Exploring the underlying mechanisms mediated by FKBP51, GR and *Tac2* in anxiety-related behaviour

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Clara Engelhardt

Munich, Germany

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Erster Gutachter: PD Dr. Mathias Schmidt

Zweiter Gutachter: Prof. Dr. Christian Leibold

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Abstract

Excessive or chronic stress can lead to maladaptive anxiety. Anxiety disorders are highly prevalent and there is a pressing demand for a more comprehensive understanding of the molecular mechanisms that underlie such disorders. Dysregulation of the stress response and genetic risk factors can contribute towards an increased susceptibility to maladaptive anxiety. The co-chaperone FKBP51 is an immunophilin protein best known as a regulator of the glucocorticoid receptor (GR) and the stress response system. Human and animal studies have shown that aberrant GR signalling as well as genetic variants within the gene encoding FKBP51, FKBP5, can precede stress-related pathology. Notably, the effects of FKBP51 are highly region- and cell type specific. The central extended amygdala, which comprises the central amygdala (CeA) and the bed nucleus of the stria terminalis (BNST), more specifically the oval BNST (ovBNST), is a promising limbic structure of the forebrain that has been repeatedly associated with the modulation of anxiety states. However, the region-specific function of FKBP51 in this nucleus has not been explored yet. Moreover, Tachykinin 2 (*Tac2*) is an emerging neuropeptide that is also expressed in the BNST and highly likely involved in mediating anxiety-like behaviour. In this thesis, we aimed to elucidate molecular mechanisms mediated by the GR, FKBP51 and *Tac2* that underlie stress-induced anxiety states. Initially we addressed whether GR signalling in forebrain neurons contributes to fear and anxiety-related behaviour. Interestingly, we found that forebrain glutamatergic, but not GABAergic, neurons mediate the anxiogenic effects of the GR. Moreover, virally-mediated GR deletion revealed that fear-related behaviour is regulated exclusively by GRs in the glutamatergic neurons of the basolateral amygdala (BLA). We then proceeded to explore whether the lack of FKBP51 would lead to alterations in brain architecture and connectivity in mice. Using in vivo structural magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) we reported two clusters of significantly larger volumes in the hypothalamus, periaqueductal grey (PAG), and dorsal raphe (DR) region of wildtype (WT) animals. DTI measurements, however, highlighted statistically higher fractional anisotropy (FA) values for FKBP51 knockout (51KO) animals in locations including the anterior commissure, fornix, and posterior commissure/superior colliculus region. In order to dissect the role of FKBP51 in anxiety, we characterised the function of FKBP51 in the ovBNST and assessed its impact on HPA axis function and anxiety-related behaviour. Notably, our data suggests that stress-induced increase of FKBP5 in the ovBNST may in fact have a protective role, leading to decreased anxiety and suppression of a future stress-induced HPA axis activation. Finally, we convey a first impression of the function of *Tac2* within the ovBNST and its implication in anxiety-like behaviour. Here we show that *Tac2* is upregulated by acute stress in the ovBNST and that ovBNST *Tac2* positive neurons are involved in anxiety circuitry and behaviour. The collective findings of the

current thesis provide novel evidence for the contribution of GR, *Tac2* and FKBP51 towards the underlying molecular mechanisms of stress-induced anxiety-like states.

Abbreviations

ACT	adrenocorticotrophic hormone
51 KO	FKBP51 knockout
adBNST	anterodorsal bed nucleus of the stria terminalis
AR	androgen receptor
AVP	arginine-vasopressin
BA	basal amygdala
BLA	basolateral amygdala
BM	basomedial amygdala
BNST	bed nucleus of the stria terminalis
CeA	central amygdala
CeL	lateral central amygdala
CeM	medial central amygdala
CNS	central nervous system
CRH	corticotrophin releasing hormone
CRHR1	corticotrophin releasing hormone R1 receptor
CSDS	chronic social defeat stress
Cyp40	cyclophilin 40
DALI	dark light test
Dex	Dexamethasone
DMH	dorsomedial hypothalamus
DR	dorsal raphe
DTI	diffusion tensor imaging
ELS	early life stress
EPM	elevated plus maze
ESARE	enhanced synaptic activity-responsive element
FA	fractional anisotropy
FKBP4	FK506 binding protein 4
FKBP5	FK506 binding protein 5
FKBP51	FK506 binding protein 51
FKBP52	FK506 binding protein 52
fMRI	functional magnetic resonance imaging
GAD	general anxiety disorder
GC	glucocorticoid
GR	glucocorticoid receptor
GREs	glucocorticoid response elements
hop	Hsp70/90 organizing protein
HPA	hypothalamic pituitary adrenal
Hsp70	heat-shock protein 70
Hsp90	heat-shock protein 90
LA	lateral amygdala
LH	lateral hypothalamus
LS	lateral septum
mPFC	medial prefrontal cortex
MR	mineralocorticoid receptor

MRI	magnetic resonance imaging
NK3R	neurokinin 3 receptor
NkA	neurokinin A
NkB	neurokinin B
NPY	neuropeptide Y
ovBNST	oval bed nucleus of the stria terminalis
p23	p23
PACAP	pituitary adenylate cyclase-activating polypeptide
PAG	periaqueductal grey
PB	parabrachial nucleus
PD	panic disorder
PFC	prefrontal cortex
POMC	proopiomelanocortin
PP5	protein phosphatase 5
PPIase	peptidyl-prolyl cis-trans isomerase
PR	progesterone receptor
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
SIS	social isolation stress
SNP	single nucleotide polymorphism
SNS	sympathetic nervous system
SOM	somatostatin
SP	substance P
Tac2	tachykinin 2
TPR	tetratricopeptide repeat
UF	uncinate fasciculus
VTA	ventral tegmental area
WT	wild type

Publications

Chapter 2.1

Hartmann, J., Dedic, N., Pöhlmann, M. L., Häusl, A., Karst, H., Engelhardt, C., Westerholz, S., Wagner, K. V., Labermaier, C., Hoeijmakers, L., Kertokarijo, M., Chen, A., Joëls, M., Deussing, J. M., & Schmidt, M. V. (2017). Forebrain glutamatergic, but not GABAergic, neurons mediate anxiogenic effects of the glucocorticoid receptor. *Molecular Psychiatry*, *22*(3), 466–475. https://doi.org/10.1038/mp.2016.87

Chapter 2.2

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Chapter 2.3

Engelhardt, C., Tang, F., Bordes, J., Brix, L.M., van Doeselaar, L.,Häusl, A.S., Pöhlmann, M.L., Schraut, K.G., Yang, H., Chen, A., Deussing, J., & Schmidt, M.V. (2021). FKBP51 in the ovBNST regulates anxiety-like behaviour. *eNeuro*. Submitted.

Chapter 2.4

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Additional Contributions

Dournes, C., Dine, J., Lopez, J.-P., Brivio, E., Anderzhanova, E., Roeh, S., Engelhardt, C., Kuehne, C., Holzapfel, M., Huettl, R.-E., Stoffel, R., Tietze, L., Eggert, C., Schieven, M., Jakovcevski, M., Deussing, J. M., & Chen, A. (2021). Hypothalamic glucocorticoid receptor in CRF neurons is essential for HPA axis habituation to repeated stressor. *biorxiv*. https://doi.org/10.1101/2020.11.30.402024

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Declaration of Contributions

I hereby certify that I contributed my own work to the current thesis, entitled "Exploring the underlying mechanisms of FKBP51, GR and *Tac2* in anxiety-related behaviour" in the following way:

Chapter 2.1

Designing and Planning the Study –
In collaboration with JH and MVS

Conducting the Experiments –
In collaboration with JH, ND, MP, HK, SW, KW, CL, LH and MK

Analysing the Data –
Independently executed - Figure 5

Preparing the Manuscript –
In collaboration with JH and MVS

Chapter 2.2

Designing and Planning the Study –
In collaboration with MVS and MC

Conducting the Experiments –
In collaboration with MC

Analysing the Data –
In collaboration with BB and MC

Preparing the Manuscript –
In collaboration with MVS and MC

Chapter 2.3

Designing and Planning the Study –
In collaboration with MVS

Conducting the Experiments –
In collaboration with FT, JB, LB, LD, HA, MP, KS, HY

Analysing the Data –
Independently executed

Preparing the Manuscript –
In collaboration with MV

Chapter 2.4

Designing and Planning the Study –
In collaboration with FT and MVS

Conducting the Experiments –
In collaboration with FT, LB, LB, LD and YH

Analysing the Data –
In collaboration with FT

Preparing the Manuscript –
In collaboration with FT and MVS

Munich, 28th April 2021

Clara Engelhardt

Unterschrift

Hiermit bestätige ich die von Frau Clara Engelhardt angegebenen Beiträge zu den einzelnen Publikationen.

München, 16. April 2021

Mathias V. Schmidt

1. Introduction

1.1. Anxiety Disorders

Mental health disorders are complex and associated with immense health care costs and a high burden of disease. Recent estimates from the Global Burden of Disease study reported that an approximate 10.7 % of the world population is affected by a mental health condition, with anxiety disorders taking the lead (James et al., 2018). Anxiety disorders are stress-induced and arise in a number of forms. The *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* divides pathological anxiety into three main categories of disorders, including obsessive-compulsive and related disorders, trauma-and stressor-related disorders, and anxiety disorders (American Psychiatric Association, 2013). Well known derivatives of these categories are phobias, post-traumatic stress disorder (PTSD), and general anxiety disorder (GAD).

While the symptoms and diagnostic criteria for each subset of anxiety disorders are unique, they all involve excessive and persistent fear and anxiety, impairing every day functioning and performance (Craske and Stein, 2016). The prevalence of anxiety disorders across the world varies from 2.5 to 7 percent by country, affecting an estimated 25 million people in the EU alone (James et al. 2018; OECD). Notably, women are twice more likely than men to develop an anxiety disorder, revealing a strong gender bias. Comorbidity among anxiety disorders is common, and they often co-occur with major depression, alcohol and other substance-use disorders, and personality disorders (Craske and Stein, 2016). The economic fallback of this reality results in a huge economic burden, with an estimated 4% of the EU's annual GDP (more than EUR 600 billion) spent on mental health care costs (Health at a Glance: Europe 2020). A combination of pharmacological treatments, particularly selective serotonin-reuptake inhibitors and serotonin-noradrenaline-reuptake inhibitors, and psychological treatments, especially cognitive behavioural therapy, has been shown to be the most effective in treating anxiety disorders. Nevertheless, the need for more accessible mental health interventions and increasingly personalised treatment has significantly heightened the demand for a more comprehensive understanding of such disorders.

1.2. Anxiety and Fear

Anxiety is critical for survival since it functions to signal potential threats or dangers in the environment, and facilitates accompanying coping strategies (Kalin, 2020). It can therefore be defined as a sustained emotional state induced by uncertain or diffuse threats (Ahrens et al., 2018). Pathological or maladaptive anxiety, however, is characterised by anxiety that is overly intense or occurs in situations or contexts that otherwise would not be expected to elicit an anxious reaction. Features of pathological

anxiety include hypervigilance, excessive worry, physiological arousal, and avoidance behaviour. Though distinct, anxiety is strongly intertwined with fear. Fear is the phasic emotional state induced by acute and explicit threats that dissipates quickly once the threat is removed (Davis et al., 2010). Moreover, fear is a rapid behavioural response that leads to active avoidance (e.g. fight or flight) or other automatic responses, such as increased heart rate or freezing/paralysis. Anxiety almost always follows a fearful experience and can in turn influence fear responses to a threat. Moreover, exposure to acute and intense fearful experiences, especially those that are life threatening, often lead to maladaptive anxiety, or anxiety disorders, such as PTSD (Ahrens et al., 2018). Additionally, prolonged exposure to distressing stimuli may eventually cause fear to bridge into anxiety. Early research on fear and anxiety tended towards a theoretical model in which anxiety (sustained) and fear (phasic) are putatively separate processes (Davis, 1998; Barlow, 2002; Sylvers et al., 2011; Robinson et al., 2019). Nevertheless, more recent and nuanced research indicates comparable clinical symptomology of fear and anxiety as well as considerable overlap in physiological substrate, circuitry, and neural mechanisms (Tovote et al., 2015). Evidently, fear and anxiety go hand-in hand, and therefore, it is crucial to take into account the commonalities of these physiological states and brain structures.

1.3. Structures and Circuitry Involved in Mediating Anxiety and Fear

The substrates of fear and anxiety have long been the subject of many research endeavours, leading to a surge of key insights in the past two decades to address one of the most pressing psychiatric concerns to date. Early research focused primarily on the role of particular brain areas in generating fear and anxiety, and the contribution of neuromodulatory and synaptic processes within identified brain areas to these internal states. However, owing to the development of optogenetics, pharmacogenetic tools and improved imaging techniques, the emphasis has shifted towards more causal investigations of specific neural circuit elements and networks.

Fear and anxiety are primitive states, which provide adaptive survival responses to threat (LeDoux, 2000; Porges, 1995; Sylvers et al., 2011). Consequently, the brain structures implicated in mediating fear and anxiety are part of the limbic system, the oldest part of the brain, and conserved across species, which allows researchers to extrapolate provisional biological findings from non-human mammals to humans (MacLean, 1952; MacLean, 1949; Roxo et al., 2011). Although there are species-specific fearful and anxious behaviours, translational research in this area is continuously supported by complementary human studies (Sylvers et al., 2011). Nevertheless, the majority of animal research in this field has been conducted using rodents.

1.3.1. The Amygdala

Early work in lesion studies and fear conditioning in animal models highlighted the key role of the amygdala in fear and anxiety (Blanchard & Blanchard, 1972). Traditionally, the amygdala has long been associated with emotion processing, specifically fear and other emotions related to both aversive and rewarding environmental stimuli. The amygdala is an intricate structure embedded in the medial temporal lobe, comprised of numerous subregions and distinct neuronal populations. The most studied sub-regions are the basolateral complex of the amygdala (BLA; made up of the basal (BA), basomedial (BM) and the lateral (LA) cell groups) and the central nucleus of the amygdala (CeA; made up of a medial (CeM) and a lateral (CeL) subdivision) (see Fig. 1A). The BLA consists of glutamatergic principal neurons and inhibitory interneurons, and robustly projects to the CeA (CeL and CeM) which is composed of ~ 95% GABAergic medium spiny neurons (McDonald, 1982). The CeL in turn projects to the CeM, which is also the main output of the amygdala (Janak and Tye, 2015).

A major determinant of whether environmental stimuli are interpreted as threatening occurs in the amygdala, wherein sensory stimuli are imbued with emotional valence (Calhoon & Tye, 2015). The simplified view of information flow through the amygdala is as follows (adapted from Calhoon & Tye, 2015; Tovote et al., 2015; Janak & Tye, 2015; Tye et al., 2011) (see Fig. 1B). The LA receives information about the external environment from the sensory cortices and via the thalamus. The LA projects within the BLA to the BM and BA, as well as to the neighbouring CeA (Janak & Tye, 2015; Tye et al., 2011). The BLA, which is also reciprocally connected with cortical regions (medial prefrontal cortex; mPFC), the hippocampus and sensory association areas, thus then processes and integrates the sensory information which results in the formation of associations between neutral predictive stimuli and outcomes of positive or negative valence (Calhoon & Tye, 2015; Janak & Tye, 2015). Thus, cues predicting reward are themselves becoming rewarding and, likewise, those predicting threats are themselves recognized as threatening (Calhoon & Tye, 2015; Janak & Tye, 2015; Ledoux, 2000). The emotional valence of these stimuli then determines whether reward or fear pathways are recruited downstream of the BLA; in fear-or anxiety provoking circumstances, the CeA and the bed nucleus of the stria terminalis (BNST) are activated. In turn, the striatum, CeA and BNST, with the latter two structures connected via GABAergic efferents, have been considered to mediate the translation of BLA signals to behavioural output.

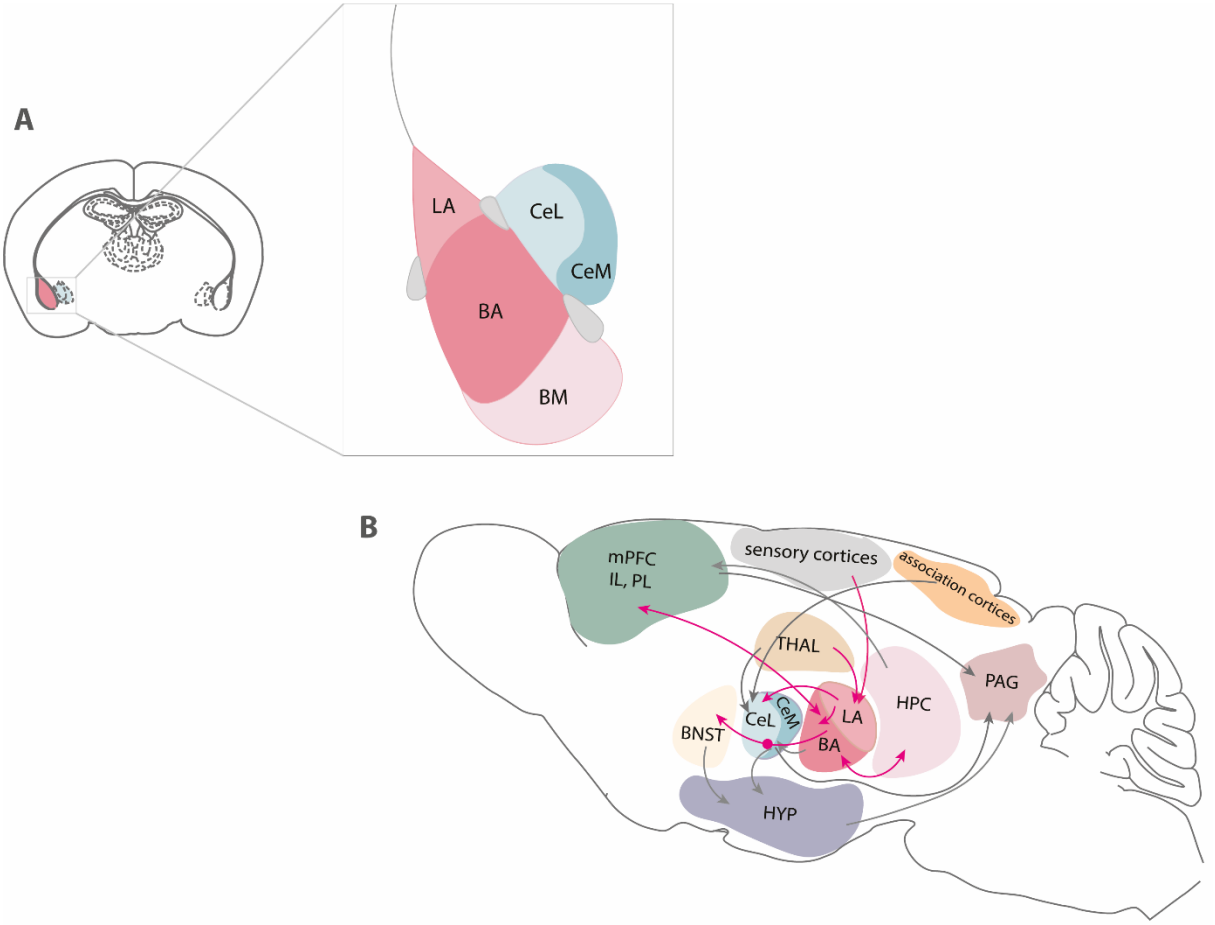

Figure 1: Anatomy of the Amygdala and Circuitry implicated in Fear- and Anxiety-related Behaviours

(A) The most studied subregions of the amygdala are the basolateral (BLA) complex of the amygdala, which consists of the lateral (LA), basal (BA) and basomedial (BM) cell groups; and the central nucleus of the amygdala (CeA), made up of the medial (CeM) and lateral (CeL) subdivision. **(B)** A sagittal view of the rodent brain including distal- and micro- circuits involved in fear-and anxiety related behaviours. Pink arrows represent a simplified information flow during threat processing; grey arrows represent projections and/or fear-anxiety circuitry. mPFC, medial prefrontal cortex; THAL, thalamus; PAG, periaqueductal gray; BNST, bed nucleus of the stria terminalis; HYP, hypothalamus; HPC, hippocampus.

Thus, while extensive research has clearly implicated the amygdala in mediating fear as well as threat and reward, its direct role in maintaining sustained anxiety symptoms has been more difficult to establish. However, previous findings have identified that the "central extended amygdala", in which the key components include the CeA and the BNST, has a central role in the generation of anxiety states (Ahrens et al., 2018; Calhoon & Tye, 2015; Gungor & Paré, 2016; Tovote et al., 2015; Robinson et al., 2019).

1.3.2. The Bed Nucleus of the Stria Terminalis

The BNST is a basal forebrain structure well-situated to integrate limbic information and regulate defensive states such as anxiety, since it is a major target of the CeA and the BLA and projects to many hypothalamic and brainstem structures that receive CeA nucleus terminals (Ledoux, 2000; Dong et al., 2001b; Adhikari, 2014). In addition, the BNST is the centre of the psychogenic circuit from the hippocampus to the paraventricular nucleus (PVN), which plays an important role in mediating the hypothalamic pituitary adrenal (HPA) stress response (Dong et al., 2001b; Lebow and Chen, 2016; Ch'ng et al., 2018). Furthermore, both the CeA and the BNST share a similar morphology and neuropeptide expression profile, including neuropeptides such as corticotrophin-releasing hormone (CRH), neuropeptide Y (NPY), and Tachykinin 2 (*Tac2*) which have also been implicated in the modulation of anxiety and the HPA axis response (Andero et al., 2016; Gafford & Ressler, 2015; McDonald, 1982; Woodhams et al., 1983; Zelikowsky, Hui, et al., 2018).

Like the amygdala, a large body of evidence associates the BNST with anxiety. Early evidence arose from the assumption of a distinct differentiation between fear and anxiety in the BNST. Spearheaded by Walker and Davis, a highly influential model theorized that the amygdala underlay "phasic" responses to threat while the BNST was thought to mediate "sustained" responses (Walker et al., 2009; Davis et al., 2010; Gungor and Paré, 2016; Goode et al., 2019). This was based on previous observations that BNST lesions did not alter conditioned fear responses elicited by discrete conditioned sensory cues (Hitchcock & Davis, 1991; LeDoux et al., 1988), unless they were very long (> 8 min) (Waddell et al., 2006; Gungor and Paré, 2016). In contrast, lesioning the BNST impaired the acquisition and recall of contextual fear responses, which might be due to the diffuse nature of contextual cues (Waddell et al., 2006; Duvarci et al., 2009). Other work indicated that lesioning the amygdala reduced fear-potentiated startle, but did not affect BNST mediated anxiety-like behaviour in the elevated plus maze (EPM) (Ventura-Silva et al., 2013), anxiety-like responses to CRH injection and bright lights (Walker & Davis, 1997) or to alarm pheromones (Fendt et al., 2003).

Clinical data has further supported the role of the BNST in anxiety. For example, patients with generalized anxiety disorder showed hyperactivation of the BNST when engaged in a gambling task with high uncertainty (Yassa et al., 2012). Furthermore, the BNST is recruited during hypervigilance caused by threat-monitoring processes in individuals with higher trait anxiety (Somerville et al., 2010). Studies in both males and females with phobias, which cause anxiety states, showed functional magnet resonance imaging (fMRI) activation of the BNST, the anterior cingulate cortex and the insula in anticipation of visual stimuli of phobia-inducing objects (for example, a spider) with no activation in the amygdala (Straube et al., 2007). Nevertheless, another study used a real tarantula during brain imaging in healthy males and females, and demonstrated that the closer the tarantula was placed to the foot of

the participant, the greater the activity in the BNST, but also in the amygdala (Mobbs et al., 2010). This indicates that diffuse states of threat apprehension are represented differently to an actual threat; while only the BNST is activated during imagery of a future threat, both the BNST and the amygdala are activated when presented with an actual threat (Lebow and Chen, 2016).

Nevertheless, these findings also suggest that both the BNST and the CeA are involved in mediating phasic and sustained responses to threat, and that a distinction between the two states- anatomically and conceptually- is an oversimplification. This was also highlighted by two studies that showed that the BNST plays a crucial role in shaping phasic responses to acute stimuli when they are encountered in potentially dangerous contexts and also contributes to the "overgeneralization" of fear and anxiety (Kheirbek et al., 2012; Lissek, 2012). In addition, in the absence of a functional BLA, the BNST was shown to act as a compensatory site in the acquisition of fear memories, although this BLA-independent fear learning required more training (Poulos et al., 2010). Overall, however, human imaging data suggests that the BNST is implicated in anxiety, and that its activity is correlated with an increase in anxiety.

More recent studies conducted with rodents have provided evidence that there are more nuanced roles of the BNST in anxiety. For example, Treit and colleagues (1998) found no effect of BNST lesions in open-arm avoidance in the EPM, whereas Duvarci et al. (2009) demonstrated anxiogenic effects of BNST lesioning on the plus maze. Moreover, Van Dijk et al. (2013) reported that electrical stimulation of the BNST did not affect anxiety-like behaviour in the plus maze, and Walker (1997) found that infusions of glutamate antagonists in the BNST were anxiolytic in the light potentiated startle paradigm (Adhikari, 2014). These data are in line with the general consensus for the involvement of the BNST in anxiety, yet they do not illustrate if this structure decreases or increases anxiety. This is perhaps unsurprising given that the BNST is small, but highly heterogenous and complex in its anatomy and neurochemical properties, likely reflecting functional differentiation among the nuclei. If different BNST subregions regulate anxiety in opposite directions, pharmacological and lesion manipulations of BNST nuclei will generate conflicting results. It is therefore essential to focus on the specific subregions, and how exactly these are implicated in mediating anxiety.

1.3.3. The Oval Bed Nucleus of the Stria Terminalis

The oval bed nucleus (ovBNST), a region considered to be the master controller of BNST outflow, is of particular interest to the topic of anxiety. In rodents, the BNST is located ventral to the lateral septum (LS) area, dorsal to the hypothalamus and surrounds the anterior commissure. A review by Lebow and Chen describes the BNST anatomy and connectivity according to the series of papers of Dong & Swanson (Dong et al., 2001a, 2001b; Dong and Swanson, 2004a, 2004b, 2006; Lebow and Chen, 2016). The BNST is primarily divided into the posterior and anterior divisions. The posterior division is comprised of three

well-characterized nuclei: the principal, the interfascicular and the transverse. The anterior division includes the anterolateral, anteromedial, fusiform, juxtacapsular, rhomboid, dorsomedial, ventral nucleus, magnocellular, and the oval BNST (see Fig.2A). Posteriorly located nuclei are involved in reproductive and social defensive behaviours via the PVN and have received little attention from fear/anxiety research. Instead, the focus has been on the anterior BNST region, because it is the main termination zone of CeA axons (Krettek and Price, 1978) and also considered a relay station between the mPFC and PVN that mediate the stress response via the HPA axis (Choi et al., 2007, 2008; Radley and Sawchenko, 2011).

The ovBNST is largely known as an integrator of mood and negative valence information. An influential study by Kim and colleagues clearly delineated the role of the ovBNST in generating anxiety, after reporting that optogenetic inhibition of dopamine-receptor-1a (DrD1a) positive cells in the ovBNST had an anxiolytic effect, whereas stimulation increased both behavioural and physiological measures of anxiety (Kim et al., 2013). In addition, anterodorsal BNST (adBNST[1])-associated activity exerted anxiolytic influences through receiving differential input from the BLA, whereby three distinct efferent projections – to the lateral hypothalamus (LH), parabrachial nucleus (PB) and ventral tegmental area (VTA) - each implemented an independent feature of anxiolysis (see Fig. 2B). These results gave a clear indication of the subregion specificity with regards to different aspects of anxiety and also emphasized the ovBNST's implication in anxiety-like behaviour.

Notably, BNST subregions and cells differ in their neurochemical profiles, suggesting functional differentiation among cells located in the same nucleus. Much evidence suggests that CRH exerts anxiogenic effects through its actions in the BNST and within the ovBNST, respectively. In fact, the ovBNST contains the highest concentration of CRH neurons in the brain (Morin et al., 1999; Daniel and Rainnie, 2016a). In line with this, a recent study confirmed that chronic stress and acute optogenetic activation of the ovBNST increased anxiogenic behaviours and cellular excitability of CRH positive cells in the ovBNST (Hu et al., 2020a). In a different study, Hu et al highlighted that early life stress (ELS) resulted in a long-lasting activation of CRH signalling in the mouse ovBNST, leading to potential maladaptive changes in ovBNST function in adulthood (Hu et al., 2020b). These results confirm that the ovBNST is not only a mediator of anxiety, but also a modulator of the stress response.

The ovBNST receives substantial input from central amygdala inhibitory afferents, with large subsets of somatostatin positive (SOM) and CRH positive CeA-to-ovBNST GABAergic projections that mediate anxiety- related behaviours (Ahrens et al., 2018; Pomrenze et al., 2019a). Additional afferents are very

[1] First defined by Ju and Swanson (1989) as the region dorsal to the anterior commissure and contiguous with the anteroventral area rostral and caudal to the commissure (Ju and Swanson, 1989). Defined by Kim et al (2013) as "the region surrounding the ovBNST". Outlined in Fig. 2A.

similar to CeA afferents; including inputs from the LA via glutamatergic neurons, the mPFC via pyramidal neurons, and from the dorsal raphe (DR) and VTA via dopaminergic neurons (Park et al., 2013). It also receives viscerosensory afferents from the insula and brainstem autonomic nuclei as well as mixed dopaminergic- glutamatergic inputs from the periaqueductal gray (PAG) (Schwaber et al., 1982; McDonald et al., 1999; Gungor and Paré, 2016; Li et al., 2016). These afferents project onto GABAergic populations within the ovBNST that co-express a wide selection of neuropeptides such as CRH, encephalin, dynorphin, NPY, SOM, *Tac2* and pituitary adenylate cyclase-activating polypeptide (PACAP) (Walter et al., 1991; Poulin et al., 2009; Hammack et al., 2010; Lebow and Chen, 2016; Ahrens et al., 2018; Zelikowsky et al., 2018b; Pomrenze et al., 2019a; Hu et al., 2020b).

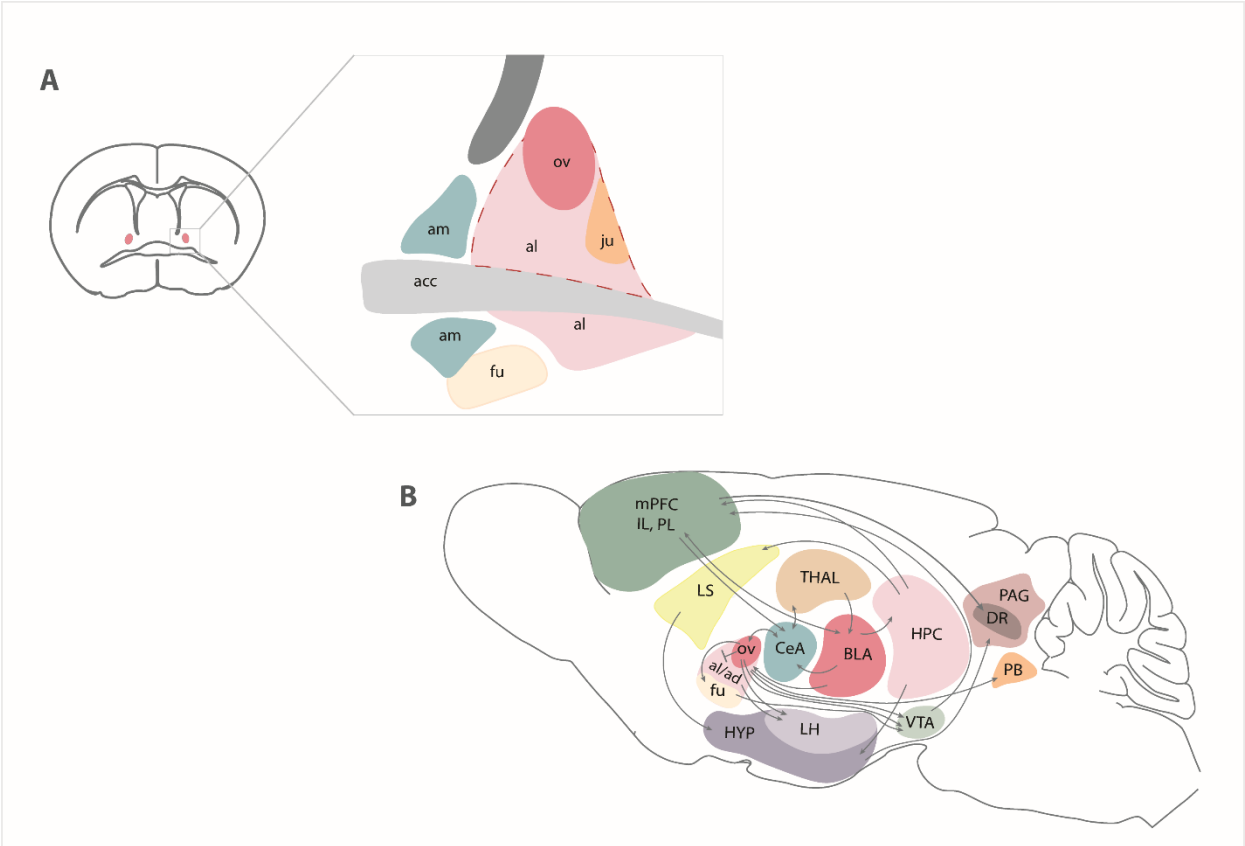

Figure 2: Anatomy of the anterior BNST and BNST Circuitry implicated in Anxiety-related Behaviours

(A) Anterior division of the rodent BNST depicting the anterior commissure (acc), anteromedial (am), anterolateral (al), juxtacapsular (ju), fusiform (fu) and oval (ov) nuclei. The red outline represents the region referred to as "anterodorsal (ad)". **(B)** A sagittal view of the rodent brain with focus on the BNST and other structures that are implicated in mediating anxiety states. Grey arrows represent neural circuits implicated in anxiety-related behaviours. mPFC, medial prefrontal cortex; LS, lateral septum; THAL, thalamus; ov, oval BNST; CeA, central amygdala; BLA, basolateral amygdala; HPC, hippocampus; LH, lateral hypothalamus; HYP, hypothalamus; VTA, ventral tegmental area; PB, parabrachial nucleus; DR, dorsal raphe; PAG, periaqueductal gray.

Interestingly, the ovBNST is devoid of direct inputs from the BLA and only receives a very weak projection from the adBNST (Dong, Petrovich, & Swanson, 2001; Kim et al., 2013). Studies using anterograde tracing illustrated that the ovBNST, similar to the CeA, projects heavily to HPA axis-related structures, and not surprisingly, also projects dense inhibitory feedback to the CeA. In fact, the ovBNST and the fusiform nucleus, which the ovBNST projects to very densely, along with the CeA, form a highly interconnected network that reportedly mediates aspects of the stress response (Dong et al., 2001b). Taken together, these data show that subregion specificity, neurochemical composition and anatomical connectivity are all features to be considered when studying the role of the BNST in anxiety. Moreover, the CeA and the ovBNST share strikingly similar circuitry and neurochemical profiles, which strongly support their interconnective roles in mediating anxiety-states. Lastly, the ovBNST has a distinct role in the modulation of anxiety.

1.4. Structural Abnormalities associated with Pathological Fear and Anxiety

The intricate interplay of structures, circuitry and neurochemical profiles that contribute to processing emotional stimuli can be disrupted in pathological anxiety and fear. Clinical and animal research has repeatedly shown that stressful and anxious experiences can induce alterations in fear/anxiety neurocircuitry and functioning. For example, ELS and anxiety has been linked to enlarged amygdala, particularly the BLA nuclei, through the interplay of prolonged exposure to stress hormones and experience- dependent plasticity in the animal brain (Mitra and Sapolsky, 2008; Lupien et al., 2009). In humans, higher amygdala volume has been reported in adults with GAD as well as healthy adults with high trait anxiety (Etkin et al., 2009; Baur et al., 2012). Furthermore, patients with social anxiety disorder showed increased amygdala activation during anticipatory anxiety relative to healthy subjects (Boehme et al., 2013). In addition, Quin and colleagues demonstrated that children with high levels of anxiety showed enlarged amygdala and functional hyper-connectivity with distributed regions (Qin et al., 2014).

In line with this, diffusion tensor imaging (DTI) studies have highlighted micro-architectural alterations in the structural organization and integrity of certain brain regions or white matter tracts across a range of psychiatric disorders including affective disorders such as anxiety. For example, a study conducted with twins discordant for lifetime GAD showed reduced fractional anisotropy (FA) values in the left uncinate fasciculus (UF) and in the right inferior longitudinal fasciculus in the affected twins (Hettema et al., 2012). The UF connects the amygdala and the orbitofrontal cortex (Ebeling and Cramon, 1992). These findings are consistent with studies in GAD in which abnormalities in connectivity between limbic and frontal structures have been described (Etkin et al., 2009, 2010; Ayling et al., 2012). Evidently, stress and anxiety, potentially in interaction with genetic predispositions and exposure to environmental adversity, have an enormous impact on structural and functional integrity within the brain.

On the molecular level, these changes have been associated with greater dendritic arborization and aberrant pruning of synapses, leading to an increased rate of growth in certain structures such as the amygdala (Davidson & McEwen, 2012; Vyas et al., 2002). Such increases have been linked to prolonged activation of stress hormones, like cortisol, which act directly on the BLA and also lead to heightened fear and anxiety (Vyas et al., 2004; Lupien et al., 2009; Roozendaal et al., 2009). Excessively elevated levels of cortisol however can also have the opposite effects, for example in the hippocampus and prefrontal cortex (PFC), leading to inhibition of cell proliferation and neurogenesis, which can eventually result in structural volume loss and functional impairments (Magariños et al., 1996; Sandi et al., 2003; Hall et al., 2015). Thus, abnormalities in the structures and circuitry that mediate anxiety-and fear-related states are an important element of pathological or maladaptive anxiety. Together with genetic predispositions and environmental factors, dysregulation of the stress system response, especially in the form of aberrant glucocorticoid (GC) signalling, poses a major risk factor to develop an anxiety disorder. It is therefore crucial to understand the complex neural and physiological underpinnings of stress and identify how an adaptive stress response can shift towards a maladaptive reaction that may ultimately contribute towards the pathogenesis of anxiety.

1.5. Stress and the HPA axis

1.5.1. The Concept of Stress

The physiologist Claude Bernard noted that the maintenance of life is critically dependent on keeping our internal milieu constant in the face of a changing environment (Bernard, 1957). Walter Cannon, also a physiologist, referred to this as “homeostasis”(Cannon, 1915). Hans Selye (1956), an endocrinologist heavily influenced by the latter two scientists, used the term “stress” to represent the “non-specific response of the body” to anything that threatens homeostasis (Selye, 1956). The actual or perceived threat to an organism is referred to as the “stressor” and the response to the stressor is called the “stress response”. Selye pioneered the field of stress research and provided convincing arguments that although stress responses evolved as adaptive processes, severe and/or prolonged stress responses might have an impact on health and lead to disease. Nevertheless, attempts to identify stress, which is an inferred state (such as anxiety, pleasure, fear, etc.) as a biological construct have suffered the same fate as other such discussions and a consensus definition still eludes the field. Over the recent years, many experimental approaches brought forth new evidence that the neuroendocrine response is in fact stressor-specific. However, although different categories of stressors (i.e. physical or neurogenic; acute or chronic; external or internal; severe or light; etc.) require different brain networks, all stressors converge to activate the HPA axis (Herman et al., 2003).

1.5.2. The HPA axis

The HPA axis, together with the sympathetic nervous system (SNS), comprise the stress system which mediates the stress response. Upon stress, the SNS stimulates rapid release of adrenaline and noradrenaline from the adrenal medulla. Simultaneously, monoaminergic neurons in brain stem areas are activated, such as the noradrenergic neurons of the locus coeruleus (Valentino and Van Bockstaele, 2008). Peripherally and centrally released monoamines then exert rapid, but short-lasting effects on neuronal activity and brain function, promoting a "fight-or-flight" response and the mobilisation of resources to compensate for adverse effects of stressful stimuli (Smith and Vale, 2006). In the HPA axis, the parvocellular neurosecretory neurons within the PVN of the hypothalamus release CRH and arginine-vasopressin (AVP) (see Fig. 3). CRH and AVP are released from the median eminence into the hypophysial portal blood system, which connects the hypothalamus and the anterior part of the pituitary gland. At the pituitary gland, CRH and AVP act synergistically to stimulate the release of adrenocorticotrophic hormone (ACTH), whereby CRH binds to G-protein coupled corticotrophin releasing hormone R1 receptors (CRHR1) which activates adenylate cyclase, and AVP to AVP1B receptors. The binding of CRH also enhances transcription of the proopiomelanocortin (POMC) gene, which encodes ACTH. Once ACTH is synthesized, it is packaged into vesicles and released into the peripheral circulation. At the adrenal cortex, ACTH stimulates the release of GCs (cortisol in humans and corticosterone in rodents). GCs are recognised as the major end products of the HPA axis and subsequently act on various targets to modulate the effects in almost every system of the body (e.g. metabolic, immune, cardiovascular, nervous, etc.). Most importantly, however, GCs are involved in negative feedback mechanisms whereby they operate at different levels of the HPA axis and the hippocampus to terminate the stress response and restore baseline levels.

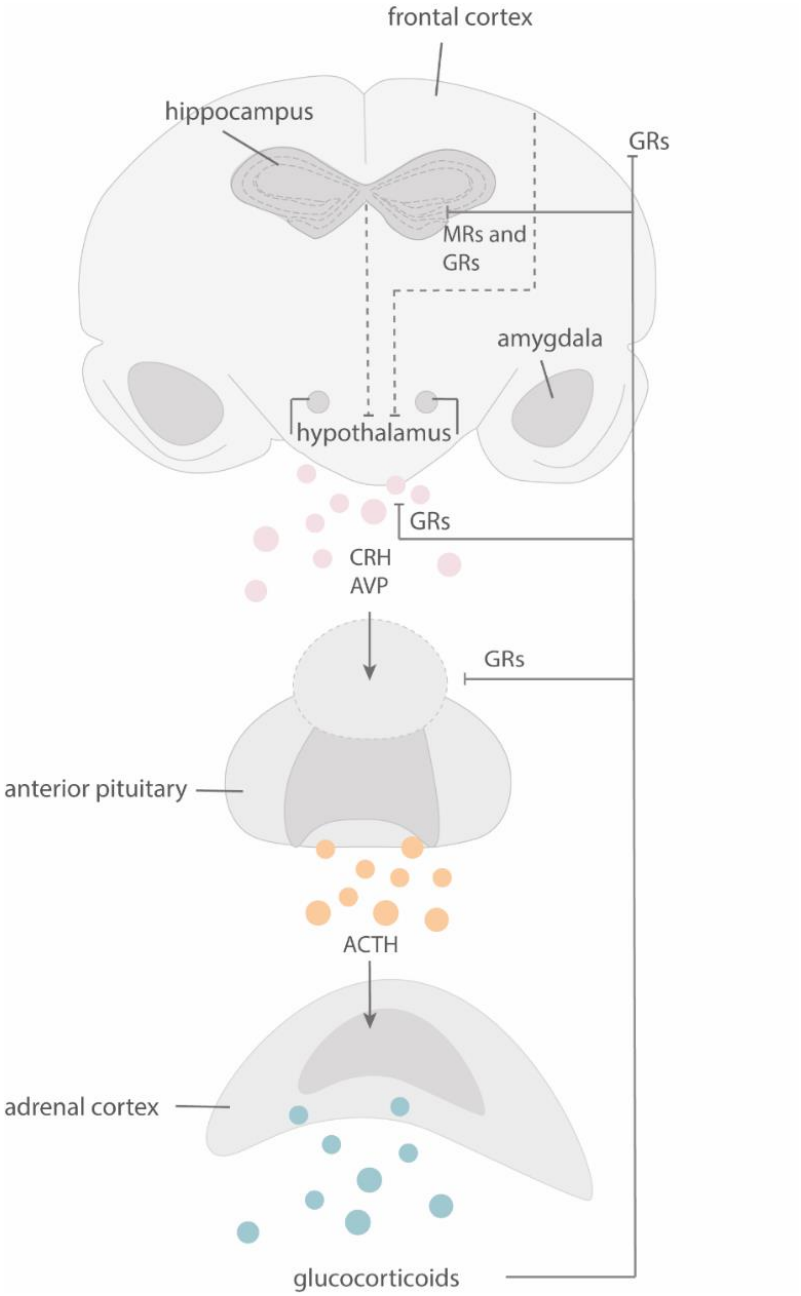

Figure 3: The stress response as mediated by the hypothalamus-pituitary-adrenal (HPA) axis

A key system in the mediation of the stress response is the HPA axis. Neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). Subsequently, this triggers the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, leading to the production of glucocorticoids by the adrenal cortex, which then bind to the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Following activation of the system, and once the perceived stressor has subsided, feedback loops at various levels of the system (from the adrenal gland to hypothalamus and other brain regions such as the frontal cortex and hippocampus) shut the HPA axis down to return to a set homeostatic point (adapted from Lupien et al (2009)).

The actions of GCs are mediated by two receptor systems, the type I, high affinity mineralocorticoid receptor (MR), encoded by the *NR3C2* gene, and the type II, low affinity glucocorticoid receptor (GR), encoded by the *NR3C1* gene. Based on differences in location and binding-affinity, the MR and GR have distinct roles. MRs are abundantly expressed in limbic circuits, whereas GRs are widely expressed in neurons and glial cells, and have their densest expression in brain regions that control the physiological and behavioural stress response, such as the amygdala, hippocampus and hypothalamus (specifically, in PVN-CRH neurons and anterior or pituitary POMC cells) (de Kloet et al., 2020). Moreover, MRs bind corticosterone with very high affinity, which means they are substantially occupied at basal circulating corticosterone levels. The GR, however, is virtually devoid of ligand at low basal corticosterone levels and only becomes activated after stress and at the circadian peak, and is therefore particularly important for negative feedback and terminating the stress response (Reul and De Kloet, 1985; de Kloet et al., 2020). Overall, the GR and MR complement each other in the control of the initiation and termination of the stress response. Moreover, proper GR signalling and HPA axis regulation is crucial for homeostasis and health.

In the absence of GC binding or other activating signals, steroid receptors such as the GR are sequestered in the cytoplasm in a heteromeric protein complex (Cheung and Smith, 2000). This complex, which involves several chaperones and co-chaperones, folds the GR to a conformation with high-affinity hormone binding competence and tightly regulates GR translocation to the nucleus. Briefly, the chaperone heat-shock protein 70 (Hsp70) binds to and unfolds the GR in the cytosol (see Fig. 4). The Hsp70-heat shock protein 90 (Hsp90) organizing protein (hop) promotes the transfer of the partially folded GR to the Hsp90-based folding platform. Hop is later dislodged from the Hsp90-GR complex upon Hsp90 binding ATP and subsequent association of the co-chaperones FK506 binding protein 51 (FKBP51), FKP506 binding protein 52 (FKBP52), cyclophilin 40 (CyP40) and protein phosphatase 5 (PP5). Hsp90 keeps the GR in a hormone binding competent state, stabilized by the co-chaperone p23. The co-chaperone FKBP51 inhibits nuclear transactivation of GR, while FKBP52 and other co-chaperones promote translocation. Subsequently, the GR binds corticosterone, dimerizes in a homo-or heterodimer and translocates to the nucleus where it binds to specific DNA sequences located in the promoter region of target genes, termed glucocorticoid response elements (GREs), stimulating or inhibiting the expression of target genes (Mifsud and Reul, 2016; Fries et al., 2017; Baker et al., 2019).

Notably, FKBP51 has a dissimilar regulatory function to all the other co-chaperones of the GR-complex. FKBP51 is upregulated by GR activity, which directly promotes the transcription of FK506 binding protein 5 (FKBP5), the gene that encodes FKBP51, and which contains functional GREs. Increased FKBP51 then hinders GR translocation, at least in part, by impairing the interaction between the GR heterocomplex

with dynein, which results in reduced GR activity. The proposed mechanism of action is a short, negative feedback loop whereby FKBP51, in a Hsp90-dependent mechanism, decreases GR ligand binding affinity, causing resistance to GC feedback (Zannas et al., 2016; Baker et al., 2019). FK506 binding protein 4 (FKBP4), the gene that encodes the highly similar FKBP51 homologue, FKBP52, has the opposite effect on GR activity, which may be a combination of increased dynein binding as well as through displacing the inhibitory effects of FKBP51 from the Hsp90-heterocomplex, However, conflicting evidence suggests that FKBP52 does not alter GR nuclear translocation (Riggs et al., 2003, 2007; Wochnik et al., 2005; Baker et al., 2019).

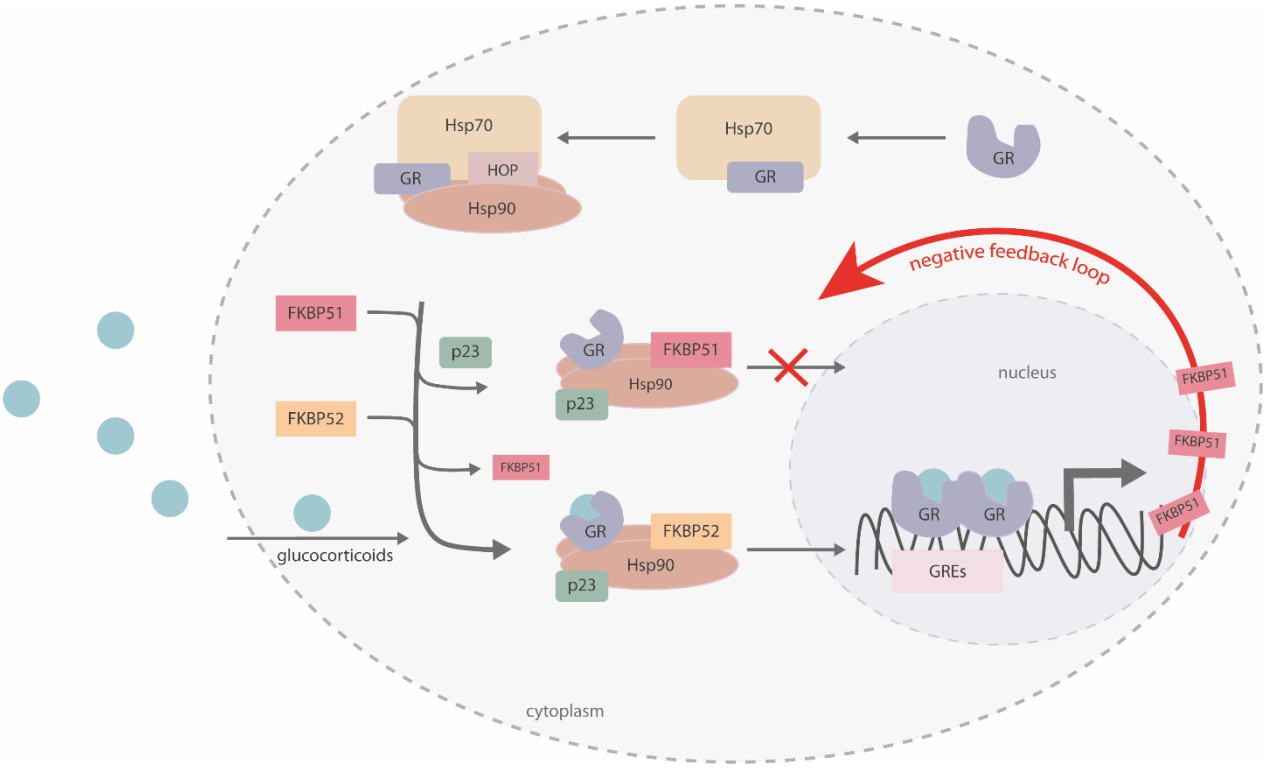

Figure 4: Schematic of glucocorticoid receptor (GR) nuclear transactivation and FKBP51-mediated negative feedback loop

Glucocorticoids secreted from the adrenal cortex crosses the plasma membrane of the cell. Inside the cell, Hsp70 binds and unfolds the GR in the cytosol; hop then recruits GR:Hsp70 to Hsp90. Co-chaperones such as FKBP51 and FKBP52 bind to Hsp90 as hop is released. p23 stabilizes the GR:Hsp90 heterocomplex. Upon glucocorticoid binding to the GR, FKBP51 (which inhibits GR nuclear transactivation) is replaced by FKBP52. FKBP52 facilitates the translocation of the GR complex into the nucleus, where it binds to glucocorticoid response elements (GREs). FKBP5 is one of the target genes of the GR, resulting in an ultra-short feedback loop that decreases GR activity.

Overall, the HPA axis response to stress is a highly complex and fine-tuned procedure, orchestrated by the balanced interplay of many subordinate components, their interconnected mechanisms, and down-stream pathways. Proper termination of the stress response through functioning negative feedback mechanisms on several levels of the HPA axis that are mediated by GR signalling and intracellular modifications of co-chaperones such as FKBP51, is crucial to maintaining homeostasis and health. Dysfunction of the HPA axis and aberrant GR signalling are believed to contribute to the development of anxiety disorders (De Kloet et al., 2005; Joëls, 2011; Joëls & Baram, 2009). It is thus important to investigate the underlying mechanisms that can lead to HPA axis dysregulation, possibly as a combination of genetic predisposition and external factors, and that pose a risk factor to maladaptive anxiety.

1.5.3. HPA axis Dysregulation in Anxiety Disorders

If the stress response is inadequate or excessive and prolonged, the cost of maintaining homeostasis may become too high, which is a condition referred to as "allostatic load" (McEwen, 2003). Traumatic experiences in childhood or acute and chronic stress challenge an organism to cope. The inability to cope with stressful life events, which may lead to the hypersecretion of corticosteroids, imposes an increased risk for mood disorders, including anxiety disorders (De Kloet et al., 2005).

In acute anxiety, the activation of the HPA axis is adaptive. Conversely, in chronic anxiety the gradual development of a disconnection between the stressor and its behavioural consequences is the main coping mechanism which allows for continuous everyday normal functioning. When this mechanism fails, the HPA axis is consistently activated, which results in a sustained increase of cortisol levels, impaired coping mechanisms, and lower tolerance to subsequent stressors. In fact, animal studies that utilized "chronic stress models" that are based on several weeks of daily immobilization or repeated social stressors such as daily exposure to a dominant male, reported severe deficits in hippocampus-related memory, increased fear-motivated behaviour, and significant neural correlates of these behavioural deficits (Conrad et al., 1999; Kim and Diamond, 2002; Bowman et al., 2003; Sandi, 2004) Specifically, in the amygdala, the hippocampus, and PFC, including aspects of structural remodelling and cell proliferation, as well as changes in amine, neuropeptide and corticosteroid systems (De Kloet et al., 2005). Most likely, the effects of chronic stress are mediated through MR- and GR- related actions, whereby negative feedback resistance of the GR results in elevated circulating GC concentrations. Consequently, MR and GR gene regulation are altered and may result in increased or decreased expression levels within different neural structures, such as the hippocampus or hypothalamus, further exacerbating deficient feedback of the HPA system. Moreover, abnormal levels of GRs and MRs have been associated with the cognitive and emotional disturbances resembling symptoms of mood

disorders. For example, deleting GR expression in the limbic system (except for hypothalamic PVN) resulted in a depression-like phenotype, whereas forebrain specific overexpression of the GR in mice resulted in a state of emotional lability (Wei et al., 2004; Boyle et al., 2005). Thus, chronic stress, such as observed during prolonged exposure to stressful life events, along with an inadequacy to cope or the perceived loss of control, may lead to persistent activation of the HPA axis, aberrant GR/MR signalling and subsequently to a constant increase of cortisol levels.

Interestingly, patients with a primary diagnosis of Cushing's disease, defined as Cushing's syndrome associated with an ACTH-secreting pituitary tumour and characterized by hypercortisolism, often suffer from anxiety, specifically GAD (Loosen et al., 1992). Successful treatment of Cushing's syndrome by correction of hypercortisolism was associated with the gradual reduction of symptoms in atypical depression and anxiety (Dorn et al., 1997). A considerable number of patients suffering from anxiety disorders exhibit hyperactivity of the HPA axis, with the consequent hypercortisolism. This has been described in patients with panic disorder (PD) or GAD, however, hypercortisolism in patients has not been observed in other studies (Tafet et al., 2001; Risbrough and Stein, 2006; Abelson et al., 2007; Hek et al., 2013). This may not come as a surprise, however, since hypercortisolism is neurochemically not the same as chronic stress or an anxiety disorder. In fact, chronic stress is characterised by prolonged release of GCs, yet the secretion is triggered by neuropeptides (for example, CRH). In healthy individuals, neuropeptides facilitate adaptation, which they fail to do so in individuals affected by anxiety disorders, which precipitate psychopathology (De Kloet et al., 2005). In Cushing's disease, or even corticosteroid therapy, the prime cause is excessive GC, but not peptide concentrations, which have vastly different effects that range from cognitive impairment to psychosis, and even to mania and delusions (De Kloet et al., 2005). Nevertheless, confirming previous observations regarding the effects of chronic stress, HPA axis dysregulation and increased levels of GCs seem to be implicated in the psychopathology of anxiety disorders. Furthermore, restoring or normalizing HPA disturbance has been shown to be a prerequisite for successful treatment and persistence in mood disorders.

Along with HPA axis hyperactivity, exposure to ELS (such as childhood abuse or adverse parenting experience) has been considered a major risk factor for anxiety disorders later in adulthood (Kendler et al., 1992; Bandelow et al., 2004; Lochner et al., 2010; Gal et al., 2011; Sauro et al., 2012; Lähdepuro et al., 2019). For example, children who experienced permanent or long-term separations from parents, or parental death, exhibited a hyperactive HPA axis, with increased basal cortisol levels and cortisol non-suppression after the Dexamethasone (Dex) Suppression Test (Breier et al., 1988; Tyrka et al., 2008). Furthermore, Pfeffer et al. (2007) noted that children who had lost their parent in the September 11th, 2011 terror attack had significantly increased rates of anxiety disorders, as well as significantly higher morning and afternoon baseline cortisol compared to non-bereaved children. At last, Carpenter and

colleagues reported that childhood sexual abuse was a significant predictor of higher cortisol curves in adulthood (Carpenter et al., 2009). Thus, it seems like severe ELS is associated with persistent sensitization of the HPA axis, which can result in HPA axis hyperactivity to subsequent stressors, eventually resulting in an increased risk for maladaptive anxiety later in life.

While the vast majority of studies demonstrate HPA hyperactivity as a risk factor for anxiety disorders, compared to the extensive literature of HPA axis reactivity in patients with depressive illness, the evidence base relating to HPA axis function in patients with anxiety disorders is relatively limited and often discordant (Arborelius et al., 1999; Heim and Nemeroff, 2001; Sauro et al., 2012; Elnazer and Baldwin, 2014; Tafet and Nemeroff, 2020). Particularly, it is important to keep in mind that anxiety is highly comorbid with depression. In fact, Vreeburg et al (2010) observed that a modest but significantly higher 1- hour cortisol awakening response among anxiety patients was driven mainly by those with comorbid depression, and similar data was confirmed by de Kloet et al. (2008). Nevertheless, Vreeburg also found that–independently of the presence of comorbid depression- panic disorder (PD) combined with agoraphobia was strongly associated with an increased cortisol awakening response, whereas PD, GAD and social phobia were not as much associated (Vreeburg et al., 2010). In addition, Graeff (2007) demonstrated that anticipatory anxiety and GAD both activate the HPA axis, whereas panic attack causes major sympathetic activation, but has little effect on the HPA axis. Thus, these results indicate that HPA axis dysregulation can be considered a general feature of anxiety disorders, but hyperactivity itself is no unifying disturbance of HPA axis function across all anxiety disorders (Sauro et al., 2012; Sotnikov et al., 2014).

Taken together, HPA axis dysregulation due to excessive or prolonged GC exposure, as exemplified by hypercortisolism in Cushing's disease or chronic stress or ELS, and subsequent sensitisation of the HPA axis, poses a vulnerability factor for anxiety disorders. However, hypercortisolism is not equivalent to chronic stress or exposure to ELS or even an anxiety disorder. Nevertheless, the same endocrine conditions that are elicited by these conditions might induce a susceptibility to anxiety in individuals that carry a genetic risk.

1.6. The co-chaperone FKBP51 as a Risk Factor for Anxiety Disorders

Co-chaperone variants and altered expression levels have been linked to psychiatric illness. The co-chaperone FKBP51 has been implicated in several pathological processes and diseases including depression, PTSD, anxiety disorders, Alzheimer's disease and even cancer (Koren et al., 2011; Hou and Wang, 2012; Zannas and Binder, 2014; Blair et al., 2015; Sabbagh et al., 2018). Variations in the function of FKBP51 can be caused by changes in FKBP5 levels due to genetic, epigenetic and environmental factors.

FKBP51 displays peptidyl-prolyl isomerase activity and classifies as an immunophilin due to its binding to the immunosuppressants FK506 and rapamycin. FKBP51 consists of three important domains; first, a peptidyl-prolyl cis-trans isomerase (PPIase) domain (called FK1), located at the N-terminus, which is recognized as the primary regulatory domain for steroid receptor signalling and furthermore as the binding site of the immunosuppressive drugs FK506 and rapamycin (Riggs et al., 2003; Sinars et al., 2003; Kozany et al., 2009). The second domain, FK2, is less well characterised and understood than FK1. Regardless, the FK2 domain possesses a scaffolding function and contributes to protein-protein interactions (Sinars et al., 2003; Hähle et al., 2019). The C-terminal region- the third functional domain- contains a three- unit repeat of the tetratricopeptide repeat (TPR) domain which mediates Hsp90 binding within steroid receptor complexes (Pratt and Toft, 1997; Kumar et al., 2017).

FKBP51 is a multifunctional protein that is not only involved in stress regulation and GR modulatory functions, but also implicated in pathways such as NF_KB, AKT/ mTOR and AKT/PHLPP signalling, as well as immune function, autophagy, apoptosis, cell growth, cytoskeleton dynamics, epigenetic remodelling, and metabolism (see Zgajnar et al., 2019 and Hähle et al., 2019 for review). FKBP51 is also a protein that is regulated at multiple layers. The FKBP5 gene consists of 13 exons which are located on the short arm of chromosome 6. The most important hallmark of the gene, at least with respect to GR regulation, is the presence of several GREs in the promoter region and intron 2,5 and 7 (Zannas et al., 2016; Hähle et al., 2019). Notably, FKBP5 transcription is not only induced by GR, but also by androgen receptor (AR) and progesterone receptor (PR) activation (Magee et al., 2006; Jääskeläinen et al., 2011; Stechschulte and Sanchez, 2011). Nevertheless, upon binding of activated GRs, these enhancer elements promote transcription via the formation of three-dimensional chromatin loops and the recruitment of RNA polymerase to the transcription start site.

Several polymorphisms have been identified within the FKBP5 gene that allow for stronger FKBP51 induction by GCs. For example, the rs1360780 short nucleotide polymorphism (SNP) is located in intron 2 close to a functional GRE, and the rarer T-allele was demonstrated to lead to increased GR-mediated FKBP51 expression, likely supported by the ability to bind TATA-box binding protein (Bertolino and Singh, 2002). FKBP5 has also been shown to be regulated by DNA methylation, directly mediating the effects of environmental stimuli on the gene. Furthermore, DNA methylation changes across different regions of the FKBP5 gene can influence its expression and interact with specific polymorphisms (Fries et al., 2017). In line with this, Klengel et al (2013) found reduced DNA methylation specifically in the intron 7 of the FKBP5 gene in rs1360780 T allele carriers that had been exposed to childhood trauma, but not individuals with alternate genotypes. Furthermore, reporter gene assays showed that decreased methylation in this specific region was linked to disinhibited GR-induced transcription of FKBP5 and GR resistance. Moreover, these effects were observed only with childhood but not adult trauma,

emphasizing a complex interplay between genotype, DNA methylation and environmental influences, specifically ELS, on FKBP5 gene transcription.

FKBP5 transcription has also been shown to be controlled in a region and tissue specific manner, possibly through a combination of the aforementioned mechanisms (Fries et al., 2017). In a study performed with mice, Scharf et al (2011) showed that FKBP51 is ubiquitously expressed throughout the entire brain, with higher expression levels in specific brain areas. In particular, regions with low basal FKBP51 expression, such as the PVN or the CeA, showed a higher increase in FKBP5 mRNA following induction via dexamethasone treatment than regions with high baseline expression levels, like the hippocampus, which showed only a modest induction of FKBP5 expression. These findings strongly support the role of FKBP51 as a negative regulator of GR sensitivity.

Notably, common FKBP5 SNPs, such as the allelic variant rs1360780, have been associated with an increased risk of developing a mood disorder (Binder et al., 2004). Accordingly, Ising et al (2008) demonstrated that healthy controls that were homozygote for the high-induction alleles showed significantly slower recovery from stress-related increases in cortisol levels as well as more anxiety symptoms in the recovery phase than healthy controls with the other allelic variants. More recently, FKBP5 polymorphisms were also associated with an increased risk of anxiety in patients with cancer (Kang et al., 2012). In addition, carriers of the same rs1360780 risk variant were more susceptible to anxiety and other mental health disorders when exposed to maltreatment as a child (Klengel et al., 2013; Scheuer et al., 2016). Similarly, other FKBP5 allelic variations have been linked to several mental disorders such as depression, PTSD, bipolar disorder, schizophrenia, suicidal behaviour and psychosis (Binder et al., 2008; Fujii et al., 2014; Mihaljevic et al., 2017; Stamm et al., 2016; Szczepankiewicz et al., 2014; for review Zannas et al., 2016). Interestingly enough, however, at least four SNPs (rs1360780, rs9470080, rs9296158, rs3800373) have been reported to be significantly implicated in anxiety (Criado-Marrero et al., 2018).

Taken together, substantial evidence from mice and human genetics has confirmed FKBP51 as a key regulator of GR sensitivity and the HPA stress response. Specifically, its regulatory mechanisms and direct interaction with stress make FKBP5 a candidate gene in the pathology of anxiety disorders.

1.6.1. FKBP51 in the Amygdala and its implication in Anxiety

The role of FKBP5 in the development and susceptibility to anxiety disorders has long been overshadowed by research into the implication of FKBP5 and depressive illness. However, recent studies in rodent models have provided supporting evidence and further insight into the link of FKBP5 and anxiety. Naturally, the focus of research has been on the amygdala. While a global knockout of FKBP5

did not affect anxiety-like behaviour under basal and stress conditions, selectively reducing FKBP51 in the amygdala with viral vectors alleviated stress-induced anxiety-like behaviour in mice (Attwood et al., 2011; Touma et al., 2011; Hartmann et al., 2012b; JC et al., 2013). Likewise, pharmacological disruption of FKBP51 signalling locally in the amygdala also demonstrated an anxiolytic effect (Hartmann et al., 2015). In line with this, viral-mediated overexpression of FKBP51 in the BLA or the CeA enhanced anxiety- like behaviour (Hartmann et al., 2015). However, anxiety was not altered by overexpressing FKBP51 in the dorsal hippocampus of mice (Hartmann et al., 2015) or by knocking down FKBP5 in the prelimbic cortex of rats (Criado-Marrero et al., 2017). This reconfirms the region- specific regulation of FKBP51 and how these effects in the amygdala seem to be critical for anxiety regulation.

As a matter of fact, interaction with stress-related mechanisms has also been shown to modulate FKBP51 in the amygdala. Attwood and colleagues found that chronic stress increases amygdalar FKBP51 in mice through a pathway involving neuropsin dependent cleavage of the tyrosine receptor kinase, EphB2 (Attwood et al., 2011). More recently, a study showed that microRNA-15a inhibits amygdalar FKBP51 expression, which decreased anxiety levels in mice. Reciprocally, reducing amygdalar microRNA-15a increased FKBP51 and anxiety-like behaviour after exposure to a chronic stress paradigm (Volk et al., 2016). Overall, these findings indicate that FKBP51 in the amygdala does in fact play an important role in the regulation of anxiety. This makes FKBP51 a key therapeutic target in stress- related anxiety disorders.

1.7. The Neuropeptide Tachykinin 2 and its implication in Stress and Anxiety

Neuromodulators such as biogenic amines and neuropeptides have long been implicated as mediators of internal states (Harris-Warrick and Marder, 1991; Marder, 2012; Kennedy et al., 2014; Zelikowsky et al., 2018a). In particular, the family of tachykinins has been identified to be involved in the regulation of emotional processes, and possibly in the modulation of stress, anxiety and mood responses. Mammalian tachykinins including the main members substance P (SP), neurokinin A (NkA) and neurokinin B (NkB) are a group of neuropeptides that are almost exclusively expressed in neurons, acting as neurotransmitter and/or neuromodulators in the central nervous system (CNS). Most studies on tachykinins have been carried out in the rat brain and have primarily focused on SP (Ebner et al., 2009). Thus, the role of NkB, which is encoded by the *Tac3* gene in humans and *Tac2* gene in rodents, in the stress response and its effects on anxiety-related phenotypes has been overlooked. NkB has long been accepted as a robust modulator for neuronal activity (Otsuka and Yoshioka, 1993; Mar et al., 2012). Interestingly, first evidence for a role of NkB in the control of stress and anxiety-related behaviours came from reports that intracerebral injections of NkB or adequate agonists modulate emotional behaviour (Ribeiro & De Lima, 2002; Ribeiro et al., 1999; Ribeiro & De Lima, 1998). For example, anxiolytic-like

effects were observed after central NK3 receptor activation in the EPM and, vice versa, anxiogenic effects after inhibition of NK3R activity (Ribeiro et al., 1999).

More recent studies have implicated *Tac2* directly in the stress response as well as in fear learning and memory (Andero et al., 2016; Andero et al., 2014). In fact, Andero and colleagues (2014) reported that increased *Tac2* expression in the CeA following virally mediated overexpression or via a stress-induced animal model for PTSD was sufficient to enhance fear consolidation. Likewise, Zelikowsky and colleagues (2018) demonstrated that social isolation stress caused a distributed upregulation of *Tac2* in regions including the adBNST and the CeA, and consequently linked *Tac2* expression to an anxiogenic phenotype. Moreover, systemic administration of Osanetant, a NK3 receptor antagonist, attenuated the SIS induced anxiety-like behaviour. Chemogenetic silencing of *Tac2* positive neurons in the adBNST, CeA and dorsomedial hypothalamus (DMH) blocked SIS specific behaviour, whereas chemogenetic activation in combination with *Tac2* overexpression mimicked the effects of SIS, respectively. Thus, *Tac2* is a relatively novel and unexplored neuropeptide that is clearly implicated in the regulation of stress and anxiety. Furthermore, it is expressed in the BNST and the CeA which makes it a promising, complimentary gene to explore with regard to the function of FKBP5.

1.8. Rational and Thesis Objectives

Anxiety disorders are highly prevalent and complex, and there is a pressing demand for a more comprehensive understanding of the mechanisms underlying such disorders. Dysregulation of the stress response and genetic risk factors can contribute towards a susceptibility to maladaptive anxiety. Human and animal studies have shown that aberrant GR signalling as well as variants and altered levels of FKBP51 precede stress-related pathology. The BNST, specifically the ovBNST, is a promising limbic structure with regard to anxiety disorders and the region-specific effects of FKBP51 in this region have not been explored yet. Furthermore, *Tac2* is an emerging neuropeptide expressed in the adBNST and highly likely involved in mediating stress-induced anxiety-like behaviour. The main objective of the current thesis was to establish how FKBP51 in its function as a GR co-chaperone is implicated in anxiety. To this end, we formulated explicit research questions to address whether, and to what extent, FKBP51 and its interaction partners might be contributing to specific pathologies that underly anxiety disorders.

Research Questions

I. How does the GR and GR signalling in forebrain neurons of the major neurotransmitter systems - GABAergic and glutamatergic- contribute to anxiety- like behaviour?
II. Does lack of FKBP51 shape brain structure and connectivity in male mice?

III. How does stress-induced FKBP51 in the ovBNST regulate HPA axis activity and anxiety-related behaviour?

IV. What is the role of *Tac2* in the ovBNST and how is it implicated in maladaptive anxiety?

2. Research Articles

2.1. Forebrain glutamatergic, but not GABAergic, neurons mediate anxiogenic effects of the glucocorticoid receptor

Hartmann, J., Dedic, N., Pöhlmann, M. L., Häusl, A., Karst, H., Engelhardt, C., Westerholz, S., Wagner, K. V., Labermaier, C., Hoeijmakers, L., Kertokarijo, M., Chen, A., Joëls, M., Deussing, J. M., & Schmidt, M. V.

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ORIGINAL ARTICLE

Forebrain glutamatergic, but not GABAergic, neurons mediate anxiogenic effects of the glucocorticoid receptor

J Hartmann[1,2], N Dedic[1], ML Pöhlmann[1], A Häusl[1], H Karst[3], C Engelhardt[1], S Westerholz[1], KV Wagner[1], C Labermaier[1], L Hoeijmakers[1], M Kertokarijo[1], A Chen[1], M Joëls[3], JM Deussing[1] and MV Schmidt[1]

Anxiety disorders constitute a major disease and social burden worldwide; however, many questions concerning the underlying molecular mechanisms still remain open. Besides the involvement of the major excitatory (glutamate) and inhibitory (gamma aminobutyric acid (GABA)) neurotransmitter circuits in anxiety disorders, the stress system has been directly implicated in the pathophysiology of these complex mental illnesses. The glucocorticoid receptor (GR) is the major receptor for the stress hormone cortisol (corticosterone in rodents) and is widely expressed in excitatory and inhibitory neurons, as well as in glial cells. However, currently it is unknown which of these cell populations mediate GR actions that eventually regulate fear- and anxiety-related behaviors. In order to address this question, we generated mice lacking the receptor specifically in forebrain glutamatergic or GABAergic neurons by breeding $GR^{flox/flox}$ mice to Nex-Creor Dlx5/6-Cremice, respectively. GR deletion specifically in glutamatergic, but not in GABAergic, neurons induced hypothalamic-pituitary–adrenal axis hyperactivity and reduced fear- and anxiety-related behavior. This was paralleled by reduced GR-dependent electrophysiological responses in the basolateral amygdala (BLA). Importantly, viral-mediated GR deletion additionally showed that fear expression, but not anxiety, is regulated by GRs in glutamatergic neurons of the BLA. This suggests that pathological anxiety likely results from altered GR signaling in glutamatergic circuits of several forebrain regions, while modulation offear-related behavior can largely be ascribed to GR signaling in glutamatergic neurons of the BLA. Collectively, our results reveal a major contribution of GRs in the brain's key excitatory, but not inhibitory, neurotransmitter system in the regulation offear and anxiety behaviors, which is crucial to our understanding of the molecular mechanisms underlying anxiety disorders.

Molecular Psychiatry(2017) 22, 466–475; doi:10.1038/mp.2016.87; published online 31 May 2016

INTRODUCTION

Anxiety disorders, such as generalized anxiety disorder, panic disorder, obsessive-compulsive disorder and posttraumatic stress disorder, represent the most common psychiatric illnesses, with a lifetime prevalence of approximately 30% in the United States.[1–3]

Dysfunctions of the major excitatory (glutamate) and inhibitory (gamma aminobutyric 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.[4–8]

The major environmental risk factor for anxiety disorders is exposure to traumatic and stressful life events, such as threats and social stress.[9,10] These situations activate the hypothalamic–pituitary–adrenal (HPA) axis, which ultimately leads to the enhanced secretion of glucocorticoids (GCs). GCs alter neuronal activity in various brain regions, including the hippocampus and the basolateral amygdala (BLA), which are implicated in attention, vigilance and the selection of appropriate behavioral strategies.[11] This mechanism allows the body to optimally face stress challenges and adapt to environmental stimuli.

GCs act via glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), and proper GR signaling is critical for a healthy stress response. Thus dysfunction of the HPA axis and altered GR signaling are believed to contribute to the development of anxiety disorders.[11–14] Earlier pharmacological and genetic approaches have clearly implicated the GR in the modulation of stress-related behaviors.[15–21] Both central nervous system (CNS)- and forebrain-specific GR ablation result in decreased anxiety.[16,21] However, the underlying brain regions and specific cell types that modulate GR action on fear and anxiety still remain largely unknown.

Given the reports of previous studies,[16,20–22] and the importance of the glutamate–GABA balance for the etiology of anxiety disorders, we argued that particularly GR expression in excitatory (that is, glutamatergic) neurons would impact fear- and anxiety-related behavior. To test this, we generated conditional mouse mutants lacking the receptor in glutamatergic neurons ($GR^{Glu\text{-}CKO}$ mice) and contrasted this with mice lacking GR in the majority of GABAergic neurons ($GR^{GABA\text{-}CKO}$ mice). Subsequently, we assessed whether fear- and anxiety-related behavior are regulated by GRs in glutamatergic neurons of the BLA ($GR\text{-}BLA^{Glu\text{-}CKO}$ mice). Thus our models have the unique potential to specifically dissect the role of the GR in the major excitatory and inhibitory neurotransmitter system within distinct brain regions of the CNS.

MATERIALS AND METHODS

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

[1]Department of Stress Neurobiology and Neurogenetics, Max Planck Institute of Psychiatry, Munich, Germany;[2]Department of Psychiatry, Harvard Medical School and McLean Hospital, Belmont, MA, USA and [3]Department of Translational Neuroscience, Brain Center Rudolf Magnus, UMC Utrecht, Utrecht, The Netherlands. Correspondence: Dr MV Schmidt, Department of Stress Neurobiology and Neurogenetics, Max Planck Institute of Psychiatry, Kraepelinstr. 2-10, Munich 80804, Germany
E-mail: mschmidt@psych.mpg.de
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Generation of neurotransmitter-specific conditional GR KO lines

The generation of *GR*-floxed mice was previously described.[16] Conditional *GR* mutant mice in glutamatergic or GABAergic cells were obtained by breeding $GR^{flox/flox}$ mice to *Nex-Cre* mice or *Dlx5/6-Cre* mice, respectively, using a three-generation breeding scheme (detailed information in Supplementary Materials and Methods).

Generation of Cre-driver-specific LacZ reporter lines

Mice reporting the expression of *Camk2α-*, *Nex-* and *Dlx5/6-Cre* drivers were generated by breeding hemizygous or heterozygous *Camk2α-*, *Nex-* and *Dlx5/6-Cre* mice to $Rosa26^{+/flopCrh\text{-}IRES\text{-}LacZ}$ reporter mice (referred to as $R26^{LacZ}$), flop: floxed stop.[23,24]

AAV-mediated deletion of GR in the BLA

Conditional deletion of the GR in the BLA was induced with adeno-associated virus (AAV) vectors, expressing an enhanced green fluorescent protein (EGFP) reporter and Cre recombinase under the control of the Camk2α promoter (AAV-Camk2α::EGFP-Cre, #PV1917, Penn Vector Core, University of Pennsylvania, Philadelphia, PA, USA). For the control group, AAV vectors lacking the Cre recombinase were used (AAV-Camk2α::EGFP, #PV2521, Penn Vector Core, University of Pennsylvania). Virus production, amplification and purification were performed by GeneDetect (Auckland, New Zealand). $GR^{flox/flox}$ mice were anesthetized with isoflurane, and 0.5 µl (BLA) of either AAV-Camk2α::EGFP-Cre or AAV-Camk2α::EGFP were bilaterally injected in the BLA at 0.06 µl min^{-1} by glass capillaries with tip resistance of 2–4 MΩ in a stereotactic apparatus. The following coordinates were used: BLA: 1.0 mm posterior to bregma, 3.5 mm lateral from midline, and 3.8 mm below the surface of the skull. After surgery, mice were treated for 5 days with Metacam. Behavioral testing started 4 weeks after virus injection. Successful knockout (KO) of the GR in Camk2α neurons of the BLA was verified by immunofluorescence. Animals that were not infected bilaterally in the BLA were excluded from the analysis.

Single *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 (*GR* and corticotropin-releasing hormone (*CRH*)) was performed as described previously.[25]

Double *in situ* hybridization

Frozen brains were sectioned at − 20 °C in a cryostat microtome at 20 µm, thaw mounted on Super Frost Plus slides, dried and stored at − 80 °C. Double *in situ* hybridization (DISH) enabling the simultaneous detection of two different mRNA markers was performed as previously described.[26]

Immunohistochemistry

Immunofluorescence was performed on free-floating sections as described previously[27] (detailed information in Supplementary Materials and Methods).

Neuroendocrine parameters

To determine basal corticosterone and adrenocorticotropic hormone levels, blood sampling was performed in the early morning (0830–0930 hours) and afternoon (0430–0530 hours, 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 s. For evaluation of the corticosterone response to stress, we collected blood samples 30 min (response levels) after an acute stressor (a forced swim test (FST)) by tail cut.[28]

Behavioral testing

All behavioral tests were recorded using a videotracking system (Anymaze 4.20; Stoelting, Dublin, Ireland), unless otherwise stated. The following behavioral tests were performed in the morning between 0830 and 1230 h in the same room in which the mice were housed: open field (OF), elevated plus maze (EPM), dark–light (DaLi) box, FST, and fear conditioning paradigms. Home cage activity was assessed during the dark and light cycle. The testing procedures were performed as described in Supplementary Materials and Methods.

mEPSC recordings

Neurons in the BLA were selected for recording if they displayed a pyramidal-shaped cell body. All miniature excitatory postsynaptic current (mEPSC) 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 ≈20 min via the perfusion medium. All data were acquired, stored and analyzed on a PC using pClamp 9.0 and Clampfit 9.2 (Axon Instruments, Berkshire, UK). Minimal cutoff for mEPSC analysis was 6 pA (experimental details in Supplementary Materials and Methods).

Statistical analysis

The data presented are shown as means+s.e.m. and were analyzed by the commercially available software SPSS 17.0 (SPSS, Chicago, IL, USA) and Sigma Plot 11.0 (Systat, Erkrath, Germany). The sample size was chosen such that with a type 1 error of 0.05 and a type 2 error of 0.2 the effect size should be at least 1.2-fold of the pooled s.d. 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. For four-group comparisons (chronic social defeat stress), two-way analysis of variance was performed. 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 the fear conditioning paradigms were analyzed by repeated-measure analysis of variance. *P*-values of < 0.05 were considered significant. All data were tested for outliers using the Grubbs' test. Homogeneity of variances was tested using the Bartlett's test. Animals were allocated to experimental groups in a semirandomized manner, and data analysis was performed blinded to the group allocation.

RESULTS

Neurotransmitter identity of GR-expressing neurons

In order to assess the distribution of GR expression within the major limbic excitatory and inhibitory brain circuits, we performed DISH. Simultaneous detection of ^{35}S-labeled GR and digoxigenin-labeled vesicular glutamate transporter 1 (Vglut1) riboprobes revealed predominant expression of GR in glutamatergic neurons of the hippocampus, BLA and throughout the cortical layers. Minimal-to-no co-expression was observed for the central amygdala (CeA), confirming earlier findings, which demonstrate a prominent expression of GABAergic markers in this region[29–31] (Figure 1a). In support, DISHs performed against GR and the GABAergic markers glutamic acid decarboxylase 65 and 67 (Gad65/67) revealed a strong co-localization in the CeA. In addition, GR mRNA was also present in GABAergic interneurons of the hippocampus and cortex and few Gad65/67 cells of the BLA (Figure 1b). In summary, GR expression within the cortex, hippocampus and BLA is predominantly confined to Vglut1-positve glutamatergic neurons, whereas expression in the CeA is largely restricted to Gad65/67-positive GABAergic neurons.

Next we used conditional mutagenesis to genetically dissect the specific involvement of GRs in glutamatergic and GABAergic neuronal subpopulations. We crossed $GR^{flox/flox}$ mice with *Nex-Cre* or *Dlx5/6-Cre* mice to generate the following lines: $GR^{Glu\text{-}CKO}$ mice, where *GR* is deleted in forebrain glutamatergic neurons, and $GR^{GABA\text{-}CKO}$ mice, carrying a *GR* deletion in forebrain GABAergic neurons. *In situ* hybridization demonstrated that lack of GR mRNA in $GR^{Glu\text{-}CKO}$ mice was most prominent in the cortex and limbic regions, including the BLA and dorsal and ventral hippocampus. We did not observe a significant loss of GRs in the dentate gyrus, which might be caused by the repopulation of GR-expressing newborn neurons during adulthood, considering that the NEX promoter is only transiently active in the dentate gyrus granule cells.[32] In addition, the absence of GR was also detected in a number of paraventricular nucleus (PVN) neurons (Figure 2a).

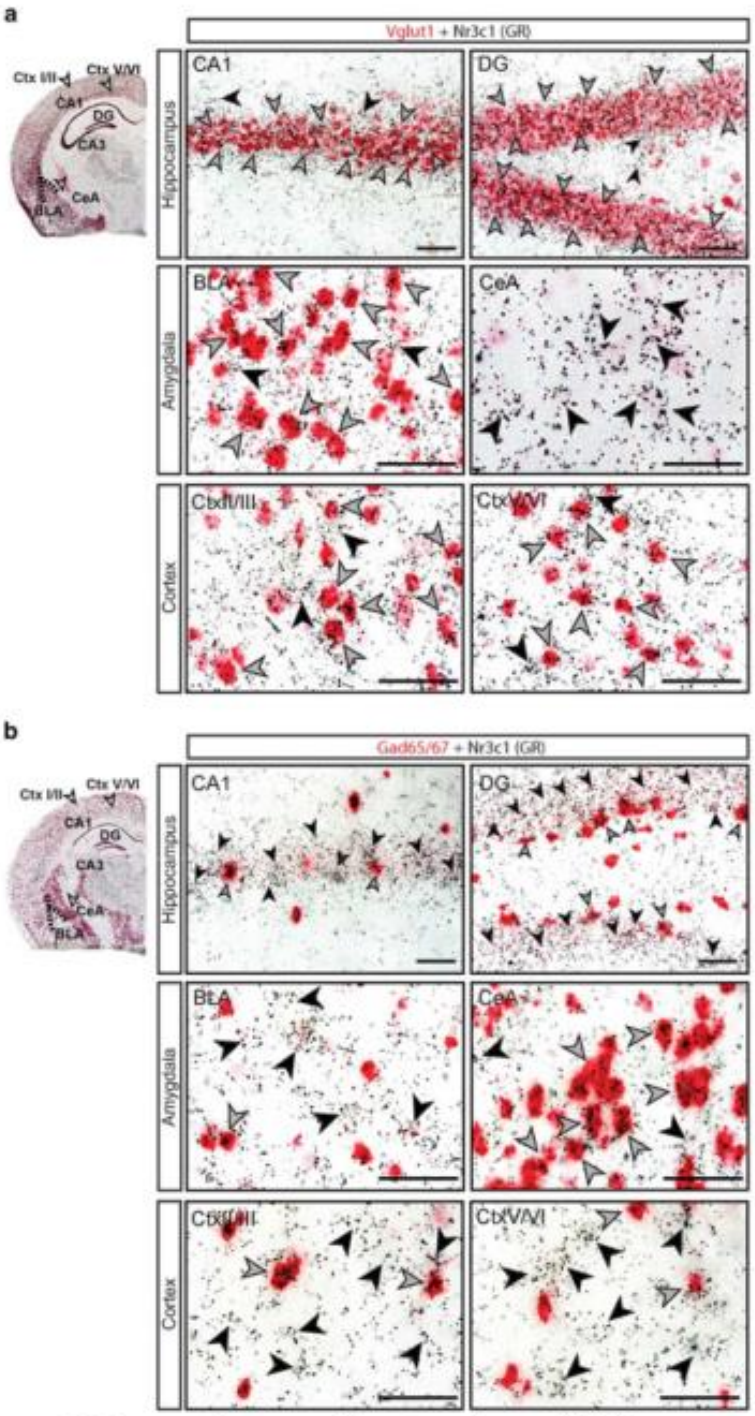

Figure 1. Glucocorticoid receptor (GR) is expressed in neurons of the major excitatory and inhibitory neurotransmitter systems within the brain. GR was co-localized with neurotransmitter-specific markers by double *in situ* hybridization using wild-type mice. (**a**) There is a predominant expression of GR in glutamatergic (Vglut1) neurons of the hippocampus, the basolateral amygdala (BLA) and throughout the cortex. Minimal-to-no co-expression was observed for the central amygdala (CeA). (**b**) GR strongly co-localized with GABAergic (Gad65/67) neurons in the CeA. In addition, GR mRNA was also present in GABAergic neurons of the hippocampus and cortex, and few Gad65/67 cells of the BLA. Black arrowheads indicate examples of cells expressing only GR (silver grains). Gray arrowheads indicate cells co-expressing GR and the respective markers (red staining). Scale bar, 50 μm. DG, dentate gyrus.

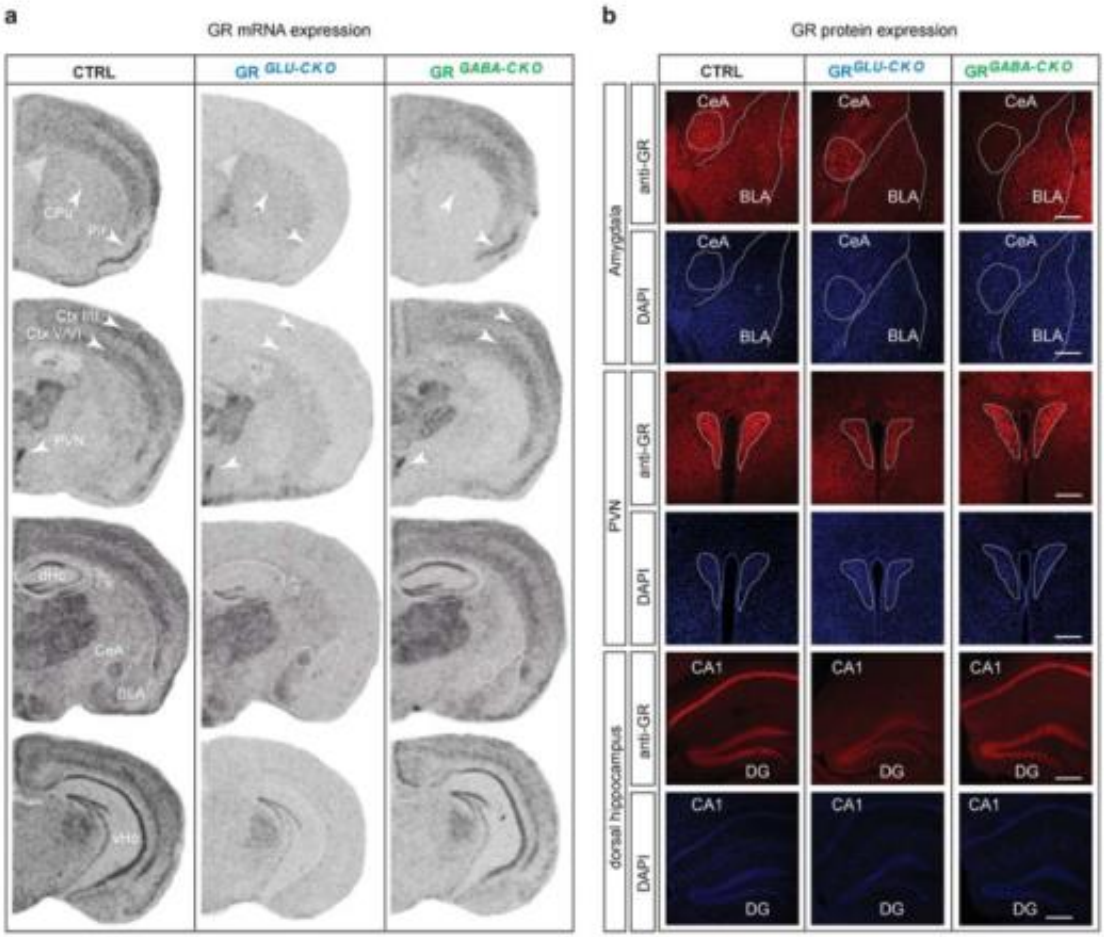

Figure 2. Neurotransmitter-specific *GR-CKO* lines lack *GR* expression in a cell type-specific manner. (**a**) Expression of *GR* mRNA was assessed by *in situ* hybridization in wild-type and neurotransmitter-specific *GR-CKO* lines. Autoradiographs of glucocorticoid receptor (GR) mRNA expression pattern 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; CeA, central amygdala; BLA, basolateral amygdala; vHc, ventral hippocampus. (**b**) Coronal sections of control and mutant mice were stained for GR protein and DAPI (4,6-diamidino-2-phenylindole). DG, dentate gyrus. 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.

Notably, mice (non-specifically) lacking the GR in principal forebrain neurons have previously been generated by breeding $GR^{flox/flox}$ mice with *Camk2α-Cre* mice. Although Camk2α is predominately expressed in excitatory projection neurons, it is also found in GABAergic medium spiny neurons of the striatum.[33–36] In addition, GABAergic Camk2α-positive neurons have recently been identified in the bed nucleus of the stria terminalis and shown to modulate anxiety-related behavior.[37] Consequently, *Nex-Cre*-mediated inactivation represents a much more selective approach to assess GR deletion specifically in glutamatergic neurons. In fact, analyses of *LacZ* mRNA expression in *Camk2α-Cre* and *Nex-Cre* reporter mice showed that, in contrast to Nex-mediated Cre activity, *Camk2α-Cre* is additionally expressed in a subset of neurons of the caudate putamen, CeA, septum and bed nucleus of the stria terminalis (Supplementary Figure S1). Moreover, the *LacZ* expression pattern in *Nex-Cre* reporter mice ($R26^{LacZ}Nex$) strongly resembles endogenous *Vglut1* expression, which is largely absent from the striatum, as well as from the thalamic and hypothalamic nuclei.[30,38,39] However, lack of GR in a subset of PVN neurons in $GR^{Glu\text{-}CKO}$ mice also suggests recombination in *Vglut2*-containing neurosecretory PVN neurons.[39–41]

Deletion of GR mRNA in $GR^{GABA\text{-}CKO}$ mice was most obvious not only in GABAergic neurons of the caudate putamen and CeA but also detected in hippocampal and cortical interneurons (Figure 2a and Supplementary Figure S2). The latter only constitute a small fraction of cortical and hippocampal neurons, and hence of GR-expressing cells, which is reflected in the apparent lack of the GR mRNA deletion pattern in $GR^{GABA\text{-}CKO}$ mice. However, scattered expression of *LacZ* mRNA in the cortex and hippocampus of *Dlx-Cre* reporter mice ($R26^{LacZ}Dlx5/6$) clearly shows Dlx5/6-Cre-mediated recombination in these regions (Supplementary Figure S1). In addition, previous work has confirmed the exclusive GABAergic identity of Cre-expressing neurons in *Dlx5/6-Cre* mice.[26,42,43] Lack of GR expression in $GR^{Glu\text{-}CKO}$ and $GR^{GABA\text{-}CKO}$ mice was also evident at the protein level (Figure 2b and Supplementary Figure S2). Overall, the pattern of GR deletion in both conditional KO lines nicely mirrored the co-expression patterns

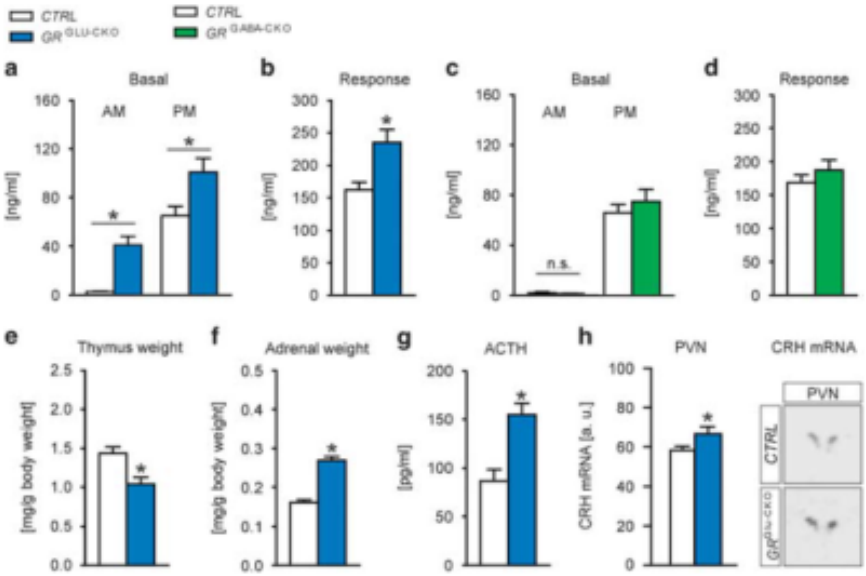

Figure 3. Glucocorticoid receptor (GR) signaling in glutamatergic, but not GABAergic, neurons is necessary for appropriate hypothalamic–pituitary–adrenal axis activity. (**a**) Basal am and pm corticosterone levels were increased in $GR^{Glu\text{-}CKO}$ mice (am: Mann–Whitney test (MW-test), $T = 153.00$, $P < 0.001$, ctrl $n = 12$, $GR^{Glu\text{-}CKO}$ $n = 9$; pm: *t*-test, $T_{21} = -2.697$, $P < 0.05$, ctrl $n = 12$, $GR^{Glu\text{-}CKO}$ $n = 11$). (**b**) 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 $n = 12$, $GR^{Glu\text{-}CKO}$ $n = 9$). (**c** and **d**) Basal am and pm, as well as response, corticosterone levels did not differ between $GR^{GABA\text{-}CKO}$ and control littermates (ctrl $n = 12$, $GR^{GABA\text{-}CKO}$ $n = 10$). (**e**) Thymus weight was decreased in $GR^{Glu\text{-}CKO}$ mice (*t*-test, $T_{19} = 3.190$, $P < 0.01$, ctrl $n = 12$, $GR^{Glu\text{-}CKO}$ $n = 9$). (**f**) Adrenal gland weight was increased in $GR^{Glu\text{-}CKO}$ mice (*t*-test, $T_{19} = -9.484$, $P < 0.01$, ctrl $n = 12$, $GR^{Glu\text{-}CKO}$ $n = 9$). (**g**) Basal adrenocorticotropic hormone (ACTH) levels were increased in $GR^{Glu\text{-}CKO}$ mice (MW-test, $T = 171.00$, $P < 0.001$, ctrl $n = 12$, $GR^{Glu\text{-}CKO}$ $n = 9$). (**h**) (left) Corticotropin-releasing hormone (CRH) mRNA levels in the paraventricular nucleus (PVN) were increased in $GR^{Glu\text{-}CKO}$ mice (*t*-test, $T_{17} = -2.226$, $P < 0.05$, ctrl $n = 11$, $GR^{Glu\text{-}CKO}$ $n = 8$); (right) Representative *in situ* hybridization images of CRH mRNA expression in the PVN. *$P < 0.05$, data are expressed as mean+s.e.m.

observed with DISH, highlighting the selective neurotransmitter type-specific deletion properties of the generated mouse mutants.

Absence of GRs in forebrain glutamatergic neurons results in HPA axis hyperactivity

$GR^{Glu\text{-}CKO}$ mice exhibit absence of GR expression not only in glutamatergic neurons of the PVN (an important feedback site for GCs) but also in the hippocampus and the BLA, which represent additional key players and regulators of the HPA axis. We therefore investigated whether neurotransmitter-specific deletion of GR in forebrain glutamatergic or GABAergic circuits 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 with $GR^{Glu\text{-}Ctrl}$ mice (Figure 3a). Moreover, response levels taken 30 min after an acute stressor (FST) showed significantly increased corticosterone levels in $GR^{Glu\text{-}CKO}$ (Figure 3b), in line with impaired negative feedback via GRs. Remarkably, we did not observe any changes in corticosterone levels in the $GR^{GABA\text{-}CKO}$ mice (Figures 3c and d). Consequently, we analyzed adrenal and thymus gland weight, as alterations in these organs are often associated with changes in HPA axis activity. Indeed, adrenal gland weight was significantly increased, and thymus weight was significantly decreased in $GR^{Glu\text{-}CKO}$ mice compared with controls (Figures 3e and f), while there were no changes at all in $GR^{GABA\text{-}CKO}$ mice (Supplementary Figures S3A and B). Similarly, basal adrenocorticotropic hormone levels were increased in $GR^{Glu\text{-}CKO}$, but not in $GR^{GABA\text{-}CKO}$ mice, supporting a predominant central HPA-hyperdrive in $GR^{Glu\text{-}CKO}$ animals (Figure 3g, Supplementary Figure S3C). Moreover, we detected significantly increased CRH mRNA levels in the PVN of $GR^{Glu\text{-}CKO}$ mice (Figure 3h). Although there were no differences in body weight of $GR^{Glu\text{-}CKO}$ mice, we found significantly reduced body weight in $GR^{GABA\text{-}CKO}$ mice (Supplementary Figures S3D and E). Because of the robust endocrine phenotype of $GR^{Glu\text{-}CKO}$ mice, we continued to check for potential differences in circadian behavior. To investigate baseline activity in a familiar environment that is not compromised by novelty, home cage activity was monitored during the dark and light phase by an automated infrared tracking system. However, we observed no differences in locomotor activity in $GR^{Glu\text{-}CKO}$ mice compared with littermate controls (Supplementary Figure S3F).

GR activity in forebrain glutamatergic neurons controls fear and anxiety

Next we assessed the behavioral consequences of GR deletion in forebrain GABAergic or glutamatergic neurons on anxiety and fear. In the EPM test, $GR^{Glu\text{-}CKO}$ mice showed reduced anxiety-like behavior as compared with control littermates, which is evident from increased open arm time and entries (Figure 4a). The low-anxiety phenotype of $GR^{Glu\text{-}CKO}$ mice was further confirmed in the DaLi 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 with $GR^{Glu\text{-}Ctrl}$ mice (Figure 4b). The anxiolytic phenotype was independent of alterations in general locomotion, as total distance traveled in the OF test (Supplementary Figures S4A and B) and home cage activity (Supplementary Figure S3F) did not differ between the two genotypes. Moreover, stress-coping behavior in the FST was not altered in $GR^{Glu\text{-}CKO}$ mice (Supplementary Figures S4C and D). No changes in anxiety-related and stress-coping behavior were observed in $GR^{GABA\text{-}CKO}$ mice (Figures 4c and d; Supplementary Figures S4G and H); however, general locomotion was increased in

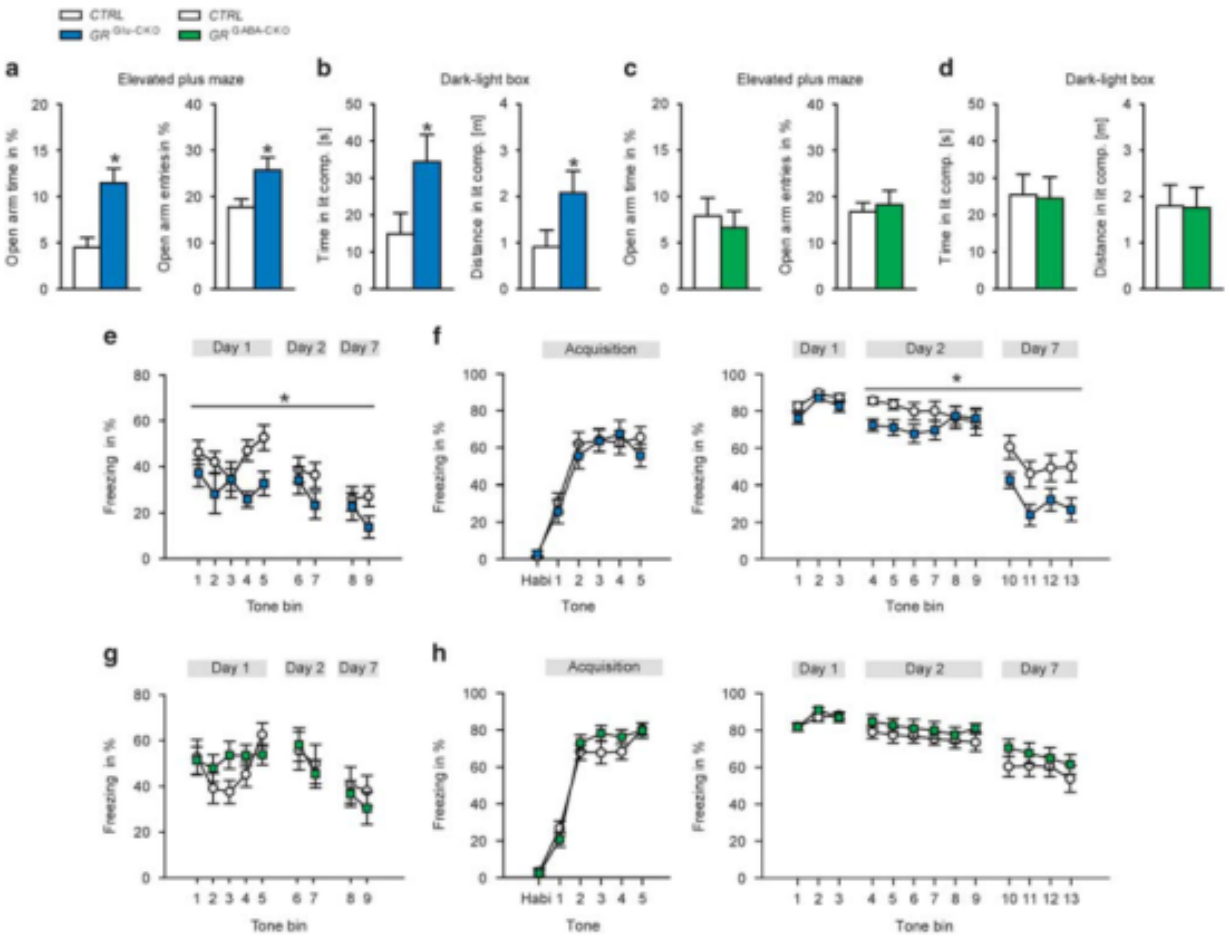

Figure 4. Glucocorticoid receptor (GR) in glutamatergic, but not in GABAergic, neurons selectively drives anxiety-related behavior and fear. (**a**) $GR^{Glu\text{-}CKO}$ mice spent more time in (*t*-test, $T_{17} = -3.823$, $P < 0.001$) and showed more entries into the open arms (*t*-test, $T_{17} = -2.602$, $P < 0.05$) of the elevated plus maze (EPM) (ctrl $n = 11$, $GR^{Glu\text{-}CKO}$ $n = 8$). (**b**) $GR^{Glu\text{-}CKO}$ mice spent more time in (Mann–Whitney test (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 dark–light (DaLi) box (ctrl $n = 12$, $GR^{Glu\text{-}CKO}$ $n = 9$). (**c** and **d**) Anxiety-related behavior was not altered in $GR^{GABA\text{-}CKO}$ mice in the EPM and DaLi (ctrl $n = 12$, $GR^{GABA\text{-}CKO}$ $n = 11$. (**e**) $GR^{Glu\text{-}CKO}$ mice showed a reduced freezing response to the conditioned stimulus (tone) in a neutral environment during the course of all extinction trials (repeated-measure analysis of variance (ANOVA), $F_{1,\ 22} = 4.976$, $P < 0.05$) after conditioning with a single tone–footshock pairing (ctrl $n = 13$, $GR^{Glu\text{-}CKO}$ $n = 12$). (**f**) No differences in freezing were found between $GR^{Glu\text{-}CKO}$ mice and control mice in the acquisition and consolidation (day 1) of another aversive fear conditioning paradigm with five tone–footshock pairings in a different batch of mice ($n = 12$ per group). During the extinction sessions (day 2 and 7), $GR^{Glu\text{-}CKO}$ mice showed a significantly reduced freezing response to the conditioned stimulus (repeated-measure ANOVA, $F_{1,\ 22} = 4.670$, $P < 0.05$ ($n = 12$ per group). (**g** and **h**) Freezing behavior did not differ in $GR^{GABA\text{-}CKO}$ mice in any of the two fear conditioning paradigms (ctrl $n = 12$, $GR^{GABA\text{-}CKO}$ $n = 11$); $^*P < 0.05$, data are expressed as mean+s.e.m.

$GR^{GABA\text{-}CKO}$ mice compared with control littermates in the OF test (Supplementary Figures S4E and F). As the contribution of forebrain GABAergic GRs to neuroendocrine and behavioral alterations may only be apparent under severe stress conditions, we subjected $GR^{GABA\text{-}CKO}$ mice to 3 weeks of chronic social defeat stress. However, we still observed no genotype-dependent differences with regard to corticosterone levels, anxiety, social behavior or stress coping (Supplementary Figure S5).

A key feature of clinical anxiety disorders is a failure to appropriately inhibit, or extinguish, fear.[44] Pavlovian fear conditioning represents one of the best rodent models to assess cognitive processes related to fear. It consists of the pairing of a conditioned stimulus (CS) with an aversive unconditioned stimulus (US; electric footshock), which mainly induces increased freezing as a conditioned fear response.[10] Thus we subjected both $GR^{Glu\text{-}CKO}$ and $GR^{GABA\text{-}CKO}$ mice to a fear conditioning paradigm with a single CS–US pairing. In order to subsequently assess the freezing response to the tone (cued fear) without confounding influences of contextual memory, conditioned mice were only tested in a neutral environment. $GR^{Glu\text{-}CKO}$ mice did not show any significant differences during fear retrieval (first three tone bins of session 1) compared with controls. However, $GR^{Glu\text{-}CKO}$ mice demonstrated enhanced fear extinction compared with $GR^{Glu\text{-}Ctrl}$ mice, as depicted in the time freezing over the course of the three extinction sessions (Figure 4e). To resolve whether the enhanced fear extinction in $GR^{Glu\text{-}CKO}$ mice may be the result of altered fear learning, we subjected another batch of mice to a paradigm with five CS–US pairings,[45] allowing the analysis of fear acquisition, consolidation and extinction in more detail. $GR^{Glu\text{-}CKO}$ mice did not show any alterations in fear learning compared with $GR^{Glu\text{-}Ctrl}$ mice (Figure 4f, left). However, while there were also no alteration during the fear consolidation session (tone bins 1–3), $GR^{Glu\text{-}CKO}$ mice demonstrated significantly enhanced fear extinction compared with $GR^{Glu\text{-}Ctrl}$ mice during the extinction phase (tone

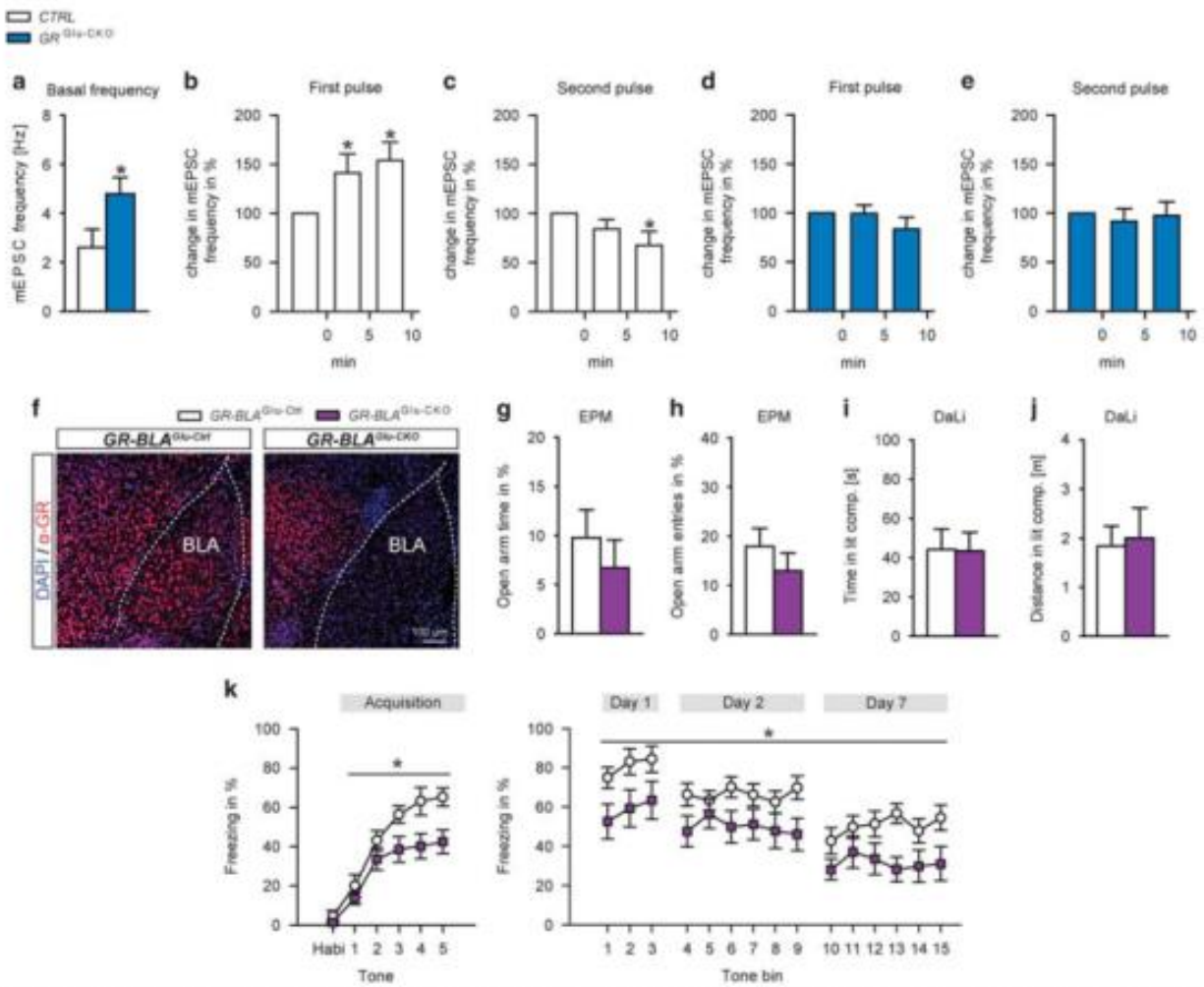

Figure 5. Glucocorticoid receptor (GR) signaling in glutamatergic neurons of the basolateral amygdala (BLA) is essential for responses to corticosterone, and cued fear conditioning. (**a**) $GR^{Glu\text{-}CKO}$ compared with control mice showed an increased basal miniature excitatory postsynaptic current (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 (20 min, 100 nM) of corticosterone with increased mEPSC frequency ($P < 0.05$, $n = 8$) and with a decreased mEPSC frequency to a second pulse (20 min, 100 nM) applied 1 h later ($P < 0.05$, $n = 6$). (**d** and **e**) BLA responses to a first corticosterone pulse were not present in $GR^{Glu\text{-}CKO}$ mice ($n = 8$). BLA responses to a second pulse of corticosterone were not present in $GR^{Glu\text{-}CKO}$ mice ($n = 8$). (**f**) Representative images of $GR^{flox/flox}$ mice injected with AAV-Camk2α::GFP-Cre (*GR-BLA*$^{Glu\text{-}CKO}$) or AAV-Camk2α::GFP (*GR-BLA*$^{Glu\text{-}Ctrl}$) in the BLA. Green fluorescent protein (GFP) in green, GR immunostaining in red. (**g**–**j**) Anxiety-related behavior was not affected in *GR-BLA*$^{Glu\text{-}CKO}$ mice (*GR-BLA*$^{Glu\text{-}Ctrl}$ $n = 10$, *GR-BLA*$^{Glu\text{-}CKO}$ $n = 12$). (**k**) *GR-BLA*$^{Glu\text{-}CKO}$ mice showed significantly attenuated fear learning during conditioning (repeated-measure analysis of variance (ANOVA), $F_{1,\ 20} = 6.620$, $P < 0.05$) as well as decreased fear expression (day 1) and enhanced extinction (days 2 and 7) (repeated-measure ANOVA, $F_{1,\ 20} = 6.199$, $P < 0.05$) following fear conditioning (*GR-BLA*$^{Glu\text{-}Ctrl}$ $n = 10$, *GR-BLA*$^{Glu\text{-}CKO}$ $n = 12$). *$P < 0.05$, data are expressed as mean ± s.e.m. DAPI, 4,6-diamidino-2-phenylindole; EPM, elevated plus maze.

bins 4–13) of days 2 and 7 after conditioning, reflected in significantly reduced freezing responses (Figure 4f, right). Thus the initial findings of an enhanced fear extinction phenotype in $GR^{Glu\text{-}CKO}$ mice is independent of fear learning during the acquisition session, which was confirmed using a different paradigm in a separate batch of mice. Interestingly, $GR^{GABA\text{-}CKO}$ mice did not exhibit any alterations in fear learning, consolidation and fear extinction (Figures 4g and h) in any of the two fear conditioning paradigms.

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

Persistence of fear involves the BLA.[46] Corticosterone strengthens (BLA-dependent) cue-related fear in C57Bl/6 mice,[47] and rapid GR-dependent effects in the BLA are important for stable emotional memory.[48] We reasoned that impaired GR-dependent signaling in the BLA may contribute to the less stable fear phenotype of $GR^{Glu\text{-}CKO}$ mice. To probe the underlying neurobiological substrate, we focused on GR-sensitive signaling specifically in BLA glutamatergic cells, that is, the frequency of mEPSCs.[49] Thus BLA cells rapidly respond to a 20-min application (pulse) of 100 nM corticosterone with increased mEPSC frequency via MRs[49]; yet, a second pulse (20 min, 100 nM, 1 h after the first pulse) causes decreased mEPSC frequency—a phenomenon called 'metaplasticity'—via a GR-dependent mechanism.[49] This GR-dependent decrease does not occur in the hippocampus, where the second pulse causes mEPSC frequency enhancement, similar to the first.[49]

Basal mEPSC frequency was significantly increased in the principal BLA neurons of $GR^{Glu\text{-}CKO}$ compared with $GR^{Glu\text{-}Ctrl}$ mice (Figure 5a), comparable to mice with GR ablation in all BLA cells.[49] As expected, BLA cells in $GR^{Glu\text{-}Ctrl}$ mice responded to a first pulse of corticosterone with increased mEPSC frequency (Figure 5b; Supplementary Table S2), whereas exposure to a second pulse caused a decrease in mEPSC frequency, earlier shown to be GR dependent (Figure 5c). In $GR^{Glu\text{-}CKO}$ mice, this GR-dependent reduction in mEPSC frequency in response to the second corticosterone pulse did not occur (Figure 5e). Notably, the (MR-dependent) response to the first pulse was also attenuated (Figure 5d), which was somewhat unexpected as we did not observe alterations in MR mRNA levels in the BLA of $GR^{Glu\text{-}CKO}$ mice (Supplementary Figure S6). Hippocampal cells (not liable to metaplasticity) of $GR^{Glu\text{-}CKO}$ mice showed a clearly enhanced mEPSC frequency to the second pulse (prior to corticosterone: 0.36+0.05 Hz, during corticosterone: 0.51+0.08 Hz, $n = 9$; $P = 0.004$).

GR deletion in glutamatergic neurons of the BLA alters fear but not anxiety behavior

Based on the electrophysiology results, we elaborated to which extent the fear-suppressing and anxiolytic phenotype of $GR^{Glu\text{-}CKO}$ mice is mediated by GRs in the glutamatergic neurons of BLA. For this, we injected AAV vectors expressing either Camk2α::GFP-Cre (*GR-BLA*$^{Glu\text{-}CKO}$) or Camk2α::GFP (*GR-BLA*$^{Glu\text{-}Ctrl}$) constructs into the BLA of $GR^{flox/flox}$ mice (Figure 5f and Supplementary Figure S7A). Thus Cre-expression is driven by the Camk2α promoter and therefore largely restricted to excitatory neurons.[23,24,26] Moreover, in contrast to the CeA, the BLA primarily contains excitatory glutamatergic neurons (Figure 1).[50–52] Consequently, Cre-mediated GR deletion in the BLA of $GR^{flox/flox}$ mice should almost entirely be restricted to glutamatergic neurons. Following viral injections and recovery for 4 weeks, *GR-BLA*$^{Glu\text{-}Ctrl}$ and *GR-BLA*$^{Glu\text{-}CKO}$ mice were subjected to the OF, EPM, DaLi box and the auditory fear conditioning paradigm with five CS–US pairings. Interestingly, we did not observe any differences in anxiety between *GR-BLA*$^{Glu\text{-}Ctrl}$ and *GR-BLA*$^{Glu\text{-}CKO}$ mice in the EPM or DaLi box test (Figures 5g-j) and no changes in general locomotion in the OF (Supplementary Figure S7B). In contrast, disruption of GR expression in glutamatergic neurons of the BLA resulted in significantly reduced fear learning during acquisition (Figure 5k, left). Along these lines, *GR-BLA*$^{Glu\text{-}CKO}$ mice also showed significantly reduced fear expression and enhanced fear extinction compared with control mice in the subsequent test sessions on days 1, 2 and 7 after conditioning (Figure 5k, right).

DISCUSSION

In this study, we employed a unique set of transgenic mice to delineate whether the GR modulates neuroendocrine regulation as well as fear- and anxiety-related behavior primarily via excitatory (glutamatergic) or inhibitory (GABAergic) forebrain circuits. Our results provide substantial evidence that GR signaling in the forebrain glutamatergic, but not in the GABAergic, neurotransmitter system is crucially involved in regulating stress system activity, fear and anxiety.

Several conditional GR KO mouse studies previously contributed to our understanding of GR-mediated control of HPA axis activity. In particular, conditional deletion of GR in the CNS (GR^{NesCre} mice) resulted in HPA axis hyperactivity, possibly owing to GR deletion in the PVN.[16] Moreover, disruption of GR limited to adult forebrain neurons (forebrain-specific GR KO (*FBGRKO*) mice (Camk2α-Cre), which primarily, but not exclusively, lack GR in forebrain excitatory neurons) led to a mild form of HPA axis hyperactivity.[53] More recently, hypercorticosteroidism was observed in PVN-specific GR KO mice (*Sim1Cre-GRe3Δ* mice).[54] Our observations of HPA axis hyperactivity in $GR^{Glu\text{-}CKO}$ mice, with GR deletion in limbic structures and the PVN, are in line with these results. We detected an upregulation of *Crh* mRNA in the PVN of $GR^{Glu\text{-}CKO}$ mice, which might result from disrupted negative-feedback control in glutamatergic, CRH-expressing GR neurons, and thus further potentiate HPA axis activity. Along these lines, a recent study demonstrated that the great majority of PVN CRH neurons co-express VGLUT2.[55] Thus it is likely that Nex-Cre-mediated recombination, and hence GR deletion, occurred in VGLUT2-expressing CRH neurons of the PVN. However, the precise percentage of glutamatergic CRH/GR-positive neurons in the PVN remains to be determined. Notably, glutamatergic and GABAergic forebrain neurons in $GR^{Glu\text{-}CKO}$ and $GR^{GABA\text{-}CKO}$ mice, respectively, are 'protected' against the raised corticosterone levels owing to GR deletion and, if anything, will show enhanced MR activation.[56] From our study, we can conclude that especially the GR in forebrain glutamatergic neurons has a prominent role in mediating the negative feedback on the HPA axis; however, this does not exclude a GR-dependent role of GABAergic neurons in HPA axis regulation, for instance, as an interface between GR-carrying excitatory extrahypothalamic neurons and CRH-expressing neurons in the PVN.[57]

Genetic mouse models of altered GR expression have also highlighted the crucial involvement of brain GR in emotional behavior.[15] Specifically, conditional overexpression of the GR in the forebrain and the limbic system (*GRov* mice) led to increased anxiety-like behavior.[18] Fittingly, CNS-specific GR KO mice (GR^{NesCre} mice) and FBGRKO, as well as $GR^{Glu\text{-}CKO}$ mice in this study, demonstrated reduced anxiety-related behavior.[16,20,21] However, the interpretation of the anxiolytic phenotype in FBGRKO is confounded by the observed increase in locomotion during the DaLi box and EPM test. In contrast to our results, despair-related behavior was enhanced in *FBGRKO* mice.[20,53] Interestingly, PVN-specific GR KO mice (*Sim1Cre-GRe3Δ* mice) and mice lacking GR in the CeA demonstrated no alterations in anxiety-related or (in the case of *Sim1Cre-GRe3Δ* mice) despair behavior.[54,58] Along these lines, we observed no significant changes in anxiety upon specific deletion of the GR in glutamatergic neurons of the BLA. This potentially suggests that GR action in glutamatergic neurons of other limbic structures, such as the hippocampus or prefrontal cortex, or in glutamatergic circuits of several brain regions is primarily involved in modulating anxiety-related behavior.

Although many of the previous studies examined the effects of GR deletion on anxiety in relatively non-homogeneous populations of neurons, we were able to isolate the specific contribution of the GR in forebrain glutamatergic neurons. Not only are these behavioral effects not observed when GR is lacking in GABAergic neurons 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 an overt anxiety-related phenotype.[17] Also an additional challenge of the system by chronic stress did not reveal a role of GABAergic GRs in stress system regulation and anxiety. Nonetheless, we cannot rule out a potential contribution of non-*Dlx5/6* expressing GABAergic interneurons in anxiety-related phenotypes. In addition, GRs in forebrain GABAergic neurons could be of relevance for other behavioral domains. In fact, the slight hyperlocomotion in $GR^{GABA\text{-}CKO}$ mice might be a consequence of the pronounced receptor deletion throughout the striatum/CPu, a structure which is highly relevant for the coordination of movement.

An inability to properly extinguish fear is often observed in patients suffering from anxiety disorders, such as posttraumatic stress disorder and phobias.[44] Rodent studies have shown that corticosterone can facilitate and is necessary for fear extinction.[47,58–60] However, while most of these studies applied acute pharmacological approaches or targeted GR deletion in a non-cell type-specific manner, we were able to specifically dissect the contribution of GRs over a prolonged period of time (similar to the timeframe in disease development) in glutamatergic and GABAergic neurons. Lack of GR in forebrain glutamatergic, but not

GABAergic, neurons led to enhanced tone-fear extinction in two different fear conditioning paradigms, while fear learning remained unaffected. Interestingly, no overt fear conditioning phenotype was reported in *FBGRKO* mice.[58] The discrepancies in fear conditioning between *FBGRKO* and $GR^{Glu\text{-}CKO}$ mice are most likely related to differences in deletion patterns and deletion time points and the subsequently triggered compensatory mechanism. Nex-Cre-induced deletion is exclusively restricted to glutamatergic neurons (mostly Vglut1-positive), most prominently of the cortex, hippocampus and BLA, and initiated during early development (E11.5).[32] On the other hand, Camk2α-induced deletion is initiated postnatally (P16–20)[24] and primarily, but not exclusively, not only observed in excitatory neurons of the cortex, hippocampus and BLA but also detected in neurons of the CeA, striatum and thalamus. Importantly, our results suggest that GRs in glutamatergic neurons of the BLA mediate conditioned fear but not anxiety. Notably, whereas $GR^{Glu\text{-}CKO}$ mice only displayed enhanced extinction, AAV-mediated deletion of the GR in glutamatergic neurons of the BLA ($GR\text{-}BLA^{Glu\text{-}CKO}$ mice) additionally resulted in decreased fear learning and fear expression. The more drastic effect on conditioned fear in $GR\text{-}BLA^{Glu\text{-}CKO}$ mice might be explained by the more instant (viral-mediated) deletion process of the GR during adulthood specifically in glutamatergic neurons of the BLA, as opposed to the gradual GR deletion process, which occurs in $GR^{Glu\text{-}CKO}$ mice throughout development within the entire excitatory circuit, and might thus be more prone to induce compensatory changes. Our results are in line with previous studies, which have repeatedly implicated GR signaling in the BLA with the formation and consolidation of fear memories.[22,61,62] Along these lines, GR antagonist application into the BLA attenuates fear-related behavior and disrupts traumatic memories.[63–65] Interestingly, viral-induced deletion of GR in the CeA (which is predominantly GABAergic) was shown to reduce contextual as well as auditory cued freezing following fear conditioning.[58] Collectively, these and our results support a role for the GR in the BLA and CeA in the regulation of fear-related behavior during adulthood. The fact that we observed no alterations in fear conditioning in $GR^{GABA\text{-}CKO}$ mice (in which GR is also deleted in the CeA) might be the result of compensatory mechanisms owing to developmental GR deletion and/or absence of GR in GABAergic neurons throughout the brain. Of course, it cannot be entirely excluded that viral spread outside the CeA in the previous study[58] or the BLA in our study might have partially influenced the behavioral outcomes.

In search of a possible mechanism whereby the lack of GR expression in glutamatergic BLA neurons could lead to less stable fear memory, we assessed mEPSCs—which reflect the spontaneous release of a glutamate-containing vesicle—in principal neurons of the BLA in $GR^{Glu\text{-}CKO}$ and control mice. High levels of corticosterone quickly and long-lastingly enhance glutamatergic transmission in BLA neurons, via nongenomic actions requiring MRs, possibly allowing an extended timeframe for encoding of emotional aspects during stressful events.[49] A second pulse of corticosterone applied 1 h later, though, leads to a quick suppression of glutamatergic transmission, a GR-dependent phenomenon. This GR-dependent reduction by a second pulse was completely abolished in $GR^{Glu\text{-}CKO}$ mice. Interestingly, even the response to the first pulse, which is MR-dependent, was absent in $GR^{Glu\text{-}CKO}$ mice, although MR mRNA levels were not altered in $GR^{Glu\text{-}CKO}$ mice. Possibly, protein level and localization of MRs (for example, availability in the plasma membrane) or systems downstream of the MR were changed, owing to prolonged GR knockdown and/or the associated hypercorticosteroidism. Regardless of the mechanism, the results clearly show attenuated GC signaling related to BLA glutamatergic transmission in $GR^{Glu\text{-}CKO}$ mice.

Taken together, our study supports that GR signaling in forebrain glutamatergic, but not GABAergic, neurons mediates fear and anxiety behavior and has a critical role in the regulation of HPA axis activity. Moreover, we were able to further disentangle GR-mediated anxiety- and fear-related behaviors. Our results suggest that pathological anxiety might result from alterations in GR signaling in glutamatergic circuits of several forebrain regions, while modulation of fear-related behavior can largely be ascribed to GR signaling in glutamatergic neurons of the BLA. Our study provides a clear dissection of GR action in phenotypically distinct neuronal populations, which adds significant clarity to its role in stress-related emotional behavior. These findings further underline the importance of GR-dependent glutamatergic pathways in the development of psychopathologies related to fear and anxiety, which is of relevance to future pharmacological approaches.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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2.2. Lack of FKBP51 shapes brain structure and connectivity in male mice

Engelhardt C., Boulat B., Czisch M., and Schmidt M.V.

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ORIGINAL RESEARCH

Lack of FKBP51 Shapes Brain Structure and Connectivity in Male Mice

Clara Engelhardt, MRes,[1,3*] Benoit Boulat, PhD,[2] Michael Czisch, PhD,[2] and Mathias V. Schmidt, PhD[1*]

Background: Stress exposure as well as psychiatric disorders are often associated with abnormalities in brain structure or connectivity. The co-chaperone FK506-binding protein 51 (FKBP51) is a regulator of the stress system and is associated with a risk to develop stress-related mental illnesses.
Purpose: To assess the effect of a general FKBP51 knockout on brain structure and connectivity in male mice.
Study Type: Animal study.
Animal Model: Two cohorts of FKBP51 knockout (51KO) and wildtype (WT) mice. The first cohort was comprised of n = 18 WT and n = 17 51KOs; second cohort n = 10 WT and n = 9 51KOs.
Field Strength/Sequence: 9.4T/3D gradient echo (VBM), DTI-EPI (DTI).
Assessment: Voxel-based morphometry (VBM) and diffusion tensor imaging (DTI). For VBM, all procedures were executed in SPM12. DTI: FMRIB Software Library (FSL) Tract Based Statistics (TBSS) were integrated within DTI-TK, allowing the creation of a mean FA skeleton. A voxelwise statistical analysis was applied between WT and 51KO mice.
Statistical Test: Volumetric differences were collected at a threshold of $P < 0.005$, and only clusters surviving a familywise error correction on the cluster level (pFWE, cluster <0.05) were further considered. VBM data were analyzed using a two-sample t-test. The Threshold Free Cluster Enhancement (TFCE) method was used to derive uncorrected-P statistical results at a P-level of 0.01.
Results: The structural analysis revealed two clusters of significantly larger volumes in the hypothalamus, periaqueductal gray, and dorsal raphe region of WT animals. DTI measurements, however, demonstrated statistically higher fractional anisotropy (FA) values for 51KO animals in locations including the anterior commissure, fornix, and posterior commissure/superior colliculus commissure region.
Data Conclusion: This study used in vivo structural MRI and DTI to demonstrate that a lack of FKBP51 leads to alterations in brain architecture and connectivity in male mice. These findings are of particular translational relevance for our understanding of the neuroanatomy underlying the interaction of FKBP5 genetic status, stress susceptibility, and psychiatric disorders.
Level of Evidence: 1
Technical Efficacy Stage: 1

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ABNORMALITIES in the structure and connectivity of the brain appear to characterize individuals with abnormal mood disruptions.[1,2] Clinical research has repeatedly reported gray matter (GM) deficits in the hippocampus of patients with major depressive disorder (MDD), as well as alterations in cortical thickness and surface area.[1,3] In line with this, diffusion tensor imaging (DTI) studies have shown microarchitectural deficits in white matter (WM) tracts across a range of psychiatric disorders, including schizophrenia, autism spectrum disorder, and affective disorders such as bipolar disorder.[2,4]

Accumulating evidence suggests that the development of GM architecture and WM integrity are largely under the influence of genetics.[5] The FK506 binding protein 51 (FKBP51; encoded by the *FKBP5* gene) is therefore of particular interest due to its function as a regulator of the stress system and its association with psychopathology.

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*Address reprint requests to: M.V.S., Kraepelinstr. 2-10, 80804 Munich, Germany. E-mail: mschmidt@psych.mpg.de
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From the [1]Department of Stress Neurobiology and Neurogenetics, Research Group Neurobiology of Stress Resilience, Max Planck Institute of Psychiatry, Munich, Germany; [2]Max Planck Institute of Psychiatry, Munich, Germany; and [3]Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.

FKBP51 is a heat shock protein co-chaperone that is involved in glucocorticoid signaling. It is ubiquitously expressed throughout the brain, but enriched in the hippocampus, hypothalamus, cortex, and amygdala.[6] Together with the heat shock protein 90, FKBP51 suppresses glucocorticoid receptor (GR) activity by decreasing the affinity of the GR to glucocorticoids and inhibiting nuclear transportation of the GR complex.[7] However, once the GR enters the nucleus, *FKBP5* expression is upregulated via glucocorticoid response elements, thereby creating a short negative feedback loop that mediates hypothalamus–pituitary–adrenal (HPA) axis activity. Interestingly, the single nucleotide polymorphism (SNP) rs1360780 in the *FKBP5* gene has been associated with psychiatric disorders, such as MDD and posttraumatic stress disorder.[8,9] Individuals carrying the risk T-allele of rs1360780 are prone to higher FKBP51 expression through cortisol, which induces a prolonged cortisol response, eventually leading to altered GR signaling and HPA axis dysregulation.[8] Aberrant GR signaling in turn has been linked to inhibition of cell proliferation and neurogenesis, resulting in structural volume loss and abnormalities in function.[10] These findings imply that there may be a neurobiological interplay of *FKBP5* genetic status, dysregulated stress system response, and psychiatric disorders, leading to alterations in brain anatomy and function.

Numerous studies have now corroborated the hypothesis that allelic differences in the *FKBP5* gene contribute to abnormalities in the function or volume of specific brain regions.[11,12] Frodl and O'Keane showed that exposure to early life stress in MDD patients with the *FKBP5* risk variant was associated with structural changes in brain structures affected by the disease.[13] Zobel et al demonstrated a smaller mean volume of the right hippocampus in patients affected by MDD and carrying the risk T-allele.[14] This was further confirmed cross-culturally in a sample of traumatized African-American women whose global and local shape analysis revealed morphological differences in the hippocampus between T and non-T carriers.[15] In addition, a study by Tozzi et al using functional magnetic resonance imaging (fMRI) and diffusion tensor imaging (DTI) found that MDD patients expressing the T allele and exposed to childhood maltreatment showed lower functional activity and mean diffusivity, but higher fractional anisotropy (FA) in brain areas involved in emotional processing in MDD.[12] More recently, Mikolas et al focused on the effects of the *FKBP5* rs13607080 polymorphism in patients with MDD in combination with early-life stress, highlighting lower volumes within the hippocampus–amygdala-transition area compared to healthy controls.[16] Even in a nonclinical population, Fujii et al could demonstrate that carrying the T allele of *FKBP5* is associated with smaller GM volume in the dorsal anterior cingulate cortex (dACC) as well as altered WM integrity both in the dACC and posterior cingulate cortex (PCC).[17] Overall, these findings indicate that allelic variants of *FKBP5*, potentially in combination with stressful environmental factors, manifest in distinct changes in brain architecture and function, thus representing an increased vulnerability for the development of psychiatric disorders.

Comparative in vivo neuroimaging using mice is probably the method most conducive to translate between the human evidence for altered brain states in psychiatric disorders and animal models thereof.[18,19] Moreover, the availability of genetically engineered mice provides a unique opportunity to investigate underlying functional and structural effects, as well as the consequences of genetic hallmarks specific to certain psychiatric disorders. This also includes finding functional correlates of genetic- or drug-related manipulations and valuable biomarkers. For example, Grandjean et al observed that chronic psychosocial stress in mice induced functional connectivity and cerebral metabolism states, as well as WM pathway alterations analogous to those in human MDD.[20] Similarly, Laine et al demonstrated that mice exposed to chronic social defeat stress showed significantly more activation in several brain regions known to regulate depressive and anxiety-like behavior.[21] In a recent review, McIntosh et al concluded that increased lateral ventricular volume and decreased hippocampal volume are relatively consistent hallmarks in both patients with MDD and animals model.[22]

Thus, based on the strong clinical evidence regarding the manifestation of *FKBP5* SNPs on human brain architecture and function, and the promising potential of MRI as a technique, it would be of great translational interest to explore whether a conventional FKBP51 knockout (KO) in mice had an effect on the connectivity and structure of the brain.

The FKBP5 KO (51KO) mouse line is well established and has provided unique insight into the behavioral, molecular, and neuroendocrinological effects of FKBP51 and its involvement in psychopathology. Mice lacking the *FKBP5* gene have repeatedly been shown to be more resilient to acute and chronic stress paradigms,[23,24] metabolic stress, and sleep deterioration.[23–26] More specifically, Hartmann et al showed that 51KO mice displayed a diminished physiological and neuroendocrine response to chronic social defeat stress.[23] Furthermore, after exposure to acute restraint stress, FKBP51 KO mice demonstrated enhanced active stress coping behavior in the forced swim test.[23] In addition, 51KO mice were also less responsive to the treatment with antidepressants, which complements clinical findings that showed faster treatment response in patients who carried the risk allele of rs1360780.[8] Overall, KO of FKBP51 seems to have a protective influence on stress physiology and stress-coping behavior, thereby potentially reducing the susceptibility towards the development of a stress-related psychiatric disorder.

Using in vivo high-resolution neuroimaging to explore the effects of FKBP51 KO in the mouse brain has unique

potential to provide a first translational perspective on the functional and structural changes analogous to the human brain. This study therefore aimed to explore the effect of a conventional FKBP51 KO on the in vivo status of the mouse brain, with a specific focus on connectivity and structural architecture.

Materials and Methods

Animals, Preparation, and Anesthesia

The experiment was carried out in the MRI animal facility of the Max Planck Institute of Psychiatry (Munich, Germany) and performed in accordance with the European Communities Council Directive 2010/63/EU. The protocols were approved by the Committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany. The animals used for this study were young adult 51KO mice.[27] Male mice from the age of 10–16 weeks were used and group housed in standard cages (21 × 15 × 14 cm, Plexiglas) on an inverted 12-hour day–night cycle, with food and water ad libitum. Two different cohorts of mice were used for the experiment, one for DTI measurements comprising n = 35 animals, resulting in n = 18 wildtype (WT) and n = 17 51KO mice, and a second cohort for voxel-based morphometry (VBM), with n = 10 WT and n = 9 51KO.

All mice were sedated in a preparation box using 2.5 vol% isoflurane. Animals were then fixed in prone position on an MR-compatible animal bed using a stereotactic device, while anesthesia was delivered via a breathing mask. Mice were kept anesthetized with an isoflurane/air mixture (1.5–1.8 vol %, with an air flow of 1.2–1.4 L/min). Respiration and body temperature were monitored using a pressure sensor placed below the mouse's chest and a rectal thermometer, respectively. Body temperature was kept between 36.5 °C and 37.5 °C using a heating pad. The first cohort of animals was subjected to DTI measurements; the second cohort underwent VBM measures.

Data Acquisition

MRI experiments were run on a BioSpec 94/20 animal MRI system (Bruker BioSpin, Rheinstetten, Germany) equipped with a 9.4T horizontal bore magnet of 20 cm diameter and a BGA12S HP gradient system capable of a maximum gradient strength of 420 mT/m with a 140 µs rise time. For DTI studies, a linear volume resonator coil for excitation and a 3 × 1 elements phased-array RF coil for reception of the signal were used, while for VBM studies, a two-channel cryogenic transmit–receive RF coil was utilized.

For VBM studies, anatomical images were recorded using a 3D gradient echo sequence with a repetition time (TR) of 34.1 msec, echo time (TE) = 6.25 msec, flip angle = 10°, number of averages (NA) = 3, matrix dimension = 256 × 166 × 205, isotropic pixel resolution 77 µm.

For DTI studies, diffusion-weighted images were recorded using a 2D multishot, four segments DTI-echo-planar imaging (EPI) sequence with TR = 5000 msec, TE = 19.26 msec, excitation pulse angle = 90°, NA = 1, matrix dimension = 128 × 92, pixel dimension 0.14 × 0.15 mm, slice thickness = 0.5 mm, number of slices = 35. Using diffusion encoding gradients with the diffusion gradient duration δ = 2.5 msec and a diffusion time Δ = 10.84 msec, seven image datasets were acquired with b-value, b = 0 s/mm^2 (B_0 images), followed by 90 gradient-encoded images with b = 1020 s/mm^2.

Data Processing

Imaging data for each individual mouse were first converted to NIFTI format.

Brain extraction was performed in a two-step procedure: In a first preprocessing step, mouse data were segmented and bias corrected using Statistical Parametric Mapping software (SPM12, Wellcome Department of Cognitive Neurology, London, UK) and the Hikishima BL6 template.[28] The initial GM, WM, and cerebrospinal fluid (CSF) probability maps were summed and binarized for image intensities larger 0.3, to create a first brain mask. This mask was dilated by five voxels and applied to the bias-corrected images. In a second step, the resulting images were again segmented, this time using a modified version of the Hikishima template for which the original CSF template was divided into two subtemplates: ventricular inner CSF and surface cortical CSF. For this second segmentation step, GM and WM were summed, the ventricular holes filled using MatLab's (MathWorks, Natick, MA) imfill function, slightly dilated, and binarized using an intensity threshold of >0.1. Finally, the resulting mask was applied to the original raw images.

VBM

3D images were segmented using the SPM12 old segment function and the spmmouse templates. Subsequent DARTEL normalization first imported the brain extracted images and two segments (GM, WM), and a study-specific template based on GM and WM was generated in seven iterations. GM tissue probability maps were normalized and modulated to the template space. Brain extracted images were transferred to the template space unmodulated, and a mean image was calculated for display purposes. Finally, the modulated GM images were smoothed using a Gaussian kernel of 4 mm. Total intracranial volume was approximated as the sum of all modulated tissue probabilities, excluding the olfactory bulb and the cerebellum due to a lower signal-to-noise ratio in these regions as a consequence of the surface coil characteristics.

DTI

For each individual in the cohort, a brain mask was derived by segmenting out GM, WM, and CSF compartments from a B_0 image, and adding back these three segments together as a mask. Multiplication of the mask with the data resulted in a brain extracted dataset. These steps were performed using SPM12 running on MatLab release 2017a. Noise in the data were removed with the procedure "dwidenoise" in the software package *MR*trix3. Individual data were each corrected for residual motions and eddy current artifacts with the FMRIB Software Library[29] subpackage "eddy_correct." A diffusion tensor component could be obtained for each individual in the cohorts using FSL's fdt procedure, from which quantities like medial diffusivity, radial diffusivity, and FA could be calculated. To derive a template for group analysis, the software tool DTI-TK was used.[30,31] DTI-TK contains an optimized DTI spatial normalization and atlas construction tool, which was shown to perform superior registration when compared to scalar registration methods.[32] Diffusion tensor datasets of six representative individuals

in each cohort with similar spatial alignment were averaged to create an initial tensor template. Then a rigid alignment of each individual tensor dataset to this initial tensor template was performed, iteratively optimizing the template for the next iteration as an average of the normalized images. Similarly, a subsequent affine registration step followed by a diffeomorphic registration step were used to refine both the alignment of each individual tensor data to the tensor template and to optimize the latter. At each iteration step, the tensor quantities associated with diffusion were transformed in a consistent manner by the registration algorithms. Finally, the process diffusion maps like medial diffusivity map, radial diffusivity map, FA map, and mode of anisotropy map could be created at the template level. The first steps of FSL's Tract Based Spatial Statistics (TBSS) were integrated within DTI-TK. These allowed the creation of a mean FA skeleton, which represented the WM skeleton from the high-resolution FA map of the DTI template derived earlier. The FA threshold was set to 0.3. The next steps in the TBSS analysis consisted of projecting all subjects' aligned fractional anisotropic data onto the mean FA skeleton.

Statistical Analysis

VBM. Data were analyzed using a two-sample *t*-test. The smoothed modulated GM maps were entered. Global differences in skull size of the individual animals were considered by adding the total intracranial volume (sum of GM, WM, and CSF) to the statistical model, applying proportional scaling. Volumetric differences were collected at a threshold of $P < 0.005$, and only clusters surviving a familywise error correction on the cluster level ($p_{FWE,cluster} < 0.05$) were further considered.

DTI. Following the final steps of FSL's TBSS procedure, a voxelwise statistical analysis was conducted that uncovered the FA skeleton voxels that were significantly different between the two different groups of subjects, WT and FKBP51 KOs. For this purpose, a nonparametric randomized procedure was followed. The Threshold Free Cluster Enhancement (TFCE) method[35] was used to derive uncorrected-*P* statistical results at a *P* level of 0.01.

Results

VBM

Total intracranial volume (TIV) did not significantly differ between the two groups, nor did the three individual tissue compartments (TIV: $P = 0.359$; GM: $P = 0.434$; WM: $P = 0.094$; CSF: $P = 0.156$). VBM revealed two clusters with significantly larger volume in the WT animals compared to the 51KO animals: the bilateral thalamus and the periaqueductal gray and dorsal raphe nucleus (Fig. 1). Thalamic differences comprised the complete length of the thalamus in the anterior–posterior direction. No suprathreshold clusters were detected for the inverse contrast (WT < 51KO). These results were confirmed using statistical nonparametric mapping (warwick.ac.uk/snpm; v. SnPM13) applying a permutation test with 5000 repetitions, which resulted in virtually identical clusters of volumetric differences.

DTI

Differences in the FA were found between the WT and the 51KO animals (Fig. 2). Specifically, FA values were significantly larger in 51KO animals than in WT animals ($P < 0.01$) in the following locations: the anterior commissure, the temporal limb, the lateral olfactory tract area, the fornix, the posterior commissure, and the superior colliculus commissure region. Changes in concomitant mean diffusivity were larger in WT animals, but only in the fornix region ($P < 0.05$). Concomitant axial diffusivity changes at $P < 0.05$ were found to be significantly larger in a location straddling the posterior commissure / superior colliculus commissure region in 51KO animals, but also significantly larger in WT animals in close by location in the periaqueductal gray. There was no significant difference in radial diffusivity between the two groups of animals. In the fornix and posterior commissure regions, there were statistically significant differences for the mode of anisotropy: at an uncorrected *P*-level of 0.02 for the fornix region and uncorrected *P*-level of 0.01 for the posterior commissure region, this metric had a higher value in the 51KO animals than in the WT ones.

Discussion

This study used in vivo structural MRI and DTI to demonstrate effects of FKBP51 KO on the connectivity and structural integrity of the mouse brain. These findings are of particular translational relevance for our understanding of the neuroanatomy underlying the interaction of *FKBP5* genetic status, stress susceptibility, and psychiatric disorders.

The structural analysis revealed two clusters with significantly larger volume in the WT animals compared to those with *FKBP5* KO: the thalamus as well as the periaqueductal gray and the dorsal raphe nucleus region. In general, the thalamus is a critical component of the frontal cortical–basal–ganglia thalamic circuits that integrate inputs related to emotion, cognition, motivation, and motor function to modulate behaviors.[34] Most important, however, thalamic areas are involved in information processing after exposure to stress, thus playing a central role in the regulation of the HPA axis stress response. Specifically, the paraventricular nucleus of the thalamus (PVT) was reported to be uniquely involved in regulating neuroendocrine and behavioral adaptations to severe or chronic stress.[35,36] Penzo et al established the PVT as a putative stress sensor that detects imminent threat and regulates fear expression and memory together with the central amygdala.[35] In response to chronic stress, corticosterone binds to the GR in the PVT in rats to promote feedback inhibition of the HPA axis stress response and habitation to stress.[37] Moreover, an association between glucocorticoid levels and thalamus volume was reported by imaging studies.[38,39] It was further highlighted that the thalamus is one of

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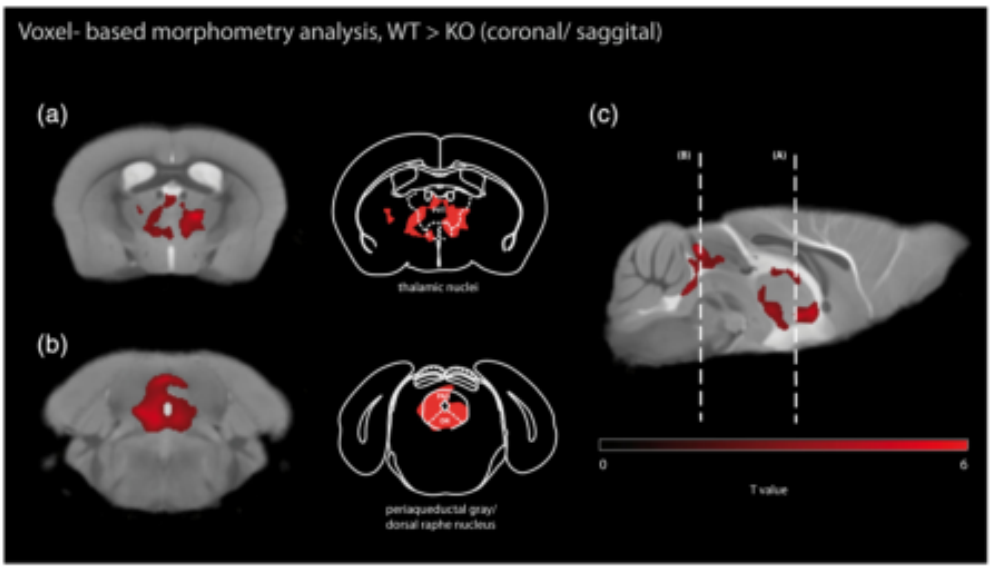

FIGURE 1: Results of the voxel-based morphometry analysis, depicting gray matter (GM) regions of larger local volume in wildtype (WT) animals as compared with 51KO mice (WT > KO). Coronal sections showing volumetric differences in the thalamus (a) as well as the periaqueductal gray (PAG) and the dorsal raphe (DR) nucleus (b); thalamic nuclei include the paraventricular thalamic nucleus (PVT), anterior part, the mediodorsal thalamic nucleus, the anterodorsal thalamic nucleus, the central medial thalamic nucleus, paratenial thalamic nucleus, and the reuniens thalamic nucleus. Positions of the coronal slices are indicated on a mid-sagittal slice (c). No significant voxels appear in the inverse contrast (WT < KO). Clusters are significant at $p_{FWE,cluster} < 0.05$, with a collection threshold of $P < 0.005$.

the areas that express *FKBP5*, and the distribution of *FKBP5* is similar to that of the GR under baseline conditions.[6]

Thus, considering the central role of the thalamus in regulating the stress system response and the numerous studies that have repeatedly highlighted an effect of the *FKBP5* genotype on subcortical structures, our findings are congruent with previous data. Furthermore, an effect of the rs1360780 genotype and maternal acceptance on thalamic GM volume (GMV) was reported,[40] suggesting that the effect of maternal acceptance on brain development was different depending on the rs1360780 genotype.[40] Interestingly, higher maternal acceptance predicted larger thalamic volume among the T-carriers and smaller thalamic volume among C/C homozygotes.

A possible explanation for this phenomenon was delivered by Sheikh et al, who showed that the association between positive parenting and WM integrity was modulated by inherent cortisol levels.[41] In girls with higher cortisol levels at the age of 3 years, a negative correlation was found between positive parenting and FA in the ACC at the age of 6 years, whereas a positive correlation was revealed in girls with lower cortisol levels.[41] Thus, Sheik et al argued that the developing brains of children might be differentially affected by positive parenting, depending on the child's inherent level of cortisol reactivity to stress. In line with this, Lau et al demonstrated positive associations between serum cortisol levels, cognitive processing speed, and regional brain volume in the thalamus of elderly patients, implying a facilitating role of cortisol in cognitive function via this brain region.[38] While these studies are not directly applicable to the results of the present study, 51KO animals have been shown to be more stress resilient than their WT controls.[23–26,42] Thus, a smaller thalamic volume in the 51KOs might be causally linked to the stress-resilient phenotype of these animals.

Moreover, a larger volume in WT animals in the periaqueductal gray (PAG) and dorsal raphe region was observed. The PAG is a fundamental structure in the hierarchical top-down control of defensive and aggressive strategies, and best known for its involvement in panic and pain.[43] The dorsal raphe nucleus, however, is critically involved in regulating serotonergic tone throughout the brain; and together with the ventral PAG, it has recently been considered as a single functional unit that is reciprocally connected, playing a role in appetitive states, anxiety, and depression.[43] Similar to the findings in this study, Anacker et al highlighted anatomic differences associated with stress susceptibility and resilience in mice.[44] Specifically, susceptible mice exhibited a reduced volume of the raphe nuclei and the thalamus, and more important, increased volume of the PAG, indicating that stress-integrative brain regions shape the neural architecture underlying individual differences in susceptibility and resilience to stress. However, while the current results demonstrated volumetric changes in similar brain regions, the volumes observed in the susceptible mice (WT) were always larger. Such differences may be attributed to the dynamic model used by Anacker et al, which comprised a social defeat

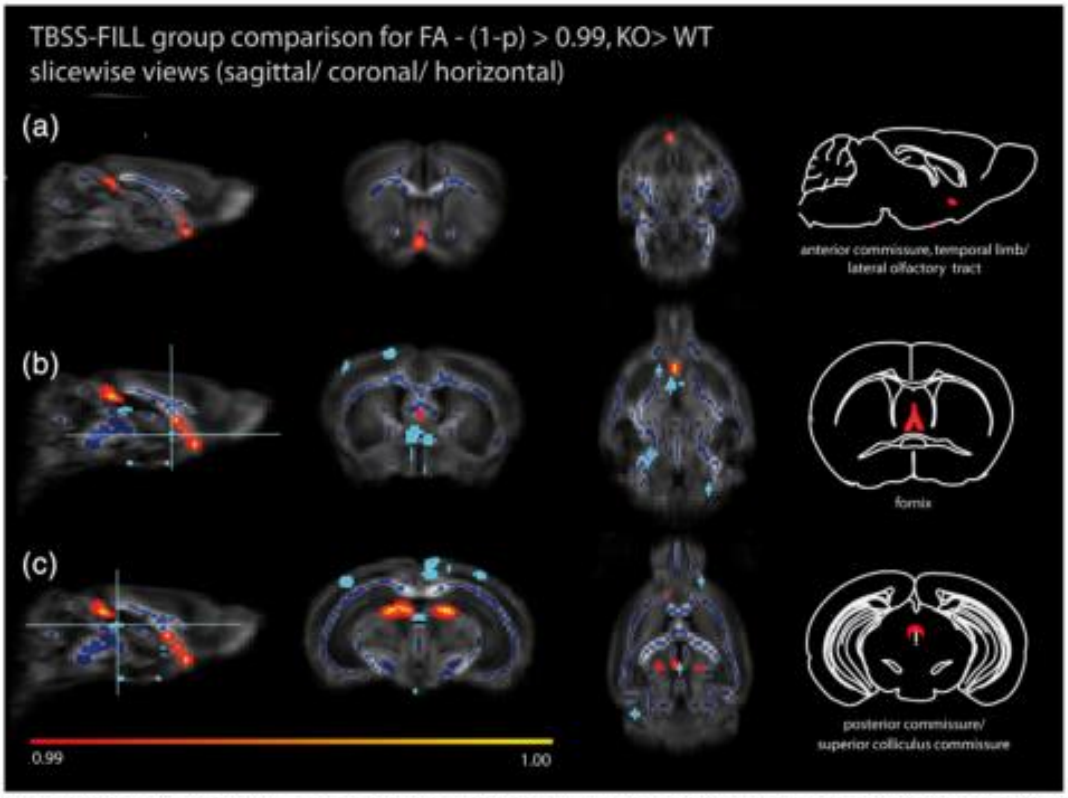

FIGURE 2: Results from the TBSS-FILL group comparison for fractional anisotropy (FA) and mode of anisotropy (MO) in wildtype (WT) and 51KO mice. Hot colors represent voxels that were significantly different for FA at a *P*-level of 0.01 between the two cohorts of mice; red implies FA in 51KO > WT. Cold colors depict voxels for the MO, which were significantly different at a *P*-level of 0.01 (posterior commissure) and 0.02 (fornix); blue implies MO in 51KO > WT. Significant differences were identified in (a) the anterior commissure, temporal limb, and the lateral olfactory tract area; (b) the fornix, marked by a cross, and (c) the posterior commissure and superior colliculus commissure region, also marked by a cross.

protocol and a subsequent social avoidance test in C57BL/6 mice, rather than a more trait-like genetic model, as used in this study.[44]

Overall, these data highlight neuroanatomic differences due to FKBP51 deletion in brain structures that are traditionally associated with the stress system. However, in other regions with high FKBP51 expression that are central to the stress response, eg, the hippocampus, no robust neuroanatomic changes were observed. One might speculate that the structural effects observed following FKBP51 deletion are strongest in regions with a highly dynamic FKBP51 regulation, rather than in regions with a consecutively high FKBP51 expression. In addition, a lack of a structural alteration in a specific brain region does not allow the conclusion that FKBP51 is of little importance in that region. The data merely illustrate that while there may be a high functional relevance of FKBP51 in a given brain region, this does not necessarily lead to changes in structure or connectivity. Finally, an important consideration is the choice of the cutoff *P* value for the analysis, and a more moderate cutoff of $P < 0.05$ would have resulted in more brain regions being affected, including the hippocampus. However, the current analysis aims to be more stringent and reports only the effects with the highest statistical confidence.

A general consideration for further evaluating local volume differences associated with a general FKBP51 KO is the underlying tissue microstructure. This multiparametric approach of combining MRI and DTI measurements of 51KO and WT mice may provide some first insight into structural differences associated with changes in white microstructure between resilient and susceptible brains. Generally, a larger FA indicates more pronounced tissue anisotropy and integrity, possibly as a result of increased axonal density and myelination, decreased interfering processes (eg, glial cells, vasculature, neuritis) and increased organization along the dominant axis (eg, alignment of neurites).[44,45] In the anterior commissure (temporal limb) and the lateral olfactory tract region this might imply a greater integrity of the microstructural architecture in the 51KO animals. However, in the posterior commissure / superior colliculus region the results demonstrated a higher FA together with an increase of MO, which represents a transition of WM from a planar to a more

linear diffusion tensor. This combination of measurements most likely reflects a selective sparing (or selective degeneration) of one fiber population among the crossing fibers,[46,47] suggesting that the 51KO animals might possibly lack a selective fiber population in the fornix and the posterior commissure / superior colliculus region in contrast to the WT animals.

Limitations

First, the animals used in this experiment were exclusively male, and thus do not provide an appropriate representation of the population. Stress-related psychiatric disorders in particular are highly sexually dimorphic in their pathology, with significant clinical implications for guiding diagnosis, treatment, and prevention in an individualized manner.[48] Second, whether the observed differences in volume and WM integrity are the basis for or the result of the previously established higher stress resilience in 51KO mice remains elusive. This is further exacerbated by potential developmental effects of the *FKBP51* KO and the lack of corresponding, cohort specific behavioral data. In addition, the effects reported might be mediated by numerous other interacting protein partners of FKBP51 or due to the disruption of cellular pathways that involve FKBP51, such as, for example, the NF-κB, Akt, and MAPK pathways.[25,42,49] Finally, animals were imaged under basal conditions only, disregarding the interesting implications of FKBP51 on the brain and behavior once activated by exposure to stress. Future research should therefore take these considerations into account and strive towards a more sex-specific and causal understanding of the effects of FKBP51 on WM integrity and brain architecture.

Conclusion

Taken together, this study provides evidence that a lack of the psychiatric risk factor FKBP51 leads to alterations in brain connectivity and architecture in male mice, which may underlie some of the functional and behavioral phenotypes observed in these animals.

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2.3. FKBP51 in the ovBNST regulates anxiety-like behaviour

Engelhardt, C., Tang, F., Bordes, J., Brix, L.M., van Doeselaar, L.,Häusl, A.S., Pöhlmann, M.L., Schraut, K.G., Yang, H., Chen, A., Deussing, J., & Schmidt, M.V.

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Abstract

The co-chaperone FKBP51 and the oval bed nucleus of the stria terminalis (ovBNST) have both been implicated in stress-induced anxiety. However, the role of *Fkbp5* in the ovBNST and its impact on anxiety-like behavior have remained unknown. Here we show that *Fkbp5* in the ovBNST is reactive to acute stress and co-expressed with the stress-regulated neuropeptides *Tac2* and *Crh*. Subsequently, results obtained from viral-mediated manipulation indicate that *Fkbp5* overexpression (OE) in the ovBNST results in an anxiolytic tendency regarding behavior and endocrinology, whereas a knockout exposed a clear anxiogenic phenotype, indicating that native ovBNST expression and regulation is necessary for normal anxiety-related behavior. Notably, our data suggests that a stress-induced increase of *Fkbp5* in the ovBNST may in fact have a protective role, leading to a transient decrease in anxiety and suppression of a future stress-induced hypothalamic-pituitary-adrenal (HPA) axis activation. Together, our findings provide a first insight into the previously unknown relationship and effects of *Fkbp5* and the ovBNST on anxiety-like behavior and HPA axis functioning.

Significance Statement

Both the co-chaperone FKBP51 and the oval bed nucleus of the stria terminalis (ovBNST) have been implicated in anxiety, yet their combined effects of mediating anxiety-like states have not been explored. Here we provide a first characterization of the role of *Fkbp5* in the ovBNST on HPA axis function and anxiety-related behavior. Our findings suggest that stress induction of *Fkbp5* in the ovBNST may have a protective role, leading to decreased anxiety and suppression of a future stress-induced HPA axis activation. Overall, this study constitutes a basic step towards understanding the underlying mechanisms of *Fkbp5* signaling in the ovBNST and their role in stress-induced anxiety disorders.

Introduction

Stress exposure can trigger maladaptive behavioral responses and induce mood disorders, such as anxiety (De Kloet et al., 2005). Anxiety is characterized by non-adaptive hypervigilance and threat overestimation in uncertain situations (Sylvers et al., 2011). The FK506 binding protein 51 (FKBP51; encoded by the *FKBP5* gene), a heat shock protein 90 kDa (Hsp90) co-chaperone, is a regulator of the stress system and a risk factor for anxiety disorders (Binder, 2009). Together with Hsp90, FKBP51 regulates glucocorticoid receptor (GR) activity via a short negative feedback loop. This signaling pathway rapidly restores homeostasis in the hypothalamic-adrenal-pituitary (HPA) axis after exposure to stress. Altered HPA axis regulation mechanisms, specifically impaired signaling of GR, have been causally implicated in the pathogenesis of anxiety (Holsboer, 2000).

Interestingly, single nucleotide polymorphisms (SNPs) in FKBP5 have been associated with increased FKBP51 expression and GR resistance, leading to differential HPA axis activation after stress exposure (Binder, 2009). Healthy controls homozygote for the high-induction alleles show significantly slower recovery of stress-related increases in cortisol levels as well as more anxiety symptoms in the recovery phase than healthy controls with other genotypes (Ising et al., 2008). Furthermore, carriers of the same risk variant were more susceptible to anxiety when exposed to childhood maltreatment (Scheuer et al., 2016).

Rodent studies have provided further insights into the role of FKBP51 and anxiety. Traditionally, anxiety is associated with the amygdala (Felix-Ortiz et al., 2016; Tye et al., 2011). While a global knockout (KO) of *Fkbp5* did not affect anxiety-like behavior, reducing FKBP51 in the amygdala decreased stress-induced anxiety-like behavior (Attwood et al., 2011). Likewise, pharmacological disruption of FKBP51 signaling in the amygdala demonstrated an anxiolytic effect, whereas FKBP51-OE enhanced anxiety-like behavior. However, anxiety was not altered by FKBP51-OE in the dorsal hippocampus of mice (Hartmann et al., 2015). These findings suggest that FKBP51 in the amygdala regulates stress-induced anxiety-like behavior, and that these effects are highly region-specific.

Like the amygdala, a large body of evidence implicates the bed nucleus of the stria terminalis (BNST) in anxiety. The BNST receives information from limbic structures (e.g. amygdala, hippocampus, mPFC) and projects to autonomic and neuroendocrine systems located in hypothalamus and brainstem regions that regulate the HPA axis (Dong et al., 2001b; Ch'ng et al., 2018). Clinical imaging data suggest that BNST activity is positively correlated with increased anxiety (Somerville et al., 2010; Yassa et al., 2012). The rodent literature however has presented conflicting results (Walker and Davis, 1997; Treit et al., 1998; Duvarci et al., 2009; Van Dijk et al., 2013; Luyck et al., 2017), confirming that the BNST in fact modulates anxiety, but not specifying if this structure increases or decreases anxiety. These discrepancies might be accounted for by the anatomical and neurochemical heterogeneity of the BNST. The BNST consists of 18 sub-nuclei, which likely regulate anxiety in different, sometimes opposing directions (Bota et al., 2012). For instance, optogenetically inhibiting the oval nucleus of the BNST (ovBNST) elicited an anxiolytic effect, whereas light-induced decreases in neuronal activity in the anterodorsal BNST (adBNST) were anxiogenic (Kim et al., 2013). Likewise, chronic stress and acute optogenetic activation of the ovBNST increased anxiogenic behaviors (Hu et al., 2020a). Moreover, early life stress resulted in a long-lasting activation of CRH signaling in the mouse ovBNST, leading to potential maladaptive changes in ovBNST function in adulthood (Hu et al., 2020b). The ovBNST is therefore a promising region with regard to anxiety disorders, since it is strongly involved in mediating anxiety-like behavior.

Given FKBP51's role in stress-induced anxiety, an understanding of its function in the ovBNST is of great relevance to further decipher maladaptive anxiety as a psychiatric disease. Here, we describe FKBP51 expression and regulation in the ovBNST under basal conditions and after stress. We further explore the neuropeptide expression profile of FKB51 positive cells within the ovBNST. Finally, we delineate the effects of *Fkbp5* manipulation in the ovBNST on anxiety-like behavior and neuroendocrinology.

Materials and Methods

Animals and Animal housing

Experiments were carried out with C57Bl/6n, $Fkbp5^{lox/lox}$ (Häusl et al., 2019) and $Fkbp5^{KO}$ (Tranguch et al., 2005) mice obtained from the in-house breeding facility of the Max Planck Institute of Psychiatry, Munich. All animals used during the experiments were male and between 8-12 weeks old. Initially, mice were group housed and then single-housed at least 1 week prior to the start of the experiment. Housing parameters in the holding and testing rooms were kept constant on a 12h light: 12h darkness cycle, with controlled temperature (22± 2°C) and humidity (45± 10%). Food (Altromin 1324, Altromin GmBH, Germany) and water (tap water) was provided *ad libitum*. 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. Protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Stress paradigms

Acute Restraint Stress

Mice were restrained manually and guided into a 50 ml falcon tube. The falcon contained holes in the top and the lid to allow for sufficient ventilation and tail movement. The restraint lasted 4 hours and animals were either sacrificed immediately after or allowed to recover in their home cage for 4 hours. Animals that underwent a FKBP51 KO were restrained for half an hour before the sacrifice in order to facilitate subsequent virus validation.

Chronic Social Defeat Stress

The Chronic Social Defeat Stress (CSDS) paradigm lasted for 21 days and was conducted as described previously (Wagner et al., 2012). Briefly, experimental mice were placed in the home cage of a dominant CD1 resident mouse. Interaction was permitted until the experimental mouse was attacked and defeated by the CD1 aggressor. Mice were subsequently separated by a wire mesh that prevented physical contact but maintained sensory contact for 24h. Each day, for 21 days, the experimental mouse was paired with another unfamiliar CD1 mouse. Both control and stressed mice were handled daily during the course of the stress exposure.

Acute Fear Conditioning

Animals were habituated for two days by placing them into the conditioning chamber (Bioseb, France) for 5 min with the chamber lights switched on. On the consecutive day (D3), mice either received five tones (30s, 9kHz, 80 dB) paired with a foot shock (500 ms, 0.7 mA) and a 5- min inter-trial, or they were exposed to the tones only. Animals remained in the shock/ tone-shock context for an additional 60 sec, before they were returned to their home cages. Control animals remained in their home cages at all times. Stimuli (light, tone, shock) were operated with commercially available software (Packwin V2.0; Panlab, Spain).

Behavioral paradigms

All behavioral testing was carried out in the animal facility of the Max Planck Institute of Psychiatry, Munich. Tests were performed during the light phase between 7am and 1pm to avoid potential behavioral alterations due to circadian variation of corticosterone levels. The behavioral testing was conducted in the following order: elevated plus maze (EPM), dark-light box (DALI), open field (OF) and acute stress response. Recording, tracking and scoring of animal behavior was carried out using the automated video tracking system ANY-maze (ANY-maze 6.18; Stoelting Co, Wood Dale, IL, USA). All tests were performed by an experienced, blinded researcher and according to established protocols.

EPM

The EPM was performed as previously described (Santarelli et al., 2014). Briefly, animals were 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). 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. An increase in open arm activity (duration and/or entries) is interpreted as a decrease in anxiety-like behavior.

DALI

The apparatus was comprised of a dark and protected compartment (15 x 20 x 25 cm, dimly lit under 10 lux) and a brightly illuminated compartment (30 x 20 x 25 cm, lit with 700 lux); both compartments were connected by a tunnel of 4 cm in length. Mice were placed in the dark compartment, facing towards a wall and recorded for 5 min. To assess anxiety-related behavior, the time spent, number of entries and latency to first entry into the lit compartment were measured.

OF

Mice were placed in a corner of a 50 x 50 x 50 cm plastic arena. Fifteen-minute trials were video recorded by an overhead camera. The test was performed under conditions of a highly lit center zone (20 lux; 20x 20 cm) and a dimly lit (16 lux) outer zone. Parameters of interest regarding anxiety-like behavior were time spent and number of entries to the center zone. For more detailed analysis, the time total was divided into three segments of 5 min.

Stress response after acute restraint stress (ARS)

Mice were restrained for 15 min and blood was immediately collected by a tail cut to examine response corticosterone levels. Mice were then released back into their home cage. 30 min and 60 min after the onset of the stressor additional blood samples were again collected via the tail vein in order to assess corticosterone levels. Plasma was collected and stored at -20°C. Levels of plasma corticosterone were subsequently determined using a commercially available radioimmunoassay kit with ^{125}I-labeled anti-corticosterone antibody (MP Biomedicals Inc.; sensitivity: 12.5 ng/ml; intra-assay coefficient of variation (CV): 7%; inter-assay CV: 7%).

Tissue collection and processing

Animals were anesthetized with isoflurane and sacrificed by decapitation. Basal trunk blood was collected and processed (as described above). Brains were removed, snap frozen and stored at -80°C. Adrenals and thymus gland were removed, dissected from fat and weighed. Alternatively, mice were anesthetized with isoflurane and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% (v/v) paraformaldehyde (PFA) fixative in PBS. Brains were rapidly removed and post-fixed in PFA overnight at 4°C. The following day, post-fixed brains were transferred to a cryoprotectant solution (30% sucrose in 0.1 M PBS) for two additional overnight incubations at 4°C. Brains were stored at -4°C until processed.

Stereotactical Microinjections

Manipulation of FKBP51 was performed using adeno-associated bicistronic AAV1/2 vectors (AAV1/2-HA-CAG-FKBP5-1 and AAV1/2-CAG-null, GeneDetect; AAV1-CMV-Cre and AAV1/2-CAG-empty-IRES-EGFP, Addgene; AAV1/2-ERT2-Cre-ERT2). Stereotactic injections were performed as described

previously (Schmidt et al. 2011) Briefly, an injection volume of 0.3 µl using a glass capillary with a tip resistance of 2 -3 MΩ over 6 min (0.05 µl/min) was used to deliver the virus. For bilateral microinjections into the ovBNST, mice were anesthetized with isoflurane and fixed in a stereotaxic frame in order to target the following coordinates relative to Bregma: posterior 0.8 mm; 1.2 mm lateral; 4.3 mm ventral. After surgery, mice remained in their home cage for 3 weeks until the start of behavioral experiments. Successful virus expression was verified by *in situ* hybridization (*ISH)*.

4-hydroxytamoxifen (4OHT) Treatment

To induce activity-dependent Cre expression, 4OHT (H6278, Sigma-Aldrich) 50 mg/ml 4OHT dissolved in DMSO (D8148, Sigma-Aldrich) and diluted 10x in saline containing 2% Tween80 (P1754, Sigma-Aldrich) and 10x in saline; final concentration: 2.5 mg/ml 4OHT, 5% DMSO and 1% Tween80 in saline) was injected immediately in $Fkbp5^{lox/lox}$ mice before a 4 h restraint (final dose: 25 mg/kg). The restraint ensured viral Cre expression, thus *FKBP5*-KO, but only in those neurons in the ovBNST that had been previously activated by the restraint stress.

Immunohistochemistry

Immunohistochemistry was used to detect beta-gal distribution and thus indirectly visualize cellular location of FKBP51. In addition, virus injections were validated using immunohistochemical staining. Briefly, serial free-floating sections were washed in 0.1 M PBS and incubated in blocking solution (10% normal goat serum (NGS)/ 1% Triton/ 0.1 M PBS) for 30 min at room temperature. Sections were incubated with the primary antibody (diluted 1:500 -1000 in 1% NGS/ 0.3% Triton/ 0.1 M PBS) overnight at 4°C. Sections were washed with 0.1 M PBS and incubated with a secondary antibody (1:500-1000) for 2 h in a light protected environment. After washes with 0.1 M PBS, the sections were mounted with DAPI medium (Fluoromount-G Cat. No. 0100-20) and cover-slipped.

Fresh-frozen sections used for virus validation were treated with an adapted version of the protocol described above. A hydrophobic barrier PAP pen (Sigma-Aldrich) was used to encircle the sections. Blocking and antibody solutions were pipetted onto the encircled region. Furthermore, slides were covered with parafilm (neoLab, Germany) during antibody incubation to prevent drying out and ensure constant coverage of the sections.

In Situ Hybridization

Fkbp5 gene expression in the BNST was examined by ISH. Coronal whole-brain slices were cryosectioned at a thickness of 20 µm and directly mounted onto Superfrost Plus Slides as 6 sequential series. A cRNA anti-sense riboprobe was transcribed from linearized plasmid DNA (for forward primer*: 5'CTTGGACCACGCTATGGTT;* reverse primer*: 5'GGATTGACTGCCAACACCTT).* ISH was performed as previously described (M. Schmidt et al. 2003; Schmidt et al. 2007). For signal detection, slides were exposed to a Kodak Biomax MR film (Eastman Kodak Co). Exposure times varied according to the radioactive properties of each riboprobe. Autoradiographic densities were quantified using the NIH ImageJ Software (NIH, Bethesda, MD, USA) to both validate and quantify expression of the gene.

Double ISH

Double ISH (DISH) was used for the co-localization of *Fkbp5* mRNA with the GABAergic marker *Gad65*/*Gad67* within the ovBNST. Briefly, ^{35}S UTP-labelled *Fkbp5* riboprobe was used with a combination of digoxigenin (DIG)-labelled *Gad65* and DIG-labelled *Gad67* riboprobe. The antisense riboprobes were transcribed from a linear plasmid for *Fkbp5* (forward primer: 5'- CTT GGA CCA CGC TAT GGT TT -3'; reverse primer: 5'-GGA TTG ACT GCC AAC ACC TT-3'), *Gad65* (forward primer: 5'- TAA TAC GAC TCA CTA TAG CG -3'; reverse primer: 5'-CCC TTT AGT GAG GGT TAA TT- 3') and *Gad67* (forward primer: 5′-ATG ACG TCT CCT ACG ATA CA-3′; reverse primer: 5′-CCC CTT GAG GCT GGT AAC CA-3′). DISH was performed as previously described (Refojo et al., 2011). Briefly, sections were fixed in 4 % PFA. After several washing steps, endogenous peroxidase was quenched in 1% H_2O_2. Background was reduced in 0.2 M HCl, followed by 2 additional washing steps (1 x PBS). The slides were then acetylated in 0.1 M triethanolamine, washed (1 x PBS) and dehydrated through increasing concentrations of ethanol. After that, tissue sections were saturated with 90 µl of hybridization buffer containing approximately 50,000 cpm/µl ^{35}S-labelled riboprobe and 0.2 µg/ml DIG-labelled riboprobe. Brain sections were cover-slipped and incubated overnight at 55°C. The following day, coverslips were removed, and sections were washed several times in decreasing concentrations of SSC/formamide buffers under stringent temperature settings. After SSC washes, sections were treated with RNAse A in 1 x NTE at 37°C and washed in 1x NTE/0.05% Tween20 (2x times) followed by a blocking step in 4% BSA for 1 hour. After additional washing steps, sections were blocked in NEN-TNB for 30 min. In a final step, slides were incubated with Roche's anti-DIG (FAB) (1:400, Roche Molecular Diagnostics) at 4°C overnight. On the last day, sections were washed several times in TNT at 30°C followed by a signal amplification step in which sections were incubated for 15 min in tyramide-biotin. Thereafter, additional washing steps were performed (Roche washing buffer, Roche Molecular Diagnostics). Sections were then incubated for 1 h with Roche streptavidin-AP (1:400, Roche Molecular Diagnostics). Afterwards, sections were washed in Roche washing buffer and subsequently prepared for Vector red staining in 100 mM Tris/HCL (Vector Laboratories, Burlingame, CA, USA). Slides were immersed in Vector red solution under unlit conditions for 15-30 min depending on staining. When staining was sufficient, the reaction was stopped in 1 x PBS followed by a fixation step in 2.5% glutaraldehyde. Finally, sections were washed in 0.1 x SSC and dehydrated through a graded series of ethanol solutions (30, 50, 70, and 96%).

RNAscope

RNAscope is a novel ISH assay for detection of target RNA within intact cells. This approach allows for multiplex detection of up to four target genes. The procedure was performed according to manufacturer's specifications and with the RNAscope Flourescent Multiplex Reagent Kit (Cat. No. 320850, Advanced Cell Diagnostics, Newark, Ca, USA). The probes used for detection were; Fkbp5 (Mm-Fkbp5-No-XHs), Tac2 (Mm-Tac2-C2/C3), Gad65 (Mm-Gad2-C3) and Crh (mm-Crh-C2).

Briefly, sections were fixed in 4% PFA for 30 min at 4°C and dehydrated in increasing concentrations of ethanol. Next, tissue sections were incubated with protease IV for 30 min at room temperature. The probes were mixed at a ratio of 1:1:50 and hybridized for 2 h at 40°C followed by four hybridization steps of the amplification reagents 1 to 4. The sections were then counterstained with DAPI, cover-slipped and stored at 4°C. Images were acquired with experimenter blinded to probes used.

Sixteen-bit images of each section were taken on a Zeiss confocal microscope using a 20x, 40x, and 63x objective. Within a sample, images were acquired with identical settings for laser power, detector gain, and amplifier offset.

Experimental Design and Statistical Analyses

Data were analysed using IBM SPSS Statistics 25 software (IBM SPSS Statistics, IBM, Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software, San Diego, California, USA). For the comparison of two groups, the independent Student's t-test was applied. If the data were not normally distributed, the non-parametric Mann-Whitney (MW-test) was used. Data with more than two groups were tested by the appropriate analysis of variance (ANOVA) model followed by Bonferroni post-hoc analysis to determine statistical significance between individual groups. In the event of multiple time points, a repeated-measures ANOVA was performed. If a single value was missing from the data, mixed model analysis was applied. All data are shown as means +/- standard error of the mean (SEM). The level of statistical significance was set at $p < 0.05$.

Results

Expression and regulation of Fkbp5 in the ovBNST

To gain a deeper understanding of the possible function of FKBP51 in the BNST, we first explored its regulation following exposure to different stressful situations. Since visualization of FKBP51 protein regulation is hindered by the lack of specific antibodies, we utilized the $Fkbp5^{KO}$ line. Here, the insertion of the *lacZ* gene in the *Fkbp5* locus of this line results in changes in beta-gal expression, which directly reflect expression changes of FKBP51. Beta-gal free-floating IHC indicated a strong upregulation of FKBP51 after exposure to acute restraint stress predominantly in the ovBNST (**Fig 1A, B**). This finding was confirmed using ISH to quantify *Fkbp5* mRNA expression in C57Bl/6n mice at basal level (n=7) and after exposure to 4h of acute restraint stress. Exposure to restraint stress resulted in a significant increase in *Fkbp5* mRNA expression within the ovBNST ($t(12)= 6.61$, $p < 0.0001$, unpaired t-test) (n = 8; **Fig. 1C**). To further characterize the *Fkbp5* positive neuronal population activated by acute restraint stress within the ovBNST, co-expression with the neuronal GABAergic markers GAD65/67 was determined using DISH at basal level and after restraint stress (**Fig. 1D**). From the results it could be concluded that *Fkbp5* is expressed and regulated in the majority of GABAergic neurons in the ovBNST. This finding was further confirmed using RNAscope (**Fig. 1E**), demonstrating a clear co-expression of the marker GAD65 and *Fkbp5* within the ovBNST and on a single cellular level. Interestingly, quantification of *Fkbp5* mRNA regulation after exposure to CSDS did not result in a significant difference between the control (n= 10) and stressed group (n= 8) in the ovBNST (**Fig. 1F**), indicating that ovBNST *Fkbp5* upregulation might be reactive predominantly to acute stress exposure. Consequently, mice were exposed to a different kind of acute stressor – a modified and acute version of classical fear conditioning that included a home cage only group (n = 7), tone only (n=8), and shock and tone combined group (n=5). A one-way ANOVA demonstrated a significant difference between the three groups [$F(2, 17) = 4.644$, $p= 0.025$] (**Fig.1G**) and Bonferroni post-hoc analysis revealed a significant difference between the home cage group and the tone and shock combined group ($t(17)= 2.835$, $p= 0.034$). Finally, to gain information on the dynamics of *Fkbp5* upregulation within the ovBNST after exposure to acute restraint stress, a time course was performed (**Fig. 1H).** *Fkbp5* upregulation differed significantly within the

different time points [F (4, 21) = 5.767, p= 0.0027, ANOVA] and peaked as observed previously after 4 hours (Bonferroni basal vs 4h, t(21)= 3.848, p= 0.0093).

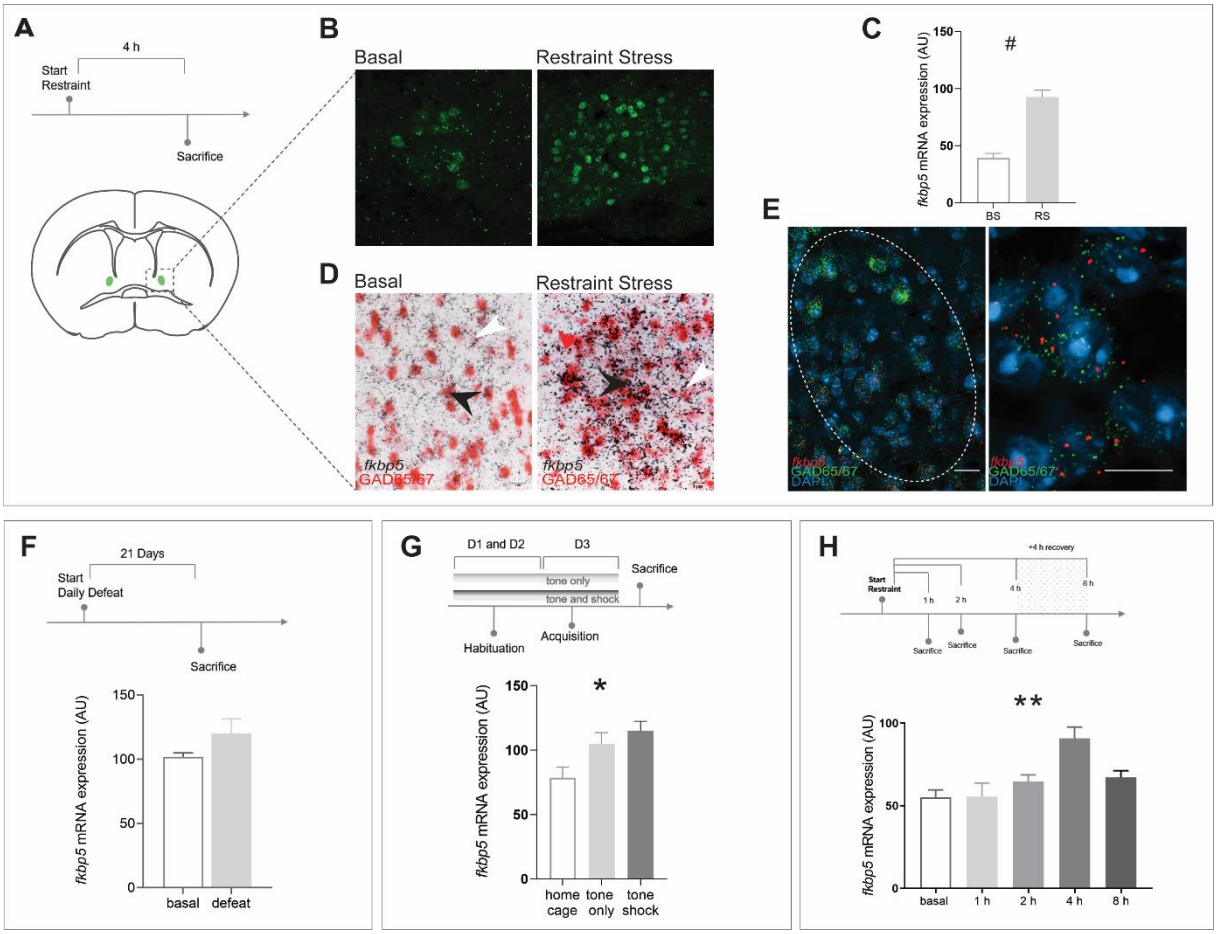

Figure 1: Expression and regulation of *Fkbp5* in the ovBNST. (**A**) *Fkbp5* is expressed predominantly in the ovBNST at basal level and highly upregulated after exposure to acute stress. Stress regulation of FKBP51 is shown though beta-gal upregulation (**B**) and on mRNA level (**C**). Furthermore, *Fkbp5* is expressed and regulated in the majority of GABAergic neurons (**D**). Black arrow: GABAergic cell expressing *Fkbp5*; white arrow: non-GABAergic cell expressing *Fkbp5*. This was further confirmed by RNAscope (**E**), demonstrating a clear co-expression of *Fkbp5* and GAD65 in a cell. (**F**) *Fkbp5* was not significantly upregulated after exposure to chronic social defeat stress. However, exposure to a different type of acute stress – acute fear conditioning – reliably resulted in a significant upregulation of *Fkbp5* in the ovBNST (**G**). A time-course of *Fkbp5* revealed a significant upregulation, with *Fkbp5* regulation peaking at its highest after 4h (**H**). Data are mean ± SEM. * P< 0.05; **P < 0.01; # P < 0.001.

Co-expression of Fkbp5 with Crh and Tac2

Next, we wanted to explore the neurochemical profile of *Fkbp5* positive neurons in the ovBNST. Previous studies have demonstrated that various stress- and anxiety-related neuropeptides are richly expressed in the BNST (Walter et al., 1991; Adhikari, 2014; Zelikowsky et al., 2018b; Hu et al., 2020b). In fact, the highest concentration of Crh neurons in the brain is located in the ovBNST (Morin et al., 1999; Daniel and Rainnie, 2016a). Another neuropeptide of interest that has been recently associated with the BNST and stress is Tachykinin 2 (*Tac2*) (Zelikowsky et al., 2018b). Thus, here we investigated the potential co-expression of *Fkbp5* with *Crh* and *Tac2* in the ovBNST (**Fig.2**). Our results indicated that *Fkbp5* and *Tac2* are strongly co-expressed in the ovBNST (**Fig. 2A,D**). This also applies to *Fkbp5* and *Crh*, which are similarly co-expressed in ovBNST cells (**Fig. 2B,E**). However, apart from these two distinct populations, co-expression patterns of *Fkbp5*, *Tac2* and *Crh* also seemed to overlap within the ovBNST (**Fig.2C,F**). Specifically, using RNAscope we could visualize *Fkbp5* positive cells co-expressing both *Crh* and *Tac2* (**Fig. 2F**, violet arrow), *Fkbp5* positive cells strongly co-expressing *Tac2* (**Fig.2F**, violet outline arrow), *Fkbp5* positive cells weakly co-expressing *Tac2* (**Fig.2F**, grey arrow) and *Fkbp5* positive cells strongly co-expressing *Crh* (**Fig.2F**, grey outline arrow).

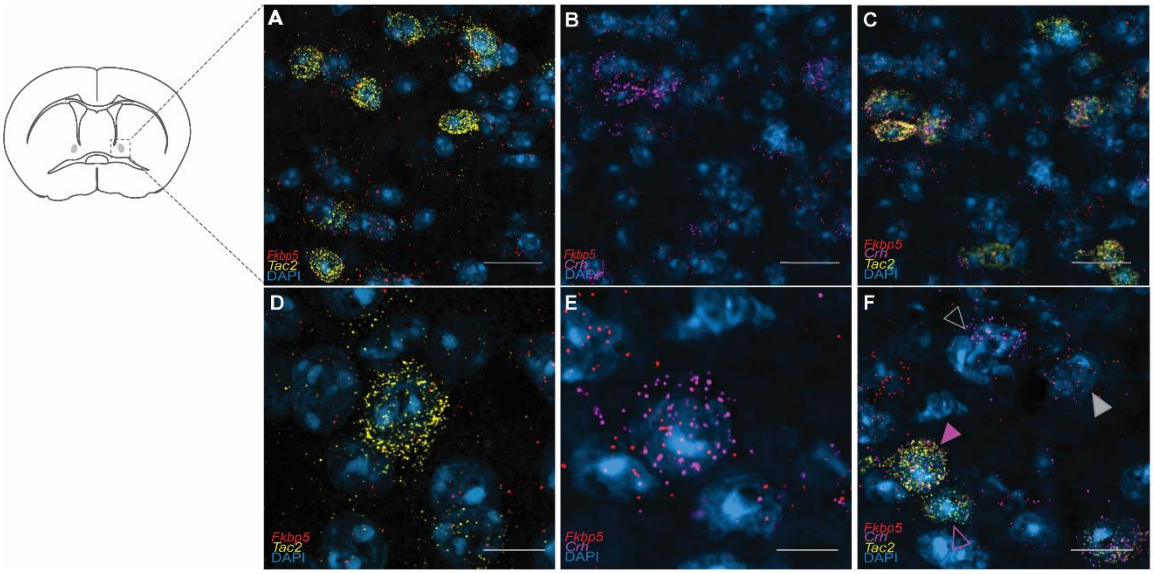

Figure 2: *Fkbp5* is co-expressed with the neuropeptides *Crh* and *Tac2* in the ovBNST. (**A**) *Fkbp5* and *Tac2* are strongly co-expressed in the ovBNST, as can be seen in detail (**D**). (**B**) *Fkbp5* and *Crh* are also co-expressed in the ovBNST as shown in detail (**E**). (**C**) Expression patterns of *Fkbp5* with *Tac2* and *Crh* in the ovBNST come in various combinations. (**F**) Violet arrow: *Fkbp5* positive cell co-expressing both *Crh* and *Tac2*. Violet outline arrow: *Fkbp5* positive cell strongly co-expressing *Tac2*. Grey arrow: *Fkbp5* positive cell weakly co-expressing *Tac2*. Grey outline arrow: *Fkbp5* positive cells strongly co-expressing *Crh*. Scalebar (A-C): 25 µm; (D-F): 10 µm.

Overexpression of Fkbp5 in the BNST

Following the characterization of *Fkbp5* expression and regulation after exposure to stress within the ovBNST, we investigated whether manipulation of *Fkbp5* would result in a behavioral phenotype and affect endocrinological parameters. Thus, viral-mediated gene transfer to the ovBNST was used to overexpress FKBP51, thereby mimicking a stress-related upregulation (**Fig.3A**). ISH of coronal sections was used to validate *Fkbp5*-OE and to exclude mice who presented off-target injection sites (**Fig. 3B**). Mice that were not infected bilaterally in the BNST were excluded from all analyses.

In total, 13 control and 13 FKBP51$^{BNST-OE}$ mice were used for further analysis. There were no significant differences between control and FKBP51$^{BNST-OE}$ animals in the time spent and the number of entries to the open arms in the EPM (**Fig. 3C**). However, FKBP51$^{BNST-OE}$ animals entered the lit zone of the DALI test significantly more often than control animals [T(23)= 2.114, p= 0.046, unpaired t-test], yet did not significantly differ in the distance covered within the light department (**Fig. 3D**). In addition, FKBP51$^{BNST-OE}$ and control animals did not differ in the total distance covered during the OF test (**Fig. 3E**). However, FKBP51$^{BNST-OE}$ animals demonstrated significantly lower corticosterone levels after a 15 min acute restraint stress, [F(3, 68) = 4.310], p=0.008, ANOVA] (**Fig. 3F**). While this experiment did not yield a clear behavioral phenotype, it alluded to the idea that *Fkbp5*-OE might have a mild anxiolytic effect and suppress HPA axis reactivity.

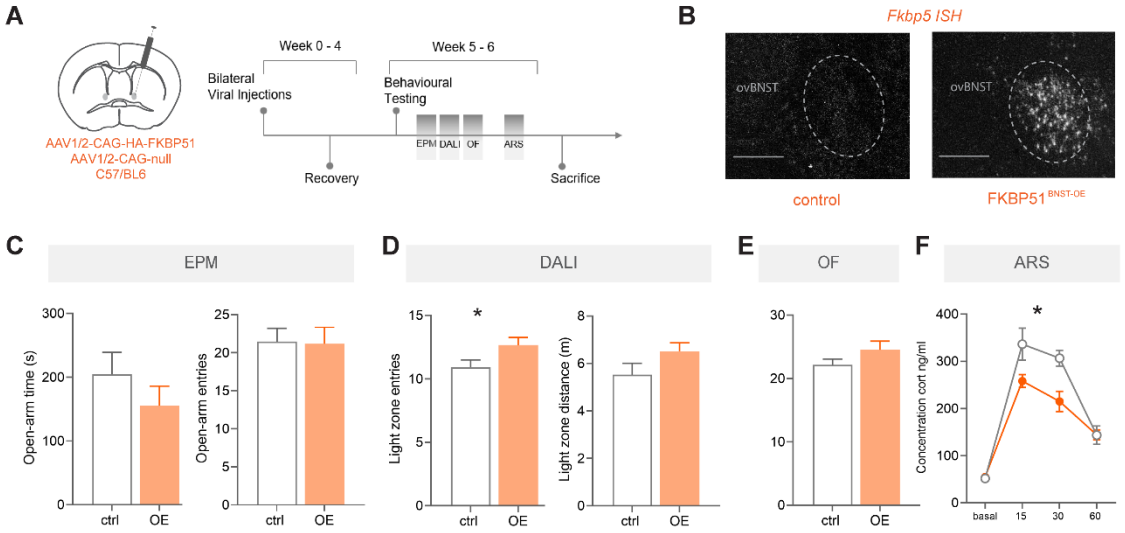

Figure 3: Overexpression of *Fkbp5* in the BNST. Virally mediated overexpression of *Fkbp5* (OE) did not yield a clear behavioral phenotype but indicated a tendency towards anxiolysis (**C-F**). (**A**) Schematic representation of viral manipulation and experimental timeline including testing battery. (**B**) *Fkbp5 ISH* demonstrating correct viral expression and appropriate targeting of the BNST. Scale bar: 250 µm. (**C**) There were no significant differences in open-arm time spent and number of open-arm entries in the elevated plus maze (EPM). (**D**) FKBP51$^{BNST-OE}$ animals entered the lit compartment of the dark-light box (DALI) more frequently than control animals, but there was no significant difference in the distance covered within the light zone. FKBP51$^{BNST-OE}$ and control animals did not differ in the total distance covered during the open field (OF) test (**E**). FKBP51$^{BNST-OE}$ animals demonstrated significantly lower corticosterone levels after a 15 min acute restraint stress (ARS) (**F**). Data are mean ± SEM. * P< 0.05; **P < 0.01.

Knockout of Fkbp5 in the BNST

The next experiment therefore explored the necessity of FKBP51 in the ovBNST on anxiety-related behavior and HPA axis regulation by specific *Fkbp5* deletion. ovBNST *Fkbp5*-KO was induced by viral manipulation and animals underwent the exact same testing battery as in the previous experiment (**Fig. 4A**). *Cre* ISH was used to validate *Fkbp5*-KO and correct targeting of the ovBNST (**Fig. 4B**). Mice that were not infected bilaterally in the ovBNST were excluded from all analyses.

Interestingly, FKBP51$^{BNST-KO}$ animals spent significantly less time in the open arms [U= 22, p= 0.001, Mann-Whitney test] and entered open arms of the EPM significantly less than control animals [T(20)= 3.171, p= 0.005, unpaired t- test] (**Fig. 4C**). Furthermore, whilst there was no significant difference for the number of entries into the light zone of the DALI test, FKBP51$^{BNST-KO}$ animals covered significantly less distance within the light zone [T (19)= 2.097, p= 0.049,unpaired t-test] (**Fig. 4D**). In addition, the two groups did not differ in the total distance covered during the OF test, indicative of unaltered locomotor behavior (**Fig. 4E**). Lastly, FKBP51$^{BNST-KO}$ animals exposed significantly higher corticosterone levels after a 15 min acute restraint stress [time: F (2.895, 60.80) = 111.1, p < 0.0001; condition: F(1, 21) = 5.308, p= 0.032] (**Fig. 4F**). Overall, these data confirm that *Fkbp5* manipulation in the BNST has a specific effect on stress-induced anxiety and highlight an anxiogenic phenotype for *Fkbp5*-KO in the ovBNST.

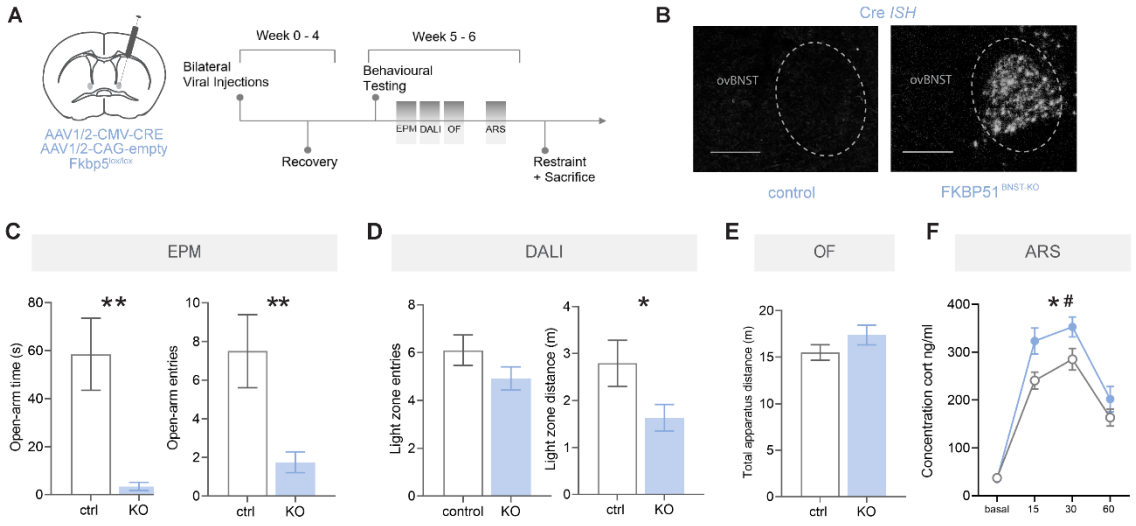

Figure 4: Knockout of *Fkbp5* in the BNST. Virally mediated knockout of *Fkbp5* (KO) resulted in a clear anxiogenic phenotype. (**A**) Schematic of viral manipulation as well as experimental timeline and testing battery. (**B**) Example of virus expression and correct targeting through *Cre ISH*. Scale bar: 250 µm (**C**) In the elevated plus maze (EPM), FKBP51$^{BNST-KO}$ animals (n=13) spent significantly less time in the open arms and entered open arms less frequently than control animals (n= 10). In line with this, FKBP51$^{BNST-KO}$ animals covered significantly less distance within the light area of the dark-light box (DALI) test (**D**). Total distance covered within the open field (OF) test increased for KO animals, albeit not significant. (**F**) FKBP51$^{BNST-KO}$ animals demonstrated a matching neuroendocrine phenotype, exposing significantly higher corticosterone levels after a 15 min acute restraint stress (ARS). Data are mean ± SEM. * P< 0.05; **P < 0.01; # P < 0.001.

Activity-dependent knockout of Fkbp5 in Fkbp5+ neurons of the ovBNST

Next, we investigated whether the lack of FKBP51 only in stress-activated cells of the ovBNST can recapitulate the anxiogenic phenotype observed in the KO experiment. Thus, *Fkbp5* was deleted in *Fkbp5* positive neurons that had been previously activated by acute restraint stress (**Fig. 5A**). Validation of correct targeting and KO was performed though *Fkbp5 ISH* (**Fig. 5B**). As in previous experiments, animals that were not infected bilaterally were excluded from all analysis.

Similar to the *Fkbp5*-KO in the BNST, activity-dependent KO of *Fkbp5* in the ovBNST indicated an anxiogenic phenotype. FKBP51$^{ovBNST-KO}$ animals spent significantly less time in the open arms [T(21)= 2.142, p=0.044, unpaired t-test)], but did not differ to the control group regarding the number of entries to the open arms (**Fig. 5C**). Furthermore, there was no significant difference in light zone entries or distance travelled during the DALI box (**Fig. 5D**). This was also observed for the total distance travelled in the OF test (**Fig. 5E**). Finally, there was no effect of *Fkbp5* ovBNST KO on endocrinological parameters following acute restraint stress (**Fig. 5F**). Thus, a more precise KO of *Fkbp5* in the ovBNST demonstrated an anxiogenic phenotype specific to the exploration of the unprotected arms of the EPM, aligning with the previous anxiety phenotype of *Fkbp5*-KO in the BNST.

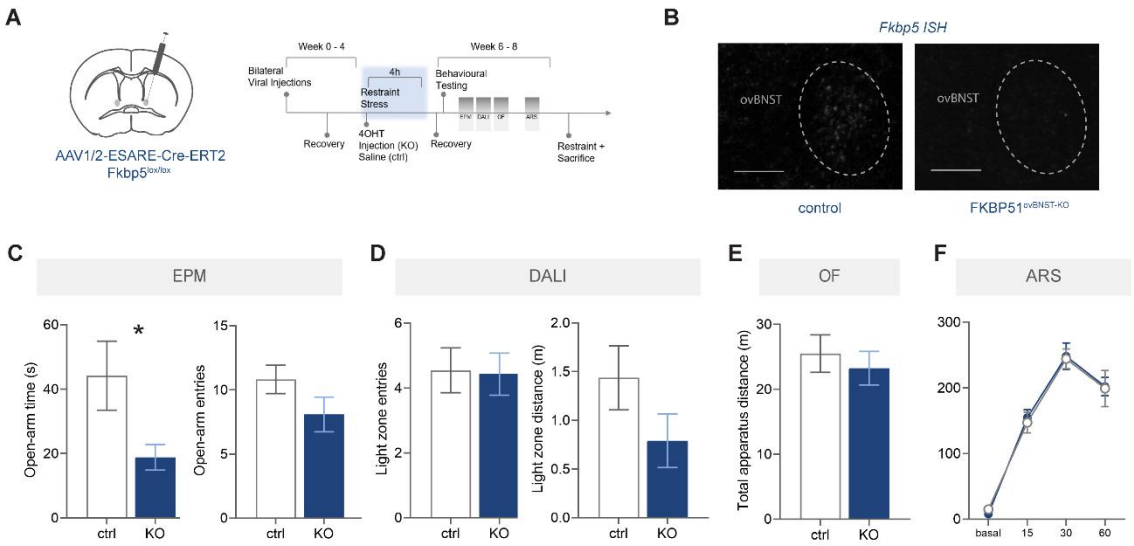

Figure 5: Activity-dependent knockout of *Fkbp5* in the BNST. Activity-dependent knockout of *Fkpb5* in the ovBNST tended towards an anxiogenic phenotype. **(A)** Schematic representation of ESARE promoter driven and 4OHT dependent conditional knockout of previously activated neurons in the ovBNST, as well as subsequent experimental timeline. (**B**) *Fkbp5 ISH* to validate correct viral manipulation. Scale bar: 250 µm (**C-F**) Activity-dependent knockout of *Fkpb5* in the ovBNST indicated an anxiogenic phenotype. (**C**) FKBP51$^{ovBNST-KO}$ (KO) animals spent significantly less time in the open arms of the elevated plus maze (EPM). In addition, they showed a tendency to travel less distance within the light zone of the dark-light box (DALI). The open field (OF) test (**E**) and the acute restraint stress response (**F**) did not show any significant differences between the two groups. Data are mean ± SEM. * P< 0.05; **P < 0.01.

Timeframe of anxiety-like behavior after exposure to acute restraint stress

The data on FKBP51 regulation in the ovBNST and anxiety-related behavior suggest that stress-induced increase of FKBP51 in this region might in fact have a protective role, thus leading to decreased anxiety. Consequently, we hypothesized that acute stress exposure should have a transient anxiolytic effect following the stress-induced FKBP51 upregulation within the ovBNST. To explore this hypothesis, C57Bl/6n mice were subjected to the EPM and OF test 12 hours after exposure to the acute restraint stress paradigm (Fig. 6A). Notably, animals that had been previously restrained spent increased time in the open arms of the EPM [T(18)= 2.306, p= 0.033, unpaired t-test] compared to controls (Fig. 6B). Furthermore, there were no differences in total distance travelled within the OF apparatus test (Fig.6C). These findings suggest that indeed acute stress exposure might lead to a transient anxiolytic phenotype due to the specific increase of FKBP51 in the ovBNST.

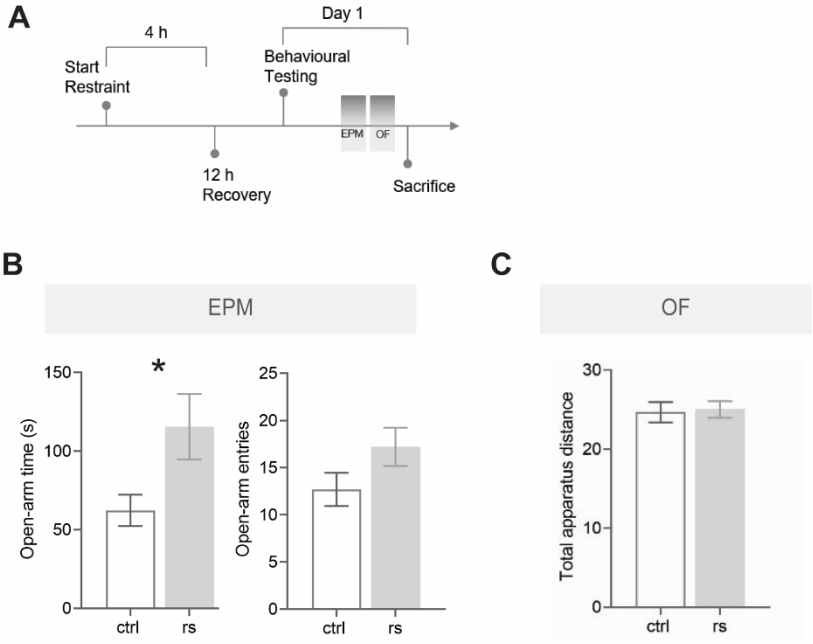

Figure 6: Timeframe of anxiety-like behavior after exposure to acute restraint stress. Exposure to acute restraint stress caused an anxiolytic phenotype. (A) Schematic representation of experimental timeline. (B) In the elevated plus maze (EPM), restrained animals (rs) spent significantly more time in the open arms. However, there was no difference regarding the number of entries to the open arms between the two groups. (C) The open field (OF) test did not show any significant differences between the two groups. Data are mean ± SEM. * P< 0.05; **P < 0.01.

Discussion

FKBP51 and the ovBNST play a pivotal role in stress-induced anxiety disorders (Ising et al., 2008; Kang et al., 2012; Kim et al., 2013; Klengel et al., 2013; Scheuer et al., 2016; Hu et al., 2020a, 2020b). However, the precise function of FKBP51 in the ovBNST and its role in anxiety remained unknown. Here we explore for the first time the function of FKBP51 in the ovBNST upon stress, and the subsequent effects on maladaptive anxiety behaviors, revealing ovBNST FKBP51's important role in anxiety. We demonstrate that ovBNST *Fkbp5* expression is upregulated after exposure to acute stress and co-expressed with

relevant stress-related neuropeptides. Furthermore, manipulation of *Fkbp5* in the BNST has an effect on anxiety-like behaviors. Remarkably, our results indicate that stress-induced increase of *Fkbp5* in that region might have a protective role regarding anxiety-like behavior.

Fkbp5 expression and regulation is highly region- and cell type-specific

Exposure to acute restraint stress significantly upregulated *Fkbp5* in the ovBNST. This upregulation seems reactive to acute stress only, since exposure to CSDS did not result in a significant upregulation. Another acute stressor confirmed this, showing *Fkbp5* upregulation relative to stressor severity. As the ovBNST is a predominantly GABAergic nucleus (Lebow and Chen, 2016), we confirmed *Fkbp5* expression and regulation in the majority of ovBNST GABAergic neurons, which reaches a maximum at around 4 hours after stress onset. The short-lasting but robust upregulation of ovBNST *Fkbp5* suggested a functional consequence of this regulation in the aftermath of an acute or traumatic stress exposure.

Furthermore, the ovBNST expresses a number of neuropeptides relevant for emotional regulation (Hammack et al., 2009; Lebow and Chen, 2016; Hu et al., 2020a), with CRH linked to anxiety-like states in the BNST (Butler et al., 2016; Faria et al., 2016; Pomrenze et al., 2019b; Hu et al., 2020a). CRH is most strongly expressed in the ovBNST and responsive to stress exposure (Dabrowska et al., 2013). Similarly, neurokinin B (encoded by Tachykinin 2 (*Tac2)* was recently related to the BNST, stress and anxiety-like behavior. Studies of *Tac2* in the central amygdala have previously implicated the peptide in fear memory and learning (Andero et al., 2016; Andero et al., 2014). Zelikowsky and colleagues demonstrated that social isolation stress resulted in a robust increase of *Tac2* mRNA expression in the CeA and the anterior dorsal BNST, linking *Tac2* expression to an anxiogenic phenotype (Zelikowsky et al., 2018b). We therefore assessed potential co-expression of *Fkbp5* with *CRH* and *Tac*2. Our results indicated that *Fkbp5* positive neurons in the ovBNST strongly co-express *Tac2* and *Crh*. Interestingly, expression patterns of *Fkbp5* with *Crh* and *Tac2* also partially overlapped and the presence of *Fkbp5/Tac2/Crh*-containing neurons suggest that these two neuropeptides might work in tandem with *Fkbp5* to mediate anxiety-like behaviour.

Tonic *Fkbp5* expression in the ovBNST shapes HPA axis responsivity to acute challenges

The anterior BNST acts as relay station between the mPFC and the PVN, mediating HPA responses to stress to activate the HPA axis to release corticosterone (Radley and Sawchenko, 2011; Lebow and Chen, 2016). The ovBNST expresses neuropeptides that innervate CRH-expressing neurons, and stimulate activation of the HPA axis (Hammack et al., 2009; Roman et al., 2014). Here, we observed reduced or suppressed HPA axis activity as a result of long-term ovBNST *Fkbp5*-OE and enhanced HPA axis response due to stable ovBNST *Fkbp5*-KO. Since there was no effect on HPA axis responsivity to acute stress when *Fkbp5*-KO was exclusively in ovBNST stress-activated neurons, we assume however that the long-term HPA axis impact of *Fkbp5* manipulation is not carried by the stress-activated neuronal population of the ovBNST, but possibly by a different distinct cellular population within this nucleus (Hammack et al., 2009; Roman et al., 2014). These findings point towards differential long-lasting changes in the responsivity of the HPA axis that potentially depend on *Fkbp5* status in the BNST.

The HPA response might be different in the immediate aftermath of a stressor that leads to transient ovBNST *Fkbp5* upregulation. Homotypic stress often results in habituation of the HPA axis whereas heterotypic stress can result in its sensitization (Kirschbaum et al., 1995; McEwen, 2003; Wüst et al.,

2005; Gianferante et al., 2014). However, these processes are highly time-dependent, as a blunted HPA axis response was previously observed if stressors were applied within 90 minutes (De Souza, 1982), but not after 150 minutes (Dallman and Jones, 1973) of the initial stress exposure. Recently GR signaling in CRH neurons of the PVN has been implicated in long-term stress habituation (Dournes et al.,2020.). Taken together, our data suggest that ovBNST *Fkbp5* likely plays a key role in adaptive HPA axis responses to repeated stressors.

Native ovBNST *Fkbp5* expression and regulation is necessary for normal anxiety-related behavior

Our genetic manipulation of native *Fkbp5* expression in the ovBNST demonstrates the central role of this co-chaperone in regulating anxiety-related behavior. While a stable and long-lasting *Fkbp5*-OE only led to a modest anxiolytic phenotype, likely due to the ectopic expression of *Fkbp5* in various cell types and adjacent BNST structures, a deletion of *Fkbp5* in the ovBNST or specifically in stress-activated cells of the ovBNST resulted in anxiogenesis. These findings are in contrast to most of the findings in other brain regions, where virally-mediated *Fkbp5*-OE resulted in an anxiogenic phenotype (Attwood et al., 2011; Hartmann et al., 2015; Criado-Marrero et al., 2019), whereas *Fkbp5*-KO or pharmacological inhibition were associated with more resilient behavior (Hartmann et al., 2015; Volk et al., 2016). However, these previous results were reported mainly from *Fkbp5* manipulations in the amygdala (Attwood et al., 2011; Hartmann et al., 2015; Volk et al., 2016; Criado-Marrero et al., 2019). Manipulations of *Fkbp5* in other regions, such as the dorsal hippocampus of mice, did not alter anxiety-like behavior (Hartmann et al., 2015). In addition, different BNST subregions are known to regulate anxiety in opposite directions (Kim et al., 2013) and there is likely even a functional differentiation among cells located in the same BNST sub-nucleus (Jennings et al., 2013). Thus, the ovBNST seems to play a special role in the extended amygdala network and *Fkbp5* in this region may act to reduce stress-induced anxiety. This might also explain the lack of anxiety-related phenotypes in the full *Fkbp5*-KO mice (Hartmann et al., 2012a), where *Fkbp5* is neither expressed in the ovBNST nor in the amygdala.

Stress-induced *Fkbp5* in the ovBNST may control transient stress-related anxiolysis

Our data suggest that the stress-induced increase of *Fkbp5* in the ovBNST might be protective, leading to temporary anxiolysis. While this might seem counterintuitive given the reported anxiogenesis following stress exposure (MacNeil et al., 1997; Mechiel Korte et al., 1999; Chotiwat and Harris, 2006; Grillon et al., 2007), anxiolysis has been reported before (Radulovic et al., 1998; Laxmi et al., 2003; Albrecht et al., 2013). Specifically, anxiolytic behavior in the EPM was shown after highly aversive context conditioning (Radulovic et al., 1998; Laxmi et al., 2003) and after single fear conditioning (Albrecht et al., 2013). Here, the observation of anxiolysis was always time-restricted and followed a delay after the stress exposure, possibly matching the time course of *Fkbp5* expression in the ovBNST. This hypothesis is supported by our data showing that a single episode of restraint stress leads to reduced anxiety 12 hours later.

Interestingly, the phenomenon of alleged anxiolytic or resilient behavior after a stressful or traumatic event has also been reported clinically. Moreover, the BNST has been repeatedly associated with PTSD-like-behavior (Buff et al., 2017; Awasthi et al., 2020). Several studies have identified exposure to traumatic events and subsequent symptoms of PTSD as risk factors for increased impulsivity and risk-taking behavior (Ben-Zur and Zeidner, 2009; James et al., 2014). Furthermore, acute stress has been

shown to modulate decision-making processes, increasing individuals' risk taking behavior in a time-dependent manner (Bendahan et al., 2017). In animal research, the EPM measures exploratory drive and anxiety in a novel environment, which is potentially useful for new niche discovery (i.e., food resources and reproductive partners) but also dangerous (i.e., predators). An increase in the open arm time or entries could therefore also be interpreted as an increase in risk-taking exploration (Macrì et al., 2002; Cortese et al., 2010; Högman, 2014). While comprehensive proof of the role of *Fkbp5* in the ovBNST on stress-induced anxiolytic behavior is still lacking, our data support this notion.

In summary, we present the first characterization of *Fkbp5*'s role in the ovBNST on HPA axis function and anxiety-related behavior. Our findings suggest that here, stress induction of *Fkbp5* may have a protective role, leading to decreased anxiety and suppression of future stress-induced HPA axis activation. Further in-depth investigations of the causality will help to understand the full extent of the underlying mechanisms and lead to a better understanding of ovBNST's role in stress-induced anxiety disorders.

Author contributions

C.E. and M.V.S. conceived the project and designed the experiments. C.E. managed the mouse lines and genotyping. C.E. and F.T. performed animal experiments and surgeries. J.B., L.B., L.v.D., A.S.H., M.L.P., K.S. and H.Y. assisted with the experiments. C.E. and F.T. analyzed the data. A.C. and J.M.D. provided scientific expertise for establishing *Fkbp5* mouse lines. C.E. and M.V.S. wrote the initial version of the manuscript. All authors revised the manuscript.

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2.4. The role of the neuropeptide *Tac2* in the oval bed nucleus of the stria terminalis and its implication in stress-induced anxiety-related behaviour

Engelhardt, C.*, Tang, F*., Bordes, J., Brix, L.M., van Doeselaar, L., Yang, H., Klein, R., Deussing, J.M., & Schmidt, M.V.

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Abstract

The neuropeptide Tachykinin 2 (*Tac2*) and the oval bed nucleus of the stria terminalis (ovBNST) are both emerging key players in stress-induced maladaptive anxiety. However, the role of *Tac2* in the ovBNST and its implication in anxiety-related behaviour has not been explored yet. Here we demonstrate that *Tac2* in the ovBNST is upregulated by exposure to acute stress. Moreover, *Tac2* positive neurons of the ovBNST project to brain structures that modulate anxiety, therefore pointing towards functional participation of this neuronal population in anxiety circuitry. In line with this, chemogenetic inhibition of ovBNST *Tac2* positive neurons resulted in a mild anxiogenic-like phenotype. Together, our findings lay the groundwork for further research focusing on the relationship and mechanistic effects of *Tac2* and the ovBNST in stress-induced anxiety states.

Introduction

Anxiety is critical for survival as it enables assessment of the environment while minimizing exposure to potential threats. However, exposure to extended or acute and intense stressors, particularly those that are life-threatening, can lead to maladaptive anxiety, or anxiety disorders such as post-traumatic stress disorder (PTSD) (Etkin et al., 2010; McLaughlin et al., 2014; Fox et al., 2015). Anxiety disorders are highly prevalent and the neural mechanisms underlying maladaptive anxiety remain ambiguous.

Previous studies have identified that the central extended amygdala, comprised of the bed nucleus of the stria terminalis (BNST) and the central amygdala (CeA), plays a key role in the generation of fear and anxiety states (Davis et al., 2010; Calhoon and Tye, 2015; Tovote et al., 2015; Gungor and Paré, 2016; Ahrens et al., 2018). Early research in fear (the phasic emotional state induced by explicit and acute threats) and anxiety proposed a model in which anxiety (sustained) and fear (phasic) were putatively separate processes (Walker and Davis, 1997; Barlow, 2002; Sylvers et al., 2011). More recent evidence, however, from humans and animals demonstrates that both structures are activated by potential and explicit threats (Mobbs et al., 2010; Fox et al., 2015; Gungor and Paré, 2016). These findings suggest that the BNST and CeA coordinate to orchestrate both acute and chronic responses to various kind of stressful stimuli, reinforcing a more nuanced conceptual and physiological understanding of fear and anxiety states.

The BNST in particular is a highly complex structure comprised of many subregions and heterogenous neuronal populations, which likely form discrete neural circuits subserving distinct aspects of anxiety-related behavior (Kim et al., 2013; Lebow and Chen, 2016). For instance, Kim and colleagues reported decreased measures of anxiety- like behavior upon optogenetically inhibiting dopamine-receptor-1a (DrD1a) positive cells in the oval BNST (ovBNST), whereas light-induced decreases in neuronal activity in the anterodorsal BNST (adBNST) DrD1a resulted in an anxiogenic phenotype (Kim et al., 2013). Likewise, a recent study provided evidence that chronic stress in male mice increased anxiogenic behaviors and cellular excitability of corticotrophin releasing hormone (CRH) positive cells in the ovBNST (Hu et al., 2020a). The ovBNST is therefore likely to be highly involved in the mediation of anxiety- like

states. Interestingly, a substantial portion of ovBNST input originates from CeA inhibitory afferents, with subsets of CeA CRH+ (Pomrenze et al., 2019a) and somatostatin (SOM) positive (Ahrens et al., 2018) GABAergic projections that provide an anatomical basis between these two extended amygdala nuclei for the coordinated modulation of anxiety.

Neuropeptides, most notably CRH, have been implicated in mediating the stress response and mal-/adaptive anxiety (Kash et al., 2015; Chen, 2016; Hu et al., 2020a, 2020b). Exposure to stress induces CRH, which is abundantly expressed in the hypothalamic paraventricular neurons and responsible for HPA axis regulation and cortisol/corticosterone release. CRH is also highly expressed in the BNST (Pomrenze et al., 2015; Sanford et al., 2017); in fact, the highest concentration of CRH neurons in the brain is located in the ovBNST (Daniel and Rainnie, 2016b). Consequently, Hu and colleagues observed that early life stress resulted in a long-lasting activation of CRH signaling in the mouse ovBNST, leading to increased anxiogenic behaviors later in adulthood (Hu et al., 2020b). In general, the ovBNST and the CeA have a very similar neurochemical profile, expressing a wide range of neuropeptides such as CRH, dynorphin, neuropeptide Y, enkephalin, neurokinin B, etc (Lebow and Chen, 2016; Zelikowsky et al., 2018b) .

The effects of one key neuropeptide in particular and its implication in stress-induced anxiety however have been largely overlooked; neurokinin B (NkB), encoded by the *Tac2* gene in rodents and the *Tac3* gene in humans, belongs to the family of tachykinin peptides and has long been accepted as a robust modulator for neuronal activity (Otsuka and Yoshioka, 1993). More recent studies of *Tac2* /NkB in the CeA have linked the peptide to fear memory consolidation, suggesting a role in fear learning and expression (Andero et al., 2016; Andero et al., 2014). Likewise, Zelikowsky and colleagues demonstrated that chronic social isolation stress (SIS) resulted in a significant increase of *Tac2* mRNA expression in both the CeA and the adBNST, consequently linking *Tac2* upregulation to an anxiogenic phenotype (Zelikowsky et al., 2018b). Subsequently, systemic administration of Osanetant, a NkB receptor (Nk3R) antagonist, reversed SIS induced anxiety- like behavior. Moreover, chemogenetic activation of *Tac2*+ neurons combined with virally mediated *Tac2* overexpression mimicked the effects of SIS, and region-specific chemogenetic silencing of *Tac2* positive neurons blocked distinct behavioral responses to SIS. These results point towards a promising role of *Tac2* in the BNST and CeA regarding its impact on stress-related anxiety-like behaviors. However, the BNST is comprised of 18 sub-nuclei, which regulate anxiety in different, sometimes opposing directions (Bota et al., 2012). Specifically, the ovBNST and the adBNST exert opposite effects on the anxious state (Kim et al., 2013). In addition, the activity of *Tac2*+ neurons is reportedly differentially required in different brain regions for different features of anxiety-like behavior (Zelikowsky et al., 2018b, 2018a). Evidence on the role of *Tac2* in the ovBNST, and to a certain extent in the CeA, and whether manipulation of ovBNST *Tac2* would have an impact on stress-induced anxiety-like behavior is still missing.

Here, we explore expression and regulation of *Tac2* after acute stress exposure in the ovBNST. Furthermore, we identify projections sites of ovBNST *Tac2*+ neurons. Lastly, chemogenetic

manipulation of *Tac2*+ ovBNST neurons points towards their implication in anxiety-related behavior.

Materials and Methods

Animals and Animal housing

Experiments were carried out with C57Bl/6n and Tac2-Cre mice (Cai et al., 2014; Zelikowsky et al., 2018a). obtained from the in-house breeding facility of the Max Planck Institute of Psychiatry, Munich. All animals used during the experiments were male and between 8-14 weeks old. Initially, mice were group housed and then single-housed at least 1 week prior to the start of the experiment. Housing parameters in the holding and testing rooms were kept constant on a 12h light: 12h darkness cycle, with controlled temperature (22± 2°C) and humidity (45± 10%). Food (Altromin 1324, Altromin GmBH, Germany) and water (tap water) was provided *ad libitum*. 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. Protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Stereotactic viral injection and guide cannula/fiberoptic cannula implantation

Chemogenetic manipulation of *Tac2* positive cells was performed using adeno-associated bicistronic AAV1/2 vectors (AAV2-eSyn-GFP and AAV1/2-hSyn-DIO-hM4D-mCherry; Addgene). For the tracing of neuronal *Tac2* positive axonal projections AAV-EF1a-eNPH3.0-mcherry (UNC, Karl Deisseroth) was used.

Stereotactic injections were performed as described previously (Schmidt et al. 2011). Briefly, an injection volume of 0.3 µl using a glass capillary with a tip resistance of 2 -3 MΩ over 6 min (0.05 µl/min) was used to deliver the virus. For bilateral microinjections into the ovBNST, mice were anesthetized with isoflurane and fixed in a stereotaxic frame in order to target the following coordinates relative to Bregma: posterior 0.8 mm; 1.2 mm lateral; 4.3 mm ventral.

All mice recovered in their home cage for 3 weeks until the start of behavioural experiments. Successful virus expression and correct targeting in all mice was validated through immunohistochemistry (IHC). Mice that were not infected bilaterally in the ovBNST were excluded from all analyses.

Clozapine N-oxide (CNO) Treatment

CNO (BML-NS105, Enzo LifeSciences, Brussels) (final dose: 5 mg/kg, injection volume 10 µl/g body weight) was injected intraperitoneally 30 min before each behavioral test and before the sacrifice in experimental mice. Control mice were injected the same amount of vehicle (saline).

Stress Paradigms

Acute Restraint Stress

Mice were restrained manually and guided into a 50 ml falcon tube. The falcon contained holes in the top and the lid to allow for sufficient ventilation and tail movement. The restraint lasted 4 hours and animals were sacrificed immediately after.

For the timecourse of *Tac2* regulation, the acute restraint stress paradigm was modified, so that mice were restrained for 1h, 2h, and 4h, respectively. They were all sacrificed immediately after the restraint or after a 4h recovery period in their homecage.

Behavioral Paradigms

All behavioral testing was carried out in the animal facility of the Max Planck Institute of Psychiatry, Munich. Tests were performed during the light phase between 7am and 1pm to avoid potential behavioral alterations due to circadian variation of corticosterone levels. The behavioral testing for Experiment 3 (DREADD manipulation) was conducted in the following order: elevated plus maze (EPM), dark-light box (DALI), open field (OF) and acute stress response (ASR).

Recording, tracking and scoring of animal behaviors was carried out using the automated video tracking system ANY-maze (ANY-maze 6.18; Stoelting Co, Wood Dale, IL, USA). All tests were performed by an experienced, blinded researcher and according to established protocols.

EPM

The EPM was performed as previously described (Santarelli et al., 2014). Briefly, animals were 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). 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 5-minute trial.

DALI

The apparatus was comprised of a dark and protected compartment (15 x 20 x 25 cm, dimly lit under 10 lux) and a brightly illuminated compartment (30 x 20 x 25 cm, lit with 700 lux); both compartments were connected by a tunnel of 4 cm in length. Mice were placed in the dark compartment, facing towards a wall and recorded for 5 min. To assess anxiety-related behavior, the time spent, number of entries and latency to first entry into the lit compartment were measured.

OF

Mice were placed in a corner of a 50 x 50 x 50 cm plastic arena. Fifteen-minute trials were video recorded by an overhead camera. The test was performed under conditions of a highly lit center zone

(20 lux; 20x 20 cm) and a dimly lit (16 lux) outer zone. Parameters of interest regarding anxiety-like behavior were time spent and number of entries to the center zone during the first 5 min.

Tissue collection and processing

Animals were anesthetized with isoflurane and sacrificed by decapitation. Brains were removed, snap frozen and stored at -80°C. Alternatively, mice were anesthetized with isoflurane and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% (v/v) paraformaldehyde (PFA) fixative in PBS. Brains were rapidly removed and post-fixed in PFA overnight at 4°C. The following day, post-fixed brains were transferred to a cryoprotectant solution (30% sucrose in 0.1 M PBS) for two additional overnight incubations at 4°C. Brains were stored at 4°C until processed.

Immunohistochemistry

Virus injections were validated using immunohistochemical staining. Antibodies used were: anti-mcherry rabbit (abcam, ab167453), anti-cfos rat (Synaptic Systems, 226017), anti-cfos guinea pig (Synaptic Systems, 226004), anti- GFP rabbit (MBL, 11072720), alexa 488 anti-rabbit (abcam, ab150077), alexa 594 anti-rat (abcam, ab150160), alexa 647 anti-guinea pig (abcam, ab150187). Briefly, serial free-floating sections were washed in 0.1 M PBS and incubated in blocking solution (10% normal goat serum (NGS)/ 1% Triton/ 0.1 M PBS) for 30 min at room temperature. Sections were incubated with the primary antibody (diluted 1:500 -1000 in 1% NGS/ 0.3% Triton/ 0.1 M PBS) overnight at 4°C. Sections were washed with 0.1 M PBS and incubated with secondary antibody (1:500-1000) for 2 h in a light protected environment. After washes with 0.1 M PBS, the sections were mounted with DAPI medium (Fluoromount-G Cat. No. 0100-20) and cover-slipped.

Fresh-frozen sections were treated with an adapted version of the protocol described above. A hydrophobic barrier PAP pen (Sigma-Aldrich) was used to encircle the sections. Blocking and antibody solutions were pipetted onto the encircled region. Furthermore, slides were covered with parafilm (neoLab, Germany) during antibody incubation to prevent drying out and ensure constant coverage of the sections.

In Situ Hybridization (ISH)

Tac2 gene expression in the ovBNST was examined by ISH. Coronal whole-brain slices were cryosectioned at a thickness of 20 µm and directly mounted onto Superfrost Plus Slides as 6 sequential series. A cRNA anti-sense riboprobe was transcribed from linearized plasmid DNA (all primer details are available upon request). ISH was performed as previously described (M. Schmidt et al. 2003; 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°C. On the next day, the sections were rinsed in 2 × SSC (standard saline citrate), treated with RNAse A (20 mg/l). After several washing steps with SSC solutions at room temperature, the sections were washed in 0.1 × SSC for 1 h at 65°C and dehydrated through increasing concentrations of ethanol. Finally, the slides were

air-dried and exposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) for up to 14 days. ISH autoradiographs were digitized, and expression was determined by optical densitometry utilizing ImageJ. 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.

RNAscope

RNAscope is a novel ISH assay for detection of target RNA within intact cells. This approach allows for multiplex detection of up to four target genes. The procedure was performed according to manufacturer's specifications and with the RNAscope Flourescent Multiplex Reagent Kit (Cat. No. 320850, Advanced Cell Diagnostics, Newark, Ca, USA). The probes used for detection was Tac2 (Mm-Tac2-C2). Briefly, fresh frozen sections at a thickness of 20 µm were fixed in 4% PFA for 30 min at 4°C and dehydrated in increasing concentrations of ethanol. Next, tissue sections were incubated with protease IV for 30 min at room temperature. The probes were mixed at a ratio of 1:1:50 and hybridized for 2 h at 40°C followed by four hybridization steps of the amplification reagents 1 to 4. The sections were then counterstained with DAPI, cover-slipped and stored at 4°C. Images were acquired with experimenter blinded to probes used.

Microscopy and Image Processing

For RNAscope, sixteen-bit images of each section were taken on a laser scanning confocal microscope (LSM 800, Zeiss, Jena, Germany) with a frame size of 1024px x 1024px (8 bits per pixel) using a 20x, 40x, and 63x objective. Within a sample, images were acquired with identical settings for laser power, detector gain, and amplifier offset.

Fluorescent images of IHC treated brain sections were obtained using a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) and the AxioVision 3.2 software (Zeiss,Jena, Germany). Black and white images of the ovBNST were taken with the 2.5x, 10x, and 20x objectives. Images were processed using ImageJ (National Institute of Health,USA).

ISH slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY) and exposed at 4°C for 2-3 weeks for better visibility of the gene expression and regulation. Slides were developed and examined with the Zeiss light microscope (5x objective) to show mRNA expression.

Experimental Design and Statistical Analyses

Data were analysed using IBM SPSS Statistics 25 software (IBM SPSS Statistics, IBM, Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software, San Diego, California, USA). For the comparison of two groups, the independent Student's t-test was applied. If the data were not normally distributed, the non-parametric Mann-Whitney (MW-test) was used. Data with more than two groups were tested by the appropriate analysis of variance (ANOVA) model followed by Bonferroni post-hoc analysis to determine statistical significance between individual groups. In the event of multiple time points, a repeated-measures ANOVA was performed. If a single value was missing from the data, mixed model

analysis was applied. All data are shown as means +/- standard error of the mean (SEM). Significance was accepted if $p < 0.05$, trends were observed at $0.05< p<0.1$.

Results

Expression and regulation of Tac2 in the ovBNST

To gain an understanding of the possible functions of *Tac2* in the ovBNST, we first characterized its expression and regulation pattern under basal conditions and after exposure to stress (Fig. 1 and 2). Using RNAscope (*Tac2*: yellow), we observed expression of *Tac2* predominantly within the ovBNST region under basal conditions (Fig.1A,B). To gain more information on the dynamics of *Tac2* upregulation within the ovBNST after exposure to acute stress, a time course was performed (Fig. 2). An *ISH* revealed that *Tac2* was significantly upregulated after exposure to acute restraint stress [$F(4, 17) = 5{,}879$, $p=0.0037$, ANOVA], with significant differences between 1h vs 2h [$t(17)= 3.661$, $p= 0.0194$; Bonferroni] and 1h vs 4h [$t(17)= 3.661$, $p= 0.0135$; Bonferroni], and peaking after 4 hours (Fig. 2A,B).

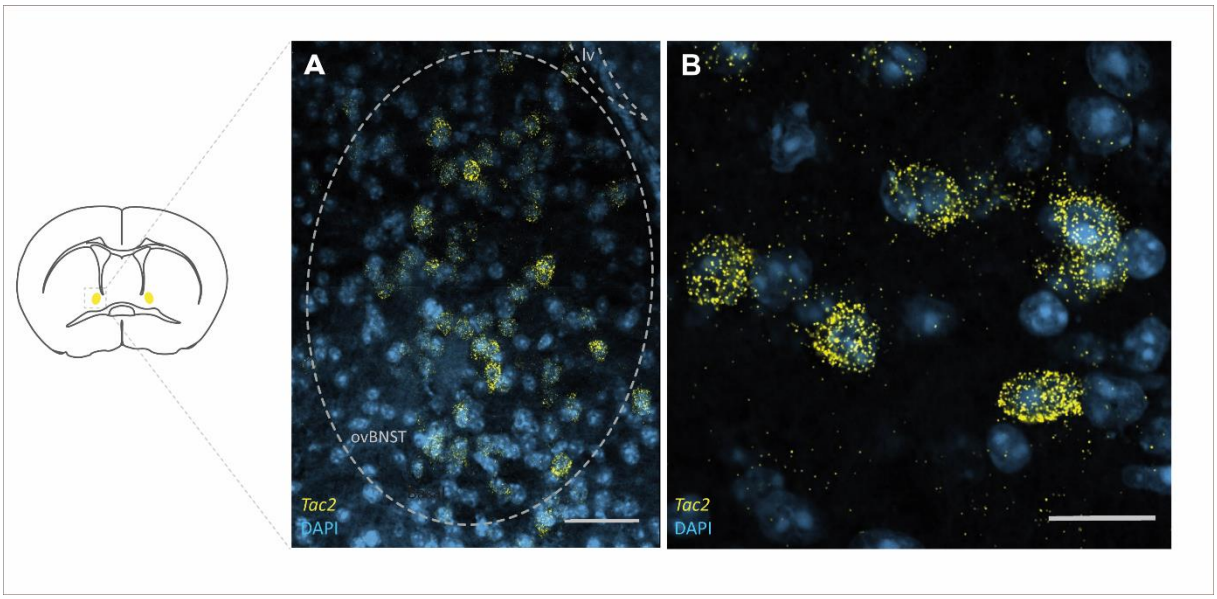

Figure 1: Expression of *Tac2* in the ovBNST. (A) *Tac2* is expressed in the ovBNST at basal level, as shown through RNAscope (yellow: Tac2). Scalebar: 50 µm. **(B)** Detail showing ovBNST *Tac2* mRNA expression. Scalebar: 20 µm

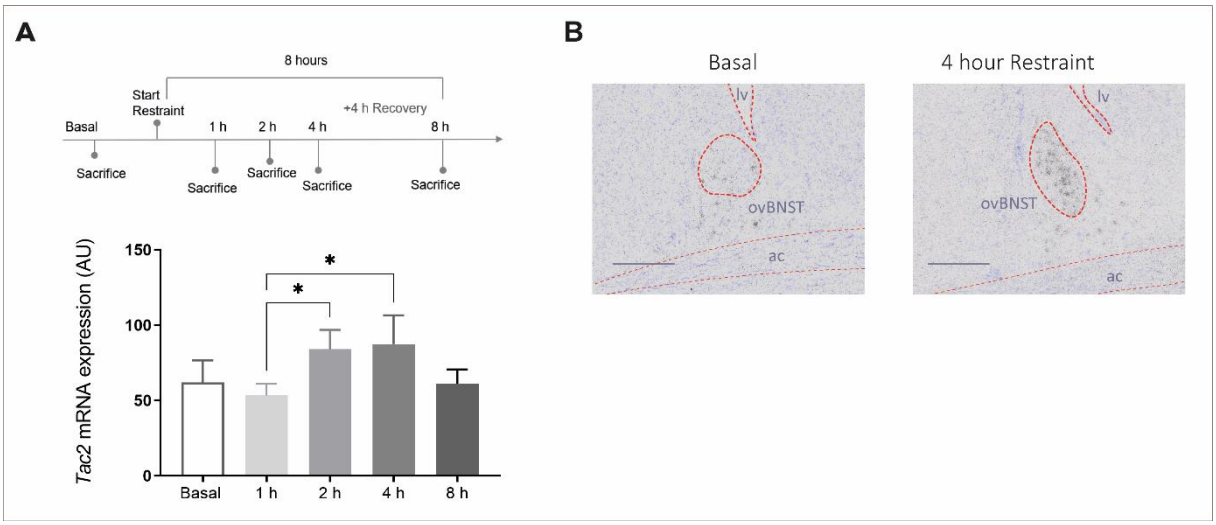

Figure 2: Upregulation of *Tac2* in the ovBNST after exposure to acute stress. (A) Timecourse of *Tac2* after exposure to varying times of restraint stress revealed a significant upregulation of *Tac2* in the ovBNST. Specifically, *Tac2* upregulation peaked after 4 hours. Data are mean ± SEM. * P< 0.05. **(B)** Example of ovBNST *Tac2* mRNA expression at basal level and after 4h of acute restraint stress. Scalebar: 200 µm.

Projections of ovBNST Tac2 positive neurons

Since the BNST is a highly heterogenous and interconnected structure with specific subregions coordinating different features of anxiety-like behavior (Kim et al., 2013), we were interested in the projection sites of *Tac2* positive neurons of the ovBNST. *Tac2*- Cre mice were injected in the ovBNST with a halorhodopsin virus (AAV-EF1a-eNPH3.0-mcherry) to trace axonal projections. The regions identified were the prefrontal cortex, in particular the infralimbic (IL) and prelimbic cortex (PL) (Fig. 3A); the lateral septal nucleus, ventral part (LS) (Fig. 3B) and the ovBNST (as injection site) and the adBNST (Fig. 3C). Moreover, projection terminal sites were visible in the thalamic nuclei (Fig. 3D) and the amygdala, specifically the BLA (Fig. 3E) as well as the lateral (CeL) and medial division (CeM) of the CeA. These findings are in line with previous results regarding BNST macro- and micro-circuitry (Lebow and Chen, 2016), but also provide a new insight into the unique circuitry of ovBNST *Tac2* neurons that might be implicated in mediating anxiety-related behaviours.

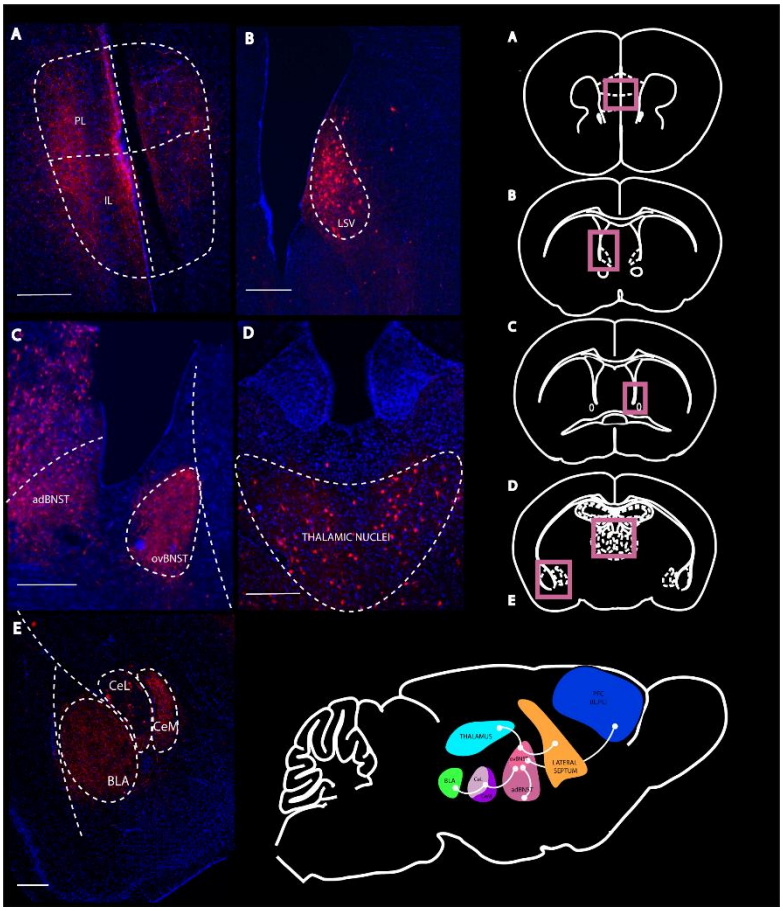

Figure 3: *Tac2* positive neurons of the ovBNST project to various structures. Labeling of axonal projections after injection of AAV-EF1a-eNPH3.0-mcherry in the ovBNST of Tac2- Cre mice. Regions identified were **(A)** the prefrontal cortex, specifically the pre-limbic (PL) and infra-limbic (IL) cortex; **(B)** the lateral septal nucleus, ventral part (LSV); **(C)** the oval bed nucleus (ovBNST) as injection site and the anterodorsal bed nucleus (adBNST); as well as the thalamic nuclei **(D)** and the amygdala **(E)**, specifically the basolateral (BLA), central, lateral division (CeL) and central, medial division (CeM). **(F)** Schematic illustrating structures implicated in ovBNST *Tac2* circuitry. Scale bars: 250 µm.

Chemogenetic inhibition of Tac2 positive neurons in the ovBNST

Following the characterization of ovBNST *Tac2* expression, regulation and projection sites, we investigated whether manipulation of *Tac2* positive neurons would result in a behavioral phenotype. Thus, viral-mediated gene transfer to the ovBNST was used to chemogenetically inhibit *Tac2* positive neurons of the ovBNST (Fig. 4A). IHC of coronal sections was used to validate ovBNST virus expression and targeting, as well as appropriate virus function in terms of inhibiting *Tac2* neurons (Fig. 4B). In total, 9 experimental (hm4D) and 15 control animals were used for further analysis. There were no significant differences between control and hm4D animals in the time spent and the number of entries to the open arms in the EPM (Fig. 4C). However, hm4D animals entered the lit zone of the DALI test significantly fewer times than control animals [t(22)= 2.336, p= 0.029, unpaired t-test], yet did not significantly differ

in the distance covered within the lit department (Fig.4D). In addition, hm4D and control animals did not differ in the total distance covered during the OF test (Fig. 4E). While this experiment did not yield a clear behavioral phenotype, it alluded to the idea that chemogenetically inhibiting *Tac2* positive neurons in the ovBNST might have a mild anxiogenic effect on behavior.

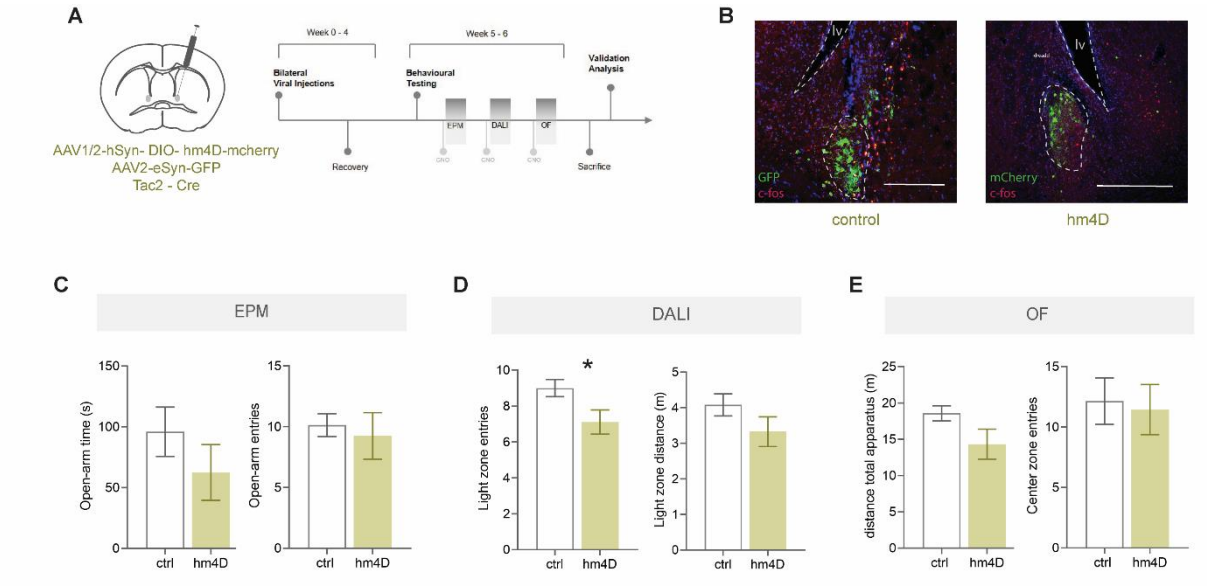

Figure 4: DREADD inhibition of *Tac2* positive neurons in the ovBNST. Chemogenetic inhibition tended towards a mild anxiogenic phenotype. **(A)** Schematic representation of viral manipulation via inhibitory DREADDs and subsequent experimental timeline. **(B)** IHC to validate correct targeting and sufficient inhibitory activity of the virus. Scalebar: 200 µm. **(C)** Chemogenetic inhibition of *Tac2* neurons in the ovBNST did not result in any significant differences between control and hm4D animals in the elevated plus maze (EPM). **(D)** However, hm4D animals entered the light zone of the dark-light test (DALI) significantly fewer times than controls **(E)**. This tendency could not be observed in the open field (OF) test, where no significant difference between the two groups was reported. Data are mean ± SEM. * P< 0.05; **P < 0.01.

Discussion

Both the ovBNST and more recently the neuropeptide *Tac2* have been associated with stress-induced anxiety-like behaviour. However, the specific role of *Tac2* in the ovBNST and its implication in anxiety have not been explored in detail yet. The majority of studies reported *Tac2* mRNA upregulation after exposure to chronic stress paradigms (Zelikowsky et al., 2018b; Hook et al., 2019). Here, for the first time we observed region-specific induction of *Tac2* in the ovBNST after exposure to acute restraint stress. Moreover, we identified structures such as the CeA and the LS as projection sites of ovBNST *Tac2* positive neurons, that have been consistently linked with neuronal circuits of anxiety. Subsequently,

chemogenetic manipulation of ovBNST *Tac2* positive neurons alluded towards their involvement in anxiety-related behaviour.

Tac2 is expressed in the ovBNST and reactive to acute stress exposure

Tac2 expression within the BNST has been reported previously (Zelikowsky et al., 2018b). Specifically, Zelikowsky et al observed expression and upregulation of *Tac2* in the adBNST and other brain structures including the dorso-medial hypothalamus (DMH) and the CeA. In general, *Tac2* upregulation has been demonstrated in response to chronic stress (e.g. two weeks of social isolation or chronic mild stress (Zelikowsky et al., 2018b; Hook et al., 2019)), but not acute stress (e.g. 2h restraint stress or 24h of social isolation (Andero et al., 2014; Zelikowsky et al., 2018b)). Our data showed that *Tac2* is expressed under basal conditions in the ovBNST. Remarkably, ovBNST *Tac2* mRNA was significantly upregulated after exposure to a 4h acute restraint stress. Moreover, we demonstrate that *Tac2* upregulation reaches its maximum at around 4 hours after stress onset and returns back to basal after 4 hours restraint and an additional 4 hours of recovery. This short-lasting but strong upregulation of ovBNST *Tac2* might indicate a functional consequence of this mechanism in the aftermath of an acute or traumatic stress exposure.

Tac2 positive ovBNST neurons project to brain structures implicated in anxiety circuitry

The ovBNST integrates information about mood and negative valence via CRH, PACAP, dynorphin, dopamine and encepahlin projections while modulating anxiety-like behaviour (Lebow and Chen, 2016). In line with this, our findings confirmed that *Tac2* positive neurons within the ovBNST project to brain structures that are principal components of the anxiety circuitry (Calhoon and Tye, 2015; Tovote et al., 2015; Lebow and Chen, 2016). While the extended amygdala has dominated research on the neural circuitry of fear and anxiety, the LS and the PFC were shown to be directly involved in stress-induced behavioural and endocrinological dimensions of anxiety states (Anthony et al., 2014; Parfitt et al., 2017). We further observed fluorescent signal in the adBNST and the thalamic nuclei; the intrinsic circuitry of the BNST is highly complex , with a large amount of connectivity in and between the smaller BNST nuclei (Dong et al., 2001b; Dong and Swanson, 2004b). The ovBNST and the adBNST were demonstrated to act as opposite players in the modulation of anxiety states (Kim et al., 2013). The same study also revealed that ovBNST Drd1a neurons, which are also expressed by CRH neurons of the ovBNST, send an inhibitory projection to undifferentiated adBNST regions (Kim et al., 2013; Daniel and Rainnie, 2016b). Likewise, Engelhardt et al (2021) illustrated that *Tac2* and CRH are co-expressed in the ovBNST. More recently, Bruzsik and colleagues also observed that somatostatin and CRH positive BNST neurons projected to the thalamic nuclei region identified in our results, suggesting an involvement in learned fear responses (Bruzsik et al., 2021). Thus, the current findings are coherent with previous results. Overall, *Tac2* positive neurons of the ovBNST and their projections pose a substantial component of anxiety circuitry.

Chemogenetic inhibition of ovBNST *Tac2* positive neurons has a mild anxiogenic behavioural effect

Our chemogenetic manipulation of *Tac2* positive neurons in the ovBNST further suggested a role of this neuropeptide in the modulation of anxiety-like behaviour. More specifically, inhibition of ovBNST *Tac2* neurons through the means of DREADDs revealed a mild anxiogenic phenotype. These findings are contrary to Zelikowsky and colleagues', who observed a significant anxiolytic effect when they

chemogenetically inhibited *Tac2* neurons in the adBNST after exposure to three weeks of chronic social isolation stress. However, it is important to keep several aspects in mind: first of all, we only targeted the ovBNST. Since there is likely functional differentiation among BNST sub-regions and even among cell populations within the same BNST sub-nucleus, a different anxiety-like phenotype may not be completely excluded. Second, our mice were not previously stressed by three weeks of single housing (SIS paradigm), but instead were group housed until 1 week prior to the start of experiments. Furthermore, we initially observed *Tac2* mRNA upregulation in the ovBNST after exposure to acute restraint stress. Thus, possibly, there is a difference of effect due to the type of stressor (social vs non-social) and the dose (SIS exposure before chemogenetic manipulation) which may contribute to a more distinct anxiety phenotype. Therefore, it would be interesting to explore how an acute restraint stress prior to ovBNST *Tac2* chemogenetic inhibition may affect anxiety behaviour. Another factor to consider is the timeline of anxiety behavioural assessment after the chemogenetic manipulation. For example, Andero and colleagues found that systemic injections of the *Tac2* antagonist Osanetant both before and after conditioning impair fear expression 24 hours later (Andero et al., 2016; Abed et al., 2021). Our observation of *Tac2* mRNA upregulation in the ovBNST may imply that the time course of *Tac2* regulation is important when predicting anxiety-like behavioural effects.

In summary, we present the first characterization of *Tac2* and its role in the ovBNST, specifically after exposure to acute stress. Our findings indicate that ovBNST *Tac2* positive neurons are heavily implicated in anxiety circuitry and behaviour. This was further alluded to by the results obtained from chemogenetic manipulation of ovBNST *Tac2* neurons. However, further in-depth investigations of the parameters of the stressor (e.g. timing, type, dose) and a focus on the causality underlying the mechanisms of ovBNST *Tac2* on anxiety like behaviour are absolutely crucial in the future.

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3. General Discussion

3.1. Summary

The main objective of the current thesis was to further unravel the underlying mechanisms of maladaptive anxiety. Accordingly, we specifically focused on the implication of the co-chaperone FKBP51 and its direct and indirect interaction partners in anxiety-related behaviours. In particular, we initially addressed the question of how lack of GR - as a steroid receptor regulated by FKBP51 and a direct FKBP51 interaction partner- in forebrain glutamatergic neurons mediates anxiogenic behaviours (Chapter 2.1). We then explored whether the general lack of FKBP51 has an effect on brain structure and connectivity in male mice (Chapter 2.2). Next, we focused on FKBP51 expression in the ovBNST and how acute stress exposure induces FKBP51, and subsequently regulates HPA axis activity and anxiety-like behaviour (Chapter 2.3). Finally, we explored the role of the neuropeptide *Tac2* in the ovBNST and how it might be implicated in anxiety-related behaviour (Chapter 2.4). Specifically, we observed regulation of *Tac2* after acute stress and chemogenetically manipulated *Tac2* positive neurons in the ovBNST.

These data comprehensively examine how FKBP51 and its interaction partners contribute to specific pathological mechanisms that underly stress-induced anxiety. Our findings indicate that the GR as well as FKBP51 and *Tac2* are all implicated in maladaptive anxiety, but under highly specific conditions. The effects observed depended - amongst other factors - on the following overarching aspects: brain region and/or structure specificity, the type of stressor, HPA axis reactivity, and cell-type specificity.

3.2. FKBP5 and *Tac2* are reactive to acute stress in the ovBNST and key mediators of anxiety states

We tested the hypothesis that FKBP5 in the ovBNST mediates the crosstalk between the regulation of the stress response and maladaptive anxiety. This hypothesis was based on the notion that FKBP51 is already known to regulate the stress response (Touma et al., 2011; Hartmann et al., 2012b) and on previous findings that demonstrated a clear role of FKBP51 in the amygdala and anxiety-related behaviours (Attwood et al., 2011; Hartmann et al., 2015; Volk et al., 2016). Furthermore, the BNST, and the ovBNST specifically, were consistently associated with the modulation of stress-induced anxiety states, yet the interaction of ovBNST FKBP51 and stress on anxiety-like behaviours remained unexplored. Therefore, as a first step we measured whether FKBP5 gene expression in the ovBNST is responsive to acute stress exposure. Using a standard restraint stress paradigm, we found indeed that FKBP5 is highly reactive to acute restraint stress in the ovBNST. To further validate this effect, we utilised a different type of acute stressor (fear conditioning) and also exposed male mice to a chronic social

defeat stress (CSDS) paradigm. Remarkably, we observed the same ovBNST FKBP51 upregulation after the acute stressor, but not after exposure to chronic stress. Although these findings did not yet establish FKBP51 as a target at the interface between stress reactivity and maladaptive anxiety per se, they highlight the fact that FKBP5 in the ovBNST is responsive to acute stress, and thus very likely involved in the modulation of behavioural states associated with this specific structure. This hypothesis was later confirmed since viral manipulation of ovBNST FKBP5 had an effect on anxiety states.

Subsequent characterisation of FKBP51 positive neurons in the ovBNST revealed co-expression with the neuropeptide *Tac2*. Since recent studies had suggested a role of *Tac2* within the BNST in stress-induced anxiety (Zelikowsky et al., 2018b) and we were interested in targeting and manipulating the ovBNST more precisely, we proposed that *Tac2* and FKBP5 in the ovBNST might work in tandem to mediate anxiety. For this purpose, we first examined the role of *Tac2* and stress in the ovBNST and its implication in anxiety separately (Chapter 2.4).

Interestingly, we observed a similar significant upregulation of *Tac2* mRNA in the ovBNST to FKBP5 after exposure to acute restraint stress. Moreover, *Tac2* also exposed a similar timeline of regulation to FKBP5. Subsequently, chemogenetic manipulation of *Tac2* positive neurons in the ovBNST revealed a mild anxiogenic behavioural phenotype. These results demonstrate that *Tac2* in the ovBNST is implicated in stress-induced anxiety states.

While together these data provide a first indication towards a coordinated mediation of stress-induced anxiety states by *Tac2* and FKBP5, further evidence is needed to disentangle the more causal roles of *Tac2* and FKBP5 in the ovBNST and anxiety states. Nevertheless, we show clearly that *Tac2* and FKBP5 in the ovBNST are responsive to acute stress, regulated in the same time-dependent manner, co-expressed in the same cells, and likewise involved in the modulation of stress-induced anxiety-like behaviours.

3.3. The effects of the GR, FKBP51 and *Tac2* on anxiety are dependent on brain region and/or structure

In all of the chapters (Chapter 2.1- 2.4) it became evident that the effects of the GR as well as of FKBP51 and *Tac2* on anxiety- like behaviours are specific to the different brain regions and/or structures.

In particular, in Chapter 2.1 we hypothesised based on reports of previous studies and the importance of the glutamate-GABA balance in the etiology of anxiety disorders that GR expression in excitatory (that is, glutamatergic) neurons of the forebrain would have an impact on anxiety- related behaviour.

For this, male $GR^{Glu\text{-}CKO}$ mice that lack GR in the cortex and limbic regions, including the BLA, dorsal and ventral hippocampus, and to a certain extent in the PVN, were used. Interestingly, these mice indeed showed reduced anxiety-like behaviour in the EPM and the DALI test compared to their control littermates. When trying to specify to which extent the anxiolytic phenotype of $GR^{Glu\text{-}CKO}$ mice was mediated by GRs in the glutamatergic neurons of the BLA - the structure most obviously associated with anxiety-like states-, we observed no significant changes in anxiety-like behaviours. Thus, the initial effects on anxiety are potentially mediated by the GR in glutamatergic neurons of other limbic structures, such as the hippocampus, PFC, or PVN. Moreover, when using $GR^{GABA\text{-}CKO}$ mice that carry a GR deletion in forebrain GABAergic neurons, we observed no changes in anxiety-like behaviour at all. Since the BNST is mainly GABAergic, this may imply that a lack of GR in regions including the BNST does not have an impact on anxiety-like behaviour, unless our results were due to compensatory mechanisms owing to the developmental GR deletion and/or absence of GR in GABAergic neurons throughout the brain. Overall, however, these findings highlight how selectively the effects on anxiety states are regulated.

In line with this, we observed changes in brain architecture and connectivity in specific brain regions in mice that lacked FKBP51 entirely (51 KO) (Chapter 2.2). More precisely, analysis of MRI data revealed two clusters with significantly larger volume in the WT animals compared to those with FKBP5 knockout: the thalamus as well as the PAG and the DR region. In addition, 51KO animals displayed potential greater integrity of microstructural architecture in the anterior commissure, but possibly a selective sparing (or degeneration) of fibers in the fornix and posterior commissure/superior colliculus region compared to control animals. The 51 KO line has been repeatedly characterised as more resilient in terms of stress physiology and stress-coping behaviour (Touma et al., 2011; Hartmann et al., 2012b; Albu et al., 2014; Balsevich et al., 2017), and is thus potentially less susceptible to stress-related psychiatric disorders. While a general knockout of FKBP51 resulted in distinct changes in specific stress-relevant brain regions and fibre bundles, we can not specify which aspect of the general FKBP51 knockout eventually lead to these differences. Unlike in chapter 2.1 we did not consolidate our initial observation by singling out the effects of FKBP51 (or knockout of FKBP51) in one or several specific brain regions. However, this was the first study of its kind to investigate whether there are any effects of FKBP5 knockout on brain connectivity and tissue architecture. Therefore, it is a matter of good scientific strategy to start more general and narrow down the research question while gaining more knowledge during the process. Consequently, based on our original findings, mice with conditional FKBP51 knockout (FKBP51-Nex-Cre/ FKBP51-Dlx-Cre) in the forebrain are now studied with a focus on more specific cause-effect differences in brain structure and connectivity.

In our third study (Chapter 2.3) we asked whether the manipulation of FKBP51 in the ovBNST has an impact on anxiety-related behaviour. To address this question, we first performed a virally-mediated

overexpression of FKBP51 and conversely, a virally-mediated knockout of FKBP51. The overexpression exposed a tendency towards an anxiolytic behavioural phenotype, whereas the knockout delivered a solid anxiogenic behavioural phenotype. The phenotype observed for the overexpression experiment may have been moderated by ectopic expression of FKBP51 in various other cell types that usually do not express FKBP51 or in adjacent BNST nuclei (such as the adBNST). This is less likely to occur in a viral knockout experiment, since FKBP51 will only be deleted in cells that already express FKBP51. Thus, the strong anxiogenic phenotype of ovBNST FKBP51 knockout is most likely highly accurate. Since we observed a remarkable inversion of anxiety phenotypes compared to results reported for other structures like the amygdala (Attwood et al., 2011; Hartmann et al., 2015; Criado-Marrero et al., 2019), in which FKBP51 overexpression usually resulted in anxiogenic behaviour and a knockout in an anxiolytic behavioural phenotype, we wanted to refine our viral manipulation by targeting only those neurons of the ovBNST that are activated during acute stress. This was based on our initial observation of FKBP51 induction in the ovBNST after exposure to acute restraint stress. Subsequently, a selective knockout in the ovBNST produced a similar anxiogenic behavioural phenotype to the more general FKBP51 knockout observed earlier. Thus, these data show that the effects of FKBP51 in the ovBNST on anxiety- like states are highly unique and contrary to anxiety-like effects reported in other brain regions or structures. This applies to even those structures that are implicated in the circuitry of anxiety, such as the amygdala and the dorsal hippocampus (Hartmann et al., 2015), as well as the prelimbic cortex (Criado-Marrero et al., 2017).

The last study (Chapter 2.4) also highlighted that the influence of *Tac2* on anxiety-like behaviour is in fact region- specific. We were particularly interested in the role of *Tac2* in the ovBNST on anxiety-related behaviours. This was based on our observation of ovBNST *Tac2* upregulation after acute stress as well as Zelikowsky and colleagues' study (2018), which reported an anxiogenic effect of adBNST *Tac2* overexpression in combination with chemogenetic activation and, vice versa, an anxiolytic behavioural phenotype due to *Tac2* knockdown, NK3R antagonist application or chemogenetic inhibition. For this purpose, we chemogenetically inhibited *Tac2* positive neurons of the ovBNST in male mice and consequently reported a mild anxiogenic behavioural phenotype. While this finding does not suffice to draw any definite conclusions regarding the role of *Tac2* in the ovBNST, it nevertheless indicates that ovBNST *Tac2* is implicated in anxiety states and that, not surprisingly, *Tac2* in the ovBNST has a potentially different role to *Tac2* in the adBNST. Thus, future studies should perform further manipulations of *Tac2* in the ovBNST, e.g. chemogenetic stimulation, optogenetic manipulations, overexpression/knockout, etc., to pinpoint the exact role of ovBNST *Tac2* in anxiety-related behaviours.

3.4. The effects of FKBP51, *Tac2* and the GR on anxiety are cell-type specific

While examining the function of FKBP51 and its interaction partners in the different brain regions and their impact on anxiety states, it became very obvious that the underlying mechanisms of the effects observed were not only mediated by the different brain structures but are also highly specific depending on the cell-type or population.

For example, in our first study we initially set out to investigate GR action on fear- and anxiety-related behaviours in both excitatory (glutamatergic) and inhibitory (GABAergic) neurons. To test this, we generated conditional mouse mutants lacking the receptor in glutamatergic neurons ($GR^{Glu\text{-}CKO}$) and contrasted this with mice lacking the GR in the majority of GABAergic neurons ($GR^{GABA\text{-}CKO}$). First baseline characterisations of both lines revealed that an absence of GRs in forebrain glutamatergic neurons resulted in HPA axis hyperactivity, while $GR^{GABA\text{-}CKO}$ mice did not display any changes at all apart from a significantly reduced body weight. In line with the endocrinological phenotype, $GR^{Glu\text{-}CKO}$ animals showed reduced anxiety- like behaviour as well as significantly enhanced fear extinction. $GR^{GABA\text{-}CKO}$ mice, however, did not exhibit any changes in anxiety-and fear- related behaviours, not even after exposure to severe stress conditions (CSDS). These results indicate that especially the GR in forebrain glutamatergic neurons, but not in GABAergic neurons, plays a prominent role in mediating HPA axis activity, as well as fear- and anxiety- like behaviour.

In this context, we wanted to identify which glutamatergic neuronal population might be responsible for the fear-supressing and anxiolytic phenotype of the $GR^{Glu\text{-}CKO}$ animals. Due to some promising electrophysiology results and the prominent role of the amygdala in anxiety-and fear-related behaviours, we used a viral construct to knock out GR exclusively in glutamatergic neurons of the BLA. Remarkably, we did not observe any differences in anxiety, but in fear learning and extinction. Since the BLA contains numerous sub-populations of GR positive glutamatergic neurons, one can speculate that the GR anxiety-like effects were possibly driven by a different population, potentially involving FKBP51, CRH or *Tac2*. However, this needs to be investigated more thoroughly in future research. Taken together, these results illustrate that GR signalling in forebrain glutamatergic, but not in the GABAergic neurotransmitter system are essential in regulating stress system activity, fear and anxiety. More specifically, the GR glutamatergic neuronal population of the BLA is substantially involved in mediating fear learning and memory. Finally, neuronal glutamatergic GR populations in the other structures of the forebrain (cortex, hippocampus, PVN) or specific glutamatergic GR sub-populations in the BLA might mediate anxiety-related behaviours.

Similar to the GR, the effects of FKBP51 on stress reactivity and anxiety-like behaviour are also highly cell-type specific. For instance, when we first characterised the FKBP51 positive neurons of the ovBNST that are activated by acute restraint stress, our results highlighted that this specific neuronal population

did not only express FKBP51 but also co-expressed CRH, *Tac2* and Gad65/67. Moreover, expression patterns were quite heterogenous and partially overlapped, possibly representing specific sub-populations of FKBP51 positive ovBNST neurons that are responsive to acute restraint stress. Nevertheless, further investigation and quantification is required to systematically categorize these different neuronal sub-/populations that are involved in the ovBNST FKBP51 mediated stress-response.

While the overexpression of FKBP51 in the ovBNST may have led to expression of FKBP51 in cells or populations that usually do not express FKBP51, the FKBP51 ovBNST knockout yielded a relatively cell-specific result, since FKBP51 can only be deleted from cells that express the co-chaperone anyway. However, this may have not been the FKBP51 positive neuronal population that is sensitive to acute restraint stress. Thus, we decided to tailor our manipulation specifically to the ovBNST restraint stress-activated neuronal population by knocking out FKBP51 in these neurons only. For this purpose, we utilized a virus construct with a synthetic promoter, the enhanced synaptic activity-responsive element (ESARE), coupled with a drug-inducible Cre recombinase downstream of the promoter. Remarkably, we obtained a similar anxiogenic-like behavioural phenotype to the previous, more general FKBP51 knockout. Interestingly enough, however, this time we did not detect any endocrinological effect on HPA axis activity which had previously accompanied the FKBP51 knockout and overexpression. Thus, we predict that the HPA axis impact of FKBP51 manipulation is not specifically mediated by the stress-activated FKBP51 positive neurons within the ovBNST, but potentially by a different distinct population within the nucleus. Taken together, these results perfectly illustrate the cell-type specific contributions of FKBP51 and advance our understanding of the role of FKBP51 within the ovBNST. However, this is still preliminary evidence which needs to be systematically pursued in the future.

Our findings concerning the GR and FKBP51 regarding their cell-type specificity were also applicable to *Tac2* and its function in the ovBNST. For example, *Tac2* positive neurons within the ovBNST were co-expressed with FKBP51 and CRH. Similar to ovBNST FKBP51 positive neurons, this could imply that there are several sub-populations of *Tac2* positive neurons within the ovBNST. However, again, this needs to be determined using a more systematic, quantified approach in follow up studies. Nevertheless, we could identify several projection sites of the *Tac2* positive neuronal population in the ovBNST, which had been previously associated with anxiety circuitry (Calhoon and Tye, 2015; Tovote et al., 2015; Lebow and Chen, 2016). Moreover, chemogenetic inhibition of this specific neuronal population resulted in a tendency towards anxiogenic-like behaviour. Overall, the cell-specific properties of *Tac2* positive neurons within the ovBNST are evident, yet there remains significant potential for more detailed investigations in upcoming experiments.

3.5. The effects of the GR and FKBP51 impact HPA axis re-/activity

Maladaptive anxiety and anxiety disorders are often characterised by altered HPA axis re-/ activity (Sotnikov et al., 2014). In both the first and the third study (Chapter 2.1 and 2.3) we reported changes in anxiety-like behaviour- either due to GR or FKBP51 manipulation-, and consequently noticed differences in HPA axis re-/activity. For instance, an absence of GRs in forebrain glutamatergic neurons resulted in HPA axis hyperactivity. This could be observed at basal levels, but also after exposure to an acute stressor. Moreover, $GR^{Glu\text{-}CKO}$ animals exposed significantly increased adrenals and significantly smaller thymus glands compared to controls. Since $GR^{Glu\text{-}CKO}$ mice exhibit absence of GR expression in glutamatergic neurons of the PVN, which is an important feedback site for GRs, and in the hippocampus and the BLA, which constitute additional regulators of the HPA axis, we were not surprised by the observed differences in HPA axis activity.

We also showed differences in HPA axis re-/activity depending on FKBP5 status in the ovBNST. Specifically, we reported blunted HPA axis activity as a result of long-term ovBNST FKBP5 overexpression and enhanced HPA axis response due to stable ovBNST FKBP5 knockout. Knockout of FKBP51 in ovBNST restraint stress activated neurons only did not affect HPA axis activity, indicating that long-term HPA axis impact of FKBP5 is not carried by this specific ovBNST neuronal population, but possibly a different distinct cellular population within the nucleus.

Several nuclei of the anterior BNST send GABAergic projections to the PVN and also project to the CeA, making the anterior BNST a relay station that mediates HPA axis responses to stress. In fact, the ovBNST contains a very high density of CRH-producing cells which project directly to the PVN (Dong et al., 2001a). Thus, parallel to the HPA axis hyperactivity in the $GR^{Glu\text{-}CKO}$ mice, which is potentially influenced by the lack of glutamatergic GRs in the PVN, we observed similar, possibly PVN-mediated effects on HPA axis activity after ovBNST FKBP51 manipulation. As discussed previously (see Chapter 1.5.3) anxiety disorders are often, but not exclusively, characterised by a hyperactive HPA axis. Moreover, Sotnikov and colleagues even reported a blunted HPA axis reactivity due to GC system disbalance in mice with high-anxiety related behaviour (Sotnikov et al., 2014).

Overall, both the ovBNST FKBP51 knockout and the GR knockout in forebrain glutamatergic neurons resulted in higher corticosterone levels after exposure to acute stress and an anxiogenic phenotype. The ovBNST FKBP51 overexpression, however, resulted in significantly lower corticosterone levels compared to control animals and a mild anxiolytic behavioural phenotype. These results perfectly illustrate the complexity and multifactorial reality of interplay between candidate gene manipulation, HPA axis re-/activity, and anxiety-related behavioural phenotypes. While it is difficult to establish a precise cause-effect relationship from these observations, they do provide several important and differentiated insights with regard to this matter. First, maladaptive anxiety can be characterised by

both a hyper- and/or hypoactive HPA axis. Second, high FKBP51 expression or upregulation does not automatically imply an anxiogenic behavioural phenotype and HPA axis hyperactivity, and vice versa, FKBP51 reduction or knockout is not always associated with anxiolytic behaviour and a decrease in HPA axis activity (Hartmann et al., 2015; Häusl et al., 2021). Instead, this is dependent on the brain structure, cell-type, and possibly the timing of the stressor and the timing of its assessment. In fact, long-term ovBNST FKBP51 overexpression or knockout may result in the reported endocrinological HPA axis phenotypes, yet these, together with anxiety-like phenotypes, might be different after an acute stress-induced increase (or potentially decrease/inhibition) of FKBP5 in the ovBNST (see Chapter 2.3). For example, subjects with PTSD reportedly expose a hyperactive HPA axis shortly after the traumatic event but a hypoactive HPA axis in the long-term aftermath. However, findings regarding pattern of HPA alterations in PTSD also remain highly inconsistent (Young et al., 2018; Schumacher et al., 2019; Speer et al., 2019). Therefore, rather than trying to categorise HPA axis activity, gene status, and anxiety phenotype accordingly, it is important to account for the dynamic properties of such a versatile system as the HPA stress response.

These factors should also be considered in potential future pharmacological treatment. SAFit2, a selective antagonist ligand of FKBP51, has successfully been shown to enhance feedback inhibition of the HPA axis, stress coping and glucose tolerance (Gaali et al., 2015; Balsevich et al., 2017; Hähle et al., 2019). Moreover, it had anxiolytic properties, protected from weight gain and alleviated pain states (Hartmann et al., 2015; Maiarù et al., 2016, 2018; Balsevich et al., 2017). When applied together with the antidepressant escitalopram, SAFit2 reduced the anxiolytic effect of escitalopram, but improved stress-coping behaviour (Pöhlmann et al., 2018). However, administration of SAFit2 in the ovBNST to regulate HPA axis activity and anxiety-like behaviour would have to be highly region- and time-specific.

Taken together, both the GR in forebrain glutamatergic neurons and FKBP51 in the ovBNST are implicated in HPA axis regulation. Based on our results we can conclude that their effects are highly dynamic and dependent on GR/FKBP5 status as well as the timing and the type of stress exposure.

3.6. Limitations and Future Directions

The current thesis contributes important insights into the molecular mechanisms underlying maladaptive anxiety. We demonstrate that GR signalling in forebrain glutamatergic neurons, but not GABAergic neurons, mediates anxiogenic behaviour. For the first time, we show that FKBP51 plays an important role in the ovBNST and is implicated in the regulation of anxiety-like behaviour. Moreover, a complete lack of FKBP51 significantly affects brain architecture and connectivity. Finally, we are the first to delineate a role of the neuropeptide *Tac2* in the ovBNST and anxiety. Overall, we present compelling data that identify particularly FKBP51 and *Tac2* in the ovBNST as novel

molecules involved in the regulation of anxiety-like behaviour. This opens a new avenue of research, which has the potential to advance current therapeutic and medical practices. Despite laying the groundwork for future research regarding FKBP51 and *Tac2* ovBNST function, there remain outstanding research questions and limitations to our current findings.

The brain region and cell- type specificity of candidate genes associated with stress and anxiety such as GR, FKBP5 and *Tac2* have been pressed before. This concept was successfully considered and applied in the first study; the GR was deleted from forebrain GABAergic or glutamatergic neurons first, and eventually we could pinpoint the observed effects on fear- related behaviour down to GR action in glutamatergic neurons of the BLA, whereas anxiety-related behaviour was assigned to glutamatergic neuronal GR signalling in limbic structures such as the hippocampus, PFC or PVN. The anxiety-like effects could have been defined further, however, this might be more appropriate for future publications.

In order to explore how the lack of FKBP51 might affect brain architecture and connectivity, we imaged the whole brain of total 51 KO male mice. Although conventional 51KO mice provided a means to establish whether FKBP51 is involved in brain plasticity and connectivity or not, there are many shortcomings associated with any conventional knockout mouse line. As discussed before (see Chapter 2. 2 and General Discussion), there is inevitably a lack of spatial and temporal control of gene expression, and compensatory mechanisms during development may distort the reported findings. Thus, the contribution of individual FKBP51 positive tissue types, or specific cell types, must be addressed in the future.

For our third and fourth study, which focused on the role of FKBP51 and *Tac2* in the ovBNST and their implication in anxiety-like behaviours, similar shortcomings apply. First, the ovBNST is so complex and heterogenous in terms of the numerous neuronal populations expressed within the nucleus, that further rigorous quantitative characterisation of FKBP51 and *Tac2* positive neuronal populations is crucial. Moreover, there needs to be a more distinct differentiation between those FKBP51/ *Tac2* positive neurons that are activated by acute restraint stress and those that are simply expressed within the ovBNST.

Additional characterisation would also enable more appropriate viral targeting and manipulation. For example, while we observed a robust anxiogenic phenotype when we virally knocked out FKBP51 in the ovBNST, a more selective manipulation by chemogenetically inhibiting only those FKBP51 positive neurons that had been previously activated by restraint stress, eventually provided a more nuanced picture.

Although further systematic characterisation and specification will help to generate more knowledge on the contributions of FKBP51 and *Tac2* in the ovBNST, it may lead to very compartmentalised

knowledge without sufficient contextual and behavioural framework. Thus, it may be of advantage to focus more on the circuitry of FKBP51/*Tac2* in the ovBNST and explore how the various components orchestrate anxiety-related behaviour. For example, we demonstrated that *Tac2* positive neurons in the ovBNST project to several anxiety-relevant structures such as the LS and the CeA. Therefore, it would be highly interesting to do more circuit- based manipulations, such as retrograde stimulation (e.g. through the means of optogenetics) of neurons projecting from the ovBNST to the CeA or the LS. Alternatively, manipulating FKBP51/ *Tac2* positive neurons in several structures simultaneously, e.g. ovBNST and CeA, may provide a more realistic impression of circuit-mediated anxiety-like behaviour. Taken together, ideally further stringent characterisation of *Tac2*/FKBP51 ovBNST neurons should go hand in hand with more circuit-based manipulations in order to disentangle the influence of the two genes on anxiety-related behaviour.

Another limitation of all our studies that needs to be discussed is the lack of inclusion of female animals. In general, anxiety disorders are more prevalent in women and including females in the future will provide data that is more representative of the general population. Moreover, *Tac2*/ neurokinin B is an important mediator of the reproductive axis and FKBP51 also regulates sex steroid receptors such as the androgen and the progesterone receptor (Magee et al., 2006; Topaloglu et al., 2009; Jääskeläinen et al., 2011; Stechschulte and Sanchez, 2011). Furthermore, the ovBNST is sexually dimorphic, which may also account for the gender disparity in prevalence and treatment of stress-related psychiatric diseases (Lebow and Chen, 2016) .

3.7. Closing Remarks

To conclude, we provide new insights into the underlying molecular mechanisms of stress-induced maladaptive anxiety. Our findings concerning the function of FKBP51 and *Tac2* in the ovBNST will lay the groundwork for a new avenue of research that is not yet widespread in literature. Future studies will be able to provide an even clearer picture in order to understand the causal mechanisms behind the observed effects. Ultimately, we hope to contribute towards the development and implementation of pharmacological treatment and therapy of stress-related psychiatric disorders.

4. Bibliography

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Assertion/ Eidesstattliche Erklärung

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

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München, den 28.04.2021

Clara Engelhardt

Unterschrift