST	Social-threat-safety test
TSS	Transcription start site
VTA	Ventral tegmental area
WHO	World Health Organization

Publications

Chapter 2.1

van Doeselaar L, Yang H, Bordes J, Brix L, Engelhardt C, Tang F & Schmidt MV. (2021). Chronic social defeat stress in female mice leads to sex-specific behavioural and neuroendocrine effects. *Stress*, 24(2), 168–180. https://doi.org/10.1080/10253890.2020.1864319

Chapter 2.2

van Doeselaar L, Stark T, Mitra S, Yang H, Bordes J, Stolwijk L, Engelhardt C, Kovarova V, Narayan S, Brix LM, Springer M, Deussing JM, Lopez JP, Czisch M & Schmidt MV. (2023) Sex-Specific and Opposed Effects of FKBP51 in Glutamatergic and GABAergic Neurons: Implications for Stress Susceptibility and Resilience. *PNAS*, 120(23), e2300722120. https://doi.org/10.1073/pnas.2300722120

Chapter 2.3

van Doeselaar L, Abromeit A, Stark T, Menegaz D, Mitra S, Yang H, Rehawi G, Huettl R, Bordes J, Narayan S, Czisch M, Deussing JM, Arloth-Knauer J, Eder M, Lopez JP & Schmidt MV. FKBP51 in glutamatergic forebrain neurons plays a role in beneficial effects of moderate early life stress exposure on hippocampal function and structure in female mice via a TCF4-mediated pathway. *Manuscript in preparation*.

Additional Contributions

Yusupov N*, van Doeselaar L*, Röh S, Wiechmann T, Ködel M, Sauer S, Rex-Haffner M, Schmidt MV & Binder EB. (2023). Extensive evaluation of DNA methylation of functional elements in the murine Fkbp5 locus using high accuracy DNA methylation measurement via targeted bisulfite sequencing. *EJN under review*.

**Authors contributed equally to the work*

Engelhardt C, Tang F, Elkhateib R, Bordes J, Brix LM, van Doeselaar L, Häusl AS, Pöhlmann ML, Schraut K, Yang H, Chen A, Deussing JM & Schmidt MV. (2021). FKBP51 in the Oval Bed Nucleus of the Stria Terminalis Regulates Anxiety-Like Behaviour. 8(6) *ENEURO* .0425-21.2021 1–15. https://doi.org/10.1523/ENEURO.0425-21.2021

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Brix LM, Häusl AS, Toksöz I, Bordes J, van Doeselaar L, Engelhardt C, Narayan S, Springer M, Sterlemann V, Deussing JM, Chen A & Schmidt M.V. (2022). The co-chaperone FKBP51 modulates HPA-axis activity and age-related maladaptation of the stress system in pituitary proopiomelanocortin cells. *Psychoneuroendocrinology*, 138:105670. https://doi.org/10.1016/j.psyneuen.2022.105670

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Declaration of contributions

I hereby certify that I contributed my own work to the current thesis, entitled “FKBP51 in a dynamic environment: How FKBP51 shapes stress resilience in a sex- and cell type-specific manner” in the following way:

Chapter 2.1

Designing and planning the study
In collaboration with MVS

Conducting the experiments
In collaboration with YH, JB, LB, CE and FT

Analysing the data
Independently executed

Preparing the manuscript
In collaboration with MVS

Chapter 2.2

Designing and planning the study
In collaboration with MVS

Conducting the experiments
In collaboration with TS, SM, HY, JB, LS, CE, VK, SN, LB, and MS

Analysing the data
In collaboration with TS and MC

Preparing the manuscript
In collaboration with MVS

Chapter 2.3

Designing and planning the study
In collaboration with MVS

Conducting the experiments
In collaboration with AA, TS, DM, SM, HY, RH, JB and SN

Analysing the data
In collaboration with TS, MC, GR, JAK and ME

Preparing the manuscript
In collaboration with MVS

München 31st May 2023

Lotte van Doeselaar

Signature

I hereby confirm the by Lotte van Doeselaar above mentioned contributions to the publications.

München 31st May, 2023

Mathias Schmidt

Signature

1. General introduction

Stress, it is an inevitable part of our modern, rushed lives and our constantly changing environments require a dynamic bodily response to cope with frequent life challenges. Imbalances in this carefully regulated stress system may result in detrimental health outcomes (de Kloet et al., 2005). The recent COVID-19 pandemic has for example illustrated how quick the experience of stressful events can negatively impact our mental health (Santomauro et al., 2021). However, stress is not a modern-day phenomenon – stressful situations have been part of life throughout evolution. The activation of stress systems is sometimes even an essential element to deal with life-threatening confrontations, as it alerts us and prepares us to "fight or "flight". How we cope with stress differs for each individual and depends on various factors. This doctoral thesis will address the importance of a number of these factors in dealing with our dynamic environment.

1.1 Stress and stress related disorders

Stress exposure comes in many different forms and can occur at any stage throughout life. Distinctive types of stress exposure may result in differential health outcomes, however, generally an overload in stressful experiences or occurrence of extreme traumatic events can lead to the development of mental health or, so-called, stress-related disorders. It is therefore important to understand how different type and timing of stress exposure can impact our mental health and how we can study the consequences of different types of stress in rodent models.

1.1.1 Stress-related disorders

A recent report of the World Health Organization (WHO) (World Health Organization (WHO), 2022) describes that approximately 970 million people world-wide suffer from mental health disorders, making it a major global health threat. Costs for diagnosis, treatment, prevention but also for more indirect societal costs, have already exceeded 2.5 trillion US dollars, affecting the world economy and causing it to be a primary burden to society (World Health Organization (WHO), 2022). The term "mental health disorders" comprises a broad collection of disorders that are elaboratively described in two main classification systems, the International Classification of Disease (ICD)–11 (World Health Organization (WHO), 2019) and the Diagnostic and Statistical Manual of Mental Disorders (DSM)-V (American Psychiatric Association & American Psychiatric Association, 2013). In both systems, stress-related disorders are characterized as a separate class of disease, either as "disorders specifically associated with stress" (ICD-11) or as "trauma- and Stressor-Related Disorders" (DSM-V). In this, exposure to a traumatic or stressful event is listed explicitly as a diagnostic criterion. This class of disorders amongst others includes post-traumatic stress disorder (PTSD). However, mood disorders, such as major depressive disorders (MDD) or anxiety disorders, are also largely impacted by stressful life events (Kessler, 1997; Miloyan et al., 2018). Even though PTSD, MDD and anxiety disorders are divided into separate classes of disease they all affect mental and physical health on overlapping domains, including dysfunctions in emotional regulation, deficits in cognitive functioning (eg. memory function) and somatic symptoms.

1.1.2 Stress and regulation of the stress response

The recurrent experience of stressful events may eventually lead to the development of psychiatric disorders. However, stress is an inevitable part of life and not each single stressful experience will necessarily lead to negative health outcomes. In fact, in certain cases, stress can be a positive experience and it therefore encompasses a broad and complex concept. The concept of stress and

stress coping has first been described by Hans Selye in 1936 and since then it has largely been extended.

Generally, exposure to a physiological or psychological stressor threatens the bodily equilibrium and this triggers the activation of an adaptive physiological, endocrine and behavioural response that strives to return to a status of homeostasis. The stress response starts with the activation of the fast-acting sympathetic adrenal medullary system. Inputs from the amygdala and the hypothalamus activate the autonomic nervous system within seconds, releasing noradrenaline from wide-spread synapses and stimulating the adrenal medulla to produce adrenaline. This results in a quick status of arousal, that helps to deal with potential environmental threats (eg. increased heart rate, dilation of the pupils) and is also known as the "fight or flight" response. Simultaneously, the activation of the hypothalamic-pituitary-adrenal (HPA) axis is initiated (Figure 1). The HPA axis is a slower-acting endocrine response that starts with the release of the neuropeptides corticotropin-releasing hormone (CRH) and vasopressin (AVP) from the parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus into the hypophyseal portal bloodstream. Once CRH and AVP arrive at the anterior pituitary, they stimulate the synthesis and release of the adrenocorticotropic hormone (ACTH) from the pro-opiomelanocortin (POMC) neurons. In its turn, ACTH activates the secretion of glucocorticoids from the adrenal gland cortex. In humans, cortisol is the primary glucocorticoid, whereas in rodents this is corticosterone (CORT). Optimal coping with a stressor is not purely achieved by a successful activation of the stress response, but proper termination of the stress response is of equal importance to reinstating the homeostatic environment. Glucocorticoids provide negative feedback to the pituitary and the hypothalamus, thereby initiating the termination of the stress response. In addition to this, glucocorticoids have an effect on many different other brain regions, amongst others on the hippocampus and the prefrontal cortex (PFC), that provide additional negative feedback to the hypothalamus. Apart from its actions in the central nervous system (CNS), glucocorticoids travel further through the circulation where it can influence peripheral processes like immune regulation and metabolism.

To exert their actions, glucocorticoids bind two different receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (de Kloet & Joëls, 2020). MR and GR are present in both the periphery and the CNS. In the periphery, MR is highly expressed in cell types such as cardiac myocytes, smooth muscle cells of the vascular system and the epithelial cells of the kidney and colon (H. Yang et al., 2023). MR binds its two ligands, aldosterone and glucocorticoids, with equally high affinity, but due to much larger fold concentration of circulating glucocorticoids, this is often the preferentially bound ligand. In the epithelial cells of the kidney and colon however, the presence of the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) - that converts glucocorticoids to their inactive forms – allows for increased binding to aldosterone in these tissues (Gomez-Sanchez & Gomez-Sanchez, 2014). Unlike MR, GR is expressed in almost every cell type of the periphery (Oakley & Cidlowski, 2013). In the brain, MR is mostly distributed in the limbic brain regions, whereas GR is more widely spread throughout. Generally, glucocorticoids bind the MR with a much higher affinity than GR and because of this and the lack of 11β-HSD2 in most brain regions, the majority of MRs are occupied with glucocorticoids under baseline conditions. The GR on the other hand, has a lower affinity for glucocorticoids and these receptors therefore only get occupied following stress-induced elevation of glucocorticoids or during the circadian peak levels. This makes the GR a particularly important player in the termination of the stress response. Due to the liposolulability of glucocorticoids, they can easily diffuse through the cell-membrane where they bind MR and GR that are located in the cytoplasm (Figure 1). MR and GR are nuclear receptors that can migrate to the nucleus and regulate transcriptional activity of numerous genes (Gray et al., 2017). However, it is thought that apart from nuclear MR and GR, membrane-bound MR and GR exist that are responsible for more rapid, non-genomic glucocorticoid actions. Examples of these non-genomic mechanisms are modulation of

glutamate release into the presynaptic neurons or regulation of mitochondrial function (Gray et al., 2017). In the cytoplasm, MR and GR are comprised of complexes of different chaperone and co-chaperone molecules that regulate the sensitivity of the GR. Whenever GR is bound to its ligand and the co-chaperone molecule FK506-binding protein of 52-kDa (FKBP52) is attached, the dynein-dynactin motor complex is recruited and the GR is translocated to the nucleus (Zgajnar et al., 2019). Once inside the nucleus, it can form homodimers or heterodimers with MR and bind to glucocorticoid-responsive elements (GREs), specific binding site sequences that are present in a wide range of genes. By binding to the GREs, GR can regulate transcriptional activity. One gene that has a large number of GREs is *FKBP5*, that encodes the co-chaperone FKBP5 protein of 51-kDA (FKBP51). *FKBP5* mRNA transcription is induced immediately upon GR binding to the GREs on the *FKBP5* gene, resulting in increased intracellular levels of FKBP51. FKBP51 and FKBP52 have a largely homologous structure and therefore compete for binding to the GR complex. Once intracellular levels of FKBP51 rise and replace FKBP52 at the GR complex, translocation of GR into the nucleus is hampered. This cascade results in an ultrashort negative feedback loop in which FKBP51 can determine GR sensitivity (Figure 1) (Hähle et al., 2019). By orchestrating transcriptional regulation of various genes, GR stimulates a number of processes that contribute to the termination of the HPA axis activation and re-stabilization of the body to pre-stress homeostasis. In addition to this, a number of other processes are set into motion, such as the promotion of memory storage, in anticipation of future challenges.

In order to deal with acute stressors, a carefully balanced adaptive response is essential, a process that has been referred to as “allostasis” (McEwen, 1998). However, with each new stressful experience, the body and brain are brought to a new set-point in order to prepare for future stress exposure. Prolonged activation of the stress response due to a cumulative exposure to life experiences may eventually result in negative behavioural outcomes as the previously balanced endocrine response loses its adaptive nature. This concept has been described by Bruce McEwen and Elliot Stellar as “allostatic load” (McEwen & Stellar, 1993). Allostatic load may reach different thresholds for separate individuals, principally defining how resilient an individual may be to recurrent or traumatic stress.

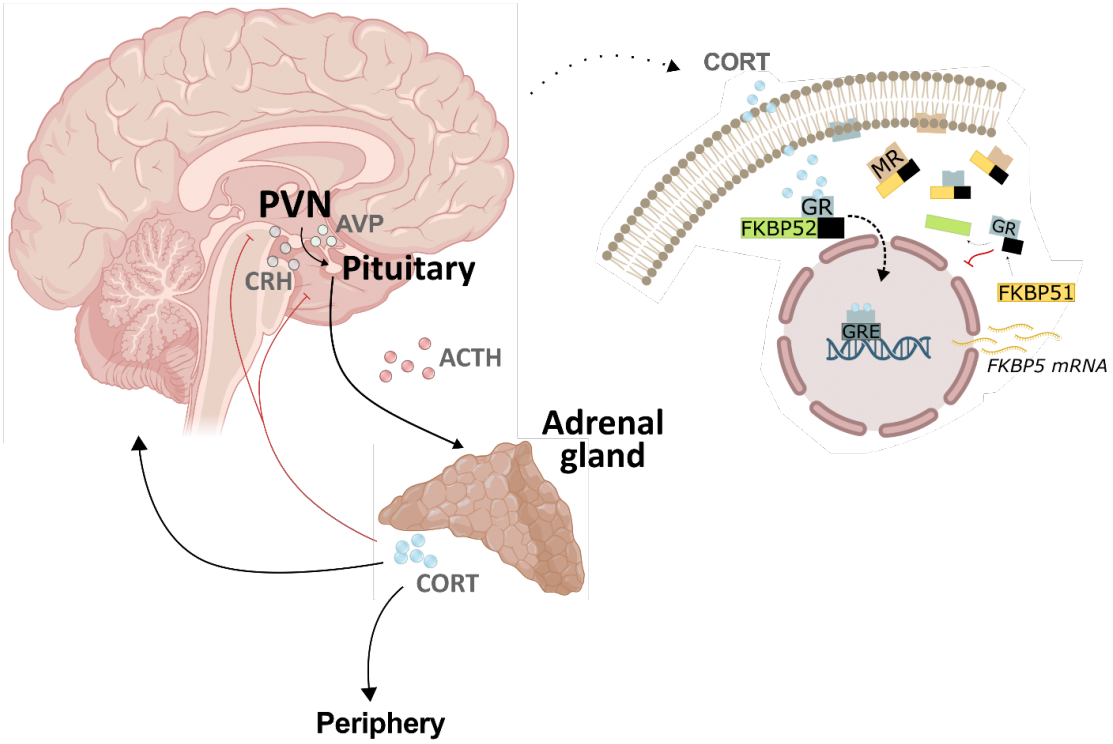

Figure 1. Regulation of the stress response via the hypothalamic-pituitary-adrenal axis
As part of the response to a physiological or psychological stressor, the hypothalamic-pituitary-adrenal (HPA) axis is activated. The neuropeptides corticotropin-releasing hormone (CRH) and vasopressin (AVP) are released from the paraventricular neucleus (PVN) in the hypothalamus and stimulate the production and release of adrenocorticotropic hormone (ACTH) from the pro-opiomelanocortin (POMC) neurons in the anterior pituitary. In turn, ACTH stimulates the secretion of glucocorticoids from the adrenal cortex, with corticosterone (CORT) being the primary glucocorticoid in rodents. CORT provides negative feedback to the pituitary and PVN. In addition, it has various effects throughout the brain and periphery, where it can regulate immune-related and metabolic functions. Due to its liposoluble nature, CORT can travel through the cell membrane and bind the cytosolic mineralocorticoid receptor (MR) with high affinity and the cytosolic glucocorticoid receptor (GR) with a lower affinity. Apart from their non-genomic effects, MR and GR act as nuclear receptors that are organized in complexes comprised of a number of chaperone and co-chaperone molecules. When bound to CORT and attached to the co-chaperone FKBP52, GR translocate to the nucleus where it forms homodimers that bind to glucocorticoid-responsive elements (GREs) and thereby regulate transcription. The *FKBP5* gene, encoding the FKBP51 co-chaperone, has a number of GREs resulting in GR-activated *FKBP5* transcription. FKBP51 competes with FKBP52 for binding the GR and once bound to the complex, FKBP51 prohibits its translocation into the nucleus. This results in an ultrashort feedback loop, in which FKBP51 regulates GR sensitivity. Careful regulation of the HPA axis is required for an adaptive response to stress and an imbalance in or overstimulation of this endocrine response to stress may result in inadequate coping.

1.1.3 The effects of stress on the brain

Stress can impact the brain on many different modalities, starting with changes on the molecular level. Numerous studies have shown that stress can influence epigenetic mechanisms, including processes of DNA methylation or histone modification (Griffiths & Hunter, 2014; Hunter et al., 2015; Lee et al., 2010). Changes in the epigenetic landscape evidently lead to shifts in transcriptional profiles. Moreover, stress can regulate gene expression more directly via actions of the MR and GR, either by binding to GREs located in various genes or by interacting with transcription factors that are already bound to the DNA. Stress can also influence brain structure. There is abundant evidence for the effects of stress on neuronal morphology and neurogenesis, particularly in the hippocampus (Chen et al., 2008; Korosi et al., 2012; Sapolsky et al., 1985), but also in the amygdala and PFC (Joëls et al., 2007; McEwen et al., 2016). Apart from this, data from magnetic resonance imaging (MRI) studies have shown that (early) stressful life experiences have an effect on brain volume (Smith & Pollak, 2020; Thomason & Marusak, 2017). Exposure to stress also results in functional changes in the brain. Electrophysiological studies have demonstrated changes in long-term potentiation (LTP) following stress exposure or glucocorticoid treatment (Ahmed et al., 2006; Karst & Joëls, 2005; McEwen, 1999) and in humans, functional brain changes following stressful life experiences have been revealed using functional MRI (fMRI) (Holz et al., 2023). Interestingly, these structural and functional alterations by stress and glucocorticoid exposure often have a biphasic nature that follow an inverted U-shaped curve. Moderate, short-term activation of the stress system leads to beneficial effects, whereas long-term excessive activation leads to maladaptive responses (McEwen et al., 2015). Ultimately, excessive stress and its associated changes on the molecular, structural and network level can lead to behavioural alterations. The effects of stress on emotional and cognitive behaviour have been extensively studied in rodents (Schwabe et al., 2012; Tran & Gellner, 2023) and these deficits in emotional regulation and cognitive functioning are reflected in the symptoms of patients suffering from psychiatric disorders.

1.1.4 Timing of stress

An individual can be exposed to challenges at any stage throughout its life and not only the duration or type of stressor, but also the timing of stress exposure is of vital importance in determining the long-

term consequences. There is a specific "sensitive window" during (early) development in which the brain is particularly susceptible to changes by environmental challenges (Andersen, 2003). During this time, the brain is still largely developing and is highly plastic. This sensitive window can be divided into three phases: the prenatal phase, the early life phase and the adolescent phase. However, also during aging the brain is increasingly susceptible to the effects of stress. Moreover, it is proposed that specific brain regions are more or less sensitive to the effects of stress during certain periods in life, depending on whether they are particularly developing at the time of stress exposure (during early stages in life) or whether they are undergoing the most profound age-related changes (during aging)(Andersen & Teicher, 2008; Lupien et al., 2009). Thus, during prenatal phases, stress may have a programming effect on many different brain regions that contribute to HPA-axis regulation (eg. hippocampus, PFC and amygdala)(Kapoor et al., 2008), whereas stress exposure in early life could have a particular impact on the developing hippocampus (Giedd et al., 1996; Gogtay et al., 2006; Knickmeyer et al., 2008). In adolescence, the PFC may be specifically susceptible to stress (Sharma et al., 2013). However, during aging, the hippocampus is again the region that is most affected by stress exposure.

A substantial amount of research has demonstrated that cumulative exposure to stressful life experiences will lead to negative health outcomes (Taylor, 2010; A. K. Walker et al., 2009). However interestingly, there is also evidence for the so-called "inoculation stress hypothesis" (Champagne et al., 2008; Lyons et al., 2009; Parker et al., 2004). This theory explains how moderate exposure to stress in early life can actually prepare the individual for later life challenges. The early life experiences have "programming" effects on the HPA-axis and brain and these changes can have an adaptive nature, a concept referred to as predictive adaptive responses (PARs) (Gluckman et al., 2005). This means, in case the individual encounters a similar type of challenge in later life (a matched environment) it "remembers" the previously applied coping strategies and can respond in a quicker fashion. However, these early life challenges may still result in maladaptation if the individual has an "unmatched environment" in later life (Nederhof & Schmidt, 2012).

1.1.5 Rodent models of stress

Even though the general research community is making continuous efforts to strongly reduce animal numbers, the rodent model currently remains an indispensable tool for studying the underlying neurobiological mechanisms of stress and stress resilience. Various models have been developed to address different types of stress exposure, including acute stress, chronic stress or early life stress (ELS) (Atrooz et al., 2021). Examples of models for acute stress are the restrained stress model and the forced swim test, of which the latter is often also used to study the choice of coping strategy (de Kloet & Molendijk, 2016). Consequences of chronic stress exposure on the other hand, can for example be investigated using the chronic unpredictable stress, chronic mild stress or chronic social defeat stress (CSDS) paradigms (Golden et al., 2011; Karamihalev et al., 2020; Larsen et al., 2010; Wagner et al., 2011). The CSDS paradigm is a very commonly used chronic stress model that specifically addresses a social aspect of stress (Gururajan et al., 2019; Hollis & Kabbaj, 2014) and it relies on the aggressive behaviour of a male mouse to its intruder. A number of paradigms have also been established to study stress during early development. Two of the most routinely used models for ELS exposure are the maternal separation (MS) and the limited bedding and nesting material (LBN) paradigm (Rice et al., 2008; Schmidt et al., 2011). In the MS paradigm, pups are separated from their dams for recurrent periods during the first 2 weeks of postnatal life. Conversely, in the LBN paradigm, pups remain with the dam in a poorly enriched environment with limited bedding and nesting material available, and a metal grid on the cage bottom. This situation leads to increased stress through fragmented and unpredictable nurturing behaviour towards the pups, resulting in moderate ELS exposure.

One important aspect with regards to stress models is their applicability in both sexes. The necessity of studying different sexes has rightfully been emphasized in the past decades, revealing

certain limitations to a number of rodent stress paradigms. Some models, like the ELS or acute stress models are easily applicable in both sexes. However, other tests are less directly translatable to the female sex and first require substantial adjustments. One example of such a test is the CSDS paradigm, that relies on the naturally aggressive behaviour of a male resident to its male intruder. Under normal circumstances, such aggressive behaviour is not observed towards female mice. Recently, a model of CSDS has been developed for females, that is highly identical to the classical CSDS paradigm that has widely been used in males, increasing comparability between different sexes (Harris et al., 2018). However, the behaviour of females after CSDS exposure still requires more extensive characterization and this calls upon further investigation.

1.2 Stress in early life

During the early phases of life, the brain and its development are in a "sensitive window" in which stressful experiences can leave a lasting mark on the individual (Andersen, 2003). The consequences of ELS have been extensively studied in the past decades (Bonapersona et al., 2019; Chen & Baram, 2016; Krugers et al., 2017; D. Wang et al., 2020) and this has revealed the wide range of effects it can have on the brain. In part depending on the severity or type of stressor, ELS cannot only have long-lasting disadvantageous, but can also lead to beneficial effects. This paragraph further discusses the consequences that may result from ELS exposure on different modalities.

1.2.1 Epigenetic and transcriptomic effects of early life stress

A bulk of research in humans and animal models has demonstrated the effects of ELS on the epigenetic and transcriptomic level (Li et al., 2020; Malave et al., 2022). Stress in early life can influence epigenetic processes, such as DNA methylation or histone modification, resulting in altered gene expression profiles. Post-mortem studies in individuals with a history of maltreatment and studies in rodents revealed that, within the hippocampus, ELS exposure increases methylation and reduces histone acetylation of the *NR3C1* gene, lowering the accessibility of the GR promotor (Kember et al., 2012; McGowan et al., 2009; Weaver et al., 2004). These processes result in decreased GR expression and are linked to altered HPA-axis function. Moreover, *NR3C1* methylation changes have been linked to depression (Palma-Gudiel et al., 2018; Tyrka et al., 2016). However, other brain regions implicated in HPA-axis regulation, such as the hypothalamic PVN and the pituitary, are also epigenetically affected by ELS. Rodent studies have shown hypomethylation of the *Avp* gene and the *Pomc* gene, leading to HPA axis hyperactivation (Murgatroyd et al., 2009; Wu et al., 2014). In addition to this, the *CRH* gene, serotonin transporter gene solute carrier family 6 member 4 (*SLC6A4*) and the brain-derived neurotrophic factor (*BDNF*) gene have been found to undergo epigenetic and transcriptomic alterations as a result of ELS exposure (Li et al., 2020). Interestingly, DNA methylation in the *Bdnf* gene in rats was shown to be affected by ELS in a sexually dimorphic manner (Blaze et al., 2013; Roth et al., 2014). At last, an important stress-regulatory gene that is highly susceptible to ELS-induced epigenetic changes is the *FKBP5* gene (Klengel et al., 2013). The implications of FKBP51 in stress-resilience mechanisms will be discussed in more detail in paragraph 1.3.

1.2.2 Effects of early life stress on brain structure and volume

In addition to molecular alterations, the structural consequences of ELS exposure have been universally studied (Krugers et al., 2017; C.-D. Walker et al., 2017). Brain structure is a broad concept and amongst others includes neuronal morphology, neuronal cell numbers, neurogenesis or brain volumes. The use of different ELS models in rodents or different type of childhood traumatic experiences in humans may eventually lead to different outcomes on behaviour. One of the structures that is mainly affected by ELS exposure is the hippocampus. Numerous studies have reported changes in dendritic complexity in the CA1, CA3 and dentate gyrus (DG) (Bagot et al., 2009; Brunson et al., 2005;

Champagne et al., 2008). This includes reduced numbers of dendritic branches, dendritic length of pyramidal neurons and a reduced number of spines following different ELS paradigms. In addition to these morphological neuronal changes, neurogenesis is altered in ELS-exposed animals. Interestingly, the effects on neurogenesis are sex-specific (Oomen et al., 2009) and depend largely on the timepoint of measurement. Right after chronic ELS exposure with the LBN paradigm, neurogenesis is increased at P9 in both sexes, however, in later life, newly born cell survival and DG volume was reduced in male mice only (Naninck et al., 2015). Furthermore, MRI studies in rats that underwent the LBN ELS paradigm revealed decreases in hippocampal volume (Molet et al., 2016). This is in line with results from MRI studies in humans that have largely reported hippocampal volumetric changes following childhood maltreatment (Dannlowski et al., 2012; Hanson et al., 2015; Opel et al., 2014; Teicher et al., 2012). Remarkably, recent use of high-resolution structural MRI revealed sub-region differential volumetric effects in the hippocampus, in which adolescents that had experienced childhood trauma presented larger volumes in the DG of the hippocampus (Picci et al., 2022).

However, morphological changes following ELS do not only occur in the hippocampus, but ELS-induced structural changes have also been found in the PFC and amygdala region. In male mice, dendritic development of pyramidal neurons in layers II/III and V of different regions of the PFC was hampered following an LBN ELS exposure (X.-D. Yang et al., 2015). Interestingly, MS stress led to increased infralimbic mPFC dendritic branch number and length in female rats (Farrell et al., 2016). Furthermore, morphological changes were found in rat pyramidal neurons of the basolateral amygdala (BLA) after LBN ELS exposure (C.-D. Walker et al., 2017). Structural MRI in humans has also demonstrated volumetric changes in various additional regions, amongst others in the PFC and amygdala (Dannlowski et al., 2012; Hanson et al., 2015; Picci et al., 2022; Teicher et al., 2016; Veer et al., 2015).

1.2.3 Effects of early life stress on brain function

Epigenetic, transcriptomic and structural changes that are a result of ELS exposure are likely to lead to functional changes in the brain. A large number of studies have addressed these functional consequences of ELS in humans and rodents. Synaptic plasticity in the CA1, CA3 and DG of the dorsal hippocampus (DHC) was typically reduced following ELS in rodents, using different types of models (Bagot et al., 2009; Brunson et al., 2005; Champagne et al., 2008; Ivy et al., 2010; X.-D. Wang et al., 2011). In the ventral part of the hippocampus (VHC) on the other hand, synaptic plasticity was enhanced (Nguyen et al., 2015). Interestingly, when rats were exposed to ELS, LTP under baseline conditions was impaired, however, in the context of a high CORT concentration *in vitro*, mimicking a stressful event, LTP was significantly strengthened (Champagne et al., 2008). Synaptic plasticity was also altered in the amygdala, where ELS resulted in increased evoked synaptic function in the BLA (C.-D. Walker et al., 2017). Evidence that ELS exposure affects brain functionality also comes from human fMRI studies (Holz et al., 2023; Teicher & Samson, 2016). Reduced hippocampal activity has been reported in adults who grew up in poverty (Liberzon et al., 2015) and childhood maltreatment also differentially altered mPFC and dorsolateral PFC activation, dependent on the task individuals were exposed to (Dannlowski et al., 2012; Fonzo et al., 2016; van Harmelen, Hauber, et al., 2014; van Harmelen, van Tol, et al., 2014). Moreover, many studies have demonstrated an enhanced amygdala reactivity in response to emotional faces, during the processing of threatening situations and during fear and anger in individuals that experienced childhood stress (Fonzo et al., 2016; Maheu et al., 2010; Teicher & Samson, 2016; van Harmelen et al., 2013). Importantly, not only single brain regions are affected by ELS, but also the activation of whole neuronal networks is altered. Human fMRI studies found that particularly functional connectivity between the amygdala and mPFC, the amygdala and the hippocampus and two other cognitive cortical circuits, the frontoparietal and the default-mode network, were affected by ELS.

Ultimately, these functional alterations will lead to changes in behaviour. An extensive amount of literature has addressed the effects of ELS exposure on emotional regulation and cognitive functioning (Chen & Baram, 2016). A number of rodent studies using different ELS paradigms have reported increased anxiety-like behaviour following ELS of which the majority of studies was performed in male mice or rats (Krugers et al., 2017; Loi et al., 2017; C.-D. Walker et al., 2017; D. Wang et al., 2020). However, several studies did not report any changes in anxiety-like behaviour following ELS and some studies even demonstrated reduced anxiety-like behaviours (Bonapersona et al., 2019; McIntosh et al., 1999; Savignac et al., 2011; D. Wang et al., 2020). Interestingly, these studies specifically included females and in one study the effect on anxiety-like behaviour also depended on the duration of the ELS exposure. For cognitive functioning, a large number of studies have shown that ELS leads to spatial or recognition memory impairments (Chen & Baram, 2016; Krugers et al., 2017; C.-D. Walker et al., 2017). Nevertheless, outcomes can be different dependent on the setting in which the test takes place. That is, several studies found that ELS resulted in improved memory performance in a stressful environment (Bonapersona et al., 2019).

Even though it is clear that ELS affects brain structure and function, results on directionalities of effect seem to depend on a number of factors. Amongst these factors are the type of early life stress, its severity or duration, type of species involved in the test and age of the animal at testing. Moreover, sex is a strongly determining factor and it is known that consequences of ELS exposure on hippocampal structure and function can strictly differ between males and females (Loi et al., 2017; Samplin et al., 2013). Remarkably, particularly data in females is largely under-represented (Chen & Baram, 2016; Joëls et al., 2022). A recent meta-analyses on ELS effects on the brain revealed that data in females as compared to males was too scarce to perform a quantitative analyses in females (Joëls et al., 2022). This clearly illustrates the remaining gap in information on ELS-related mechanisms in females.

1.3 Gene by environment interactions: The *FKBP5* gene

Outcomes of stress on mental and physical health are heavily dependent on a number of genetic and environmental aspects. One major interactive factor determining health outcomes of stress is the genetic background of an individual. A study by Belsky and colleagues proposed that specific genes or genetic variants, referred to as plasticity genes, determine the susceptibility of an individual to environmental influences (Belsky et al., 2009). Moreover, a “three-hit model of stress” has been described, in which the genetic background as a “first hit” modulates adaptive coping with a “second hit” early life stressor and “third hit” later life stressors (Daskalakis et al., 2013). One gene that has received special attention in the light of these gene by environment (GxE) interactions is the *FKBP5* gene (Matosin et al., 2018). In addition to the influence of genetic factors on stress coping, environmental factors, such as age and sex, also play an important role in determining an individual’s resilience to stress. This paragraph further discusses how GxE interactions, in particular those of the *FKBP5* gene, shape stress resilience (Figure 2).

1.3.1 FKBP51 structure and function

Ever since it was first discovered that FKBP51 could be a potential risk factor for developing psychiatric disease (Binder et al., 2004), an expanding amount of research has focussed on studying this stress-responsive protein. FKBP51 is a molecular co-chaperone molecule to the GR complex and it is encoded by the *FKBP5* gene that is located on chromosome 6 of the human genome or by *Fkbp5* on chromosome 17 of the mouse genome. Both FKBP51 and FKBP52, another co-chaperone molecule with a highly homologous structure, belong to the protein family of immunophilins. These two co-chaperones have antagonistic properties and because they share 75% similarity in structure, they compete for binding at the GR (Zgajnar et al., 2019). At the extreme N-terminal of these two proteins,

the peptidyl-prolyl-(cis/trans)-isomerase (PPIase) domain, or FK1 domain, is located. This domain has PPIase activity, which catalyzes the exchange of protein structure from the *trans*- to the *cis*-conformation (Zgajnar et al., 2019). This *cis-trans* isomerization is highly important for protein folding mechanisms and thereby FKBP51 and FKBP52 likely contribute to organizing protein complexes in various signalling pathways (Rein, 2016). A second important domain of both immunophilins is the sequence that contains tetratricopeptide repeats (TPR). The TPR domain, together with the attached C-terminal motif, enables associations to the heat-shock protein 90 (Hsp90) chaperone molecule dimers, that are also directly bound to the GR (Hähle et al., 2019; Zgajnar et al., 2019). A short sequence, called the FK-linker region, links the FK1 domain to the FK2 region. The FK2 domain is highly similar in structure to the FK1 domain, but does not possess PPIase activity and instead is thought to be involved in protein-protein interactions (Hähle et al., 2019). Even though FKBP51 and FKBP52 both possess PPIase activity, their FK1 domains have unique conformational properties, which leads to the differential qualifications of these co-chaperones. Although the PPIase activity of the two molecules is important for their antagonistic functionality, it does not exclusively contribute to their differences. Also their association to Hsp90 influences the way in which the FK1 domain interacts with the ligand-binding domain of the GR, affecting the GR conformation and its ligand-binding affinity (Zgajnar et al., 2019). Furthermore, FKBP52 binding to the GR activates recruitment of the dynein-dynactin motor complex. In absence of glucocorticoids, GR is predominantly cytoplasmic, but when binding its ligand and in association with FKBP52, the entire complex translocates to the nucleus. Only here, Hsp90 and FKBP52 dissociate from GR, dimerization takes place and it can act as a transcriptional regulator. FKBP51 does not bind dynein and thereby it prevents translocation into the nucleus. At last, FKBP51 can reduce GR sensitivity by decreasing stimulatory phosphorylation of GR (Rein, 2016). Other steroid receptors, such as the progesterone receptor (PR), MR and to a lesser extent the androgen receptor (AR) are also bound by FKBP51 (Schülke et al., 2010). Apart from its interactions with steroid receptors, FKBP51 is involved in additional pathways. It was for example found that FKBP51 acts as a scaffolding protein to facilitate dephospohorylation of the kinase Akt via PH domain leucine-rich repeat phosphatase (PHLPP) recruitment (Balsevich et al., 2017; Hähle et al., 2019). The Akt pathway is mediating several signaling pathways involved in growth and proliferation, but also glucose homeostasis (Balsevich et al., 2017). Moreover, Gassen and colleagues showed that FKBP51 interacts with a substrate of AKT, the autophagic marker Beclin 1 (Gassen et al., 2014, 2019). It was found that FKBP51 can change the protein interactions and phospohorylation of Beclin 1, thereby activating autophagic pathways. Autophagy is a process that is implicated in synaptic functioning and it was proposed that FKBP51, via shared autophagic pathways, mediates the treatment response to antidepressants (Gassen et al., 2014). Furthermore, autophagy is linked to whole-body metabolism. A study by Häusl and colleagues demonstrated that FKBP51 acts as a master mediator of the AMPK-mTOR network, particularly upon a metabolic challenge, and proposed a model in which FKBP51 is an important regulating switch between autophagy initiation and mTOR signaling (Häusl et al., 2022). Apart from its implications in autophagy-related processes, FKBP51 regulates nuclear factor binding near the κ light-chain in B-cells (NF-κB), a family of transcription factors that is heavily involved in immune regulation (Hähle et al., 2019).

Because of its ability to regulate GR sensitivity, FKBP51 is strongly involved in stress-related pathways and indirectly contributes to HPA-axis regulation. It is therefore not surprising that FKBP51 plays a role in the risk for developing stress-related disorders (Binder, 2009; Binder et al., 2004; Zannas et al., 2016). A large number of rodent studies have also confirmed its direct involvement in stress-coping mechanisms (Engelhardt et al., 2021; Hartmann et al., 2012, 2015; Hoeijmakers et al., 2014; Touma et al., 2011).

1.3.2 FKBP51 and early life stress

FKBP5 is a highly stress-inducible gene due to its various GREs, located in the promotor region, intron 2, 5 and 7. Upon binding of the ligand bound-GR, distal GREs in the *FKBP5* gene function as enhancers by recruiting RNA polymerase to the transcription start site (TSS). A number of genetic variations have been found for the *FKBP5* gene, which augment the transcriptional response upon glucocorticoid stimulation. The most commonly described genetic variation is the single nucleotide polymorphism (SNP) rs1360780, with a protective C-allele and less common risk T-allele, located in intron 2, in close proximity of a functional GRE. The risk T-allele of the rs1360780 SNP leads to a conformational change, which brings the distal GRE in contact with the TSS in the promotor region. In addition, it creates a TATA box, which binds TATA box binding proteins and other transcriptional activators. This eventually results in a stronger FKBP51 induction upon GR binding and an increased GR resistance (Klengel et al., 2013). Interestingly, *FKBP5* polymorphisms often require an additional trigger to result in adverse health outcomes (Matosin et al., 2018). Klengel and colleagues found that the rs1360780 SNP interacted with childhood adversity to increase the risk for developing PTSD (Klengel et al., 2013). Due to their augmented FKBP51 induction, carriers of the T-risk allele of rs1360780 have an increased GR resistance, which leads to prolonged cortisol exposure after stress. When risk allele carriers are exposed to childhood trauma, the prolonged presence of cortisol results in a demethylation of cytosine-phosphate-guanine-dinucleotides (CpGs) near GREs located in intron 7 of the *FKBP5* gene. This in turn leads to an even further enhancement of FKBP51 transcription (Figure 2). Remarkably, in carriers of the protective C-allele, a normal HPA-axis termination prevents the ELS-induced demethylation from happening (Klengel & Binder, 2015).

MRI studies in humans have already linked ELS-induced demethylation at intron 7 to structural changes in the thalamus (Womersley et al., 2022). However, they did not observe any interactions with the rs1360780 genotype. Another MRI study did demonstrate GxE interactive effects of *FKBP5* as they found T-risk allele carriers to have increased grey matter volumes (GMV) in the thalamus following positive parenting experiences, whereas C-allele carriers had reduced thalamus GMV (Matsudaira et al., 2019). Apart from the epigenetic changes, few rodent studies have tried to investigate underlying mechanisms of the interactive effects of FKBP51 levels and ELS on brain and behaviour (Criado-Marrero et al., 2019, 2020). Nonetheless, a more detailed characterization on molecular pathways and structural and functional underlying changes is still needed.

1.3.3 FKBP51 and aging

Apart from genetic predisposition, age is a factor that can also strongly influence consequences of stress exposure and contribute to the development of stress-related disease (McEwen, 2002). Illustrating this, anti-depressant treatment resistance is worsened in elderly MDD patients and depressive symptoms exacerbate with increasing age (Glaesmer et al., 2011; Lenze et al., 2005; Naismith et al., 2012). Intriguingly, in the light of GxE interactions, FKBP51 was also found to specifically interact with age. Data from post-mortem human brain tissue and rodent studies have demonstrated that FKBP51 levels rise over the lifespan (Blair et al., 2013; Matosin et al., 2023; Sabbagh et al., 2014; Shannon Weickert et al., 2016). Remarkably, this elevation in FKBP51 was associated with de-methylation of intron 7, the same epigenetic mechanism that has been proposed for ELS-induced upregulation of FKBP51 in rs1360780 risk allele carriers (Blair et al., 2013). In addition, rodent studies revealed an age-dependent effect of knockout of FKBP51 on depressive-like behaviours and restraint stress-induced CORT serum levels (Sabbagh et al., 2014). This emphasizes the importance of age when it comes to GxE interactions (Figure 2).

1.3.4 FKBP51 and sex differences

A topic of debate that has been overlooked for many years and has, rightfully so, gained an expansive amount of attention in recent time, is the topic of sex differences. Strangely, sex differences in psychiatric disease have always been obvious, with MDD and anxiety disorders being twice as common in women as in men (Eid et al., 2019; Ferrari et al., 2013; Heo et al., 2008; Kessler, 1994). Extensive amounts of studies in humans and rodents have now confirmed that sex is a strongly determining factor when it comes to stress-coping mechanisms and it was repeatedly shown that sex modulates the effects of stress and glucocorticoids on brain and behaviour (Bangasser & Valentino, 2014; Bourke et al., 2012; Brivio et al., 2020; Dalla et al., 2005; Hodes & Epperson, 2019; Rincón-Cortés et al., 2019). Even though studies in both sexes are on the rise, a large gap in literature on females remains. For studies that investigated the role of FKBP51 in stress resilience mechanisms, only a number also included females. Up till now, effects of full body knockout of FKBP51 have been reported in male and female samples, but only in separate studies (Hoeijmakers et al., 2014; Touma et al., 2011). These data surprisingly did not reveal any major sex differences, however, studies differed in set-up and used different types of stressors to test for stress-induced changes, making direct sex comparisons difficult. Other work, using an overexpression model of FKBP51, did demonstrate differences between sexes on anxiety-like behaviours and baseline CORT levels (Criado-Marrero et al., 2019, 2020). Without a doubt, there is still a major scarcity in information on female data on FKBP51 functionality and this is a gap that needs to be filled to provide a better understanding of female FKBP51-mediated stress resilience.

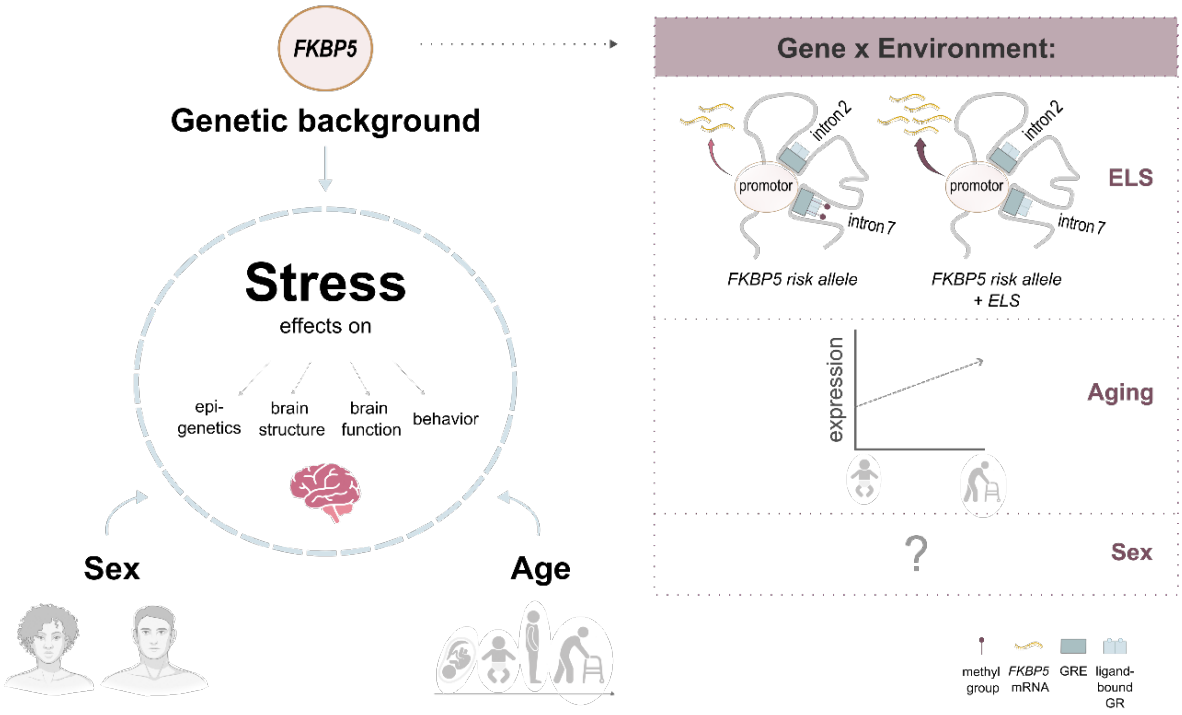

Figure 2. Genetic and environmental factors influence stress-induced effects on the brain

The way in which stress impacts the brain on various levels is not identical for each individual and a number of factors can heavily influence the eventual outcomes of stress on brain and behaviour. The genetic background of an individual plays a large role in determine consequences of stress exposure, but also environmental factors such as age or sex can be of large influence. So-called plasticity genes have been described that determine the susceptibility of an individual to environmental influences. One gene that has been studied in the light of these gene by environment (GxE) interactions is the stress-responsive *FKBP5* gene, encoding the FKBP51 protein. Different genetic variations of this gene have been described of which the single nucleotide polymorphism (SNP) rs1360780, located in intron 2, in close proximity of a glucocorticoid-responsive element (GRE), is the most

studied. This SNP results in a conformational change, which causes the GRE to come in close contact with the promotor region and lead to an enhanced glucocorticoid-induced FKBP51 transcriptional response. However, only in combination with ELS exposure, which leads to a further demethylation of the GRE in intron 7, it increases the risk of developing psychiatric disorders. Furthermore, FKBP51 interacts with age as FKBP51 levels rise over the lifespan, due to a similar demethylation of intron 7. Sex differences have also been reported for FKBP51 functionality, however evidence is limited and it requires more detailed investigation to determine the exact sex-dependent effects of FKBP51.

1.4 Brain regions involved in stress coping

The brain is a highly complex organ, with many different regions that each contribute to specific brain functions, but are also working together in carefully regulated brain networks. The limbic system consists of a number of brain structures that are thought to be particularly involved in emotional regulation and stress-related activity and it includes the hippocampal formation, the (extended) amygdala, including the bed nucleus of the stria terminalis (BNST), the limbic cortex, the septal area and the hypothalamus (RajMohan & Mohandas, 2007). Although all these regions are important in stress-related functions, in this thesis, two regions deserve special attention: the hippocampus and the BNST (Figure 3).

1.4.1 The hippocampus

The hippocampal formation is a relatively large brain structure that is located within the temporal lobe of the human brain and in the caudal forebrain of rodents (Figure 3). The principle components of the hippocampal formation are the hippocampus, subdivided in the CA1, CA2 and CA3 region, the DG and the attached subiculum. The hippocampal formation has a highly divergent cell profile, including neuronal, glial and vascular cells. The two main types of neuronal cell populations found within the hippocampal regions are excitatory glutamatergic neurons (85–90%) and GABAergic interneurons (10–15%) (Fink, 2019). Excitatory neuronal cells of the hippocampus and subiculum are called pyramidal neurons and their pyramidal cell bodies are organized in a C-shape pyramidal layer. Another principal cell type of the hippocampal formation are the granule cells of the DG, of which the cell bodies form a V-shaped granule layer. In addition to this, there is a population of glutamatergic neurons that is located in the hilus of the DG, called mossy cells, and these neurons connect to the granule cells. Dendrites and axonal segments of the principal neurons are extended into the surrounding neuropil zone and dendritic trees of the principal neurons are largely covered with spines that mostly form excitatory post-synaptic sites. In addition to the excitatory neurons, there are different types of inhibitory, GABAergic interneurons. The inhibitory interneurons are predominantly located in the neuropil zone and the majority of these interneurons connects to the pyramidal or granule cells, thereby directly inhibiting excitatory activity. Another type of GABAergic interneuron instead targets other inhibitory interneurons, resulting in disinhibition of principal neuronal activity. Together with the associated entorhinal cortex (EC), the regions of the hippocampal formation are in close communication with each other via an intra-hippocampal neuronal network called the trisynaptic circuit (Yeckel & Berger, 1990). The DG receives input from the pyramidal neurons of the EC, that forms synapses with the granule neuron dendrites. This is called the perforant pathway. Granule neurons then project towards dendrites of CA3 pyramidal cells, via the mossy fibre pathway. From the CA3 pyramidal neurons a branch of collateral axons is formed, called the Schaffer collaterals, that project towards CA2 (Dudek et al., 2016) and CA1 pyramidal neurons. At last, final output from the CA1 pyramidal neurons goes to the subiculum, back to the EC. Initial input to the EC comes from numerous other cortical regions and the EC is hereby thought to provide the hippocampal formation with sensory input of the ongoing experience. Alongside its connections to the DG, the EC can also directly innervate the CA3 and CA1 region, which allows these regions to integrate processed information (via the DG)

and unprocessed information from the EC. Apart from the EC, the hippocampus receives innervations from numerous other types of neurons. Cholinergic neurons from the medial septal nucleus mostly form synapses with hippocampal interneurons, dopaminergic afferents from the ventral tegmental area (VTA) and the substantia nigra innervate the subiculum, CA1, CA3 and the hilus and granule zone of the DG, serotonergic projections originating from the raphe nuclei connect primarily to GABAergic interneurons, histamine neurons from the hypothalamus project towards CA1, CA3 and DG granule neurons and norepinephrine innervations from the locus coeruleus to the hippocampus have been found (Fink, 2019). Output from CA1 pyramidal neurons largely goes via the EC back to the medial PFC (mPFC) and other cortical regions. However, it can also directly, or via the subiculum, connect to the mPFC and subcortical structures, such as amygdala, hypothalamus, septum and the thalamus. In addition to the trisynaptic pathway, there are other intra-hippocampal connections, mostly originating from non-Schaffer collateral branches of CA3 pyramidal neurons and contributing axons from the CA2 pyramidal cells and mossy cells.

The hippocampal formation is largely known for its implications in memory formation. Interestingly, the rodent hippocampus extends in a longitudinal manner (Figure 3) and it is thought to follow differential functionality along its longitudinal axis (Fanselow & Dong, 2010). The DHC plays a particularly important role in spatial memory function, whereas the VHC is believed to be more involved in emotional memory formation. An important underlying mechanism for memory formation is the process of synaptic plasticity, in which cellular processes can lead to modifications in synaptic transmission. This process has been extensively described for the hippocampus and depends on two different cellular processes, LTP and long-term depression (LDP) (Malenka & Bear, 2004). Either one strong experience, or a repetition of experiences can lead to high frequency stimulation (HFS) at the excitatory synapse. On the long-term, this can provoke an increased synaptic response to a future stimulation. This process is dependent on the large Ca2+ influx as a result of the HFS, which leads to an increased AMPA and NMDA receptor recruitment to the post-synaptic membrane. Ultimately, this makes the post-synaptic terminal more sensitive to future stimulation. In LDP on the other hand, a low frequency stimulation results in a low Ca2+ influx and an enhanced AMPA and NMDA receptor endocytosis, thereby decreasing the post-synaptic membrane sensitivity.

The hippocampus is extremely sensitive to the effects of stress (Kim & Diamond, 2002; Sapolsky et al., 1985). It has been repeatedly shown that glucocorticoid stimulation or acute and chronic stress exposure can alter hippocampal structure (eg. neuronal dendritic complexity and spine numbers), neurogenesis, but also functional processes such as synaptic plasticity in the CA1, CA3 and DG. The hippocampus is developing strongly during the early stages of life and is therefore particularly impacted by ELS exposures. Furthermore, it is also the brain region that is the most sensitive to age-related changes (Lupien et al., 2009). Structural and functional changes have also recurrently been found in patients suffering from MDD or other psychiatric disorders (Campbell & Macqueen, 2004; Gilbertson et al., 2002; McKinnon et al., 2009; Stockmeier et al., 2004). It is therefore no surprise that apart from the depressed mood and anxiety, depressive patients often suffer from symptoms in the cognitive domain.

1.4.2 The bed nucleus of the stria terminalis

Another limbic brain region, that is located in the mouse basal forebrain, is the BNST. The BNST is sometimes referred to as the extended amygdala and it is highly innervated by projections from this region. Like the amygdala, it is also thought to be majorly implicated in anxiety- and stress-related behaviour (Feola et al., 2023). The structure of the BNST is highly heterogenous as it is comprised out of multiple nuclei (Figure 3). Principally, the BNST can be divided into a posterior and an anterior division. The posterior division has three well-defined nuclei, the principal, interfascicular and transverse nucleus, and this part plays a role in social defence and reproductive behaviours. The

anterior region on the other hand, has received most attention when it comes to its role in regulating anxiety. This anterior division is made up out of a large number of nuclei (anterolateral, anteromedial, fusiform, juxtacapsular, rhomboid, dorsomedial, ventral nucleus, magnocellular and the oval BNST) and receives strong input from the central amygdala (CeA). The CeA largely sends CRH-containing projections, via the BNST to the hypothalamic PVN, thereby initiating HPA-axis activation. Further output of the BNST goes to the VTA and the mPFC. Primarily, the BNST has an important integrative role in processing information with negative or positive valence, mood, energy and motivation status and accordingly shifts from homeostasis to stress activation or *vice versa* (Lebow & Chen, 2016). Interestingly, whereas the amygdala is highly involved in more "phasic" fear responses, the BNST is considered to play a role in the "sustained" responses and anticipation to threat (Lebow & Chen, 2016). Data from fMRI studies in patients suffering from either generalized anxiety disorder (Yassa et al., 2012) or specific phobias have also demonstrated hyperactivate responses of the BNST in response to respectively a gambling tasks or presentation of the phobic stimulus. However, the highly heterogenous anatomy of the BNST leads to strong sub-regional directionality of effects on anxiety states.

One subregion of the BNST that is particularly interesting in the light of stress-regulation is the oval BNST (ovBNST), a subregion located in the dorsal and anterolateral part of the BNST. For example, it was found that *Crh* mRNA was upregulated in the ovBNST following corticosterone exposure and an acute foot shock (Daniel & Rainnie, 2016). Moreover, both chronic stress and ELS was found to induce long-lasting alterations in CRH signaling in the ovBNST (Hu, Liu, et al., 2020; Hu, Maita, et al., 2020). Apart from CRH neurons, this region is highly rich in GABAergic neurons, but also integrates information from dopaminergic neurons and expresses the neuropeptides pituitary adenylate cyclase-activating polypeptide (PACAP) and Tachykinin 2 (Tac 2) (Engelhardt et al., 2021; Lebow & Chen, 2016).

1.4.3 FKBP51 in the brain

FKBP51 is widely distributed throughout the brain. Interestingly though, it has been found that FKBP51 has distinct functionality in different brain regions and responds in a unique way towards various types of stress exposure. Scharf and colleagues demonstrated that the hippocampus has a particularly high baseline expression of FKBP51 (Scharf et al., 2011). Hippocampal FKBP51 expression was also selectively increased by stress exposure in the form of a 24h food deprivation, but not by a 4h restraint stressor. Notably, only the CA1 and DG regions showed a stress-induced FKBP51 elevation. In contrast to the hippocampus, two other stress-related brain regions, the CeA and the hypothalamic PVN, did not have particularly high baseline levels of FKBP51. Nevertheless, both regions showed a significant upregulation in *Fkbp5* mRNA in response to both restraint and food deprivation stress and this response was stronger than the stress-induced FKBP51 induction in the hippocampus. FKBP51 was also found to be expressed in the BNST, where it was reactive to a 4h acute restraint stressor and where it co-localizes with the stress neuropeptides CRH and Tac2 (Engelhardt et al., 2021).

Remarkably, different rodent studies have already demonstrated that FKBP51 can have opposing effects on stress endocrine responses and behaviour, depending on the region or cell-type that it is expressed in. For example, a study by Hartmann and colleagues showed that FKBP51 inhibition by systemic administration of the antagonist SaFit2 led to a reduced anxiety-like behaviour. Conversely, overexpression of FKBP51 in the BLA, but not in the CeA, led to an increased anxiety-like behaviour (Hartmann et al., 2015). Surprisingly and in contrast to the previous findings by Hartmann and colleagues, a selective overexpression of FKBP51 in the ovBNST, resulted in a contrasting anxiolytic-like behaviour, whereas loss of FKBP51 in the same region led to an anxiogenic behaviour. In addition to this, a hyper-reactive HPA-axis was observed upon stress exposure in animals with a knockout in the ovBNST (Engelhardt et al., 2021). When FKBP51 is lacking in the hypothalamic PVN or in the POMC

neurons of the pituitary however, an improved HPA axis regulation was observed (Brix, 2023). Moreover, different types of nuclei and neurons in the hypothalamus have alternative effects on metabolism (Brix, 2023; Häusl et al., 2022). These data evidently indicate the cell-specific fashion in which FKBP51 can modify various bodily processes.

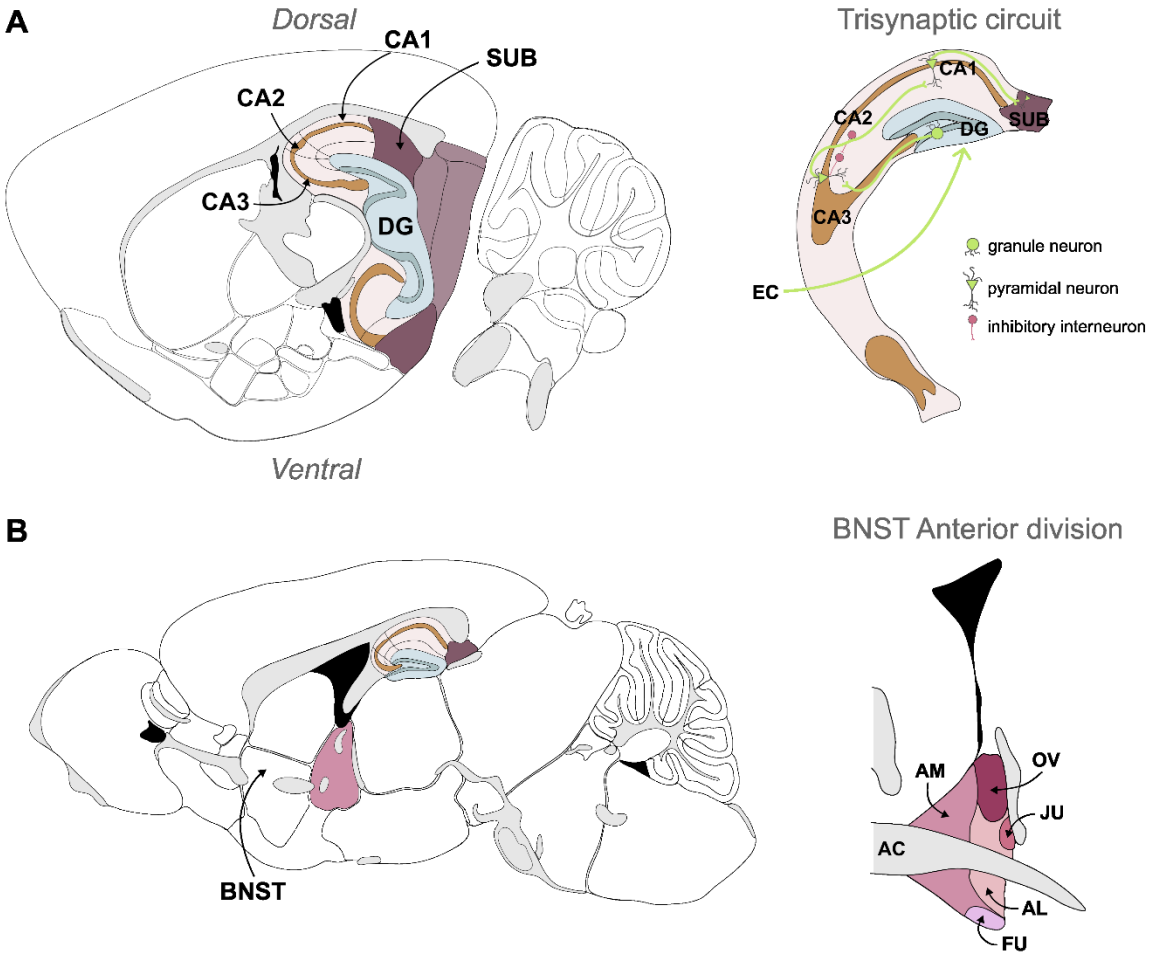

Figure 3. Hippocampus and the Bed Nucleus of the Stria Terminalis
On the left in panel A, a sagittal image of the mouse hippocampal formation is depicted, including the hippocampus (CA1, CA2, CA3 region), dentate gyrus (DG) and subiculum along its dorsoventral longitudinal axis. On the right, a coronal section of the hippocampal formation shows the trisynaptic circuit in which neurons of the entorhinal cortex (EC) connect to the glutamatergic granule cells in the DG, called the perforant pathway. Via the mossy fibre pathway, granule neurons connect to post-synaptic synapses on the dendrites of the glutamatergic CA3 pyramidal neurons. Different CA3 pyramidal neurons form Shaffer collateral axons that make synaptic connections to the pyramidal neurons of the CA1. At last, CA1 pyramidal neurons connect back to the subiculum and provide output to cortical regions via the EC and to other subcortical regions. In the neuropil zone, GABAergic interneurons connect to the glutamatergic pyramidal and granule cells to inhibit excitatory action, or connect to other inhibitory interneurons for disinhibition. In panel B on the left, a sagittal section of the bed nucleus of the stria terminalis (BNST) and dorsal hippocampus (DHC) is displayed. The anterior division of the BNST plays an important role in fear and anxiety states. On the right, the heterogenous structure of the anterior BNST is illustrated of which a number of different subnuclei are shown. In this coronal section of the anterior division of the BNST, subnuclei are located dorsally and ventrally of the anterior commissure (AC) white matter tract and include the anteromedial area (AM), anterolateral area (AL), juxtacapsular nucleus (JU), fusiform nucleus (FU) and oval nucleus (OV). The latter is known to be particularly involved in stress-related behaviour.

1.4 Rationale and thesis objectives

It is clear that the outcomes of stress exposure can be influenced by a variety of environmental factors including sex, age and genetic background. The *FKBP5* gene is a particularly interesting genetic factor that largely impacts stress vulnerability and resilience mechanisms. Moreover, *FKBP5* is known for its GxE interactions with ELS exposure and with age and sex as relevant modifying factors. Ever since the discovery of *FKBP5* and its role in psychiatric disease development, human and rodent studies have aimed to unravel the underlying mechanisms of how FKBP51 impacts stress resilience and vulnerability. However, FKBP51 has a highly region- and cell-specific functionality. Therefore, there is still a strong need for a broader and more in-depth characterization of the cell-specific effects of FKBP51. In addition to this, sex differences are a long-overlooked, but highly important aspect of stress-related processes, which are in part due to a lack in stress paradigms that are translational to both sexes. A large knowledge gap is also evident for FKBP51-mediated mechanisms of stress coping in females. This dissertation addresses the need for sex-independent rodent models of stress and provides a broader and more in-depth characterization of the cell- and region-specific impact of FKBP51 on (early life) stress coping and how it interacts with the environmental factors sex and age. This led to the formation of the following research questions that were answered in three separate studies:

1. Does a model of CSDS in female mice lead to sex-specific behavioural and neuroendocrine effects? (Chapter 2.1)
2. Does FKBP51 in glutamatergic and GABAergic neurons have cell-type specific and sex-specific effects on brain structure and function and what are the underlying mechanisms for this? (Chapter 2.2)
3. Does FKBP51 in glutamatergic forebrain neurons interact with moderate ELS exposure to affect brain structure, function and behaviour and what are the underlying pathways for this? (Chapter 2.3).

2. Research articles

2.1 Chronic social defeat stress in female mice leads to sex-specific behavioural and neuroendocrine effects

Van Doeselaar L, Yang H, Bordes J, Brix L, Engelhardt C, Tang F & Schmidt MV.

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Chronic social defeat stress in female mice leads to sex-specific behavioral and neuroendocrine effects

Lotte van Doeselaar, Huanqing Yang, Joeri Bordes, Lea Brix, Clara Engelhardt, Fiona Tang and Mathias V. Schmidt

Research Group Neurobiology of Stress Resilience, Max Planck Institute of Psychiatry, Munich, Germany

ABSTRACT

Over the years, it has become increasingly clear that males and females respond differently towards environmental stressors, highlighting the importance of including both sexes when studying the effects of stress. This study aims to provide further insight into the detailed consequences of exposing female mice to 21 days of chronic social defeat stress (CSDS). We used a protocol that relies on the ability of odorants and pheromones in male urine to trigger male mouse aggressive behavior. Collected male C57Bl/6n urine was applied to female C57Bl/6n mice who were then attacked by a novel male CD1 mouse each day according to the CDSD protocol. Control females were pair-housed and handled daily. Physiological, neuroendocrine and behavioral changes were evaluated during the experiment. CSDS exposure resulted in number of physiological changes, such as body weight gain, enlarged adrenals and reduced thymus weight, exaggerated HPA-axis negative feedback and increased anxiety-like behavior. However, no generalized social avoidance behavior was observed. This study provides important insights in the physiological, neuroendocrine and behavioral responses of female mice to CSDS, which are partially dependent on estrous cycle stage. This protocol will allow direct comparison of male and female responses to CSDS and enable sex-specific study of mechanisms underlying individual stress resilience.

LAY SUMMARY

- In this study we found that there are differences in the way that female and male mice respond towards chronic social stress conditions when it comes to behavior and hormonal changes.

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Stress; chronic social defeat; sex; female; estrous cycle; behavior

Introduction

Over the past few decades, mental health disorders have become a wide-spread health concern, and a leading cause of disability world-wide (World Health Organisation, 2020). The risk for developing a mental health disorder is dependent on the interaction between underlying genetic predispositions and environmental factors (Ron de Kloet et al., 2005). One of the main environmental factors modulating disease vulnerability is chronic exposure to stressors over the life time.

It is well known that stress-related disorders, such as anxiety disorders and major depressive disorder, are more common in women than men (Gutiérrez-Lobos et al., 2002; Kessler, 1994; WHO International Consortium in Psychiatric Epidemiology 2000) In addition, male and female responses to stress exposure differ critically on several dimensions, which has become particularly evident from a large number of rodent studies (Dalla et al., 2005; Galea et al., 1997; Hodes et al., 2015; Sachs et al., 2014; Westenbroek et al., 2004). Findings of these differential stress responses argue the importance of including both sexes when it comes to studying the effects of stress.

Nonetheless, for a long-time, comparison of male and female mice has been difficult in experimental chronic stress conditions, especially with regard to social stressors. Application of one of the most widely used chronic stress paradigms in mice, chronic social defeat stress (CSDS), strongly relies on aggressive behavior (Kudryavtseva et al., 1991; Russo & Nestler, 2013). In CSDS, male mice attack male intruders with a high likelihood, however, such aggressive behavior towards female mice is usually absent. Furthermore, female-to-female aggression is less likely to occur under standard laboratory conditions making it challenging to apply such CSDS protocols in females. Efforts to replicate effects of CSDS in female rodents were for a long time hampered by unavailable or particularly complex methods (Bourke & Neigh, 2012; Haller et al., 1999; Takahashi et al., 2017). In the past few years there have been attempts to design a hands-on protocol in which social defeat of females is achieved either using male-to-female or female-to-female aggression

CONTACT Mathias V. Schmidt mschmidt@psych.mpg.de Research Group Neurobiology of Stress Resilience, Max Planck Institute of Psychiatry, Kraepelinstr. 2-10, Munich, 80804, Germany

Supplemental data for this article can be accessed here.

(Harris et al., 2018; Logan, 2019; Newman et al., 2019). One of these attainable CSDS models was recently described by Harris et al. This CSDS model relies on the odorants and pheromones in male urine to increase male mouse aggressive behavior towards female mice and has proved to be successful in establishing a stress phenotype (Harris et al., 2018).

The aim of the current study was to reproduce and extend the findings of the model described by Harris and colleagues, but with an adjustment to the length of the chronic defeat to 21 days, as a longer defeat period is also commonly used in many male CSDS studies (Haenisch et al., 2009; Wagner et al., 2011). This has previously been demonstrated to result in robust depression-like phenotypes in male mice (Kudryavtseva, 2000; Kudryavtseva et al., 1991) and offers better possibilities for pharmacological interventions (Gassen et al., 2014). This study provides additional insights on the different physiological, neuroendocrine and behavioral responses towards CSDS stress in females. The establishment of a CSDS protocol in female mice will allow researchers to test gene × environment interactions in both sexes. Ultimately, this will lead to increased knowledge on the differential sex effects in stress resilience mechanisms.

Methods

Animals and housing conditions

C57Bl/6n mice were bred in an in-house colony of the Max Planck Institute of Psychiatry (Munich, Germany) and used as experimental animals (females, 12 weeks old) or for the training of resident mice and urine sampling (males). Experimental animals were pair-housed in standard individually ventilated caging (IVC) system cages at least 2 weeks prior to the start of the experiment. In addition, CD1 mice (purchased from Janvier Labs, Germany) were used as residents (males, 12 weeks old, single-housed before the experiment) or social interaction partner (females, 6 weeks old, group-housed). CD1 males were allowed to habituate to the social defeat cage for two weeks before the start of the experiment.

Housing and experiments were performed under a 12 h light, 12 h dark cycle (lights on at 07.00 a.m.), constant temperature (23 ± 2 °C, humidity 55%) conditions and mice had access to food (Altromin 1324, Altromin GmbH, Germany) and water ad libitum.

Experiments were performed in accordance with the European Communities Council Directive 2010/63/EU. At all times efforts were made to minimize animal suffering during the course of the experiment. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Experimental design

The 24 female C57Bl/6n mice were randomly divided into two groups (n = 12 CSDS; n = 12 control). The CSDS group was exposed to 21 consecutive days of the CSDS paradigm; control animals were handled daily. From day 18 until day 21 of the experimental period, behavior of CSDS and control animals was evaluated using a number of behavioral tasks assessing locomotor activity, anxiety-like behavior, social avoidance (SA) behavior and coping strategy selection (Figure 1(A)). One day after the last defeat all animals were sacrificed under basal conditions. A second cohort of female mice (C57Bl/6n, n = 11) was subjected to the same CSDS paradigm for a better quantification of the aggressive encounters during the CSDS exposure.

Chronic social defeat stress paradigm

Prior to the start of the CSDS experiment, resident CD1 males were tested on likelihood to attack an intruder C57Bl/6n male and the 12 most aggressive CD1 mice were selected for the CSDS experiment. Based on a protocol by Harris et al. (2018) females were covered in previously collected urine (at room temperature) from C57Bl/6n male mice, in order to trigger an attack by the CD1 residents. Using a brush, urine was applied to the head, back, tail and at the vaginal orifice. The CSDS paradigm was performed as previously described (Wagner et al., 2011). In short, following application of male urine, female mice were immediately placed into the home-cage of a CD1 male resident. Animals were separated as soon as the aggressive confrontation was achieved, or after a maximum of 5 min. Subsequently, animals spent 24 h in the same cage (45 × 25 cm) as the resident CD1 male, separated by a transparent divider, to prevent any physical contact, but allow continuation of the social threat via visual and olfactory cues. Each day, experimental animals were introduced (after fresh urine application) into a new cage with a novel resident (Figure 1(A)). The defeat occurred between 11 a.m. and 4 p.m. each day. Starting times of the defeat varied each day in order to reduce predictability of the stressor and thereby decreasing the chances of a potential habituation effect. Control animals remained pair-housed in their home-cage throughout the experimental period and were exposed to male odor via bedding material once a week. All animals were handled on a daily basis.

In order to allow qualitative evaluation and to further describe the intensity, frequency and duration of the aggressive behavior of the CD1 males towards the female mice, videos were recorded within a second cohort of animals (n = 11 CSDS female C57Bl/6n) on day 1, day 8 and day 14 of the CSDS experiment. The number of attacks and chasing by the CD1 male during the 5-min defeat period was quantified.

Urine collection

Urine from C57Bl/6n male mice was either collected in a tube while manually restraining the mouse, or by placing mice in an empty cage on top of a metal grid. After 1 h, urine was then collected from the bottom of the cage. Fresh urine of 15–20 mice was collected every 3–4 days, mixed together and stored at 4 °C.

Fur status and body weight

Fur status was determined every 2–5 days prior to the social defeat and body weight was measured weekly for both

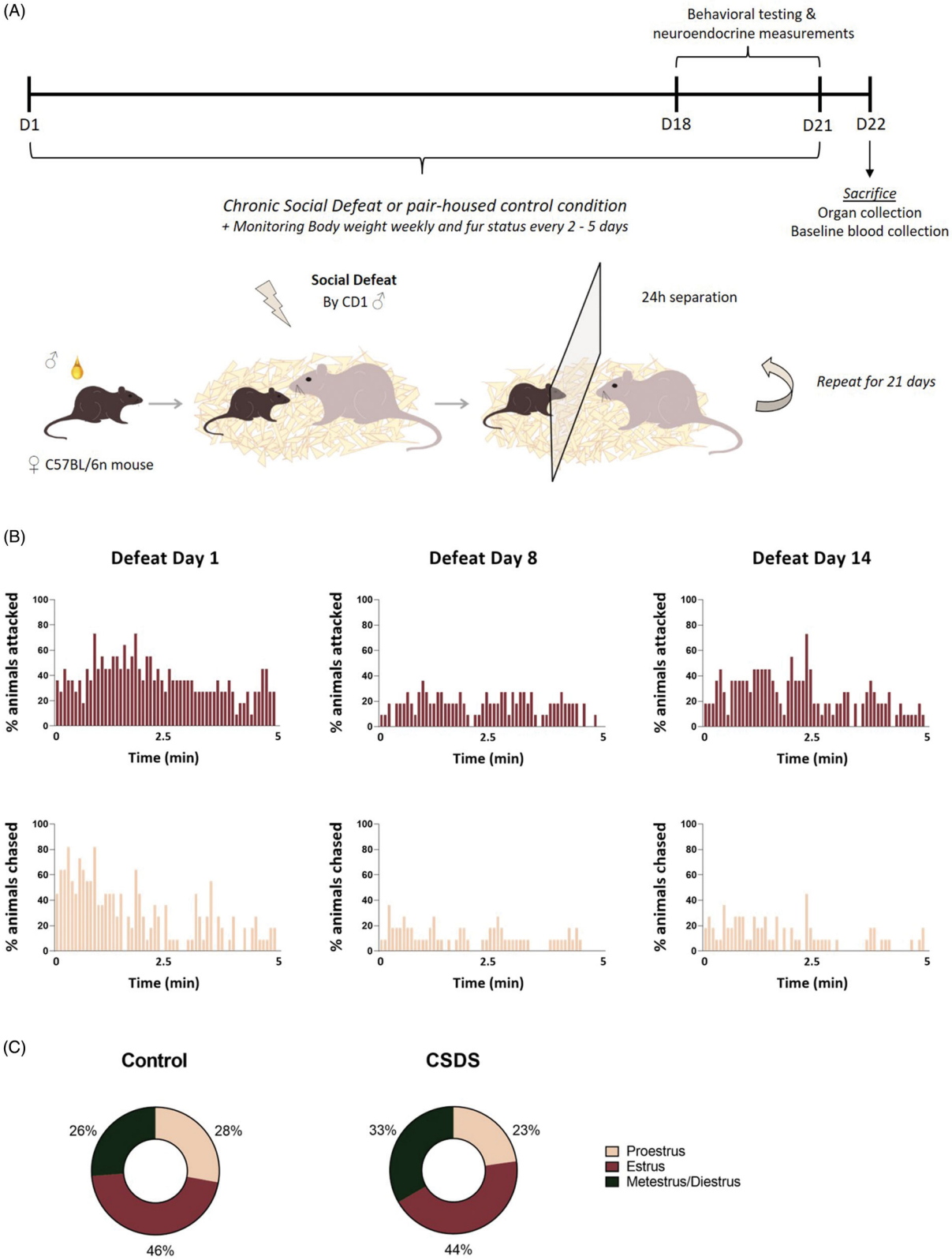

Figure 1. Female C57Bl/n mice are socially defeated by a CD1 male mouse over a period of 21 days when covered in male C57Bl/6n urine. (A) Over the course of 21 days female C57Bl/6n mice were exposed to a social chronic defeat stress (CSDS) paradigm. In this CSDS paradigm female mice were covered in male C57Bl/6n urine after which they were immediately exposed to a social defeat of 5 min by a CD1 male mice, subsequently separated by a transparent divider and housed together with the CD1 male for 24 h. This procedure was repeated with a novel CD1 each day. (B) In a separate cohort of animals, this 21-day CSDS paradigm led to a substantial number of attacks and chasing by the CD1 male as quantitively illustrated for the 5-min defeat on day 1, day 8 and day 14 of the experiment. (C) In this cohort, the percentages in which estrus, proestrus or metestrus/diestrus cycle phases occur from day 10 to day 18 of the experiment are comparable for control and CSDS mice. D: day; CSDS: chronic social defeat stress.

control and CSDS mice. The condition of the fur was assessed by an experienced investigator as described previously (Mineur et al., 2003). In short, scores were classified according to a 4-point scale, where 1 stands for a perfect, clean fur, while 4 represents a disheveled, scruffy fur, often including traces of wounds and scurf. Ratings of 2 and 3 demonstrate intermediate fur states, respectively.

Behavioral assessment

Tests were performed between 08.00 a.m. and 12.00 p.m. and took place in a specially equipped behavioral room, adjacent to the housing room. After performance of the test, control mice were put back in their homecage and CSDS females returned to the same cage with the same CD1 as prior to test performance, separated by the transparent divider. On the days of behavioral testing chronic social defeat took place in the afternoon. All tests were recorded and tracked using the automated video-tracking system Anymaze 4.99z (Stoelting, Dublin, IE). In case manual scoring was necessary, this was performed by an experienced observer that was blinded to the experimental condition.

Open field

On day 18 of the experiment, the open field (OF) was conducted as previously performed (Schmidt et al., 2007; Sterlemann et al., 2008) to assess locomotor activity and anxiety-like behavior. The test was executed in an empty open field arena (50 cm × 50 cm × 50 cm), made out of gray polyvinyl chloride (PVC), under constant lighting conditions of approximately 20 lux. Total test duration was 10 min, in which total distance travelled and the number of entries, time spent and distance travelled within the inner zone of the OF were analyzed.

Elevated plus maze

The elevated plus maze (EPM) was performed on day 19 of the experiment to measure anxiety-like behavior as previously described (Schmidt et al., 2007; Sterlemann et al., 2008). The apparatus was comprised of a plus shaped platform, with two opposing open arms (30 cm × 5 cm × 0.5 cm) and two opposing enclosed arms (30 cm × 5 cm × 15 cm), made of gray PVC, which were connected by a central area (5 cm × 5 cm × 0.5 cm). The whole apparatus was elevated 50 cm above the floor. Lighting conditions were set at 20 lux in the open arms and less than 10 lux in the closed arms. At the start of the test, animals were placed in the central zone, facing an enclosed arm. The test lasted for 5 min, in which the following parameters were measured: number of entries, time spent and distance travelled in the open and closed arms of the EPM.

Social avoidance task

To test whether CSDS led to avoidance of a social encounter with an adolescent female CD1 mouse, the SA task was conducted on day 20 of the experimental period (according to Tsankova et al., 2006). The SA task consisted of two phases: the acquisition phase and the retrieval phase, which all animals underwent subsequently. In the acquisition phase animals were placed in the OF arena with a small empty cage placed in a fixed position of the arena. The acquisition phase lasted for 2.5 min. While animals remained in the OF arena, the empty cage was replaced with a cage containing a female adolescent CD1 mouse, allowing experimental animals to socially interact or avoid interaction with the newly introduced mouse. This retrieval phase lasted for another 2.5 min. A "social zone" was defined, which included an area closely surrounding the inserted cage. Time spent in this social zone was manually scored when the experimental mouse was also directing its head towards the cage.

Forced swim task

The forced swim task (FST) was performed on day 21 of the experiment in order to assess choice of coping strategy upon exposure to a stressful and unescapable environment (as described by Hartmann et al., 2012). For this, animals were put in a 2 liter glass beaker (diameter: 13 cm; height: 24 cm) filled with tap water (21 ± 1 °C) up to 15 cm height, so that the mouse could not touch the bottom of the beaker with its hind paws or tail, nor climb out of the beaker. The test lasted for 6 min and after completion of the test, mice were dried with a towel to prevent hypothermia. The following parameters were manually scored: time spent swimming, struggling and floating.

Vaginal smear sampling

Wet vaginal smear samples were taken on each day of behavioral testing. Smears were taken in between 11 a.m. and 1 p.m., right after completion of the behavioral task. As previously described (Caligioni, 2009), 40 µl of sterile PBS (room temperature) was carefully pipetted up and down the vaginal canal multiple times, without penetrating the vaginal canal. The collected fluid was then placed on a glass slide and air-dried for at least 1 h. Subsequently, slides were stained with a Giemsa-Wright staining (Sigma-Aldrich) for 30 s and washed with distilled water for 3–7 min. Afterwards, slides were analyzed using a light microscope at 10x magnification. It was decided to divide the cycle in a biphasic manner with proestrus/estrus (P/E) as stages where estradiol levels are usually high and with metestrus/diestrus (M/D) as stages where estradiol levels are usually lower (McLean et al., 2012). When samples were ambiguous, they were excluded from analysis.

Stress neuroendocrine assessment

After completion of the FST, all animals were placed in a novel cage to recover from the acute stressor. Neuroendocrine response profiles were assessed by collecting blood samples in 1,5 mL EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany) via a tailcut (Fluttert et al., 2000) 30 min after onset of the stressor (stress response) and 90 min following onset of the FST (stress recovery). After

completion of blood sampling, animals returned to their home-cage (for controls) or their CSDS cage. Blood samples were kept on ice and subsequently centrifuged at 8000 rpm, for 15 min at 4 °C. At least 5 µl of plasma was collected and stored at −20 °C. Later, corticosterone levels (ng/ml) were quantified by radioimmunoassay following the manufacturer's instructions (MP Biomedicals Inc.; sensitivity 12.5 ng/ml).

Sampling procedure

One day after the last defeat, animals were weighed and subsequently sacrificed by decapitation following quick anesthesia by isofluorane. Baseline trunk blood was collected in 1.5 mL EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). Blood samples were saved on ice and subsequently centrifuged at 8000 rpm, for 15 min at 4 °C. Blood plasma was collected and stored at −20 °C. In addition, the adrenal glands and the thymus were removed, dissected from fat and weighed.

Statistical analyses

Data were analyzed using the IBM SPSS statistics v25 package and graphs were prepared using Graphpad Prism v8.3.

Physiological and neuroendocrine measures were analyzed using independent *t*-tests. In case data were not normally distributed the non-parametric Mann–Whitney *U* test was performed. In time-course analyses, a repeated measures analysis of variance (ANOVA) with time as a within-subject factor and condition (CSDS vs. control) as a between-subject factor was applied. For fur status results the non-parametric Friedman test, followed by Chi Square post-hoc testing, was performed. All behavioral measures were analyzed using a two-way ANOVA with condition (CSDS vs. control) and estrous cycle (P/E vs. M/D) as fixed factor. If no effect of estrous cycle and no interaction effect were found, the data were analyzed using a one-way ANOVA with condition as fixed factor and estrous cycle stage as co-variate. In case behavioral data were not normally distributed, a log transformation was performed to normalize data before analyses. If significant main or interaction effects were found, post-hoc independent sample *t*-tests were performed. Correlation analyses were performed using the Pearson's correlation coefficient. ANOVA significance levels for main effects were set to $p < 0.05$ and for interaction effects to $p < 0.1$. For all post-hoc tests, the significance level was set to $p < 0.05$. Values outside a margin of two times the standard deviation were considered outliers and excluded from analyses. Data are visualized as the mean ± the standard error of the mean (SEM), including the individual data points. If main effects for estrous cycle or an interaction effect between estrous cycle and condition were found, data were additionally illustrated as separated by cycle stage.

Results

CSDS procedure

The encounter of the resident CD1 male with the urine-covered experimental C57Bl/6n female led to a robust physical attack by the CD1 resident in 60% of the cases (in 150 of the confrontations out of a total 252 confrontations female mice were attacked by the CD1 male). Even though animals were not attacked by a CD1 male on every day of the experiment, overall there was no separation in the cohort with regard to the frequency of aggressive encounters throughout the 21 days. If animals were not attacked, we still observed physical contact between the CD1 resident and experimental mouse in the form of chasing. In most cases, experimental mice displayed a defensive posture upon approach by the CD1 resident, even if this encounter did not lead to an attack. For a more qualitative evaluation of the aggressive behavior of the CD1 males, defeats were recorded on day 1, day 8 and day 14 in a separate CSDS cohort (Supplementary Videos 1–3). The number of attacks and chasing by the CD1 male during the 5-min defeat period on these three days of the experiment were quantified (Figure 1(B)), illustrating a robust exposure of females to aggressive behavior by the CD1 males in this paradigm. In this cohort, CSDS did not affect cycling (Figure 1(C)).

Physiological parameters

At the start of the experiment, animals did not significantly differ in body weight. A repeated measures ANOVA revealed a significant within-subjects effect for time ($F_{(2.2,44.3)} = 148.732$, $p < 0.000$), as well as time × condition interaction ($F_{(2.2,44.3)} = 6.168$, $p < 0.01$) and an in-between-subjects effect for condition ($F_{(1,20)} = 5.017$, $p < 0.05$; Figure 2(A)). Post-hoc tests indicated that from day 15 of the CSDS paradigm onwards, CSDS mice had an increased body weight compared to control animals ($t_{(20)} = -3.597$, $p < 0.01$) and this effect remained until sacrifice at day 22 of the experiment ($t_{(20)} = -3.345$, $p < 0.01$).

Fur condition scores also did not differ at the start of the experiment, but the non-parametric Friedman test detected differences in fur condition ($\chi^2_{(5)} = 54.459$, $p < 0.001$; Figure 2(B)). Post-hoc tests revealed that already at day 7 of the defeat paradigm, CSDS mice had a higher fur state score ($\chi^2_{(2)} = 11.169$, $p < 0.01$) and this difference continued to be present at day 12 ($\chi^2_{(2)} = 12.103$, $p < 0.01$), day 15 ($\chi^2_{(2)} = 9.214$, $p < 0.05$) and day 19 ($\chi^2_{(2)} = 18.333$, $p < 0.001$) of the experiment.

One day after the last defeat, animals were sacrificed and organs were collected and weighed. Following 21 days of CSDS the weight of the adrenal glands corrected for body weight was significantly increased when compared to control animals ($t_{(21)} = -3.761$, $p < 0.01$) and although not statistically significant, relative thymus weight tended to be reduced ($t_{(22)} = 1.917$, $p = 0.07$) vs. control mice (Figure 2(C,D)).

Neuroendocrine measures

Basal and stress neuroendocrine profiles of CSDS and control mice were further investigated by quantifying basal morning levels of corticosterone (at day 22 of the experiment) and corticosterone response and recovery levels upon exposure to an acute stressor (on day 21 of the experiment; respectively 30 min or 90 min following the onset of the FST). We

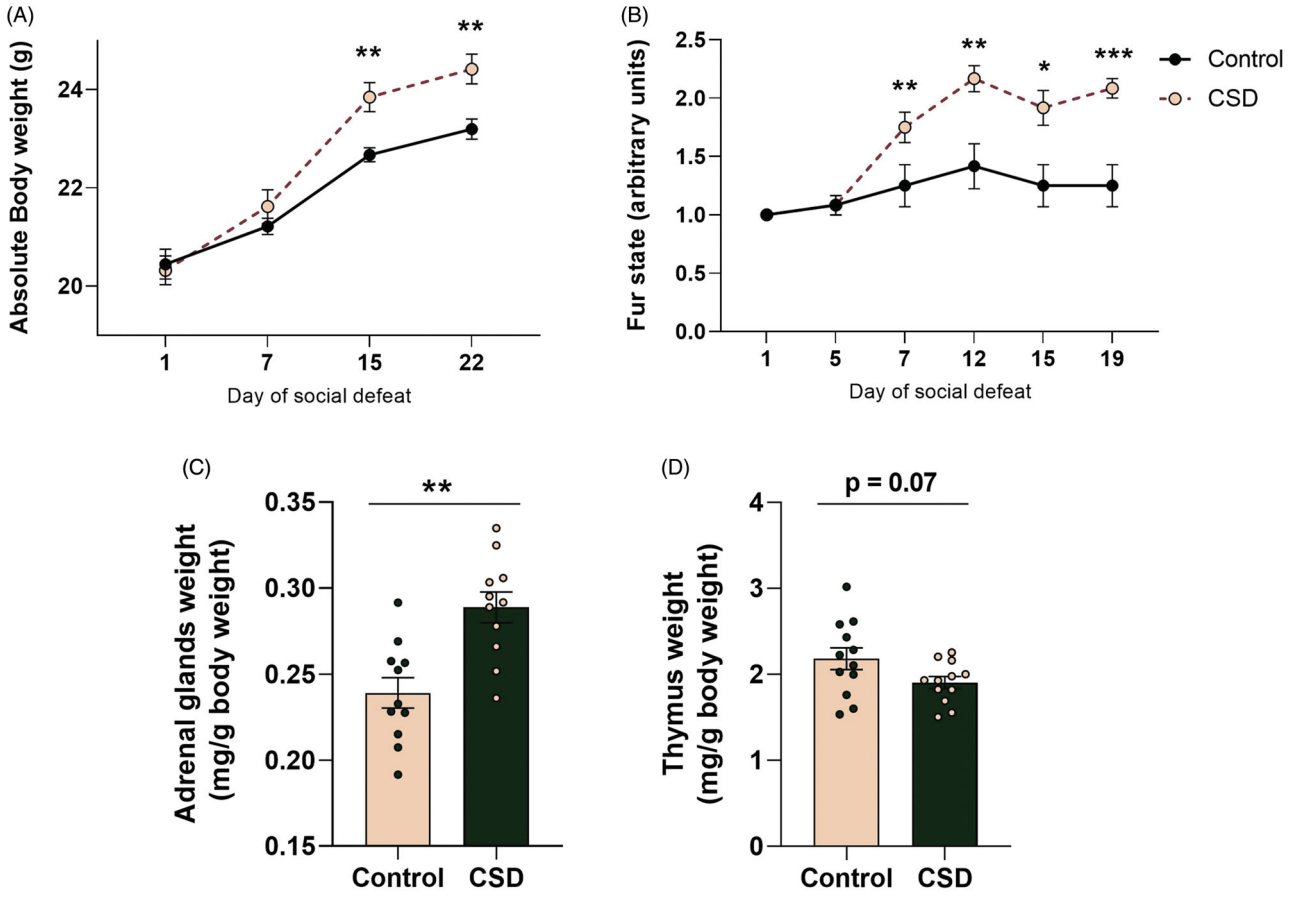

Figure 2. Chronic social defeat stress leads to changes in body weight, organ weight and fur condition in female C57Bl/6n mice. Exposure to a 21-day social defeat led to (A) a significant increase in body weight from day 15 of the defeat onwards. In addition, (B) fur condition was worsened in socially defeated mice compared to control animals from day 7 of the defeat onwards. Chronic social defeat stress exposure led to (C) a significant increase in relative adrenal gland weight and (D) a tendency for reduced relative thymus weight. CSDS: chronic social defeat stress; data represent mean ± SEM; $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

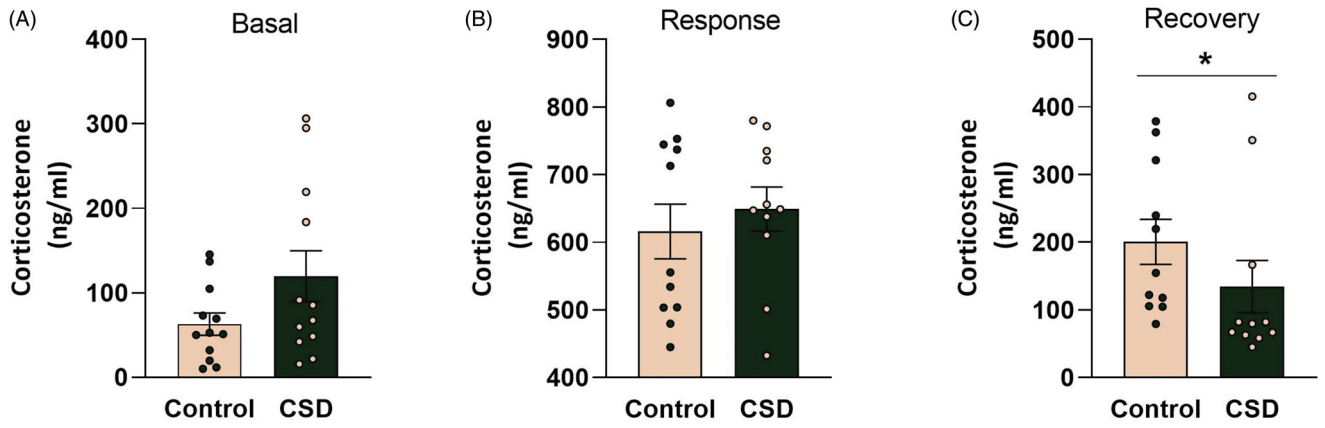

Figure 3. Chronic social defeat stress affects corticosterone recovery levels following a stressor in female C57Bl/6n mice. (A) Basal morning corticosterone levels are unaffected following 21 days of social defeat. Following the stressor (forced swim test; FST) the corticosterone response (B) 30 min after FST onset was affected by chronic social defeat (CSDS) exposure, whereas the corticosterone recovery (C) 90 min after FST onset was reduced in CSDS mice compared to controls. CSDS: chronic social defeat stress; data represent mean ± SEM; $^{*}p < 0.05$.

did not observe differences in basal morning corticosterone levels, nor any differences in the corticosterone response 30 min following an acute stressor (Figure 3(A,B)). However, the Mann–Whitney *U* test revealed significantly reduced corticosterone concentrations in the plasma of CSDS mice vs. control mice, during the recovery phase following an acute stressor ($U = 29$, $p < 0.05$; Figure 3(C)).

Behavioral assessment

Open field

The OF was used to assess whether CSDS exposure affects locomotor activity and anxiety-like behavior. Mice did not present any changes in overall locomotor activity, as total distance travelled did not differ between CSDS and control

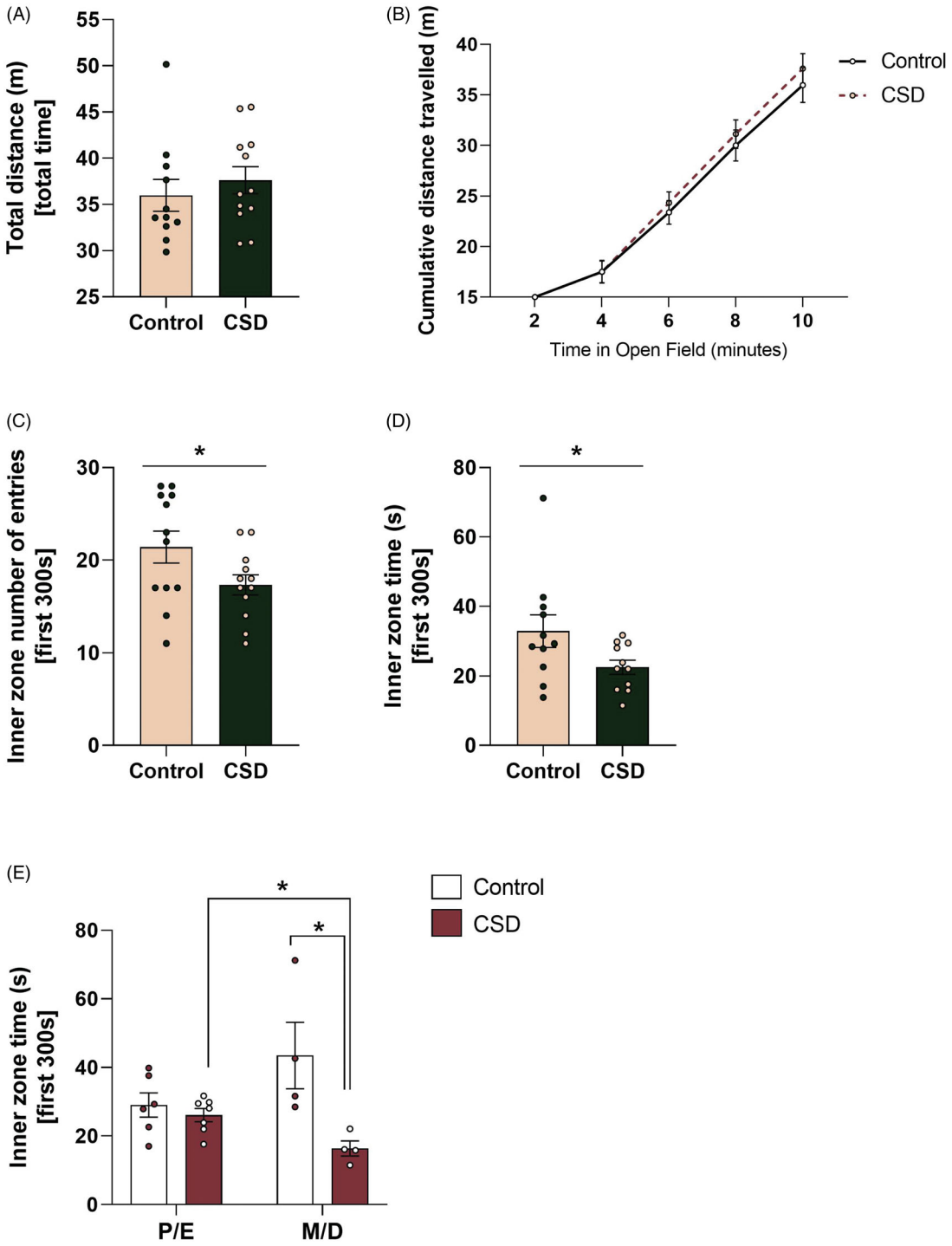

Figure 4. Chronic social defeat stress exposure increases anxiety-like behavior in female C57Bl/6n mice and this effect is most prominent in the metestrus/diestrus phase of the cycle. Chronic social defeat stress (CSDS) exposure does not affect (A) total distance travelled after 10 min in the Open Field (OF), nor could (B) any changes be observed during the course of the test between CSDS mice and controls. However, exposure to CSDS increases anxiety-like behavior in female C57Bl/6n mice as demonstrated by (C) a decreased number of entries into the inner zone of the OF and (D) a decreased amount of time spent in the inner zone of the OF. The effects on time spent in the inner zone of the OF (E) were most prominent in mice that were in the metestrus/diestrus cycle phase. CSD: chronic social defeat stress; P/E: proestrus/diestrus; M/D: metestrus/diestrus; data represent mean ± SEM. $^{*}p < 0.05$.

mice ($F_{(1,20)} = 0.077$, $p = 0.785$; Figure 4(A)). A cumulative representation of the distance travelled in the total area of the OF arena is illustrated in Figure 4(B), for which a repeated measures ANOVA revealed no differences between CSDS and control animals ($F_{(1,20)} = 0.085$, $p = 0.774$).

To assess anxiety-like behaviour in the OF, the first 5 min of OF exposure were analyzed. Here, the total number of entries into the inner zone of the OF ($F_{(1,20)} = 6.717$, $p < 0.05$) and the time spent in the inner zone of the OF ($F_{(1,18)} = 5.718$, $p < 0.05$) was reduced in CSDS mice vs. control mice (Figure 4(C,D)). In addition, a two-way ANOVA showed an condition × estrous cycle interaction effect for the parameter time spent in seconds in the inner zone of the OF during the first 300 s ($F_{(1,17)} = 7.110$, $p < 0.05$; Figure 4(E)).

Differences between CSDS and control mice for time in the inner OF zone were only present within mice that were in the M/D phase ($t_{(6)} = 2.719$, $p < 0.05$). Furthermore, within the CSDS group, animals within the M/D phase spent even less time in the inner zone of the OF than CSDS animals in the P/E phase ($t_{(9)} = 3.196$, $p < 0.05$). No effect for estrous cycle was found for number of entries into the inner zone of the OF ($F_{(1,19)} = 0.797$, $p = 0.383$).

Elevated plus maze

In contrast to data from the OF, no differences were found between CSDS and control mice for time spent ($F_{(1,22)} = 1.938$, $p = 0.178$), nor for distance travelled ($F_{(1,22)} = 0.818$, $p = 0.376$) on the open arms of the EPM. In addition, no estrous cycle effects were found for time spent or distance travelled on the open arms of the EPM (respectively $F_{(1,19)} = 0.536$, $p = 0.473$ and $F_{(1,19)} = 0.514$, $p = 0.482$).

Social avoidance

Data from the SA task revealed that socially defeated females spent less time in close proximity (time in social zone) of an inanimate object (empty cage) than control mice ($F_{(1,20)} = 4.575$, $p < 0.05$; Figure 5(A)). A two-way ANOVA showed a condition × estrous cycle interaction effect ($F_{(1,19)} = 3.030$, $p < 0.1$) and from post hoc tests it became clear that avoidance of an inanimate object is stronger in CSDS mice within the P/E phase and therefore the effect of CSD is mostly driven by mice in the P/E phase (Figure 5(B)). However, even though CSDS mice avoided an inanimate object, no differences were found for time spent in the social zone when an adolescent CD1 female mouse was placed in the cage ($F_{(1,19)} = 0.217$, $p = 0.647$).

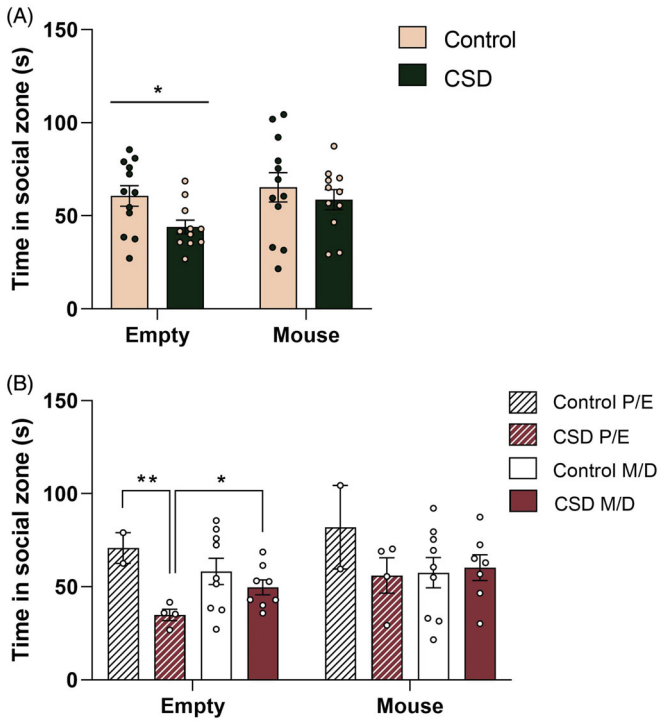

Figure 5. Chronic social defeat stress exposure leads to avoidance of an inanimate object, but not to social avoidance in female C57Bl/6n mice and this process is influenced by the cycle phase. Chronic social defeat stress (CSDS) exposure (A) leads to a decreased approach of an inanimate object, but did not affect the approach of a CD1 female adolescent mouse. The avoidance of an inanimate object (B) was stronger in CSDS mice within the proestrus/estrus (P/E) phase and therefore the effect of CSDS is mostly driven by mice in this P/E phase. CSD: chronic social defeat stress; P/E: proestrus/diestrus; M/D: metestrus/diestrus; data represent mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$.

Forced swim task

Finally, the FST was performed to investigate whether CSDS exposure leads to different coping strategy choices upon exposure to a stressful and unescapable environment. As these data were not normally distributed, a log transformation was first applied to the parameters time spent struggling, time spent swimming and time spent floating. An ANOVA with estrous cycle as a covariate subsequently revealed no differences between CSDS and control mice in either time spent struggling ($F_{(1,19)} = 0.777$, $p = 0.389$; Figure 6(A)), time spent swimming ($F_{(1,19)} = 0.230$, $p = 0.637$; Figure 6(B)) or time spent floating ($F_{(1,19)} = 2.097$, $p = 0.164$; Figure 6(C)) in seconds in the FST.

Correlations between physiological and neuroendocrine parameters

A Pearson's correlation revealed interesting correlations between a number of physiological and neuroendocrine parameters (Figure 7). Most interestingly, basal morning corticosterone levels at sacrifice and corticosterone recovery levels, 90 min after onset of the stressful FST, were positively correlated with body weight gain after 15 days of CSDS ($r = 0.593$, $n = 24$, $p < 0.01$; Figure 7(A)) and negatively correlated with actual body weight following 15 days of CSDS ($r = -0.512$, $n = 20$, $p < 0.05$; Figure 7(B)), respectively. Relative adrenal weight was positively correlated with body weight after 15 days of CSDS ($r = 0.535$, $n = 21$, $p < 0.05$; Figure 7(C)) and negatively correlated with corticosterone recovery levels, 90 min following onset of the FST ($r = -0.460$, $n = 21$, $p > 0.05$; Figure 7(D)).

Discussion

Over the past decades it has become more and more evident that male and female responses towards environmental stressors critically differ (Dalla et al., 2005; Hodes et al., 2015; Kessler, 1994; Westenbroek et al., 2004) and this has emphasized the importance of including both sexes within stress research. Here, we extend our knowledge on the detailed consequences of CSDS in females, showing that 21 days of CSDS in female C57Bl/6n mice induced many of the physiological, neuroendocrine and behavioral changes often described in males. At the same time, important differences in the response of females to CSDS in comparison to males were uncovered.

In male mice, CSDS commonly causes various physiological changes. Previous studies frequently reported body weight gain, deterioration of fur condition, adrenal glands enlargement and shrinkage of the thymus (Chuang et al., 2010; Hartmann et al., 2012; Wagner et al., 2011). Apart from

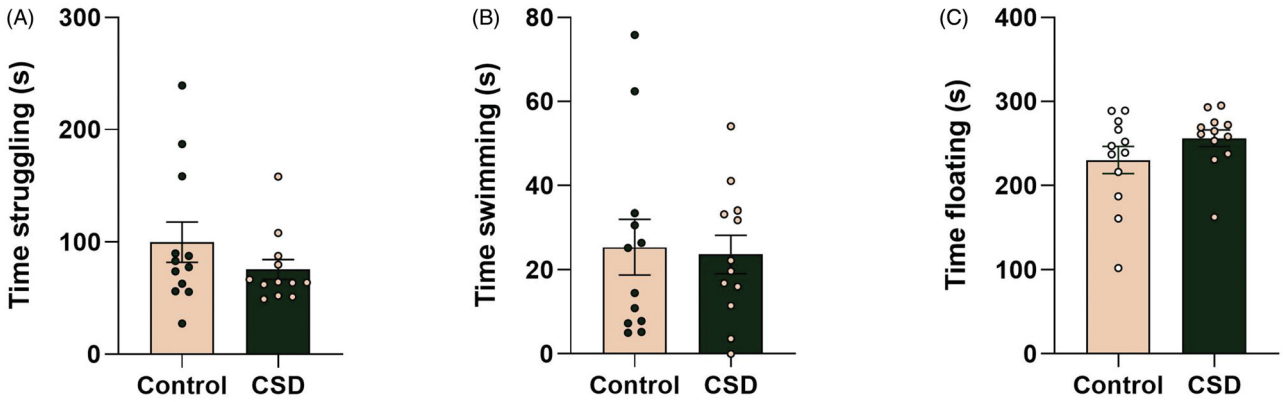

Figure 6. Chronic social defeat does not affect coping strategy selection in female C57Bl/6n mice. Chronic social defeat exposure does not significantly affect (A) time swimming, (B) time struggling or (C) floating behavior in the forced swim task (FST). CSD: chronic social defeat; data represent mean ± SEM.

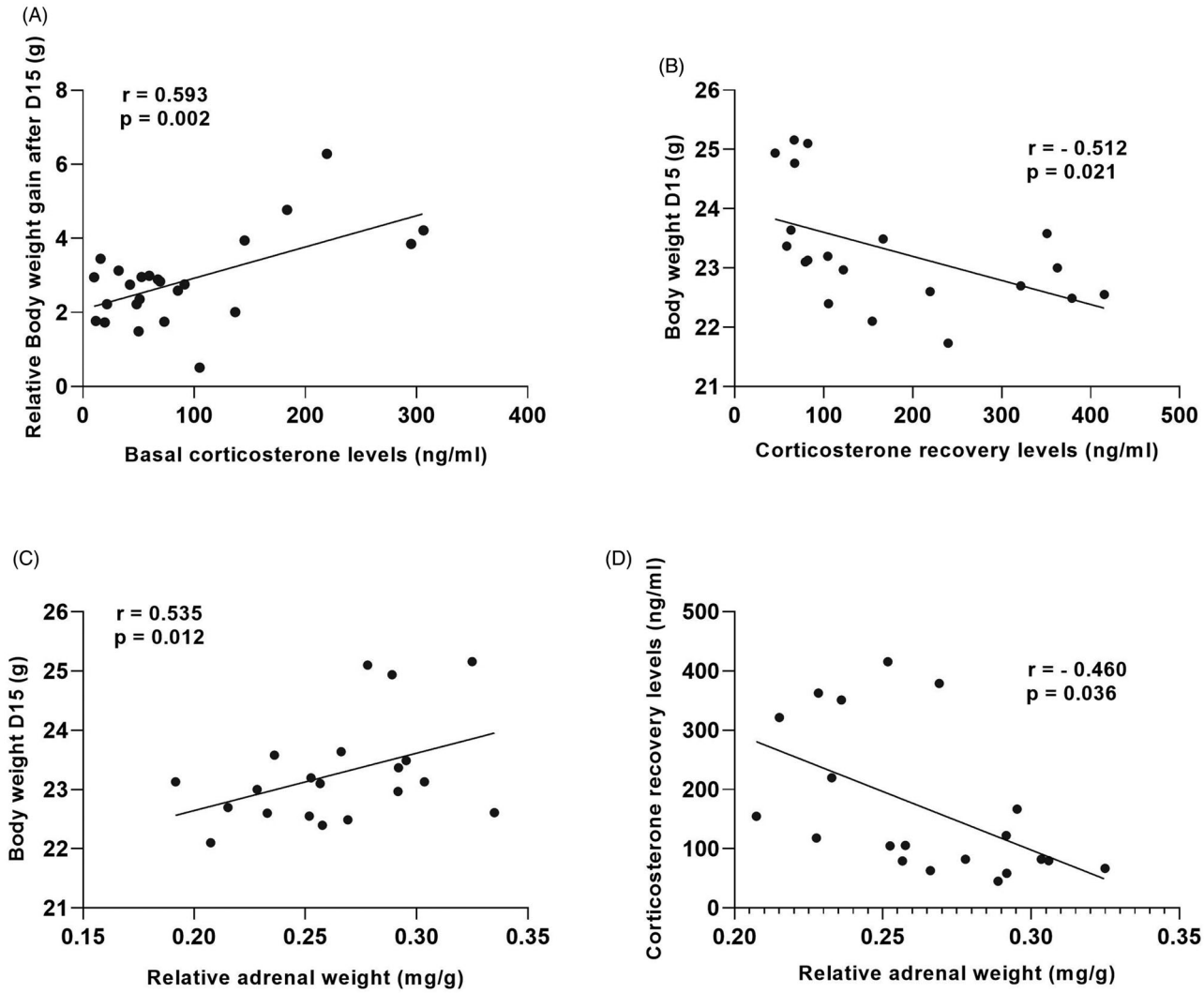

Figure 7. Physiological parameters of stress are correlated with each other. (A) Basal corticosterone levels at sacrifice and (B) corticosterone recovery levels, 90 min after onset of the stressor (forced swim task; FST) were respectively positively correlated with body weight gain after 15 days of chronic social defeat stress (CSDS) and negatively correlated with actual body weight following 15 days of CSDS. Relative adrenal weight was (C) positively correlated with body weight after 15 days of CSDS and (D) negatively correlated with corticosterone recovery levels, 90 min following onset of the FST. CSDS: chronic social defeat stress. Data represent mean ± SEM. r = Pearson's correlation, p = significance level.

an increase in adrenal weight in one study (Haller et al., 1999), most studies using an alternative method for CSDS in females remarkably do not indicate any of these classical physiological changes (Bourke & Neigh, 2012; Newman et al., 2019; Takahashi et al., 2017; Yohn et al., 2019; 2019). Harris et al. (2018) neither reported any alterations in physiological parameters as a result of their CSDS protocol. Unlike these earlier studies in females, we show that 21 consecutive days

of exposure to CSDS does indeed lead to the physiological changes that are generally found upon CSDS exposure in males, underlining that the stress procedure is effective and that the general physiological adaptations to CSDS are very similar in males and females.

In addition to physiological modifications, alterations in basal and stress-induced neuroendocrine profiles are commonly observed in males that underwent CSDS. As a result of chronic stress exposure, male mice often display elevated basal corticosterone levels as well as dysregulation of hypothalamic-pituitary-adrenal (HPA) axis negative feedback (Hartmann et al., 2012; Ron de Kloet et al., 2005; Schmidt et al., 2010; Sterlemann et al., 2008; Wagner et al., 2011). In contrast to studies in male mice, no changes in basal neuroendocrine profiles were found in previous chronic stress studies with females and also in our study we did not observe such changes. An explanation for this differential pattern could be that females have higher baseline corticosterone levels than male mice under non-stress conditions (Viau, 2002) and this may mask a further slight increase following chronic stress exposure. In the current study we did not observe the in males commonly detected overshoot of the corticosterone response to a novel stressor, which may in part be due to the large variability in the two groups. Interestingly, during the recovery phase, CSDS females displayed significantly reduced corticosterone levels, which may point towards an improved negative feedback regulation. However, other systems like the sympathetic nervous system or the immune system also affect corticosterone feedback regulation and further studies will need to address this phenotype mechanistically.

Adding to these findings, a number of physiological and neuroendocrine parameters of stress were correlated with each other. As previously mentioned, body weight gain can be an indicator of chronic stress exposure and increased basal corticosterone levels or HPA axis activity changes are often associated with vulnerability to stress (Schmidt et al., 2010). We found that animals with greater body weight gain during the experiment also had higher basal corticosterone levels. Conversely, body weight during the experiment was inversely correlated with HPA axis activity during the recovery phase of an acute stressor. Furthermore, higher adrenal weight – one of the classical indicators of a hyperactive HPA axis – was associated with increased body weight during the experiment and animals with larger adrenals also had lower HPA axis recovery activity. Altogether, these correlations further underline that the physiological and neuroendocrine responses to CSDS in females are reflective of a stressful state and that we observe individual variability in stress vulnerability and resilience.

Chronic stress also consistently results in a marked behavioral phenotype. Anxiety-like behaviors are often observed in both males and females following chronic stress exposure (Harris et al., 2018; Hartmann et al., 2012; Schmidt et al., 2007; Sterlemann et al., 2008; Takahashi et al., 2017; Wagner et al., 2011; Yohn et al., 2019; 2019). Here we found that CSDS mice display more anxious-like behavior in the OF test. Moreover, when tested during the SA task, female mice avoided an inanimate object in the open field, suggesting an aversion for novel environments or environmental changes. Interestingly, the estrous cycle phase also modulates anxiety-related behavior in females and therefore plays a significant role in the degree of stress-induced anxiety.

Another behavior that is typically affected following CSDS exposure is social behavior. A large number of studies has found a decrease in social interaction or increase in SA behavior following CSDS exposure and reduced social interaction is often presented as one of the key phenotypic features of vulnerability to chronic stress (Golden et al., 2011; Harris et al., 2018; Krishnan et al., 2007; Newman et al., 2019; Russo & Nestler, 2013; Takahashi et al., 2017; Yohn et al., 2019). Remarkably, we did not find such social behavioral changes in our study. One explanation for this could be the choice of the social target. Often a male CD1 mouse, with an identical appearance and age as the initial attacker, is used. However, we selected a female adolescent CD1 mouse as the social target to investigate the generalized nature of a potential SA behavior. We can therefore conclude from our data that in female mice, SA following CSDS is at least not generalized to mice from another sex or age as the initial attacker. It will therefore be important for future studies to differentiate SA behavior based on the sex and age of the tested conspecific.

Our study design was based on the CSDS protocol as described by Harris and colleagues (Harris et al., 2018), with only applying an adjustment to the length of the chronic defeat to 21 days. Even though we have reported findings that add to the stress phenotype as described by Harris et al. we have also found some discrepancies between the studies. First of all, Harris and colleagues report increased corticosterone levels, 30 min following the first defeat and 30 min following the defeat on day 10 of the experiment compared to the control condition. In our study, we did not find any changes in corticosterone levels at baseline, nor at 30 min following exposure to the forced swim test. Even though these results may look contrary at first sight, the environmental exposure and/or type of stressor were different in both studies and a direct comparison of the neuroendocrine results can therefore not be made. Moreover, in our study the stressor was applied to both the control and CSDS group, explicitly investigating differential stress reactivity, whereas Harris et al. studied the acute effect of a social defeat (with and without prior CSDS exposure) versus no stress exposure at all. On the behavioral level, one finding that we could not replicate was the presence of SA behavior following CSDS stress, as discussed above. Interestingly, Harris and colleagues further investigated the resiliency or vulnerability towards CSDS in their sample and subsequently stratified for this, which was not possible in our study due to insufficient statistical power. On a further note, the behavioral assessment in the current study was limited to a few tests and we would like to avoid an over-interpretation based on the current results. In fact, a lack of observed phenotype in a specific assay (e.g. the social avoidance task) does not mean that socially stressed females do not show alterations in social behavior. The current available data on the effects of social stress in female mice is – compared to the males – unfortunately still limited, again arguing for the inclusion of females

in future studies and a deeper and more thorough phenotyping.

Even though this study and the former report by Harris et al. show that increased male-to-female aggressive behavior, by introducing male odorants and pheromones, leads to a substantial stress phenotype in female mice, there are a few limitations to the study design. Firstly, the number of attacks towards female mice is less than the days of aggression reported in male studies (Golden et al., 2011). While all females were attacked regularly during the 21-day defeat period there were variations in attack duration and frequency, including individual days were no aggressive encounter occurred. Even though attack variations over the 21 days was similar for all experimental females, we still cannot rule out that variations in attack frequency across the CSDS procedure may have contributed to the variability in the behavioral and physiological outcomes. However, the presence of an overall robust stress phenotype may reduce this concern. Another concern may be that application of male odors and pheromones alone may affect behavior in female mice. In this study, the sole effect of urine application was not tested. However, control mice were also exposed to male odors on a weekly basis by introducing CD1 male bedding into their homecage. Furthermore, Harris et al. (2018) did include an additional control sample in their study and showed that urine application alone does not affect behavioral outcomes. A last important point of debate is whether a model utilizing male-to-female aggression can be considered etiologically relevant, as compared to a female-to-female aggression models (Logan, 2019; Newman et al., 2019). It is clear that rendering females to be perceived as males comes along with questions on how etiologically relevant the model is. However, the same is true for CSDS models that rely on female-to-female aggression, where a behavior is elicited that is not observed under (semi)naturalistic conditions and requires substantial experimental manipulation. In fact, also the male CSDS model is very artificial and adapted to a laboratory setting, as in natural mouse populations subordinate males will quickly leave the territory of an alpha male. We believe that having the availability of a simple paradigm that is reproducible across laboratories and simulates a chronic social stress situation in female mice under standard laboratory conditions is of high scientific value.

An important aim of this study was to further describe the differential responses of females to CSDS as compared to male mice. Even though the absence of data from a contemporary, non-historic male sample makes it harder to draw definite conclusions on potential sex differences, the establishment of the CSDS phenotype in males has been repeatedly reported (Golden et al., 2011; Hartmann et al., 2012; E. J. Nestler & Hyman, 2010; Sterlemann et al., 2008; Wagner et al., 2011), which allows for a more general comparison. When studying female cohorts, the estrous cycle and their corresponding estradiol levels may largely contribute to the variability in neuroendocrine and behavioral outcomes (Green et al., 2018; Shansky & Woolley, 2016) and in-depth analyses revealed that in our sample the estrous cycle indeed modulated the CSDS induced anxiety-like behavior. However, even though the biphasic estrous cycle assessment was based on predictive estradiol levels (usually high estradiol levels in proestrus/estrus cycle phase vs. the usually lower estradiol levels in the metestrus/diestrus phase; McLean et al., 2012), we did not quantify blood estradiol levels. Behavioral effects may therefore just as well be the result of alternative alternating hormone levels, such as progesterone. Moreover, chronic stress may influence the regularity of the female cycling. Even though, based on the observations in our sample there is no indication that in this particular protocol CSDS females stop cycling, the sample size of collected smears was insufficient to exclude more moderate effects of the stress exposure on the female cycle. Nevertheless, it should be clear that the study of the estrus cycle is not a prerequisite when including female cohorts and data of females without the information on cycle stage are equally valid. Many of the effects of CSDS are expected to occur in females independent of the estrous cycle, and this is supported by the data in this study. Therefore, the current model does not require the monitoring of the estrous cycle and the behavioral consequences of CSDS in females can also be studied without taking the cycle of the females in to account.

Taken together, this study provides further insights in the typical physiological, neuroendocrine and behavioral responses of female mice to CSDS. The results pave the way for a direct comparison of the responses of both males and females to chronic social stress and will ultimately allow the sex-specific study of mechanisms underlying individual stress resilience.

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Author contributions

LvD and MVS conceived the study. LvD, HY, JB, LB, CE and FT performed the study. LvD analyzed the data and wrote the first draft of the manuscript. All authors contributed to the revision of the manuscript.

Disclosure statement

The authors declare no conflict of interest.

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2.2 Sex-Specific and Opposed Effects of FKBP51 in Glutamatergic and GABAergic Neurons: Implications for Stress Susceptibility and Resilience

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Sex-specific and opposed effects of FKBP51 in glutamatergic and GABAergic neurons: Implications for stress susceptibility and resilience

Lotte van Doeselaar[a,b], Tibor Stark[c], Shiladitya Mitra[a], Huanqing Yang[a], Joeri Bordes[a], Linda Stolwijk[a], Clara Engelhardt[a], Veronika Kovarova[a,b], Sowmya Narayan[a,b], Lea M. Brix[a,b], Margherita Springer[a], Jan M. Deussing[d], Juan Pablo Lopez[e], Michael Czisch[c], and Mathias V. Schmidt[a,1]

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Mental health disorders often arise as a combination of environmental and genetic factors. The *FKBP5* gene, encoding the GR co-chaperone FKBP51, has been uncovered as a key genetic risk factor for stress-related illness. However, the exact cell type and region-specific mechanisms by which FKBP51 contributes to stress resilience or susceptibility processes remain to be unravelled. FKBP51 functionality is known to interact with the environmental risk factors age and sex, but so far data on behavioral, structural, and molecular consequences of these interactions are still largely unknown. Here we report the cell type- and sex-specific contribution of FKBP51 to stress susceptibility and resilience mechanisms under the high-risk environmental conditions of an older age, by using two conditional knockout models within glutamatergic ($Fkbp5^{Nex}$) and GABAergic ($Fkbp5^{Dlx}$) neurons of the forebrain. Specific manipulation of Fkbp51 in these two cell types led to opposing effects on behavior, brain structure and gene expression profiles in a highly sex-dependent fashion. The results emphasize the role of FKBP51 as a key player in stress-related illness and the need for more targeted and sex-specific treatment strategies.

FKBP51 | sex differences | aging | stress-related disorders | behavior

To cope well with the physical and psychological stressors that we are exposed to throughout our life span, an adequate response to stress is required. Insufficient coping may result in the development of stress-related disorders, such as depressive or anxiety disorders (1–4), which are one of the most pressing and costly burdens of modern society (5–8). In the past decades, it has become evident that mental health problems often arise as a combination of environmental and genetic factors (9–12) and genome wide association studies identified risk genes that play a role in psychiatric disorders (13–15). One gene that has been uncovered as a key genetic risk factor for stress-related illness is *FKBP5* (16). As a co-chaperone to the glucocorticoid receptor (GR) directly affecting its sensitivity to circulating glucocorticoids (17), a central function of the encoded FKBP51 protein is the regulation of stress system activity (18–21). Emphasizing this central role, single nucleotide polymorphisms (SNPs) in the *FKBP5* gene modulate the risk to psychiatric disease development in interaction with (early) environmental stress exposure (13, 22, 23). Moreover, pharmacological modulation of FKBP51 or genetic manipulation of *Fkbp5* in rodents has already demonstrated its implications in stress resilience mechanisms (19, 20, 24–27). However, the contribution of FKBP51 to stress resilience processes may vary largely between different brain regions, or even so, between specific cell types, which has already been highlighted by previous work from our group and others (25–31).

Fkbp5 is expressed widely throughout the brain with especially high baseline expression levels in the hippocampus (32). The hippocampus is a brain region that is particularly sensitive to the effects of stress (33, 34), and it has been extensively implicated in the pathophysiology of major depressive disorder (MDD) (35–39). Glutamatergic neurons make up the vast majority of the highly divergent cell type profile of the hippocampus, which further comprises GABAergic interneurons, different glial and vascular cell populations (40, 41). The hippocampus is known to have a differential functionality along its longitudinal axis, in which the dorsal hippocampus (DHC) is mostly involved in (spatial) memory and learning processes, whereas the ventral hippocampus (VHC) is particularly involved in emotional regulation (42, 43). Furthermore, a region that has largely been associated with sustained fear and anxiety states is the bed nucleus of the striatum terminalis (BNST) (44, 45). In contrast to the hippocampus, the BNST is a structure that is particularly rich in GABAergic neurons (45) and a previous study from our group already demonstrated the presence of *Fkbp5* messenger RNA (mRNA) within these GABAergic neurons, specifically in the oval BNST (ovBNST) (27).

Significance

The *FKBP5* gene is a key genetic risk factor for stress-related disorders. The highly divergent symptomology of psychiatric disorders highlights that genetic risk factors like FKBP5 do not simply affect stress-coping mechanisms in a unilateral manner. Rather, we here demonstrate that FKBP5 affects cognitive and emotional behavior, brain structure, and brain region-specific gene expression profiles in a highly cell-type and sex-specific manner. Our results underline that labelling a stress—sensitive factor as FKBP5 as "risk factor" is too simplistic, as loss of FKBP5 can have opposite effects on brain and behavior, dependent on which cell type and sex the loss is taking place. The results are of high importance for the development of targeted intervention strategies for psychiatric disorders.

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[1]To whom correspondence may be addressed. Email: mschmidt@psych.mpg.de.

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Apart from genetic predispositions, there are also a number of other factors that contribute to the development of stress-related pathology. One of these contributing factors is age. Elderly people that suffer from MDD have increased treatment resistance and depressive symptoms worsen over the years, which is a predictor for deteriorating disability amongst the elderly population (46–48). Interestingly, with age, FKBP51 imposes a higher risk on developing stress-related illness. Data from both human and rodent studies demonstrated that epigenetic mechanisms cause an age-dependent elevated *Fkbp5* induction (49–52), resulting in augmented intracellular FKBP51 levels, similar to what has been observed in individuals carrying the *FKBP5* psychiatric risk allele (23). Adding to this, knockout (KO) of *Fkbp5* in mice had an accumulating antidepressant effect across the lifespan (50). In addition to age, sex heavily impacts stress vulnerability and its associated diseases. Stress-related disorders such as MDD or anxiety disorders are about twice as common in women as in men (53–56). Although research from the past decades increasingly shed a light on the highly sex-dependent stress coping mechanisms (57–64), research in female subjects is still largely underrepresented. Even more so, data on the interaction between sex, age, and genetic risk factors in the context of stress-related disorders is extremely limited.

To tackle this scarcity in information, this study aimed to investigate the region, cell type- and sex-specific contribution of FKBP51 to stress vulnerability and resilience mechanisms under the high-risk environmental conditions of an older age. Using two different conditional KO models, that lack FKBP51 in either glutamatergic or GABAergic neurons of the forebrain, we demonstrated that this psychiatric risk factor affects behavior, brain structure and gene expression in a highly sex-dependent and cell type-divergent manner.

Results

Validation of *Fkbp5* KO in Glutamatergic and GABAergic Neurons of the Forebrain and its Physiological Consequences in Male and Female Older Aged Mice. $Fkbp5^{lox/lox}$ mice were bred with *Nex-Cre* mice in order to achieve KO of *Fkbp5* in glutamatergic neurons of the forebrain (Fig. 1*A*) (65, 66) or bred with *Dlx5/6-Cre* mice to induce loss of FKBP51 in GABAergic forebrain neurons (Fig. 1*B*) (67). Two separate RNAscope experiments were performed for *Fkbp5,* with well-known markers of glutamatergic and GABAergic neurons (*Vglut1* and *Gad1*), to validate the successful KO of *Fkbp5* mRNA in the cortex and DHC of glutamatergic and GABAergic neurons, respectively. The quantification of the RNAscope confocal images showed a significant reduction in the percentage of *Fkbp5* positive cells within *Vglut1* positive cells in $Fkbp5^{Nex}$ mice compared to $Fkbp5^{lox/lox}$ controls in cortical regions [$t(4) = 4.11$, $P < 0.05$; Fig. 1*A*]. *Fkbp5* positive cells within *Gad1* positive cells in $Fkbp5^{Dlx}$ mice and $Fkbp5^{lox/lox}$ controls revealed a significant reduction of *Fkbp5* within GABAergic neurons of the DHC [$t(6) = 7.07$, $P < 0.001$; Fig. 1*B*]. As the RNAscope *Fkbp5* probe targets the whole *Fkbp5* mRNA and not only the deleted exon 9, the residual *Fkbp5* signals in both lines in the targeted cell populations likely detect truncated mRNA that does not result in a functional FKBP51 protein. Representative confocal images of the dorsal CA1 of $Fkbp5^{lox/lox}$ and $Fkbp5^{Nex}$ mice and of the DHC for $Fkbp5^{lox/lox}$ and $Fkbp5^{Dlx}$ mice are illustrated (Fig. 1 *A* and *B*). *Fkbp5* mRNA levels in off-target cell types for $Fkbp5^{Nex}$ and $Fkbp5^{Dlx}$ mice did not differ from $Fkbp5^{lox/lox}$ mice [$Fkbp5^{Nex}$: $t(4) = 1.0$, $P > 0.05$; $Fkbp5^{Dlx}$: $t(4) = 0.173$, $P > 0.05$; *SI Appendix*, Fig. S1].

We then further investigated the functional, structural, and molecular consequences of the loss of *Fkbp5* in these two distinct neuronal cell populations in the context of sex and older age. To this end, cohorts of older aged male and female $Fkbp5^{Nex}$, $Fkbp5^{Dlx}$ and their wildtype (WT) $Fkbp5^{lox/lox}$ litter mates were generated and tested on different modalities (experimental timeline in Fig. 1*C*). Interestingly, physiological features were afflicted in a sex- or in a FKBP51-manipulated cell type-dependent manner. Body weight at baseline was only affected in female mice, in which $Fkbp5^{Nex}$ mice [$F_{(1,\ 18)} = 3.393$, $P = 0.082$] and $Fkbp5^{Dlx}$ mice [$t(25) = -2.01$, $P = 0.055$] showed a trend towards an increased body weight, as compared to their $Fkbp5^{lox/lox}$ controls (Fig. 1*D*). However, relative adrenal weight was affected in a FKBP51-manipulated cell type-specific manner, where both $Fkbp5^{Nex}$ males [$F_{(1,\ 18)} = 4.24$, $P = 0.054$] and females [$F_{(1,\ 16)} = 4.86$, $P < 0.05$] had a reduced relative adrenal weight as compared to their WT control group. Adrenal weight of $Fkbp5^{Dlx}$ mice remained unaffected.

Loss of FKBP51 in Glutamatergic and GABAergic Neurons Leads to Differential and Sex-Dependent Effects on Behavior. In addition to the physiological consequences of loss of FKBP51, we explored the effects of reduced FKBP51 on several behavioral parameters. First, effects of loss of FKBP51 on general locomotor behavior were excluded as measured by the total distance travelled in the open field (OF) (Fig. 2*A*). Interestingly, we did observe a sex-specific effect on anxiety-like behavior, in which female mice were solely affected (Fig. 2*B*). Moreover, apart from the sex-specific nature of the effect, the directionality of the effect on anxiety behavior was also dependent on the cell-type in which loss of FKBP51 took place. Lack of FKBP51 led to opposing effects on anxiety-like behavior depending on the cell type involved. A reduction of FKBP51 in glutamatergic neurons induced an increased anxiety-like behavior as measured by open arm distance in the elevated plus maze (EPM) [$F_{(1,\ 18)} = 5.44$, $P < 0.05$], whereas mice that lack FKBP51 in GABAergic neurons showed an anxiolytic phenotype [$t(25) = -2.77$, $P < 0.05$].

Furthermore, cognitive behavior was assessed using the novel object recognition (NOR) and spatial object recognition (SOR) task, testing memory performance in a neutral environment, and using the conditioned context retrieval for memory performance under stressful conditions. The loss of FKBP51 did not lead to any changes on cognitive behavior in a neutral context (*SI Appendix*, Fig. S2). Remarkably though, again a sex- and cell type-specific effect was observed for cognitive functioning in a stressful environment. In contrast to anxiety-like behaviors, changes in memory performance could only be observed in male mice (Fig. 2*C*). $Fkbp5^{Nex}$ male mice took a longer time to show freezing behavior in a familiar aversive environment than their $Fkbp5^{lox/lox}$ littermates, indicating a worsened memory of the aversive spatial context [$F_{(1,\ 17)} = 6.10$, $P < 0.05$]. $Fkbp5^{Dlx}$ mice, on the other hand, displayed a more rapid freezing response as compared to their WT controls upon exposure to the aversive context [$F_{(1,\ 22)} = 20.97$, $P < 0.01$], indicating an enhanced memory of the aversive location. To further emphasize the sex-dependent nature of the effects on physiological and behavioral parameters, additional information on main effects of sex and sex × genotype interactions can be found in *SI Appendix*, Table S1.

Loss of FKBP51 in Glutamatergic and GABAergic Neurons Leads to Pronounced Structural Brain Changes in $Fkbp5^{Dlx}$ Mice. To investigate whether the observed sex- and cell-type–dependent behavioral changes upon loss of FKBP51 in either glutamatergic or GABAergic neurons are accompanied by structural brain differences, mice underwent a MRI scan succeeding the behavioral protocol.

$Fkbp5^{Dlx}$ vs. $Fkbp5^{lox/lox}$. A two-way ANOVA of the deformation-based morphometry analyses revealed a main effect of genotype with

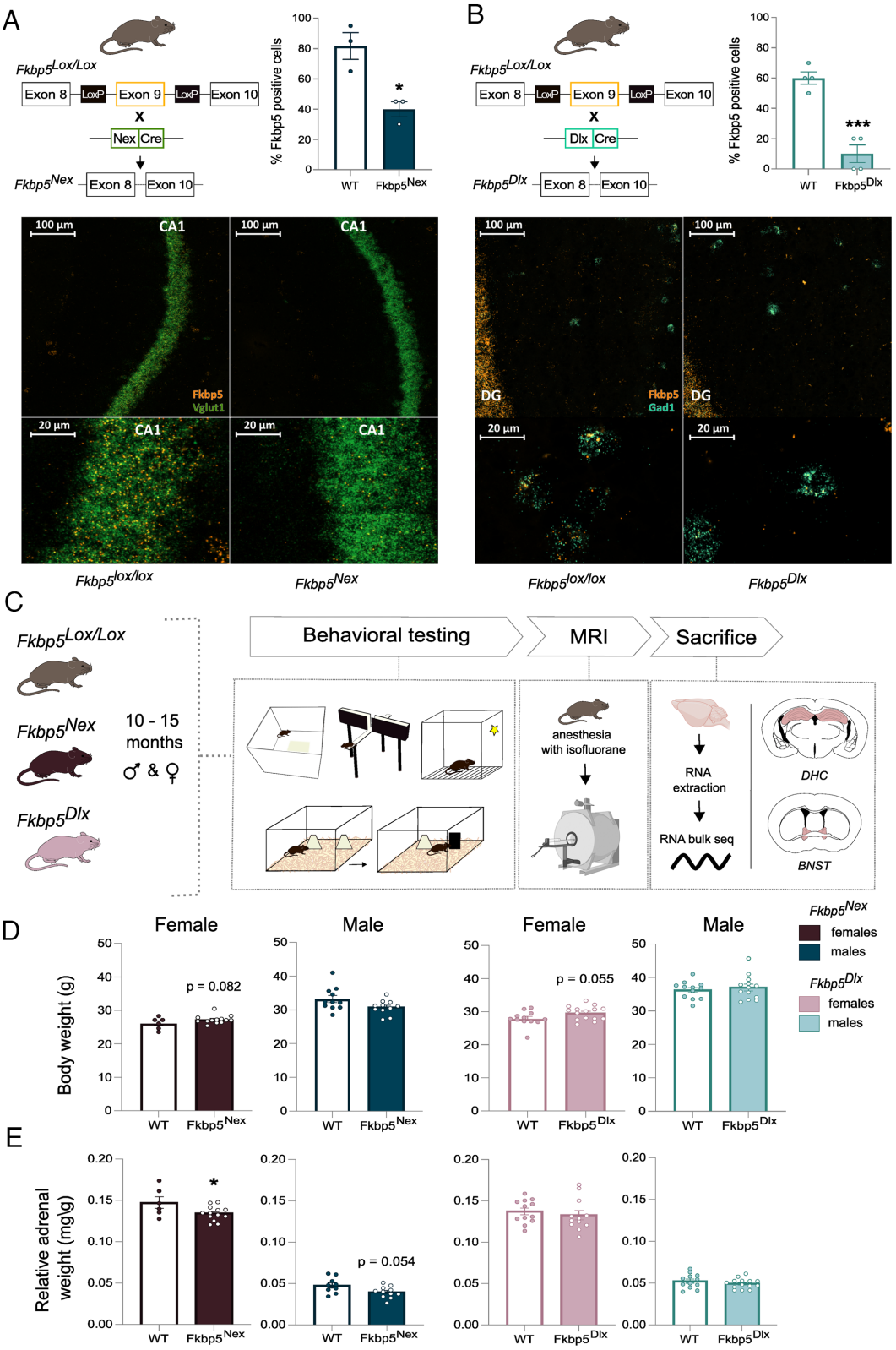

Fig. 1. Experimental setup and physiological measurements. By breeding *Fkbp5*$^{lox/lox}$ mice, in which exon 9 of the *Fkbp5* gene is flanked by two *loxP* sites, with (*A*) *Nex-Cre* mice, a significant reduction of *Fkbp5* mRNA in glutamatergic forebrain neurons was achieved. (*B*) Breeding of *Fkbp5*$^{lox/lox}$ mice with *Dlx5/6-Cre* mice resulted in a significant loss of *Fkbp5* mRNA in GABAergic forebrain neurons. RNA scope confocal images of the DHC illustrated these significant reductions of *Fkbp5* (orange panel) in glutamatergic CA1 neurons (*Vglut1*; green panel) and GABAergic interneurons (*Gad1*; blue panel). Ten- to fifteen-month–old *Fkbp5*^{Nex} and *Fkbp5*^{Dlx} mice of both sexes were exposed to a behavioral paradigm including tasks for anxiety, neutral and stressed cognition, followed by an MRI scanning procedure (C). After completion of the experiments, mice were sacrificed and tissue from the DHC and the BNST was collected for RNA bulk sequencing purposes. Analyses of physiological parameters revealed sex specific effects for body weight (D) and cell dependent changes in relative adrenal weight (E). Error bars represent mean ± SEM. **P* <0.05, ****P* < 0.001.

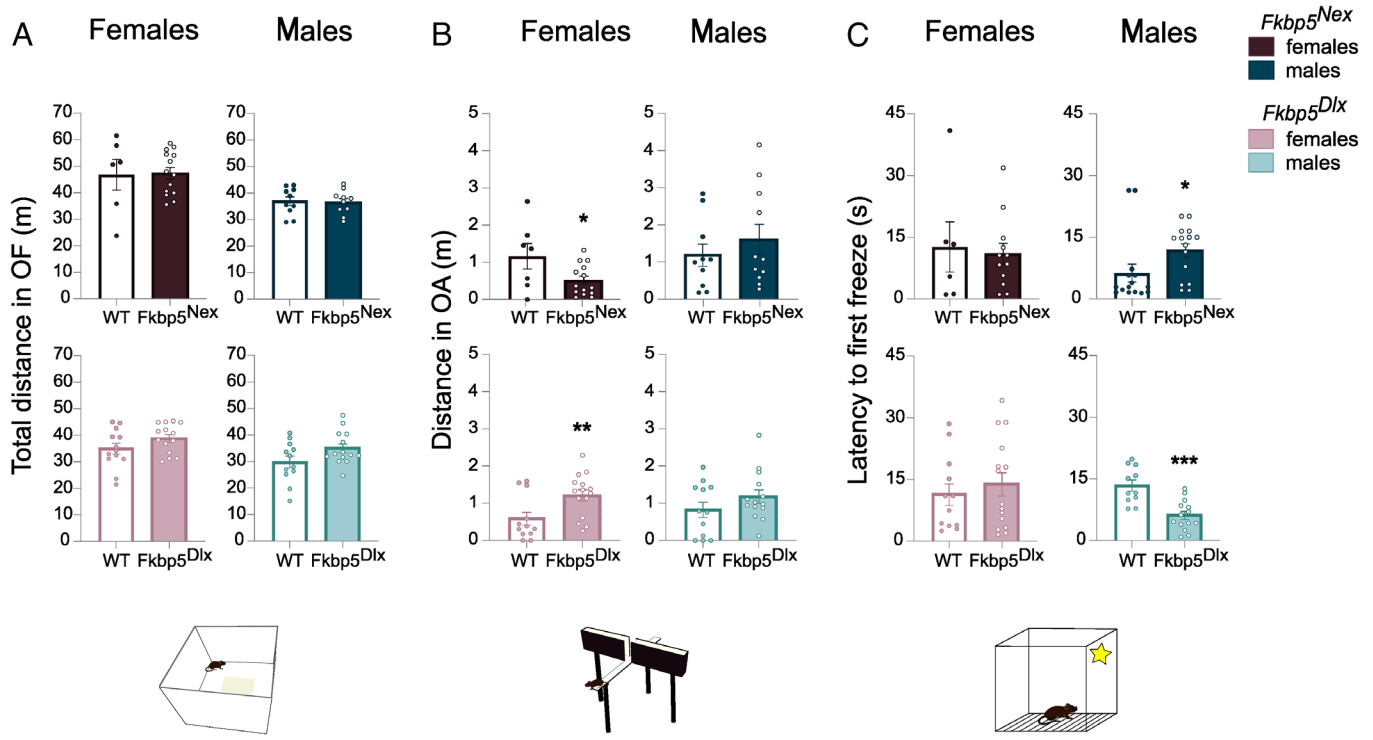

Fig. 2. Loss of *Fkbp5* leads to cell- and sex-specific effects on behavior that are unique to certain behavior domains. No significant changes in locomotion (A) as measured by a 15-min Open Field test could be observed as a result of loss of *Fkbp5* in older aged mice. However, loss of *Fkbp5* in glutamatergic neurons leads to (*B*) an anxiety-like phenotype on the EPM, where loss in GABAergic neurons leads to anxiolytic behavior. These effects on anxiety are solely observed in female mice. On the contrary, cognitive behavior under stressful conditions is mainly affected in older aged male mice (*C*). Where loss of *Fkbp5* in glutamatergic neurons reduces the memory of an adverse environment, a reduction in *Fkbp5* in GABAergic neurons enhances the memory of an aversive event. Error bars represent mean ± SEM.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

strongest volumetric differences in the caudoputamen, thalamus, and hippocampus, but was otherwise restricted to white matter (WM) and cerebrospinal fluid regions (*SI Appendix*, Fig. S3). Interaction effects (genotype × sex) revealed clusters in the right DHC, and in the left primary and supplemental somatosensory areas ($p_{FWE,cluster} < 0.005$, *SI Appendix*, Fig. S3). In addition, the left DHC and the right piriform area showed an interaction effect, although not surviving cluster FWE correction. These regions show higher volume differences in male mice ($Fkbp5^{Dlx} > Fkbp5^{lox/lox}$) than in female animals (*SI Appendix*, Fig. S3). The inverse interaction (female $Fkbp5^{Dlx} > Fkbp5^{lox/lox}$ larger than male $Fkbp5^{Dlx} > Fkbp5^{lox/lox}$) showed clusters in the left pretectal region ($p_{FWE,cluster} = 0.025$), and left caudoputamen, right pretectal region, right inferior colliculus and left ventral subiculum (no cluster correction, *SI Appendix*, Fig. S3). For male $Fkbp5^{Dlx}$ mice, post hoc analysis for genotype effects showed larger volumes in the right hippocampus ($p_{FWE,\ cluster} = 0.002$) compared to $Fkbp5^{lox/lox}$, with similar, though smaller, effects in the left hippocampus (Fig. 3). $Fkbp5^{Dlx}$ also had larger volumes in the hypothalamus; however, these clusters were only significant at an uncorrected threshold ($p_{uncorr} < 0.001$). Smaller volumetric differences at the border of the brain volume were not considered, as they most likely stem from individual differences during the digital brain extraction step. For the contrast $Fkbp5^{Dlx} < Fkbp5^{lox/lox}$, significant clusters were found in the right caudoputamen ($p_{FWE,cluster} = 0.029$) and the left lateral ventricle ($p_{FWE,cluster} = 0.029$; Fig. 3). Further underlying sex differences, brain volume was differentially affected by genotype in female mice as compared to male mice. For female mice, two clusters in the thalamus were found for the contrast $Fkbp5^{Dlx} > Fkbp5^{lox/lox}$ [$p_{FWE,cluster} < 0.001$ (left thalamus) and $p_{FWE,cluster} = 0.07$ (right thalamus)]. Furthermore, a strongly significant grey matter (GM) cluster in the somatosensory cortex, claustrum and left auditory cortex was found for the cluster $Fkbp5^{Dlx} < Fkbp5^{lox/lox}$ ($p_{FEW,cluster} < 0.001$). In addition, several $Fkbp5^{Dlx} > Fkbp5^{lox/lox}$ clusters in the WM (e.g., corpus callosum, anterior commissure) and clusters in GM areas surrounding the BNST and the nucleus accumbens regions occurred, although these did not survive clustfewFWE correction (Fig. 3).

$Fkbp5^{Nex}$ vs. $Fkbp5^{lox/lox}$. Main effect of genotype showed nominal volumetric differences in the left caudoputamen, bilateral regions of the thalamus-related to both the polymodal association cortex and to the sensory motor cortex, and in retrosplenial and visual cortical areas, however, not survfewng FWE correction (*SI Appendix*, Fig. S3). No significant interaction clusters were revealed. In post hoc analyses for genotype, male mice showed larger volumes for $Fkbp5^{Nex} > Fkbp5^{lox/lox}$ in the left caudoputamen, right piriform area, bilateral dorsal thalamus and left ventral subiculum (uncorrected, Fig. 3). For $Fkbp5^{Nex} < Fkbp5^{lox/lox}$, we observed a cluster in the bilateral motor cortex extending to the anterior cingulate area ($p_{FWE,cluster} = 0.029$) and right barrel field (uncorrected). Again, sex-specific effects on brain volume were demonstrated. Female $Fkbp5^{Nex}$ mice showed a cluster in the right anteriormedial visual area for the contrast $Fkbp5^{Nex} < Fkbp5^{lox/lox}$ (trend: $p_{FWE,cluster} < 0.072$), as well as a nonsignificant cluster in the left dorsal thalamus and left anteriormedial visual area (Fig. 3).

Loss of FKBP51 in GABAergic Neurons Leads to Most Prominent Molecular Changes in the BNST of Females and in the DHC of Males. To elaborate further on the molecular pathways that may underly the sex- and FKBP51-manipulated cell type-dependent functional and structural changes that were observed as a result of loss of FKBP51, RNA was extracted from two brain regions: the BNST and the DHC. The BNST is known to be involved in anxiety disorders (44, 45) and as behavioral analyses indicated opposing

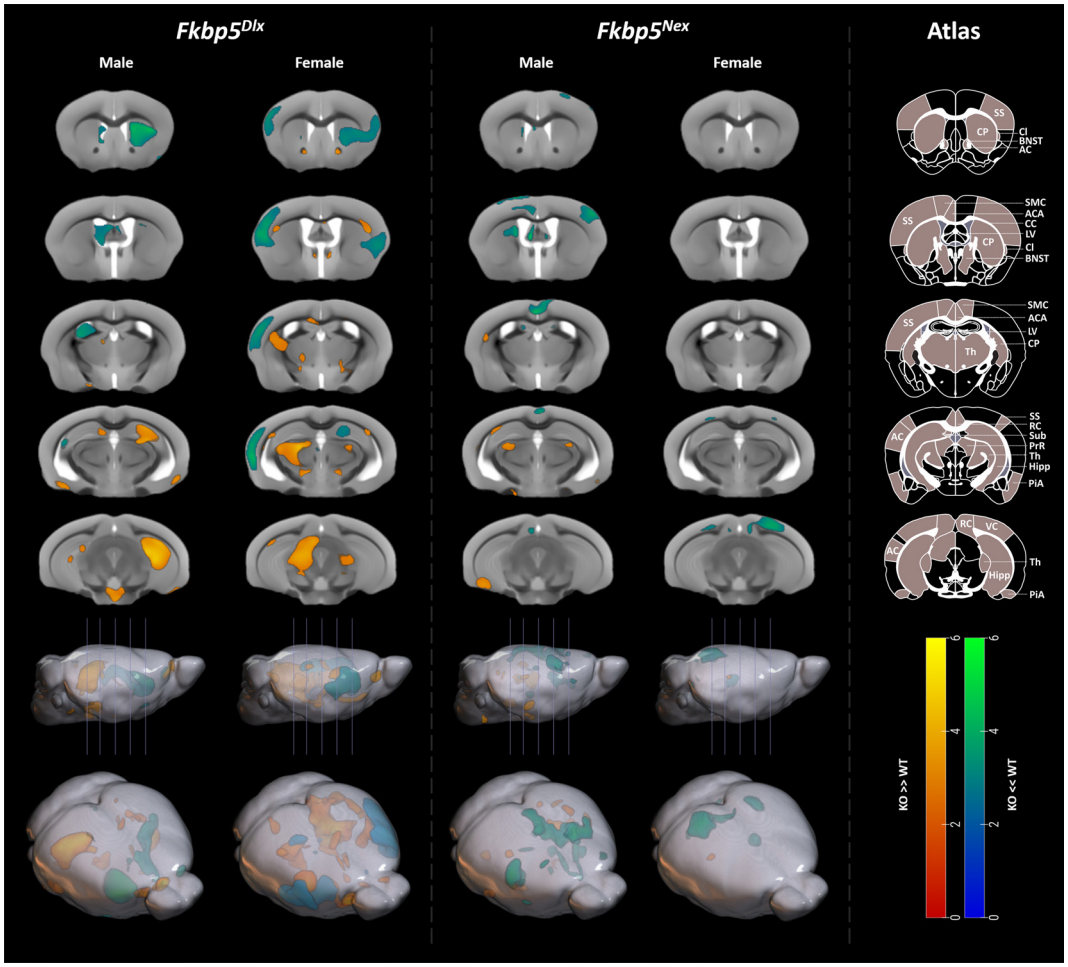

Fig. 3. Loss of *Fkbp5* leads to the largest structural changes when restricted to GABAergic neurons of the forebrain. Data from the T2*-weighted MRI scan revealed underlying structural consequences of loss of *Fkbp5* in either glutamatergic or GABAergic neurons of the forebrain, with the largest and most significant changes in $Fkbp5^{Dlx}$ male and female mice. For $Fkbp5^{Dlx}$ male mice, large and strongly significant increases in GM volume were found in the bilateral hippocampus compared to $Fkbp5^{lox/lox}$ controls, whereas GM volumes were significantly decreased in the caudoputamen and lateral ventricle. Female $Fkbp5^{Dlx}$ mice had larger GM volumes in the thalamus than their $Fkbp5^{lox/lox}$ controls and strongly significant smaller GM volumes in the somatosensory cortex, claustrum, and auditory cortex. In addition, a number of WM structures were altered such as the corpus callosum and some GM areas around the BNST, although these clusters did not survive FWE correction. Furthermore, $Fkbp5^{Nex}$ male mice had significantly smaller GM volumes in bilateral regions of the motor cortex extending to the anterior cingulate area vs. $Fkbp5^{lox/lox}$ controls and uncorrected increased GM volumes were found in the left caudoputamen, right piriform area, bilateral dorsal thalamus and left ventral subiculum. Female $Fkbp5^{Nex}$ mice had decreased GM volumes in the right anteriormedial visual area. Scales represent Z-scores. Yellow-red scale: KO > WT, Greenblue scale: KO < WT. AC = Auditory Cortex, ACA = Anterior Cingulate Area, CC = Corpus Callosum, Cl = Claustrum, CP = Caudoputamen, Hipp = Hippocampus, LV = Lateral Ventricle, PiA = Piriform Area, PrR = Pretectal region, RC = Retrosplenial Cortex, SMC = Sensorimotor cortex, SS = Somatosensory Cortex, Sub = Subiculum, Th = Thalamus, VC = Visual Cortex.

changes in anxiety-like behavior for $Fkbp5^{Nex}$ and $Fkbp5^{Dlx}$ female mice and the structural analyses revealed deformations around the BNST in female $Fkbp5^{Dlx}$ mice, this region was selected to explore underlying molecular changes. In addition, loss of FKBP51 affected spatial memory performance in an aversive context, exclusively in male mice. Since the DHC is known to be the major brain region involved in spatial memory formation and the MRI analyses indicated large volumetric increases in male $Fkbp5^{Dlx}$ vs. $Fkbp5^{lox/lox}$ mice, it was chosen as the second region of interest. Following the RNA extraction, BNST and DHC samples of all conditions were sequenced. Bioinformatic analyses of the sequencing data revealed a differential expression pattern for each of the eight comparisons. However, only a selection of these differentially expressed genes (DEGs) survived additional statistical correction (Table 1). In line with the structural changes mentioned above and once more emphasizing the sex and cell-type dependency, the most profound significant DEG profiles were found within $Fkbp5^{Dlx}$ (vs. $Fkbp5^{lox/lox}$) female mice in the BNST area and in $Fkbp5^{Dlx}$ (vs. $Fkbp5^{lox/lox}$) male mice within the DHC (Fig. 4*A*). Male and female $Fkbp5^{Dlx}$ mice had respectively a 17-fold larger (52 vs. 3 significant DEGs) and 30-fold larger (91 vs. 3 significant DEGs) differential transcriptional profile than $Fkbp5^{Nex}$ mice of matching sex in the same region. Gene expression profiles also were unique for $Fkbp5^{Dlx}$ and $Fkbp5^{Nex}$ mice as for females in the BNST there was only a 3% overlap of DEGs and for male mice in the DHC a 1.5% overlap of differentially regulated transcripts was found (Fig. 4*B*). $Fkbp5^{Dlx}$ female mice had a regulatory transcriptional signature in the BNST with 70% upregulated genes and 30% downregulated genes, whereas $Fkbp5^{Dlx}$ male mice had 83% upregulated and only 17% downregulated transcripts. This was reflected in the top 12 most significant DEGs which contained both up- and downregulated genes for female $Fkbp5^{Dlx}$ mice, but for male $Fkbp5^{Dlx}$ mice only consisted of downregulated genes (Fig. 4*C*).

To further explore the underlying pathways related to the transcriptional profiles that were found in the BNST of $Fkbp5^{Dlx}$ female and the DHC of male $Fkbp5^{Dlx}$ mice, a gene ontology (GO) enrichment analysis was performed on all DEGs. In Fig. 4*D*, a dot bar illustrates the most significantly enriched GO terms for biological processes, cellular components, and molecular functions for each

Table 1. Differential expression profile for all brain regions, sexes and genetic conditions

Brain region	Sex	Comparison	No. of ↑ genes corrected (Uncorrected)	No. of ↓ genes corrected (Uncorrected)
BNST	Female	*Fkbp5*^{Dlx} vs. *Fkbp5*$^{lox/lox}$	**64** (629)	**27** (515)
		Fkbp5^{Nex} vs. *Fkbp5*$^{lox/lox}$	2 (404)	1 (407)
	Male	*Fkbp5*^{Dlx} vs. *Fkbp5*$^{lox/lox}$	0 (436)	0 (448)
		Fkbp5^{Nex} vs. *Fkbp5*$^{lox/lox}$	0 (159)	0 (119)
DHC	Female	*Fkbp5*^{Dlx} vs. *Fkbp5*$^{lox/lox}$	1 (202)	0 (316)
		Fkbp5^{Nex} vs. *Fkbp5*$^{lox/lox}$	2 (591)	2 (589)
	Male	*Fkbp5*^{Dlx} vs. *Fkbp5*$^{lox/lox}$	**43** (1,024)	**9** (650)
		Fkbp5^{Nex} vs. *Fkbp5*$^{lox/lox}$	2 (310)	1 (326)

Notes: ↑ = upregulated; ↓ = downregulated; corrected = p adjusted < 0.02; uncorrected = p < 0.05

of the transcriptional profiles of the *Fkbp5*^{Dlx} male and female mice. This GO enrichment analyses revealed that the BNST transcriptional signature for *Fkbp5*^{Dlx} females is associated with immune-related functions [leukocyte migration, leukocyte-mediated immunity, myeloid leukocyte activation, and mechanisms of phagocytosis, including (plasma) membrane invagination] but also with blood pressure regulation and sex differentiation. Moreover, these functions were reflected in the top DEGs, as a number of these genes are known to modulate immune function (*S100a13*, *Isoc1, Gpr174*, *Wnt4*, *Ildr2*, *Cck*; Fig. 4*C*) (68–73). Pathways that were enriched in the DHC DEG profiles for *Fkbp5*^{Dlx} males on the other hand were predominantly related to cell metabolic processes and mitochondrial and ribosomal structural and functional mechanism. Significant DEGs in the DHC *Fkbp5*^{Dlx} male sample are implicated in age-related cognitive impairments (*Gadd45g*, *Smo*) (74) or play a role in memory and learning (*Asic4*) (75) or pre-synaptic and autophagic alterations (*Sipa1l2*) (76).

Within our genetically manipulated mouse lines, loss of FKBP51 was exclusively present in either gamma-aminobutyric acid (GABA) or glutamatergic neurons of the forebrain. However, lack of FKBP51 in a select population of neurons may also alter properties of other cell types in the same brain region. In order to further identify cell types that might be additionally affected by the selective loss of FKBP51, we examined in which cell types the significant DEGs of our samples are enriched. For this, we made use of an at-hand previously obtained hippocampus single-cell RNA sequencing dataset (77). Interestingly, we found that significant DEGs in the *Fkbp5*^{Dlx} female BNST are generally also highly expressed in microglia (Fig. 4*E*). This finding is in line with the associated immune function-related pathways that we observed from the GO enrichment analyses and top significant DEGs. In addition to microglia, groups of significant DEGs of the female *Fkbp5*^{Dlx} BNST also had a relative high expression in neural progenitor cells, glutamatergic cells, astrocytes, oligodendrocyte precursor cells (OPCs), committed OPCs, mature oligodendrocyte, macrophages, ependymal cells, meningeal and vascular cells. These findings indicate that even though *Fkbp5* is only lacking in the GABAergic neurons of the forebrain of female mice it may also affect other cell types of the brain, in particular microglia, and could thereby lead to neuroimmune function-related alterations. For the DHC of *Fkbp5*^{Dlx} male mice we found that the significant DEGs are also generally expressed in cell types such as dentate gyrus and mossy glutamatergic neurons, ependymal, meningeal, choroid plexus, and vascular cell types (Fig. 4*E*).

Discussion

Since the discovery of *FKBP5* as a key genetic risk factor for psychiatric disease (13), a vast amount of work has been put in trying to unravel the exact mechanisms by which it contributes to stress susceptibility and resilience (18–20, 22–27, 31, 32, 49, 50). Part of this work has already highlighted that FKBP51 differentially implements its functions based on the cell type it is expressed in. Moreover, previous studies have shown that sex and age, two strongly determining risk factors for psychiatric illness, can influence FKBP51 functionality. However, there is still a gap in information on the contribution of FKBP51 in specifically glutamatergic or GABAergic neurons to stress coping behavior and how it interacts with sex and age. With this study we have gained more insight in this by demonstrating that the glutamatergic or GABAergic loss of FKBP51 in the forebrain of older aged animals opposingly affects behavior, brain structure and gene expression profiles in a highly sex-dependent fashion.

In line with results from earlier studies, we found that loss of FKBP51 resulted in changes in emotional regulation and cognitive functioning in a stressful environment. Previous studies demonstrated that systemic FKBP51 inhibition with the selective inhibitor SAFit2(25) and manipulation of FKBP51 in the ovBNST affected anxiety-like behavior (27). Other studies have also emphasized the role of FKBP51 in cognitive functioning. For example, overexpression of *Fkbp5* resulted in diminished reversal learning in the Morris Water Maze (30) and led to a reduction in neuronal numbers in the hippocampus (31). Moreover, in humans, increased FKBP51 levels were associated with Alzheimer's disease progression, a neurological disorder that is well known for its devastating progressive memory decline(49). One notable finding in our study was that loss of FKBP51 affected memory function under stressful conditions, but not in a neutral environment. An extensive amount of literature has already described the versatile relationship between stress and memory and learning (78, 79), and as a stress-responsive gene, it is therefore not surprising that our results accentuate the context-dependent nature of FKBP51 actions. Apart from the effects on behavior, loss of FKBP51-induced changes in brain structure. Deformations were found in GM volumes in limbic structures, areas of the cortex and basal ganglia and in a number of WM structures. Structural brain changes have been demonstrated before in relation to FKBP51. A previous study involving *Fkbp5*$^{-/-}$ mice exposed volumetric changes in limbic, periaqueductal grey and dorsal raphe nuclei regions as a result of full body loss of FKBP51, but, in line with our findings, also detected changes in WM structures such as the anterior commissure (80). In our study, the thalamus was also affected in both *Fkbp5*^{Dlx} and *Fkbp5*^{Nex} mice. Interestingly, an MRI study in humans revealed that individuals with decreased methylation at intron 7 of the *FKBP5* gene, associated with increased FKBP51 levels (81), have larger right thalamus volumes (82). Moreover, carriers of the *FKBP5* SNP risk allele rs13060780 had reduced thalamic GM volumes when they are growing up in a positive parenting environment (83). The thalamus is an important integrator of sensory inputs and behaviors and it has previously shown to be sensitive to the effects of stress (84, 85). Although the exact

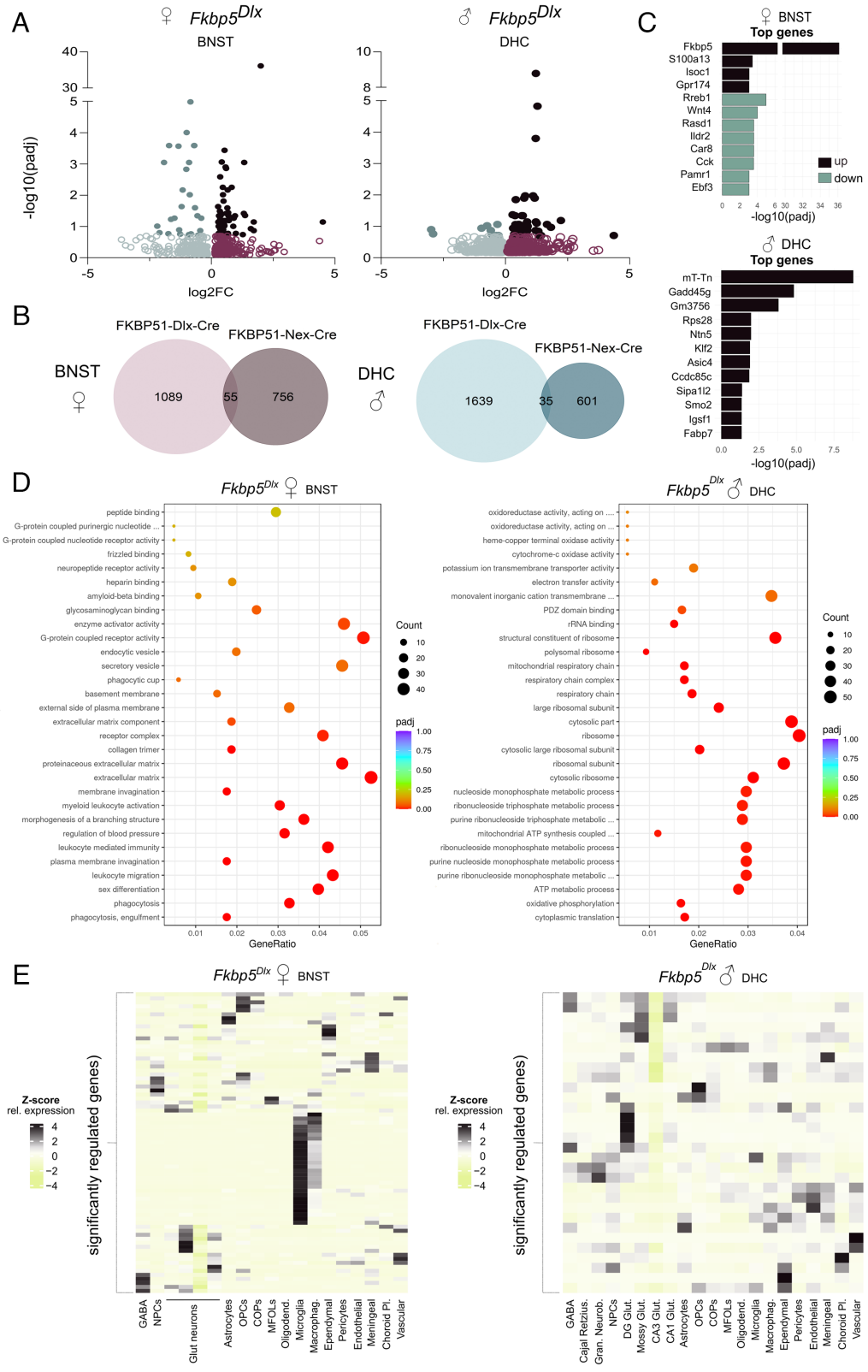

Fig. 4. Loss of *Fkbp5* leads to cell-specific and sex-dependent molecular profiles that are highly region distinctive. (*A*) A large differential expression profile could be found within the BNST of female *Fkbp5*^{Dlx} mice and the DHC of male *Fkbp5*^{Dlx} older aged mice. (*B*) These regulated transcriptional profiles were unique for *Fkbp5*^{Dlx} vs. *Fkbp5*^{Nex} mice. (*C*) Within the BNST of *Fkbp5*^{Dlx} female mice, the top significantly DEGs consisted of up- and downregulated genes, whereas the top DEGs for the DHC of *Fkbp5*^{Dlx} male mice only included downregulated genes. (*D*) GO-enrichment analyses revealed enriched biological processes, cellular components and molecular functions in samples of our two separate transcription profiles. For the BNST of *Fkbp5*^{Dlx} female mice, regulated genes were particularly related to immune functions. Regulated genes of the DHC of *Fkbp5*^{Dlx} male mice were on the other hand predominantly associated with metabolic processes and ribosomal structural and functional mechanisms. Using a VHC single-cell sequencing dataset (*E*), we found that significant DEGs in the BNST of *Fkbp5*^{Dlx} female mice are highly enriched in microglia.

mechanisms by which *Fkbp5* manipulation affects thalamic brain volume still remain unclear, these results suggest that the thalamus may be involved in the emotional regulatory actions of FKBP51. Two other brain regions that are likely involved in the mechanisms by which *Fkbp5* manipulation affects behavior, are the hippocampus and the BNST. Not only did we observe volumetric differences in the bilateral hippocampus and GM regions around the BNST, but a robust transcriptomic profile was also found in both regions, substantiating their importance in *Fkbp5*-mediated effects.

Remarkably, our study revealed that loss of FKBP51 had opposing effects on behavior when it was restricted to different neuronal cell populations. We found that glutamatergic loss led to anxiogenic behaviors and enhanced the memory of an aversive spatial context, whereas reduced FKBP51 in GABAergic neurons resulted in an anxiolytic phenotype and diminished aversive spatial memory formation. This cell-divergent profile was not only observed on a behavioral level, but was also reflected in changes in brain volume and downstream gene expression levels. In general, the strongest effects were found for $Fkbp5^{Dlx}$ mice on all three dimensions. Since glutamatergic neurons principally exert excitatory functions, while GABAergic projections have inhibiting effects, it is understandable that loss of FKBP51 in either cell population leads to opposing functionality. Supporting this, cell-specific effects of genetic manipulation in relation to stress and behavior have already been reported before. Our group, for example, showed that KO of GR in glutamatergic or GABAergic neurons differentially affected fear- and anxiety-like behavior and hypothamalic-pituitary-adrenal axis reactivity (66). Remarkably, loss of FKBP51 in one specific cell type can indirectly lead to molecular changes in other cell populations. We demonstrated that specific manipulation of *Fkbp5* in GABAergic neurons resulted in a transcriptomic profile in the BNST that was particularly enriched in microglia. Interestingly, microglia have a high *Fkbp5* mRNA expression on their own (52) and an FKBP51-dependent link between neuroimmune regulation and GABAergic neurons has already been suggested (86). Our data further endorse this communication between GABAergic neurons and microglia, with FKBP51 as a mediating factor. Notably, in our study, we found that GABAergic loss of FKBP51 can lead to anxiolytic behavior and improved cognitive functioning and is associated with increased volumes of the hippocampus. This demonstrates that targeted manipulation of a stress-regulating factor like FKBP51 can have beneficial effects on behavior and brain structure. A recent MRI study, using high-resolution structural imaging, showed that exposure to early life trauma led to volumetric increases in specific subregions of the hippocampus and amygdala and proposed that these subregion-specific increases were associated with beneficial outcomes on behavior (87). It is interesting to speculate on that cell-type or region-specific changes in GR-mediated pathways might be underlying these more protective neurobiological mechanisms following exposure to stress in early life. However, without a doubt, it underlines the importance of more region and cell-type specific approaches when studying stress resilience mechanisms.

Another important observation of this study was the clear presence of a sex-dependent phenotype. Basic phenotyping of global KO of FKBP51 has been done previously in male and female mice (18, 20) and in contrast to our cell-type specific approach, it identified no drastic differences in baseline and stress-induced phenotypes between sexes. However, data on male and female global FKBP51 KO comes from separate studies and have used different type of stressors to test for stress reactivity phenotypes. In our study with conditional KO models, however, loss of FKBP51 in females evidentially induced changes in anxiety-like behavior, whereas for male mice, it led to alterations on the cognitive domain. Our data support demographic studies in humans, which have shown on a large scale that anxiety disorders are twice as common in women than in man (55, 88). The sex-dependent distinction that we found in behavior was supported by the observed structural brain changes. Male $Fkbp5^{Dlx}$ mice had a strongly increased volume of the bilateral hippocampus, a brain region that is majorly implicated in spatial memory and learning. Female $Fkbp5^{Dlx}$ mice, on the other hand, had volumetric differences in the areas around the BNST, which is highly associated with fear and anxiety states. To continue along this line, robust differential expression profiles for males were found in the DHC, though for females, downstream gene expression was most strongly altered in the BNST. Matching with the previously mentioned behavioral changes, some of the top regulated genes in male mice lacking FKBP51 in GABAergic neurons were either directly implicated in memory and learning, or were associated with presynaptic function and autophagic changes. The most significant DEGs in females were however involved in immune function regulation, endorsing the enrichment of the transcriptional signature in microglia. Even though sex-dependent transcriptomic profiles following acute or chronic stress exposure have been demonstrated before, particularly data from high-throughput studies is still limited (59). It is therefore extremely important that increasingly more studies highlight sex-divergent effects on many different levels. One limitation of this study was that cohorts of male and female animals did not come from identical breeding pairs or were not tested at the exact same timepoint. Therefore, the additional, direct sex-specific analyses as carried out for physiological and behavioral, data could not be extended for the RNA sequencing data.

Apart from sex, age is a strongly contributing psychiatric risk factor. Therefore, we tested KO of *Fkbp5* under the high-risk environment of an older age. Previous studies have investigated the consequences of loss of FKBP51 in younger male and female mice and did not observe changes in emotional regulation or cognitive functioning under baseline conditions (18, 20). Even though these studies investigated behaviors in full-body KO animals, while in our sample loss of FKBP51 was restricted to the glutamatergic or GABAergic neurons, it is plausible that the lack in effect on behavior can be subscribed to the younger age of the animals. This conception is substantiated by a study from Sabbagh and colleagues that have demonstrated an additive antidepressant effect of KO of *Fkbp5* with increasing age (50). This observation may in part be explained by underlying epigenetic changes that lead to accumulated intracellular FKBP51 levels, which impose a higher risk for developing psychiatric symptoms (30, 31, 81).

In summary, we demonstrated that under the high-risk environment of an older age, loss of FKBP51 in GABA or glutamatergic neurons led to unique and strongly sex-dependent outcomes on multiple levels. The outcomes of this study once again corroborate the importance of FKBP51 in emotional regulation and cognitive functioning, even under baseline conditions. As our data highlight that manipulation of FKBP51 leads to highly unique phenotypes dependent on the cell type, this emphasizes the need for cell-specific target treatments. Even more so, it underlines the extreme importance to consider sex when studying stress resilience mechanisms and to ultimately recognize this differential profile in treatment strategies.

Materials and Methods

Animals and Housing Conditions. The genetic mouse lines $Fkbp5^{Nex}$ and $Fkbp5^{Dlx}$ were bred in-house at the breeding facility of the Max Planck Institute of Psychiatry in Munich, Germany. Male $Fkbp5^{Nex}$ and $Fkbp5^{Dlx}$ mice that were used for RNAScope validation were 9 to 10 mo old at sacrifice. All experimental animals of both mouse lines and sexes were between 10 and 15 mo of age at the onset of the experiments. Mice were group-housed in individually ventilated cages

(IVCs; 30 cm × 16 cm × 16 cm), serviced by a central airflow system (Tecniplast, IVC Green Line–GM500) in a stably controlled environment (12h:12h light/dark cycle, temperature of 23 ± 2 °C and humidity of 55%). Water and food (standard research diet by Altromin 1318, Altromin GmbH, Germany) were provided to the animals ad libitum. Two weeks before the start of experimental testing male mice were single-housed and female mice were pair-housed. All experiments and protocols were performed in accordance with the European Communities' Council Directive 2010/63/EU and were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria. All effort was made to minimize any suffering of the animals throughout the experiments.

Generation of *Fkbp5^{Nex}* and *Fkbp5^{Dlx}* Mouse Lines. Conditional KO of *Fkbp5* in glutamatergic neurons (*Fkbp5^{Nex}*) of the forebrain was achieved by crossing *Fkbp5$^{lox/lox}$* mice with Nex-Cre mice (65), where *Cre* is highly expressed in differentiating neurons of the dorsal telencephalon and is active in the adult mouse brain in glutamatergic neurons of the neocortex, amygdala, olfactory bulb and hippocampus, but not in the dentate gyrus (65, 66). *Fkbp5^{Nex}* offspring therefore selectively lack *Fkbp5* expression in the forebrain glutamatergic neurons, starting from embryonic day 11.5 (65). For a conditional KO of *Fkbp5* in GABAergic neurons (*Fkbp5^{Dlx}*), *Fkbp5$^{lox/lox}$* mice were crossed with Dlx5/6-Cre mice, in which *Cre* is expressed in essentially all GABAergic neurons of the forebrain during development (67). *Fkbp5$^{lox/lox}$* littermates were used in as a WT control group in all experiments.

Experimental Setup. Older aged male and female *Fkbp5^{Nex}* (males: n = 11 vs. n = 11 *Fkbp5$^{lox/lox}$*; females: n = 15 vs. n = 7 *Fkbp5$^{lox/lox}$*) and *Fkbp5^{Dlx}* mice (males: n = 15 vs. n = 13 *Fkbp5$^{lox/lox}$*; females: n = 15 vs. 13 *Fkbp5$^{lox/lox}$*) were tested as separate cohorts (separated per sex and strain), but with the same experimental timeline. In the week prior to testing, animals were weighed and handled twice to familiarize them with the experimenter. During 4 consecutive days, mice underwent a number of behavioral tests in the following sequence: the OF test, EPM test, and NOR and SOR test. Following 2 to 4 rest days, mice were exposed to a 2-d fear-conditioned context retrieval paradigm. Subsequently, 4 to 7 wk succeeding behavioral testing, animals underwent a structural MRI scan. Finally, all mice were weighed and sacrificed 2 wk after the MRI scan.

Behavioral Protocol. A 6-d behavioral protocol was set up in order to study the effects of loss of FKBP51 in either glutamatergic or GABAergic forebrain neurons on a number of behavioral domains, including tests assessing locomotor activity (OF), anxiety-like behaviors (EPM) and cognitive functioning (NOR and SOR, contextual fear conditioning). All behavior tests were performed in the light phase between 7 AM and 1 PM. During all behavioral tests, animals were recorded with an external camera device and behaviors were later tracked using the advanced video tracking software ANY-maze v.7.15 (Stoelting). In case manual tracking was necessary, an experienced observer was blinded to the group allocation. Please find a more detailed description of each of the behavioral tests in *SI Appendix*.

MRI. Structural MRI was performed in a horizontal BRUKER Biospec 94/20 animal scanner (Bruker BioSpin), operating at 9.4 Tesla and using a transmit/receive cryo-coil with two coil elements, as described previously (89). For more details see *SI Appendix*.

Tissue Sampling. Animals were sacrificed by decapitation immediately following anesthesia with isofluorane. Baseline trunk blood was collected in 1.5 mL EDTA-coated microcentrifuge tubes (Kabe Labortechnik), centrifuged for 15 min at 8,000 rpm at 4 °C and stored at −20 °C. Furthermore, adrenals and brains were dissected. After collection, adrenals were washed in 9% NaCl, dried and weighed. Brains were snap-frozen in methyl butane on dry-ice and stored at −80 °C.

RNAScope mRNA In Situ Hybridization. RNAScope mRNA in situ hybridization was performed on male mice of both conditional KO lines and *Fkbp5$^{lox/lox}$* controls (3 *Fkbp5^{Nex}* vs. 3 *Fkbp5$^{lox/lox}$* and 4 *Fkbp5^{Dlx}* vs. 4 *Fkbp5$^{lox/lox}$*). For details see *SI Appendix*.

RNA Sequencing.

RNA extraction. Frozen brains were mounted in a cryostat microtome and punches of the BNST and DHC were collected in 1.5 mL DNA LoBind Safe-lock Eppendorf tubes, using a punching tool with a diameter of 1 mm. Tissue of six biological replicates per condition was immediately saved on dry ice and later stored at −80 °C. RNA extraction was then achieved by making use of the miRNeasy Mini Kit (cat. no. 1038703, QIAGEN) according to the manufacturer's protocol.

RNA sequencing and differential expression analysis. RNA quality control, library preparation, transcriptome sequencing, and bioinformatic analyses were performed on-site by the company Novogene UK (Novogene Europe) according to their standardized protocols. For the differential expression analyses, eight comparisons were setup (a KO vs. WT comparison was performed for each of the genetic mouse lines, the different sexes and two separate brain regions). Genes with adjusted *P* value (q) < 0.02 and log2 fold change were referred to as significantly differentially regulated genes.

Enrichment analyses. GO enrichment analysis of DEGs was implemented by the clusterProfiler R package. GO terms with a corrected *P* value < 0.05 were considered significantly enriched by DEGs. At last, a single-cell RNA sequencing dataset of the mouse hippocampus was used to check in which cell types significant DEGs are enriched (77).

Statistical Analyses. Statistical analyses for physiological, behavioral and RNAScope data were performed in R studio (R.4.2.0). Statistical assumptions were then checked by using a Shapiro-Wilk test for Normality and a Levene's test to check for equality of variances. If data violated these assumptions, nonparametric statistical tests were used or a boxcox transformation was applied to normalize the data. Subsequently, two-group comparisons were performed with an independent *t* test or a nonparametric Wilcoxon test. As cohorts of the female *Fkbp5^{Nex}* and male *Fkbp5^{Nex}* and *Fkbp5^{Dlx}* mice had varying age at baseline in control and conditional KO groups, for these cohorts a one-way analysis of covariance (ANCOVA) with age in weeks at baseline as a covariate was conducted. Before analyses, additional assumption checks for the one-way ANCOVA were done, including checks for linearity between covariate and dependent variable, homogeneity of the regression slopes and a normality check for the residuals. As cohorts of male and female animals were tested at different timepoints and these cohorts of animals resulted from different breeding pairs, the initial analysis did not include "sex" as a factor. However, in order to further emphasize interesting sex effects, an additional two-way ANCOVA with sex and genotype as independent factors was applied to the data of the *Fkbp5^{Nex}* and *Fkbp5^{Dlx}* cohorts. Results from these analyses are given in *SI Appendix*, Table S1. Values that were greater or smaller than two times the SD from the mean (M) were considered outliers and were excluded from analyses. Graphs were created with GraphPad Prism 9 and all remaining data illustrations were composed in R studio. Part of the figures was composed with the help of Biorender.com. *P* values of less than 0.05 were considered statistically significant and a statistical trend was recognized for *P* values of $0.1 \geq P \geq 0.05$.

For MRI data a two-way ANOVA (sex × genotype and interaction) with Tukey post hoc testing was used on the total brain volume (TBV) and on the tissue compartments to evaluate differences in brain tissue composition and total brain size. Smoothed Jacobian deformation fields were compared in SPM12 in an independent two-factorial model (genotype × sex) for *Fkbp5^{Dlx}* and *Fkbp5^{Nex}* mice vs. their *Fkbp5$^{lox/lox}$* controls, respectively. Analyses included TBV as a covariate. As TBV differed for sexes, we introduced the TBV covariate split according to sex (i.e., independent group mean values for male and female values). If not stated otherwise, reported results survive an FWE correction at the cluster level ($p_{FWE,cluster} < 0.05$), with a cluster collection threshold of $P < 0.005$ uncorrected.

Data, Materials, and Software Availability. RNAseq data have been deposited in Gene Expression Omnibus under accession number GSE232460 (90). All other data are included in the manuscript and/or *SI Appendix*.

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Author affiliations: [a]Research Group Neurobiology of Stress Resilience, Max Planck Institute of Psychiatry, 80807 Munich, Germany; [b]International Max Planck Research School for Translational Psychiatry, 80807 Munich, Germany; [c]Core Unit Neuroimaging, Max Planck Institute of Psychiatry, 80807 Munich, Germany; [d]Research Group Molecular Neurogenetics, Max Planck Institute of Psychiatry, 80807 Munich, Germany; and [e]Department of Neuroscience, Karolinska Institute, Solna 171 77, Sweden

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2.3 FKBP51 in glutamatergic forebrain neurons plays a role in beneficial effects of moderate early life stress exposure on hippocampal function and structure in female mice via a TCF4-mediated pathway.

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FKBP51 in glutamatergic forebrain neurons mediates beneficial effects of moderate early life stress exposure on hippocampal function and structure in female mice via a Tcf4-mediated pathway

Lotte van Doeselaar[1,2], Alexandra Abromeit[1], Tibor Stark[3], Danusa Menegaz[4], Shiladitya Mitra[1], Huanqing Yang[1], Ghalia Rehawi[5], Rosa Huettl[6], Joeri Bordes[1], Sowmya Narayan[1,2], Jan M. Deussing[7], Michael Czisch[3], Janine Arloth-Knauer[5], Matthias Eder[4], Juan P. Lopez[8] & Mathias V. Schmidt[1].

[1] Research Group Neurobiology of Stress Resilience, Max Planck Institute of Psychiatry, Munich, Germany

[2] International Max Planck Research School for Translational Psychiatry, Munich, Germany

[3] Core Unit Neuroimaging, Max Planck Institute of Psychiatry, Munich, Germany

[4] Core Unit Electrophysiology, Max Planck Institute of Psychiatry, Munich, Germany

[5] Department Genes & Environment, Max Planck Institute of Psychiatry, Munich, Germany

[6] Core Unit Virus Production, Max Planck Institute of Psychiatry, Munich, Germany

[7] Research Group Molecular Genetics, Max Planck Institute of Psychiatry, Munich, Germany

[8] Department of Neuroscience, Karolinska Institute, Stockholm, Sweden

Corresponding author:

PD. Dr. Mathias V. Schmidt

Research group Neurobiology of Stress Resilience

Max Planck Institute of Psychiatry

Kraepelinstrasse 2-10

80804, Munich, Germany

Abstract

Early life stress (ELS) has often been described as a risk factor for developing psychiatric disease. However, moderate exposure to ELS can also lead to adaptive changes on brain and behavior. Moreover, the *FKBP5* gene, encoding the FKBP51 co-chaperone, has been associated with increased risk for developing psychiatric disorders, specifically in interaction with ELS exposure. However, the underlying mechanisms behind the interaction of FKBP51 and moderate ELS exposure are still not completely understood and particularly data in the female sex are scarce. In this study, the contribution of FKBP51 in glutamatergic forebrain neurons to the long-term consequences of moderate ELS was investigated in male and female mice, by using the *Fkbp5*^{Nex} conditional knockout line and the limited bedding and nesting material paradigm. Data showed that, particularly in female mice, ELS exposure led to an anxiolytic phenotype and improved memory performance in a stressful context in *Fkbp5*$^{lox/lox}$ wild-type mice and these effects were absent in *Fkbp5*^{Nex} mice. Interactive effects of FKBP51 in glutamatergic forebrain neurons and moderate ELS exposure in female mice were also reflected on brain volume of different cortical regions, the subiculum and white matter structures. Furthermore, similar interactive effects were observed for structural and functional electrophysiological cell-properties of the CA1 pyramidal neurons of the dorsal hippocampus. RNA sequencing of the hippocampus revealed the transcription factor 4 (TCF4) as a potential regulator of these interactive effects. Cre-dependent viral overexpression of TCF4 in Nex-Cre female mice led to similar beneficial effects as the moderate ELS exposure on anxiety-like behavior and spatial memory performance in a stressful environment. This study shows that FKBP51 in glutamatergic forebrain neurons mediates adaptive effects of moderate ELS exposure on emotional regulation, cognitive behavior, neuronal structure and function. Moreover, it proposes TCF4 as an underlying target that drives the FKBP51-mediated effects of moderate ELS on brain and behavior.

Introduction

Patients with psychiatric disorders, such as major depressive disorder (MDD) or anxiety disorders, suffer from mood-related and cognitive symptoms, often disabling them to fully participate in society. As a consequence, psychiatric disorders are an important and costly global health problem, for which the biological underpinnings are still poorly understood. In the past decades, it has become clear that psychiatric disease often arises as a combination of genetic and environmental factors (Belsky et al., 2009; Caspi & Moffitt, 2006). Environmental stress exposure can occur at any stage in an individual's life and not only the duration, type or severity, but also the timing of stress exposure can be determining for the long-term health outcomes. A wide number of studies have uncovered a time window during early development in which the brain is particularly sensitive to environmental challenges (Andersen, 2003; Gilbertson et al., 2002; Lupien et al., 2009; Schmidt, 2010). Interestingly, stress exposure during early life can not only lead to maladaptive outcomes, but there has also been evidence for the so-called "inoculation theory of stress" (Champagne et al., 2008; Lyons et al., 2009; Parker et al., 2004, 2005) that proposes that moderate exposure to stress in early life may prepare an individual to cope with future challenges in adulthood. This process of "early programming" may therefore act as a long-lasting adaptive mechanism (Gluckman et al., 2005; Nederhof & Schmidt, 2012).

Generally, careful regulation of the stress response is required for adequate stress coping (de Kloet et al., 2005) and the hypothalamic pituitary adrenal (HPA) axis is important for keeping this balanced response. Upon perceiving a stressor, the endocrine cascade of the HPA-axis leads to the production of cortisol in humans or corticosterone (CORT) in rodents. CORT binds its two receptors that are located in the periphery and brain: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), of which the latter is particularly important in dampening the acute response to stress (de Kloet et al., 2005). As nuclear receptors, MR and GR can influence transcription of a wide number of genes, by binding to glucocorticoid responsive elements (GREs) that are present on the DNA of numerous genes. One gene that is under strict regulation by GR is the *Fkbp5* gene, encoding the chaperone protein FKBP51. FKBP51 can influence GR sensitivity by binding to the GR complex, thereby hampering GR's transcriptional activity, which results in an ultra-short feedback loop (Hähle et al., 2019; Häusl et al., 2019). One particular brain region of interest in this regard is the hippocampus. The hippocampus has a specifically high expression of FKBP51 (Scharf et al., 2011), it is highly sensitive to the effects of stress (Sapolsky, 2000) and studies in MDD patients have demonstrated structural and cellular alterations in this region (Campbell & Macqueen, 2004; Malykhin et al., 2010; Stockmeier et al., 2004). Moreover, the hippocampus is known to play a role in both emotional and (spatial) memory functions (Jimenez et al., 2018; Moser et al., 1995), two domains that are reflected in the symptomology of patients suffering from psychiatric disorders, such as MDD. The hippocampus has a diverse cell-type profile, but the excitatory glutamatergic pyramidal neurons make up the vast majority (Erö et al., 2018). Notably, FKBP51 is particularly strongly expressed in excitatory neurons (Matosin et al., 2021). Moreover, the effects of FKBP51 on stress resilience and vulnerability are highly sex-dependent (Criado-Marrero et al., 2020; van Doeselaar et al., 2023).

Previous work in human studies has identified *FKBP5* as a genetic risk factor for psychiatric disease (Binder, 2009; Binder et al., 2004) and studies in rodents have extensively described its role in stress vulnerability and resilience processes (Blair et al., 2019; Engelhardt et al., 2021; Hartmann et al., 2015; Häusl et al., 2021; Touma et al., 2011). Interestingly, polymorphisms in the *FKBP5* gene were found to interact with childhood trauma to increase the risk for developing psychiatric disorders (Klengel & Binder, 2015; Q. Wang et al., 2018; Zannas & Binder, 2014). The risk allele of the most studied *FKBP5* single nucleotide polymorphism (SNP) rs1360780 leads to a conformational change of the *FKBP5* DNA structure and this causes the GRE in intron 2 to come in close contact with the transcriptional start site in the promotor region. Ultimately, this results in an enhanced glucocorticoid-induced FKBP51 induction and endured circulating glucocorticoid concentrations after stress (Klengel et al., 2013).

When exposed to childhood adversity, glucocorticoid levels rise and, only in combination with the already augmented FKBP51 induction in rs1360780 risk allele carriers, the endured glucocorticoid stimulation can lead to demethylation of a GRE in intron 7. This leads to an even further enhanced FKBP51 induction upon stress and increases the risk for developing psychiatric disease (Klengel & Binder, 2015).

In mice, overexpression of FKBP51 in forebrain glutamatergic neurons led to differential effects of maternal separation on anxiety behaviour and hippocampal neurogenesis (Criado-Marrero et al., 2019, 2020). However, the mechanism underlying the interaction of FKBP51 variations with early life adversity is not yet completely understood. Importantly, while stress-related psychiatric disorders are highly predominant in women (Ferrari et al., 2013; Heo et al., 2008; Kessler, 1994), the consequences of early life stress (ELS) in females is severely understudied. To address this paucity, we here exposed female mice with a deletion of FKBP51 in glutamatergic neurons to moderate early adversity and investigated the long-term behavioural, structural, functional and molecular consequences. Together the data uncover a novel mechanism by which FKBP51 can contribute to a pro-resilient phenotype following ELS exposure.

Methods

Animals and housing conditions

All animals were bred at the in-house breeding facility of the Max Plank Institute of Psychiatry in Martinsried, Munich, DE. Unless specifically stated otherwise, animals were group-housed in individually ventilated cages (IVC; 30 cm x 16 cm x 16 cm), serviced by a central airflow system (Tecniplast, IVC Green Line—GM500), under standard housing conditions (stably controlled 12h:12h light/dark cycle, temperature of 23 ± 2 °C, humidity of 55% and sufficient bedding ad nesting material) and were provided with a standard research diet (Altromin 1318, Altromin GmbH, Germany) and water *ad libitum* at all times. Two weeks prior to the experimental testing phase, male mice were single housed and female mice were pair housed. All experiments and protocols were performed in accordance with the European Communities' Council Directive 2010/63/EU and were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria. All effort was made to minimize any suffering of the animals throughout the experiments.

Generation of developmental *Fkbp5^{Nex}* and virally induced *Nex^{Tcf4OE}* mouse lines

The *Fkbp5^{Nex}* genetic mouse line was generated by breeding *Fkbp5$^{lox/lox}$* mice with Nex-Cre mice (Goebbels et al., 2006), as previously described in detail (van Doeselaar et al., 2023). This resulted in loss of FKBP51 in glutamatergic neurons of the forebrain (including the neocortex, amygdala, olfactory bulb and hippocampus, but not the dentate gyrus) from embryonic day 11.5 onwards (Goebbels et al., 2006; van Doeselaar et al., 2023).

TCF4 overexpression (OE) in glutamatergic neurons of the CA1 (*Nex^{Tcf4OE}*) was achieved by injecting a cre-dependent AVV-CMV-DIO-mTcf4 virus (Vector Biolabs, Malvern, PA, USA) bilaterally into the CA1 of Nex-Cre female mice. Female mice of the control condition were injected with an AVV-CMV-DIO-eGFP control virus (Vector Biolabs, Malvern, PA, USA) in the same region. Injections were performed via stereotaxic surgeries as described previously (Häusl et al., 2021). In short, 5-month-old female mice were anesthetized with isofluorane and fixated in a stereotaxic apparatus. Following preparatory actions, 700 nL of the Tcf4 OE or control viruses were bilaterally injected by using a glass capillary (tip resistance of 2 – 4 MΩ) at a flow rate of 100 ng/min in the CA1. The CA1 was targeted by using the following coordinates: for right injections 2.3 mm posterior, 2.15 mm lateral and 1.5 mm dorsal from

Bregma; for left injections 2.3 mm Posterior, 2.15 mm Lateral and 1.6 mm dorsal from Bregma. Following surgeries, animals received the painkiller meloxicam (2 mg/kg for three days in the drinking water) and were monitored closely up till 7 days post-surgery.

Limited Bedding and Nesting Material paradigm

In order to investigate the long-term consequences of a moderate ELS exposure, mice were exposed to the limited bedding and nesting material (LBN) paradigm that was originally described by Rice and colleagues (Rice et al., 2008). Male *Fkbp5*^{Nex} mice were paired with *Fkbp5*$^{lox/lox}$ females for breeding purposes. Throughout pregnancy, females were single-housed and monitored daily for birth of pups. The day of birth of the litter was considered postnatal day 0 (P0) and dams and litter were then assigned to either the ELS or control condition. At P2, dams and pups were checked and put in a fresh cage. If assigned to the control condition, dams and pups were returned to standard housing conditions, with a regular amount of Nestlets (Ancare, Bellmore, NY, USA; 2 full pieces). Dams and pups in the ELS condition were however put back in an IVC with a metal grid, placed on the bottom of the cage, and were only provided a very limited amount of bedding and Nestlets material (half a piece) for a period of 7 days. At P9, pups of either condition were weighed and all dams and pups were put in fresh IVCs with standard housing conditions. At P26, animals were weaned, group-housed and left undisturbed into adulthood until the start of the experimental procedure.

Behaviour analyses

Behavioural testing was performed to study anxiety-like behaviour and cognitive functioning in a neutral and a stressful environment. The open field (OF) test, elevated plus maze (EPM) test, novel object recognition (NOR) and spatial object recognition (SOR) tests were performed subsequently over a period of 6 days between 8 AM and 1 PM. The Morris Water Maze (MWM) tasks started 4 days after the SOR. The behaviour of the animals was recorded and later tracked using the advanced video tracking software ANY-maze v.7.15 (Stoelting, Dublin, IE). In case manual tracking was required, this was performed by an experienced observer that was blinded to group allocations.

Open Field

In order to asses anxiety-like behaviours and general locomotor activity, the OF test was performed. In this test, mice could freely explore a OF arena (50 cm x 50 cm x 50 cm), made out of grey polyvinyl chloride material, for a period of 15 minutes under dimmed light conditions (30 lux). Total distance travelled in the entire OF arena during the full 15 minutes was taken as a measure for general locomotor activity. Other parameters that were measured were total distance travelled in meters, time spent in seconds and number of entries into the inner zone (dimensions: 26 cm x 26 cm) of the OF, to assess anxiety-like behavior. For analyses of anxiety-like behaviour, data was separated in bins of 300 seconds.

Elevated Plus Maze

As an additional measure for anxiety-like behaviour mice underwent the EPM test. For this, animals were placed on an elevated EPM apparatus that consisted out of an elevated (50 cm above the ground) cross maze with two open (30 cm x 5 cm x 0.5 cm) and two closed arms (30 cm x 5 cm x 15 cm). Dimmed light conditions were set to less than 10 lux in the closed arms and approximately 20 lux in the open arms. Mice were located in the centre of the cross maze and were allowed to freely explore the maze for 10 min. Anxiety like-behavior was measured as amount of entries into the open arms, time spent in seconds and distance travelled in meters in the open arms. Data was analysed in 300 seconds-time bins.

Novel object and spatial object recognition

In order to evaluate memory performance in a neutral context we applied two tests, assessing memory function on different domains: the NOR (for recognition memory) and the SOR (for spatial memory). During these tests, the ability of the mice to discriminate between a familiar and unfamiliar object and a familiar and unfamiliar location of objects was evaluated. To this end, two separate objects were built out of black and white Lego© blocks, that were unique enough to allow discrimination, but not too distinct that it could create a potential bias based on preference for one specific object. Lego blocks were placed in a square OF arena and mice were allowed to explore objects or their locations for a period or 15 min. Following an inter trial interval of 30 min, the type of object or the object location was changed and animals were placed back into the arena where they could explore the novel objects or their locations during a 5-min retrieval phase. Exploration of the objects was assessed manually and time spent in seconds exploring the objects was analysed.

Morris Water Maze

The MWM is a task that is widely used to assess spatial memory performance in mice under a stressful environment (Vorhees & Williams, 2006). The MWM was performed in dimly lit square room with 4 unique spatial cues surrounding the pool, in order to ensure spatial navigation. The pool, that was elevated 110 cm above the floor, had a diameter of 150 cm and a height of 41 cm and was filled with water up to the top, leaving an edge of about 5 cm long. The pool was divided into four quadrants (northwest (NW), northeast (NE), southwest (SW) and southeast (SE) quadrant) and an invisible platform was located in a fixed position in the SW quadrant 0.5 – 1 cm below the water surface. The MWM spatial learning task consisted out of two phases, the training phase and the probe trial testing phase. The training phase included 5 consecutive training days for males and 6 consecutive training days for females, in which the mice were placed in randomized starting locations in the opposite quadrant (NE) from the platform location. During the training phase, animals were allowed to find the location of the invisible platform within a 90-seconds learning trial. Upon finding the platform, mice were taken off the platform immediately. If animals did not find the platform location before the end of the training trial, they were guided towards the platform and left to explore the platform area for 10 seconds, before being removed. Animals were then quickly dried and returned to their home cage. Each day, mice performed 4 consecutive training trials, with an inter-trial interval of 12 to 16 minutes. During this phase of the MWM, the time in seconds it took the mice to find the platform location was measured. One day subsequent to the training phase, the probe trial testing phase started. For the testing phase, the platform was removed from the pool and animals were allowed to explore the pool area for 60 seconds. During this test, the relative distance travelled in meter in the original platform quadrant (SW) versus the adjacent (NW and SE) and opposite (NE) quadrants was evaluated as a measure of spatial memory performance.

Magnetic Resonance Imaging

A horizontal BRUKER Biospec 94/20 animal scanner (Bruker BioSpin, Rheinstetten, Germany), operating at 9.4 Tesla and using a transmit/receive cryo-coil with two coil elements, was used to apply structural magnetic resonance imaging (MRI) as previously described (van Doeselaar et al., 2023). In short, animals were sedated using 2.5% isoflurane and stereotactically fixated in a prone position on an MR-compatible animal bed, on top of a warm water silicon pad, where they were held under constant inhalational anesthesia with isoflurane (1.5 - 2.5% in pressured air, with a flow of 1.5 l/min). Bepanthen cream (Bayer, Leverkusen, DE) was applied in order to prevent drying of the eyes. Bodily signs, such as body temperature and respiration, were consistently checked and remained at a constant value, by either adjusting the temperature of the warm water silicon pad or the depth of isoflurane anesthesia. For the collection of MR images, first, general adjustments of the system and

collection of localizer scans were performed, after which a 3D T2*-weighted image was acquired using a FLASH sequence with TE=6.25 ms, TR=34.1 ms, flip angle 10°, matrix size 256 x 166 x 205 points, resolution 0.077 mm isotropic, 2 averages, with fat and outer volume suppression. Acquisition time for the 3D was 41 minutes 8 seconds.

Image processing

Images processing was handled as previously described (van Doeselaar et al., 2023). In short, brain extraction was based on a three-step procedure, including segmentation using the Hikishima templates (Hikishima et al., 2017), smoothing and spatial filtering. Filtering was then performed on the bias corrected images from the first segmentation. A second segmentation step was performed with a modified Hikishima version, in which the inner CSF and skull bones is represented in different probability maps. This separation was necessary because in our T2*-weighted images (different from the typical T1-weighted images, for which Hikishima templates were generated), ventricular CSF shows high image intensities. The resulting brain-masked images, created from grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) compartments and corrected for the high image intensities of the ventricular CSF in our T2*-weighted images, were then co-registered to the Hikishima T2-weighted reference image. The olfactory bulb and the cerebellum were cut out (due to lower signal intensities caused by the geometry of the surface coil). At last, an SPM12 old segmentation step was performed using the GM, WM and inner CSF compartment tissue templates. Resulting tissue probability maps for GM and WM were imported to DARTEL and normalized with isotropic voxel size 0.7 mm, to create a study specific template. Flow fields were transformed into jacobian deformation fields for later deformation-based morphometry (DBM) analysis (Ashburner et al., 1998) and were smoothed with a Gaussian kernel of 4 mm. Both total brain volume (TBV) and volume of the individual tissue compartments were defined from the DARTEL imported images (native space), by summation of the tissue probability values in GM, WM and CSF compartments. The anatomical images were also normalized using the DARTEL flow fields, and a mean image was calculated.

Tissue Collection

At sacrifice animals were anesthetized using a lethal dose of isoflurane and subsequent immediate decapitation. Trunk blood was then collected in 1,5 mL EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Nümbrecht-Elsenroth, DE) and saved on ice until further processing. Plasma separation was later achieved by centrifugation (15 min, 8 000 RPM at 4 °C) and samples were stored at -20 °C. In addition, adrenal glands and brains were extracted. Brains were immediately snap frozen in isopentane on dry-ice and later stored at -80 °C. Following collection, adrenal tissue was washed in 9% NaCl, dried and weighed.

Hippocampal dendritic morphology

In order to further evaluate structural hippocampal consequences of ELS in *Fkbp5*^{Nex} females, the Golgi-Cox staining was applied to visualise and quantify dendritic tree morphology as well as spine density of the dorsal CA1 of 8-month old female *Fkbp5*^{Nex} and *Fkbp5*$^{lox/lox}$ mice that either underwent the LBN ELS procedure or a control condition. First, mice were sacrificed by a lethal dose of isoflurane, after which transcardial perfusion was conducted, using a perfusion pump and ice-cold phosphate buffered saline (PBS) with 0.1% heparin for approximately five minutes. Following subsequent decapitation, brains were extracted and the left hemisphere was dissected.

Golgi-Cox staining procedure

Golgi-Cox staining was then performed with help of the Bioenno superGolgi Kit (Bioenno Tech, LLC, Santa Ana, CA, USA) according to the manufacturer's protocol. In short, brains were first impregnated

for 12 – 14 days in the provided impregnation solution. After rinsing with distilled water, they were then transferred to a post-impregnation buffer for 2 days. Subsequently, CA1 brains sections (150 µm) were collected on a vibratome (HM650V, Thermo Scientific) in a 6% sucrose collection buffer and mounted on gelantine-covered slides (6% gelantine). Following, sections were incubated in staining solution and incubated in the post-staining solution. After the staining procedure was completed, slides were imaged.

Imaging and analyses

After the Golgi-cox staining procedure was completed, dorsal CA1 sections were imaged for dendritic length and branching and spine analyses. For dendritic length and branching, Z-stacked images (100 µm stacks) were collected with the Olympus BX61VS slide scanner microscope (Olympus, Hamburg, DE) at 40 x magnification. For dendritic spine analysis, images were made at 100 x magnification, using the Zeiss AXIO Imager M2 with the camera Zeiss Axiocam506 (Zeiss, Oberkochen, DE) and the software Neurolucida (MBF Bioscience, Williston, VT, USA).

A Sholl analysis was performed to determine dendritic branching length with the help of the Simple Neurite Tracer (SNT) plugin from the ImageJ software on 121 collected neurons. The dendritic branch was investigated for a total length of 300um (starting from the soma) and the number of intersections were measured for each 10um section. Dendritic spine analysis was done with the image J software for 3 animals in each condition, with 4 – 6 apical and 4 – 6 basal dendritic segments. Length of the individual dendritic segments were measured, spines per segment were counted and finally a score per 10 µm of each dendritic segment for each animal was calculated. The average number of spines/10 µm dendritic segments for each condition was used for statistical analyses.

Electrophysiology

Mice were anesthetized with isoflurane and immediately decapitated, after which the brain was rapidly removed from the cranial cavity. Subsequently, 350 µm-thick coronal slices of the dorsal hippocampus were collected using a vibratome, in an ice-cold carbogen gas (95% O2/5% CO2)-saturated solution consisting of (in mM): 87 NaCl, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 0.5 CaCl2, 7 MgCl2, 10 glucose, and 75 sucrose. Brain slices were then incubated in carbogenated physiological saline (containing 125 mM NaCl, 2.5 mM KCl, 25 mM NaHCO3, 1.25 mM NaH2PO4, 2 mM CaCl2, 1 mM MgCl2, and 10 mM glucose) for 30 min at 34°C, followed by an incubation at room temperature (23-25°C) for at least 1 h. All electrophysiological measurements were conducted at room temperature. Slices assigned to the CORT condition (*Fkbp5*^{Nex} CORT and *Fkbp5*$^{lox/lox}$ CORT) were stored for 1 h in carbogenated phyisiological saline, containing a 1 µM CORT solution (Sigma-Aldrich Corticosterone, product nr. 27840, dissolved in 0.01 % EtOH; Merck KGaA, Darmstadt, DE). Brian slices in all other conditions (Ctrl and ELS conditions) were pre-incubated with a carbogen physiological saline vehicle solution, containing 0.01% EtOH. Following pre-incubation with CORT or vehicle solution, slices were washed for 30 min in pure carbogenated physiological saline. Slices were then transferred to the recording chamber, where they were superfused with carbogenated physiological saline (4-5 ml/min flow rate). Field excitatory postsynaptic potentials (fEPSPs) at CA3 - CA1 synapses were evoked by square-pulse electrical stimuli (50 µs pulse width) delivered via a bipolar tungsten electrode (50 µm pole diameter, ∼0.5 MΩ nominal impedance) to the Schaffer collateral-commissural pathway. fEPSPs were recorded using glass microelectrodes (filled with physiological saline, ~1 MΩ open-tip resistance) that were placed into the CA1 stratum radiatum. Voltage stimulation intensity was adjusted accordingly to produce a fEPSP of ∼50% of the amplitude at which a population spike appeared. Recording data were low-pass filtered at 1 kHz and digitized at 5 kHz. Before and after LTP induction, which was induced by high-frequency stimulation (HFS, 100 Hz for 1 s), a single stimulation pulse was delivered every 15 s to the neural tissue.

RNA sequencing

RNA extraction

Hippocampal tissue of 6 mice per condition (female *Fkbp5^{Nex}* and *Fkbp5$^{lox/lox}$* mice of the ELS and Ctrl condition) was collected from frozen brains via punches, using a 1 mm-diameter punching tool. Tissue was then immediately transferred into 1.5 mL DNA LoBind Safe-lock Eppendorf tubes that were kept on dry-ice. Following collection, tissue was again stored at - 80 °C. RNA isolation was then later achieved with help of the miRNeasy Mini Kit (cat. no. 1038703, QIAGEN, Venlo, NL) RNA extraction kit, according to the manufacturer's protocol.

RNA sequencing

For all steps up until filtering, samples were analysed together with samples form a different experiment. All subsequent analyses were separately conducted for the hippocampus tissue samples of this study. RNA quality control, library preparation, transcriptome sequencing, and RNA sequencing analyses were performed on-site by the company Novogene UK (Novogene Europe, Cambridge, UK) according to their standardized protocols. For cleaning of the data, reads containing adapter, reads containing poly-N and low-quality reads were removed from the raw data. At the same time, Q20, Q30 and GC content were calculated from the clean data. Index of the reference genome was built using Hisat2 v2.0.5 and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. FeatureCounts v1.5.0-p3 was used to count the reads numbers mapped to each gene. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. The subsequent analysis was performed in R version 3.6.1. Genes with less than 10x coverage across all samples in each experimental group in each brain region were removed (n = 4 experimental groups: *Fkbp5$^{lox/lox}$* and control; *Fkbp5^{Nex}* and control; *Fkbp5$^{lox/lox}$* and ELS; *Fkbp5^{Nex}* and ELS). 16,621 genes were left after this filtering step and 48 samples. To identify outliers, we performed a principal component analysis (PCA). Samples with a distance of more than 2.5 standard deviations from the mean in the first principle component were excluded, which lead to the removal of one sample. Surrogate variable analysis (SVA) was applied to account for unwanted variation in the data.

Differential expression analysis

Significant surrogate variables were included as covariates in the DE analysis. DE analysis between 4 comparisons (ELS: Fkbp5^{Nex} vs. Fkbp5$^{lox/lox}$; Ctrl: Fkbp5^{Nex} vs. Fkbp5$^{lox/lox}$; ^{Fkbp5Nex}: ELS vs. Ctrl and Fkbp5$^{lox/lox}$: ELS vs. Control) each brain region was set up. The expression data was normalized and transformed using the vst function of DESeq2 v1.24. We tested for DE with DESeq2 and reported the genes with a false discovery rate (FDR) below 2% as significant.

*Constructing gene networks and e*nrichment analyses

In order to detect underlying pathways, containing networks of co-expressed genes, a weighted correlation network (WGCNA) analyses was performed, in addition to the differential expression analysis. We used R package WGCNA with a soft threshold of 10, deep split of 4, min. module size of 30 and merge cut height of 0.15. to construct the co-expression network. In addition to this, hub genes of the revealed co-expressed subnetworks or modules were identified. Furthermore, a transcription factor enrichment analyses for selected WGCNA subnetworks was carried out with the online enrichment analysis tool WEB-based Gene SeT AnaLysis Toolkit (WebGestalt). Using the software Knowing^{01} (Knowing^{01} Gmbh, Munich, Germany), we then further identified which genes of the significant DEGs, WGCNA subnetworks or enriched transcription factors and its regulated genes were enriched in publicly available human GWAS datasets for psychiatric diseases. For this, we selected GWAS datasets of childhood traumatic events in both sexes and in females only (UK Biobank) (Warrier & Baron-Cohen, 2021), PTSD in both sexes or in females only (PGC-PTSD Freeze 2 GWAS) (Nievergelt

et al., 2019), MDD (Wray et al., 2018), that were obtained from the psychiatric genomic consortium website (PCG).

Statistical analyses

Statistical analyses for behavioural, CA1 dendritic spine density and branching and electrophysiological analyses were carried out in R studio (R.4.2.0) or GraphPad Prism 9. Statistical assumptions were checked by using a Shapiro-Wilk test for Normality and a Levene's test for equality of variances. In case these assumptions were violated, non-parametric statistical tests were performed or a boxcox transformation was conducted to normalize the data. Data including 4 groups (*Fkbp5*^{Nex} ELS and Ctrl; *Fkbp5*$^{lox/lox}$ ELS and Ctrl) were analysed using a two-way ANOVA for ELS exposure by genotype to test for main differences in ELS exposure and genotype or their interaction (ELS x genotype), with post-hoc t-test. For analysis of field potential recordings and hippocampal dendritic branching, an average value per group was precedingly calculated. For data with 2 groups (*Nex*^{Tcf4OE} vs. Ctrl), an independent sample t-test or non-parametric Mann Whitney U test was applied to test for group differences. Outliers were identified as values greater than 2 times the standard deviation (SD) from the mean (M) and excluded from analyses. Graphs were constructed with GraphPad Prism 9 or R studio and part of the figures was created with the help of Biorender.com. *P* values of less than 0.05 were considered statistically significant and a statistical trend was recognized for *p* values of $0.1 \geq P \geq 0.05$.

MRI data was analysed with a two-way ANOVA (ELS x genotype and interaction if statistically significant) with Tukey post-hoc testing for TBV and tissue compartments to detect differences in brain tissue composition and total brain size. An independent 2- factorial model in SPM12 (ELS x genotype) for *Fkbp5*^{Nex} animals vs. *Fkbp5*$^{lox/lox}$ animals and for ELS vs. control mice, respectively was used to compare smoothed jacobian deformation fields. Analyses included TBV as a covariate. If not stated otherwise, reported results survive an FWE correction at the cluster level ($p_{FWE,cluster} < 0.05$), with a cluster collection threshold of $p < 0.005$ uncorrected.

Results

***Fkbp5*^{Nex} genotype and ELS exposure have interactive effects on behaviour in female mice**

In order to study the contribution of FKBP51 in glutamatergic forebrain neurons to the long-term consequences of moderate ELS exposure, male and female *Fkbp5*^{Nex} and their *Fkbp5*$^{lox/lox}$ littermate wild types were exposed to an ELS paradigm from P2 to P9 (Males: n = 9 *Fkbp5*^{Nex} and n = 8 *Fkbp5*$^{lox/lox}$; Females: n = 10 *Fkbp5*^{Nex} and n = 11 *Fkbp5*$^{lox/lox}$) or a control condition (Males: n = 8 *Fkbp5*^{Nex} and n = 18 *Fkbp5*$^{lox/lox}$; Females: n = 10 *Fkbp5*^{Nex} and n = 9 *Fkbp5*$^{lox/lox}$) and underwent a behavioural experimental test battery in adulthood and subsequent structural MRI scanning (Figure 1A). For females, ELS drastically reduced BW at P9 at the end of the LBN paradigm ($F_{(1, 36)} = 4.50$ $p < 0.001$), however no changes were found in body weight at the start of the experimental procedure, in adrenal weight or in baseline CORT levels at sacrifice (Figure S1A). For males, ELS also reduced body weight at P9 ($F_{(1, 40)} = 21.18$ $p < 0.001$) and in addition to this, a main effect of genotype was found for adrenal weight ($F_{(1, 29)} = 9.97$ $p < 0.01$) and CORT levels at baseline ($F_{(1, 29)} = 7.30$, $p < 0.05$.; Figure S1B).

The behavioural protocol included tests assessing anxiety-like behaviour (OF and EPM) and tests for (spatial) memory performance in a neutral environment (NOR and SOR) or in a stressful context (MWM). Data from the total distance travelled in the entire OF arena, for the complete 15-min trial, revealed an increased locomotor behaviour for female *Fkbp5*^{Nex} compared to *Fkbp5*$^{lox/lox}$ wild types, regardless of ELS exposure ($F_{(1,35)} = 7.38$, $p < 0.05$; Fig. 1B). Male mice were unaffected on general locomotor behaviour (Figure S2A). However, on the anxiety domain, behavioural changes were

predominantly ELS-induced. Female mice that were exposed to ELS travelled a longer distance in the inner zone of the OF, for the first 10 minutes, independent of genotype ($F_{(1, 34)} = 7.50$, $p < 0.01$; Fig 1B). Moreover, ELS-exposed females showed a strongly increased distance travelled in meters ($F_{(1, 35)} = 24.34$, $p < 0.001$) and time spent in seconds ($F_{(1,33)} = 24.84$, $p < 0.001$) in the open arms of the EPM (Figure 1C). Notably, calculating the fold change (FC) of the ELS effect (vs. their respective control group) on EPM parameters revealed that the beneficial effect of ELS was significantly stronger in WT mice than in *Fkbp5*^{Nex} mice (FC ELS effect EPM OA distance: $t_{(17)} = 2.30$, $p < 0.05$; Figure 1D). These data suggest that ELS exposure leads to an anxiolytic phenotype in females, which is significantly dampened in *Fkbp5*^{Nex} mice. In contrast to females, the phenotype induced by ELS and FKBP51 deletion was less pronounced in males. For males, a significant interaction effect was found on distance travelled in the inner zone of the OF ($F_{(1,38)} = 12.91$, $p < 0.05$: Figure S2A), but otherwise no significant effects on anxiety-like behaviour were found (Figure S2B).

Interestingly, interaction effects between ELS exposure and *Fkbp5* genotype were also found for tests assessing memory performance in female mice. In a neutral environment, ELS exposure in females led to a worsened recognition memory, as defined by a lower discrimination of the novel object ($F_{(1, 25)} = 5.65$, $p < 0.05$; Figure 1E), and an interaction effect between ELS and genotype was found for spatial memory function ($F_{(1, 31)} = 4.98$, $p < 0.05$; Figure 1E). This was illustrated by a reduced discrimination between a familiar and novel location of the object (SOR), in which the effect of ELS on spatial memory was only present in *Fkbp5*^{Nex} female mice. Furthermore, for the MWM, a spatial memory task performed under stressful circumstances, a clear interaction effect was observed as well. Remarkably, ELS exposure in females led to beneficial effects on spatial memory performance, but only in *Fkbp5*$^{lox/lox}$ mice (probe trial interaction effect: $F_{(1, 30)} = 4.63$, $p < 0.05$; Probe trial ELS effect: $F_{(1,30)} = 3.65$, $p = 0.067$; 1F). This interaction effect was also reflected in the average latencies to finding the platform location during the 5 training days. In *Fkbp5*$^{lox/lox}$ mice, ELS-exposed females spent significantly less time to finding the platform on the last three training days (post-hoc day 3: $p < 0.05$; day 4: $p < 0.05$; day 5: $p < 0.05$; Figure 1F). This strongly improved memory function following ELS was not observed in *Fkbp5*^{Nex} mice (difference only on training day 3: $p < 0.05$). Male mice, did not show differences on memory performance following ELS exposure, nor any interactive effects were observed (Fig S2C).

Fkbp5^{Nex} genotype interacts with ELS exposure to affect brain volume in female mice

Since the effects of ELS and the interaction between ELS exposure and *Fkbp5* genotype was most prominently present in the female behavioural phenotype we next probed potential structural and functional differences in these mice. To determine whether the observed changes in behaviour are accompanied by volumetric brain differences, female mice underwent an MRI structural scan (ELS: *Fkbp5*$^{lox/lox}$ n = 7; *Fkbp5*^{Nex} n = 10; control: *Fkbp5*$^{lox/lox}$ n = 11 and *Fkbp5*^{Nex} n = 8) subsequent to behavioural testing. A two-way ANOVA DBM analysis was performed and revealed main effects for genotype in GM structures in the right somatosensory cortex, the right visual cortex and in the ventral part of the subiculum ($p_{FWE, cluster} < 0.001$; Figure 2A). Furthermore, WM deformations were found in the bilateral dorsal hippocampal commissure ($p_{FWE} < 0.001$; Figure 2A). In addition to this, a main interaction effect was found within these regions, with exception of the somatosensory cortex (right hemisphere: $p_{FWE, cluster} = 0.007$; left hemisphere: $p_{uncorrected\ clusteer} = 0.024$; Figure 2A). Post-hoc tests showed that ELS exposure, within the *Fkbp5*^{Nex} female mice only, led to reductions in GM volume in the right somatosensory cortex ($p_{FWE, cluster} < 0.007$), visual cortex (right: $p_{FWE, cluster} = 0.007$; left: $p_{FWE, cluster} = 0.005$) and retrosplenial area ($p_{FWE, cluster} = 0.005$) and minor effects within the right caudal hippocampus ($p_{FWE, cluster} = 0.007$; Figure 2B). ELS however, showed a tendency to increase the volume of the third ventricle ($p_{uncorrected, cluster} = 0.039$). Additionally, within female mice that were exposed to ELS, *Fkbp5*^{Nex} mice compared to *Fkbp5*$^{lox/lox}$ mice, had reduced volumes in the right somatosensory cortex, bilateral visual cortex, left retrosplenial area ($p_{FWE, cluster} = 0.007$) and the ventral subiculum ($p_{FWE,}$

$_{cluster}$ < 0.001; Figure 2C). Fkbp5^{Nex} mice that underwent ELS also had smaller volumes in WM structures of the dorsal hippocampal commissure ($p_{FWE, cluster}$ < 0.001; Figure 2C).

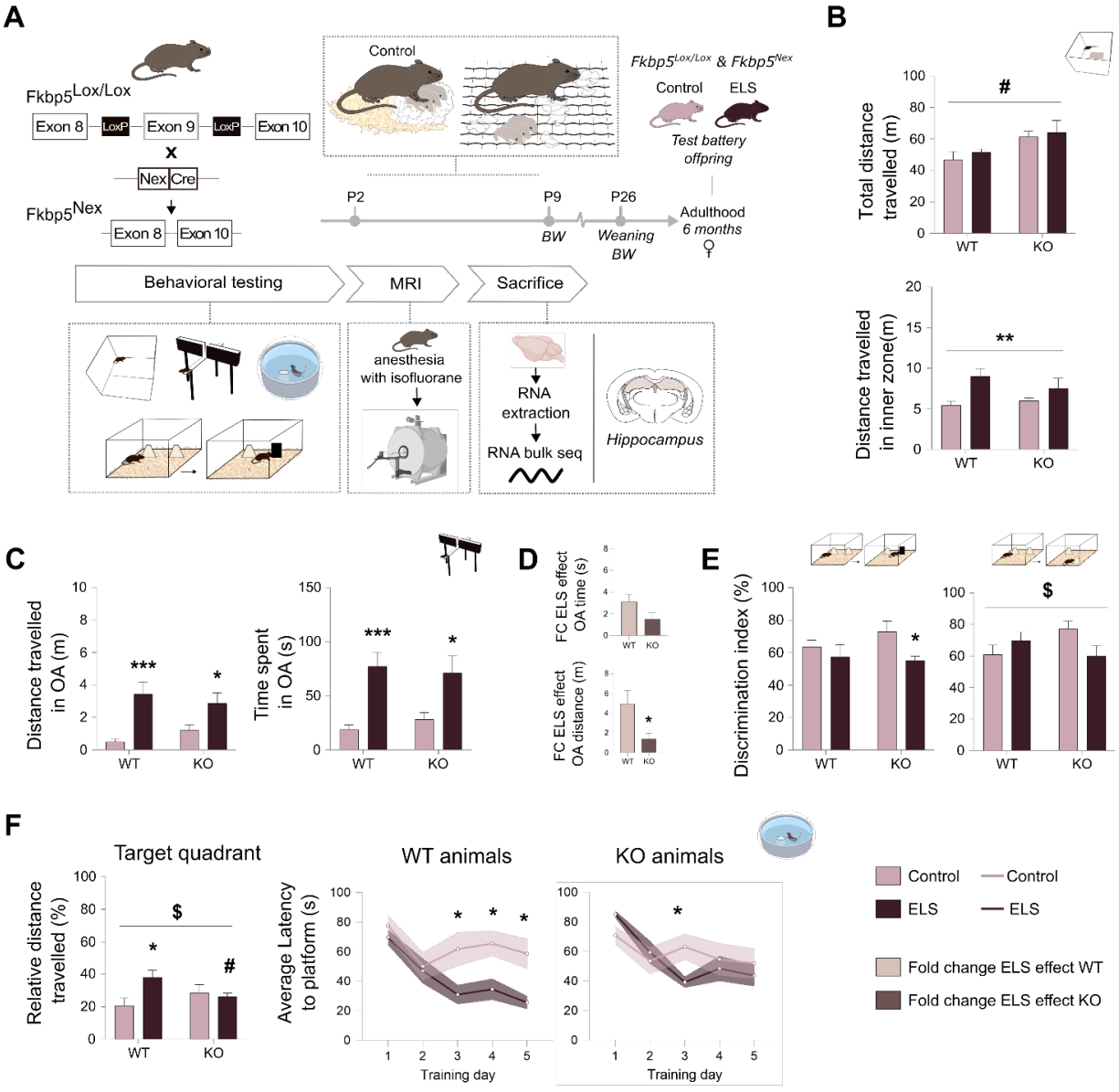

Figure 1. FKBP51 in glutamatergic forebrain neurons and early life stress exposure have an interactive effect on behaviour in female mice.

(A) Female offspring of *Fkbp5$^{lox/lox}$* wild type mice and mice lacking FKBP51 in glutamatergic forebrain neurons (*Fkbp5^{Nex}*) were exposed to a limited bedding and nesting (LBN) early life stress (ELS) paradigm from postnatal day 2 (P2) to P9. At 6 months of age, mice were exposed to a behavioural protocol, including tests assessing anxiety-like behaviour and (spatial) memory performance in a neutral or stressful context. Genotype affected locomotor behaviour of female mice independent of ELS exposure (B). However, data from the open field (OF) test (B) and elevated plus maze (EPM) test (C) revealed a strong beneficial effect of ELS exposure on anxiety-like behaviour. The fold change of the ELS effect (vs. the control condition) on EPM parameters was significantly larger in WT mice than in KO mice (D). Further, memory performance in a neutral context (E) revealed a deteriorating effect of ELS, in interaction with FKBP5 genotype. Interestingly, for assessing memory function in a stressful context (F) with the Morris water maze (MWW), ELS had a beneficial effect on spatial memory performance in Fkbp5$^{lox/lox}$ mice only. Error bars represent mean + S.E.M. *effect of ELS p < 0.05; **effect of ELS p < 0,01; *** effect of ELS p < 0.001; $^{\#}$ effect of genotype p < 0.05; $^{\$}$ interaction effect ELS x genotype p < 0.05.

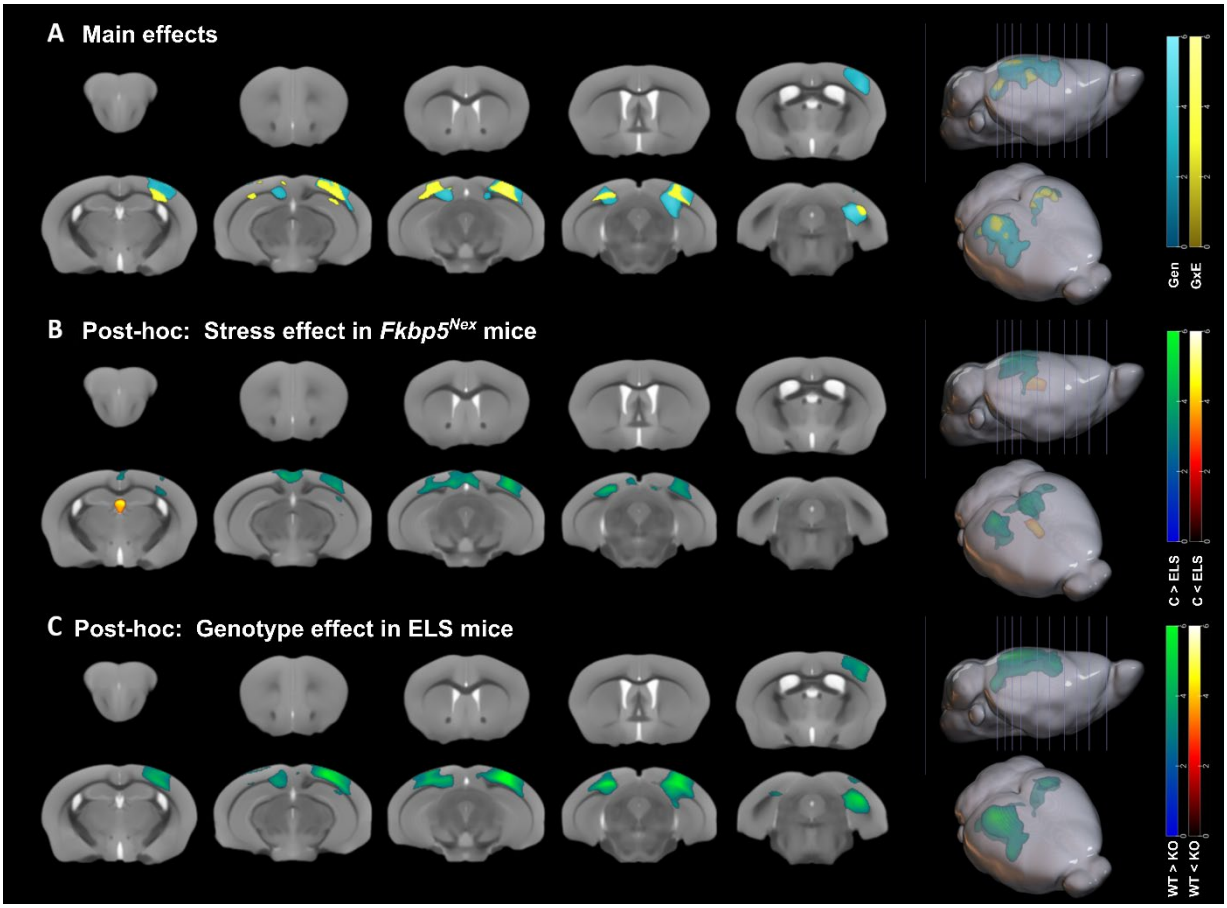

Figure 2. ELS and *Fkbp5* genotype lead to separate and interactive changes in brain volume in female mice.

Deformation based morphology (DBM) analyses of the female brains revealed main effects of genotype (Gen) in GM volumes of the cortex (right somatosensory cortex and right visual cortex) and in the ventral subiculum. Overall WM deformations were found for genotype in the bilateral dorsal hippocampal commissure. In addition, interactive effects of *Fkbp5* genotype and early life stress (ELS) exposure (GxE), were found in various cortical regions (right somatosensory cortex, bilateral visual cortex and bilateral retrosplenial cortex). Post-hoc tests revealed reductions in brain volume following ELS, in $Fkbp5^{Nex}$ mice (KO) only, within bilateral cortical areas and increased volumes in the third ventricle. Furthermore, within mice that underwent ELS, $Fkbp5^{Nex}$ mice had smaller GM volumes in cortical areas and reduced volumes in WM structures of the dorsal hippocampal commissure. Scales represent Z-scores. *WT = wild-type*.

$Fkbp5^{Nex}$ genotype and ELS exposure have interactive effects on hippocampal dendritic morphology and cell-physiological properties in female mice

To investigate the underlying mechanisms of the interactive effects between ELS exposure and *Fkbp5* genotype, that we observed particularly on memory performance, we investigated the neuronal structure of pyramidal neurons in the CA1 area of the hippocampus, by staining brains of a separate cohort of female $Fkbp5^{Nex}$ and $Fkbp5^{lox/lox}$ mice that underwent ELS exposure or a control condition (Figure 3A) and defined both the spine densities (ELS: $Fkbp5^{Nex}$ n = 15 vs. $Fkbp5^{lox/lox}$ n = 12; Control: $Fkbp5^{Nex}$ n = 15 vs. $Fkbp5^{lox/lox}$ n = 15) and the dendritic complexity (ELS: $Fkbp5^{Nex}$ 30 neurons vs. $Fkbp5^{lox/lox}$ 40 neurons; Control: $Fkbp5^{Nex}$ 18 neurons vs. $Fkbp5^{lox/lox}$ 33 neurons). Interestingly, a very similar interactive effect as was observed for cognitive behaviour in a stressful context was also reflected in the CA1 pyramidal neuron structure (apical spines: interaction effect $F_{(1, 48)} = 8.84$, $p < 0.01$, main effect ELS $F_{(1, 48)} = 30.99$ $p < 0.001$; basal spines: interaction effect $F_{(1, 48)} = 5.09$, $p < 0.05$, main effect ELS $F_{(1, 48)} = 23.98$, $p < 0.001$; Figure 3A). ELS exposure increased the number of both apical (p <

0.001) and basal spines ($p < 0.001$) compared to the control condition in *Fkbp5*$^{lox/lox}$ mice, but did not change the number of spines in *Fkbp5*^{Nex} mice. ELS exposure also increased complexity in dendritic branching in the CA1 region of the hippocampus, measured as the number of dendritic intersections (sum of dendritic intersections: main effect of ELS $F_{(1, 117)} = 55.37$, $p < 0.001$; Figure 3B) in both *Fkbp5*$^{lox/lox}$ ($p < 0.001$) and *Fkbp5*^{Nex} mice ($p < 0.01$).

Next, we performed field potential recordings following HFS in the CA1 to test for functional alterations. At first, we investigated the interactive effects of *Fkbp5* genotype and an *ex vivo* stress exposure (Figure 3C), in the form of a corticosterone application to the brain sections in artificial CSF (*Fkbp5*^{Nex}: n = 4 mice, n = 11 brain slices; *Fkbp5*$^{lox/lox}$: n = 5 mice, n = 11 brain slices), vs. a vehicle condition (*Fkbp5*^{Nex}: n = 5 mice, n = 9 brain slices; *Fkbp5*$^{lox/lox}$: n = 6 mice, n = 9 brain slices). Again, we observed an interactive effect between stress exposure and genotype (interaction effect genotype x CORT: $F_{(1, 32)} = 16.84$, $p < 0.001$). When looking at the average fEPSP slope 70 – 80 min following HFS, we found a reduced LTP induction profile following CORT application in *Fkbp5*$^{lox/lox}$ mice ($p < 0.001$), that was absent in *Fkbp5*^{Nex} mice. Following up on this, we studied the effects of ELS exposure (*Fkbp5*^{Nex}: n = 4 mice, n = 11 brain slices; *Fkbp5*$^{lox/lox}$: n = 5 mice, n = 11 brain slices) vs. control mice (*Fkbp5*^{Nex}: n = 5 mice, n = 9 brain slices; *Fkbp5*$^{lox/lox}$: n = 6 mice, n = 9 brain slices) on the LTP induction profiles. Strikingly, the interactive effect between *Fkbp5* genotype and ELS exposure that was already observed on the behavioural and structural neuronal level, was again confirmed on the level of electrophysiological properties. When analysing the average fEPSP slope of the last 10 min of recording (Figure 3D), we found a main effect of ELS exposure on reducing LTP induction profiles ($F_{(1, 36)} = 7.63$ $p < 0.01$) and an interaction effect between ELS exposure and genotype ($F_{(1, 36)} = 6.49$ $p < 0.05$). Post-hoc tests revealed that ELS reduced LTP in *Fkbp5*$^{lox/lox}$ mice ($p < 0.01$), but not in *Fkbp5*^{Nex} mice (Figure 3D). In summary, we observed robust changes upon ELS exposure in wild-type *Fkbp5*$^{lox/lox}$ mice, with beneficial outcomes on the behavioural level, that were not present in mice lacking *Fkbp5* in the glutamatergic forebrain neurons. This interactive nature between *Fkbp5* genotype and (beneficial) ELS effects implies that FKBP51 in glutamatergic forebrain neurons may play a role in the changes that are happening in the brain during stress exposure in early developmental stages.

RNA bulk sequencing reveals TCF4 as a potential target for molecular pathways underlying the interactive effects between *Fkbp5*^{Nex} genotype and ELS exposure

Analyses revealed beneficial effects of ELS exposure on behaviour in female wild type *Fkbp5*$^{lox/lox}$ mice, that were absent in mice lacking FKBP51 in the glutamatergic neurons of the forebrain and the interaction between the *Fkbp5* genotype and ELS exposure was further emphasized in CA1 neuronal structure and electrophysiological properties. These findings suggested an important mediating role for glutamatergic forebrain FKBP51 in the alterations that exposure to stress during early life has on the brain. However, the exact underlying molecular mechanisms still remained unclear. To further unravel molecular processes that may underly these observed changes, we performed a bulk mRNA sequencing in the hippocampus of female *Fkbp5*^{Nex} and *Fkbp5*$^{lox/lox}$ mice. The data revealed a differential expression pattern that was associated with the effect of genotype (13 DEGs upregulated in *Fkbp5*^{Nex} and 33 DEGs downregulated in *Fkbp5*^{Nex}; Figure 4A). For the main effect of ELS, only three significantly downregulated genes were found. However, it has been implicated before that not only the impact of specific individually significant DEGs are important to consider when studying downstream biological effects, but networks of co-expressed genes, that do not individually reach significance, may still be of large importance in impacting behaviour, brain structure and function (Gerstner et al., 2022).

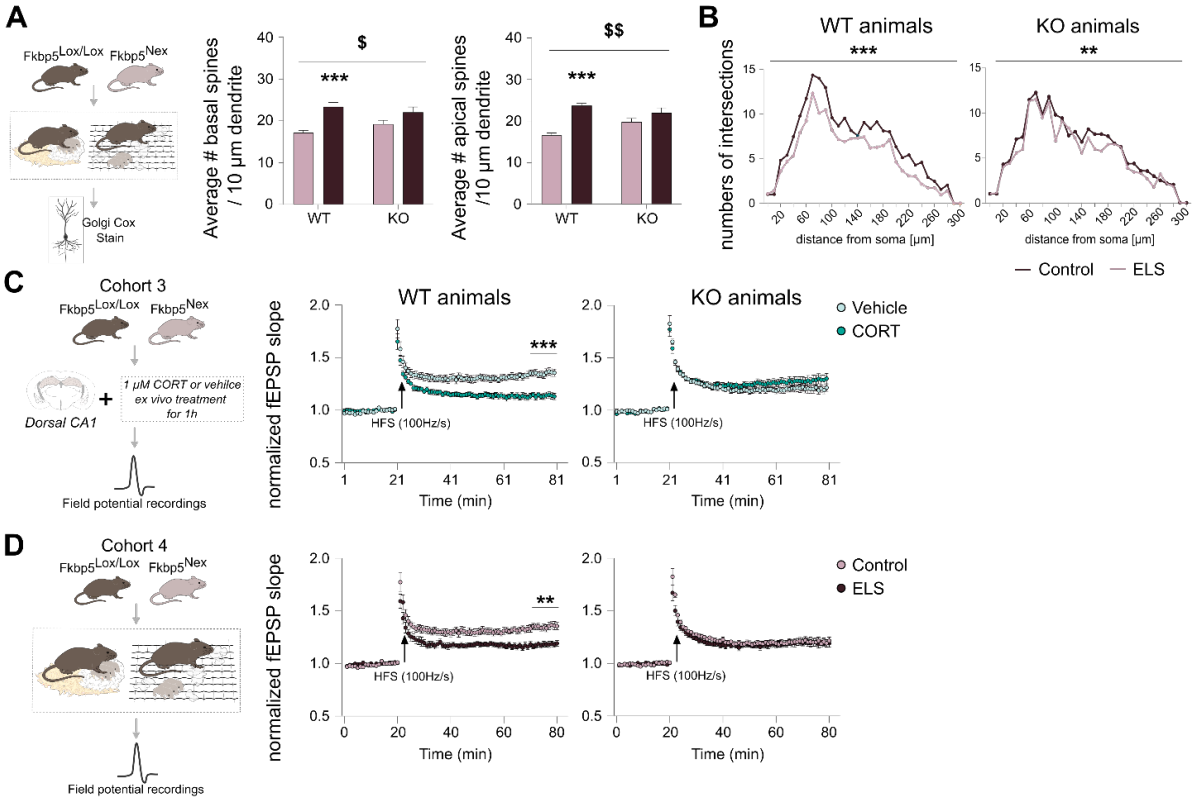

Figure 3. FKBP51 in glutamatergic forebrain neurons and early life stress exposure have an interactive effect on brain structure and function in female mice.

A second cohort (A) of *Fkbp5*^{Nex} and *Fkbp5*$^{lox/lox}$ offspring was exposed to a limited bedding and nesting (LBN) early life stress (ELS) paradigm and a Golgi Cox staining was performed at the hippocampus of 8-month old female mice. A very similar interaction effect as was observed for behaviour, was also found for apical spine densities in pyramidal neurons of the CA1. Furthermore, dendritic complexity (B) of pyramidal CA1 neurons was increased following ELS exposure. In addition to this, data from electrophysiological measurements testing LTP induction profiles, again demonstrated interactive effects of *Fkbp5* genotype with an *ex vivo* stress exposure, in the form of a corticosterone application (C), and with ELS exposure (D). **effect of ELS $p < 0,01$; *** effect of ELS $p < 0.001$; $^{\$}$ interaction effect ELS x genotype $p < 0.05$; $^{\$\$}$ interaction effect ELS x genotype $p < 0.01$.

The previous interactive findings between *Fkbp5* genotype and ELS exposure on behavior, brain structure and function were mainly driven by the ELS effect within wild type *Fkbp5*$^{lox/lox}$ mice and absence of this effect in *Fkbp5*^{Nex} mice. Therefore, the darkorange co-expressed gene network was particularly interesting and was selected for further in-depth analyses. Most genes in this network had a similar direction of effect, with increased expression levels upon ELS exposure in *Fkbp5*$^{lox/ox}$ mice, but no increases in expression within *Fkbp5*^{Nex} mice (illustrated by expression hub genes; Figure S3). Subsequently, we then performed a transcription factor enrichment analyses in the darkorange network (Figure 4C) to find transcription factors that may be the driving force behind this important network of genes. This revealed a total of 10 transcription factors that at least regulated 8 of the 176 genes in the network (Figure S4). To decide which of these transcription factors could be the most important driver of the network in the light of our previous findings, we compared our datasets with other relevant human GWAS datasets from PTSD patients and individuals (both sexes and females only) that had suffered from childhood trauma (Figure 4D). Furthermore, we also overlaid the selected genes with our list of hub genes of the darkorange network, to identify genes that are highly

interconnecting with other genes in the network and may therefore have a strong driving force. From the genes that were regulated by any of the enriched transcription factors, 8 had an overlap with any of the GWAS datasets and hub genes dataset (Figure 4D, Figure S4). The transcription factor that regulated most of the genes that had an overlap with the selected datasets (5 overlap hits; *Foxp2*, *Slc17a6*, *Tcf7l2*, *Zic1* and *Zic4*), was the transcription factor 4 (TCF4) (Figure 4D). Moreover, this transcription factor regulated the only gene that was associated with childhood trauma in females (*Slc17a6*) and it regulated three hub genes in the darkorange network (*Tcf7l2* and *Zic1* and *Zic 4*). Based on these findings, TCF4 could be a potential interesting factor, responsible for underlying mechanisms of the interactive effects that we observed between ELS exposure and *Fkbp5* genotype.

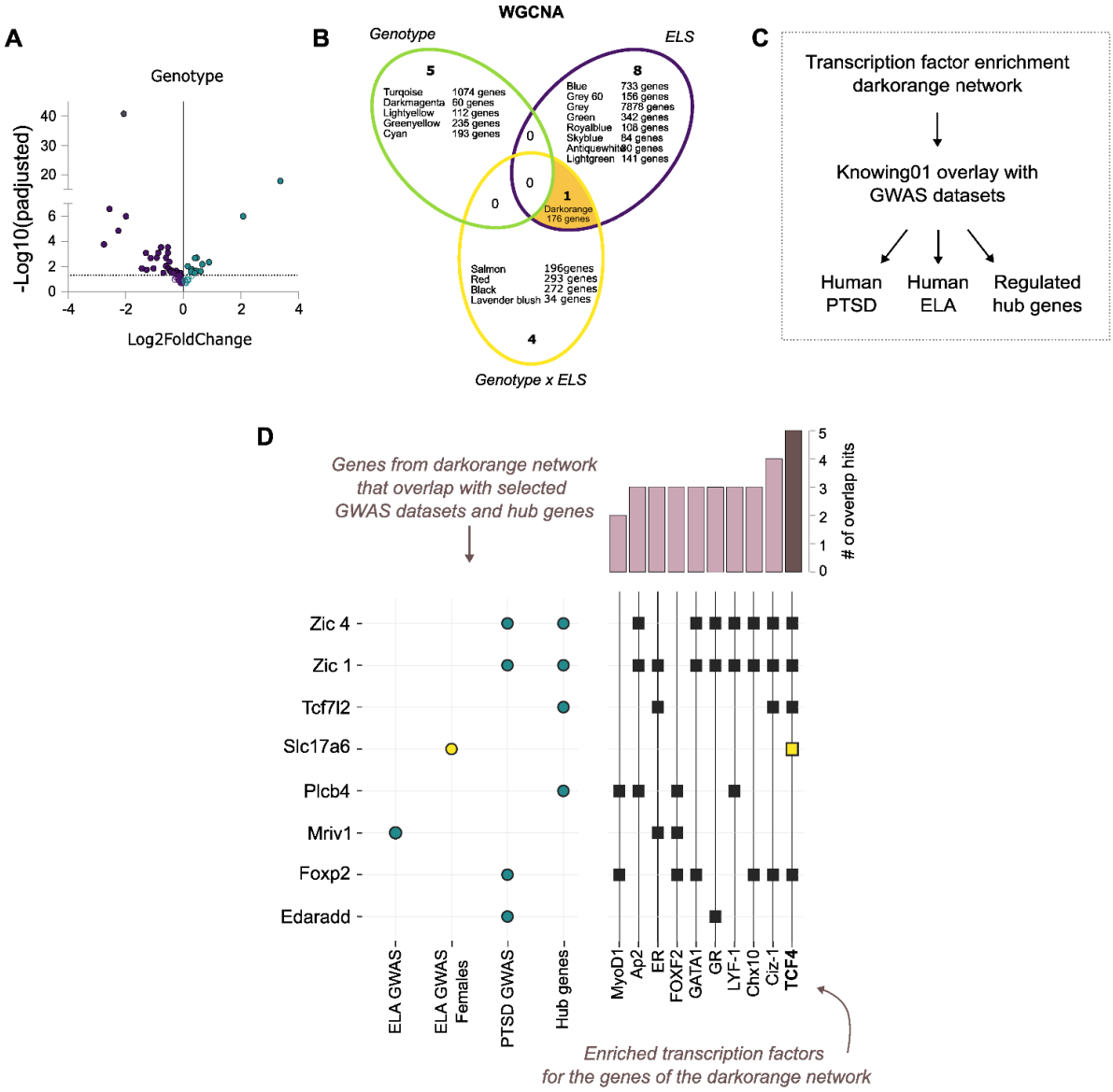

Figure 4: RNA bulk sequencing reveals transcription factor 4 as a potential regulator of early life stress-induced effects on the brain that interact with FKBP51 in glutamatergic forebrain neurons.

RNA bulk sequencing was performed on the hippocampus of female mice of the first cohort. (A) A clear differential expression profile was found for the effects of genotype. Furthermore, a weighted gene co-expression analysis (WGCNA) revealed 18 co-expressed gene networks that were associated with effects of genotype, ELS exposure or their interaction (B). One of these networks was associated not only with ELS

exposure, but was also associated with the interaction of ELS and *Fkbp5* genotype. (C) Subsequently, a transcription factor enrichment analysis was performed for the darkorange network, which resulted in 10 enriched transcription factors. Using the software Knowing01, all genes that are regulated by the enriched transcription factors were overlaid with datasets from human psychiatric GWAS studies and the hub genes of the darkorange network (D). The right panel D shows which genes are regulated by specific enriched transcription factors and their resulting (indirect) overlap with the datasets. This revealed that the transcription factor 4 (TCF4) regulates the largest number of genes that had an overlap with any of the datasets. Moreover, it is the only enriched transcription factor that regulates a gene that was associated with early life adversity in females (Slc17a6; yellow dot).

Viral overexpression of *Tcf4* in glutamatergic neurons of the hippocampus leads to changes on behaviour

From the RNA bulk sequencing data and follow-up analyses, TCF4 was identified as a potentially important underlying regulator of the ELS-induced effects that were observed in *Fkbp5*$^{lox/lox}$ mice, but were absent in *Fkbp5*^{Nex} mice, by leading to a hyperstimulation of (at least part) of the darkorange co-expressed gene network. However, a direct link between the ELS induced- phenotype and over-stimulation of the darkorange network by TCF4, remained speculative. Based on the strongest interactive phenotype in stressed cognition in the previous cohort, we overexpressed Tcf4 in the CA1, specifically in the glutamatergic neurons by bilateral injections with a Cre-dependent *Tcf4* OE AVV virus (n = 15) vs. a GFP expressing control AVV virus (n = 15) in 5 months-old female mice. Interestingly, in line with what we found for *Fkbp*$^{lox/lox}$ mice that were exposed to a moderate ELS exposure, we found that mice that had an OE of *Tcf4* in the glutamatergic neurons of the hippocampus showed reduced anxiety-like behaviour. This was reflected by an increased time spent in seconds in the open arms on the EPM ($W = 64$, $p < 0.05$) and a reduced latency towards the first entry into the open arm in seconds ($W = 149.5$, $p < 0.001$), whereas locomotor behaviour was unaffected. Moreover, Tcf4 OE also had a similar effect on spatial memory performance under stressful conditions as moderate ELS exposure. We found that on the 3rd training day of the MWM Tcf4OE mice had significantly shorter latencies to finding the platform location than mice injected with the control virus (repeated measures ANOVA; post hoc (t): $p < 0.05$), indicating an improved learning of the spatial location. However, control animals did catch up to Tcf4 OE mice on the 4th training day. These data indicate that TCF4 in the glutamatergic neurons of the hippocampus, at least in part, contribute to the effects that ELS exposure has on behaviour.

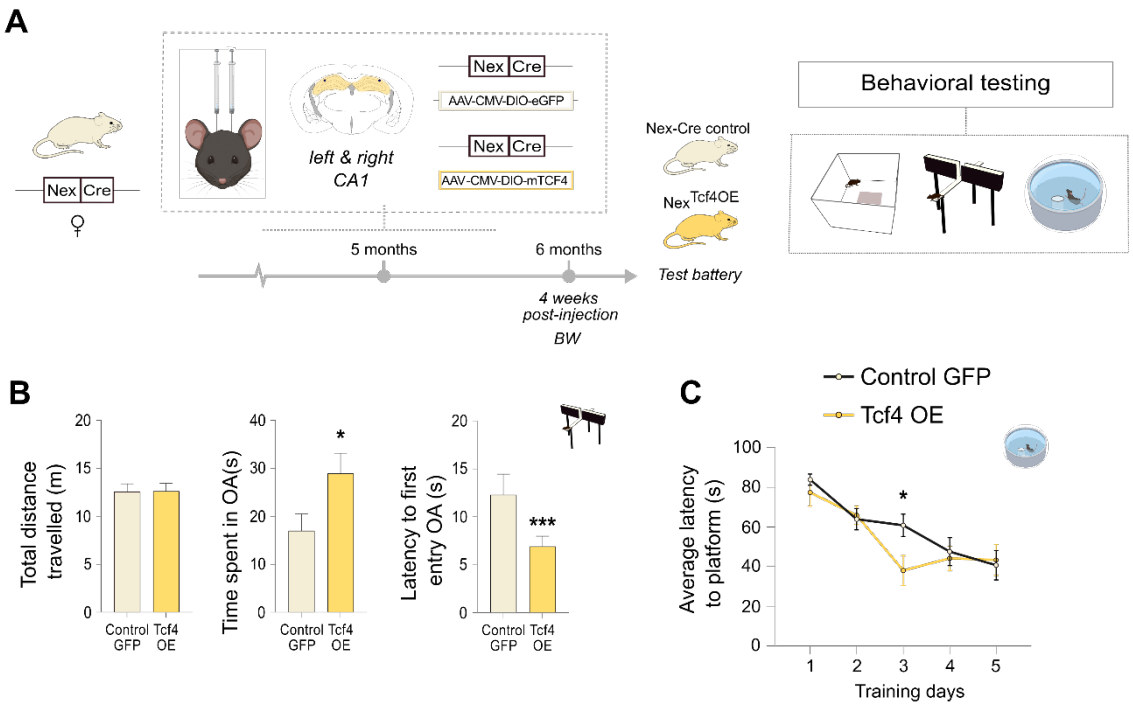

Figure 5. Tcf4 overexpression leads to similar beneficial effects on behaviour as ELS exposure in *Fkbp5*$^{lox/lox}$ mice.

To investigate whether TCF4 in glutamatergic neurons of the hippocampus is indeed the underlying molecular target regulating the beneficial effects of ELS exposure on behaviour, we overexpressed *Tcf4* in these neurons by injecting an AVV Cre-dependent *Tcf4* overexpression (OE) virus in Nex-Cre female mice of 6 months of age and compared them to female mice that were injected with an AVV GFP control virus (A). Interestingly, TCF4 OE indeed leads to (B) an anxiolytic phenotype on the elevated plus maze (EPM) test, like was observed upon ELS exposure in *Fkbp5*$^{lox/lox}$ mice. Furthermore, data from the MWM showed that TCF4 OE mice had an improved memory of the platform location on the 3rd training day, which was a less strong, but similar effect, to as was observed for ELS-exposed female *Fkbp5*$^{lox/lox}$ mice. Error bars represent mean + S.E.M. *p < 0.05; **p < 0,01; ***p < 0.001.

Discussion

Psychiatric disorders often arise as a combination of environmental and genetic factors and early life adversity has frequently been described as a risk factor for developing psychiatric disease (Caspi & Moffitt, 2006; McKay et al., 2021). Nevertheless, ELS can also lead to adaptive changes that prepare an individual to cope with future life events (Gluckman et al., 2005; Nederhof & Schmidt, 2012). The *FKBP5* gene is a psychiatric risk factor that is known to interact with ELS exposure (Klengel et al., 2013; Zannas & Binder, 2014). However, the exact underlying mechanisms behind this interactive effect are still poorly understood. Moreover, FKBP51 functionality is cell-type specific and largely dependent on sex (van Doeselaar et al., 2023). Unfortunately, up to date, there is still a scarcity in information on the effects of ELS and FKBP51 functionality in the female sex. This study demonstrated that FKBP51 in glutamatergic forebrain neurons mediates (beneficial) effects of a moderate ELS exposure on emotional regulation, cognitive functioning and brain volume, particularly in females, and that this was associated with similar interactive effects on neuronal structure and function. Furthermore, we propose that TCF4 is an underlying regulator of the FKBP51-mediated effects of ELS exposure on brain and behavior.

In this study, we provide evidence that moderate ELS results in anxiolytic behavior and improves spatial memory performance in a stressful context and this effect is dependent on the presence of FKBP51 in glutamatergic forebrain neurons. Interestingly, the FKBP51-dependent beneficial effects of ELS are predominantly present in female mice. A bulk in research is available on the effects of ELS exposure on brain and behavior (Bonapersona et al., 2019; Chen & Baram, 2016; Krugers et al., 2017; Walker et al., 2017; D. Wang et al., 2020) and the majority of these findings show that ELS can lead to negative outcomes on brain structure, brain function and behavior. However, effects of ELS are highly dependent on a number of factors, amongst others the age of the animal, the type, severity or duration of the early life stressor or the context in which the test takes place (Champagne et al., 2008; Naninck et al., 2015; D. Wang et al., 2020). A number of studies have shown that moderate exposure to ELS can also result in beneficial alterations in brain function, neuroendocrine responses and behavior (Champagne et al., 2008; Lyons et al., 2009; Parker et al., 2004, 2005). Moreover, an extremely important factor to consider is the sex of the animal (Loi et al., 2017; Oomen et al., 2009; Samplin et al., 2013). In the past decades it has become increasingly clear that sex can have a tremendous effect on stress resilience and vulnerability. Nevertheless, there is still a large gap in female research and studies investigating the effect of ELS exposure are no exception. In a recent meta-analysis on early life adversity, Joëls and colleagues were unable to perform a quantitative analysis on the female data, due to a too scarce availability of female studies (Joëls et al., 2022). It is therefore not strange that ELS in females might result in differential outcomes as traditionally described in males. FKBP51 functionality has also been shown to be heavily dependent on sex (van Doeselaar et al., 2023). In line with our results, previous work has already demonstrated a sex-dependent interaction of FKBP51 and ELS on emotional behavior. A study by Criado-Marrero and colleagues found that overexpression of FKBP51 amplified anxiogenic effects of maternal separation stress and this effect was more pronounced in female mice (Criado-Marrero et al., 2019). Unlike the study by Criado-Marrero and colleagues, we found beneficial effects on anxiety and cognition in a stressful context following ELS exposure, but we also found the effects to be predominantly present in female mice. The differences in outcomes on behavior between our study and the study by Criado-Marrero and colleagues might be explained by the use of a different ELS paradigm. Interestingly, we found opposing effects of ELS exposure on cognitive behavior, depending on the context the test was performed in. Unlike for spatial memory functioning in a stressful context, we found that in a neutral environment ELS lead to a worsened memory function. Notably, opposing to findings from the stressful memory task, effects were exacerbated in *Fkbp5*^{Nex} mice. Such dependency on the environmental context has been described previously in relation to cognitive behavior (Abrari et al., 2009; Joëls et al., 2006). In fact,

these findings would be in line with the stress inoculation hypothesis that proposes that moderate ELS prepares for future matching life events. Illustrating this, in this study the moderate ELS exposure resulted in detrimental effects in an "unmatched environment" (memory task in neutral context), but in beneficial effects in a "matched environment" (stressful memory task)(Nederhof & Schmidt, 2012) compared to the unstressed control group.

In addition to the changes on behavior, we also observed interactive effects of ELS and FKBP51 in glutamatergic forebrain neurons on brain volume in female mice. ELS was found to result in volumetric reductions in several GM and WM structures, amongst others, in different cortical regions, the ventral subiculum and the dorsal hippocampal commissure and these effects of ELS were exclusively present in *Fkbp5*^{Nex} mice. Spatial memory function is strongly linked to activity in the dorsal hippocampus (Moser et al., 1995). However, the MRI results indicated that our behavioral effects in female mice could not directly be linked to GM volume changes in this brain region. Nevertheless, volumetric alterations in the fiber tracts of the dorsal hippocampal commissure in stressed *Fkbp5*^{Nex} mice can also be linked to memory function (Postans et al., 2020). The dorsal hippocampal commissure is a WM structure that is responsible for interhemispheric connections between the temporal regions (Postans et al., 2020). It therefore plays an important role in the communication between the hippocampus and other temporal lobe regions, such as the amygdala, a brain region that is heavily implicated in fear and anxiety behavior. It was previously demonstrated that the dorsal hippocampal commissure was associated with recognition memory (Postans et al., 2020). Therefore, volumetric reductions in this WM tract would match with the observed impaired memory performance on the novel object recognition task, an effect that was specifically present in ELS exposed *Fkbp5*^{Nex} female mice. Alternatively, one could speculate that the adaptive changes by ELS, resulting in enhanced spatial memory performance in a stressful context, are not occurring in *Fkbp5*^{Nex} mice and this is reflected by the observed reduced GM and WM volumes. Another interesting finding was the reductions in the ventral subiculum in ELS exposed *Fkbp5*^{Nex} female mice. The subiculum is most commonly known as an integrator for the output of hippocampal information to other brain regions, however, it has a segregated functionality along the dorsoventral axis (O'Mara, 2005). Where the dorsal part of the subiculum is thought to be involved in the processing of spatial memory information, the ventral subiculum is implicated in HPA-axis feedback. More specifically, via glutamatergic ventral subiculum output neurons, the hippocampus dampens the stress-induced glucocorticoid release, by connecting to neurons in the paraventricular nucleus of the hypothalamus (Herman & Mueller, 2006). It is therefore unsurprising that interactions between ELS exposure and FKBP51 in glutamatergic neurons, which primary function is to regulate GR sensitivity, are associated with changes in this region.

Complementing the interactive findings of ELS and FKBP51 in glutamatergic forebrain neurons on memory performance, we found highly similar interactive patterns on dorsal CA1 pyramidal neuronal structure and function. ELS improved spatial memory performance in a stressed context in wild-type female mice and, strikingly, ELS also exclusively increased the spine density in CA1 pyramidal neurons in *Fkbp5*$^{lox/lox}$ mice. Furthermore, we found that both an *ex vivo* glucocorticoid administration and ELS exposure reduce LTP activity in the dorsal CA1 in WT mice, but not in *Fkbp5*^{Nex} mice. Even though at this point, the observed improved stress-related cognition, increased spine density and decreased synaptic plasticity remains elusive, the direct dependence of these stress-induced alterations on glutamatergic FKBP51 function is highly apparent. Our data clearly indicate that, independent of the directionality, early life adversity effects in female mice are dependent on FKBP51 in glutamatergic neurons. The data further support previous findings that reduced synaptic transmission can be associated to enhanced spatial memory performance (Hung et al., 2008) and that ELS effects on cognition and LTP are highly dependent on the test context (Champagne et al., 2008).

Based on the findings from the RNA sequencing, we identified one network of genes that could be the driving force behind of the FKBP51-mediated effects of ELS on the behavior of female mice.

Interestingly, we found TCF4 to be an important enriched transcription factor of this network of genes. A follow up study showed that enhanced TCF4 activity in glutamatergic neurons of the hippocampus, on its own, is sufficient to induce highly similar beneficial effects on anxiety and spatial memory in a stressful context as was observed with moderate ELS exposure. Thus, we propose TCF4 as an underlying regulator of the observed beneficial FKBP51-mediated ELS effects. TCF4 belongs to the helix-loop-helix protein family that can bind DNA as homo- or heterodimers at the E-box sites and thereby regulate transcription of a number of target genes (Teixeira et al., 2021). It has amongst others been implicated in neurogenesis (Fischer et al., 2014), been shown to affect neuronal morphology (D'Rozario et al., 2016) and is involved in memory and learning processes and associated neuronal activity (Kennedy et al., 2016). More specifically, mice with a knockdown of TCF4 were presented with spatial memory deficits on the MWM and this was accompanied by improved LTP in the CA1 of the hippocampus. Furthermore, TCF4 has been associated with oligodendrocyte functioning and myelination processes (Phan et al., 2020), which could reflect the observed changes in WM structures. TCF4 has already frequently been implicated in a number of psychiatric and neurological disorders such as schizophrenia, bipolar disorder, MDD, PTSD and autism (Teixeira et al., 2021; Zavala et al., 2009). Interestingly, childhood maltreatment is a major risk factor for MDD and PTSD (Kessler, 1997; Koenen et al., 2007). Thus, we here provide compelling evidence that TCF4-mediated transcriptional regulation, specifically in females, might drive a pro-resilient phenotype.

Taken together, this study showed that, particularly in females, ELS has adaptive effects on behavior by inducing structural and functional changes in the hippocampus. These underlying alterations in neuronal morphology and electrophysiological properties of pyramidal CA1 neurons and GM and WM changes in cortical and subcortical regions are dependent on the presence of FKBP51 in glutamatergic neurons. In part, these FKBP51-dependent changes are regulated via an augmented transcriptional drive of a network of genes by the TCF4 transcription factor. This study provides novel insights in how ELS can affect behavior in an adaptive manner and proposes highly interesting targets for further research towards mechanisms of ELS resilience.

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Contributions

LvD and MVS conceived the study and designed the experiments. LvD performed all animal and MRI experiments and was responsible of analyses and/or interpretation of the behavioural and molecular data. AA, TS, DM, SM, HY, RH, JB, SN and JPL assisted in the (animal) experiments. TS and MC assisted in the MRI animal work, analysed the MRI data and provided scientific advice on the technical aspects and experimental design of the MRI experiments. JMD provided scientific expertise for establishing genetic mouse lines. GH and JAK performed analysis of the RNA sequencing data and JPL provided scientific advice for the interpretation of the RNA bulk sequencing data. LvD wrote the initial draft of the manuscript and MVS supervised the research.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary figures

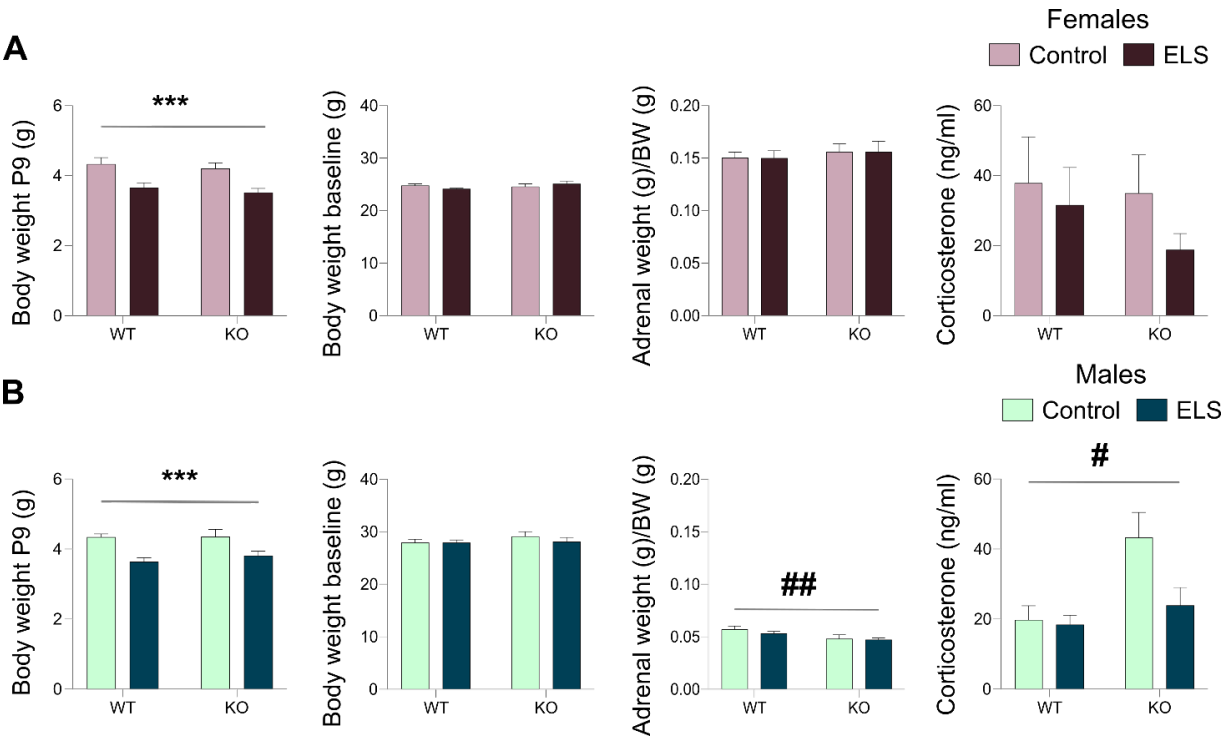

Figure S1. Differential physiological effects of early life stress exposure and loss of FKBP51 in glutamatergic forebrain neurons in male and female mice.

Male and female offspring, including *Fkbp5*^{Nex} and *Fkbp5*$^{lox/lox}$ mice, underwent a limited bedding and nesting (LBN) early life stress (ELS) exposure from postnatal day 2 (P2) to P9 and in female this led to a reduced body weight at the end of the procedure in both genotypes (A). In adulthood however, body weight at the start of the experiment and adrenal weight and baseline corticosterone concentrations remained unaffected by both ELS exposure and genotype. Male mice also had reduced body weight as a result of ELS exposure at the end of LBN paradigm, but in adulthood body weight was unaffected. A main effect of genotype was however found in both adrenal weight and for baseline corticosterone levels. Error bars represent mean + S.E.M. *** effect of ELS $p < 0.001$, $^{\#}$ effect of genotype $p < 0.05$; $^{\#\#}$ effect of genotype $p < 0.01$.

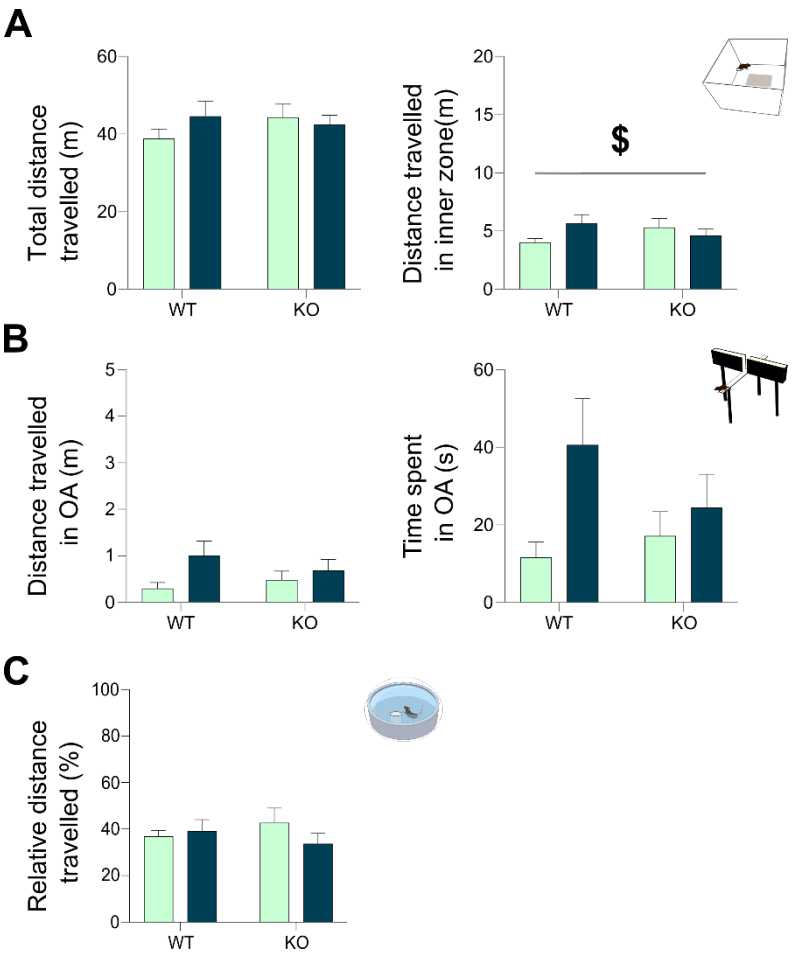

Figure S2. **Effects of early life stress and loss of FKBP51 in glutamatergic forebrain neurons on anxiety-like behaviour and cognitive memory function in male mice.**

Male offspring, including *Fkbp5*^{Nex} and *Fkbp5*$^{lox/lox}$ mice, underwent a limited bedding and nesting (LBN) early life stress (ELS) exposure from postnatal day 2 (P2) to P9 and were tested on a number of behavioral tests in adulthood. Locomotor behaviour of male mice remained unaffected (A), however an interaction effect between ELS exposure and *Fkbp5* genotype was found for distance travelled in the inner zone of the open field test. However, (B) when further investigating anxiety-like behaviour in the elevated plus maze (EPM), no significant differences of ELS exposure and genotype or their interaction were found. Neither ELS exposure, nor genotype affected spatial memory performance under stressful environments (C). Error bars represent mean + S.E.M. $^{\$}$ interaction effect ELS x genotype $p < 0.05$.

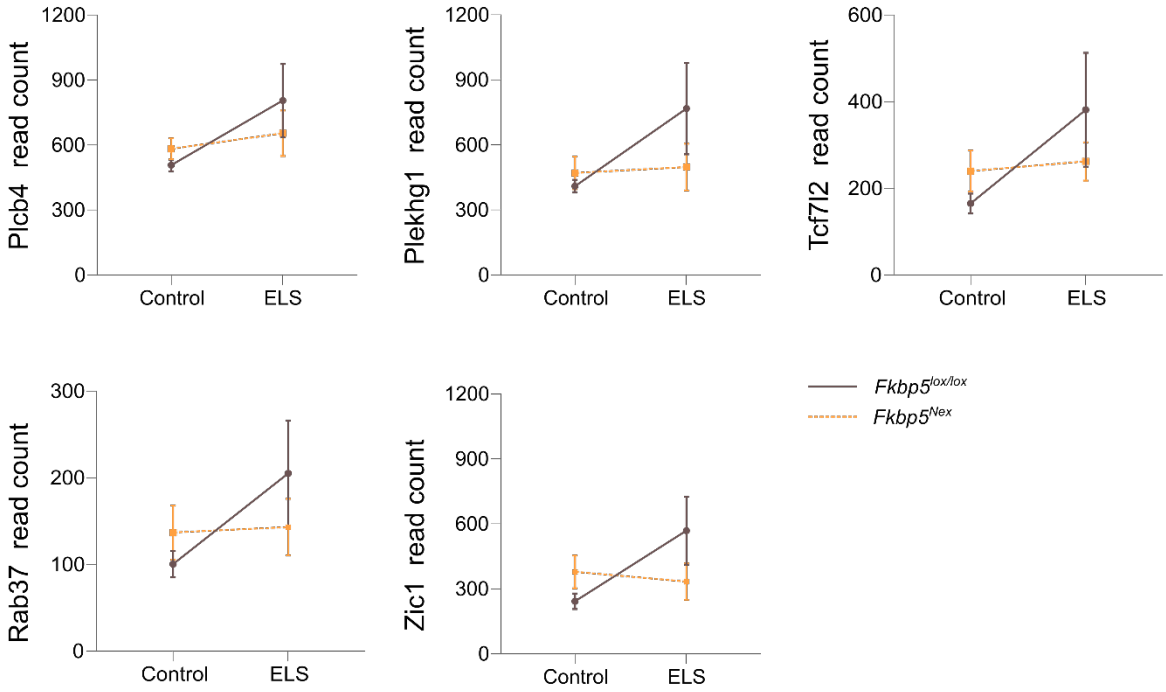

Figure S3. Hub genes of the darkorange network.

The five hubgenes of the darkorange network from the WGCNA analyses, that was associated to early life stress (ELS) exposure and ELS x genotype interaction, showed a similar expression pattern. Upon ELS, gene expression was upregulated in wild-type *Fkbp5*$^{lox/lox}$ animals, whereas gene expression in *Fkbp5*^{Nex} animals was more stable following ELS.

Transcriptional regulator	Genes from darkorange network regulated by transcriptional regulator
Lymphoid transcription factor 1 (LYF-1)	Smyd2, Rasa3, Zic1, Ephb1, Zic4, Ptpn3, Plcb4, Rnf152, Grm4, Tnnt1
Chx10	Adarb1, Cacna2d4, Dcc, Ephb1, Foxp2, Fzd10, Grm4, Opn3, Prkch, Rasa3, Rgs16, Rora, Secisbp2l, Slitrk6, Wnt3, Zic1, Zic4
Forkhead box F2 (FOXF2)	Ephb1, Foxp2, Mrvi1, Plcb4, Prkch, Rasgef1b, Rora, Slitrk6, Zfhx3
Glucocorticoid receptor (GR)	Adgrg6, Ank3, Edaradd, Ephb1, Pcdh9, Rnf152, Rora, Zic1, Zic4
Estrogen receptor (ER)	Ccdc136, Hcn4, Kcnj12, Mrvi1, Rasa4, Tcf7l2, Wnt3, Zfhx3, Zic1
Erythroid transcription factor (GATA1)	Ank3, Foxp2, Grm4, Plcxd2, Rora, Slc8a3, Zic1, Zic4
Transcription factor 4 (TCF4)	Foxp2, Nexn, Pcdh9, Slc17a6, Tcf7l2, Wnt3, Zic1, Zic4
AP2	Ccdc136, Ephb1, Grm4, Plcb4, Rgs16, Slitrk6, Wnt3, Zic1, Zic4
Myoblast determination protein 1 (MyoD1)	Ank3, Cacna2d4, Dbndd1, Ephb1, Foxp2, Fzd10, Hhatl, Kcnj12, Lef1, Pcdh9, Plcb4, Rasa4, Rasgef1b, Rgs16, Slc8a3, Wnt3, Zfhx3, Zscan25
Cas interacting zinc finger protein (CIZ)	Foxp2, Grm4, Pcdh9, Rims3, Slc8a3, Tcf7l2, Zic1, Zic4

Figure S4. Enriched transcription factors of the darkorange network and their regulated genes

A transcription factor enrichment analysis of the darkorange network revealed 10 enriched transcription factors. This figure shows the different enriched transcription factors and their targets from the darkorange network. These target genes from the darkorange network were later overlaid with different human psychiatric GWAS datasets and the dataset with hub genes from the darkorange network. The genes that are highlighted were found to have an overlap with any of the selected datasets.

3. General discussion

Resilience to stress depends on numerous different factors that can be of genetic or environmental nature, leading to strong inter-individual differences in how one deals with a certain life challenge. In the past decades, increasing focus has been put on the interactions between stress, genetics and other environmental factors in the risk for developing stress-related disease (Belsky et al., 2009; Matosin et al., 2018). In the light of these GxE interactions, FKBP51 has gained special attention as an important modulator of (early life) stress outcomes. In addition, FKBP51 is also known to be greatly influenced by age (Blair et al., 2013; Klengel & Binder, 2015; Sabbagh et al., 2014). Another factor that strongly impacts the effects of stress on brain and behaviour is sex. The topic of sex differences has gained rising attention in recent years, but it has long been overlooked, leaving a large gap in information on sex-specific stress resilience mechanisms, particularly within the female sex. In this doctoral thesis, the urgency of applying stress models in rodent research that are comparable between sexes was addressed (Chapter 2.1). Sex-differential effects were further emphasized in the second study (Chapter 2.2) in which not only sex-dependent, but also cell-specific effects of the stress-responsive co-chaperone FKBP51 were demonstrated in an aged sample. At last, the cell-specific contributions of FKBP51 to the consequences of a moderate ELS exposure were investigated. It was demonstrated that glutamatergic forebrain FKBP51 interacts with moderate ELS to increase stress resilience, particularly in females, and structural, functional and molecular mechanisms that are - at least in part - responsible for this interactive effect were proposed (Chapter 2.3). Overall, this work underlines the importance of FKBP51 in (early life) stress resilience and vulnerability mechanisms. It also particularly emphasizes the cell-type specific nature of these interactions and the strong impact of sex on these processes.

3.1 The importance of considering sex differential effects in stress research

With MDD and anxiety disorders being twice as common in woman as in man (Eid et al., 2019; Ferrari et al., 2013; Heo et al., 2008; Kessler, 1994) it seems obvious that sex is a strongly modulating factor in the context of stress-related disorders. Nonetheless, the topic of sex differences has been drastically under-addressed in the stress research field for a long period of time. A bulk of evidence can be found on the effects of stress exposure on the brain and behaviour, but especially in the field of preclinical neuroscience, only 20% of the studies reports to include both sexes (Beery, 2018). There might be several reasons for this lack in female-inclusive studies. Firstly, it has long been thought that adding females would increase variability to the data as compared to male-only samples, because of the interference of the estrus cycle. However, recent studies have shown that this is a false conception as, in fact, males have a slightly, but significantly higher variability than females (Beery, 2018). Another reason for only including males, is that certain universally applied stress paradigms are not easily applicable in the female sex. An example of such a stress model is the CSDS paradigm, that is a well-established model in males and has widely been used in the field to test for consequences of social chronic stress (Golden et al., 2011; Karamihalev et al., 2020; Nestler & Hyman, 2010; Wagner et al., 2011). This stress model relies strongly on the naturally aggressive behaviour of a dominant, resident male mouse towards its male intruder. Since male-female or female-female aggression is typically absent under standard laboratory settings, the practical applicability of this paradigm in females was long difficult. Even though CSDS models have been applied in females before, they often required complex adjustments to the paradigm (Bourke et al., 2012; Haller et al., 1999; Takahashi et al., 2017). However, in 2018, Harris and colleagues introduced a hands-on protocol of CSDS in female mice, with a highly identical procedure as the CSDS paradigm that is traditionally applied in males (Harris et al., 2018). Nonetheless, a broad characterization of the female CSDS-induced phenotype was still lacking. In chapter 2.1 we further established sex-differential effects of CSDS. We could demonstrate that

female mice showed classical physiological changes, comparable to males, following CSDS exposure, such as an increased body weight and relative adrenal weight. This confirmed once more that the novel paradigm indeed leads to a stressed phenotype in female mice. Notably, neither studies using other CSDS paradigms in females, nor the study by Harris and colleagues, could demonstrate such classic changes as a result of CSDS (Bourke et al., 2012; Harris et al., 2018; Newman et al., 2019; Takahashi et al., 2017). Interestingly, we also observed clear sex-specific changes upon CSDS exposure that had not been previously identified. Females had no basal endocrine changes, following CSDS stress, nor an elevated CORT-response towards an acute stressor. Remarkably, female mice even had an improved negative feedback regulation after acute stress. Moreover, unlike males, social behaviour of the females was not affected by CSDS. Some discrepancies with the originally described model were however observed, for example in social behaviour. To study social avoidance behaviour, Harris and colleagues used a classic social avoidance test in which the experimental mouse was placed in an open field and was able to interact with or to avoid a novel CD1 male mouse, that looked identical to the actual aggressor. This test is often used to make a distinction between CSDS-resilient (interactors) and CSDS-vulnerable (avoiders) mice. Interestingly, a very recent study suggested that a more detailed discrimination between actual resilient, adaptive behaviour and susceptible, generalized avoidance behaviour can be made by using a modified version of the social avoidance test, called the social-threat-safety test (STST) (Ayash et al., 2023). In the original social avoidance test as used by Harris et al. mice only have the option to explore a CD1 mouse (considered resilient behaviour) or avoid the mouse (considered susceptible behaviour). In the STST test, the mouse gets the choice to explore a CD1 male that is very similar to the aggressor in one chamber (considered impaired aversive learning), but is additionally able to explore a mouse from a different strain in another chamber (considered discriminative avoidance and resilient behaviour) or to not explore any of the mice (considered indiscriminative, generalized avoidance and susceptible behaviour). It would be highly interesting to gain further insights in CSDS resilience behaviour in females with this new model. Altogether, this chapter clearly underlines the need for establishing stress protocols that are applicable in both sexes, so that sex-differential effects of stress resilience and vulnerability mechanisms can continue to be investigated.

Sex differences were further emphasized in Chapter 2.2., which revealed strong sex-differential outcomes of manipulation of the stress-responsive co-chaperone FKBP51. In this chapter, older-aged male and female mice of two different conditional knockout lines underwent a number of behavioural tasks and structural MRI scanning. Adding to this, mechanisms underlying sex-specific changes were followed up with molecular analyses. Fascinatingly, we found a clear sex-dependent phenotype of loss of FKBP51 in the older-aged sample, that was reflected on several levels in the brain. Results from behavioural tests showed that loss of FKBP51 in select neurons of the brain predominantly resulted in changes in anxiety-like behaviour in females, whereas males had specific alterations in the cognitive domain. It is not surprising that female mice were more affected on the anxiety domain, as findings from demographic studies in humans have extensively shown that anxiety disorders are twice as common in women (Kessler, 1994; Otten et al., 2021). Interestingly, these sex-dependent effects were also evident in structural brain analyses of mice lacking FKBP51 in GABAergic neurons of the forebrain. Alongside a number of cortical areas, the thalamus and white matter structures, structural brain deformations were found in the BNST, a brain region that has been prominently associated with sustained fear and anxiety states (Davis et al., 2010; Lebow & Chen, 2016; Yassa et al., 2012), of female mice. Moreover, strong volumetric changes in the bilateral hippocampus were found in males. This brain region is known to be heavily involved in (spatial) memory formation (Andersen, Per et al., 2007) and this matches the observed predominant cognitive phenotype within the male sex. Strikingly, when investigating the molecular mechanisms behind this sex-dependent phenotype, the sex-distinctive effects were once more emphasized. In female mice that lacked FKBP51 in GABAergic neurons of the

forebrain, a robust differential expression pattern was explicitly found in the BNST, whereas extensive differential gene expression in males was strictly present in the DHC. Notably, the differential transcriptomic profile of females was primarily associated with immune-related functioning, whereas the top regulated genes in the male sample were implicated in memory and learning or linked to presynaptic function and autophagy changes, in line with the behavioural findings. The role of FKBP51 in stress resilience and vulnerability has been widely studied before. Studies using the FKBP51 inhibitor SAFit2 (Hartmann et al., 2015) or region-specific genetic manipulation of FKBP51 (Engelhardt et al., 2021) had already demonstrated its implications in anxiety-like behaviour. In addition, it was also previously described that FKBP51 manipulation could result in memory and learning deficits (Criado-Marrero et al., 2019) and resulted in decreased neuronal cell numbers in the hippocampus (Criado-Marrero et al., 2020). Nevertheless, only a limited amount of studies addressed the sex-specific nature of FKBP5 manipulation-driven effects (Criado-Marrero et al., 2019, 2020; Nold et al., 2022). The consequences of full-body knockout of FKBP51 were investigated in male and female mice in two separate studies (Hoeijmakers et al., 2014; Touma et al., 2011). In contrast to our cell-specific approach, these studies did not reveal major differences in baseline and stress reactivity phenotypes. However, the experimental set up of these separate studies were not identical and therefore direct comparison between the male and female sample is difficult. Other recent studies, investigating the consequences of FKBP51 overexpression, did directly compare male and female mice and revealed sex-specific effects on basal CORT levels (Criado-Marrero et al., 2020) and sex-specific interactions with ELS exposure on anxiety-like behaviour (Criado-Marrero et al., 2019). In line with the latter, it was demonstrated in Chapter 2.3 that moderate ELS exposure, in the form of a LBN paradigm, had more robust effects on anxiety-like behaviour in females than in males. Moreover, interactions between ELS and specific FKBP51 knockout in glutamatergic neurons of the forebrain on spatial memory function in a stressful environment were only observed in female mice and were absent in males.

It would be highly interesting to speculate on the exact underlying processes that lead to these sex-differential effects in stress-related disorders or stress resilience and vulnerability mechanisms. During their reproductive period, females exclusively have high fluctuating levels of female steroid sex hormones, primarily estrogen and progesterone, that are likely the initial ground for the observed sex-differential effects (Choleris et al., 2018; Galea et al., 2017). Estrogen binds the steroid receptors estrogen receptor (ER) α and β and the G-protein coupled estrogen receptor 1 and progesterone binds its steroid receptor, the PR. Interestingly, studies in breast cancer cell lines showed that estrogen can reduce GR mRNA expression. Moreover, it was proposed that an activated-ER can also inhibit GR function by inducing transcription of phosphatase 5, which mediates dephosphorylation of GR and thereby hampers its binding to GRE elements (Krishnan et al., 2001; Zhang et al., 2009). Another study in the rat ventromedial hypothalamus, suggested interactions of estrogen with GR chaperones Hsp70 and Hsp90 (Olazábal et al., 1992). Consequential disturbed chaperone binding to GR might lead to reduced ligand or co-chaperone binding and this may hinder its translocation to the nucleus. In addition, it was suggested that progesterone directly competes with glucocorticoids for GR binding (Kontula et al., 1983). In addition to the effects on GR functionality, expression of *Fkbp5* is also directly regulated by PR and binding of progesterone to GR can augment FKBP51 levels (Hubler et al., 2003; Jääskeläinen et al., 2011). Reversely, FKBP51 is a co-chaperone to the PR and could thereby influence PR functionality, which may impact the regulatory loops in steroid receptors. Unlike other steroid receptors, FKBP51 does not bind ER with high affinity (Schülke et al., 2010). However, via the effects of ER on the GR, it may still influence FKBP51-mediated stress resilience and vulnerability mechanisms.

One brain region that has received much attention in the light of sex-differences is the hippocampus. The hippocampus has an abundant number of steroid receptors, including PR and ERα and ERβ, and sex differences have extensively been described for hippocampal-based memory and learning abilities, morphology and electrophysiology of hippocampal neurons and adult neurogenesis (Yagi & Galea,

2019). Furthermore, it has been well-established that estrogens can mediate neurogenesis and neuronal plasticity (including spine and synapse formation), particularly within the hippocampus (Sheppard et al., 2019). Estrogens are mostly thought to play a neuroprotective role. Illustrating this, the reduction of estrogens during menopause are suggested to mediate the observed increased vulnerability of (hippocampal) neurons to Alzheimer's pathology (Choleris et al., 2018). Thus, our sex-specific effects observed specifically in relation to improved hippocampal function might be heavily related to the presence or absence of female sex hormones.

Taken together, the chapters in this thesis highlight the strong presence of sex-differential effects in (FKBP51-mediated) stress resilience and vulnerability mechanisms and demonstrate the importance of sex-independent applicable stress models. Since much is still unknown about sex-specificity in the stress research field, it should be once more urged that considering the inclusion of both the male and female sex is of vital importance in finding personalized solutions to stress-related disorders in the future.

3.2 Cell-specificity in FKBP51 functionality

In addition to the sex-dependent nature of FKBP51 functionality, it was demonstrated in Chapter 2.2 that FKBP51 function can strongly differ dependent on the cell-type it is expressed in. In this study, loss of FKBP51 was achieved in either the glutamatergic neurons of the forebrain or in GABAergic neurons specifically, by using two different conditional knockout lines. Interestingly, the data showed that where sex was strongly determining the type of behavioural domain that was affected by FKBP51 manipulation, the cell-type specificity of the manipulation was rather defining the directionality of the effect. Both mice with loss of FKBP51 in forebrain glutamatergic neurons and loss in GABAergic neurons had changes in anxiety-like behaviour in females and differences in the cognitive domain in males. However, glutamatergic loss of FKBP51 led to increased anxiety like behaviours, whereas loss of FKBP51 in GABAergic neurons resulted in an anxiolytic phenotype. For male mice, FKBP51 knockout in glutamatergic neurons led to a reduced memory of the aversive environment, while selective loss of FKBP51 in GABAergic neurons improved memory function in a stressful context.

Glutamatergic neurons are known for their excitatory synaptic function and GABAergic neurons primarily have inhibitory effects, even though GABAergic interneurons can also synapse with other inhibitory neurons and can therefore contribute to disinhibitory effects (Fink, 2019). Due to their generally opposing functionality it is not surprising that they lead to contrasting effects on behaviour, brain structure and underlying molecular functions. Surprisingly, it was found that effects on brain structure and molecular profiles were largest in animals that lack FKBP51 in GABAergic neurons. For example, mice with a knockout of FKBP51 in GABAergic neurons had strong volumetric changes in the bilateral hippocampus when compared to wild-type animals. Furthermore, differential expression patterns were clearly more robust than in animals lacking FKBP51 in glutamatergic neurons. Remarkably, GABAergic neurons only comprise about 10 - 15% of the cells of the hippocampus, whereas the glutamatergic neurons make up the vast majority. Apparently, the inhibitory signalling is of vital importance for hippocampal functionality and, regardless of the fewer numbers, it still contributes to a larger extend to the structural and molecular consequences of altered FKBP51 functionality than the glutamatergic neurons. Nevertheless, the brain is not only comprised of neuronal cell populations. Different glial cell populations and vascular cell populations can be found in large numbers in the brain (Erö et al., 2018; Zeisel et al., 2015) and these cell types have supportive or unique, vital functions (Colonna & Butovsky, 2017; Sofroniew & Vinters, 2010). FKBP51 is known to be highly expressed in a number of these different cell populations, amongst others in microglia and astrocytes (Matosin et al., 2023). Interestingly, in Chapter 2.2 it was observed that, in females, loss of

FKBP51 in GABAergic neurons led to a BNST-specific differential gene expression profile that was enriched in microglia. Supposedly, loss of FKBP51 in GABAergic neurons can indirectly lead to molecular changes in other cell-types. Since microglia, considered the macrophages of the brain, have an important function in the clearance of microbes, apoptotic cells, excessive synapses and protein aggregates and are essential in mediating neuroinflammatory processes (Colonna & Butovsky, 2017), it is not surprising we found immune-related functions to be enriched in the BNST of females with a lack of FKBP51 in GABAergic neurons. Interestingly, a link between FKBP51, GABAergic neurotransmission and the immune system had been observed before. A study by Gan and colleagues demonstrated that FKBP51 mediates LPS-triggered upregulation of glutamic acid decarboxylase 65, a GABA-synthesizing enzyme in the hippocampus (Gan et al., 2022). Moreover, it was shown in another study that FKBP51 overexpression promoted neuronal cell loss in the hippocampus and simultaneously increased microglial markers in older-aged mice (Criado-Marrero et al., 2020). Speculatively, it might be suggested that, in females, FKBP51 in GABAergic neurons mediates neuroinflammatory processes and a lack of FKBP51 in these neurons may promote an adaptive neuroimmune response and have beneficial outcomes on anxiety-like behaviours. Opposingly, an overshoot in FKBP51 in these neurons could potentially lead to increased neuroinflammatory responses, neuronal cell damage and might result in negative health outcomes. However, future research would need to clarify exact underlying mechanisms.

Cell-type specificity has been demonstrated before in terms of FKBP51 functionality. Systemic inhibition of FKBP51 with the SAFit2 inhibitor was for example shown to reduce anxiety-like behaviours and overexpression of FKBP51 in the BLA led to an anxiogenic phenotype (Hartmann et al., 2015). Surprisingly, specific overexpression of FKBP51 in the ovBNST opposingly led to anxiolytic effects and knockout of FKBP51 resulted into anxiogenic behaviours (Engelhardt et al., 2021). Altogether this illustrate that cell-type specific approaches are, without a doubt, an important approach for future research. Conditional knockout mouse lines or conditional viral genetic approaches are one way to study cell-type distinctive effects of genetic factors like FKBP51. However, at least for now, straight-forward drug administration into specific cell types in the brain remains difficult and it would be interesting to unravel the cell-specific downstream effects of potential pharmacological interventions, such as the FKBP51 inhibitor SAFit2. In addition to conditional genetic manipulations, the development of single-cell sequencing techniques in the past decades has provided the opportunity to unravel cell type-specific alterations resulting from environmental challenges or drug administrations (Chehimi et al., 2023; Van De Sande et al., 2023). Studies with post-mortem tissue of human patients have already investigated cell-specific FKBP51-mediated effects (Matosin et al., 2023). However, it would also be interesting to apply such techniques in stably controlled *in vivo* models, to provide further insights on the cell-specific functionality of FKBP51. At last, even though neuronal cell populations are a popular target, our results indicated that it would also be highly important to look beyond neuronal cell populations and investigate the specific contribution of FKBP51 to different glial cells.

3.3 Glutamatergic FKBP51 modulation of beneficial effects of moderate early life stress exposure via a TCF4 mediated pathway

Polymorphisms of the *FKBP5* gene have repeatedly found to be associated with the risk for psychiatric disorders such as MDD and anxiety disorders (Binder, 2009; Binder et al., 2004). Interestingly, one of the most commonly described SNPs of the *FKBP5* gene, the rs1360780 SNP, specifically interacts with childhood adversity, to increase the risk for psychiatric disease (Klengel et al., 2013). Much is however still unknown about the exact mechanisms behind this interaction between FKBP51 and early life adversity. In Chapter 2.3 the contribution of FKBP51 in glutamatergic

neurons of the forebrain to the long-term consequences of ELS exposure and its underlying mechanisms were studied. As demonstrated in Chapter 2.2, FKBP51 functionality is highly cell-type and sex specific, therefore it was decided to investigate the contribution of FKBP51 within a particular neuronal cell-type and conduct this study in both male and female mice.

In this chapter, it was demonstrated that ELS exposure led to adaptive changes on anxiety-like behaviour and memory performance, particularly in the female sex, and these beneficial effects were dependent on the presence of FKBP51 in glutamatergic forebrain neurons. Moreover, we identified alterations in brain volume and neuronal morphology and synaptic plasticity of dorsal CA1 neurons to be underlying these FKBP51-mediated effects. The consequences of ELS on brain and behaviour have been investigated extensively (Bonapersona et al., 2019; Chen & Baram, 2016; Joëls et al., 2022; Krugers et al., 2017; C.-D. Walker et al., 2017) and these data, of which the majority only included males, have predominantly shown negative outcomes of ELS. However, there is a large gap in information on ELS effects in females, which may result in differential outcomes as compared to males. Aside from the described disadvantageous consequences of ELS, some studies have also demonstrated adaptive changes as a result of mild or moderate ELS exposure, establishing the inoculation stress hypothesis (Dienstbier, 1989; Lyons et al., 2009; Parker et al., 2004, 2005). In fact, the data from chapter 2.3 are an excellent illustration of the so-called “match-mismatch” theory, as we observed a worsened memory following ELS exposure when this was tested in a neutral context, but ELS led to improved spatial memory performance in a stressful context. Interestingly, we observed several deformations in GM and WM structures upon interaction of ELS and FKBP51 in glutamatergic forebrain neurons, amongst others in different cortical regions, the ventral subiculum and the dorsal hippocampal commissure. These brain regions describe interesting underlying pathways as the ventral subiculum contains the output neurons through which the hippocampus connects with the PVN to inhibit HPA-axis activation upon stress-induced glucocorticoid release (Herman & Mueller, 2006) and the dorsal hippocampal commissure WM structure is largely important for the communication between the hippocampus and other temporal lobe regions and has implications in memory function (Postans et al., 2020). In line with the interactive findings on behaviour, particularly as observed in the stressful Morris Water Maze learning and memory task, we found highly similar interactions of glutamatergic FKBP51 and ELS on dorsal CA1 pyramidal neuron morphology. Spatial memory in a stressful context was improved following ELS exposure, but this beneficial effect was absent in female mice that lack FKBP51 in glutamatergic forebrain neurons. Similarly, apical and basal spine densities were increased after ELS exposure and this increase was absent in *Fkbp5*^{Nex} female mice. Interactive effects of ELS and FKBP51 on synaptic functioning were also observed, as ELS exposure altered LTP in the dorsal CA1, but only in wild-type female mice.

RNA sequencing data revealed that there is one distinct network of genes that is heavily associated with the interaction of glutamatergic FKBP51 and ELS exposure in female mice. Matching our previous findings on brain and behaviour, the genes in this network were upregulated upon ELS but no changes in expression were observed in mice lacking FKBP51 in glutamatergic neurons. Interestingly, follow up analyses revealed one enriched transcription factor, TCF4, that regulates a number of specifically important genes within the network. This selection of genes was particularly relevant, because they were either hub genes of the network, or they were associated with data from human GWAS studies in women that experienced childhood trauma or individuals that suffered from PTSD. Subsequent specific overexpression of TCF4 in glutamatergic neurons of the forebrain confirmed that TCF4 is a regulator of the FKBP51-mediated ELS effects on behaviour, as this resulted in highly similar beneficial effects on anxiety-like and spatial memory function in a stressful context as ELS exposure in wild-type mice. TCF4 is a helix-loop-helix protein that, by binding to E-box sequences on the DNA, can regulate transcription of genes. Previous work on TCF4 has already demonstrated its importance in processes like neurogenesis, neuronal morphology, memory and

learning and associated neuronal plasticity in the hippocampus (D'Rozario et al., 2016; Fischer et al., 2014; Kennedy et al., 2016; Teixeira et al., 2021). Moreover, TCF4 was also associated with oligodendrocyte function and myelination (Phan et al., 2020), relating to the observed WM alterations that were described in Chapter 2.3. Furthermore, TCF4 seems to be an important factor in different psychiatric or neurological disorders, as it was repeatedly found in GWAS studies of disorders such as schizophrenia, autism, MDD and PTSD (Teixeira et al., 2021; Zavala et al., 2009). Altogether, this confirmed the TCF4 as an important regulatory factor, driving the upregulation of a network of genes that are needed for the adaptive effects of moderate early life stress exposure, mediated by glutamatergic forebrain FKBP51.

The question remains in which way the presence of FKBP51 is linked to enhanced TCF4 activity following ELS exposure. FKBP51 might be directly linked to TCF4 activity via its various protein-protein interactions (Hähle et al., 2019), but it might also affect TCF4 activity more indirectly via the GR and its transcriptional regulatory actions. Hypothetically, ELS-induced elevated glucocorticoid levels may induce epigenetic changes in the *TCF4* gene or affect other genes or proteins that can indirectly lead to TCF4 upregulation or increased TCF4 activity. In the absence of FKBP51 however, GR sensitivity is increased, and the ELS-induced augmented glucocorticoid levels can potentiate GR activation. With strong reductions in FKBP51 levels, GR is more likely to translocate to the nucleus. If the *TCF4* gene has GREs with repressing activity, GR might be able to prevent an ELS-induced upregulation of *TCF4* by binding such GREs. Alternatively, GR might bind enhancers or repressors that can regulate *TCF4* transcription and thereby mediate TCF4 activation. The mechanisms by which GR and TCF4 may interact remain unclear for now, but a study by Gerstner and colleagues has already demonstrated that *Tcf4* expression in the brain can be downregulated by direct GR activation via a dexamethasone administration in mice (Gerstner et al., 2022). Another plausible mechanism for linking FKBP51 to Tcf4 activity would be via interactions with the AR. Prostate cancer research has already demonstrated associations between FKBP52, AR and TCF4 activity (Storer Samaniego et al., 2015) and binding of FKBP51 to AR, or an altered FKBP52/FKBP51 balance, could potentially effect TCF4 activity. For now, the direct link between glutamatergic FKBP51 and TCF4 activity remains speculative and further research is needed to identify the exact underlying mechanisms.

3.4 FKBP51 as a dynamic stress-responsive factor that interacts with its environment

Ever since the discovery that FKBP51 polymorphisms are implicated in different stress-related disorders (Binder, 2009; Binder et al., 2004; Zannas et al., 2016), FKBP51 has primarily been described as a risk factor for causing psychiatric disease. Risk alleles of SNPs of the *FKBP5* gene are associated with elevated intracellular levels of FKBP51 and consequential reduced GR sensitivity, sustained glucocorticoid responses to stress and increased ELS susceptibility (Binder et al., 2004; Klengel et al., 2013). Therefore, augmented FKBP51 levels are classically thought to be disadvantageous for (mental) health and lower levels of FKBP51 are typically considered beneficial. Nevertheless, as clearly illustrated in Chapter 2.2, FKBP51 functionality strongly differs dependent on the cell type it is expressed in. Loss of FKBP51 in GABAergic neurons indeed resulted in beneficial outcomes on anxiety-like behaviours and cognitive functioning. However, in contrast to this, specific loss of FKBP51 in glutamatergic neurons of the forebrain actually led to detrimental effects on anxiety-like behaviour and memory function. A similar contradiction to historical conceptions was found in Chapter 2.3. Opposing to frequently described phenotypes in males, exposure to moderate ELS was actually observed to result in advantageous effects on the anxiety domain and stressful spatial memory performance in female mice. Furthermore, it was demonstrated that FKBP51 in glutamatergic neurons

of the forebrain plays an important mediating role in these ELS-induced beneficial effects. Interestingly, it was shown that beneficial effects of ELS on memory performance were exclusively present when mice were in acute stressful environments during the learning phase. In a neutral environment however, memory performance was actually negatively impacted by moderate ELS exposure and, in contrast to what was observed in a stressful context, FKBP51 reduction augmented the consequences of ELS on memory performance.

Age is another determining factor when it comes to the impact of FKBP51 on stress-related disease. From previous work we know that FKBP51 levels rise with age (Blair et al., 2013; Sabbagh et al., 2014) and a recent study in animals has showed age-related differential outcomes of FKBP51 overexpression on depressive-like behaviours (Criado-Marrero et al., 2020). In chapter 2.2 we observed robust behavioural, structural and molecular consequences as a result of lack of FKBP51 in an older-aged sample. However, we did not include younger-aged animals in this study, so a direct age-comparison could not be made. Nonetheless, in contrast to our findings, previous studies with full-body knockout of FKBP51 in younger animals did not show baseline changes in emotional and cognitive behaviour (Hoeijmakers et al., 2014; Touma et al., 2011). Even though its needs to be taken in consideration that the lack in effect could also be subscribed to cell-type independent approach, it might also well be that the older age of the mice in our study augmented the effect on brain and behaviour.

In conclusion, this thesis clearly demonstrates that FKBP51 functionality is highly responsive to its environment. Environmental factors such as sex, age and exposure to (early life) stress, but also the regions or cell-types that FKBP51 is expressed in, all contribute to the way in which FKBP51 shapes stress resilience. Therefore, in contrast to its more traditional view of FKBP51 as a risk factor, this thesis rather proposes a model in which FKBP51 should be viewed as a dynamic stress-responsive factor, that, depending on changing environmental circumstances, may dynamically impact stress resilience and related mental health (Figure 4).

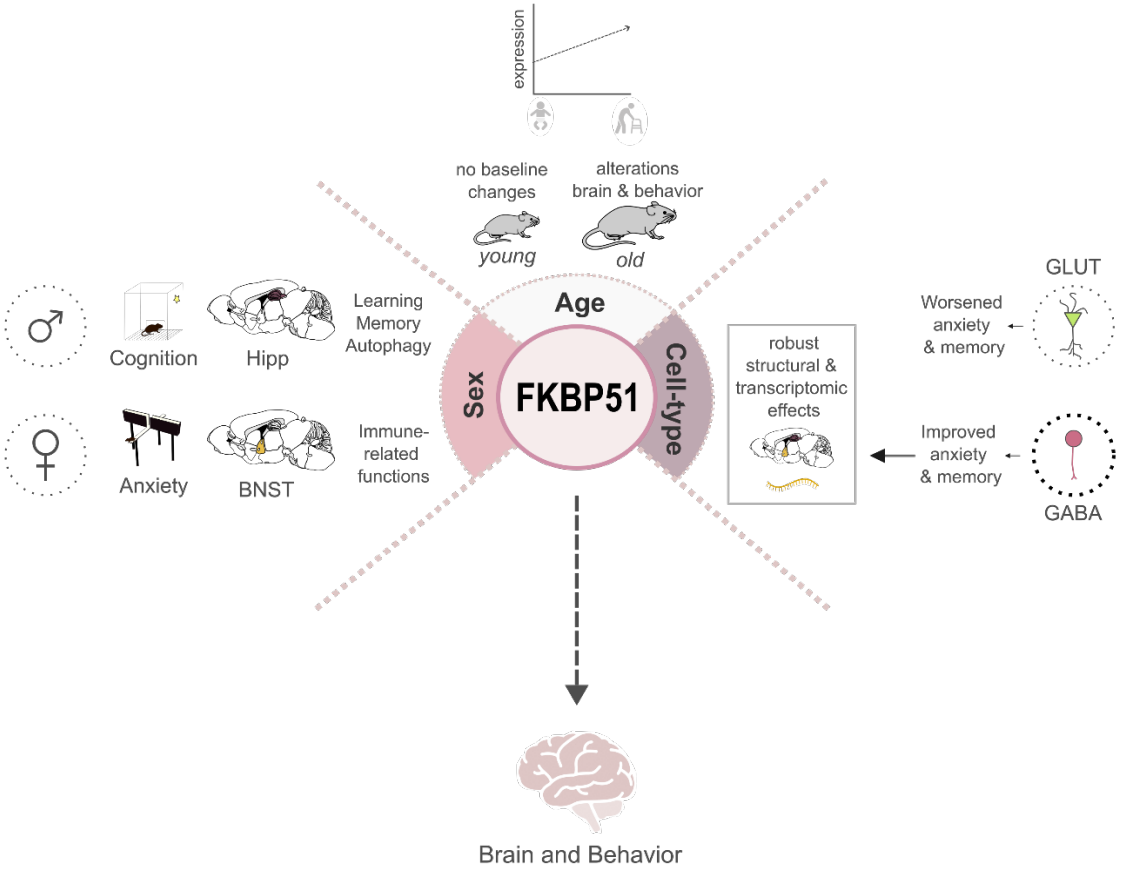

Figure 4. The sex-, age- and cell-type specific effects of FKBP51
The dynamic effects of the stress-responsive co-chaperone FKBP51 on brain and behaviour are highly influenced by several biological and environmental factors. Three factors can have a strong impact on FKBP51 functionality: sex, age and the cell-type specific expression. Male mice that lack FKBP51 in specific neuronal populations were predominantly affected on the cognitive domain, had strong volumetric alterations in the hippocampus (Hipp), the brain region that is strongly implicated in memory and learning, and robust differential expression profiles were associated with processes of learning and memory, pre-synaptic transmission and autophagy. Females with specific loss of FKBP51 on the other hand, were mostly affected on the anxiety domain, had structural alterations in the bed nucleus of the stria terminalis (BNST), an anxiety-regulating brain region, and had a transcriptomic profile that was enriched in microglia and immune-related functions. Furthermore, it is known that FKBP51 protein levels aggregate with age. Studies in younger animals with a global FKBP51 knockout model did not have any alterations under baseline conditions, whereas older animals with a conditional knockout of FKBP51 in neuronal cell populations were found to have changes in emotional regulation and memory function. This might be an indication that aging can enhance FKBP51-mediated changes. At last, cell-type specificity can strongly influence FKBP51 functionality. Loss of FKBP51 in glutamatergic neurons (GLUT) of the forebrain leads to an anxiogenic phenotype and worsened memory function. However, loss of FKBP51 in GABAergic neurons leads to the strongest changes in the brain. GABAergic loss of FKBP51 has anxiolytic effects, enhances memory function and leads to robust structural and transcriptomic changes.

3.5 Future directions and closing Remarks

This thesis corroborates the highly dynamic role that the stress-responsive gene *FKBP5* plays in shaping stress resilience. Genetic factors, biological factors, and challenges throughout life all contribute to the way in which FKBP51 mediates stress-related processes. Moreover, cell specificity has proven to be an unneglectable factor in understanding FKBP51 functionality. FKBP51 drug targets, such as SAFit2 are promising future strategies to tackle stress-related pathology (Gaali et al., 2015; Schmidt et al., 2012). However, psychiatric disorders are a largely heterogenous group of disease, with a high percentage of women affected. In order to work towards more targeted, personalized treatment strategies, the understanding of specific FKBP51 functionality needs to be extended. It is also essential that sex-differential effects are taken in consideration and a necessity that the body of work in the female sex continues to grow. In addition, single cell-specific tools could provide an interesting approach in elucidating further cell-specific mechanisms of FKBP51 or FKBP51 drug targets and it is important that research goes beyond neuronal cell populations and additionally focusses on other cell types in the brain, such as different glial cell populations. Furthermore, this thesis proposes an FKBP51-mediated model for ELS resilience with novel contributing pathways. As it is becoming increasingly clear that psychiatric disorders have a poly-gene-environmental nature (Uher & Zwicker, 2017) it is important to further identify the different networks of genes and environmental exposures that FKBP51 mediates or works together with towards the ultimate goal, increasing stress resilience.

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Grants, conferences and courses

Grants

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Federation of European Neuroscience Societies (FENS) travel award for the Society for Neuroscience (SfN) conference 2021

International Max Planck Research School for Translational Psychiatry (IMPRS-TP) post-doc wrap up grant

Conferences

Federation of European Neuroscience Societies (FENS) Forum 2022, Paris, France

Society of Neuroscience (SfN) conference 2021, online

49th meeting of the European Brain and Behaviour Society (EBBS) 2021, Lausanne, Switzerland

3rd Winter conference on Stress 2021, Garmisch-Partenkirchen, Germany

Federation of European Neuroscience Societies (FENS) Forum 2020, online

48th meeting of the European Brain and Behaviour Society (EBBS) 2019, Prague, Czech Republic

2nd Winter conference on Stress 2019, Garmisch-Partenkirchen, Germany

Courses

Proposal writing, June 2021

Introduction to R statistics, January 2021

Statistical Genomics, October 2020

Good Scientific Practice, July 2020

Electrophysiology workshop, February 2020

Clinical lecture series IMPRS-TP, January 2019

Deutsche Sprachkurs B2 Ludwig Maximilian Universität, winter semester 2018

Methods in Neuroscience lecture series IMPRS-TP, October 2018

Medical Munich Research School lecture series 2018 - 2021

Affidavit

Hiermit versichere ich eidesstattlich, dass ich die vorliegende Dissertation selbstständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet.
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München, den 31.05.2023

Lotte van Doeselaar