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# Unraveling the Functional Contribution of Fkbp5 to Stress Vulnerability

## Shaking up Molecular Links Between Stress and Disease

MAX · PÖHLMANN


DISSERTATION

der Fakultät für Biologie

der Ludwig · Maximilians · Universität München

1. Gutachter: PD Dr. Mathias Schmidt
2. Gutachterin: Prof. Dr. Laura Busse
3. Gutachter: Prof. Dr. Christof Osman
4. Gutachterin: Prof. Dr. Gisela Grupe

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*In my opinion, we don't devote nearly enough scientific research to finding a cure for jerks.*

Calvin

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# II. Abstract

Despite tremendous efforts and decades of research, we are still far from finding effective treatments for most psychiatric diseases. Since we can impact the environment we are living in only to a certain degree, one promising approach to fight mental diseases is the identification of genetic risk factors for said disorders. One of these risk factors is the gene *FKBP5* that has been associated with conditions like major depression and post-traumatic stress disorder in humans. The respective protein, FKBP51, is an essential regulator for the secretion of stress hormones and occupies a powerful position within the molecular pathway that terminates the stress response. In order to further our understanding of the role and biological relevance of *FKBP5*, I conducted an extensive set of experiments using different mouse models. I was able to identify two monoaminergic nuclei as main regions of *Fkbp5* expression and could show that the regulation of the gene in these nuclei is stress dependent. Both, viral up-regulation as well as a genetic knock-out of *Fkbp5* resulted in behavioral alterations, as well as changes in the activity of the hypothalamic-pituitary-axis, one of the major physiological systems of the stress response. Pharmacological blockage of the co-chaperone FKBP51 is another promising approach on the path of medication development and was improved on in a set of studies that utilized the specific antagonist SAFit2. This drug evokes behavioral and endocrine effects that mimic effects observed in genetic knock-out lines for *Fkbp5*. I could show that treatment with SAFit2 was able to negate very specific aspects of the stress response when applied exclusively or in combination with a commonly available antidepressant. In addition to the identification of functional properties of the drug, I conducted a number of *in vivo* studies that could help to develop novel delivery methods for substances like SAFit2 in the future. Taken together, the findings of my thesis provide a strong foundation for any work on *Fkbp5* in the monoaminergic system and the use of specific antagonists in the future. Manipulations of the *Fkbp5* systems remain a very promising alternative to the available targets for pharmacological treatment of mental disorders.

# III. Zusammenfassung

Trotz jahrzehntelanger Bemühungen gibt es noch immer eine Vielzahl psychiatrischer Erkrankungen, für die uns keine effektiven Behandlungsmöglichkeiten zur Verfügung stehen. Da wir den Einfluss unserer Umwelt auf die psychische Gesundheit nur zu einem gewissen Grad beeinflussen können, ist die Identifikation von Risikogenen für eben solche Krankheiten besonders wichtig. Einer der Kandidaten, der in den letzten Jahren große Beachtung gefunden hat ist dabei das *FKBP5*-Gen, welches beim Menschen in starker Verbindung zu Depression oder Posttraumatischer Belastungsstörung steht. *FKBP5* ist ein wichtiger Regulator für die Ausschüttung von Stress-Hormonen und an essenzieller Stelle in den molekularen Signalweg der körpereigenen Stressantwort eingebunden. Um unser Verständnis des Gens zu erweitern, habe ich im Rahmen dieser Arbeit eine weitreichende Charakterisierung verschiedener Mausmodelle durchgeführt. Dabei konnte ich zwei monoaminerge Regionen im Gehirn identifizieren, die eine sehr hohe Grundexpression des Gens aufweisen und in denen *Fkbp5* stressabhängig reguliert wird. Sowohl virale Überexpression, als auch das genetische Ausschalten des Gens hatten Veränderungen im Verhalten und der hormonellen Stressantwort der Tiere zur Folge. Im nächsten Schritt habe ich SAFit2, einen spezifischen Antagonisten für *Fkbp5,* eingesetzt und konnte dabei wieder Veränderungen im Verhalten und der endokrinen Reaktion auf einen externen Stressor beobachten. Auch die Kombination mit einem etablierten Antidepressivum hatte verhaltensbasierte Auswirkungen und sollte in zukünftigen Untersuchungen noch erweitert werden. Im Rahmen meiner Versuche konnte ich zudem einen erheblichen Beitrag zur Entwicklung und Erprobung neuer Applikationsmöglichkeiten von chemischen Substanzen leisten. Dabei habe ich eine Reihe von *in vivo* Tests mit einem neu entwickelten, Gel-basierten Trägerstoff für SAFit2 durchgeführt. Insgesamt bilden die Ergebnisse dieser Arbeit eine hervorragende Basis für die weitere Erprobung von *Fkbp5*-basierten Behandlungsmethoden. Die Manipulation von *FKBP5* ist immer noch eine vielversprechende Alternative zu etablierten Behandlungsmethoden für psychiatrische Erkrankungen.

# IV. List of Abbreviations

| | |
|---|---|
| 5-HT | serotonin |
| AAV | adeno-associated virus |
| ACTH | adrenocorticotrophic hormone |
| ANOVA | analysis of variance |
| ANS | autonomic nervous system |
| ASDS | acute social defeat stress |
| BLA | basolateral amygdala |
| BSA | bovine serum albumin |
| CAN | central autonomic network |
| CID | collision-induced dissociation |
| CRH | corticotropin-releasing hormone |
| CSDS | chronic social defeat stress |
| DA | dopamine |
| DaLi | dark-light box test |
| DG | dentate gyrus |
| dISH | double *in situ* hybridization |
| DR | dorsal raphe nucleus |
| EPM | elevated plus maze test |
| FKBP51 | FK506 binding protein 5 |
| *Fkbp5* | FK506 binding protein 5 (gene) |
| FST | forced swim test |
| GR | glucocorticoid receptor |
| GRE | glucocorticoid response elements |
| HPA | Hypothalamic-Pituitary-Adrenocortical |

| | |
|---|---|
| HPLC/MS-MS | high-performance liquid chromatography/mass spectrometry |
| HSP | heat shock protein |
| i.p. | intraperitoneal |
| IL | infralimbic cortex |
| ISH | *in situ* hybridization |
| ITI | inter trial interval |
| IVC | individually ventilated cage |
| KO | knock-out |
| LC | locus coeruleus |
| MR | mineralocorticoid receptor |
| MRM | multiple reaction monitoring |
| *Nat* | norepinephrine transporter |
| NE | norepinephrine |
| OE | over-expression |
| OF | open field test |
| OLT | object relocation test |
| ORT | object recognition test |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| *Pet* | *Pet*-1, FEV transcription factor , ETS family member |
| PFA | paraformaldehyde |
| PL | prelimbic cortex |
| PPI | peptidylprolyl isomerase |
| PTSD | Post-traumatic stress disorder |
| PVC | polyvinyl chloride |
| PVN | paraventricular nucleus |

| | |
|---|---|
| s.c. | subcutaneous |
| SA | social avoidance test |
| SAFit | selective antagonist for FKBP51 by induced fit |
| SEM | standard error of the mean |
| *Sert* | serotonin transporter |
| SSRI | selective serotonin re-uptake inhibitor |
| *Th* | tyrosine hydroxylase |
| TPR | tetratricopeptide-repeats |
| VPG | vesicular phospholipid gel |
| WT | wild-type |

# 1 Introduction

## 1.1 Mental disorders - a worldwide burden

Mental disorders like depression, Alzheimer's disease or schizophrenia are extremely prevalent in our modern world. Not only are they a burden for the affected person, but also for health care systems and the economy in general. According to recent studies, almost one fifth of the adult population in developed countries suffers from a mental illness at least once in their lifetime. In the United States alone, this accounts for 46.6 million cases in the year 2017 (NIH, 2017). Worldwide, an estimated number of 300 million people are suffering from depression each year, with about twice as many women affected than men. However, due to the relatively high amount of un-diagnosed individuals, the estimated number of unreported cases is likely to be even higher than that. Therefore, depression is one of the major causes of disability nowadays (WHO, 2018). Unfortunately, psychiatric conditions are still stigmatized to a certain degree, which might prevent many people from seeking professional help. Mental illnesses include a wide variety of conditions and diseases, affecting different aspects of a patients' life. This could range from relatively mild impairments in the day-to-day running (like phobias or mild anxiety) to extremely severe cases in which patients could become a serious threat towards themselves or their environment. In any case, the treatment of mental disease is complicated by several factors. First of all is the problem of a correct diagnosis. While physiological diseases are oftentimes relatively clear in their clinical picture, many psychological conditions are hard to diagnose since some symptoms might be detectable while others are not or, to make matters worse, they might even overlap among several types of mental disorders. Mental diseases are generally characterized by a combination of abnormal thoughts, perceptions, feelings or behaviors. Up until now, most mental illnesses are diagnosed using rather rudimentary techniques, mainly relying on observations and questioning. Another difficulty derives from the lack and inefficacy of medication to treat many of these diseases.

Even though huge amounts of money go into research and development each and every year, the amount of new drugs that make it to the market is very marginal. Most of the time physicians have to rely on substances that have been identified many decades ago and more often than not, their actual mode of action is not precisely known either. All these factors make psychiatric diseases a very costly and hard to combat burden of our modern society.

## 1.2 Genetic predisposition and environmental impact

Since psychiatric diseases are so difficult to diagnose and treat, understanding their biological background and the triggers is of utmost importance. Many studies have shown that most mental issues go hand in hand with abnormal neural transmission (Pan et al., 2017; Crawford et al., 2013). These abnormalities can be the result of a plethora of different, mostly dysregulated, processes in the brain. In recent years, studies revealed certain genetic aberrations that are heavily linked with specific mental illnesses (D'Souza and Rajkumar, 2019; Gill et al., 2019; Howard et al., 2019; Dedic et al., 2017; Zalsman, 2012; Krishnan et al., 2007; Binder et al., 2004). Some of these defects are single nucleotide polymorphisms that, on their own, do not automatically result in a diseased state, but only marginally increase the risk to develop such a condition. One example here would be the *Fkbp5* gene, which encodes for a protein that is an endogenous regulator of the stress system. Alterations in genetic and epigenetic regulation of the Fkbp5 gene have been shown to contribute to the risk of developing mental disease (Binder et al., 2004). Up-regulation of *Fkbp5* is thought to impact stress-related behavior (Matosin et al., 2018) and studies demonstrated decreased stress-coping, elevated anxiety levels and impaired extinction learning in mice (Zannas et al., 2016; Hartmann et al., 2012). On the other hand, there are also genetic predispositions and molecular adaptations that are thought to hold protective properties for the carrier (Shrivastava and Desousa, 2016; Krishnan et al., 2007). However, it is not just a genetic background that causes mental diseases. One very important factor here is the environment an individual is or was living in. The impact of environmental factors and experiences becomes clear when looking at diseases like post-

traumatic stress disorder (PTSD). In that specific case, exposure to a traumatic event like war experiences or physical abuse might trigger long-lasting effects such as flashbacks, anxiety or isolation (Bisson et al., 2015; Fullerton et al., 2004). Taken together, it becomes clear that many mental disorders are not the result of one single factor, but rather a combination of a certain genetic predisposition and an environmental trigger. The multifactorial nature of these diseases is what makes them so hard to identify and fight from a medical point of view. It is quite possible that there are several layers to the cause of mental diseases, which makes it very unlikely that there is an easy solution to the problem. When looking at the environmental factors, a number of different options have been identified in the past. Factors that are heavily linked to psychiatric diseases include nutrition (Kelly et al., 2015, 2017), drug exposure (Khosravani et al., 2019; Fluharty et al., 2017; Kruckow et al., 2016), early development (Groenman et al., 2017), defects in the immune system (Chrousos, 2009), and most prominently stress.

## 1.3 The concept of stress

Everybody knows the feeling of being 'stressed'. However, when one is asked to describe this feeling in more detail, it becomes obvious how washed-out the meaning of the word really is. Most of the time, it is commonly used in a wide variety of situations and contexts, without giving it a second thought. However, when it comes down to a precise definition of the term, it becomes clear how diverse the perception of stress can be. For some, a daily commute in a crowded subway is stressful, while others remain calm and sometimes even enjoy the busy world around them. Some people only live up to their full potential when being on stage, speaking in front of a large crowd, whereas others break out in a sweat the moment they think about a similar situation. The questions of 'why' and 'how' kept doctors and scientists puzzling for decades and still do so today in many ways. Nowadays, it is clear that stress is a biological phenomenon but it was not until the 1930s when Canadian-Hungarian scientist Hans Selye (1907-1982) linked stress to physiological conditions that could be described and explained by biological processes. Expanding the work of the American

physiologist Walter Cannon (reviewed in Ulrich-Lai and Herman, 2009), who coined the term homeostasis, Selye proposed his famous hypothesis of the general adaptation syndrome (reviewed in Szabo et al., 2017). Selye stated that the body always tries to retain a balance in its physiological parameters, namely pH, glucose levels, etc., in order to maintain an overall healthy condition. Once this so-called homeostasis is disturbed by external factors, the body starts to send alarm signals in order to restore its original state. Selye described the three steps involved in the process as alarm reaction, resistance stage and ultimately an exhaustion stage. Going further, Selye demonstrated that every organism is equally affected by both, positive (eustress) and negative (distress) stressors. In fact, positive stress (e.g. the stress of creation, be it in art or science) as he describes it impacts motivation, alertness as well as attention and is actually an essential factor for health and survival, while distress – just as the name suggests – goes along with negative phenomena like fear or anxiety, potentially leading to pathology. This relationship was originally described by psychologists Robert Yerkes and John Dodson in 1908 as a hypothetical concept (Figure 1) and later worked on by others (Anderson et al., 1989; Duffy, 1957). An early definition that was used by Selye in an interview with Jerry Jarvis in 1974 described stress as "the non-specific response of the body to any demand made on it". Since then, these very fundamental observations have long been expanded and elaborated by a number of stress researchers over the years. Sterling and Eyer postulated that the needs and optimal conditions of an organism tend to change over the course of its lifespan and named this phenomenon "allostasis" (Sterling, 1988). In the following years, Bruce McEwen was one of the most influential scientists to further develop the ideas of an organisms' adaptation to stress and its coping strategies for future stressors (McEwen, 1998, 2003, 2005; McEwen and Stellar, 1993).

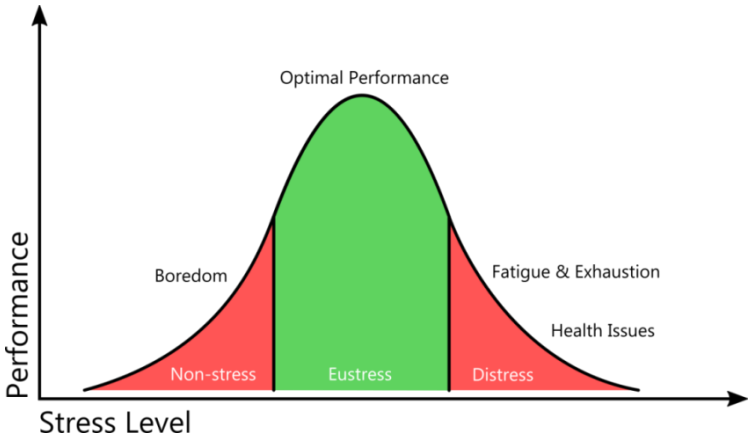


**Figure 1: The Yerkes-Dodson performance curve - Impact of stress on body performance**
Stress causes changes in different body parameters and thereby impacts the performance of an individual. Optimal performance requires moderate levels of stress, so called eustress. Once it surpasses a critical point, stress can harm the body (distress) and lead to a drop in performance and the development of health problems.

Another important focus of interest was the classification of different kinds of stressors and their impact on health and disease. Most of them differ in causality, severity, and modality. Physical stressors, for example, trigger different pathways than anticipatory stressors, thereby evoking a variety of adaptive responses from the body (Dayas et al., 2001; Pacák and Palkovits, 2001). Taken together, these processes include a number of essential steps, performed by the body in order to execute the best possible response to the stressor. First, the body needs to judge a situation using sensory stimuli as well as stored information. Second, it needs to initiate the appropriate physiological response by activating one of its 'emergency systems' and lastly, it is absolutely essential to return to a normal non-stress state once the impact of the stressor abates.

## 1.4 The stress response

In a biological sense, an organisms' response to stress is mainly driven by two interdependent pathways, the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenocortical (HPA) axis. Both of these pathways are regulated by the central autonomic network (CAN), a

network of several brain regions which control - amongst other things - homeostasis, the "fight-or-flight" response, and reproduction (Kandel et al., 2013). The main purpose of the ANS is the appropriate allocation of energy resources in a potentially life-threatening situation. It is controlled and regulated by several areas including hypothalamus, medulla, pons and specific neurons in the spinal cord (Benarroch, 1993). The ANS itself is divided into a sympathetic and a parasympathetic branch, controlling different and often opposing aspects of the energy allocation within the system. Via the use of mediators like epinephrine and norepinephrine (NE), the ANS orchestrates parameters like heart rate, respiration, glucose release or digestion (Kandel et al., 2013). That way, stress can induce a redirection of resources away from energy consuming regions like the digestive tract or gonads towards areas that are more important in terms of immediate survival in that instance. The natural stress response includes enhanced oxygen and glucose delivery to specific muscles, increased glycogenesis and glycolysis as well as renal sodium retention in the liver in order to expand the blood volume. As a result, the body is able to devote all available resources towards behavioral adaptations that can ultimately maximize its chances of survival (Figure 2). While the ANS mainly regulates the response to immediate threats ('fight-or-flight') via the release of fast acting transmitters like epinephrine and NE, actions by the HPA-axis are much slower, but long lasting in comparison (Herman et al., 2016). As the name suggests, the response of the HPA-axis is also triggered by the hypothalamus, which innervates the pituitary gland both, directly and indirectly. While large magnocellular neurons send their axons to the posterior part of the pituitary, where they directly release peptide hormones like vasopressin and oxytocin, neurons from the parvocellular neuroendocrine system send their axons onto a venous portal system, located in the median eminence of the hypothalamus. These portal veins then transport corticotropin-releasing hormones (CRH) to the anterior part of the pituitary where they initiate the release of the adrenocorticotrophic hormone (ACTH) into the general blood circulation. ACTH is transported to the adrenal cortex of the adrenal glands via the blood stream. In the adrenal cortex, ACTH stimulates synthesis and release of stress hormones, namely corticosteroids, which are then secreted into the blood circulation.

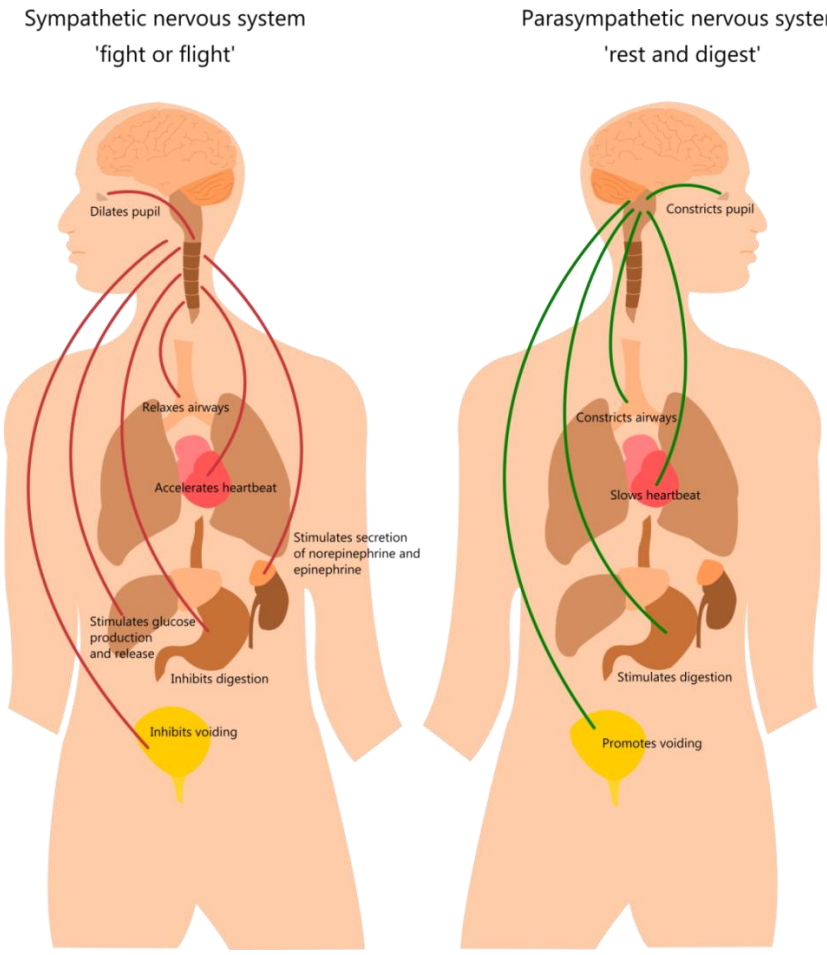


**Figure 2: The autonomic nervous system**
The ANS consists of two opposing branches, the sympathetic and the parasympathetic nervous system. They are activated upon demand and control body functions in the periphery. While the sympathetic system aims to increase activity and alertness by redirecting energy resources from organs that are not needed in a specific situation ('fight or flight' reaction), the parasympathetic system initiates the so called 'rest and digest' state in which the body is focused on energy production and regeneration.

Stress hormones in the blood target a variety of different tissues in the body where they initiate respective adaptations. Very importantly, corticosteroids also target the brain, where they bind variants of corticoid receptors, like the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Kandel et al., 2013; Nestler et al., 2009). These receptors are major components of a negative feedback loop and heavily involved in the shutdown of the stress response (Figure 3).

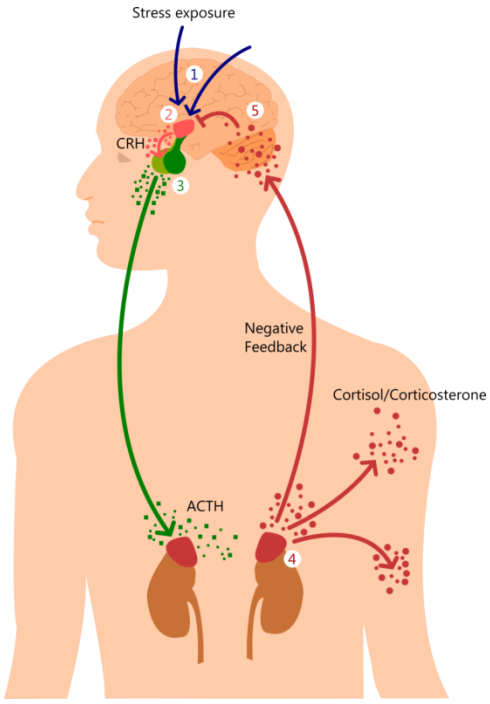


**Figure 3: The hypothalamic-pituitary-adrenal-Axis**
The HPA-axis is controlling the endocrine reaction to stress by ultimately controlling stress hormone levels. Upon stress exposure (1), the paraventricular nucleus (PVN) of the hypothalamus initiates the release of CRH (2). Subsequently, CRH causes the pituitary gland to release ACTH into the bloodstream (3). Once ACTH reaches the adrenal glands, stress hormones (cortisol in humans, corticosterone in rodents) are released into the body where they initiate adaptive processes in the periphery (4). The same hormones also provide negative feedback to the brain and ultimately cause the whole pathway to shut-down after a certain level of saturation is achieved (5).

The hormonal cascade can result in immediate effects, but also long-lasting changes in the system, some of which being detectable for several months and even years (Van Holland et al., 2012). In some cases, molecular changes evoked by specific experiences, such as domestic violence, war or accidents can have life-long implications that might cause pathologic states like PTSD (Barak and Szor, 2000).

## 1.5 From stress to disease - Stress-related diseases

Since stress evokes such an extensive response from the body, it is easy to understand how it can be a massive contributor to health problems when present over a longer period of time. Even

though stress exposure causes long term changes that are supposed to prepare the body for future encounters of a similar stressor, the stress response itself is a short term solution that needs to be terminated once the immediate danger has passed. Since energy resources need to be re-allocated towards systems that are essential for an appropriate response, other functions are suppressed in the meantime. If prolonged, down-regulation of the immune system for example can expose the body to secondary threats. However, not just long-term stress exposure is problematic. Just like almost any biological system, the stress response is finely tuned and therefore rather error-prone once the neural mechanism or one of its many regulators is not operating properly. Many psychiatric diseases go along with over-activation of stress pathways or malfunctioning feedback loops that cause the system to constantly flood the body with stress signals, evoking a chronic state of endocrine emergency for the organism similar to the chronic exposure to an actual stressor (Herman et al., 2016; Jokinen and Nordström, 2009; Pariante and Lightman, 2008). This emphasizes the importance of regulatory mechanisms within the stress response. Independent of the underlying causality, studies confirmed the negative impact of chronic activation of the HPA-axis and the associated presence of increased stress hormone levels on the pathological mental condition of patients (Van Holland et al., 2012). In addition, some of the immunophilins that have been associated with the aforementioned psychiatric disorders have also been found to be aberrantly expressed in diseases like breast and prostate cancer (Ward et al., 1999), as well as diabetes and various metabolic disorders (Ratajczak, 2015). Given the importance of strict regulation, proteins involved in the process are of utmost significance for both, understanding potential triggers for diseases, as well as finding new courses of action for the treatment of such. Even though the stress response has been studied for decades, there are many players and pathways that are either unknown or poorly understood. One first step towards a better understanding is the identification of genetic risk factors in patients. These risk factors do not only act as markers for specific disorders but may also enable us to understand certain aspects of the stress response better and develop new treatment options on a molecular level. This bottom-up approach has yielded a number of genes and proteins that are significantly involved in stress-dependent diseases like major

depression or PTSD. In order to manipulate the system and utilize this knowledge, it is important to develop accurate concepts for these candidates and, if possible, decipher their exact role in the development and pathology of a disease.

## 1.6 How the brain deals with stress - Neurotransmitters

In order to understand the impact of stress, it is important to know the biological background of the underlying processes. Independent of the activated system, stress evokes a finely tuned response in the body and the brain. Due to its complexity and the plethora of molecular players involved, Joëls and Baram called this response the 'neuro-symphony of stress' (Joëls and Baram, 2009). Even though most of this 'symphony' is still poorly understood, the glimpses of knowledge that we acquired so far allow for an insight into the complicated ways the brain can deal with challenges. Since those challenges vary in nature, severity, and timing, the interplay between different classes of mediators (e.g. neurotransmitters, peptides, and steroid hormones) is essential for the acquisition and classification of individual stressors and the execution of an appropriate behavioral response. That way, the range of molecular transmitters allows the body to respond to acute threats by facilitating strategic decision making and immediate attention, as well as storing information about the situation for future encounters. An acute threat like an approaching car requires rapid neurotransmission and neuronal activation, mainly achieved by the release of monoamines like NE. Even though such an event can cause long term alterations, a return to baseline happens relatively fast. Chronic stressors like an aversive work environment, an upcoming deadline or verbal/physical abuse trigger different transmission pathways, operated by slower acting peptides or steroid hormones. Functionality and specificity of the stress response substantially depend on spatial and temporal differences of the involved mediators as well as their receptors. Stress mediators are synthesized and released in certain brain areas but since they only affect neurons that express specific receptors, the spatial resolution of this system is extremely high. It can be observed that these receptors tend to accumulate in stress-relevant 'hot spots' in the brain where they help

to connect networks that are relevant for different aspects of the stress response (reviewed in Joëls and Baram, 2009). That way, both, the site of transmitter release and that of the equivalent receptors contribute to the overall resolution of the stress response. At the same time, the effects of various mediators differ widely in their timing, ranging from milliseconds to hours or even days, providing an additional level of complexity for the system. While monoamines like NE and serotonin evoke fast synaptic effects, mainly as an immediate response to an acute stressor, peptides like CRH and steroid hormones like corticosteroids are capable of causing long-term genomic and structural effects, allowing the body to prevent future effects of a changing environment (Joëls and Baram, 2009).

## 1.7 Corticoids and corticoid receptors

As the name suggests, corticosteroids are a class of steroid hormones. Mineralocorticoids and glucocorticoids are the two major sub-classes of corticoids, synthesized and released from the adrenal cortex. While the physiological mineralocorticoid is called aldosterone and mainly regulates $Na^+$ transport across the epithelium, the respective glucocorticoid in most mammals is called cortisol (or corticosterone in rodents). In both, the periphery and the brain, corticoids bind to so-called corticoid receptors. However, more than one type of steroid hormone can bind to each receptor, with high variability in binding affinity. The different receptors vary in their affinity to aldosterone and are therefore named after their main ligand. The receptors with a high aldosterone-affinity are called mineralocorticoid receptors, and the ones with lower binding-affinity are named glucocorticoid receptors. However, the concentration of aldosterone and corticosterone in the circulation is vastly different. Under normal conditions, concentrations of corticosterone are several orders of magnitude higher than aldosterone. This results in important functional differences of said receptors and allows for very effective fine-tuning of the resulting actions. In the absence of ligands, most receptors are bound to protein complexes and reside in the cytosol of the cell. Upon activation, the receptor is usually trafficked into the nucleus where it controls gene

transcription (Kandel et al., 2013). Both corticoid receptors are ligand-dependent transcription factors that bind DNA-sequences, called glucocorticoid response elements (GREs). Once bound to the GRE they enhance or repress the transcription of a large number of genes. Notably, both receptors also possess non-genomic properties. However, these actions are not directly related to the stress response and hence not described in more detail here. It is important to note that under baseline conditions steroid hormones are released in a pulsatile and circadian fashion (Figure 4).

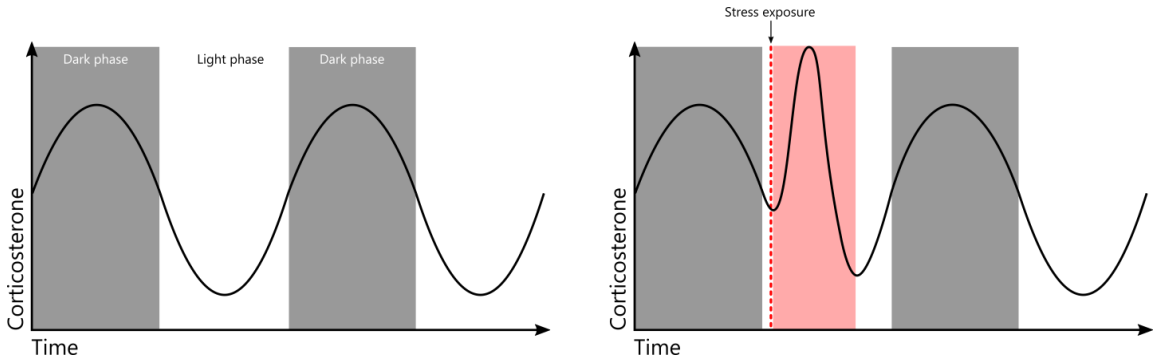


**Figure 4: Release of stress hormones over time**
Stress hormones like cortisol or corticosterone are not only released in response to a stressor but rather follow characteristic release-pattern over the course of a 24h day-night cycle (simplified depiction). In nocturnal animals like mice, corticosterone levels rise during the active, dark phase and are lowest during their inactive phase (left). Exposure to stress leads to a quick burst in stress hormone release that can reach different levels depending on the time of day (right).

This rhythm is crucial in order to maintain appropriate hormone levels during different phases of the day and night cycle. Upon stress exposure, however, a large burst of corticosterone release triggers effects of the stress response (Joëls and Baram, 2009).

## 1.8 Hypothalamic-Pituitary-Adrenal-axis regulation

Because stress hormones are such effective modulators of molecular pathways and behavior, their secretion needs to be controlled tightly. Regulation of the HPA-axis is mainly operated by corticoid receptors and achieved by negative feedback. Excessive amounts of free corticosteroids that enter the brain can bind to those receptors which in turn initiate the shutdown of the stress response

once a big enough swing in the equilibrium is surpassed. This system is extremely important to protect the organism from excessive exposure to stress hormones and a return to healthy homeostatic levels. For one, regulation is controlled by the amount of available corticoids in the brain. One of the key factors in this regard is the sensitivity of the receptors, which is mainly controlled by secondary proteins. The GR for example is predominantly bound to the so called HSP90-complex. This complex includes a number of regulatory proteins, thereby controlling the binding capacity of the receptor to its ligands. One of these proteins is the co-chaperone FK506 binding protein 5 (FKBP51), which, as long as it is bound to the complex, reduces the sensitivity of the GR and therefore prevents its activation and transfer to the nucleus (Zgajnar et al., 2019). Heat shock proteins (HSP) are molecular chaperones which can aid with protein assembly, folding, transport or disposal by binding and stabilizing proteins that might not be in a stable state on their own. They hold protective properties towards external stressors like heat shock (hence the name) and protect cells from potential damage. Those chaperones are highly conserved and ubiquitous proteins, widely expressed across species (Csermely et al., 1998). Chaperones are usually classified by their molecular weight. As mentioned before, the 90kD protein HSP90 plays a pivotal role in the regulation of the stress response as it forms a stable complex with the GR and several regulators of GR function. One of the direct regulators of the GR is FKBP51, which is encoded by the *Fkbp5* gene. Mechanistically, FKBPs are both, immunophilins (capable of binding immunosuppressants like FK506 and rapamycin) as well as peptidylprolyl isomerases (PPIs), which, as the name suggests, can catalyze the *cis-trans* isomerization of proline-peptide bonds. In FKBP51 in particular, these actions are executed via the N-terminal FK506 binding domain (FK1 domain) of the protein. Furthermore, a so-called FKBP-like domain (FK2) has been identified. Even though the FK2 shares about 19% homology with the FK1 domain, it does lack some key residues necessary to bind immunosuppressants (Schmidt et al., 2012). FKBP51 in particular functionally interacts with mature corticoid receptor hetero-complexes like the aforementioned HSP90. Pirkl and Buchner, as well as Schmidt and colleagues, stated that two respective PPIase monomers bind one HSP 90 dimer via tetratricopeptide-repeats (TPR), located at the C-terminus (Schmidt et al., 2012; Pirkl and

Buchner, 2001). In that role, FKBP51 is known to act as a potent co-chaperone, involved in protein trafficking and folding (Sinars et al., 2003). When FKBP51 is bound to the HSP90-complex, it reduces GR sensitivity and inhibits translocation to the nucleus and thus the GRs function as a transcription factor. Transport of the GR from the cytosol into the nucleus, on the other hand, is facilitated by a protein called FKBP52 (encoded by the *Fkbp4* gene), a close homolog to FKBP51. Despite sharing about 75% of its sequence, the functions of these two proteins are contrary due to conformational differences (Zgajnar et al., 2019). Riggs and colleagues identified a single residue in the FK1 domain as one of the critical differences between the two proteins (Riggs et al., 2007). While FKBP51 is the more potent chaperone of the two, FKBP52 is showing a higher affinity for HSP90 dimers (Pirkl and Buchner, 2001). However, it has been shown that when levels of free ligand (corticosterone) are low, the binding pocket of the HSP90-complex is predominantly occupied by FKBP51. Therefore, the complex is likely located in the cytosol. Upon exposure to corticoids, a conformational change of the receptors causes the balance to rapidly shift towards FKBP52 and the complex is translocated into the nucleus (Zgajnar et al., 2019). This translocation is thought to be driven by FKBP52 and is possibly mediated by co-recruitment and binding of dynein, a motor-protein that is known to be involved in intracellular transport (Figure 5). However, both, hormone-free as well as hormone-bound receptor-complexes have been found in the cytosol, as well as in the nucleus. This finding makes it clear that ligand binding does not necessarily determine the location of the complex but much rather shift the equilibrium of its movement from and into the nucleus (Echeverria et al., 2009; Davies et al., 2002; Galigniana et al., 2001). Especially in large cells like neurons, the intracellular movement might be one of the rate-limiting factors (Schmidt et al., 2012). Once the complex is translocated into the nucleus and dissociated, the activated receptor can bind to the DNA and act as a transcription factor. Noteworthy, while the GR controls a number of genes that are important for long term adaptations to stress, it also regulates the transcription of *Fkbp5* itself. That way, low cellular levels of FKBP51 cause an increase in transcription, forming an ultra-short feedback loop within the regulatory feedback mechanism of the stress response (Fries et al., 2017).

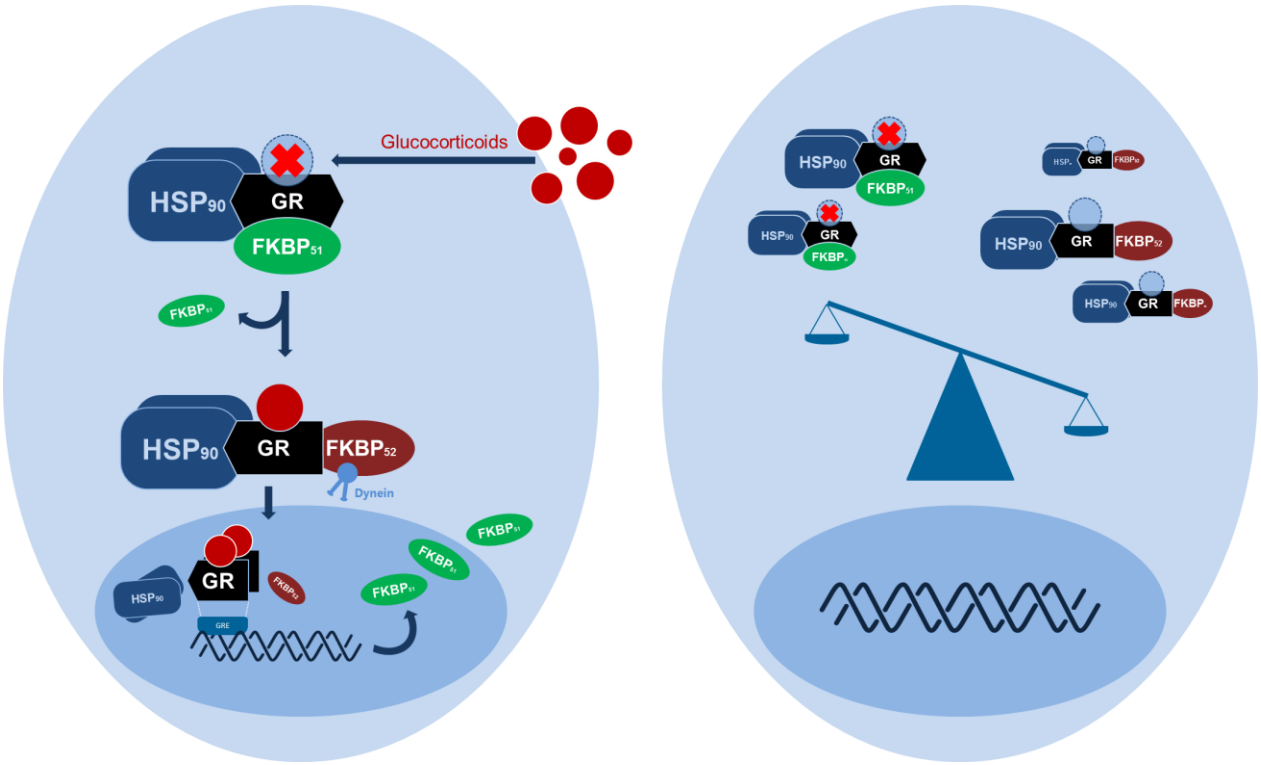


**Figure 5: FKBP51 – one of the molecular regulators of the stress response**
*Left*: Once glucocorticoids reach the brain, they initiate the termination of the stress response by binding intracellular GRs. Those receptors are bound to other proteins like HSP90 or FKBP51, forming regulative hetero-complexes in the cytosol. FKBP51 is one of the major regulators of GR sensitivity. It keeps the GR from entering the nucleus where it could act as a transcription factor for genes that are involved in stress response termination. Upon binding of a ligand to the GR, FKBP51 is replaced by its close homologue FKBP52 which facilitates the translocation of the HSP90-complex into the nucleus (most likely via a motor-protein called dynein). Within the nucleus, the GR can bind to glucocorticoid-response elements (GRE) and initiate gene transcription. *Fkbp5* is one of the target genes of the GR, resulting in an ultra-short feedback loop to retain protein balance.
*Right*: The cytosol contains HSP90-complexes of different compositions (bound to FKBP51 or FKBP52 respectively) at all times. Termination of the stress response depends on the availability of one or the other variant. Therefore, the balance between the two players is a determining factor in this molecular pathway.

Taken together, glucocorticoids not only initiate a number of physiological and molecular adaptations to stress, but ultimately the termination of the stress response. However, individual differences in the molecular response to glucocorticoid activation are linked to differences in the

stress response itself. This might lead to changes not only on a cellular, but also at brain circuitry and systemic levels (Matosin et al., 2018).

## 1.9 Genetic modulation of *Fkbp5*

Since FKBP51 and FKBP52 play such a crucial role in the stress response, both immunophilins have been the focus of many studies in the past. While some interesting features of FKBP51 and FKBP52 are well described, the majority of their properties are still poorly understood. The usage of genetically modified mouse models can help to shed light into the many aspects controlled by this set of proteins. While the role of *Fkbp4* in early development makes its genetic modulation extremely difficult, *Fkbp5* has been successfully removed in conventional knock-out (KO) mouse lines ($Fkbp5^{KO}$). Hartmann and colleagues revealed that a full body KO of *Fkbp5* results in a more stress resilient phenotype, most likely due to an increase in sensitivity of the GR. Animals in these studies showed reduced baseline corticosterone levels, as well as an attenuated stress response and enhanced negative feedback (Hartmann et al., 2012). Other studies have confirmed that corticosterone is especially low in these animals during the natural peak at the onset of the active phase when compared to wild-type (WT) controls ($Fkbp5^{WT}$) (Albu et al., 2014). A lack of FKBP51 also leads to a change in stress coping behavior when animals were subjected to the forced swim test (FST) (Touma et al., 2011). However, these results do not allow for any speculation about the site specific role of FKBP51 since it is usually transcribed in a wide range of cells in the brain and the periphery.

## 1.10 Pharmacological modulation of FKBP51

While genetic approaches bear their own set of problems, pharmacological modulation has proven to be difficult due to the similarity of the FKBPs. The fact that the respective mode of action of FKBP51 and FKBP52 is often opposing highlights the importance of specificity in this case. Up until recently, most antagonists have failed to be specific enough for one or the other FKBP. Even

non-immunosuppressive analogs of FK506 or rapamycin have proven to be pan-selective within the FKBP family. Studies have shown that inhibition of FKBP52 causes a number of problematic side effects, specifically on the reproductive system of the animals (reviewed in Schmidt et al., 2012). This goes along with a high embryonic lethality observed in *Fkbp4*$^{KO}$ animals (Hartmann et al., 2012). For these reasons, FKBP51 has been a more promising target for pharmacological intervention. A couple of years ago, Hausch and colleagues managed to design a chemical modulator with high specificity for FKBP51. A highly selective induced-fit mechanism makes the ligands SAFit1 and SAFit2 (short for selective antagonist for FKBP51 by induced fit; SAFit2 is a brain-permeable analog of SAFit1) much less favorable for FKBP52 (>10.000-fold selectivity for FKBP51 over FKBP52) and has been shown to alter neurite growth, neuroendocrine feedback and stress-coping behavior in mice. Although the exact binding mechanics of the drug are still unclear, it is hypothesized that SAFit2 can bind unbound FKBP51 and prevent it from its interaction with the HSP90-complex (Figure 6). Studies in animals have also demonstrated that SAFit2 increased the suppression of corticosterone levels after Dexamethasone or CRF treatment. This effect is in line with observations in *Fkbp5*$^{KO}$ animals that show a higher sensitivity of corticosteroid receptors (Touma et al., 2011). Both ligands also possess higher affinity compared to the FKBP ligand FK506 without showing any of the FK506-like immunosuppressive properties that might be undesirable in many applications (Gaali et al., 2015).

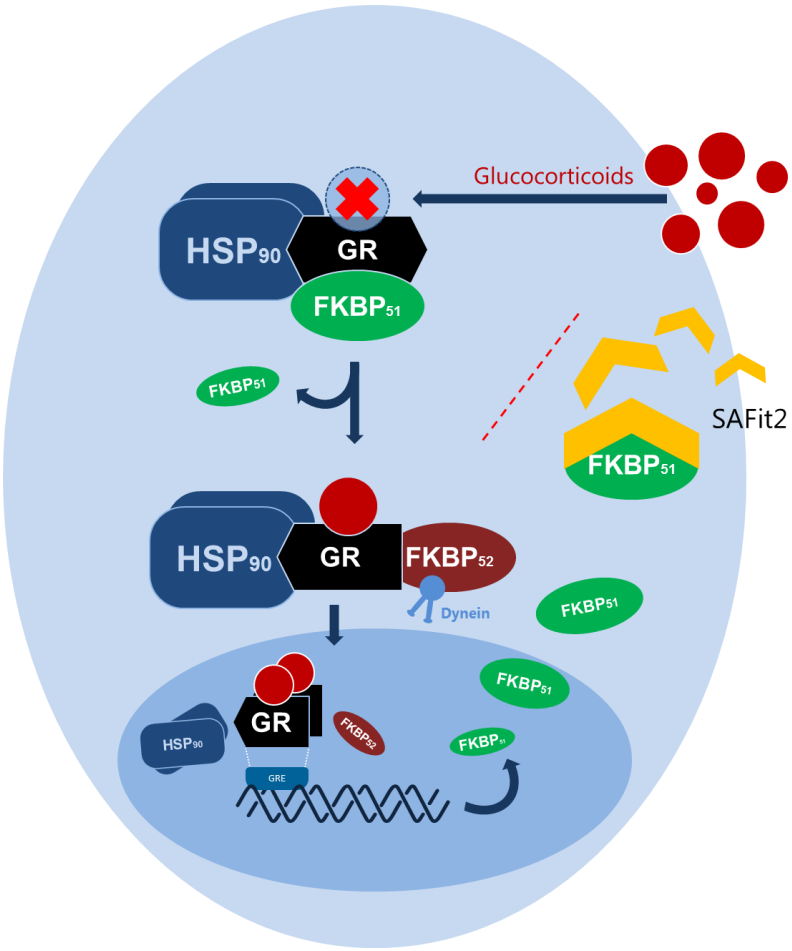


**Figure 6: SAFit2 inhibits FKBP51 functions**
SAFit2 is a modulator, specifically developed to interfere with FKBP51 functionality. Its hypothesized mode of action lets it bind to free FKBP51 where it initiates a conformational change that prevents FKBP51 from binding to the HSP90-GR-complex. Whether it can also target fully assembled complexes is still unknown.

## 1.11 The monoaminergic system

Because of the ubiquitous distribution across a wide variety of cell types and tissues, levels of the immunophilin expression might hold regulatory properties for the biological functions of the associated receptors (Zgajnar et al., 2019). *Fkbp5* in particular is expressed in a wide range of cell types and brain areas. Studies show that the expression of the *Fkbp5* gene is highly enriched in stress-relevant areas of the mouse brain and also regulated in a stress-dependent manner (Scharf et

al., 2011). While *Fkbp5* received a tremendous amount of attention in many of the 'classical' stress regions, its' role in areas like the dorsal raphe nucleus (DR) or the locus coeruleus (LC) has not been studied to the same extent. However, the monoaminergic system is not only heavily involved in the stress response (Benarroch, 2018; Puglisi-Allegra and Andolina, 2015) but also one of the hot-spots of *Fkbp5* mRNA expression and regulation. In that regard, the DR and the LC are heavily affected by stress exposure, which makes them interesting targets for stress research.

### 1.11.1 The dorsal raphe nucleus

The DR is a relatively large area located in the midbrain and pons on the midline of the brainstem. Its projections are widely spread throughout several brain areas and reach as far as the olfactory bulb. In rats, a major part of the neurons have been found to project to the amygdala (Ma et al., 1991), however, there are also strong connections to other areas like the caudate putamen. While the DR utilizes a multitude of neurotransmitters, the monoamine serotonin (5-HT) is the most abundantly released substrate of DR neurons (Michelsen et al., 2008) making the DR the largest serotonergic nucleus of the brain. Monoamines are particular sorts of chemical substances that are crucial neurotransmitters in specific areas of the brain. The term arises from their chemical structure, as all monoamines are defined by one amino group and an aromatic ring (Kandel et al., 2013). Like other neurotransmitters, they are synthesized in the presynaptic neuron and mostly derive from aromatic amino acids, which are processed by aromatic amino acid decarboxylases. Monoamines can be divided into several sub-groups, depending on their precursor substrates. Serotonin, for example, is a derivate of the essential amino acids tryptophan and histamine, the product of histidine. Biosynthesis of Serotonin is a two-step reaction, catalyzed by tryptophan hydroxylase and aromatic amino acid decarboxylase. It plays an important role in regulating physiological processes of the stress response including pain control, the cardiovascular system, and the gastrointestinal tract, as well as complex cognitive processes like the encoding of reward signals (Li et al., 2016) or attention and mood (Kandel et al., 2013; Saxena, 1995). The DR is known to be heavily involved in psychiatric disorders like depression (Matthews and Harrison, 2012) and

a lot of the most effective antidepressants target the serotonergic system by regulating the amount of available serotonin in the synaptic cleft.

### 1.11.2 The locus coeruleus

The LC has been of special interest for a long time since it is the main area of NE synthesis. The LC has been found to be a highly organized brain area with system-wide projections (Levitt et al., 1984; Lindvall et al., 1984). It receives a majority of its input from medullary nuclei like the nucleus paragigantocellularis and the nucleus prepositus hypoglossi, as well as other monoamine areas like the ventral tegmental area and raphe nucleus (Benarroch, 2018). Bodies of NE neurons are located within the LC and, ranging from there, those neurons mainly innervate cortical and subcortical areas and the spinal cord. Like its close relative epinephrine, NE is a catecholamine that derives from the non-essential amino acid tyrosine. In that process, tyrosine hydroxylase (TH) and DOPA-carboxylase are the most important enzymes which convert L-tyrosine to L-DOPA and L-DOPA into dopamine (DA). DA on its own regulates cognitive processes and motivational behavior and the reward system (Tzschentke, 2001) but can be processed further. It is stored in neurons of the LC and, upon the event of an action potential, transformed to NE within the vesicles by dopamine-B-hydroxylase and released into the synaptic cleft via exocytosis (Kuhar et al., 1999). NE neurons promote synaptic activity and NE is a crucial part of the adrenergic nervous system. It controls cardiovascular effects (heart rate, blood pressure and blood flow), energy distribution (glucose availability) and behavioral stress adaptations like concentration and arousal (Ressler and Nemeroff, 1999). In general, release of NE mobilizes the brain and body for action and initiates an appropriate response to stress. Dysregulated NE circuits are known to be very prevalent in a number of affective disorders, potentially by disrupting neurotransmission in cortical areas that control states like concentration, attention, memory, arousal and sleep regulation. For that reason, researchers have been linking the LC to mental diseases for centuries now, without disentangling its exact role (Benarroch, 2018; Bissette et al., 2003; Klimek et al., 1997; Weiss et al., 1994). However, some studies propose that the NE system might be part of the symptomatology rather than the

causality (Ressler and Nemeroff, 1999). All in all, the features of the monoaminergic system make it a very interesting and promising target in regard of stress related mental diseases.

## 1.12 Aims of the thesis

In order to develop and improve treatment methods in the future, it is essential to have a comprehensive understanding of all the underlying biological mechanisms. Even though, some functions of important regulators of the stress response like FKBP51 are well understood (O'Leary et al., 2013), we have barely any knowledge about its exact role in specific brain areas or cell types. We are still lacking clear understanding of the interplay of steroid hormone regulators and the chaperone-complexes they bind. And even though complexes like the HSP90-complex are in such a pivotal position for the termination of the stress response, we lack the understanding and knowledge to use them for directed pharmacological intervention. Here, I therefore wanted to address the following open questions:

1) Can we replicate the described stress-dependent up-regulation of *Fkbp5* after acute stress, utilizing a social stressor?
2) Does chronic social stress alter the overall *Fkbp5* expression pattern in areas where it is regulated after an acute challenge?
3) Which are the brain areas where *Fkbp5* is regulated the most?
4) Since we found two of the monoaminergic nuclei to be heavily affected by stress-dependent *Fkbp5* regulation, is there a behavioral adaptation linked to the level of *Fkbp5* expression in said regions?
5) More precisely, does site-specific *Fkbp5* over-expression (OE) in the LC or DR affect behavioral aspects like locomotion, anxiety-like behavior or stress coping when tested under baseline conditions?
6) Likewise, does a conditional genetic *Fkbp5* KO in the same areas result in a more anxiolytic phenotype, similar to the effect reported in full body KOs?

7) Do we see behavioral changes when we pharmacologically inhibit FKBP51 functionality and if so, are these effects likely to increase resilience to stress?
8) What effects do we observe when the inhibition of FKBP51 is combined with common antidepressants?
9) Can we improve the comparability of a genetic manipulation (or KO) and a pharmacological intervention by observing the animals' home cage behavior before and after stress?

In order to find answers to these questions, I first investigated *Fkbp5* mRNA expression using *in situ* hybridization (ISH). Next, I extensively tested different genetic and pharmacological approaches and conducted an in-depth characterization of the mouse lines and cohorts in the process. I aimed to provide a comprehensive behavioral description of both, region specific *Fkbp5* OE as well as conditional *Fkbp5* KO lines, lacking the gene in either the LC or the DR. Second, I applied the FKBP51-specific antagonist SAFit2 in different setups in order to decipher its effects on behavior and stress coping while improving and simplifying the application of the drug.

# 2 Animals, Materials and Methods

## 2.1 Short summary of experiments

### 2.1.1 Experiment 1: *Fkbp5* mRNA expression after stress

Experiment 1 was designed to provide an extensive insight into the stress induced regulation of the psychiatric risk gene *Fkbp5* in mice. *Fkbp5* mRNA levels were assessed by ISH after either acute social defeat stress (ASDS) or a prolonged exposure to chronic social defeat stress (CSDS). The experimental design allowed for a comprehensive overview of stress-relevant regions with significant *Fkbp5* mRNA expression, as well as the detection of up- or down-regulation following different durations of stress exposure.

### 2.1.2 Experiment 2: *Fkbp5* over-expression in monoaminergic nuclei

Following the mapping, we selected two monoaminergic regions (LC and DR) that showed significant mRNA regulation after acute stress for continuative manipulation studies. As an initial step, we validated the co-localization of *Fkbp5* with specific regional markers. The main aim of experiment 2 was to further improve our knowledge about region specific roles of FKBP51 in brain areas with high mRNA expression and stress-induced regulation, identified in experiment 1. It also allowed for better understanding of viable ways to manipulate the system and - in this case - mimic stress conditions in pre-defined brain regions exclusively. Instead of an external stressor, artificial up-regulation was achieved via viral over-expression. Following surgery and an incubation period, animals were tested for behavioral effects of the local increase in *Fkbp5* expression in either the LC or the DR.

### 2.1.3 Experiment 3: Knock-out of *Fkbp5* in monoaminergic nuclei

Building on experiment 2, which revealed potential effects of an abundance of FKBP51 in certain brain areas, experiment 3 was designed to explore the effects of a contrary approach. By cross-

breeding *Fkbp5* KO animals with region specific *Cre*-lines, *Fkbp5* was knocked-out in either the LC or the DR. Subsequently, animals underwent an extensive test battery in order to provide a comprehensive characterization of potential behavioral, physiological or endocrine implications of *Fkbp5* deletion in these areas. Behavioral tests were carefully selected to cover locomotion, explorative behavior, anxiety-like behavior, cognition, sociability, social avoidance and stress coping behavior. All animals were weighed several times during the experiment and the endocrine stress response was measured at the end of the behavioral assessments.

### 2.1.4 Experiment 4: Effects of SAFit2 in a chronic social stress model

In order for our findings to be of any translational relevance, pharmacological tools to manipulate FKBP51 are needed. One of the most promising drugs manipulating FKBP51 is SAFit2, which was used in experiment 4. SAFit2 is known to have inhibitory properties towards FKBP51 functionality and comparable effects to full body KOs have been shown in some tests (Hartmann et al., 2015). In this experiment, animals received a drug-loaded, slow-releasing pellet to guarantee steady SAFit2 levels over the course of a 3 week stress paradigm. After being exposed to social stress for 21 consecutive days, animals were tested, for explorative and anxiety like behavior in order to examine effects of FKBP51 inhibition in this context.

### 2.1.5 Experiment 5: Co-medication of SAFit2 and Escitalopram

Another essential factor during the development of pharmacological treatments for psychiatric disorders is the potential for co-medication. While simultaneous drug administration can benefit the outcome, new combinations of drugs require extensive testing in order to guarantee their safety and efficacy. As a first step towards a possible implication of FKBP51 into a combined treatment routine, a combination of FKBP51 inhibition via SAFit2 and a commonly used selective serotonin re-uptake inhibitor (SSRI) was tested in mice. Animals received one dose of a SAFit2-loaded vesicular phospholipid gel (VPG) in addition to a daily injection of Escitalopram (a commonly used SSRI). A standard testing battery was conducted to monitor any behavioral implications caused by

each individual drug, as well as the combination of treatments. Additionally, the endocrine stress reaction in treated animals was tested via corticosterone measurements from blood plasma.

### 2.1.6 Experiment 6: Improvements to the application of SAFit2

Experiment 6 was conducted to increase efficiency and applicability of SAFit2 treatment in rodents. After extensive testing of different application methods, it was postulated that intraperitoneal (i.p.) injections - while yielding dose-effective drug levels in the brain - are too invasive and stressful and therefore not suitable for chronic treatment. Furthermore SAFit2-loaded pellets like the ones used in experiment 4 simply lack loading capacity to result in high enough drug release needed for baseline studies. To avoid any of these problems, a novel formula was tested where SAFit2 was implemented into a VPG. Due to the low viscosity and high loading capacity of the formula, it was possible to inject the VPG into the neck area of the mice without causing any hindrance or impairment while ensuring steady release of the drug. To generate a comprehensive overview and optimize the approach, blood and brain samples were collected at a number of time points up to 16 days after application. SAFit2 levels were then measured via mass spectrometry.

### 2.1.7 Experiment 7: FKBP51 modulation and acute stress

While basic knowledge about the comparability of a full body *Fkbp5* KO and SAFit2 treatment exists, most of it originates from independent studies. Experiment 7 was designed to provide an in depth comparison of the two groups, mainly focused on natural home cage behavior, both, under baseline and stress conditions. For that, home cage activity was recorded and analyzed before and after exposure to restraint stress. As a result, a total of 8 distinguishable behaviors could be observed over the recorded time period.

## 2.2 Animals

### 2.2.1 Animal strains

Animals used in all of the experiments were based on a C57Bl/6n background. Conventional Fkbp5$^{KO}$ animals were obtained from the in-house breeding facility of the Max-Planck Institute of Psychiatry in Munich, Germany. All animals used for the generation of the conditional KO lines were kindly provided by Dr. Jan Deussing and bred in the in-house breeding facility of the Max-Planck Institute of Psychiatry in Munich, Germany. Aggressive residents were mice of the CD1 strain, obtained from Charles River Europe.

### 2.2.2 Standard housing conditions

Animals were either kept in an open cage system with a standard cage size of (21cm x 15cm x 14cm, Plexiglas) or in individually ventilated cages (IVC; 30cm x 16cm x 16cm; 501cm$^2$), serviced by a central airflow-system (Tecniplast, IVC Green Line – GM500). The system used for each individual experiment is indicated accordingly. Housing conditions in the holding and testing rooms were kept constant at 23±2°C and humidity 55% at all times. Light conditions were also kept continual with a 12h : 12h light-dark-cycle (lights on at 08:00 am). All cages were equipped with sufficient bedding and nesting material to provide species-appropriate animal housing and allow natural behaviors like digging and nest building. While not in an acute experiment, all animals had access to environmental enrichment in form of a wooden tunnel. However, the tunnels were removed for the duration of any experimental procedure. If not stated elsewise, food (Altromin 1324, Altromin GmbH, Germany) and water (tap water) was available *ad libitum* in all our experiments. All experiments were carried out in accordance with the European Communities Council Directive 2010/63/EU. All animal suffering was minimized during testing. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany. The number of animals was kept at a minimum and all efforts were made to minimize animal suffering.

## 2.3 Experimental design and background

### 2.3.1 Experiment 1

All animals used in this study were aged between 10 and 12 weeks and bred on a C57Bl/6n background in our in-house breeding facility. Mice were housed in the holding room for at least 2 weeks to properly acclimate. Both, control (n=12) and stress (n=13) animals of the acute stress cohort were kept in groups of 3-4. Animals of the CSDS cohort (control: n = 16; stress: n = 16) were single housed (control group) or in a cage setup with a CD1 resident (stress group) during the course of the experiment. A total of 17 trained adult CD1 animals were used as aggressive residents. Animals in this experiment were kept in IVC cages. The experiment was split in two parts to ensure fast and effective processing of animals and tissue. First, mice were exposed to an acute social defeat and sacrificed 4 hours later in order to obtain any effects of an acute stressor. Second, we exposed an additional cohort to CSDS and sacrificed them after 21 days. The last defeat was conducted 24h before the sacrifice to avoid any acute stress effects. Animals from all groups were deeply anesthetized and decapitated. In both cases, tissue (brain, thymus, adrenals) was collected for validation of the stress paradigm and further gene-expression analysis (Figure 7).


**Figure 7: Timeline experiment 1**
Experiment 1 was split into two separate cohorts, acute (blue) and chronic (red) stress. Each of the two cohorts itself was divided into a control and a stress group respectively. Animals from the stress groups were sacrificed at the time-points depicted in this graph and tissue was collected for further analysis.

### 2.3.2 Experiment 2

Double *in situ* hybridization

We used eight week old, naïve animals (n=5) that were bred on a C57Bl/6n background in our in-house animal facilities to collect brain-tissue for dISH. Animals were acclimatized to their holding environment for 2 weeks, deeply anesthetized and decapitated under baseline conditions. Animals in this experiment were group housed in IVC cages (Figure 8).

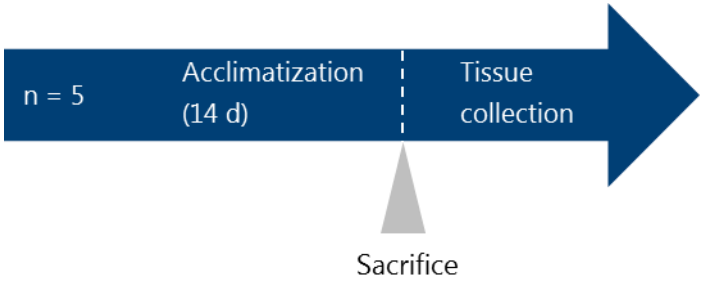


**Figure 8: Timeline double *in situ* hybridization**
A total of 5 animals were sacrificed after a 14 day acclimatization period. Brains were cryo-sectioned and used for double ISH.

Over-expression

All animals used for the over-expression studies were aged between 8 and 10 weeks at the beginning of the experiment and bred on a C57Bl/6n background in our in-house breeding facility. Due to possible losses during surgery, animal group sizes were adjusted accordingly. Experimental groups were split in 3 cohorts to allow faster processing. Cohort 1 received a bilateral injection (300nl) of either control virus (LC *Fkbp5*-Ctrl; n=19) or *Fkbp5* over-expressing adeno-associated virus (AAV) (see virus details described by Hartmann et al., 2012; Schmidt et al., 2011) (LC *Fkbp5*-OE; n=12) into the LC (coordinates: AP: +5.5; L: ±0.9; DV: +4.5). Similarly, animals from cohort 2 received a bilateral injection of the same two viruses into the LC (LC *Fkbp5*-Ctrl; n=15; LC *Fkbp5*-OE; n=11), however, the volume was decreased to 200nl to minimize spread of the virus. Subsequently, cohort 3 (DR *Fkbp5*-Ctrl; n=13; DR *Fkbp5*-OE; n=19) was injected unilaterally with identical viruses into

the DR (300nl; coordinates: AP: +4.5; L: ±1.0; DV: +3.7). All animals that underwent surgery were single housed and kept in the surgery room for 3 consecutive days while being monitored closely. After that, they were transferred back into the regular holding rooms and allowed to recover for 4 more weeks. Both rooms were equipped with an IVC system.

Due to limitations in holding and testing capacity, the LC over-expression experiment was split in two cohorts, each undergoing a different subset of behavioral tests. Animals of the first cohort underwent the following tests: open field test (OF), elevated plus maze test (EPM), dark-light box test (DaLi) and FST. Animals from the second batch were tested in the OF, Y-Maze test, object relocation test (OLT), social avoidance test (SA) and FST. The OF was repeated in each batch to exclude any locomotive impairments. The SA was conducted two times, once under baseline conditions and once 24h after subthreshold defeat stress. The FST was conducted twice as well. A first time under baseline conditions and a second time after the animals received an injection of a common SSRI (Escitalopram).

Just like the animals in the LC *Fkbp5*-OE experiments, mice from the DR cohort were allowed to recover for 4 weeks after surgery. The order of tests was as follows: OF, EPM, DaLi, SA, FST and OLT. Appropriate resting time was assured after the FST to avoid any impact on later tests. Again, the SA was repeated 24h after a subthreshold defeat and the FST was conducted twice, once under baseline conditions and once after an i.p. Escitalopram injection (Figure 9). Animals from all groups were deeply anesthetized and sacrificed on the day following the last behavioral test. Brains and organs were removed and frozen up until further analysis. Successful OE of *Fkbp5* was verified by ISH. Animals that were not infected in the targeted areas were eliminated from the analysis.

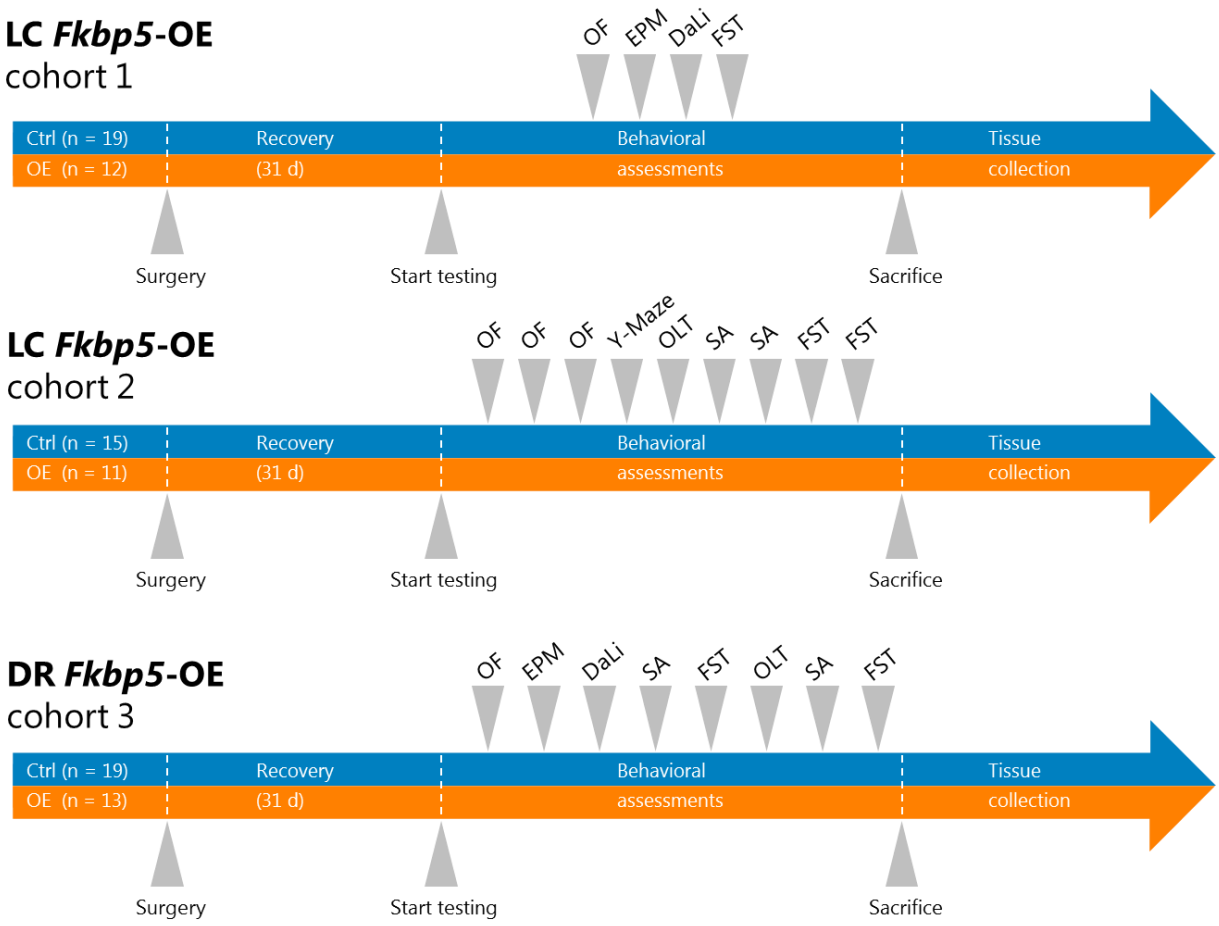


**Figure 9: Timeline experiment 2**
Experiment 2 was divided in 3 subsequent cohorts. Cohort 1 and 2 received an injection of *Fkbp5* over-expressing AAV into the LC, while cohort 3 received the same injection into the DR. All three cohorts were allowed to rest for 31 days after surgery and tested as depicted in the timeline. After the last test, animals were sacrificed and tissue was collected for further analysis.

### 2.3.3 Experiment 3

Genetic knock-out

Conditional KO of *Fkbp5* was achieved by utilizing the *Cre*/loxP system. This system is a commonly applied site-specific recombinase technology, originating from P1 bacteriophages that allows for specific modification of chromosomal DNA. In order to remove a sequence of DNA, it needs to be flanked by two loxP sites. Excision of the sequence is then catalyzed by an enzyme *Cre* -

recombinase. The section between the detection sites is cut out of the chromosomal DNA, forms a circular product and is degraded within the cell. In order to achieve a cell type or area specific excision of a gene, expression of the *Cre*-recombinase needs to be regulated very tightly. By coupling *Cre* with a promoter that is only active in a target area or cell type, the recombinase is solely transcribed and active in those cells. For that reason, animals carrying the flanked target gene can be cross-bred with a mouse line that expresses *Cre* only in certain cell types. As a result, the floxed sequence of DNA is only excised in the cells that show *Cre*-activity (Metzger and Chambon, 2001) (Figure 10).

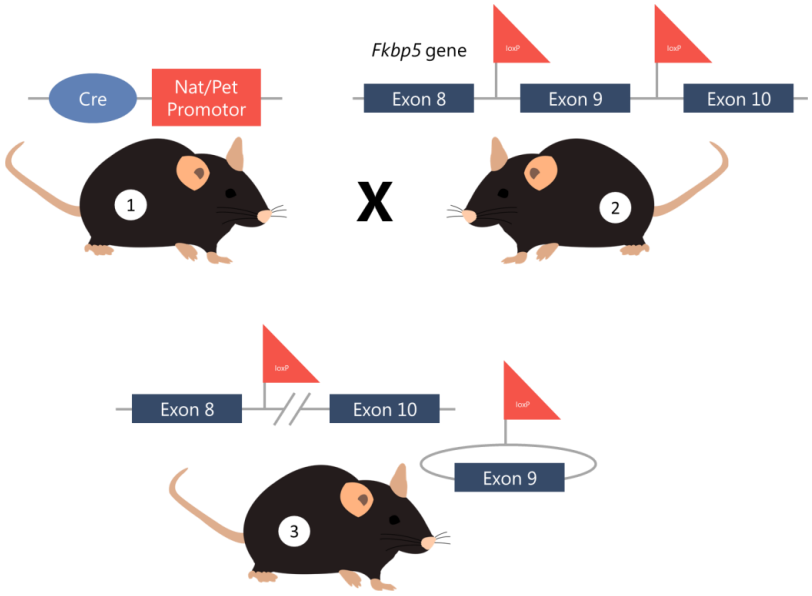


**Figure 10: Generation of conditional *Fkbp5* KO lines**
In order to obtain a genetic KO of *Fkbp5* in monoaminergic cell populations, two different mouse lines were crossed. Animals with *Cre*-recombinase linked to either the *Nat*- or *Pet*-promotor (1) were bred with a line that had exon 9 of the *Fkbp5* gene floxed (2). Exon 9 was cut out by *Cre*-recombinase in cells containing an active *Nat*- or *Pet*-promotors, resulting in a dysfunctional FKBP51 protein in these cells (3).

For the baseline characterization of a *Fkbp5* KO in monoaminergic areas, we used two genetically engineered lines: *Nat-Cre* animals to target cells expressing the norepinephrine transporter (*Net* aka *Nat*) (Fentress et al., 2013) in the LC and a *Pet-Cre* line (aka *Pet-1*) specific for serotonergic cells (Deneris, 2011; Scott et al., 2005; Hendricks et al., 2003) of the DR. These animals were crossed

with another line containing a floxed *Fkbp5* locus (*Fkbp5*$^{lox/lox}$) in order to achieve a conditional elimination of *Fkbp5* in cells with active *Cre*-recombinase. In experiment 3, only litter mates from homozygous *Fkbp5*$^{lox/lox}$ parents with or without *Cre* were used. From here on, the conditional KO lines are referred to as *Fkbp5*$^{LC\ KO}$ and *Fkbp5*$^{DR\ KO}$ respectively. Control animals were *Cre*-negative littermates from now on referred to as *Fkbp5*$^{LC\ WT}$ and *Fkbp5*$^{DR\ WT}$. Group sizes were as follows: WT controls: *Fkbp5*$^{LC\ WT}$; n=13 as well as *Fkbp5*$^{DR\ WT}$; n=28, and littermate KOs: *Fkbp5*$^{LC\ KO}$; n=13 as well as *Fkbp5*$^{DR\ KO}$; n=12. Both lines were tested separately.

All mice were 3 to 5 months of age at the time of testing. They were allowed to acclimate to the holding conditions for 2 weeks and were single housed in an IVC system one week prior to the start of the experiment.

We tested all cohorts for a wide range of behaviors. The following tests were included: OF (3x), OLT, object recognition test (ORT), DaLi, EPM, SA, FST and a second SA after exposure to a subthreshold defeat. The OF test was repeated 3 times to make sure the animals were properly acclimated to the testing conditions, as well as test for possible effects on spatial memory. To avoid any crossover effects, all tests were conducted in a specific order, starting with the least stressful and finishing with the most stressful paradigm. Blood samples for endocrine measurements of the stress response and recovery rate were taken 30 and 90 minutes after the onset of the stressor (FST) respectively. Baseline corticosterone levels were assessed in trunk blood, obtained on the day of sacrifice. Additionally, brains were removed for further analysis. In order to confirm the successful KO of *Fkbp5* in the targeted areas, we performed ISH and evaluated mRNA expression levels (Figure 11).

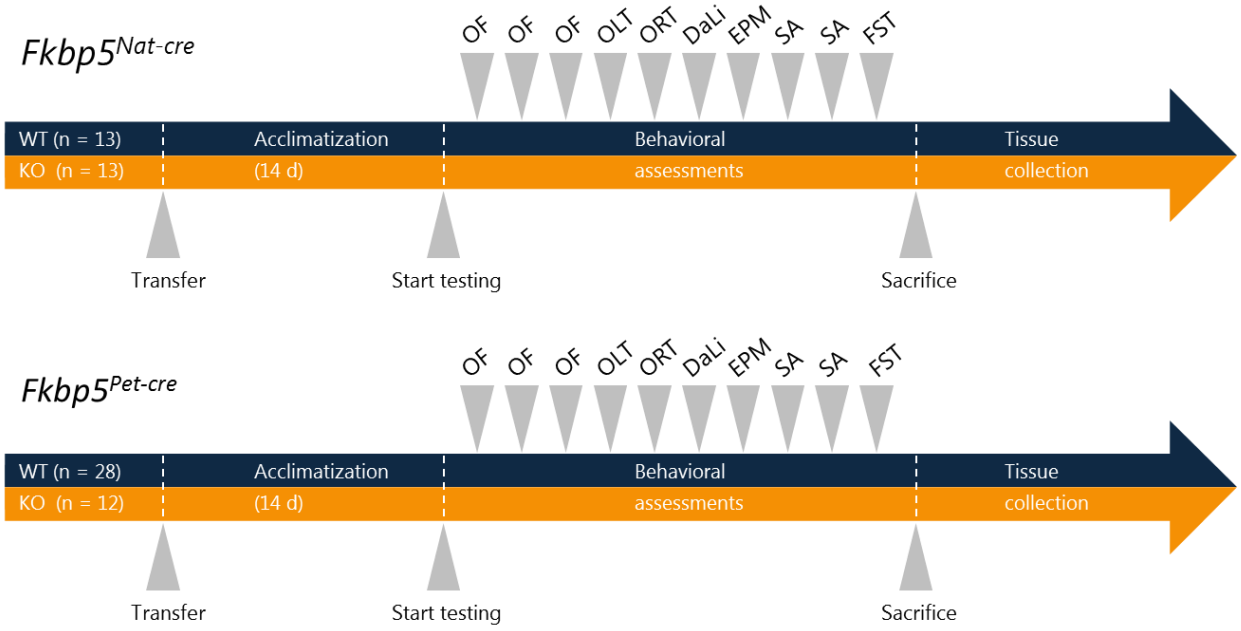


**Figure 11: Timeline experiment 3**
For experiment 3, two lines of conditional *Fkbp5* KOs were bred up and tested after a 14 day acclimatization period. Both lines were tested individually in order to ensure fast processing and testing. After the last behavioral test, animals were sacrificed and tissues were collected for further analysis.

### 2.3.4 Experiment 4

Mice for experiment 4 were bred on a C57Bl/6n background in our in-house animal facilities and 8-10 weeks old. A 2 x 2 design was chosen to provide appropriate controls for all groups: (1) Vehicle Basal (n=14), (2) SAFit2 Basal (n=10), (3) Vehicle Stress (n=11) and (4) SAFit2 Stress (n=10). All animals underwent a quick surgical procedure to implant either vehicle pellet, or a SAFit2-loaded pellet. The incision was localized in the neck area of the animals to pose as little interference as possible. After surgery, wounds were carefully stitched and animals were allowed to recover for one day. Controls were single-housed in open standard cages, while animals of the stress group were housed in bigger cages together with aggressive residents. Animals from the stress group underwent CSDS for a total of 21 days. Behavioral testing of all groups was conducted during the third week of the CSDS. All tests were performed in a specific order to minimize possible cross-

over effects: OF, EPM, DaLi, SA and FST. All animals were sacrificed on day 22, brains and tissues were collected for further analysis (Figure 12).

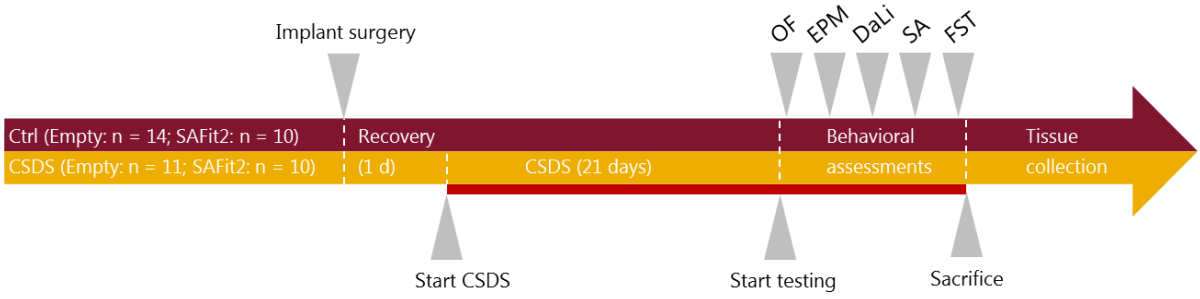


**Figure 12: Timeline experiment 4**
Animals from experiment 4 underwent a quick surgery under anesthesia one day prior to the start of the CSDS protocol to implant either empty or SAFit2 loaded pellets subcutaneously. In the third week of the CSDS paradigm, animal behavior was tested. After the last test, all mice were sacrificed and tissue was collected for further analysis.

### 2.3.5 Experiment 5

Mice for experiment 5 were 8-10 weeks old and bred on a C57Bl/6n background in our in-house animal facilities. A 2 x 2 design with 4 experimental groups was used: (1) control animals (n=10) injected with empty VPG and Vehicle, (2) SAFit2 animals (n=10) injected with SAFit2 VPG and Vehicle, (3) Escitalopram animals (n=10) injected with empty VPG and Escitalopram, and (4) SAFit2+Escitalopram animals (n=10) receiving both drugs. On day 1 of the experiment, half of the animals received SAFit2 treatment (10mg/kg corrected for bodyweight) and the other half received an injection of empty VPG. Animals were allowed to recover from the injection for 24 hours. On the subsequent days, half of each group received either the SSRI Escitalopram (10mg/kg corrected for bodyweight) or vehicle (saline) 30 minutes before behavioral testing. The following tests were performed with a 24-hour inter trial interval (ITI): OF, EPM, DaLi and FST. Blood samples for corticosterone measurements were assessed 30 (response) and 90 (recovery) minutes after the FST. The animals were sacrificed 24 hours after the last behavioral test and brain tissue was collected for

further analysis. Baseline corticosterone levels were measured from trunk blood samples, obtained during the sacrifice of the animals (Figure 13).

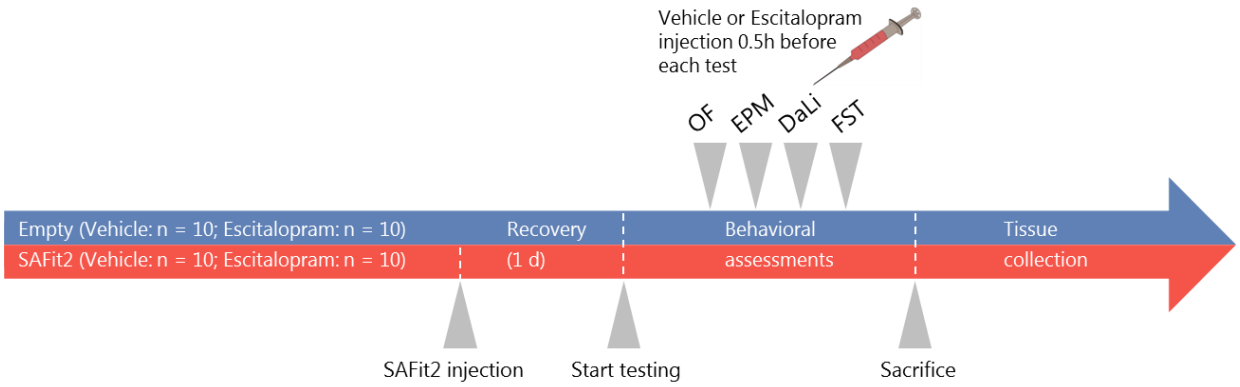


**Figure 13: Timeline experiment 5**
In experiment 5, animals were assigned to four different groups. Depending on their group, animals received an injection of empty or SAFit2 loaded VPG. After one day of recovery, animals were tested for behavior. On each consecutive day, half of each group received a vehicle injection while the other half was injected with Escitalopram 30 minutes prior to the start of testing. After the last behavioral test, animals were sacrificed and tissues were collected for future analysis.

### 2.3.6 Experiment 6

All animals for experiment 6 were bred on a C57Bl/6n background in our in-house animal facilities and 8-10 weeks old at the time of testing. Animals were housed in groups of 3-4 in an IVC system and split into 2 cohorts in order to allow for a comprehensive comparison of immediate-release drug delivery via s.c. injection (n=27) to a newly developed sustained-release VPG (n=15). The FKBP51 modulator SAFit2 was used to test the chronic effects on plasma substance-levels after a single injection in each of the two delivery methods. Two different drug concentrations were tested in the VPG group (30%: n=7; 50%: n=8) to allow for a more in depth analysis of the pharmacokinetics of the gel. Each control groups received a similar vehicle solution or empty VPG lacking SAFit2. Animals from all groups were weighed and the injection volume of both, the SAFit2 solution and the VPG was adjusted to a dose of 10mg/kg bodyweight accordingly. Blood samples were obtained from the tail vein at 8 pre-defined points in time post injection and plasma levels of

SAFit2 were quantified by ultra-performance liquid chromatography at the Max-Planck Institute of Psychiatry in Munich, Germany as described earlier (Gaali et al., 2015) (Figure 14).

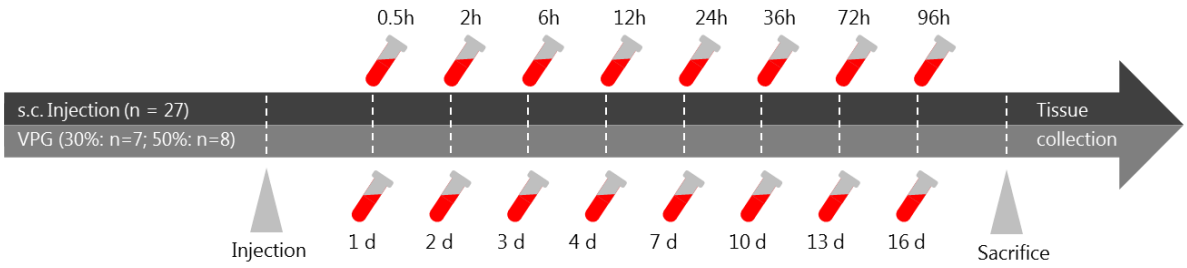


**Figure 14: Timeline experiment 6**
At the start of experiment 6, animals received either an s.c. injection of dissolved SAFit2 in a fluid solution, or an injection of VPG, loaded with 30% or 50% SAFit2. Blood samples were obtained at the indicated time-points and blood plasma was extracted for analysis of SAFit2 content.

### 2.3.7 Experiment 7

Experiment 7 was specifically designed to allow a direct comparison between pharmacological inhibition and genetic KO of *Fkbp5* in a home cage setup. Animals for the pharmacology group were 12 week old male mice, bred on a C57Bl/6n background in our in-house facilities. The conventional *Fkbp5* KO line was generated and described previously (Gassen et al., 2014; Hartmann et al., 2012; Touma et al., 2011) and backcrossed to C57Bl/6n. Only 12-16 week old homozygous males derived from heterozygous breeding were used for the experiments. To ensure sufficient acclimatization animals were single housed and moved from our holding rooms to the test room one week prior to the experiment. All animals were housed in standard cages. In this study, a 2x2 design with 4 experimental groups was used: (1) control animals (n = 12), injected with empty VPG, (2) SAFit2 animals (n = 12) injected with SAFit2-loaded VPG, (3) WT ($Fkbp5^{WT}$) animals (n = 14) and (4) KO ($Fkbp5^{KO}$) animals (n = 10). On day 1 of the experiment, half of the animals from the pharmacology group received SAFit2 treatment, and the other half received an injection of empty VPG. Recording of the animals' home cage behavior started 4 days after the injection to avoid any acute stress effects. Right before the start of the experiment, all mice were

weighed to ensure they were not sleeping and then recorded for a total of 15 minutes. After that, they underwent a 15 minute restraint. Following stress, animals were placed back in their home cage and recorded for another 15 minutes (Figure 15).

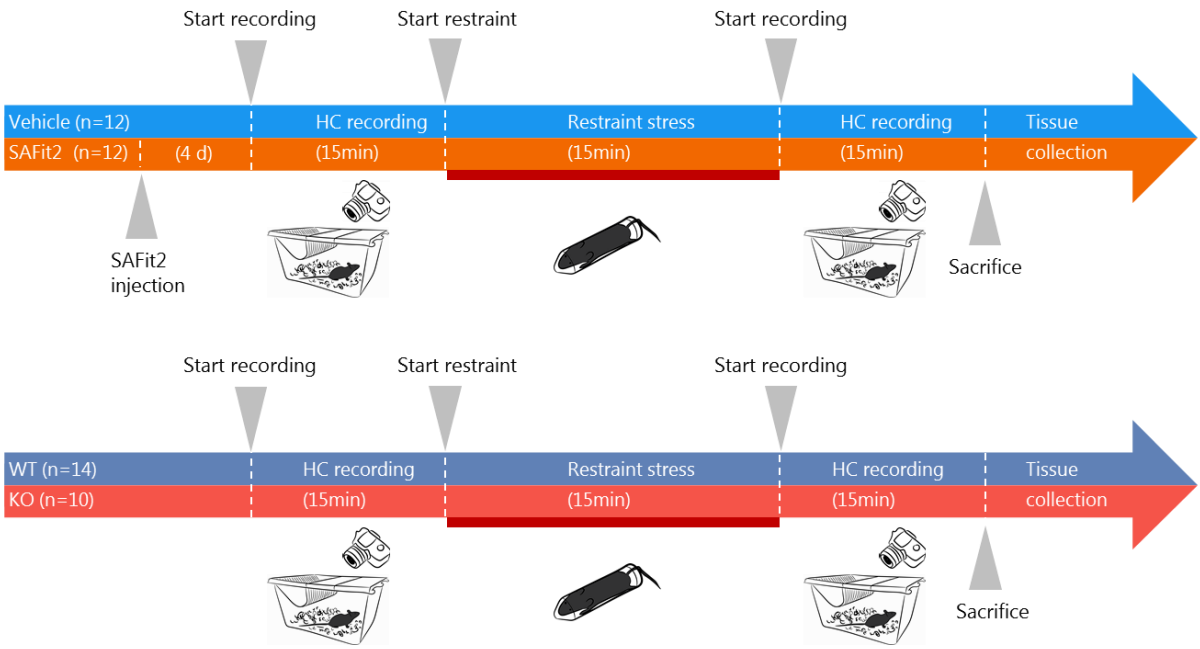


**Figure 15: Timeline experiment 7**
Animals from the pharmacology group were treated with either empty or SAFit2 loaded VPG 4 days prior to the experiment. At test day, animals were recorded in their home setting for 15 minutes. After the baseline recording, they were restrained in 50ml falcon tubes for 15 minutes before they were allowed to return to their home cage, in which they were video-taped for another 15 minutes. Following the last session, animals were sacrificed and tissue was collected for future analysis.

## 2.4 Stress paradigms

### 2.4.1 Social defeat stress (acute, chronic and subthreshold)

A number of studies have demonstrated the negative impact of social defeat stress as one of the most severe stressors for social rodents, including mice, especially in regard of their endocrine reaction (Koolhaas et al., 1997). The social defeat stress paradigm is based on the aggressive behavior that single housed male mice display against intruders into their territory (Bartolomucci

et al., 2001). In order to achieve the best possible results, resident mice were trained for 3-4 days to staunchly defend their home cage against any other intruder animal. Even though play-fighting is a common behavior in social groups, attacks by a physically superior and aggressive resident pose a severely stressful situation for the introduced experimental animal. One of the main factors for that response is the inevitable and therefore potentially life-threatening nature of the attack. Conducted as a single defeat, social stress was utilized as an acute stressor.

Depending on the experimental design, the social defeat was also used in a more long-term setup. In order to test the effects of chronic, rather than acute stress, a similar protocol was employed, which consisted of daily social defeats, repeated for a total of 21 days. After each defeat, both animals were separated by a wire mesh, allowing sensory and visual, but not physical contact. Every day, the experimental animals were rotated through the cages so they never faced the same resident twice. The so called CSDS paradigm was originally described by Sam Golden and colleagues (Golden et al., 2011) and adapted according to the specific scientific questions of our experiments (Figure 16).

**Figure 16: Social defeat stress - Male aggressiveness towards intruders as a severe stressor for experimental mice**
Specimen of a physically superior, more aggressive strain (CD1) compared to the experimental animals (C57Bl/6n background) were trained to defend their home cage against intruders. Once an experimental mouse was introduced, they were allowed to attack for up to 3 minutes, depending on severity of the defeat (right). The animals were separated before any serious injuries could occur.

Some scenarios require a rather mild form of stress to separate vulnerable from resilient individuals within the same experimental group. Because a single defeat generates a very short-lived, but severe stress effect, a different protocol was used to ensure only vulnerable animals would be affected. As

part of the so called subthreshold defeat (Krishnan et al., 2007), all experimental mice underwent a social defeat twice for a maximum of 5 minutes each. Between the two social defeats the experimental animals were placed back into their home cage for a total of 15 minutes. The animals were separated before serious injuries could occur. The subthreshold defeat was performed 24 hours pre-testing.

### 2.4.2 Restrain stress

To test the effects of a robust, non-social stressor, the animals' movement was restrained by placing them in 50ml falcon tubes. Each tube had 2 holes drilled into in the front, as well as the lid to allow the animals to breathe normally and move their tail. The animals remained in the container for 15 minutes before they got placed back into their home cage.

### 2.4.3 Forced swim stress

The FST was not only used for the behavioral read-out, but also as a stressor in order to evoke an endocrine reaction. After 6 minutes in the water, mice were dried and placed back in their home cage. After 30 minutes, a first blood sample was taken to capture the peak of the hormonal reaction. The recovery rate was assessed by a second sample, 90 minutes after stress onset.

## 2.5 Utilized drugs

### 2.5.1 SAFit2 – a pharmacological modulator of FKBP51

SAFit2 (CAS Number: 1643125-33-0) is a highly selective antagonist for FKBP51 functionality and was developed, tested and described in detail by Hausch and colleagues (Kolos et al., 2018; Gaali et al., 2015). Any SAFit2 used in our studies was synthesized and purified as a soluble powder in the Hausch laboratory at the Max-Planck Institute of Psychiatry in Munich. Due to the hydrophobic properties of SAFit2, it was dissolved in 20% Ethanol, 40% Propylenglycol, 5% Tween80, and 5% PEG 400 in 0.9% Saline (conc.: 10mg/ml) when utilized as a solvent solution.

### 2.5.2 Escitalopram – a commonly used antidepressant

Escitalopram was obtained as a crystalline powder from ChemCruz (Santa Cruz Biotechnology, Inc, Dallas, TX, USA) and dissolved in purified water (Ampuwa Water; Fresenius Kabi Deutschland GmbH) (10mg/10ml) as described by the manufacturer. The Drug was administered into the intraperitoneal cavity (10mg/kg bodyweight) and the injection volume was corrected to the animal's bodyweight accordingly.

## 2.6 Application methods for utilized drugs

### 2.6.1 Intraperitoneal injection

Unless stated otherwise, the administration of all liquid systemic drugs was achieved by i.p. injection. For the application, animals were placed on a wire cage top, carefully fixated and turned upside down to inject into the intraperitoneal cavity at an approximate angle of 10°. In order to avoid any injuries to underlying organs, the head of the animals was kept lower than the body and the needle was held almost parallel to the vertebral column (Figure 17) (Shimizu, 2004).

**Figure 17: Injection methods**
Left: For i.p. injections, animals were restrained and held in a supine position with its head lower than the body. The injection was aimed to the lower quadrant of the abdomen at a slight angle of approximately 10°. The needle was inserted through the abdominal wall without puncturing any underlying organs.
Right: Animals are carefully restrained in order to prevent sudden movement. Then the needle is inserted into the skin fold in the neck area.

### 2.6.2 Subcutaneous injection

Subcutaneous (s.c.) injections were applied in the animals' neck area. In general the absorption rate is lower than from i.p. injections and allows for a longer term treatment if the applied solution is stable enough. The mouse was manually restrained on a wire cage top to safely inject the substance of choice into the skin fold between the shoulder blades (Figure 17) (Shimizu, 2004).

### 2.6.3 Vesicular phospholipid gels

In order to minimize stress for the animals while maintaining a sustained release of drug into the bloodstream, VPGs were utilized that were either empty or loaded with SAFit2 (concentration: 10mg/ml VPG). Each SAFit2 gel was loaded with a total amount of 10mg/ml of substance to guarantee sufficient drug-levels over the course of at least 2 weeks. The formula allows for s.c. injections without the necessity of additional anesthesia. Due to the high viscosity of the gel above a temperature of 25°C it dispenses uniformly underneath the skin and does not interfere with locomotion of the animals. Production and loading of the VPGs was carried out by the Winter group at the pharmaceutical faculty of the Ludwig-Maximilians-University in Munich (Breitsamer and Winter, 2019; Tian et al., 2010; Brandl, 2007).

## 2.7 Drug level assessment

Drug levels in the animals' blood plasma were analysed by an in-house analytics core-facility at the Max-Planck Institute of Psychiatry using the combined high-performance liquid chromatography/mass spectrometry (HPLC/MS-MS) technique. Analysis was performed using a Shimadzu Nexera X2 (Shimadzu, Duisburg, Germany) liquid chromatograph that was interfaced to the ESI source of a Sciex QTrap 5500 (Sciex, Darmstadt, Germany) triple quadrupole mass spectrometer. All samples were prepared using Ostro protein precipitation and phospholipid removal plates (Waters, Eschborn, Germany).

Chromatography was accomplished using an gradient elution in a Accucore RP-MS column (100 x 2,1mm, 2,6µm Thermo Scientific, Dreieich, Germany) at a flow rate of 0.5 ml/min and 30 °C. The composition of eluent B was methanol with 10mM ammonium formate with 0.1% formic acid and water with 10mM ammonium formate with 0.1% formic acid as eluent A. The gradient was 0-3 min 60% B, 3-4,5 min 60-90% B, 0,5 min held at 90% B, 5-5,2 min 90-60% B and 5,2-6 min 60% B. The total run time was 6 min and the injection volume was 2µl. The ion source was operated in the positive mode at 500°C, and multiple reaction monitoring (MRM) collision-induced dissociation (CID) were performed using nitrogen gas as the collision gas. Deuterated SAFit2 (SAFit2-D3) was used as internal standard. The retention time and transitions monitored during analysis for the analytes were as shown in the following table (Table 1).

**Table 1: Overview retention time and transitions**
This table shows the different retention time and transitions monitored during the analysis of the SAFit2 probes

| Compound | Used as | Q1_Mass | Q3_Mass | RT [min] | DP [V] | EP [V] | CE [V] | CXP [V] |
|---|---|---|---|---|---|---|---|---|
| SAFit2 | Quantifier | 803.2 | 384.2 | 4.95 | 141 | 10 | 41 | 18 |
| SAFit2 | Qualifier | 803.2 | 114.2 | 4.95 | 141 | 10 | 67 | 8 |
| SAFit2-D3 | Internal Standard | 806.4 | 384.3 | 4.95 | 106 | 10 | 41 | 26 |

## 2.8 Physiological assessments (bodyweight and fur state)

Animals were weighed on a standard laboratory scale. In case of the CSDS, bodyweight and fur state of all animals were assessed on days 1, 4, 8, 11, 15, 18 and 21 of the respective experiment. Fur state was ranked on a scale from 1 to 4 (Mineur et al., 2003). A score of 1 equals a healthy state of the animal with smooth and shiny fur and no visible wounds or imperfections. In case the fur was dull or rough, animals received a score of 2. Once an animal had visible wounds, the fur state score

was ranked at 3. Mice with serious wounds, infections or other circumstances that could cause unnecessary suffering received a rating of 4. After two consecutive days with a score of 4, an animal was excluded from the experiment.

## 2.9 Surgeries

Prior to surgery, animals were anesthetized with isoflurane (CP-Pharma®, Burgdorf, Germany) and received an i.p. injection of Meloxicam (Metacam®, Boehringer Ingelheim, Ingelheim am Rhein, Germany). Mice were supplied with oxygen-enriched isoflurane during surgery, ensuring constant anesthesia. Subsequently, their skull was firmly fixed in a stereotaxic frame to prevent any head movement during surgery. The skin between both ears was cleaned and sanitized before it was opened with a scalpel. Coordinates of the injection holes were calculated relative to Bregma. Depending on the target area, we injected the virus either unilaterally or bilaterally using micro-syringes (Hamilton®, Bonaduz, GR, Switzerland). The surrounding skin was disinfected with iodide and carefully sewn together. Mice were treated with Metacam® injections daily for 3 consecutive days. Bodyweight, overall appearance and status of the wound were monitored closely for 7-10 days post-surgery. Animals were allowed to recover for at least 4 weeks before behavioral testing.

## 2.10 Utilized viruses

For viral OE, we used an adeno-associated bicistronic AAV1/2 vector (GeneDetect, Auckland, New Zealand) as described previously (Hartmann et al., 2015, 2012; Schmidt et al., 2011). The vector contained aCAG-HA-tagged-FKBP51-WPRE-BGH-polyA expression cassette (containing the coding sequence of human Fkbp51 NCBI CCDS ID CCDS4808.1; titers: 1.3 1012 genomic particles/ml). For the control group, we used the same vector construct without expression of FKBP51 (CAG-Null/Empty WPRE-BGH-polyA). Virus production, amplification, and purification were performed by GeneDetect.

## 2.11 Behavioral tests

All behavioral tests were carried out between 08:30am and 12:30pm in the animal room inside the holding facility of the Max-Planck Institute of Psychiatry in Munich. All tests were analyzed by an experienced, blinded researcher utilizing the commercially available automated video-tracking software AnyMaze (Anymaze 4.99, Stoelting, Wood Dale, IL, USA). All tests were conducted according to established protocols (Hartmann et al., 2015; Wagner et al., 2012; Hartmann et al., 2012).

### 2.11.1 Open field test (OF)

The OF test was the first test employed in our experiment in order to asses locomotion as well as anxiety-like or explorative behavior. Testing was conducted under low light conditions (~15 lux) in an empty arena (50 cm x 50 cm x 50 cm) made from gray polyvinyl chloride (PVC). At the start of the test, animals were placed in one of the four corners of the apparatus (Figure 18).

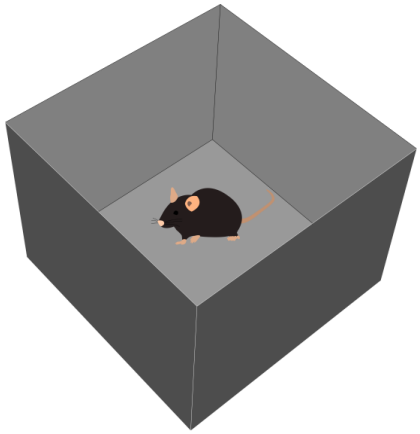

**Figure 18: The open field test**
In the OF, animals were allowed to freely explore a square arena, made from gray PVC. Factors of interest are the distance covered and the time, mice spend in the more aversive central area of the box.

Total test time was 15 minutes, subsequently divided into three time bins of 5 minutes each for a more in depth analysis. Parameters of interest for locomotor activity were the overall distance

traveled in the arena, as well as the time animals were immobile. The time animals spent investigating the exposed, highly illuminated and therefore aversive mid-section of the arena (25 cm x 25 cm) was used as a measurement of anxiety-like or explorative behavior. Additionally, the OF was conducted to ensure habituation to the arena as well as handling by the experimenter.

### 2.11.2 Object recognition test (ORT)

As part of their investigative nature, rodents show a preference for unfamiliar objects when exposed to simple discrimination tasks, spending more time with a novel object when given the choice (Ennaceur, 2010). During the ORT, animals are exposed to objects for a short amount of time and then asked to discriminate said objects from an unfamiliar one in a retrieval phase. Number of acquisition trials, as well as the ITI can be altered to address short- or long-term memory. All objects were built from 13 LEGO®-bricks to allow a consistent volume, while shape and color could be varied to create distinguishable objects. A 10 minute acquisition phase was chosen in which the mice were placed into the arena (50 cm x 50 cm x 50 cm) made of gray PVC together with two identical objects placed in the rear corners 10 cm away from each corner. After an ITI of 30 minutes, one of the two identical objects was replaced by a novel one. In the retrieval phase the mouse was allowed to explore the new setting for 5 minutes. Any approach towards the object was considered an interaction as soon as the mouse touched the object with its nose, forepaws or vibrissae or once it clearly sniffed in the direction of the object within close proximity. During the retrieval phase, interaction time with the familiar as well as the interaction time with the novel object was measured. Animals that did not interact with both the novel and the familiar object during retrieval were excluded from the analysis. At the start of each phase, the experimental mouse was placed in the lower left corner. Two different types of objects were used for the test, for none of which the animals showed any preference. The type of object that was chosen as familiar or novel respectively was counterbalanced across the groups accordingly. Spatial clues (geometric symbols) were attached to the walls of the testing arena to allow better orientation (Figure 19).

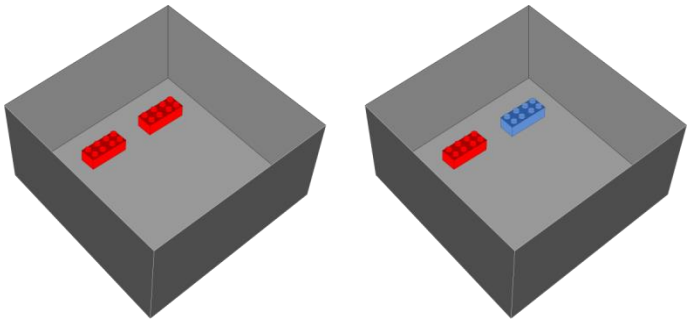

**Figure 19: The object recognition test**
The ORT consists of (several) acquisition phases in which the animals are allowed to familiarize themselves with two identical objects and a retrieval phase. During the retrieval phase, one of the objects is replaced by an unfamiliar one. The animals' ability to discriminate between the objects and remember the familiar one is determined by the times they investigate either object.

### 2.11.3 Object relocation test (OLT)

The capability to memorize spatial cues and the capacity in which the position of certain objects can be recalled is an important parameter that is often disturbed in psychiatric disorders. To investigate the spatial memory of the animals in this study, an OLT was conducted. Similar to the animals' preference for novel objects, they also show increased interests in objects that are placed in an unfamiliar location. Two acquisition phases of 10 minutes each with an ITI of 15 minutes were used to familiarize the animals with the location of both objects in the arena. During both acquisition phases, the animals were allowed to freely explore the arena (50 cm x 50 cm x 50 cm) made of gray PVC in which two identical objects were placed 10 cm away from the two rear corners. After another 30 minutes ITI, one of the objects was moved into one of the front corners for the retrieval phase. The mice were able to explore the new setting for 5 minutes in which all their movement was tracked. Again, the interaction time with the object in the new position as well as the old, familiar, position was measured. Whenever an animal touched the object with its nose, forepaws or vibrissae or if it sniffed in the direction of the object while in close proximity, the behavior was considered an interaction. Animals that did not interact with each of the objects at least once during retrieval were excluded from analysis. At the test start of each phase, the

experimental mouse was placed in one of the lower corners. Again, all objects were built from 13 LEGO®-bricks to allow a consistent volume, while shape and color could be varied. Objects were altered between test setups. The object displaced was counterbalanced among the four different apparatuses.

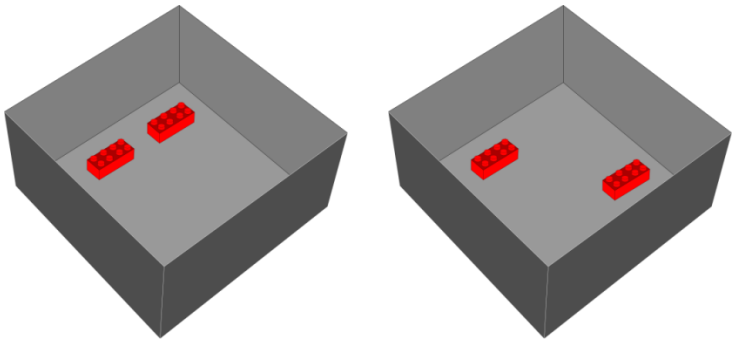

**Figure 20: The object relocation test**
Similar to the ORT, this test consists of (several) acquisition phases and a retrieval trial. During the acquisition, animals can freely explore the arena that contains two identical objects. During the retrieval, one of the objects is moved to a different location within the box. Interaction times are measured in order to determine the animals' capacity to recall the original placement of the objects.

One spatial cue (4 different geometric symbols) was attached to each of the four walls of the testing box respectively to allow for easy orientation. For the *Fkbp5*$^{\text{DR KO}}$ and the respective WT control mice one single brick of LEGO® was placed into each home cage three days before testing to provide habituation to the LEGO® bricks for the OLT and the ORT. Animals that displayed less than 1 second of interaction with one or the other object were excluded. Similarly, animals that showed a total interaction time of less than 4 seconds were considered low-responders and eliminated from the analysis (Figure 20).

### 2.11.4 Dark-light box test (DaLi)

Since mice are nocturnal, they tend to prefer dimly lit areas and avoid bright, exposed places. The DaLi was employed to measure anxiety-related behavior by providing a dark and safe, but relatively small area, which was connected to a bigger part of the arena that was brightly lit. The arena itself

consisted of a rectangular box, split into 2 separate compartments. The compartments were connected by a 4 cm long tunnel, allowing the animals to freely choose between both sides.

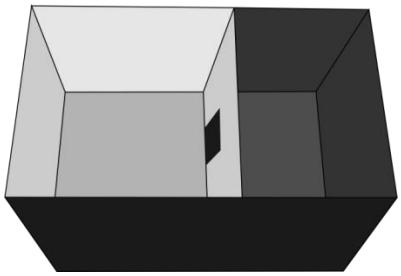

**Figure 21: The dark-light box test**
The DaLi is a test for anxiety-like behavior. The arena consists of two compounds, connected by a tunnel. While the bigger side of the box is brightly illuminated (and therefore aversive for nocturnal animals) the smaller compartment is dark. Levels of anxiety-like behavior can be determined based on the time animals spend in each of the two compartments and the latency until they first enter the aversive side.

The lit compartment (30 cm x 20 cm x 25 cm) was brightly illuminated by an external light source (~700 lux) while the dark compartment (15 cm x 20 cm x 25 cm) was not illuminated (~5 lux). During the 5 minutes of testing time animals spent in the lit zone, distance traveled in the lit zone as well as the number of entries into the lit zone was assessed (Figure 21).

### 2.11.5 Elevated plus maze test (EPM)

The EPM is based on the aversive nature of an elevated and exposed platform. It consists of two opposed open arms (30 cm × 5 cm × 0.5 cm) and two opposed enclosed arms (30 cm × 5 cm × 15 cm) made of gray PVC, which were connected by a central platform (5 cm × 5 cm) shaping a plus sign. Because the whole arena was elevated 50 cm above the floor, animals usually refrain from jumping off the open arms. At the start of the test, animals were placed in the center of the plus maze facing one of the closed arms and allowed to explore the maze for 5 minutes. Total time spent in the respective arms as well as the number of open arm entries was analyzed. Any animals that fell off the open arms during testing were excluded from the analysis (Müller et al., 2003) (Figure 22).

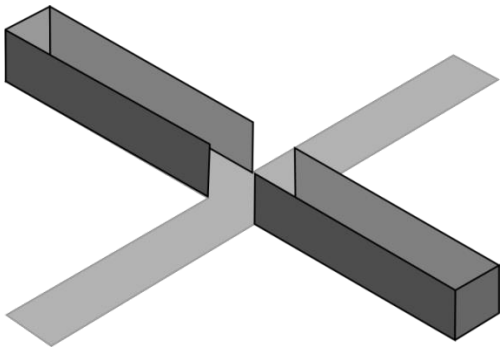

**Figure 22: The elevated plus maze test**
Similar to the DaLi, the EPM is considered a classical test for anxiety-like behavior. The plus-shaped arena is elevated to a height that generally prevents animals from jumping off. While two opposing arms are surrounded by a wall to all sided (and therefore considered safe), the two remaining arms are open and

### 2.11.6 Social avoidance test (SA)

The SA test provides measurable evaluation for the level of social interaction or aversion of the experimental animal towards an unfamiliar social specimen. The test was performed in the same arena as the OF, ORT and OLT. A small wire mesh cage (10cm diameter; 10cm in height) was placed at the arena back wall. The mesh cage allowed for sensory interaction between the two animals but refrained any of the two animals from attacking the other. The test consisted of two consecutive 150 second long trials. While the experimental animal was allowed to explore and interact with the empty wire mesh cage, an unfamiliar male CD1 mouse was placed inside the cage for the second trial. At the beginning of the first trial the experimental mouse was placed in one of the lower corners of the arena. While changing the cages between trial 1 and trial 2 the experimental animal was allowed to remain in the arena to prevent additional stress. The interaction time with the cage or animal in the cage was measured respectively. The ratio of interaction time between trial 2 and trial 1 was the main readout of the test. Alternatively, the interaction with the social cue as percentage of total interaction time was calculated (Figure 23).

**Figure 23: The social avoidance test**
The SA test consists of two short trials. In the first trial, a small empty cage is placed in the test arena. The experimental animal is then allowed to explore the setup and the interaction time with the cage is measured. In the second trial, a social cue (juvenile mouse of the same sex but a different strain) is placed in the cage and the interaction time is assessed again. Mice that show higher interaction times with the social compared to the non-social cue are considered 'social' animals.

### 2.11.7 Forced swim test (FST)

Since it is considered to be the most stressful of all the tests, the FST was conducted on the last day of any experiment. For the FST, cylindrical 2-liter glass beakers filled with 1.5 liters of water at room temperature were used. Water depth was chosen so mice were neither able to touch the bottom of the container, nor escape from it, therefore creating an inevitable stressful situation (Figure 24).

**Figure 24: The forced swim test**
The FST was used as a stressor, as well as a test to determine stress coping behavior. Animals were placed in a beaker filled with water and their behavior was filmed. As mice are quite accomplished swimmers, they show three different types of behavior: struggling, swimming and floating. The amount of time spent executing each behavior was used to determine their ability to cope with the stressful situation.

Test duration was 6 minutes and the animals were dried with a towel afterwards to prevent hypothermia. Scored parameters were the respective times spent struggling, swimming and floating, as well as the immobility response. The transition from an active state into floating behavior is thought to showcase despair as a direct consequence of learned helplessness (Molendijk and de Kloet, 2019).

### 2.11.8 Assessment of home cage behavior

Most behavioral tests present extremely artificial challenges to the animals, likely evoking reactions that are very hard to compare to any naturally occurring situation or behavior. In order to obviate this problem as much as possible, the undisturbed behavior of animals is best observed in an environment they are most comfortable with. For laboratory-raised rodents, the closest resemblance to a nest is the home cage. Home cage behavior was recorded from one side of the home cage in 15 minute sessions. Recording started 30 minutes after the start of the light cycle to ensure optimal visibility. After the first session of baseline recording, animals were restrained for 15 minutes, followed by another 15 minute period of home cage recording. Prior to the first session, every animal was taken out of the cage and weighed to ensure they were not sleeping. Food and water was removed for the duration of the recordings in order to prevent excessive differences caused by variable food or water intake during the dark phase. The following behaviors were assessed during both, the baseline and the stress session: surveying (the animal was standing still with a slightly raised head, watching its surroundings), walking (the animal moved in the cage without any other visible purpose), rearing (the animal was standing on its rear legs, sniffing the area), grooming (self-directed cleaning behavior, the animal was licking its front paws and fur), climbing (the animal was using the cage top to climb), digging (the animal was actively moving the bedding around with its front or rear legs) and chewing (the animal was chewing an object). All behaviors were scored manually (Füzesi et al., 2016).

### 2.11.9 Y-maze test

Besides the ORT and OLT, the Y-Maze was used as an additional test to evaluate the cognitive capacity of mice. The maze consists of three identical arms (30cm x 10cm) made of gray PVC, all connected by a central area. Each arm was lined with a different color pattern to allow the animals to visually distinguish them. The test was split in two trials. During the first trial, only two of the three arms were open for the animals to explore for a total of 10 minutes. During the ITI, the animals were placed back in there home cage for 30 minutes before they were put back into the arena for a 5 minute retrieval phase in which they were allowed to explore the entire 3-armed maze.

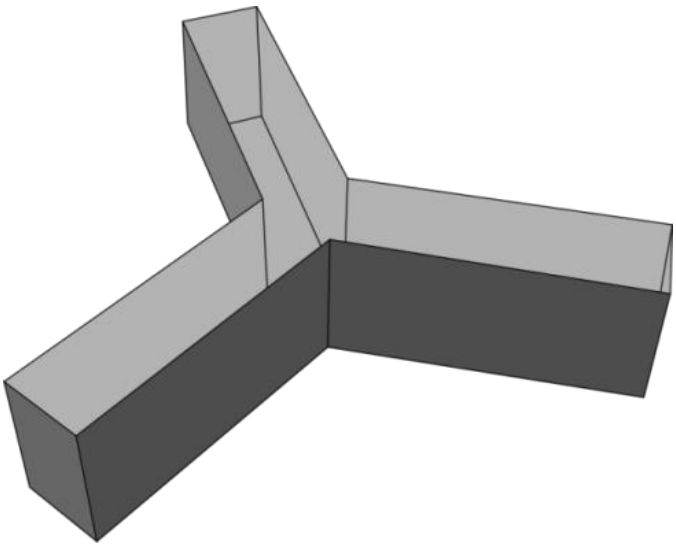

**Figure 25: The Y-maze test**
The arena for the Y-maze consists of three arms connected by a central area. In the first trial, the animals were allowed to explore two of the arms while one of them was blocked. In the second trial all arms were open to explore and the animals' ability to recognize and memorize the familiar arms was measured.

The session was recorded and the time an animal spent in any of the three respective arms was measured. The amount of time an animal spent in the novel arm opposed to the familiar arms was used to determine whether they could still memorize which arms they had explored in the first trial (Figure 25).

## 2.12 Tissue processing

### 2.12.1 Tissue collection and further processing

Until stated elsewise, all animals that were sacrificed at the end of an experiment and specific tissues or organs were extracted for further analysis. All mice were deeply anesthetized with isoflurane and immediately decapitated. After decapitation, the brains were carefully removed from the skull, snap frozen in -40°C cold methylbutane and stored at -80°C until further use.

Because hormonal glands like the thymus or the adrenal glands change in size depending on their activity they were used as a read-out in some of our stress experiments. Both organs were collected after sacrifice, freed of any residual tissues and weighted.

Any blood samples from living animals were obtained from the dorsal tail vein via a small incision with a razor blade. Trunk blood was obtained after animals were decapitated. All blood samples were collected in 1.5 ml EDTA-coated micro-centrifuge tube (Microvette CB 300 K2E, Sarstedt) and centrifuged for 15 minutes at 8000 rpm and 4°C. Plasma was transferred to 1.5 ml tubes and stored at 4°C until further use.

Whenever genetically modified mouse lines were used, small tail biopsies were collected after sacrifice. The tissue was then stored at -20°C until it was used for genotyping.

### 2.12.2 Radioimmunoassay

RIA was used to measure concentrations of stress hormones from blood plasma samples. For the assay, we used a Double Antibody Kit from MP Biomedicals (ImmunoChemTM Double Antibody Corticosterone $^{125}$I RIA Kit, MP Biomedicals, LLc, Orangeburg, NY, USA), strictly following the manufactures' instructions. Blood samples were diluted in order to ensure they were in range of the standard curve (basal 1:50; response 1:200; recovery 1:100). The radioactive precipitate was measured in a gamma counter (Packard Cobra II Auto Gamma, Perkin-Elmer, Waltham, MA,

USA) and the exact corticosterone concentrations were calculated based on the standard curve provided by the kit (12.5, 25, 50, 100, 250, 500, 100 ng/ml).

### 2.12.3 Brain sectioning

Frozen brains were mounted onto a metal disk with water. For ISH, brains were cut at a thickness of 18µm utilizing a cryostat microtome (HM569, Microm, Walldorf, Germany). We collected brain sections with a fine brush and thaw-mounted them on Super Frost Plus Slides (Menzel GmbH, Braunschweig, Germany). Slides were stored at -20°C until further use.

### 2.12.4 *In situ* hybridization

All ISHs were performed as described earlier (Schmidt et al., 2003, 2007). To prepare the samples, thawed brain sections were fixed and dehydrated. We used $^{35}$S UTP-labelled ribonucleotide probes (forward primer: 5'-CTTGGACCACGCTATGGTTT-3'; reverse primer: 5'-GGATTGACTGCCAACACCTT-3'). RNase-free DNase I (Roche Applied Science, Mannheim, Germany) was used to destroy any remaining DNA template within the reaction samples and purification of the riboprobes was accomplished using a Qiagen RNeasy Kit (Qiagen, Hilden, Germany). After the brain sections were saturated with the $^{35}$S-labeled riboprobe, incubated over-night and washed in decreasing concentrations of saline-sodium citrate (SSC) and Ethanol, the slides were exposed to Kodak Biomax MR Films (Eastman Kodak Co., Rochester, NY, USA) and stored in a lightproof environment. Depending on the radioactive properties of the riboprobe, the films were developed after 7-10 days of exposure. After development, autoradiographs were digitized and we determined mRNA expression by densitometry using the open source software NIH ImageJ (NIH, Bethesda, MD, USA). To avoid faulty signal caused by artificial fluctuation of exposure on the film, a background value was assessed and subtracted from each measurement before the data was analyzed by a blinded experimenter.

### 2.12.5 Double *in situ* hybridization

In order to confirm co-localization of *Fkbp5* mRNA and markers for monoaminergic cell populations (serotonin transporter (*Sert*) for DR and *Th* for LC), dISH was performed as described earlier (Refojo et al., 2011). The $^{35}$S UTP-labelled ribonucleotide probe for *Fkbp5* (forward primer: 5'-CTTGGACCACGCTATGGTTT-3'; reverse primer: 5'-GGATTGACTGCCAACACCTT-3') was combined with either digoxigenin (DIG)-labelled *Sert* riboprobe or DIG-labelled *Th* riboprobe. Coronal brain sections were collected and cryosectioned at 18µm thickness from a test-naïve cohort of C57Bl/6n mice. The sections were thaw mounted onto Super Frost Plus Slides and fixed in 4% paraformaldehyde (PFA) before undergoing several washing steps. Before the background was reduced in 0.2 M HCl, endogenous peroxidase was quenched in 1% $H_2O_2$. This was followed by two washing steps in 1 x phosphate buffered saline (PBS) before the slides were acetylated in 0.1 M triethanolamine, another wash in 1 x PBS and the dehydrated through increasing concentrations of ethanol. Then sections were saturated with 90µl of hybridization buffer (approximately 50,000 cpm/µl $^{35}$S-labelled riboprobe and 0.2µg/ml DIG-labelled riboprobe respectively). The slides were then cover-slipped and incubated overnight at 55°C. Coverslips were removed the following morning before the slides were again washed several times (decreasing concentrations of SSC/formamide buffers under stringent temperature settings). Following the washes, brain sections were exposed to RNAse A in 1 x NTE (NaCl, Tris-HCl, EDTA) at 37°C and washed in 1 x NTE/0.05% Tween20 two times. This was followed by a 1 hour blocking step in 4% bovine serum albumin (BSA). After being washed again, brain sections were blocked in NEN-TNB (Tris-Hcl, NaCl, Blocking reagent; NEN® Life Science Products, Boston MA, USA) for a total of 30 minutes. During the last step, the slides were incubated with Roche's anti DIG (FAB) (1:400, Roche Molecular Diagnostics) at 4°C overnight. On the final day, sections were washed several times in TNT (Tris-HCl, NaCl, Tween20) at 30°C followed by a 15-minute incubation in tyramide-biotin in order to amplify the signal. Sections were washed in Roche washing buffer (Roche Molecular Diagnostics) before they were incubated for 1 hour with Roche streptavidin-AP (1:400, Roche

Molecular Diagnostics). Afterwards, sections were again washed in Roche washing buffer and prepared for Vector red staining in 100mM Tris-HCl (Vector Laboratories, Burlingame, CA, USA). Then, slides were immersed in Vector red solution in the dark for approximately 30 minutes. When the staining was sufficient, the chemical reaction was stopped using 1 x PBS followed by a fixation step in 2.5% glutaraldehyde. In the final step, all sections were washed in 0.1 x SSC and then dehydrated in a graded series of ethanol solutions (30, 50, 70 and 96%) before they were air dried in a dust-free environment.

The prepared slides were then exposed to pre-warmed KODAK NTB2 silver emulsion and dried overnight in a light-proof cabinet. Once the silver solution was dry, the slides were moved to a light-tight box and 4°C during exposure time. After sufficient exposure to the solution, the slides were dipped into KODAK D 19 developer, followed by KODAK fixer. Subsequently, they were rinsed with tap water for about 20 minutes and air dried.

For better conservation and protection, the dipped slides were then cover-slipped using DPX (Diethyl procarbonate Xylol). If not stated otherwise, chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Hamburg, Germany).

## 2.13 Genotyping

Genotyping of all genetically modified mouse lines was conducted with lysed tail clips. For DNA amplification a standardized polymerase chain reaction (PCR) was used. All details and primers for conventional, as well as conditional *Fkbp5* KO, as well as the lox/lox lines are listed in Table 2. Gel-electrophoresis (2% agarose gel; Sigma Aldrich, Merck KGaA, Darmstadt, Germany; with ethidium bromide 0.1 µg/ml; Carl Roth GmbH, Karlsruhe, Germany) was used to determine the genotype of the probes from the amplified DNA products respectively.

**Table 2: Genotyping of genetically modified mouse lines – Primers and details**
The table lists all used primers and the PCR conditions for the genotyping of conditional and conventional *Fkbp5* KOs.

| ***Fkbp5*$^{KO}$** | |
|---|---|
| **Primer** | **Sequence** |
| 51Ex2-fw | 5'AAAGGACAATGACTACTGATGAGG3' |
| 51Int2/3-re | 5'AAGGAGGGGTTCTTTTGAGG3' |
| B-gal-rev2 | 5'GTTGCACCACAGATGAAACG3' |
| **PCR product** | **PCR condition** |
| 363bp (WT); 510-550bp (KO) | 95°C 4min [35x (95°C 30s, 55°C 30s, 72°C 40s)] 72°C 5 min, 4°C ∞ |
| **lox/lox** | |
| **Primer** | **Sequence** |
| Fkbp5-GT-1-c : | 5'-ACTATCTCACAAGCCGTCCA-3' |
| Fkbp5-GT-2-nc: | 5'ATGAAGGTCACGTGCTCAGG-3' |
| Fkpb5-GT-Flp-nc | 5`-AATAAAGCCTAGGACCCGCC-3´ |
| **PCR product** | **PCR condition** |
| 249bp (WT); 504bp (Mutant) | 95°C 5min [35x (95°C 30s, 61°C 30s, 72°C 1:30min)] 72°C 5 min, 4°C ∞ |
| ***Cre*-recombinase** | |
| **Primer** | **Sequence** |
| *Cre* R | 5´-AATCGCCATCTTCCAGCAG-3´ |
| *Cre* F | 5´-GATCGCTGCCAGGATATACG-3´ |
| Thy-R | 5´-CCACTGGTGAGGTTGAGG-3´ |
| Thy-F | 5´-TCTGAGTGGCAAAGGACCTTAGG-3´ |
| **PCR product** | **PCR condition** |
| 372bp (Thy control band)<br>574bp (*Cre* band) | 95°C 5min [35x (95°C 15s, 57°C 30sek, 72°C 1min)] 72°C 5 min, 4°C ∞ |

## 2.14 Statistical analysis

The data are shown as means ± standard error of the mean (SEM), analyzed by the commercially available software GraphPad Prism 7.03. Student's t-test was employed for comparison of two independent groups. A one-sample t-test was used to test against a hypothetical mean if applicable. For all other parameters, two-factorial analysis of variance (ANOVA) was employed if normally distributed. A significance level of $p < 0.05$ for main effects and $p < 0.1$ for interaction effects was followed by Tukey's post hoc test, with a nominal level of $p < 0.05$ considered significant. All values outside a margin of 2 times standard deviation were considered outliers and excluded from the analysis. Investigators were blinded to the experimental groups during the experiments and data analysis. In case two experimental groups were tested more than once (see experiment 7) repeated measures ANOVA was applied in order to detect stress or genotype/treatment effects. If a single value from one of the tests was missing, mixed model analysis was applied.

# 3 Results

## 3.1 Experiment 1

### 3.1.1 *Fkbp5* mRNA expression after acute stress

The expression level of *Fkbp5* mRNA was assessed in response to an acute stressor. Brain areas of interest were the hippocampus, the basolateral amygdala (BLA), the LC and the DR.

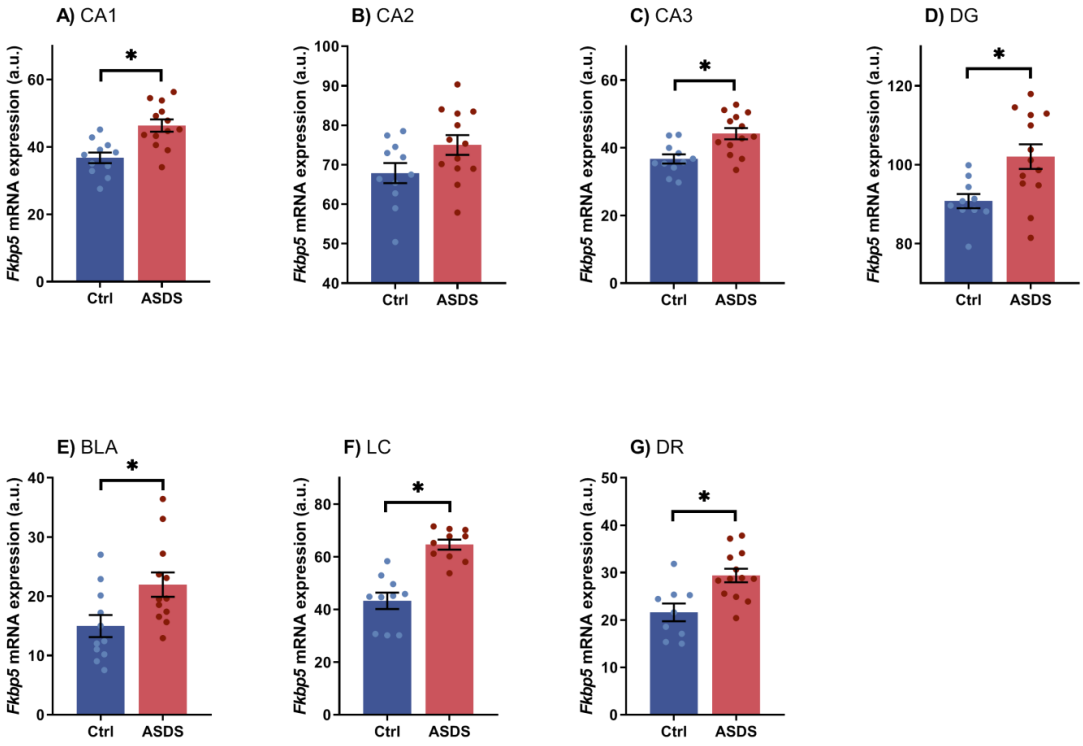


**Figure 26: *Fkbp5* mRNA expression after exposure to acute stress**
Animals from the stress group (ASDS) did show higher *Fkbp5* mRNA expression levels than unstressed controls in all selected brain areas (A, C-G), except the CA2 region of the hippocampus (B). Data are represented as mean ± SEM. * significantly different from the control condition.

A 5 minute ASDS treatment resulted in significant up-regulation of *Fkbp5* mRNA in a number of hippocampal sub-regions like the CA1 ($p = 0.0007$), CA3 ($p = 0.0028$) and dentate gyrus (DG) (p

= 0.0085). However, stress dependent regulation did not reach significance in the CA2 of the dorsal hippocampus (p = 0.0598) (Figure 26 B). Additionally, we found highly significant effects of acute stress on *Fkbp5* expression in the BLA (p = 0.0205) and two monoaminergic regions, namely the LC (p < 0.0001) and DR (p = 0.0031).

### 3.1.2 *Fkbp5* mRNA expression after chronic stress

Next I assessed whether the effects of an acute challenge on *Fkbp5* mRNA expression are also observed following a prolonged, chronic stress exposure. For this, I applied the CSDS paradigm before the mRNA expression levels were measured in the same regions as before.

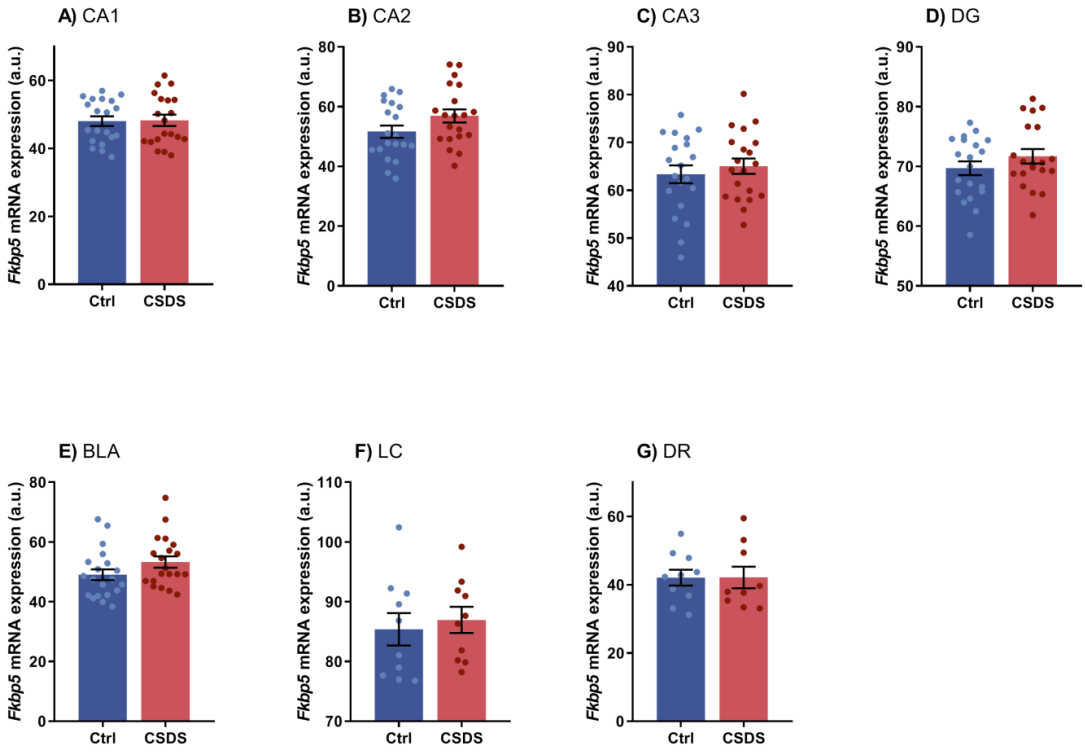


**Figure 27: *Fkbp5* mRNA expression after exposure to chronic stress**
*Fkbp5* mRNA expression was not altered in any of the selected regions after a three week exposure to CSDS (A-G). Data are represented as mean ± SEM.

Contrary to the effects of an acute stressor, *Fkbp5* mRNA is not up-regulated after chronic stress exposure (CA1: p = 0.9125; CA2: p = 0.0880; CA3: p = 0.4883; DG: p = 0.2421; BLA: p = 0.1139; LC: p = 0.6609; DR: p = 0.9903), while other chronic stress-related parameters were significantly altered. Specifically, adrenal glands were lighter (p < 0.0001) while the thymus was heavier in the stress group (p < 0.0001) compared to controls (Figure 28).

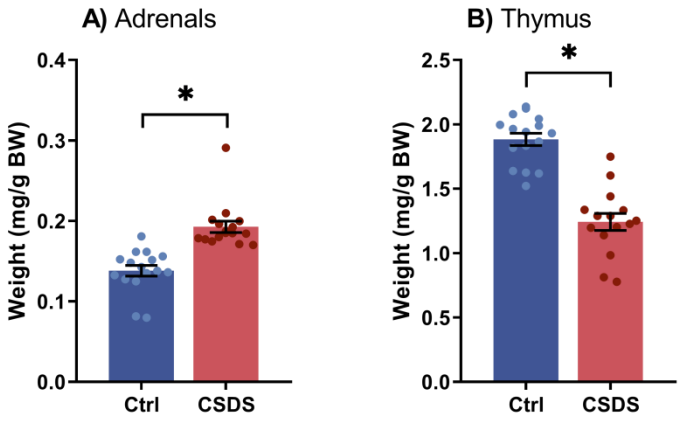


**Figure 28: Organ weight after CSDS**
Adrenal glands (A) were significantly enlarged while the size of the thymus (B) was decreased in stressed animals. Data are represented as mean ± SEM. * significantly different from the control condition.

## 3.2 Experiment 2

Given the pronounced regulation of *Fkbp5* in monoaminergic nuclei, I next focused on studying the effects of *Fkbp5* specifically in the LC and the DR.

### 3.2.1 Co-localization of *Fkbp5* with monoaminergic markers

First, I confirmed a co-localization of *Fkbp5* with specific markers for the LC and the DR via dISH. *Fkbp5* was found co-localized with *Th* in the LC (Figure 29), as well as in cells that express *Sert*, a commonly used marker for the DR (Figure 30).

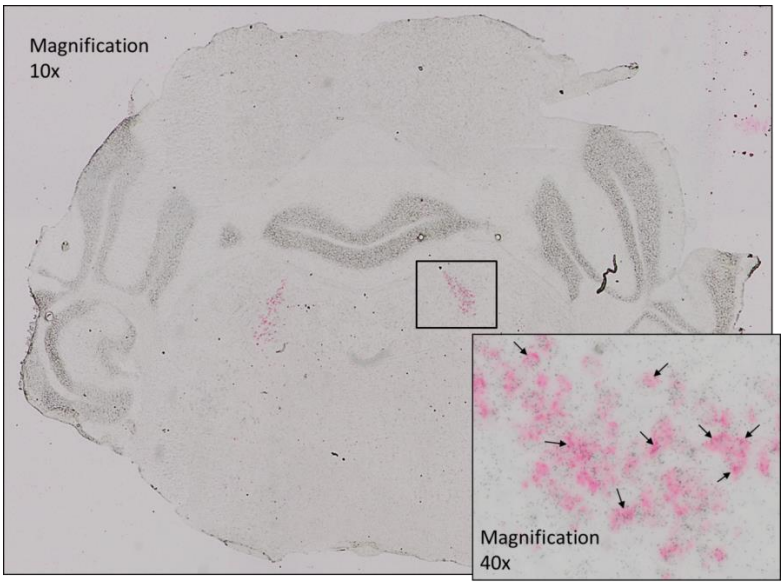


**Figure 29: Co-localization of *Fkbp5* and *Th* in the locus coeruleus**
*Fkbp5* (silver grains) and *Th* (red staining) expression pattern determined by dISH. Double positive neurons were observed in the LC (exemplary indication by black arrows).

Since we measured expression levels independently, co-localization was not quantified in this experiment.

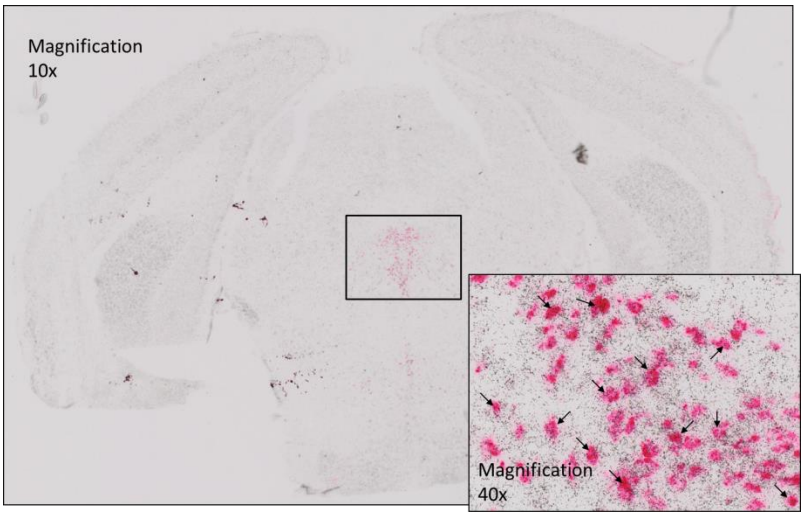


**Figure 30: Co-localization of *Fkbp5* and *Sert* in the dorsal raphe nucleus**
*Fkbp5* (silver grains) and *Sert* (red staining) expression pattern determined by dISH. Overlap of both stainings indicates double positive neurons in the DR (exemplary indication by black arrows).

### 3.2.2 *Fkbp5* over-expression in the locus coeruleus (cohort 1)

Next, I tested whether I could mimic behavioral effects of acute stress exposure by increasing *Fkbp5* expression in the LC.

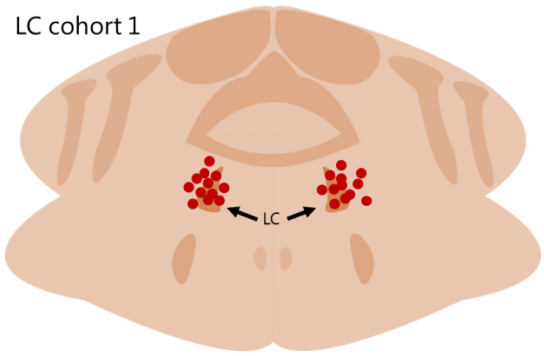


**Figure 31: AAV-injections into the locus coeruleus**
This figure shows the estimated center-point of each successful injection. Individuals that did not show expression in the targeted areas were excluded from the analysis.

Following the experiment the viral injection location was confirmed by ISH (Figure 31). Animals that did not show increased *Fkbp5* mRNA expression in the LC were excluded from the analysis.

Open field test

The OF was the first test conducted in this experiment. There was a significant decrease in distance traveled in animals that over-express *Fkbp5* in the LC ($p = 0.0448$) even though they did not show significantly increased immobility ($p = 0.0885$). There was no difference in the time animals spent in the aversive central area of the arena during the 15 minutes test time ($p = 0.2018$). However, when the test was split in 3 sections of 5 minutes each, the initial exploration of the inner zone by the LC *Fkbp5*-OE group is increased ($p = 0.0469$), shown by the time they spent in the inner zone during the first time bin. After this initial phase of testing, there is no difference in the second and third section ($p = 0.4646$; $p = 0.4700$) (Figure 32).

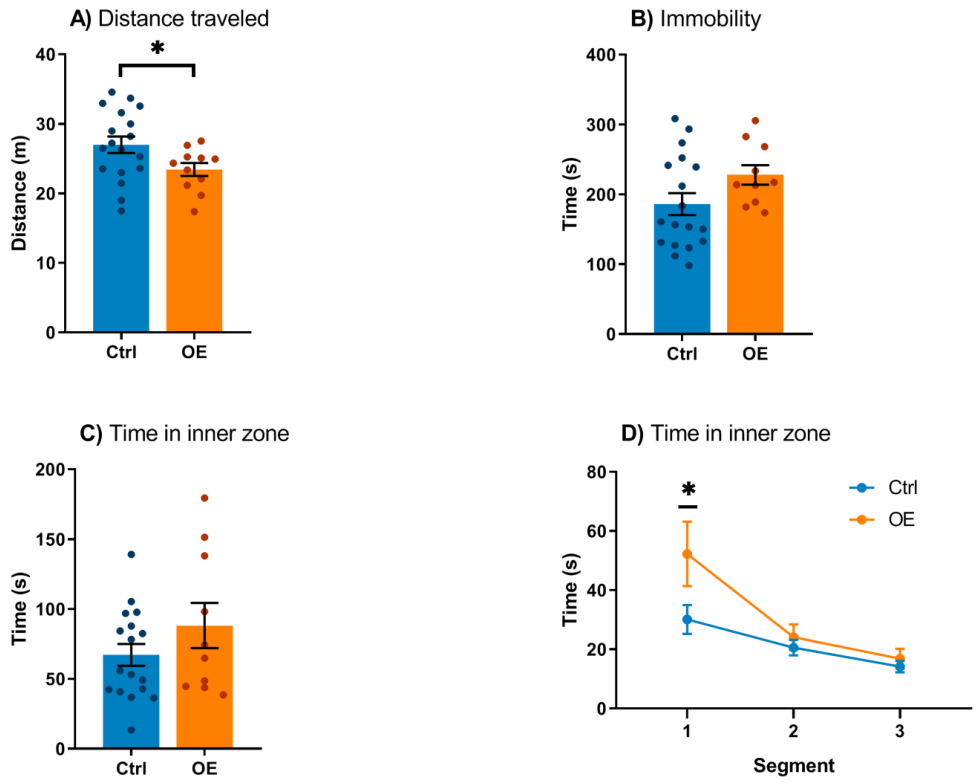


**Figure 32: Open field test results (experiment 2, cohort 1)**
OE of *Fkbp5* in the LC did reduce the distance traveled (A) without significantly affecting immobility (B). Animals of the LC *Fkbp5*-OE group did spend more time in the aversive inner zone during the first 5 minutes of the test (D), however, overall time in the center was not altered (C). Data are represented as mean ± SEM. * significantly different from the control condition.

Elevated plus maze test

In the EPM, there was no significant difference between both groups. *Fkbp5* OE affected neither the latency until the animals first entered the open arm ($p = 0.6951$) nor the time animals spent in the open arms as a percentage of the total arm time ($p = 0.1314$) (Figure 33).

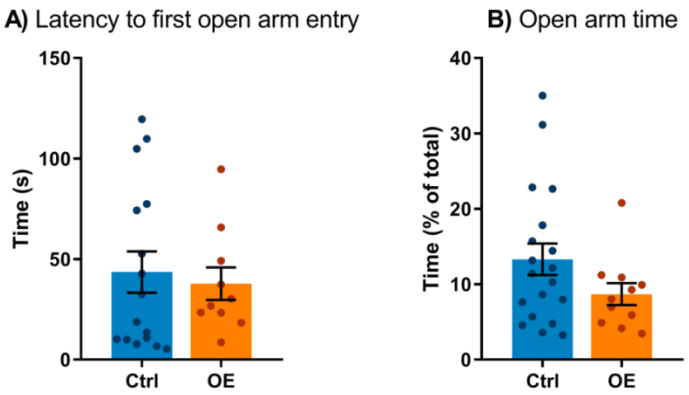


**Figure 33: Elevated plus maze test results (experiment 2, cohort 1)**
Neither the latency until animals entered the open arm for the first time (A), nor the total open arm time (B) was affected by OE of *Fkbp5* in the LC. Data are represented as mean ± SEM.

## Dark-light box test

Latency to the first entry into the lit compartment of the DaLi test did not differ between controls and the LC *Fkbp5*-OE group (p = 0.1625). Overall percentage of time the animals spent in the lit zone was not altered by the OE of *Fkbp5* (p = 0.1837) (Figure 34).

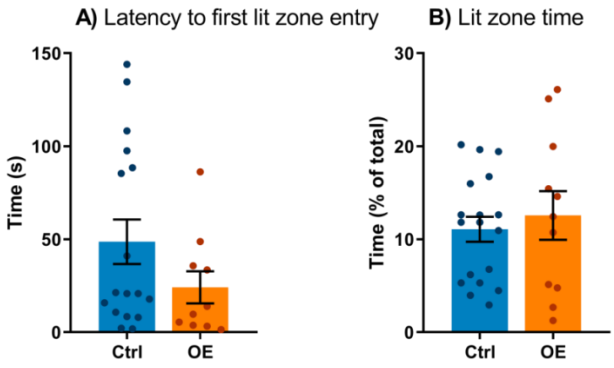


**Figure 34: Dark-light box test results (experiment 2, cohort 1)**
Neither the latency until animals entered the lit zone for the first time (A), nor the time in the lit zone (B) was affected by OE of *Fkbp5* in the LC. Data are represented as mean ± SEM.

Forced swim test

There was no difference between the groups in the percentage of struggling, swimming and floating behavior (% struggling: p = 0.3418; % swimming: p = 0.3322; floating: p = 0.8544) (Figure 35).

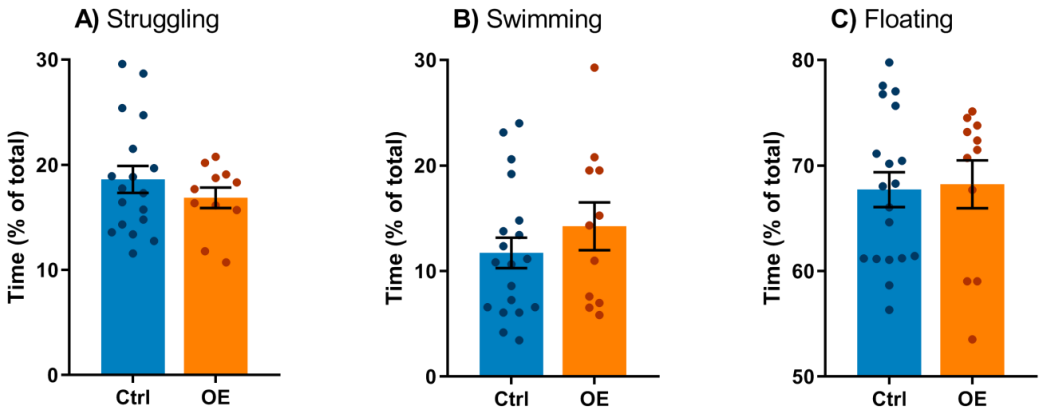


**Figure 35: Forced-swim test results (experiment 2, cohort 1)**
*Fkbp5* OE in the LC did not affect struggling (A), swimming (B) or floating (C) behavior. Data are represented as mean ± SEM.

Physiology

There were no observable differences in bodyweight during the course of the experiment. Since the animals were still growing, bodyweight increased in the same rate in both groups (day 5: p = 0.8574; day 63: p = 0.9070) (Figure 36).

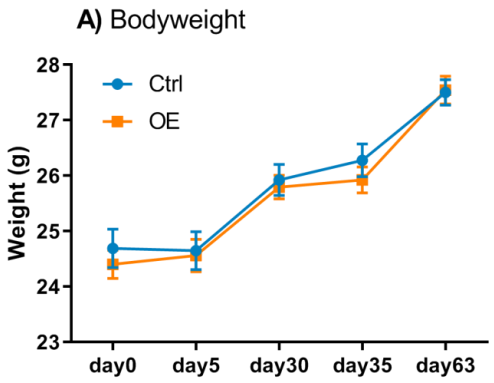


**Figure 36: Bodyweight over time (experiment 2, cohort 1)**
Bodyweight did not differ between the two groups. All animals increased in weight due to their growth over the 63 day testing period. Data are represented as mean ± SEM.

Organs

The adrenal glands and the thymus of each animal were weighed as a measurement of endocrine functionality. Usually, these organs change in size and weight in response to chronic hyper-activity of the HPA-axis. Adrenal glands were enlarged in the LC *Fkbp5*-OE group, while there was no difference in the relative weights of the thymus (adrenals: p = 0.0130; thymus: p = 0.8527) (Figure 37).

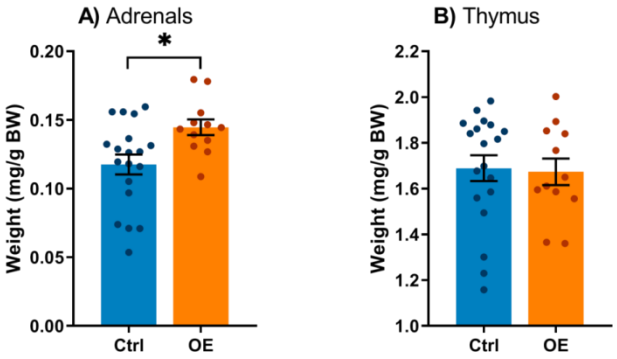


**Figure 37: Organ weight (experiment 2, cohort 1)**
Adrenal glands (A) were significantly enlarged while thymi (B) did not differ in weight between the controls and the LC *Fkbp5*-OE animals. Data are represented as mean ± SEM. * significantly different from the control condition.

### 3.2.3 *Fkbp5* over-expression in the locus coeruleus (cohort 2)

I extended our characterization of *Fkbp5* OE in the LC in a second cohort of animals, in order to cover a wider variety of behaviors, including several cognitive tasks mainly focusing on spatial memory. For that reason, a second batch of animals received an injection of *Fkbp5* over-expressing AAV or the respective control virus into the LC.

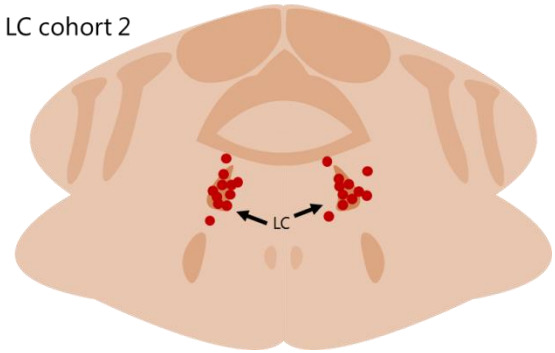


**Figure 38: AAV-injections into the locus coeruleus**
This figure shows the estimated center-point of each successful injection. Individuals that did not show expression in the targeted areas were excluded from the analysis.

The injection site was again verified after the experiment (Figure 38) and animals without a significant OE of *Fkbp5* in the target area were excluded from analysis. In order to allow for sufficient habituation to the test arena before the more sensible tests, animals underwent 3 OF tests of 15 minutes each.

Open field test (x3)

During the first of the 3 OF tests, I found no difference in distance traveled ($p = 0.7518$), time immobile ($p = 0.1619$) or the time animals spent in the aversive, inner zone ($p = 0.1596$) (Figure 39).

In the second OF, there was no difference in regard of the distance traveled ($p = 0.1952$) or the time animals were immobile ($p = 0.0813$). I found a significant reduction of time in the inner zone in the LC *Fkbp5*-OE group ($p = 0.0144$) (Figure 39).

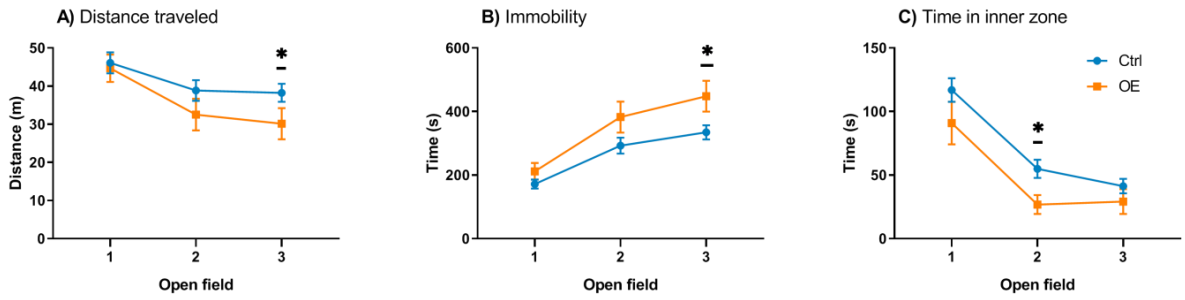


**Figure 39: Open field test results (experiment 2, cohort 2)**
In cohort 2, the OF test was repeated three times. There was no difference in distance traveled in the first two tests, however, OE animals showed a decrease in distance traveled (A) and an increase in immobility (B) during the third OF. The time they spent exploring the inner zone of the arena was significantly different in the second test with OE animals showing a decrease (C). Data are represented as mean ± SEM. * significantly different from the control condition.

When repeating the test for a third time, I found that animals from the LC *Fkbp5*-OE group adapted quicker to the environment, which was represented by a decrease in locomotion (distance traveled: $p = 0.0367$) and mobility ($p = 0.0280$). However, there was no difference in inner zone time ($p = 0.2678$) (Figure 39).

Y-maze test

During the retrieval phase of the test, a one sample t-test revealed that both groups spent more than 33.3% of the time in the novel arm of the three-armed maze (Ctrl: $p < 0.0001$; OE: $p = 0.0012$). Therefore, all animals did learn to discriminate between the familiar arms and the novel arm. However, there was no difference between the groups ($p = 0.2913$) (Figure 40).

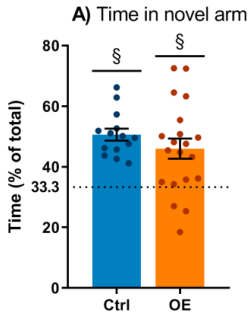


**Figure 40: Y-maze test results (experiment 2, cohort 2)**
Animals from both groups showed intact memory, indicated by their ability to recognize the novel arm of the maze. There was no treatment effect between the groups. Data are represented as mean ± SEM. § significantly different from chance level.

Object relocation test

Animals did not learn to discriminate the object in the novel location from the one that was located in the same place as during the acquisition phase. When I calculated the discrimination index for the time spent with the novel compared to the familiar location, both groups failed to show significant discrimination between the objects (LC *Fkbp5*-Ctrl: p = 0.0832; LC *Fkbp5*-OE: p = 0.9671). When I compared both groups directly, there was no significant difference between the indices (p = 0.1743) (Figure 41).

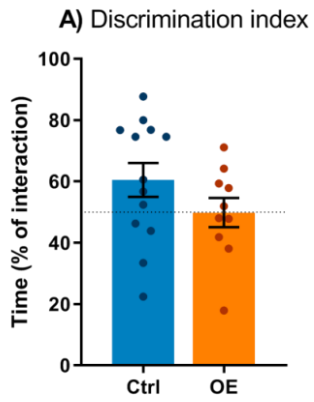


**Figure 41: Object relocation test results (experiment 2, cohort 2)**
None of the two groups did show a significant preference for the object in the novel location. Data are represented as mean ± SEM.

Social avoidance test (x2)

The SA was conducted twice in order to compare sociability under baseline conditions and social avoidance after a subthreshold defeat.

During the first SA, animals of the OE group showed less interaction with the empty cage in trial 1 ($p = 0.0007$), as well as the social animal in trial 2 ($p = 0.0110$). When comparing the trials, both groups did show a significant preference for the social animal compared to the empty cage demonstrated by a value higher than 50% when looking at the interaction time during trial 2 in regard to the overall interaction time in both trials combined (LC *Fkbp5*-Ctrl: $p = 0.0002$; LC *Fkbp5*-OE: $p = 0.0019$). However, here was no significant difference between the two experimental groups ($p = 0.2063$) (Figure 42).

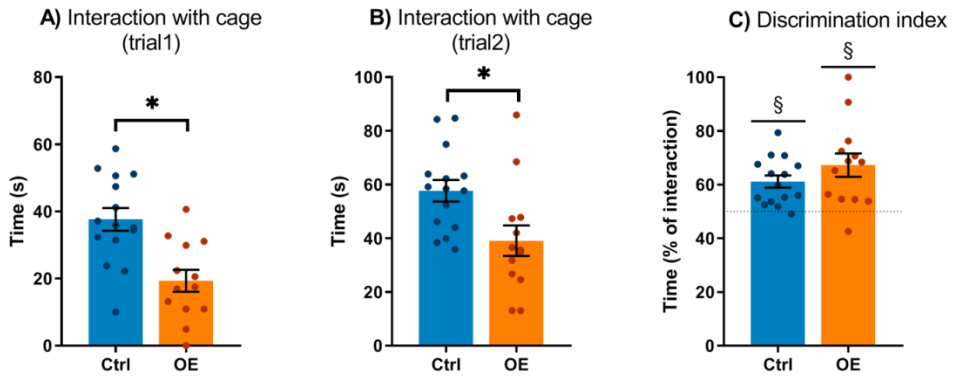


**Figure 42: Social avoidance test 1 results (experiment 2, cohort 2)**
Animals that OE *Fkbp5* in the LC did show lower interaction times in trial 1 (A) and trial 2 (B). Both groups did show a preference for the social cue compared to the non-social cue but there was no difference between controls and OE animals (C). Data are represented as mean ± SEM. * significantly different from the control condition. § significantly different from chance level.

The second SA test was conducted 24h after the animals were exposed to a subthreshold defeat.

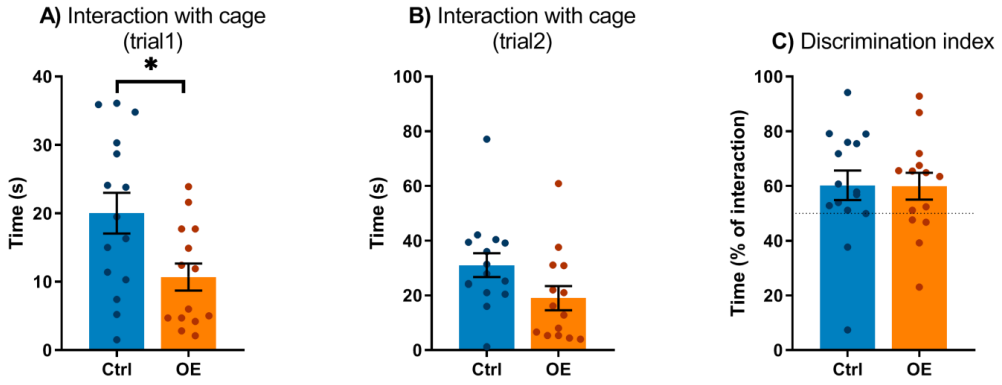


**Figure 43: Social avoidance test 2 results (experiment 2, cohort 2)**
During the second SA, animals of the LC *Fkbp5*-OE group did interact significantly less with the non-social cue than controls (A). There was no difference in the second trial (B). Both groups showed a loss of social preference after the subthreshold defeat, without revealing any differences between them (C). Data are represented as mean ± SEM. * significantly different from the control condition.

Again, animals from the LC *Fkbp5*-OE group did interact less with the empty cage (p = 0.0162) but not with the social animal (p = 0.625). The defeat led to a loss of preference for the social cue compared to the static object in both groups (LC *Fkbp5*-Ctrl: p = 0.0773; LC *Fkbp5*-OE: p = 0.0639). Again, there was no difference between the two conditions (p = 0.9618) (Figure 43).

Forced swim test (x2)

Similar to the SA, the FST was conducted under baseline conditions and one more time after Escitalopram treatment to test the effects of an SSRI when combined with FKBP51 manipulation.

Under baseline conditions, both groups spent a similar time struggling (p = 0.3964), swimming (p = 0.0945) and floating (0.0953) (Figure 44).

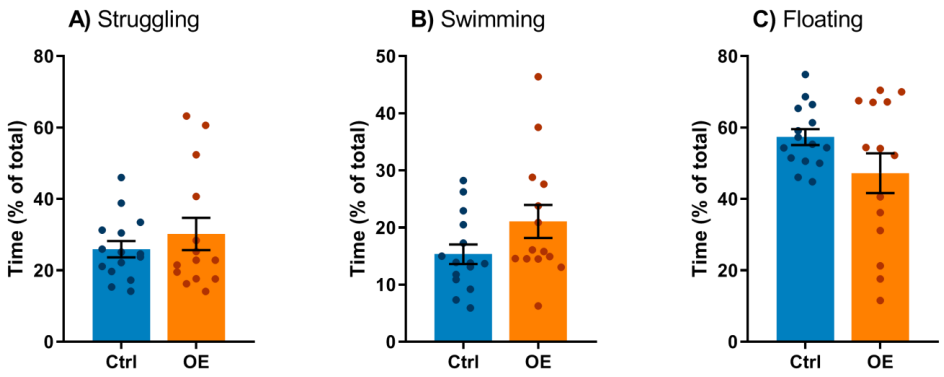


**Figure 44: Forced swim test 1 results (experiment 2, cohort 2)**
There was no difference between the two groups in struggling (A), swimming (B) or floating behavior (C). Data are represented as mean ± SEM.

Using ANOVA, I found no main effect of LC *Fkbp5*-OE ($F_{(1,25)}$ = 1.070; p = 0.3109) or Escitalopram treatment ($F_{(1,25)}$ = 0.5418; p = 0.4686) as well as no interaction effect ($F_{(1,25)}$ = 0.5190; p = 0.4780) for struggling behavior in the second FST. The same was true for swimming (OE: $F_{(1,25)}$ = 2.233; p = 0.1476; Esc: $F_{(1,25)}$ = 0.9062; p = 0.3502; interaction: $F_{(1,25)}$ = 0.0009871; p = 0.9752) and floating behavior (OE: $F_{(1,25)}$ = 2.869; p = 0.1027; Esc: $F_{(1,25)}$ = 0.0007741; p = 0.9780; interaction: $F_{(1,25)}$ = 0.3314; p = 0.5700) (Figure 45).

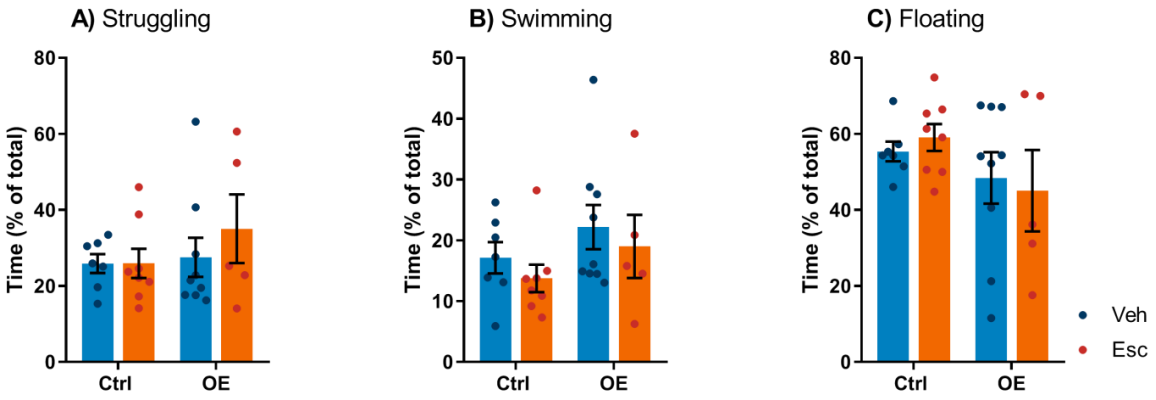


**Figure 45: Forced swim test 2 results (experiment 2, cohort 2)**
Treatment with Escitalopram had no effect on behavior in the second FST. Data are represented as mean ± SEM.

## Endocrinology

To detect potential alterations of the endocrine stress response, I measured corticosterone levels under baseline conditions, in response to stress and after 90 minutes of recovery. However, there was no difference at any of the three points in time between the groups (baseline: $p = 0.1866$; response: $p = 0.0882$; recovery: $p = 0.8175$) (Figure 46).

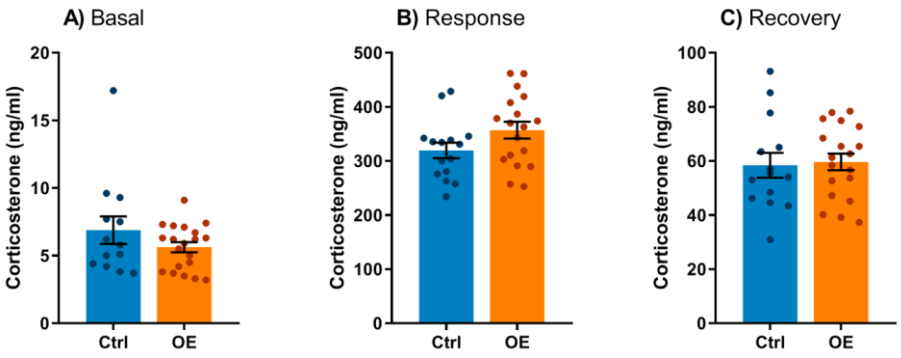


**Figure 46: Endocrinology (experiment 2, cohort 2)**
Over-expression of *Fkbp5* in the LC did not affect baseline corticosterone levels (A). The stress response (B) and recovery rate (C) remained unchanged as well. Data are represented as mean ± SEM.

### 3.2.4 *Fkbp5* over-expression in the dorsal raphe nucleus (cohort 3)

I next tested the effects of *Fkbp5* OE in the DR. Again, the injection site was verified after the experiment (Figure 47) and animals without a significant OE of *Fkbp5* in the target area were excluded from analysis.

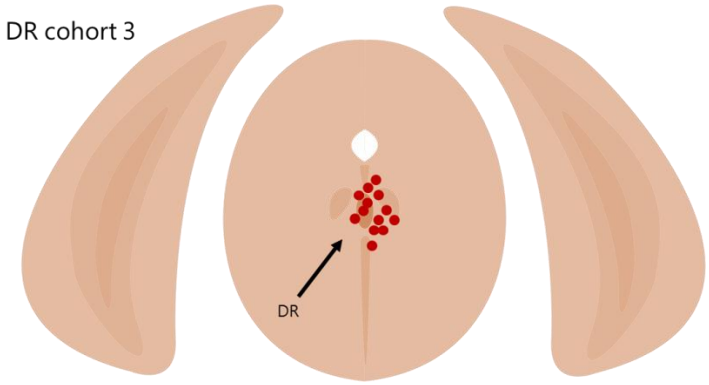


**Figure 47: AAV-injections into the dorsal raphe nucleus**
This figure shows the estimated center-point of each successful injection. Individuals that did not show expression in the targeted areas were excluded from the analysis.

Animals over-expressing *Fkbp5* in the DR were characterized utilizing a standard testing battery.

Open field test

I did not see any differences in locomotor activity. Neither the distance traveled ($p = 0.1805$), nor the time animals were immobile ($p = 0.5309$) did differ between the DR *Fkbp5*-Ctrl and the DR *Fkbp5*-OE group. Animals also showed the same level of aversion towards the central area of the arena ($p = 0.2733$) (Figure 48).

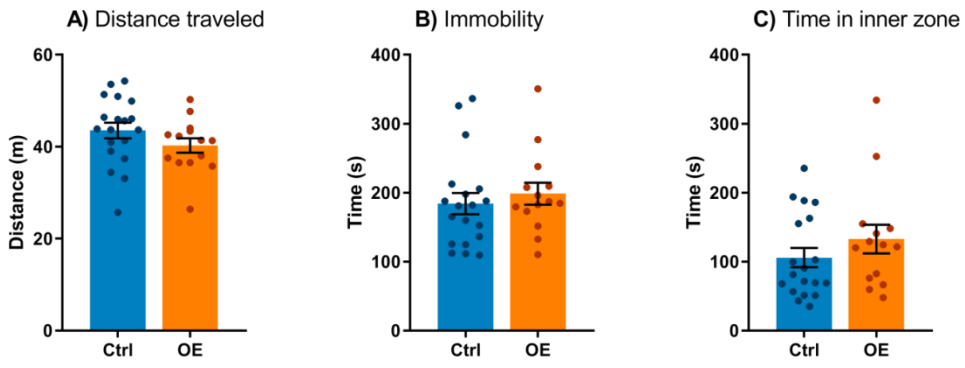


**Figure 48: Open field test results (experiment 2, cohort 3)**
Neither the traveled distance (A), nor immobility (B) or the time animals spent in the inner zone of the arena (C) were affected by the OE of *Fkbp5* in the DR. Data are represented as mean ± SEM.

Elevated plus maze test

In the EPM, I did not detect any significant differences between both groups. DR *Fkbp5*-OE had no effect on the latency until the animals first entered the open arm ($p = 0.3080$) or the time animals spent in the open arms as a percentage of the total arm time ($p = 0.9439$) (Figure 49).

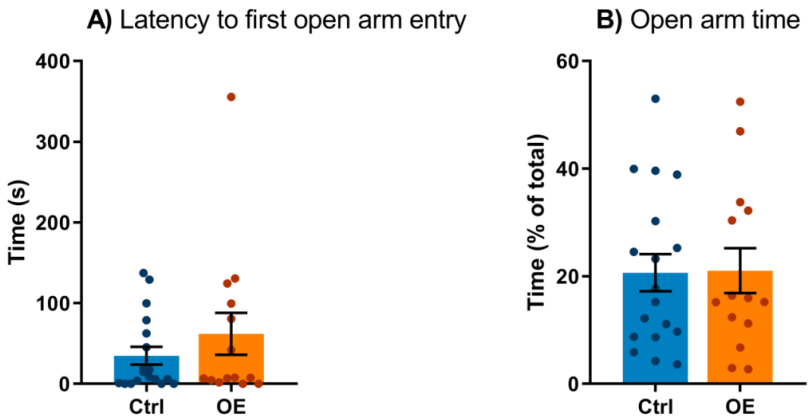


**Figure 49: Elevated plus maze results (experiment 2, cohort 3)**
There was no detectable difference between the two groups in latency to the first entry into an open arm (A) or the total time spent in the aversive area of the maze (B). Data are represented as mean ± SEM.

Dark-light box test

The latency to the first entry into the lit compartment of the arena did not differ between controls and the DR *Fkbp5*-OE group (p = 0.9079). Also, the overall time the mice explored the lit zone was not affected by the DR *Fkbp5*-OE (p = 0.5042) (Figure 50).

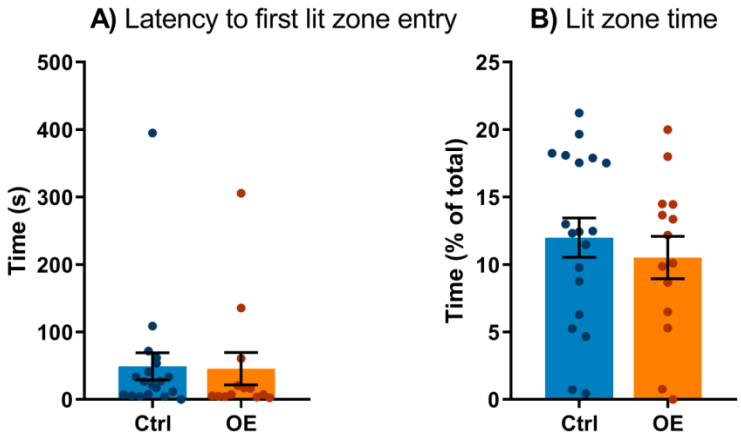


**Figure 50: Dark-light box test results (experiment 2, cohort 3)**
There was no significant difference between the two groups in latency to the first entry into the lit zone (A) or the total time spent in the aversive area of the arena (B). Data are represented as mean ± SEM.

Social avoidance test (x2)

Like in the LC OE experiment the SA was conducted twice in order to compare sociability under baseline conditions and social avoidance after a subthreshold defeat.

During the first SA, there was no difference in interaction time with the respective cue during trial 1 (p = 0.1059), as well as in trial 2 (p = 0.5018). When comparing the trials, both groups failed to show a significant preference for the social cue compared to the empty cage (one sample t-test: DR *Fkbp5*-Ctrl: p = 0.4528; DR *Fkbp5*-OE: p = 0.2752). However, there was no significant difference between the two experimental groups (p = 0.7587) (Figure 51).

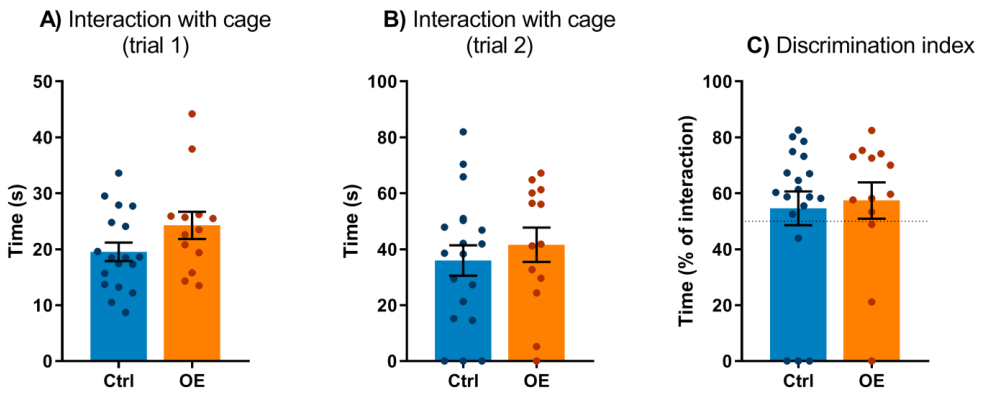


**Figure 51: Social avoidance test 1 results (experiment 2, cohort 3)**
Interaction times with the cues did not differ between the groups in trial 1 (A) or trial 2 (B). Both groups failed to prefer the social cue over the non-social one (C). Data are represented as mean ± SEM.

The second SA test was conducted 24h after the animals were exposed to a subthreshold defeat. Again, there was no difference in interaction time with the empty cage ($p = 0.5240$) as well as the social animal ($p = 0.8732$). The defeat led to a significant avoidance of the social cue compared to the static object in both groups (DR *Fkbp5*-Ctrl: $p = 0.0001$; DR *Fkbp5*-OE: $p < 0.0003$). Again, there was no difference between the two treatments ($p = 0.8525$) (Figure 52).

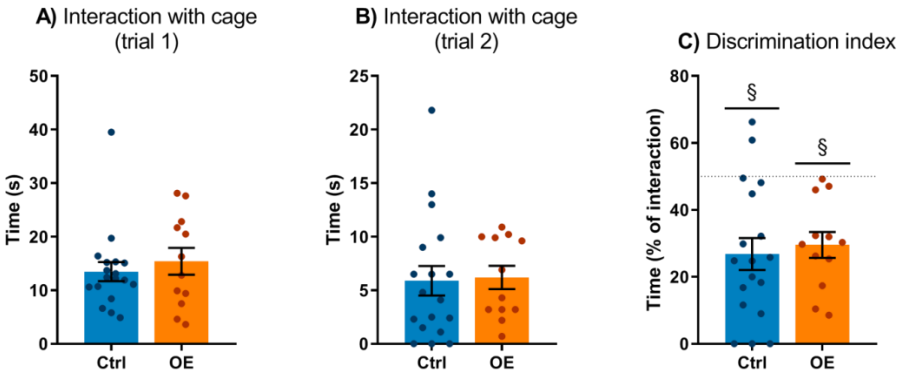


**Figure 52: Social avoidance test 2 results (experiment 2, cohort 3)**
Both groups showed similar interaction times with the cues in trial 1 (A) and trial 2 (B). Subthreshold defeat induced active avoidance of the social cue in both groups (C). Data are represented as mean ± SEM. § significantly different from the control condition.

Object relocation test

Animals from the control group were able to discriminate the object in the novel location from the one that was in the same place as during the acquisition phase. When I calculated the discrimination index, they did show a value significantly larger than 50 (one sample t-test: p = 0.0147). Mice over-expressing *Fkbp5* in the DR on the other hand did not show a preference for either of the locations (p = 0.1661). However, when I compared both groups directly, there was no difference in the indices (p = 0.1499) (Figure 53).

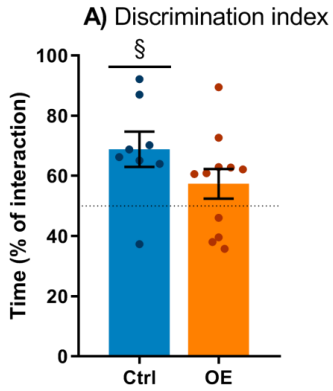


**Figure 53: Object relocation test results (experiment 2, cohort 3)**
Control animals were able to discriminate between the objects in the familiar and the novel location, demonstrated by the preference for the unfamiliar one. Animals over-expressing *Fkbp5* in the DR did not show any discrimination between the objects in the retrieval phase. Data are represented as mean ± SEM. § significantly different from the control condition.

Forced swim test (x2)

Like the SA, the FST was conducted under baseline conditions and one more time after Escitalopram treatment to test the effects of an SSRI when combined with FKBP51 manipulation.

Under baseline conditions, both groups spend the same percentages of the test struggling (p = 0.2801), swimming (p = 0.4472) and floating (0.2350) (Figure 54).

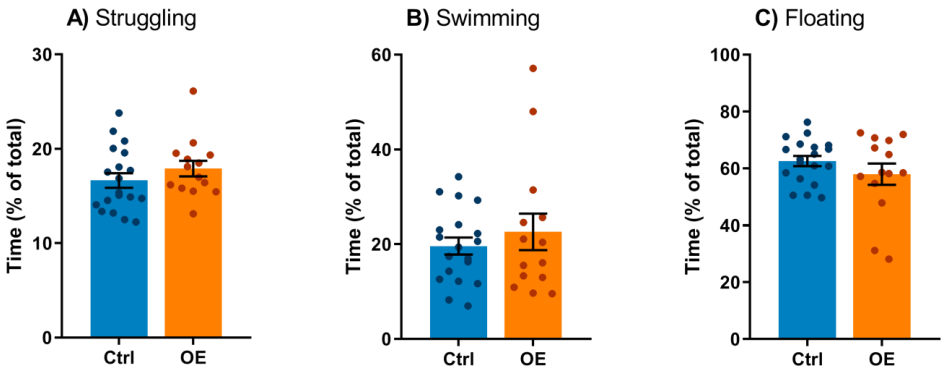


**Figure 54: Forced swim test 1 results (experiment 2, cohort 3)**
There was no detectable difference between struggling (A), swimming (B) or floating (C) times between the groups in the first FST. Data are represented as mean ± SEM.

A single injection with Escitalopram did result in an increase in struggling behavior in both groups with no difference between controls and DR *Fkbp5*-OE animals, as well as no interaction (OE: $F_{(1,25)} = 0.8060$; $p = 0.3779$; Esc: $F_{(1,25)} = 9.511$; $p = 0.0049$; interaction: $F_{(1,25)} = 1.511$; $p = 0.2305$).

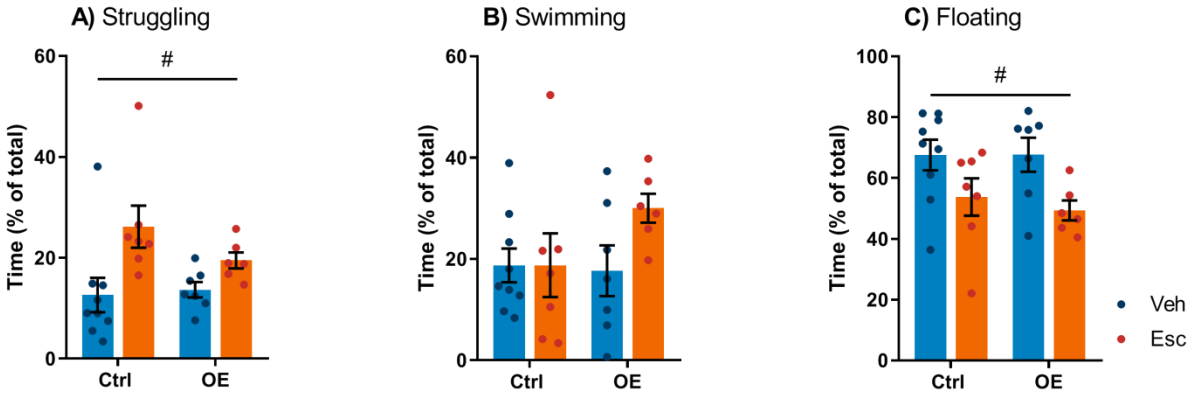


**Figure 55: Forced swim test 2 results (experiment 2, cohort 3)**
Treatment with Escitalopram did increase struggling behavior (#), while OE had no effect (A). Swimming behavior was not affected by Escitalopram treatment or the OE (B). Floating was reduced by Escitalopram (C). Data are represented as mean ± SEM. # significant Escitalopram main effect.

I did not find any interaction or changes in swimming behavior (OE: $F_{(1,25)} = 1.233$; $p = 0.2775$; Esc: $F_{(1,25)} = 1.809$; $p = 0.1907$; interaction: $F_{(1,25)} = 1.796$; $p = 0.1923$), however two-way ANOVA revealed that floating was significantly reduced by Escitalopram while not being affected by DR *Fkbp5*-OE (OE: $F_{(1,25)} = 0.1638$; $p = 0.6892$; Esc: $F_{(1,25)} = 9.121$; $p = 0.0058$; interaction: $F_{(1,25)} = 0.1794$; $p = 0.6755$) (Figure 55).

## Physiology

There were no observable differences between the groups in bodyweight during the course of the experiment. Since the animals were still growing, bodyweight increased in the same rate in both groups (day 9: $p = 0.2240$; day 63: $p = 0.9731$) (Figure 56).

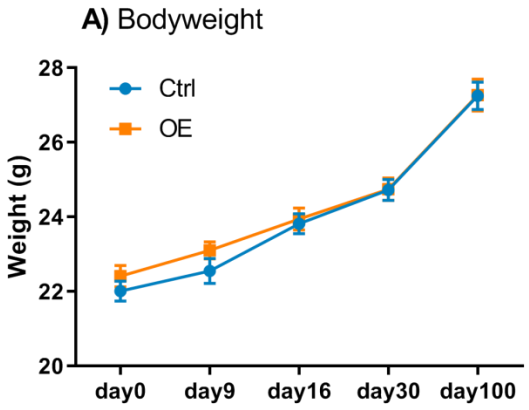


**Figure 56: Bodyweight over time (experiment 2, cohort 3)**
There was no difference in bodyweight between control and OE animals. Data are represented as mean ± SEM.

## Endocrinology

In order to check whether the endocrine stress response was altered in the DR *Fkbp5*-OE group, I measured corticosterone levels under baseline conditions, in response to stress and after 90 minutes of recovery. However, there was no difference under baseline conditions ($p = 0.0620$), as well as 30 minutes after stress ($p = 0.3504$). Mice from the DR *Fkbp5*-OE group had a significantly quicker

recovery after 90 minutes with lower levels of corticosterone compared to controls (p = 0.0360) (Figure 57).

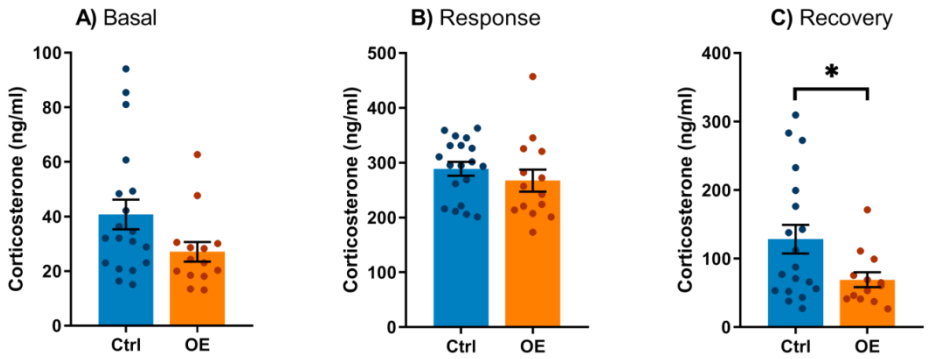


**Figure 57: Endocrinology (experiment 2, cohort 3)**
No difference in baseline corticosterone levels was present between controls and OE animals (A). While the stress response was also not changed (B), OE animals recovered quicker 90 minutes after stress (C). Data are represented as mean ± SEM. * significantly different from the control condition.

## 3.3 Experiment 3

### 3.3.1 *Fkbp5* knock-out in *Nat*-positive cells of the locus coeruleus

Next, I tested whether loss of *Fkbp5* specifically in noradrenergic neurons would affect the stress-related phenotype of mice. For the conditional $Fkbp5^{LC\ KO}$ line, $Fkbp5^{lox/lox}$ mice were bred with a previously generated *Nat-Cre* line. In this case, exon 9 of the *Fkbp5* is marked by the loxP sites and its excision results in transcription of a truncated product and therefore no functional FKBP51 protein activity in all *Nat*-expressing cells respectively. With the LC being one of the major outputs of noradrenaline, a vast majority of cells expressing this type of transporter are located there. In order to confirm the successful deletion of Fkbp5 in the LC, ISH was applied (Figure 58).

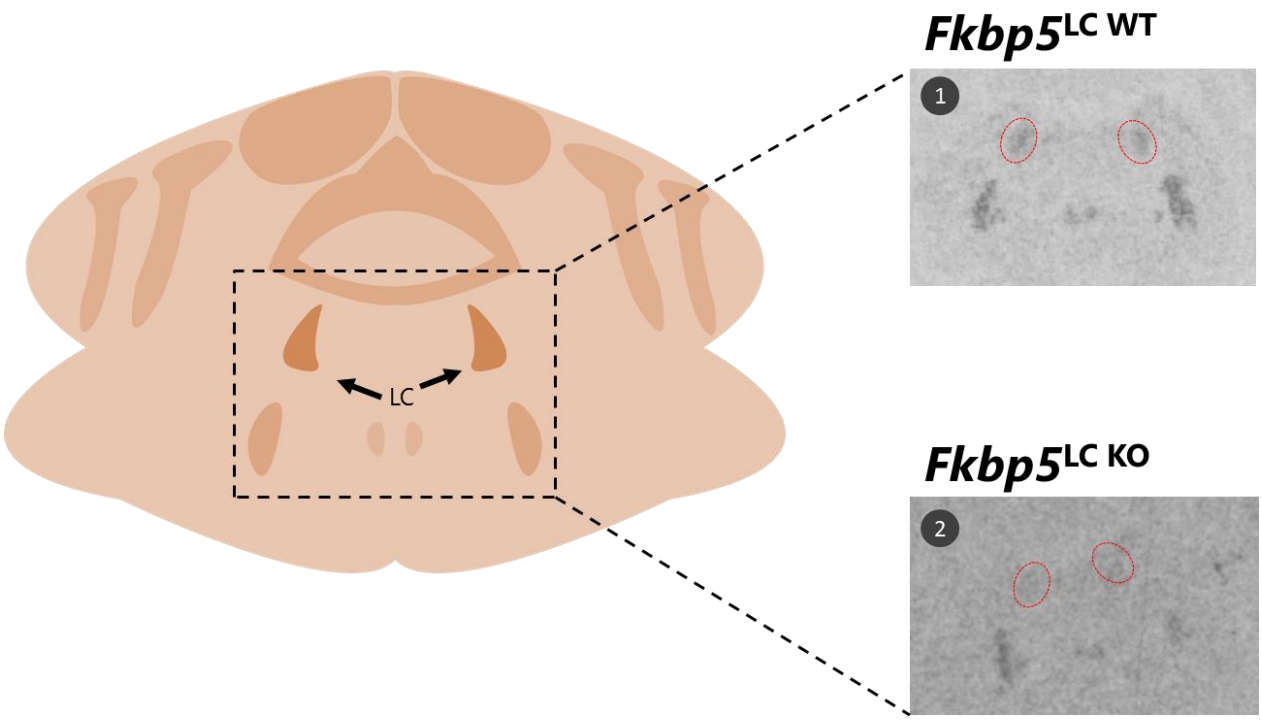


**Figure 58: Conditional knock-out of *Fkbp5* in the locus coeruleus**
The conditional KO of *Fkbp5* in the LC was confirmed by ISH. Red circles indicate the location of the LC in *Fkbp5*$^{LC\ WT}$ (1) and *Fkbp5*$^{LC\ KO}$ (2) animals.

Open field test (3x)

Animals were tested in the OF for three consecutive days in order to track locomotion as well as to habituate them to the arena for future tests. Animals habituated well to the test, which was represented by a steady decrease in overall mobility and exploration in consecutive OFs. However, there was no difference between *Fkbp5*$^{LC\ WT}$ and *Fkbp5*$^{LC\ KO}$ animals in terms of distance traveled (OF1: $p = 0.5963$; OF2: $p = 0.6792$; OF3: $p = 0.5013$), time immobile (OF1: $p = 0.9010$; OF2: $p = 0.9476$; OF3: $p = 0.4827$) or the time they spent in the aversive mid-zone of the arena (OF1: $p = 0.3983$; OF2: $p = 0.8117$; OF3: $p = 0.4608$) (Figure 59).

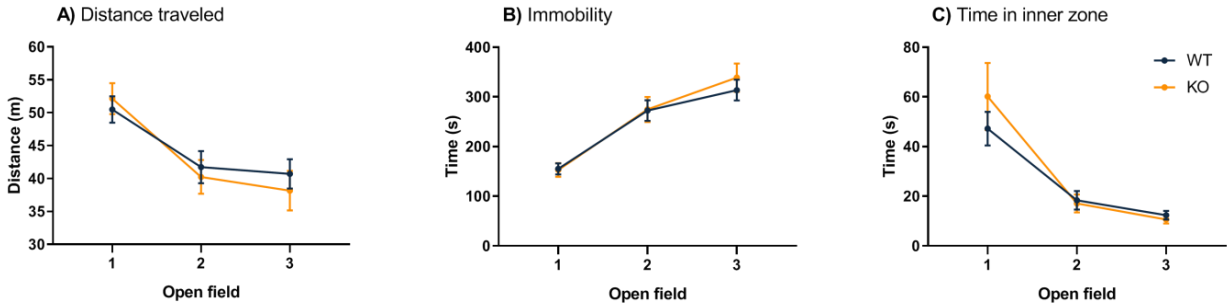


**Figure 59: Open field test results (experiment 3, *Nat- Cre*)**
Conditional KO of *Fkbp5* in the LC did not affect locomotor behavior in three consecutive OF tests. Both groups showed the same amount of distance traveled (A), similar levels of immobility (B) and no difference in the time they spent in the aversive mid-section of the arena (C). Data are represented as mean ± SEM.

Object relocation test

Subsequently, the OLT was conducted to test spatial short-term memory in the animals. Both genotypes did learn to discriminate between the object in the old location and the one that was displaced during the retrieval phase and spent subsequently more time investigating the one in the novel location (*Fkbp5*$^{LC\ WT}$: p = 0.0002; *Fkbp5*$^{LC\ KO}$: p = 0.0295). I was able to detect significant differences between the groups when comparing the discrimination indices for the objects in the two locations with *Fkbp5*$^{LC\ WT}$ animals showing higher interest in the displaced object than *Fkbp5*$^{LC\ KO}$ (p = 0.0040). However, it hast to be noted that some animals were excluded from the analysis due to their low level of overall interaction with the objects which might impact the reliability of the results (Figure 60).

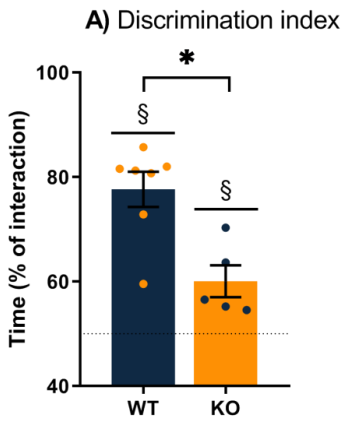


**Figure 60: Object relocation test results (experiment 3, *Nat- Cre*)**
Both, WTs and KOs were able to memorize the positions of the objects and spent more time investigating the one in the unfamiliar location. Animals from the KO group however did show significantly lower levels of recognition. Data are represented as mean ± SEM. * significantly different from the WT control condition. § significantly different from chance level.

Object recognition test

The ORT was used to test recognition memory in the animals. Unfortunately, the interaction times with the objects were too low to allow for any confident and reliable analysis of the data.

Dark-light box test

The DaLi was used to measure anxiety-like behavior. *Fkbp5*$^{\text{LC KO}}$ animals spent significantly less time in the aversive compartment of the arena than *Fkbp5*$^{\text{LC WT}}$ ($p = 0.0325$). I did not find a significant effect on the latency to the first entry into the lit compartment ($p = 0.4719$) (Figure 61).

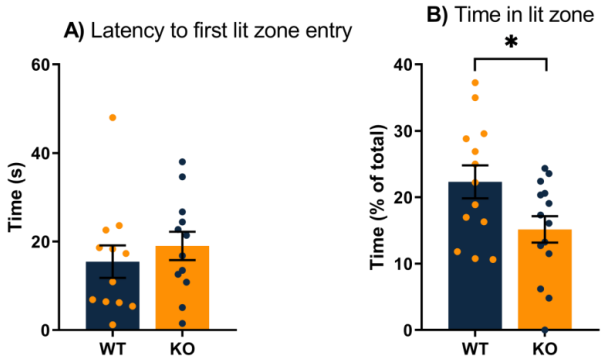


**Figure 61: Dark-light box test results (experiment 3, *Nat-Cre*)**
There was no difference between controls and KOs in terms of the time between the start of the test and their first entry into the lit compartment of the arena (A). KOs did spend significantly less time in the lit zone compared to WT controls (B). Data are represented as mean ± SEM. * significantly different from the WT control condition.

Elevated plus maze test

The EPM was a second anxiety task used. There was no detectable difference in anxiety-related behavior in the EPM. Both groups showed about the same level of anxiety when I looked at the number of open arm entries (p= 0.2072) and the time, they spent exploring the open arms of the maze (p = 0.8277) (Figure 62).

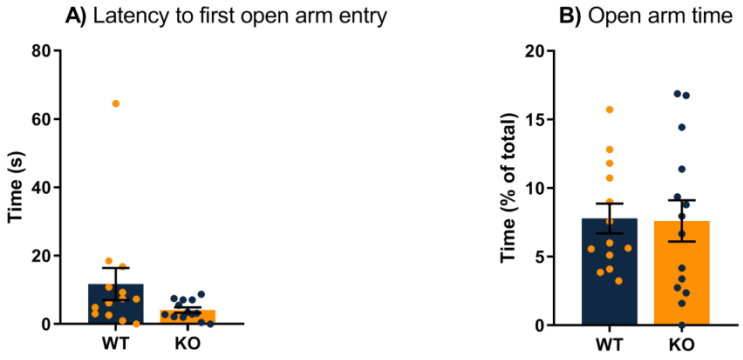


**Figure 62: Elevated plus maze test results (experiment 3, *Nat-Cre*)**
There was no difference between conditional KOs and WT controls when looking at the latency to their first entry into to open arms (A) or the time they spent on the aversive, open sections of the maze (B). Data are represented as mean ± SEM.

Social avoidance test (2x)

The first of two SA tests was conducted under baseline conditions to test for any impact of a disrupted FKBP51 system on sociability. There was no significant difference in interaction time during trial 1 ($p = 0.8103$) or trial 2 ($p = 0.0675$). When looking at the interaction time during trial 2 in regard to the overall interaction time in both trials combined, a one-sample t-test revealed a value significantly higher than 50% for the *Fkbp5*$^{LC\,WT}$ ($p = 0.0062$), but not the *Fkbp5*$^{LC\,KO}$ group ($p = 0.8848$). However, I did not find a significant difference between groups ($p = 0.0671$) (Figure 63).

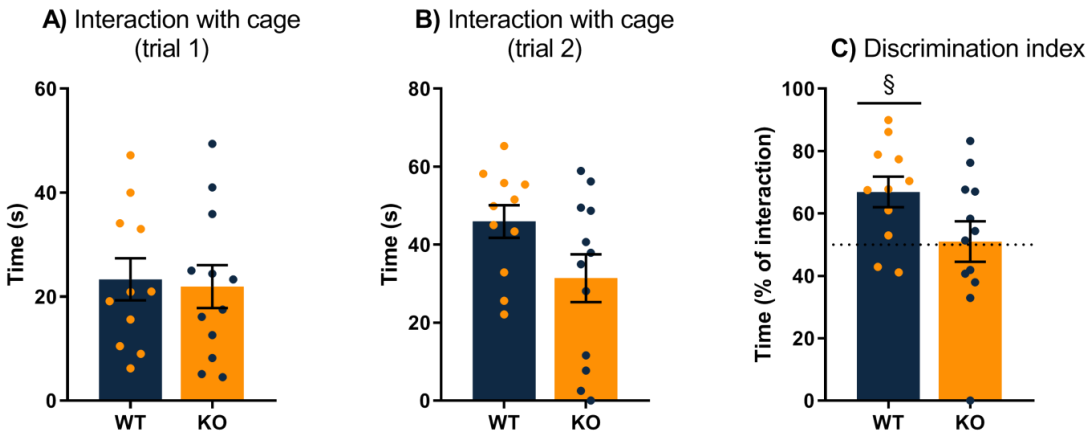


**Figure 63: Social avoidance test 1 results (experiment 3, *Nat-Cre*)**
Both groups spent similar times interacting with the non-social and the social cue in trial 1 (A) and trial (2). When both trials were compared, WT controls did show social preference while KOs did no show preference for any of the cues (C). Data are represented as mean ± SEM. § significantly different from chance level.

The second SA test was conducted 24h after the animals were exposed to a subthreshold defeat. There was no difference in interaction with the non-social cue during trial 1 ($p = 0.0772$), as well as with the social cue in trial 2 ($p = 0.4962$). Overall, both groups failed to spend more than 50% of their interaction time with the social cue and hence did not show social preference (WT: $p = 0.5916$; KO: $p = 0.4055$) (Figure 64).

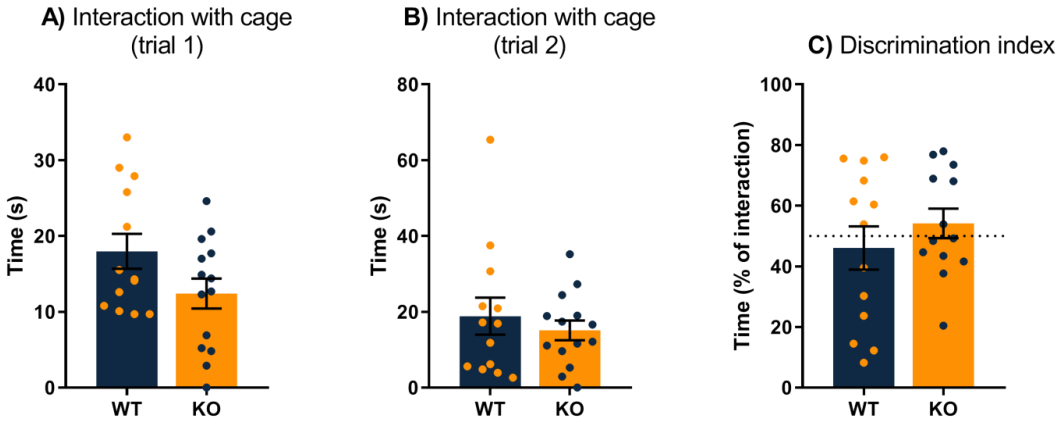


**Figure 64: Social avoidance test 2 results (experiment 3, *Nat-Cre*)**
Similar to the first SA, there was no difference between the groups in time spent with any of the two cues during both trials (A, B). When considering the discrimination index, WT controls lost their social preference after the subthreshold defeat and showed no preference for any of the cues. Data are represented as mean ± SEM.

However, the response of the *Fkbp5*$^{\text{LC WT}}$ group was particularly heterogeneous with 6 animals showing strong social aversion while 7 mice clearly preferred contact to an unfamiliar conspecific. Splitting the groups into susceptible (less than 50% of interaction with the social cue) and resilient (more than 50% of interaction with the social cue) reveals that some animals from both groups show social avoidance even under baseline conditions, which is to be expected. After a subthreshold defeat, some animals switch from social preference to social avoidance. This portion is larger in *Fkbp5*$^{\text{LC WT}}$ than in *Fkbp5*$^{\text{LC KO}}$ (Figure 65).

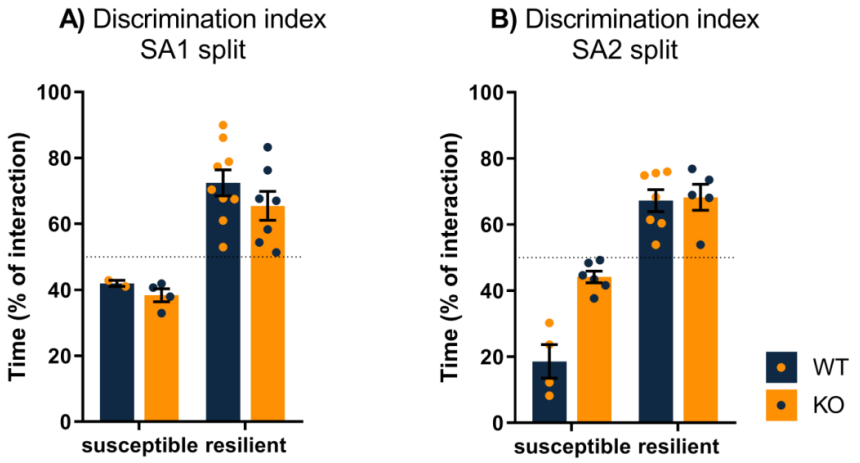


**Figure 65: Social avoidance test 2 split (experiment 3, *Nat-Cre*)**
This graph shows the same data as Figure 64, however split into susceptible and resilient animals based on their discrimination index in SA1 (A) and SA2 (B). While KO animals remain at a similar level after the subthreshold defeat, WT controls that were susceptible in SA1 showed less interaction during SA2. Data are represented as mean ± SEM.

Forced swim test

I used the FST to test for stress coping behavior. Both groups spend the same percentages of the test struggling ($p = 0.7954$), swimming ($p = 0.6440$) and floating (0.6853) (Figure 66).

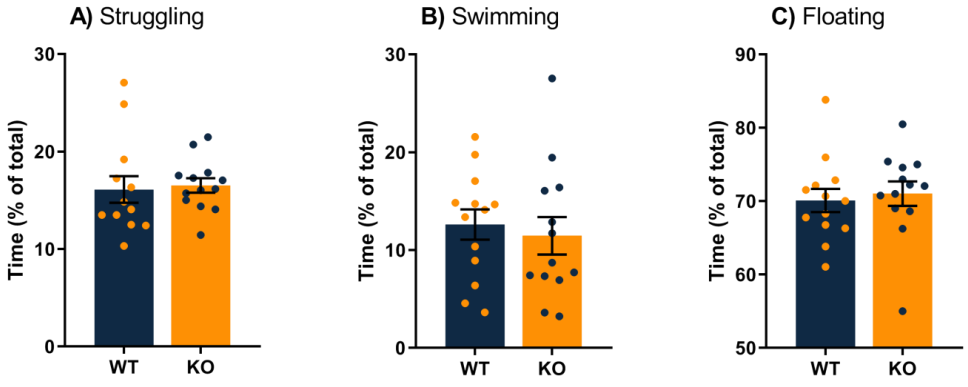


**Figure 66: Forced swim test results (experiment 3, *Nat-Cre*)**
There was no genotype effect on struggling (A), swimming (B) or floating behavior (C). Data are represented as mean ± SEM.

## Physiology

Mice were weighted during the course of the experiment. *Fkbp5* KO in the LC did not impact bodyweight of the animals (p = 0.7899) (Figure 67).

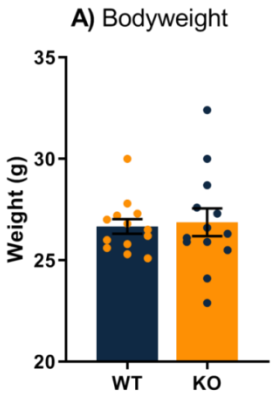


**Figure 67: Bodyweight (experiment 3, *Nat-Cre*)**
There was no difference in bodyweight between WT controls and conditional KOs. Data are represented as mean ± SEM.

There was no difference in organ size when I compared the $Fkbp5^{LC\,WT}$ and $Fkbp5^{LC\,KO}$ adrenals (p = 0.7404) as well as the thymus (p = 0.4331) of $Fkbp5^{LC\,WT}$ animals to the $Fkbp5^{LC\,KO}$ (Figure 68).

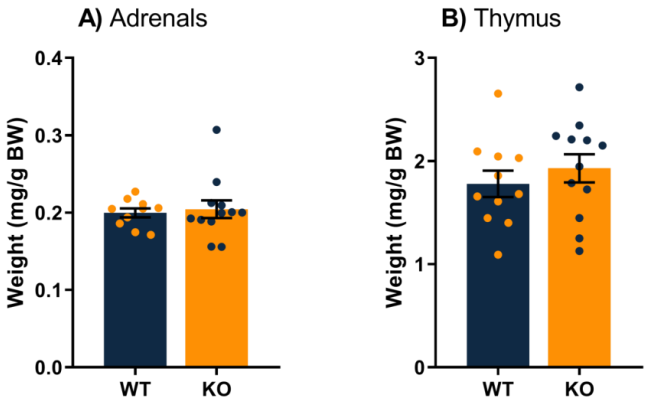


**Figure 68: Organs (experiment 3, *Nat-Cre*)**
Genotype did not affect the relative weight of the adrenal glands (A) or the thymus of the animals (B). Data are represented as mean ± SEM.

Endocrinology

Corticosterone levels were measured in blood plasma probes under baseline conditions, shortly after stress and a third time after 90 minutes recovery time. Corticosterone concentrations did not differ in any of the three measurements (basal: p = 0.2375; response: p = 0.0990; recovery: p = 0.9240) (Figure 69).

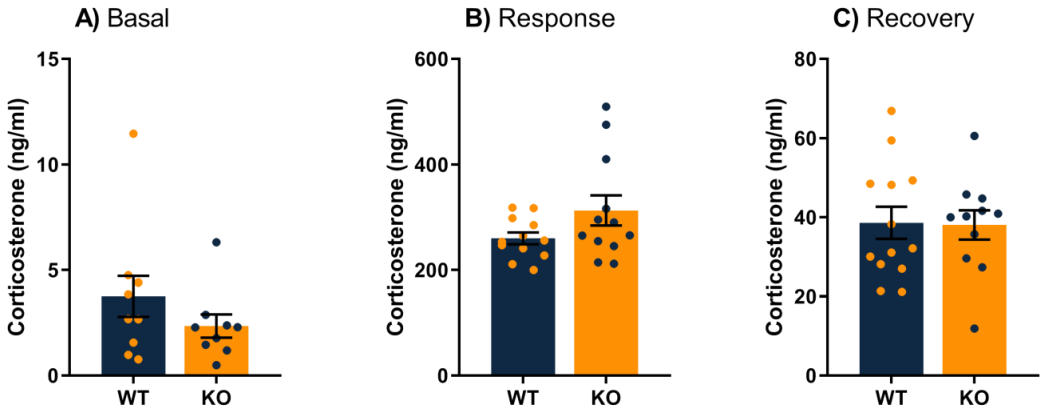


**Figure 69: Endocrinology (experiment 3, *Nat-Cre*)**
Corticosterone levels were not affected by genotype neither under baseline conditions (A), nor in response to stress (B) or in the recovery rate after the stressor (C). Data are represented as mean ± SEM.

### 3.3.2 *Fkbp5* knock-out in *Pet*-positive cells of the dorsal raphe nucleus

For the conditional DR KO line, *Fkbp5*$^{lox/lox}$ mice were bred with a previously generated *Pet-Cre* line. In this case, exon 9 of the *Fkbp5* is marked by the loxP sites and its excision results in transcription of a truncated product and therefore no functional FKBP51 protein activity in all *Pet*-expressing cells respectively. *Cre*-recombinase in the *Pet-Cre* animals is driven by a promotor for the transcription factor *Pet-1*, which regulates the differentiation of serotonergic neurons, predominantly in the DR. In order to confirm the successful deletion of Fkbp5 in the DR, ISH was applied (Figure 70).

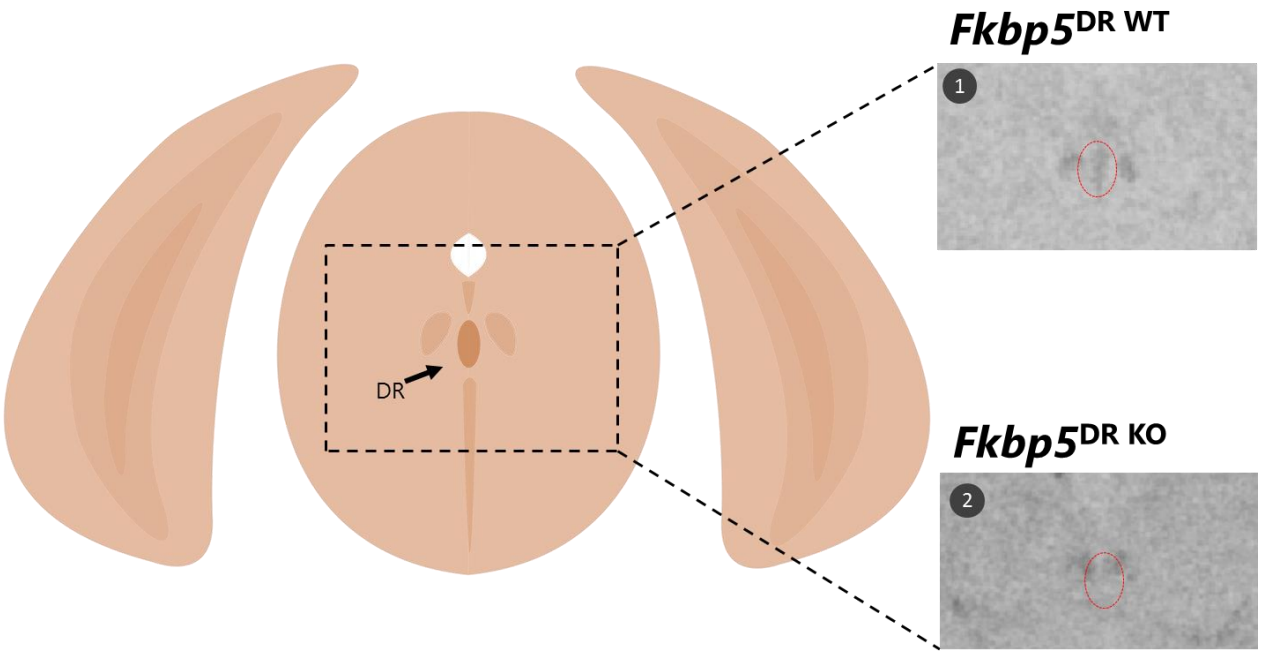


**Figure 70: Conditional knock-out of *Fkbp5* in the dorsal raphe nucleus**
The conditional KO of *Fkbp5* in the DR was confirmed by ISH. Red circles indicate the location of the LC in *Fkbp5*$^{DR\ WT}$ (1) and *Fkbp5*$^{DR\ KO}$ (2) animals.

OF (3x)

Animals were tested in the OF for three consecutive days in order to track locomotion as well as habituate them to the arena and test situation. *Fkbp5*$^{DR\ WT}$ animals habituated well to the test which was represented by a steady decrease in overall mobility and exploration in consecutive OFs. However, there was a significant difference between *Fkbp5*$^{DR\ WT}$ and *Fkbp5*$^{DR\ KO}$ animals in terms of distance traveled in the third OF (OF1: $p = 0.4980$; OF2: $p = 0.0912$; OF3: $p = 0.0165$). Time immobile was also altered in the second and third test session (OF1: $p = 0.3331$; OF2: $p = 0.0149$; OF3: $p = 0.0391$). I did not find any differences in the time the animals spent in the aversive mid-zone of the arena (OF1: $p = 0.9623$; OF2: $p = 0.0686$; OF3: $p = 0.4871$) (Figure 71).

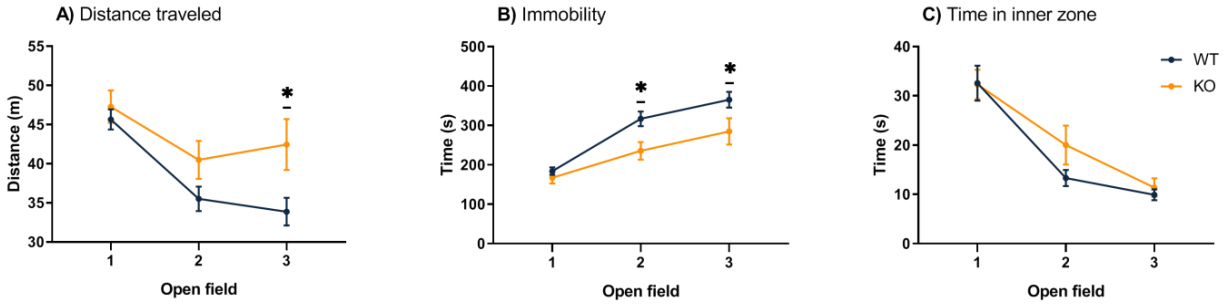


**Figure 71: Open field test results (experiment 3, *Pet-Cre*)**
There was no difference in behavior in the first of three OF tests. However, conditional KOs failed to adapt to the test setup and stayed more active in the second and third OF, indicated by higher levels of distance traveled (A) and lower immobility (B) when compared to WT controls. The time in the inner zone was not affected by genotype (C). Data are represented as mean ± SEM. * significantly different from the WT control condition.

## Object relocation test

Subsequently, the OLT was conducted to test spatial memory in the animals. I was not able to detect significant differences between the groups when comparing the discrimination indices of both genotypes ($p = 0.8369$).

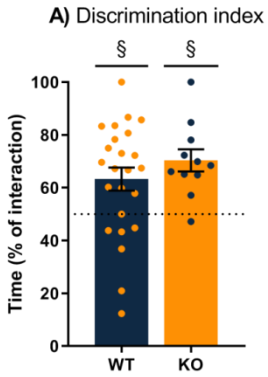


**Figure 72: Object relocation test results (experiment 3, *Pet-Cre*)**
Both genotypes were able to discriminate between the object in the familiar and the one in the novel location, indicated by the preference for the latter. There was no difference between the two genotypes however. Data are represented as mean ± SEM. § significantly different from chance level.

Both groups did show a significantly higher index when compared to the theoretical mean of 50 (*Fkbp5*$^{DR\ WT}$: $p < 0.0001$; *Fkbp5*$^{DR\ KO}$: $p < 0.0001$), meaning that the animals learned to discriminate between the object in the novel location and the one in the same location as before (Figure 72).

Object recognition test

The ORT was used to test recognition memory in the animals. There was no difference in between groups when looking at the discrimination index ($p = 0.1199$), as both equally learned to identify the novel object, shown by an index significantly larger than the theoretical mean of 50% (*Fkbp5*$^{DR\ WT}$: $p = 0.0014$; *Fkbp5*$^{DR\ KO}$: $p = 0.0019$) (Figure 73).

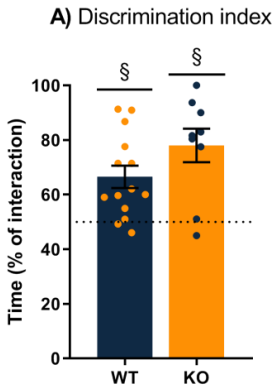


**Figure 73: Object recognition test results (experiment 3, *Pet-Cre*)**
Similar to the OLT, animals of both genotypes showed a preference for the novel object. Again, there was no difference between the groups. Data are represented as mean ± SEM. § significantly different from chance level.

Dark-light box test

I was not able to detect differences that would distinguish *Fkbp5*$^{DR\ KO}$ from *Fkbp5*$^{DR\ WT}$ controls in regard of anxiety-related behavior. All animals frequented the aversive lit zone at comparable rates ($p = 0.4996$) and would remain there for the same amount of time ($p = 0.0884$). The delay until the animals entered the lit compartment for the first time was also indifferent between both experimental groups ($p = 0.3831$) (Figure 74).

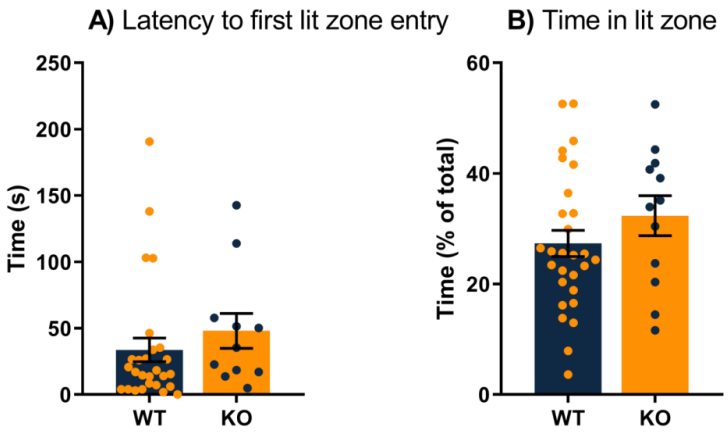


**Figure 74: Dark-light box test results (experiment 3, *Pet-Cre*)**
No differences in the latency to the first entry into the lit zone (A) or the overall time spent in the lit zone (B) were observed. Data are represented as mean ± SEM.

## Elevated plus maze test

Anxiety-related behavior in the animals was not altered by a *Fkbp5*$^{\text{DR KO}}$ when they were tested in the EPM. Neither the latency to the first entry onto the open arms ($p = 0.1694$), nor the time the animals spent there ($p = 0.4851$) was significantly different between the two groups (Figure 75).

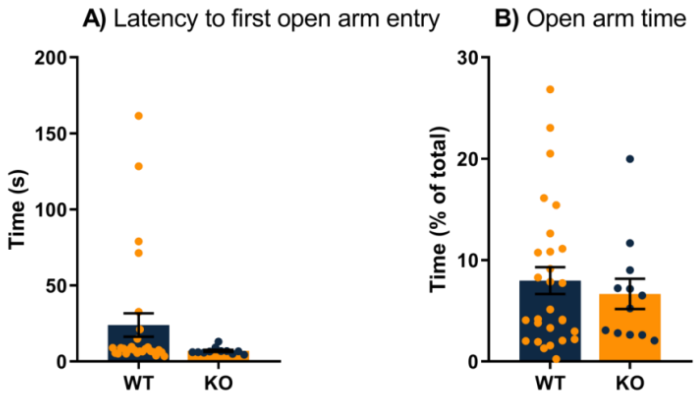


**Figure 75: Elevated plus maze test results (experiment 3, *Pet-Cre*)**
There was no difference in the latency to first open arm entry (A) or the time they spent on the open arms of the arena (B). Data are represented as mean ± SEM.

Social avoidance test (2x)

During the baseline SA, I did not observe different interaction times with the non-social cue (p = 0.7564) or the social cue (p = 0.7566). Neither of the two genotypes showed social preference (discrimination index indifferent from 50, one sample t-test) under baseline conditions (*Fkbp5*$^{DR\ WT}$: p = 0.6768; *Fkbp5*$^{DR\ KO}$: p = 0.4659). There was no significant difference between the discrimination indices of both groups (p = 0.5652) (Figure 76).

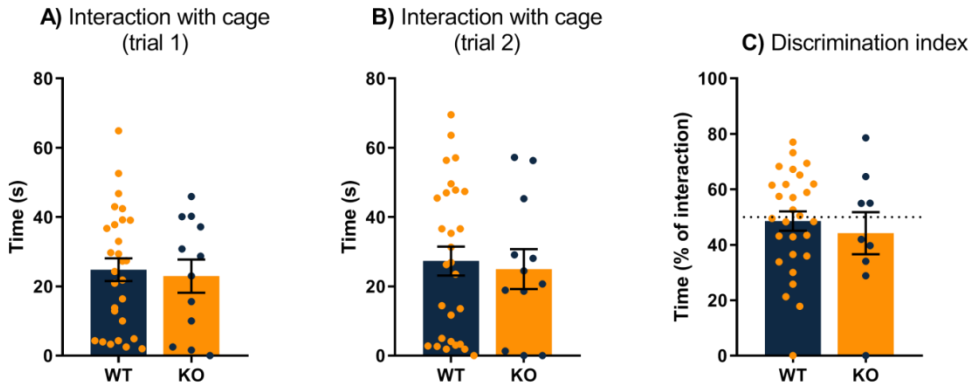


**Figure 76: Social avoidance test 1 results (experiment 3, *Pet-Cre*)**
Both groups showed similar levels of interaction with the non-social cue (A) and the social cue (B). Neither of the two genotypes showed preference for the social cue compared to the non-social one. Data are represented as mean ± SEM.

After subthreshold defeat, both groups did not differ in interaction time with the two different cues (*Fkbp5*$^{DR\ WT}$: p = 0.2253; *Fkbp5*$^{DR\ KO}$: p = 0.3126). *Fkbp5*$^{DR\ WT}$ animals showed a trend towards a pro-social phenotype (p = 0.0523), while *Fkbp5*$^{DR\ KO}$ remained indifferent towards a social cue (p = 0.4874). There was no significant difference between the groups when I compared the discrimination indices (p = 0.6659) (Figure 77).

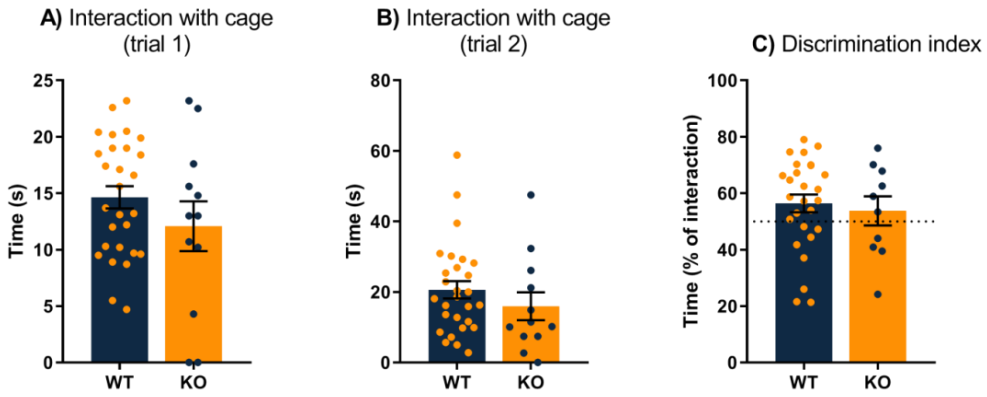


**Figure 77: Social avoidance test 2 results (experiment 3, *Pet-Cre*)**
Both groups showed similar levels of interaction with the non-social cue (A) and the social cue (B). After the subthreshold defeat, neither of the two genotypes showed preference for the social cue compared to the non-social one. Data are represented as mean ± SEM.

Forced swim test

I did not find any significant differences in stress coping behavior. Animals showed the same percentages of struggling ($p = 0.5424$), swimming ($p = 0.1452$) and floating ($p = 0.1220$) behavior (Figure 78).

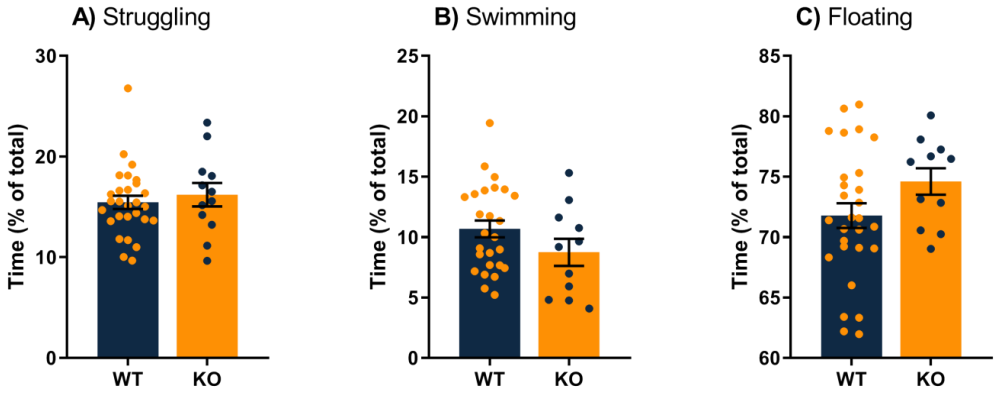


**Figure 78: Forced swim test results (experiment 3, *Pet-Cre*)**
There was no difference between genotypes in struggling (A), swimming (B) or floating (C) times in the FST. Data are represented as mean ± SEM.

Physiology

Mice were weighted during the course of the experiment. A *Fkbp5* KO in the DR did impact overall physiology of the animals, resulting in a significantly higher bodyweight (p = 0.0013) (Figure 79).

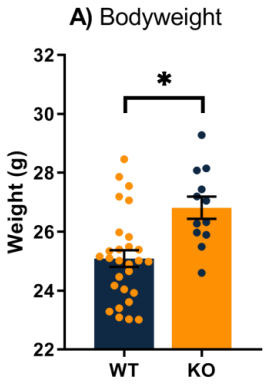


**Figure 79: Bodyweight (experiment 3, *Pet-Cre*)**
Conditional KOs were significantly heavier than WT controls. Data are represented as mean ± SEM. * significantly different from the WT control condition.

There was no difference in organ size when I compared the *Fkbp5*$^{DR\ WT}$ and *Fkbp5*$^{DR\ KO}$ adrenals (p = 0.3636) as well as the thymus (p = 0.3886) of *Fkbp5*$^{DR\ WT}$ animals to the *Fkbp5*$^{DR\ KO}$ (Figure 80).

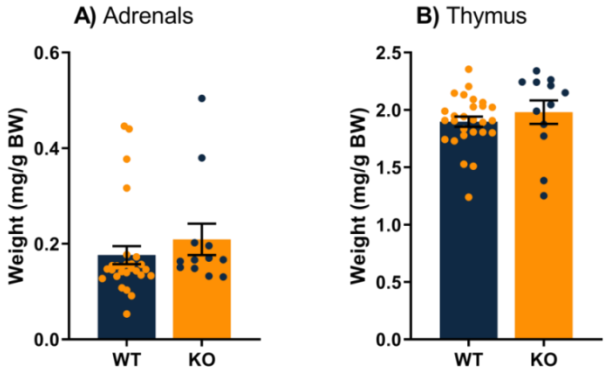


**Figure 80: Organs (experiment 3, *Pet-Cre*)**
Genotype did not affect the relative weight of the adrenal glands (A) or the thymus of the animals (B). Data are represented as mean ± SEM.

Endocrinology

Corticosterone levels were measured in blood plasma probes under baseline conditions, shortly after stress and a third time after 90 minutes recovery time. Corticosterone concentrations did not differ in any of the three measurements (basal: p = 0.9801; response: p = 0.6690; recovery: p = 0.5543) (Figure 81).

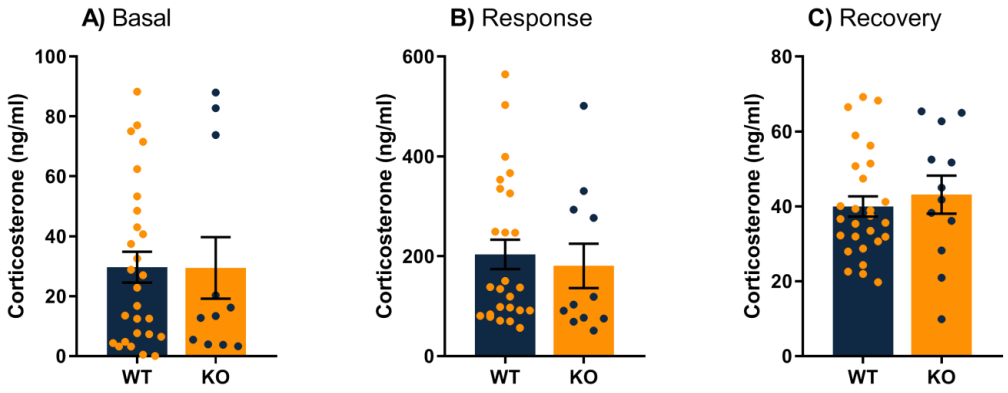


**Figure 81: Endocrinology (experiment 3, *Pet-Cre*)**
Corticosterone levels were not affected by genotype neither under baseline conditions (A), nor in response to stress (B) or in the recovery rate after the stressor (C). Data are represented as mean ± SEM.

## 3.4 Experiment 4

Behavioral effects of the FKBP51 modulator SAFit2 on chronically stressed animals were tested in order to further our knowledge about the potential of the drug in stress treatment.

### 3.4.1 Blood plasma levels of SAFit2

SAFit2 levels were assessed on days 7 and 21. On average, both SAFit2 groups displayed stable drug concentrations between 100 and 200 ng/ml over the course of the 21 day defeat (basal d7 mean: 151.5 ng/ml; stress d7 mean: 134.8 ng/ml; basal d21 mean: 179.5 ng/ml; stress d21 mean: 120.5 ng/ml) (Figure 82).

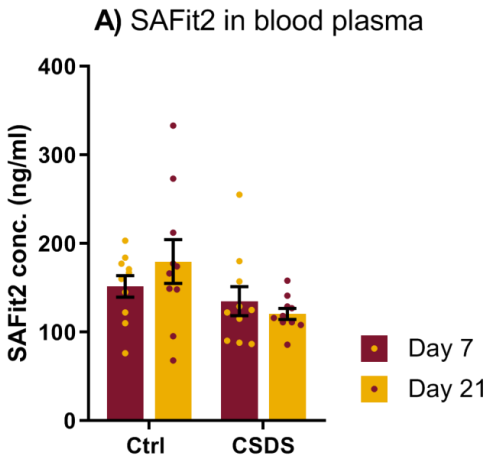


**Figure 82: Blood plasma levels of SAFit2 after chronic treatment via slow-releasing subcutaneous pellets (experiment 4)**
SAFit2 levels remained stable in all four groups over the course of the 21 day experiment. There was no difference in SAFit2 plasma concentration between the groups. Data are represented as mean ± SEM.

### 3.4.2 Open field test

Locomotion was assessed using the OF test. I found a significant stress effect on the distance animals traversed during the 15 minutes of testing ($F_{(1,37)} = 21.67$; $p < 0.0001$). SAFit2 treatment had no effect ($F_{(1,37)} = 0.001440$; $p = 0.9699$) and there was no interaction between the treatments and conditions ($F_{(1,37)} = 1.241$; $p = 0.2724$). Likewise, stressed animals showed an increase in immobility compared to unstressed groups ($F_{(1,37)} = 17.70$; $p = 0.0002$), with no difference regarding SAFit2 treatment ($F_{(1,37)} = 0.09515$; $p = 0.7595$) and no interaction ($F_{(1,37)} = 0.1428$; $p = 0.7076$). Stress exposure not only had an impact on mobility but also decreased the amount of time animals explored the more aversive mid-section of the OF arena ($F_{(1,37)} = 5.934$; $p = 0.0198$). ANOVA revealed that SAFit2 did not alter explorative behavior in this setup (SAFit2: $F_{(1,37)} = 0.02346$; $p = 0.8791$; interaction: $F_{(1,37)} = 0.04873$; $p = 0.8265$) (Figure 83).

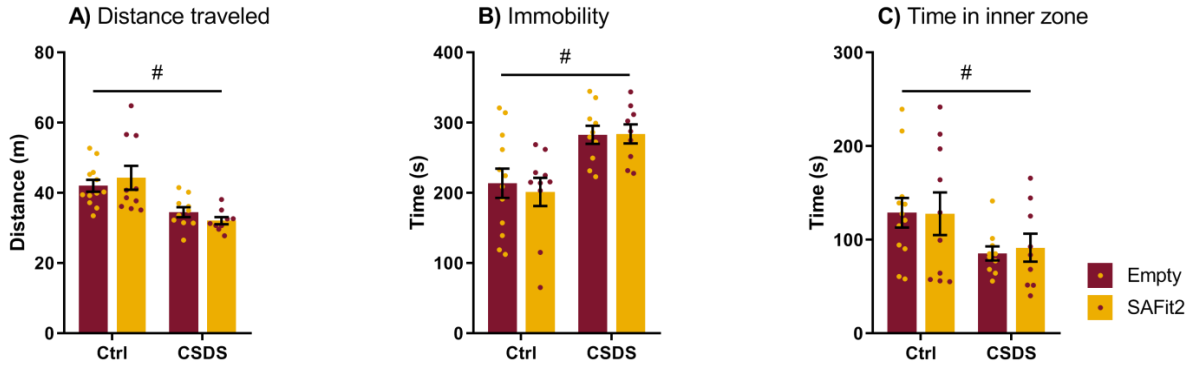


**Figure 83: Open field test results (experiment 4)**
Chronically stressed animals did cover less distance (A) and showed increased immobility (B) during the OF test when compared to unstressed controls. They spent less time in the aversive center zone of the maze (C), indicating higher levels of anxiety and reduced explorative behavior. SAFit2 treatment did not affect behavior in this test. Data are represented as mean ± SEM. # significant main stress effect.

### 3.4.3 Elevated plus maze test

I measured anxiety-like behavior by exposing the mice to the EPM test. In line with the results of the OF, the EPM revealed a significant main effect of stress in both, entries into the open arms ($F_{(1,38)} = 18.52$; $p < 0.0001$) as well as the portion of test time the animals explored the open arms ($F_{(1,38)} = 19.08$; $p < 0.0001$). No main effects for SAFit2 treatment was found for entries ($F_{(1,38)} = 1.405$; $p = 0.2433$) or time spent in the open arms ($F_{(1,38)} = 0.0002765$; $p = 0.9868$). No interaction between the two factors -stress and SAFit2- were found (entries: $F_{(1,38)} = 1.230$; $p = 0.2743$; time: $F_{(1,38)} = 0.01342$; $p = 0.9084$) (Figure 84).

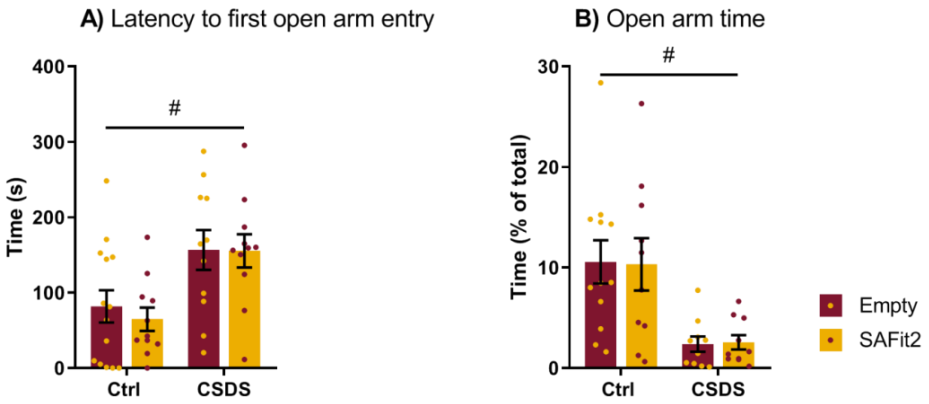


**Figure 84: Elevated plus maze test results (experiment 4)**
There was a significant stress effect in both, the latency to the first entry into the open arms (A) and the overall time, animals spent exploring the open arms (B). SAFit2 treatment had no effect on anxiety-like behavior in the EPM. Data are represented as mean ± SEM. # significant main stress effect.

### 3.4.4 Dark-light box test

The DaLi test was used as an additional measure of anxiety-like and explorative behavior. Two-way ANOVA revealed significant main effects of stress in the relevant parameters like the number of entries into the lit zone ($F_{(1,39)}$ = 12.61; p = 0.0010), the time animals spent in the lit zone ($F_{(1,39)}$ = 8.393; p = 0.0061) and the latency until they first entered the zone ($F_{(1,39)}$ = 8.176; p = 0.0068). However, SAFit2 did not alter behavior in this paradigm (SAFit2: entries: $F_{(1,39)}$ = 1.332; p = 0.2555; time: $F_{(1,39)}$ = 0.1216; p = 0.7292; latency: $F_{(1,39)}$ = 1.991; p = 0.1661) nor was I able to find an interaction in any of the measured parameters (entries: $F_{(1,39)}$ = 0.1199; p = 0.7310; time: $F_{(1,39)}$ = 1.257; p = 0.2691; latency: $F_{(1,39)}$ = 0.04219; p = 0.8383) (Figure 85).

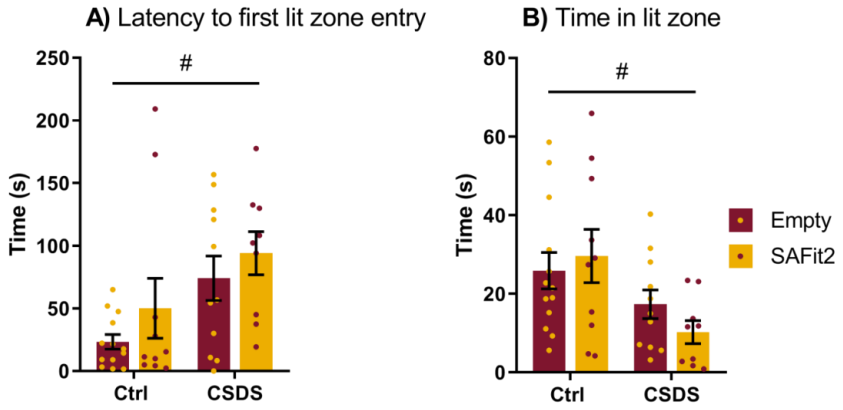


**Figure 85: Dark-light box test results (experiment 4)**
There was an overall stress effect on behavior in the DaLi. Animals from the CSDS group did enter the lit zone later than unstressed controls (A) and did spend less time in the lit compartment (B). SAFit2 treatment had no effect on behavior in the DaLi. Data are represented as mean ± SEM. # significant main stress effect.

### 3.4.5 Social avoidance test

When looking at the overall interaction times with the non-social cue during trial 1, there was no effect of stress ($F_{(1,39)} = 0.1134$; $p = 0.7381$) or treatment ($F_{(1,39)} = 0.6256$; $p = 0.4338$). During the second trial with the social cue, stressed animals did show a decrease of interaction time when compared to unstressed controls ($F_{(1,39)} = 16.95$; $p = 0.0002$). However, there was no effect of SAFit2 treatment ($F_{(1,39)} = 1.351$; $p = 0.2522$). Social avoidance behavior was assessed as a natural response to a social stressor in rodents. There was neither a main effect of stress ($F_{(1,38)} = 2.530$; $p = 0.1200$) or SAFit2 treatment ($F_{(1,38)} = 0.01417$; $p = 0.9059$) nor an interaction effect ($F_{(1,38)} = 0.6195$; $p = 0.4361$) on the discrimination index when ANOVA was applied. However, when calculating the discrimination index one-sample t-test revealed a preference for the social cue in both control groups, represented by the significant difference to a theoretical mean of 50, which would imply no preference for either of the cues (Ctrl Empty: $p < 0.0001$; Ctrl SAFit2: $p = 0.0002$). While animals of the CSDS Empty group did not show social preference ($p = 0.2749$), SAFit2 treated mice of the stress group showed a trend towards social preference (CSDS SAFit2: $p = 0.0771$) (Figure 86).

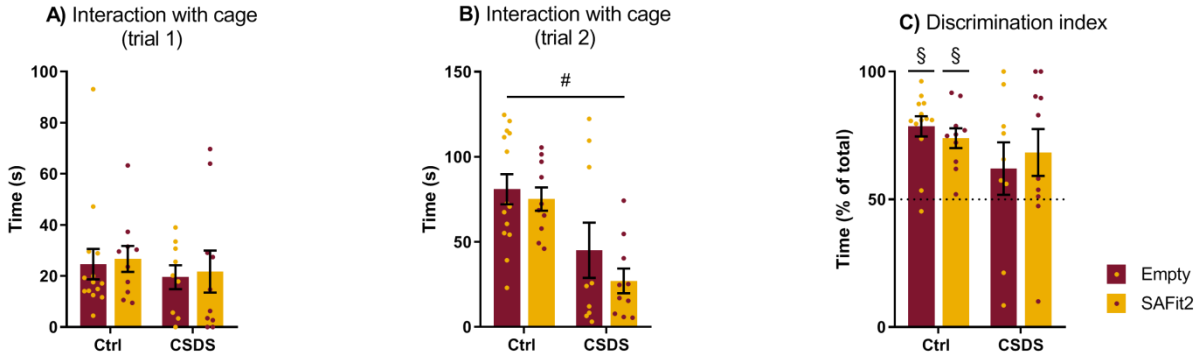


**Figure 86: Social avoidance test results (experiment 4)**
All 4 groups showed similar levels of interest in the non-social cue during trial 1 (A). In trial 2, stressed animals interacted significantly less with the cue when compared to non-stressed controls (B). SAFit2 had no effect on interaction times with the cues. Unstressed animals of both treatment groups did show social preference, while stressed mice did not prefer the social over the non-social cue (C). SAFit2 treatment did result in a trend towards social preference, but the effect was not significant. Data are represented as mean ± SEM. § significantly different from chance level. # significant main stress effect.

### 3.4.6 Forced swim test

The FST was used to test for differences in stress coping capability. While there were no significant stress or drug induced main effects in struggling (CSDS: $F_{(1,38)} = 1.184$; $p = 0.2834$; SAFit2: $F_{(1,38)} = 0.02799$; $p = 0.8680$; interaction: $F_{(1,38)} = 0.9255$; $p = 0.3421$) or swimming (CSDS: $F_{(1,38)} = 3.002$; $p = 0.0913$; SAFit2: $F_{(1,38)} = 0.01322$; $p = 0.9091$; interaction: $F_{(1,38)} = 0.01589$; $p = 0.9004$) behavior, ANOVA revealed a significant increase in floating behavior in stressed animals ($F_{(1,38)} = 4.594$; $p = 0.0385$). SAFit2 did not alter floating any further ($F_{(1,38)} = 0.04829$; $p = 0.8273$; interaction: $F_{(1,38)} = 0.2091$; $p = 0.6500$) (Figure 87).

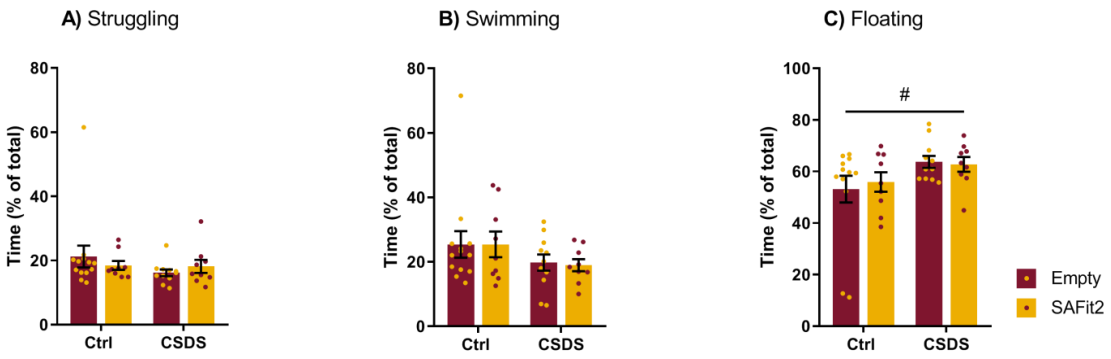


**Figure 87: Forced swim test results (experiment 4)**
Struggling (A) and swimming behavior (B) was not affected by stress or SAFit2 treatment. Stress exposure did increase the time animals spent floating when compared to unstressed controls (C). However, SAFit2 had no effect on floating behavior in the FST. Data are represented as mean ± SEM. # significant main stress effect.

### 3.4.7 Endocrine organs

Since activation of the endocrine system is known to affect the size of related secretion organs, adrenal glands as well as the thymus of the animals were weighted to assess treatment-related changes.

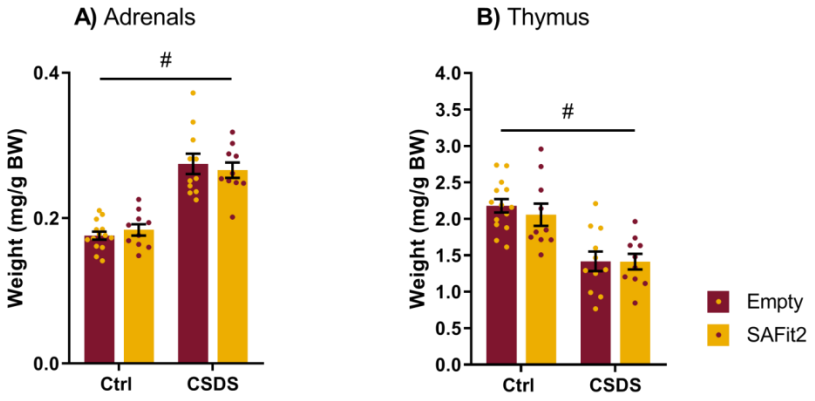


**Figure 88: Organs (experiment 4)**
Stress did induce a very robust effect on the size of endocrine organs. Relative adrenal weights of stressed animals were significantly increased (A) while the thymi were smaller, compared to unstressed controls (B). SAFit2 treatment did not affect organ weight. Data are represented as mean ± SEM. # significant main stress effect.

The relative adrenal and thymus weight was altered in a stress dependent manner (adrenals: $F_{(1,41)}$ = 87.71;p < 0.0001; thymus: $F_{(1,41)}$ = 33.42; p < 0.0001) while SAFit2 did not affect these organ weights (adrenals: $F_{(1,41)}$ = 0.001286; p = 0.9716; thymus: $F_{(1,41)}$ = 0.2763; p = 0.6020). In neither of the organs ANOVA detected an interaction effect (adrenals: $F_{(1,41)}$ = 0.7466; p = 0.3926; thymus: $F_{(1,41)}$ = 0.2352; p = 0.6303) (Figure 88).

## 3.5 Experiment 5

### 3.5.1 Open field test

The OF is classically used to examine locomotor activity and anxiety-like behavior. For the total distance traveled, ANOVA revealed no main effect of SAFit2 treatment ($F_{(1,36)}$ = 3.567, p = 0.067), but a main effect of Escitalopram ($F_{(1,36)}$ = 56.03, p < 0.001) as well as an interaction effect ($F_{(1,36)}$ = 3.196, p = 0.082). Respectively, immobility was significantly decreased in the Escitalopram groups ($F_{(1,36)}$ = 32.77, p < 0.001) but not affected by SAFit2 ($F_{(1,36)}$ = 0.4293, p = 0.5165). There was no interaction effect in immobility ($F_{(1,36)}$ = 0.5909, p = 0.4471). Under vehicle treated conditions, SAFit2 had no effect on overall locomotion. Escitalopram treatment significantly increased locomotor activity in both groups. However, the Escitalopram effect was significantly reduced in SAFit2 treated animals compared to empty VPG-treated controls (p < 0.05). When the data were split up in three 5-minute time bins, it becomes clear that the moderating effect of SAFit2 treatment on the Escitalopram effect is mainly evident in the 2$^{nd}$ (p < 0.05) and 3$^{rd}$ (p < 0.05) time bin for both, distance traveled and immobility respectively (Figure 90).

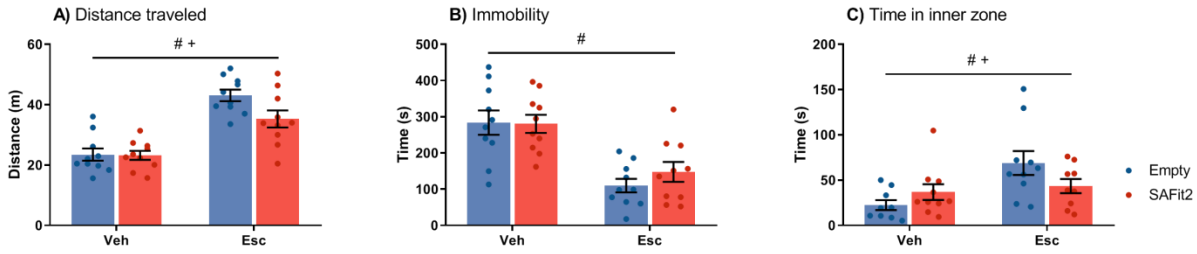


**Figure 89: Open field test results (experiment 5)**
Escitalopram did increase the distance traveled in the OF and co-medication with SAFit2 did result in an interaction effect for this parameter (A). Immobility was also reduced by Escitalopram application (B). There was a significant effect of Escitalopram as, well as an Escitalopram x SAFit2 interaction effect on the time, animals spent in the aversive inner zone of the arena (C). Data are represented as mean ± SEM. # significant main stress effect. # significant Escitalopram main effect. + significant interaction effect.

Regarding the time the animals spent in the inner zone of the OF, ANOVA revealed no main effect of SAFit2 ($F_{(1,34)} = 0.3393$, $p = 0.564$), but a main effect of Escitalopram ($F_{(1,34)} = 7.862$, $p = 0.008$) as well as a significant interaction effect ($F_{(1,34)} = 4.402$, $p = 0.043$). In animals without SAFit2, Escitalopram application resulted in a marked anxiolytic effect, indicated by the increased time spent in the inner zone of the OF ($p = 0.0083$). This effect was absent in animals treated with SAFit2 (Figure 90).

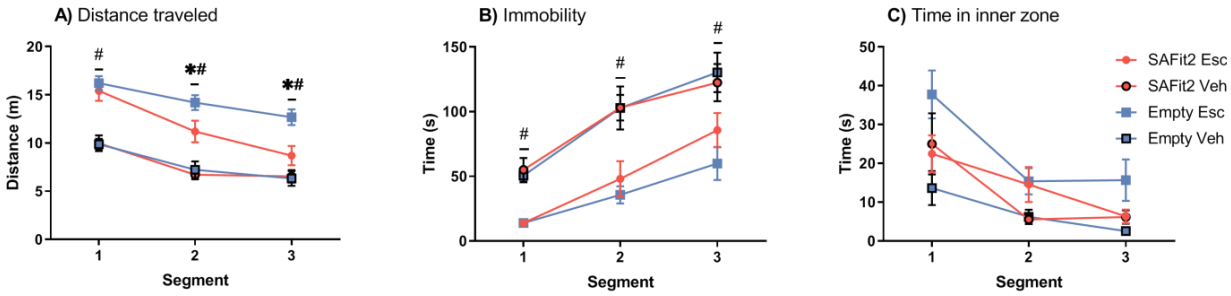


**Figure 90: Open field test results (experiment 5, 5 minute time segments)**
In order to better analyze locomotion in the OF, this graph shows behavior in each of the three 5-minute time segments of the test. Distance traveled was higher in Escitalopram treated animals in general, however, after the first 5 minutes of the test, combination with SAFit2 results in a significantly faster drop in activity (A). Immobility on the other hand was solely affected by Escitalopram with no changes induced by SAFit2 application (B). Even though there was a significant interaction effect on inner zone time in regard of the whole 15 minute test, there were no differences in one of the three segments when tested individually (C). Data are represented as mean ± SEM. # significant Escitalopram main effect. * significant SAFit2 main effect.

### 3.5.2 Elevated plus maze test

To test for anxiety-related and explorative behavior the EPM-test was employed. Here, ANOVA revealed that SAFit2 ($F_{(1,33)} = 7.26$, $p = 0.011$, $p < 0.001$) and Escitalopram ($F_{(1,33)} = 31.41$, $p < 0.001$) treatment resulted in significant main effects in distance traveled. However, there was no interaction between the four treatment groups ($F_{(1,33)} = 0.2775$, $p = 0.602$). In vehicle-treated animals, SAFit2 had no significant effect on locomotion ($p = 0.181$), but in Escitalopram treated mice SAFit2 significantly reduced locomotor activity compared to empty gel treated mice ($p < 0.05$).

SAFit2 did not induce a significant main effect when looking at anxiety-related parameters e.g., the number of entries into the open arm ($F_{(1,33)} = 1.521$, $p = 0.226$) or the time animals spent on the open arms ($F_{(1,33)} = 0.658$, $p = 0.423$). As expected, animals treated with Escitalopram had a significant increase in open arm entries ($F_{(1,33)} = 39.45$, $p < 0.001$) and time spent ($F_{(1,33)} = 12.39$, $p < 0.001$). Again, this anxiolytic effect was moderately dampened when Escitalopram was combined

with SAFit2, as depicted by a trend towards a lower number of entries into the open arms (p = 0.075) (Figure 91).

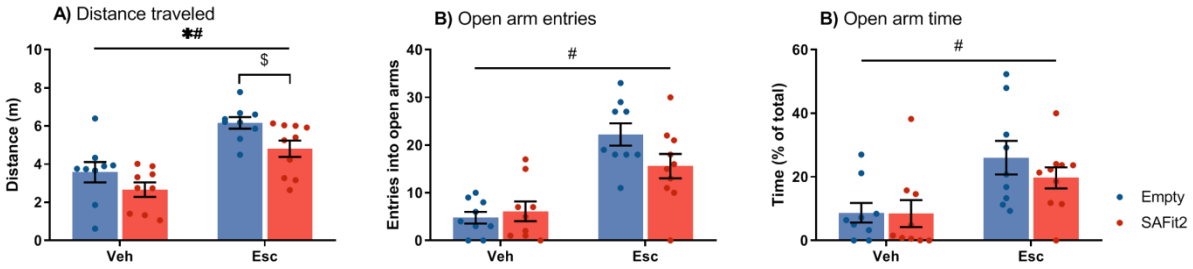


**Figure 91: Elevated plus maze test results (experiment 5)**
SAFit2 as well as Escitalopram treatment resulted in significant main effects when regarding the distance, animals traveled on the EPM (A). In addition, SAFit2 significantly hampered the effect of Escitalopram when applied together. Open arm entries were only affected by Escitalopram with no SAFit2 effect (B). The same was true for the time the mice spent on the open arms of the arena (C). Data are represented as mean ± SEM. $ significant SAFit2 effect. # significant Escitalopram main effect. * significant SAFit2 main effect.

### 3.5.3 Dark-light box test

Neither SAFit2, nor Escitalopram induced significant main effects on explorative behavior in the DaLi.

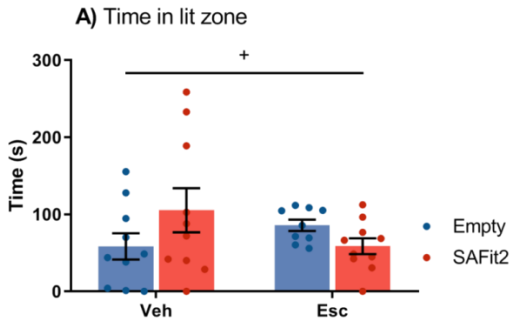


**Figure 92: Dark-light box test results (experiment 5)**
There was a significant interaction effect for the time animals spent in the more aversive lit zone of the arena. Data are represented as mean ± SEM. + significant interaction effect.

Nonetheless, I saw a significant interaction effect, using Two-Way ANOVA ($F_{(1,33)}$ = 4.138, p = 0.049). In line with our former observations, SAFit2 co-medication induced a trend towards a reduced anxiolytic effect of Escitalopram treatment (p = 0.051) (Figure 92).

### 3.5.4 Forced swim test

The FST is a recognized test to evaluate stress coping behavior in rodents, as it confronts them with an unescapable stressful situation. ANOVA revealed significant main effects for SAFit2 ($F_{(1,32)}$ = 7.21, p = 0.011) and Escitalopram ($F_{(1,32)}$ = 19.55, p < 0.001), as well as an interaction effect ($F_{(1,32)}$ = 4.474, p = 0.042) when looking at the time animals spent struggling. SAFit2 alone had no effect on struggling behavior. As expected, Escitalopram treatment significantly increased time spent struggling in both groups. Interestingly, SAFit2 treatment significantly enhanced this effect (Figure 93). Swimming behavior was only affected by Escitalopram ($F_{(1,32)}$ = 17.95, p < 0.001) while SAFit2 had no effect ($F_{(1,32)}$ = 0.3903, p = 0.5366). There was no detectable interaction effect on swimming either ($F_{(1,32)}$ = 0.7596, p = 0.3900). ANOVA revealed significant SAFit2 ($F_{(1,32)}$ = 5.961, p = 0.0203), as well as Escitalopram ($F_{(1,32)}$ = 5.652, p = 0.0236) effects for floating behavior, however there was no statistical interaction between the two factors ($F_{(1,32)}$ = 3.081, p = 0.0888). It has to be noted that the main effect for Escitalopram is most likely driven by the SAFit2 group and might not be a biologically relevant effect.

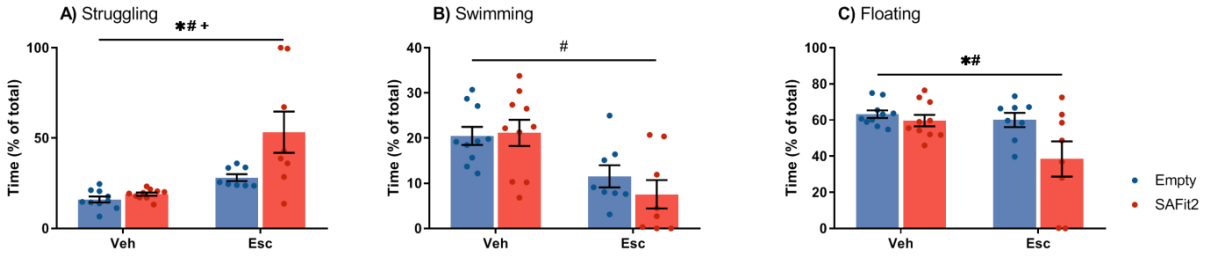


**Figure 93: Forced swim test results (experiment 5)**
Struggling behavior in the FST was increased by both, Escitalopram as well as SAFit2 application. This effect was mainly driven by the drastic increase in struggling when both drugs were injected together (A). The amount of swimming was only affected by Escitalopram with no effects caused by SAFit2 treatment (B). Floating behavior was significantly changed after both, SAFit2 as well as Escitalopram treatment. Notably, the Escitalopram effect could be mainly driven by the SAFit2 group and might not be biologically relevant (C). Data are represented as mean ± SEM. # significant Escitalopram main effect. * significant SAFit2 main effect. + significant interaction effect.

### 3.5.5 Endocrinology

Corticosterone levels were measured in blood plasma probes under baseline conditions, shortly after stress (30 minutes after FST) and a third time after 90 minutes recovery time. Pharmacological treatment did not alter baseline levels of corticosterone. In response to stress, ANOVA revealed a significant interaction effect ($F_{(1,34)} = 3.239$, $p = 0.0808$), as well as a SAFit2 effect on animals that were not treated with Escitalopram ($p < 0.05$). After 90 minutes of recovery, ANOVA revealed a significant main effect of SAFit2 ($F_{(1,35)} = 5.481$, $p = 0.0250$) and a significant reduction by SAFit2 pre-treatment in the Escitalopram-treated animals ($p < 0.05$) while it did not reach *post hoc* significance in vehicle treated animals.

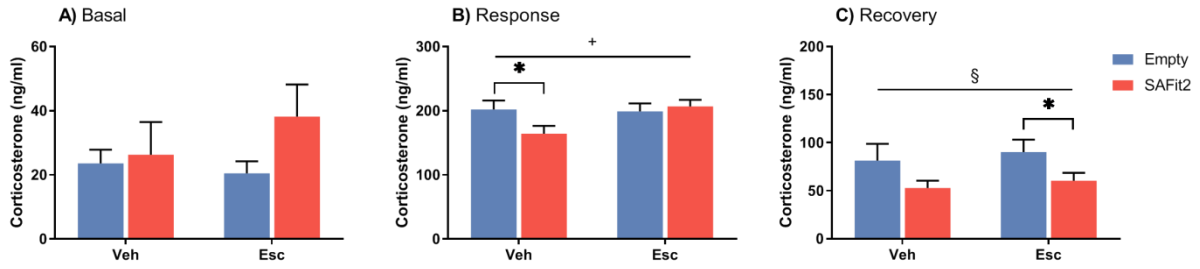


**Figure 94: Effects on hypothalamic-pituitary-adrenal-axis function**
(A) Basal corticosterone secretion is unaffected by SAFit2 or escitalopram treatment. (B) SAFit2 reduces corticosterone secretion in response to an acute stressor in vehicle treated animals, while no effect is observed under Escitalopram treatment. (C) Ninety minutes after the onset of the stressor (recovery), SAFit2 suppression of the HPA axis function is observed in both vehicle and Escitalopram-treated animals, but only reaches post hoc significance in the Escitalopram-treated group. Data are represented as mean ± SEM. * significant SAFit2 effect. § significant SAFit2 main effect. + significant interaction effect.

## 3.6 Experiment 6

### 3.6.1 Subcutaneous injection of SAFit2 solution

A single s.c. injection of SAFit2 solution resulted in a burst of SAFit2 release 30 minutes post application with a maximum concentration of 5374.3 ng/ml. I was able to observe a fast, non-linear drop in drug levels, with an initial half-life of approximately 12 hours. After 96 hours, the last point of measurement, only 404.4 ng/ml of drug was left in the plasma on average. Because the depletion rate becomes rather linear after about 36 hours, it can be assumed that SAFit2 is completely washed out after about 112 hours (Figure 95).

### 3.6.2 SAFit2 application via vesicular phospholipid gels

Two different concentrations of SAFit2 VPGs were tested in this experiment. As a result of the encapsulation in VPG, both, the 30% VPG, as well as the 50% VPG yielded a lower initial plasma concentration than the SAFit2 solution that was applied via s.c. injection (30%: 1601.6ng/ml; 50%: 1171.0ng/ml). The 30% VPG reached its maximum concentration after 6 hours, while the release

was slightly delayed to 24 to 36 hours for the 50% VPG. Although the overall drug level reached with the VPGs is lower compared to the injection as solution, the release rate is very steady and sustained over at least 7 days (Figure 95).

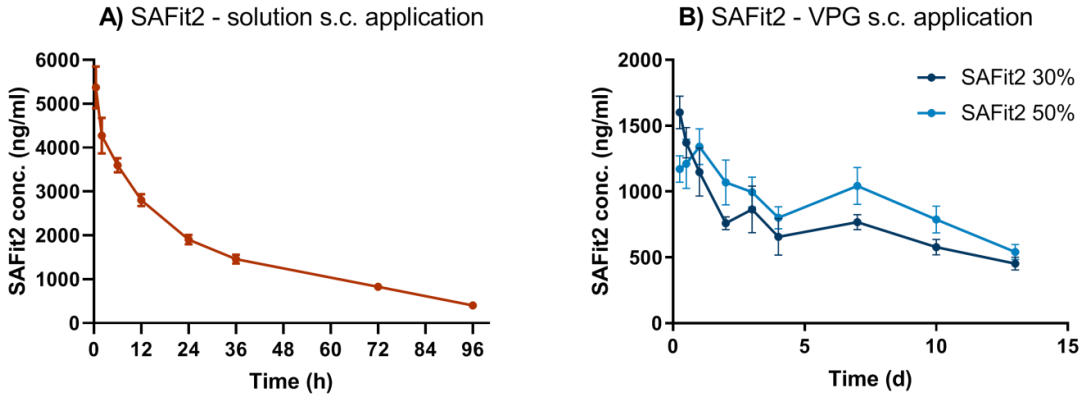


**Figure 95: SAFit2 concentrations in blood plasma (experiment 6)**
Injection with SAFit2 solution did result in a massive spike of the drug in the blood plasma. However, levels dropped relatively quickly over the course of 96 hours (A). Using SAFit2 loaded VPGs on the other hand evoked lower total levels of SAFit2 in the plasma initially, however levels stayed at a comparatively high level for up to 14 days post injection (B).

## 3.7 Experiment 7

Home cage behavior of *Fkbp5*$^{WT}$ and *Fkbp5*$^{KO}$ animals (from here on referred to as cohort 1) was recorded and manually scored under baseline conditions, as well as after 15 minutes of restrain stress. In parallel, a second cohort (cohort 2) of non-modified C57Bl/6n mice was treated with either empty VPG or SAFit2 loaded VPG and tested in the same experimental paradigm.

### 3.7.1 Explorative/active behavior

Walking, rearing and climbing were considered explorative behaviors by which the animal actively assesses its direct environment. In general, all these behaviors were predominantly present under basal conditions and substantially reduced after stress.

Walking

Walking was scored whenever the animal was moving through the cage for the sole purpose of relocation.

Under basal conditions, there was a trend towards an increase in walking behavior in *Fkbp5*$^{KO}$ animals when compared to the *Fkbp5*$^{WT}$ ($F_{(1, 22)} = 3.318$; $p = 0.0822$). There was a significant main effect after the restrain ($F_{(1, 22)} = 33.01$; $p < 0.0001$), however, there was no detectable interaction effect ($F_{(1, 22)} = 1.651$; $p = 0.2122$).

There was no main effect of SAFit2 treatment between the groups ($F_{(1, 42)} = 0.3788$; $p = 0.5416$), however, mixed model analysis revealed a significant stress effect ($F_{(1, 42)} = 15.87$; $p = 0.0003$), as well as a treatment x stress effect ($F_{(1, 42)} = 4.875$; $p = 0.0328$) (Figure 96).

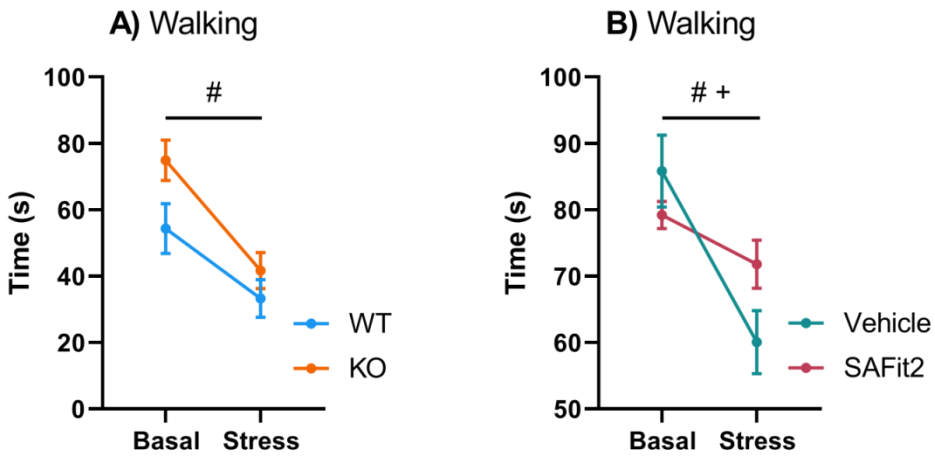


**Figure 96: Home cage behavior – walking (experiment 7)**
When looking at home cage behavior pre and post stress, the time animals from cohort 1 spent walking was significantly reduced by the exposure to a stressor (A). In cohort 2, there was a significant main effect for stress, as well as a significant treatment x stress interaction (B). Data are represented as mean ± SEM. # significant stress main effect. + significant interaction effect.

Rearing

Whenever the animal actively observed its surroundings while standing on its hind legs, the behavior was considered rearing. I also included incidents in which the animal stood up against the walls of the cage, sniffing and observing the area.

Genotype had no effect on the amount of rearing under basal conditions ($F_{(1, 22)} = 0.03310$; $p = 0.8573$). There was a stress induced main effect ($F_{(1, 19)} = 17.17$: $p = 0.0006$), however no genotype x stress effect ($F_{(1, 19)} = 0.9156$; $p = 0.3507$).

In the pharmacology group, I found both, a significant treatment effect ($F_{(1, 22)} = 11.41$; $p = 0.0027$), as well as a stress effect ($F_{(1, 19)} = 52.15$; $p < 0.0001$), however no treatment x stress interaction ($F_{(1, 19)} = 0.2832$; $p = 0.6008$) (Figure 97).

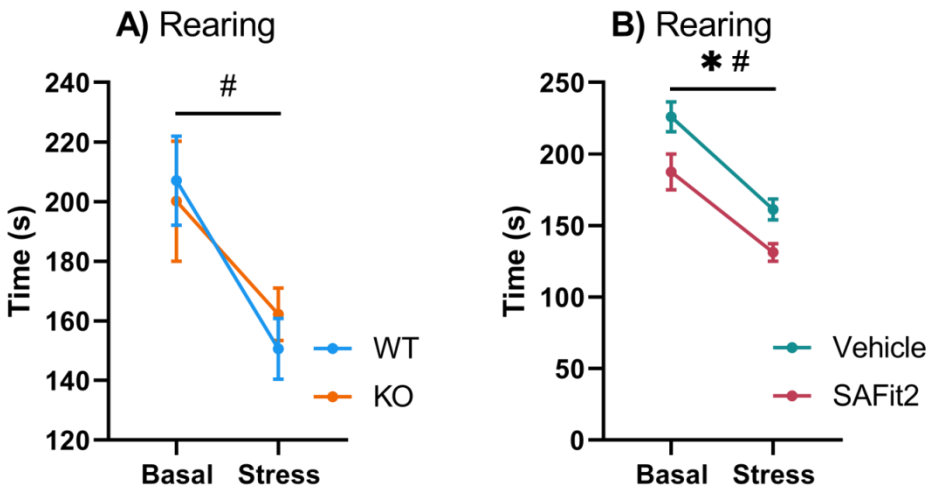


**Figure 97: Home cage behavior – rearing (experiment 7)**
Rearing behavior was significantly reduced by stress in both genotypes of cohort 1 (A). SAFit2 treated animals showed reduced levels of rearing when compared to controls that received a vehicle injection, both pre and post stress. Restraining the mice did reduce rearing behavior in both groups of cohort 2 (B). Data are represented as mean ± SEM. # significant stress main effect. * significant genotype/treatment main effect.

Climbing

Any time, the animal was grapping and moving along the cage lid, this behavior was considered climbing.

In the genetically altered cohort climbing was not affected by genotype ($F_{(1, 22)} = 0.8726$; $p = 0.3604$), however stress did significantly reduce it in both groups ($F_{(1, 22)} = 25.16$; $p < 0.0001$). There was no significant interaction ($F_{(1, 22)} = 0.1113$; $p = 0.7418$).

In the pharmacology group, mixed model analysis resulted in a treatment effect ($F_{(1, 22)} = 9.418$; $p = 0.0056$), as well as a stress effect ($F_{(1, 21)} = 89.26$; $p < 0.0001$). There was no treatment x stress interaction ($F_{(1, 21)} = 0.09704$; $p = 0.7585$) (Figure 98).

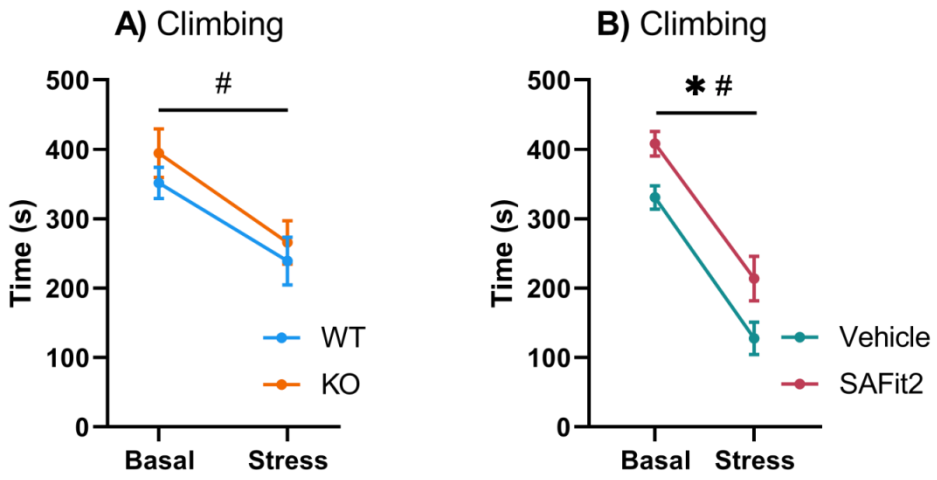


**Figure 98: Home cage behavior – climbing (experiment 7)**
Climbing behavior was significantly reduced by stress in both genotypes of cohort 1 (A). SAFit2 injected animals showed reduced levels of rearing when compared to controls treated with vehicle, both pre and post stress. Restraining the animals did reduce rearing behavior in both groups of cohort 2 (B). Data are represented as mean ± SEM. # significant stress main effect. * significant genotype/treatment main effect.

### 3.7.2 Self-directed/passive behavior

Self-directed and more passive behaviors, namely surveying, grooming, digging and chewing, were rarely observed under basal conditions. After restrain however, these types of behavior were highly present and conducted in very rapid intervals.

Surveying

Surveying behavior was scored when the animal was not moving but actively observing and sniffing the area.

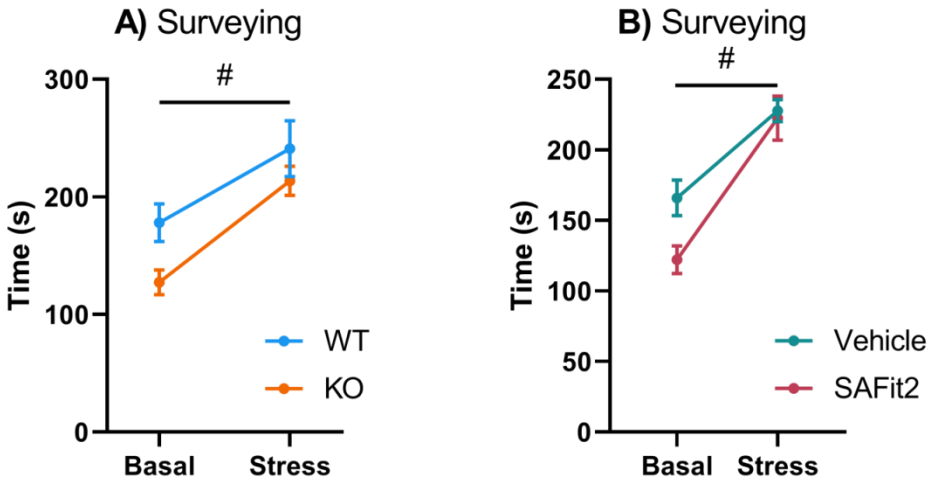


**Figure 99: Home cage behavior – surveying (experiment 7)**
Surveying behavior in the home cage was neither impacted by genotype in cohort 1 (A), nor affected by SAFit2 treatment in cohort 2 (B). However, stress increased the amount of surveying behavior in both cohorts. Data are represented as mean ± SEM. # significant stress main effect.

There was no significant effect of genotype when mixed model analysis was applied ($F_{(1, 21)} = 3.351$; $p = 0.0814$). However, there was a significant stress effect ($F_{(1, 20)} = 28.92$; $p < 0.0001$), yet no genotype x stress interaction ($F_{(1, 20)} = 0.7396$; $p = 0.4$).

SAFit2 treatment did not result in a significant change in surveying behavior ($F_{(1, 22)} = 3.796$; $p = 0.0642$), however, there was a significant increase after stress ($F_{(1, 20)} = 50.70$; $p < 0.0001$). No genotype x stress interaction was observed ($F_{(1, 20)} = 2.797$; $p = 0.11$) (Figure 99).

Grooming

Any self-directed cleaning or licking behavior was considered grooming.

Genotype did not affect grooming behavior ($F_{(1, 42)} = 0.05350$; $p = 0.8182$), although stress induced it overall ($F_{(1, 42)} = 77.27$; $p < 0.0001$). There was no genotype x stress interaction ($F_{(1, 42)} = 0.02321$; $p = 0.8797$).

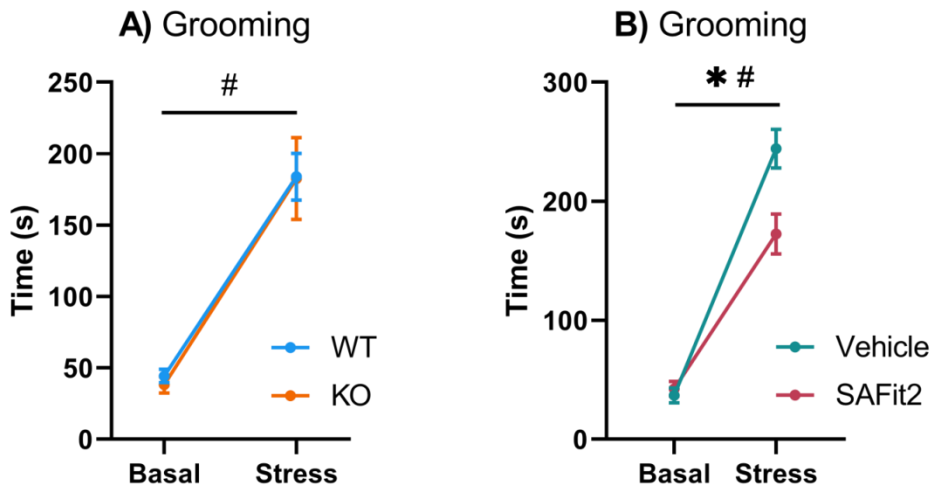


**Figure 100: Home cage behavior – grooming (experiment 7)**
Grooming behavior in the home cage was not impacted by genotype in cohort 1 (A). SAFit2 treatment in cohort 2 reduced the amount of grooming overall, however this effect is mainly driven by a reduction following the 15 minute stress-period (B). Stress significantly increased the amount of grooming behavior in both cohorts. Data are represented as mean ± SEM. # significant stress main effect. * significant genotype/treatment main effect.

Grooming was affected by both, SAFit2 treatment ($F_{(1, 22)} = 6.781$; $p = 0.0162$) as well as stress exposure ($F_{(1, 18)} = 187.3$; $p < 0.0001$). There was also a significant treatment x stress interaction when mixed model analysis was applied ($F_{(1, 18)} = 9.891$; $p = 0.0056$) (Figure 100).

Digging

Digging was scarcely observed across all groups. There was no effect of genotype ($F_{(1, 21)} = 1.535$; $p = 0.229$), however stress did alter digging behavior in the genetically altered cohort ($F_{(1, 19)} = 9.871$; $p = 0.0054$). There was no genotype x stress interaction ($F_{(1, 19)} = 0.6103$; $p = 0.4443$).

The pharmacology group did not show any treatment ($F_{(1, 22)} = 0.04298$; $p = 0.8377$) or stress induced changes ($F_{(1, 20)} = 1.869$; $p = 0.1867$), neither did I detect a treatment x stress interaction ($F_{(1, 20)} = 1.913$; $p = 0.1819$) (Figure 101).

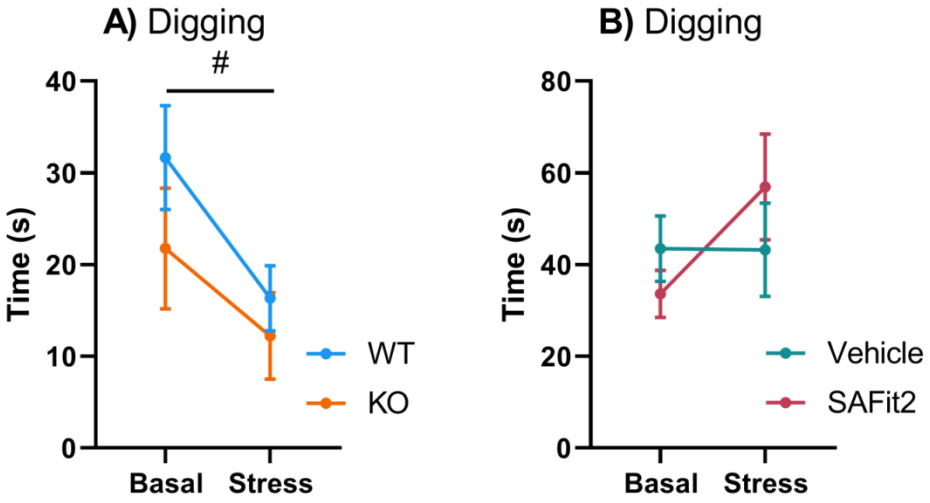


**Figure 101: Home cage behavior – digging (experiment 7)**
Stress did reduce digging behavior in cohort 1 (A). There was no effect of genotype. Digging was neither affected by stress, nor by treatment with SAFit2 in cohort 2 (B). Data are represented as mean ± SEM. # significant stress main effect.

Chewing

Since all food was removed from the cage, chewing was almost absent in all groups and therefore, no meaningful effects were observed.

# 4 Discussion

In my thesis, I conducted several studies that aimed to better understand the role of the psychiatric risk-gene *Fkbp5* in stress vulnerability. Therefore, I utilized different mouse models in order to study the effects of brain region-specific *Fkbp5* modulation on behavior and physiology.

## 4.1 *Fkbp5* mRNA expression after acute or chronic stress

The goal of the first set of experiments was to detect differences in *Fkbp5* expression following acute and chronic social stress. Furthermore, I used the results to identify the LC and the DR as areas of high *Fkbp5* regulation, which is of interest for a number of follow-up research projects.

To study the role of a gene or protein, one of the very first steps is to localize its main site of action. In the case of *Fkbp5*, the range is very broad due to the many tissues in the body that all express the gene to some extent. Even though *Fkbp5* is mostly known for its prevalent role in the development of mental diseases, one has to keep in mind that it is a widely expressed co-chaperone that fulfills a plethora of vastly different cell-type specific functions, not only in the brain (Balsevich et al., 2017). Here, however, I want to exclusively focus on the role of *Fkbp5* in the brain. Expression patterns in the mouse brain have been studied before, both under baseline conditions as well as after different types of (acute) stress like restraint or food deprivation. In both cases, mRNA was up-regulated in response to stress hormone release (Scharf et al., 2011).

In order to extend the available data-set, I introduced two additional stressors in the form of an acute and a chronic social defeat by a physically superior specimen. Because *Fkbp5* is expressed in a wide variety of neurons all over the brain, both studies focused on regions that are classically considered stress relevant. The most notable effects were found in the hippocampus, the BLA and several nuclei that belong to the monoaminergic system. Interestingly, many of these regions showed elevated *Fkbp5* baseline expression in contrast to most of the 'non-stress' areas. However,

when Scharf and colleagues compared the expression shift of *Fkbp5* between their stressors (restrained stress and food deprivation), it became apparent that the more severe stressor (food deprivation) resulted in an even higher increase compared to the milder stressor (restraint). This observation is in line with the current model of the *Fkbp5* mode of action. Higher amounts of glucocorticoids entering the brain after a more stressful event can bind more receptors, shift the equilibrium towards FKBP52 and in turn, activate the transcription of target genes, including *Fkbp5*. A similar pattern was present in the recent study after the animals were exposed to an aggressive resident and defeated for 5 minutes. Again, *Fkbp5* transcription was activated in stress-relevant brain areas (hippocampus, BLA, monoaminergic nuclei) four hours after stress exposure. Unfortunately, a quantitative inter-study comparison of our results with the previously published Scharf study is difficult due to the variability in radioactive signal between the individual ISH films.

The fast up-regulation of *Fkbp5* is in line with the commonly accepted concept of an ultra-short feedback loop that self-regulates the termination of the stress response (Klengel and Binder, 2015). In general, there are a number of plausible options when talking about the effects of acute or chronic stress on the circadian steroid hormone rhythm and *Fkbp5* regulation. In the case of acute stress, the process is well understood: hormones fluctuate over the course of the 24h day-night-cycle. When disrupted by stress exposure, HPA-axis activation causes a momentary spike in corticosterone levels (Figure 102), which, in turn, results in up-regulation of *Fkbp5* mRNA expression. As mentioned before, this effect is extremely stressor-dependent and together with measures of the endocrine reaction can be helpful when rating the severity of a specific stressor. As an example, Scharf and colleagues (2011) reported the elevation of corticosterone levels in animals after food deprivation but failed to observe an effect four hours after a restraint. While this is likely to be an issue of timing due to the fact that corticosterone gets removed from the system relatively quickly, the respective genetic effects were visible for both of their stress groups several hours after stress exposure.

Interestingly, I discovered that the LC and the DR are amongst the regions with the highest up-regulation of *Fkbp5* mRNA percentage-wise after acute stress. In contrast, none of the areas that showed regulation after acute stress were changed in the chronic stress group when compared to controls. In that regard, it is not unreasonable to assume that the 24-hour time difference between the last stress episode and the harvest of the brain was too long to still detect mRNA up-regulation in the chronic stress group. Unlike in former studies by Hartmann and colleagues (Hartmann et al., 2012), baseline corticosterone levels in the chronically stressed animals did not differ from controls. However, baseline measurements of corticosterone levels can be error-prone due to the time of sampling, which is usually carried-out at the beginning of the light-phase, when corticosterone blood levels are lowest. During the trough of the circadian rhythm, any measurement can be easily disturbed by the slightest exposure to stress. Interestingly, in my own study the adrenal glands were still enlarged following chronic stress, an effect that is widely used as a marker for chronically enhanced HPA-axis activity. Nevertheless, the inconsistency of endocrine results and *Fkbp5* regulation opens up the question whether enlarged adrenals secrete higher baseline levels of stress hormone or if they rather amplify the spiking-capacity, (or output) in case of a stressful event. As it stands now, the results of this study favor the latter scenario in which the potential for adrenal activity is enhanced, while baseline output remains unchanged (Figure 102). In this case, there is no 1:1 relationship between the regulation of *Fkbp5* expression and corticosterone levels in the blood plasma. Alterations in *Fkbp5* expression regulation might be due to epigenetic changes at the *Fkbp5* locus and could explain the discrepancy observed in this experiment. In the CSDS cohort however, the feedback mechanism itself seems to be intact since expression levels were back at baseline 24 hours after the last stress exposure. To ultimately answer this question, measures of a time point closer to the last encounter of a stressor (e.g. four hours after the last defeat) in a chronically stressed cohort would be required. This could not only help to determine whether chronic defeat alters the stress-induced hormonal response, but also answer the question to which extent the magnitude of *Fkbp5* expression relates to the excessive presence of corticosterone in the brain.

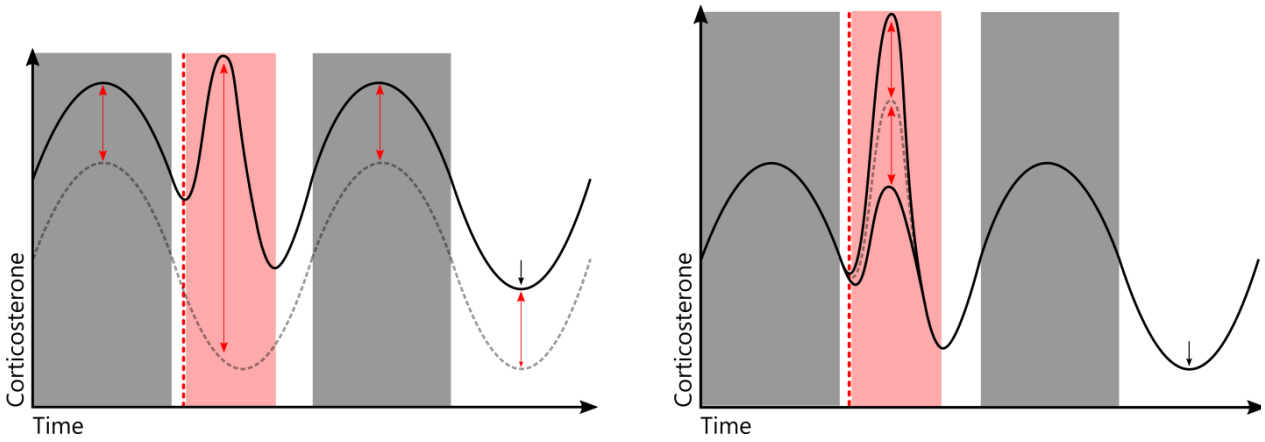


**Figure 102: Hypothetical impact of chronic stress on corticosterone release**
This figure depicts two hypothetical ways chronic stress could impact the circadian rhythm of corticosterone release. One possibility is a general elevation (red arrows) of baseline glucocorticoid levels triggered by chronic activation of the HPA-axis (left). In that case an acute stressor (shaded in red) would cause a spike of corticosterone release on top of already increased baseline levels. The second option (right) is characterized by normal corticosterone levels over the course of 24 hours, however sensitivity of the hormonal reaction is increased in case of a stressful event (red arrows). Black arrows indicate the time of sacrifice in experiment 1.

Unfortunately, the current study designs do not allow conclusions regarding the underlying kinetics of hormonal secretion or mRNA regulation. How long does up-regulation of *Fkbp5* take in response to the onset of a stressor? How long is the increased expression maintained while animals recover from stress? These are questions that would require larger cohorts in order to increase the number of sampling points.

Another issue that limits research on *Fkbp5* at the moment is the lack of any specific antibody, at least for immunohistochemistry. As a result, most conclusions have to be drawn based on mRNA expression studies rather than observations on a protein level. Even though gene expression and protein expression overlap to some degree, the current understanding of the co-chaperone (FKBP51) – receptor (GR) – ligand (corticosterone) interactions within a cell is only rudimental.

In order to improve on previous studies in the future, it might also be worth looking at the receptors that bind corticosterone as well as affiliated proteins like CRH and ACTH. Comprehensive studies that specifically test the balance of these players and how it might change in response to manipulations or challenges are very much needed. A possible scenario would be the down-regulation of GR to buffer the effects of excessive corticosterone exposure. A lot more attention needs to be paid to the interplay of different proteins in order to understand their exact role and develop effective ways to manipulate them when the system is imbalanced or dysfunctional.

In summary, ASDS produces the same effects on *Fkbp5* expression as other (acute) stressors. The most notable effects could be observed in two of the monoaminergic nuclei, the LC and the DR. This finding was the foundation for several subsequent experiments with a more specific focus on these regions. In contrast, chronic stress did not result in a baseline regulation of *Fkbp5* expression. Although the increase in mRNA expression is heavily stress-dependent, it does not seem to be a long-lasting effect. Overall, the observations in this study are confirming the hypothesis that *Fkbp5* expression is (directly) correlated to corticosterone levels. However, further investigation is needed in order to draw any comprehensive conclusions about the effects of chronic stress on *Fkbp5* expression.

## 4.2 Manipulation of *Fkbp5* in the monoaminergic system

Based on the strong up-regulation of *Fkbp5* in the LC and the DR in response to stress exposure, I aimed to explore its role in both regions more specifically. Both nuclei are major hubs for monoamine synthesis, activated upon stress exposure and recognized for their relevance in controlling behavioral adaptations to stress. Aspects like alertness, arousal, attention and memory formation are modulated by serotonin and NE neurocircuitry and alterations in these systems are known to be involved in diseases like anxiety disorders, PTSD, ADHD, depression and Alzheimer's (Moret and Briley, 2011; Benarroch, 2009; Bracha et al., 2005; Ressler and Nemeroff, 2001). However, even though we know that disturbances in serotonin and NE neurotransmission in the

central nervous system are contributing to the development of these diseases, the cause for the disruption is unclear. Stress is known to cause an increase of neural activity in the infralimbic cortex (IL), the lateral habenula and the amygdala, as well as a down-regulation in other parts of the brain, namely prelimbic cortex (PL), the hippocampus and the monoamine systems. Brain regions involved in regulating the stress response are strongly interconnected and the dynamic change of activity in all of them is interdependent. The IL and the PL, for example, are both modulating the monoaminergic systems via synaptic inputs and therefore act as upstream regulators. They either send hypoactive projections directly to the DR (PL), or hyperactive connections (IL) onto GABAergic neurons in the DR that suppress serotonergic neurons (Figure 103 ) (Lee and Han, 2019).

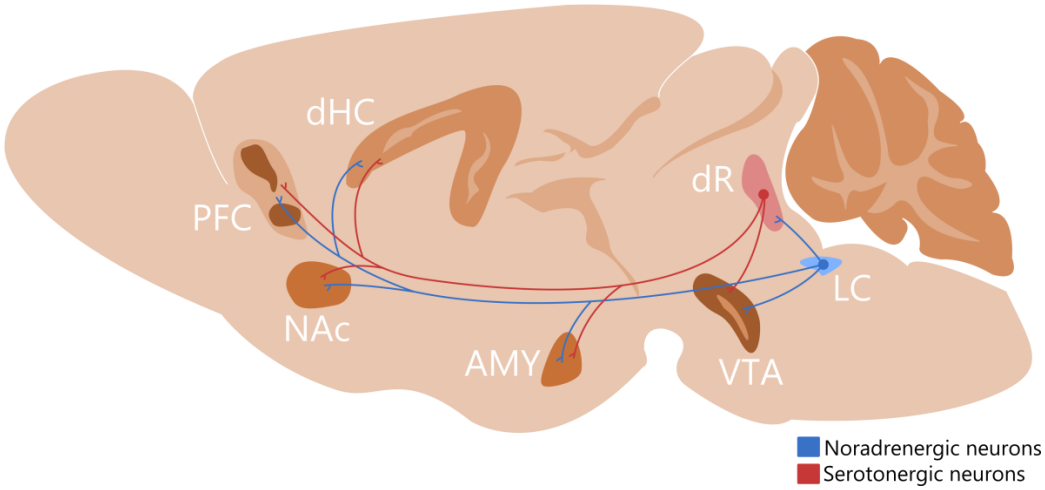


**Figure 103: Connectivity of the monoaminergic system**
Both monoaminergic nuclei, the LC and the DR, are interconnected with a number of different regions all over the brain. This figure depicts a selection of areas that are innervated by either noradrenergic neurons originating from the LC (red) or serotonergic neurons controlled by the DR (blue). Adapted from (Lee and Han, 2019)

For that reason, dysregulation of regulatory proteins in one segment can change the balance of the whole system if not controlled adequately. I therefore hypothesized that the upregulation of *Fkbp5* following acute stress exposure in the DR and LC might shape the function and activity of these brain regions and thereby contribute to the (mal)adaptive consequences of stress exposure. To

model a scenario of malfunctioning feedback control, I utilized viral *Fkbp5* OE and conditional, *Cre*-dependent genetic KOs. Viral vectors are a potent method to induce momentous up-regulation and protein abundance without any detrimental effects on development. However, a malfunctioning system could not only be a result of 'too much' but also 'not enough'. Therefore, it is crucial to look at both sides of the coin and an opposite approach of tuning down gene transcription is just as important to provide a comprehensive picture of the model. Eliminating a gene is one of the most powerful tools in the process of risk-gene assessment, especially when combined with reciprocal OE studies. As a first step, animals were characterized by a physiological and behavioral level without the addition of a stressor.

OE in either of the two regions of interest did not result in a change in bodyweight, as did a KO in the LC. Animals of the *Fkbp5*$^{\text{DR KO}}$ group, however, were significantly leaner than controls. This very robust phenotype could be induced by one of two options: (1) Activity of neuronal interconnections between the DR and the PVN, an area that directly controls aspects like appetite, is possibly disrupted by missing FKBP51-driven regulation. (2) KO in the *Fkbp5*$^{\text{DR KO}}$ line is the result of *Pet*-promoter driven *Cre*-expression. The promoter is predominantly active in serotonergic neurons of the DR. However, earlier studies have reported active *Cre*-recombinase in beta-cells of the pancreas (Deneris, 2011). Therefore, an additional KO of *Fkbp5* in these cells and a potential impact on insulin secretion cannot be excluded. Further investigation of *Fkbp5* expression in the pancreas and the insulin balance in these animals is needed.

Regarding the behavioral phenotype, neither the abundance of *Fkbp5* nor its' absence in these areas wide-spread and robust effects on behavior under baseline conditions. Even though specific behavioral phenotypes were significantly affected in OE and KO animals when compared to controls, no clear overall phenotype emerged.

Over-expressing animals did show a reduction in their initial exploration of the OF arena, however, this effect could not be replicated in the second group of animals. Since all behavioral tests are relatively sensitive to parameters like lighting, noise, smells, air pressure and so on, some effects,

especially when not reproducible, have to be considered a potential artifact. However, animals from the same group also showed less interaction with non-social and social cues. This could be another hint towards a mild alteration in attention/arousal levels of these animals but further investigation is needed to confirm this. *Fkbp5* reduction does not alter locomotor activity, which is an important precondition for many behavioral paradigms. In our setup, the OF test was not only used to control for mobility but also as a way to habituate the mice to the test setup. Since behavioral adaptations like arousal, alertness and memory formation are partially controlled by monoaminergic circuits, animals were exposed to the OF arena on 3 consecutive days and their exploration of the environment was monitored. Interestingly, while mice lacking *Fkbp5* in the LC did not show any abnormal behavior when compared to $Fkbp5^{LC\,WT}$ controls, the same manipulation in the DR results in disrupted habituation during repeated OF testing. Usually, animals explore their environment less, once it is familiar. However, during the second and third exposure to the arena, animals of the $Fkbp5^{DR\,KO}$ group maintained the same activity levels as on the first day. Diminished habituation could be caused by different processes in the brain. For one, the deletion of *Fkbp5* in the DR might lead to increased arousal which causes the animals to investigate their environment, even though it is familiar. The observed effect could also originate from disrupted memory formation, induced by the KO. In this scenario, animals would be unable to remember important spatial cues and the arena would appear to be a novel setting during each of the three trials. Interestingly, deficits in spatial memory could not be verified in the OLT, a test that is specifically designed to investigate an animals' ability to remember cues in a clearly defined environment. Independent of overall activity, KO animals were clearly able to distinguish between the locations of two objects in the arena. Since this task is considered rather demanding for mice, it can be assumed that the increased exploration during the $2^{nd}$ and $3^{rd}$ OF was not a result of disrupted spatial orientation and memory formation per se. Furthermore, LC animals behaved similar to controls in the OF, however, when specifically tested for spatial memory their ability to distinguish between different object locations is impaired.

When creating an animal model for a stress-related-disease, aspects like anxiety-like behavior, sociability and stress coping are amongst the most interesting parameters to look at, as many of these behaviors are typically affected in mood disorder patients. However, since there was no significant difference in most of the classical anxiety-related tests between neither of the groups, it is rather safe to conclude that anxiety-like behavior is not affected by *Fkbp5* manipulation in monoaminergic cells, as long as there is no additional challenge to the system. Only a KO in the LC did result in a more anxious phenotype in the DaLi, demonstrated by the decreased exploration of the lit compartment. Since this effect was not present in other anxiety-related tests, it seems to be very specific to a high-light, high-exposure situation and yet another clue that Fkpb5 driven effects in the monoaminergic system are highly situational and brain area dependent. As mentioned before, changes in neural activity within the monoaminergic nuclei can have a massive impact on the number of monoamines in the system, which seems to alter very specific aspects of animal behavior. Stress coping was unchanged across all groups when the animals were exposed to an unescapable stressful situation in the FST.

Interestingly, *Fkbp5* OE or KO had no effect on corticosterone levels under baseline conditions and in response to stress. The only aspect affected by the over-expression was the recovery rate in the DR group, which was increased 90 minutes after stress. Hartmann and colleagues discovered that conventional *Fkbp5*$^{KO}$ animals - just like the DR *Fkbp5*-OE animals - do not show a difference during the peak of their endocrine reaction but rather a faster return to baseline levels when compared to controls (Hartmann et al., 2012). In the case of the *Fkbp5*$^{KO}$, the widely accepted model of *Fkbp5* functionality makes sense. The magnitude of the stress response is independent of *Fkbp5*, while the lack of FKBP51 results in an increased sensitivity of the GR, which, in turn, results in higher transcriptional activity and an earlier shutdown of the stress response. In the case of the DR *Fkbp5*-OE cohort however, this effect is harder to explain. It rather shows that *Fkbp5* manipulation does not impact HPA-axis activity directly. Inhibited feedback in serotonergic neurons could possibly affect innervations to other areas like the PVN (Goel et al., 2011) and therefore result in

altered steroid release. In this case, one would also expect the conditional *Fkbp5*$^{DR\ KO}$ to impact corticosterone recovery. However, these animals did react just like their WT counterparts. Another option could be, that the FST as a stressor evokes a strong enough endocrine reaction to over-saturate the cells with steroids. Therefore, the equilibrium of FKBP51 and FKBP52 might not be as relevant under certain conditions.

Another point is the difficult comparability of a genetic KO and a viral OE and the fact that there is no knowledge about the cell-specific function of FKBP51. While the conventional *Fkbp5*$^{KO}$ described by Hartmann and colleagues affects all cells in the body, AAV OE only involves a small subset of cells in the infected area. Again, more research is needed to disentangle the exact impact of global and local *Fkbp5* manipulations on the kinetics of the stress response. Usage of a *Cre*-dependent virus could be beneficial to improve specificity in future studies.

One major drawback of the OE study was the necessity of overall adjustments to the behavioral protocols. After running the first cohort of LC *Fkbp5*-OE animals, habituation times to some of the more delicate (cognitive) test setups were increased in order to minimize anxiety-related effects in these paradigms. Unfortunately, these changes impede comparability between the cohorts.

Up until now, the exact role of *Fkbp5* in the LC and the DR is not as clear as it is in regions like the PVN, where we know that the gene alters HPA-axis activity. Taken together, the results of this study demonstrate that manipulation of *Fkbp5* in the LC and the DR does not impact the overall baseline behavior of mice. Nevertheless, the fact that very distinct parameters are indeed altered shows how complex and explicit any manipulation of a stress regulated gene with as many brain region specific functions as *Fkbp5* can be. Because *Fkbp5* is thought to mainly be regulated by the feedback system of the HPA-axis, the lack of a clear phenotype might as well be due to the fact that baseline expression levels in the WTs are very low. Therefore, protein levels in the controls might be close to the KO condition. Since FKBP51 controls the expression of GR-responsive genes, a number of different pathways could also be affected. A recent study by Gassen and colleagues for example (Gassen et al., 2015) demonstrated a prominent role of *Fkbp5* in autophagy, another

system that could impact neuronal activity in monoaminergic areas. While this is just speculation, a paradigm that includes up-regulation after stress exposure might help to reveal potential alterations in neuronal activity in these animals. Therefore, the findings of this study provide a good baseline and an ideal foundation for future experiments. If *Fkbp5* plays a role in monoaminergic neurotransmission, challenging the system is likely to evoke functional consequences for neurons and detectable behavioral effects when combined with the manipulations introduced here.

## 4.3 Inhibition of FKBP51 - effects on chronically stressed animals

Pharmacological modulation of FKBP51 is a promising alternative to the available treatment methods for psychiatric diseases. This study was designed to assess the potential effects of persistent SAFit2 treatment on chronically stressed mice. First studies with SAFit2 have shown effects on neurite outgrowth in cell culture (Gaali et al., 2015) or behavioral alterations after an acute dosage of the drug (Hartmann et al., 2015). Here I tried to use a slow releasing application method for the first time in order to simulate a situation closer to a human medication scenario.

Overall, chronic dosage of SAFit2 produced up to 30-times lower plasma concentration compared to an acute injection. In this case, the drug was not able to protect animals from CSDS on a behavioral level. Neither locomotor nor anxiety-like behavior was altered by the drug and none of the stress-induced effects could be rescued. The only effect that was observed was the absence of social aversion in SAFit2 treated, stressed animals. Social avoidance is one of the key parameters observed in chronically defeated animals. However, this effect was mild and needs to be replicated in order to confirm the finding.

An acute injection of SAFit2 solution was potent enough to alter anxiety-like behavior while the up to 30-times lower plasma concentration following a 3-week application of the pellet specifically affected social avoidance. There are a number of scenarios that could account for the observed differences in drug effectiveness: 1) the drug content of the slow-releasing pellets was below the

therapeutic dose. 2) SAFit2 did not reach the brain regions involved in e.g. anxiety-like behavior and this is why I was not able to detect significant treatment effects. 3), the applied dose was sufficient but CSDS-effects are not FKBP51 dependent. 4) FKBP51 plays varying roles in different brain regions (e.g. Amygdala vs. bed nucleus of the stria terminalis in case of anxiety) and global pharmacological blockage cancels potential effects. Following my findings, it is safe to conclude that any effects of the drug are at least dose-dependent to a high degree. Finding the right dosage for the right situation will be absolutely crucial for all future tests to come. The upside of this observation is the fact that it might be possible to address different aspects of behavior by choosing the appropriate dose. Another important factor that is not sufficiently documented is the optimal treatment duration. Up until now, there are no studies directly comparing the effects of acute and chronic SAFit2 exposure.

Unfortunately, the application method in this study was not adjustable enough to draw comprehensive conclusions about dosage related questions. The supplier of the pellet was also not willing to share in-depth details about the composition or properties of the product. The drug was delivered via depot-pellets placed subcutaneously into the neck area of the animals. Due to the size of the pellet, mice had to be anesthetized before the incision, adding a source of the disturbance. Drug effects could also be altered by mild inflammatory reactions following surgery. For that reason, repeating the experiment with SAFit2 loaded VPG could improve the accuracy of the results as animals are less disturbed by the injection and release of the drug is relatively stable.

A lot of work is still required to fully understand the mode of action of SAFit2. So far, one hypothesis is that SAFit2 specifically binds FKBP51 and changes its confirmation. This switch in molecular shape could prevent the protein from binding the HSP90 complex and therefore 'inhibit' some of its functionality. However, it is unclear whether this would result in decoupling of already formed complexes or rather affects unbound FKBP51. In that case, efficacy of the drug would be much different after stress exposure, when *Fkbp5* transcription is increased.

In summary, low levels of SAFit2 did not show protective effects in chronically stressed animals when looking at anxiety-related tests alone. However, it specifically reverted the natural avoidance behavior typically observed after exposure to CSDS.

## 4.4 Co-medication of SAFit2 and Escitalopram

The following study has been aiming to further our knowledge about the interaction between the newly developed *Fkbp5* modulator SAFit2 and commonly used SSRIs in a mouse model and the results of the study have been published recently (Pöhlmann et al., 2018). The medication of psychiatric diseases heavily depends on substances that modulate the availability of monoamines for neuronal transmission. Since scientists and doctors alike are still struggling to predict their efficacy for individual patients, co-medication is a common way to treat many diseases like depression or PTSD (Kukreja et al., 2013). For that reason, the development of drugs that target different molecular mechanisms in the brain is extremely important. SAFit2 is a good example of a potential addition to the arsenal of treatments in the future. While drugs like SSRIs directly affect the availability of neurotransmitters in the synaptic cleft, SAFit2 modulates (amongst other things) the termination of the stress response, an often dysfunctional process in psychiatric patients. Several studies have also revealed the regulation of protein-protein interactions by FKBP51, some of which are likely to be affected by SAFit2 treatment (Balsevich et al., 2017; Gassen et al., 2015). Even though modulation of *Fkbp5* has shown promising results in the past, further research is required to improve its effectiveness and scope of application. In this study, I combined chronic SAFit2-treatment with acute Escitalopram injections and performed a basic behavioral characterization.

As expected, Escitalopram resulted in an increase in overall mobility, risk-seeking behavior as well as stress coping. Again, SAFit2 was used in a relatively low dose that did not trigger a behavioral response on its own. However, in the combined treatment, SAFit2 did impact the efficacy of Escitalopram. The SSRI typically acts anxiolytic when applied before an anxiety-related behavioral

test. This effect was dampened but not absent in animals that received both drugs. “While an increase in serotonin levels via SSRI action reduces anxiety and fear, simultaneous disruption of FKBP51 functionality seems to counteract this effect. This finding is in line with the clinical observation that *Fkbp5* risk allele carriers with high *Fkbp5* levels show an improved response to SSRI treatment (Ellsworth et al., 2013). In addition, *Fkbp5* KO mice were previously shown to respond less to SSRI treatment (Gassen et al., 2014)“ (Pöhlmann et al., 2018). Stress coping is another behavioral adaptation that is enhanced after SSRI application (Can et al., 2011). Contrasting the influence on anxiety-like behavior, co-medication with SAFit2 had an additive effect on stress coping in the FST. On an endocrine level, SAFit2 did alter the response to acute stress and the rate of recovery afterwards, hinting towards an effect on the overall reactivity of the HPA-axis.

The results of this study suggest a functional interaction between Escitalopram and SAFit2, thereby once again emphasizing the relevance of *Fkbp5* as a target for the treatment of mental disorders. The study also shows that combination of serotonin reuptake inhibition and blockade of FKBP51 could be beneficial for the treatment of symptoms that relate to stress coping, while a positive, antidepressant-driven, effect on anxiety-related parameters can be hampered by FKBP51 inhibition. A possible option would be that separate pathways or systems in the brain mediate the distinct challenges set by the different behavioral tests and that these systems are differentially affected by the combination of SSRI and SAFit2. A number of studies have shown that SSRIs like Escitalopram impact a multitude of cellular pathways, not necessarily only dependent on their function as SSRI (Einoch et al., 2017; Eskelund et al., 2017). On the other hand, FKBP51 is known to have functions that, by far, exceed its role as HSP90/GR co-chaperone (Hamilton et al., 2018; Balsevich et al., 2017; Fries et al., 2017). It seems rather likely that some but not necessarily all of these functions are also modified by SAFit2. This, however, could also mean that FKBP51-modulating drugs might be able to target different aspects of the protein’s function, which could enhance the applicability of co-medication with SSRIs.

One of the major limitations of the study is the lack of a group that received long-term medication with SSRIs. Here, all the consequences of SSRI treatment were tested following an acute injection, while some SSRI-mediated effects on anxiety-related behavior are known to occur predominantly following chronic application (Burghardt and Bauer, 2013). In future experiments, it will be important to try a long-term application of the drugs, as well as to add more antidepressants in order to gather in-depth information about beneficial (or non-beneficial) combinations. Due to the positive effects on active stress coping, drugs that target the DA or NE system could prove to be promising candidates for future experiments (Bardal et al., 2011).

In summary, the results of this study highlight the potential promise, but also limitations of FKBP51 modulators in co-medication in the medication of mental disorders. The fact that beneficial effects were limited to stress coping shows that a combination of these drugs is highly specific and pre-clinical trials are absolutely crucial in order to determine potential benefits of such an approach for patients. In conclusion, this study provides an important characterization of the therapeutic capabilities of SAFit2 and its potential significance for pharmacological treatments in the future.

## 4.5 SAFit2 application via vesicular phospholipid gels

So far, there are no studies that directly compared either doses or routes of administration of SAFit2. Therefore, any data about pharmacokinetics and blood plasma content of the drug is extremely valuable. The goal of this study was to examine the SAFit2 release rate from VPGs with varying drug concentrations in comparison to a single, short-term release injection of a liquid SAFit2-solution. This approach could help to develop a better understanding of the substance properties and answer a couple of important questions for future usage. First of all, how long do drug levels in blood plasma stay consistently above a level that we know causes behavioral effects? How can we maximize the duration of stable SAFit2 release by using different VPG concentrations?

And last, is the acute application of SAFit2 in a liquid form feasible for specific paradigms and questions?

As expected, initial SAFit2 levels in the blood were highest after application of the SAFit2 solution, however, drug levels did drop a lot quicker compared to both VPGs used in this approach. The concentration of SAFit2 in the gel did marginally affect the release rate. While the 30% gel showed a higher initial release than the 50% gel, its drop-off was also faster and overall plasma levels were slightly lower over time. However, both VPGs retained stable SAFit2 concentrations of about 500ng/ml for at least 14 days.

Animals also did not seem to be hindered by the gel, since it usually disperses uniformly underneath the skin. However, the usage of VPGs also comes with its own minor drawbacks. Residuals of the gel can still be found underneath the skin of the animals after 14 days. So far, it is unclear if the phospholipid component of the VPGs can cause any unwanted long-term effects. However, reiterated injection of the gel has been shown to disturb glucose and insulin regulation, rendering this application method suboptimal for metabolic studies (Häusl & Balsevich, personal communication). More testing is needed to disentangle the exact background and behavioral impact of metabolic changes induced by VPGs. In general, more experiments are required to test the consequences of VPG re-application. So far, it is unknown whether SAFit2 levels can stack or if the effect becomes prolonged by a second injection. Due to the chemical properties of SAFit2, it is not possible to increase the drug concentration in the gels beyond 50%. Even though the gel provides a stable release that way, overall drug levels are lower than in any study that used acute application so far (Hartmann et al., 2012). In the future, further development could hopefully lead to a higher drug load, in order to provide better flexibility for the experimenter and the design of the studies.

Overall, VPGs are a very good solution for long-term application of a drug like SAFit2, as they facilitate a comparatively non-invasive chronic treatment with a chemical substance in mice. The desired depot-like effect provides reliable substance release and uptake into the bloodstream for at

least two weeks. Drug concentration in the gel did change the pharmacokinetics slightly. However, this needs to be investigated further in order to determine the most effective gel composition. Acute injection of SAFit2 solution is only suitable for short-term manipulation of the FKBP51 system and depends on the injection location. In earlier experiments, SAFit2 was injected i.p. while this study used s.c. injections in order to remain comparable to the VPGs.

The next step would be to use the results of this study and compare the molecular and behavioral effects of an acute (over-) dose of SAFit2 and a more subtle, steady exposure to the drug.

## 4.6 FKBP51 inhibition impacts home-cage behavior after stress

Behavioral tests in animals are often suffering from their extremely artificial nature. Lights, smells, sounds and textures, even temperature are all factors that differ massively between holding cages and test arenas. Even though we try to account for all of these deviations by acclimating animals and keeping parameters as uniform and consistent as possible, they are undeniably impacting all behavioral paradigms. For that reason, animals behave a lot differently in these setups than they would in a more familiar environment. In order to take the first step into a more 'natural' assessment of behavior, I recorded mice in their home cage and tracked the range of behaviors they exhibit under baseline conditions (Füzesi et al., 2016). The same animals were then stressed and observed again, always remaining in their own cage. This basic setup was utilized to compare different modulations of the stress feedback system and gather insight into behavioral readouts asides from the classic approaches that have not been changed or improved for many years by now.

Stress induces very significant changes in behavior. When undisturbed, animals tended to spend a lot of time actively exploring their environment by walking and climbing around in their cage. Once exposed to stress, however, behavioral patterns shifted towards more self-directed activities. Animals from all groups exhibited extensive grooming, paired with careful surveillance of the environment. Understandably, a bad experience reduces risk-taking and increases alertness but at the same time seems to induce replacement-behaviors that do not serve any obvious purpose in

that particular situation. Effects like this are a well-known, yet poorly understood phenomenon (Breed et al., 2016). The focus of this study was not only to establish home cage based recordings as part of a behavioral characterization routine but also the comparison of mice that were either pharmacologically treated with SAFit2 or genetic conditional *Fkbp5* KOs as well as their respective controls. Here, differences were surprisingly minor, especially in full body KOs. While SAFit2 treatment induced a tendency towards behavioral patterns resembling baseline observations after stress in some parameters, KOs, for the most part, did not behave differently from their WT counterparts.

One could speculate that all previously reported genotype effects (Touma et al., 2011) were discovered under more stressful conditions and that the familiar environment might mask some of the more subtle differences. While some behavioral aspects depend on novelty and arousal and are therefore best observed in an artificial setup, a lot of insight can be gained by approaches that do focus on naturalistic behavior. This study was partially used as a pilot for more in-depth analyses in the future. Some of the limitations here were the amount of manual labor that was necessary and the choice to use restrain stress as a challenge. For one, tracking all behaviors manually was not efficient enough to process large numbers of animals. The necessity to define a set range of behaviors beforehand also introduced a secondary bias to the analysis. We can surely distinguish some behavioral actions but might lose a great amount of information by limiting the analysis to pre-defined cues.

Overall, the field of behavioral (neuro-) science is likely to move away from limiting itself to tests that are conducted in extremely sterile environments. Even though simplification is necessary to a certain degree, some questions cannot be answered by one-dimensional readouts. Behavioral adaptations to external threats are multi-dimensional processes, controlled by complex neuronal pathways and transmitter cascades that require scientists to better adapt the ways they research them in the future. A number of recent studies have utilized deep neuronal networks and machine learning to automatically extract and analyze data from video material (de Chaumont et al., 2019;

Mathis et al., 2018). If combined with such technologies, observation in a home-cage-environment of either one or several individuals could proof to be a very powerful method for animal research in the future. In that regard, the present study was an important initial step to establish high-resolution behavioral phenotyping in the laboratory.

## 4.7 Synopsis

Stress greatly impacts our everyday life. Most of this impact seems to have negative effects on how we feel, perceive our surrounding or interact with others. However, it is important to understand that an appropriate stress response to at least moderate levels of stress is absolutely vital for an organism to cope and adapt to its environment. This adaptation is mainly based on functional (hormonal) feedback from the periphery to the brain, which controls the stress response. Many of these processes are finely tuned and very dependent on correct molecular signaling. When is it supposed to activate? When is it safe to switch it off? What part of the experience contains important information that is worth storing? All these questions need to be addressed from a biological point of view when we want to find novel ways to treat patients suffering from stress-related disorders in the future. Especially since stress is not a negative thing per se it is of utmost importance to understand the molecular background and the causality behind stress-related diseases. How much of it is too much? How can we support our body and increase resilience? And how can we influence the way stress is processed in our brain in case of a mental disease? In this regard, it is important to utilize all the translational tools that enable us to identify risk genes in humans, bring them into a model organism that can help to understand their molecular roles and ultimately develop treatment methods that can potentially be brought back to the human patient.

In this set of studies, I aimed to contribute to the understanding of the genetic risk factor *Fkbp5* by dissecting its role within the monoaminergic system and advancing our understanding of potential pharmacological inhibitors.

In stress-relevant areas, *Fkbp5* is highly up-regulated in response to stress but does not seem to change its expression patterns after chronic stress exposure. This finding is important to understand the molecular difference between a single stress event and long term over-stimulation of the system.

The LC and the DR were amongst the brain regions with the highest *Fkbp5* regulation after stress, meaning that gene transcription is extremely active while corticosterone levels are elevated. Considering their central role in many behavioral adaptations as well as memory, I went on to test whether behavioral traits can be affected by manipulation of the stress response feedback system. So far, baseline characterizations of a viral OE mouse model and a conditional *Fkbp5* KO mouse line for both regions are complete. Interestingly, neither abundance nor depletion of the important co-chaperone did alter overall behavior. These findings pave the way for future experiments ideally using the same animal models to investigate whether the manipulations affect the response to a stressful challenge. Depending on the kind of behavioral or cognitive changes, one can start looking into specific neuronal populations and connections to other brain regions in order to further disentangle the exact role of *Fkbp5* in these cells.

When we try to relate our approaches back to humans, genetic editing, as well as viral injections are no viable options (yet). Therefore, the development of the specific FKBP51 inhibitor SAFit2 is a promising step towards a future transition into the actual treatment of patients. Implementing SAFit2 into VPGs helped to assure reliable release from the depot-like chemical structure and enabled me to conduct the first long-term studies using the FKBP51 modulator. While the first results showed promising similarities to the conventional KO situation, a lot of improvement is needed in order to refine the dosage and pharmacokinetics of the drug. Treating chronically stressed animals with the drug did not affect anxiety-like behavior but specifically protected animals against social avoidance following CSDS. This study however likely suffered from low SAFit2-dosage and needs to be confirmed by future replication.

Lastly, in order to improve the resolution of our future analyses, I initiated a transition from classical behavioral testing to a more unbiased observation of natural behavior in a low-stress environment. Animals like mice display a very different array of behaviors when in a familiar cage compared to the artificial nature of most test setups. This helps to improve resolution and enables the experimenter to better account for individual differences between the animals. Stress had a massive impact on animals' behavior, represented by an increase in self-directed behaviors and a decrease of explorative drive in the first minutes after the challenge. While the design of the experiment worked well, inhibition of FKBP51 functionality only affected minor aspects of the behavioral response to stress. SAFit2 treated mice did show less drastic changes in behavior, hinting towards a somewhat protective effect of the substance.

## 4.8 Future directions

Even though many of the described experiments did help to describe specific aspects of the stress feedback system better, future work and improvement are needed to fully decipher the contributions of its molecular players. In order to translate this knowledge back to the human situation and potentially help patients, it is absolutely crucial to fully understand the consequences of any intervention we inflict.

Looking at the results of the *Fkbp5* mRNA expression study, no finite conclusion can be drawn for the effect of chronic stress on gene transcription. The study was designed in a way that would allow me to detect any changes in baseline gene activity caused by chronic exposure to corticosteroids. As there was no difference between controls and CSDS animals, the next step would be to look for abnormalities in the acute stress response after CSDS. In that sense, corticosterone levels in chronically stressed animals should be measured 4 hours after the last defeat in order to compare their stress response to controls. Even though chronic stress did not alter overall *Fkbp5* expression, it could very well impact the immediate transcriptional response to the challenge.

Manipulation of *Fkbp5* levels within the monoaminergic system did not drastically alter behavior under baseline conditions. Since there were no behavioral abnormalities, these findings provide a solid foundation for the following experiments. Introducing acute or chronic stress could be an interesting way to measure changes in stress vulnerability, cognitive capacity and stress coping behavior of these animals.

The overall goal in future experiments needs to be to improve the complexity and scale of studies. Since we know that the systems we are looking at are highly interconnected and might affect a wide range of behavioral adaptations, our analysis needs to match these pretensions as best as possible. So far, technical limitations really held back the development of tests that take factors into account that cannot be investigated in most current setups. One example is the problem that mice are social animals however most research is conducted in single-housed animals, taking away any behavioral adaptations that would change e.g. group dynamics or factors like social status. Modern camera and recording technologies are leading the field into a direction where complex analyses of groups of animals within enriched environments can reveal traits and effects that have been hidden in the past. In line with this way of thinking is the idea to use behavioral tests that can be conducted within the animals' cages. That way, cross-over effects (e.g. anxiety-like behavior in a novel setup affecting cognitive performance) could be kept to a minimum which might help to improve the liability and reproducibility of behavioral experiments.

Overall, *Fkbp5* is still one of the most promising candidates for the development of new treatment methods for mental diseases. The results of my thesis work can help understand the molecular background of the stress response regulatory system better and supports a push towards the implementation of respective pharmacological treatments in the future.

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# 8 Publications

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| 2018 | **Pöhlmann ML**, […], Schmidt MV; *Pharmacological Modulation of the Psychiatric Risk Factor FKBP51 Alters Efficiency of Common Antidepressant Drugs.* Frontiers of Behavioral Neuroscience |
| 2018 | Maiarù M**,** Morgan OB**,** Mao T**,** Breitsamer M**,** Bamber, H**, Pöhlmann ML**, […], Géranton SM; *The Stress Regulator Fkbp51: A Novel and Promising Druggable Target for the Treatment of Persistent Pain States Across Sexes.,* PAIN |
| 2018 | Dedic N, Kühne C, Jakovcevski M, Hartmann J, Genewsky AJ, Gomes KS, Anderzhanova E, **Pöhlmann ML**,[…], Deussing JM. *Chronic CRH depletion from GABAergic, long-range projection neurons in the extended amygdala reduces dopamine release and increases anxiety.* Nature Neuroscience |
| 2017 | Balsevich G, Häusl AS, Meyer CW, Karamihalev S, Feng X, **Pöhlmann ML**, […], Schmidt MV; *Stress-responsive FKBP51 is a novel regulator of metabolic function and AKT2-AS160 signalling.* Nature Communications |
| 2017 | Dedic N, **Pöhlmann ML**, […], Deussing JM; *Cross-disorder risk gene CACNA1C differentially modulates susceptibility to psychiatric disorders during development and adulthood.* Molecular Psychiatry |
| 2017 | Smith CJ, **Pöhlmann ML**, […], Veenema AH; *Age and sex differences in oxytocin and vasopressin V1a receptor binding densities in the rat brain: focus on the social decision-making network.* Brain Structure & Function |
| 2017 | Hartmann J, Dedic N, **Pöhlmann ML**, […], Schmidt MV; *Forebrain glutamatergic, but not GABAergic, neurons mediate anxiogenic effects of the glucocorticoid receptor.* Molecular Psychiatry |
| 2014 | Wagner KV, Häusl AS, **Pöhlmann ML**, […], Schmidt MV; *Hippocampal homer1 levels influence motivational behavior in an operant conditioning task.* PLoS One |

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# 10 Assertion/Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation mit dem Titel „Unraveling the Functional Contribution of Fkbp5 to Stress Vulnerability“ selbstständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet.

Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die vorliegende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

München, den 28.02.2020

Max Pöhlmann