FKBP51 at the interface of stress and psychiatric disorders

Dissertation

an der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

Vorgelegt von

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Juni 2014

Dissertation eingereicht am: 23. Juni 2014

Mündliche Prüfung am: 03. November 2014

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Summary

Chronic stress is a crucial risk factor for the development of numerous psychiatric diseases. One major finding in many depressed patients is an impaired regulation of the hypothalamic-pituitary-adrenal (HPA) axis, which is mediated via the glucocorticoid receptor (GR). FK506-binding protein 51 (FKBP51) has been shown to decrease ligand binding sensitivity of the GR, thereby directly affecting stress system (re)activity. Single nucleotide polymorphisms of FKBP51 have been linked to psychiatric disorders and antidepressant treatment response. Thus, FKBP51 has emerged as a novel potential drug target for the treatment of psychiatric disorders. This thesis aimed to investigate the function of FKBP51 as a mediator of the stress response system, its role in the development of stress related diseases and antidepressant responsiveness, as well as its potential as a novel treatment approach in psychiatric disorders. Therefore, I first subjected FKBP51 knockout mice to acute and chronic stress paradigms. Lack of FKBP51 resulted in a more stress-resistant phenotype, especially with regard to neuroendocrine parameters, which was most likely due to an enhanced GR sensitivity. Interestingly, mice lacking one allele of FKBP52 (FKBP52$^{+/-}$ mice), which is FKBP51's close homologue and functional counter-player, demonstrated a mixed phenotype of stress susceptibility and resilience. Furthermore, FKBP51 knockout mice were less responsive to acute and chronic treatment of the antidepressant drug paroxetine. Searching for novel molecular actions of FKBP51 linked to antidepressant effects, we found that FKBP51 is involved in priming autophagic pathways, which are necessary for full antidepressant potency. In addition, I also investigated brain-region specific functions of FKBP51, by overexpressing the gene via virus-mediated gene transfer. While FKBP51 overexpression in the dorsal hippocampus did not result in a strong phenotype, mice with an overexpression in the basolateral amygdala showed an increased anxiety-related behavior. Interestingly, I could also demonstrate that pharmacological inhibition of FKBP51 resulted in an anxiolytic phenotype in mice. Thus, the findings presented in this thesis provide a strong basis for further research investigating the role of FKBP51 in psychiatric disorders and might pave the way for drug development of selective FKBP51 inhibitors.

Zusammenfassung

In den letzten Jahren wurde eine Reihe von Studien veröffentlicht, die genetische Varianten (Einzelnukleotid-Polymorphismen) des Gens FKBP51 mit dem Auftreten von psychiatrischen Erkrankungen wie der Depression, sowie mit der Responsivität für Antidepressiva assoziieren. Das molekulare Co-Chaperon FKBP51 reguliert die Signaltransduktion von Steroidhormonrezeptoren, wie z. B. dem Glukokortikoidrezeptor (GR), welcher ein wichtiger Mediator der hormonellen Stressantwort über die Hypothalamus-Hypophysen-Nebennieren (HHN) Achse des Körpers ist. Dabei verringert FKBP51 die Bindungsaffinität des GR für Stresshormone (Cortisol bei Menschen und Corticosteron bei Mäusen). Ein wiederholt auftretender Befund bei vielen psychiatrischen Erkrankungen wie der Depression ist eine Störung der HHN-Achse. FKBP51 rückte deshalb als potentielles neues Zielprotein zur Behandlung von psychiatrischen Erkrankungen vermehrt in den Fokus der Forschung. In der vorliegenden Arbeit untersuche ich die zugrundeliegenden Effekte von FKBP51 auf das Stresssystem im Körper und seine Verwicklung in psychiatrischen Erkrankungen. Hierfür habe ich transgene Mäuse, denen das FKBP51 Gen fehlt (FKBP51 knockout Mäuse), verschiedenen akuten und chronischen Stressoren ausgesetzt. Ich konnte zeigen, dass FKBP51 knockout Männchen und Weibchen eine geringere Stressanfälligkeit aufweisen als Kontrollmäuse, was vor allem durch die stets sehr niedrigen Corticosteronwerte deutlich wurde und auf eine verbesserte negative Rückkopplung der HHN-Achse schließen lässt. Um das molekulare Umfeld von FKBP51 weiter zu verstehen, habe ich außerdem seinen wichtigsten molekularen Gegenspieler und engsten Verwandten, das FKBP52, näher untersucht. Ich konnte zeigen, dass Mäuse mit nur einem Allel für FKBP52 (ein kompletter FKBP52-Knockout ist letal), in manchen Parametern stressanfälliger waren, bemerkbar z. B. durch erhöhtes Angstverhalten, gleichzeitig zeugten geringe basale Corticosteronwerte aber auch von einer gewissen Stressresistenz. Auf Grund der genetischen Assoziation zwischen FKBP51 Einzelnukleotid-Polymorphismen und Antidepressiva-Responsivität in klinischen Studien, unterzog ich FKBP51 Knockout Mäuse außerdem einer akuten und chronischen Behandlung des Antidepressivums Paroxetin. Hierbei konnte ich zeigen, dass FKBP51 knockout Mäuse weniger auf Paroxetin reagieren. Dieses Ergebnis spiegelt die klinische Situation wider, in der depressive Patienten mit erhöhten FKBP51 Werten schneller auf Antidepressiva reagieren. Wir konnten daraufhin

neue FKBP51-abhänginge Signalwege der Autophagie identifizieren, über die Antidepressiva (neben ihrer gängigen Funktion zur Verstärkung der Wirkung von Neurotransmittern) alternativ wirken können. Um FKBP51 als mögliches Zielprotein zur Behandlung von psychiatrischen Erkrankungen weiter zu validieren, habe ich außerdem Hirnregion-spezifische Funktionen des Co-Chaperons untersucht. So konnte ich zum Beispiel zeigen, dass eine Virus-vermittelte Überexpression von FKBP51 in der basolateralen Amygdala zu erhöhtem Angstverhalten in Mäusen führt. Ich konnte zudem zeigen, dass die Verabreichung von kürzlich entwickelten FKBP51 Antagonisten zu verringertem Angstverhalten in Mäusen führt. Die vorliegende Arbeit bietet daher eine exzellente Grundlage für weitere Forschungsarbeiten, die sich mit der Rolle von FKBP51 in psychiatrischen Erkrankungen beschäftigen und ebnet den Weg für die Entwicklung von Antidepressiva mit neuen Wirkmechanismen.

List of Abbreviations

^{35}S	isotope 35 of sulfur
51KO	FKBP51 knockout
51OE	FKBP51 overexpression
AAV	adeno-associated virus
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
AD	antidepressant
AMI	amitriptyline
Amy	amygdala
ANOVA	analysis of variance
ATG	autophagy related gene
AVP	arginine vasopressin
BLA	basolateral amygdala
BGH	bovine growth hormone
BSA	bovine serum albumin
CA1	cornu ammonis region 1
CA2	cornu ammonis region 2
CA3	cornu ammonis region 3
CCDS	consensus coding sequence
cDNA	complementary DNA
CNS	central nervous system
CoIP	coimmunoprecipitation

Cort	corticosterone
CRH	corticotropin-releasing hormone
cRNA	complementary RNA
CSDS	chronic social defeat stress
DaLi	dark light box
DBD	DNA-binding domain
DEX	dexamethasone
DG	dentate gyrus
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraaceticacid
EPM	elevated plus maze
EtOH	ethanol
FKBP	FK506 binding protein
FKBP51	FK506 binding protein 51
FKBP52	FK506 binding protein 52
FLX	fluoxetine
FST	forced swim test
GABA	gamma aminobutric acid
GCs	glucocorticoids
GFP	green fluorescent protein
GLU	glutamate
GR	glucocorticoid receptor
GRE	glucocorticoid response element

$GR^{GABA\text{-}CKO}$	conditional GR knockout in GABAergic neurons
$GR^{Glu\text{-}CKO}$	conditional GR knockout in glutamatergic neurons
HC	hippocampus
HPA axis	hypothalamic-pituitary-adrenal axis
Hsp	heat shock protein
KO	knockout
LBD	ligand-binding domain
MDD	major depressive disorder
MEF	mouse embryonic fibroblast
mEPSC	miniature excitatory postsynaptic currents
mRNA	messenger RNA
MR	mineralocorticoid receptor
OE	overexpression
OF	open field
PAR	Paroxetine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFC	prefrontal cortex
POMC	pro-opiomelanocrotin
PPIase	peptidyl-prolyl isomerase
PTSD	post-traumatic stress disorder
PVC	polyvinyl chloride
PVN	paraventricular nucleus of the hypothalamus

RNA	ribonucleic acid
SA	social avoidance
SAFit2	selective antagonist of FKBP51 by induced fit
SEM	standard error of the mean
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SSC	saline sodium citrate
SSRI	selective serotonin reuptake inhibitor
TPR	tetratricopeptide repeat
UTP	uridine triphosphate
WT	wild type

1. General introduction

1.1. Mood and anxiety disorders

Every year more than a third of the population in the European Union (EU) suffers from brain disorders. Adjusted to age and comorbidity, this corresponds to 164.8 million persons affected, with mood disorders (7.8 %), such as major depression, and anxiety disorders (14.0 %), such as generalized anxiety disorder and post-traumatic stress disorder (PTSD), being the most frequent disorders (Wittchen et al., 2011). Interestingly, women are more susceptible to develop these diseases than men (Solomon and Herman, 2009; Bangasser, 2013). The total EU cost burden of disorders of the brain for both genders was estimated at €798 billion in 2010, of which €113.4 billion were attributed to mood disorders and €74.4 billion to anxiety disorders. These numbers highlight that disorders of the brain must be considered to be Europe's foremost health care challenge of the 21st century (Gustavsson et al., 2011).

Clinical depression refers to a condition of intense sadness, melancholia or despair, accompanied by anhedonia (loss of interest or pleasure), irritability, cognitive disturbances and abnormalities in appetite and sleep (Krishnan and Nestler, 2008). The condition can be chronic, with periods of good health interspersed with relapses; the recurrence of depressive episodes tends to increase over time, with increasing severity of each successive bout of illness (Yu et al., 2008). The diagnosis of depression is based on the documentation of a certain number of symptoms that significantly impair functioning for a certain duration. These diagnostic criteria overlap with other conditions such as anxiety disorders, which have substantial co-morbidity with depression (Nestler et al., 2002; Rocha et al., 2013).

Although antidepressant drugs are the most effective treatment for depressive disorders, pharmacotherapy still suffers from modest efficacy, the delayed onset of clinical improvement and high relapse rates. Despite intensive efforts in the development of antidepressants, major breakthroughs have only been achieved on the reduction of side effects of these drugs. Treatment efficacy can be enhanced by a combination of different

antidepressant drugs, but adequate therapy response rates, i.e. full remission, are yet to be reached (Binder and Holsboer, 2006; Thase, 2006).

Epidemiologic as well as family and twin studies show that there is a substantial genetic risk to develop depression with an estimated heritability, i.e. genetic factors, of 30% to 40% (Fava and Kendler, 2000). However, depression is a complex phenomenon, as these studies also point to a polygenetic nature of the disorder, which implies that individual genes are likely to exert only a relatively small effect and that the interaction of several genes with each other determines the phenotype (Nestler et al., 2002; Klengel and Binder, 2013b). In addition, susceptibility to depression is only partly genetic, with environmental factors also being important. Exposure to aversive life events, such as traumatic experiences and chronic stress have consistently been established in the pathophysiology of this disorder (McEwen, 2004; Chrousos, 2009). The childhood period seems to be particularly sensitive to negative environmental factors, such as early life stress or child abuse, that increase the risk to develop depression later in life (Heim et al., 2010; Rocha et al., 2013).

It is important to note that depression is a multifactorial disease. A genetic predisposition or environmental risk factor *per se* is not sufficient to cause depression. It is generally accepted that in most people, depression is caused by interactions between a genetic predisposition and some environmental factors. Thus, studies investigating gene x environment interactions that aim at assessing the joint contribution of genetic and environmental factors for developing depression are an important focus of recent research in the field of mood disorders (Nestler et al., 2002; Klengel and Binder, 2013b). However, knowledge about the molecular neurobiology of depression is rather rudimentary compared to knowledge of other common chronic and potentially fatal multifactorial diseases such as type 2 diabetes, due to the fact that observing pathological alterations within the brain remains markedly more difficult than for all other organs (Krishnan and Nestler, 2008). To further elucidate the complex nature of mood disorders, mouse models have proven extremely useful not only regarding the validation of neurobiological underpinnings, but also with respect to the identification and improvement of therapeutic substances (Nestler and Hyman, 2010).

1.2. Modeling mood disorders in mice

It is extremely difficult to envision a mouse model that perfectly mimics the human condition of depression with respect to its etiology, symptomatology, treatment and biological basis. For instance, many of the symptoms used to diagnose depression in humans, such as sadness, guilt and suicidality, cannot be convincingly assessed in animals (Deussing, 2006). Thus, it is highly unlikely that an animal model can mirror all the salient characteristics of human mood disorders. Nonetheless, these models can be successfully used if questions related to specific key symptoms prevalent in human depression are addressed (Müller and Holsboer, 2006). Therefore the scientific community established a framework of the three minimal requirements for a valid disease model. An animal model should fulfill (1) face validity, i.e. the similarity of disease symptoms in humans and rodents, (2) construct validity, which requires that the symptoms produced in the mouse model are based on the same underling neurobiological mechanisms as in humans, and (3) predictive validity, referring to the ability of the animal to respond to pharmacological treatments in a manner that predicts the effects of those treatments in humans (McKinney, Jr. and Bunney, Jr., 1969; Nestler and Hyman, 2010; Dedic et al., 2011).

Most mouse models of mood disorders rely either on targeted genetic manipulations or on environmental challenges. One possibility is to use the intrinsic heterogeneity of whole populations (outbred strains). Hereby, one or more characteristics are investigated and the animals are selected into subpopulations based on molecular or behavioral parameters, which would also reflect genetically or defined populations (Schmidt et al., 2010b; Scharf and Schmidt, 2012). Such subpopulation selection can further be used as a particular factor for selective breeding to presumably enhance the differences in the subgroups and isolate the genetic component transferred over the germ line (Touma et al., 2008). However, one of the most common approaches is to modify the murine genome, resulting in knockout (KO) or transgenic (knock-in) mice specific for selected candidate genes (Müller and Holsboer, 2006). The refinement of techniques for directed genetic mutations or gene targeting, such as the propagation of the Cre-Lox recombination technology, has allowed an increasingly precise control of spatial (e.g. brain region or cell-type specific) and temporal (e.g. during or after development) gene expression (Dedic et al., 2011). Other popular and frequently used methods are

conditional manipulations including RNAi technology and virus-mediated gene transfer which also enable the control over spatial and temporal gene expression, as well as optogenetically mediated stimulation or inhibition of distinct cell populations in specific brain regions (Glaser et al., 2005; Fenno et al., 2011).

Besides genetic manipulations, many animal models of mood disorders focus on environmental risk factors, such as chronic stress, which have crucially been implicated in the cause of depression in humans. There are a number of mouse models that have been designed on the basis of chronic stress, which fulfill the criteria of face, construct and predictive validity. Such chronic stress paradigms comprise, among others, restraint-based chronic stress (Kim and Han, 2006), chronic mild stress (Willner, 1997), intruder stress (Tornatzky and Miczek, 1994), prey and predator stress (Adamec and Shallow, 1993), sleep-deprivation stress (Pokk et al., 2000), conditioned emotional stress (Gomita et al., 1983; Katsura et al., 2002), chronic social stress (Schmidt et al., 2007; Sterlemann et al., 2008) and chronic social defeat (Berton et al., 2006). Especially the chronic social defeat paradigm is very useful (Figure 1.1), since the animals are exposed to a permanent stress situation, which is not limited to a defined time period per day. Moreover, there are no adaptations in response to the stressor as observed in other paradigms such as the chronic restrained stress. Furthermore, the stressor of the defeat paradigm is of social nature, taking into account the etiology of human stress-associated disorders (Golden et al., 2011).

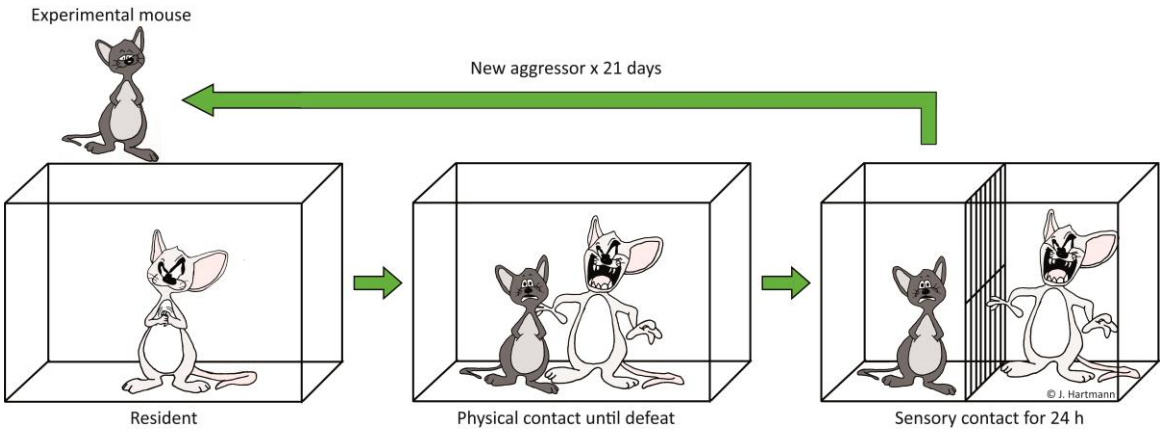

Figure 1.1: Chronic social defeat stress paradigm. Schematic overview of the social defeat procedure. The experimental mouse is placed into the cage of a dominant resident mouse. After physical defeat, a divider allows only sensory contact. After 24 hours experimental animals are introduced to another unknown resident.

It is important to note that some individuals are more susceptible to chronic stress, while others show a marked resilience. Along this line, it is rather unlikely that a single genetic variant is responsible for a specific disorder. However, the combination of environmental challenges and genetic predispositions are thought to play a central role for the development of depression (Gillespie et al., 2009; Pitchot et al., 2012; Klengel and Binder, 2013a; Klengel and Binder, 2013b). Therefore many studies apply the concept of gene x environment interactions in preclinical research, which is more likely to reflect the pathophysiological mechanisms of mood disorders. One approach to address such gene x environment interactions is to expose mouse lines with a genetic manipulation of a gene hypothesized to be a risk factor for the development of stress-related disorders to situations that induce symptoms of psychopathologies in mice. Indeed, such studies could show that disease associated genetic alterations *per se* do not necessarily have to be beneficial or pathological under basal conditions. However, in combination with chronic stress, these genetic alterations turned out to enhance resilience or susceptibility towards the development of depression-like phenotypes in mice (Berton et al., 2006; Vialou et al., 2010; Wagner et al., 2011; Wang et al., 2011a; Donahue et al., 2014).

1.3. Stress and stress response systems

Stress is a term often used to describe situations that are emotionally and physiologically challenging. In biological terms, stress is characterized as a state of disturbed body homeostasis. Hans Selye, a pioneer of stress research, defined stress as the 'non-specific response of the body to any demand for change' and introduced the so called 'General Adaptation Syndrome' (Selye, 1936), which is comprised of three stages. First, an alarm reaction to prepare the body to fight or flight is initiated, after which a resistance to the stress is built and finally it comes to an exhaustion state due to a too long stress exposure. Later on, Sterling and Eyer introduced the state of allostasis as the process of achieving stability, or homeostasis, through physiological or behavioral changes (Sterling and Eyer, 1988; McEwen, 1998). The body's adaptive systems respond to the internal state (e.g. sleeping or exercising) and to the external environment. Those mechanisms promote adaptation to activities such as locomotion but also to aversive stimuli including noise, isolation, hunger and threats. Usually, the mediation of a stress response is beneficial, as

long as the adaptive systems are activated for a short period of time and efficiently shot off again, without being chronically overstimulated. Nevertheless, several circumstances that lead to a permanent overstimulation or an inactivity of allostatic systems that are normally involved in adaptation to environmental challenges can be maladaptive. McEwen introduced such a condition as 'allostatic load', which can lead to impaired immune system function, obesity and atrophy of nerve cells in the brain (McEwen, 1998). Taken together, it should be highlighted that averse experiences and excessive challenges such as chronic stress clearly impose a major risk factor for the development of depression and other stress-mediated disorders (Holsboer, 2000; van Praag, 2005; de Kloet et al., 2005a). In order to completely understand the mechanisms that cause depression, it is necessary to study the systems mediating the stress response in great detail.

1.4. The HPA axis

The major control module of the stress response in mammals, besides the autonomic nervous system, is the hypothalamic-pituitary-adrenal (HPA) axis. Upon stress, the HPA axis is activated and thereby cortiocotropin-releasing hormone (CRH) and vasopressin (AVP) are secreted from neurons of the paraventricluar nucleus into the portal blood system that is connected to the anterior part of the pituitary gland (Sapolsky et al., 2000). At the pituitary, CRH and AVP bind to their respective receptors and synergistically trigger the synthesis and release of pro-opiomelanocortin (POMC), the precursor for andreno-corticotropic hormone (ACTH). Circulating ACTH reaches the secretory cells of the *zona fasciculate* and the *zona reticluaris* of the adrenal cortex via the blood stream and stimulates them to synthesize and secrete glucocorticoids (GCs) into the systemic circulation. Glucocorticoids (cortisol in humans and corticosterone in rodents), the main hormonal end products of the HPA axis, are then acting on numerous organ systems, including the brain, to modulate physiology and behavior (Figure 1.2). Among other things they mediate cardiovascular activation, energy mobilization from storage sites, anti-inflammatory effects and suppress reproductive and digestive functions (Sapolsky et al., 2000; Sorrells and Sapolsky, 2007).

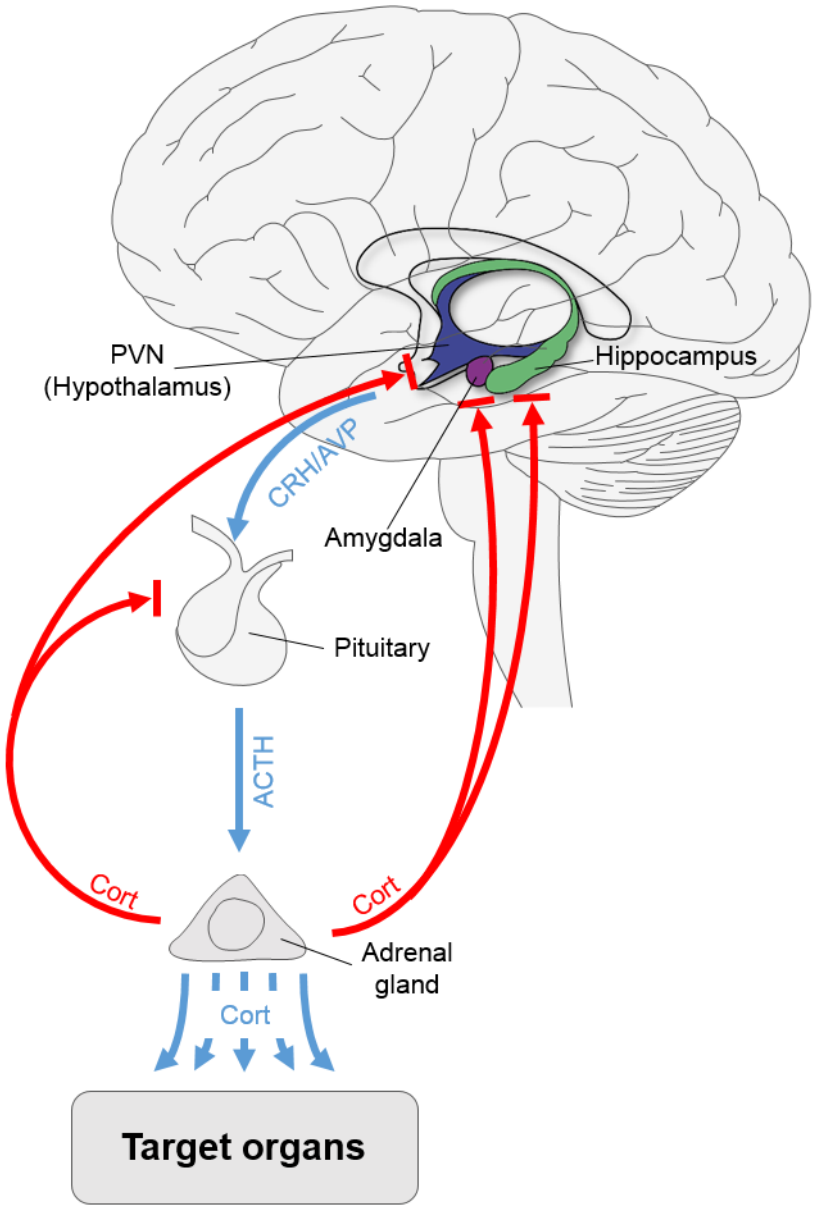

Figure 1.2: Hypothalamic-pituitary-adrenal axis. Activation of the HPA axis leads to the secretion of hypothalamic corticotropin-releasing hormone (CRH) and vasopression (AVP), which in turn trigger the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH simulates the secretion of glucocorticoid hormones (cortisol in humans and corticosterone in rodents). GCs target various tissues and organs in the body, but also regulate HPA axis activity via corticosteroid receptors (GR and MR).

The nature of a stressor can differ largely and therefore the stress response, mediated via the HPA axis, arises from interactions of several distinct stress-sensitive brain circuits and neuronal populations of the PVN. This nucleus appears to be a major player in the central

regulation of the HPA axis, since lesion studies of the PVN revealed markedly decreased portal CRH- and stress-induced ACTH and corticosterone levels (Herman and Cullinan, 1997). The stress-sensitive pathway of the catecholaminergic system projects directly to the CRH-containing neurons of the PVN (Cunningham et al., 1990), is mainly stimulated by direct threats like hemorrhage, hypotension and respiratory distress, and might play a role in the ATCH responses to immune challenges (Ericsson et al., 1994). Furthermore, it has been suggested that serotonin can also affect local neuronal circuits projecting to the PVN, given the fact that serotonergic neurons project to regions adjacent to the PVN (Leonard, 2005). Additionally, psychological stressors that require interpretation and processing by higher brain structures are mainly channeled through limbic structures like the amygdala and the hippocampus (Sinha et al., 2004). These central brain regions are associated with emotion as well as learning and memory and are connected to the bed nucleus of the stria terminalis (BST), which in turn projects to the PVN (Cullinan et al., 1993; Choi et al., 2008). It is suggested that the amygdala activates the HPA axis, since lesion studies displayed decreased ACTH and corticosterone secretion following stress, whereas amygdala stimulation led to increased HPA axis output (Herman et al., 2005). Persistent stress that is not resolved through coping or adaptation (termed distress) can lead to a dysregulation of the HPA axis and may cause depression-like behavior (Holsboer, 2000). In order to recover the HPA axis to its basal activity and to prevent it from being pathologically over-activated it is necessary to terminate the stress response on time. Several negative feedback mechanisms are involved in normalizing the activity of the stress system (Figure 1.2). Numerous studies have shown that hippocampal stimulation reduces glucocorticoid secretion, whereas hippocampal lesion increases GC release (Dunn and Orr, 1984; Herman et al., 2005). These lesion effects are most pronounced during the recovery phase of stress-induced glucocorticoid secretion, suggesting a critical role of the hippocampus in the feedback inhibition of the HPA axis (Jacobson and Sapolsky, 1991). Moreover, GCs act directly on different levels of the HPA axis, like the PVN and the pituitary, in order to repress CRH and ACTH release, respectively, and therefore regulate their own secretagogues (Sapolsky et al., 2000). GCs exert these negative feedback effects on the HPA axis by binding to different receptor subtypes, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR).

1.5. Corticoid receptors

The corticoid receptors GR and MR are both members of the ligand-dependent transcription factor family (De Kloet and Reul, 1987; Mangelsdorf et al., 1995). MRs bind GCs with a 10-fold higher affinity than GRs, suggesting different roles for each receptor type in the regulation of HPA axis activity. Hippocampal MRs are already extensively bound to their ligands under basal levels of GCs and are therefore mainly responsible for basal regulation of the HPA axis, whereas GR occupation occurs at the circadian peak of glucocorticoid secretion and following stress (De Kloet et al., 1993; Joels and De Kloet, 1994). Furthermore, both receptors are expressed in different tissues of the body's periphery. In the brain, the main function of both receptors, among others, is the mediation of the negative feedback control of the HPA axis. MRs and GRs mediate such regulation mainly in the hypothalamus and the pituitary, but also on the level of the hippocampus (De Kloet et al., 1998; Ulrich-Lai and Herman, 2009). The proper negative feedback via the GR appears to be critical for a healthy stress response and prolonged or excessive activation of this system have been implicated in the pathogenesis of mood and anxiety disorders (Holsboer, 2000; Pariante and Miller, 2001). One of the most robust biological abnormalities observed in such mood disorder is an impaired signaling via GR, which often leads to an impaired negative feedback regulation and thus to partial glucocorticoid resistance. In order to better understand the negative feedback loop of the HPA axis and its mediation via MRs and GRs it is necessary to obtain a better understanding of the molecular mechanisms underlying those receptors.

Corticoid receptors are intracellular transcription factors that bind to DNA following activation by means of ligand-binding in order to regulate the expression of specific target genes. They contain a DNA-binding domain (DBD), located in the central part of the polypeptide chain, a ligand-binding domain (LBD) which is a large C-terminal domain and a N-terminal domain, that contains a transcriptional activation function (Ma et al., 1999). In the absence of a ligand, steroid receptors are typically associated with a dimer of the heat shock protein 90 (hsp90) and additional components of the molecular chaperone machinery (Cheung and Smith, 2000). Upon ligand activation, the receptor is subjected to several conformational changes that lead to its dissociation from the cytoplasmic chaperones. After transportation to the nucleus by a group of nuclear translocation

proteins, the receptor dimerizes. The formed homodimers bind to their cognate DNA sequences, called glucocorticoid response elements (GREs), and induce the transcription of GRE-containing genes. Moreover, transcription can be decreased by interaction of the dimer with other transcription factors or binding to negative GREs (Beato et al., 1995; De Bosscher et al., 2003; Liberman et al., 2007).

1.6. Molecular chaperones and the hsp90 complex

Molecular chaperones are proteins that assist other proteins (both newly synthesized polypeptide chains and assembled subunits) in various steps of protein maturation to avoid misfolding. Moreover, they participate in the ubiquitin-proteasome and autophagy systems, which mediate the timely removal of irreversibly misfolded and aggregated proteins. The protein quality control network and the maintenance of proteome homeostasis are crucial for cellular and organismal health. (Hartl and Martin, 1995; Hartl et al., 2011). Hartl and colleagues defined a molecular chaperone as any protein that interacts with, stabilizes or helps another protein to acquire its functionally active conformation, without being present in its final structure (Hartl, 1996; Hartl and Hayer-Hartl, 2009).

Molecular chaperones belong to the heat-shock proteins (hsps), since they are synthesized in dramatically increased amounts after a short exposure of cells to an augmented temperature (Georgopoulos and Welch, 1993). The main groups of chaperones in mammals are the hsp60, hsp70 and hsp90-families, which are usually classified according to their molecular weight (Pratt and Toft, 1997; Fink, 1999; Hartl et al., 2011). Hsp90 is one of the most abundant proteins in the cytoplasm of eukaryotes, representing up to 1 % of soluble protein even in the absence of heat-stress (Welch and Feramisco, 1982). Chaperones exist in the cytosol as multiprotein heterocomplexes independent of their association with receptors and are the center of a dynamic, multifunctional and multicomponent chaperone machinery (Buchner, 1999).

In the absence of a ligand the GR resides in the cytoplasm. Numerous molecular chaperones assemble the receptor in a successive and ATP-dependent manner to a conformational state capable of binding a hormone with high affinity (Pratt and Toft, 1997). The mature GR complex consists of an hsp90 dimer, hsp70 and the hsp90-binding

protein p23. Moreover, several co-chaperones bind via their tetratricopeptide repeat (TPR) domains to a universal TPR binding domain on hsp90 during GR complex maturation. These include among others the mammalian FK506-binding proteins (FKBPs), FKBP51 (also called FKBP5) and FKBP52 (also called FKBP4) (Tai et al., 1992; Cheung and Smith, 2000; Wochnik et al., 2005). One important task of the hsp90-heterocomplex is to assist the folding of the LBD of the receptors into a high-affinity ligand-binding state. The direct interaction of hsp90 with the LBD of the receptors appears to account for an inhibition of receptor function that is relieved upon subsequent binding of the ligand (Pratt and Toft, 1997).

1.7. The immunophilins FKBP51 and FKBP52

Immunophilins are ubiquitous and conserved proteins that are targeted by immunosuppressant drugs, like FK506, rapamycin and cyclosporine (Schreiber, 1991). There are two immunophilin subfamilies: the cyclophilins (CyPs) bind cyclosporine A, whereas the FKBPs represent binding proteins for compounds such as FK506 and rapamycin. Immunophilins have been identified in many eukaryotes from yeast to humans and all members of this family exhibit a peptidyl-prolyl isomerase (PPIase) activity, suggesting that they may be involved in protein folding in the cell (Trandinh et al., 1992).

In mammals, FKBP51 and FKBP52 share more than 70% sequence identity, but they contrary regulate GR signaling via hormone binding and nuclear translocation (Wochnik et al., 2005; Binder, 2009; Schmidt et al., 2012). The murine FKBP51 is a 51 kDa protein encoded by the Fkbp5 gene (also known as Fkbp51) and its expression is strongly hormone-dependent (Hubler et al., 2003; Vermeer et al., 2003). FKBP51 was first identified in the progesterone receptor complex (Smith et al., 1990) and has co-chaperone activity, since it is participating in the hsp90-complex (Schiene-Fischer and Yu, 2001). Increased levels of FKBP51 in COS cells have been reported to diminish the binding affinity of GR and therefore reduce the transcriptional activity of GR after hormone exposure (Denny et al., 2000). In addition to this reduction of hormone binding it has been shown in mammalian cells that the mechanism for the inhibitory action of FKBP51 on GR involves

an impairment of nuclear translocation (Wochnik et al., 2005). Furthermore, FKBP51 may also regulate the nuclear translocation of the nonactive beta-isoform of the GR, thereby diminishing overall GR signalling (Zhang et al., 2008). In neurons, siRNA knockdown of the gene encoding for FKBP51 was associated with elevated baseline GR nuclear localization (Tatro et al., 2009a). Taken as a whole, FKBP51 acts as a negative regulator of GR activity. However, it is important to note that FKBP51 is also involved in various other intracellular processes, besides its regulatory function of the GR (Pei et al., 2009; Blair et al., 2013).

The murine FKBP52, a 52 kDa protein, is encoded by the Fkbp4 gene (also referred to as Fkbp52) and its expression can be increased via exposure to heat or chemical stress, determining itself as heat shock protein (Sanchez, 1990). Like FKBP51, it is associated with the hsp90-heterocomplex (Tai et al., 1992). Moreover, it is localized in the cytoplasm as well as in the nucleus (Czar et al., 1997). Upon hormone binding, FKBP51 is replaced by FKBP52 in the cytosolic GR-hsp90-complex (Davies et al., 2002). Subsequently, FKBP52 interacts with the motor protein dynein, which is part of a transportosome, a protein heterocomplex that leads the GR along cytoskeletal tracts to the nucleus (Galigniana et al., 1998; Silverstein et al., 1999; Galigniana et al., 2004). Moreover, Rein and colleagues showed that elevated levels of FKBP52 attenuated the impairment of GR nuclear translocation induced by FKBP51 (Wochnik et al., 2005). Furthermore, siRNA knockdown of FKBP52 inhibited cortisol-activated GR nuclear translocation in neurons (Tatro et al., 2009a).

Taken together, FKBP51 binds to hsp90 during the maturation of the GR-complex, which leads to a decreased GR-complex affinity for glucocorticoids (Figure 1.3). Upon hormone binding, FKBP51 is replaced by FKBP52, which recruits dynein into the complex, facilitating its nuclear translocation and transcriptional activity (Binder, 2009; Storer et al., 2011). As mentioned previously the expression of FKBP51 is stimulated by steroids, such as GCs, as part of an intracellular ultra-short negative feedback loop for GR activity (Vermeer et al., 2003; Hubler et al., 2003). Therefore, augmented transcription and translation of FKBP51 following corticoid receptor activation reduces GR sensitivity.

The suggested role of immunophilins in GR signal transduction also involves novel potential possibilities to elucidate GR impairment associated with many psychiatric disorders, like PTSD and depression (Holsboer, 2000). It is well established that

malfunction of GRs due to low affinity ligand binding can be the consequence of mutations of the GR receptor itself or of limited hsp90 action (Picard et al., 1990; Bohen and Yamamoto, 1993; Brönnegard et al., 1996). Since FKBP51 can contribute to augmented GC levels and thereby modulating GR sensitivity, it was suggested to mediate gene-environment interactions relevant in mood and anxiety disorders.

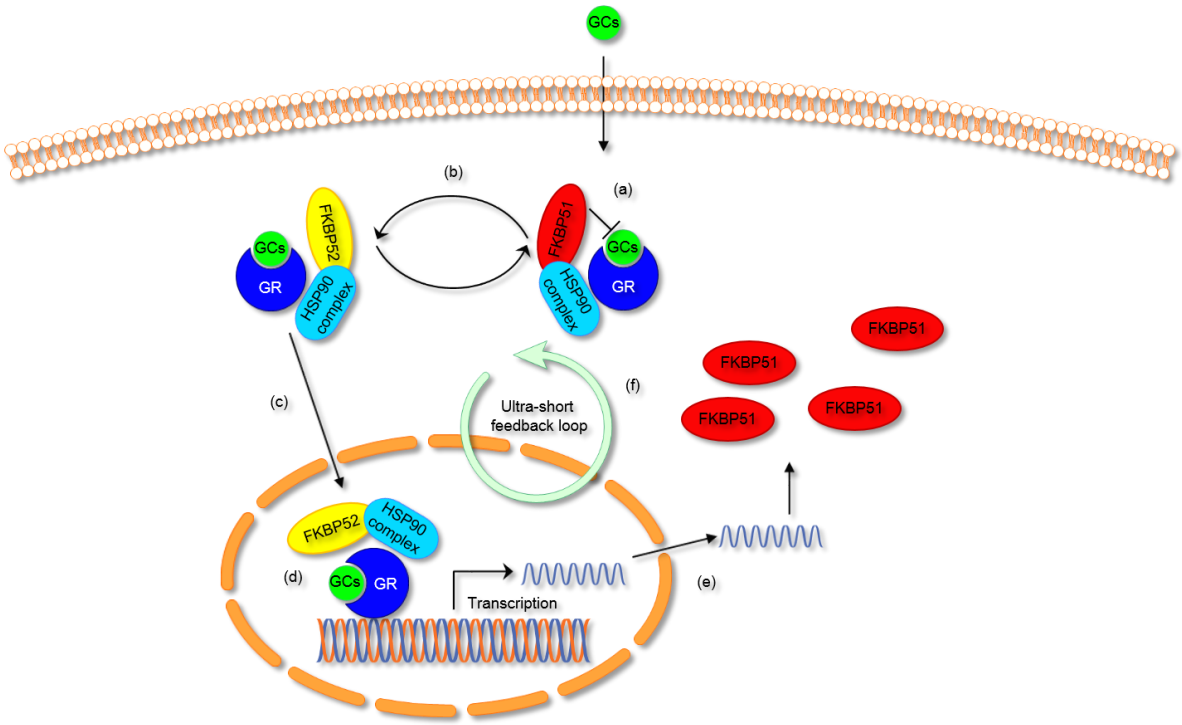

Figure 1.3.: Interaction and function of FKBP51 and FKBP52 with the GR-complex. (a) When FKBP51 is bound to the GR-complex via hsp90, the receptor has lower affinity for glucocorticoids. (b) Upon ligand binding, FKBP51 is replaced by FKBP52, (c) which promotes the translocation into the nucleus and (d) subsequent DNA binding. (e) The GR can then increase FKBP51 transcription and translation via intronic response elements. (f) Increased FKBP51 levels confers higher GR resistance, completing an ultra-short negative feedback loop on GR sensitivity.

1.8. FKBP51 in human studies

In 2004, Binder and colleagues were the first that put genetic variants of FKBP51 into the context of psychiatric disorders. They could show that risk allele carriers of the single nucleotide polymorphism (SNP) rs1360780 in the gene encoding FKBP51 were linked to elevated FKBP51 protein expression and increased recurrence of depressive episodes (Binder et al., 2004). Since then, numerous independent studies confirmed the association

of FKBP51 polymorphisms with mood disorders such as major depression (Lekman et al., 2008; Tatro et al., 2009b; Zobel et al., 2010; Lavebratt et al., 2010; Velders et al., 2011; Menke et al., 2013) or bipolar disorder (Willour et al., 2009; Leszczynska-Rodziewicz et al., 2014). Other studies also reported associations of FKBP51 SNPs with suicidal behavior (Brent et al., 2010; Supriyanto et al., 2011) as well as interactions with childhood trauma on suicide attempt (Roy et al., 2010; Roy et al., 2012). Along these lines, it has been shown that adverse early life experiences, such as child abuse, moderate the effects of FKBP51 SNPs on vulnerability to adult depression (Appel et al., 2011) and the interaction of early trauma and FKBP51 gene variants can furthermore predict onset of depression (Zimmermann et al., 2011). Klengel et al. reported that childhood trauma-dependent allele-specific FKBP51 DNA demethylation increased the risk of developing stress-related psychiatric disorders in adulthood. In more detail, they addressed the functionality of FKBP51 SNP rs1360780, showing differential chromatin folding of the risk allele. Altered three dimensional folding of the gene enhances interaction of a GRE positioned in FKBP51 intron 2 with RNA polymerase at the transcription start site of the gene. This elevates FKBP51 transcription, decreasing GR sensitivity and finally resulting in decreased efficiency of the negative feedback loop of the HPA axis (Klengel et al., 2013).

Several studies also report that FKBP51 polymorphisms affect the response to antidepressant drug treatment. The initial analysis by Binder et al. demonstrated that depressed patients that were homozygous carriers of the risk allele of rs1360780 responded faster to antidepressant treatment than depressed individuals with the other genetic variations (Binder et al., 2004). Thereby, differences in response to antidepressants seemed to be independent of the type of drug, since it was observed in groups of patients treated with either mirtazapine, selective serotonin reuptake inhibitors or tricyclic antidepressants. Although three smaller studies could not replicate such associations, probably due to lack of statistical power (Papiol et al., 2007; Tsai et al., 2007; Sarginson et al., 2009), numerous other studies reported significant associations between FKBP51 SNPs and enhanced response to antidepressants in independent samples (Lekman et al., 2008; Kirchheiner et al., 2008; Zou et al., 2010; Horstmann et al., 2010; Ellsworth et al., 2013).

Besides their significant roles in mood disorders and antidepressant treatment response, FKBP51 polymorphisms have also been associated with an increased risk to develop post-traumatic stress disorder. It has been shown that adults abused during their childhood who carry risk alleles of FKBP51 SNPs are at a higher risk for developing PTSD to subsequent trauma exposures (Binder et al., 2008; Xie et al., 2010). PTSD has been associated with enduring changes in HPA axis reactivity, especially with enhanced sensitivity of the GR (Yehuda et al., 2004; Yehuda et al., 2006). In line with this, people exposed to the World Trade Center attacks on 9/11 demonstrated reduced expression of FKBP51 in patients with PTSD, which is consistent with enhanced GR responsiveness (Yehuda et al., 2009; Sarapas et al., 2011).

Studies performed in healthy volunteers further underline the importance of FKBP51 polymorphisms in stress physiology. The Trier Social Stress test was performed with healthy individuals, homozygous for the previously described FKBP51 risk alleles, to evaluate their psychosocial stress response (Ising et al., 2008). Risk allele carriers show significantly slower recovery of stress related elevations of cortisol levels as well as more anxiety symptoms in the recovery phase than healthy controls with different alleles, suggesting that these subjects are at a greater risk to develop stress-related psychiatric disorders. Moreover, associations of FKBP51 SNPs and mother-infant attachment have been shown to predict cortisol reactivity in infants (Luijk et al., 2010). Genetic variants of FKBP51 have also been suggested to affect personality traits such as harm avoidance and cooperativeness in a gender-specific fashion in healthy volunteers (Shibuya et al., 2010).

Taken together, genetic variations of FKBP51 seem to be crucially involved in stress physiology, the susceptibility to mood and anxiety disorders, as well as antidepressant treatment response.

1.9. FKBP51 in animal studies

The profound involvement of FKBP51 in psychiatric disorders further encouraged preclinical research to study the mechanistic role of FKBP51 in HPA axis functioning and disease using rodent models.

Initially, the role of FKBP51 in stress regulation has been discovered in Squirrel monkeys. Compared to other monkeys and primates, including humans, these New World primates have markedly increased plasma cortisol levels, but they lack signs of detrimental glucocorticoid excess. Endogenes overexpression of FKBP51 and thus, decreased hormone-binding affinity of the GR has been demonstrated to be a reason of glucocorticoid resistance in these animals (Denny et al., 2000; Scammell et al., 2001; Westberry et al., 2006).

In the mouse brain, there is an ubiquitous basal FKBP51 mRNA expression, with increased expression levels in specific GR-rich brain areas, such as the hippocampus (Scharf et al., 2011). Both in rats and mice, FKBP51 mRNA expression and protein levels can be induced by the glucocorticoid agonist dexamethasone, by corticosterone and by stress paradigms such as restraint stress, food deprivation, chronic mild stress and chronic social defeat stress (Lee et al., 2010; Scharf et al., 2011; Lee et al., 2011; Wagner et al., 2012; Yang et al., 2012; Guidotti et al., 2013). Thus, elevated GCs levels alter FKBP51 levels as part of the ultra-short feedback loop (Figure 1.3), thereby decreasing GR sensitivity. Along these lines, Scharf et al. could also show that regions with low basal FKBP51 expression such as the PVN and the amygdala showed a higher increase in FKBP51 mRNA following induction than regions with a high basal expression such as the hippocampus, further supporting the hypothesis that GR sensitivity is modulated by FKBP51 (Scharf et al., 2011).

Chronic exposure to corticosterone also caused significant demethylation within fkbp51 in blood as well as in the hippocampus and hypothalamus of mice (Lee et al., 2010). Furthermore, the same authors reported an interaction of corticosterone induced fkbp51 demethylation and anxiety, as demethylation in the intron 1 region of fkbp51 correlated with reduced time spent in the closed arms of the elevated plus maze (Lee et al., 2011). Interestingly, virus-mediated knockdown of FKBP51 in the basolateral amygdala of mice, led to reduced anxiety-related behavior, when these mice were previously restraint stressed for 6 hours (Attwood et al., 2011).

A conventional knockout mouse line lacking the FKBP51 gene has previously been generated (Tranguch et al., 2005) and young adult FKBP51 knockout ($FKBP51^{-/-}$, also called 51KO) males have been characterized under basal and acute stress conditions for neuroendocrine reactivity as well as in several tests on anxiety- and depression-related

behavior. (Touma et al., 2011). Lack of FKBP51 decreased HPA axis reactivity and GR expression changes in response to acute stressors, shaping the neuroendocrine profile of these mice. Under basal conditions, ablation of FKBP51 did not reveal an overt behavioral phenotype compared to wild type mice. However, after an intense stressor (restraint stress), FKBP51 knockout mice showed an enhanced active stress coping behavior in the forced swim test (FST). This is in line with findings of O'Leary and colleagues, who demonstrated that aged mice of this mouse line displayed the same anti-depressant phenotype in the tail suspension test and FST (O'Leary, III et al., 2011). Interestingly, FKBP51 deletion also resulted in a pro-resilience sleep phenotype, as sleep architecture of knockout mice was nearly opposite to sleep profiles observed in human depression (Steiger and Kimura, 2010; Albu et al., 2013).

The genetic associations of FKBP51 with psychiatric disorders in humans as well as the protective effect of FKBP51 knockout and knockdown on stress physiology, stress-coping behavior and sleep architecture in animal models, promoted the emergence of FKBP51 as an interesting therapeutic approach in the context of stress-related psychiatric diseases.

1.10. FKBP51 as a drug target

In recent years, FKBP51 arose as a promising novel drug target for psychiatric disorders. However, the design of efficient ligands has proven extremely challenging in drug development. The inability to pharmacologically differentiate against the highly homologues functional counter-player FKBP52, substantially complicated drug discovery for FKBP51 and all known FKBP ligands such as rapamycin or FK506 lack selectivity and show strong off-target effects (Gopalakrishnan et al., 2012). A high-throughput screening of 350 000 compounds also did not provide any suitable chemical starting points, raising doubts on the feasibility of FKBP51 as a drug target (Wang et al., 2013b). However, Hausch and colleagues including us, recently reported the development of the first potent and highly selective inhibitors of FKBP51, SAFit and SAFit2. Using these ligands to selectively inhibit FKBP51, we were able to enhance neurite elongation in neuronal cultures as well as improve neuroendocrine feedback and stress-coping behavior in mice. Thus, these compounds may trigger a new line of antidepressant drug development, the first truly based on basic scientific insight (Gaali and Kirschner et al., unpublished data).

2. Aim of the thesis

The current thesis aimed to further unravel the role of FKBP51 in mood and anxiety disorders. To achieve this, we first investigated the involvement of FKBP51 in the behavioral and neuroendocrine effects of chronic stress using a conventional FKBP51 knockout mouse line (Chapter 1). In addition, we studied female FKBP51 knockout mice under basal and acute stress conditions, to elucidate potential gender differences in FKBP51-dependent modulation of the HPA axis (Chapter 2). Next, we investigated the behavioral and neuroendocrine relevance of FKBP52, the close homologue but functional counter-player of FKBP51, with respect to chronic stress in a FKBP52 knockout mouse line (Chapter 3). To further elucidate the function of FKBP51 in stress recovery and antidepressant response, we studied the ability of FKBP51 knockout mice to recover from chronic stress and examined the effects of a chronic antidepressant (paroxetine) treatment during the 3-week recovery period (Chapter 4). Unraveling the molecular mechanism of antidepressant-FKBP51 interaction, we continued to study the acute effects of paroxetine in FKBP51 knockout mice and elucidated a possible FKBP51-dependent mechanism priming antidepressant efficacy (Chapter 5). Finally, we investigated the amygdala-specific role of FKBP51 with regard to anxiety-related behavior by genetic and pharmacological intervention of FKBP51 (Chapter 6). In summary, this thesis gives novel and substantial insight into the involvement of FKBP51 in mood and anxiety disorders and provides a profound basis for future studies aiming to develop novel treatment strategies in the context of personalized medicine.

3. Research articles

Chapter 1: *The involvement of FK506-binding protein 51 (FKBP5) in the behavioral and neuroendocrine effects of chronic social defeat stress*
Neuropharmacology 2012 Jan; 62(1):332-9

Chapter 2: *Depletion of FKBP51 in female mice shapes HPA axis activity*
PLOS ONE 2014 Apr; 9(4): e95796

Chapter 3: *Fkbp52 heterozygosity alters behavioral, endocrine and neurogenetic parameters under basal and chronic stress conditions in mice*
Psychoneuroendocrinology 2012 Dec; 37(12):2009-21

Chapter 4: *FKBP51 critically impacts antidepressant action and chronic stress recovery*
Manuscript in preparation

Chapter 5: *FKBP51 directs autophagic pathways, enabling prediction of antidepressant treatment response*
Manuscript in preparation

Chapter 6: *Pharmacological FKBP51 inhibition reduces anxiety-related behavior in mice*
Manuscript in preparation

3.1. The involvement of FK506-binding protein 51 (FKBP5) in the behavioral and neuroendocrine effects of chronic social defeat stress

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Originally published in:

Neuropharamcology 2012 Jan; 62(1):332-9

Abstract

Chronic stress is increasingly considered to be a main risk factor for the development of a variety of psychiatric diseases such as depression. This is further supported by an impaired negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis, which has been observed in the majority of depressed patients. The effects of glucocorticoids, the main hormonal endpoint of the HPA axis, are mediated via the glucocorticoid receptor (GR) and the mineralocorticoid receptor. The FK506-binding protein 51 (FKBP5), a co-chaperone of the Hsp90 and component of the chaperone-receptor heterocomplex, has been shown to reduce ligand sensitivity of the GR. This study aimed to investigate the function of FKBP5 as a possible mediator of the stress response system and its potential role in the development of stress-related diseases. Therefore, we assessed whether mice lacking the gene encoding FKBP5 (51KO mice) were less vulnerable to the adverse effects of three weeks of chronic social defeat stress. Mice were subsequently analyzed with regards to physiological, neuroendocrine, behavioral and mRNA expression alterations. Our results show a less vulnerable phenotype of 51KO mice with respect to physiological and neuroendocrine parameters compared to wild-type animals. 51KO mice demonstrated lower adrenal weights and basal corticosterone levels, a diminished response to a novel acute stimulus and an enhanced recovery, as well as more active stress-coping behavior. These results suggest an enhanced negative glucocorticoid feedback within the HPA axis of 51KO mice, possibly modulated by an increased sensitivity of the GR.

Introduction

In daily life, humans are repeatedly exposed to periods of stress. The short-term effects of stress are mostly beneficial by promoting adaptation, as long as the stress response is efficiently shut off. In contrast, excessive challenges like chronic stress clearly impose a major risk factor for the development of a variety of psychiatric diseases such as anxiety related disorders and depression (McEwen, 2004; de Kloet et al., 2005a).

The major control module of the stress response in mammals, besides the autonomic nervous system, is the hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoids (GCs), which are the main hormonal endpoint of the HPA axis, regulate the activity of the HPA axis through negative feedback via the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). MRs and GRs mediate this regulation mainly in the hypothalamus and the pituitary, but also on the level of the hippocampus and other limbic structures (De Kloet et al., 1998; Ulrich-Lai and Herman, 2009). The proper negative feedback via the GR is critical for a healthy stress response. One of the most robust biological abnormalities observed in the majority of depressed patients is altered signalling via the GR, which often leads to an impaired negative feedback regulation and thus to partial glucocorticoid resistance (Holsboer, 2000; Pariante and Miller, 2001).

The GR is a member of the ligand-dependent transcription factor family (De Kloet and Reul, 1987). Upon ligand binding, the receptor undergoes a conformational change, translocates from the cytosol to the nucleus and modulates gene transcription. The process of GR activation, trafficking and subsequent GR action on gene transcription is regulated by a multiprotein complex that assembles around the molecular chaperone heatshock protein 90 (hsp90) (Pratt et al., 2006). The hsp90 complex is crucial for proper folding, maturation, translocation and DNA binding of the GR and consists of numerous co-chaperones (Grad and Picard, 2007). These include, among others, the immunophilins FK506 binding protein 51 (FKBP5) and FK506 binding protein 52 (FKBP4). Recent studies have shown that FKBP5 is a key player of the GR-hsp90 complex. Once FKBP5 is bound to the GR complex via hsp90, the receptor's affinity for cortisol is decreased (Riggs et al., 2003; Wochnik et al., 2005). Upon ligand binding, FKBP5 is replaced by FKBP4, which in turn binds to dynein. This promotes the translocation of the GR complex into the nucleus and subsequent DNA binding (Davies et

al., 2002; Wochnik et al., 2005; Binder, 2009). In neurons, siRNA knockdown of the gene encoding FKBP5 is associated with elevated baseline GR nuclear translocation (Tatro et al., 2009a). The endogenous overexpression of FKBP5 in squirrel monkeys has been demonstrated to be causative for their glucocorticoid resistance (Denny et al., 2000; Scammell et al., 2001; Westberry et al., 2006). Moreover, the expression of FKBP5 is stimulated by steroids, such as GCs, as part of an intracellular ultra-short negative feedback loop for GR activity (Vermeer et al., 2003; Hubler and Scammell, 2004). Therefore, augmented transcription and translation of FKBP5 following corticoid receptor activation reduces GR sensitivity. In addition, FKBP5 mRNA expression has recently been shown to be increased in brain regions such as the hippocampus, central amygdala and PVN of mice, after a single dexamethasone treatment (Scharf et al., 2011).

The suggested role of FKBP5 in GR signal transduction may represent a new possibility to elucidate GR impairment associated with many psychiatric disorders such as depression (Holsboer, 2000). It is well established that malfunction of GRs due to low affinity ligand binding can be the consequence of mutations of the receptor itself or of limited hsp90 action (Picard et al., 1990; Bohen and Yamamoto, 1993; Brönnegard et al., 1996). In humans, genetic variations of FKBP5 have been associated with a major risk factor for the development of posttraumatic stress disorder (PTSD) (Segman et al., 2005; Binder et al., 2008; Yehuda et al., 2009). Furthermore, the same alleles have been linked with the regulation of the HPA axis in depression, with an enhanced recurrence of depressive episodes, and with a faster antidepressant treatment response. In addition, these polymorphisms predispose individuals to increased sensitivity to psychosocial stress (Binder et al., 2004; Ising et al., 2008; Kirchheiner et al., 2008). Specifically, it has been shown that the risk alleles result in a higher FKBP5 expression, thereby lowering GR sensitivity, which is suggested to be the underlying mechanisms for the increased sensitivity to psychosocial stress.

It is still largely unclear why some individuals are more susceptible to develop a stress-related disorder, while others turn out to be resilient. Recent studies suggest that genetic predispositions interact with environmental demands such as chronic stress (Pezawas et al., 2005; Binder, 2009; Gillespie et al., 2009; Schmidt et al., 2010a). However, the molecular mechanisms underlying individual susceptibility or resilience to chronic stress are still poorly

understood. Given the fact that FKBP5 has been suggested to be a possible mediator of the stress response system and its potential role in the development of stress-related disorders, we hypothesised that mice lacking the FKBP5 gene may show a lower vulnerability to chronic stress exposure. To test this hypothesis, we analyzed the response of conventional FKBP5 knockout (51KO) mice to a chronic social defeat stress (CSDS) paradigm at the behavioral, neuroendocrine and molecular level.

Materials and Methods

Animals and housing conditions

The FKBP5 knockout mouse line (51KO) was previously generated (Tranguch et al., 2005). Since *fkbp5* is too large to efficiently target all coding sequences, a *lacZ*/neomycin cassette was inserted in place of sequences from exons 1-2. The partially disrupted gene retains the endogenous FKBP5 promoter and expresses β-galactosidase in-frame with the FKBP5 initiation codon (Supplemental Fig. 1A). Details of the generation of the 51KO mice are provided in the supplemental material. Genotypes were verified by PCR and Southern analysis of tail DNA, and the absence of FKBP51 protein was verified by fluorescence immunohistochemistry and western blotting of mouse tissue extracts (Supplemental Fig. 1B and C). Since most of the *fkbp5* exons were retained and could potentially encode a truncated protein in transgenic mice, Westerns were repeated with four anti-FKBP5 monoclonal antibodies having distinct epitopes and with a rabbit polyclonal antibody prepared against full-length FKBP5; none of these antibodies detected an FKBP5 protein product (results not shown). 51KO mice did not show overt reproductive failures and no abnormalities in the survival rate. The genotypes of the offspring corresponded approximately to the Mendelian ratios and sex was equally distributed. Only male mice were used for the experiment, obtained from heterozygous breeding pairs. Animals were 13 weeks old at the start of the experiment. Mice were singly housed as adults at least two weeks prior to the experiment. Maintenance and experiments were performed under a 12 h light, 12 h dark cycle (lights on at 7:00 am) and constant temperature (23 ± 2 °C) conditions. Food and water were provided *ad libitum*.

Male CD1 mice (19 - 21 weeks old) serving as residents were held under the same conditions as described above and were allowed to habituate to the social defeat cage for two weeks before the onset of the experiments. The experiments were performed in accordance with European Communities Council Directive 86 / 609 / EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Chronic social defeat stress paradigm

The CSDS paradigm was performed as described previously (Wagner et al., 2011). In short, mice were subjected to daily bouts of social defeat by a resident mouse, which was physically superior and specifically trained for aggressive behavior towards intruders. The animals were separated as soon as the aggressive confrontation was accomplished, or after a maximum of 5 min. Subsequently, the animals spent 24 h in the same cage (45 cm x 25 cm), which was divided by a mesh partition, to prevent physical, but allow sensory contact. Every day, stressed animals were introduced to a new resident cage, in order to exclude a repeated encounter with the same resident throughout the experiment. Experimental mice were always defeated by resident males during the course of the experiment. Control mice were held in their home cages for the entire experimental period. All mice were handled daily; fur status and weight were determined every 3-4 days before social defeat was enforced.

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

Experimental design

Initially, 24 wild-type (WT) and 21 51KO mice were split into four groups (n = 13 WT control, n = 11 51KO control, n = 11 WT stress and n = 10 51KO stress) and subjected to the CSDS paradigm for 21 days. The daily defeat occurred between 12:00 pm and 4:00 pm; varying starting times limited the predictability of the stressor and therefore minimized a potential habituation effect. During the third week of the procedure, all behavioral tests were performed.

Behavioral analysis

Behavioral tests were carried out between 08:00 am and 12:00 pm in the same room where the animals were housed. All tests were performed using an automated video-tracking system (Anymaze 4.20, Stoelting, IL, USA).

Open-field test

The open-field (OF) test was conducted on day 15 of the CSDS paradigm. Testing was performed in an empty open-field arena (50 cm x 50 cm x 50 cm) made of gray polyvinyl chloride (PVC), which was evenly illuminated with 15 lux. Testing duration was 15 min, divided into three segments of 5 min each. Parameters of interest included the total distance traveled and the time of immobility.

Elevated plus-maze

On day 16 of the stress procedure, the elevated plus-maze (EPM) test was performed. The apparatus consisted of a plus-shaped platform with two opposing open arms (30 cm x 5 cm x 0.5 cm) and two opposing enclosed arms (30 cm x 5 cm x 15 cm), made of gray PVC, which were connected by a central area (5 cm x 5 cm). The whole device was elevated 50 cm above the floor. Illumination was less than 10 lux in the enclosed arms and 25 lux in the open arms. The trial duration was set to 5 min and mice were placed into the center zone facing one of the closed arms at the beginning of the test. The time spent in the open arms compared to the time spent in the closed arms and the total distance traveled were analyzed.

Forced swim test

The forced swim test (FST) was performed on day 19 of the CSDS procedure. Each mouse was placed into a 2 liter glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing time was 6 min. The parameters floating, swimming and struggling were scored by an experienced observer, blind to genotype, condition or treatment of the animals.

Acute stress response

The FST on day 19 was used as acute stressor in order to determine the stress response by measuring corticosterone plasma concentrations. After the FST, all mice were placed into a novel cage to recover from the acute stressor. Blood samples were collected by tail cut 15 min (stress response) and 90 min (stress recovery) after the onset of the FST (Fluttert et al., 2000). Samples were gathered in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). After the recovery period and the second tail cut, mice were returned to their initial home (for controls) or social defeat (for stressed animals) cages. All

blood samples were kept on ice and later centrifuged for 15 min at 8000 rpm and 4 °C. Plasma was transferred to new, labelled microcentrifuge tubes and stored at -20 °C until further processing.

Sampling procedure

All animals were sacrificed by decapitation following quick anaesthesia by isolflurane. Trunk blood was collected in labelled 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). All blood samples were kept on ice until centrifugation at 4 °C and 8000 rpm for 15 min. After centrifugation, the blood plasma was transferred to a new, labelled 1.5 ml microcentrifuge tube. All plasma samples were stored frozen at -20 °C until the determination of corticosterone by radioimmunoassay (MP Biomedicals Inc; sensitivity 6.25 ng/ml). Brains were removed, snap-frozen in isopentane at -40 °C, and stored at -80 °C for *in-situ* hybridization. Adrenal and thymus glands were removed, dissected from fat and weighed.

In-situ hybridization

Frozen brains were sectioned at -20 °C in a cryostat microtome at 18 µm, thaw mounted on Super Frost Plus slides, dried and stored at -80 °C. *In-situ* hybridization using ^{35}S UTP labeled ribonucleotide probes (CRH, AVP, GR, MR) was performed as described previously (Schmidt et al., 2007). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA probes were transcribed from a linearized plasmid. Tissue sections were saturated with 100 µl of hybridization buffer containing approximately 1.5 × 10^6 cpm ^{35}S labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55 ºC. The following day, the sections were rinsed in 2 × SSC (standard saline citrate), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 × SSC for 1h at 65 ºC and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software. The mean of four measurements of two different brain slices was calculated for each animal. The data were

analyzed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest from the measurements.

For AVP, slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY) and exposed at 4 °C for 8 h. Slides were developed and examined with a light microscope with both bright and dark field condensers. Expression of AVP mRNA in the parvocellular part of the PVN was determined by optical densitometry as described above.

Statistical analysis

The data presented is shown as means ± standard error of the mean (SEM), analyzed by the software SPSS 16.0. For body weight and fur status progression as well for the time-dependent behavioral parameters assessed in the open field tests, 3-factorial analyses of variance (ANOVA) with repeated measures was performed. Thereby genotype and condition were two between-subjects factors and time a within-subjects factor. Two factorial multivariate ANOVAs were employed for thymus, adrenal gland weight, neuroendocrine, and other behavioral variables and for gene expression contrasts as well. Whenever significant main or interaction effects were found in the ANOVAs univariate F-tests or tests with contrasts followed for specifying and locating simple effects. For some simple comparisons, t-tests for dependent or independent samples were performed. As nominal level of significance was alpha = 0.05 accepted and adjusted according to Bonferroni correcture by all posteriori tests (univariate F-tests, test of simple effects or contrasts).

Results

Physiological parameters

At the start of the experiment, statistical analysis showed significantly lower body weights for 51KO mice compared to WT animals (T_{43} = 2.279, $p < 0.05$) (Fig. 1A). For body weight progression, ANOVA with repeated measures revealed a significant effect of time ($F_{6, 36}$ = 17.613, $p < 0.0001$) as well as a time x condition interaction ($F_{6, 36}$ = 2.593, $p < 0.05$). Subsequent analysis of simple effects identified a significant increase especially at the days 8, 11 and 21 in the body weight of stressed WT mice compared to their littermate controls (tests with contrasts in ANOVA, $p < 0.05$). A significant increase at the days 8, 11 and 18 in body weight was also revealed in stressed 51KO mice compared to their littermate controls (Fig. 1B).

For the animals fur state, ANOVA with repeated measures found a time effect ($F_{6, 36}$ = 7.403, $p < 0.0001$), a condition effect ($F_{1, 41}$ = 29.828, $p < 0.0001$) and a time x condition interaction ($F_{6, 36}$ = 7.805, $p < 0.0001$). Further investigation of the simple effects revealed a significant decrease in fur state quality of stressed animals compared to their respective genotype controls throughout the experiment from day 4 to day 21 (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 1C). No differences between both genotypes were observed.

Organ weights were measured on the day of sacrifice. Adrenal gland weight showed a condition effect ($F_{1, 41}$ = 95.170, $p < 0.0001$) as well as a genotype effect ($F_{1, 41}$ = 18.651, $p < 0.0001$). Following chronic social stress, significantly increased adrenal gland size could be shown regardless of genotype (Mean ± SEM: control: WT = 0.134 ± 0.004, 51KO = 0.117 ± 0.005; defeat: WT = 0.226 ± 0.013, 51KO = 0.176 ± 0.008). However, a significant decrease in adrenal gland weights was revealed in 51KO animals compared to WT mice under control and stress conditions (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 1D).

For thymus weight, ANOVA revealed a condition ($F_{1, 41}$ = 67.769, $p < 0.0001$) and a genotype effect ($F_{1, 41}$ = 53.140, $p < 0.0001$). Chronic stress resulted in a significant decrease in thymus weight in both WT and knockout animals (Mean ± SEM: control: WT = 1.545 ± 0.054, 51KO = 1.184 ± 0.058; defeat: WT = 1.131 ± 0.059, 51KO = 0.680 ± 0.067). In addition, thymus weight was significantly lower in 51KO mice compared to WT animals under control and stress conditions (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 1E).

Figure 1

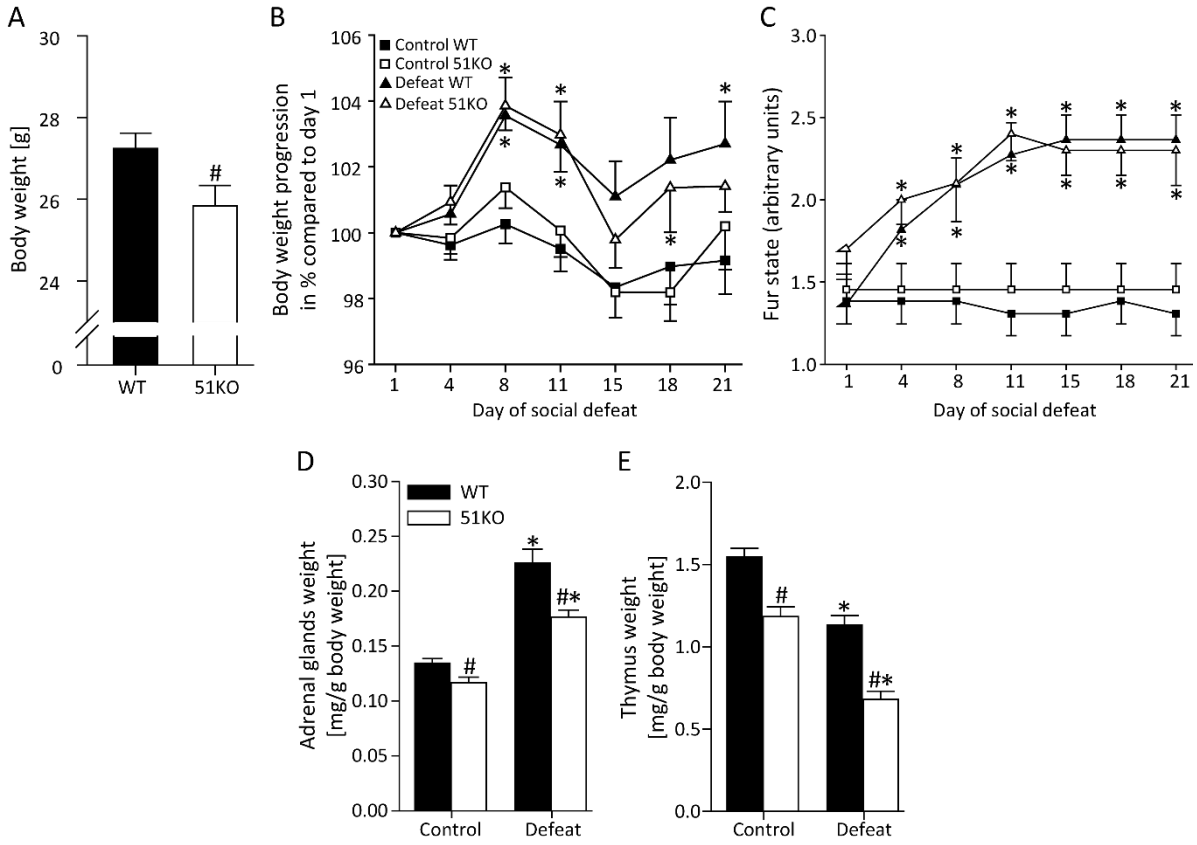

Figure 1: Physiological phenotype of 51KO and WT mice. (A) Body weight before the start of the CSDS. (B) Body weight gain over the course of the experiment. (C) Progression of fur quality during the stress procedure. (D) Adrenal and (E) thymus weight at the end of the stress exposure. Data are represented as mean ± SEM. * significantly different from the control condition of the same genotype, $p < 0.05$; # significantly different from the WT of the same condition, $p < 0.05$.

Neuroendocrine analysis

Basal levels of corticosterone were assessed in blood plasma during the circadian nadir in the morning. Furthermore, response and recovery blood samples were taken 15 min and 90 min after the onset of the FST. ANOVA showed a genotype effect for basal corticosterone levels ($F_{1, 38} = 7.619$, $p < 0.01$) as well as a genotype by condition interaction ($F_{1, 38} = 9.800$, $p < 0.01$). Analyzing the simple effects i.e. condition differences for each genotype and genotype differences for each condition we found that chronic stress led to increased corticosterone levels in WT animals compared to their control littermates. In contrast, stressed 51KO mice revealed lower basal levels of corticosterone compared to control 51KO animals. Furthermore, significantly lower corticosterone levels were seen in stressed 51KO mice compared to stressed WT mice (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 2A).

Corticosterone response levels (15 min after the FST) indicated a genotype effect ($F_{1, 39} = 4.428$, $p < 0.05$), a condition effect ($F_{1, 39} = 10.777$, $p < 0.005$) and a genotype by condition interaction ($F_{1, 39} = 4.489$, $p < 0.05$). In WT animals, chronic stress resulted in significantly higher corticosterone levels compared to non-stressed controls. This effect of chronic stress exposure was not observed in 51KO mice, leading to a reduced stress response, with significantly lower corticosterone levels in stressed 51KO mice compared to stressed WT animals (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 2B).

For corticosterone recovery levels (90 min after the FST), ANOVA revealed a genotype effect ($F_{1, 37} = 11.072$, $p < 0.005$). WT animals of both conditions showed no difference in recovery levels. In 51KO mice, CSDS resulted in higher corticosterone values 90 min after the FST. However, 51KO mice displayed an increased ability for negative feedback regulation with significantly lower corticosterone levels under control and stress conditions compared to WT animals (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 2C).

Figure 2

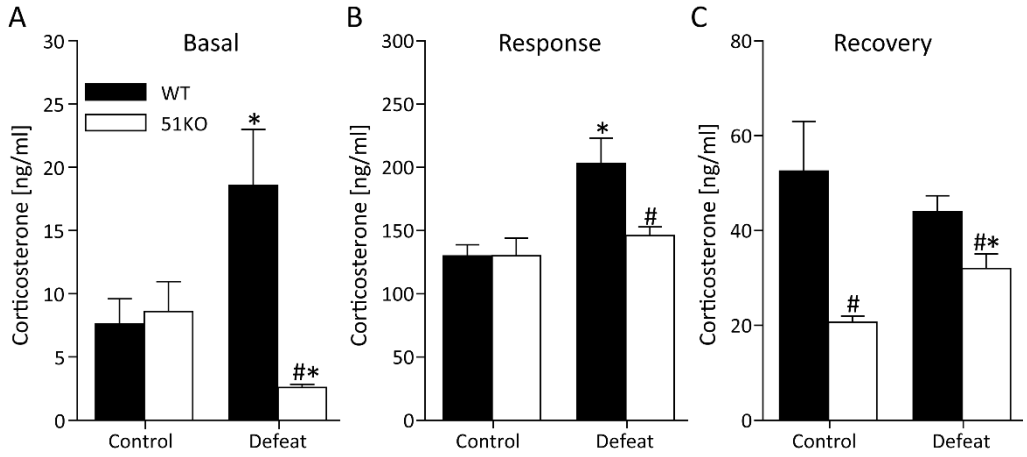

Figure 2: Neuroendocrine profile of 51KO and WT animals. (A) Basal morning corticosterone levels at the end of the chronic stress procedure. (B) Corticosterone response 15 min after the FST. (C) Corticosterone recovery 90 min after the onset of the FST. Data are represented as mean ± SEM. * significantly different from the control condition of the same genotype, $p < 0.05$; # significantly different from the WT of the same condition, $p < 0.05$.

Behavioral analysis

Three tests were performed in order to investigate exploration, anxiety and stress-coping behavior (OF, EPM, FST).

Open-field test

The OF was used to quantify behavioral responses such as locomotor activity and explorative behavior. We subdivided the 15 min test interval in three segments of 5 min each. ANOVA of repeated measures revealed a time x condition interaction ($F_{2, 40}$ = 14.867, $p < 0.0001$) concerning the general locomotor activity. In WT animals, CSDS resulted in a significant decrease of the total distance traveled compared to their control littermates in segment two and segment three (segment 2: $F_{1, 22}$ = 17.12, $p < 0.0001$; segment 3: $F_{1, 22}$ = 5.46, $p < 0.05$). Under control conditions, 51KO mice showed a significant increase in the distance traveled compared to WT animals for segment two ($F_{1, 22}$ = 8.21, $p < 0.01$). Furthermore, a significant decrease of locomotor activity in stressed 51KO animals compared to control 51KO mice was revealed in all three segments (segment 1: $F_{1, 22}$ = 6.93, $p < 0.05$; segment 2: $F_{1, 22}$ = 35.71, $p < 0.0001$; segment 3: $F_{1, 22}$ = 20.27, $p < 0.0001$) (Fig. 3A).

For the time spent immobile, ANOVA with repeated measures illustrated a time x condition interaction ($F_{2, 40}$ = 8.770, $p < 0.005$). Since genotype did not reveal a main or interaction effect we pooled the data of the two genotypes for the following simple effect analysis. The time immobile was significantly higher in all three segments of stressed mice compared to non-stressed animals (segment 1: $F_{1, 43}$ = 5.92, $p < 0.05$; segment 2: $F_{1, 43}$ = 61.38, $p < 0.0001$; segment 3: $F_{1, 43}$ = 26.38, $p < 0.0001$). There was no difference between the two genotypes (Fig. 3B).

Elevated plus-maze test

The EPM was used to investigate anxiety-related behavior. For the time spent in the open arms, there was no significant effect of condition or genotype (Fig. 3C). The total distance traveled revealed a condition effect ($F_{1, 37}$ = 23.026, $p < 0.0001$). Irrespective of genotype chronically stressed animals showed significantly lower locomotor activity compared to their respective control group (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 3D).

Forced swim test

We examined the animals' stress coping behavior using the FST. Regarding the time spent struggling, ANOVA revealed only a significant condition effect. Irrespective of genotype chronically stressed mice spent significantly less time struggling compared to mice under control conditions ($F_{1, 42}$ = 8.912, $p < 0.01$) (Fig. 3E).

For the time swimming, only a genotype effect was revealed. Stressed 51KO animals spent more time swimming compared to stressed WT mice ($F_{1, 42}$ = 4.788, $p < 0.05$) (Fig. 3F). Concerning the parameter time floating, ANOVA did not reveal any significant effects (Fig. 3G).

Figure 3

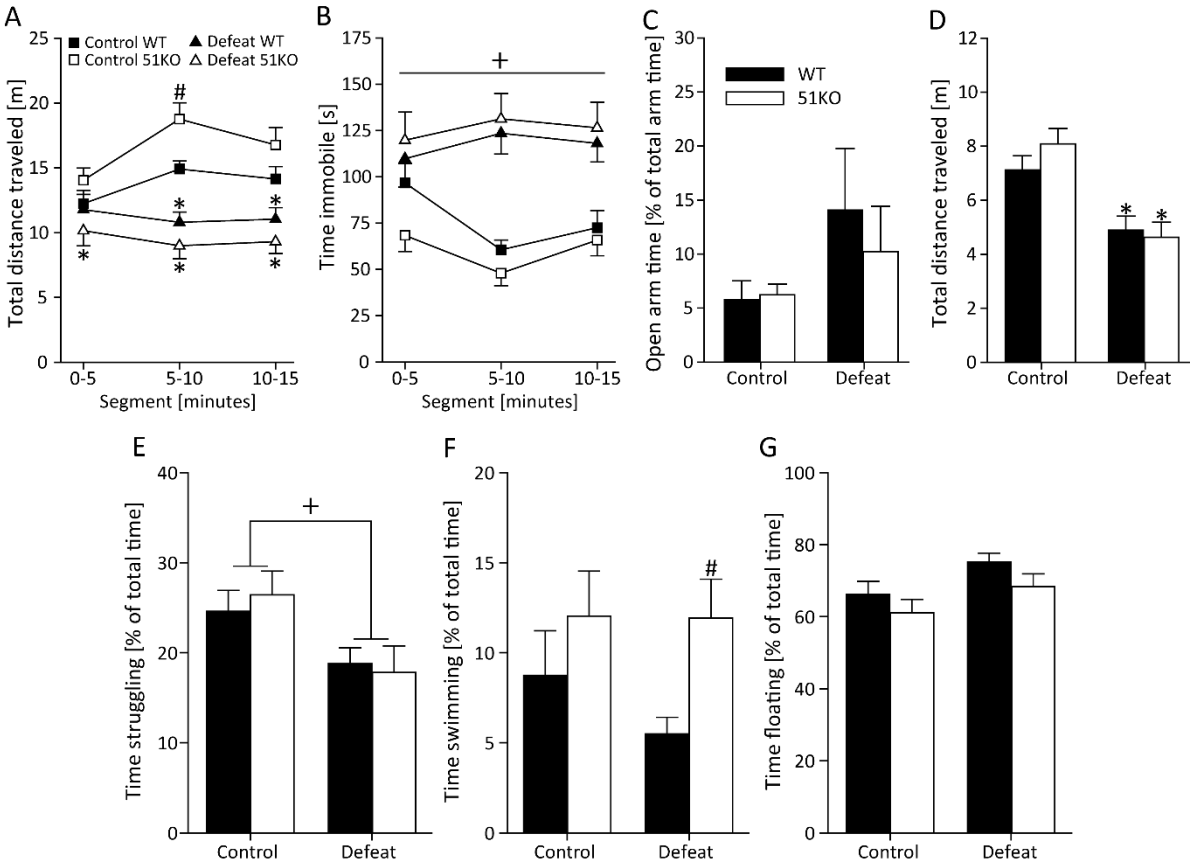

Figure 3: Behavioral profile of 51KO and WT mice. (A) Total distance traveled in the OF. (B) Time spent immobile in the OF. (C) Time spent in the open arms of the EPM. (D) Total distance traveled in the EPM. (E) Time spent struggling in the FST. (F) Time spent swimming in the FST. (G) Time spent floating in the FST. Data are represented as mean ± SEM. * significantly different from the control condition of the same genotype, $p < 0.05$; # significantly different from the WT of the same condition, $p < 0.05$; + significant condition effect, $p < 0.01$.

Gene expression analysis

We performed *in-situ* hybridization in the hippocampus and PVN in order to examine expression patterns of genes involved in the major regulation and mediation of the HPA axis. For CRH mRNA expression in the PVN, ANOVA showed a condition effect. Chronically stressed animals showed higher CRH expression levels than their respective controls of the

same genotype ($F_{1, 38}$ = 12.886, p < 0.005) (Fig. 4A and B). No significant changes in mRNA expression could be shown for AVP (Fig. 4C and D) and GR in the PVN and MR and GR in the hippocampus (data not shown).

Figure 4

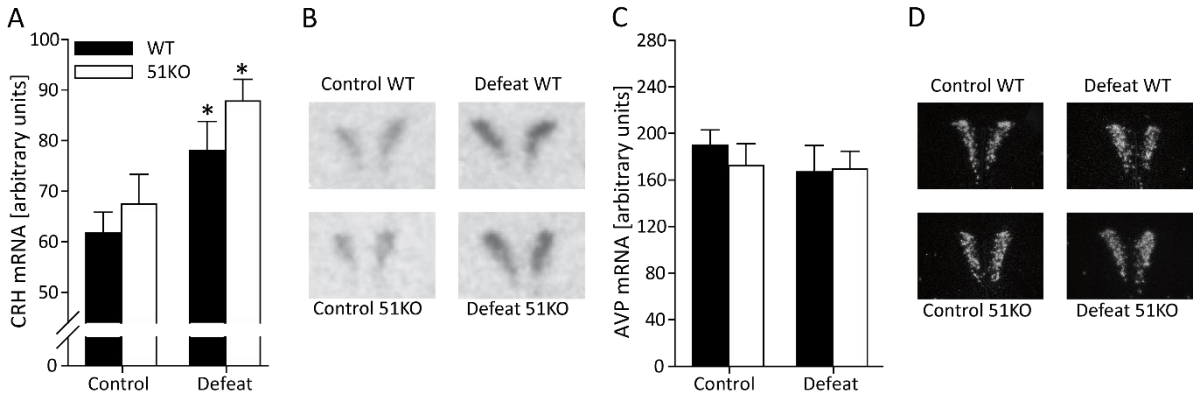

Figure 4: Gene expression in the PVN of 51KO and WT animals. (A) CRH mRNA expression in the PVN. (B) Representative CRH mRNA autoradiograms in the PVN. (C) AVP mRNA expression in the PVN. (D) Representative AVP mRNA dark field photomicrographs in the PVN. Data are represented as mean ± SEM. * significantly different from the control condition of the same genotype, p < 0.05.

Discussion

Chronic stress represents a key risk factor for developing many psychiatric diseases (de Kloet et al., 2005a; de Kloet et al., 2005b; Bale, 2006), and the CSDS paradigm has been frequently used to modulate depression in rodents (Nestler and Hyman, 2010). However, susceptibility to stress-related disorders like depression is determined by a combined effect of (epi-)genetic predispositions and environmental influences (Caspi and Moffitt, 2006; Krishnan and Nestler, 2008; El Hage et al., 2009). By using mice with a conventional deletion of the FKBP5 gene, we were able to provide evidence that the co-chaperone FKBP5 significantly influences the animals' stress response under basal and chronic stress conditions.

The chronic social defeat stress paradigm resulted in a number of robust physiological alterations in WT and 51KO animals, such as adrenal gland enlargement and thymus atrophy, which have repeatedly been demonstrated before (Klein et al., 1992; Karst and Joëls, 2003; Keeney et al., 2006; Schmidt et al., 2007; van den Brandt et al., 2007). However, we found significantly lower adrenal gland weights in 51KO mice compared to WT animals under both conditions. Therefore, we cannot exclude an aberrant impairment of organ development due to the genetic deletion of the gene. Nonetheless, the decrease in adrenal weight may also implicate a reduced HPA axis activity and an improved negative feedback regulation under stress and even under control conditions. FKBP5 has previously been shown to decrease GR sensitivity in cellular model systems (Wochnik et al., 2005; Westberry et al., 2006). Our findings support the hypothesis that FKBP5 reduces GR responsiveness also in physiological settings relevant for psychiatric diseases. Our data of lower thymus weights in 51KO mice compared to WT animals under both conditions may partly provide further evidence for this line of reasoning, as studies of adrenolectomized mice with a thymus-specific inducible GR transgene reported a decrease in thymocyte number after induced GR overexpression, which could be prevented by the application of a GR antagonist (Pazirandeh et al., 2005). In addition, transgenic rats, overexpressing a mutant GR in the thymus with increased ligand affinity caused massive thymocyte apoptosis at physiological hormone concentrations, which was reversible by adrenalectomy (van den Brandt et al., 2007). However, the observed lower thymus weights in 51KO mice may very well be a combination of an impaired organ development, chronic stress and increased GR sensitivity.

In addition, all chronically stressed mice showed a significant decrease in fur quality regardless of genotype. The degradation of the fur state can be ascribed to an impairment of an animal's grooming behavior, which has been associated with a depression-like phenotype (Ducottet et al., 2003; Mineur et al., 2003; Cryan and Holmes, 2005; Denmark et al., 2010). Changes in body weight upon chronic stress are well documented, but rather contradictory and still not fully understood. In mice, detailed studies indicate an increase in body weight depending on the kind of stressor and the animals' social status (Bartolomucci et al., 2009; Wagner et al., 2011). Our findings are in line with these studies, revealing a significant increase in body weight progression in all chronically defeated animals. Therefore, both body weight progression during CSDS as well as fur state deterioration may be GR-independent phenomena. However, 51KO mice were lighter at the beginning of the stress procedure, possibly due to the enhanced GR sensitivity, as elevated levels of GCs are observed in most strains of genetically obese mice and adrenalectomy and glucocorticoid antagonists tend to attenuate obesity (Friedman and Halaas, 1998). Taken together, the chronic social defeat stress paradigm led to a number of physiological changes, including lower thymus weight and fur quality as well as higher adrenal weight and body weight gain. Furthermore, differences in 51KO mice compared to WT animals, such as lower thymus and adrenal weights as well as the initially lower body weight are in line with the predicted higher GR activity in those mice.

Circulating GCs are another robust indicator of stress and HPA axis activity. It has repeatedly been shown that chronic stress results in elevated circulating GCs and in a dysregulation of the HPA axis regulatory feedback (Sapolsky, 1992; Bartolomucci et al., 2005; Keeney et al., 2006; Schmidt et al., 2007). In line with these studies we found significantly elevated basal corticosterone levels in stressed WT animals compared to controls of the same genotype. In contrast, we detected significantly lower basal corticosterone levels in 51KO mice compared to WT animals under chronic stress conditions. Moreover, it has been shown, that rodents exposed to chronic stress exhibit an enhanced HPA axis response to a novel, acute stimulus (Akana et al., 1992; Koolhaas et al., 1997; Bhatnagar and Dallman, 1998). Indeed, we were able to detect a significantly higher corticosterone response 15 min after the FST for chronically stressed WT mice compared to controls of the same genotype. However, this chronic stress effect was not detected in 51KO mice. Corticosterone levels, measured 90

min after the FST were significantly higher in chronically stressed 51KO mice compared to controls of the same genotype, indicating that chronic stress still impairs the recovery in 51KO mice. However, both groups of 51KO mice still had significantly lower corticosterone levels compared to WT animals of the respective condition, implicating an enhanced recovery. Thus, our neuroendocrine data suggest an enhanced negative feedback within the HPA axis of 51KO mice and consequently a lower vulnerability to stress in this parameter, which is likely mediated by the enhanced GR sensitivity.

Chronic stress also affects the behavioral phenotype of animals, leading to higher anxiety-related behavior and diminished locomotor activity (Choleris et al., 2001; Berton and Nestler, 2006; Schmidt et al., 2007; Wagner et al., 2011). In the current study, CSDS resulted in lower locomotor activity in a novel environment (OF / EPM). The data of the EPM in terms of open arm time are not conclusive and may be compromised by the differences in locomotor activity. However, in contrast to the neuroendocrine and physiological data, we did not detect differences for anxiety-related behavior in 51KO mice. In the OF the magnitude of the stress effect compared to baseline was even greater in 51KO mice compared to WT animals. It is therefore likely that locomotion and anxiety-related behavior as assessed in the current study is largely independent of the FKBP5 modulation of GR signaling.

In order to determine potential differences in stress coping, 51KO mice and WT animals were subjected to a FST. It has been shown that chronic stress increases immobility and antidepressant sensitivity in mice subjected to the FST (Karolewicz and Paul, 2001). In line with this study, we could show a diminished active stress-coping behavior in chronically stressed mice compared to control animals. Interestingly, chronic stress led to a significant increase in time swimming in 51KO mice compared to WT mice. In contrast, there were no significant genotype differences in the other two core parameters of the FST. However, our data is in line with findings from Touma and colleagues, who observed a more active stress-coping behavior in 51KO mice 24 h following restraint stress (Touma et al., 2011).

Chronic stress has previously been shown to affect gene expression of central HPA axis related genes, such as MR and GR in the hippocampus as well as CRH and AVP in the PVN (Makino et al., 1995; Albeck et al., 1997; Herman and Spencer, 1998; Bartolomucci et al., 2003; Figueiredo et al., 2003; Keeney et al., 2006; Schmidt et al., 2007; Lachize et al., 2009).

In the current study we observed an increase of CRH expression in the PVN after chronic stress, which is in line with previous reports. The lack of AVP, MR and GR regulation may be ascribed to the length, nature or intensity of the applied stress protocol. However, gene expression regulation was not affected by the lack of FKBP5, which may point at compensatory mechanisms.

A limitation of the current study may be the general involvement of FKBP5 in the maturation of other steroid receptors (Pratt and Toft, 2003). Therefore, the observed effects may not exclusively be due to increased GR binding, but might partially be a combination of various other receptor types. Furthermore, we used mice with a conventional knockout of FKBP5, thus potential and yet unidentified compensatory mechanisms or adaptive processes in 51KO mice cannot be excluded. A spatial and temporal restriction of FKBP5 expression using conditional knockouts might reveal a clearer picture of the involvement of this gene in stress related disorders and may be a useful tool to provide further insight of the utility of FKBP5 as a therapeutic target. Nevertheless, our data clearly show the importance of FKBP5 as a mediator of the stress response and its involvement in the development of stress related disorders.

In summary, we demonstrate that animals with a conventional FKBP5 deletion are less responsive to the adverse effects of chronic stress with regards to physiological and neuroendocrine parameters. 51KO mice displayed a diminished physiological and neuroendocrine response to chronic social defeat stress, evidenced by lower adrenal weights as well as lower basal corticosterone levels, an attenuated response to a novel acute stimulus and an enhanced recovery, as well as more active stress coping. In contrast, no protective effects were observed for locomotor activity and anxiety-related behavior or central gene expression. Thus, the current data support the hypothesis that 51KO mice are less affected by chronic social stress on a number of parameters, due to an enhanced sensitivity of the GR. Pharmacological inhibition of FKBP5 might thus constitute a novel therapeutic approach to ameliorate the neuroendocrine alterations observed in patients with stress-related psychiatric disorders.

Supplementary data

Generation of 51KO mice

Bacterial artificial chromosome (BAC) clones containing genomic regions for FKBP5 were isolated by PCR screening the 129SvJ mouse BAC library (Genome Systems, St. Louis, MO). Restriction fragments were subcloned into pBluescript (pBS; Stratagene, La Jolla, CA) or pZero (Invitrogen, Carlsbad, CA) for further analysis and sequencing. PCR products amplified from the BAC clones were used to construct a targeting vector in pPGK*neo* (kindly provided by James Lee, Mayo Clinic Scottsdale). As shown in supplemental Fig. 1, the targeting vector contained a beta-galactosidase expression and neomycin resistance cassette flanked upstream by a 3 kb PCR product containing parts of intron A and the first 12 amino acids of FKBP5 and downstream by a 2.4 kb PCR product containing parts of intron B, exon 3 and parts of intron C. Homologous recombination using the final construct resulted in removal of most of exon 2 from FKBP5. Sequences for PCR primers or hybridization probe used in these studies are available upon request. ES cells isolated from 129SvJ mouse were cultured in Knockout DMEM (Invitrogen) supplemented with 10% FBS, penicillin/streptomycin, essential amino acids, ESGRO (103 U/ml; Chemicon, Temecula, CA) with irradiated embryonic fibroblast feeder cells. ES cells were electroporated at 0.2 kV, 950µF (Gene Pulser II; Bio-Rad, Hercules, CA) with linearized targeting vectors and selected with G418 (300µg/ml). DNA from G418-resistant clones was isolated for Southern blot analysis. A DNA probe was used to distinguish *Pst*I restriction fragments from wildtype (~7.5kb) and mutant (~10kb) alleles. Appropriate homologous recombination in ES cell clones was confirmed by PCR using primers complementary to sequences within the neomycin cassette and to 3' FKBP5 sequences downstream from the recombination site. ES cell clones containing a mutant FKBP5 allele were injected into C57BL/6 blastocysts and implanted into pseudopregnant 129SvJ females. Chimeric offspring were identified by coat patterns and mated to C57BL/6 mice.

Western blot

Cortex were homogenized in ice-cold lysis buffer (50mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton 100, protease inhibitor cocktail, Sigma, Deisenhofen, Germany) and centrifuged

(30 min, 12.000 rpm, 4°C) to eliminate cell debris. Protein concentrations were determined using a detergent compatible protein assay kit (Bio-Rad, Hercules, CA, USA). Samples containing 100 µg of protein per lane were loaded on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred onto nitrocellulose membranes (Invitrogen, Karlsruhe, Germany). The membranes were blocked with 5 % milk in TBS containing 0.1% Tween 20 (TBST-T) for 3h at room temperature and incubated with goat-anti-FKBP51 (1:500, 5% milk in TBS-T, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-actin (1:1000, 1% Milk in TBS-T, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies overnight at 4°C. Subsequently, membranes were washed 3x 10min in TBS-T. Following incubation with horseradish peroxidase-conjugated rabbit anti-goat (1:500, 5% Milk in TBS-T, overnight at 4°C, DAKO, Glostrup, Denmark) or donkey anti-goat (1:2000, 1% milk in TBST-T for 2h at room temperature, Santa Cruz Biotechnology, Santa Cruz, CA, USA**)** secondary antibodies, membranes were washed 3x10min in TBS-T and bands were visualized using an enhanced chemiluminescene system according to the manufacturer's protocol (Amersham Biosciences, Freiburg, Germany).

Fluorescence immunohistochemistry

Perfused brains were sectioned at -18°C in a cryostat at 30 µm in the coronal plane through the level of the dorsal hippocampus. Immunohistochemistry was performed on free-floating sections. After several washes (3x10min) with 0.1 M PB containing 0.3% Triton X-100 (PB-T), sections were treated with 1% donkey serum in PB-T for 1h to block nonspecific sites. Subsequently, sections were incubated overnight at 4°C with rabbit anti-FKBP51 (1:250, Abcam, Cambridge, UK) in PB-T containing 1% donkey serum and washed in PB-T (3x10min). Sections were incubated in donkey anti-rabbit Alexa Fluor 488 (1:500; Invirtogen, Darmstadt, Germany) in 1% donkey serum in PB-T for 3h. After washing (3x10min) with PB, sections were transferred onto slides with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector laboratories, Burlingame, CA, USA).

The fluorescent images (1600 × 1600 pixels) of area CA3 were obtained with an Olympus IX81 confocal microscope (Olympus, Tokyo, Japan) at 20× magnification using the Kalman filter and sequential scanning mode under identical settings for laser power, photomultiplier gain and offset. Images were imported into the NIH ImageJ software, converted to 8-bit

grayscale and thresholded uniformly. For visualization of FKBP51, sections from wild type and 51KO mice were incubated and developed in the same wells to ascertain identical conditions.

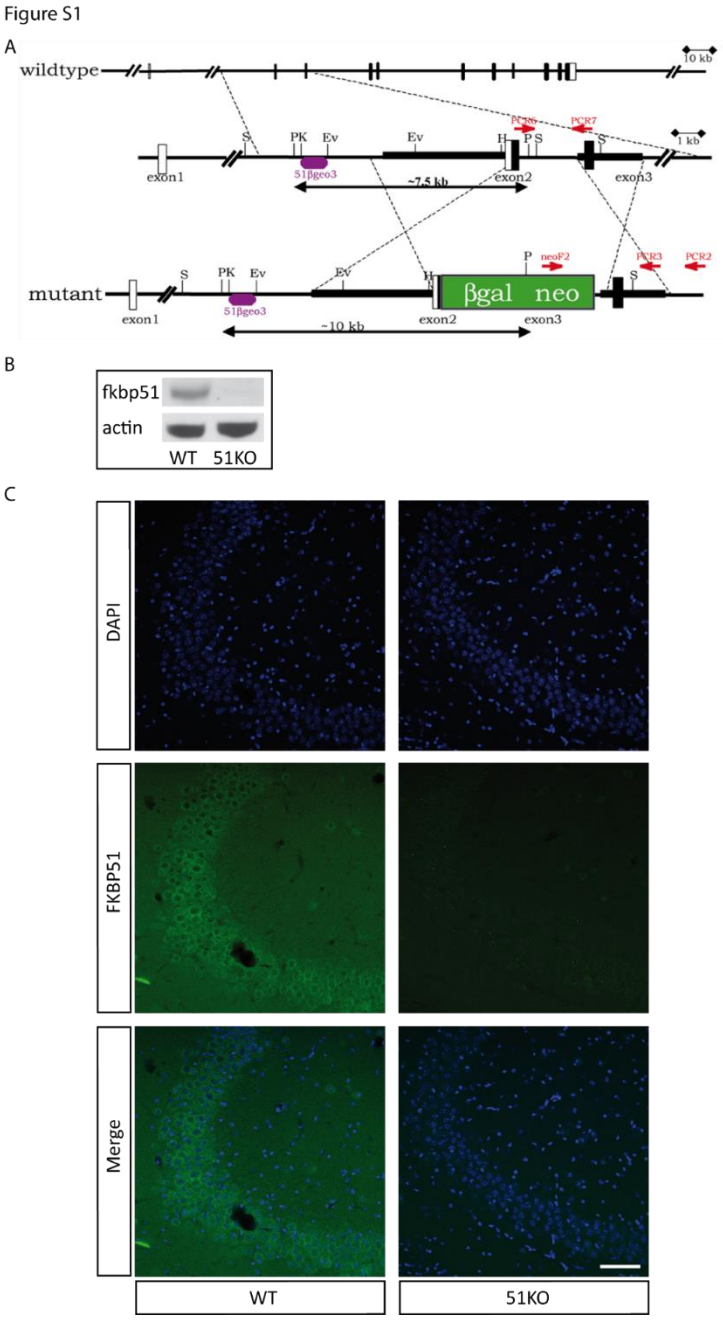

Figure S1: **A**, Targeting strategy. Mouse *fkbp5* is ~100 kb in size. Exon 2, the first coding exon, is replaced by an in-frame bgal/neo cassette, which permits monitoring *fkbp5* expression from the native 5' promoter. The 10 coding exons in the wildtype (wt) allele are depicted as *solid boxes*; the final exon of the mutant (mut) allele contains both coding and noncoding (*open box*) sequences. The βgal/neomycin resistance cassette (βgal/*neo, green*) is indicated as are restriction enzyme sites for *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), *Sac*I (S), and EcoRV (EV). The *bar* near the 5‘ end of either allele indicates the probe used for Southern blots (51βgeo2). *Short*, *bold arrows* on the 3‘ half indicate PCR primers used for genotyping. **B**,

Western blot analysis of cortex cytosol confirmed the absence of FKBP51 protein in homozygous mutant tissue. **C**, Representative confocal images of hippocampal area CA3 immunostained for DAPI and FKBP51. 51KO mice do not express FKBP51. Scale bar = 100 µm.

3.2. Depletion of FKBP51 in female mice shapes HPA axis activity

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Originally published in:

PLOS ONE, 2014 Apr; 9(4): e95796

Abstract

Psychiatric disorders such as depressive disorders and posttraumatic stress disorder are a major disease burden worldwide and have a higher incidence in women than in men. However, the underlying mechanism responsible for the sex-dependent differences is not fully understood. Besides environmental factors such as traumatic life events or chronic stress, genetic variants contribute to the development of such diseases. For instance, variations in the gene encoding the FK506 binding protein 51 (FKBP51) have been repeatedly associated with mood and anxiety. FKBP51 is a negative regulator of the glucocorticoid receptor and thereby of the hypothalamic–pituitary–adrenal axis that also interacts with other steroid hormone receptors such as the progesterone and androgen receptors. Thus, the predisposition of women to psychiatric disorders and the interaction of female hormones with FKBP51 and the glucocorticoid receptor implicate a possible difference in the regulation of the hypothalamic–pituitary–adrenal axis in female FKBP51 knockout (51KO) mice. Therefore, we investigated neuroendocrine, behavioral and physiological alterations relevant to mood disorders in female 51KO mice. Female 51KOs and wild type littermates were subjected to various behavioral tests, including the open field, elevated plus maze and forced swim test. The neuroendocrine profile was investigated under basal conditions and in response to an acute stressor. Furthermore, we analysed the mRNA expression levels of the glucocorticoid receptor and corticotrophin release hormone in different brain regions. Overall, female 51KO mice did not display any overt behavioral phenotype under basal conditions, but showed a reduced basal hypothalamic–pituitary–adrenal axis activity, a blunted response to, and an enhanced recovery from, acute stress. These characteristics strongly overlap with previous studies in male 51KO mice indicating that FKBP51 shapes the behavioral and neuroendocrine phenotype independent of the sex of the individual.

Introduction

Anxiety and mood related disorders such as major depressive disorder (MDD), are a major burden for society (Solhaug et al., 2012) affecting more than 1 billion people worldwide (Wittchen et al., 2011). Women are more susceptible to develop these disorders than men, which may relate to sex-dependent gene x environment interactions (Breslau, 2002; Solomon and Herman, 2009; Bangasser, 2013).

Besides the contribution of sex-specific factors, stress is a major environmental risk factor for depression, whose effects in mammals are mainly mediated by the hypothalamus–pituitary–adrenal (HPA) axis (Holsboer, 2000; de Kloet et al., 2005a). The secretion of glucocorticoids, the main end product of the HPA axis, is regulated by the activation of the mineralocorticoid receptor and glucocorticoid receptor (GR) in different brain regions, including the hypothalamus, pituitary and hippocampus (De Kloet et al., 2009). An impairment of this negative feedback loop results in an aberrant stress response and is associated with the development of MDD and anxiety disorders (van Rossum et al., 2006; Heim and Nemeroff, 2009; Tatro et al., 2009a; Bangasser, 2013; Menke et al., 2013).

The co-chaperone FK506 binding protein 51 (FKBP51; encoded by the gene *fkbp5*) acts as an inhibitor of GR activity, determines GR binding affinity to glucocorticoids and thus determines the negative feedback sensitivity of the HPA axis (Pratt et al., 2006; Cheung-Flynn et al., 2007; Tatro et al., 2009a). Moreover, translocation of the GR into the nucleus induces activation of an intracellular ultra-short feedback loop by directly enhancing *fkbp5* gene transcription, thus ultimately inhibiting GR activity (Hubler and Scammell, 2004; Binder, 2009; Storer et al., 2011). FKBP51 also acts as an inhibitor of other steroid hormone receptors, such as the progesterone and androgen receptors (Hubler and Scammell, 2004; Febbo et al., 2005), thereby potentially linking the stress effects to gonadal hormone signalling.

The involvement of FKBP51 in stress related disorders was first indicated by studies illustrating an association between multiple genetic variants of *fkbp5* and the risk of MDD and PTSD development in humans (Koenen et al., 2005; Binder et al., 2008; Kirchheiner et al., 2008; Binder, 2009). This relationship was further investigated in animal models to determine the mechanistic role of FKBP51 in HPA axis functioning. In both rats and mice,

mRNA expression and protein levels of FKBP51 in GR-rich brain regions can be inducedby administration of the glucocorticoid agonist dexamethasone, corticosterone (CORT) or by subjecting animals to stress paradigms namely restraint, food deprivation or chronic mild stress (Lee et al., 2010; Scharf et al., 2011; Yang et al., 2012; Guidotti et al., 2013). Depletion of FKBP51 in males did not reveal robust behavioral differences under basal conditions (Touma et al., 2011). However, the lack of FKBP51 in stressed mice led to enhanced stress-coping behavior and a less vulnerable neuroendocrine state (Touma et al., 2011; Chapter 1). To date, only male rodents have been investigated in relation to FKBP51 while the potential influence of gonadal hormones like oestrogens has been largely ignored.

Beyond regulatory function of FKBP51 on GR hormone affinity, FKBP51 lowers ligand affinity of other gonadal steroid receptors such as the progesterone receptor and androgen receptor (Hubler et al., 2003; Hubler and Scammell, 2004; Febbo et al., 2005). Thus, FKBP51 functions not only as a regulator of glucocorticoid-induced effects, but also as a negative feedback regulator for progesterone and androgens in the brain. This implicates a possible sex-specific regulation of the HPA axis and behavior in respect to FKBP51 (Bourke et al., 2013), which has not been investigated yet in previous studies on FKBP51 mediated HPA axis activity. Moreover, the exclusion of female hormones in previous studies of FKBP51 in mice is unfortunate as the susceptibly to develop psychiatric disorders is mediated in a sex-dependent gene x environment fashion (Kendler et al., 2002).

The predisposition of women to psychiatric disorders and the interaction of female hormones such as progesterone or oestradiol with both FKBP51 and GR, implicate a possible differential regulation of the HPA axis in female 51KO mice. Therefore, the aim of this study was to characterize female 51KO and wild type (WT) littermates under basal conditions, with regard to possible behavioral, neuroendocrine and physiological alterations relevant to mood disorders. In this regard, we subjected female 51KO and WT littermates to a series of behavioral tests, examining exploratory, cognitive, anxiety-related and stress-coping behavior. Furthermore, measurements of CORT and ACTH circulating levels, physiological parameters and gene expression levels were taken to assess the neuroendocrine profile and physiological differences in these animals.

Materials and Methods

Animals

Female wild type Fkbp51+/+ (WT) and knockout Fkbp51-/- (51KO) mice of 3 to 4 months of age were used for the experiment. The 51KO mouse line was previously generated (Tranguch et al., 2005) and described (Chapter 1). Females were pair housed under standard housing conditions two weeks prior to the start of the experiment. Standard housing consisted of sufficient saw-dust bedding, cage enrichment, *ad libitum* access to food and water, a 12h day-night cycle (lights on at 8:00 am) and constant temperature (23± 2 °C) conditions.

All animal experiments were performed in accordance to European Communities Council Directive 2010/63/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Experimental design

Female 51KO mice (n=14) and WT littermate controls (n=14) were subjected to a battery of behavioral tests, conducted on 5 consecutive days in the following order: open field (OF), elevated plus maze (EPM), dark light box (DaLi), Y-maze and forced swim test (FST). Additionally, the FST served as acute stressor for a stress response test in order to determine CORT levels. The animals were sacrificed by decapitation 2 days after the FST. Trunk blood, brain, thymus and adrenal glands were collected for further analysis.

Determination of estrous cycle phase

Determination of the estrous cycle phase of the females was performed by gentle flushing of the vaginal opening with 20 mL 7% standard saline solution, in order to collect vaginal cells. Samples containing the cells were transferred to a dry glass slide and coverslipped. The samples were viewed shortly after collection of the sample under the microscope at 400x magnification. Determination of the cycle phase was based on the presence and absence of leukocytes and appearance of epithelial cells, according to standard characteristics as

described previously (Goldman et al., 2007). Ultimately, females were classified to be in estrous or non-estrous.

Behavioral assessment

Behavioral tests were carried out between 08:00 am and 12:00 pm in the same room where the animals were housed. All behavioral tests have been described and validated previously (Touma et al., 2011; Wang et al., 2011b; Chapter 1). They were performed using an automated video-tracking system (Anymaze 4.20, Stoelting, IL, USA). Collection of vaginal cells for determination of estrous cycle phase was performed daily after finalizing the behavioral test.

Open field (OF)

On day 1, general locomotor activity and exploratory behavior was assessed in the OF for 15 min. Briefly, the apparatus consisted of an empty, enclosed, square arena (50 x 50 x 50 cm) made of grey polyvinylchloride (PVC), virtually divided into two areas, an inner zone (25 x 25 cm) and an outer zone. The arena was evenly and weakly illuminated (< 10 lux). Parameters of interest included total distance travelled and inner zone time.

Elevated plus maze (EPM)

On the second day, anxiety-related behavior was investigated using the EPM. The animal was placed in an elevated (50 cm) plus-shaped platform made of grey PVC, with two opposing open arms (30 x 5 x 0.5 cm) and two opposing enclosed arms (30 x 5 x 15 cm), which are connected by a central area (5 x 5 cm). Illumination was less than 10 lux in the enclosed arms and 25 lux in the open arms. Animals were placed in the center zone facing one of both closed arms at the beginning of a 10 minute trial. Parameters of interest included the distance travelled and relative open arm time.

Dark light box (DaLi)

The DaLi test is an additional setup to measure anxiety-related behavior and was performed on day 3. Briefly, the apparatus consisted of a rectangular box with two compartments, the dark compartment (15 cm x 20 cm x 25 cm, lit with <10 lux) and the larger, more aversive and brightly lit compartment (30 cm x 20 cm x 25 cm, lit with approximately 700 lux), connected by a 4 cm long tunnel. Animals were placed in a corner of the dark compartment

at the beginning of the test. Exploration of dark and lit compartment was allowed during a 10 minute period. The mice were considered to have entered the lit compartment after entering the zone with the hind paws. Behavior of the animal was scored manually. Parameters of interest were latency to first lit compartment entry, number of entries and time spent in the lit compartment.

Y-maze

On day 4, hippocampal-dependent spatial memory was investigated in the Y-maze. The grey PVC apparatus consists of a platform of three identical arms, 30 cm long, 10 cm wide, and sides 15 cm high, connected by a center zone. The angle between each arm is 120°. The inner walls of each arm are marked with distinct, easy recognizable visual cues, using white adhesive tape. The illumination was 15 lux in all three arms. The test comprises two trials, the acquisition and retrieval trial. During the acquisition trail, one arm was blocked with a grey PVC board. The board is removed during the retrieval trail and this arm is further referred to as the novel arm. At the start of trial one, the mouse is placed into the central zone facing of one of both accessible arms and is allowed to explore the apparatus for a duration of 10 minutes. After 30 minutes inter-trial time spent in their home cage, the animals were reintroduced to the apparatus by placing them in the same starting position. In this retrieval phase, the mouse was allowed to explore all three arms for 5 minutes. Spatial memory performance was assessed by analysis of the distance travelled in the novel arm compared to distance travelled in the other arms as well as the number of entries into the novel arm.

Forced swim test (FST)

On the fifth day, analysis of escape-orientated behavior and stress coping was investigated during the six minute FST trial. The animals were placed in a two litre glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21° C) to a height of 15 cm, so that the mouse could not touch the bottom of the beaker with its hind paws or tail. After the test trial, the animals were dried with a towel and put back into their home cage. Scoring of animal behavior was performed by an experienced observer who was blind to the genotype of the animals, and who classified the following behavioral patterns: floating, swimming and struggling.

Sample processing

Females were sacrificed in the beginning of the light phase (between 08.00 and 10.00 am), 2 days after the last behavioral test (FST). After quick body weight measurement and anaesthesia by isoflurane, the animals were sacrificed by decapitation. Collection of vaginal cells was performed after sacrifice of the animal to minimize the time handling the animal prior to sacrifice. Brains were removed, snap-frozen in isobutene at -40 °C and stored till further use at -80 °C for *in situ* hybridization. Thymus and adrenal glands were removed, dissected from fat and weighted. Trunk blood was collected in 1.5 ml EDTA-coated tubes (Kabe Labortechnik, Germany) for basal CORT and ACTH level determination. All blood samples were kept on ice until centrifugation for 15 minutes at 8000 rpm and at 4 °C. Blood plasma was transferred to new tubes; 100 µl blood plasma for ACTH analysis and 10 µl blood plasma for CORT analysis, stored at -20 °C until further usage.

Investigation of endocrine profile

The FST was used as an acute stressor in order to determine the stress response by measuring CORT blood plasma concentrations. After the FST, all mice were placed into their home cage to recover from the acute stressor. Blood samples for the stress response were collected by a tail cut 30 minutes (stress response) and 90 minutes (stress recovery) after the onset of the FST (Fluttert et al., 2000). Blood was collected in 1.5 ml EDTA-coated tubes (Kabe Labortechnik, Germany) and processed as described above.

CORT and ACTH concentrations were determined by radioimmunoassay using a Corticosterone double antibody 125I RIA kit (sensitivity: 12.5 ng/ml, MP Biomedicals Inc) and Adrenocorticotropic double antibody hormone 125I RIA kit (sensitivity: 10 pg/ml, MP Biomedicals Inc) and were used following the manufacturers' instructions. Radioactivity of the pellet was measured with a gamma counter (Packard Cobra II Auto Gamma; Perkin-Elmer). Final CORT and ACTH levels were derived from the standard curve.

In-situ hybridization

Brain tissue was sectioned at -20 °C in a cryostate microtome at 18 µm in the coronal plane. Sections were thaw mounted on Super Frost Plus slides, air dried at a 28 °C warming plate

and stored at -20 °C. Hippocampal sections were further divided in dorsal (bregma > -2.70 mm) and ventral (bregma < -2.70 mm). Expression levels of the GR and corticotrophin release hormone (CRH) genes were investigated by *in-situ* hybridization of different brain regions, dorsal hippocampus, ventral hippocampus and paraventricular nucleus (PVN) of the hypothalamus, with 35S UTP labelled ribonucleotide probes as previously described (Schmidt et al., 2007). Briefly, sections were fixed in 4% para- formaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/ HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol and treated with 100% chloroform. The antisense cRNA probes were transcribed from a linearized plasmid. Tissue sections were saturated with 100 ml of hybridization buffer containing approximately 3-5 million cpm 35S labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55 °C. The following day, the sections were rinsed in 2 x SSC solution containing 20 mg/l RNAse A and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 x SSC for 1 h at 65 °C and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software. The mean of four bilateral measurements (PVN) or two unilateral measurements (hippocampal subregions) was calculated for each animal, subtracting the background signal of a nearby structure not expressing the gene of interest from the measurements.

Statistical analysis

Data graphs are presenting the means ± standard error of the mean. Analysis was performed using the commercially available software SPSS 17.0. Significance was accepted with $P < 0.05$. All data were tested for outliers using the Grubbs' test (GraphPad software). If the equality of variance was violated in Levene's test ($P < 0.05$), the modified t-values were used for parametric measurements. Body weight, thymus, adrenal glands and gene expression data were analyzed with the unpaired Student's t-test (independent factor genotype). The non-parametric Mann-Whitney test was applied in case of non-normality of these datasets. The cycle phase (estrous or non-estrous) was assessed after each behavioral test to correct for the estrous cycle phase dependent effects on behavioral parameters and

neuroendocrine levels. Behavioral and neuroendocrine data were analyzed using the analysis of covariance (ANCOVA) model with genotype as independent factor and the estrous cycle as covariate. Data of Y-maze novel arm entries were analysed after natural logarithmic transformation to meet the assumption of normality for statistical analysis.

Results

Physiological parameters of female 51KO mice

Body weight measurements were taken before sacrifice and used for normalization of the organ weights. Student's t-test analysis detected that body weight of female 51KOs was significant lower than of their WT litter mates (*t26 = 3.026, P = 0.006,* fig 1A). In addition, both relative adrenal glands weight (Student's t-test: *t25 = 7.025, p < 0.001*, fig 1B) and relative thymus weight (Mann-Whitney: *U = 10.00, p < 0.001*, fig 1C) were reduced in the female 51KO mice in comparison to the WT females.

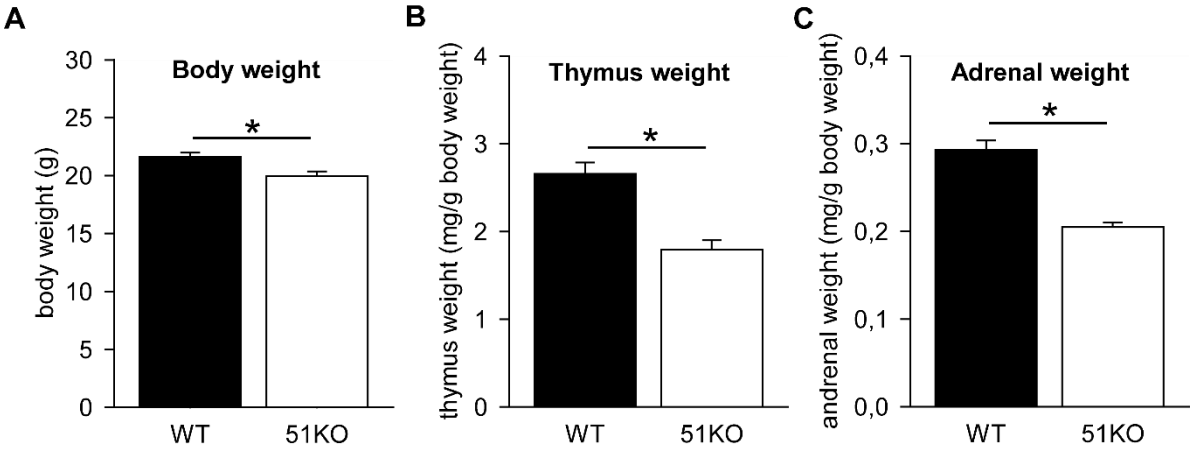

Figure 1: Physiological parameters of the female 51KO mice and WT littermates. (A) Bodyweight in 51KOs is reduced. (B) The thymus and (C) adrenal glands weights are reduced in 51KOs. ** P < 0.05.*

Neuroendocrine profile

The endocrine profile of the females was investigated by analysis of the CORT blood plasma levels after an acute stressor and CORT and ACTH blood plasma levels under basal conditions.

Measurements under basal conditions were made using trunk blood at sacrifice. The ACTH levels in blood plasma did not differ between female WT and 51KO mice under basal conditions (ANCOVA: *F1 = 2.583, P = 0.121,* fig 2A). ANCOVA indicated lower CORT levels under basal conditions in female 51KOs (*F1 =6.472, P = 0.018,* fig 2B). Upon stress, as induced by the FST, ANCOVA indicated an effect of genotype (*F1 = 35.514, P < 0.001*). The CORT response levels of female WTs were elevated in comparison to the female 51KOs

(fig 2C). In addition, ANCOVA revealed that CORT levels of female 51KOs were lower than of female WTs 90 minutes after the onset of the FST (*F1 = 5.392, P = 0.029,* fig 2D).

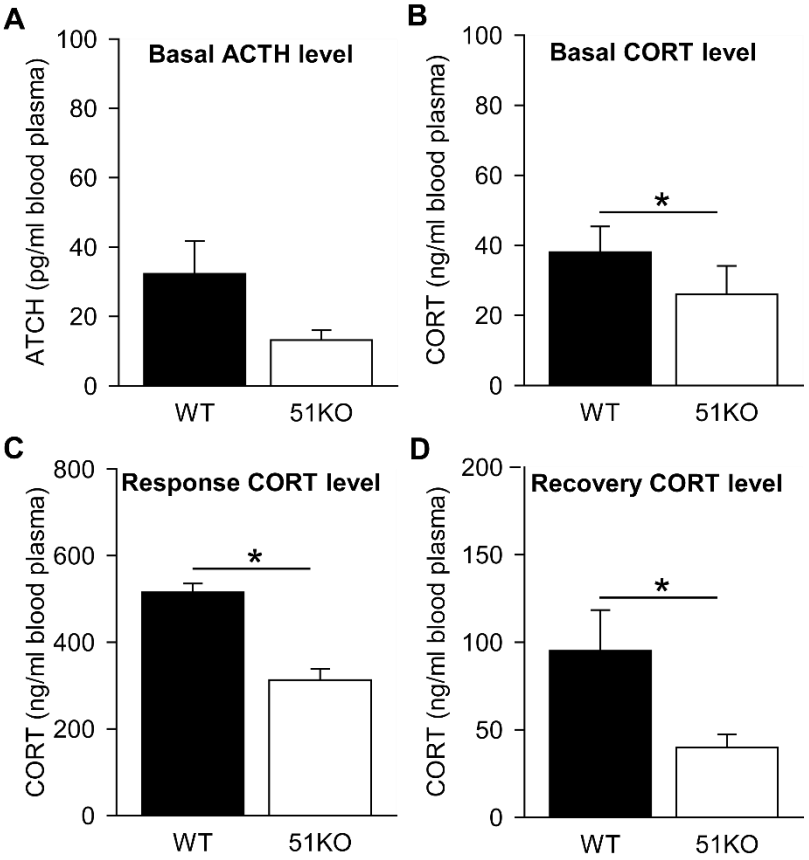

Figure 2: The neuroendocrine profile of female mice under basal conditions and after an acute stressor (FST). (A) ACTH levels under basal conditions are similar in the females. CORT levels in 51KOs are lower (B) under basal conditions, (C) 30 minutes after the onset of the FST (response) and (D) 90 minutes after the onset of the FST (recovery). ** P < 0.05.*

Behavioral characterization of female 51KO mice

Female WT and 51KO mice were subjected to a series of 5 behavioral tests (OF, EPM, DaLi, Y-maze, FST) to investigate locomotion, anxiety and stress-coping behavior as well as cognitive function.

Female 51KO and WT mice behaved similar in respect to exploratory behavior during the test. ANCOVA did not reveal an effect of genotype on locomotion (*F1 = 0.238, P = 0.629,* fig 3A*)* or on the time spent in the inner zone of the arena (*F1 = 0.440, P = 0.513,* fig 3B*)*.

During the EPM, analysis of anxiety-related behavior using ANCOVA revealed no effects of genotype. No differences were present in the distance travelled in the EPM (*F1 = 0.001, P = 0.979887,* fig 3C) or the relative time spent in the open arm (*F1 = 0.448, P = 0.510,* fig 3D*)*.

Animals were tested in the DaLi as an additional measurement of anxiety-related behavior. ANCOVA revealed no differences in the time spent in the lit compartment by 51KO mice (*F1 = 2.993, P = 0.097,* fig 3E) or in the number of entries to the lit compartment between 51KO and WT (*F1 = 1.207, P = 0.283,* fig 3F).

Cognitive functioning of the animals was tested in the hippocampal dependent spatial memory related Y-maze. Female WT and 51KO mice did not perform differently on any of the parameters. ANCOVA revealed no genotype effect on the number of entries to (*F1 = 0.048, P = 0.829,* fig 3G) or the distance travelled in the novel arm (*F1 = 0.024, P = 0.879,* fig 3H). Both the distance travelled in the novel arm and the number of entries of the novel arm exceeded the chance level of 33% in all animals (fig 3H,I).

The female mice were subjected to the FST to assess stress-coping behavior. Analysis showed an increase in the swimming time in female 51KOs (*F1 = 7.466, P = 0.012*), but revealed no genotype effects on the time spent struggling (*F1 = 0.018, P = 0.893*) or floating (*F1 = 0.981, P = 0.331,* fig 3I). In addition, ANCOVA of the latency till the first floating moment revealed no effect of genotype (*F1 = 0.670, P = 0.411,* fig 3J).

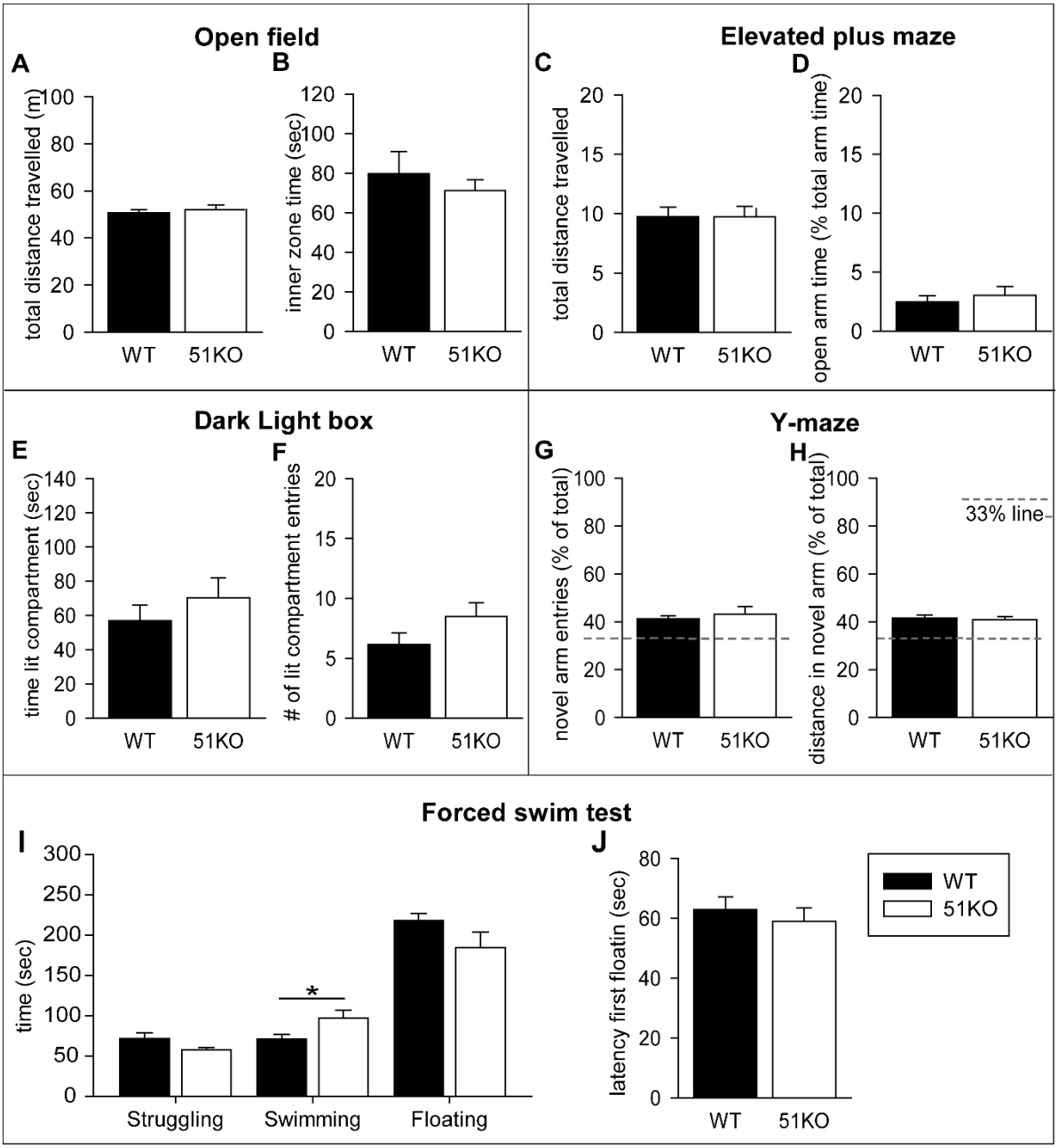

Figure 3: Behavioral profile of female 51KO and WT. Overall, female 51KOs and WTs performed similar in all presented behavioral tests. Open field: (A) Total distance travelled and (B) time in inner zone. Elevated plus maze: (C) Total distance travelled and (D) percentage of the relative time in the open arm. Dark light box: (E) Time in the lit compartment and (F) number of entries to the lit compartment. Y-maze: (G) Percentage of novel arm entries of total arm entries and (H) the percentage of distance travelled in the novel arm. The striped lines in the graphs of the Y-maze represent the 33% change level of the novel arm measurements. Forced swim test: (I) Time spent struggling, swimming or floating and (J) latency till the first observed floating episode. ** P < 0.05.*

Gene expression

Expression of GR mRNA in the hippocampus is similar in female 51KOs and WTs. Student's t-test analysis revealed no effect of genotype in the different subregions of the dorsal hippocampus (CA1: *t26= -0.617, P = 0.542,* CA3: *t26 = -1.045, P = 0.306,* DG: *t26 = -0.466, P = 0.645,* fig 4A,B) or the ventral hippocampus (CA1: *t26 = -0.034, P = 0.973*, CA3: *t21,257 = -0.487, P = 0.631*, DG: *t26 = -0.064, P = 0.949,* fig 4C,D).

Student's t-test analysis of GR mRNA expression in the PVN showed no genotype effect (*t21 = 0.636, P = 0.532,* fig 4E,F), but revealed an increased level of CRH mRNA expression in the PVN of female 51KOs (*t18 = -2.529, P = 0.021*, fig 4G,H).

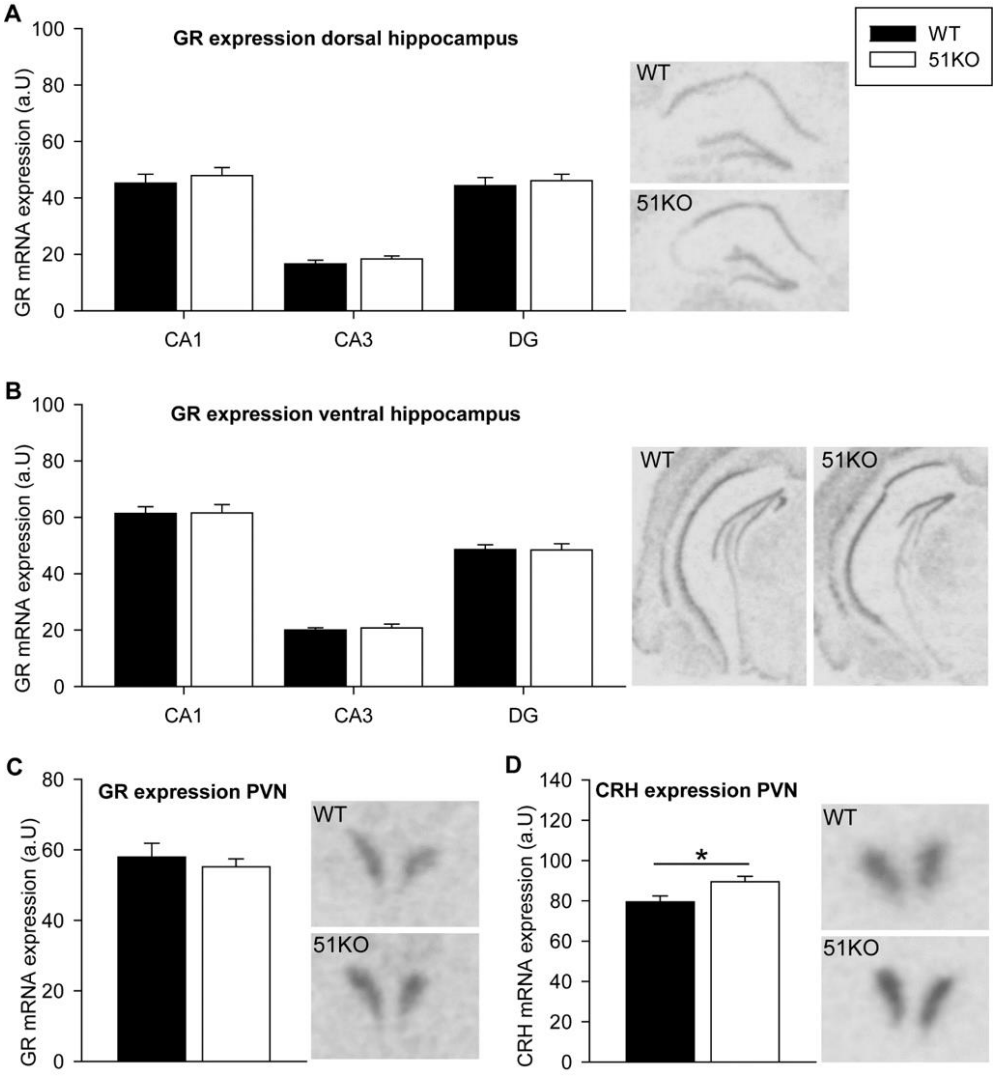

Figure 4. GR and CRH mRNA expression in the hippocampus and PVN. The hippocampus is split in different subregions of (A) the dorsal and (B) the ventral area for analysis of GR mRNA expression. (C) GR mRNA expression is similar in the PVN between genotype groups. (D) CRH expression in the PVN is elevated in the female 51KOs. Representative autoradiograph images are always depicted on the right side of the respective figure panel. ** P < 0.05.*

Discussion

In this study, we investigated the effects of FKBP51 deletion in female mice on behavioral, physiological and neuroendocrine parameters. Overall, female 51KO mice presented a reduced basal HPA axis activity as well as a blunted response to, and an enhanced recovery from, an acute stressor. Nevertheless, 51KO female behavior was not affected by the lack of FKBP51 under basal conditions. The physiological and endocrine profile of the female 51KOs differs from WT mice and is similar to that of male 51KO mice described previously.

Physiological alterations in female 51KO mice

Similar to male 51KO mice, several physiological alterations, including reduced thymus and adrenal gland weights, are observed in female 51KOs compared to WT animals. Considering FKBP51 is known to reduce GR sensitivity in cellular models (Wochnik et al., 2005; Westberry et al., 2006; Albu et al., 2013), an enhanced GR sensitivity most likely explains the thymus involution in female 51KO mice. Indeed, a study on mutant GR overexpressing rats shows an increased affinity for glucocorticoids in the thymus, resulting in strong thymocyte apoptosis, which can be reversed by adrenalectomy (van den Brandt et al., 2007). Along this line of reasoning, a report of adrenolectomized mice with a thymus-specific inducible GR transgene, showed a reduction in thymocyte number after induced GR overexpression, which could again be inhibited by a GR antagonist (Pazirandeh et al., 2005). Chronic stress and HPA axis hyperactivity have been consistently associated with adrenal gland enlargement (Wagner et al., 2011; Wagner et al., 2012; Chapter 1) with considerable consequences for brain structure and function (Lucassen et al., 2014). However, we found that female 51KO mice showed rather decreased adrenal gland weight compared to WTs even under basal conditions. This may implicate attenuated HPA axis activity and enhanced negative feedback regulation in absence of FKBP51.

Next to the alterations in organ weights, body weight of the female 51KOs was also reduced, as it was in the males. Studies of obesity risk in psychiatric disorders relate enhanced glucocorticoid levels to the level of obesity (Rebuffé-Scrive et al., 1992; Mustillo et al., 2003). Hence, reduced circulating CORT levels and lower female 51KO body weight may point to the reversed situation, possibly as a result of the hypothesized GR sensitivity. The specific

underlying biomechanism of the association between body weight and glucocorticoid levels is not identified to-date, and requires future research (Touma et al., 2011).

FKBP51 depletion shapes levels of circulating CORT

The present profile of basal and stress-induced CORT secretion in female 51KO mice supports the hypothesis of an enhanced GR sensitivity. This could result from the depleted FKBP51 mediated inhibition of hormone binding to the GR. Under basal condition, as well as shortly after an acute stressor and during the recovery of this stressor, female 51KOs show reduced circulating CORT, thereby exhibiting a highly similar phenotype to male 51KO mice. However, ACTH levels do not show a significant modulation in female 51KOs at the present time point. It cannot be ruled out that over the course of the circadian rhythm (e.g. at the onset of the dark period) differences in ACTH secretion might be apparent. Overall, the neuroendocrine profile of the female 51KO mice is modulated in a similar manner as the previously described male 51KOs (Touma et al., 2011; Chapter 1). Beyond its role on GR and thus HPA axis activation, it is known that FKBP51 inhibits gonadal hormone receptors in the brain (Hubler et al., 2003; Hubler and Scammell, 2004; Febbo et al., 2005; Bourke et al., 2013), suggesting that FKBP51 may have distinct effects in male and female mice. However, the presented neuroendocrine profiles of female and male 51KOs strongly overlap and no genotype x estrous cycle interaction was observed.

Expression levels of GR and CRH

Next to the circulating glucocorticoids, expression of GR is a key factor affecting HPA axis feedback and regulation. GR mRNA levels in the PVN of the hypothalamus and in different subregions of the hippocampus were similar in female 51KOs and WT animals. Interestingly, the observed increase in CRH mRNA in the female 51KOs, might be due to a reduced GR-mediated negative feedback on CRH expression as a consequence of the lower circulating CORT levels in these animals. In male 51KOs, no differences of CRH mRNA in the PVN are described (Chapter 1), indicating a sex-specific difference in HPA axis regulation at the level of the PVN in 51KO animals. Overall, possible compensatory mechanisms in the 51KO females might involve other components of the HPA axis or steroid hormones. At least in

human brain, sex specific differences in GR protein expression in depression are present (Wang et al., 2012a).

FKBP51 deletion in female mice does not affect behavior under basal conditions

In order to determine the behavioral profile of female mice depleted of FKBP51, mice were subjected to a battery of behavioral tests. The behavioral phenotype of female 51KO mice appears to be unaffected by the depletion of the FKPB51 under basal conditions. Female 51KOs perform adequate and similar to WT littermates, in all performed behavioral tests. The increase in swimming time during the FST is the only difference between 51KOs and WTs. This type of behavior is considered to be neutral, whereas no differences were observed in the determinative types of behavior during this test (i.e. attempting to escape and despair-like types of behavior). Overall the behavior of female 51KOs is in line with previous studies in male 51KOs that also did not display behavioral alterations under basal conditions. Yet, FKPB51 deletion led to marked alterations in males that were challenged by chronic stress (Touma et al., 2011; Chapter 1). Whether this is the case for female mice as well remains to be elucidated.

In accordance to the regulation of CORT and ACTH in female 51KOs, no behavioral differences under basal conditions or in stress-coping behavior during the FST were observed nor could they be subscribed to the effects of gonadal hormones in female 51KOs and WTs. Although FKBP51 interacts with steroid hormones besides CORT (Hubler et al., 2003; Hubler and Scammell, 2004; Febbo et al., 2005; Bourke et al., 2013), no interaction of FKBP51 with the estrous cycle was present in the current study. Future investigation is needed to reveal whether this is only the case under basal conditions. Studies of chronic stress models in female mice at least illustrate regulatory effects of oestrogens and androgens on HPA axis activity and behavior (Walf and Frye, 2006; McCormick and Mathews, 2007; Weiser and Handa, 2009). Therefore, even though no interaction of FKBP51 in female 51KOs is indicated in the present study, it is possible that mediating effects of female gonadal hormones in absence of FKBP51 are present under other conditions, namely after exposure to chronic stress.

3.3. Fkbp52 heterozygosity alters behavioral, endocrine and neurogenetic parameters under basal and chronic stress conditions in mice

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Originally published in:

Psychoneuroendocrinology 2012 Dec; 37(12):2009-21

Abstract

Aversive life events represent one of the main risk factors for the development of many psychiatric diseases, but the interplay between environmental factors and genetic predispositions is still poorly understood. One major finding in many depressed patients is an impaired regulation of the hypothalamic-pituitary-adrenal (HPA) axis. The negative feedback loop of the HPA axis is mediated via the glucocorticoid receptor (GR) and the mineralocorticoid receptor. The co-chaperones FK506-binding protein 51 (FKBP51) and FK506-binding protein 52 (FKBP52) are components of the heat shock protein 90-receptor-heterocomplex and are functionally divergent regulators of both receptors. Here, we characterized heterozygous Fkbp52 knockout ($Fkbp52^{+/-}$) mice under basal or chronic social defeat stress (CSDS) conditions with regards to physiological, neuroendocrine, behavioral and mRNA expression alterations. $Fkbp52^{+/-}$ mice displayed symptoms of increased stress sensitivity in a subset of behavioral and neuroendocrine parameters. These included increased anxiety-related behavior in the elevated plus-maze and an enhanced neuroendocrine response to a forced swim test (FST), possibly mediated by reduced GR sensitivity. At the same time, $Fkbp52^{+/-}$ mice also demonstrated signs of stress resilience in other behavioral and neuroendocrine aspects, such as reduced basal corticosterone levels and more active stress-coping behavior in the FST following CSDS. These contrasting results are in line with previous reports showing that FKBP52 is not involved in all branches of GR signaling, but rather acts in a gene-specific manner to regulate GR transcriptional activation.

Introduction

Susceptibility to psychiatric disorders such as major depression is determined by the relationship between genetic predisposition and environmental factors including negative life events and chronic stress (Hammen, 2005; El Hage et al., 2009; Gillespie et al., 2009; Hyde et al., 2011). However, the molecular mechanisms underlying individual vulnerability or resilience to chronic stress are still poorly understood.

The hypothalamus-pituitary-adrenal (HPA) axis represents one of the major stress-systems in mammals, which ultimately leads to the release of glucocorticoids (GCs) from the adrenal cortex. GCs are then acting on numerous organ systems, including the brain, to modulate metabolism and behavior. In parallel, GCs activate a negative feedback circuit via glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) at different levels of the HPA axis, thereby terminating the stress response (De Kloet et al., 1998; Ulrich-Lai and Herman, 2009).

GRs and MRs are ligand-dependent transcription factors (De Kloet and Reul, 1987). The process of receptor folding, maturation, activation, trafficking and subsequent receptor action on gene transcription is regulated by a multiprotein complex that assembles around the molecular chaperone heat shock protein 90 (hsp90) (Pratt et al., 2006; Grad and Picard, 2007). The FK506-binding protein 51 (FKBP51, encoded by the FKBP5 gene) and FK506-binding protein 52 (FKBP52, encoded by the FKBP4 gene) are components of the chaperone-receptor heterocomplex and differentially regulate the GR or MR at two levels: hormone binding and nuclear translocation. FKBP51 has been shown to decrease ligand binding sensitivity of the receptor and efficiency of nuclear translocation (Denny et al., 2000; Scammell et al., 2001; Wochnik et al., 2005; Westberry et al., 2006). Upon ligand binding, FKBP51 is replaced by FKBP52, which in turn binds to dynein. This promotes the translocation of the receptor-complex into the nucleus and enhances subsequent DNA-binding (Davies et al., 2002; Wochnik et al., 2005; Gallo et al., 2007; Galigniana et al., 2010; Schülke et al., 2010). For a detailed illustration and description of the mechanism see review of Binder 2009.

In numerous clinical studies, single nucleotide polymorphisms of FKBP51 have repeatedly been associated with stress-related diseases such as posttraumatic stress disorder and

major depression (Yehuda et al., 2009; Zobel et al., 2010; Zou et al., 2010; Mehta et al., 2011). In addition, we could recently show that Fkbp51 knockout (51KO) mice were less affected by chronic social defeat stress (CSDS), in a subset of neuroendocrine and behavioral parameters (Chapter 1). Furthermore, 51KO mice displayed enhanced active stress coping behavior following an acute severe stress exposure which was independently also shown in aged 51KO mice, likely due to a more sensitive GR (O'Leary, III et al., 2011; Touma et al., 2011).

In contrast, most studies with Fkbp52 knockout ($Fkbp52^{-/-}$) mice focused on developmental and molecular aspects. In this regard it is important to know that $Fkbp52^{-/-}$ mice show an increased embryonic lethality, while the Mendelian ratio of heterozygous Fkbp52 ($Fkbp52^{+/-}$) mice is normal (Cheung-Flynn et al., 2005; Tranguch et al., 2005; Warrier et al., 2010). Nonetheless, several studies revealed an androgen, progesterone, and glucocorticoid insensitivity in $Fkbp52^{-/-}$ mice (Cheung-Flynn et al., 2005; Tranguch et al., 2005; Yang et al., 2006; Yong et al., 2007; Warrier et al., 2010). Furthermore, it has been shown that female $Fkbp52^{-/-}$ mice are infertile as a result of embryonic implantation and decidualization failure caused by uterine defects and progesterone insensitivity (Tranguch et al., 2005; Yang et al., 2006; Tranguch et al., 2007). Male $Fkbp52^{-/-}$ mice display several defects in reproductive tissues such as ambiguous external genitalia and dysgenic prostate consistent with partial androgen insensitivity (Cheung-Flynn et al., 2005; Yong et al., 2007). In addition, $Fkbp52^{+/-}$ mice showed a susceptibility to high-fat diet induced hepatic steatosis, due to a state of glucocorticoid resistance arising from liver-specific loss of GR activity (Warrier et al., 2010).

All these findings suggest that FKBP52 plays an important role in steroid receptor function. However, a comprehensive behavioral, endocrine and neurogenetic characterization combined with challenging conditions such as chronic stress is still lacking. To fill this gap, we investigated the function of FKBP52 and its potential role in the development of stress-related disorders. We characterized heterozygous Fkbp52 knockout mice under basal and chronic stress conditions. Mice were then analyzed for alterations in physiological, neuroendocrine, behavioral and mRNA expression parameters. We hypothesized that $Fkbp52^{+/-}$ mice would display a higher stress sensitivity due to a partial glucocorticoid insensitivity.

Materials and Methods

Animals and housing conditions

The Fkbp52 knockout mouse line was previously generated as described by Cheung-Flynn et al., (2005) and were kept on a mixed 129SvJ x C57BL/6 background. Due to the low viability of null embryos, we used Fkbp52$^{+/-}$ mice in this study, obtained from heterozygous breeding pairs. Genotypes were verified by PCR of tail DNA. Only male mice were used for the experiment. Animals were 12 weeks old at the start of the experiment. Mice were singly housed two weeks prior to the experiment. Maintenance and experiments were performed under a 12 h light, 12 h dark cycle (lights on at 0800 h) and constant temperature (23 ± 2 °C) conditions. Food and water were provided *ad libitum*.

Male CD1 mice (12 - 16 weeks old) serving as residents were allowed to habituate to the social defeat cage for two weeks before the onset of the experiments. Male juvenile Balb/c mice (5 weeks old) serving as social targets in the sociability test were housed in groups of 4. The experiments were performed in accordance with the European Communities Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Chronic social defeat paradigm

The CSDS paradigm was conducted as described previously (Wagner et al., 2011; Wang et al., 2011a; Chapter 1). Briefly, the experimental mice were introduced into the home cage (45 cm x 25 cm) of a dominant resident mouse and defeated shortly after. After the defeat was achieved, the animals were separated by a wire mesh, preventing physical but allowing sensory contact for 24 h. Each day, stressed animals were defeated by another unfamiliar, dominant resident mouse, in order to exclude a repeated encounter throughout the experiment. Experimental mice were always defeated by resident males during the entire experimental period. Control mice were housed in their home cages during the course of the experiment. All mice were handled daily; body weight and fur quality were assessed every 3-4 days prior to the social defeat.

The fur state was rated by an experienced investigator as previously described (Mineur et al., 2003). Briefly, ratings were classified according to a 4-point scale, where 1 represents a perfect, clean fur, while 4 stands for disheveled, scruffy fur, often including traces of wounds and scurf. Scores of 2 and 3 demonstrate intermediate fur states, respectively.

Experimental design

24 wild-type (WT) and 24 Fkbp52$^{+/-}$ mice were divided into four groups (WT control, Fkbp52$^{+/-}$control, WT stress and Fkbp52$^{+/-}$ stress) of 12 animals each and subjected to the CSDS paradigm for 21 days. The daily defeat was performed between 1200 h and 1600 h; varying starting times reduced the predictability of the stressor and therefore minimized a potential habituation effect. All behavioral tests were conducted during the third week of the stress paradigm. In case of serious injury or death, mice were excluded from the experiment.

Behavioral analysis

Behavioral tests were carried out between 0830 h and 1230 h in the same room in which the mice were housed. All tests were performed using an automated video-tracking system (Anymaze 4.20, Stoelting, IL; USA).

Sociability test

On day 15 of the stress paradigm, the sociability test was performed as previously described (Moy et al., 2004; Sankoorikal et al., 2006; O'Tuathaigh et al., 2008), with slight modifications. The test was employed to assess the preference between a social target (unfamiliar juvenile Balb/c mouse) and a non-social object (a toy mouse). The apparatus, a three chamber box (50 x 25), contained one center and two outer compartments (left and right chamber 19 x 25 x 40 cm; center chamber 12 x 25 x 40 cm). Two small openings with doors served as access points from the center chamber to the others. The apparatus was filled with bedding and evenly illuminated with 3-5 Lux. The test consisted of two separate trials. In the initial habituation trial, the animals were allowed to freely explore the apparatus on two consecutive days for 10 min. Only the empty wire cages were present in the chambers at this time. On the third day of testing, the sociability trial took place. This

time an unfamiliar male juvenile BALB/c mouse was introduced into one of the chambers, enclosed in the wire cage, while a toy mouse was placed in the opposite chamber (alteration occurred after 3 consecutive trials). The test mouse was placed in the middle chamber for 5 min with both trap doors being shut. Afterwards, the doors were opened and the test animal was allowed to explore the rest of the apparatus for an additional 10 min. An experienced observer scored the time the mouse spent in the zone with the social target and the time spent in the zone with the non-social object. The discrimination index in % was calculated as follows:

[Time in zone with social target (s) / (Time in zone with social target (s) + Time in zone with non-social object (s))]*100.

Elevated plus-maze

The elevated plus-maze (EPM) was conducted on day 16 of the stress procedure. The device consisted of a plus-shaped platform with two opposing open arms (length: 30 cm x width: 5 cm x height: 0.5 cm) and two opposing enclosed arms (length: 30 cm x width: 5 cm x height: 15 cm), made of gray polyvinyl chloride (PVC), which were connected by a central area (5 cm x 5 cm). The whole device was elevated 50 cm above the floor. The illumination was 25 Lux in the open arms and less than 10 Lux in the closed arms. Testing duration was 5 min and all mice were placed into the center zone facing one of the enclosed arms at the start of the test. The time spent in the open arms compared to the total arm time as well as the number of open arm entries were analyzed.

Open-field test

On day 17 of the stress paradigm, the open-field test (OF) was performed. Testing was carried out in an empty open-field arena (50 cm x 50 cm x 50 cm) made of gray PVC, which was evenly illuminated with 15 Lux. Testing time was 15 min, split into 3 segments, 5 min each. Parameters of interest were the total distance traveled as well as the inner zone time (inner zone size 25 cm x 25 cm).

Forced swim test

The forced swim test (FST) was performed on day 19 of the CSDS procedure. Each mouse was put into the a 2 l glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21

± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 6 min. The parameters time floating, time swimming, time struggling and latency to 1st floating were scored by an experienced observer, blind to genotype or condition of the animals.

Acute stress response

The FST was performed on day 19 and served as an acute challenge in order to determine the stress response by measuring corticosterone plasma concentrations. Blood samples were taken by tail cut 15 min (stress response) and 90 min (stress recovery) after the onset of the FST (Fluttert et al., 2000). Samples were collected in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). All blood samples were kept on ice and later centrifuged at 8000 rpm at 4 °C for 15 min. Plasma was transferred to new, labeled microcentrifuge tubes and stored at -20 °C until further processing.

Sampling procedure

All animals were sacrificed 24 h after the last defeat in the circadian nadir by decapitation following quick anaesthesia by isoflurane. Trunk blood was collected in labeled 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany) for basal corticosterone levels. All blood samples were kept on ice until centrifugation at 4 °C and 8000 rpm for 15 min. After centrifugation, the blood plasma was transferred to a new, labeled 1.5 ml microcentrifuge tube. All plasma samples were stored at -20 °C until the determination of corticosterone by radioimmunoassay (MP Biomedicals Inc; sensitivity 6.25 ng/ml). Brains were removed, snap-frozen in isopentane at -40 °C, and stored at -80 °C for *in-situ* hybridization. Adrenal and thymus glands were removed, dissected from fat and weighed.

In-Situ hybridization

Frozen brains were sectioned at -20 °C in a cryostat microtome at 18 µm at the level of the paraventricular nucleus (PVN) and the dorsal hippocampus, thaw mounted on Super Frost Plus slides, dried and stored at -80 °C. *In-situ* hybridization using ^{35}S UTP labeled ribonucleotide probes (Corticotropin-releasing hormone (CRH), vasopressin (AVP), GR, MR,

androgen receptor (AR), FKBP51, FKBP52) was performed as described previously (Schmidt et al., 2007). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA probes were transcribed from a linearized plasmid. Tissue sections were saturated with 100 µl of hybridization buffer containing approximately 3-5 × 10^6 cpm ^{35}S labeled riboprobe. Brain sections were coverslipped and incubated overnight (14 h) at 55 ºC. The following day, the sections were rinsed in 2 × SSC (standard saline citrate), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 × SSC for 1h at 65 ºC and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software (http://rsbweb.nih.gov/ij/). We performed two measurements (left and right for the hippocampus and PVN) for each brain slice and assessed two brain slices per animal. The data was analyzed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest.

For AVP, slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY) and exposed at 4 °C for 8 h. Slides were developed and examined with a light microscope with both bright and dark field condensers. Expression of AVP mRNA in the parvocellular part of the PVN was determined by optical densitometry as described above.

Statistical analysis

All results are shown as mean ± SEM and were analyzed by the software SPSS 16.0. Values outside the 95 % confidence interval were defined as outliers and excluded from the analysis. For body weight and fur status progression as well as for the time-dependent behavioral parameters assessed in the open field test, 3-factorial analysis of variance (ANOVA) with repeated measures was performed. Thereby, genotype and condition were two between-subjects factors and time a within-subjects factor. Two factorial multivariate ANOVAs were employed for thymus, adrenal gland weight, neuroendocrine, and other behavioral variables as well as for gene expression contrasts. Whenever significant main (p

< 0.05) or interaction ($p < 0.1$) effects were found in the ANOVAs, univariate F-tests or tests with contrasts followed for specifying and locating simple effects. As a nominal level of significance $p < 0.05$ was accepted and corrected according to Bonferroni (univariate F-tests, test of simple effects or contrasts).

Results

Verification of reduced Fkbp52 expression in Fkbp52$^{+/-}$ mice

As expected, we observed an overall reduction of Fkbp52 mRNA expression with *in-situ* hybridization which was specifically quantified in the hippocampus. Statistical analysis showed significantly lower Fkbp52 mRNA expressions in the hippocampus for Fkbp52$^{+/-}$ mice compared to WT animals under control (CA1: $T_{21} = 9.710$, $p < 0.0001$; CA3: $T_{21} = 9.461$, $p < 0.0001$; DG: $T_{21} = 10.341$, $p < 0.0001$) (Fig. 1A and B) as well as under stress conditions (CA1: $T_{22} = 6.942$, $p < 0.0001$; CA3: $T_{22} = 7.607$, $p < 0.0001$; DG: $T_{22} = 7.953$, $p < 0.0001$). There were no CSDS-dependent effects on Fkbp52 mRNA expression either in WT or in Fkbp52$^{+/-}$mice (data for stress condition not illustrated).

Figure 1

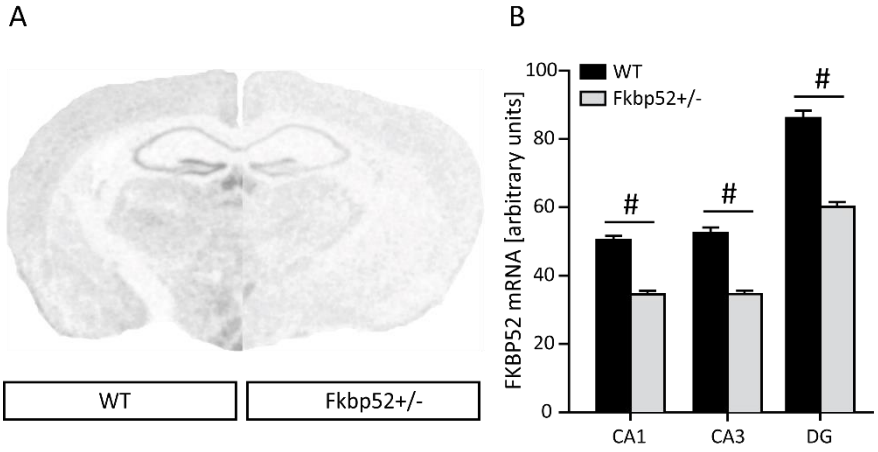

Figure 1: FK506-binding protein 52 (Fkbp52) mRNA expression. (A) Representative *in-situ* hybridization images show Fkbp52 mRNA expression in the hippocampus of wild type (WT) and Fkbp52$^{+/-}$ mice under control (ctrl) conditions. (B) Heterozygous deletion of Fkbp52 reduces hippocampal Fkbp52 mRNA expression ($N_{WT\ Ctrl} = 11$, $N_{Fkbp52^{+/-}\ Ctrl} = 12$). Data are represented as mean + SEM. # significantly different from the WT, $p < 0.05$.

Physiological parameters

CSDS evoked robust physiological changes in both WT and Fkbp52$^{+/-}$ animals. A significant increase in body weight was observed in all stressed animals independent of genotype (ANOVA with repeated measures: time x condition interaction; $F_{6, 47} = 10.505$, $p < 0.001$). Body weight of stressed WT mice at days 8, 18 and 21 was significantly increased compared

to their littermate controls (tests with contrasts in ANOVA, $p < 0.01$). Similarly, a significant body weight increase was also identified in stressed Fkbp52$^{+/-}$ mice at days 4, 11, 15, 18 and 21 compared to their respective controls (Fig. 2A).

A significant decrease in the animal's fur state was observed in all stressed animals independent of genotype (ANOVA with repeated measures: time x condition interaction; $F_{6, 47} = 19.918$, $p < 0.0001$). Fur quality of stressed Fkbp52$^{+/-}$ mice was significantly reduced in comparison to Fkbp52$^{+/-}$ controls from day 4 to day 21 and from day 8 to day 21 in stressed WT mice compared to their controls (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 2B).

Organ weights were measured on the day of sacrifice. Adrenal gland weights were significantly increased in chronically stressed WT and Fkbp52$^{+/-}$ mice (ANOVA: condition effect $F_{1, 45} = 63.586$, $p < 0.0001$) (Fig. 2C). Further, we observed a significant reduction in thymus weights in both WT and Fkbp52$^{+/-}$ stressed animals (ANOVA, condition: $F_{1, 46} = 94.535$, $p < 0.0001$) (Fig. 2D).

Figure 2

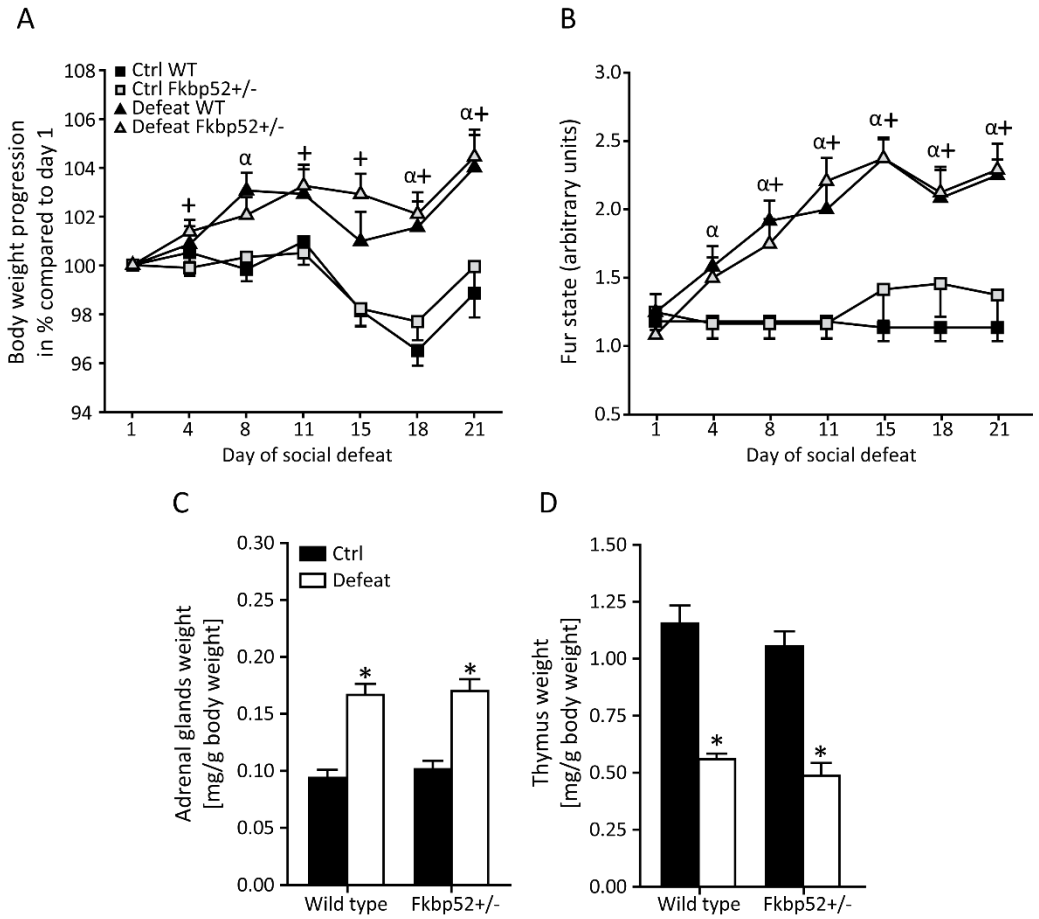

Figure 2: Physiological phenotype evoked by chronic social defeat stress (CSDS). (A) Body weight gain over the course of the experiment ($N_{WT\ Ctrl}$ = 11, $N_{Fkbp52^{+/-}\ Ctrl}$ = 12, $N_{WT\ Stress}$ = 12, $N_{Fkbp52^{+/-}\ Stress}$ = 12). (B) Fur state progression during the stress exposure ($N_{WT\ Ctrl}$ = 11, $N_{Fkbp52^{+/-}\ Ctrl}$ = 12, $N_{WT\ Stress}$ = 12, $N_{Fkbp52^{+/-}\ Stress}$ = 12). 1 represents a perfect, clean fur, 2 and 3 stand for intermediate fur states, 4 demonstrates a disheveled, scruffy fur. (C) Adrenal ($N_{WT\ Ctrl}$ = 10, $N_{Fkbp52^{+/-}\ Ctrl}$ = 12, $N_{WT\ Stress}$ = 12, $N_{Fkbp52^{+/-}\ Stress}$ = 11) and (D) thymus weight ($N_{WT\ Ctrl}$ = 11, $N_{Fkbp52^{+/-}\ Ctrl}$ = 12, $N_{WT\ Stress}$ = 11, $N_{Fkbp52^{+/-}\ Stress}$ = 12) at the end of CSDS. Data are represented as mean ± SEM. α significant condition effect in WT animals, $p < 0.05$; + significant condition effect in $Fkbp52^{+/-}$ mice, $p < 0.05$; * significantly different from the control condition of the same genotype, $p < 0.05$.

Neuroendocrine analysis

Basal levels of corticosterone were measured in blood plasma at circadian nadir. Moreover, response and recovery blood samples were collected 15 min and 90 min after the onset of the FST. ANOVA identified a genotype x condition interaction ($F_{1, 45} = 9.214$, $p < 0.01$) for basal corticosterone levels. Analyzing condition differences for each genotype and genotype differences for each condition, we found that chronic stress led to increased corticosterone levels in WT mice compared to WT controls. Moreover, significantly lower corticosterone levels were seen in stressed $Fkbp52^{+/-}$ mice compared to stressed WT animals (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 3A).

A condition ($F_{1, 45} = 23.887$, $p < 0.0001$) and a genotype ($F_{1, 45} = 5.619$, $p < 0.05$) effect were identified for corticosterone response values (15 min after the FST). $Fkbp52^{+/-}$ mice had significantly higher corticosterone levels compared to WT mice under control conditions. Furthermore, chronic stress led to a significant increase in corticosterone levels in WT and in $Fkbp52^{+/-}$ mice compared to non-stressed controls (Fig 3 B).

Regarding corticosterone recovery levels (90 min after the FST), ANOVA revealed a condition effect ($F_{1, 44} = 21.777$, $p < 0.0001$) and a marginal genotype effect ($F_{1, 44} = 3.626$, $p = 0.064$), but not a significant interaction. Both chronically stressed groups had significantly lower corticosterone levels 90 min after the FST compared to their control groups. However, chronically stressed $Fkbp52^{+/-}$ mice showed significantly higher corticosterone levels compared to stressed WT animals (Fig. 3C).

Figure 3

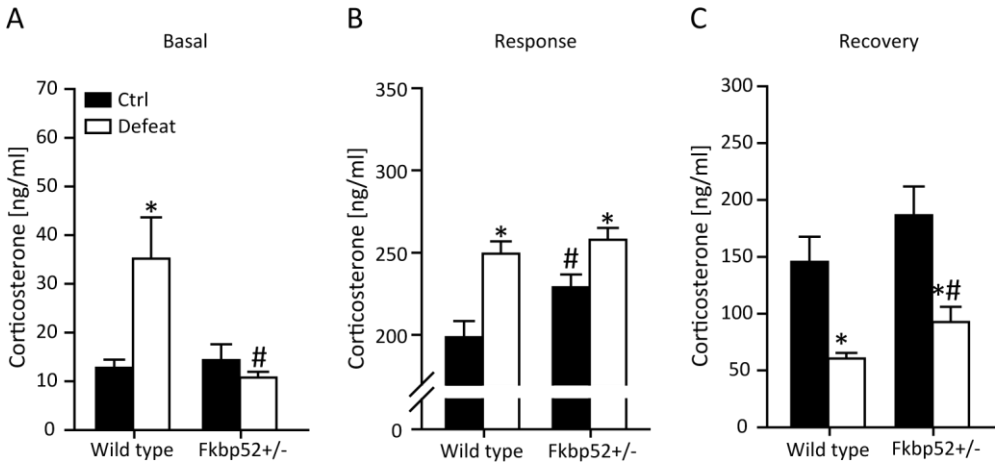

Figure 3: Corticosterone profile of Fkbp52$^{+/-}$ and WT mice. (A) Basal morning corticosterone levels at the end of the stress exposure ($N_{WT\ Ctrl}$ = 10, $N_{Fkbp52^{+/-}\ Ctrl}$ = 12, $N_{WT\ Stress}$ = 12, $N_{Fkbp52^{+/-}\ Stress}$ = 11). (B) Corticosterone response 15 min after the forced swim test (FST) ($N_{WT\ Ctrl}$ = 11, $N_{Fkbp52^{+/-}\ Ctrl}$ = 12, $N_{WT\ Stress}$ = 11, $N_{Fkbp52^{+/-}\ Stress}$ = 11). (C) Corticosterone recovery 90 min after the onset of the FST ($N_{WT\ Ctrl}$ = 11, $N_{Fkbp52^{+/-}\ Ctrl}$ = 12, $N_{WT\ Stress}$ = 10, $N_{Fkbp52^{+/-}\ Stress}$ = 11). Data are represented as mean + SEM. * significantly different from the control condition of the same genotype, $p < 0.05$; # significantly different from the WT of the same condition, $p < 0.05$.

Behavioral analysis

Four tests were conducted in order to investigate social preference, anxiety, exploration and stress-coping behavior (Sociability, EPM, OF, FST).

Sociability test

The sociability test was performed to assess the social preference between an unfamiliar juvenile Balb/c mouse and a non-social object. The time spent in the zone with the social target revealed a condition effect ($F_{1, 45}$ = 7.495, $p < 0.01$). Chronically stressed Fkbp52$^{+/-}$ mice spent significantly less time in the zone with the social target compared to their control group of the same genotype (Fig. 4A), while this effect was not significant in WT controls.

Elevated plus-maze test

The EPM was used to investigate anxiety-related behavior. Animals which fell off the open arms were excluded from the analysis (2 WT Ctrl and 2 Fkbp52$^{+/-}$ Ctrl mice). For the time spent in the open arms, ANOVA identified a genotype x condition interaction ($F_{1, 39}$ = 3.352, $p = 0.076$). Under control conditions, Fkbp52$^{+/-}$ mice spent significantly less time in the open

arms compared to WT mice. Furthermore, chronic stress resulted in a significant decrease in open arm time in stressed WT mice compared to WT controls (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 4B, left panel).

For the open arm entries, ANOVA revealed a condition effect ($F_{1, 39} = 9.483$, $p < 0.01$). Chronic stress led to significantly less open arm entries in stressed WT mice compared to their controls of the same genotype (Fig. 4B, right panel).

Figure 4

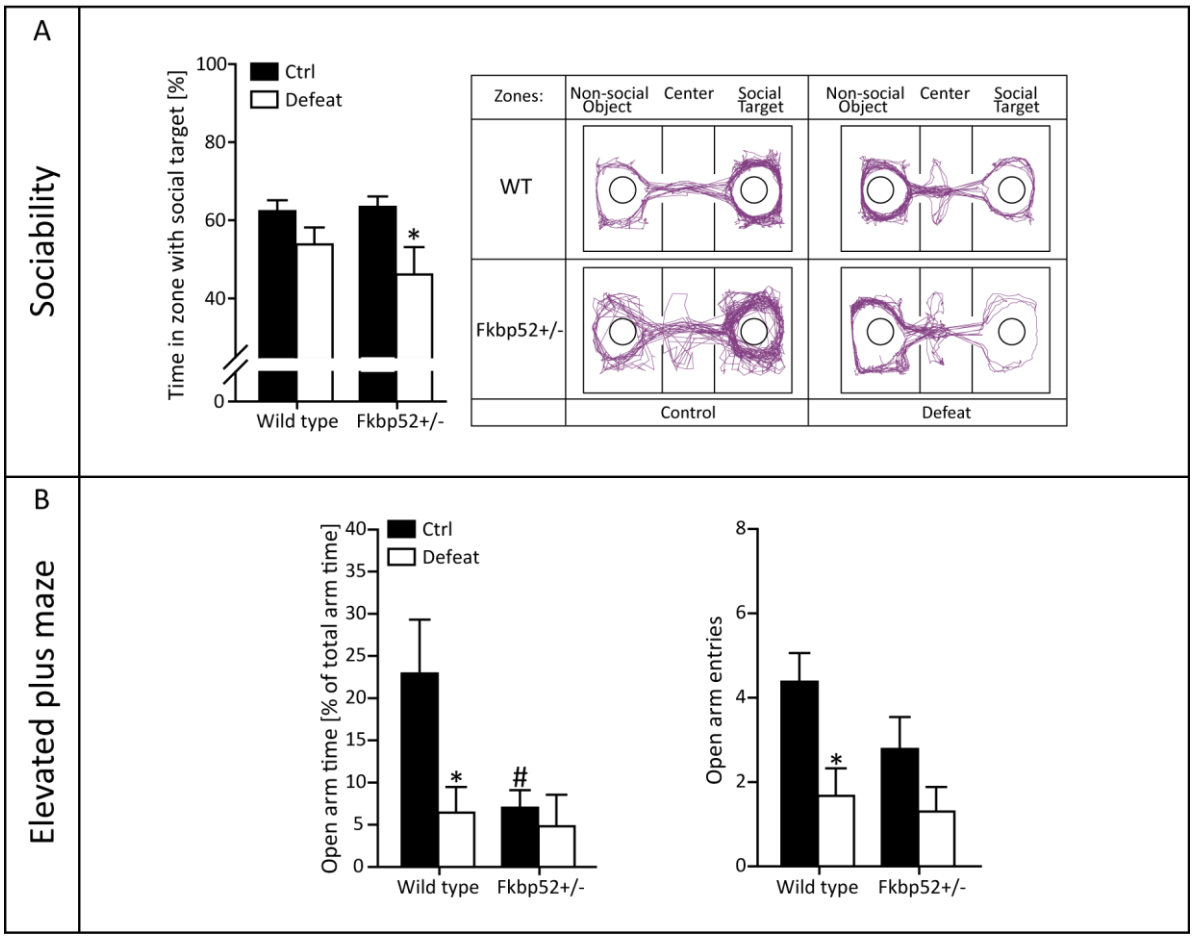

Figure 4: Behavioral profile of Fkbp52$^{+/-}$ and WT mice (part 1). (A) Sociability test. Time in the zone with social target (left panel) ($N_{WT\ Ctrl} = 11$, $N_{Fkbp52^{+/-}\ Ctrl} = 11$, $N_{WT\ Stress} = 11$, $N_{Fkbp52^{+/-}\ Stress} = 12$) and representative track plots of Fkbp52$^{+/-}$ and WT mice in the sociability apparatus (right panel). (B) Elevated plus-maze. Time spent in the open arms (left panel) and open arm entries (right panel) ($N_{WT\ Ctrl} = 8$, $N_{Fkbp52^{+/-}\ Ctrl} = 9$, $N_{WT\ Stress} = 12$, $N_{Fkbp52^{+/-}\ Stress} = 10$). Data are represented as mean + SEM. * significantly different from the control condition of the same genotype, $p < 0.05$. # significantly different from the WT of the same condition, $p < 0.05$.

Open-field test

The open-field test was used to assess behavioral responses such as locomotor activity and explorative behavior. The 15 min test interval was subdivided in three segments of 5 min each. For the total distance traveled, ANOVA with repeated measures revealed a time effect ($F_{2, 47}$ = 109.260, $p < 0.0001$) and a time x condition interaction ($F_{2, 47}$ = 12.176, $p < 0.0001$). Post hoc analysis with contrast tests showed a significant decrease of locomotor activity in chronically stressed WT mice compared to WT controls in segment 3. A significant decrease of locomotor activity in stressed Fkbp52$^{+/-}$ mice compared to Fkbp52$^{+/-}$ controls could be shown in all 3 segments (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 5A, left panel).

Regarding the time spent immobile, ANOVA with repeated measures demonstrated a time effect ($F_{2, 47}$ = 30.309, $p < 0.0001$) and a condition effect ($F_{1, 47}$ = 22.987, $p < 0.0001$). Chronic stress resulted in a significantly higher immobility in both groups compared to their respective controls of the same genotype in all three segments (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 5A, middle panel).

For time in the inner zone, ANOVA with repeated measures indicated a condition effect ($F_{1, 47}$ = 13,429, $p < 0.001$). Chronically stressed Fkbp52$^{+/-}$ mice spent significantly less time in the inner zone compared to Fkbp52$^{+/-}$ controls in all three segments. This effect did not reach significance in WT animals (Fig. 5A, right panel).

Forced swim test

We investigated the animals' stress coping behavior using the FST. For the time spent struggling, ANOVA identified a genotype x condition interaction ($F_{1, 46}$ = 3.742, $p = 0.06$). Chronic stress led to a significant decrease in time struggling in WT mice compared to WT control animals (tests with contrasts in ANOVA, $p < 0.05$). This effect was not observed in Fkbp52$^{+/-}$ mice (Fig. 5B, left panel).

Concerning the parameter time floating, ANOVA showed a condition effect ($F_{1, 46}$ = 22.844, $p < 0.0001$). Chronically stressed WT and Fkbp52$^{+/-}$ mice spent significantly more time floating compared to their respective control group of the same genotype (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 5B, middle panel).

For the latency to 1st floating, ANOVA found a significant genotype x condition interaction ($F_{1, 46}$ = 8.559, $p < 0.01$). Chronic stress lead to a significant decrease in the latency to 1st floating in WT mice compared to WT control animals. This effect was not seen in Fkbp52$^{+/-}$ mice. In contrast, chronically stressed Fkbp52$^{+/-}$ animals had a significantly increased latency to 1st floating compared to WT mice of the same condition (tests with contrasts in ANOVA, $p < 0.05$) (Fig. 5B, right panel).

Figure 5

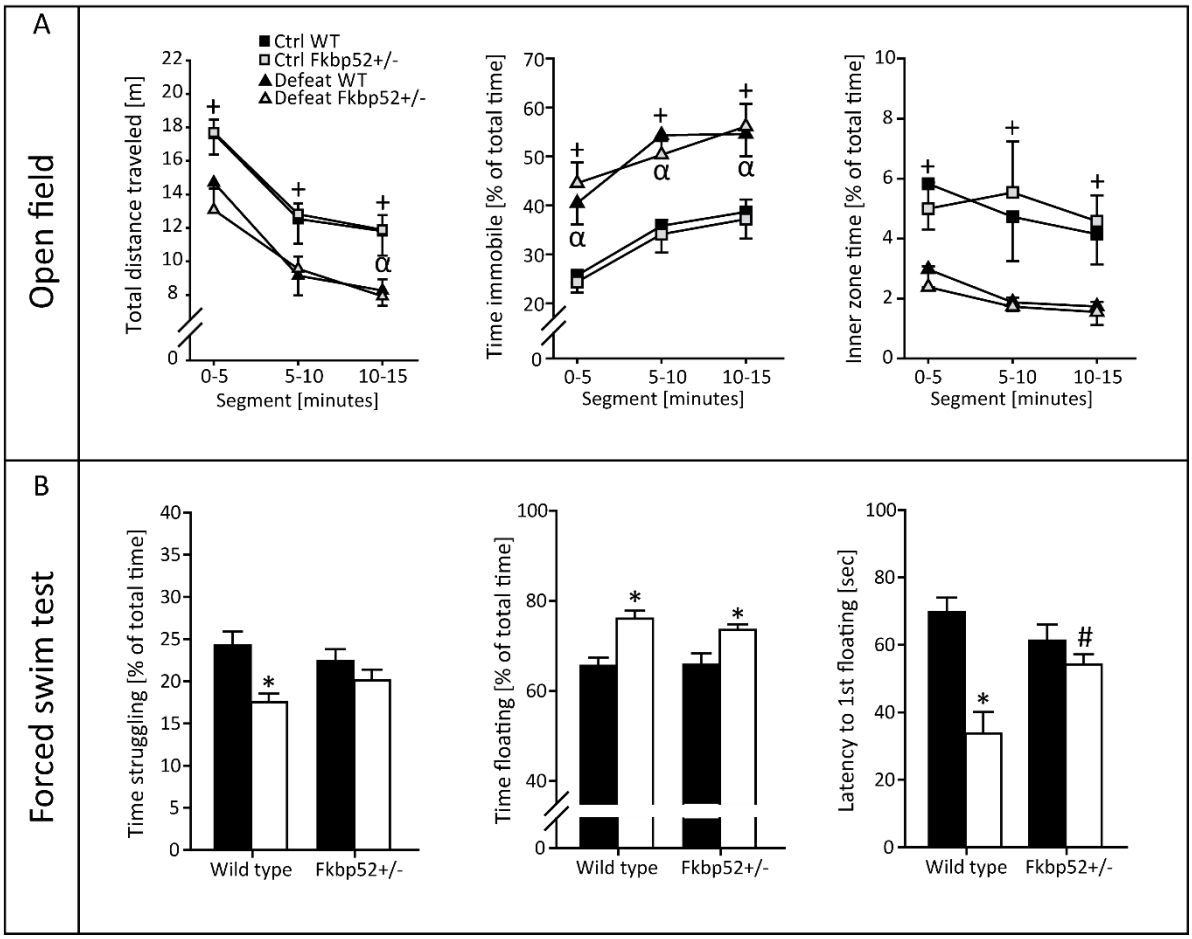

Figure 5: Behavioral profile of Fkbp52$^{+/-}$ and WT mice (part 2). (A) Open field. Total distance traveled (left panel), time immobile (middle panel) and time in the inner zone (right panel) ($N_{WT\ Ctrl}$ = 11, $N_{Fkbp52^{+/-}\ Ctrl}$ = 12, $N_{WT\ Stress}$ = 12, $N_{Fkbp52^{+/-}\ Stress}$ = 12). (B) FST. Time spent struggling (left panel), time spent floating (middle panel) and latency to 1st floating (right panel) ($N_{WT\ Ctrl}$ = 11, $N_{Fkbp52^{+/-}\ Ctrl}$ = 12, $N_{WT\ Stress}$ = 12, $N_{Fkbp52^{+/-}\ Stress}$ = 11). Data are represented as mean ± SEM. α significant condition effect in WT animals, $p < 0.05$; + significant condition effect in Fkbp52$^{+/-}$ mice, $p < 0.05$; * significantly different from the control condition of the same genotype, $p < 0.05$. # significantly different from the WT of the same condition, $p < 0.05$.

Gene expression analysis

We conducted *in-situ* hybridization in order to investigate expression patterns in the hippocampus and PVN of genes involved in the major regulation and mediation of the HPA axis. Expression of CRH mRNA in the PVN was significantly increased in chronically stressed WT mice compared to their respective controls of the same genotype (ANOVA condition effect: $F_{1, 44} = 6.330$, $p < 0.05$). This effect was not observed in Fkbp52$^{+/-}$ mice (Fig. 6A).

A significant effect in hippocampal MR mRNA expression was shown by ANOVA. In the CA1, CA3 and the dentate gyrus (DG) region, condition effects were found (CA1: $F_{1, 44} = 6.637$; CA3: $F_{1, 44} = 9.437$; DG: $F_{1, 44} = 4.865$, $p < 0.05$). In Fkbp52$^{+/-}$ mice, chronic stress led to significantly higher MR mRNA expression levels in all three hippocampal regions compared to Fkbp52$^{+/-}$ control animals (tests with contrasts in ANOVA, $p < 0.05$). This effect was not observed in WT mice (Fig. 6B). No significant condition or genotype changes in mRNA expression could be shown for GR, AR and FKBP51 in the hippocampus and GR, AVP and FKBP51 in the PVN (data not shown).

Figure 6

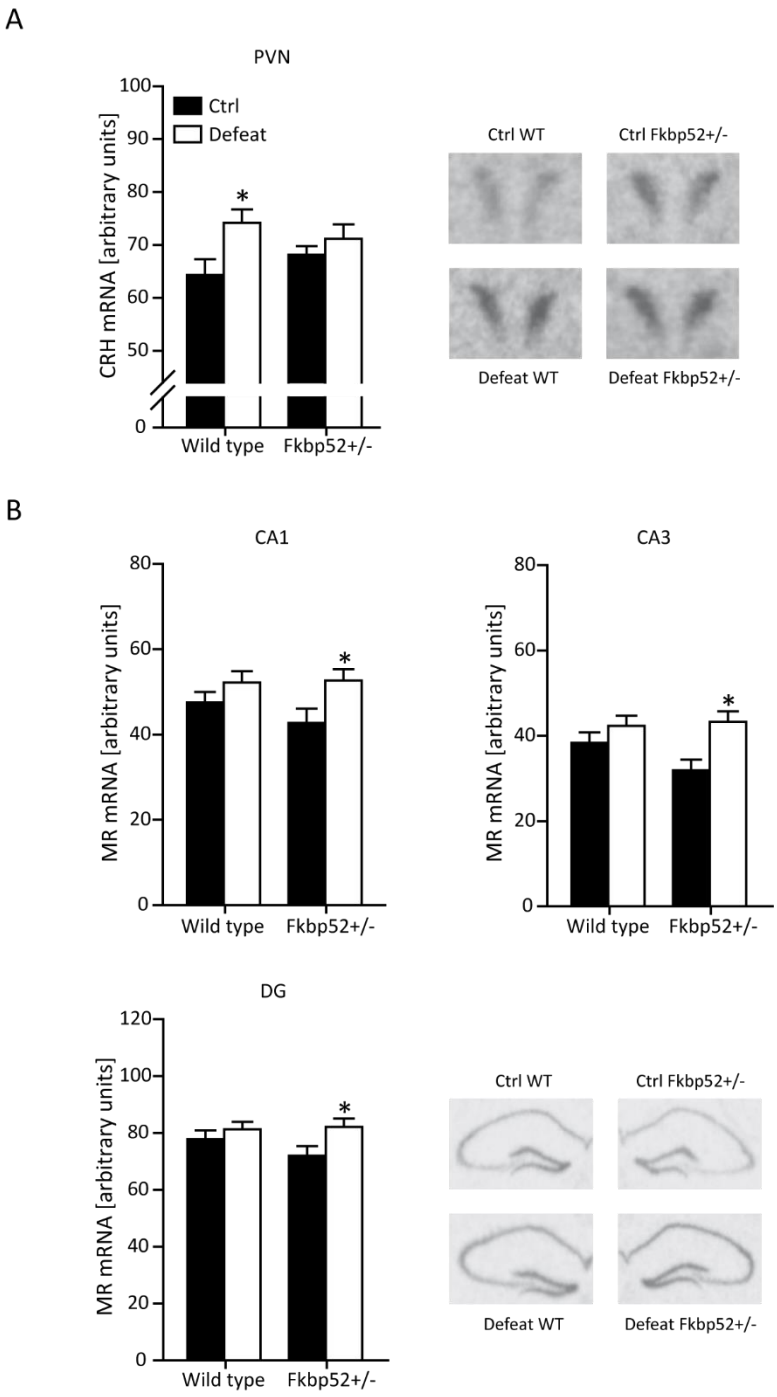

Figure 6: Gene expression in the paraventricular nucleus (PVN) and hippocampus of Fkbp52$^{+/-}$ and WT mice. (A) Corticotropin-releasing hormone (CRH) mRNA expression in the PVN (left panel) ($N_{WT\ Ctrl}$ = 10, $N_{Fkbp52^{+/-}\ Ctrl}$ = 11, $N_{WT\ Stress}$ = 11, $N_{Fkbp52^{+/-}\ Stress}$ = 12). Representative *in situ* hybridization images in the PVN (right panel). (B) Mineralocorticoid receptor (MR) mRNA expression in the hippocampus (CA1, CA3 and dentate gyrus (DG)) ($N_{WT\ Ctrl}$ = 11, $N_{Fkbp52^{+/-}\ Ctrl}$ = 11, $N_{WT\ Stress}$ = 10, $N_{Fkbp52^{+/-}\ Stress}$ = 12). Representative *in situ* hybridization images in the hippocampus (lower right panel). Data are represented as mean + SEM. * significantly different from the control condition of the same genotype, $p < 0.05$.

Discussion

By characterizing Fkbp52$^{+/-}$ mice generated in the Cox/Smith lab, we were able to provide evidence that the co-chaperone FKBP52 significantly alters the animal's phenotype on behavioral and neuroendocrine levels under basal and chronic stress conditions. Intriguingly, these animals show a higher stress vulnerability in some parameters, while at the same time being less affected by the stress procedure in other parameters tested.

Loss of one functional Fkbp52 led to a significant decrease of Fkbp52 expression. Independent of genotype, CSDS led to robust physiological changes, such as higher adrenal gland and reduced thymus weights, implicating an increased HPA axis activity which has repeatedly been reported before (Karst and Joëls, 2003; Keeney et al., 2006; Schmidt et al., 2007; Wagner et al., 2011; Wang et al., 2011a; Chapter 1). Moreover, exposure to chronic stress caused a reduction in fur quality for both genotypes, which can be ascribed to an impaired grooming behavior, and has been associated with a depression-like phenotype (Ducottet et al., 2003; Mineur et al., 2003; Cryan and Holmes, 2005; Denmark et al., 2010). Alterations in body weight following chronic stress are frequently documented, but nonetheless a rather inconsistent finding. The outcome mainly depends on the type of stressor, its intensity, diet, as well as the paradigm duration, mouse strain and social status of the animal (Bartolomucci et al., 2009; Finger et al., 2011). In line with previous reports concerning body weight change in this CSDS paradigm, stressed mice gained more body weight than controls (Wagner et al., 2011; Wang et al., 2011a; Chapter 1). Taken together, the exposure to CSDS led to a number of profound physiological alterations, independent of genotype.

One of the most robust characteristics found in many depressed patients is an HPA axis overdrive (Holsboer, 2000). In mice, HPA axis hyperactivity caused by chronic stress has repeatedly been shown to increase circulating corticosterone (Bartolomucci et al., 2005; Keeney et al., 2006; Schmidt et al., 2010a; Schmidt et al., 2010b; Komatsu et al., 2011). Therefore, we assessed plasma corticosterone levels under control and chronic stress conditions. Basal corticosterone levels in control mice did not differ between genotypes, which has been reported previously (Warrier et al., 2010). And while CSDS led to increased basal circulating GCs in WT animals, Fkbp52$^{+/-}$ mice showed an attenuated response to

chronic stress, reflected by a lack of increased basal corticosterone levels. This finding is counterintuitive considering the reported glucocorticoid insensitivity in the Fkbp52 knockout mouse line generated by Yang et al., 2006. However, the increased hippocampal MR mRNA expression levels observed in chronically stressed Fkbp52$^{+/-}$ mice may account for a potential explanation for the blunted basal corticosterone levels to CSDS, due to a subtle shift in MR/GR balance towards increased MR signaling in chronically stressed Fkbp52$^{+/-}$ mice. This might be further supported by the fact that forebrain specific MR overexpressing female mice exhibit a moderate suppression of corticosterone response to restraint stress (Rozeboom et al., 2007), while conventional MR knockout mice show elevated plasma corticosterone levels (Gass et al., 2001).

HPA axis reactivity to an acute challenge was increased after CSDS, independent of genotype, which is in line with previous reports (Akana et al., 1992; Koolhaas et al., 1997; Bhatnagar and Dallman, 1998). Interestingly, even under control conditions Fkbp52$^{+/-}$ mice showed an increased HPA axis response to the acute stress challenge and a blunted recovery under chronic stress conditions. In contrast to the data under basal conditions, these findings are in line with our initial hypothesis of a higher vulnerability of Fkbp52$^{+/-}$ mice to chronic stress. Given the fact that FKBP52 has been shown to be a positive regulator of GR function *in vitro* (Wochnik et al., 2005) and the reported increased susceptibility to high fat diet-induced glucocorticoid resistance in Fkbp52$^{+/-}$ mice (Warrier et al., 2010), our findings of an increased HPA axis response and an attenuated negative feedback in Fkbp52$^{+/-}$ mice following an acute stress challenge might be most likely due to reduced GR sensitivity.

Numerous acute and chronic stressors have previously been shown to alter the expression of central HPA axis related genes, such as CRH, AVP, FKBP51, MR and GR in the PVN and/or the hippocampus (Makino et al., 1995; Albeck et al., 1997; Herman and Spencer, 1998; Bartolomucci et al., 2003; Figueiredo et al., 2003; Keeney et al., 2006; Schmidt et al., 2007; Lachize et al., 2009; Scharf et al., 2011). In line with these reports, CSDS resulted in increased CRH mRNA expression in the PVN of WT mice. Interestingly, this was not observed in Fkbp52$^{+/-}$ mice. However, the reduced corticosterone levels and the increased hippocampal MR mRNA expression in chronically stressed Fkbp52$^{+/-}$ mice, might explain why CRH mRNA levels in the PVN were not increased as observed in WT mice following CSDS. The lack of AVP, GR and FKBP51 regulation may be ascribed to the nature, duration and intensity of the

applied stressor. In addition, the absence of genotype alterations in the analyzed genes may be due to compensatory effects.

It has repeatedly been shown that chronic stress can have a major impact on the behavioral phenotype of animals, often resulting in diminished locomotor activity and higher anxiety-related behavior (Choleris et al., 2001; Berton et al., 2006; Schmidt et al., 2007; Wagner et al., 2011; Chapter 1). Similarly, in the current study CSDS led to lower general locomotor activity in the OF and increased anxiety-related behavior in the EPM, independent of genotype. Interestingly, Fkbp52$^{+/-}$ mice displayed this stress-related phenotype already under non-stressed conditions in the EPM, which could possibly be due to reduced glucocorticoid sensitivity. In addition, only chronically stressed Fkbp52$^{+/-}$ mice developed a deficit in social behavior, resulting in a reduced social preference for an unfamiliar juvenile conspecific in the sociability test. A reduction of social preference is often reminiscent of core endophenotypes of depression (Nestler and Hyman, 2010; Dedic et al., 2011). Thus, in these behavioral parameters Fkbp52$^{+/-}$ mice seem to display a more stress sensitive phenotype. On the other hand, chronic stress has also been shown to lower the animals' active stress-coping behavior in the forced swim test (Karolewicz and Paul, 2001; Zhang et al., 2012). While we detected this effect in WT animals, this was not observed in Fkbp52$^{+/-}$ mice. This finding is of high importance, as it not only suggests a stress-resilience in this test, but also mimics the phenotype of Fkbp51 deficient mice in the FST (O'Leary, III et al., 2011; Touma et al., 2011). We did not observe a reduction of FKBP51 mRNA in Fkbp52$^{+/-}$ mice in the hippocampus and PVN. However, the deletion of FKBP52 may have resulted in a down regulation of hormone-induced FKBP51 expression in other brain regions or peripheral tissue (Yong et al., 2007), since it has been shown that FKBP51, but not FKBP52, is hormone-regulated. In addition, the lack of a reduced active stress-coping behavior in Fkbp52$^{+/-}$ mice might be related to the observed decrease of basal corticosterone levels and the increased MR mRNA expression in chronically stressed Fkbp52$^{+/-}$ mice. However, the causal relationship between stress system activity, GR sensitivity and stress-coping behavior is still unclear and awaits further investigation.

The interplay of FKBP52 with other steroid receptors (Gallo et al., 2007; Schülke et al., 2010; Sanchez, 2011; Storer et al., 2011) does not allow for a simple explanation of our results. The observed effects may not exclusively be due to increased glucocorticoid insensitivity,

but could at least be partially the result of a combination of effects on various other receptor types. In addition, we used mice with a heterozygous ablation of Fkbp52, allowing for counter-regulatory effects. Nevertheless, our approach is more closely related to the natural situation of variations in Fkbp52 expression. Furthermore, using a spatial and temporal restriction of Fkbp52 expression instead of conventional knockouts may be a useful tool to provide further insight of the utility of FKBP52 as a therapeutic target. To our knowledge this data demonstrates for the first time the importance of FKBP52 as a mediator of the stress response and its close involvement in GR signaling in the context of stress related-disorders in an *in vivo* model.

Taken together, we demonstrate that Fkbp52$^{+/-}$ mice display symptoms of increased stress sensitivity in a subset of behavioral and neuroendocrine parameters, probably due to reduced GR sensitivity, and stress resilience in another subset of these parameters. This seeming discrepancy is in line with previous work showing that FKBP51 can be down-regulated in certain tissues of Fkbp52$^{+/-}$ mice (Yong et al., 2007), which may then partially mimic the phenotype of 51KO mice. Therefore, the observed effect in Fkbp52$^{+/-}$ mice may have been a complex mixture, resulting from the deletion of FKBP52 and a potential down-regulation of FKBP51, especially under conditions where GR activity was enhanced. In addition, FKBP52 is not involved in all branches of GR signalling, but instead acts in gene-specific manner to regulate GR transcriptional activation (Wolf et al., 2009). Therefore, our findings clearly underline the potential of FKBP52 for the treatment of diseases that are involved in functional steroid receptor signaling pathways.

3.4. FKBP51 critically impacts antidepressant action and chronic stress recovery

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Manuscript in preparation

Abstract

Aversive life events represent one of the main risk factors for the development of numerous psychiatric diseases, but the interplay between environmental factors and genetic predispositions is still poorly understood. One major finding in many depressed patients is an impaired regulation of the hypothalamic-pituitary-adrenal (HPA) axis. The negative feedback loop of the HPA axis is mediated via the glucocorticoid receptor (GR) and the mineralocorticoid receptor, whose ligand binding sensitivity has been shown to be decreased by the FK506-binding protein 51 (FKBP51) . In humans, polymorphisms in the FKBP5 gene have been linked to psychiatric diseases. Recently, we could show that FKBP51 knockout mice (51KO mice) are less vulnerable to three weeks of chronic social defeat stress (CSDS). In a follow up study, we now aimed to investigate the ability of 51KO mice to recover from CSDS and to examine the effects of chronic paroxetine treatment during the 3-week recovery period. Mice were subsequently analyzed with regard to physiological, neuroendocrine and behavioral alterations. Our results show an enhanced recovery in 51KO mice with respect to physiological, neuroendocrine and behavioral parameters compared to wild-type animals. 51KO mice demonstrated lower adrenal weights and corticosterone levels, as well as no alteration in social behavior as observed in stressed WT mice in the social avoidance test. These results suggest an enhanced negative glucocorticoid feedback within the HPA axis of 51KO mice, likely modulated by an increased sensitivity of the GR. Moreover, chronic paroxetine treatment did not alter the behavioral phenotype of 51KO mice as observed in WT animals in the forced swim test and the social avoidance test. These results are in line with clinical studies, showing that patients with increased FKBP51 protein levels in lymphocytes, experience increased numbers of depressive episodes, but also respond faster to antidepressants.

Introduction

Psychiatric disorders, such as major depression (MD) and post-traumatic stress disorder (PTSD) are a significant disease burden worldwide, but the underlying molecular mechanisms are still poorly understood (Tennant, 2001; Krishnan and Nestler, 2008; Nestler and Hyman, 2010). One of the most robust characteristics in many depressed patients is an altered glucocorticoid receptor (GR) signaling, which often leads to a disturbed negative feedback loop of the hypothalamic-pituitary-adrenal (HPA) axis and thus to partial glucocorticoid resistance (Holsboer, 2000; Pariante and Miller, 2001). In recent years, genome-wide association studies identified several HPA axis related genes that are contributing to the development of psychiatric diseases (Engineer et al., 2013; Rogers et al., 2013; Guillaume et al., 2013).

One interesting candidate gene is the co-chaperone FK506 binding protein 51 (FKBP51), which has been shown to alter GR sensitivity. When it is bound to GR via hsp90, the receptors affinity for cortisol (in humans), or corticosterone (in rodents), is decreased (Riggs et al., 2003). Upon hormone binding, FKB51 is replaced by its close homologue and functional opponent FKBP52, which in turn enhances the receptor's translocation to the nucleus and subsequent DNA binding (Davies et al., 2002; Wochnik et al., 2005; Binder, 2009). Single nucleotide polymorphisms in the FKBP51 gene have repeatedly been associated with suicidal behavior, bipolar disorder, MD and PTSD (Binder et al., 2008; Brent et al., 2010; Horstmann et al., 2010; Lavebratt et al., 2010; Pérez-Ortiz et al., 2012; Klengel et al., 2013). Interestingly, risk allele carriers of FKBP51 have elevated FKBP51 protein levels in lymphocytes, an increased recurrence of depressive episodes, but also an enhanced selective serotonin reuptake inhibitor (SSRI) treatment response (Binder et al., 2004; Lekman et al., 2008; Laje et al., 2009; Zou et al., 2010).

In addition to the genetic contribution to psychiatric disorders, the environment plays a significant role in the development of mood disorders. It is generally accepted, that trauma and chronic stress represent key risk factors that are able to trigger (in interaction with a genetic predisposition and previous life history) the development of psychiatric diseases (McEwen, 2004; de Kloet et al., 2005a; Chrousos, 2009).

To further elucidate the complex nature of these disorders, mouse models and stress paradigms have proven extremely useful not only regarding the validation of neurobiological underpinnings, but also with respect to the identification and improvement of therapeutic substances (Nestler and Hyman, 2010). Indeed, we could recently show that FKBP51 knockout (51KO) mice are less affected by three weeks of chronic social defeat stress (CSDS), especially with regard to neuroendocrine parameters (Chapter 1).

The genetic link of increased FKBP51 levels, increased numbers of depressive episodes and an enhanced antidepressant treatment response in humans, made us question whether we would see similar results in the FKBP51 knockout mouse line. We hypothesised that mice lacking the FKBP51 gene may show an enhanced recovery from the long lasting effects of CSDS and may be less responsive to antidepressant treatment. We therefore analyzed the ability of 51KO mice and wild-type littermates to recover from three weeks of CSDS and the effects of chronic paroxetine treatment during a 3-week recovery period, thereby closely mimicking the clinical treatment situation.

Materials and Methods

Animals

The Fkbp51 knockout (51KO) mouse line was previously generated (Tranguch et al., 2005) and fully backcrossed to C57/Bl6. Genotypes were verified by PCR of tail DNA. Only male mice were used for the experiment, obtained from heterozygous breeding pairs. Animals were between 10 and 16 weeks old at the start of the experiment. Mice were held under standard conditions (12L: 12D light cycle, lights on at 08:00 am, temperature 23±2°C), were singly housed and acclimated to the room for one week before the beginning of the experiments. Male CD1 mice (16–18 weeks of age) served as resident mice, which were held under the conditions described above. They were allowed to habituate to the social defeat cage for 2 weeks before the experiment. Food (Altromin 1314, Altromin GmbH, Germany) and tap water were available ad libitum.

All experiments were carried out in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 86/609/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Experimental design

We analyzed the ability of 51KO mice to recover from CSDS and the effects of chronic paroxetine treatment during a 3-week recovery period. Initially, wild-type and 51KO mice were split into 2 x 2 groups (control WT, control 51KO, defeat WT, defeat 51KO) and subjected to the chronic stress procedure described below. After cessation of the stressor, groups were subdivided into vehicle-treated and paroxetine-treated animals, resulting in 8 groups (n = 7-13 per group). The treatment phase lasted for three weeks. The behavioral tests took place during the last week of the paroxetine treatment.

Chronic Stress Procedure and Physiological Parameters

The CSDS paradigm lasted for 21 days and was conducted as described previously (Wagner et al., 2011). Briefly, the experimental mice were introduced into the home cage (45 cm x 25 cm) of a dominant resident mouse and defeated shortly after. When the defeat was achieved, the animals were separated by a wire mesh, preventing physical but allowing sensory contact for 24 h. Each day, stressed animals were defeated by another unfamiliar, dominant resident mouse in order to exclude a repeated encounter throughout the experiment. The daily defeat was performed between 1100 and 1600 h; varying starting times reduced the predictability of the stressor and therefore minimized a potential habituation effect. Experimental mice were always defeated by resident males during the entire stress period. Control mice were housed in their home cages during the course of experiment. Both stress and control animals were handled daily during the stress procedure; body weight for all mice was assessed at the beginning of the experiment, after the cessation of the stress period, and on the day of killing. Animals that underwent the stress procedure were subsequently single housed in standard cages.

Paroxetine treatment

Paroxetine (GlaxoSmithKline, Munich, Germany) was administered via drinking water as described previously (Wagner et al., 2012). Briefly, the paroxetine solution was diluted in tap water to a final concentration of 0.16 mg/ml. With average water consumption of 5ml/mouse/day, the daily dose of paroxetine was ~20 mg/kg body weight. Fluid intake was monitored daily and the variation of fluid intake was found to be <10% over the course of experiment.

Behavioral Analysis

The behavioral tests were carried out between 0830 and 1230 h in the same room in which the mice were housed. The testing order was as follows: Open-field (OF), social avoidance (SA), forced swim test (FST), and acute stress response. All tests were analyzed using an automated videotracking system (Anymaze 4.20, Stoelting, Wood Dale, IL). All animals underwent the same testing battery in the same order of tests. To minimize possible

carryover effects of the different behavioral tests, the sequence of tests was arranged from the least stressful to the most stressful, with a minimum of 24 h between tests (McIlwain et al., 2001).

Open-field test

The open-field test was performed to investigate locomotion differences. Testing was carried out in an empty open-field arena (50 cm x 50 cm x 50 cm) made of gray polyvinyl chloride (PVC), which was evenly illuminated with 15 Lux. The low illumination of the open field arena was chosen to specifically investigate locomotion behavior and not create an aversive center region that may induce anxiety-related behavior. Testing time was 10 min and the main parameter of interest was the total distance travelled.

Social avoidance test

The social avoidance test was performed as described previously (Golden et al., 2011). Briefly, animals were allowed to explore the open field arena for 2.5 min with an empty wire mesh cage placed at one side of the apparatus. In a second stage, the animals were confronted with an unfamiliar CD1 resident mouse in the wire mesh cage for another 2.5 min. The ratio between the time in the interaction zone of the no-target trial and the time in the interaction zone of the target trial serves as a marker for disturbed social behavior associated with depressive disorders. Animals that did not explore the interaction zone at all were excluded from the analysis (wt ctrl veh = 1; 51KO ctrl veh = 1; wt ctrl PAR = 2; 51KO ctrl PAR = 1; wt stress veh = 1; 51KO stress veh = 1; wt stress PAR = 1).

Forced swim test

In the forced swim test, each mouse was put into the a 2 l glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 5 min. Time spent immobile (floating) and time spent struggling was scored by an experienced observer, blind to treatment or condition of the animals.

Acute stress response

The FST also served as an acute stressor in order to determine the stress response by measuring corticosterone plasma concentrations. After the FST, all mice were towel-dried and placed into their home cage to recover from the acute stressor. Blood samples were taken by tail cut (Fluttert et al., 2000) 30 min (stress response) and 90 min (stress recovery) after the onset of the FST. Samples were collected in 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). All blood samples were kept on ice and later centrifuged at 8000 rpm at 4 °C for 15 min. Plasma was transferred to new, labeled tubes and stored at -20 °C until determination of corticosterone by radioimmunoassay (MP Biomedicals Inc; sensitivity 12.5 ng/ml).

Sampling Procedure

24 hours after the last defeat, blood samples were taken by tail cut as described above, in order to assess the plasma corticosterone levels immediately after the chronic stress paradigm. 3 weeks after the last defeat, thus following 3 weeks of treatment, all animals were killed by decapitation following quick anesthesia by isoflurane at the end of the experiment. Basal trunk blood samples were processed as described above. Adrenal and thymus glands were removed, dissected from fat, and weighed.

Statistical Analysis

The data presented are shown as means+SEM, analyzed by the commercially available software SPSS 16.0. When two groups were compared, the student's *t*-test was applied. For four or more group comparisons, 2-way or 3-way analysis of variance (ANOVA) was performed. If interaction effects ($p < 0.05$) were observed Tukey post-hoc tests were applied. A nominal level of significance $p < 0.05$ was accepted.

Results

We aimed to investigate the ability of 51KO mice to recover from CSDS and to examine the effects of chronic paroxetine treatment during the 3-week recovery period (Figure 1).

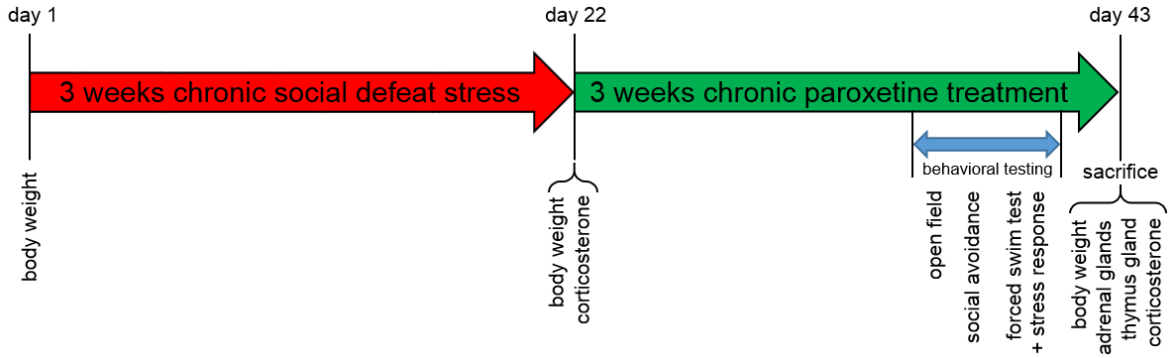

Figure 1: Time course of the experiment. Treatment with paroxetine commences after the stress procedure. Body weight was assessed on day 1, day 22 and day 43. Basal corticosterone was measured on day 22 and day 43. The behavioral testing (open field, social avoidance, forced swim test) and the stress response test (corticosterone samples 30 min and 90 after the FST) is performed in the last week of the treatment phase.

Physiology

51KO mice were significantly leaner compared to wild-type mice before the start of the experiment ($T_{84} = 3.458$, $p < 0.001$) (Figure 2A). After 3 weeks of CSDS, all chronically stressed animals demonstrated a significant increase in body weight (2-way ANOVA, condition effect: $F_{1,81} = 4.865$, $p < 0.05$). Independent of condition, 51KO mice were still significantly leaner than wild-type animals (2-way ANOVA, genotype effect: $F_{1,81} = 17.794$, $p < 0.001$) (Figure 2B). On the day of sacrifice (e. g. day 43, after 3 weeks treatment), we found a main genotype effect (3-way ANOVA, $F_{1,66} = 30.177$, $p < 0.001$) and a condition x treatment interaction (3-way ANOVA, $F_{1,66} = 3.296$, $p = 0.074$) for body weight (Figure 2C). In order to isolate genotype-dependent stress and treatment effects, we proceeded to normalize the data to either non-stressed controls or vehicle treatment. First, we normalized all stressed groups by assessing the difference in body weight compared to their corresponding unstressed control of the same genotype and treatment. We found a significant treatment effect (2-way ANOVA, $F_{1,36} = 5.557$, $p < 0.05$), because stress-induced increase of body weight was significantly more pronounced in vehicle treated mice than in paroxetine treated mice (Figure 2D). To isolate stress effects as well as genotype-dependent treatment effects, we

then normalized all paroxetine treated groups by assessing the difference in body weight compared to their corresponding vehicle treated control of the same genotype and condition. 2-way ANOVA revealed a significant condition ($F_{1,36}$ = 6.000, $p < 0.05$) and genotype effect ($F_{1,36}$ = 4.458, $p < 0.05$). Paroxetine-induced body weight increase was significantly enhanced in mice under basal conditions compared to stressed animals. In addition, 51KO mice were significantly less affected by paroxetine-induced body weight increase compared to wild-type mice (Figure 2E). For adrenal gland size, 3-Way ANOVA revealed a main genotype effect ($F_{1,69}$ = 51.909, $p < 0.001$), a main condition effect ($F_{1,69}$ = 60.813, $p < 0.001$), a genotype x condition interaction ($F_{1,69}$ = 51.909, $p < 0.05$) and a genotype x treatment interaction ($F_{1,69}$ = 4.838, $p < 0.05$) (Figure 2F). To analyze simple treatment effects and genotype-dependent stress effects, we normalized all stressed groups by assessing the difference in adrenal gland weight compared to their corresponding unstressed control of the same genotype and treatment. Stress-induced increase of adrenal gland weight was significantly less pronounced in paroxetine-treated mice compared to vehicle treated animals (2-way ANOVA, $F_{1, 38}$ = 4.407, $p < 0.05$). In addition, 51KO mice were significantly less affected by stress-induced adrenal gland enlargement compared to wild-type mice (2-way ANOVA, $F_{1, 38}$ = 12.609, $p < 0.001$) (Figure 2G). To isolate stress effects and genotype-dependent treatment effects, we normalized all paroxetine treated groups by assessing the difference in adrenal weight compared to their corresponding vehicle treated control of the same genotype and condition. 2-way ANOVA revealed a condition effect ($F_{1, 36}$ = 8.620, $p < 0.01$) and a genotype effect ($F_{1, 36}$ = 22.198, $p < 0.001$) (Figure 2H). Paroxetine treatment induced adrenal gland enlargement in wild-type mice under control conditions, but not under defeat conditions (Figure 2H). The lack of paroxetine-induced enlargement of the adrenals in defeated wild-type mice, might however be a ceiling effect, due to the strong stress-induced adrenal gland increase (Figure 2G). In contrast to the wild-types, adrenal gland weight of 51KO mice under control conditions was not affected by paroxetine. However, under stress conditions paroxetine treatment led to a decrease in adrenal gland size (Figure 2H). For the thymus weight, stressed animals showed significantly reduced thymus gland size, with 51KO mice having a decreased overall thymus gland weight (3-Way ANOVA: main condition effect: $F_{1,70}$ = 6.546, $p < 0.05$; main genotype effect: $F_{1,70}$ = 63.415, $p < 0.001$) (Figure 2I). We did not find any genotype-dependent treatment or stress effects (data not shown).

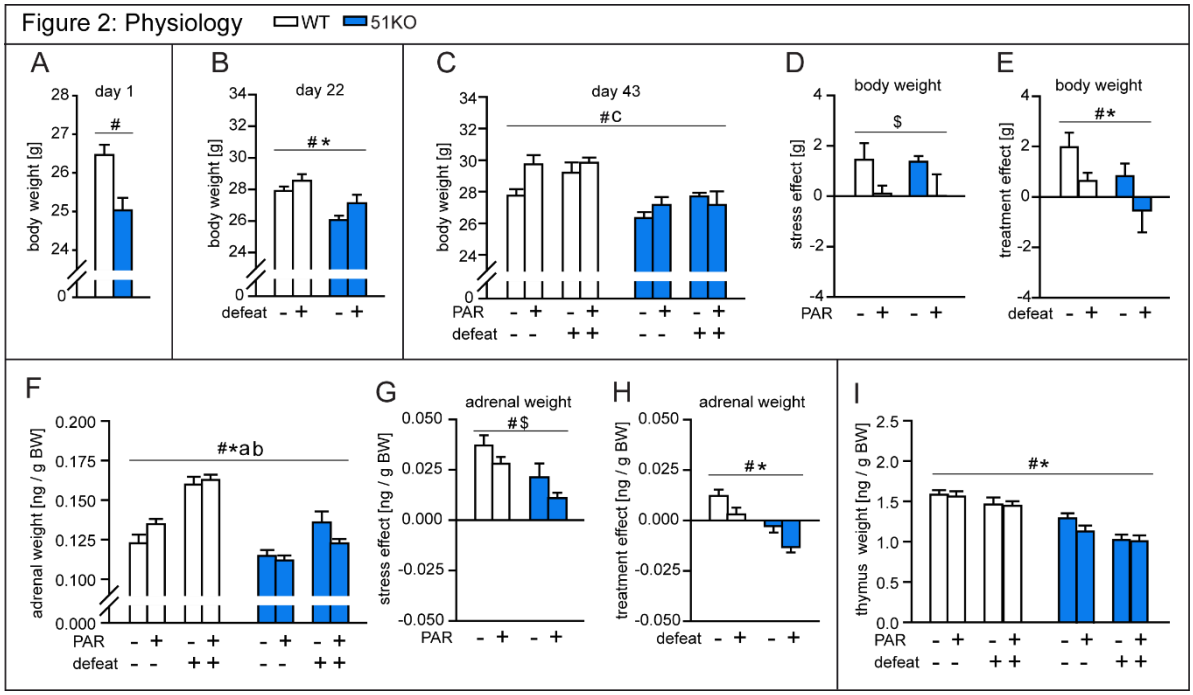

Figure 2: FKBP51 shapes the physiological effects of stress and paroxetine. Whenever we found an interaction effect ($p < 0.1$) in the 3-way ANOVA, we additionally isolated genotype-dependent stress and treatment effects, by normalizing the data to either non-stressed controls or vehicle treatment. A, Body weight was significantly reduced in 51KO mice before the start of the first defeat. B, CSDS induced a significant increase of body weight. 51KO showed still significantly reduced body weight, independent of condition. C-E, Stress-induced body weight increase was still present after 3 weeks of recovery. Paroxetine-induced increase of body weight was most pronounced under control conditions. 51KO mice were less affected by paroxetine-induced body weight increase. F-H, 51KO mice showed overall reduced adrenal gland size compared to wild-type mice. Adrenal gland weight was significantly increased in stressed animals. The stress effect was less pronounced in 51KO mice compared to wild-type animals. Paroxetine led to adrenal gland enlargement in WT and reduced size in 51KO mice. I, Thymus size was significantly reduced following CSDS. 51KO mice demonstrated significantly lower thymus weight than wild-type mice. # main genotype effect, * main condition effect, $ main treatment effect, *a* genotype x treatment interaction, *b* genotype x condition interaction, *c* condition x treatment interaction; for #, *, $, a, b: $p < 0.05$; for *c* $p < 0.1$; data are expressed as mean + SEM.

Neuroendocrinology

Directly after the cessation of the stressor on day 22, 2-way ANOVA revealed a condition effect ($F_{1,76} = 16.226$, $p < 0.001$), a genotype effect ($F_{1,76} = 6.465$, $p < 0.05$) and a genotype x condition interaction ($F_{1,76} = 4.519$, $p < 0.05$) for basal corticosterone levels. Tukey post-hoc tests showed that corticosterone levels were significantly increased in stressed wild-type mice compared to control wild-type mice ($p < 0.05$). This stress effect was not observed in 51KO, instead they showed significantly reduced corticosterone levels compared wild-type mice under defeat conditions ($p < 0.05$) (Figure 3A). On day 43, 51KO mice showed an overall

reduction of basal corticosterone levels compared to wild-type animals (3-way ANOVA: main genotype effect: $F_{1,68}$ = 18.256, $p < 0.001$). 3-way ANOVA also revealed a genotype x treatment interaction ($F_{1,68}$ = 2.991, p = 0.088) (Figure 3B). To isolate simple treatment effects and genotype-dependent stress effects, we normalized all stressed groups by assessing the difference in corticosterone compared to their corresponding unstressed control of the same genotype and treatment. 2-way ANOVA only revealed a significant genotype effect ($F_{1, 35}$ = 7.407, $p < 0.01$); corticosterone levels in 51KO were not affected by the previous stress period as observed in wild-type mice (Figure 3C). To isolate stress effects and genotype-dependent treatment effects, we normalized all paroxetine treated groups by assessing the difference in corticosterone compared to their corresponding vehicle treated control of the same genotype and condition. Paroxetine treatment reduced corticosterone levels especially in 51KO mice, independent of condition (2-way ANOVA, genotype effect, $F_{1, 36}$ = 4.924, $p < 0.05$) (Figure 3D). In response to a novel acute stressor, lack of FKBP51 largely decreased the corticosterone response, independent of condition or treatment (3-Way ANOVA: main genotype effect: $F_{1,71}$ = 85.594, $p < 0.001$) (Figure 3E). At 90 min after the acute stressor, all defeated animals recovered faster from the acute challenge (3-Way ANOVA: main condition effect: $F_{1,73}$ = 10.703, $p < 0.01$). In addition, 51KO mice demonstrated significantly lower corticosterone recovery levels than wild-type animals (3-Way ANOVA: genotype effect: $F_{1,73}$ = 67.998, $p < 0.001$) (Figure 3F).

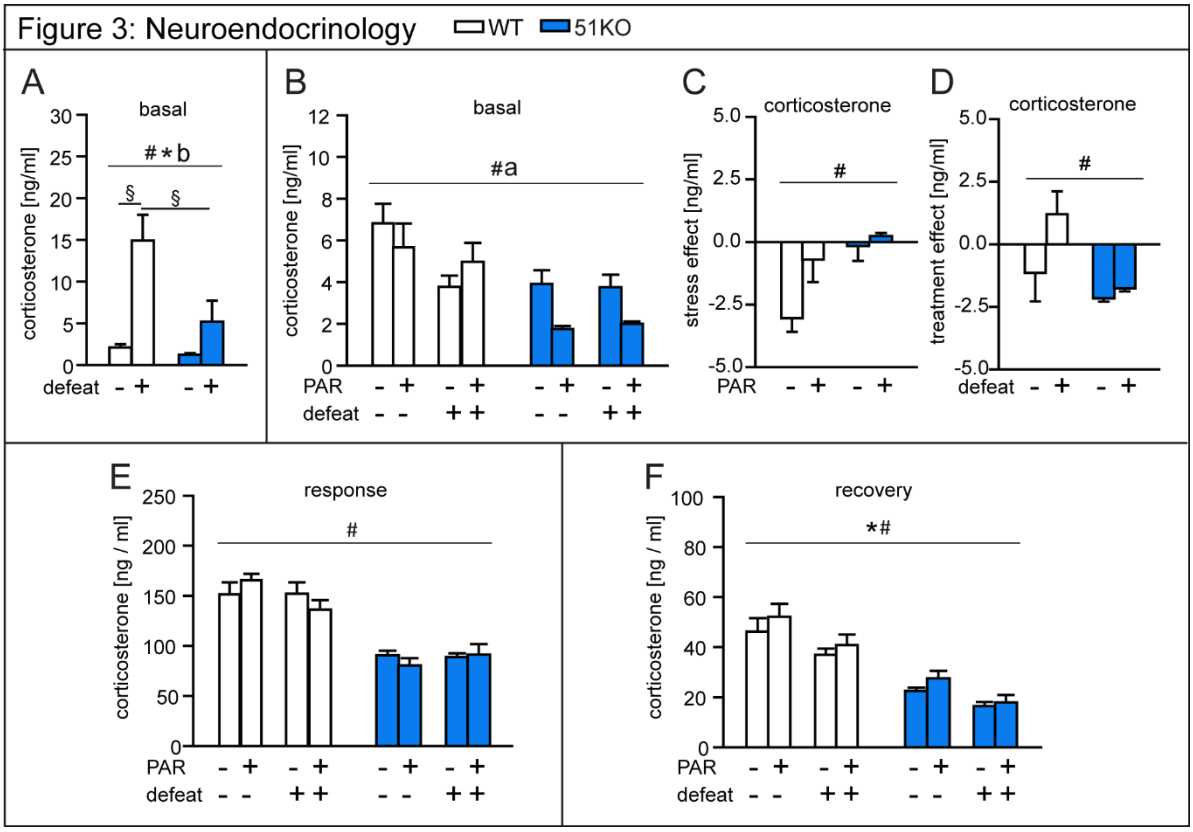

Figure 3: FKBP51 shapes the neuroendocrine effects of stress. Whenever we found an interaction effect ($p < 0.1$) in the 3-way ANOVA, we additionally isolated genotype-dependent stress and treatment effects, by normalizing the data to either non-stressed controls or vehicle treatment. **A**, CSDS resulted in significantly increased basal corticosterone levels in wild-type, but not in 51KO mice. **B**, Basal corticosterone levels assessed three weeks after the stressor were significantly lower in 51KO mice. **C**, Basal corticosterone levels of 51KO mice were less affected by the long lasting effects of CSDS. **D**, Paroxetine decreased basal corticosterone especially in 51KO mice. **E** and **F**, Circulating corticosterone was significantly decreased in 51KO mice in response to an acute stressor as well as after a 90 min recovery period. # main genotype effect, *a* genotype x treatment interaction, *b* genotype x condition interaction, § Tukey post-hoc; for #, b, §: $p<0.05$; for *a*, $p < 0.1$; data are expressed as mean + SEM.

Behavior

In the third week of the treatment phase, general locomotion in the open field did not differ between genotype, condition or treatment groups (Figure 4A). In the social avoidance test, 3-Way ANOVA revealed a main treatment effect ($F_{1,66} = 6.084$, $p < 0.05$), a genotype x condition interaction ($F_{1,66} = 4.271$, $p < 0.05$) and a genotype x treatment interaction ($F_{1,66} = 3.221$, $p = 0.077$) (Figure 4B). To isolate treatment effects and genotype-dependent stress effects, we normalized all stressed groups by assessing the difference in the social interaction ratio compared to their corresponding unstressed control of the same genotype and treatment. We found that CSDS significantly reduced social behavior in wild-type, but

not in 51KO mice (2-way ANOVA, $F_{1,33} = 10.677$, $p < 0.01$) (Figure 4C). To isolate stress effects and genotype-dependent treatment effects, we normalized all paroxetine treated groups by assessing the difference in the social interaction ratio compared to their corresponding vehicle treated control of the same genotype and condition. Paroxetine significantly enhanced social behavior in wild-type, but not in 51KO mice, both in CSDS-exposed and control animals (2-way ANOVA, $F_{1,31} = 5.663$, $p< 0.05$) (Figure 4D). In the forced swim test, we observed a genotype x treatment interaction for the parameters struggling and floating (3-Way ANOVA; struggling: $F_{1,72} = 3.777$, $p = 0.056$; floating: $F_{1,72} = 4.784$, $p < 0.05$) (Figure 4E and F). To isolate stress effects and genotype-dependent treatment effects, we normalized all paroxetine treated groups by assessing the difference in struggling and floating compared to their corresponding vehicle treated control of the same genotype and condition. In both parameters, 51KO mice were significantly less affected by paroxetine treatment compared to wild-type mice, independent of condition (2-way ANOVA, genotype effect, struggling: $F_{1,35} = 5.105$, $p < 0.05$, floating: $F_{1,35} = 11.740$, $p < 0.01$) (Figure 4G and H). The parameter swimming did not differ between genotype, condition or treatment groups (data not shown).

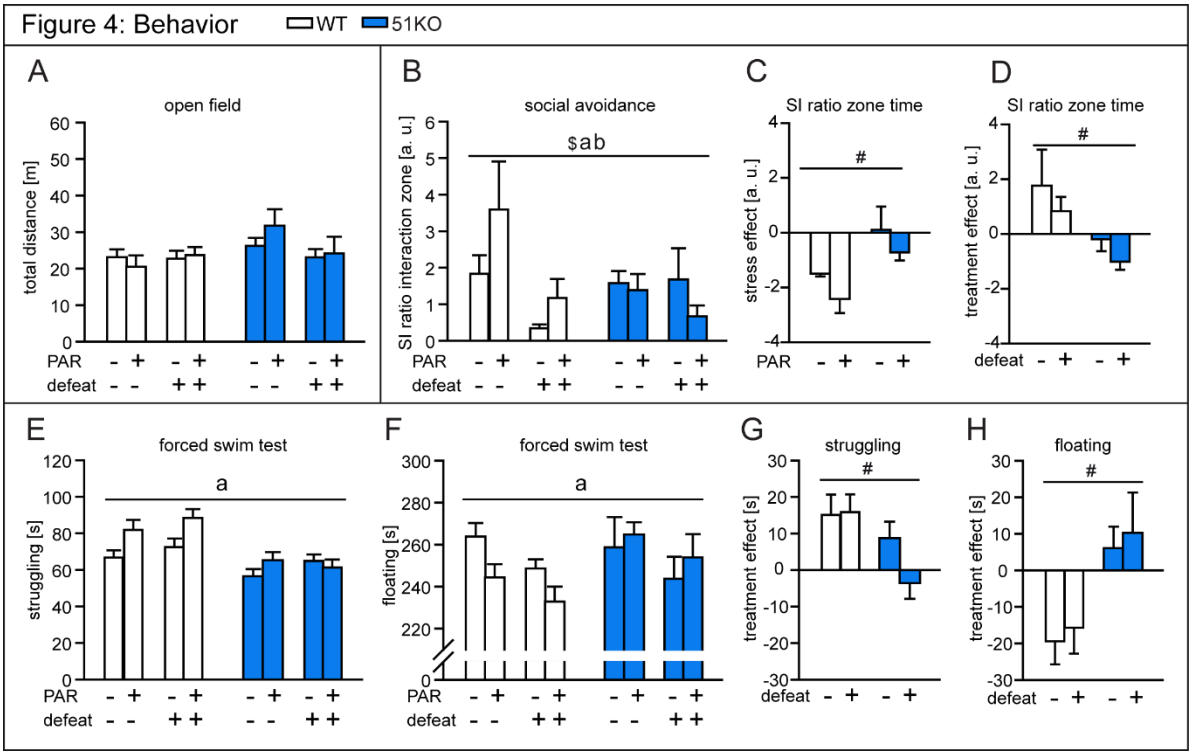

Figure 4: FKBP51 shapes the behavioral effects of stress and paroxetine. Whenever we found an interaction effect ($p < 0.1$) in the 3-way ANOVA, we additionally isolated genotype-dependent stress and treatment effects, by normalizing the data to either non-stressed controls or vehicle treatment. **A**,

General locomotion in the open field did not differ, independent of genotype, condition or treatment of the mice. **B** - **D**, Stressed 51KO mice demonstrated no alteration in social behavior, as observed in stressed WT mice in the social avoidance test. Paroxetine treatment could enhance social behavior in wild-type, but not in 51KO mice. **E-H**, Paroxetine treatment significantly increased the time struggling and decreased the time floating of wild-type mice in the forced swim test. 51KO mice were significantly less affected by paroxetine-induced FST behavior. # main genotype effect, $ main treatment effect, *a* genotype x treatment interaction, *b* genotype x condition interaction; for #, $: $p<0.05$; for *a*, *b*: $p < 0.1$; data are expressed as mean + SEM.

Discussion

In this study, we show that FKBP51 critically impacts the long lasting effects of chronic stress as well as paroxetine responsiveness in mice. CSDS resulted in robust physiological, neuroendocrine and behavioral alterations in wild-type mice that were still detectable after a 3 weeks recovery period. These stress effects included increased body weight and adrenal gland size, as well as increased social withdrawal. Interestingly, these long lasting alterations were less pronounced in 51KO mice. Treatment with the commonly used SSRI paroxetine could ameliorate the stress-induced phenotype in wild-type mice, especially with regard to social behavior. Moreover, chronic paroxetine treatment evoked substantial changes in physiology and forced swim test behavior even under control conditions. However, 51KO mice were less responsive to paroxetine treatment with regard to physiological and behavioral parameters, independent of condition.

In order to confirm the stress effects of the paradigm, we assessed a couple of physiological and neuroendocrine parameters immediately after the cessation of the stressor. CSDS resulted in significantly increased body weight at day 22, which has previously been reported for this stress paradigm (Wagner et al., 2011; Chapter 1 and 3). In addition, elevated circulating glucocorticoids are a robust indicator of chronic stress and an impaired negative feedback regulation of the HPA axis. Thus, we measured basal corticosterone 24 hrs after the last defeat. In line with previous reports, CSDS evoked significantly elevated corticosterone levels in wild-type mice compared to control mice of the same genotype, confirming the effectiveness of stress paradigm (Wagner et al., 2011; Wagner et al., 2012; Chapter 3). As previously shown, we also detected significantly lower corticosterone levels in 51KO mice compared to wild-type mice under CSDS conditions (Chapter 1).

We were especially interested in the long lasting effects of chronic stress and the influences of a subsequent paroxetine treatment. Stress-induced physiological alterations were still detectable after the three weeks recovery period especially in WT mice. Body weight at day 43 was still increased in stressed mice compared to controls in vehicle-treated groups. Paroxetine treatment led to significantly increased body weight in wild-type mice, especially under control conditions, which is in line with previous findings (Wagner et al., 2012). Interestingly, 51KO mice were not affected by the paroxetine-induced body weight gain.

Adrenal glands were enlarged in all groups of stressed animals, implicating an increased HPA axis activity, which has repeatedly been reported before (Karst and Joëls, 2003; Keeney et al., 2006; Wang et al., 2011a; Wagner et al., 2012). However, the stress-induced adrenal gland enlargement was significantly lower in 51KO mice than in wild-type animals. This points to a less vulnerable phenotype of 51KO mice, which is most-likely meditated by a reduced HPA axis activity and an improved negative feedback regulation, due to an increased GR sensitivity (Wochnik et al., 2005; Tatro et al., 2009b; Chapter 1). This is in line with our previous report that 51KO mice were already less affected by the immediate effects of CSDS, reflected in a stress-resistant HPA system (Chapter 1). Interestingly, SSRI treatment shaped adrenal gland size in an opposing manner in both genotypes. While paroxetine increased adrenal gland weight in wild-type mice, it led to a decrease in 51KO mice, highlighting the impact of FKBP51 on paroxetine actions. Thymus weight was significantly decreased in 51KO mice compared to wild-type animals, independent of condition or treatment, which is in line with previous findings of this mouse line, possibly mediated by an enhanced GR sensitivity (Chapter 1).

Basal corticosterone on day 43, as well as response and recovery levels after the FST, were mainly shaped by FKBP51, independent of condition or treatment. 51KO always demonstrated significantly lower corticosterone values compared to wild-types of the same condition and treatment group. This is in line with previous reports, implicating FKBP51 as regulator of neuroendocrine stress physiology (Touma et al., 2011; Albu et al., 2013; Chapter 1).

Disturbed exploratory and social behavior are phenotypes that have repeatedly been observed during or immediately after the CSDS period (Berton et al., 2006; Golden et al., 2011; Wagner et al., 2011; Chapter 3). Regarding the long-lasting effects of our CSDS model, general locomotion, as assessed in the open field test, returned to basal levels, independent of genotype or treatment. However, even after the three weeks recovery period, social behavior was still severely affected, as stressed, vehicle-treated wild-type mice showed significantly increased social withdrawal compared to vehicle-treated WTs under control condition. This long-lasting stress effect was not observed in 51KO mice, which may implicate an enhanced recovery from CSDS, especially because the behavioral phenotype of wild-type and 51KO mice was equally affected by the immediate effects of CSDS in our

previous study (Chapter 1). Paroxetine treatment reversed social avoidance behavior in stressed wild-type mice and further enhanced their social activity under control conditions, which is in line with previous findings (Berton et al., 2006). In contrast, 51KO mice did not respond to the SSRI treatment with regard to social behavior. Along these lines, paroxetine evoked the classical antidepressant effects in the forced swim test of increased activity and lower immobility time in wild-type mice (Sillaber et al., 2008; Ghasemi et al., 2009; Wagner et al., 2012; Gai et al., 2014), while 51KO mice were significantly less responsive to the antidepressant treatment in the FST.

Intriguingly, our current findings on the one hand further underline the importance of FKBP51 in GR-dependent stress physiology, due to the resilient phenotype of 51KO mice. On the other hand we demonstrate that antidepressant actions are largely absent in this mouse line. Thus, stress resilience and lack of antidepressant response in these mice may point to different molecular pathways of FKBP51. Indeed, recent studies identified FKBP51 in various cellular processes besides its regulatory function of the GR (Pei et al., 2009; Blair et al., 2013). Therefore, it is likely that FKBP51 is also implicated in GR-independent functions, which may overlap with antidepressant pathways.

In summary, we could provide evidence that FKBP51 critically shapes the long lasting effects from chronic stress, as well as antidepressant actions. 51KO mice were less affected from CSDS after a three weeks recovery period on the physiological, neuroendocrine and behavioral level, which is possibly due to an enhanced GR sensitivity. We could previously show that 51KO were already less vulnerable to the immediate effects of CSDS. Therefore, our current findings are likely the result of a less vulnerable phenotype of 51KO mice immediately after the stress period together with an enhanced recovery from CSDS during the subsequent three weeks. The other major outcome of this study demonstrated that FKBP51 is necessary for the physiological and behavioral effects of the commonly used SSRI paroxetine. Our results strikingly mirror findings of human genetic studies, linking FKBP51 levels to the recurrence of depressive episodes as well as to antidepressant response rate (Binder et al., 2004; Lekman et al., 2008; Laje et al., 2009; Zou et al., 2010). Future studies are necessary to further investigate and understand the underlying molecular mechanisms. In conjunction with previous studies, our findings highlight the important role of FKPB51 in

the development of stress-associated psychiatric disorders and its potential as a drug target for future treatment options.

3.5. FKBP51 directs autophagic pathways, enabling prediction of antidepressant treatment response

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Manuscript in preparation

Abstract

Background

FK506 binding protein 51 (FKBP51) is an Hsp90 co-chaperone and regulator of the glucocorticoid receptor, and consequently of the stress hormone axis and stress physiology. Clinical studies suggested a genetic link between the co-chaperone FKBP51 and antidepressant response in mood disorders; however the underlying mechanisms remained elusive.

Methods and Findings

We first show that antidepressant action on behavior is abolished in FKBP51 KO mice. Searching for novel molecular actions of FKBP51 linked to antidepressant effects we found that autophagic markers, such as the autophagy initiator Beclin1, were increased following antidepressant treatment in brains from wild type but not FKBP51 KO animals. Further cellular and biochemical assays revealed that FKBP51 binds to Beclin1, changes decisive protein interactions and phosphorylation of Beclin1, and triggers autophagic pathways. In addition, we demonstrate that antidepressants enhance the FKBP51-Beclin1 complex formation and affect autophagic pathways in dependency of FKBP51. Since autophagic pathways have been linked to synaptic function, we also treated hippocampal slices with antidepressants and found that they enhanced hippocampal synaptic transmission in wild type, but not FKBP51 KO mice. Finally, in human blood cells of healthy subjects, FKBP51 levels correlate with the potential of antidepressants to induce autophagic pathways. Importantly, the clinical antidepressant response of patients with depression (sample size n=43) could be predicted by the antidepressant response of autophagic markers in patient-derived peripheral blood lymphocytes cultivated and treated *ex vivo*, as well as by the lymphocyte expression levels of FKBP51 and Beclin1 at admission.

Conclusions

These findings provide the first evidence for the molecular mechanism of FKBP51 in priming autophagic pathways; this process is linked to synaptic function and is required for full antidepressant potency. These newly dicoverd functions of FKBP51 also provide novel predictive markers for treatment outcome, further illustrating their high physiological and clinical relevance.

Introduction

Depression is a devastating mental disorder affecting an estimated 350 million people worldwide (Kessler et al., 2009). While currently available antidepressants (ADs) show an overall favorable benefit/risk balance, less than 50% of patients achieve sustained remission following treatment, indicating a large inter-individual variability in treatment efficacy and response (Geddes and Miklowitz, 2013). The poor effectiveness of ADs is at least partially due to the fact that the mechanism of action of these drugs, which is responsible for their clinical potency, is still largely unknown. As a consequence, it is currently also not possible to predict treatment success in individual patients, constraining physicians and patients to lengthy trial and error procedures with uncertain success.

FK506 binding protein 51 (FKBP51) is a regulator of the glucocorticoid receptor, and consequently of the stress hormone axis and stress physiology (Wochnik et al., 2005; Attwood et al., 2011; Touma et al., 2011; Klengel et al., 2013; Chapter 1). Human genetic studies suggested a link of FKBP51 to the AD response rate (Binder et al., 2004; Lekman et al., 2008; Laje et al., 2009; Zou et al., 2010). Despite the intimate connection of stress physiology to pathophysiology and treatment of depression (Holsboer, 2000; de Kloet et al., 2005a), the mechanistic role of FKBP51 for AD response has not been elucidated.

Putative convergent molecular pathways addressed by both FKBP51 and ADs might explain and substantiate the suggested impact of FKBP51 on AD response; the modulation of these pathways by FKBP51 could influence their reactivity to ADs. We analyzed whether these pathways involve autophagic events based on reports stating that some ADs alter at least the initial processes of autophagy (Rossi et al., 2009; Zschocke et al., 2011). Given that FKBP51 can act via the central chaperone Hsp90, which controls several kinases including autophagy regulators like Akt and Beclin1 (Taipale et al., 2010), the intracellular autophagic events appeared more likely to be co-regulated by FKBP51 than the membrane targets of ADs.

Autophagy is a conserved cellular degradation process ensuring continuous removal of damaged macromolecules, and thus, functional integrity of cells and tissues (Mizushima and Komatsu, 2011). The predominant form of autophagy involves a step-wise formation of an autophagosome engulfing damaged cytosolic proteins or organelles, and subsequent fusion

with a lysosome yield to the autolysosome. This process is orchestrated by a series of autophagy-related genes (Atg) and is regulated by complex pathways, which are partly under control of the kinase mTOR (Nakatogawa et al., 2009). Based on multitude of involved factors, autophagy is commonly assessed by the determination of several autophagy executers. These include, among others, the initiators Beclin1 and Vps34 (class III phosphatidylinositol-3-kinase), Atg12 and lipidation of LC3B-I (yielding LC3B-II) involved in autophagosome membrane expansion and vesicle formation, and the association of LC3B-II with mature autophagosomes. Very recently, the mTOR-independent regulation of autophagy was reported: Akt-dependent phosphorylation of Beclin1 shifts Beclin1's action from autophagy to apoptosis (Wang et al., 2012b). As guardian of cellular homeostasis, autophagy has been established as a pivotal process in a range of (patho-) physiological conditions including infectious diseases, cancer, diabetes, and more recently neurotransmission and neurodegeneration (Levine and Kroemer, 2008; Cai et al., 2010; Harris and Rubinsztein, 2012; Welberg, 2012). Indirect evidence has been provided for a potential role of autophagy in depression; some ADs change the autophagic flux and the expression of autophagy markers (Zschocke et al., 2011; Chen et al., 2012). Moreover, the induction of autophagy by the mTOR inhibitor rapamycin was reported to exert AD-like effects in mice (Cleary et al., 2008).

In this study, we examined the FKBP51-dependency of molecular actions of ADs on signaling pathways. We revealed a novel function of FKBP51 in directing autophagic processes. FKBP51 is required for the behavioral effects of ADs in mice, for the AD effects on autophagic pathways in cells and mice, and for the AD effects on synaptic function in brain slices. In humans, treatment response in patients could be predicted by the expression of FKBP51 and Beclin1 in lymphocytes, and in particular by the response of autophagic markers to AD treatment of lymphocytes cultivated *ex vivo*.

Methods

Detailed information on experimental procedures is provided in supplementary Materials and Methods.

Animal treatments and behavioral experiments

The *Fkbp51*$^{-/-}$ mouse line was previously generated (Touma et al., 2011) and fully backcrossed to C57/Bl6. Male mice (age 10-16 weeks) were injected intraperitoneally with a single dose of either PAR (10 mg kg^{-1}) or saline vehicle. 45 min later, a subgroup of mice (WT vehicle, n=10; Fkbp51$^{-/-}$ vehicle, n=9; WT PAR, n=10; Fkbp51$^{-/-}$ PAR, n = 10; WT) was sacrificed. Hippocampus and prefrontal cortex were extracted. Another subgroup (WT vehicle, n=8; Fkbp51$^{-/-}$ vehicle, n=8; WT PAR, n=9; Fkbp51$^{-/-}$ PAR, n=7) was subjected to a forced swim test (6 min test duration) 45 min after the injection.

Electrophysiology

Brain slices were obtained from 11-18 week-old male FKBP51$^{+/+}$ and FKBP51$^{-/-}$ mice. Preparation and staining of slices with the voltage-sensitive dye Di-4-ANEPPS as well as VSDI and data analysis were performed essentially as described (von Wolff et al., 2011; Refojo et al., 2011). Four acquisitions subsequently recorded at intervals of 5 s were averaged. Neuronal activity was evoked by square pulse electrical stimuli (200 µs pulse width) delivered via a custom-made monopolar tungsten electrode to the Schaffer collateral-commissural pathway (Stepan et al., 2012). The circular ROI was placed into the CA1 stratum radiatum near the stimulation electrode. The ROI also covered a part of the adjacent CA1 stratum pyramidale (Fig. 3). The intensity of voltage stimulation was adjusted to produce FDSs with peak amplitudes of ~30% of the highest attainable value.

Analysis of GFP-LC3 in astrocytes

2x10^6 cells were transfected with 2 ng GFP-LC3 expressing plasmid or the respective cloning vector using the Amaxa Nucleofactor. Cells were grown for 48 h and analyzed by fluorescence microscopy. At least 20 cells were counted.

Human samples

Protein-protein correlations were determined in extracts from PBMCs from 21 healthy male subjects (25.8±2.7 yrs). 14 of these subjects received 1.5 mg DEX and protein changes in PBMCs were determined 6 h later. FKBP51-dependent AD effects were evaluated in cultivated PBMCs from another 20 healthy male subjects (age 34.8±6.9 yrs).

43 consecutive participants (25 females, 18 males; age: 49.7 ± 14.5 yrs) of the Munich Antidepressant Response Signature (MARS) project were selected for protein expression analyses and PBMC cultivation and treatment. MARS is an open-label prospective clinical study conducted at the hospital of the Max Planck Institute of Psychiatry Munich and collaborating hospitals in South Bavaria and Switzerland to evaluate typical depression courses under standard antidepressant (AD) treatment (Hennings et al., 2009). Patients were diagnosed according to DSM-IV criteria (American Psychiatric Association, 2000). Patients suffered from major depression, recurrent depression or from a depressive episode within a bipolar disorder (n=6). Depression severity was assessed by using the 21-item Hamilton Depression rating scale (HDRS) (Overall and Rhoades, 1982) with an average baseline score of 24.3 (SD 5.5), indicating severe depression. Patients received antidepressant treatment in a naturalistic study setting (Hennings et al., 2009). AD treatment outcome was evaluated as percent change in the HDRS scores between baseline and after six weeks of treatment. Fasting venous blood samples for extraction of PBMCs were drawn from patients between 8 and 9 a.m. within 5 days after admission.

Ethics statement

The animal experiments were carried out in accordance with the European Communities' Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments; all experiments were based on the 3R principles: reduction, refinement, replacement. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany (license number 55.2-1-54-2532-48-11).

Approval for the MARS project was received by the ethics committee in charge (submission number 318/00, ethics committee of the Medical Faculty at the Ludwig Maximilians

University Munich, Germany), and participants gave oral and written consent before study inclusion.

Statistical analysis

When two groups were compared, the student's *t*-test was applied. For three or more group comparisons, one-way or two-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test, as appropriate. All ANOVA F and p-values are reported in table S1; significant results of the contrast tests are indicated by asterisks in the graphs. Protein-protein associations were analyzed using Pearson correlations. In the clinical sample, associations between human protein expression and antidepressant treatment outcome as well as protein-protein interactions were evaluated with Pearson correlations after correction for the effects of age and gender (partial correlation). $p < 0.05$ was considered significant.

Results

FKBP51 dependent effects of paroxetine on behavior in FKBP51 knockout mice and identification of FKBP51- directed pathways of autophagy.

To assess whether FKBP51 modulates AD response in mice, we treated *Fkbp51*$^{+/+}$ and *Fkbp51*$^{-/-}$ mice with the antidepressant paroxetine (PAR). We employed a well-established test for AD action; *Fkbp51*$^{-/-}$-mice were subjected to the forced swim test to monitor the animals' response to an acute PAR injection (10 mgkg^{-1}). PAR-treated *Fkbp51*$^{+/+}$ mice spent less time floating (immobility) compared to vehicle-treated *Fkbp51*$^{+/+}$ mice, thus exhibiting the expected response to ADs. In contrast, the effect of PAR was significantly less pronounced in *Fkbp51*$^{-/-}$ mice (Fig. 1A). Similarly, PAR treatment led to an increase in the time spent struggling in *Fkbp51*$^{+/+}$ mice. This effect of PAR was significantly attenuated in *Fkbp51*$^{-/-}$ mice (Fig. 1A).

To evaluate molecular effects of ADs that may depend on FKBP51 and parallel the behavioral phenotype, we analyzed autophagy markers in protein extracts from the hippocampus and prefrontal cortex after PAR injection (10 mgkg^{-1}). In *Fkbp51*$^{+/+}$ mice, PAR treatment increased the levels of Beclin1, Atg12, and Vps34, increased the ratio of LC3B-II/I, and evoked dephosphorylation of the kinase Akt (phosphorylation at S473 in Akt1 represents the activated form, subsequently referred to as "pAkt^{S473}"). In contrast, *Fkbp51*$^{-/-}$ mice showed no alterations in protein levels of autophagy markers and pAkt^{S473} in either brain region after PAR treatment (Figs. 1B,C, S1).

The results in *FKBP51*$^{-/-}$ mice suggest a role of FKBP51 in autophagic events, which we further evaluated using biochemical and cell biological methods. Because FKBP51 has been reported to interact with Akt (Pei et al., 2009), which in turn regulates Beclin1 (Wang et al., 2012b), we initially tested whether FKBP51 interacts with Beclin1. FLAG-tagged FKBP51 was expressed in HEK cells to assess its interaction with the autophagy initiator Beclin1 by co-immunoprecipitation. FKBP51 displayed binding to Beclin1 (Fig. 1D), in addition to the previously reported interaction with Akt and the phosphatase PHLPP (Pei et al., 2009).

A direct effect of Akt on Beclin1 has been discovered as an important mechanism blocking autophagy (Wang et al., 2012b). Thus, we next tested whether FKBP51 affects the interaction between Akt and Beclin1. Increasing the levels of FKBP51 led to higher overall

levels of Beclin1 (Figs. 1D, S2) and to higher amounts of Akt co-precipitating with Beclin1 (normalized to precipitated Beclin1, Figs. 1E, S2A). In addition, FKBP51 lowered the amounts of pAkt^{S473} interacting with Beclin1 (Figs. 1E, S2A). Furthermore, FKBP51 increased the levels of autophagy promoting dephosphorylated Beclin1 (Figs. 1E, S2B). The overall level of pAkt^{T308} (conferring basal activity) was unchanged upon expression of FKBP51, while the pAkt^{S473} levels were reduced (Fig. S2B). This is in line with previous findings and has been explained by the recruitment of the phosphatase PHLPP to Akt (Pei et al., 2009). Together, the findings support the model that FKBP51 acts two-fold on Beclin1: first by increasing the overall Beclin1 levels, and second by recruiting the inactive (dephosphorylated by PHLPP) form of Akt^{S473} to Beclin1 (cf. Fig. 7).

To evaluate whether FKBP51's effects on Beclin1 go along with alterations of other autophagy markers, we compared *Fkbp51*$^{-/-}$ and wild-type (*Fkbp51*$^{+/+}$) mouse embryonic fibroblasts (MEFs). MEFs lacking FKBP51 displayed reduced levels of Beclin1 and decreased levels of two other autophagy proteins, LC3B-II/I and Atg12 (Figs. 1F, S3A). Consistent with the reduced levels of pAkt^{S473} observed in HEK cells upon ectopic expression of FKBP51 (Fig. S2B), deletion of FKBP51 in MEF cells resulted in higher levels of pAkt^{S473} (Fig. S3A). These effects of FKBP51 gene deletion were attenuated by ectopic expression of FKBP51 in Fkbp51$^{-/-}$-MEFs (Figs. 1F, S3A). Together, the data corroborate a stimulatory role of FKBP51 on autophagic pathways.

Given that FKBP51 acts on a variety of intracellular proteins, we used *Akt1/2*$^{-/-}$ MEFs to test whether FKBP51's impact on pAkt^{S473} was required for its influence on autophagic markers. In wild-type *Akt1/2* MEFs, transfection of FKBP51 increased the levels of the autophagy markers Beclin1 and LC3B-II/I (Fig. S3B). These effects of FKBP51 were not observed in *Akt1/2*$^{-/-}$-MEFs, but were restored after re-introducing Akt1 and Akt2 into *Akt1/2*$^{-/-}$-MEFs by transient transfection (Fig. S3B). Thus, the impact of FKBP51 on autophagy markers requires Akt1 and/or Akt2 (cf. also Fig. 7).

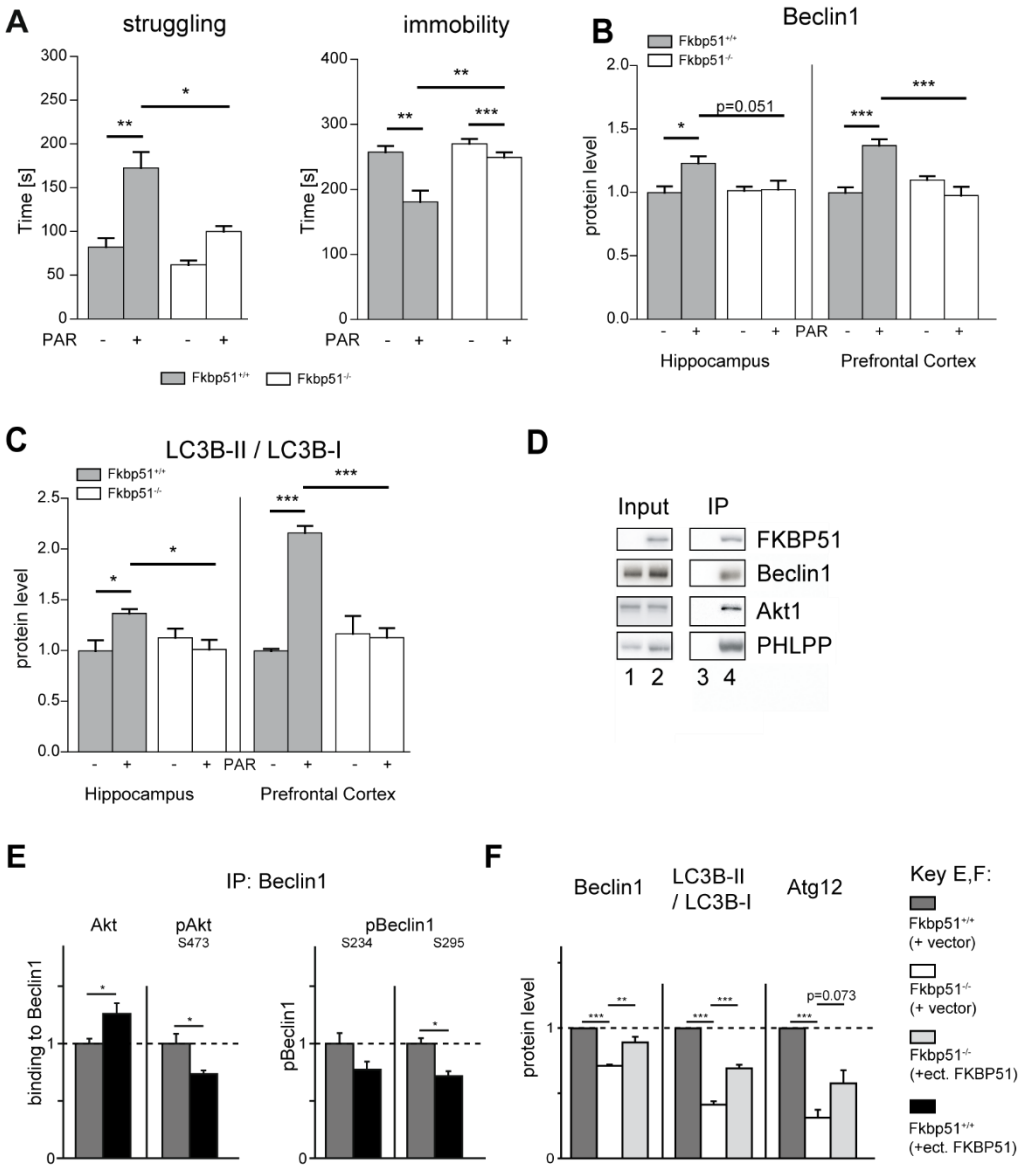


Figure 1. FKBP51 is required for the effects of PAR on behavior and directs autophagic pathways. **A,** *FKBP51*$^{-/-}$ and FKBP51$^{+/+}$ mice were treated with PAR (10 mgkg^{-1}) or vehicle and subjected to the forced swim test (n=7-9 per group). Graphs show the times of immobility and struggling. **B, C**, FKBP51$^{-/-}$ and FKBP51$^{+/+}$ mice were treated with PAR (10 mgkg^{-1}) or vehicle and sacrificed 45 mins later. Protein levels were determined in extracts of the hippocampus and the prefrontal cortex from 9-10 animals in technical triplicates. *p<0.05; **p<0.01; ***p<0.001. Statistical parameters in Table S1. **D**, HEK cells were transfected with a FLAG-tagged FKBP51 expressing plasmid and lysed 3 d later. After precipitation of protein complexes using a Flag-antibody, (co)precipitated proteins were visualized by Western blotting. **E**, quantification of pBeclin1 and Beclin1-Akt/pAkt^{S473} interaction after Beclin1-immunoprecipitation from HEK cells transfected with FKBP51/52 (details in Fig. S2). **F**, effects of FKBP51 expression in FKBP51$^{+/+}$-MEFs, FKBP51$^{-/-}$-MEFs, and FKBP51$^{-/-}$-MEFs transfected with FKBP51 on endogenous Beclin1-, LC3B-II/I-, and Atg12-levels. Graphs show the relative mean expression +SEM (n=4). *p<0.05; **p<0.01; ***p<0.001, see also Fig. S3, Table S1.

Cellular effects of ADs on autophagic markers depend on FKBP51

To determine potential effects of ADs on the FKBP51-directed autophagic events characterized above, we tested whether PAR, amitriptyline (AMI), or fluoxetine (FLX) (all at 10 µM, the concentration that proved sufficient for induction of autophagy markers (Zschocke et al., 2011)) impacted on FKBP51 heterocomplexes in HEK cells. Treatment with PAR increased the amount of Akt and Beclin1 co-precipitating with FKBP51 (Fig. 2A, S4A). Similar effects were observed with AMI, while FLX showed no effect (Fig. S4A).

Both a decrease and an increase of $pAkt^{S473}$ induced by ADs have been reported to date (Jeon et al., 2011; Huang et al., 2013). Treatment of rat primary astrocytes with PAR, AMI, or FLX decreased the levels of $pAkt^{S473}$ (Figs. 2B, S4B,C). This AD effect was significantly stronger when FKBP51 was transfected into astrocytes.

The importance of FKBP51 for AD effects on components of the autophagy pathway was further evaluated in $Fkbp51^{-/-}$- and $Akt1/2^{-/-}$-MEFs. PAR, AMI, and FLX reduced $pAkt^{S473}$ in $Fkbp51^{+/+}$-, but not in $FKBP51^{-/-}$-MEFs; ectopic expression of FKBP51 in $Fkbp51^{-/-}$-MEFs restored the effect of ADs on $pAkt^{S473}$ (Figs. 2C, S4D,E). The autophagy marker LC3B-II/I was elevated upon AD treatment in $Fkbp51^{+/+}$-MEF cells (Figs. 2D, S4D, S5A), as reported previously for astrocytes (Zschocke et al., 2011). This AD effect was blunted in $Fkbp51^{-/-}$-MEFs, and restored when FKBP51 was ectopically expressed in these cells. In $Akt1/2^{-/-}$-MEFs, AD exposure did not induce changes in the ratio of LC3B-II/I (Figs. S5B,C). When Akt1 and Akt2 were re-introduced by ectopic expression, the effects of ADs re-emerged.

The shift of LC3B from cytosolic to autophagosomal compartments is also a major hallmark of autophagy. The influence of FKBP51 on the capability of ADs to change the distribution of recombinant GFP-LC3B was monitored in primary cortical astrocytes. Ectopically expressed FKBP51 enhanced AD-induced clustering of GFP-LC3B (Figs. 2E,F, S5D,E). In the absence of ADs, FKBP51 triggered the formation of GFP-LC3B positive puncta to a lesser extent. Further, FKBP51 enhanced the ability of ADs to induce conversion from LC3B-I to LC3B-II and to elevate Beclin1 and Atg12 levels in astrocytes (Figs. 2G-I, S4B, S5F-H). Collectively, these data support the conclusion that the cellular AD effects on autophagy pathways depend on FKBP51.

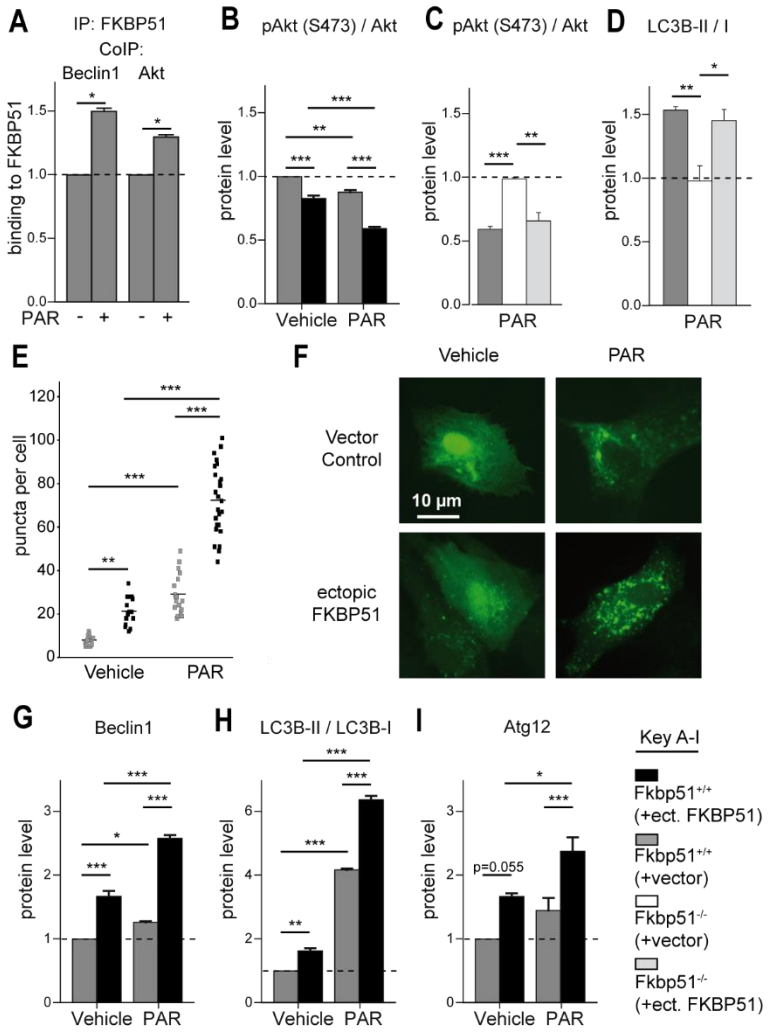


Figure 2. FKBP51 enhances cellular effects of ADs. **A,** the interaction of FKBP51 with Beclin1 and Akt in HEK cells in the presence or absence of PAR (10 µM) was analyzed by co-immunoprecipitation. **B**, primary rat cortical astrocytes were transfected with vector-control or FKBP51, treated with PAR (10 µM) for 48 h, and the $pAkt^{S473}$/Akt ratio was determined. Bars represent the mean +SEM (n=3). **C**, **D**, $FKBP51^{+/+}$-, $FKBP51^{-/-}$-, and $FKBP51^{-/-}$-MEFs transfected with FKBP51 were treated with PAR (10 µM) for 48 h; $pAkt^{S473}$/Akt (C) and LC3B-II/I (D) ratios were determined. Bars represent the mean +SEM (n=3). Protein levels in untreated $FKBP51^{+/+}$-MEFs were set to 1. **E**, primary astrocytes were cotransfected with GFP-LC3B together with vector-control or FKBP51, and treated with PAR (10 µM) for 48 h. The number of GFP-LC3B positive puncta/cell was determined in 15-25 randomly selected cells for each condition. **F**, representative fluorescence images of E. **G-I**, primary astrocytes were transfected with vector-control or FKBP51, and treated as in B; Beclin1, LC3B-II/I and Atg12 levels were determined. Bars represent the mean +SEM (n=3). *p<0.05; **p<0.01; ***p<0.001. Statistical parameters in Table S1.

FKBP51 shapes the effects of ADs on synaptic function

Irrespective of the question whether AD-induced increase of autophagic markers in all cases indicates enhanced protein turnover (Rossi et al., 2009; Zschocke et al., 2011), autophagic

pathways have been linked to synaptic function. Since the latter is more obviously linked to behavior (Luo et al., 2008) and diseases such as depression, we also tested whether FKBP51 changes the effect of PAR on neuronal function. Hippocampal CA3-CA1 synapses were electrically stimulated in brain slices from *Fkbp51*$^{+/+}$ and *Fkbp51*$^{-/-}$ mice, and CA1 neuronal activity was monitored by means of voltage-sensitive dye imaging (VSDI) (Refojo et al., 2011; von Wolff et al., 2011; Stepan et al., 2012). This approach was chosen for two reasons. First, the CA1 area is an important output subfield of the hippocampus, which has been implicated in the pathophysiology of depression and in AD action (Airan et al., 2007; Duman and Aghajanian, 2012). Second, we considered potential heterogeneity of the PAR effect at CA3-CA1 synapses; thus, VSDI measurement of neuronal activity in a larger portion of CA1 appeared more relevant than the typically more restricted electrode recording techniques. In slices from Fkbp51$^{+/+}$-mice, bath application with PAR (10 µM) enhanced the 'region of interest' (ROI)-extracted fast, depolarization-mediated imaging signals (FDSs), which reflect excitatory postsynaptic potentials as well as neuronal action potentials (von Wolff et al., 2011; Stepan et al., 2012). This effect was not observed in slices from *Fkbp51*$^{-/-}$ animals (Fig. 3A and B).

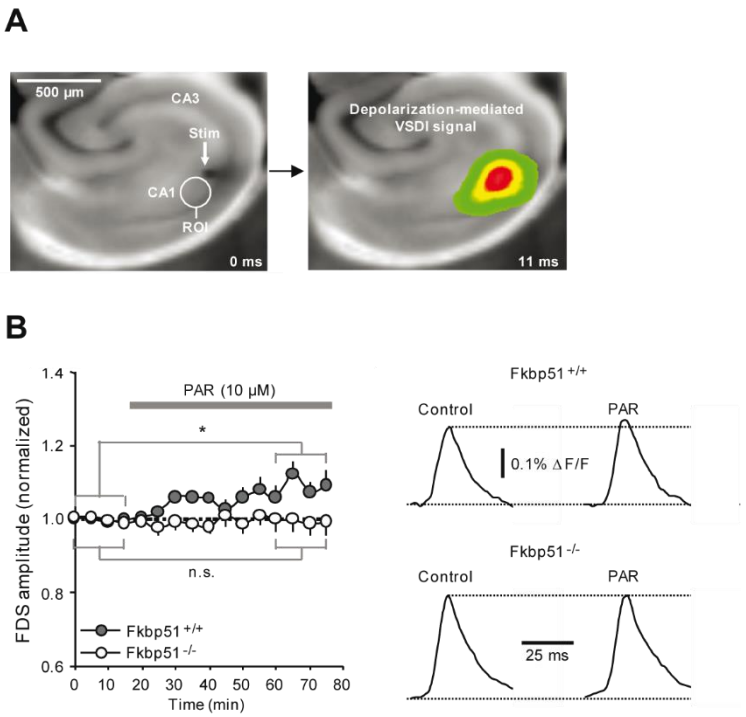


Figure 3. FKBP51 is required for the effect of PAR on evoked CA1 neuronal activity. **A,** *Left*, illustration of the position of the stimulation electrode (Stim) and ROI used for the calculation of CA1 neuronal population activity. *Right*, depolarization-mediated VSDI signal recorded 11 ms after the electrical

stimulation pulse. Warmer colors represent higher values of the fractional change in fluorescence ($\Delta F/F$) and, thus, stronger neuronal activity. **B,** *Left*, quantification of PAR's effects on CA1 FDSs (n=10 slices from 4 mice for each group). **p*=0.032 (paired *t*-test); n.s., *p*=0.912 (paired *t*-test); data are given as mean ±SEM. *Right*, representative recording traces illustrating the PAR effects on CA1 FDSs.

Effects of ADs and of dexamethasone in blood cells of healthy individuals depend on FKBP51

We next tested if the link between FKBP51 expression and AD response of autophagy markers could be confirmed in human peripheral blood mononuclear cells (PBMCs). PBMCs derived from healthy male subjects showed marked variations in basal FKBP51 expression (Figs. 4A-C, S6A). The FKBP51 expression level was positively correlated with the expression of Beclin1 and Atg12, and negatively correlated with the phosphorylation status of Akt^{S473}; LC3B-II/I displayed a trend correlation with FKBP51 protein levels (Figs. 4A-C, S6A,B).

Since FKBP51 is a stress-responsive gene [(Hubler and Scammell, 2004; Klengel et al., 2013) and glucocorticoids have been shown to induce autophagy (Laane et al., 2009), FKBP51 might also link stress to autophagy. We determined the expression of Beclin1 and FKBP51 in PBMCs from healthy male subjects before and 6 h after administration of the synthetic glucocorticoid dexamethasone (DEX, 1.5 mg). There was a significant correlation between the induction of FKBP51 and the increase in Beclin1 expression (Figs. S6C,E), and between the induction of FKBP51 and the de-phosphorylation of $pAkt^{S473}$ (Figs. S6D,E). In an animal model, DEX affected levels of Beclin1, Vps34, and $pAkt^{S473}$ in cortical astrocytes from $Fkbp51^{-/-}$, but not from $Fkbp51^{+/+}$-mice (Fig. S6F,G).

PBMCs from healthy male subjects were also cultivated *ex vivo* and treated with AMI, FLX, or PAR for 72 h. To more closely mimic the clinically relevant *in vivo* conditions, drug concentrations were chosen according to actual consensus guidelines for therapeutic drug monitoring in psychiatry (Hiemke et al., 2011). The extent of de-phosphorylation of $pAkt^{S473}$, change of LC3B-II/I ratio, and levels of Beclin1 in response to ADs correlated with the expression level of FKBP51 (Figs. 4D-F, S6H,I). Higher expression of FKBP51 was associated with AD-induced de-phosphorylation of $pAkt^{S473}$, and lower expression of FKBP51 was associated with AD-enhanced $pAkt^{S473}$. With increasing FKBP51 levels, a gradual shift from slightly inhibitory to stimulatory effects of ADs on Beclin1 expression was observed. The AD-

triggered lipidation of LC3B-I was positively correlated with the FKBP51 expression status. Atg12 expression was not significantly changed by AMI and FLX, but PAR affected Atg12 expression in an FKBP51-dependent fashion similarly to Beclin1. Taken together, FKBP51 is required for the effects of AD and of DEX on autophagic pathways in humans.

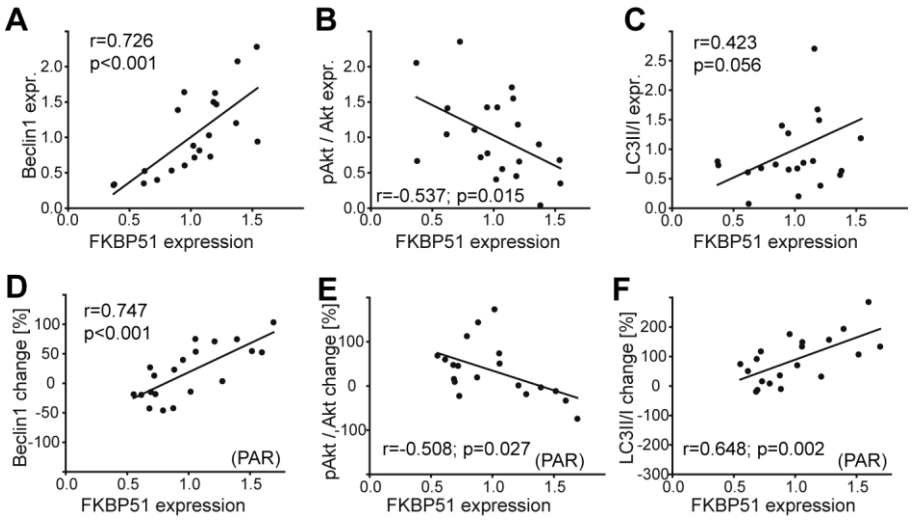


Figure 4. Correlation of FKBP51 with autophagy pathway components and with effects of ADs in human PBMCs. **A-C,** protein levels of FKBP51, Beclin1, pAkt^{S473}/Akt, and LC3B-II/I, in PBMCs from healthy male subjects (n=21). Each dot represents the levels of FKBP51 and the respective protein in PBMCs from one individual subject. Average expression levels were set to 1. **D-F,** levels of FKBP51, Beclin1, pAkt^{S473}, and LC3B-II/I in PBMCs from healthy male subjects (n=21), cultivated *ex vivo* and treated with PAR (0.365 µM, 48 h). Plots depict protein changes upon treatment with AD compared to vehicle-treated cells in correlation to FKBP51. Each dot represents the level of FKBP51 and the expression change of the respective protein in PBMCs from one individual.

Clinical improvement of depressive patients is predicted by their levels of Beclin1 and FKBP51 at hospital admission.

To further evaluate the clinical relevance of our findings we determined the expression levels of Beclin1, FKBP51, and pAkt^{S473} in PBMCs from inpatients with a current depressive episode at admission and compared them with the clinical treatment response. The clinical response after 6 weeks of treatment showed statistically significant positive correlations with the levels of Beclin1 and FKBP51, and a negative correlation with pAkt^{S473} (Figs. 5A-C).

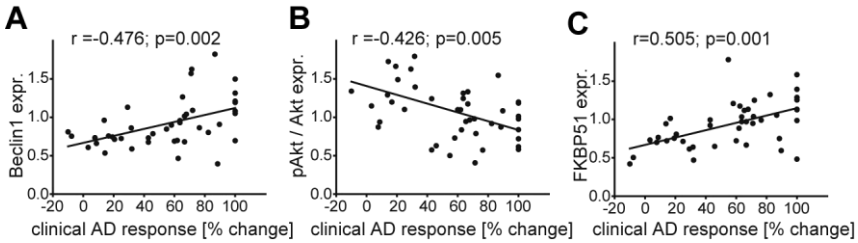


Figure 5. ***Correlation of clinical AD response with the expression of FKBP51 and Beclin1, and with the effects of ADs in PBMCs ex vivo.*** **A-C,** Beclin1, FKBP51, and pAkt^{S473}/Akt levels in PBMCs from inpatients with depression (n=43) at admission. Expression values are correlated with the response to AD-treatment expressed as percent change in the total score of the 21-items Hamilton Depression Rating scale between admission and after 6 weeks of treatment.

Reactivity of Beclin1 and pAkt of patient-derived PBMCs to AD treatment ex vivo predicts clinical improvement.

PBMCs were also collected from inpatients at admission, cultivated and treated with AMI, FLX, or PAR *ex vivo*. We observed a correlation between therapeutic outcome and the increase of Beclin1 levels in treated PBMCs for all three ADs (Figs. 6A-C). There was a significant negative correlation between therapeutic outcome and the change of pAkt^{S473} upon treatment with AMI or PAR, (Fig. 6D, E; no correlation for FLX, Fig. S7A). There was a trend for the correlation between therapeutic outcome and induction of LC3B-II/I in the case of treatment with PAR, but not with AMI or FLX (Figs . 6F, S7B,C). Thus, the levels of FKBP51 and Beclin1 at admission, as well as the response to AD treatment in PBMCs, correlate with therapeutic outcome.

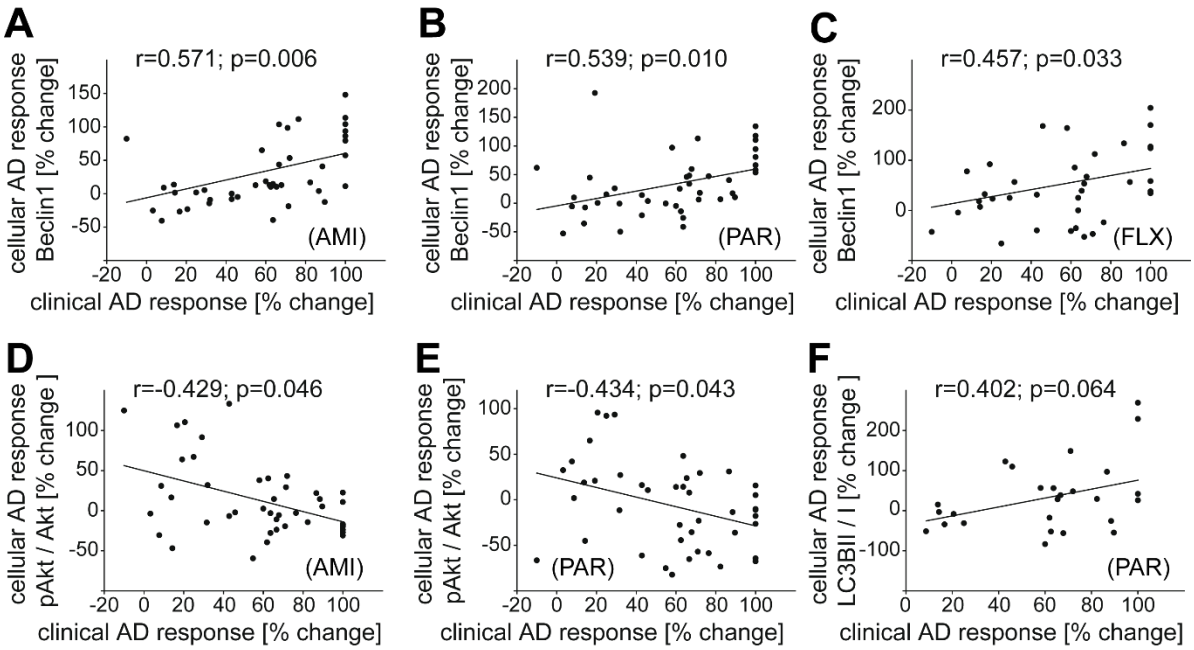


Figure 6. Correlation of clinical AD response with the effects of ADs in PBMCs ex vivo. PBMCs from depressive inpatients were cultivated *ex vivo* and treated with PAR (0.365 µM), FLX (1695 nM) or AMI (888 nM) for 48 h. The AD induced changes in expression of Beclin1, pAkt^{S473}/Akt and LC3BII/I are correlated with the clinical response to AD-treatment expressed as percent change in the total score of the 21-items Hamilton Depression Rating scale between admission and after 6 weeks of treatment.

Discussion

FKBP51 has originally been selected as a candidate for genetic analyses in depression and AD responsiveness (Binder et al., 2004), because it is involved in the regulation of the glucocorticoid receptor (Wochnik et al., 2005), and thereby the stress hormone axis (Scammell et al., 2001). Our study brings novel FKBP51-directed pathways to the fore (Fig. 7), and establishes the importance of FKBP51 in AD action at the molecular, cellular, and organismic level. It also identifies a molecular mechanism of the previously suggested (Binder et al., 2004) positive correlation between FKBP51 expression and AD treatment response.

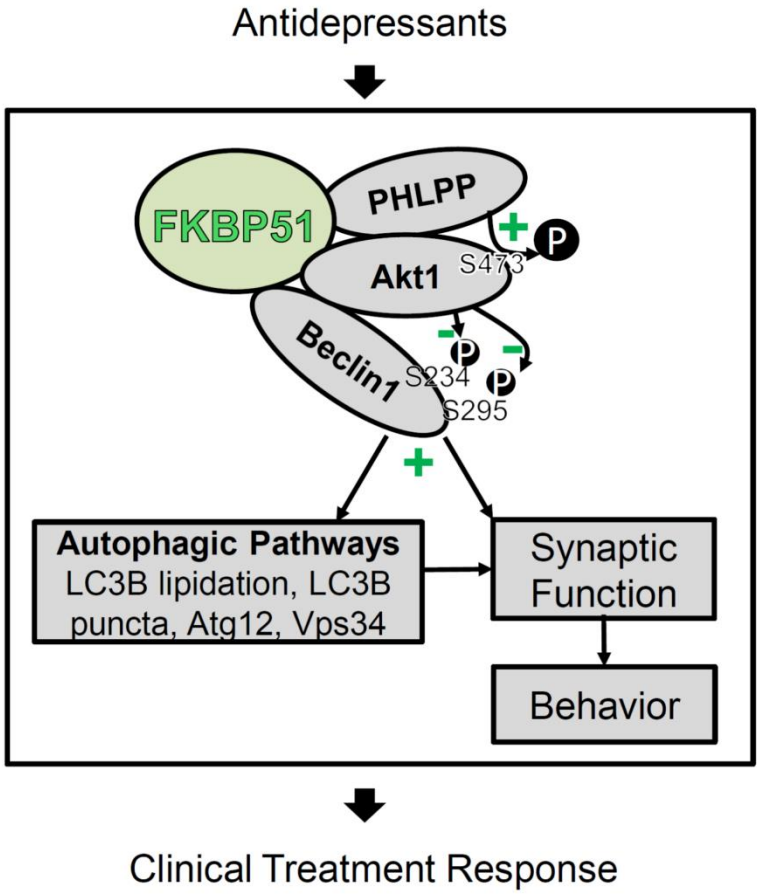


Figure 7. ***Model of FKBP51's impact on Beclin1 and autophagy pathways.*** FKBP51 interacts with PHLPP, Akt, and Beclin1. Since the PHLPP de-phosphorylates Akt (S473 in Akt1), inactive Akt is recruited to Beclin1. This results in lower phosphorylation of Beclin1 and thus the induction of autophagic pathways (Wang et al., 2012b). Autophagic pathways, and thereby also FKBP51, are linked to synaptic function (Hernandez et al., 2012) as a physiological correlate of behavior (Luo et al., 2008). ADs act on the same pathways in an FKBP51-dependent manner and change FKBP51 protein interactions; this could form the basis for the FKBP51-dependency of AD effects in cells, animals, and humans.

The impact of FKBP51 on autophagy established in this study significantly expands the range of actions of this versatile protein to be considered relevant for AD action. Our protein interaction analyses support the model (Fig. 7) that FKBP51 as scaffolding protein recruits Akt to Beclin1; since FKBP51 also binds to PHLPP (Pei et al., 2009), inactive Akt is recruited to Beclin1. This results in decreased phosphorylation of Beclin1, thereby shifting Beclin1's action to trigger autophagy (Wang et al., 2012b). FKBP51-mediated increase in overall Beclin1 levels constitutes a further route by which FKBP51 facilitates autophagy.

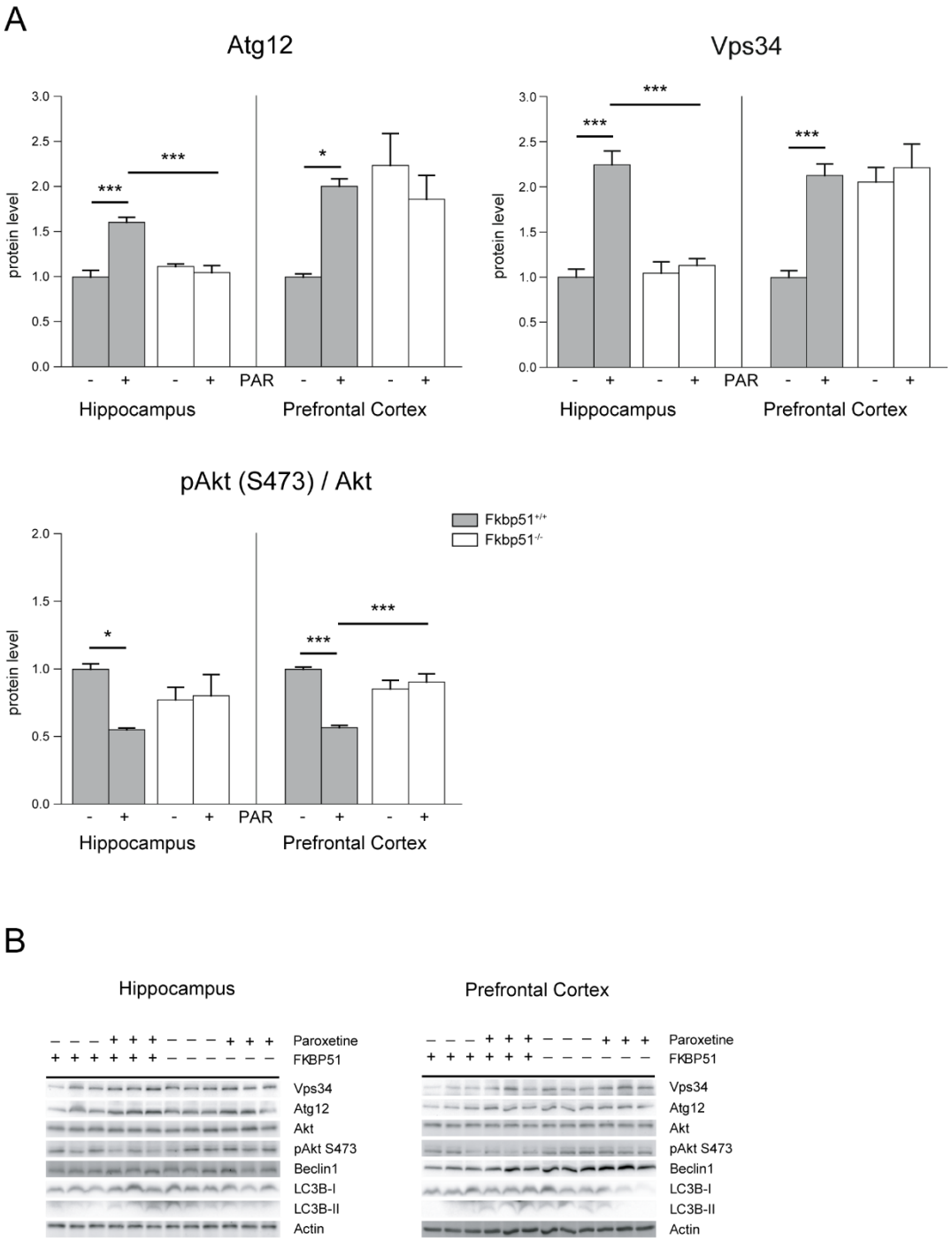
The regulation of FKBP51 by glucocorticoids (Hubler and Scammell, 2004; Klengel et al., 2013) potentially links all the molecular actions of FKBP51 to stress. Our data provide strong evidence that up-regulation of FKBP51 mediates dexamethasone-induced autophagy and de-phosphorylation of $pAkt^{S473}$ (Laane et al., 2009). Akt, in turn, has been reported as target of AD action before, and both positive and negative changes of $pAkt^{S473}$ levels have been found for different ADs in various cellular systems (Basta-Kaim et al., 2005; Jeon et al., 2011; Bhuiyan et al., 2011; Huang et al., 2013). Treatment duration and other factors may account for the divergent results; based on our findings, we propose that the FKBP51 expression status determines whether and how ADs alter $pAkt^{S473}$. In general, $pAkt^{S473}$ is under multifactorial control (Manning and Cantley, 2007) that could be the basis for both inhibitory and FKBP51-dependent stimulatory actions of ADs.

In light of the still largely unknown molecular mechanism by which ADs act, our results highlight intracellular molecular mechanism of AD effects that complement the known actions on mono-aminergic neurotransmitter systems (Berton and Nestler, 2006). Previously, a link has been discovered from autophagy to neurotransmission (Hernandez et al., 2012), and from neuronal circuit dynamics in the hippocampus to behavior in an animal model of depression (Airan et al., 2007). Thus, it is plausible that the FKBP51-governed autophagy pathways underlie the here described FKBP51 dependency of PAR's effect on neuronal activity and on behavior, and the FKBP51-dependency of clinical AD response.

While genetic studies of complex diseases, including studies on respective drug actions, have significantly benefitted from the advanced technologies of genome-wide genotyping, the necessity of complementary approaches such as molecular pathways and network analyses to move from genomic localization to mechanistic insight has come into focus only recently (Barabasi et al., 2011). Overall, our finding of FKBP51-dependent effects of ADs on

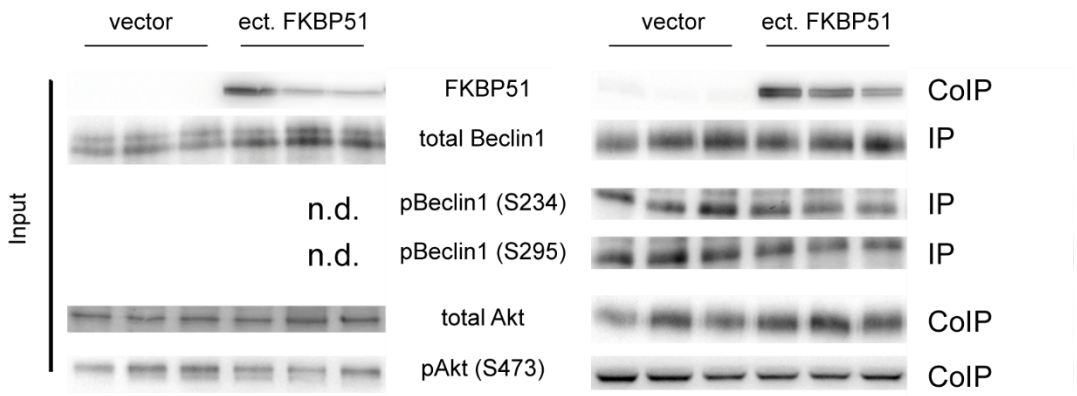
intracellular pathways and consequently brain function and behavior strengthens the relevance of the genetic association of FKBP51 with AD response and provides substantial information for more targeted genetic studies. In addition, our results suggest considering autophagy initiating mechanisms as a pharmacological target to improve treatment of depression, as discussed for other diseases (Harris and Rubinsztein, 2012). Finally, our data provide a direct basis for including the current protein expression status of FKBP51 and Beclin1 as well as the response of autophagic factors to AD treatment of *ex vivo* cultivated lymphocytes as novel biomarkers predicting antidepressant efficacy in depressed patients.

Supplemental Materials and Methods



Suppl. Fig. 1. ***The effect of paroxetine in mice depends on FKBP51.*** FKBP51$^{-/-}$- and FKBP51$^{+/+}$-mice were treated with PAR (10 mgkg^{-1}) or vehicle and sacrificed 45 mins later. Protein levels were determined in extracts of the hippocampus and the prefrontal cortex from 9-10 animals in technical triplicates. **A**, *p<0.05; ***p<0.001. Statistical parameters in supplemental Table 1. **B**, representative Western blots, also to figure 1B,C of the main text.

A

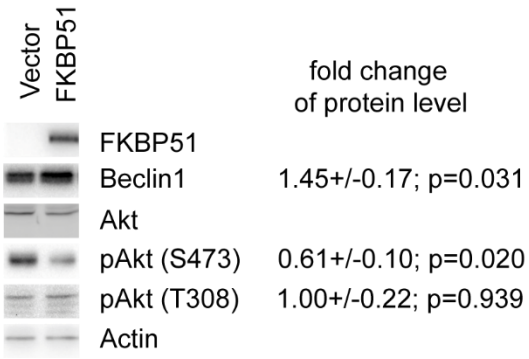


fold change				
construct (Beclin1-IP)	pBeclin1 (S234) ± SEM	pBeclin1 (S295) ± SEM	Akt ± SEM	pAkt (S473) ± SEM
vector	1 ± 0.158	1 ± 0.083	1 ± 0.074	1 ± 0.147
ect. FKBP51	0.772 ± 0.122	0.716 ± 0.074	1.259 ± 0.161	0.734 ± 0.569

p-values comparison vector*FKBP51			
pBeclin1 (S234)	pBeclin1 (S295)	Akt	pAkt (S473)
0.190 (est.)#	0.011	0.036	0.043

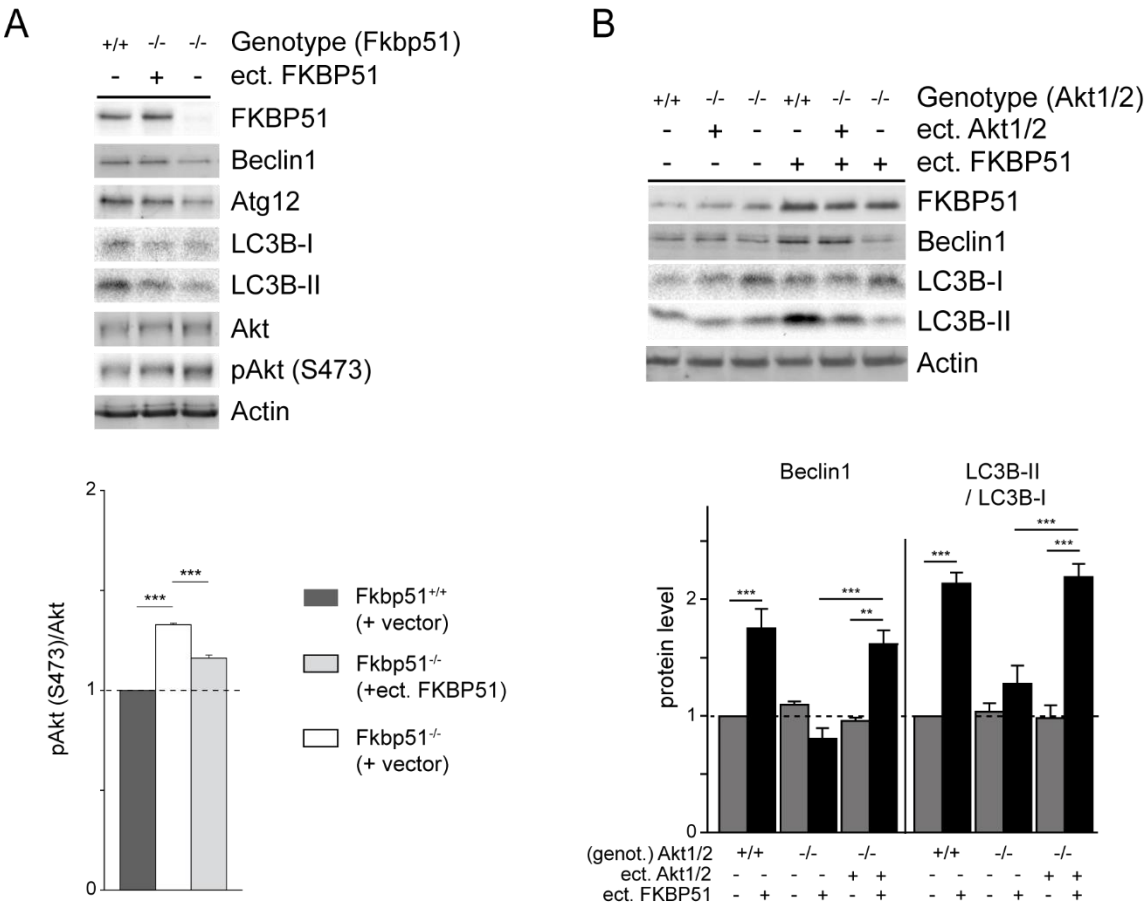
estimated p-value according to Man-Whitney Rank Sum Test

B

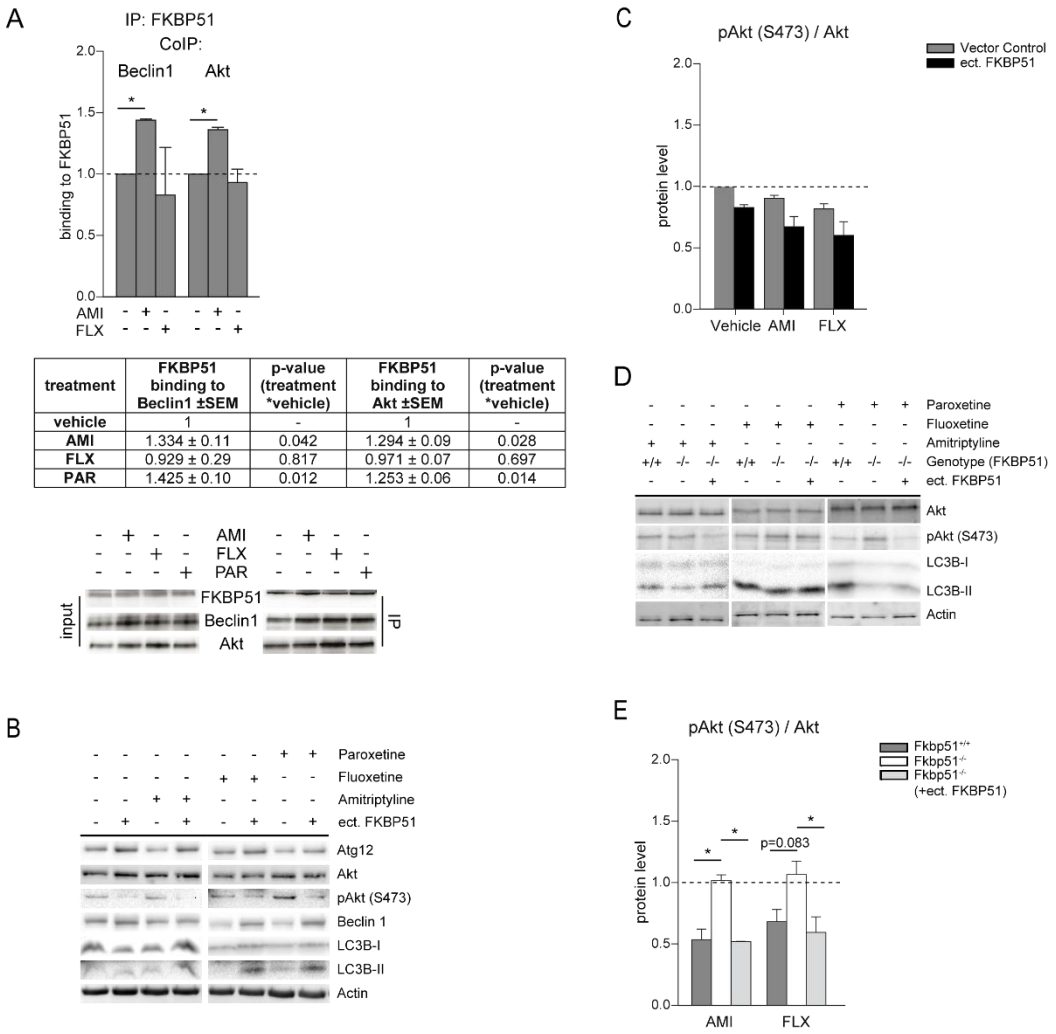


Suppl. Fig. 2. ***Functional interaction of FKBP51 with Beclin1, Akt and PHLPP.*** HEK cells were transiently transfected with vector control or a plasmid expressing FKBP51. **A**, representative Western blots of Beclin1 immunoprecipitations from transfected cells and analysis of pBeclin1^{S234,S295} and co-precipitating pAkt^{S473}/Akt are displayed (corresponding to Fig. 1E). The antibodies directed against phosphorylated Beclin only worked after immunoprecipitation (Wang et al., 2012b). The table summarizes the results of 3 independent co-immunoprecipitations with 3 technical replicates of the respective Western blots. In the case of Akt and pAkt^{S473}, numbers indicate relative intensities of interaction with Beclin1 (intensity of vector control was set to 1), including p-values of interaction differences. In the case of pBeclin1^{S234,S295}, numbers indicate the fold change in the presence or absence of ectopic FKBP51 (vector control set to 1), including p-values. **B**, representative Western blots indicating the change of total Beclin1, pAkt^{S473} and pAkt^{T308} upon expression of FKBP51. FKBP51 was detected by an antibody directed against its FLAG tag.

Numbers indicate the the effect of FKBP51 expression on the levels of Beclin1 and on the phosphorylation status of Akt^{S473} and Akt^{T308}, and reflect the results of 3 independent experiments with 3 technical replicates of the respective Western blots.

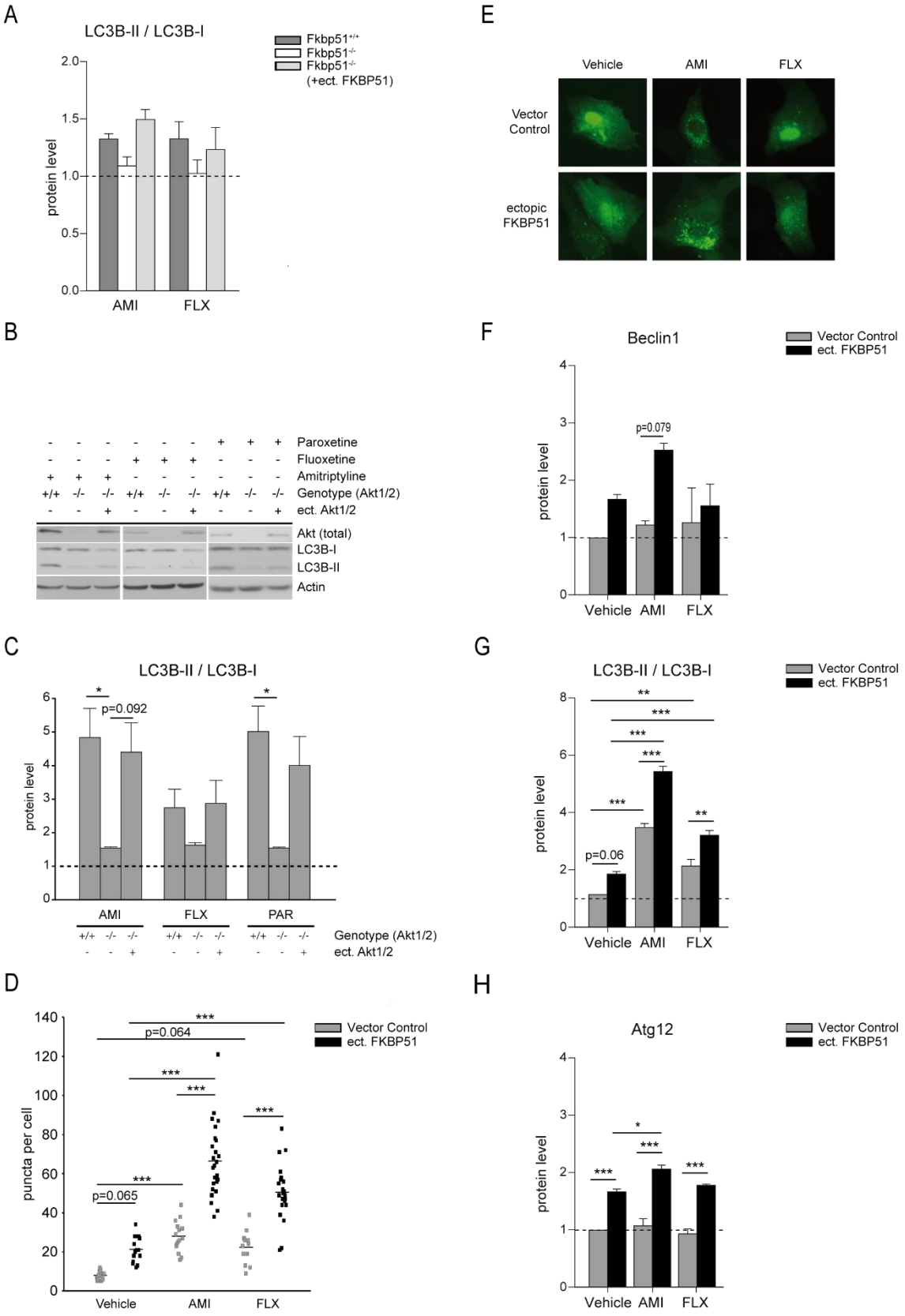


Suppl. Fig. 3. ***Effect of FKBP51 on autophagic markers in cells deficient of endogenous FKBP51 or Akt1/2.*** **A**, the impact of the FKBP51 expression status on the levels of Beclin1, Atg12, LC3B-II/I, and pAkt^{S473} was assessed by Western blot analysis in Fkbp51$^{+/+}$ MEFs, Fkbp51$^{-/-}$ MEFs, and Fkbp51$^{-/-}$ MEFs transfected with an FKBP51 expressing plasmid. Graphs show the relative mean expression +SEM of 3 different experiments; expression in Fkbp51$^{+/+}$ MEFs was set to 1. Western examples of Beclin1, Atg12 and LC3B-II/I correspond to Figure 1F of the main text. **B,** cellular effects of FKBP51 require Akt1 or Akt2. The expression of Beclin1 and LC3B-II/I was evaluated in Akt1/2$^{+/+}$ MEFs, Akt1/2$^{-/-}$ MEFs and in Akt1/2$^{-/-}$ MEFs transfected with Akt1 and Akt2 expressing plasmids. Representative Western examples are shown. Graphs display pairwise comparisons of cells cotransfected with vector or FKBP51 expressing plasmid and represent the relative mean expression + SEM of 3 different experiments; expression in Akt1/2$^{+/+}$, vector-transfected MEFs was set to 1. **p<0.01; ***p<0.001. See supplemental Table 1 for all statistical parameters.



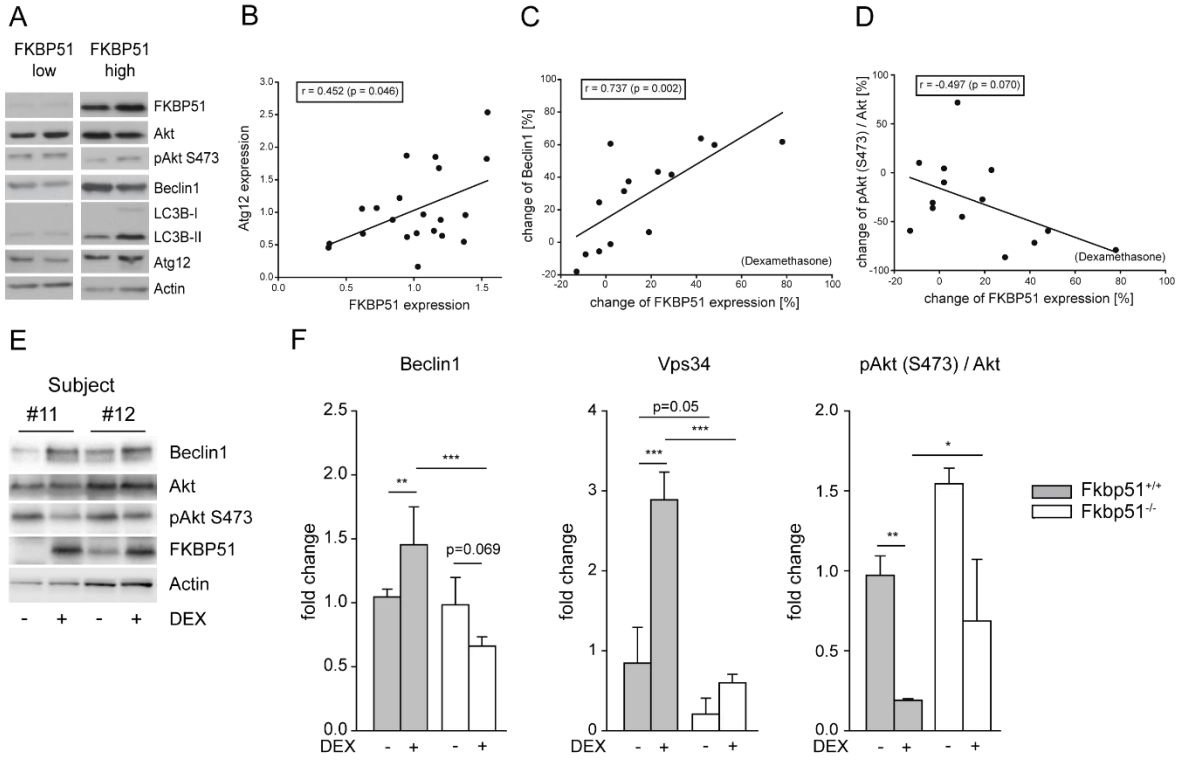
treatment	FKBP51 binding to Beclin1 ±SEM	p-value (treatment *vehicle)	FKBP51 binding to Akt ±SEM	p-value (treatment *vehicle)
vehicle	1	-	1	-
AMI	1.334 ± 0.11	0.042	1.294 ± 0.09	0.028
FLX	0.929 ± 0.29	0.817	0.971 ± 0.07	0.697
PAR	1.425 ± 0.10	0.012	1.253 ± 0.06	0.014

Suppl. Fig. 4. ***Convergent effects of FKBP51 and antidepressants in cells on protein interactions and pAkt.*** **A,** the interaction of FKBP51 with Beclin1 and Akt was analyzed by Western blotting after coimmunoprecipitation from HEK cells cultivated in the presence or absence of AMI or FLX (10 µM each). Table indicates changes of protein interaction relative to vehicle treatment. **B, C,** cortical astrocytes were transfected with an FKBP51 expression or control plasmid and treated as in A and levels of $pAkt^{S473}$/Akt were determined by Western blotting (representative blot in B). Bars represent the mean +SEM of 3 independent experiments performed in triplicate. Protein levels in wild-type, untreated cells were set to 1. **D** and **E**, $Fkbp51^{+/+}$, $Fkbp51^{-/-}$ and $Fkbp51^{-/-}$ MEFs transfected with an FKBP51 expressing plasmid were treated AMI, FLX or PAR (10 µM each) for 48 h, and the $pAkt^{S473}$/Akt (E) ratios were determined by Western blot (representative blot in D, also referring to Figs. 2C,D, S5A). Bars represent the mean +SEM of 3 independent experiments performed in triplicate. Protein levels in $Fkbp51^{+/+}$, untreated cells were set to 1. *p<0.05. See supplemental Table 1 for all statistical parameters.



Suppl. Fig. 5. ***Convergent effects of FKBP51 and antidepressants in cells on*** **LC3B-II/I, Beclin1 and Atg12.** **A**, Fkbp51$^{+/+}$, Fkbp51$^{-/-}$ and Fkbp51$^{-/-}$ MEFs transfected with an FKBP51 expressing plasmid were treated with AMI, FLX or PAR (10 µM each) for 48 h, and LC3B-II/I ratios were determined by Western blot (representative blot in Figure S4D). Bars represent the mean +SEM of 3 independent experiments

performed in triplicate. Protein levels in untreated Fkbp51$^{+/+}$ cells were set to 1. **B, C**, Akt1/2$^{+/+}$ MEFs, Akt1/2$^{-/-}$ MEFs and in Akt1/2$^{-/-}$ MEFs transfected with Akt1 and Akt2 expressing plasmids were treated with AMI, FLX or PAR (10 µM each) for 48 h. The ratio of LC3B-II/I was determined by Western blot (representative example in B). Bars represent the mean +SEM of 3 independent experiments performed in triplicate. LC3B-II/I in the respective untreated cells was set to 1 (dotted line). **D, E**, primary rat cortical astrocytes were transfected with a vector expressing GFP-LC3B, in combination with an FKBP51 expressing vector or cloning vector, and treated with AMI or FLX (10 µM each) for 48 h. The number of GFP-LC3B positive puncta was determined per cell (D). 15-25 randomly selected cells were evaluated for each condition (Representative fluorescence images in E). **F-H**, cortical astrocytes were transfected with an FKBP51 expression or control plasmid, treated as in A, and levels of Beclin1 (F), LC3B-II/I (G) and Atg12 (H) were determined by Western blotting (representative blot in Fig. S4B). Bars represent the mean +SEM of 3 independent experiments performed in triplicate. Protein levels in wild-type, untreated cells were set to 1. *p<0.05; **p<0.01; ***p<0.001. See Supplemental Data Table 1 for all statistical parameters.

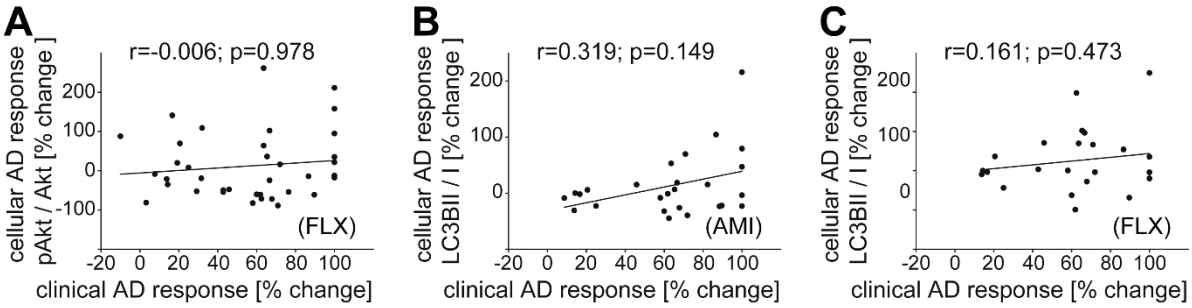


I

Change in protein level [%] vs FKBP51	Amitriptyline		Fluoxetine		Paroxetine	
	r	p-value	r	p-value	r	p-value
Beclin1	0.575	0.008	0.497	0.026	0.747*	<0.001*
pAkt (S473) / Akt	-0.479	0.038	-0.462	0.047	-0.508*	0.027*
LC3II / LC3I	0.742	<0.001	0.741	<0.001	0.648*	0.002*
Atg12	0.247	0.309	0.313	0.206	0.667	0.003

* graphs shown in Figure 4D-F

Suppl. Fig. 6. FKBP51-dependendency of autophagy pathway components in human PBMCs and of the effects of dexamethasone and antidepressants in primary astrocyte and in human PBMCs. **A,** protein extracts from PBMCs of healthy volunteers were analyzed for expression of FKBP51 and components of the autophagy pathway. Representative Western blot corresponding to Figure 4A-C and Figure S6B. **B**, correlation of protein levels of Atg12 and FKBP51 in PBMCs from healthy male subjects (n=21). Each dot represents the levels of Atg12 and FKBP51 in PBMCs from one individual subject. Average expression levels were set to 1. **C-E**, PBMCs were collected from healthy volunteers (n=14) before and 6 h after the intake of 1.5 mg dexamethasone (DEX) and proteins were analyzed. Representative Western blot is shown in E. **F, G**, primary astrocytes from FKBP51$^{+/+}$ and FKBP51$^{-/-}$ mice were treated with vehicle or DEX (10 µM for 6 h) and protein levels were determined by Western blot (representative example in G). **H,** representative Western of extracts from AD-treated PBMCs (corresponding to Figure 4D-F of the main text and table in I). **I**, summary of the correlations of the effects of ADs on autophagic markers with the expression of FKBP51 in PBMCs from healthy subjects (n=21).



Suppl. Fig. 7. ***Correlation of Clinical response to antidepressant treatment with response to antidepressant treatment of PBMCs ex vivo.*** PBMCs from depressive inpatients were cultivated and treated with FLX (1695 nM) or AMI (888 nM) for 48 h. The AD induced changes in expression of pAkt^{S473}/Akt and LC3II/I are correlated with the clinical response to AD-treatment expressed as percent change in the total score of the 21-items Hamilton Depression Rating scale between admission and after 6 weeks of treatment.

Suppl. Table 1. Details of the results of the statistical analyses. NS, non significant.

figure	parameter	ANOVA	variable 1		variable 2		variable 1 * variable 2	
1 A	struggling	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{3,27} = 25.752$	$p < 0.001$	$F_{3,27} = 13.520$	$p < 0.001$	$F_{3,27} = 4.247$	$p < 0.05$
1 A	immobility		$F_{3,27} = 15.234$	$p < 0.001$	$F_{3,27} = 10.476$	$p < 0.01$	$F_{3,27} = 4.927$	$p < 0.05$
1 B	Hip Beclin 1		$F_{3,28} = 4.944$	$p < 0.05$	$F_{3,28} = 3.167$	n.s.	$F_{3,28} = 4.299$	$p < 0.05$
1 B	PFC Beclin 1		$F_{3,28} = 6.335$	$p < 0.5$	$F_{3,28} = 8.827$	$p < 0.01$	$F_{3,28} = 25.002$	$p < 0.001$
1 C	Hip LC3B-II / LC3B-I		$F_{3,28} = 2.187$	n.s.	$F_{3,28} = 1.723$	n.s.	$F_{3,28} = 7.967$	$p < 0.01$
1 C	PFC LC3B-II / LC3B-I		$F_{3,28} = 28.243$	$p < 0.001$	$F_{3,28} = 16.671$	$p < 0.001$	$F_{3,28} = 32.117$	$p < 0.001$
1 F	Beclin 1	1 Way ANOVA variable 1 = genotype	$F_{2,8} = 32.996$	$p < 0.001$				
1 F	LC3B-II / LC3B-I		$F_{2,8} = 177.628$	$p < 0.001$				
1 F	Atg12		$F_{2,8} = 26.092$	$p < 0.001$				
2 B	pAkt (S473) / Akt	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{3,8} = 126.233$	$p < 0.001$				
2 C	pAkt (S473) / Akt	1 Way ANOVA variable 1 = genotype	$F_{2,8} = 27.702$	$p < 0.001$				
2 D	LC3B-II / LC3B-I		$F_{2,8} = 12.041$	$p < 0.01$				
2 E	punctae per cell	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{3,67} = 173.593$	$p < 0.001$	$F_{3,67} = 106.640$	$p < 0.001$	$F_{3,67} = 29.555$	$p < 0.001$
2 G	Beclin 1		$F_{2,8} = 135.813$	$p < 0.001$	$F_{2,8} = 389.709$	$p < 0.001$	$F_{2,8} = 41.657$	$p < 0.001$
2 H	LC3B-II / LC3B-I		$F_{2,8} = 2785.191$	$p < 0.001$	$F_{2,8} = 357.590$	$p < 0.001$	$F_{2,8} = 111.099$	$p < 0.001$
2 I	Atg12		$F_{2,8} = 15.001$	$p < 0.01$	$F_{2,8} = 28.481$	$p < 0.001$	$F_{2,8} = 0.776$	$p = 0.404$
S1 A	Hip Atg12	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{3,28} = 18.858$	$p < 0.001$	$F_{3,28} = 12.645$	$p < 0.001$	$F_{3,28} = 29.447$	$p < 0.001$
S1 A	PFC Atg12		$F_{3,28} = 1.942$	n.s.	$F_{3,28} = 5.887$	$p < 0.05$	$F_{3,28} = 9.383$	$p < 0.01$
S1 A	Hip Vps34		$F_{3,28} = 33.501$	$p < 0.001$	$F_{3,28} = 21.781$	$p < 0.001$	$F_{3,28} = 25.275$	$p < 0.001$
S1 A	PFC Vps34		$F_{3,28} = 14.334$	$p < 0.001$	$F_{3,28} = 11.231$	$p < 0.01$	$F_{3,28} = 8.125$	$p < 0.01$
S1 A	Hip pAkt (S473) / Akt		$F_{3,28} = 4.999$	$p < 0.05$	$F_{3,28} = 0.018$	n.s.	$F_{3,28} = 6.508$	$p < 0.05$
S1 A	PFC pAkt (S473) / Akt		$F_{3,28} = 17.239$	$p < 0.001$	$F_{3,28} = 4.354$	$p < 0.05$	$F_{3,28} = 27.685$	$p < 0.001$
S3 A	pAkt (S473) / Akt	1 Way ANOVA variable 1 = genotype	$F_{2,8} = 267.265$	$p < 0.001$				
S3 B	Beclin 1	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{5,12} = 24.605$	$p < 0.001$	$F_{5,12} = 11.728$	$p < 0.01$	$F_{5,12} = 19.634$	$p < 0.001$
S3 B	LC3B-II / LC3B-I		$F_{5,12} = 105.288$	$p < 0.001$	$F_{5,12} = 11.107$	$p < 0.01$	$F_{5,12} = 13.721$	$p < 0.001$
S4 C	pAkt (S473) / Akt	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{5,12} = 5.781$	$p < 0.5$	$F_{5,12} = 17.236$	$p < 0.001$	$F_{5,12} = 0.129$	$p = 0.880$
S4 E	pAkt (S473) / Akt		$F_{5,12} = 1.549$	n.s.	$F_{5,12} = 17.786$	$p < 0.001$	$F_{5,12} = 0.156$	$p = 0.858$
S5 A	LC3B-II / LC3B-I		$F_{5,12} = 1.208$	n.s.	$F_{5,12} = 3.835$	$p = 0.052$	$F_{5,12} = 0.635$	$p = 0.547$
S5 C	LC3B-II / LC3B-I		$F_{8,18} = 3.244$	$p = 0.063$	$F_{8,18} = 14.749$	$p < 0.001$	$F_{8,18} = 1.139$	$p = 0.370$
S5 D	punctae per cell		$F_{5,96} = 54.488$	$p < 0.001$	$F_{5,96} = 107.763$	$p < 0.001$	$F_{5,96} = 8.018$	$p < 0.001$
S5 F	Beclin 1		$F_{5,12} = 1.892$	n.s.	$F_{5,12} = 9.553$	$p < 0.01$	$F_{5,12} = 1.449$	$p = 0.273$
S5 G	LC3B-II / LC3B-I		$F_{5,12} = 182.414$	$p < 0.001$	$F_{5,12} = 95.458$	$p < 0.001$	$F_{5,12} = 8.243$	$p < 0.01$
S5 H	Atg12		$F_{5,12} = 6.645$	$p < 0.05$	$F_{5,12} = 205.529$	$p < 0.001$	$F_{5,12} = 2.627$	$p = 0.113$
S6 F	Beclin 1	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{1,8} = 0.883$	n.s.	$F_{1,8} = 11.959$	$p < 0.01$	$F_{1,8} = 15.316$	$p < 0.01$
S6 F	Vps34		$F_{1,8} = 27.295$	$p < 0.001$	$F_{1,8} = 52.357$	$p < 0.001$	$F_{1,8} = 15.796$	$p < 0.01$
S6 F	pAkt (S473) / Akt		$F_{1,8} = 15.607$	$p = 0.004$	$F_{1,8} = 10.323$	$p = 0.012$	$F_{1,8} = 0.921$	$p = 0.365$

Cell lines

Human embryonic kidney cells (HEK-293, ATCC CRL-1573) and mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FCS and 100 units/ml penicillin and streptomycin, respectively. FKBP51$^{-/-}$ and Akt1/2$^{-/-}$ MEFs (kind gift of Nissim Hay, University of Illinois, Chicago, Illinois) have been described before (Skeen et al., 2006; Touma et al., 2011).

Primary cultures of murine astrocytes

Enriched astroglial cultures were prepared from postnatal day 1 rat pups (Sprague-Dawley, Charles River, Sulzfeld, Germany) and handled as described (Franke et al., 1998; Perisic et al., 2010).

FKBP51 Rescue in MEF Fkbp51$^{-/-}$ cells

Afore detached MEF cells (2 x 10^6) were re-suspended in 100 µl transfection buffer (50 mM HEPES pH 7.3, 90 mM NaCl, 5 mM KCl, 0.15 mM $CaCl_2$) (Schumann Burkard et al., 2011). A maximal amount of 5 µg plasmid DNA was added to the cell suspension, and electroporation was carried out using the Amaxa Nucleofactor system (program # T-020). Cells were re-plated at a density of $10^5/cm^2$ and further processed for Western blot analysis.

Analysis of GFP-LC3 in astrocytes

2x10^6 cells were transfected with 2 ng GFP-LC3 expressing plasmid or the respective cloning vector using Amaxa Nucleofactor system as described above. Cells were grown for 48 h and analyzed by fluorescence microscopy. At least 20 cells were counted.

Plasmids

Plasmids expressing FKBP51-FLAG, Akt1, Akt2, and LC3B have been described (Brunet et al., 1999; Wochnik et al., 2005; Brognard et al., 2007; Kim et al., 2010).

Co-Immunoprecipitation (CoIP)

CoIPs of Flag-tagged FKBP51/52 and Flag-tagged FKBP51 endogenous Akt with were performed in HEK293 cells. 5×10^6 cells were electroporated with 5 µg of the respective expression plasmids using a GenePulser (Bio-Rad, USA) at 350 V/700 µF in 400 µl of electroporation buffer (50 mM K_2HPO_4/KH_2PO_4, 20 mM KAc, pH 7.35, 25 mM $MgSO_4$). After three days of cultivation in DMEM/10% FCS, cells were lysed in CoIP-buffer containing 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Igepal complemented with protease inhibitor cocktail (Sigma, P2714). This was followed by incubation on an overhead shaker for

20 min at 4 °C. The lysate was cleared by centrifugation, the protein concentration was determined, and 1.2 mg of lysate was incubated with 2.5 µg FLAG antibody overnight at 4 °C. 20 µl of BSA-blocked Protein G Dynabeads (Invitrogen, 100-03D) were added to the lysate–antibody mix followed by 3 h incubation at 4 °C. The beads were washed 3 times with PBS and protein-antibody complexes were eluted with 100 µl of 1× FLAG-peptide solution (Sigma, 100–200 µg ml^{-1}, F3290) in CoIP buffer for 30 min at 4 °C. 5–15 µg of the cell lysates or 2.5 µl of the immunoprecipitates were separated by SDS-PAGE.

Western Blot analysis

Protein extracts were obtained by lysing cells in 62.5 mM Tris, 2% SDS and 10% sucrose, supplemented with protease (Sigma , P2714) and phosphatase (Roche, 04906837001) inhibitor cocktail. Samples were sonicated and heated at 95 ºC for 5 min. Proteins were separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes. Blots were placed in Tris-buffered saline, supplemented with 0.05% Tween (Sigma, P2287) and 5% non-fat milk for 1 h at room temperature and then incubated with primary antibody (diluted in TBS/0.05% Tween) overnight at 4 ºC. The following primary antibodies were used: Beclin1 (1:1000, Cell Signaling, #3495), pBeclin1 (S234 and S295, both 1:1000, Phosphosolutions #p117-234 and #p117-295), Atg12 (1:1000, Cell Signaling #2010), LC3B-I/II (1:1000, Cell Signaling, #2775), FLAG (1:7000, Rockland, 600-401-383), PI3K Class III (Vps34, 1:1000, Cell Signaling, #4263), FKBP51 (1:1000, Bethyl, A301-430A), Akt (1:1000, Cell Signaling, #4691), pAkt (1:1000, Ser473, T308, 1:1000, Cell Signaling, #4058, #9275), Actin (1:5000, Santa Cruz Biotechnologies, sc-1616).

Subsequently, blots were washed and probed with the respective horseradish peroxidase- or fluorophore-conjugated secondary antibody for 1 h at room temperature. The immuno-reactive bands were visualized either using ECL detection reagent (Millipore, Billerica, MA, USA, WBKL0500) or directly by excitation of the respective fluorophore. Determination of the band intensities were performed with BioRad, ChemiDoc MP, or X-ray-films

Preparation of human PBMCs

Blood of healthy male volunteers and of depressed patients was collected via venipuncture, diluted with PBS and carefully loaded on Biocoll solution (1BioChrom AG, L6113) and centrifuged at 800 x g for 20 min (brakeless running down). PBMCs were enriched by selecting the interphase of the Biocoll gradient. PBMCs of the interphase were washed two times with ice-cold PBS. PBMCs were re-suspended in RPMI and plated at $4x10^5/cm^2$. After recovery for 6 h, cells were treated with either 888 nM (120 ng ml^{-1}) AMI, 1695 nM (500 ng ml^{-1}) FLX, 365 nM (120 ng ml^{-1}) PAR. Concentrations had been chosen to match therapeutic concentrations in the serum according to the consensus guidelines for therapeutic drug monitoring in psychiatry (Hiemke et al., 2011).

3.6. Pharmacological FKBP51 inhibition reduces anxiety-related behavior in mice

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Manuscript in preparation

Abstract

Psychiatric disorders such as major depression (MD) and posttraumatic stress disorder (PTSD) represent one of the largest health burdens worldwide, with still increasing numbers. However, a substantial group of patients does not respond to any of the currently used pharmacotherapies. Therefore, the need to focus research on personalized medicine and the development of conceptually novel drugs is stronger than ever. Single nucleotide polymorphisms of the FK506 binding protein 51 (FKBP51) gene have been associated with MD, PTSD and suicidal behavior in numerous studies in recent years. In addition it has been shown that FKBP51 is involved in stress-vulnerability by reducing the sensitivity of the glucocorticoid receptor (GR). Given the intimate relationship of stress and anxiety and the robust induction of FKBP51 in the amygdala following stress, we hypothesized that amygdala FKBP51 may mediate anxiety-related behaviors. We therefore first mimicked the stress effect by specifically overexpressing FKBP51 in the basolateral amygdala (BLA) of mice via virus-mediated gene transfer, which resulted in increased anxiety-related behavior. Next we show that blockade of an overexpressed functionally fully active FKBP51 variant (with a point mutation) in the BLA with a highly selective and specifically designed FKBP51 antagonist (bump-and-hole approach), was able to reduce the observed anxiogenic phenotype. Finally, we blocked wild-type FKBP51 with a potent and highly selective FKBP51 inhibitor in naïve mice, which led to an anxiolytic phenotype. To our knowledge this is the first study assessing a selective FKBP51 antagonist with regard to anxiety in an *in vivo* system, thereby further unraveling the role of FKBP51 as a potential drug target in the context of stress-related psychiatric diseases.

Introduction

Psychiatric diseases such as major depression (MD) and posttraumatic stress disorder (PTSD) contribute significantly to disease burden worldwide, but their underlying molecular mechanisms are still poorly understood (Tennant, 2001; Krishnan and Nestler, 2008; Nestler and Hyman, 2010). In addition, a substantial proportion of patients does not benefit from prevalent treatment options such as selective serotonin re-uptake inhibitors (SSRIs), highlighting the need for personalized medicine (Porcelli et al., 2011).

In recent years, genome wide association studies identified several genes contributing (in interaction with environmental factors) to the development of such disorders. One interesting candidate is the gene encoding FK506 binding protein 51 (FKBP51), which has been shown to alter glucocorticoid receptor (GR) sensitivity (Riggs et al., 2003; Binder et al., 2004). When it is bound to the GR via Hsp90, the receptor's affinity for glucocorticoids is reduced and the translocation to the nucleus is less efficient. Interestingly, one major characteristic of many depressed patients is an impaired negative feedback loop of the hypothalamic-pituitary-adrenal (HPA) axis, including altered GR signaling (Holsboer, 2000; Pariante and Miller, 2001).

Single nucleotide polymorphisms (SNPs) in the Fkbp51 gene have repeatedly been associated with various psychiatric diseases such as MD, bipolar disorder (Binder et al., 2004; Willour et al., 2009; Tatro et al., 2009b; Lavebratt et al., 2010) and PTSD (Binder et al., 2008; Mehta et al., 2011). In addition, FKBP51 variants have been linked with risk for suicidal behavior (Willour et al., 2009; Brent et al., 2010; Roy et al., 2010; Supriyanto et al., 2011). Interestingly, alterations in FKBP51 expression in the amygdala have been reported in suicide victims (Pérez-Ortiz et al., 2012). The amygdala plays a pivotal role in the neurocircuitry of fear and anxiety disorders (LeDoux, 2007; Shin and Liberzon, 2010; Dias et al., 2013), and was also shown to modulate the HPA-axis through negative feedback mediated by the GR (Gray, 1993; Davis, 1994; McEwen and Sapolsky, 1995).

Importantly, FKBP51 mRNA expression is strongly upregulated in the amygdala following acute stress (Scharf et al., 2011). Furthermore, we could recently show that FKBP51 knockout mice are less affected by chronic social defeat stress, reflected in a stress-resistant HPA system, possibly mediated by an increased GR sensitivity (Chapter 1).

Pharmacological inhibition of FKBP51 might thus constitute a novel therapeutic approach for the prevention and treatment of stress-related affective disorders. The recent development of the first selective and potent FKBP51 antagonists (Gaali and Kirschner et al., unpublished data), now offers the unique possibility to test this hypothesis. In the current study we therefore aimed to further unravel the role of FKBP51 as a potential drug target in the context of stress-related anxiety diseases. First, we mimicked the stress-induced FKBP51 increase in the basolateral amygdala (BLA) and studied anxiety-related behavior in mice. Second, we over-expressed a functionally fully active FKBP51 variant with a point mutation ($FKBP51^{F67V}$) in the BLA, for which a custom-designed antagonist (Ligand1) was recently developed (Gaali and Kirschner et al., unpublished data). We then tested, whether this antagonist, which does not affect wild-type FKBP51, can ameliorate the phenotype induced by $FKBP51^{F67V}$ overexpression. In a third step, we blocked wild-type FKBP51 in naïve mice, using a potent and highly selective antagonist (SAFit2) and subsequently studied anxiety-related behavior.

Materials and Methods

Animals

For all experiments, male C57Bl/6N mice (Charles River Laboratories, Maastricht, the Netherlands) at the age of 12 weeks were used. The mice were held under standard conditions (12L: 12D light cycle, lights on at 08:00 AM, temperature 23±2°C) and were single housed and acclimated to the room for 2 weeks before the beginning of the experiments. Food (Altromin 1324, Altromin GmbH, Germany) and tap water were available *ad libitum*. For all experiments a separate batch of animals was used (n = 8 – 14). All experiments were carried out in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Experimental design

Experiment 1.

We investigated the effects of a FKBP51 overexpression in the BLA or the dorsal hippocampus (dHc) with regard to anxiety-related behavior (n = 11-14 per group).

Experiment 2.

We first tested the blood-brain barrier penetration of Ligand1, a FKBP51 antagonist that specifically binds to the mutated FKBP51^{F67V} variant, but does not bind to wild-type FKBP51. FKBP51^{F67V} preserves the repressing activity of wild type FKBP51 on the GR, but can specifically be inhibited by the FKBP51 antagonist Ligand1. Animals received a single injection of either 0.1 mg/kg BW, 1.0 mg/kg BW, 10.0 mg/kg BW Ligand1 or vehicle solution (n = 4 per group). Next, we tested the ability of Ligand1 to rescue the anxiogenic effects, induced by FKBP51^{F67V} overexpression in the BLA (n = 11-13 per group). To confirm the specificity and to rule out any potential side effects of the antagonist in the control group, we performed an additional control experiment, in which naïve C57Bl/6N mice received a

single injection of 10.0 mg/kg BW Ligand1 or vehicle solution and were subsequently tested with regard to anxiety-related behavior (n = 8 per group).

Experiment 3.

We selectively blocked wild-type FKBP51 with SAFit2 in naïve mice and investigated its effects on anxiety-related behavior (n = 8 – 12 per group).

Viral overexpression of FKBP51

Viral overexpression was performed as described previously (Schmidt et al., 2011). We used an adeno-associated bicistronic AAV1/2 vector (GeneDetect). In experiment 1 the vector contained a CAG-HA-tagged-FKBP51-WPRE-BGH-polyA expression cassette (containing the coding sequence of human Fkbp51 NCBI CCDS ID CCDS4808.1). For experiment 2 the vector contained a CAG-HA-tagged-FKBP51-F67V-mutant-WPRE-BGH-polyA expression cassette (containing the coding sequence of the human Fkbp51 NCBI CCDS ID CCDS4808.1) with a F67V point mutation (Gaali and Kirschner et al., unpublished data). 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. Mice were anesthetized with isoflurane, and 0.5 µl of either AAV-FKBP51, AAV-FKBP51^{F67V} or AAV-Empty (titers: 1.3 x 10^{12} genomic particles/ml (experiment 1), 2.6 x 10^{12} genomic particles/ml (experiment 2) were bilaterally injected in the BLA or hippocampus at 0.06 µl/min by glass capillaries with tip resistance of 2–4MΩ in a stereotactic apparatus. For the amygdala the following coordinates were used: 1.0 mm posterior to bregma, 3.5 mm lateral from midline, and 3.8 mm below the surface of the skull, targeting the BLA; for the hippocampus we used: 1.9 mm posterior to bregma, 1.3 mm lateral from midline, and 1.3/1.8 mm below the surface of the skull, targeting the CA1 and dentate gyrus (DG) region of the dorsal hippocampus. After surgery, mice were treated for 5 d with Metacam via drinking water. Behavioral testing started 4 weeks after virus injection. Successful overexpression of the FKBP51-forms was verified by in situ *hybridization* and *immunofluorescence*. Animals that were not infected bilaterally in the dorsal hippocampus or BLA were excluded from the analysis. Quantification of the overexpression was achieved by *in situ* hybridization using the riboprobe described below.

Behavioral testing

All behavioral tests were recorded using a videotracking system (Anymaze 4.20; Stoelting). The following behavioral tests were performed: open field (OF), elevated plus maze (EPM), dark-light box (DaLi). The testing procedures were performed as described below.

Open field

The open-field test was performed to investigate general locomotor activity. Testing was carried out in an empty open-field arena (50 cm x 50 cm x 50 cm) made of gray polyvinyl chloride (PVC), which was evenly illuminated with 15 Lux. The low illumination of the open field arena was chosen to specifically investigate locomotion behavior and not create an aversive center region that may induce anxiety-related behavior. Testing time was 15 min and parameter of interest was the total distance traveled.

Elevated plus maze

The EPM was conducted to display changes in anxiety-related behavior. The device consisted of a plus-shaped platform with two opposing open arms (30 cm x 5 cm x 0.5 cm) and two opposing enclosed arms (30 cm x 5 cm x 15 cm), made of gray PVC, which were connected by a central area (5 cm x 5 cm). The whole device was elevated 50 cm above the floor. The illumination was 25 Lux in the open arms and less than 10 Lux in the closed arms. Testing duration was 10 min in experiment 1 and 3 and 5 min in experiment 2. Mice were placed into the center zone facing one of the enclosed arms at the start of the test. The time spent in the open arms compared to the total arm time as well as the number of open arm entries were analyzed. Animals that fell off the open arm of the apparatus during testing were excluded from the analysis.

Dark-Light box

The DaLi test was used as another paradigm to measure anxiety-related behavior. The apparatus consisted of a rectangular box with two compartments, the dark compartment (15 cm x 20 cm x 25 cm), with <10 lux and the light compartment (30 cm x 20 cm x 25 cm), lit with 700 lux. Both compartments were connected by a 4 cm long tunnel. At the beginning of the test, each mouse was placed in the center of the dark compartment facing the back wall of the apparatus. Testing duration was 7 min in experiment 1 and 10 min in experiment 2 and 3. The time spent in the lit compartment, as well as the number of lit compartment

entries, the latency to the first entry to the lit compartment and the distance traveled in the lit compartment were analyzed.

Drugs

The FKBP51 antagonists (Ligand1 and SAFit2) were solubilized in 4% EtOH, 5% Tween80 and 5% PEG400 in 0.9% saline. In experiment 2, animals received an intraperinal (IP) injection of either vehicle (4% EtOH, 5% Tween80 and 5% PEG400 in 0.9% saline) or Ligand1 (0.1 mg/kg BW, 1.0 mg/kg BW or 10mg/kg BW) solution 1 hr prior perfusion to assess the blood-brain barrier penetration. Mice that underwent surgeries and behavioral analysis in experiment 2 received an IP injection of vehicle or Ligand1 (10mg/kg BW) on 10 consecutive days. On testing days, the injection was applied 1 hr prior to the start of the test. Naïve mice (without surgery) received a single IP injection of vehicle or Ligand1 (10mg/kg BW) 1 hr prior to the start of the behavioral test. In experiment 3, animals received an IP injection of vehicle or SAFit2 (20mg/kg BW) 1hr or 16 hrs prior to the start of the test.

Sampling procedure

At the day of sacrifice, animals were deeply anesthetized with isoflurane. For animals that were used to assess the pharmacokinetics of Ligand1 in experiment 2, blood samples were taken by cardiac puncture, kept on ice and later centrifuged at 8000 rpm at 4°C for 15 min. Plasma was transferred to new, labeled tubes and stored at -20°C. These mice were perfused intracardially with saline; brains were removed and immediately stored at -80°C. The concentration of the FKBP51 antagonist in plasma and brain was quantified by LC-MS/MS. All other animals of experiment 1 and experiment 2 were perfused intracardially with 4% paraformaldehyde. Brains were removed, post-fixed overnight in 4% paraformaldehyde, followed by subsequent overnight incubation in 30% sucrose solution at 4 °C, and then stored at -80 °C until processing for *in situ* hybridization and immunohistochemistry.

In Situ hybridization and immunohistochemistry

For *in situ* hybridization and *immunohistochemistry*, frozen brains were coronally sectioned in a cryostat microtome at 25 µm and kept at -80°C. *In situ* hybridization using a ^{35}S UTP-

labeled ribonucleotide probe for Fkbp51 (Forward primer: 5'-CTTGGACCACGCTATGGTTT; Reverse primer: 5'-GGATTGACTGCCAACACCTT) was performed as described previously (Schmidt et al., 2007). Immunofluorescence (mouse anti-Ha, 1:1000, Sigma; Donkey anti-mouse Alexafluor 488, 1:500) was performed on free-floating sections (*n* =3 per mouse) as described previously (Wang et al., 2011a).

Statistical analysis

The data presented are shown as means +SEM and were analyzed by the commercially available software SPSS 17.0 and Sigma Plot 11.0. When two groups were compared, the unpaired student's *t*-test was applied. If data were not normally distributed the non-parametric Mann-Whitney test (MW-test) was used. For four group comparisons, two-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test, as appropriate. P values of less than 0.05 were considered significant.

Results

FKBP51 overexpression in the basolateral amygdala induces anxiogenic behavior

We overexpressed FKBP51 in the BLA by viral mediated gene transfer and subsequently investigated mice with regard to anxiety-related behavior. The stable FKBP51 overexpression in the BLA was quantified by *in situ* hybridization (t-test: T_{26} = 105.000, $p < 0.001$) and was additionally validated by immunofluorescence (Figure 1A-D).

Mice with a FKBP51 overexpression in the BLA ($51OE^{BLA}$ mice) showed increased anxiety-related behavior in the EPM, which was reflected in a significantly reduced open arm time (t-test: T_{22} = 2.593, $p < 0.05$) as well as in significantly decreased open arm entries (t-test: T_{22} = 2.198, $p < 0.05$) compared to control mice (Figure 1E, F). These findings were further supported by an increased latency to enter the aversive lit compartment (t-test: T_{26} = -1.781, $p = 0.087$) (Figure 1G). This anxiogenic phenotype of $51OE^{BLA}$ mice was independent of general locomotor activity, which did not differ between the groups (Figure 1H). Furthermore, these effects were BLA specific, as we did not observe any differences in anxiety-related behavior in mice with a FKBP51 overexpression in the dHc (Suppl. Figure 1).

Figure 1

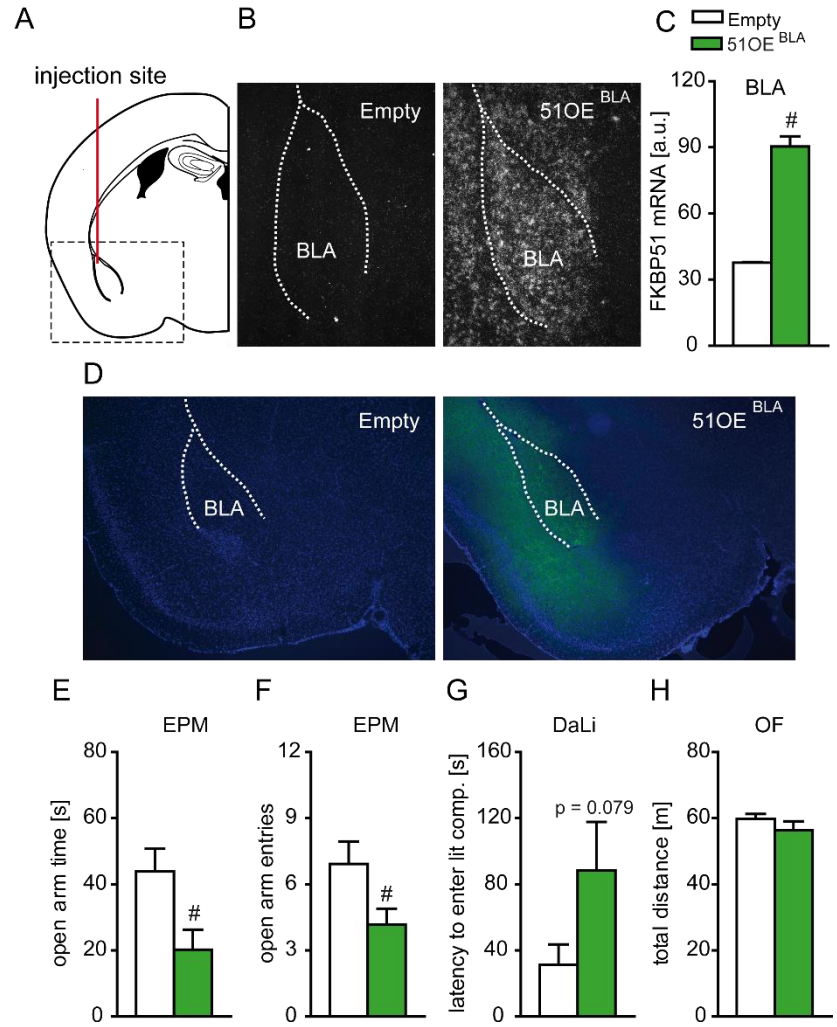


Figure 1: Overexpression of FKBP51 in the basolateral amygdala (BLA) induces anxiety-related behavior. **A**, Schematic representation of the viral injection into the BLA. **B**, Representative of autoradiographs of viral FKBP51 mRNA expression in the BLA 7 weeks after injection **C**, Quantification of FKBP51 mRNA levels in the BLA. Infection with the viral construct induced a strong increase. **D**, Representative immunohistochemistry of viral FKBP51 expression (Ha-tag Immuno) in the BLA region 7 weeks after injection. **E** and **F**, FKBP51 overexpression in the BLA leads to significantly reduced open arm time and open arm entries in the elevated plus maze (EPM). **G**, 51OE^{Amy} mice show an increased latency to enter the lit compartment in the dark light box (DaLi). **H**, General locomotion as assessed in the open field (OF) is not affected by FKBP51 overexpression in the BLA. # $p < 0.05$, data are expressed as mean + SEM.

A specific FKBP51 antagonist reduces the anxiogenic phenotype induced by FKBP51 overexpression in the BLA

We then examined whether we could rescue the anxiogenic phenotype observed in 51OE^{BLA} mice with a FKBP51 antagonist. Therefore, we overexpressed a mutant form of FKBP51with a single point mutation (51OE$^{F67V\text{-}BLA}$ mice) in the BLA and subsequently treated the animals with the specifically designed FKBP51 antagonist Ligand1 that only binds to the FKBP51-mutant.

At first we investigated the antagonist's ability to cross the blood-brain barrier in C57/Bl6N mice. Ligand1 showed a dose-dependent accumulation in the plasma and the brain 1h after the injection (Suppl. Figure 2A and B).

The stable FKBP51^{F67V} overexpression in the BLA was quantified by *in situ* hybridization (MW-test: $T = 351.000$, $p < 0.001$) and was additionally validated by immunofluorescence (Figure 2A-C).

The overexpression of FKBP51^{F67V} in the BLA induced an anxiogenic phenotype in the EPM, which was reflected in a significantly reduced open arm time (2-Way ANOVA: main genotype effect, $F_{1,41} = 4.696$, $p < 0.05$) (Figure 2D). This finding was independent of the treatment groups. In the DaLi test 51OE$^{F67V\text{-}BLA}$ mice spent significantly less time in the lit compartment than the empty control groups (2-Way ANOVA: main genotype effect, $F_{1,45} = 4.671$, $p < 0.05$). However, in further post-hoc analysis this genotype effect is only significant within the vehicle treated groups (Tukey's post-hoc: $p < 0.05$) (Figure 2E). This was further supported by a significantly reduced distance traveled in the lit compartment of 51OE$^{F67V\text{-}BLA}$ mice compared to control mice (2-Way ANOVA: main genotype effect, $F_{1,45} = 4.514$, $p < 0.05$), since further post-hoc analysis only revealed a slight genotype difference in the vehicle treated mice (Tukey's post-hoc: Trend, $p < 0.1$) (Figure 2F). The OF test served again as control, and revealed that the anxiety-related phenotype of 51OE$^{F67V\text{-}BLA}$ mice is independent of general locomotion, which did not differ between the experimental groups (Figure 2G). Moreover we found no differences in anxiety-related behavior in naïve mice treated with Ligand1 compared to the vehicle treated group (Suppl. Figure 2C-F), to rule out potential side effects.

Figure 2

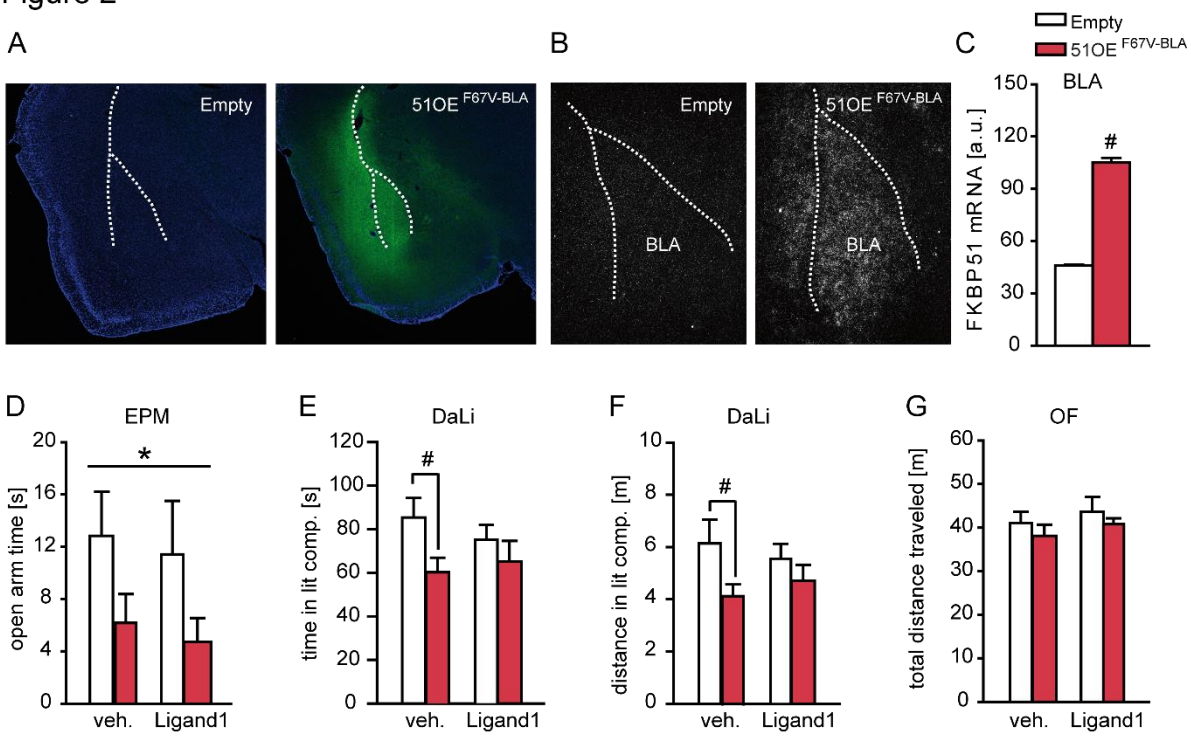


Figure 2: The FKBP51^{F67V} antagonist Ligand1 reverses the anxiogenic phenotype induced by overexpression of FKBP51^{F67V} in the amygdala. **A**, Representative immunohistochemistry of viral FKBP51^{F67V} expression (Ha-tag Immuno) in the BLA 7 weeks after injection. **B**, Representative of autoradiographs of viral FKBP51^{F67V} mRNA expression in the BLA 7 weeks after injection. **C**, Quantification of FKBP51 mRNA levels in the BLA. Infection with the viral construct induced a strong increase. **D**, FKBP51^{F67V} overexpression in the BLA leads to significantly reduced open arm time, independent of treatment group in the EPM. **E** and **F**, Vehicle treated 51OE$^{F67V\text{-}BLA}$ mice spend significantly less time and travel less distance in the lit compartment of the DaLi compared to vehicle treated control animals. This effect is abolished in the Ligand1 treated groups. **G**, General locomotion in the OF does not differ between the experimental groups. * $p < 0.05$ (ANOVA genotype effect); # $p < 0.05$ (Tukey post-hoc test); data are expressed as mean + SEM.

The specific FKBP51 antagonist SAFit2 induces anxiolytic behavior in naïve mice

When we injected the selective and potent FKBP51 antagonist SAFit2 1 hr prior to testing, we did not find any differences in anxiety-related behavior (Suppl. Figure 3). However, SAFit2 treatment induced an anxiolytic phenotype in mice injected in the afternoon, 16 hrs prior testing, which was reflected in an increased open arm time in the EPM (MW-test: $T = 79.000$, $p < 0.05$) (Figure 3A). This finding was further support by an increase in the number of entries to the lit compartment (t-test: $T_{20} = 2.593$, $p < 0.01$) as well as in the time spent in the lit compartment (t-test: $T_{20} = -2{,}275$, $p < 0.05$) in the DaLi (Figure 3B and C). Moreover, SAFit2 treatment led to a reduced latency to enter the lit compartment (MW-test: T =

161.000, $p < 0.05$) as well as an increased distance traveled in the lit compartment (t-test: $T_{20} = -2.371$ $p < 0.05$) (Figure 3D and E).

Figure 3

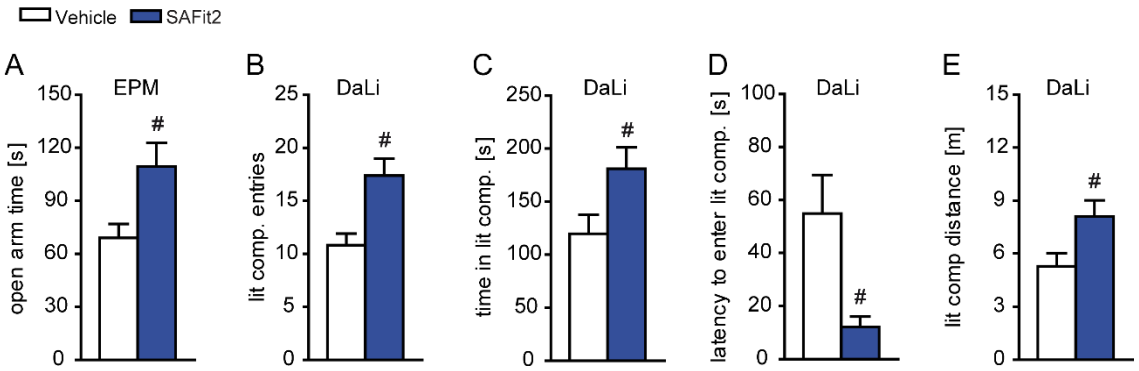


Figure 3: Inhibition of wild-type FKBP51 by SAFit2 induces anxiolytic behavior 16 hrs after injection. **A**, Mice treated with SAFit2 show a significant increase in the open arm time of the EPM. **B** and **C**, SAFit2 leads to significantly increased entries to the lit compartment and significantly more time spent in the lit compartment of the DaLi. **D** and **E**, Mice treated with SAFit2 showed a significantly decreased latency to enter and a significantly increased distance traveled in the lit compartment of the DaLi. # $p < 0.05$, data are expressed as mean + SEM.

Discussion

In our study, we show that specific FKBP51 overexpression in the BLA by viral transfection is able to mimic a stress-induced anxiogenic phenotype in mice. We then blocked an overexpressed FKBP51 variant ($FKBP51^{F67V)}$ in the BLA, with a highly selective FKBP51 antagonist (Ligand1) and could thereby reduce the induced phenotypes of increased anxiety-related behavior in the DaLi box. When applying a selective and potent FKBP51 antagonist (SAFit2), an anxiolytic phenotype in naïve mice in the EPM and the DaLi box is induced.

The first focus of the study was the investigation of anxiety-related behavior following specific FKBP51 overexpression in the amygdala. The amygdala is an important brain region for the mediation of stress responses, fear and anxiety (Joëls and Baram, 2009). Interestingly, alterations in FKBP51 levels have been observed in suicide victims (Pérez-Ortiz et al., 2012). In mice, FKBP51 mRNA levels were significantly upregulated after acute stressors or dexamethasone stimulation in the amygdala (Scharf et al., 2011). In line with this study, we found that mice with an FKBP51 overexpression in the BLA show an increase in anxiety-related behavior, suggesting that the natural induction of amygdala FKBP51 following stress functionally mediates stress-induced anxiety. Moreover, the anxiogenic phenotype was independent of locomotion as we did not find any differences in the open field test. Furthermore the increased anxiety-related effects were BLA specific, as FKBP51 overexpression in the dorsal hippocampus did not reveal any changes in such behavior.

FKBP52 is a close homologue of FKBP51 and shares more than 75% sequence similarity, but acts as functional opponent: FKBP51 decreases GR nucleus translocation and subsequent GR signaling, while FKBP52 enhances GR translocation to the nucleus (Wochnik et al., 2005). We could previously show that FKBP51 knockout mice demonstrate a reduced corticosterone response to forced swim stress, while mice lacking one allele of FKBP52 showed increased corticosterone levels to this stressor and an anxiogenic phenotype (Chapter 1 and 3). Thus, the selectivity of an FKBP51 antagonist is crucial. At first, we tackled this challenge by using a bump-and-hole approach, overexpressing a functionally fully active FKBP51 variant carrying a point mutation ($FKBP51^{F67V}$), which can selectively be blocked by the specifically designed $FKBP51^{F67V}$-antagonist, Ligand1 (Gaali and Kirschner et al.,

unpublished data). We could show that overexpression of FKBP51^{F67V} in the BLA induced a similar anxiogenic phenotype in mice as overexpression of wild-type FKBP51, thereby confirming our initial results. When we sub-chronically treated these mice with Ligand1, we could reduce the increased anxiety-related behavior in the DaLi box. However, we were not able to fully reverse the induced phenotype, especially in the EPM, indicating a potentially too low dose or unfavorable pharmacokinetics in relation to the robust AAV-mediated FKBP51 overexpression.

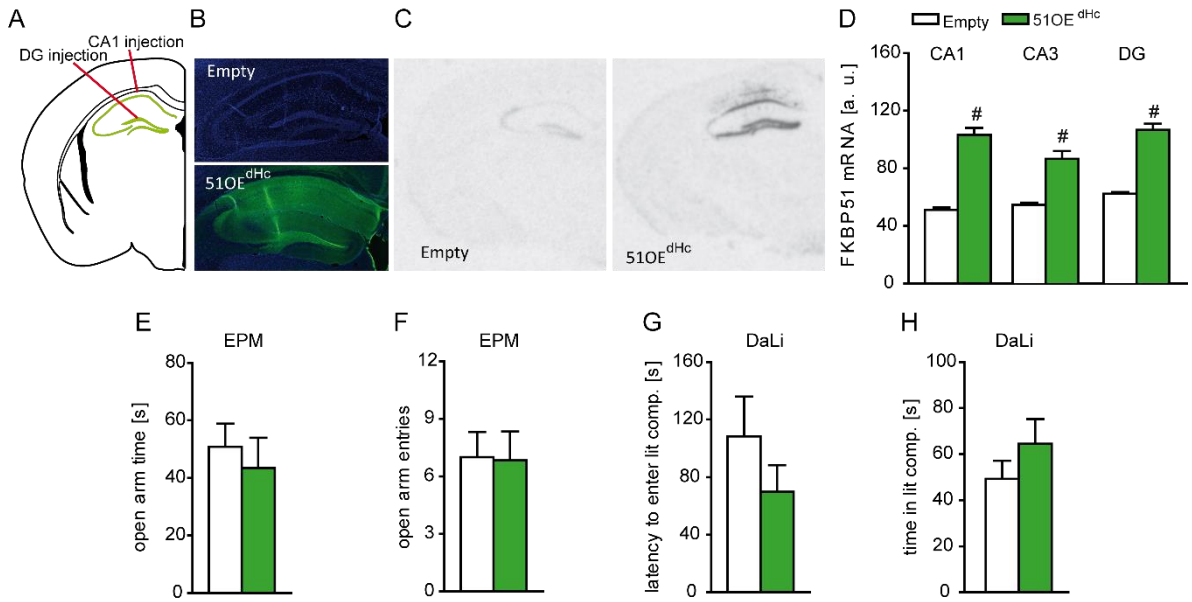
When using the selective and potent novel antagonist for wild-type FKBP51, SAFit2, we found that naïve mice injected 16 hr prior to testing with SAFit2 showed a significant anxiolytic phenotype in the EPM and DaLi. These results are in line with the observation that knockdown of FKBP51 in the BLA reduces stress-induced anxiety in mice (Attwood et al., 2011). Our pharmacological approach revealed an interesting time dependency, as injection of SAFit2 1 h prior testing was ineffective. This time dependency may point to a genomic effect, possibly mediated by enhanced GR signaling. The activity of the HPA-axis is characterized by a prominent circadian rhythm with peak glucocorticoid secretion occurring shortly before the onset of an animal's activity period and nadir levels during the beginning of the resting period (Engeland and Arnhold, 2005; Kriegsfeld and Silver, 2006). This daily variation of glucocorticoid concentration is critical for homeostatic regulation of neural processes and subsequently for behavior. We could previously show that FKBP51 regulates corticosterone levels, by attenuating the negative feedback loop of the HPA-axis (Touma et al., 2011; Chapter 1). In addition, we recently demonstrated that mice treated with SAFit2 showed reduced corticosterone levels during the circadian peak (Gaali and Kirschner et al., unpublished data). Inhibition of FKBP51 during the circadian peak of glucocorticoid secretion, may thus lead to an improved negative feedback loop of the HPA axis, mediated by enhanced GR signaling, which might explain the anxiolytic phenotype 16 hrs after the injection. However, FKBP51 has been shown to be involved in various other pathways besides its regulatory function of the GR (Pei et al., 2009; Blair et al., 2013), thus a more detailed molecular analysis may lead to further insight in this respect.

In summary, our results represent a cornerstone in the current development of FKBP51 inhibitors, because they pinpoint the potential of potent FKBP51 inhibitors to modulate

anxiety *in vivo.* These results further pave the way for the development of FKBP51 antagonists in the context of pharmacotherapy for stress-related psychiatric diseases.

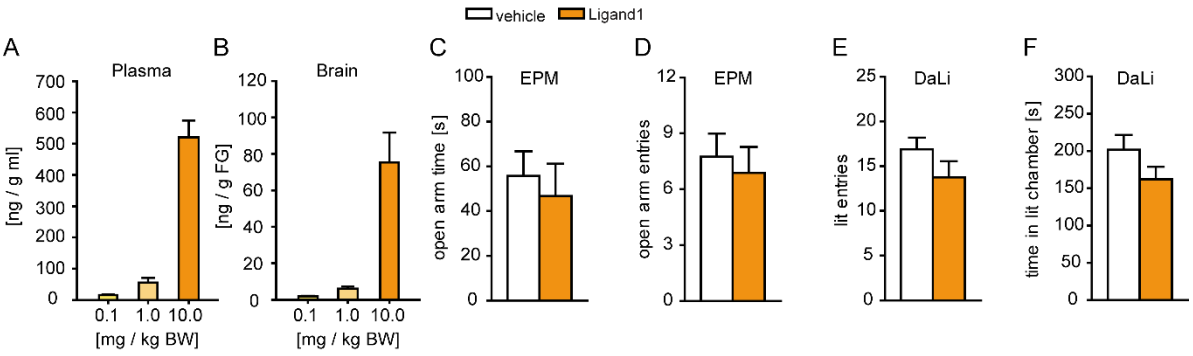
Supplemental Figures

Supplemental Figure 1



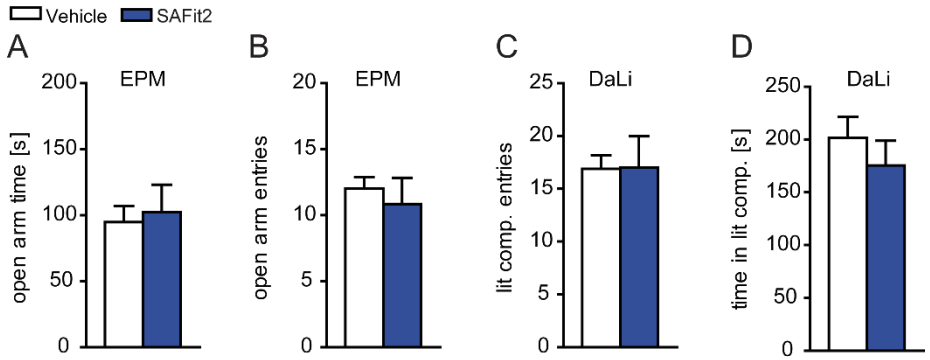
Supplemental Figure 1: Overexpression of FKBP51 in the dorsal hippocampus (dHc). **A**, Schematic representation of the viral injection sites into the CA1 and dentate gyrus (DG) of the dHc. **B**, Representative immunohistochemistry of viral FKBP51 expression (Ha-tag Immuno) in the dHc 7 weeks after injection. **C**, Representative of autoradiographs of viral FKBP51 mRNA expression in the dHc 7 weeks after injection. **D**, Quantification of FKBP51 mRNA levels in the CA1, CA3 and DG of the dHc. Infection with the viral construct induced a strong increase. **E**-**H**, No differences between 51OE^{dHc} and Empty groups were found in the EPM and Dali. # $p < 0.05$, data are expressed as mean + SEM.

Supplemental Figure 2



Supplemental Figure 2: **A** and **B**, Ligand1 pharmacokinetics. The compound was administered i.p. at the indicated dose and the plasma and brain concentrations were determined after 1h. Ligand1 accumulates in the brain in a dose-dependent manner. **C**-**G**, Ligand1 did not show any overt behavioral side effects in the EPM and DaLi in naïve mice.

Supplemental Figure 3



Supplemental Figure 3: **A**-**D**, Mice treated with SAFit2 do not show any differences to the vehicle group in the EPM and DaLi 1 h after the injection.

4. General discussion

Psychiatric disorders such as major depression are a major disease burden, affecting an estimated 350 million people worldwide (Kessler et al., 2009). Psychiatric phenotypes are of multifactorial and polygenic nature, resulting from a complex interaction of environmental and genetic factors (Zannas and Binder, 2014). However, the underlying molecular mechanisms are still not fully understood. One of the most robust biological characteristics in many depressed patients is an impaired negative feedback of the HPA axis, leading to partial glucocorticoid resistance (Holsboer, 2000; Pariante and Lightman, 2008). Therefore a growing number of clinical studies investigated gene x environment interactions occurring at genetic loci involved in the stress response. FKBP51 emerged as an interesting candidate gene, due to its role as negative regulator of GR signaling and its associations with psychiatric disorders such as major depression and PTSD.

This thesis provides further substantial evidence that FKBP51 is crucially involved in GR-dependent stress susceptibility/resilience and consequently in stress physiology and psychiatric diseases. Moreover, it significantly extends the role of GR-independent functions of FKBP51 in the context of psychiatric disorders and antidepressant actions. At first, we tested FKBP51 knockout male mice in the well-established chronic social defeat stress paradigm with respect to immediate consequences (Chapter 1). In chapter 2, we investigated the role of FKBP51 in female mice under basal and acute stress conditions to elucidate potential FKBP51-dependent gender differences. We then studied the involvement of FKBP52, the functional counter-player of FKBP51, in the immediate effects of chronic stress (Chapter 3). We continued to investigate the long term consequences of chronic social defeat stress in FKBP51 knockout males. SNPs in the FKBP51 gene have also been associated with antidepressant treatment efficacy. Thus, to mimic the clinical situation, we concurrently tested whether currently prescribed antidepressants reveal an altered treatment response in FKBP51 knockout mice (Chapter 4) and elucidated a possible FKBP51-dependent mechanism priming antidepressant efficacy (Chapter 5). FKBP51 also raised the attention as a potential novel treatment approach, however, up to date there were no potent and selective inhibitors available. By using a novel class of very recently developed ligands, we were the first that extensively investigated the potential of FKBP51

as a drug target *in vivo* with respect to psychiatric disorders and further elucidated brain-region specific roles of FKBP51 (Chapter 6). In summary, this thesis substantially contributes to the broad functions and interactions of FKBP51 in the context of psychiatric disorders (for an overview see Figure 3.1., page 166).

4.1. GR-dependent functions of FKBP51

Altered function of the GR and consequently an impaired negative feedback regulation of the HPA axis have repeatedly been shown to be key features in the precipitation, development and resolution of psychiatric disorders (Anacker et al., 2011). FKBP51 has been shown to be a major regulator of GR signaling and stress physiology. When it is bound to the GR, the receptor's affinity for glucocorticoids is reduced (Binder, 2009). Previous preclinical studies investigated FKBP51 knockout mice under basal and acute stress conditions with regard to behavioral and neuroendocrine parameters. Touma and colleagues showed that FKBP51 knockout mice spent more time struggling and less time floating in the forced swim test following an acute severe stressor, implicating an enhanced stress-coping phenotype. Moreover, lack of FKBP51 led to decreased HPA axis reactivity and GR expression changes in response to stressors (Touma et al., 2011). Along these lines, O'Leary et al. demonstrated that aged FKBP51 knockout mice revealed a similar behavioral anti-depressant like phenotype already under basal conditions, both in the forced swim test and tail suspension test, as well as a reduced corticosterone response to an acute stressor (O'Leary, III et al., 2011). In addition, 51KO mice demonstrated a pro-resilience sleep phenotype and showed reduced hippocampal corticosterone levels during the circadian peak of glucocorticoid secretion (Albu et al., 2013). To gain further insight into the complex nature of psychiatric diseases, we here investigated the role of FKBP51 in GR-dependent (chronic) stress physiology, as chronic stress is a major risk factor for the development of such disorders.

At first we subjected FKBP51 knockout mice to the chronic social defeat stress paradigm. In support of the existing literature, we could provide further evidence that FKBP51 is crucially involved in stress physiology as we convincingly demonstrated that this co-chaperone mediates the neuroendocrine and behavioral consequences of chronic stress. We found a more resilient phenotype of FKBP51 knockout mice, especially with regard to neuroendocrine parameters (Chapter 1). Chronically stressed 51KO mice demonstrated a

reduced basal HPA axis activity, a blunted corticosterone response to an acute stressor, as well as an enhanced recovery from such stressor. In addition, these mice had lower adrenal gland weights. Female 51KO mice displayed the same neuroendocrine phenotype as males under basal and acute stress conditions, pointing to gender-independent FKBP51 effects, at least under low aversive circumstances (Chapter 2). 51KO mice also demonstrated a more resilient behavioral phenotype with respect to long term consequences of chronic stress (Chapter 4). In contrast to wild type mice, social behavior assessed three weeks after the stress period was not affected in 51KO mice. Moreover, the blunted corticosterone phenotype of 51KO mice was present at numerous time points. Considering the fact that FKBP51 reduces GR sensitivity in cellular models (Wochnik et al., 2005; Westberry et al., 2006; Albu et al., 2013), an enhanced GR sensitivity is the most likely explanation for the changes in HPA axis settings assessed here. In the long run, such changes might also be able to alter the behavioral outcome and favor a more resilient phenotype of 51KO mice subjected to stress (see Figure 3.1).

Strikingly, our results mirror findings of human studies with depressed patients carrying genetic variants of FKBP51. Activation of the GR induces FKBP51 expression, which promotes an ultra-short feedback of GR sensitivity (Binder, 2009). Interestingly, polymorphisms of FKBP51 have been associated with an altered FKBP51 mRNA induction following GR activation (Binder et al., 2004). Allele-specific alterations of the 3-dimensional conformation in the FKBP51 gene leads to enhanced stressor-induced induction of FKBP51 and an impaired GR-mediated negative feedback of the HPA axis in risk allele carriers of rs1360780 (Klengel et al., 2013). Indeed, healthy subjects, carrying the high induction allele of rs1360780 were associated with a prolonged cortisol response to psycho social stress, and thus increased GR resistance (Ising et al., 2008). Furthermore, the impaired negative feedback seems to predispose individuals to psychiatric disorders, as carriers of the high induction alleles have been associated with an increased susceptibility to develop major depression and bipolar disorder in numerous studies in the past (Binder et al., 2004; Lekman et al., 2008; Willour et al., 2009; Lavebratt et al., 2010; Zobel et al., 2010; Zimmermann et al., 2011; Menke et al., 2013). We found that FKBP51 knockout mice were less affected by stress, reflected in a stress-resistant HPA system (Chapter 1, 2 and 4). Inhibition of FKBP51

might thus constitute a promising novel pharmacological approach to ameliorate the neuroendocrine alterations observed in patients with stress-related psychiatric disorders.

The selectivity of FKBP51 ligands is extremely crucial, as FKBP51 and its close homologue, FKBP52, share more than 75% sequence similarity, but act as functional opponents of GR signaling (Wochnik et al., 2005). Indeed, we could show that FKBP51 knockout mice demonstrated a reduced corticosterone response to forced swim stress (Chapter 1, 2 and 4), while mice lacking one allele of FKBP52 showed increased corticosterone levels to this stressor (Chapter 3) (see also Figure 3.1). Very recently we could show that the use of a newly developed potent and highly selective FKBP51 antagonist, SAFit2, led to significantly decreased corticosterone levels in mice during the circadian peak of glucocorticoid secretion, improved the neuroendocrine feedback following a DEX-CRH test and resulted in a more active stress-coping phenotype in the FST (Gaali and Kirschner et. al., unpublished data). These findings strongly overlap with the behavioral and neuroendocrine phenotype of FKBP51 knockout mice. We continued to comprehensively elucidate the potential of FKBP51 as a drug target *in vivo* with respect to psychiatric disorders and further studied brain-region specific roles of FKBP51 (Chapter 6). Interestingly, virus-mediated overexpression of FKBP51 in the hippocampus did not reveal an overt behavioral phenotype in mice. Although, hippocampal FKBP51 mRNA expression is rather strong under basal conditions compared to other regions of the murine brain, the induction of FKBP51 expression, following GR activation, is much more pronounced in regions with low baseline expression, such as the PVN or the amygdala (Scharf et al., 2011). Accordingly, we found that FKBP51 overexpression in the BLA led to an anxiogenic phenotype in mice (Chapter 6). Interestingly, knockdown of FKBP51 in the BLA resulted in reduced anxiety-related behavior in mice that were previously restraint stressed (Attwood et al., 2011). Along these lines, we found that pharmacological inhibition of FKBP51, using the potent and selective antagonist SAFit2, led to anxiolytic behavior 16 hrs after injection (Chapter 6). The pharmacological approach revealed an interesting time dependency, as injection of SAFit2 1 h prior testing was ineffective (see Figure 3.1). This time dependency may point to a genomic effect, possibly mediated by enhanced GR signaling.

Taken together, our findings substantially contribute to the knowledge of GR-dependent effects of FKBP51 on stress-physiology and represent a cornerstone in the current

development of FKBP51 inhibitors in the context of pharmacotherapy for stress-related psychiatric diseases.

4.2. GR-independent functions of FKBP51

Besides the well-established association of FKBP51 polymorphisms with psychiatric disorders and the corresponding involvement of FKBP51 in GR-dependent stress-physiology, clinical studies suggested a link between genetic variants of FKBP51 and antidepressant treatment response. Binder and colleagues were the first to report that patients carrying the high induction/risk allele of FKBP51 SNP rs1360780 were associated with an enhanced antidepressant efficacy (Binder et al., 2004). Since then several other studies demonstrated significant correlations between FKBP51 SNPs and treatment outcome in independent samples (Lekman et al., 2008; Kirchheiner et al., 2008; Zou et al., 2010; Horstmann et al., 2010; Ellsworth et al., 2013). However, the molecular basis for the FKBP51-dependent treatment response remained far from clear.

In line with the clinical situation, we could show that lack of FKBP51 resulted in reduced response to acute and chronic administration of the antidepressant paroxetine (Chapter 4 and 5). Paroxetine-induced bodyweight gain, as observed in wild-type mice, was absent in mice with a deletion of FKBP51. Moreover, in contrast to wild-type mice, FKBP51 knockout mice did not show an increase in time struggling and a reduction in time floating in the forced swim test. In addition, chronic paroxetine treatment was able to enhance social behavior under basal and chronically stressed conditions in wild-type mice, but showed no effects in 51KO mice (see Figure 3.1).

Since the known GR-dependent functions of FKBP51 in stress physiology were unlikely to serve as link to antidepressant action, we searched for novel FKBP51 functions. Indeed, FKBP51 has been shown to be involved in various other pathways besides its regulatory role of the GR (Pei et al., 2009; Blair et al., 2013). Based on reports that some antidepressants alter at least the initial processes of autophagy (Rossi et al., 2009; Zschocke et al., 2011), we analyzed whether putative convergent molecular pathways addressed by both FKBP51 and antidepressants might explain and substantiate the suggested impact of FKBP51 on antidepressant efficacy.

We found that FKBP51 interacts with the autophagy executer Beclin1, alters protein interactions and phosphorylation of Beclin1, and thereby promotes autophagic pathways (Chapter 5).

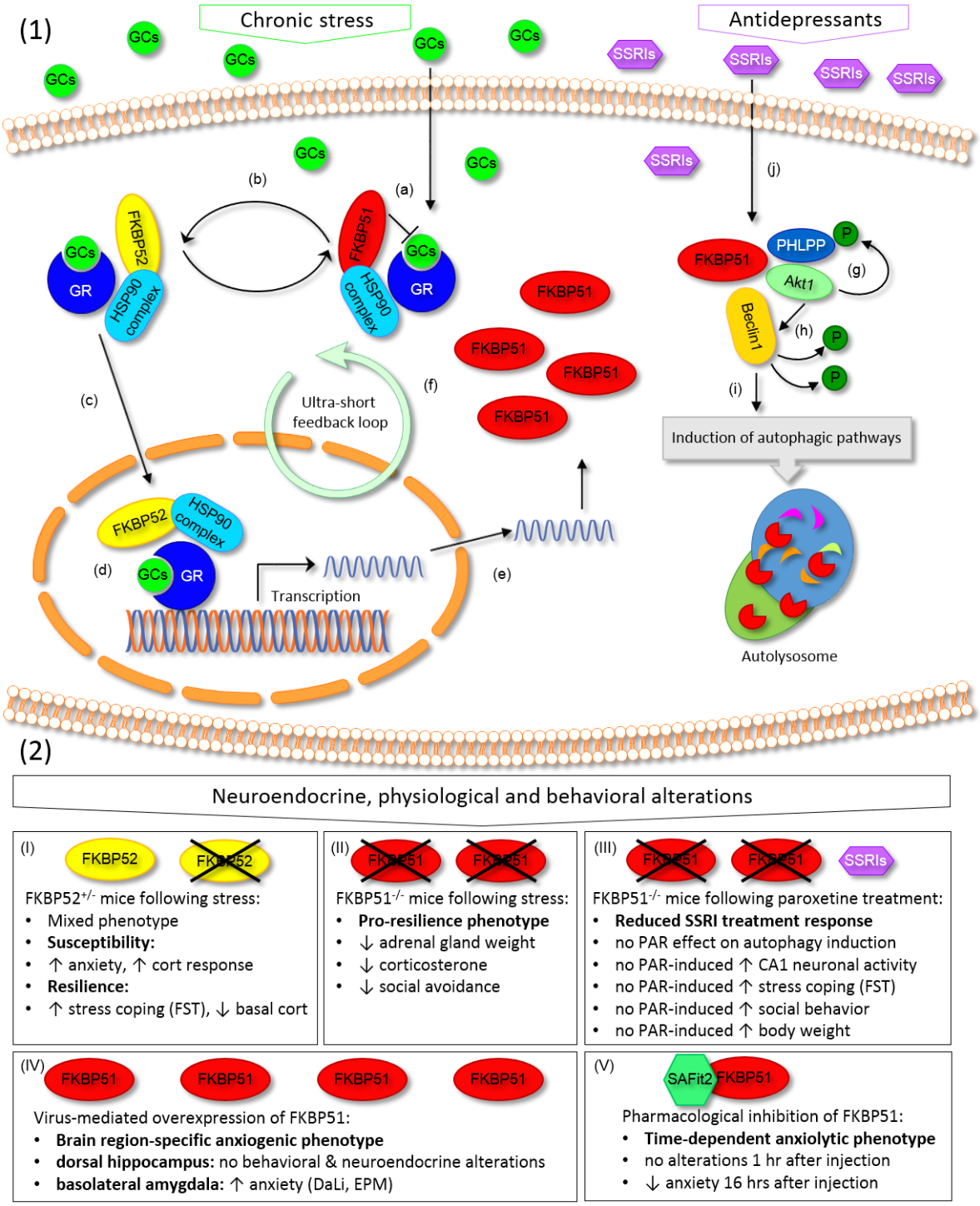


Figure 3.1: Schematic overview of major FKBP51 related pathways and their impact on different genetic and pharmacological outcomes. (1) FKBP51 is involved in both, GR signaling and autophagic pathways. (a) Following stress, circulating corticosterone binds to GR, but FKBP51 (which is important for GR maturation) lowers the receptor's affinity for the stress hormone. (b) Upon corticosterone binding, a

switch from FKBP51 to FKBP52 promotes the translocation of the GR-hsp90 machinery into the nucleus (c) and subsequent DNA binding (d). FKBP51 itself is a GR target-gene and forms and ultra-short feedback loop, leading to increased FKBP51 transcription and translation (e), following GR activation. (f) Increased FKBP51 protein results in higher GR resistance, completing the ultra-short negative feedback loop on GR sensitivity. FKBP51 also interacts with PHLPP, Akt and Beclin1. (g) PHLPP de-phosphorylates Akt in Akt1. (h) Inactive Akt is recruited to Beclin 1, which results in lower phosphorylation of Beclin1, and thus (i) leading to the induction of autophagic pathways. (j) SSRIs act on the same pathways in an FKBP51-dependent manner and change FKBP51-protein interactions. (2) Activation of both FKBP51-dependent mechanisms result in various physiological, neuroendocrine and behavioral alterations depending on the genetic and pharmacological manipulation. (I) $FKBP52^{+/-}$ mice show a mixed phenotype of stress susceptibility and resilience. (II) Following stress, $FKBP51^{-/-}$ mice demonstrate a more resilient phenotype. (III) Independent of condition, lack of FKBP51 results in lower SSRI treatment response. (IV) FKBP51 overexpression in the hippocampus has no major effects, but overexpression in the BLA leads to an anxiogenic phenotype. (V) Pharmacological inhibition of FKBP51 results in a time-dependent anxiolytic phenotype.

In line with our findings from chronic paroxetine treatment, we found that FKBP51 is not only required for the acute effects of the antidepressant paroxetine on behavior and synaptic function, but also for the effect on Beclin1 (see Figure 3.1). In human blood cells of healthy subjects, FKBP51 levels correlate with the potential of antidepressants to induce autophagic pathways. Further, data from patients suffering from depression show that the levels of both FKBP51 and Beclin1 in peripheral blood cells at admission correlate with the later antidepressant treatment outcome. Intriguingly, the antidepressant response of autophagic markers in *ex vivo* cultivated blood cells from inpatients predicts the clinical antidepressant response. Thus, we were able to unravel the first molecular correlate for the genetic association of FKBP51 with clinical antidepressant response. Moreover, the new molecular functions of FKBP51 are of high physiological and clinical relevance as they could serve as novel predictive markers.

4.3. Implications for novel treatment options

Depression is a devastating psychiatric disorder that raises the need for elaborated pharmacological interventions. Although, currently available antidepressants show an overall favorable benefit/risk balance, pharmacotherapy still suffers from modest efficacy, because less than 50% of patients achieve full remission following treatment, indicating a large inter-individual variability in treatment efficacy (Geddes and Miklowitz, 2013). Thus, the need for a personalized medicine in psychiatric disorders is stronger than ever. At its

root, personalized medicine is based on the assumption that an individual's unique characteristics play a central role in both disease susceptibility and in response to specific therapies. Such characteristics include, among others, genetic variants and epigenetic modifications, environmental factors and the individual's life history as well as observable biomarker changes. Thus, the major goal of personalized medicine is to predict the individuals disease vulnerability, achieve an accurate diagnosis, and optimize the most efficient and favorable response to treatment (Myers and Nemeroff, 2010; Ozomaro et al., 2013).

In recent years, FKBP51 has emerged as a promising new drug target for the development of mechanistically novel antidepressants. Our findings provide substantial evidence to the concept of FKBP51 as an important factor governing gene-environment interactions relevant for the etiology of psychiatric disorders and further promote the idea of FKBP51 antagonists as novel treatment strategy in psychiatric disorders. FKBP51 inhibition could be most effective in depressed patients who suffer from an HPA axis insensitivity, as enhancement of the negative feedback of the HPA axis could accelerate remission, which is another major limitation of all known antidepressant treatments (Schmidt et al., 2012).

The poor effectiveness of commonly used antidepressants, such as SSRIs, is also partially accounted for by their still largely unknown molecular mechanisms that are responsible for their clinical potency. In the search for antidepressants' mechanisms of action beyond their influence on monoaminergic neurotransmission, antidepressant drugs have been shown to stimulate autophagic pathways (Zschocke et al., 2011). We now found that FKBP51 is involved in priming autophagic pathways, which are required for full antidepressant potency. Thus, the newly discovered functions of FKBP51 also provide novel predictive markers for treatment outcome, highlighting their physiological and clinical relevance in personalized medicine.

It is important to note that FKBP51 is a multi-domain and multi-functional protein. Some of its functions such as the involvement in autophagic initiation are mediated by other domains than the PPIase pocket, in which novel FKBP51 ligands bind. Therefore, FKBP51-dependent autophagic pathways will most likely not be affected by the novel FKBP51 ligands. Indeed, it has been shown that none of the new class inhibitors of FKBP51 affected AktS473 phosphorylation or downstream targets of Akt (Fabian et al., 2013; Hausch et al., 2013).

Thus, in the clinical situation, treatment with commonly used antidepressants would not necessarily exclude simultaneous pharmacotherapy with novel FKBP51 ligands.

4.4. Summary and future perspectives

This thesis constitutes a compelling basis for the role of FKBP51 in stress-related psychiatric disorders. We thoroughly explored the implication of FKBP51 and its functional counter-player FKBP52 in the context of environmental challenges, and could provide strong evidence that FKBP51 is involved in the neuroendocrine and the behavioral response and recovery to acute and chronic stress. Moreover, we identified and elucidated a novel role of FKBP51 in antidepressant treatment efficacy and investigated its potential as a novel and alternative drug target for psychiatric disorders (see Figure 3.1). Nonetheless, many open questions remain and should be addressed in future studies. It is important to continue with the identification of FKBP51's role in brain regions other than the hippocampus and the amygdala. The PVN could be of special interest, because of its crucial involvement in the negative feedback of the HPA axis and its strong induction of FKBP51 mRNA following stress. Moreover, it is not clear which neuronal populations express FKBP51. The generation of brain region and or neurotransmitter-specific FKBP51 knockout and overexpressing mouse lines could be a useful tool to shed some light on these issues. Another important point includes experiments with chronic treatment of FKBP51 antagonists in combination with chronic stress models, as well as the simultaneous administration of FKBP51 compounds and SSRIs. In order to pursue the path of drug development of FKBP51 ligands, the underlying molecular mechanisms and actions of FKBP51 and its inhibitors need to be fully clarified, to subsequently be considered suitable to enter clinical trials.

Taken together, this thesis will help to broaden the view of FKBP51 in the context of psychiatric diseases from strictly GR-dependent / HPA axis related functions to alternative roles of FKBP51, such as its convergent impact on autophagy and antidepressant action.

5. Addendum

5.1. Glucocorticoid receptor in glutamatergic neurons controls anxiety

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Manuscript in preparation

Abstract

While anxiety disorders constitute a major disease and social burden worldwide, little is known about the underlying molecular mechanisms. Besides the involvement of the major excitatory (glutamate) and inhibitory (GABA) neurotransmitter systems in anxiety, the stress system has been directly implicated in the pathophysiology of these complicated mental disorders. Here, the main receptor for the stress hormone corticosterone, the glucocorticoid receptor (GR), seems to play a central role, but its function in glutamatergic and GABAergic neurons has not been addressed yet. We therefore generated mice with selective deletion of the GR in glutamatergic or GABAergic neurons and investigated their neuroendocrine, behavioral and neurotransmission profile. GR loss in glutamatergic, but not in GABAergic, neurons specifically promoted fear and anxiety, hypothalamic-pituitary-adrenal axis hyperactivity and electrophysiological neuroadaptations, while locomotion and stress-coping behavior remained unaffected. Our data suggests a central and specific role of GR in the major excitatory neurotransmitter system for the regulation of fear and anxiety behaviors and sheds further light on the molecular mechanisms underlying anxiety disorders.

Introduction

Anxiety disorders such as generalized anxiety disorder, panic disorder, obsessive-compulsive disorder and post-traumatic stress disorder, represent the most common psychiatric illnesses, with a lifetime prevalence of approximately 30% in the U.S. (Kessler et al., 2005; Norrholm and Ressler, 2009; Vos et al., 2012).

Dysfunctions of the major excitatory (glutamate) and inhibitory (gamma aminobutric acid, GABA) neurotransmitter circuits have been implicated in anxiety disorders. In particular, an imbalance between these neurotransmitter systems can lead to abnormal excitability of the anxiety-related neuronal network, thereby causing aberrant behavioral responses (Gross and Hen, 2004; Kalueff and Nutt, 2007; Wu et al., 2008; Sanacora et al., 2012; Zorumski et al., 2013).

The major environmental risk factor for anxiety disorders is exposure to traumatic and stressful situations such as threats and social stress. These situations activate the hypothalamic-pituitary-adrenal (HPA) axis, which ultimately leads to the enhanced secretion of glucocorticoids (GCs). GCs alter neuronal activity in brain regions, such as the hippocampus and the basolateral amygdala, which are implicated in attention, vigilance and the determination of appropriate behavioral strategies. This mechanism allows the body to optimally face the stress challenges and adapt to environmental stimuli. GCs act via glucocorticoid receptors (GR) and mineralocorticoid receptors (MR), and proper GR signaling is critical for an appropriate stress response. Dysfunction of the HPA axis and altered GR signaling can trigger the development of psychiatric disorders such as depression and pathological anxiety (de Kloet et al., 2005a; Joëls and Baram, 2009; Joëls, 2011). Molecular and genetic approaches previously implicated the GR in the modulation of stress-related behaviors (Tronche et al., 1999; Wei et al., 2004; Müller and Holsboer, 2006; Wagner et al., 2011; Barik et al., 2013).

Interestingly, the fundamental question whether GR activation in glutamatergic and/or GABAergic neurons is involved in the modulation of anxiety still remains unsolved.

Therefore, we set out to investigate the specific effect of GRs on stress system activity and emotional behavior by generating conditional mouse mutants lacking the receptor in

glutamatergic (*GR*$^{Glu\text{-}CKO}$ mice) or GABAergic neurons (*GR*$^{GABA\text{-}CKO}$ mice). Our models have the unique potential to specifically dissect the role of GR activation in the major excitatory and inhibitory neurotransmitter system of the CNS.

Materials and Methods

Animals and housing

In all experiments male mice were used, aged 2-4 month, singly housed two weeks prior to the experiment under standard conditions (12L:12D light cycle, lights on at 08:00 AM, temperature 23±2°C). Food (Altromin 1324, Altromin GmbH, Germany) and tap water were available *ad libitum*. mEPSC recordings were performed at the University Medical Center Utrecht, Rudolf Magnus Institute of Neuroscience, Utrecht, the Netherlands. All other experiments were carried out in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 2010/63/EU. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

Generation of neurotransmitter-specific GR conditional KO lines

The generation of *GR*-floxed mice was previously described (Tronche et al., 1999). Conditional *GR* mutant mice were obtained by breeding $GR^{flox/flox}$ mice to the respective Cre-expressing mouse lines using a three generation breeding scheme.

For the selective disruption of GR in forebrain glutamatergic neurons, $GR^{flox/flox}$ mice were bred to *Nex-cre* mice (Goebbels et al., 2006). The helix-loop-helix transcription factor Nex (also known as NeuroD6/Math2) is a marker of embryonic cortical neuronal precursors (Wu et al., 2005). In the adult brain Nex is expressed in mature glutamatergic cortical neurons. Accordingly, in *Nex-cre* mice Cre mediated recombination is essentially restricted to glutamatergic neurons of the telencephalon: mitral and periglomerular layers of the olfactory bulb, projection neurons of the cortex, pyramidal neurons of the CA1 and CA3 regions of the cornus ammoni, granule cells of dentate gyrus, glutamatergic mossy cells of the hilus of the dentate gyrus and the cortical-related amygdaloid nuclei (lateral and basolateral amygdala, basomedial amygdala and cortical amygdaloid nucleus) (Goebbels et al., 2006). Resulting $GR^{+/flox}$ *Nex-cre* offspring was bred to $GR^{flox/flox}$ mice and finally the line was maintained by breeding $GR^{flox/flox}$ *Nex-cre (*here called $GR^{Glu\text{-}CKO}$) mice to $GR^{flox/flox}$ (here called $GR^{Glu\text{-}Ctrl}$).

For the selective disruption of GR in forebrain GABAergic neurons $GR^{flox/flox}$ mice were bred to the *Dlx5/Dlx6 (Dlx)-cre* mice. In *Dlx-cre* mice the Cre-recombinase is expressed under the control of the regulatory sequences of the *Dlx5/Dlx6* genes, which are homeobox genes expressed in migrating forebrain GABAergic neurons during development (Stühmer et al., 2002). Indeed, in the adult brain *Dlx-cre* mice show a specific inactivation of floxed alleles in forebrain GABAergic neurons (Monory et al., 2006). $GR^{flox/flox}$ *Dlx-cre* mice (here called $GR^{GABA\text{-}CKO}$) were obtained by breeding $GR^{flox/flox}$ (here called $GR^{GABA\text{-}Ctrl}$) to *Dlx*-cre mice as described above.

Genotyping

Genotyping was performed by PCR using the following primers: GR-flox1, 5´- GGC-ATG-CAC-ATT-ACG-GCC-TTC-T-3´; GR-flox8, 5´-CCT-TCT-CAT-TCC-ATG-TCA-GCA-TGT-3´ and GR-flox4, 5´-GTG-TAG-CAG-CCA-GCT-TAC-AGG-A-3´. Standard PCR conditions resulted in a 225-bp wild-type and a 275-bp floxed PCR product. A premature deletion of the floxed allele would have been identified by the occurrence of a 390-bp PCR product. In *Nex-cre* and *Dlx-cre* mice, the presence of Cre was evaluated using the primers CRE-F 5'-GAT-CGC-TGC-CAG-GAT-ATA-CG-3', CRE-R 5'-AAT-CGC-CAT-CTT-CCA-GCA-G-3', Thy-F 5´-TCT-GAG-TGG-CAA-AGG-ACC-TTA-GG-3´ and Thy-R 5´- CCA-CTG-GTG-AGG-TTG-AGG-3´, resulting in a Cre-specific PCR product of 574 bp and a control PCR product of Thy1 of 372 bp. All mouse lines used were on a mixed 129S2/Sv×C57BL/6J genetic background. All animals used in single experiments were littermates. Experimenters were always blind to genotype.

In situ hybridization

Mice were sacrificed by decapitation in the morning (0830 to 0930 h) following quick anaesthesia by isoflurane. Brains were removed, snap-frozen in isopentane at -40 °C, and stored at -80 °C. Frozen brains were sectioned at -20 °C in a cryostat microtome at 18 µm, thaw mounted on Super Frost Plus slides, dried and stored at -80 °C. In-situ hybridization using ^{35}S UTP labeled ribonucleotide probes (GR, *MR* and *CRH*) was performed as described previously (Schmidt et al., 2007). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/ HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA

probes were transcribed from a linearized plasmid. Tissue sections were saturated with 100 ml of hybridization buffer containing approximately 1.5 x 10^6 cpm ^{35}S labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55 °C. The following day, the sections were rinsed in 2 x SSC (standard saline citrate), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 x SSC for 1 h at 65 °C and dehydrated through increasing concentrations of ethanol. The slides were exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and expression was determined by optical densitometry utilizing the freely available NIH ImageJ software. The mean of four measurements of two different brain slices was calculated for each animal. The data were analyzed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest from the measurements.

Immunohistochemistry

Mice were deeply anesthetized with isoflurane and perfused intracardially with 4% paraformaldehyde. Brains were removed, post-fixed overnight in 4% paraformaldehyde following overnight incubation in 30% sucrose solution at 4 °C, and then stored at -80 °C. Frozen brains were coronally sectioned in a cryostat microtome at 30 µm and kept at -80 °C. Double-labeling immunofluorescence (rabbit anti-GR (M-20): 1:1000, Santa Cruz; Alexafluor) was performed on free-floating sections as described previously (Wang et al., 2011a). Immunohistochemical analysis was carried out by a laser-scanning confocal microscope. Images were acquired simultaneously in two acquisition channels with the FLUOVIEW FV 1000 (version 3.3) acquisition analyzer program.

Sampling procedure

Organ sampling

Mice were sacrificed by decapitation following quick anaesthesia by isoflurane. Brains were removed, snap-frozen in isopentane at -40 °C, and stored at -80 °C for in situ hybridization. Adrenal and thymus glands were removed, dissected from fat and weighed.

Hormone measurements

To determine basal corticosterone and ACTH levels, blood sampling was performed in the early morning (0830 to 0930 h) and afternoon (0430 to 0530 h, only corticosterone) by collecting trunk blood from animals rapidly decapitated under isoflurane anesthesia, with the time from first handling of the animal to completion of bleeding not exceeding 45 sec. For evaluation of the corticosterone response and recovery to stress, we collected blood samples 30 min (response levels) and 90 min (recovery levels) after an acute stressor (a forced swim test) by tail cut (Fluttert et al., 2000). Stress experiments were performed in the morning (08:30–10:00 am). All blood samples were gathered in labeled 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany) and kept on ice until centrifugation at 4 °C and 8000 rpm for 15 min. After centrifugation, the blood plasma was transferred to a new, labeled 1.5 ml microcentrifuge tube. All plasma samples were stored frozen at -20 °C until the determination of corticosterone or ACTH by commercially available RIA kits (MP Biomedicals Inc).

Behavioral testing

All behavioral tests were recorded using a videotracking system (Anymaze 4.20; Stoelting). The following behavioral tests were performed between 0830 and 1230 h in the same room in which the mice were housed: open field (OF), elevated plus maze (EPM), dark-light box (DaLi), forced swim test (FST), fear conditioning and extinction. The testing procedures were performed as described below.

Open field

The open-field test was performed to investigate general locomotor activity. Testing was carried out in an empty open-field arena (50 cm x 50 cm x 50 cm) made of gray polyvinyl chloride (PVC), under low light conditions (5 Lux). The low illumination of the open field arena was chosen to specifically investigate locomotion behavior and not create an aversive center region that may induce anxiety-related behavior. Testing time was 30 min and the distance travelled was recorded.

Elevated plus maze

The EPM was conducted to assess changes in anxiety-related behavior. The device consisted of a plus-shaped platform with two opposing open arms (30 cm x 5 cm x 0.5 cm) and two opposing enclosed arms (30 cm x 5 cm x 15 cm), made of gray PVC, which were connected by a central area (5 cm x 5 cm). The whole device was elevated 50 cm above the floor, illumination was 5 Lux. Testing duration was 10 min. Mice were placed into the center zone facing one of the enclosed arms at the start of the test. The time spent in the open arms and the number of open arm entries were analyzed.

Dark-Light box

The DaLi was used as another test to measure anxiety-related behavior. The apparatus consisted of a rectangular box with two compartments, the dark compartment (15 cm x 20 cm x 25 cm), with <10 lux and the light compartment (30 cm x 20 cm x 25 cm), lit with 700 lux. Both compartments were connected by a 4 cm long tunnel. At the beginning of the test, each mouse was placed in the center of the dark compartment facing the back wall of the apparatus. Testing duration was 5 min. The time spent in the lit compartment, as well as the distance travelled in the lit compartment were analyzed.

Forced swim test

The FST was used to assess stress-coping behavior. Each mouse was put into the a 2 l glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 6 min. Time spent immobile (floating) and time spent struggling was scored by an experienced observer, blind to genotype of the animals.

Fear conditioning and extinction procedures

Fear conditioning: For conditioning, mice were placed into a conditioning chamber (Bioseb, France) with a cubic-like shape and a metal grid floor for shock application, and the chamber light was switched on. Three minutes later, a 20 s sine wave tone (80 dB, 9 kHz) was presented that co-terminated with a 2 s scrambled electric footshock of 0.7mA. Animals remained in the shock context for additional 60 s before they were returned to their home

cages. After each trial, the chamber was cleaned with 70% EtOH and the bedding (wood shavings) placed underneath the grid was changed.

Fear extinction: To measure the freezing response to the tone without confounding influences of contextual memory, conditioned mice were tested in a neutral environment 1 day (Ext. d1), 2 days (Ext. d2) and 7 days (Ext. d7) after conditioning, adapted from Plendl and Wotjak (Plendl and Wotjak, 2010). Briefly, mice were placed in the test context, which differed from the conditioning context in material, shape, surface texture, and odor of the cleaning solution (1% CH_3COOH). After an initial 3 min of habituation (lights on), mice were confronted either with 30 x 20 s tones (9 kHz, 80 dB, Ext. d1) or with 10 x 20 s tones of the same characteristics (Ext. d2 and Ext. d7) presented with variable tone intervals ranging from 20 to 35 s. Mice returned to their home cage 60 s after the end of the exposure protocol.

For a detailed overview of the conditioning and extinction protocols see supplemental table 1. Stimuli (Light, tone and shock) for conditioning and extinction sessions were operated with commercially available software (Packwin V2.0.01; Panlab, Spain). Freezing responses over the course of tone presentations were averaged in tone bins of 5 tones and normalized to the length of the analysis interval (5 tone bins of Ext. d1, 2 tone bins each of Ext. d2 and Ext. d7). The first tone of extinction day1 was excluded, because mice displayed escape behavior during this tone, attempting to jump out of the chamber (Riddle et al., 2013).

mEPSC recordings

Animals (12-16 weeks old) were decapitated under rest in the morning. The brain was removed from the skull and chilled (4 °C) in artificial cerebrospinal fluid (aCSF) containging 120 mmol/L NaCl, 3.5 mmol/L KCl, 5.0 mmol/L $MgSO_4$, 1.25 mmol/L NaH_2PO_4, 0.2 mmol/L $CaCl_2$, 10 mmol/L $_{D\text{-glucose}}$, and 25.0 mmol/L $NaHCO_3$ (gassed with 95% O_2 and 5% CO_2). Coronal slices (350 µm thick) containing the BLA were prepared with a vibrosclicer (Leica VT 1000S). Slices were stored at room temperature until use in recording aCSF containing 120 mmol/L NaCl, 3.5 mmol/L KCl, 1.3 mmol/L $MgSO_4$, 1.25 mmol/L NaH_2PO_4, 2.5 mmol/L $CaCl_2$, 10 mmol/L $_D$-glucose, and 25.0 mmol/L $NaHCO_3$.

One slice at a time was placed in a recording chamber mounted on an upright microscope (Axioskop 2 FS plus; Zeiss) with differential interference contrast, water-immersion objective (63x), and 10x ocular. The slices were continuously perfused with aCSF (flowrate 2-3 mL/min,, temperature 32 °C, pH 7.4) consisting of 120 mM NaCl, 3.5 mM KCl, 1.3 mM$MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.25 mM KH_2PO_4, and 10 mM $_D$-glucose; bicuculline methylchloride (20 µm; Tocris) and tetrodotoxin (o.5 µm; Latoxan) were added to block $GABA_A$ receptor-mediated signals and action potentials, respectively. For mEPSC recordings, the intracellular pipette solution contained 120 mM Cs methane sulphonate, 17.5 mM CsCl, 10 mM Hepes, 5 mM BAPTA, 2 mM MgATP, and 0.1 mM Na GTP (295 mOsm, pH 7.4 adjusted with CsOH). BAPTA was obtained from Molecular Probes; all other chemicals were purchased from Sigma. Neurons in the BLA were selected for recording if they displayed a pyramidal- shaped cell body. All mEPSCs were recorded with a holding potential of −70 mV. If the neuron under study displayed stable mEPSC properties during baseline recording (at least 10 min), corticosteroids were applied for ≈10 min via the perfusion medium. In one set of cells, we compared cells 1 h after a 10 min application of corticosterone or vehicle, as described previously (Liebmann et al., 2008). Corticosterone, was prepared weekly in a stock solution (1 mM in 95% ethanol) and diluted to its final concentration in aCSF just before application. Corticosterone was dissolved directly in aCSF and freshly prepared on the day of the experiment. Series resistance compensation was >70% in all recordings. Series resistance and capacitance were monitored during the whole recording. Responses were filtered at 5 kHz and digitized at 10 kHz (Digidata 1322A; Axon Instruments). All data were acquired, stored, and analyzed on a PC using pClamp 9.0 and Clampfit 9.2 (Axon Instruments). Minimal cutoff for mEPSC analysis was 6 pA.

Statistical Analysis

The data presented are shown as means +SEM and were analyzed by the commercially available software SPSS 17.0 and Sigma Plot 11.0. When two groups were compared, the unpaired student's *t*-test was applied. If data were not normally distributed the non-parametric Mann-Whitney test was used. Comparisons between mEPSC properties determined during the final 5 min of baseline recording and the final 5 min of recording in the presence of corticosterone in the same cells, were analyzed by one-tailed paired *t*-test.

The courses of locomotor activity in the OF and the freezing responses during fear extinction were analyzed by repeated measure analysis of variance (ANOVA). P values of less than 0.05 were considered significant.

Results

GRs are largely expressed in glutamatergic neurons of the limbic system

In situ hybridization demonstrated that lack of GR mRNA in $GR^{Glu\text{-}CKO}$ mice was most prominent in limbic regions such as the basolateral amygdala (BLA), PVN and dorsal and ventral hippocampus, but also in other regions such as cortex and piriform cortex. Deletion of GR in GABAergic neurons was mainly observed in caudate putamen, but also the central amygdala (Fig. 1 and suppl. Fig. 1).

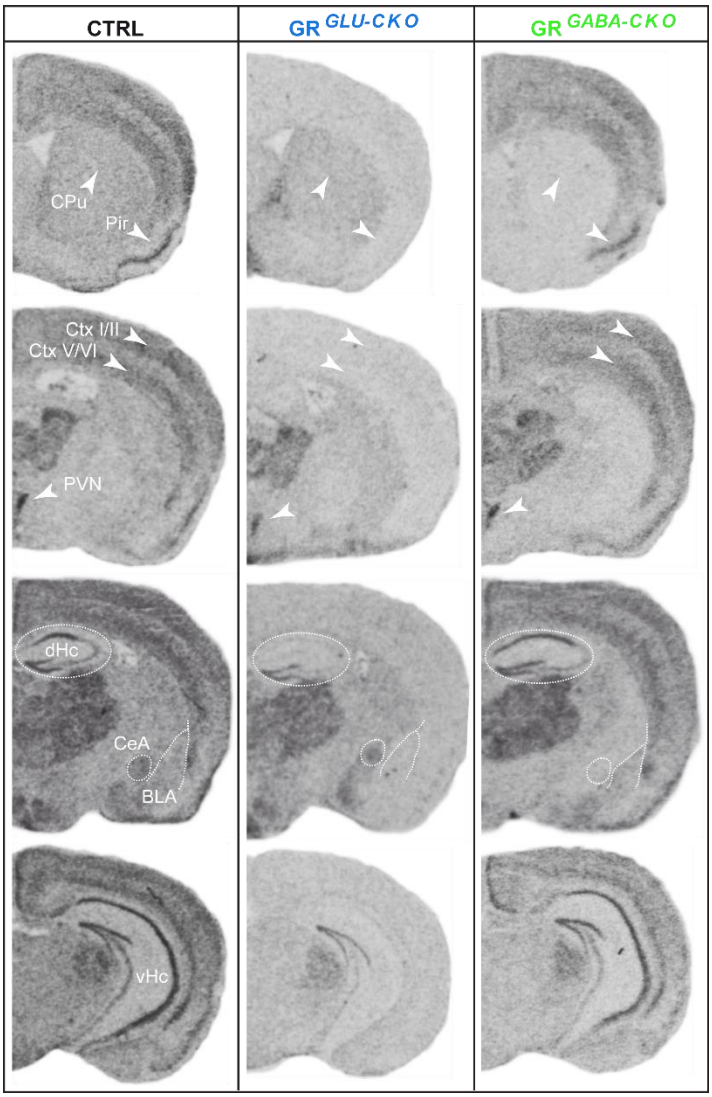


Figure 1: Neurotransmitter-specific GR-CKO lines lack GR expression in a cell type-specific manner. Expression of *GR* mRNA was assessed by ISH in wild-type and neurotransmitter-specific GR-CKO lines. Autoradiographs of GR mRNA expression pattern in brain sections of wild-type, $GR^{Glu\text{-}CKO}$ and $GR^{GABA\text{-}CKO}$ mice. Areas of interest are highlighted with arrowheads and dashed lines. CPu, caudate putamen; Pir, piriform cortex; Ctx, cortex; PVN, paraventricular nucleus; dHc, dorsal hippocampus, Coronal sections of control and mutant mice were stained for GR and DAPI. Amy, amygdala, CeA, central amygdala, BLA,

basolateral amygdala; PVN, paraventricular nucleus; dHc, dorsal hippocampus; CeA, central amygdala; BLA, basolateral amygdala; vHc, ventral hippocampus.

This expression pattern was entirely recapitulated by applying immunohistochemistry using antibodies against GR (Fig. 2 and suppl. Fig. 1) in $GR^{Glu\text{-}CKO}$ and $GR^{GABA\text{-}CKO}$ mice.

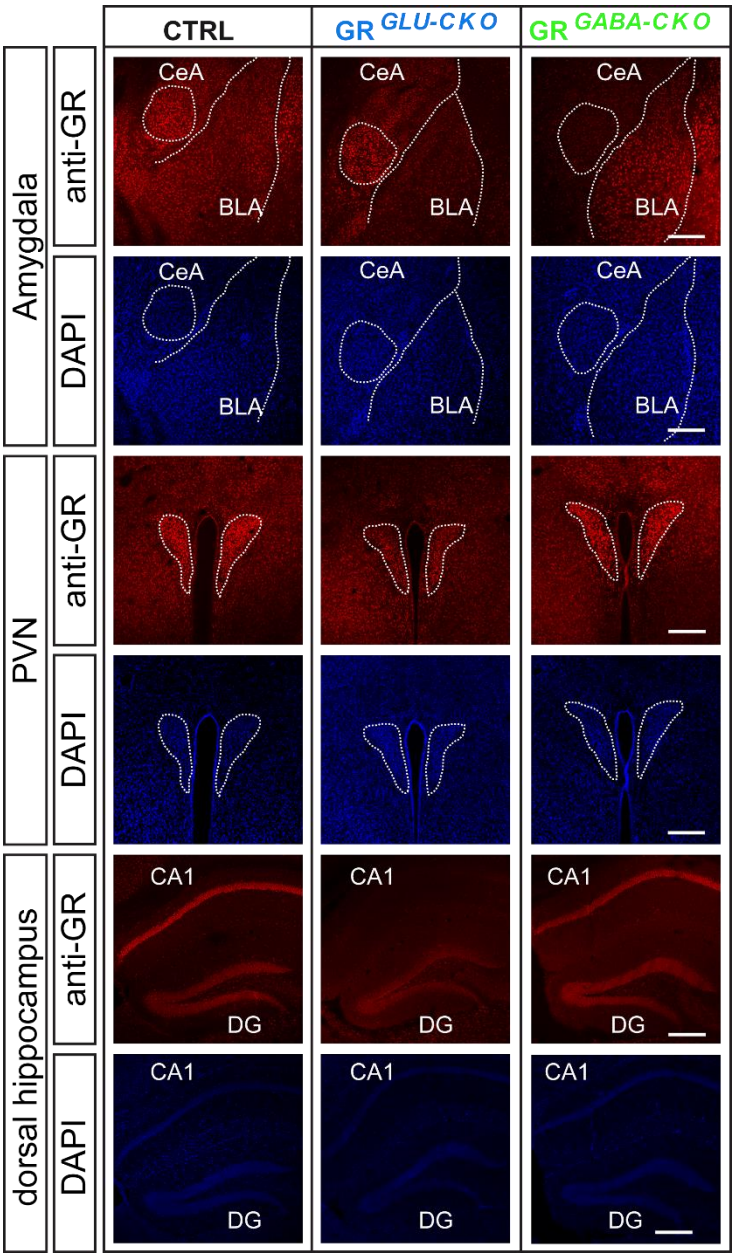


Figure 2: Coronal sections of control and mutant mice were stained for GR and DAPI. Amy, amygdala, CeA, central amygdala, BLA, basolateral amygdala; PVN, paraventricular nucleus; dHc, dorsal hippocampus; magnification x10. Scale bar, 250 µm. GR deletion in $GR^{Glu\text{-}CKO}$ mice is most prominent in limbic structures, such as the BLA, PVN and hippocampus, whereas lack of GR in $GR^{GABA\text{-}CKO}$ mice was mainly observed in the CeA.

Absence of GRs in glutamatergic neurons results in HPA axis hyperactivity

One of the most robust biological abnormalities observed in many depressed patients is an impaired negative feedback regulation of the HPA axis and thus, to partial glucocorticoid resistance (Holsboer, 2000; Pariante and Miller, 2001). The expression pattern of GR in the glutamatergic system revealed substantial overlap with key players and regulators of the HPA axis and the limbic system, such as the PVN, the hippocampus and the BLA. We therefore studied whether neurotransmitter-specific deletion of GR in gluatmatergic or GABAergic pathways would have an effect on HPA axis regulation.

We found that basal am and pm corticosterone levels were significantly increased in $GR^{Glu\text{-}CKO}$ compared to $GR^{Glu\text{-}Ctrl}$ mice (Fig. 3A and B). Moreover, response levels taken 30 min after an acute stressor showed significantly increased corticosterone levels in $GR^{Glu\text{-}CKO}$, while recovery levels assessed 90 min after the FST did not differ between the genotypes (Fig. 3C and D). Interestingly, we did not observe any changes in corticosterone levels in the $GR^{GABA\text{-}CKO}$ mouse line (Fig. 3E-H). We then checked for adrenal and thymus gland weight, as alterations in these organs are often associated with HPA axis activity. Indeed, adrenal gland weight was significantly increased and thymus weight was significantly decreased in $GR^{Glu\text{-}CKO}$ mice compared to controls (Fig. 3I and J), while there were no changes in $GR^{GABA\text{-}CKO}$ mice (Suppl. Fig. 2A and B).

To discriminate whether the increased corticosterone levels in $GR^{Glu\text{-}CKO}$ mice are resulting from peripheral effects due the increased adrenal gland weight or whether the adrenal gland weight is the consequence of central effects, we assessed ACTH levels in the $GR^{Glu\text{-}CKO}$ mouse line. $GR^{Glu\text{-}CKO}$ mice showed significantly increased ACTH levels compared to $GR^{Glu\text{-}Ctrl}$ mice (Fig. 3K), with no differences in the $GR^{GABA\text{-}CKO}$ mouse line (Suppl. Fig. 2C). Along these lines, we detected significantly increased CRH mRNA levels in the PVN of $GR^{Glu\text{-}CKO}$ mice (Fig. 3L).

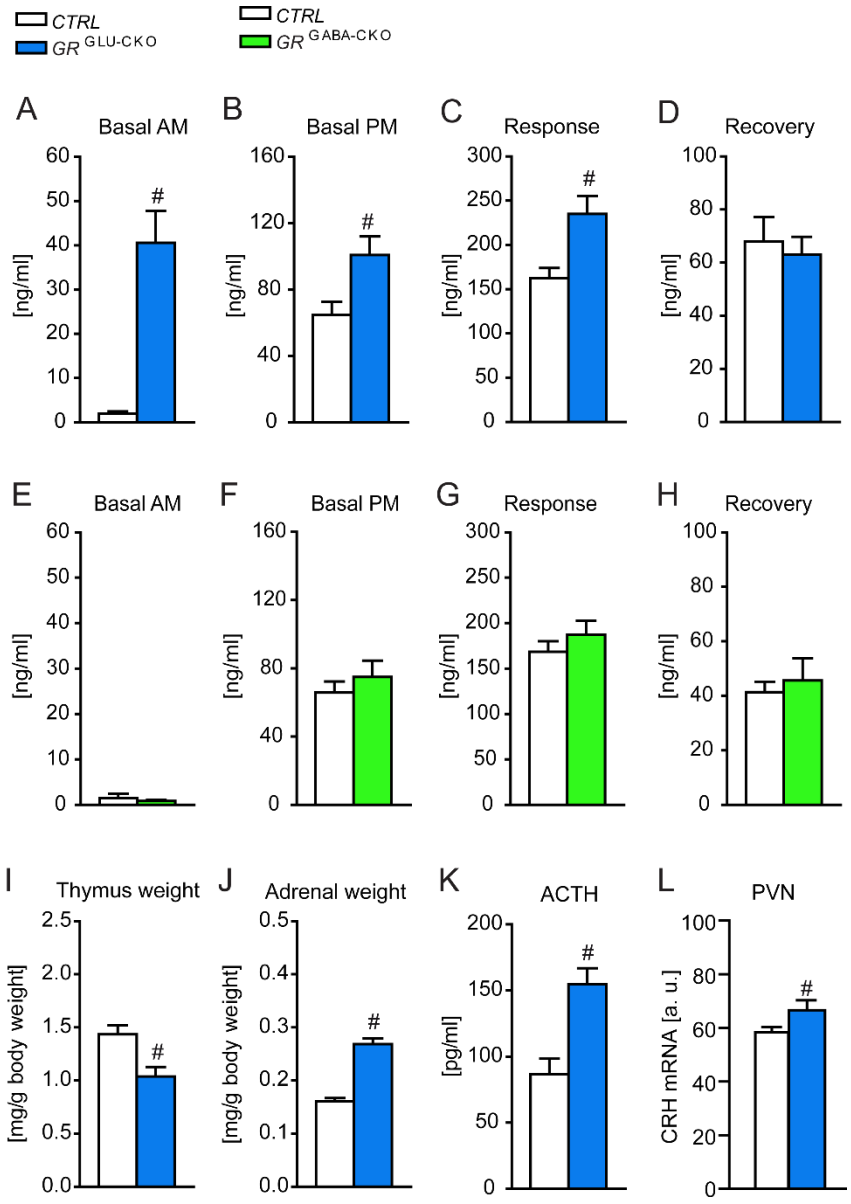


Figure 3: GR signaling in glutamatergic neurons is necessary for appropriate HPA axis activity, while GR activity in GABAergic neurons is dispensable for such parameters. A and B, Basal am and pm corticosterone levels were increased in $GR^{Glu\text{-}CKO}$ mice (am: MW-test, T = 153.00, $p < 0.001$, ctrl n = 12, $GR^{Glu\text{-}CKO}$ n = 9; pm: t-test, $T_{21} = -2.697$, $p < 0.05$, ctrl n = 12, $GR^{Glu\text{-}CKO}$ n = 11). C, Corticosterone response levels, assessed 30 min after an acute stressor, were increased in $GR^{Glu\text{-}CKO}$ mice (t-test, $T_{19} = -3.309$, $p < 0.05$, ctrl n = 12, $GR^{Glu\text{-}CKO}$ n = 9). D, Recovery levels, assessed 90 min after the stressor, did not differ in the $GR^{Glu\text{-}CKO}$ mouse line. E-H, Basal am and pm, as well as response and recovery corticosterone levels did not differ between $GR^{GABA\text{-}CKO}$ and control littermates (n = 10-12 per group). I, Thymus weight was decreased in $GR^{Glu\text{-}CKO}$ mice (t-test, $T_{19} = 3.190$, $p < 0.01$, ctrl n = 12, $GR^{Glu\text{-}CKO}$ n = 9). J, Adrenal gland weight was increased in $GR^{Glu\text{-}CKO}$ mice (t-test, $T_{19} = -9.484$, $p < 0.01$, ctrl n = 12, $GR^{Glu\text{-}CKO}$ n = 9). K, ACTH levels were increased in $GR^{Glu\text{-}CKO}$ mice (MW-test, T = 171.00, $p < 0.001$, ctrl n = 12, $GR^{Glu\text{-}CKO}$ n = 9). L, CRH mRNA levels were increased in $GR^{Glu\text{-}CKO}$ mice (t-test, $T_{17} = -2.226$, $p < 0.05$, ctrl n = 11, $GR^{Glu\text{-}CKO}$ n = 8). # $p < 0.05$, data are expressed as mean + SEM.

Glucocorticoid receptor activity in glutamatergic neurons controls anxiety

Malfunction of the HPA axis and altered GR signaling have been shown to be crucially involved in the development of psychiatric disorders characterized by pathological anxiety. In addition, imbalance of the glutamatergic and GABAergic neurotransmitter systems can critically add to aberrant behavioral responses. To functionally dissect the glutamatergic and GABAergic subpopulations of neurons that may mediate such effects of GR signaling on emotional behavior, $GR^{Glu\text{-}CKO}$ and $GR^{GABA\text{-}CKO}$ mice were subjected to a series of tests, to assess anxiety-related behavior and fear memory, stress-coping and locomotion.

In the elevated plus-maze test, $GR^{Glu\text{-}CKO}$ mice showed reduced anxiety-like behavior as compared to control littermates, which is depicted in increased open arm time entries (Fig. 4A and B). The low-anxiety phenotype of $GR^{Glu\text{-}CKO}$ mice was further confirmed in the dark-light box test, as $GR^{Glu\text{-}CKO}$ mice spent more time in the lit compartment and showed an increased distance travelled in the lit compartment compared to $GR^{Glu\text{-}Ctrl}$ *mice* (Fig. 4C and D). The anxiolytic phenotype was independent of general locomotion, as total distance traveled did not differ between the two genotypes in the open field test (Suppl. Fig. 3A and B). Moreover, stress-coping behavior in the forced swim test was not altered in $GR^{Glu\text{-}CKO}$ mice (Suppl. Fig. 3C and D). No changes in anxiety-related and stress-coping behavior were observed in $GR^{GABA\text{-}CKO}$ mice (Fig. 4E-H; Suppl. Fig. 3E and F); only general locomotion was increased in $GR^{GABA\text{-}CKO}$ mice compared to control littermates in the open field (Suppl. Fig 3G and H).

A key feature of clinical anxiety disorders is a failure to appropriately inhibit, or extinguish, fear (Graham and Milad, 2011). Therefore, we subjected $GR^{Glu\text{-}CKO}$ and $GR^{GABA\text{-}CKO}$ mice to a fear extinction paradigm to investigate their fear responses. $GR^{Glu\text{-}CKO}$ mice demonstrated an enhanced fear extinction compared to $GR^{Glu\text{-}Ctrl}$ mice, as depicted in the total time freezing during the sum of tone presentations of day 1 as well as the time freezing over the course of the 3 extinction sessions (Fig. 4I and J). $GR^{GABA\text{-}CKO}$ mice did not exhibit alterations in fear extinction (Fig. 4K and L).

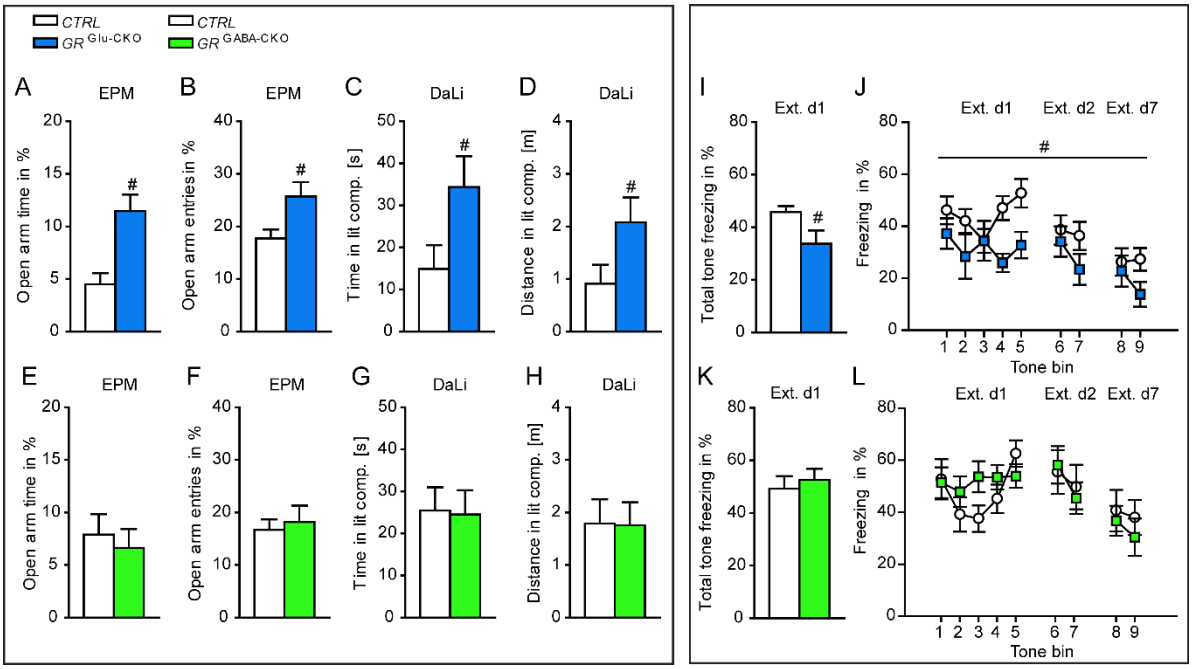


Figure 4: GR in glutamatergic, but not in GABAergic neurons, selectively drives anxiety-related behavior. **A** and **B**, $GR^{Glu\text{-}CKO}$ mice spent more time in (t-test, $T_{17} = -3.823$, $p < 0.001$) and showed more entries into the open arms (t-test, $T_{17} = -2.602$, $p < 0.05$) of the EPM (ctrl n = 11, $GR^{Glu\text{-}CKO}$ n = 8). **C** and **D**, $GR^{Glu\text{-}CKO}$ mice spent more time in (MW-test, $T = 131.00$, $p < 0.05$) and showed an increased distance traveled (MW-test, $T = 128.00$, $p < 0.05$) in the lit compartment of the DaLi box (ctrl n = 12, $GR^{Glu\text{-}CKO}$ n = 9). **E**-**H**, Anxiety-related behavior was not altered in $GR^{GABA\text{-}CKO}$ mice in the EPM and DaLi (n = 11-12 per group). **I** and **J**, $GR^{Glu\text{-}CKO}$ mice showed a reduced freezing response to the tone in a neutral environment during the first day of extinction (t-test, $T_{22} = 2.304$, $p < 0.05$) and during the course of all extinction trials (repeated measures ANOVA, $F_{1, 22} = 4.976$, $p < 0.05$) after conditioning with foot shocks (ctrl n = 13, $GR^{Glu\text{-}CKO}$ n = 12). **K** and **L**, freezing behavior did not differ in in $GR^{GABA\text{-}CKO}$ mice (n = 11-12 per group). # $p < 0.05$, data are expressed as mean + SEM.

GR deletion in glutamatergic neurons prevents metaplasticity of BLA responses to corticosterone

The fact that $GR^{Glu\text{-}CKO}$ mice showed less anxiety-like behavior and lack GR expression in the basolateral amygdala, which is a critical limbic region in the neurobiology of mood disorders, made us question, whether these animals would show any changes of glutamatergic transmission in this structure. Therefore we assessed miniature excitatory postsynaptic currents (mEPSCs) in $GR^{Glu\text{-}CKO}$ mice.

Interestingly, the basal mEPSC frequency of principal neurons of the BLA was significantly increased in coronal slices of $GR^{Glu\text{-}CKO}$ mice compared to slices of $GR^{Glu\text{-}Ctrl}$ mice (Fig. 5A). As expected, cells of control slices responded to corticosterone exposure with an increase in

mEPSC frequency (Fig. 5B). In contrast, BLA neurons of GR$^{Glu\text{-}CKO}$ mice did not respond to the first corticosterone application (Fig. 5D), which was surprising, as the rapid effect of glutamatergic transmission has previously been shown to be mineralocorticoid receptor-dependent (Karst et al., 2010). However, we did not observe alterations in MR mRNA levels in the BLA of GR$^{Glu\text{-}CKO}$ mice (Fig. 5F). On the other hand, we found significantly decreased MR mRNA levels of GR$^{Glu\text{-}CKO}$ mice compared to controls in the ventral hippocampus, which is another critical limbic region in the neurocircuitry of anxiety disorders, projecting to the BLA (Fig. 5G).

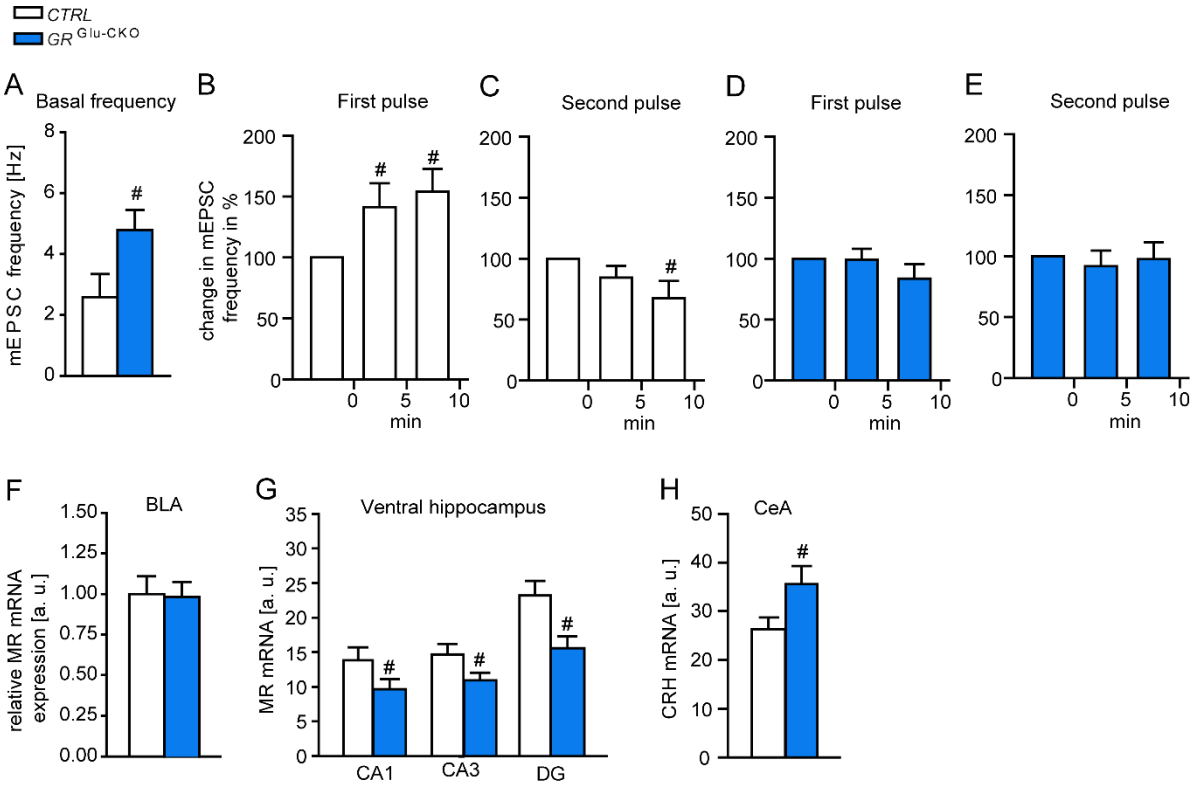


Figure 5: GR signaling in glutamatergic neurons is essential for the metaplasticity of BLA responses to corticosterone. A, GR$^{Glu\text{-}CKO}$ mice showed an increased basal mEPSC frequency (t-test, $T_{14} = -2.192$, $p < 0.05$; ctrl n = 8, GR$^{Glu\text{-}CKO}$ n = 8). **B** and **C**, BLA neurons of controls responded to the first pulse with increased mEPSC frequency ($p < 0.05$, n = 8) and with a decreased mEPSC frequency to a second pulse ($p < 0.05$, n = 6) of corticosterone. **D** and **E**, Metaplasticity of BLA responses to corticosterone was not present in GR$^{Glu\text{-}CKO}$ mice (n = 8). **F**, MR mRNA levels did not differ in the BLA of GR$^{Glu\text{-}CKO}$ mice, but were increased in the CA1, CA3 and dentate gyrus region of the ventral hippocampus (**G**) (t-test, CA1: $T_{15} = 2.616$; $p < 0.05$; CA3: $T_{15} = 3.477$, $p < 0.01$; DG: $T_{15} = 4.284$, $p < 0.001$). **H**, CRH mRNA levels were increased in the central amygdala (CeA) of GR$^{Glu\text{-}CKO}$ mice (t-test: $T_{17} = -2.185$, $p < 0.05$) # $p < 0.05$, data are expressed as mean + SEM.

Renewed exposure of BLA neurons to corticosterone caused a decrease in mEPSC frequency in controls (Fig. 5C). This quick suppression of glutamatergic transmission, which is glucocorticoid receptor dependent (Karst et al., 2010), was absent after the second corticosterone pulse in $GR^{Glu\text{-}CKO}$ mice (Fig 5E). Thus, responses of basolateral amygdala neurons to the stress hormone corticosterone might fail to switch from excitatory to inhibitory transmission, which could be a functional explanation of the anxiolytic phenotype in $GR^{Glu\text{-}CKO}$ mice. In addition, we observed elevated CRH mRNA levels in the CeA of $GR^{Glu\text{-}CKO}$, which might further potentiate the excitatory drive (Fig 5F).

Discussion

In our study, we investigated whether and how GR activation in the major excitatory and inhibitory neurotransmitter systems of the CNS is involved in the modulation of stress system activity and anxiety. Therefore, we generated conditional mouse mutants lacking the GR in glutamatergic or GABAergic neurons. GR deletion in glutamatergic neurons was strongly observed in limbic regions, while GR depletion in GABAergic neurons was mainly apparent in the CPu and the CeA, in line with previous reports on the prevalence of glutamatergic and GABAergic neurons in those regions, respectively (Refojo et al., 2011). We first investigated neuroendocrine parameters in both mouse lines, as altered HPA axis activity can have a major impact on pathogenesis of mood and anxiety disorders. We found that lack of GR in glutamatergic neurons leads to HPA axis hyperactivity, while there was no neuroendocrine effect in $GR^{GABA\text{-}CKO}$ mice. Furthermore, $GR^{Glu\text{-}CKO}$ mice demonstrated an anxiolytic phenotype in several behavioral tasks, while GR deletion in GABAergic neurons did not lead to an overt behavioral outcome. In line with the behavioral observations, we found that metaplasticity of BLA responses to corticosterone is inhibited in $GR^{Glu\text{-}CKO}$ mice. Our results provide substantial evidence that GR signaling in the glutamatergic, but not in the GABAergic neurotransmitter system is crucially involved in the modulation of stress system activity and anxiety.

The glucocorticoid receptor is widely distributed throughout the brain, especially in limbic structures. However, besides the region specificity of GR expression, little is known on which kind of neurons the receptor is expressed. By generating conditional GR knockout mice lacking the receptor either in glutamatergic or GABAergic neurons, we found that GR was present in glutamatergic neurons of the PVN, Hippocampus and BLA and in GABAergic neurons of CPu and the CeA.

Glucocorticoids regulate the activity of the HPA axis through negative feedback via the GR mainly in the PVN, but also at the level of the hippocampus and other limbic structures (De Kloet et al., 1998; Ulrich-Lai and Herman, 2009). Several conditional GR knockout mouse studies further contributed to this knowledge. In particular, conditional deletion of GR in the CNS (GR^{NesCre} mice) resulted in HPA axis hyperactivity, possibly due to GR deletion in the PVN (Tronche et al., 1999). Moreover, disruption of GR limited to adult forebrain neurons

($GR^{CaMKIIα\text{-}Cre}$ mice) led to a mild form of HPA axis hyperactivity (Boyle et al., 2005). More recently, PVN-specific GR knockout mice (*Sim1Cre-GRe3Δ* mice) demonstrated hypercorticosterolemia (Laryea et al., 2013). The deletion pattern in limbic structures such as the PVN in $GR^{Glu\text{-}CKO}$ mice and the increased HPA axis activity, are along these lines. It becomes clear that especially the GR in glutamatergic neurons plays a prominent role in mediating the negative feedback on the HPA axis.

Limbic dysfunction and HPA axis dysregulation, due to altered GR signaling, are key features of affective disorders (Herman et al., 2005). Numerous transgenic mouse models of altered GR expression have demonstrated a role for GR in mediating neuroendocrine responses, but have also highlighted its crucial involvement on emotional behavior (Müller and Holsboer, 2006). Mice with a deletion of the GR in the anterior pituitary ($GR^{POMC\text{-}Cre}$ mice) were less affected by chronic social defeat stress, which was reflected in reduced anxiety-related behavior (Wagner et al., 2011). Moreover, conditional overexpression of GR in the forebrain and the limbic system (GRov mice) led to increased anxiety-like behavior (Wei et al., 2004), while conditional GR deletion in the CNS (GR^{NesCre}) demonstrated reduced anxiety-related behavior (Tronche et al., 1999).

However, while previous studies focused on region specific influences of GR on neuroendocrine, physiological and behavioral responses, we were able to isolate the specific contribution of GR activation in glutamatergic and GABAergic neurons on neuroendocrine and behavioral outcomes. We found that $GR^{Glu\text{-}CKO}$ mice demonstrate an anxiolytic phenotype, which was reflected in decreased anxiety-related behavior in the EPM and DaLi and an enhanced fear extinction. These findings are glutamatergic system specific, as $GR^{GABA\text{-}CKO}$ mice, but also mice lacking the GR in dopamine-releasing neurons ($GR^{Dat\text{-}Cre}$ mice) or in dopaminoceptive neurons ($GR^{D1\text{-}Cre}$ mice) did not show any overt anxiety-related phenotype (Barik et al., 2013). Our data are in line with the proposed abnormalities in glutamatergic and GABAergic neurotransmitter systems in the pathophysiology of psychiatric disorders (Norrholm and Ressler, 2009; Millan et al., 2012; Murrough et al., 2013). In addition, disruption of glutamatergic neurotransmission has been implicated in increased anxiety-like behavior in mice (Wu et al., 2007).

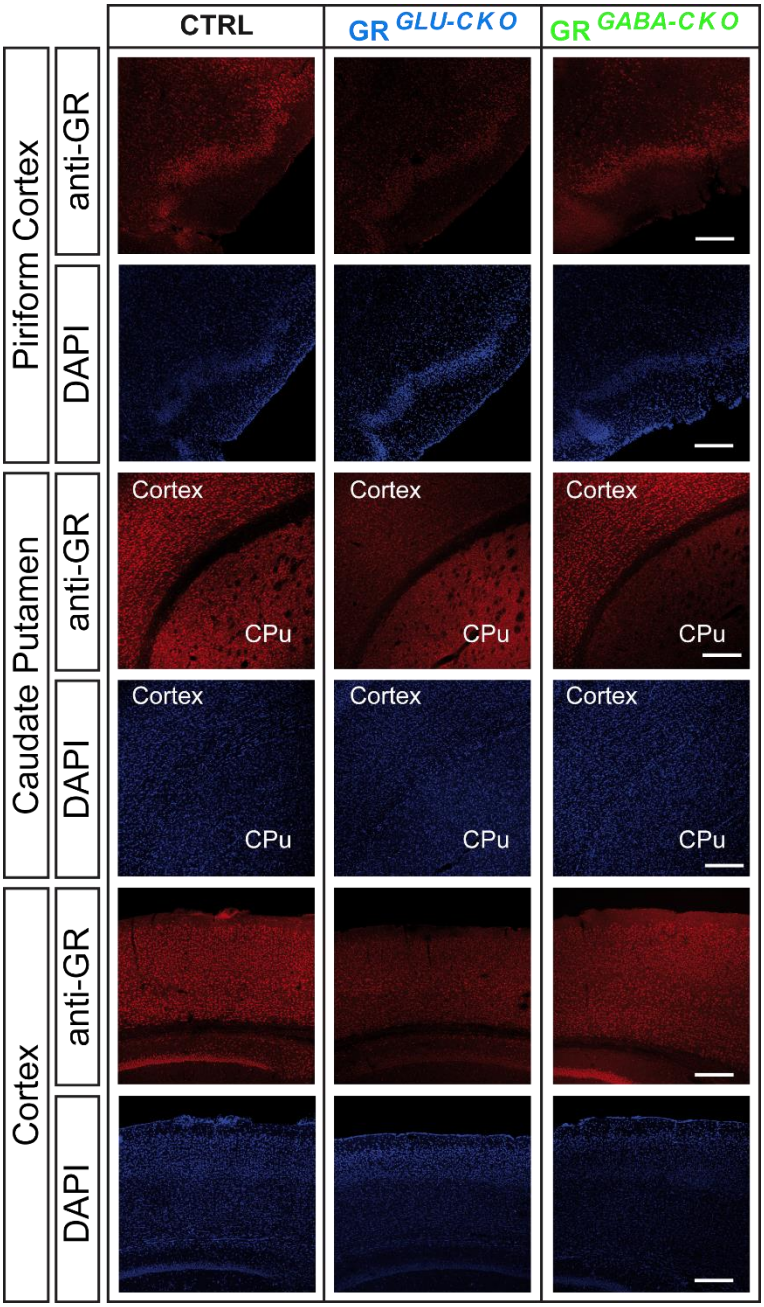
The amygdala is a key player in the neurocircuitry of fear and anxiety disorders (Davis, 1992; LeDoux, 2007; Dias et al., 2013). This brain region is also important in modulating the HPA-

axis through negative feedback mediated by the GR (Gray, 1993; McEwen and Sapolsky, 1995; Herman et al., 2005).

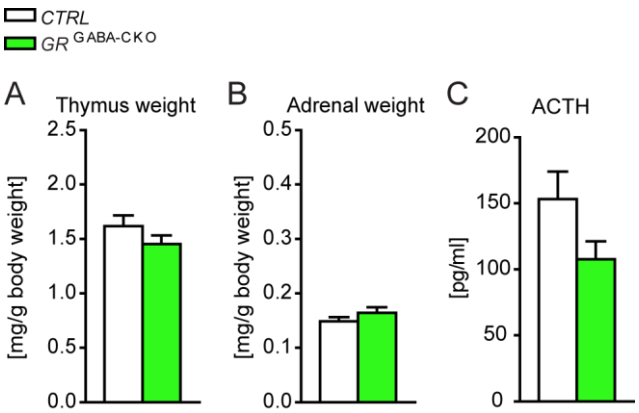
Interestingly, postmortem studies reported lower GR expression in the amygdala of patients with mood disorders (Pérez-Ortiz et al., 2012; Wang et al., 2013a). $GR^{Glu\text{-}CKO}$ mice lack GR expression in the basolateral amygdala and showed less anxiety-like behavior. Therefore we investigated a possible mechanism whereby the lack of GR expression in glutamatergic BLA neurons could lead to a decreased emotional reactivity. We assessed miniature excitatory postsynaptic currents, which reflect the spontaneous release of a glutamate-containing vesicle, in principal neurons of the BLA in $GR^{Glu\text{-}CKO}$ mice. Responses of BLA neurons to corticosterone can switch from excitatory to inhibitory, depending on the recent stress history of the organism (Karst et al., 2010). In particular, high levels of corticosterone quickly and long-lastingly enhance glutamatergic transmission in basolateral amygdala neurons, via nongenomic actions requiring mineralocorticoid receptors, possibly allowing an extended timeframe for encoding of emotional aspects during stressful events. The long-lasting change in state of BLA neurons crucially affects the responsiveness to subsequent pulses of corticosterone, leading to a quick suppression of glutamatergic transmission, which is glucocorticoid receptor-dependent. This metaplasticity of amygdalar responses to corticosterone was completely abolished in $GR^{Glu\text{-}CKO}$ mice. Interestingly, even the rapid effect of glutamatergic transmission, which has been shown to be MR-dependent, was absent in $GR^{Glu\text{-}CKO}$ mice. Although we did not observe any MR mRNA expression changes in the BLA of these mice, we found decreased MR mRNA levels in the ventral hippocampus, another limbic region involved in the neurocircuitry of anxiety, projecting to the amygdala. Thus, the increased basal mEPSC frequency and the suppression of metaplasticity responses to corticosterone in the BLA might functionally explain the anxiolytic phenotype of $GR^{Glu\text{-}CKO}$ mice.

Taken together, our study introduces the glucocorticoid receptor in glutamatergic neurons as a major mediator of fear and anxiety. We also provide evidence that GR signaling in glutamatergic neurons plays a critical role in the regulation of HPA axis activity. These findings underline the importance of GR-dependent glutamatergic pathways in the development of psychopathologies that are related to anxiety.

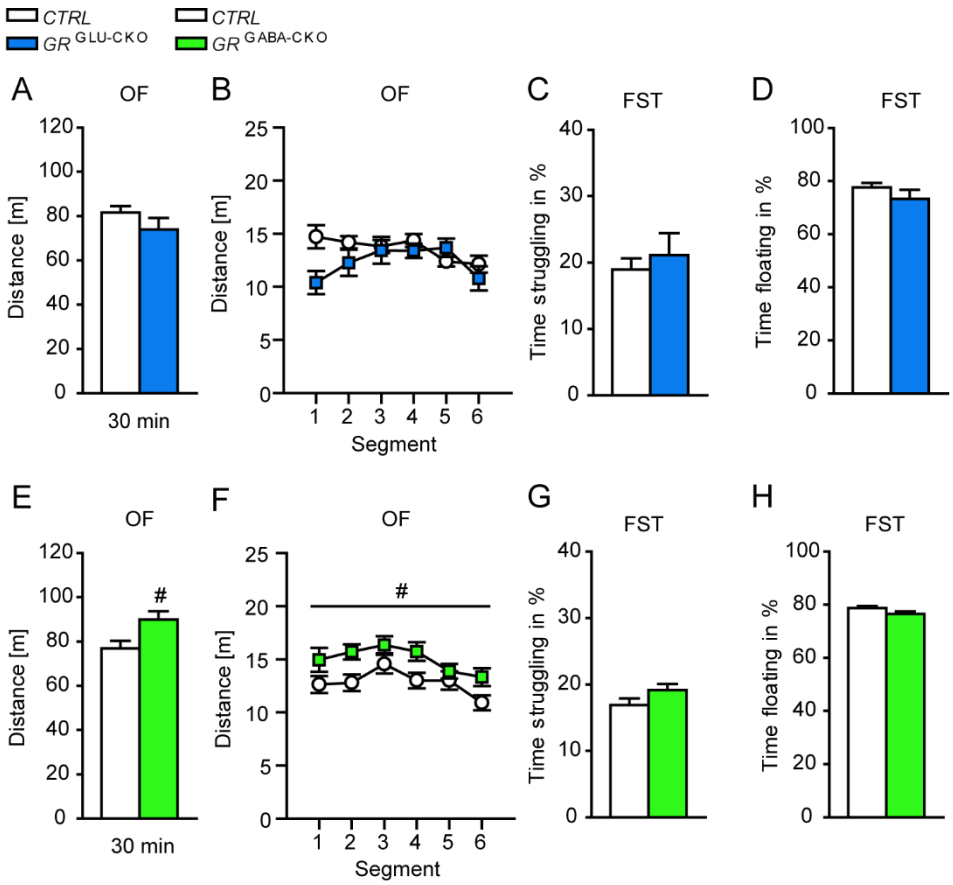
Supplemental Figures



Suppl. Figure 1: Coronal sections of control and mutant mice were stained for GR and DAPI. Piriform cortex, cortex: magnification x10; caudate putamen (CPu): magnification x20; Scale bar, 250 µm.



Suppl. Figure 2: Physiological parameters of GR$^{GABA\text{-}CKO}$ mice. Relative thymus (**A**) and adrenal gland weight (**B**), as well as ACTH level (**C**) did not differ in GR$^{GABA\text{-}CKO}$ mice (n = 11 and 14 per group). Data are expressed as mean + SEM.



Suppl. Figure 3: General locomotion in the open field test (OF) and stress-coping behavior in the forced swim test (FST). Total distance traveled (**A**) and distance traveled divided in 5 min segments (**B**) did not differ in GR$^{GLU\text{-}CKO}$ mice. Time struggling (**C**) and time floating (**D**) were not different in GR$^{GLU\text{-}CKO}$ mice (n = 9-12 per group). (**E** and **F**) GR$^{GABA\text{-}CKO}$ mice traveled significantly more in the OF (30 min: t-test, T_{20} = -2.583, $p < 0.05$; 5 min segments: repeated measures ANOVA, main genotype effect: $F_{1, 20} = 6.671$, $p < 0.05$; ctrl n = 11, GR$^{GABA\text{-}CKO}$ n = 11). (**G** and **H**) No differences in stress-coping behavior were observed in GR$^{GABA\text{-}CKO}$ mice (n = 11-12 per group). # $p < 0.05$, data are expressed as mean + SEM.

Suppl. Table 1. Fear conditioning protocol.

Fear Conditioning 1x20s 0.7mAx2s		
Time (Sec)	**Event**	**Duration (Sec)**
	Lights On	
0	Habituation	180
180	Tone 1	20
198	Shock 1	2
200	Last Interval	60
260	Animal is removed	

Fear Extinction day 1 26x20s V.I. 20-35s		
Time (Sec)	**Event**	**Duration (Sec)**
	Lights On	
0	Habituation	180
180	Tone 1	20
200	Inter-Tone interval	20
220	Tone 2	20
240	Inter-Tone interval	35
275	Tone 3	20
295	Inter-Tone interval	30
325	Tone 4	20
345	Inter-Tone interval	25
370	Tone 5	20
390	Inter-Tone interval	35
425	Tone 6	20
445	Inter-Tone interval	30
475	Tone 7	20
495	Inter-Tone interval	35
530	Tone 8	20
550	Inter-Tone interval	20
570	Tone 9	20
590	Inter-Tone interval	30
620	Tone 10	20
640	Inter-Tone interval	20
660	Tone 11	20
680	Inter-Tone interval	35
715	Tone 12	20
735	Inter-Tone interval	25
760	Tone 13	20
780	Inter-Tone interval	30
810	Tone 14	20
830	Inter-Tone interval	20
850	Tone 15	20
870	Inter-Tone interval	25
895	Tone 16	20
915	Inter-Tone interval	35
950	Tone 17	20
970	Inter-Tone interval	30
1000	Tone 18	20
1020	Inter-Tone interval	20
1040	Tone 19	20
1060	Inter-Tone interval	25
1085	Tone 20	20
1005	Inter-Tone interval	20
1025	Tone 21	20
1045	Inter-Tone interval	25
1070	Tone 22	20
1090	Inter-Tone interval	30
1120	Tone 23	20
1140	Inter-Tone interval	20
1160	Tone 24	20
1180	Inter-Tone interval	35
1215	Tone 25	20
1235	Inter-Tone interval	20
1255	Tone 26	20
1275	Last Interval	60
1335	Animal Removal	

Fear Extinction day 2 10x20s V.I. 20-35s		
Time (Sec)	**Event**	**Duration (Sec)**
	Lights On	
0	Habituation	180
180	Tone 1	20
200	Inter-Tone interval	20
220	Tone 2	20
240	Inter-Tone interval	35
275	Tone 3	20
295	Inter-Tone interval	30
325	Tone 4	20
345	Inter-Tone interval	25
370	Tone 5	20
390	Inter-Tone interval	35
425	Tone 6	20
445	Inter-Tone interval	30
475	Tone 7	20
495	Inter-Tone interval	35
530	Tone 8	20
550	Inter-Tone interval	20
570	Tone 9	20
590	Inter-Tone interval	30
620	Tone 10	20
640	Last Interval	60
700	Animal Removal	

Fear Extinction day 7 10x20s V.I. 20-35s		
Time (Sec)	**Event**	**Duration (Sec)**
	Lights On	
0	Habituation	180
180	Tone 1	20
200	Inter-Tone interval	20
220	Tone 2	20
240	Inter-Tone interval	35
275	Tone 3	20
295	Inter-Tone interval	30
325	Tone 4	20
345	Inter-Tone interval	25
370	Tone 5	20
390	Inter-Tone interval	35
425	Tone 6	20
445	Inter-Tone interval	30
475	Tone 7	20
495	Inter-Tone interval	35
530	Tone 8	20
550	Inter-Tone interval	20
570	Tone 9	20
590	Inter-Tone interval	30
620	Tone 10	20
640	Last Interval	60
700	Animal Removal	

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Acknowledgments

This thesis would not have been possible without the tremendous support from my colleagues. My deepest gratitude goes to my mentor Mathias Schmidt (the Hammer) for this great research project, his supervision, guidance and assistance. I greatly appreciate his scientific advice and his extremely supportive mentoring as well as a very pleasant working atmosphere. I will not forget how much you supported me, especially in the period of my back issues.

I would also like to thank all other reviewers for their willingness to invest their time in the evaluation of my thesis.

My gratitude goes to my long term office partner and lab buddy Klaus Wagner (It's time to leave the island, because the cake is a lie!) as well as Bianca Schmid (I'm still convinced that I don't want Glücksbärchen as background picture of my desktop, but thanks for trying so many times!) and Daniela Harbich (LOST and the Wire forever!). Thanks for your ongoing support and advice and the fun times we had!

Many thanks go to my master student Lianne Hoeijmakers that I was happy to supervise and who largely contributed to the success of my work by writing an excellent piece of work herself.

Furthermore, I would like to thank all former and current lab members that additionally contributed this work, directly or indirectly, by practical help, discussion, advice or feedback: Georgia Balsevich, Carine Dournes, Maurice Kertokarijo, Yuji Kitaichi, Christine Kohl, Christiana Labermaier, Frank Lapidus, Claudia Liebl, Daria Marinescu, Merce Masana-Nadal, Marianne Müller, Jacky Oh, Sara Santarelli, Sebastian Scharf, Yun-Ai Su, Andrés Uribe, Xiao-Dong Wang, Sören Westerholz and Miriam Wolf. Thank you also for a very pleasant working atmosphere and all the enjoyable moments!

I would also like to thank all the contributions made by our collaborators from other research groups and institutes as well as their support in integrating the data into this thesis: Nils Gassen, Theo Rein, Steffen Gaali, Alexander Kirschner, Felix Hausch, Jan Deussing, Henk Karst, Marian Joëls, Sergey Asmus, Novak Bozidar, Joyce Cheung-Flynn, Marc Cox, Matthias Eder, Kathrin Hafner, Nissim Hay, Marcus Ising, Thomas Kirmeier, Stefan Kloiber, Susanne

Lucae, Paul Lucassen, Ania Mederer, Judith Reichel, Randall Sakai, Ulrike Schmidt, David Smith, Jens Stepan, Chadi Touma, Manfred Uhr, Carsten Wotjak, Alexander Yassouridis, Jürgen Zschocke, Elisabeth Binder, Alon Chen and Florian Holsboer.

I am very grateful to you, Nina, for your permanent encouragement, advice and ongoing support as well as the great time we spent together! This thesis would not have been possible without you!

Last but not least, I would like to thank Claudio, my sister and her family and my parents for keeping faith in me and their constant support throughout my life.

Assertion / Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation

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Hartmann J, Wagner KV, Liebl C, Scharf SH, Wang XD, Wolf M, Hausch F, Rein T, Schmidt U, Touma C, Cheung-Flynn J, Cox MB, Smith DF, Holsboer F, Müller MB, Schmidt MV (2012): The involvement of FK506-binding protein 51 (FKBP5) in the behavioral and neuroendocrine effects of chronic social defeat stress. *Neuropharmacology 62:332-339*

- Studiendesign und –planung: JH, in Zusammenarbeit mit MVS
- Durchführung der Experimente: JH, in Zusammenarbeit mit KVW, CL, SHS, XDW, MW
- Datenanalyse: JH
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Hoeijmakers L, Harbich D, Schmid B, Lucassen PJ, Wagner KV, Schmidt MV, Hartmann J (2014): Depletion of FKBP51 in female mice shapes HPA axis activity. *PLOS ONE 9(4):e95796*

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Hartmann J, Wagner KV, Dedic N, Marinescu D, Scharf SH, Wang XD, Deussing JM, Hausch F, Rein T, Schmidt U, Holsboer F, Müller MB, Schmidt MV (2012): Fkbp52 heterozygosity alters behavioral, endocrine and neurogenetic parameters under basal and chronic stress conditions in mice. *Psychoneuroendocrinology 37:2009-2021*

- Studiendesign und –planung: JH, in Zusammenarbeit mit MVS
- Durchführung der Experimente: JH, in Zusammenarbeit mit KVW, ND, DM, SHS, XDW
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Hartmann J, Dedic N, Wagner KV, Harbich D, Schmid B, Deussing JM, Holsboer F, Müller MB, Schmidt MV: FKBP51 critically impacts antidepressant action and chronic stress recovery. *Manuscript in preparation*

- Studiendesign und –planung: JH, in Zusammenarbeit mit MVS
- Durchführung der Experimente: JH, in Zusammenarbeit mit ND, KVW, DH, BS
- Datenanalyse: JH, in Zusammenarbeit mit MVS
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- Studiendesign und –planung der Verhaltensexperimente: JH, in Zusammenarbeit mit MVS
- Durchführung der Verhaltensexperimente: JH, in Zusammenarbeit mit KVW, MVS
- Datenanalyse der Verhaltensexperimente: JH

- Verfassen des Manuskripts: In Zusammenarbeit mit NCG, JZ, MVS, TR

Hartmann J, Wagner KV, Gaali S, Kirschner A, Hoeijmakers L, Westerholz S, Uhr M, Chen A, Holsboer F, Hausch F, Schmidt MV: Pharmacological FKBP51 inhibition reduces anxiety-related behavior in mice. *Manuscript in preparation*

- Studiendesign und –planung der Experimente: JH, in Zusammenarbeit mit MVS
- Durchführung der Experimente: JH, in Zusammenarbeit mit KVW, LH, SW
- Datenanalyse: JH
- Verfassen des Manuskripts: JH, in Zusammenarbeit mit MVS

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- Studiendesign und –planung: JH, in Zusammenarbeit mit MVS, MJ, JMD
- Durchführung der Experimente: JH, in Zusammenarbeit mit HK, SW, ND, KVW, CL, LH, MK
- Datenanalyse: JH, in Zusammenarbeit mit MVS, HK, MJ
- Verfassen des Manuskripts: JH, in Zusammenarbeit mit MVS

I hereby confirm that the dissertation

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is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, Juni 2014

Jakob Hartmann

Hiermit bestätige ich die von Herrn Jakob Hartmann angegebenen Beiträge zu den einzelnen Publikationen.

München, Juni 2014

Mathias V. Schmidt