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Consequences of Stress on the Psycho-Immune-Neuro-Energy Network - On the Search for Novel Biomarkers and Protective Factors

PhD Thesis

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Abstract

The body constantly needs to adjust physiologic processes according to current environmental influences. Such influences could be the cycle of day and night or the appearance of a challenging situation. Glucocorticoids play a central role in these adjustments. A sudden increase in glucocorticoid levels in the morning is an integral part of the circadian rhythm and human awakening response, while an on demand secretion of glucocorticoids is typical for the stress response. Even though the rise in glucocorticoids is rather unspecific, the Psycho-Immune-Neuro-Energy (PINE) network responds with a variety of nuanced reactions that are modulated by context, timing, and dose. This flexibility is essential for health and well-being since it allows adaptation to the present situation and thus resolving the associated stress. Persisting stress due to maladaptation is thought to cause wear and tear in the long run. Resistance to glucocorticoid signalling and decreased glucocorticoid dynamics are commonly observed symptoms in a variety of disorders that have been associated with chronic stress. Moreover, co-morbid symptoms often reflect alterations in several components of the PINE network. Maintaining or restoring the flexibility of the PINE network to respond to glucocorticoids could therefore be a preventive or curative measure against stress-associated disorders and co-morbid symptoms.

To this end, a better understanding of the multiple interactions between the components of the PINE network and the influence of internal and external modulators of glucocorticoid signalling is required. The goal of this thesis was to study components of the PINE network through encounters with different stress paradigms with the aim to better resolve early changes indicating a critical transition in the stress response system towards disorder. While stress models do not lead to a (psychiatric) pathology *per se*, finding biomarkers of a type of prodromal phase, where adaptive or maladaptive responses to stress can be observed, might be possible.

In **study I**, the effects of social isolation during adolescence combined with unpredictable chronic mild stress during early adulthood on the PINE network were investigated in male Wistar Kyoto rats. An activation of the immune system in terms of blood cellular composition was observed, but with no resulting changes in cytokine levels. In the hippocampus, a brain region responsible for memory formation, improved mitochondrial respiration and increased mitochondrial density were observed. An elevated metabolism of tryptophan was demonstrated by measuring plasma and cerebrospinal fluid (CSF) levels of tryptophan catabolites (TRYCATs). The observed changes indicated lower neurostimulation via TRYCATs. In the pre-frontal cortex (PFC), a brain region responsible for decision-making, and in the hippocampus, a reduced expression of genes related to neuronal activity was measured. In total, the observed changes in the PINE network in response to the applied mild stressors indicated adaption to the situation. However, the putatively reduced activity of the central nervous system (CNS) and the observed lethargy of the animals could lead to decreased responsivity and adaptive capacity on long-term. Rats that were exposed to unpredictable chronic mild stress for a prolonged time showed depressive-like behaviours in the sucrose-preference and forced-swim tests.

Given that the PINE network is strongly regulated by glucocorticoids, **studies II** and **IV** investigated a single nucleotide polymorphism (SNP) in *FKBP5*, a gene encoding for an inhibitor of glucocorticoid receptor (GR) maturation. The genetic variant rs1360780 with thymidine instead of cytosine was shown to be stronger induced upon stress and is associated with an elevated risk to develop psychiatric, inflammatory, and metabolic disorders. These negative effects predominantly occur in combination with aversive experiences during childhood. To study causal relationships between the risk and resilience variants of *FKBP5*, environmental influences, and well-being, *Fkbp5*-humanized mice were generated and characterized within the scope of this thesis.

Within **study II**, repeated separation from mothers and peers before weaning was used to induce early life adversity (ELA) in *Fkbp5*-humanized mice and the effects on social-, anxiety-, and activity-behaviour were analysed in adulthood. Males were less active than females and displayed lower levels of glucocorticoids. Female carriers of the resiliency-associated CG-allele responded to ELA with increased activity, while carriers of the AT-allele had a lower responsivity to novel environments irrespective of early life conditioning. In parallel to the behavioural differences, CG-allele carrying females had pronounced diurnal rhythms of glucocorticoids while female AT-allele carriers had a disrupted circadian rhythm, including elevated nadir glucocorticoid levels and no significant rise towards awakening. On a molecular level, AT- vs. CG-allele carriers showed lower expression of genes related to circadian entrainment in the hypothalamus, a brain region integrating nervous and endocrine signalling. The comparison between mice with undisturbed development and those exposed to ELA revealed that ELA in addition led to a lower expression of these genes and genes related to dopaminergic and endocannabinoid signalling in the hippocampus. Moreover, lower expression of genes related to synaptic communication in both brain regions but higher expression of genes related to mitochondrial energy production and metabolism was seen in AT- vs. CG-allele carriers. Viewed together the data suggests that changed communication within the brain could, via attenuated circadian entrainment, manifest in altered alertness and activity states that hinder AT- vs. CG-allele carriers to respond and adjust to novel situations. ELA may push AT-allele carriers further in that direction by modulating gene expression, but the functional differences in glucocorticoid rhythmicity and behaviour were already elicited by the genetic predisposition of carrying the AT-allele alone. To further define the role of energy production and consumption in discriminating AT- and CG-allele carriers with or without ELA is an interesting task for future research.

Since the levels of TRYCATs have an impact on brain activity and are influenced by stress, the applicability of TRYCAT profiles as biomarkers for lifetime stress and decreased cognitive abilities was assessed. This was done in serum samples of healthy elderly humans at risk to develop neuro-cognitive disorders that were enrolled in **study III**. The levels of 3-hydroxy-kynurenine (3-HK), the precursor of the neuroactive quinolinic acid (QUIN), positively correlated with the number of stressful life events while QUIN negatively correlated with executive functions. To test whether physical and cognitive training would result in changes of the TRYCAT profiles, a 10-week intervention period was carried out and serum TRYCATs were assessed afterwards. After physical training, the levels of 3-HK decreased on trend level while after cognitive training the levels of 3-HK and of the anti-excitatory kynurenic acid (KYNA) decreased. However, no training-related effects on cognition were observed. Together, the findings could indicate that changes in cognition occur on a long-term basis while molecular changes manifest rather short-term. Whether TRYCAT profiling can be used as biomarker to monitor treatment success needs to be further investigated.

In **study IV**, the acute transcriptional response of primary murine brain cells to glucocorticoids in the context of both human SNP versions, as well as putative reasons for cell-type dependent reactivity were determined. Baseline expression levels of *Fkbp5* and *Nr3c1*, the gene encoding for the GR, suggested that neurons would rather be irresponsive to glucocorticoids since they expressed high amounts of *Fkbp5* and low amounts of *Nr3c1*. In contrast, microglia and astrocytes expressed more *Nr3c1* and less *Fkbp5* than neurons, with microglia expressing more *Fkbp5* than astrocytes. As to be expected from these findings, the transcriptional differences between the studied CNS cell types indicated that astrocytes responded the most to stimulation with glucocorticoids, followed by microglia and neurons. Moreover, the AT-allele was associated with a stronger induction of *Fkbp5* than the CG-allele. While the acute induction of other glucocorticoid-responsive genes was not further influenced by the *Fkbp5* genotype, potential effects on repeated or chronic stimulation with glucocorticoids require further investigation. Since astrocytes maintain the cerebral energy household, the findings of this study suggest that regulation of metabolism is a crucial part of the cerebral response to glucocorticoids. Furthermore, *Fkbp5*-genotype dependent reactivity of astrocytes might contribute to differences in stress coping of rs1360780 SNP carriers, making astrocytes an interesting target for further research.

This dissertation showed that internal and external factors comprising genetic make-up, lifetime experiences, and the current physiology influence the functioning of the PINE network, responsiveness to novel situations, and the presumably associated stress. These factors interact with each other, as well as with timing and dose, and can give rise to different coping strategies ranging from reduced receptiveness to increased activity. Dependent on the context, these strategies may be more or less effective in relieving stress. Thus, gene × environment interactions shape the individual resilience or vulnerability to stress-associated disorders. Although different models and stressors were used to investigate the effects of different factors influencing the PINE network, parallels in terms of increased metabolic readiness in the brain, decreased cognitive activity, and altered circadian rhythmicity were seen in the stress- and genetically-burdened groups. The matching of findings across studies suggests that negative effects of stressors converge on similar pathways and could explain why multiple hits on these pathways can accumulate and synergise in worsening the outcome of the affected individual. Moreover, the preservation of these critical pathways across species indicates that the used animal models are valid to study aspects of human pathomechanisms including altered glucocorticoid regulation exerting an impact on metabolism, CNS functioning, and the immune system. Taking actions against putatively negative effects of the repeatedly observed changes in the prodromal stage by optimizing resource allocation, establishing active and resting phases, and promoting flexibility in the mental and behavioural repertoire could represent central treatment goals. Personalization of preventive or curative attempts that account for individual risk and resilience factor interactions by tackling alterations in the PINE network from several angles appears desirable to maintain or improve quality of life. This strategy may boost the effectiveness of existing treatments and help patients that are currently considered to be treatment-resistant. The results of this thesis imply promotion of physical activity and regulation of *Fkbp5* and glucocorticoid dynamics, especially in astrocytes, to be promising treatment modalities. More research to enable stratification of patients or persons at risk into permissive treatment groups and to monitor and improve treatment effectiveness through optimal timing and contextualization of the interventions is needed.

List of Abbreviations

×	Times
3-HK	3-hydroxy-kynurenine
A	Adenine
ACTH	adreno-cortico-tropic hormone
ADP	Adenosine-di-phosphate
a.m.	*ante meridiem*
ANOVA	Analysis of variance
ARRIVE	Animal research reporting of *in vivo* experiments
ATP	Adenosine-tri-phosphate
BDNF	brain-derived neurotrophic factor
C	Cytosine
CI	Confidence interval
CIRN	Chronic illness risk network
CNS	Central nervous system
CoA-SH	coenzyme A with sulf-hydryl functional group
CS	Citratesynthase
CSF	Cerebrospinal fluid
CRH	Corticotropin-releasing hormone
DAMP	danger associated molecular pattern
df	Degrees of freedom
DMS - V	Diagnostic and statistical manual of mental disorders V
DMSO	Dimethyl-sulfoxide
DNA	Deoxyribo nucleic acid
E16.5	Embryonic day 16.5
e.g.	Exempli gratia, for example
ELA	Early life adversity
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
EU	European union
F	F-value
FCCP	Carbonyl-cyanide-4-(trifluoromethoxy)-phenylhydrazone
FK506	Tacrolimus or fujimycin, macrolide lacton CAS 104987-11-3
FKBP5	FK506 binding protein 5 (human gene)
Fkbp5	FK506 binding protein 5 (murine or humanized gene)
FKBP51	FK506 binding protein 51 (protein)
FPKM	Fragments per kilobase million
G	Guanine
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
hiPSC	human induced pluripotent stem cell
HPA	Hypothalamus pituitary adrenal
HPLC	High performance liquid chromatography
HSP	Heat shock protein
i.e.	*id est*, that is

IDO	Indolamine-di-oxygenase
IgG	Immune globulin G
IL-	Interleukin
JSH	Juvenile single housing
KAT	Kynurenine amino transferase
KMO	Kynurenine mono oxygenase
KYN	Kynurenine
KYNA	Kynurenic acid
LTD	Long term depression
LTP	Long term potentiation
min	Minute
MMSE	Mini mental state examination
MR	Mineralocorticoid receptor
MS	Maternal separation
MS/MS	Tandem mass spectrometry
mTOR	mammalian target of rapamycin
NCD	Neurocognitive disorder
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NGS	Next generation sequencing
NMDAR	N-methyl-d-aspartate receptor
NR3C1	Glucocorticoid receptor encoding gene
NR3C2	Mineralocorticoid receptor encoding gene
OXPHOS	Oxidative phosphorylation
p	p-value
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PFC	Pre-frontal cortex
PINE	Psycho immune neuro energy
p.m.	*post meridiem*
PND	Post natal day
PTSD	Post traumatic stress disorder
QUIN	Quinolinic acid
R^2	Coefficient of determination
RDoC	Research domain criteria
RMSE	Root mean square error
RNA	ribonucleic acid
ROX	Residual oxygen consumption
ROS	Reactive oxygen species
RPKM	Reads per kilobase of transcript per million
rpm	Rounds per minute
SCT	Social chamber test
SD	Standard deviation
SNP	Single nucleotide polymorphism
t	t-value
T	Thymine
TDO	Tryptophan-di-oxygenase
TCA	Tricarboxylic acid cycle
TNFα	Tumour necrosis factor alpha
TRP	Tryptophan
TRYCAT	Tryptophan catabolite
uCMS	Unpredictable chronic mild stress

Contents

1. Introduction

1.1. Stress as Tool to Tackle Major Health Burdens?

Many of the medical conditions modern society faces have multiple factors of origin, which poses severe emotional and financial pressure on the patients, their environments, and the health system, because therapeutic interventions against only one contributor often fail to cure [175, 500, 593, 263]. It is moreover difficult to identify the driving forces that caused the development of pathological symptoms in affected individuals, since other subjects with seemingly the same exposure can show resiliency [288, 626, 107, 396, 473, 550].

Even among patients with the same diagnosis, substantial differences in their symptomatology and likely the aetiology of their disorder exist. Prominent examples of opposed symptoms can be found in depression where one patient may suffer from substantial weight gain and fatigue, while another depressed patient suffers from weight loss and insomnia [14]. To tackle this, definitions of subgroups within formerly unitary diagnoses [208, 329] as well as the consideration of disease clusters e.g. reflected in the term 'autism spectrum disorders' become increasingly used.

The research domain criteria (RDoC) framework proposed by the US National Institutes of Mental Health furthermore acknowledge that the '*basic dimensions of functioning* [...] *span the full range* [...] *from normal to abnormal*' [111, 514]. This notion illustrates that diseases develop over time and that possibly not one tipping point demarks disease onset but a combination of (subtle) changes in different aspects of functioning interact during the transition to pathology.

Shared symptoms between multifactorial disorders were observed and patients often suffer from multimorbidity. For example, depression is a common psychiatric comorbidity of cardiovascular diseases, disorders of the immune system, or the metabolic syndrome [570, 291, 616, 535, 571, 48, 498, 494, 257]. These observations led to the proposition of a common chronic illness risk network (CIRN) [545, 566]. Accordingly, a deep understanding of this network could pave the way for the discovery of novel preventive, diagnostic, or therapeutic strategies against several symptoms.

Given its implication in the development and progression of both, psychiatric [602, 90, 577, 560] and somatic [55, 97, 144, 402, 436, 568, 642] disorders, stress is not only one of the major risk factors for reduced productivity and health worldwide but also a promising environmental factor to study the existence of shared pathomechanisms. Since treatment attempts against fully developed disorders might be too late to achieve full remission, this thesis elucidates the impact of repeated stress challenges during a prodromal stage to contribute to the understanding how the CIRN should function in a healthy state, to learn which alterations (in highly connected nodes of the CIRN) could serve as early warning signs for unfavourable developments and to identify factors that may modulate the transition speed from health to disease.

1.2. Allostasis Results from Healthy Stress Responses

The chronic or acute inability of an individual to cope with any demand produces stress [538, 541]. This generic definition of stress as a response to unmet requirements proposed by Hans Selye introduces the need of responding to an adverse situation to resolve the stress exerted on the affected individual. The triggers of stress can be internal or external in nature. All non-specific reactions of the body to allow for coping with challenges can be summarized under the umbrella term 'stress response'. The regular procedure after being exposed to stressors is an instant response mediated by beta-adrenergic signalling that releases catecholamines such as adrenalin and norepinephrine via the sympathetic-adrenal-medullary system as part of the so called 'fight-or-flight' response. This reaction represents the first part of the 'general adaptation syndrome' postulated by Hans Selye [540]. The associated shift in the sympathicotonus limits anabolic and restorative processes and favours catabolic and energy consuming processes like skeletal muscle activity [599].

To support this potential increase in energy expenditure and to coordinate longer-termed stress responses, the hypothalamus-pituitary-adrenal (HPA) axis is simultaneously triggered upon stress perception. As a result, glucocorticoids are secreted from the *zona fasciculata* of the adrenal glands into the blood stream to fulfil their eponymous actions on blood glucose levels [325], which involves the mobilization of fatty acids and amino acids [366], maintaining a sufficient blood flow to distribute these nutrients and oxygen [13, 398] and to trigger functional changes in mitochondrial dynamics [161, 462, 330], immune system activity [662, 133], and processing of cues in the central nervous system (CNS) [412, 282, 100, 139, 119, 39]. In sum, these actions guarantee the necessary supply of vital tissues with adenosine-tri-phosphate (ATP) to fuel the stress response and ultimately promote survival.

After resolving the stressful situation, the HPA axis is shut off via a negative feedback loop involving glucocorticoids [252, 207, 214, 255]. Furthermore, alterations in metabolism are reverted and restoration of the emptied energy depots, healing of received wounds, and mental processing of the experienced situation takes place. The body returns back to homeostasis [612, 102], a term coined by Walter Bradford Cannon that translates to 'stability through constancy' [132, 84, 83].

If certain stressors occur repeatedly, a change to these default settings might be more cost-efficient, thus a training effect can result in the permanent adaptation towards these chronic stressors. The process by which the body's physiological systems adapt to environmental changes is termed allostasis, from the greek 'stability through change' [397, 197, 530, 391]. This induced adjustment of physiology and learning after sub-critical doses of stress could be beneficial for the health and well-being of individuals in terms of fitting to the changed environmental demands [314, 315, 529].

Both the high flexibility to cope with several stressors and the ability to adapt to them were of evolutionary advantage since less fitting individuals were eliminated. Thus an efficient and tight networking of systems required for allostasis evolved of which the psycho-immune-neuro-energy (PINE) network is a part of.

1.3. Stress-Reactive Systems Form a Network

1.3.1. Interconnected Neuro-Circuits Cope with Stress

The brain is a highly adaptive organ and retains the ability to change throughout life via a process termed (neuro-)plasticity [471, 565]. In response to experiences and learnings, plasticity involves the weakening or strengthening of synapses on a cellular level and circuits between brain areas on an anatomical level. Given the individuality of experiences, this results in unique wiring of the brain and could explain why stress has a different meaning for different people under different conditions. During childhood and adolescence, the brain is still maturing and undergoes changes that require even more plasticity.

According to a systems-biology approach, systems undergoing changes are less stable and thus more vulnerable [567]. Indeed, stress and trauma have been reported to severely damage the developing brain [198, 609]. Comparisons of normally developed brain functionality, brains from individuals that suffered from early life adversity (ELA) such as abuse or neglect, or brains of psychiatric patients [43, 233, 627, 259] revealed that a defined set of brain areas determines what is threatening and thus stressful to an individual. This distributed neural circuitry plays an important role in the way individuals cope with stress and adversity. Beside other functions, the ability to stay optimistic, a controlled regulation of emotions, high levels of attentional set shifting to focus on different aspects of the current situation, the capacity to reflect on experiences and own reactions, and higher cognitive abilities required for executive functions in general were described to be protective factors [177].

All these functions are biologically linked within a network comprising the hypothalamus, hippocampus, amygdala, and pre-frontal cortex (PFC). Upon perception of sensory inputs from various brain regions such as the thalamus and sensory cortex, the medial PFC and cingulate cortex filter and process this information to initiate thoughts and previously learned behaviours in accordance with internal goals retrieved from the hippocampus. To this end, the medial PFC projects to various structures that orchestrate defensive physiological and behavioural responses [371], for example to the amygdala, the emotion regulation area of the brain, which in turn is connected to the bed nucleus of *stria terminalis* for activating stress hormones [303], but also to the periaqueductal grey for protective behaviour, and to the lateral hypothalamus for sympathetic and HPA axis activation [333]. Furthermore, the PFC sends dampening signals to the hippocampus to modulate memory formation [15, 209, 235, 115]. The amygdala also interferes with memory by changing firing rates of the hippocampus [301]. With respect to aversive memory formation, the amygdala was reported to intensify long-term memory consolidation of emotional events [531, 303, 376]. By limiting emotions produced in the amygdala which is supportive for cognitive flexibility in challenging situations, the PFC also indirectly influences learning processes in the hippocampus.

Together, these extensive sensory-defence circuits are required to perform stress operations. A high level of plasticity is required to update their connectivity based on recent experiences to enable adaptation to the current environment and to regulate the assessment of future challenges. Dependent on individual factors and particular contexts, challenges may have adaptive value and result in personal growth by remodelling the PFC-hippocampus-amygdala brain circuit to change the balance between mood control, memory, anxiety, and decision making. However, at the same time frequent

challenges can imply stress and strain-harden connections within the brain, which can be maladaptive in case of persistence and lack of reversibility due to reduced plasticity [393]. The remodelling of brain circuitry in response to challenges is not only mediated via CNS-internal processes that in a top-down manner influence the periphery, but also arises from bidirectional communication between the metabolism, immune system and state of the vegetative (neuronal) system [395]. Other than neurotransmitters and catecholamines, this cross talk is influenced by endocrine mechanisms, in which glucocorticoids play a central role.

1.3.2. Glucocorticoids are Universal Messengers with Dual Effects

Signalling of the HPA axis mutually effects the metabolism, CNS, autonomous nervous system, and the immune system in very different ways dependent on endpoint and context [516]. This is implemented by pleiotropic effects of glucocorticoids via multiple modes of actions. As example of fast-forward reactions through non-genomic modes of action, intercalation of glucocorticoids with plasma and mitochondrial membranes and interaction with membrane-associated receptors have been described [62, 75, 256, 589, 240, 588, 143, 150]. Furthermore, glucocorticoids can trigger other non-genomic effects via glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), which can interfere with cytoplasmic signalling complexes [312]. In the medium-term, both receptors may translocate into the nucleus upon ligand binding and elicit genomic effects. By interacting with other transcription factors for example at glucocorticoid response elements (GREs) in the deoxyribo nucleic acid (DNA), the GR and MR transactivate or transrepress a multitude of targets [78]. Given their ubiquitous expression, both receptors serve as ligand-activated transcription factors and are setting the stage for the omnipresence of glucocorticoid signalling in regulation of physiology. The reactions to glucocorticoids depend on time, condition, and dose.

In the brain, changes in glucocorticoids and differential expression of their target receptors influence emotional, executive and cognitive responses [285, 495] by interacting with plasticity and functioning of the amygdala [141, 302], PFC [76, 399, 412] and hippocampus [188, 549, 450, 136, 401, 439]. An inverted U-shaped association of glucocorticoids and plasticity has been observed [151, 8], starting with long-term depression (LTD), mediated via GR stimulation with higher glucocorticoid concentrations [451], and followed by long-term potentiation (LTP) due to the selective activation of MRs in the presence of lower glucocorticoid concentrations [8, 304]. Glucocorticoids thus have dual effects on cognition: they help to execute acute reactions without over-thinking possible solutions and memorizing details of the stressful situation but then contribute to learning from the event in the aftermath or, in the pathological state, are linked to rumination.

This biphasic pattern of glucocorticoid effects is also present outside of the CNS. In the acute phase of stress and high glucocorticoid exposure, the immune system is suppressed [120, 575, 38]. This reduces inflammation-associated swelling of tissue and enables flight [459]. Moreover, energy is allocated for fighting the current situation rather than pathogens. In the clinic, these immunosuppressive effects of glucocorticoid are widely used in the treatment of inflammatory diseases and autoimmune disorders [608, 298, 258]. On a molecular level, this can be explained by the GR-mediated inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) followed by a downregulation of pro-inflammatory cytokines and modulation of T-cell activity [78]. In contrast to these inhibitory effects of acute high doses of glucocorticoids, immune activating properties of glucocorticoids gearing up the immune system

in preparation for an anticipated stressor or for the post-acute phase of stress have been described [127, 73]. This function was evolutionarily relevant since the exposure to a stressor is potentially linked to subsequent tissue damage and contagion with bacteria or viruses. Catecholamines act together with glucocorticoids in this preparatory phase by triggering the mobilisation of monocytes from the bone marrow [148]. The increased perfusion of peripheral tissues during the fight-or-flight response distributes the mobilised monocytes and in the event of wounding flushes out pathogens. Extravasated from the blood vessels, monocytes differentiate into tissue macrophages which clear wounds from pathogens or debris and are involved in healing processes [524, 271, 216, 130]. After survival of the acute stressful situation, glucocorticoid levels decrease and pro-inflammatory processes are initiated as preventive line of defence against pathogen encounters [346, 190].

The adaptation to stressors is an active process consuming energy. To guarantee a sufficient energy supply during stress, glucocorticoids interfere with ATP production. Besides glycolysis and fatty acid oxidation, the majority of ATP is produced during oxidative phosphorylation (OXPHOS). The motor for the production of ATP is an inwardly rectifying proton gradient across the inner mitochondrial membrane. In the process of generating this gradient, a series of reduction and oxidation reactions occurs at complex I to IV of the electron transport chain (ETC), which consumes oxygen and substrates generated in the tri-carboxic acid cycle during glycolysis [187]. By intercalating into the mitochondrial membranes, glucocorticoids influence mitochondrial activity. Moreover, glucocorticoids regulate the expression of nuclear and mitochondrial genes [161, 268, 475, 462].

1.3.3. ... that are Strongly Regulated

In light of their broad effects on physiology, glucocorticoid levels need to be strongly regulated. Over the course of the day, the levels of glucocorticoids undergo substantial fluctuations that overall follow a diurnal rhythmicity and feature an ultradian rhythm [345]. These oscillations result from delayed responses of the different components of the HPA axis and the communication between them [563, 564, 126, 207, 622, 624, 623]. Together, the 24-hour cycle and the ultradian oscillations of glucocorticoid levels have strong influence on the functioning of the body by regulating sleep-wake states, excitability, and plasticity [440, 610, 243, 518].

Superimposed to this basal activity, challenging situations assessed to be of potential danger cause stress and stimulate HPA axis activity. This process includes activation of neurosecretory nerve terminals within the hypothalamic paraventricular nucleus to release corticotropin-releasing hormone (CRH) into the portal system of the anterior pituitary, where in response adreno-cortico-tropic hormone (ACTH) is secreted, transported across the blood-brain barrier into the peripheral circulation, and in the adrenal glands stimulates the secretion of glucocorticoids. In humans the main glucocorticoid is cortisol, while in rodents mainly corticosterone is released [283]. Determination of the responsiveness and flexibility of the HPA axis is key to fully access its functionality. In humans, this can be assessed using the Trier-Social-Stress-Test [306, 322]. Patients with psychiatric disorders often show prolonged stress responses after challenge and less inhibition of ACTH and cortisol release when compared to healthy controls [260, 404].

The activity of the HPA axis and the subsequent feedback to terminate elicited stress responses are influenced by the momentous diurnal and ultradian glucocorticoid levels [442, 517]. Differential glucocorticoid affinity and shifts in the expression patterns of

MR and GR dependent on brain regions further modulate the stimulation and inhibition of the HPA axis [92, 517, 286, 66, 488, 137]. Complementary to the regulation of the immune system via stress signalling, the immune system can feedback to the HPA axis and increase the availability of glucocorticoids. In the presence of inflammation, cortisol binding globulins in the blood undergo conformational changes and liberate glucocorticoids at the respective site [456]. Moreover, the pro-inflammatory cytokines tumour necrosis factor (TNF-) α and interleukin (IL-) 6 have been shown to induce the adrenal release of glucocorticoids [165], while pro-inflammatory signalling locally increases the expression of the enzymes 11β-hydroxy-steroid dehydrogenase 1 and 2, which are involved in the production and activation state of glucocorticoids [236].

These and many of the enzymes involved in steroidogenesis are expressed in mitochondria. The episodic transcription of the rate-limiting enzymes as well as the proteins necessary for cholesterol trafficking influence the availability of glucocorticoids and are themselves targets of glucocorticoid signalling [277]. Besides being the site of expression for the above-named proteins, mitochondria are involved in the formation of reactive oxygen species (ROS) that are a co-product of their bioenergetic activity. In a defined manner, ROS serve as important signalling molecules that can elicit immune responses and promote inflammation [434, 409]. Moreover, mitochondria are a central platform for the regulation of the immune system and neural activity through their role in energy provision [452, 612, 60] as well as their impact on programmed cell death (apoptosis) and calcium homeostasis [163, 116]. Given the connections between these systems and glucocorticoids, mitochondria can also indirectly regulate glucocorticoid signalling. Together, all these interactions enable a fine-tuning of the stress response and physiology.

1.4. Studying the Brain Allows Studying Neuronal, Immune, and Metabolic Activity

1.4.1. Microglia Represent the Immune System of the CNS

In the brain, full blown immune reactions including sudden tissue loss would be deleterious for the fine-tuned neural circuits and networks. Therefore, the brain is especially protected from wounding via the skull and a tight interface of astrocytes, pericytes and endothelial cells, termed blood-brain barrier, limits the access of blood-borne immune responses to the brain [275, 3, 2, 561, 3]. As replacement for the peripheral immune cells, the brain harbours specialized tissue-resident immune competent cells, the microglia [88]. These belong to the monocyto-phagocyting-system like macrophages and are of mesenchymal origin. Besides their phagocytic properties to clear debris, microglia contribute to the pruning of synapses during development and learning [446].

In the resting state, microglia have a ramified shape and monitor the brain parenchyma for pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs). Upon detection of such patterns, microglia become activated and change towards a more amoeboid shape that allows for increased mobility [204, 205, 447, 206]. Activation of the N-methyl-d-aspartate receptor (NMDAR) following an increase in glucocorticoids has been reported to promote microglia activation, too [419]. Subsequently, receptors like the cannabinoid receptor 2 or toll-like receptors become expressed on the surface to guide microglia via chemotactic signalling to the site where the activating signal originated from [648, 379, 300, 351, 467, 156, 369, 125]. Once activated, microglia proliferate and produce inflammatory cytokines like IL-1β,

IL-6 or TNF-α [234]. Analogous to inflammation in the periphery, these stimuli trigger clonal expansion and attract more immune cells [590, 368, 221, 146, 33, 284]. Normally, all immune cells in the brain are derived from the residual microglia pool. In case of severe inflammation, the blood-brain-barrier becomes leaky and other immune cells can enter the brain [5]. The latter implies a neurotoxic effect of proinflammatory responses initiated by microglia [576, 578].

After resolution of the inflammatory insult, returning to normal state is essential for brain physiology. This involves tightening of the blood-brain barrier [406, 77, 320], apoptosis of invaded peripheral immune cells and activated microglia [69]. Alternatively, a switchback to resting microglia or intermediate forms that are morphologically characterized by shorter extensions has been reported [515, 381, 400, 31]. The different states of appearance allow microglia a high degree of flexibility in their responses to the different challenges the brain has to face.

1.4.2. Astrocytes (Metabolically) Support Brain Functions

Astrocytes were long seen as filling material for the neuronal circuits in the brain. Indeed, they are required for the stability of neuronal networks, but their functions are more complex than serving as filling material [422]. Combined with their role in the formation of the blood-brain barrier [3, 275, 77], the homeostasis of glutamate, ions, water, and energy is a key role of astrocytes.

Astrocytes are a component in the so called 'tripartite synapse' formed between two neurons and an astrocyte [222, 611, 23]. Through the expression of a multitude of transporter proteins and channels on their surface [499, 657, 343, 99, 619], astrocytes take up substrates from the blood or synaptic cleft to keep the brain parenchyma cleared [213]. This mechanism regulates the synaptic transmission by removing neurotransmitters and restoring the membrane potential [9, 174]. Synaptic transmission is furthermore regulated by astrocytes controlling the number [603] and genesis [425] of excitatory [173, 321, 10] and inhibitory [155] synapses via direct contact [231] or secreted factors [104, 386].

Neurons and astrocytes are bioenergetically coupled, since after conversion and repackaging of substrates taken up by astrocytes, neurons are provided with a continuous supply of e.g. glutamate and lactate [181, 372, 200, 528, 254]. This ensures the neuronal energy supply and allows neurons to catabolize glucose via the pentose phosphate pathway to generate antioxidative glutathione [61, 666]. Astrocytes hence play a role in the defence against oxidative or nitrosative stress [640, 492]. In the broader context of stress, astrocytes were reported to be among the first cell types to respond to glucocorticoids by changing gene expression [89, 592]. Moreover, astrocytes were shown to influence information processing and cognition by integrating local sensory information and behavioural state [230, 347, 460, 554] and were linked to (emotional) learning through their influence on structural reorganization of neural networks [361, 555, 46].

1.4.3. The Kynurenine Pathway Exemplifies Orchestration of Inflammation, Metabolism, and Synaptic Signalling

Prokaryotic and eukaryotic cells need to metabolise the essential amino acid tryptophan for their survival. In the event of bacterial infection, pro-inflammatory cytokines induce the enzyme indole-amine-di-oxygenase (IDO) in macrophages and microglia to convert tryptophan to kynurenine, leading to a local depletion of this nutrient and the

weakening of the infiltrated pathogen [435, 591, 645, 279]. In an acute stress setting, the induction of glucocorticoids leads to an upregulation of the enzyme tryptophan-dioxygenase (TDO), which is mostly expressed in the liver [203, 558] and catalyses the same rate-limiting step of the kynurenine pathway as IDO.

Departing from kynurenine, two neuroactive substances can be produced. Under a pro-inflammatory state, kynurenine is processed in microglia by kynurenine-mono-oxygenase (KMO) to 3-hydroxy-kynurenine (3-HK) and quinolinic acid (QUIN) [557, 80, 28]. While both catabolites are oxidative modulators considered to be neurotoxic [519, 437, 533], QUIN additionally is an NMDAR agonist that can be further processed in mitochondria to produce ATP [458]. In an anti-inflammatory state, kynurenine is processed in astrocytes by kynurenine-amino-transferases (KATs) to kynurenic acid (KYNA). Given its antioxidant properties and the inhibitory capacity on NMDARs and excitatory acetylcholine receptors, KYNA is considered to be neuroprotective [363, 433].

Based on the differential inducibility of the transforming enzymes and the divergent effects of tryptophan catabolites (TRYCATs), the ratios of their levels could inform about excitation propensities, the presence of inflammation, and ongoing stress-related processes. Profiling of TRYCATs was therefore proposed as a biomarker in disorders where these components play a role in their pathomechanisms [532, 138, 159]. Moreover, the kynurenine pathway illustrates how strong PINE components (here represented by microglia, astrocytes, and neurons) network to incorporate and affect stress signalling from different angles.

1.5. Modulating the Stress Network from Inside Each Cell

To bring an agitated stress response network back into balance, inhibition of glucocorticoid signalling is required. Activity of the GR is modulated by a molecular hetero complex that comprises heat shock proteins (HSPs) 90 and 70, protein phosphatases, and (co-) chaperones [358, 196, 167, 220]. The human gene *FKBP5* encodes for the protein FKBP51, which is a co-chaperone that functionally inhibits glucocorticoid signalling by interfering with the maturation of the glucocorticoid receptor complex [142, 521]. If the GR is bound to FKBP51, the GR is in a low affinity state and glucocorticoids that entered the cytoplasm are less likely to be bound by the GR complex [643, 193]. In the presence of high abundant FKBP51, the dissociation of the complex and entry of the GR to the nucleus to influence gene expression is thus less frequent.

Among the transcriptionally induced targets are genes that auto-regulate glucocorticoid signalling, like *FKBP5* itself [266]. Other factors like the MR [244] and retinoic acid [349] were shown to further influence basal *FKBP5* levels. Via this ultra-short feedback loop, a further source of oscillation in glucocorticoid levels is defined. Since dynamic changes in glucocorticoid levels are essential for the stress response [285], differences in the levels of *FKBP5* will influence the reaction to stressors.

In humans, single-nucleotide polymorphisms (SNPs) in the *FKBP5* gene have been described to modulate the stress response [269, 659, 404, 179, 219, 53]. One of the best studied SNPs in *FKBP5* is rs1360780 located in intron 2, where the more abundant cytosine (C) on the reverse strand is exchanged with a thymine (T). The guanine (G) on the sense strand is changed with an adenine (A), respectively. This SNP was reported to lead to a higher expression of FKBP51 due to alterations in chromatin folding [309].

Phenotypically, a decreased efficiency of negative feedback on the HPA axis resulting in a higher risk for prolonged stress responses and glucocorticoid resistance have been reported in carriers of the AT-allele [269]. Moreover, altered inflammatory signalling has been observed to depend on *FKBP5*, which may be attributed to its immunophilin characteristics [295, 19, 202, 290, 52, 184, 359, 383] or to chronic latent immune system activity in the presence of glucocorticoid resistance due to a deregulation of the HPA axis. Irrespective of the molecular mechanism, inhibition of excessive *FKBP5* was suggested to be protective against symptoms associated to inadequate stress responses [195, 227, 311, 228, 269].

1.6. Lifetime Events Shape the Stress Response and Vulnerability

Besides genetic predispositions to altered stress responses, environmental influences in orchestration with timing and dose of exposure exert an impact on the HPA axis and stress response network [323, 108, 350]. Adverse experiences during developmentally sensitive periods like childhood and adolescence are known to severely alter the individual stress vulnerability and are related to disorders in later life [172, 416, 601, 547, 658, 212, 503]. In depression, a positive correlation between lifetime prevalence, severity, and chronicity with the amount of early life adversity (ELA) has been described [95, 341]. Events like the loss of a close relative, experiencing war, or childhood maltreatment including abuse or neglect are prime examples for ELA that trigger adaptation [153, 166]. Focusing on the HPA axis, increased cortisol levels at baseline and after challenge have been reported in victims of childhood maltreatment [109, 215, 87, 250]. Considering the immune system, ELA was associated with impaired immunity [127, 556] and with chronic low-grade inflammation determined by elevated levels of pro-inflammatory cytokines [112, 168, 245].

This developmental impact was found to have long-lasting consequences for health: affected individuals are not only at higher risk to develop mental illnesses [431, 548, 454, 262], but also somatic disorders [423, 408]. Mechanistically, psychological factors like the lack of social support [438, 665, 480, 449], learned behaviours [65, 64], and altered coping strategies [152, 594, 319, 287, 131] were reported to modulate the stress response. Moreover, epigenetic modifications that directly interact with genes involved in stress response regulation have been observed [365, 583]. For example, a hypermethylation of NR3C1, the gene encoding for the GR, in hypothalamic neurons [58, 445, 607] and a hypomethylation of *FKBP5* in peripheral blood mononuclear cells [600, 650, 309, 600] of individuals that experienced ELA were reported. Based on the observations in patients suffering from Cushing's syndrome where excess cortisol levels in the blood were linked to decreased methylation [486], chronic exposure to glucocorticoids secreted as response to ELA was suggested to drive demethylation of *FKBP5* [335, 474, 638]. Dependent on the cell type and gene locus, a demethylation of promotor regions is usually associated with increased gene expression [631, 336].

In combination with the increased expression of *FKBP5* in carriers of rs1360780-A/T upon stress exposure, ELA holds the potential to aggravate the risk of *FKBP5*-mediated HPA axis deregulation in genetically predisposed individuals [50, 237, 25, 309]. Indeed, rs1360780 has been described to predict the development of psychiatric disorders like post-traumatic stress disorder (PTSD) and depression as well as somatic disorders in persons with childhood trauma [338, 364, 659, 481, 52, 20, 664, 105, 22].

1.7. Aims of this Thesis

By investigating the interactions of environmental factors as well as genetic predispositions over developmental sensitive periods, this thesis aimed at better elucidating how stress exposure can contribute to the aetiology and progression of medical conditions. Moreover, light was shed on which risk or resilience factors as well as biomarkers might exist. The resulting insights could guide the development of preventive strategies for persons at risk as well as the development of novel therapeutic approaches.

The first part of the thesis is centred on the question of how environmental as well as genetic factors influence the networking of PINE-system components. **Study I** addressed how (unpredictable) chronic mild stress and social isolation interact in shaping the individual stress vulnerability in rats. Moreover, different exposures to stress were investigated to assess whether dose-dependent effects of stress on the PINE network are apparent. This could help to better understand a potential transition from healthy adaptation to pathological aberration.

In **study II**, the influence of the SNP rs1360780 was examined *in vivo* and *ex vivo* in *Fkbp5*-humanized mice that either carry the risk (AT) or the resilience (CG) allele. Within the characterization of these transgenic mice, the question of how these human gene variants mechanistically contribute to an individual's risk or resilience towards stress were adressed. Since in humans the influence of both SNP alleles to develop disorders becomes most apparent in combination with ELA, the interaction of both variants with maternal separation on the modification of stress axes and behaviour in adulthood was explored.

Complementary to confirming risk or resilience factors and getting a deeper understanding of the timely and mechanistic evolvement of physiologic or pathologic stress responses, the transfer from research to preventive or therapeutic interventions is needed to improve patients' lifes. Diagnostic tools that help to identify, among the individuals exposed to confirmed risk factors, those individuals that are most likely to benefit from preventive measures are needed. Based on the assumption that molecules which interfere with several components of the PINE network are more likely to indicate early changes in physiology, the catabolism of the essential amino acid tryptophan along the kynurenine pathway was investigated for its potential as a biomarker. Within **study III**, the association of kynurenine pathway activity with stressful lifetime events as well as the responsiveness of this pathway to physical and cognitive training was assessed in the context of cognition in elderly humans.

Along the lines of finding targets for therapeutic interventions, in **study IV** the glucocorticoid responsiveness of primary CNS cell types was investigated. Moreover, comparisons between the two *Fkbp5* variants were performed.

2. Results

2.1. Activation of the Kynurenine Pathway and Mitochondrial Respiration to Face Allostatic Load in a Double-Hit Model of Stress

2.1.1. Summary Study I

Stress is a risk factor for the development and progression of a variety of disorders but at the same time essential to promote survival of the fittest by initiating adaptation to the current situation. To ensure the latter, responses to stress evolved to be fast and efficient. This is implemented by coupling the communication between the brain, immune system, and metabolism via the PINE network. While acute stress responses may be beneficial in a temporally limited situation, chronic stress is thought to produce an allostatic load which may exceed the adaptive capacities of the affected individual. Based on this proposed continuum of stress effects over time, we hypothesized that stress would, in a dose-dependent manner, elicit differences in the functioning of PINE network components. Moreover, we hypothesized that some components would respond earlier than others, which could allow for using such alterations as biomarkers for recent or lifetime stress load.

To provide empirical evidence for these hypotheses, 45 juvenile male rats were exposed to unpredictable chronic mild stress (uCMS) including limited access to food, water, bedding, space, and sleep, as well as hostile encounters with age-matched peers. Before these five weeks of uCMS, 27 of these rats were in addition subjected to five weeks of juvenile single housing (JSH) during their early youth. In both stress groups (uCMS vs. uCMS + JSH) and in 27 control animals that experienced adolescence together with peers and without stressors, morning corticosterone levels were assessed weekly and in addition diurnal corticosterone profiles were traced before, after two and after four weeks of uCMS. Moreover, the catabolites of the essential amino acid tryptophan were measured via high performance liquid chromatography (HPLC) combined with tandem mass spectrometry (MS/MS) analytic in CSF and plasma, enzyme-linked immunosorbent assays (ELISAs) were carried out to quantify plasma levels of pro-inflammatory cytokines, the cellular composition of peripheral blood was determined, mitochondrial oxygen consumption in the hippocampus was investigated via high-resolution respirometry, and high-throughput ribonucleic acid (RNA) sequencing of the PFC and ventral hippocampus was performed. The validity of the uCMS manipulation was confirmed in a second cohort where rats were exposed to 14 weeks of uCMS and their depression-like behaviours were analyzed using sucrose preference, forced swim, and open field tests. All procedures were approved by the Regierungspräsidium Tübingen and adhere to the animal research reporting of *in vivo* experiments (ARRIVE) guidelines and the european union directive 2010/63/EU.

The diurnal corticosterone rhythms changed over time dependent on age and exposure to stressors. Controls showed a peak in fecal corticosterone levels between 10 a.m. to 2 p.m. at an age of 10 weeks that was shifted to 2 p.m. at an age of 15 weeks. Both

stress groups revealed the peak in the afternoon already at an age of 10 weeks, namely after experiencing or witnessing JSH, which was maintained after uCMS (Fig. 2.1, revised supplementary figure from [430]). The exposure to uCMS for five weeks lead to increased counts of granulocytes, lymphocytes, and monocytes, but no significant changes in cytokine levels. An increase in mitochondrial density was observed in the hippocampi of all stressed animals. Only in the double-hit group where rats were additionally subjected to JSH, additional increases in the routine respiration and oxygen consumption after blocking of ETC complex I were observed. In conformity with the absence of ongoing inflammatory processes, elevated levels of KYN and KYNA but not QUIN were detected after stress exposure. This activation of the kynurenine pathway was already present after JSH alone and remained after three weeks of uCMS. After five weeks of uCMS, the levels of QUIN even decreased while KYN and KYNA levels were indistinguishable from controls. In line with the putative decrease in excitatory signalling via NMDAR due to the relative increase of KYNA over QUIN, a lower expression of immediate early genes as markers of neural activity was detected in the PFC. Correlations between QUIN and these activity-indicating genes were detected (Fig. 2.2, published in [427]). After prolonged exposure to uCMS for 14 weeks, a lower expression of immediate early genes was detected in the hippocampus, too (Fig. 2.3, revised supplementary figure from [430]). In addition, a mild transcriptomic signal suggesting compromised cellular health was observed in the hippocampus of rats with prolonged uCMS exposure. These findings were parallelized by increased immobility in the forced swim test and shorter latency to leave the center of the open field arena. While the first is used as proxy for helplessness, the second is used to measure anxiety.

The findings of **study I** confirmed that the PINE network components show a dose- and component-dependent responsiveness to stress. The data moreover emphasize that the regulation of energy supply is an essential feature of the PINE network to adjust to chronic stress. These changes in energy regulation follow a non-linear dose-response curve: In the groups with shorter stress exposure, the expected higher energy requirements due to stress could be addressed with an increase in mitochondrial density, the activation of the kynurenine pathway with bias towards its anti-excitatory arm, and a putative reduction in neural activity as suggested by reduced immediate early gene expression in the PFC. In the double-stress group with medium duration of stress exposure, additional adjustments in ETC functioning that improve mitochondrial performance were established. Despite these early measures, cellular health in the hippocampus appeared to be compromised after prolonged stress exposure, decreased immediate early gene expression suggested decreased communication in the stress-regulating PFC-hippocampus-circuit, and depressive-like behaviours were observed. Taken together, the data suggest that initially adaptive measures enable coping with stress for a certain amount of time, but that these measures might fail in the context of stress overload. Viewed from a preventive or therapeutic perspective, supportive actions to boost effectiveness of these existing measures, promotion of alternative coping strategies, and maintenance of recovery breaks could help preventing affected individuals from the negative consequences of stress.

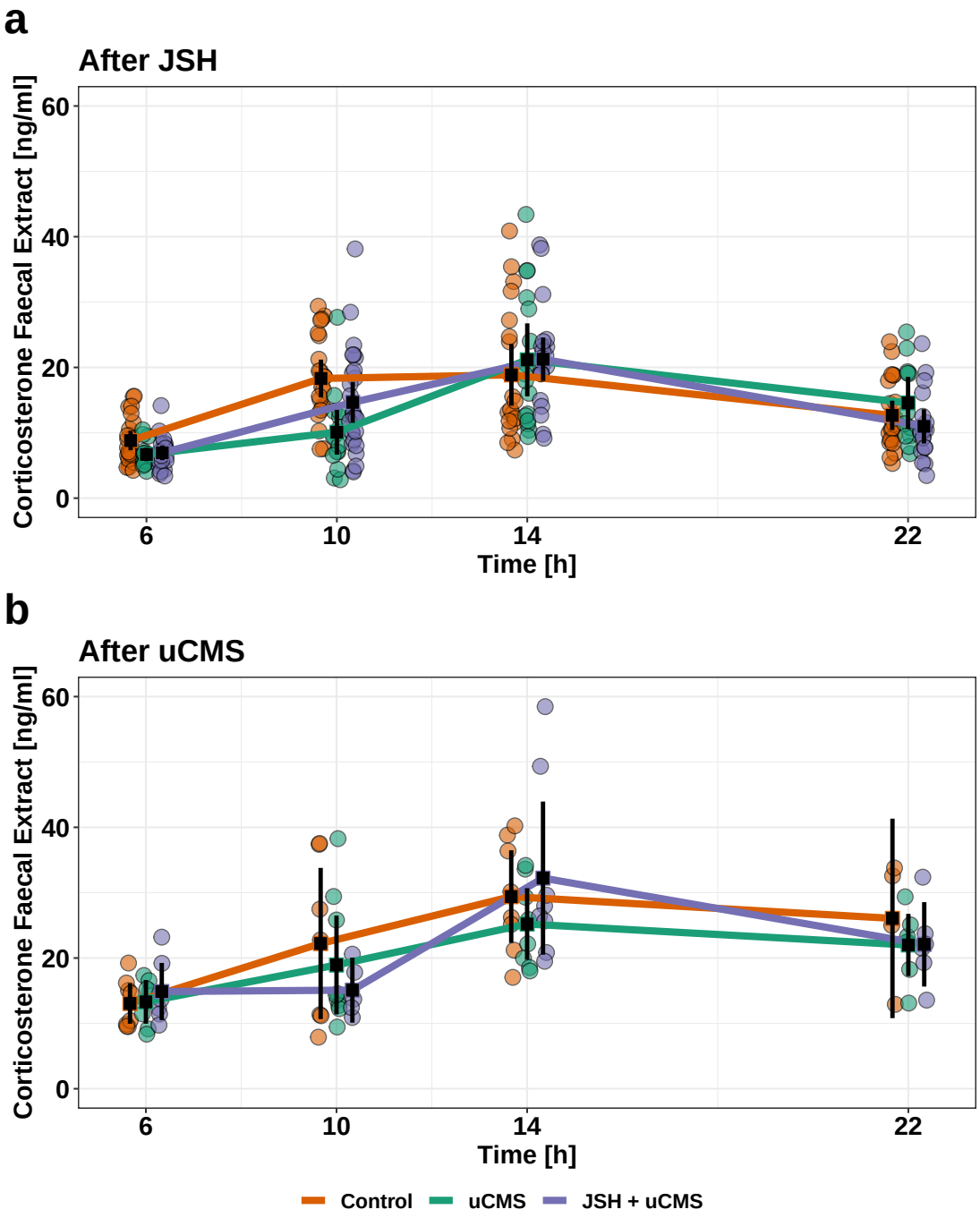

Figure 2.1.: **Faecal Diurnal Corticosterone Rhythms after JSH and uCMS**
(a) At an age of 10 weeks, after JSH, time was influencing corticosterone levels ($F(2, 65) = 17.9$, $p < .00001$) which was *post hoc* attributable to a diurnal rhythm in all groups (control: $F(3,36) = 8$, $p = .001$, JSH: $F(2,23) = 10.6$, $p = .002$, witnessing JSH (future uCMS): $F(3,15) = 5.5$, $p = .03$). Differences in the timing of the corticosterone peak per group were visible. In controls, the peak was between 10 a.m. ($p_{adj} = .00001$) to 2 p.m. ($p_{adj} = .002$), while the peak was at 2 p.m. in rats experiencing ($p_{adj} = .00005$) or witnessing ($p_{adj} = .0009$) JSH.
(b) At an age of 15 weeks, after uCMS, a diurnal rhythm was present ($F(3, 18) = 5.8$, $p = .006$) with all groups showing a peak at 2 p.m. (6-14: $p_{adj} = .00005$) and less decrease towards the night (6-22: $p_{adj} = .0002$).
Repeated measurement 2-way ANOVA using 'group' as between and 'time' as within factor. Bonferroni correction was applied to adjust the p-value. Single data points for the individual rats are shown alongside with the mean ± 95% confidence interval.

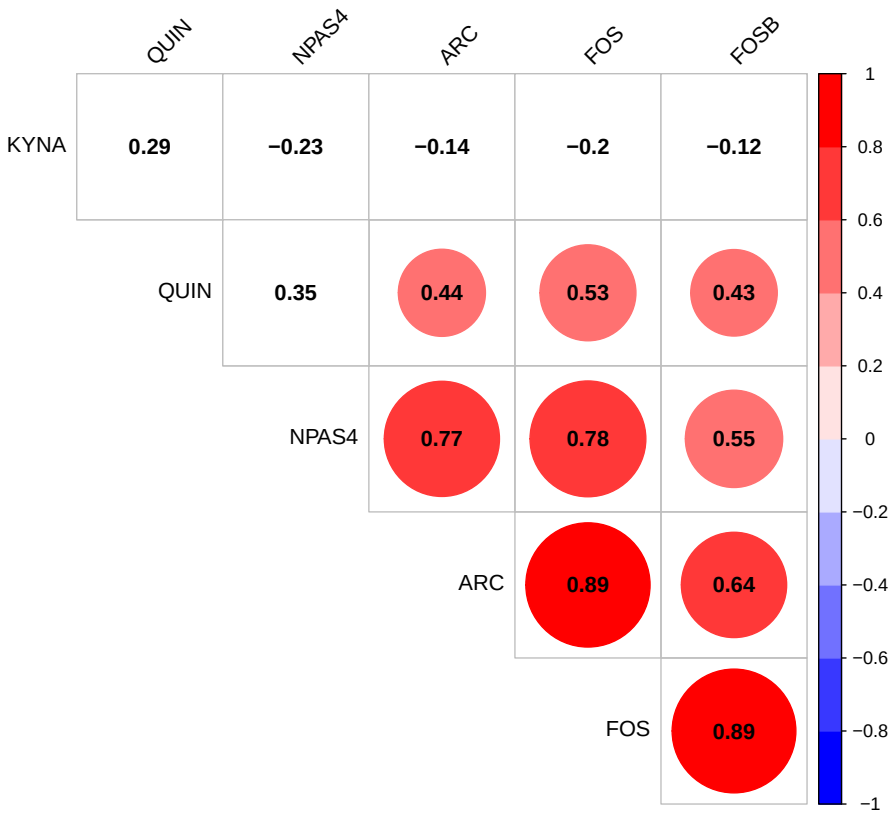

Figure 2.2.
Correlation Matrix of Neuroactive TRYCAT Levels in Plasma and Immediate Early Gene Expression in the PFC After 5 Weeks of uCMS
Values represent Pearson's correlation estimates. The presence of surrounding circles indicates statistical significance at a false positive level of α =.05. Bigger circle size illustrates a nominal bigger correlation coefficient. Warmer colours mark positive correlations.

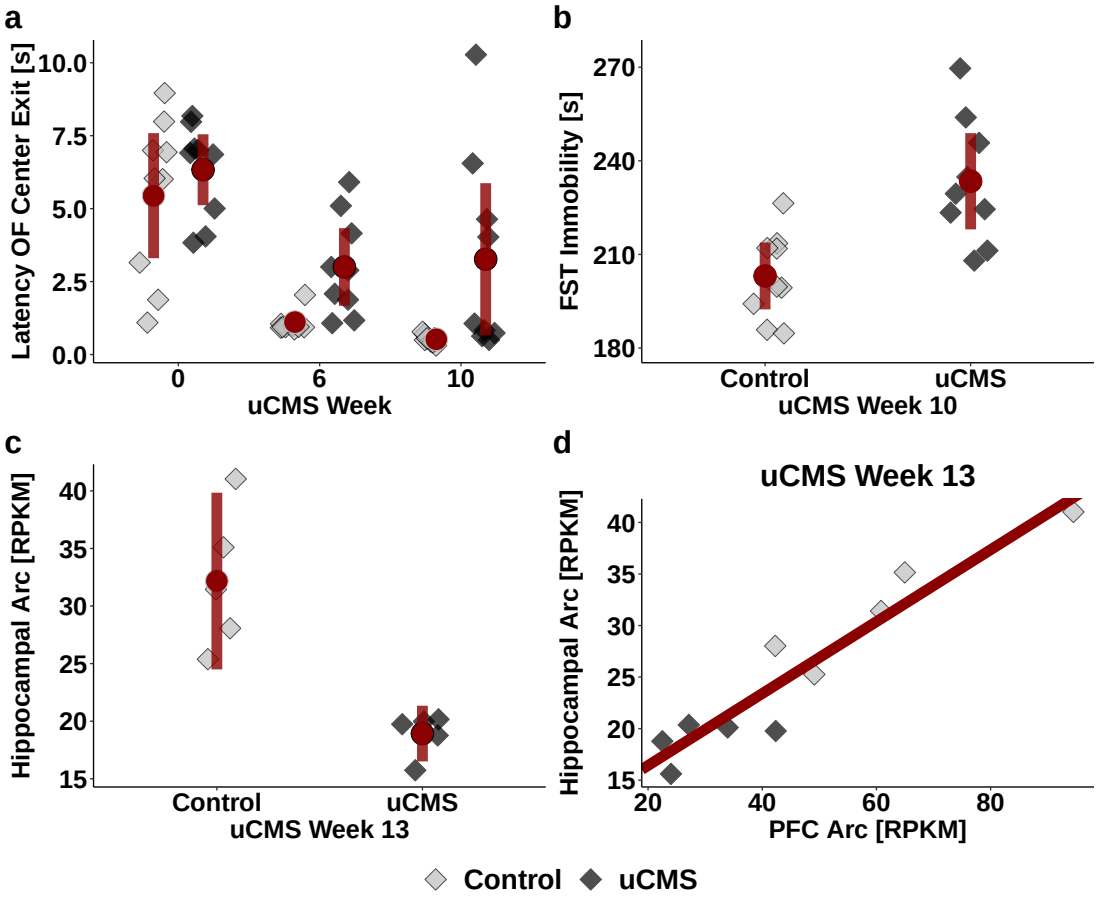

Figure 2.3.: **Effects of Prolonged Exposure to uCMS in Wistar Kyoto Rats**
(**a**) A significant influence of uCMS on the latency to leave the center of the open field arena was observed ($F(1) = 13.4$, $p = .0008$) and *post hoc* tests showed, that rats after 6 ($t(8.6) = -3.2$, $p = .01$) and 10 ($t(8.1) = -2.4$, $p = .04$) weeks of uCMS exposure remained longer in the centre than controls.
(**b**) In the forced swim test, an increased immobility was determined in the uCMS rats after 10 weeks of exposure ($t(14.3) = 3.7$, $p = .002$).
(**c**) After sacrifice in uCMS week 13, next generation sequencing of PFC and hippocampus were performed. After eliminating read outs which did not fulfill the quality criteria, the immediate early gene *Arc* ($t(4.8) = -4.6$, $p = .007$) was found to be significantly decreased in the hippocampus after uCMS.
(**d**) A correlation between hippocampal and PFC expression levels of *Arc* was detected ($\rho(20) = .88$, $p = .001$).
Single data points for the individual rats are shown alongside with the mean ± 95% confidence interval.

2.1.2. Reprint of the Original Research Article

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Activation of the kynurenine pathway and mitochondrial respiration to face allostatic load in a double-hit model of stress

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ABSTRACT

Allostasis is the process by which the body's physiological systems adapt to environmental changes. Chronic stress increases the allostatic load to the body, producing wear and tear that could, over time, become pathological. In this study, young adult male Wistar Kyoto rats were exposed to an unpredictable chronic mild stress (uCMS) protocol to increase allostatic load. First, physiological systems which may be affected by extended uCMS exposure were assessed. Secondly, 5 weeks of uCMS were used to investigate early adaptations in the previously selected systems. Adverse experiences during developmentally sensitive periods like adolescence are known to severely alter the individual stress vulnerability with long-lasting effects. To elucidate how early life adversity impacts stress reactivity in adulthood, an additional group with juvenile single-housing (JSH) prior to uCMS was included in the second cohort. The aim of this work was to assess the impact of chronic stress with or without adversity during adolescence on two domains known to be impacted in numerous stress-related disorders: mitochondrial energy metabolism and the immune system. Both, uCMS and adolescence stress increased kynurenine and kynurenic acid in plasma, suggesting a protective, anti-oxidant response from the kynurenine pathway. Furthermore, uCMS resulted in a down-regulation of immediate early gene expression in the prefrontal cortex and hippocampus, while only rats with the double-hit of adolescent stress and uCMS demonstrated increased mitochondrial activity in the hippocampus. These results suggest that early life adversity may impact on allostatic load by increasing energetic requirements in the brain.

1. Introduction

Several diseases have been found to be associated with chronic stress exposure, such as immune and metabolic dysfunction, and psychiatric or neurological disorders (Bisht et al., 2018; Chen et al., 2018a; Deak et al., 2017; Deschenes et al., 2018; Kraynak et al., 2018; Mellon et al., 2018; Ohno, 2017; Patist et al., 2018; Pearson-Leary et al., 2017; Stefanaki et al., 2018; Wirtz and von Kanel, 2017). It is commonly accepted that certain pathologies (excess oxidative stress, altered immune function, deregulated HPA-axis signaling (Rezin et al., 2008; Bauer, 2008; Maes et al., 2012; Srivastava et al., 2018) are present in such disorders and may be the result of stress history. Presumably there is a pre-pathological state and when a certain threshold is reached, allostatic load pushes these systems beyond adaptation. Our interest is to investigate physiological systems known to be disrupted in patients. The goal is to have translational biomarkers related to depressive symptoms. A secondary goal, in particular with regards to this study, is to be able to assess the mechanisms of adaptation that precede pathology, with an aim to learn how adaptation failure leads to pathology. To this end, young adult male Wistar Kyoto rats, which are thought to be sensitive to stress (Gomez et al., 1996; Redei et al., 2001; Solberg et al., 2001), were subjected to unpredictable chronic mild stress (uCMS). This model is broadly used to induce depressive-like behaviour (Armario et al., 1995; Baum et al., 2006; Will et al., 2003). However, it is difficult to say with certainty whether a behavioral phenotype in rodents truly reflects depressive behaviors in humans. Hence, we aimed to assess physiological processes occurring during adaptation to stress instead of behavior which might provide a better comparison with patients (Rezin et al., 2008).

Glucocorticoid induced immune signals resulting in the synthesis

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and release of pro-inflammatory cytokines prepare against potential tissue damage and pathogen encounter associated with a stressor, but can also be observed as an end result of long-term stress exposure and allostatic overload (Pearson-Leary et al., 2017; Wirtz and von Kanel, 2017; Bauer, 2008; Maes et al., 2012; Piskunov et al., 2016; Miller et al., 2002; Wright, 2009). Studies in rats have shown that distress induces changes in the amount and distribution of peripheral immune cells (Dhabhar et al., 2012; Swan and Hickman, 2014) and microglia (Tynan et al., 2010). Furthermore, altered bioenergetics during stress responses and inflammation were reported (Buttgereit et al., 2000; Delmastro-Greenwood and Piganelli, 2013; Rupprecht et al., 2012; Assmann and Finlay, 2016; Lopez-Armada et al., 2013; Pearce and Pearce, 2013), which conversely can increase reactive oxygen species (ROS) levels to trigger an immune response (Chen et al., 2018b). These neuro-immune and stress-related cascades provide physiological biomarkers that can be monitored. In this study, classical blood counts were measured to observe the overall circulating immune cell profiles. In addition, tryptophan catabolites (TRYCATs) of the kynurenine pathway were measured in blood and cerebrospinal fluid (CSF) because they recently have been suggested as novel biomarkers for depression (PS216, 2016; Schwarcz, 2016). The differential induction of involved enzymes makes profiling of TRYCATs a promising proxy for an inflammatory state: Stress-induced glucocorticoid release leads to an upregulation of the enzyme TDO, which converts tryptophan to kynurenine, mostly in the liver (Gibney et al., 2014; Ohta et al., 2017; Soichot et al., 2013). However, the IDO enzyme, which also converts tryptophan to kynurenine, is induced by cytokines. In the presence of inflammation, activated microglia and macrophages convert kynurenine further to quinolinic acid (due to upregulation of KMO/KYNU/HAAO enzymes), which is an NMDA-receptor agonist and potential neurotoxin due to its oxidative properties (Pérez-De La Cruz et al., 2012; Campbell et al., 2014; Asp et al., 2011; Smith et al., 2001). In an anti-inflammatory state, the excess kynurenine may be further processed to kynurenic acid, which has anti-oxidant properties and hence is considered protective (Lugo-Huitron et al., 2011). Profiling of the kynurenine pathway therefore provides insights into the inflammatory state of the immune system and the antioxidant defense system. Therefore, in an inflammatory state, quinolinic acid is increased relative to any change that might be present in kynurenic acid (Campbell et al., 2014).

Alterations in blood concentrations of TRYCATs have been detected in patients, possibly giving an indication of inflammatory or stress history (de Punder et al., 2018; Demir et al., 2015). Kynurenine was reported to promote resilience (Notarangelo et al., 2018), while decreased levels of kynurenic acid and increased levels of quinolinic acid were associated with depression (Doolin et al., 2018).

To determine new mechanisms that could help to treat or even prevent disease, tracking adaptations or the induction of pathology in brain, in addition to the peripheral immune system is critical. Our focus is in the regions of the medial prefrontal cortex (PFC) and the hippocampus. Due to its role in planning complex cognition, and moderating social behavior, by orchestrating thoughts and actions in accordance with internal goals, the PFC is a brain region largely responsible for the way how stress is perceived and responded to. Indeed, the PFC is associated with stress vulnerability and resilience, and has been largely implicated in stress-related disorders, particularly depression, post-traumatic stress disorder, and anxiety (Han and Nestler, 2017; Wang et al., 2014; Holmes et al., 2018). Chronic stress paradigms have demonstrated a myriad of changes to the PFC, including volumetric, connectivity, and electrophysiological alterations (Belleau et al., 2018; Popoli et al., 2011; Shepard and Coutellier, 2018; Goldwater et al., 2009). In order to keep its influence on connected brain regions like the hippocampus and amygdala updated to the recent experiences, a high level of plasticity is required in the PFC: by changing its hard-wiring on a synaptic level, the PFC enables adaptation to the current environment and regulates future stress assessment. The hippocampus is strongly involved in the latter since it is the CNS region where memory formation and recall occur. Stress has been associated with both enhanced or diminished memory, which can directly be linked to increased and decreased metabolic requirements in the hippocampus, respectively (Osborne et al., 2015). Our approach to investigating potential adaptive processes in the PFC and the hippocampus was two-fold: First, an extended period of 14 weeks of uCMS was applied in which standard behavioral testing (Forced Swim Test, Open Field Test, Sucrose Preference Test) was included to validate the protocol. *Ex vivo*, next generation sequencing (NGS) of the PFC and hippocampus was performed to assess which physiological systems may be affected by uCMS. Second, a 5 week uCMS exposure was used to determine the early adaptive transcriptional changes in the PFC with a focus on how plasticity may be affected. Since the hippocampus is particularly vulnerable to compromised energy metabolism and chronic stress has been demonstrated to accelerate hippocampal aging via this mechanism (Smith, 1996), hippocampal tissue from this second cohort was examined for effects of stress on bioenergetics using high-resolution respirometry. As adolescence was suggested to be a developmentally sensitive period during which the individual stress vulnerability is shaped (Zannas and Binder, 2014; Tzanoulinou and Sandi, 2017; Gomes et al., 2016; Entringer et al., 2016), a preconditioning of adolescent stress inflicted by 5 weeks of juvenile single-housing (JSH) was included in the second cohort.

Allostasis is the process by which the body's physiological systems adapt to environmental changes (McEwen, 2004). Chronic stress increases the allostatic load to the body, producing wear and tear that could, over time, become pathological. Early life adversity was suggested to accelerate this process, but the understanding how initially adaptive alterations join up into pathology is far from complete. In this study we propose two hypotheses: First, uCMS can be used to model allostasis and potentially allostatic overload. This could be evidenced by alterations in systems relevant in human depression, although not necessarily identical to changes observed in patients if the animals are still adapting to the stress. Second, we hypothesized that the double hit of JSH followed by uCMS during early adulthood could add up to a higher cumulative stress load and that aspects of allostatic stress responses would differ mechanistically between the single and the double stress group. Taken together, our aim is to provide new insights into allostatic mechanisms involved in a 'prodromal' phase of stress-induced pathology.

2. Methods

2.1. In vivo experiments

2.1.1. Animal housing

Test-naïve male Wistar Kyoto rats were obtained from Charles River with a bodyweight of about 100 g and an age of 5 weeks in March (cohort 1) or October (cohort 2). The rats were randomly assigned into the experimental groups and habituated to an inverse 12 h light cycle (lights on at 18:00, lights off at 6:00 with a ramp of 30 min) for 5 weeks. During their time in the specific pathogen free animal care facility at Boehringer Ingelheim Pharma GmbH & Co KG, the animals were housed in groups of three in type III Macrolon-cages (900 cm^2, 15 cm height, stainless steel wire cover) with enrichment or single-housed in type II Macrolon-cages (400 cm^2, 14 cm height, stainless steel wire cover) without enrichment (JSH). Enrichment consisted of a red plastic shelter, a red plastic tube, wood wool and a wooden stick for chewing. During the course of the entire experiment, animals were housed in a temperature- (25.5 ± 0.5 °C) and humidity- (50 ± 5%) controlled environment, while food (AIN-93 G basic diet 2222.PH.A05, Kliba Nafag, Switzerland) and tap water was available ad libitum if not contradicted by the uCMS protocol. Controls were continuously shielded from odors and noises of the stress rats by a Scantainer (Scanbur, Denmark). Aspen shavings were exchanged every Monday

morning by the same care taker, if not earlier as part of the uCMS protocol.

2.1.2. Unpredictable chronic mild stress

From an age of 10 weeks onwards, the uCMS protocol started. Nine formerly group-housed rats (uCMS group); and in cohort 2 additionally 9 JSH rats (JSH + uCMS group); were subjected to the uCMS procedure and single-housed, while 9 controls remained group-housed. Mild stressors used in this protocol involved e.g. wet bedding, frequent changes of the bedding, timely limited food and water restriction, intruder confinements, reduction of provided space and flashing lights and were applied in a scheduled manner using 2 designs to increase unpredictability. A detailed chart of the uCMS procedure can be found in appendix A. The stressors were applied by the same experimentor and the order in which group the stressor was performed first was randomized. In cohort 1, behavioural testing was performed in addition to the stressors of the uCMS protocol, while in cohort 2 no behavioural testing was performed to reduce potential confounding effects of excessive handling.

2.1.3. Sucrose preference test

Following 21 h of food and water deprivation, rats were placed into a cage for 1 h with access to 2 drinking bottles, attached to an automated weighing system (TSE Systems, Labmaster). One bottle contained tap water, the other a 2% sucrose solution. By measuring the weight of the bottles each minute, quantities and patterns of drinking behaviour was identified. During the first 8 sessions, the position of the 2% sucrose solution was alternated, training the rat to actively search for the sucrose solution. From training session 8 onwards the position of the sucrose solution remained constant in order to distinguish between rats that actively search for the sucrose and those displaying a side preference. Training was performed twice weekly on Tuesday and Friday until the rats reached an age of 10 weeks. After 11 training sessions, 18 of 24 trained rats actively sought the sucrose, demonstrated by drinking from both bottles before settling on drinking sucrose. These 18 rats were taken into the study and formed cohort 1.

2.1.4. Forced swim test

Is contrast to the standard Porsolt Forced Swim Test, rats were not trained the day before but were only tested once. Rats were placed into 17 cm diameter cylinders containing 30 cm of 25 °C cold water for 5 min and swimming, struggling and immobility were scored by a separate experimenter blinded to the treatment group. Rats were tested after 10 weeks of stress procedure.

2.1.5. Open field test

Rats were placed into the centre of an 83 cm diameter circular arena brightly illuminated from above the centre circle so as to prevent any areas of shadow. The arena was divided into 8 segments with a centre circle measuring 22 cm in diameter. During a period of 3 min, the time to leave the centre circle, rearing, the number of entries into the centre circle and the number of segments entered were determined. Only when all 4 paws were within one segment were counted as entry. Rats were tested at baseline and in weeks 6 and 10 of the uCMS procedure.

2.1.6. Sacrifice

To test for the effect of juvenile single-housing at an age of 10 weeks, 9 controls and 9 JSH-rats of cohort 2 were sacrificed between 7:00–11:00 in the morning under deep anaesthesia with pentobarbital (intra peritoneal injection of 100 mg/kg body weight, Narcoren®, Boehringer Ingelheim Pharma GmbH & Co KG, Germany). Pentobarbital was selected, because other narcotics were described to influence respirometric performance (Takaki et al., 1997). Since high-resoultion respirometry requires the use of fresh tissue, the final sacrifice of cohort 2 was staggered over Monday - Wednesday with 3 rats of each group being sacrificed per day in alternating order of groups, while cohort 1 was sacrificed on one day. All experiments were conducted under the approval of the Regierungspräsidium Tübingen in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2. Ex vivo analyses

2.2.1. Quantification of fecal corticosterone

Samples of feces were collected directly from the rats at 10 a.m. once weekly. Additional fecal samples were collected at 2 p.m., 10 p.m. and 6 a.m. one day prior to sacrifice. Samples were frozen at −20 °C and dried in a lyophylisator. Dried samples were pestled in a mortar pre-cooled with liquid nitrogen. Ethanolic extracts were obtained from 50 mg of fecal powder of each sample. Samples were resuspended in assay buffer and a competitive enzyme-linked immune assay (Cayman Chemicals, Ann Arbor, Michigan, USA) was performed following the manufacturer's instructions.

2.2.2. Mass-spectrometrical determination of TRYCATs

Quantification of plasma and cortico-spinal-fluid (CSF) levels of tryptophan (TRP) and its catabolites kynurenic acid (KYNA), kynurenine (KYN) and quinolinic acid (QUIN) was performed by liquid chromatography tandem mass spectrometry (HPLC-MS/MS). All analytes were quantified simultaneously in one assay. The assays comprised sample clean-up by protein precipitation with ice-cold methanol followed by reversed phase chromatography and mass spectrometric detection in the positive ion multiple reaction monitoring mode using the deuterated analogues of the analytes, namely d_5-kynurenic acid, d_4-kynurenine, and d_3-quinolinic acid as internal standards. The lower limits of quantification in plasma were 625 nM for TRP, 62.5 nM for KYN; 12.5 nM for KYNA and 25 nM for QUIN. The lower limits of quantification in CSF were 1250 nM for TRP, 1 nM for KYN, 5 nM for KYNA and 25 nM for QUIN.

2.2.3. Blood counting

Under deep pentobarbital anaesthesia, a cardiac puncture was performed to draw a critical volume of blood. An aliquot of 750 µl was used for blood count analyses, which were performed on a Siemens Advia 2120i (Siemens Healthcare GmbH, Erlangen, Germany).

2.2.4. High-resolution respirometry

High-resolution respirometry to assess mitochondrial function can give a detailed view of the activity of the electron transport chain (ETC) and its coupling to ATP production, therefore providing some hints to the energetic requirements of the investigated tissue. The rostral halves of the right hippocampi were isolated and stored in ice-cold custodial® (DR. FRANZ KÖHLER CHEMIE GMBH, Bensheim, Germany) to stabilize the tissue prior to respirometry. For measuring oxygen consumption, tissue was homogenated, diluted to a concentration of 2 mg/ml with mitochondrial respiration medium MiRO5 (Oroboros Instruments, Innsbruck, Austria) and loaded into the calibrated oxygraph chambers which were pre-warmed to 37 °C. Measurements were performed in duplicates. The respirometry was conducted following the recommendations of the manufacturer (Oroboros Instruments, Innsbruck, Austria) using the measurement protocol described in (Pesta and Gnaiger, 2012).In brief, pyruvate (5 mM), glutamate (10 mM) and malate (0.5 mM) as substrates for complex I of the ETC and cytochrome c (10µM) as indicator of mitochondrial membrane integrity were added subsequently. After the supplementation with ADP (5 mM) and the complex III substrate succinate (10 mM), the routine respiration was measured. By injecting oligomycin (2.5µM), ATP-sythase was inhibited and hence induced the LEAK state of respiration during which the oxygen is consumed to compensate for the leakage and slippage of ions and cation cycling across the inner mitochondrial membrane. Next the uncoupler FCCP was titrated in steps of 0.5µM to determine the maximal capacity of the ETC. By afterwards adding rotenone (0.5µM) the amount of oxygen which is consumed due to the activity of complex

II–IV alone was determined. Lastly, antimycin A (2.5μM) as inhibitor of complex III was injected to measure the residual oxygen consumption (ROX) outside of mitochondria. All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., St. Louis, Missouri, USA). During the measurement of one control animal, the obtained signal of oxygen concentration was not stable in one measurement chamber, so this measurement was excluded and only the values obtained in the stable chamber were used. Furthermore, we excluded 1 animal from each group, due to a lack of responsiveness after supplementation with substrates, which is an indicator of damaged samples. Lastly, one control animal was excluded, due to an increased dose of pentobarbital needed.

2.2.5. Citrate synthase assay

The activity of citrate synthase in the frozen homogenates of hippocampus was detected photometrically. By coupling the synthesis of citrate and CoA-SH from oxalacetate and acetyl-CoA with the formation of TNB out of DTNB and CoA-SH, the activity of the citrate synthase was measured as the rate of increase in absorbance. In brief, 0.1 M triethanolamine HCl buffer, oxalacetate (10 mM), DTNB (1.01 mM) and the citrate synthase standard were freshly prepared on every experimental day. Distilled water was loaded into 1 ml glass cuvettes together with 100 µl of DTNB, 50 µl oxalacetate, 25 µl acetyl CoA and 25 µl Triton and sample or standard. The absorbance was measured at 32 °C.

2.2.6. Next generation sequencing

2.2.6.1. RNA extraction, Illumina library preparation and sequencing. RNA was extracted from each tissue using the Ambion Magmax™-96 RNA isolation kit (Life Sciences) according to the manufacturer's instructions. Briefly, cells were placed in the lysis solution and homogenized in Qiagen Tissuelyzer™ for a period of 30 s. Nucleic acids were captured onto magnetic beads, washed and treated with DNase. Total RNA was then eluted in 30 µl nuclease free water. RNA integrity and concentration were assessed using the Fragment Analyzer and the Standard Sensitivity RNA kit (DNF-471, Advanced Analytical). High quality RNA samples with RIN > 7 were selected for further processing.

2.2.6.2. mRNA library preparation and sequencing. Fifty nanograms of total RNA were used as input material for the NEBNext Poly(A) mRNA Magnetic Isolation Module and the subsequent NEBNext Ultra™ II Directional RNA Library Prep Kit (New England Biolabs). NEBNext Adaptors for Illumina were diluted 100 fold prior to cDNA ligation. Adaptor ligated cDNA was amplified via 14 PCR cycles using NEBNext unique dual index primers (New England Biolabs). PCR products were cleaned up using AMPure XP Magnetic Beads (Beckman Coulter). mRNA libraries were qualitatively and quantitatively assessed using the 1–6000bp NGS kit (DNF-473, Advanced Analytical) and the Quant-iT PicoGreen dsDNA Assay kit, respectively. Final libraries yields were ˜40 nM, while fragment size were ˜ 350bp. Libraries were normalized, pooled and clustered on the cBot Instrument using the TruSeq SR Cluster Kit v3 (GD-401 − 3001, Illumina Inc, San Diego, CA). The clustered flowcells were sequenced on a HiSeq 3000 using a read length of 84 bases in single-read mode, generating an average of ˜30 million pass-filter reads per sample.

2.2.6.3. RNA-Seq data processing. RNA-Seq reads were aligned to the Rat genome using the STAR Aligner v2.5.2a with their corresponding Ensembl 84 reference genome (http://www.ensembl.org). Sequenced read quality was checked with FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and alignment quality metrics were calculated using the RNASeQC v1.18. Following read alignment, duplication rates of the RNA-Seq samples were computed with bamUtil v1.u.11 to mark duplicate reads and the dupRadar v1.4 Bioconductor R package for assessment. The gene expression profiles were quantified using Cufflinks software version 2.2.1 to get the Reads Per Kilopbase of transcript per Million mapped reads (RPKM) as well as read counts from the feature counts software package. The matrix of read counts and the design file were imported to R, normalization factors calculated using trimmed mean of M-values (TMM) and subsequently voom-normalized, before subjected to downstream descriptive statistics analysis. Genes with RPKM values > 5 in at least one group were considered in the final analyses to ensure data quality (Sollner et al., 2017). The Benjamini-Hochberg's method was used to correct for multiple testing, and only protein-coding genes with adjusted p value < 0.05, independent of magnitude of change, were considered as differentially expressed and used in the subsequent analyses. To conduct pathway analyses, the PFC and hippocampal gene lists from cohort 1 were imported into Ingenuity.

2.3. Statistical analyses

Earlier uCMS studies performed in-house or external showed sufficient discriminatory power with a sample size of 9 (Demirtas et al., 2014; Sahin et al., 2017; Foyet et al., 2017; Mo et al., 2014) for physiological parameters. This is supported by literature indicating physiological parameters are likely to be less variable than behavioural parameters (Becker et al., 2016) All data of the comparison juvenile single housing vs. control was assessed for departures from normality using the Shapiro Wilk test and heteroscedasticity using Bartlett's or Levene's test where appropriate. Parametric statistical testing was performed for the TRYCAT data with a correction for unequal variances (Welch's *t*-test) while non-parametric Mann-Whitney-U-tests were performed for the blood counts, body weight and adrenal weight. For the comparisons after the uCMS procedure, linear models were compared using one-way analyses of variance (ANOVA). Where required, the ranks were analyzed. The corticosterone data was analyzed with a repeated measurement ANOVA of a mixed linear effect model. Individual measures for different analyses are listed in the associated tables and graphs. No correction for multiple testing was performed, due to the exploratory nature of the study and the high inter-correlation of TRYCAT and respirometric data. All analyses were conducted using R 3.2.4 and tests were considered statistically significant when the *p*-value was smaller or equal to the selected alpha level of 5%.

3. Results

3.1. Behavioural validation of the uCMS protocol

In the first cohort, there were no significant changes to body weight gain, sucrose preference, or sucrose consumption. Significant influence of uCMS was observed in the forced swim test and in open field test (appendix D). In addition, alterations were observed in plasma tryptophan catabolites (appendix D). Next generation sequencing revealed 882 significantly deregulated genes in the PFC, and 431 in the hippocampus. Sixty-two of the de-regulated genes in the PFC reached at minimum a 2-fold-change, while none of the genes in the hippocampus reached this level of deregulation. Ingenuity pathway analysis was used to assess the commonalities in deregulated gene sets (appendix E). The overlap between molecular and cellular functions and physiological systems underlying behavior in hippocampus and PFC is of particular note. The immediate early gene Arc is a component of the behavioural function networks, and its deregulation is correlated between the PFC and hippocampus (appendix D).

3.2. Effect of juvenile single housing

In the second cohort, the effect of JSH alone was assessed prior to subjecting rats to uCMS. In a subset, the diurnal HPA-axis activity was traced and 9 rats of each group were sacrificed for analyses at an age of 10 weeks. No significant differences were determined in body or adrenal weight between groups. There were no significant differences

Table 1
Blood Counts and TRYCAT in CSF After 5 Weeks of Juvenile Single Housing.

	Control (n = 8)			JSH (n = 6)				
	min	median ± IQR	max	min	median ± IQR	max	*U*	*p*
White Blood Cells	0.66	1.9 ± 1.5	4.51	1.34	1.56 ± 0.36	3.21	24	1
Monocytes	0.02	0.05 ± 0.05	0.09	0.03	0.05 ± 0.01	0.14	22	0.8
Lymphocytes	0.48	1.2 ± 1.4	3.88	0.93	1.1 ± 0.3	2.58	24	1
Eosinophils	0.01	0.03 ± 0.01	0.04	0.01	0.02 ± 0.02	0.04	31	0.4
Basophils	0	0.01 ± 0.02	0.08	0	0 ± 0.008	0.01	37	0.08
Neutrophils	0.12	0.41 ± 0.12	0.54	0.31	0.38 ± 0.10	0.55	27	0.7
Platelets	183	582 ± 137	648	536	634 ± 44.8	657	14	0.2
Hematocrit	13.3	43.8 ± 9.4	63.5	43	46 ± 3.3	47.7	22	0.9
Red Blood Cells	7100	8310 ± 293	10050	8010	8345 ± 340	8610	25	1.0
Hemoglobin	12.1	14.5 ± 0.93	18.1	13.7	14.3 ± 0.3	14.6	31	0.4
Mean Peroxidase Index	2.3	9.7 ± 18.1	33.2	2.5	4.2 ± 1.7	8.2	31	0.4
Procalcitonin	0.14	0.45 ± 0.16	0.75	0.37	0.39 ± 0.02	0.42	35	0.2

	Control (n = 8)			JSH (n = 8)				
CSF	min	mean ± sd	max	min	mean ± sd	max	*t*	*p*
TRP	1880	2211 ± 299	2740	2020	2456 ± 343	3020	t(13.7) = -1.5	0.2
KYN	11.9	20.5 ± 8.2	35.2	17.6	27.1 ± 6.3	34.6	t(13.2) = -1.8	0.1
QUIN	50.9	58.6 ± 5.8	70	42.8	60.8 ± 14.3	80.5	t(9.2) = -0.4	0.7
KYN / TRP	0.005	0.009 ± 0.004	0.0155	0.0081	0.011 ± 0.002	0.015	t(11.1) = -1.1	0.3

Unit: Blood counts [1000 cells /µl], hemoglobin [g/dl], hematocrit [%], procalcitonin[%], TRYCAT [nM].

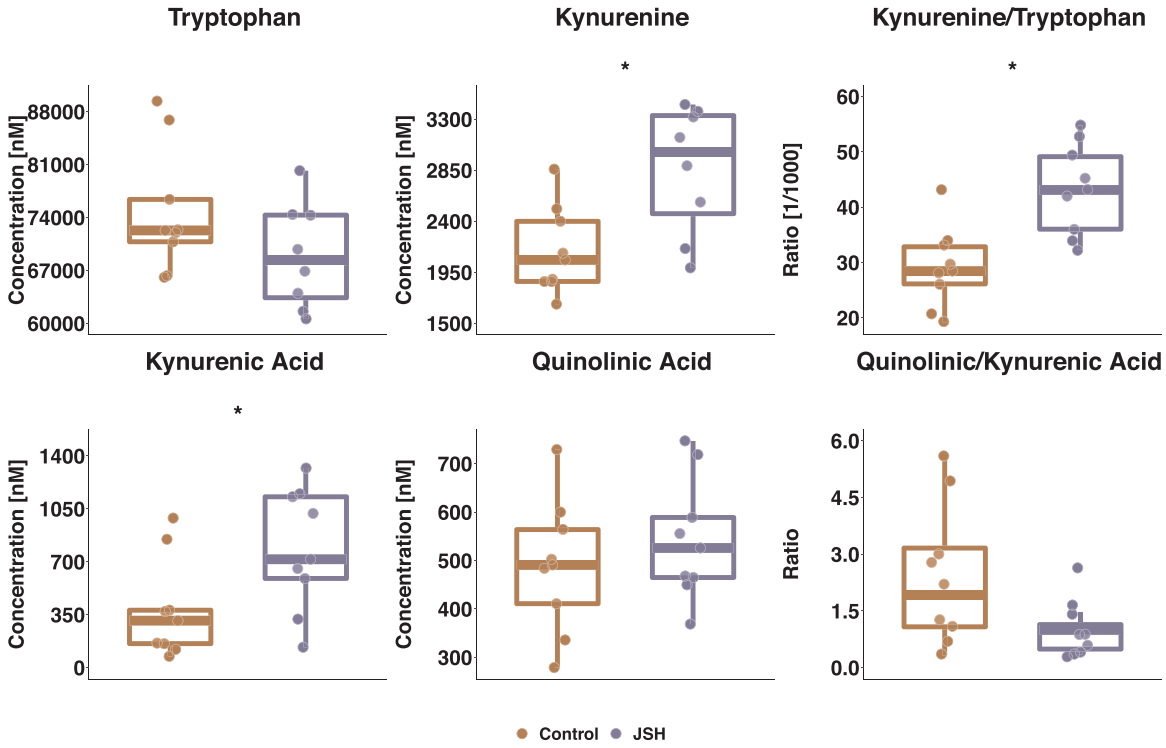

Fig. 1. TRYCAT Profiling After 5 Weeks of Juvenile Single Housing. Plasma concentrations of tryptophan and its catabolites kynurenine, kynurenic acid and quinolinic acid were measured by HPLC-MS/MS. An independent samples *t*-test was conducted to compare plasma levels in 10 weeks old male Wistar Kyoto rats after 5 weeks of single housing during adolescence (violet) and age-matched controls (orange). Results were as follows: tryptophan: t(16) = 1.9, p = .08; kynurenine: t(16) = -3.5, p = .003; kynurenic acid: t(16) = -2.3, p = .03; quinolinic acid: t(16) = -0.9, p = .4; kynurenine/tryptophan: t(16) = -3.9, p = .001; quinolinic acid /kynurenic acid: t(11) = 2.1, p = .06 * = p < .05 in independent samples *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

in fecal corticosterone at 6 a.m., 10 a.m., 2 p.m. and 10 p.m. between controls housed in groups of three and the JSH group (appendix B). Blood counts and the TRYCATs assessed in CSF did not reveal statistically significant changes attributable to group- vs. single-housing (Table 1). The results of TRYCAT assessment in plasma suggest an effect of the housing condition and are provided in Fig. 1.

3.3. Allostatic load of short-term uCMS exposure

To investigate allostatic processes, rats of the second cohort were subjected to 5 weeks of uCMS with or without JSH as preconditioning to trigger adaptive changed in stress relevant systems.

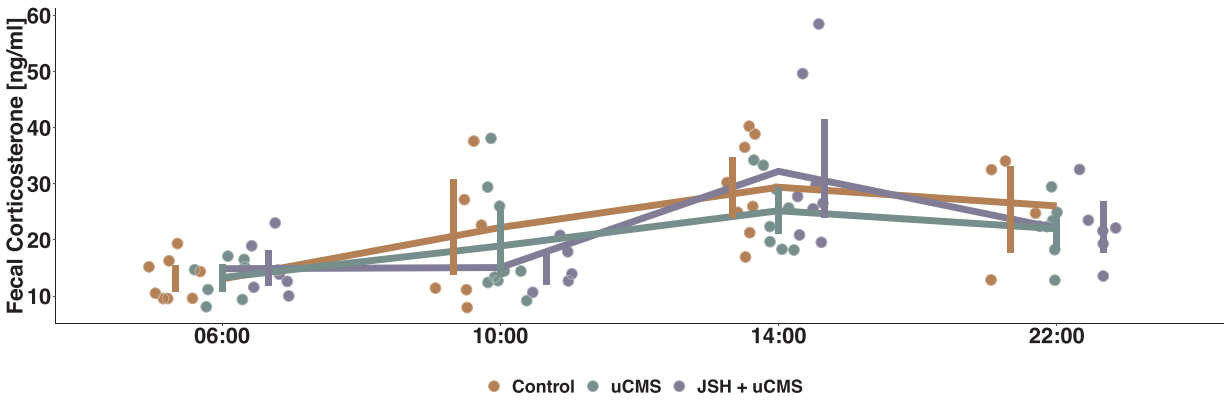

Fig. 2. Diurnal Corticosterone Profile After 5 Weeks of uCMS. At 6:00, 10:00, 14:00 and 22:00, fecal corticosterone levels were measured in rats exposed to five weeks of uCMS (green), rats exposed to 5 weeks of juvenile single housing followed by five weeks of uCMS (violet) and controls (orange). No significant effect of group or the interaction of group and daytime was found. Time showed a significant difference between levels: 6:00 - 14:00 t(54) = -6.4, p < 0.0001; 6:00 - 22:00 t (54) = -3.6, p = 0.003; 10:00 - 14:00 t(54) = -4.0, p = 0.001. Individual data points are shown alongside with the mean ± 95% confidence interval. Repeated measurement two-way ANOVA (group + time | ID), main effect of time: F(3, 48) = 14.4, p < 0.0001 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3.1. Diurnal HPA-Axis rhythmicity

The diurnal rhythm of corticosterone excretion in feces was traced after 5 weeks of uCMS exposure (Fig. 2). No statistically significant differences between groups were observed in the overall corticosterone levels as well as at different times of day. A circadian rhythm was present in all groups with a peak at 2 p.m. and a nadir at 6 a.m. in fecal corticosterone concentration.

3.3.2. Kynurenine pathway profiling

After 5 weeks of uCMS, the concentration of quinolinic acid in plasma was decreased in the uCMS group ($t(24) = 4.5$, $p = .0005$) and in the uCMS rats with additional juvenile single-housing ($t(24) = 4.8$, $p = .0002$) compared to controls. The other TRYCAT measures and ratios were not significantly changed in plasma. In CSF, an increase in the ratio of kynurenine and tryptophan was found which in the post hoc test is significant in the double-hit group ($t(24) = -3$, $p = .02$). A summary of all TRYCAT [nM] and their ratios is provided in Table 2.

3.3.3. Mitochondrial respirometry

After 5 weeks of uCMS, the activity of the mitochondrial enzyme citrate synthase was significantly affected by group ($F(2,24) = 5.71$, $p = 0.009$). Tukey HSD post-hoc testing revealed an increase in the uCMS group ($t(24) = -2.7$, $p = 0.03$) and in the group with combined juvenile single-housing combined with uCMS ($t(24) = -3.1$, $p = 0.01$). The residual oxygen consumption (ROX), which is *per definitionem* independent of mitochondrial respiration, was not different in uCMS rats compared to controls, but the amount of oxygen consumed at routine and LEAK as well as the respiration after uncoupling the ETC and after inhibiting complex I was increased after uCMS, while this increase was more pronounced in the group which additionally had experienced juvenile single housing. Since the amount of oxygen consumption is directly linked to the amount of mitochondria, we normalized the respirometric measurements to the citrate synthase activity, since this is a commonly used surrogate for mitochondrial density. The results of ROX, citrate synthase activity and the normalized high-resolution respirometry are visualized in Fig. 3. After accounting for mitochondrial density, the respiration at routine and without ETC I was still significantly increased in the double-hit group. Notably, the routine flux control, coupling control, coupling efficiency and net-routine were unaltered by stress exposure (data not shown).

3.3.4. Next generation sequencing

A significant decrease in the immediate-early genes *Arc*, *Fos*, *Fosb* and *Npas4* was detected in the PFC of rats which underwent uCMS alone or in combination of juvenile single-housing, compared to controls (Fig. 4).

3.3.5. Blood counts

Rank ANOVA of blood counts suggests that after 5 weeks of uCMS there is an increase in several white blood cells in the stress groups compared to controls. Counts for lymphocytes were increased in both, the uCMS group ($t(22) = -4.5$, $p = .0005$) and the uCMS group with additional juvenile single-housing experience ($t(22) = -2.8$, $p = .03$). Eosinophil granulocytes were as well increased in both, the uCMS group

Table 2
TRYCAT Levels and Ratios After 6 Weeks of uCMS.

	Control (n = 9)			uCMS (n = 9)			JSH + uCMS (n = 9)				
CSF	min	mean ± sd	max	min	mean ± sd	max	min	mean ± sd	max	*F (224)*	*p*
TRP	2240	2436 ± 180	2780	1980	2441 ± 382	3340	2120	2284 ± 218	2790	1.0	0.4
KYN	7.4	13.1 ± 4.7	21.5	11.1	18.8 ± 4.5	25	8.4	19.1 ± 7.4	30.8	3.1	0.06
QUIN	42.2	55.4 ± 10.0	70.5	41.2	53 ± 8.1	66.5	48.1	53.2 ± 4.7	63.3	0.3	0.8
KYN / TRP	0.003	0.005 ± 0.002	0.008	0.006	0.008 ± 0.001	0.009	0.004	0.008 ± 0.003	0.013	4.9	0.02*
Plasma											
TRP	58000	68077 ± 9041	83900	51700	65466 ± 11143	83700	49000	60133 ± 7211	69500	1.7	0.2
KYN	989	1773 ± 484	2420	1320	2154 ± 520	2970	1100	2029 ± 635	3050	1.1	0.3
KYNA	224	449 ± 162	720	46.1	510 ± 331	1210	140	489 ± 173	772	0.2	0.9
QUIN	365	476 ± 68.8	543	170	308 ± 94.7	508	166	294 ± 73.7	438	14.0	< .001*
KYN / TRP	0.01	0.027 ± 0.01	0.041	0.02	0.03 ± 0.01	0.05	0.02	0.03 ± 0.01	0.05	1.4	0.3
QUIN / KYNA	0.73	1.152 ± 0.33	1.7	0.25	1.0 ± 1.0	3.7	0.35	0.71 ± 0.42	1.8	1.0	0.4

Unit: Individual TRYCAT measures in nM.

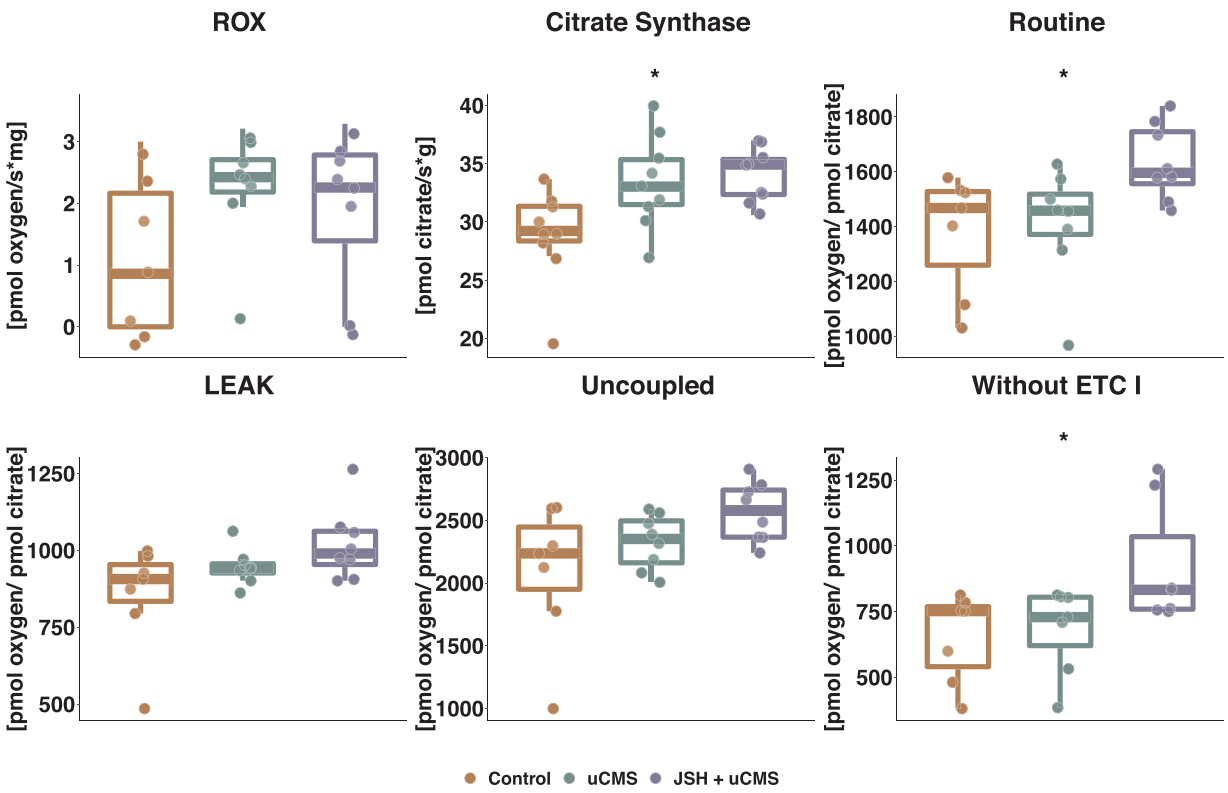

Fig. 3. Respirometry After 5 Weeks of uCMS. Hippocampal respirometric performance was determined in 16 weeks old rats which underwent 5 weeks of juvenile single housing followed by 5 weeks of uCMS (violet), 5 weeks of uCMS (green) and controls (orange). Residual oxygen consumption (ROX) was subtracted from all respirometric measurements to only report oxygen consumption associated with mitochondrial respiration. To account for overall mitochondrial increase, the oxygen consumption was divided by citrate synthase activity. Tukey honest significant difference post-hoc testing suggests an increased routine respiration in the combination of juvenile single-housing with uCMS compared to both, uCMS (p = .0301) and controls (p = .0242). Post hoc testing for respiration without ETC I suggests a borderline increase in juvenile single housed rats with uCMS compared to controls (p = .0529). 1-way-rank-ANOVA (group): Citrate Synthase $F(2,24) = 7$, p = .0042; Routine $F(2,20) = 5.39$, p = .0134;, Without ETC I $F(2,18) = 3.69$, p = .0453 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

($t(22) = -4.1$, $p = .001$) and the JSH + uCMS group ($t(22) = -4.9$, $p = .0002$) and basophil granulocytes also showed an increase in the uCMS group ($t(22) = -2.8$, p(uCMS) = .03) and the double-hit group ($t(22) = -5.1$, $p = .0001$). Neutrophil granulocytes were only increased in the double-hit group ($t(22) = -2.6$, $p = .05$). Monocytes were increased in the uCMS group ($t(22) = -5.3$, $p = .0001$) and the juvenile single-housed uCMS group ($t(22) = -5.7$, $p < .0001$). An overview of the findings in blood counts is provided in Table 3.

4. Discussion

In this study, an extensive period of uCMS was used in a first cohort to demonstrate moderate behavioural changes and to explore which physiological systems might play a role in adaptation to uCMS. This

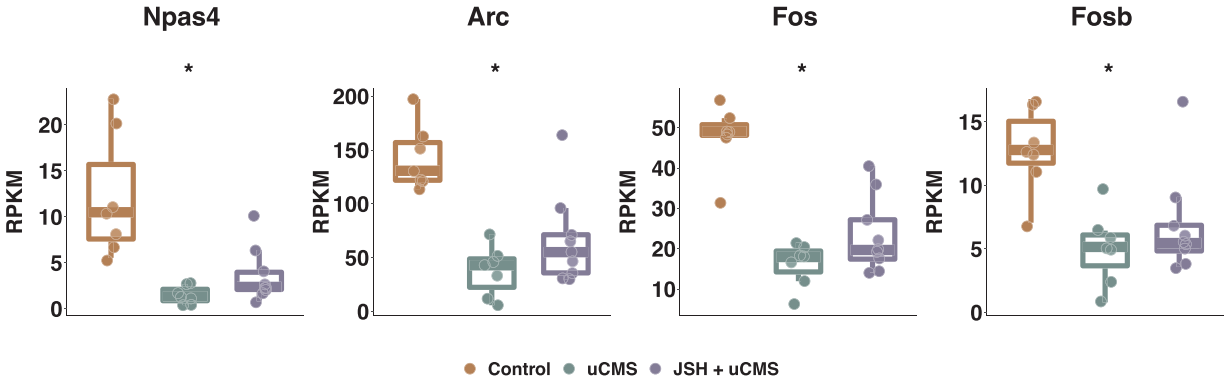

Fig. 4. PFC Gene Expression Profile After 5 Weeks of uCMS. Next-generation sequencing of the PFC was used to compare the uCMS-only group (green) and the double-hit group with juvenile single-housing prior to uCMS (violet) to controls (orange). From all significantly different genes, only the most reliable with RPKM > 5 in at least one experimental group were eligible. There was a statistically significant reduction in the expression of Npas4, Arc, Fos and Fosb in the uCMS groups compared to controls. Results of the post hoc tests in the uCMS-only group were: Npas4 (t(11) = -3.1, p = .006), Arc (t(11) = -1.7, p = .01), Fos (t(11) = -1.5, p = .002) and Fosb (t(11) = -1.2, p = .003). In the double-hit group results were as follows: Npas4 (t(12) = -2.1, p = .02), Arc (t(12) = -1.3, p = .05), Fos (t(12) = -1.2, p = .003) and Fosb (t(12) = -1.2, p = .0004). * = p < .05 in pairwise comparison of control vs. uCMS and control vs. JSH + uCMS (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
Blood Counts After 6 Weeks of uCMS.

	Control (n = 9)			uCMS (n = 8)			JSH + uCMS (n = 8)				
	min	mean ± sd	max	min	mean ± sd	max	min	mean ± sd	max	*F*(222)	*p*
White Blood Cells	0.74	1.09 ± 0.24	1.53	1.1	1.6 ± 0.5	2.7	1.01	1.6 ± 0.9	3.8	4.95	0.02*
Monocytes	0.02	0.03 ± 0.01	0.04	0.04	0.06 ± 0.04	0.17	0.03	0.07 ± 0.05	0.19	21.28	< .0001*
Lymphocytes	0.47	0.70 ± 0.14	0.84	0.77	1.1 ± 0.4	2.2	0.61	1.1 ± 0.7	2.9	10.49	0.0006*
Eosinophils	0.01	0.02 ± 0.01	0.03	0.02	0.04 ± 0.02	0.07	0.02	0.4 ± 1.1	3	13.96	0.0001*
Basophils	0	0.003 ± 0.005	0.01	0	0.006 ± 0.005	0.01	0	0.008 ± 0.005	0.01	12.92	0.0002*
Neutrophils	0.21	0.32 ± 0.12	0.62	0.2	4.5 ± 12.0	34	0.31	0.4 ± 0.1	0.6	3.48	0.05*
Platelets	389	503 ± 52.7	565	477	549 ± 75.6	695	410	502 ± 55.4	593	0.69	0.5
Hematocrit	39.6	41.9 ± 1.7	44.3	38.8	42.0 ± 2.5	45.5	39.4	43.0 ± 3.0	48.4	0.24	0.8
Red blood cells	7570	8096 ± 398	8750	7300	7922 ± 423	8640	7380	8086 ± 403	8770	0.43	0.7
Hemoglobin	12	13.1 ± 0.6	14	11.8	13.0 ± 0.7	14.1	12	13.3 ± 0.6	14.3	0.82	0.5
MPXI	4.4	9.1 ± 2.6	12.7	3.8	7.0 ± 2.6	10.9	4.4	7.5 ± 3.2	13.1	1.22	0.3
Procalcitonin	0.31	0.36 ± 0.04	0.4	0.32	0.4 ± 0.05	0.48	0.31	0.4 ± 0.06	0.5	1.01	0.4

Unit: Blood counts [1000 cells /µl], hemoglobin [g/dl], hematocrit [%], procalcitonin[%]; MPXI = mean peroxidase index.

first cohort provided evidence for changes in the kynurenine pathway which was followed up in a second cohort. Furthermore, NGS suggested neuronal activity changes due to reduced expression of the immediate early genes Arc and Fos. This deregulation was correlated between hippocampus and PFC, but was greater in PFC, which led to a focus on the transcriptome from the PFC in the second cohort and the decision to assess cellular health functionally in the hippocampus by using high-resolution respirometry. Respirometry was chosen because decreased mitochondrial functioning or evidence of slowed metabolism which was suggestive in the mild transcriptome changes from cohort 1 has also been observed in depressive patients (Maes et al., 2012; Boeck et al., 2018; De et al., 2017).

In the blood of rats which underwent uCMS for 5 weeks, we saw an increase in the amount of white blood cell populations. In line with earlier publications (Dhabhar et al., 2012), a mobilization of monocytes was present in blood of uCMS-exposed rats. As precursors of macrophages, monocytes are involved in the initiation and propagation of immune reactions and hence could be an early marker for the establishment of a pro-inflammatory milieu (Wang et al., 2015; Stotz et al., 2015; Eo et al., 2016). In our study, increased monocytes levels after stress were accompanied by elevated numbers of granulocytes and lymphocytes. This suggests alterations in the composition of the innate and adaptive immune system following stress. In line with the neuroinflammation hypothesis of depression, increased amounts of immune cells could lead to an increased secretion of pro-inflammatory signaling molecules which in turn could impact the CNS (Kitaoka and Furuyashiki, 2014; Nikolaienko et al., 2018). However, the levels of quinolinic acid were not increased, which would be the case in the presence of increased circulating cytokines. In contrast, quinolinic acid was decreased, which suggests that the activity of immune competent cells was not increased to a biologically relevant point. Nevertheless, TRYCAT profiling revealed that after 5 weeks of single-housing during adolescence, an increased catabolism of the essential amino acid tryptophan was detectable in plasma, which led to an increase in kynurenic acid and its precursor kynurenine. These metabolites are considered to be neuroprotective (Sas et al., 2007), because kynurenic acid inhibits glutamatergic NMDA-receptors and reduces the release of dopamine and glutamate (Capuron and Miller, 2011). Hence, an activation of this side of the kynurenine pathway holds the potential to decrease the excitatory signaling in the brain. Even though tryptophan catabolism was increased in the periphery, which presumably stems from activity of the liver enzyme TDO, kynurenine can exert activity in the CNS, because it can be transported by the large neutral amino acid carrier of the blood brain barrier (Fukui et al., 1991). In the absence of inflammatory processes, kynurenine is converted to kynurenic acid by the astrocytic kynurenine-amino-transferases. The correlation of kynurenine in plasma with its levels in CSF as well as the correlation with its catabolite kynurenic acid in plasma supports this (appendix C). Taken together, blood counts and TRYCAT profiling did not point towards inflammatory processes, but suggested an activation of the neuroprotective and anti-oxidant side of the kynurenine pathway.

The increased amount of NMDA-receptor inhibiting kynurenine and kynurenic acid and reduced NMDA-receptor stimulating quinolinic acid, is suggestive of reduced excitatory neurotransmission. One hypothesis is that this could be reflected as reduced synaptic plasticity (Mattson, 2008; De Pitta and Brunel, 2016; Rebola et al., 2010; Hunt and Castillo, 2012; Jay, 2003; Otani et al., 2003). Our NGS finding that the expression of immediate early genes was reduced in the PFC is supportive of this hypothesis. After 5 weeks of uCMS, immediate early genes like Arc, Fos, Fosb and Npas4 were switched off or significantly reduced in both stress groups compared to controls. Decreased levels of these genes suggest a decreased ability of the PFC to respond to incoming signals by changing its synaptic outputs (Takahashi and Miczek, 2014; Minatohara et al., 2015). Interestingly, synaptic weakening in the PFC has been associated with resilience to stress (Wang et al., 2014). This observation would fit with our interpretation the rats in this study were still coping and undergoing allostasis. An alternative hypothesis could be raised, that reduced plasticity in the PFC might increase the likelihood of insufficient provisions for future challenges, which could render the body more prone to accumulate damage. From a circuit perspective, this could be relevant for the interactions of the PFC and hippocampus under healthy conditions, the hippocampus receives dampening signals from the PFC (Anderson et al., 2016; Godsil et al., 2013). If the hippocampal activity is increased and the plasticity of the PFC is reduced, inhibitory outputs from the PFC may be insufficient, leading to excess hippocampal activity. Excess hippocampal activity could result in the mild transcriptome signature from the first cohort indicating changes to cellular health, and might also be detectable as increased energy consumption.

Indeed, after 5 weeks of uCMS an increased activity of the mitochondrial enzyme citrate synthase was found in hippocampus. This enzyme is a common proxy for the cellular density of mitochondria (Larsen et al., 2012). Since the main energy currency of the body, adenosine-tri-phosphate (ATP), is produced in mitochondria, an increase in their number as measured by citrate synthase, suggests increased energy consumption and hence activity in the rat hippocampus after uCMS. Increased hippocampal activity is directly linked to enhanced memory formation and recall, which has been associated with stress (Osborne et al., 2015). In the pathologic state, such an over-encoding of memory presents as rumination of adverse thoughts in depression and strengthening of the fear-network in post-traumatic stress disorder. Additionally to the increased number of mitochondria, the activity of the ETC was found to be altered in rats exposed to uCMS. However, after normalizing the oxygen consumption to the citrate

synthase activity, only an increased routine respiration and oxygen consumption without ETC complex I in rats which underwent the double stress of JSH followed by uCMS was observed. In turn, this steady energy consumption in the hippocampus might require reallocation of resources, which could be at expense of the PFC. Reduced availability of resources in combination with decreased expression of immediate early genes would render the PFC even less able to inhibit hippocampal activity and a vicious circle is formed, that could over time cumulate in allostatic overload and functional consequences.

If the suggested energetic linkage hypothesis between hippocampus and PFC holds true, our observation that ETC activity was increased only in the double stress group might indicate that these animals are already in the transition from adaptation to pathology. After a certain type or duration of stress, their mitochondria might decompensate due to a mitochondria inherent susceptibility to ROS (Indo et al., 2007; Yakes and Van Houten, 1997; Jendrach et al., 2008) and hippocampal cells could suffer from insufficient energy supply. In our previous uCMS study with triple the duration of uCMS exposure (15 weeks), NGS analyses of hippocampus and PFC both revealed a reduction of immediate early genes (appendix D). This could be a way to adjust energetic demands in order to survive, but is on a slippery slope because it does not solve the original problem. On the long run, even this adjustment might not be enough to rescue hippocampal activity. In depressive patients, reduced mitochondrial respiration as sign for decompensation (Karabatsiakis et al., 2014) and an atrophy of the hippocampus (Opel et al., 2014; Sheline et al., 1996) have indeed been described. Interestingly, a reduction in hippocampal volume in depressive patients has been shown to correlate with TRYCAT profiles, suggesting that perhaps the tipping point revolves around the balance of multiple measures.

Taken together, the alterations observed by respirometry point towards higher energetic demands of the hippocampus after a certain dose or duration of stress. An elevated respiration after inhibition of ETC I with rotenone in the double-hit group compared to controls is indicative for an increased utilization of ETC complex II to fuel ATP production. ETC II serves as alternative entry point for hydrogen abstracted from Flavin-adenin-dinucleotide-dihydrogen while at ETC I NADH is utilized. Interestingly, complex I activity is associated with more production of reactive oxygen species (ROS) than complex II (Liu et al., 2002), so a shift towards complex II might be a possibility to reduce ROS overload and to evade the risk of oxidative stress which is inseparably linked to increased mitochondrial activity. Our observation of decreased quinolinic acid levels at the same time point when mitochondrial changes are present and the moderate negative correlation of quinolinic acid and citrate synthase activity (appendix C) strengthens this idea, because quinolinic acid is a precursor for the ETC I substrate NAD. Whether a reduced availability of quinolinic acid and hence NAD leads to the alternative usage of ETC complex II or whether a mitochondrial mechanism to avoid excessive ROS reduces the quinolinic acid side of the kynurenine pathway is an exciting question for future studies. Either way, the 5 weeks of uCMS, even when combined with juvenile single-housing stress has not induced mitochondrial pathology, since the respirometric control ratios remained unaltered. There was no difference in the relation between oxygen consumption used for ATP production at routine compared to ETC activity solely accounting for leakage of electrons and cation slippage in the LEAK stage. Furthermore, the coupling efficiency of controls and uCMS animals did not differ after 5 weeks of exposure, suggesting that no damage or functional disintegration of the mitochondria had occurred yet. In sum, the alterations in mitochondrial activity observed in this study indicate a healthy coping mechanism, in line with the concept of allostasis being an adaptive process.

In context with our findings from profiling immune cells, TRYCATs and gene expression in the PFC, our mitochondrial data supports the hypothesis that uCMS can be used to model allostasis and potentially allostatic overload. By including juvenile single-housing as preconditioning and using a short-term exposure to uCMS, we extended the uCMS model to better elucidate the initial alterations in physiology as part of an allostatic response and how early life adversity moderates this process. As hypothesized, the double hit of JSH followed by uCMS during early adulthood seemed to add up to a higher cumulative stress load leading to mechanistically different allostatic stress responses in some aspects, since ETC functioning was only altered in the double stress group. Overall, the physiological alterations observed in these studies could be interpreted as origin of allostatic overload. With allostasis being an “active” process, first mitochondrial activity could increase proportional to the amount of adaptation necessary, but later might decompensate due to the inherent vulnerability of mitochondria to ROS (Indo et al., 2007; Yakes and Van Houten, 1997; Jendrach et al., 2008). While decreased mitochondrial functioning has been detected in blood of depressive patients, it is possible that such changes in metabolic processes will be observed in the central nervous system as well. Given the sensitivity of the hippocampus to compromised energy metabolism (Karabatsiakis et al., 2014; Smith, 1996), this brain region is a good starting point to test for this hypothesized link between peripheral and central energy metabolism. The findings in our second cohort of rats support this energetic linking hypothesis. Using a unique combination of innovative readouts in addition to classical readouts used in the clinics, we have discovered that a functional link between TRYCAT and adaptive mechanisms involving mitochondrial respiration and synaptic plasticity might exist. This helps to better bring into context the essential networking of stress responsive systems during allostasis. The interconnectedness of the systems studied can be seen in Fig. 5. Importantly, allostatic processes in one system can influence the functioning of all other systems. This is beneficial if quick adaptations are vital, but conveys the risk of building up disturbances which cause instability of the network, transition of feedback loops from inhibitory regulation to promotion and subsequently switching to a pathological state (Stapelberg et al., 2018). Our findings on TRYCATs and IEGs substantiate the linking of the immune system with neuronal activity. Based on our respirometry findings, we propose the hypothesis of an energetic linkage between hippocampus and PFC which could help to explain further how disturbances in one system spread to the other and advance the likelihood to develop pathology. However, follow-up studies to verify this hypothesis are needed and some limitations should be considered when interpreting the presented results.

One limitation of our study is, that it was set up to investigate allostatic processes occurring early after onset of stress exposure, and so cannot make any firm conclusions as to eventual pathology. Testing the proposed suggestion that these allostatic mechanisms eventually fail and lead to pathology needs to be studies further. Profiling of the diurnal rhythm of corticosterone excretion indicated no changes in basal HPA-axis activity after 5 weeks of juvenile single-housing or after 5 additional weeks of uCMS exposure, suggesting that HPA-axis activity was not pathologically altered by the stress manipulation. However, no stress challenge was performed, since this could confound readouts from the other parameters accessed. Therefore, we cannot rule out that the responsiveness of the HPA-axis might have suffered during the stress manipulation. Importantly, appendix C indicates numerous correlations between different systems; ideally causal relationships should be demonstrated to support the interpretations. Dependent on the research question, amendment further improvement may be the use of brain parenchyma instead of CSF. By using CSF, we provide insights into a clinically applicable bio specimen, but studies aiming at elucidating mechanistically whether TRYCATs can effectively modulate plasticity via NMDA-receptor, tissue health, and mitochondrial function should detect catabolite levels within the tissue itself.

Currently 50–60 % of depressive patients do not respond to the first-line treatment (Fava and Davidson, 1996) and 30% do not reach remission even after several treatment attempts (Rush et al., 2006; Thase et al., 2007). Despite this unmet need for alternative treatments, no mechanistically new treatment options were reported lately and the

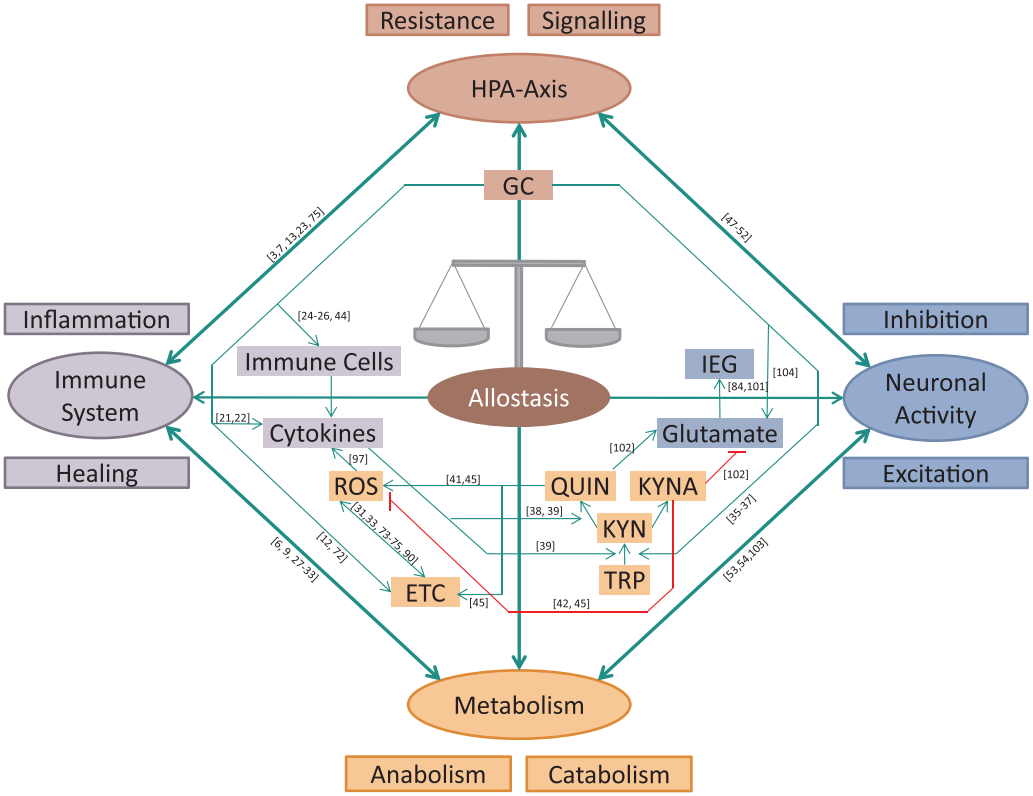

Fig. 5. Schematic of the Psycho-Immune-Neuro-Endocrine Network. Stress responsive systems interact with each other to adequately respond to stressors via allostatic processes. At the boarders, the HPA-axis (red), neuronal activity (blue), metabolism (orange) and the immune system (violet) are depicted as sentinel network nodes alongside with two extremes of their setting options. In the centre, the networking of representatives of these systems is visualized, while green arrows symbolize activation or feed-forward mechanisms and red arrows indicate inhibition. The numbers adjacent to the arrows represent selected publications included in the reference list which are further describing the link (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

potential of classical approaches investigating the pathological state alone appeared to be exhausted. Hence, we selected the novel strategy to determine biomarkers pathology evidenced in patients in animals undergoing chronic stress. The potential to add stress-related 'prodromal' biomarkers can contribute to the mechanistic understanding of how pathology evolves. This could help to develop early diagnostic markers or even preventive treatment. In conclusion, this study demonstrated that allostatic processes allow the endurance of chronic exposure to mild stressors for a certain time and that juvenile single-housing stress has an influence on these processes. Both stress groups showed an increase in circulating immune cells, a decrease in the expression of immediate early genes in the PFC, and an increase in mitochondrial density in the hippocampus. However, only the double-hit of juvenile single housing stress and uCMS resulted in increased mitochondria respiration in the hippocampus. This increased metabolism might represent a cornerstone for later pathology, because increased mitochondrial activity goes hand in hand with increased ROS production, priming immune cells to pro-inflammatory actions (Naik and Dixit, 2011; Nakahira et al., 2011). This has extensively been described as starting point for functional disintegration of mitochondria (Yakes and Van Houten, 1997; Jendrach et al., 2008). From an evolutionary perspective, it is advantageous that adverse experiences during childhood and adolescence induce stress reactions are stronger and faster, to promote survival in inhospitable environments. However, this comes at the cost that individuals with adverse experiences during childhood or adolescence have an increased cumulative stress load. In a setting of chronic stress exposure, individuals with such a preconditioning may come closer to pathology and show signs of premature ageing than individuals without it. The increased mitochondrial respiration and amount of immune cells could be the beginning of the transition towards pathology, since these could lead over time to the development of oxidative stress and a pro-inflammatory milieu. To this end it would be interesting to know whether a longer duration of uCMS exposure would be sufficient to reach the tipping point, after which key physiological systems are out of balance and diseases develop. In such a longitudinal study, the transition from allostasis to pathology could be elucidated and a better timely resolution between the changes in energy metabolism and the immune system could help to understand the mechanistic linkage between both.

Declaration of interest

T. Bretschneider, N. Lawless, and KA Allers are employees of Boehringer Ingelheim Pharma GmbH KG.

Role of the funding source

The funding for this study was provided by Boehringer Ingelheim Pharma GmbH KG, to provide a master's thesis project to V. Nold. The company had no further influence on this work.

Submission declaration and verification

The hippocampal respirometry, blood counts and corticosterone measurements have been published in part as the Master's degree thesis of V. Nold, University of Ulm. The NGS analyses and TRYCAT measures as well as the statistical analyses were not part of the thesis.

CRediT authorship contribution statement

V. Nold: Conceptualization, Investigation, Writing - original draft, Formal analysis. **C. Sweatman:** Investigation, Conceptualization, Resources, Methodology. **A. Karabatsiakis:** Writing - review & editing, Investigation. **C. Böck:** Writing - review & editing, Investigation. **T. Bretschneider:** Resources, Methodology. **N. Lawless:** Resources, Formal analysis. **K. Fundel-Clemens:** Formal analysis, Resources. **I.-T. Kolassa:** Supervision, Writing - review & editing. **K.A. Allers:** Conceptualization, Project administration, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.04.006.

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2.2. Impact of Fkbp5 × Early Life Adversity × Sex in Humanized Mice on Multidimensional Stress Responses and Circadian Rhythmicity

2.2.1. Summary Study II

Interactions of ELA and the SNP rs1360780 within the human *FKBP5* gene were reported to increase the risk to suffer from psychiatric symptoms and to predict treatment outcome in later life [628, 659, 52, 270]. Influences on the responsiveness to challenges and glucocorticoid signaling have been demonstrated for both factors of this gene × environment interaction and are thought to be a core driver during the transition from adaptation to stress-associated disorders [602]. Adversity during early life was shown to trigger aberrations in brain development that can prime the HPA axis activity [384], manifest in cognitive alterations [98, 420], and trigger abnormal social behaviour [64, 625]. The glucocorticoid-induced expression of *FKBP5* constitutes a regulatory feedback loop to the HPA axis [142, 465, 521, 193, 265] and is a hub for integrating lifetime and recent stressful experiences [297, 122, 659] that is modulated by single nucleotide polymorphisms in the gene [54, 309]. Naturally occurring *Fkbp5*-SNPs in laboratory rodents do not feature comparable functional effects as rs1360780 in humans, which hindered further elucidation of diathesis for disorders.

The aim of **study II** was to determine the translational capacity and validity of the *Fkbp5*-humanized mice × ELA model by characterizing behavioral and physiological aspects of adult stress responsiveness. Based on this framework of findings in the literature, we hypothesized that a valid animal model which shall allow for studying the molecular mechanisms that causally link *Fkbp5*, ELA, and stress regulation to pathology would need to incorporate these factors (1). We expected ELA to cause alterations in the adult behaviour and physiology compared to individuals with an undisturbed development (2) and hypothesized that AT-allele carriers would respond differently to ELA than CG-allele carriers (3). Mice harbouring the AT-allele were expected to be more at risk to acquire alterations that render their stress response less flexible and the individuals more likely to develop disorders. Mice carrying the CG-allele were expected to be more resilient to the negative effects of ELA and stress, which would protect them from developing aberrant PINE network activities over time. Since the hypothalamus and hippocampus are brain regions that strongly regulate stress responses [394], we hypothesized that these brain regions would show *Fkbp5*-genotype × ELA dependent differences in gene expression (4) that would be related to the functioning of PINE network components (5) and thus could provide insights into underlying molecular mechanisms of the elevated prevalence of disorders in AT- vs. CG-allele carriers with additional stressful life experiences.

To address these hypotheses, *Fkbp5*-humanized mice homozygous for the AT- or CG-allele were timely mated and litters were allocated to ELA or control condition in such a way that group sizes were balanced as far as possible. No offspring was culled or swapped between litters to avoid uncontrolled additional stress effects, resulting in five to eleven animals per group defined by early life condition, *Fkbp5*-genotype, and sex. While the control groups were left undisturbed, mice in the ELA groups were separated from mothers and littermates for three hours per day at different times a day from postnatal day two until weaning. Behavioural and physiological examination started at an age of ten weeks. Since exploration of novel environments offers an easily accessible measure of mild stress in rodents [81], the mice were challenged with novel

situations to probe their stress coping strategies. To this end, mice were placed in open field arenas at dusk to trace locomotion during the murine active phase, T-mazes to measure spatial orientation and working memory, dark-light boxes to determine preference for the dark as surrogate measure of anxiety, and a compartmentalized arena with an unfamiliar mouse to quantify sociability. In addition, diurnal, stimulated, and suppressed HPA axis activity was assessed by measuring plasma corticosterone levels in the morning, noon, and evening, after five minutes of restraint stress, and six hours after intraperitoneal injection of dexamethasone, respectively. At an age of 30 weeks, mice were sacrificed in the morning and tissues for *ex vivo* analyses were collected.

The analyses revealed that female AT- vs. CG-allele carriers with or without ELA showed elevated nadir glucocorticoid levels and a lower diurnal amplitude. In parallel, female AT-allele carriers were less responsive to novel environments, as seen during dark-light box testing, locomotor habituation, and the social chamber test. This was the case irrespective of their early life condition. Nevertheless, ELA-exposed AT-allele carrying females had a lower body weight than controls (Fig. 2.4). In contrast, female CG-allele carriers with ELA were more active than controls but showed no differences in body weight. Moreover, female CG-allele carriers with ELA spent less time directly interacting with an unfamiliar mouse, but spent more time in its surrounding. Males were less active than females, weighed more, and displayed lower levels of glucocorticoids. Spontaneous alternations in the T-maze were similar between all groups but ELA-exposed female mice completed the task quicker than controls (Fig.2.5, supplementary figure from [428]). On a molecular level, more genes differed in their expression between AT- and CG-allele carriers in the subset of ELA-exposed mice than controls. The effects of ELA vs. control measured by the count of differentially expressed genes was higher in the CG- vs. AT-allele carriers. On a functional level, the differentially expressed genes in hypothalamus and hippocampus were enriched in pathways associated with synaptic communication, circadian entrainment, and energy production. While the expression of genes related to OXPHOS was higher in AT- vs. CG-allele carriers, a lower expression of genes related to synaptic communication and circadian entrainment was detected in AT- vs. CG-allele carriers. In addition, ELA was associated with reduced hippocampal expression of genes related to dopaminergic signalling as well as circadian entrainment. An initial indication for the translatability of the findings regarding circadian entrainment in the *Fkbp5*-humanized mice was obtained by qualitatively comparing gene expression in the mouse hypothalamus to astrocytes and neurons derived from human induced pluripotent stem cells (hiPSCs) of rs1360780 carriers (Fig.2.6, supplementary figure from [428]).

Taken together, the findings of **study II** indicate that the *Fkbp5* × ELA mouse model shows face and mechanistic validity to psychiatric symptomatology. Behavioural aberrations in AT- vs. CG-allele carriers like decreased exploration of novel environments, which may be attributable to decreased curiosity or increased anxiety, and decreased sociability when additionally exposed to ELA, resemble observations in psychiatric patients. Mechanistically, reduced circadian entrainment, elevated nadir, and flattened diurnal glucocorticoid rhythmicity in AT- vs. CG-allele carrying females match with the use of HPA axis profiling as diagnostic tool in the clinic. However, other pathological findings in psychiatric patients like impaired negative feedback to the HPA axis after dexamethasone challenge were not recapitulated in this first *in vivo* study carried out with the *Fkbp5*-humanized mice. Future studies that add chronic stress exposure as third hit to the *Fkbp5* × ELA model might shed more light on pathological transitions potentially occurring after the states investigated within this study.

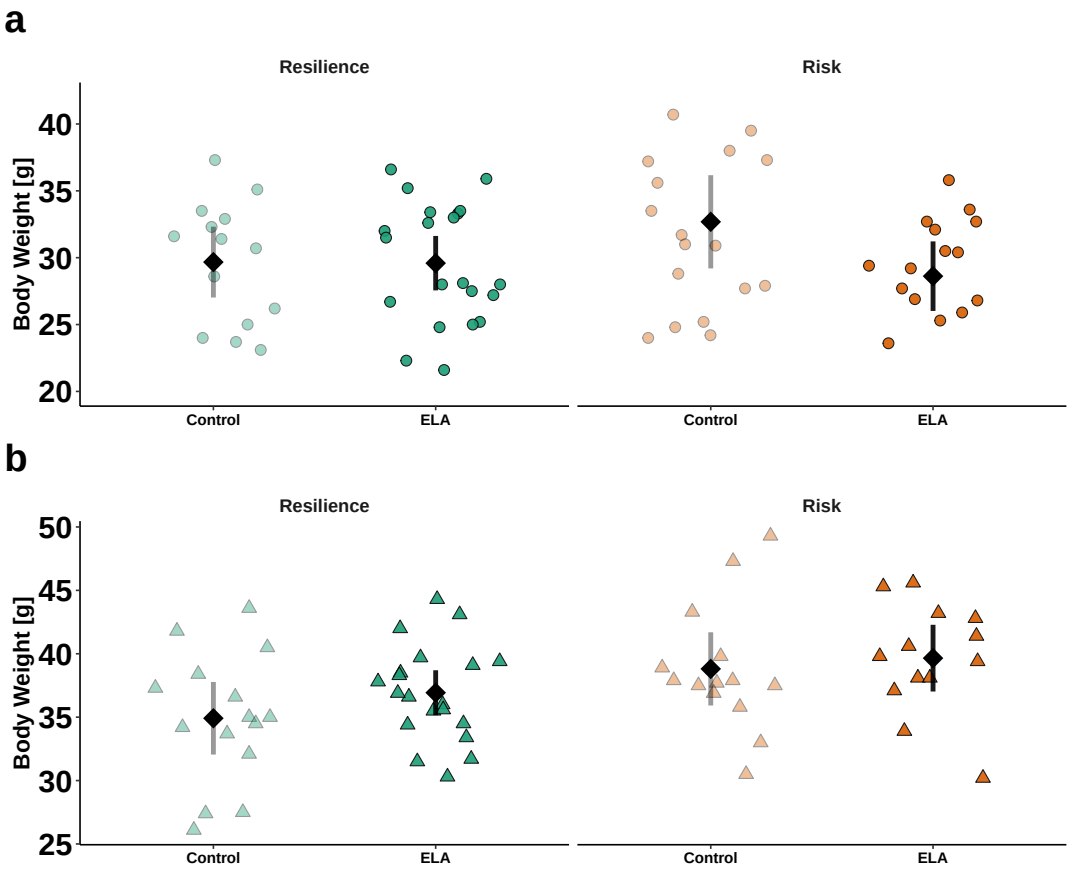

Figure 2.4.
The Genotype Modulates Body Weight in *Fkbp5*-Humanized Mice
Data of females (**a**) and males (**b**) is shown separately since females weigh less than males ($F(1) = 71$, $p < 1^{-5}$).
A significant effect of *Fkbp5*-genotype was detected using ANOVA with AT-allele carriers weighing more than CG-allele carriers ($F(1) = 5$, $p = .02$).
In addition, an interaction of early life condition with sex was suggestive ($F(1) = 4$, $p = .055$) that *post hoc* was attributable to female AT-allele carriers showing reduced body weight when exposed to ELA compared to controls ($t(123) = 2.4$, $p = .02$). Data of individual mice are shown alongside with the mean (black diamond) ± 95% confidence interval to indicate significant differences between subgroups.

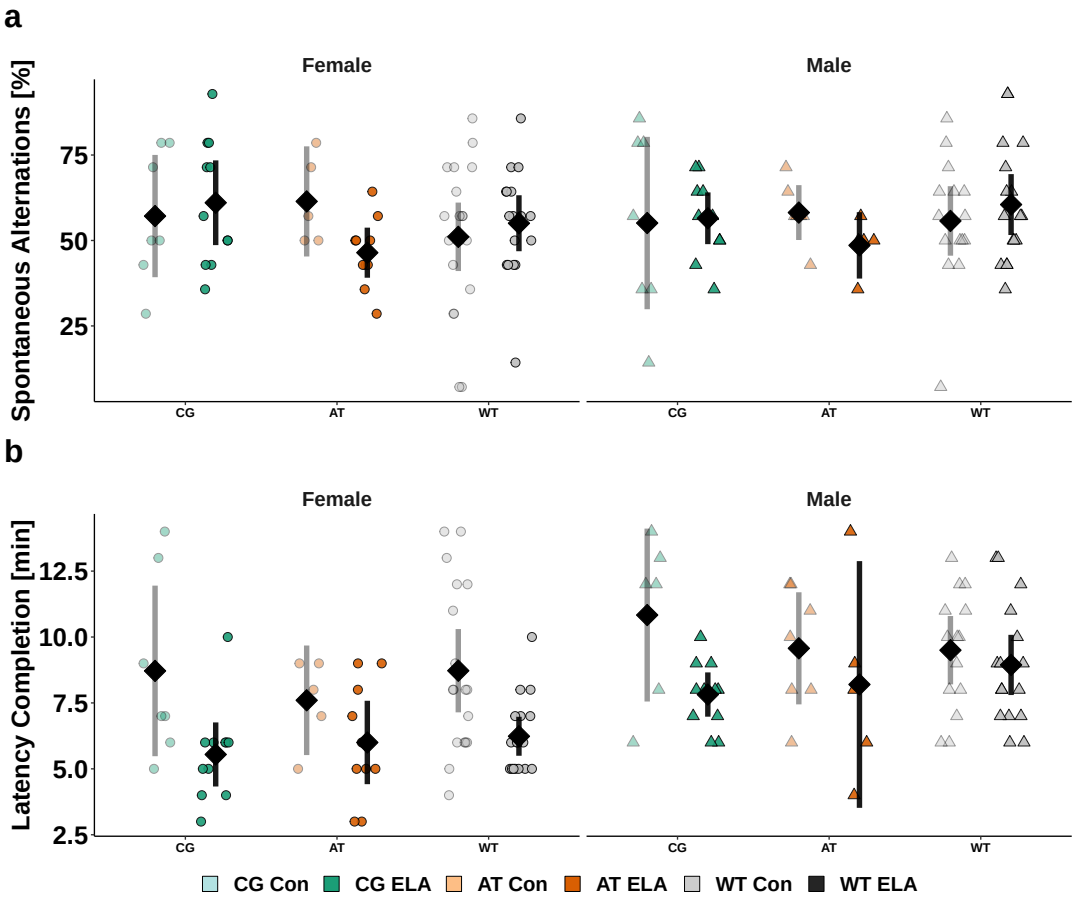

Figure 2.5.
ELA Shortens the Time to Completion of the T-Maze Test in *Fkbp5*-Humanized and Wild Type Mice
Data of individual mice are shown alongside with the mean (black diamond) ± 95% confidence interval to indicate significant differences between subgroups.
(**a**) The percentage of spontaneous alternations between the arms of the T-shaped arena was performed at chance level with no statistically significant effect of early life condition, *Fkbp5*-genotype or sex.
(**b**) The time [min] needed to complete the 15 trials differed between controls and ELA-exposed mice ($p < 1^{-5}$) and was further modulated by sex with females being faster than males ($p < 1^{-5}$).

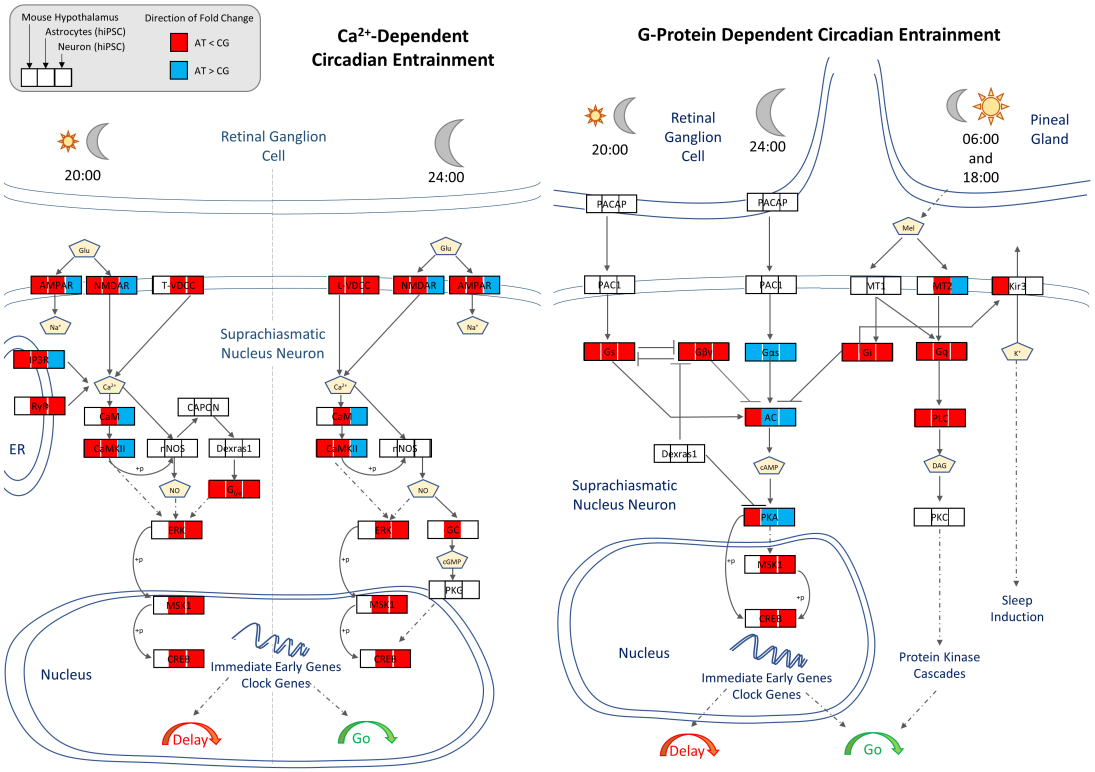

Figure 2.6.
Differential Expression of Genes Related to Circadian Entrainment in *Fkbp5*-Mice and in hiPSC-Derived Astrocytes and Neurons of Human *FKBP5*-SNP Carriers
Visualization of significant fold changes between AT- vs. CG-allele shown for mouse hypothalamus, hiPSC-derived astrocytes, and neurons. The calcium-dependent and the G-protein dependent arm of the circadian entrainment KEGG pathway is shown on the left and right, respectively.

2.2.2. Reprint of the Original Research Article

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Impact of *Fkbp5* × early life adversity × sex in humanised mice on multidimensional stress responses and circadian rhythmicity

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The cumulative load of genetic predisposition, early life adversity (ELA) and lifestyle shapes the prevalence of psychiatric disorders. Single nucleotide polymorphisms (SNPs) in the human *FKBP5* gene were shown to modulate disease risk. To enable investigation of disease-related SNPs in behaviourally relevant context, we generated humanised mouse lines carrying either the risk (AT) or the resiliency (CG) allele of the rs1360780 locus and exposed litters of these mice to maternal separation. Behavioural and physiological aspects of their adult stress responsiveness displayed interactions of genotype, early life condition, and sex. In humanised females carrying the CG- but not the AT-allele, ELA led to altered HPA axis functioning, exploratory behaviour, and sociability. These changes correlated with differential expression of genes in the hypothalamus, where synaptic transmission, metabolism, and circadian entrainment pathways were deregulated. Our data suggest an integrative role of *FKBP5* in shaping the sex-specific outcome of ELA in adulthood.

Molecular Psychiatry; https://doi.org/10.1038/s41380-022-01549-z

INTRODUCTION

Stress responses are essential to adjust physiology and behaviour to recurrently changing environmental demands [1], but corrupted stress responses are a hallmark feature of psychiatric conditions [2]. The susceptibility or resilience to develop psychiatric disorders can be attributed to interactions of genetic predispositions and environmental factors [3]. Among environmental factors, early life adversity (ELA) is found to be especially detrimental given that aberrations during development will influence the affected individuals throughout life [4]. Childhood maltreatment is common in the history of many psychiatric patients and comprises experiences of physical, sexual, and emotional abuse, as well as physical and emotional neglect [5]. Such experiences during development shape disease prevalence in later life through alterations in HPA axis programming, stress coping strategies, and brain connectivity [6]. With respect to genetic predispositions, the regulation of glucocorticoid signalling is a prominent research target since glucocorticoids are a key messenger for the spread and initiation of stress-responsive signalling. This regulation is finetuned in a timing- and dose-dependent manner and depends on the individual cellular set-up such as the relative expression of glucocorticoid receptors and its regulators [7]. Expression levels of *FKBP5*, a potent negative regulator of glucocorticoid signalling, is part of this cellular identity and is itself a target of glucocorticoid-mediated gene transcription [8]. Single nucleotide polymorphisms (SNPs) inside the human *FKBP5* gene are associated with differential induction of the FKBP51 protein upon glucocorticoid stimulation [9] and add to the variability of stress perception and response in the population [10]. Carriers of the high induction allele rs1360780-A/T of *FKBP5* who suffered from ELA are more prone to develop psychiatric symptoms in later life than individuals without such preconditioning [11]. Importantly, sex-dependent differences in the interaction of *FKBP5* and life adversities have been associated to a higher prevalence of depression in females [12]. Despite the strong negative impact of psychiatric disorders on quality of life and productivity, the underlying processes linking *FKBP5* genotypes, stress regulation and pathological transitions are not fully understood. Animal models offer a possibility to investigate gene × environment interactions in a timely resolved manner. In depth analyses of laboratory mouse sequences in-house indicated numerous *Fkbp5* SNPs that vary by strain. However, no SNPs at the same location or with the same functional impact as found in humans occur naturally in rodents. This lack of an animal model suited to exploring human *FKBP5* SNPs hinders elucidation of causal relationships and mechanisms underlying disease development and progression. Therefore, we previously generated *Fkbp5*-humanised mice carrying either the risk-associated high induction AT-allele of rs1360780 or the resiliency-associated CG-allele. Initial characterisation of primary CNS-cell types derived from these mice revealed that the presence of the AT-allele results in the increased expression of *Fkbp5* upon stimulation of the glucocorticoid receptor compared to the CG-allele [7]. This initial characterisation prompted us to exploit this new model to examine the *Fkbp5* × ELA interactions on the stress response system in adulthood. We exposed AT- and CG-allele carrying mice to prolonged maternal separation stress, since this paradigm is broadly used to mimic ELA in rodents [13]. When mice reached adulthood, the performance of the HPA axis and behavioural response of *Fkbp5*-humanised mice to mild stressors were measured. Furthermore, we investigated the transcriptomic profiles in several brain regions engaged in stress

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processing. Lastly, astrocytes and neurons derived from human induced pluripotent stem cells (hiPSCs) were analysed for SNP-based differences in their expression profiles.

The goals of the study were to validate the *Fkbp5* × ELA model by (1) determining whether ELA would cause alterations in the offspring's adult behaviour and physiology compared to controls, (2) determining whether risk AT-allele carriers would respond differently to ELA than CG-allele carriers, (3) assessing which pathways are involved in the adaptation to ELA in context of risk and resilience associated SNPs. A more far-reaching aim was to demonstrate that the humanised *Fkpb5* × ELA mouse model can be used to further investigate the influence of the human *FKBP5* gene variants on the risk and resilience to stress and to further elucidate their contribution to psychiatric disorders.

RESULTS

Prolonged separation from mothers and peers was performed for the first three weeks of postnatal life to model ELA. In parallel, control mice were housed with littermates and received undisturbed maternal care until weaning. An overview of the group sizes of the cohort is provided in Table 1. On postnatal day 21, pups were weaned and grown to adulthood with physiological and behavioural examination starting at 10 weeks of age (Fig. 1). Exploration of novel environments offers an easily accessible measure of mild stress in rodents [14]. Therefore, we challenged control and ELA-exposed mice with novel situations to probe for their stress coping strategies. The same procedures were simultaneously carried out in wild type mice of both sexes to control effectiveness of the manipulations. Since the focus of this study is on the differences between the human SNPs and how these interact with ELA, the data on wild type HPA axis functioning and behaviour are visualised in Supplementary Figs. 1–5. Statistical analyses were performed jointly for males and females of all three lines to address differences between sex, ELA exposure, *Fkbp5*-genotypes and the interactions thereof. Details of the descriptive analyses, model summaries and analysis of variance (ANOVA) results are provided in Supplementary Table 1–40. Only the significant findings are indicated in the following paragraphs. A significant effect of sex × genotype × treatment interaction and significant two-way interactions in the vast majority of measured parameters were detected and are detailed in the following paragraphs.

Table 1. Overview of the Study Cohort.

Genotype	Early Life	Sex	*N*	Litters
CG	Control	Male	7	2
CG	Control	Female	7	3
CG	ELA	Male	11	3
CG	ELA	Female	11	3
AT	Control	Male	7	4
AT	Control	Female	5	4
AT	ELA	Male	5	2
AT	ELA	Female	10	3
WT	Control	Male	15	6
WT	Control	Female	20	5
WT	ELA	Male	17	5
WT	ELA	Female	17	6

Early life adversity and *Fkbp5*-genotype shift and attenuate diurnal HPA axis rhythmicity

To measure the impact of *Fkbp5* SNPs in combination with ELA on the diurnal performance of the HPA axis, the plasma corticosterone concentration of samples collected at three time points was assessed. As confirmed in the wild type mice (Supplementary Fig. 1, Supplementary Tables 1–3), these timepoints were reflecting the diurnal nadir (morning), peak (evening) and one intermediate state (noon).

In control females carrying the CG-allele, the expected increase of plasma corticosterone over the course of the day was observed, with a clear peak towards the evening (Fig. 2a). Following ELA exposure, the highest concentration was instead measured at noon. The increase of plasma corticosterone levels in AT-allele carrying control females was not statistically significant, regardless of ELA exposure. In addition, the morning corticosterone levels in AT- vs. CG-allele carrying females were higher, suggesting that the levels did not fully decrease to low levels for the murine resting phase. In *Fkbp5*-humanised males, the diurnal plasma corticosterone concentration peaked towards noon with CG- vs. AT-allele carriers showing a decrease towards the evening, regardless of ELA exposure (Fig. 2c). Given that the shift in corticosterone peak was present in males of both humanised lines, this effect is likely a

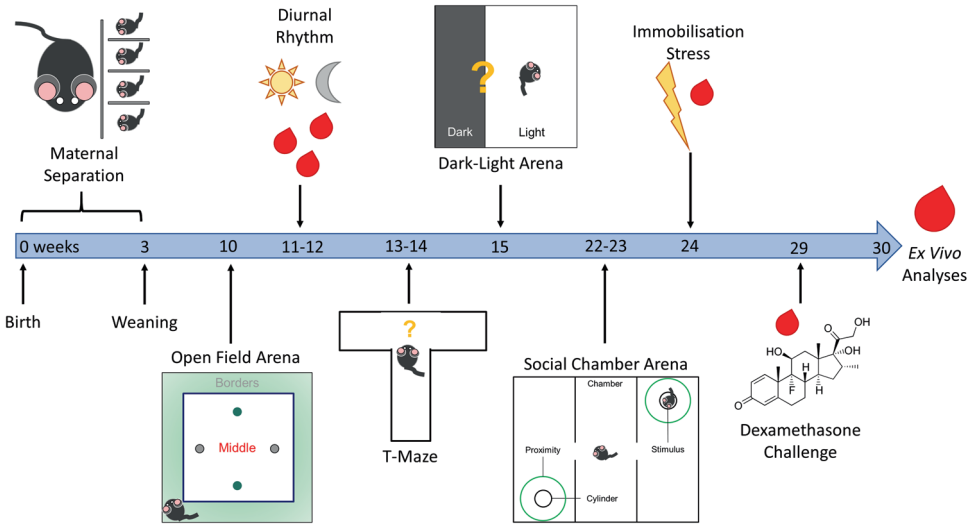

Fig. 1 Timeline of experiments. Study overview of in vivo and ex vivo experiments during the lifetime of *Fkbp5*-humanised mice with ELA. The same timeline, except for the maternal separation, was applied to control mice in parallel.

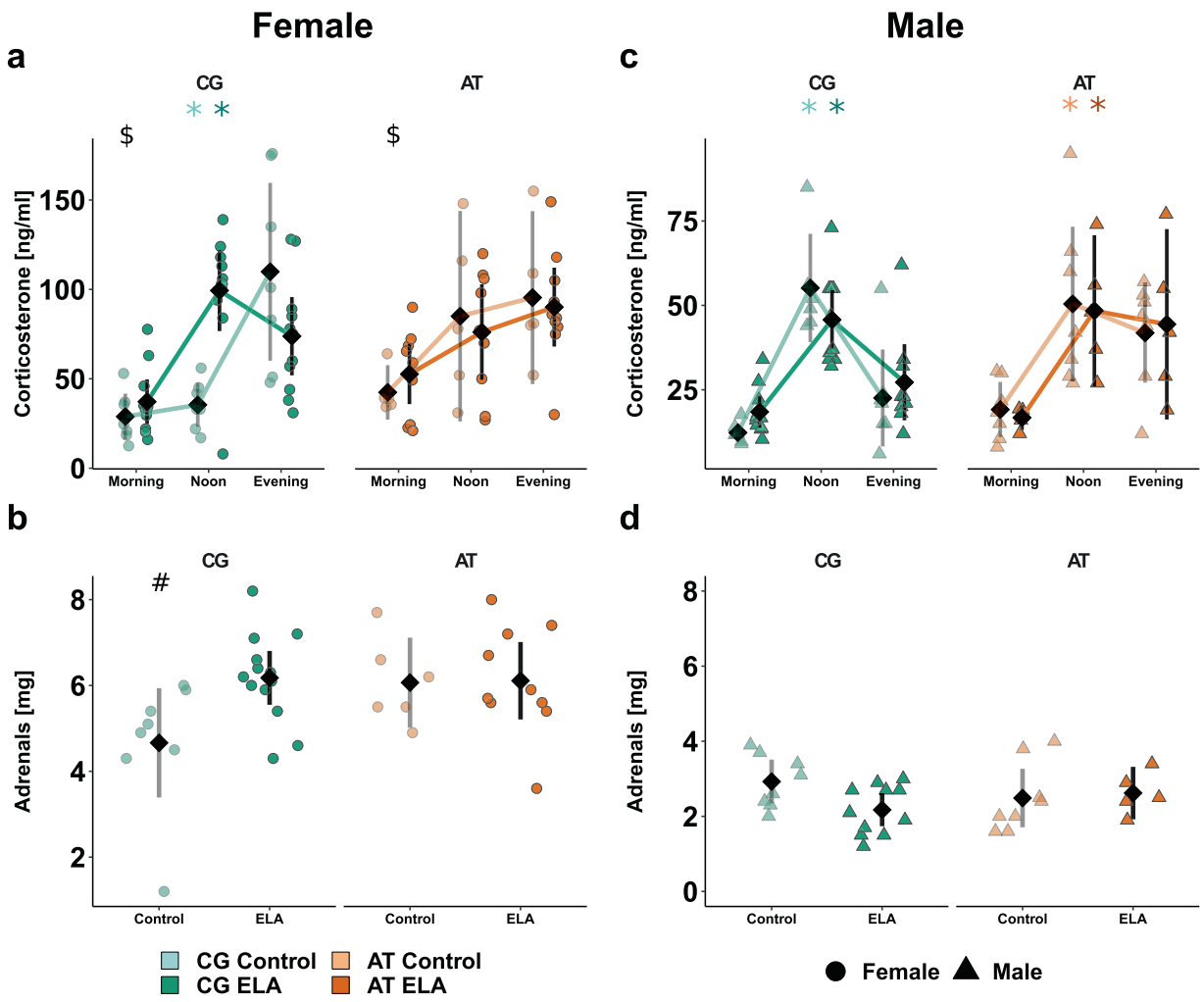

Fig. 2 ***Fkbp5*-genotype × ELA influence the unstimulated HPA axis activity in a sex-dependent manner.** Individual animal data is shown alongside with the mean (black diamond) ± 95% confidence intervals to indicate statistical differences among the subgroups. Selected results of the ANOVA at group level are indicated. All descriptive statistics, model summaries, and ANOVA results are provided in Supplementary Table 1–6. Diurnal rhythmicity of corticosterone plasma levels in female (**a**) and male (**c**) *Fkbp5*-humanised controls or ELA-exposed mice. A different scale for males than females was used to make the pattern better visible. Significant diurnal rhythm was seen in CG-allele carrying female and male controls (*) and ELA-exposed mice (*), as well as in AT-allele carrying control (*) and ELA-exposed males (*). Morning corticosterone was higher in AT- vs. CG-allele carrying females ($p = 0.03$, $). Comparison of adrenal weights in females (**b**) and males (**d**). Female CG-allele carrying controls differ from most other subgroups (SNP × ELA × sex $p = 0.04$, #).

feature of the human gene and not of the transgenic modification. The detected diurnal amplitude of corticosterone was smaller in males than females. The adrenal weight in female AT-allele carriers and CG-allele carrying females after ELA compared to CG-allele carrying controls was increased (Fig. 2b).

No significant differences in the adrenal weights were observed among males (Fig. 2d), but male vs. female adrenal weights were significantly lower.

Taken together, female AT- vs. CG-allele carriers are genetically predisposed to less pronounced diurnal HPA axis rhythmicity and elevated corticosterone levels at time points when mice usually would rest. Lower diurnal corticosterone amplitudes and adrenal weights in males vs. females suggest a different corticosterone secretion capacity between sexes.

Early life adversity increases responsiveness to novel environments dependent on *Fkbp5* genotype and sex

Exposure to novel environments as mild stress was applied to determine natural behaviour and coping strategies. First, behaviour in open field test arenas was assessed to obtain a measure of locomotor activity before (17:00–18:30), throughout (18:30–05:30) and after (05:30–06:00) the murine active phase. Overall activity within the first 15 minutes, including running and rearing, was assessed by measuring the frequency of crossing light beams (Fig. 3a). During this period, the activity decreased over time with early life condition and sex showing an interaction with time. As in wild type females (Supplementary Fig. 3, Supplementary Tables 11–16), CG control females displayed habituation in the shape of a strong decrease in activity, while the exposure to ELA led to flattening of the 15 minutes activity profile and thus slower habituation (Fig. 3a). AT-allele carrying females tended to decrease their activity less than CG-controls, regardless of early life condition. Analyses of the total nocturnal distance revealed main effects of sex and early life condition, as well as an interaction effect of ELA × genotype (Fig. 3c). While ELA-exposed female CG-allele carriers were more active than controls, AT-allele carrying females with ELA experience were indistinguishable from controls. In males, the activity measured in the open field arena (Fig. 3b, e) were similar among groups.

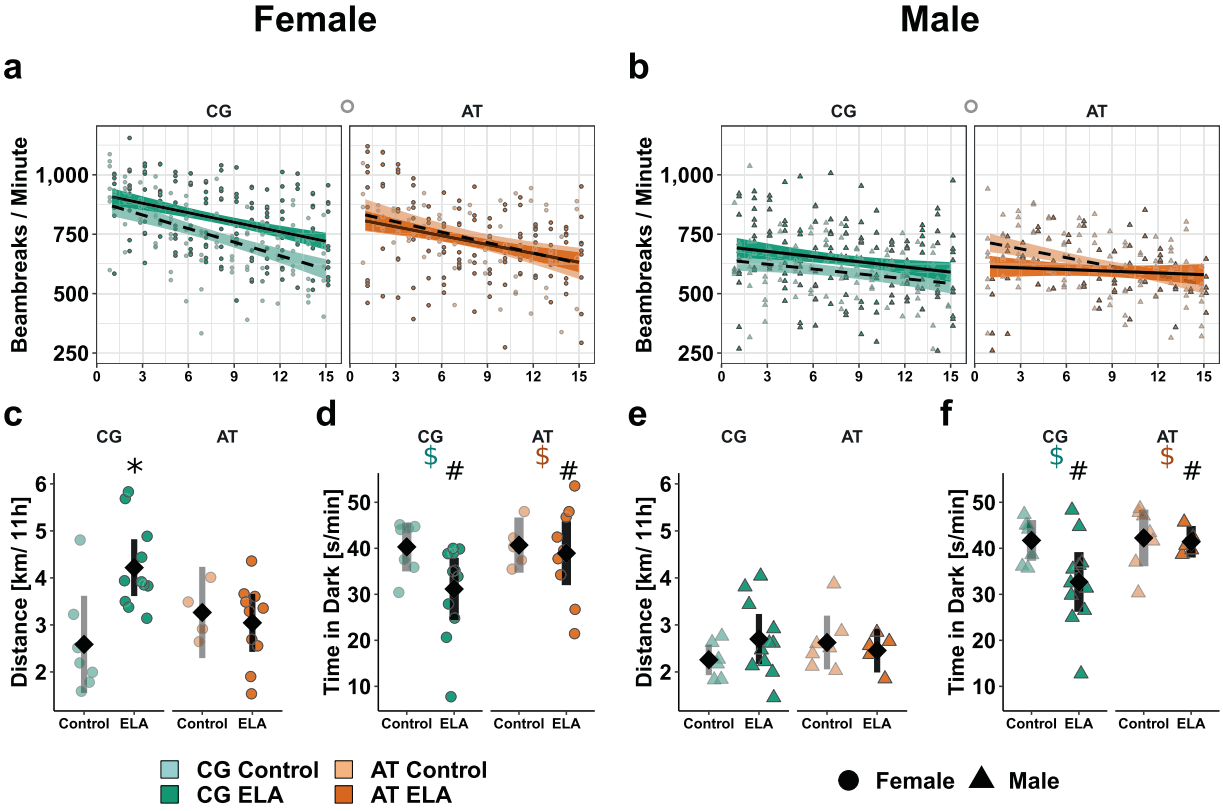

Fig. 3 Sex × *Fkbp5*-genotype × ELA interactions alter activity in humanised mice. Individual data is shown alongside with the mean ± 95% confidence intervals to indicate statistical differences among subgroups. Selected results of the ANOVA at group level are indicated. Descriptive statistics, model summary, and ANOVA results are provided in the Supplementary Tables 11-19. Exploration activity (light beams crossing / minute) during the first 15 minutes in a novel environment in females (**a**) and males (**b**). The decrease in activity was lower in ELA-exposed than control mice ($p < 0.05$) and less in males than females ($p = 1^{-5}$) given lower initial activity. AT- vs. CG-allele carrying controls tended to remain more active (°, $p = 0.07$). Total distance [km] females (**c**) and males (**e**) moved during the night. The CG-allele and ELA showed significant interaction ($p = 0.01$) that was most visible in females (∗), since females were more active than males ($p < 1^{-5}$). Average time [s/min] females (**d**) and males (**f**) spent in the dark compartment. ELA-exposed mice were less in the dark than controls (#, $p < 1^{-5}$), with the CG- vs. AT-allele tending to decrease the time in the dark ($, $p = 0.08$).

In the spontaneous alternations T-maze, ELA did not affect the fraction of alternations between left or right side of the maze, irrespective of genotype or sex (Supplementary Fig. 4, Supplementary Tables 20–25), suggesting no impact on working memory performance. However, ELA-exposed mice performed the task significantly faster than the respective control group. Females were quicker than males.

In the dark-light test, ELA decreased the mean time spent in the dark compartment (Fig. 3d, f). This was rather the case in CG- than AT-allele carriers but not strong enough to be detected as ELA × genotype interaction. Instead, a trend for *Fkbp5*-genotype related effects was detected, with CG- vs. AT-allele carriers spending less time in the dark.

Finally, we measured social preference in the social chamber test in *Fkbp5*-humanised (Fig. 4) and wild type mice (Supplementary Fig. 5, Supplementary Tables 26–31). Pairwise comparisons of compartment effect separated by early life conditions, genotype, and sex revealed significant differences. CG-allele control females showed social preference, measured by the time the mouse spent in the nearest vicinity of the cylinder with the social stimulus (Fig. 4a). The exposure to ELA led to a decrease of this parameter, while simultaneously we observed a significant increase in the time spent in 5 cm distance from the social stimulus (Fig. 4c). Matching with the time spent, CG-allele carrying females that experienced ELA moved more distance in the area surrounding the unfamiliar mouse than controls (Supplementary Fig. 6, Supplementary Tables 32–37). Moreover, they moved faster than controls on the social, but not on the reference side. AT vs. CG-allele carrying control females spent less time interacting with the unfamiliar mouse. With ELA, AT-allele carrying females did not show social preference. The time in 'social distance' was similar in the AT-allele carrying controls and ELA-exposed females and indicated no preference for the social side. In contrast to females, the social preference was not affected by ELA in male CG-allele carriers. In AT-allele carrying males with ELA vs. controls, less time was spent with or close by the social stimulus (Fig. 4b, d).

Overall, the data on behavioural responses to mild stress suggest that the effects of ELA on these read outs depend on the genetic variants of *Fkbp5* × sex.

HPA axis responses are stronger in females than males

To probe the HPA axis reactivity to acute induction and negative feedback, we measured plasma corticosterone after five minutes of restraint stress and six hours after a single intraperitoneal injection of the synthetic glucocorticoid dexamethasone, respectively. In all mice, corticosterone increased in response to restraint stress without a differential effect of genotype. The slope was steeper in females (Fig. 5a) compared to males (Fig. 5b). Overall corticosterone levels were higher in ELA-exposed mice than controls and higher in females than males. Similarly, all mice responded to dexamethasone with reduced corticosterone levels, suggesting a suppression of the endogenous corticosterone secretion. *Post hoc* analyses revealed that the slope of decrease was overall steeper in females than males (Fig. 5c, d).

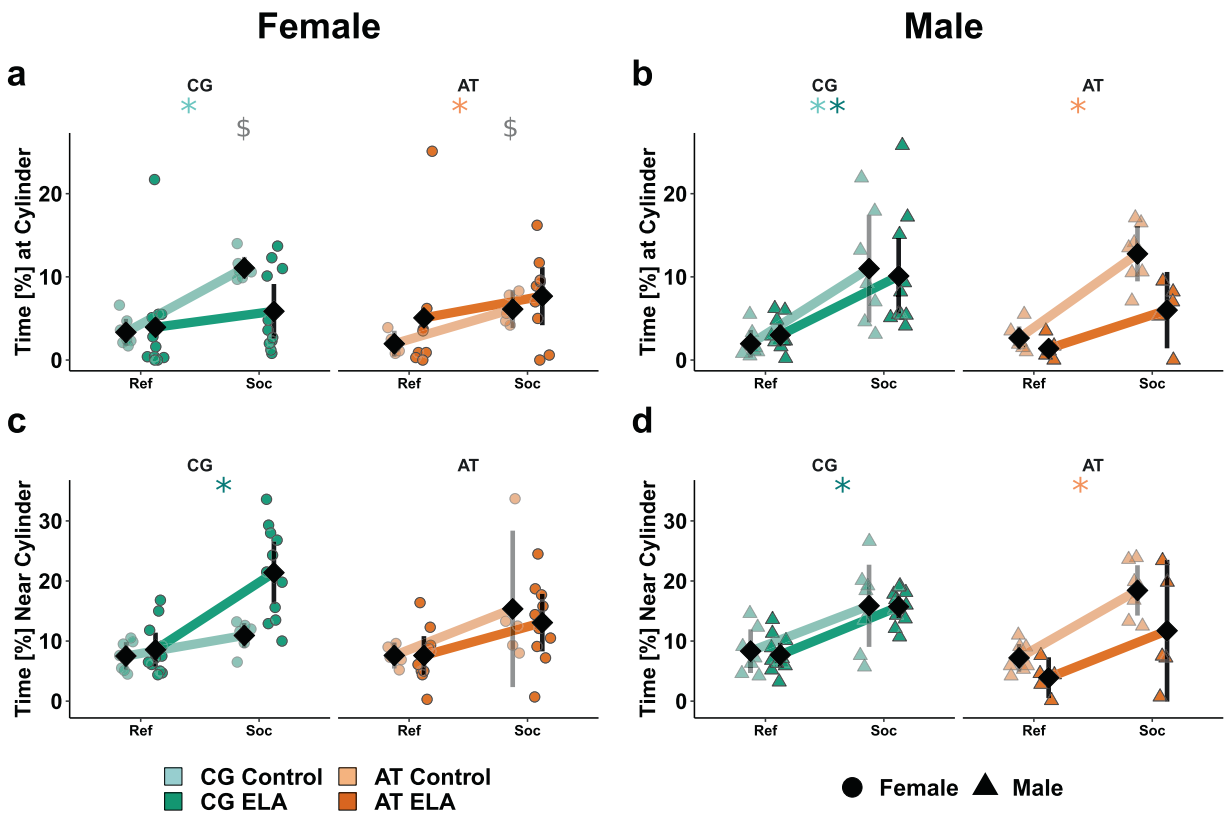

Fig. 4 Sex × *Fkbp5*-genotype × ELA interactions alter social behaviour. Individual data is shown alongside with the mean ± 95% confidence intervals to indicate statistical differences among subgroups. Selected results at group level are indicated. Descriptive statistics, model summary, and ANOVA results are provided in the Supplementary Tables 26–31. Time [%] females (**a**) and males (**b**) spent at the cylinder with (Soc) or without (Ref) an unfamiliar mouse. Significant social preference is indicated in CG-allele carrying controls (∗), ELA-exposed males (∗), and in AT-allele carrying controls (∗). The preference for the social compartment ($p < 1^{-5}$) was more pronounced in males ($p = 0.01$) since only AT-allele carrying males with ELA lost the preference, while both AT- and CG-allele carrying females with ELA discriminated less between the social and reference side (ELA × SNP × sex $p = 0.02$). AT- vs. CG-allele carrying female controls spent less time in social interaction ($, $t(7) = 5$, $p = 0.001$). Time [%] females (**c**) and males (**d**) spent in the area surrounding the cylinder with or without a stimulus mouse. An overall preference for the social side was present ($p < 1^{-5}$) that was seen in CG-allele carriers with ELA (∗) and AT-allele carrying male controls (∗). AT- vs. CG-allele carriers ($p = 0.01$) and males vs. females ($p = 0.04$) with ELA spent less time on the social side. Both effects are attributable to CG-allele carrying females with ELA spending more time on the social side.

In summary, the responsiveness of the HPA axis is preserved in *Fkbp5*-humanised mice.

Transcription in stress-responsive brain regions is affected by *Fkbp5* × ELA

To identify transcriptional differences that could be related to differences in behaviour and HPA axis physiology of *Fkbp5*-humanised mice × ELA, mRNA sequencing and analyses of differential gene expression were carried out. Given the behavioural and physiological findings that female AT- vs. CG-allele carriers differ, while little to no effects were seen in males, next generation sequencing was limited to *Fkbp5*-humanised females to identify potential transcriptomic correlates of the differences in vivo. The analyses focused on hypothalamus, ventral and dorsal hippocampus as brain regions engaged in stress regulation [15]. In the SNP-comparison among controls, more differentially expressed genes (DEGs) were found in the hypothalamus (579), followed by ventral (41) and dorsal (2) hippocampus (Table 2). Among ELA-exposed individuals, more DEGs between the SNPs were detected than in controls, underscoring the interaction of ELA × *Fkbp5*-genotype. Looking at the effect of ELA, fewer differences were detected in AT-allele carriers (114) than in CG-allele (903) carriers. This matches to the behaviour and HPA axis data, where few additional impact of ELA to the differences introduced by the AT-allele were seen.

Adopting knowledge from the SNP effects in humans, the overlap and uniqueness of the identified DEGs were analysed for nomination of potential resiliency- or vulnerability-related genes. Genes linked to CNS-development such as *Mab21l2*, *Gart* and *Lipt2* were spotted as potentially vulnerability-related and were changed in opposite directions, with AT- vs. CG-allele carriers displaying a lower expression.

A second analysis focussing on gene clusters related to neurological disorders using a two-step core and comparison analysis of the commercial software Ingenuity (Qiagen) confirmed that the ELA-responsive DEGs in both mouse lines have an impact on neurological and psychiatric symptoms (Supplementary Fig. 7). In eight of the shown 30 deregulated clusters e.g., comprising 'congenital neurological disorder' or 'learning', the effects were opposite between AT- vs. CG-allele carriers.

In sum, the counts of DEGs and their accordant vs. discordant overlap suggest that the *Fkbp5* × ELA interaction on gene expression may have relevance for neurologic and psychiatric symptomatology.

The AT-allele and ELA reduce CNS communication but increase metabolism

To identify how the DEGs might be linked to disorders via their role in cellular pathways, their over-representation in metabolism and signalling-related pathways listed in the Kyoto Encyclopaedia

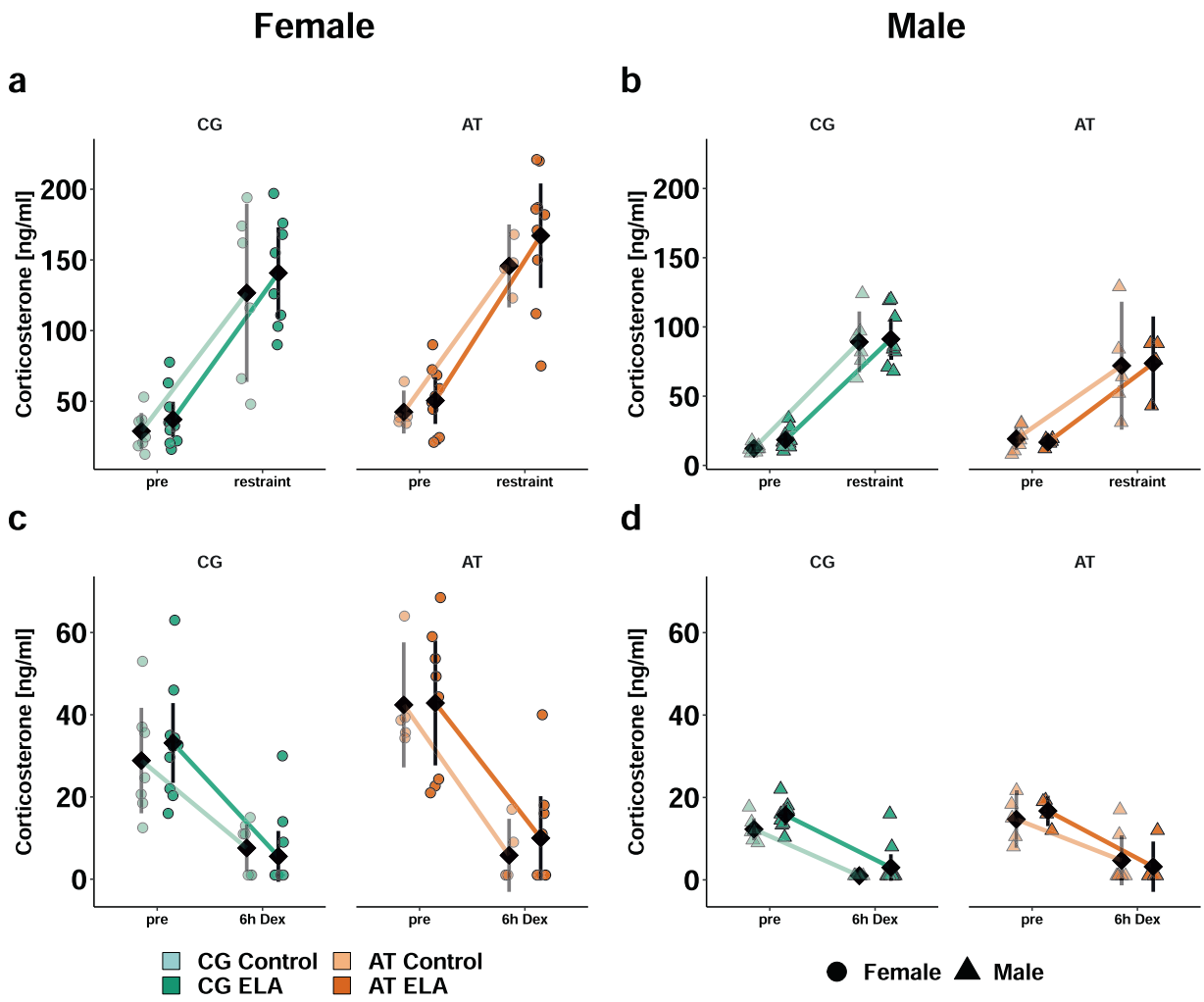

Fig. 5 Stimulated HPA axis reactivity in *Fkbp5*-humanised females is greater than in males. Individual animal data are shown alongside with the mean ± 95% confidence intervals (black) to indicate statistical differences among subgroups. Selected results at group level are indicated. Descriptive statistics, model summaries, and ANOVA results are provided in the Supplementary Tables 1 and 7-10. Acute responsiveness of the HPA axis assessed by comparison of plasma corticosterone levels before and five minutes after restraint stress in females (**a**) and males (**b**). Stress induced an increase in corticosterone ($p < 1^{-5}$) with females responding more than males ($p < 1^{-5}$). Overall, females vs. males ($p < 1^{-5}$) and mice with ELA vs. controls had higher corticosterone levels ($p = 0.04$). Suppression of endogenous corticosterone production six hours after dexamethasone injection ($p < 1^{-5}$) was more pronounced in females (**c**) than males (**d**, $p < 1^{-5}$).

of Genes and Genomes (KEGG) was assessed. The analyses revealed significantly altered pathways in the hypothalamus and ventral hippocampus (Table 3). The direction of change between *Fkbp5*-genotypes differed dependent on function, with pathways related to neuronal communication rather showing a down-regulation, and pathways related to metabolism rather showing an upregulation in AT- vs. CG-allele carriers. In the hypothalamus, the most significantly downregulated pathways included circadian entrainment, regulation of synaptic plasticity via long-term potentiation and depression as well as activity of dopaminergic and cholinergic synapses together with changes in calcium, cAMP, and oxytocin signalling. In the ventral hippocampus, reduced expression of genes related to synaptic communication in AT- vs. CG-allele carriers was repeated. Especially in the ELA subgroup, lower expression of genes related to cAMP signalling and dopaminergic synapses were found in AT-allele carriers compared to CG-allele carriers. Independent of strain, ELA was linked to lower expression of transcripts related to endocannabinoid and circadian entrainment relative to controls. For genes in pathways related to metabolism, such as 'protein digestion and absorption' in the hypothalamus or 'ribosome' activity and 'oxidative phosphorylation' in the ventral hippocampus of controls, higher expression in AT- vs. CG-allele carriers was observed.

The mRNA of neurons and astrocytes derived from hiPSCs of rs1360780 carriers was used to qualitatively validate the SNP-dependence of the observed differences in an independent expression system. In both cell types, similar SNP-based expression differences like in the *Fkbp5*-humanised mice were seen, which could indicate that less synaptic communication in AT- vs. CG-allele carriers is not an artefact from the process of generating the transgenic mice. However, the distribution within the pathways differed between hiPSC and mouse derived samples. More DEGs in the upstream vs. downstream members of the circadian entrainment pathway were seen in the *Fkbp5*-humanised mice, while in the hiPSCs rather downstream targets were changed (Supplementary Fig. 8). The expression patterns in astrocytes vs. neurons were more similar to the patterns seen in mice.

The KEGG pathway analyses imply that ELA and the AT-allele both lead to less entrainment of diurnal HPA axis and sleep-wake rhythmicity. This may interact with the decreased ability of AT- vs. CG-allele carriers to process incoming inputs via synaptic communication.

Table 2. Counts of differentially expressed genes in subgroups of *Fkbp5*-humanised female mice.

Comparison	Tissue	Direction	Control	ELA
Fkbp5-genotype	Hypothalamus	AT > CG	349	561
	Hypothalamus	CG > AT	230	855
	Ventral Hippocampus	AT > CG	18	468
	Ventral Hippocampus	CG > AT	23	457
	Dorsal Hippocampus	AT > CG	1	798
	Dorsal Hippocampus	CG > AT	1	844
Comparison	**Tissue**	**Direction**	**CG**	**AT**
Early life condition	Hypothalamus	ELA > Con	410	4
	Hypothalamus	Con > ELA	195	29
	Ventral Hippocampus	ELA > Con	16	0
	Ventral Hippocampus	Con > ELA	10	0
	Dorsal Hippocampus	ELA > Con	145	27
	Dorsal Hippocampus	Con > ELA	127	54

Table 3. Enriched KEGG pathways in *Fkbp5*-humanised females.

Tissue	Group	Comparison	KEGG Pathway	*p*	Mean
Hypothalamus	Overall	AT vs. CG	Dopaminergic synapse	0.002	−0.012
			Circadian entrainment	0.003	−0.943
			ECM-receptor interaction	0.007	2.702
			Oxytocin signalling pathway	0.020	−0.115
			Long-term potentiation	0.022	−0.189
			Ras signalling pathway	0.025	−0.028
			Protein digestion & absorption	0.029	2.051
			Cholinergic synapse	0.030	−0.958
			Long-term depression	0.037	−0.893
			Calcium signalling pathway	0.041	−0.779
			cAMP signalling pathway	0.049	1.689
Ventral Hippocampus	overall	AT vs. CG	Ribosome	0.007	2.496
			Phosphatidylinositol signalling	0.012	−0.470
			Inositol phosphate metabolism	0.014	−0.418
			cAMP signalling pathway	0.024	−0.043
			Oxytocin signalling pathway	0.042	−0.804
			Aldosterone synthesis & secretion	0.043	−0.818
	Controls	AT vs. CG	Ribosome	0.014	2.252
			Oxidative phosphorylation	0.030	1.950
	ELA	AT vs. CG	Dopaminergic synapse cAMP	0.042	−0.794
			signalling pathway	0.043	−0.781
	overall	ELA vs. Con	Dopaminergic synapse	0.019	−0.218
			Circadian entrainment	0.021	−0.173
			Endocannabinoid signalling	0.032	−0.912

The DEGs detected in the dorsal hippocampus were not significantly overrepresented in individual KEGG pathways.
p = *p*-value of geometric mean, *Mean* = mean difference of fold changes, *ECM* = extracellular matrix, *Ras* = rat sarcoma, *cAMP* = adenosine 3′,5′-cyclic monophosphate, *AT* = *Fkbp5* rs1360780-A/T (high induction) allele, *CG* = *Fkbp5* rs1360780-C/G (resilience) allele.

Lower glucocorticoid sensitivity of the Hippocampus is modulated by *Fkbp5*

To estimate how much impact the potentially altered glucocorticoid exposure due to differences in circadian entrainment and synaptic signalling might exert on the hypothalamus, ventral hippocampus, and dorsal hippocampus, the expression levels of genes related to glucocorticoid signalling were compared (Fig. 6). This analysis provides insights in the likelihood of the brain regions to respond to glucocorticoid stimulation. While expression levels of the glucocorticoid receptor (*Nr3c1*) and heat shock protein 90 (*Hsp90ab1*) were comparable between all three brain regions, the mineralocorticoid receptor (*Nr3c2*) and *Fkbp5* were less expressed in hypothalamus than hippocampus. Moreover, the AT- vs. GC-allele was associated with a lower *Fkbp5* expression in the hippocampus and less *Hsp90ab1* in all three brain regions.

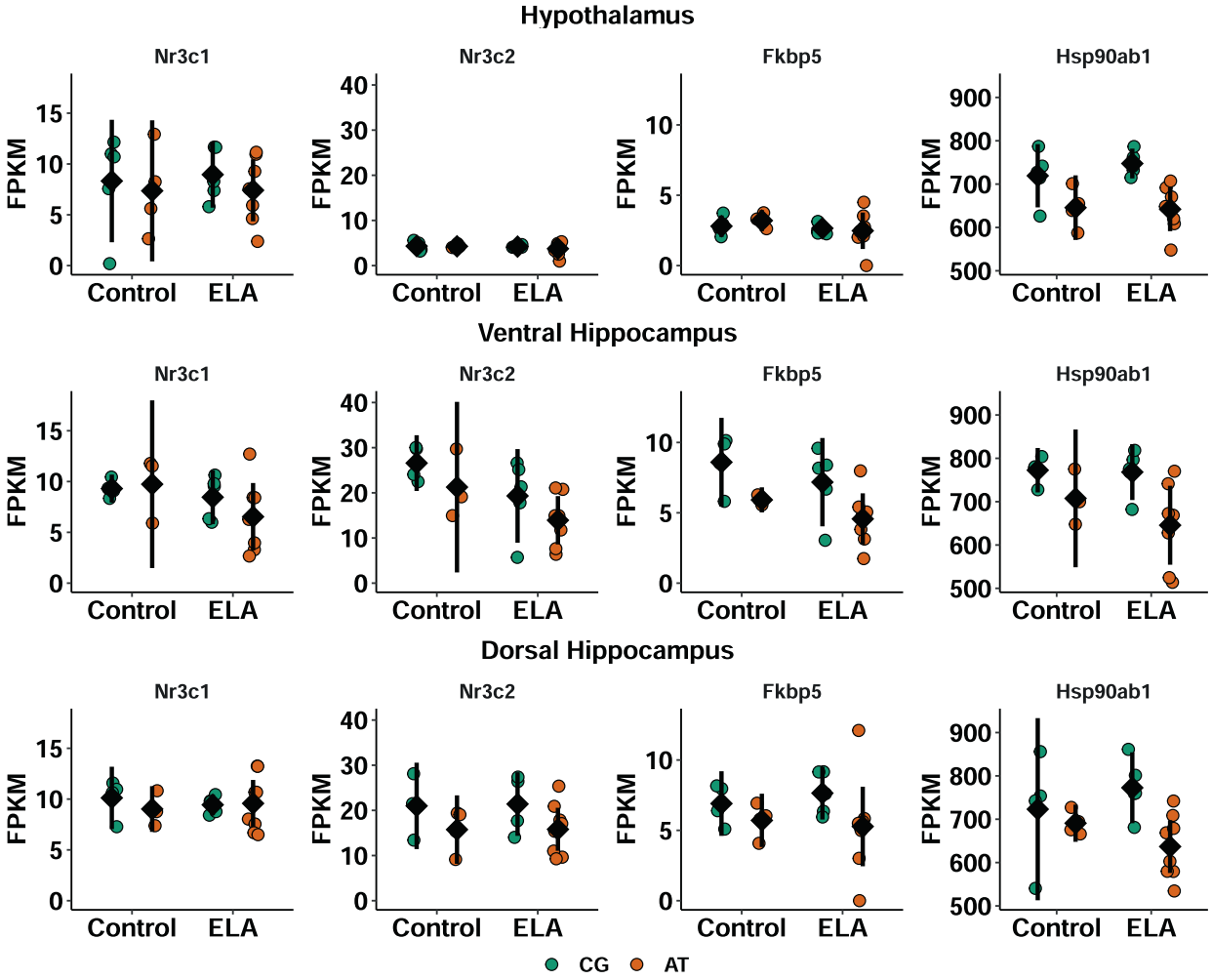

Fig. 6 Brain region specific expression levels of glucocorticoid signalling regulators. Expression levels [FPKM] of the glucocorticoid receptor (*Nr3c1*, GR), mineralocorticoid receptor (*Nr3c2*), *Fkbp5* and heat shock protein 90 (*Hsp90ab1*) of individual female AT- or CG-allele carriers that experienced ELA or undisturbed maternal care (control) are visualised alongside with the mean ± 95% confidence intervals. Plots are shown separate for hypothalamus (**top**), ventral hippocampus (**middle**) and dorsal hippocampus (**bottom**). Descriptive statistics and an overview of the significant model terms in the ANOVA are provided in the Supplementary Tables 38 and 39. In all three regions, AT- vs. CG-allele carriers expressed less *Hsp90* (hypothalamus $p = 0.0006$, ventral hippocampus $p = 0.009$, dorsal hippocampus $p = 0.009$). *Fkbp5* was lower expressed in the hippocampi of AT- vs. CG-allele carriers ($p = 0.01$) and *Nr3c2* was lower expressed in the ventral hippocampus of ELA-exposed vs. control mice ($p = 0.03$) as well as lower expressed in the dorsal hippocampus of AT- vs. CG-allele carriers ($p = 0.03$).

Considering the gene functions, the hypothalamus appears to be more sensitive to glucocorticoid receptor mediated signalling than the hippocampus, with CG- vs. AT-allele hippocampi being more protected.

DEGs in *Fkbp5*-humanised mice are related to differences in vivo

The decreased cerebral expression of genes related to synaptic communication in AT- vs. CG-allele carriers might be a compensatory mechanism to prevent excessive excitation. To test whether the expression levels of the identified DEGs and *Fkbp5* could be linked to the observed behavioural and physiological differences, tissue-wise correlation analyses were carried out. For each brain region, the top 10 correlations are provided in the supplements (Supplementary Fig. 9, Supplementary Table 40, the full list of correlations will be provided upon request). In all three brain regions, the majority of DEGs correlated with *Fkbp5*. In the hypothalamus, gap junction protein β 1 (*Gjb1*) showed a correlation with the time spent in the dark compartment of the test arena, while the membrane-associated tyrosine-specific kinase 1 (*Pkmyt1*) and the nicotinic acetylcholine receptor subunit 7 (*Chrna7*, regression shown in Supplementary Fig. 10) were linked to morning corticosterone levels. This could indicate an association between some hypothalamic DEGs and differences in HPA axis functioning and behaviour. The limitation of the transcriptomic analyses to females was useful in identifying physiological correlates of the transcriptome.

The correlation analyses suggest a linkage between expression levels of *Fkbp5* and DEGs in brain regions relevant for stress processing.

DISCUSSION

The present study has demonstrated a gene × environment interaction in novel *Fkbp5*-humanised mice, indicating that the model is suited to investigate the effects of ELA in the context of risk- and resiliency-related SNPs. Early life adversity elicited by maternal separation has differential impact on adult physiology and behaviour based on genetic predisposition imparted by *Fkbp5* alleles. This is demonstrated by changes in locomotor, social, and anxious behaviour. Additionally, diurnal corticosterone rhythmicity is moderately altered as seen at a functional level via HPA axis

profiling and on molecular levels through altered gene expression in the circadian entrainment pathway. Differential gene expression in brain regions relevant to stress regulation shows an enrichment for pathways linked to neural communication and brain disorders. Many of the DEGs are correlated with *Fkbp5* levels. In the tests utilised here, the impact of *Fkbp5* SNPs and ELA was greater in females than males.

These stronger effects of *Fkbp5* × ELA in female compared to male mice match previously reported sexual dimorphism in responsiveness to ELA in animals [16] and was discussed in humans [17]. Notably, ELA and sex hormones both influence maturation kinetics and thus the development of cerebral regions implicated in glucocorticoid regulation [18]. The interactions of the SNP rs1360780, sex, and ELA observed in the mice presented here and in humans [11, 12] could thus be explained by the regulatory capacity of *Fkbp5* on glucocorticoid signalling. Based on sex-dependent correlations between *FKBP5* levels and depression and anxiety scores as well as with nadir cortisol levels, *FKBP5* was suggested as a female-specific biomarker for prolonged cortisol load and the associated risk of psychiatric disorders [19]. In line with this correlation, we observed associations between genotype and nadir corticosterone levels in *Fkbp5*-humanised mice, with AT-allele carrying females displaying higher morning glucocorticoid levels than CG-allele carrying females. The sexual dimorphism in the effect of ELA indicate that the novel *Fkbp5*-humanised mouse model offers the possibility to further investigate the networking of ELA, sex, and disease-related SNPs.

In addition, the data provide mechanistic insights into how *Fkbp5* SNPs may contribute to the shaping of overall physiology and the stress response system. As negative modulator of glucocorticoid receptor maturation, *Fkbp5* holds the potential to inhibit glucocorticoid signalling. At the same time, its expression depends on recent glucocorticoid exposure since *Fkbp5* itself harbours glucocorticoid response elements [8]. The higher induction of the AT-allele in CNS cell types of *Fkbp5*-humanised mice upon glucocorticoid stimulation could thus be expected to result in stronger or longer inhibition of subsequent glucocorticoid signalling [7]. In vivo, this stronger induction of inhibitory potential via *Fkbp5* in AT-allele carriers could lead to dampened negative feedback to the HPA axis and a prolonged interval of elevated glucocorticoid levels, as reflected by elevated morning corticosterone levels in AT- vs. CG-allele carrying females. The negative feedback loop is furthermore critical for the maintenance of oscillation patterns and function [20]. The reduction in the complexity of ultradian fluctuation and the resulting decreased variability of HPA axis reactivity in AT-allele carriers could decrease their flexibility to respond to novel environments. Behavioural evidence of this differential responsiveness could include the alterations in light-dark box testing, locomotor habituation, and abnormal social behaviour as seen in this study. In humans, differences in HPA axis responsiveness to environmental stimuli, e.g., in the Trier Social Stress Test, between human AT- and CG-allele carriers has been demonstrated [10]. The findings imply that *Fkbp5* genotype dependent regulation of ultradian HPA axis activity might be a core molecular mechanism that contributes to the variability seen in human stress responsiveness, which ultimately plays a role in distinction between healthy adaptation or pathological alteration in the aftermath of stress [21].

Another environmental stimulus that can affect glucocorticoid rhythms is the light-dark cycle [22]. One commonly investigated manifestation of this circadian rhythmicity is the pronounced increase of glucocorticoids prior to awakening [23]. Mechanistically, the ability to detect light in the retinal ganglia and to signal this via the suprachiasmatic nucleus to the periphery is a crucial trigger for the awakening response [24]. In AT- vs. CG-allele carriers, flatter diurnal glucocorticoid profiles were paralleled by lower expression of circadian entrainment related genes even though histological analyses of the eyes (data not shown) indicated no differences in the ability to detect light. This underscores the relevance of self-maintaining feedforward and feedback loops in regulating overall physiology throughout the day. While external light signals can synchronise individuals to a 24 hours cycle [25], the internal gene expression driven clock seems to define the shape of the circadian glucocorticoid profile and thus when and how strong individuals are likely to respond to challenges. In humans, modulation of the cortisol awakening response was reported to influence their performance during the upcoming day and was dependent on the anticipation of challenges [26]. The awakening response is used clinically to identify individuals with certain personality traits that are vulnerable to develop psychiatric disorders [27], and for the diagnosis of depression [28]. Besides impaired awakening responses, differences in kinetic and responsiveness of the HPA axis, e.g. to acute stress or dexamethasone exposure, between psychiatric patients and healthy controls have been demonstrated [29]. In the present study, no disfunction of HPA axis responsiveness was observed, which indicates that the combination of ELA and genetic predisposition via the AT-allele of *Fkbp5* alone might not be sufficient to cause full pathology. This is in agreement with the Research Domain Criteria framework proposing a continuum between'normal' and'pathological' which needs to be better understood in order to alleviate symptoms. Accordingly, the transition to pathology occurs over a lifetime and is a multidimensional process shaped by numerous genetic and environmental factors that introduce subtle changes which jointly alter networking of physiological systems [30]. As in humans, the *Fkbp5*-humanised mouse model demonstrates changes in basal HPA axis activity dependent on genotype and early life experience, with more prominent effects in females than males. These alterations in non-stimulated HPA axis functioning were suggested to have an impact on sleep-wake states, responsiveness to environmental stimuli and vice versa [31]. In the long run, insufficient adaptation could contribute to allostatic load and finally development of disorders [21]. However, the cumulative stress load in this study was low since the animals were not exposed to any severe or chronic stressors during later life.

Nevertheless, the *Fkbp5* × ELA model shows indications of changes in the psycho-immune-neuro-endocrine system that are commonly seen in response to chronic stress. Reduced expression of immediate early genes as markers of plasticity in the prefrontal cortex and hippocampus as well as elevated mitochondrial respiration in response to repeated mild stress during adulthood was previously reported [32]. In the present study, the increased expression of genes related to oxidative phosphorylation in the hippocampus of AT- vs. CG-allele carriers is an interesting parallel, as is the reduction of genes related to synaptic communication. Reduced neural communication and plasticity might become maladaptive since dendritic retraction has been described to render the hippocampus more vulnerable to neurotoxic or metabolic challenges [33, 34]. The longer the time window of decreased plasticity and increased vulnerability exists, the higher is the likelihood of a co-incidental high metabolic demand. Stressful situations only transiently elevate energetic demands while simultaneously decreasing the neuronal supply with glucose [35]. Unique stress events may thus not cause irreversible harm to the hippocampus, and AT-allele carriers might even benefit from their inherent higher expression of mitochondrial genes. Under prolonged exposure to glucocorticoids, increased oxidative phosphorylation in AT-allele carriers might produce excessive amounts of neurotoxic reactive oxygen species which may damage the hippocampus. Findings of this study imply more glucocorticoid signalling in the hippocampus of AT- relative to CG-allele carriers since the glucocorticoid signalling inhibitor *Fkbp5* had a lower expression level while nadir corticosterone levels were

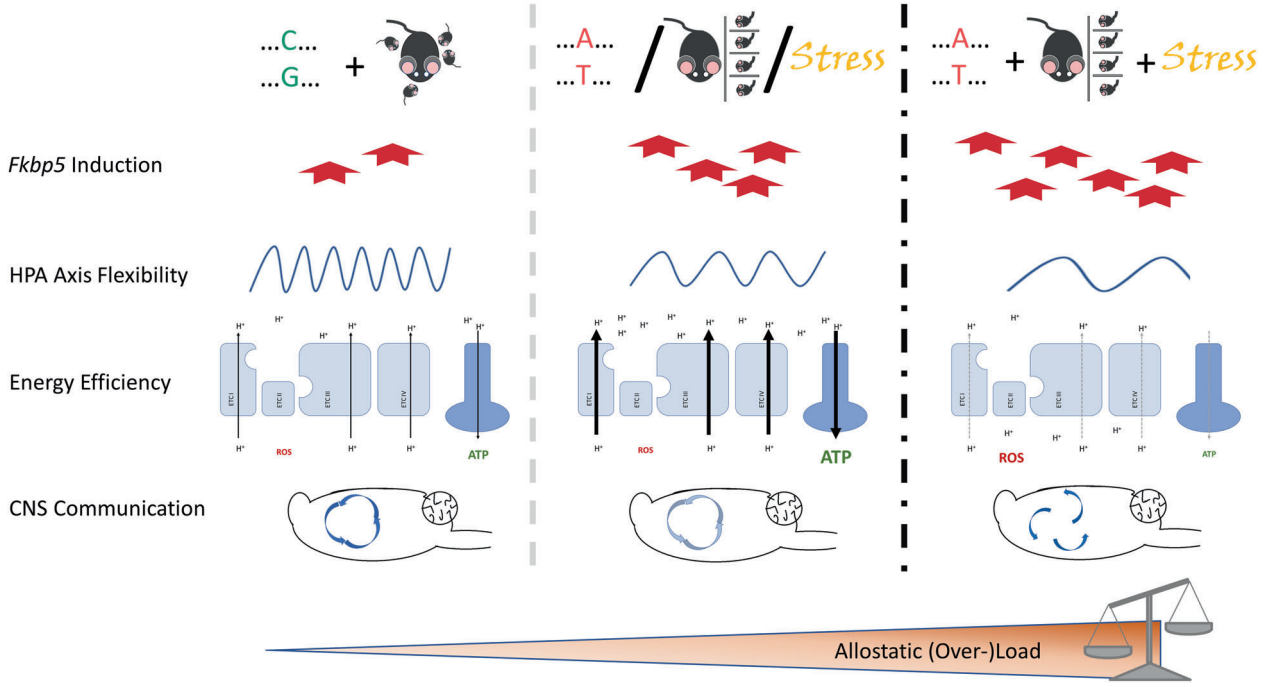

Fig. 7 Proposed sequence of alterations in the stress response system on cellular and brain circuit level in health, allostasis, and allostatic overload. The normal induction of *Fkbp5* upon challenge in CG-allele carriers with undisturbed maternal care allows for dynamic ultradian and circadian rhythms of the HPA axis (**left**). In parallel, the electron transport chain (ETC) in the mitochondrial membrane produces energy in the form of adenosine-triphosphate (ATP) and few reactive oxygen species (ROS), while brain regions involved in stress regulation such as hypothalamus, hippocampus, pre-frontal cortex and amygdala engage in interconnected communication. Carriers of the AT-allele, or individuals exposed to early life adversity or mild chronic stress show signs of allostatic load (**centre**). The affected individuals display a higher induction of *Fkbp5* and an attenuated rhythmicity of the HPA axis. The associated increase in nadir glucocorticoid levels is linked to higher expression of genes related to oxidative phosphorylation, resulting in elevated mitochondrial respiration and ATP production, and to a lower expression of genes involved in synaptic communication. In the proposed triple-hit condition, a further increase in the levels of *Fkbp5* could interfere with the negative feedback to the HPA axis and delay the termination of the stress response (**right**). As consequence of prolonged stress, the ETC might suffer from wear and tear resulting in a decreased efficiency in ATP production combined with elevated ROS generation and oxidative stress. Moreover, the reduced communication between stress-regulating brain regions could manifest in uncoupling of the brain circuits and asynchronous neural signalling. The here described *Fkbp5*-humanised mice will support future work to validate this scenario.

increased in female AT- vs. CG-allele carriers. Cumulatively, this mechanism could contribute to the loss of hippocampal volume in stress-related disorders such as depression and would explain why AT-allele carriers are more prone to develop disorders than CG-allele carriers [36]. The proposed sequence of alterations on cellular and circuitry level from healthy to allostatic load and allostatic overload conditions is outlined in Fig. 7. Assessment of behaviour and physiologic read outs in *Fkbp5*-humanised mice that experienced both, ELA and more severe or chronic stress paradigms, would resolve these questions.

Moreover, the combination of *Fkbp5* SNPs and ELA with simultaneous or sequential stress hits could enable prediction of and intervention at critical transition points during the development and progression of psychiatric symptoms.

CONCLUSION

The cumulative load of genetic predisposition, unfavourable environmental influences during development, and repeated exposure to stressful events increases the prevalence of psychiatric disorders in affected individuals. The glucocorticoid-induced expression of *Fkbp5* is a hub for integrating lifetime and recent stressful experiences. Simultaneously, *Fkbp5* modulates responsiveness to acute stressors as negative modulator of glucocorticoid signalling. The naturally occurring *Fkbp5*-SNPs in laboratory rodents do not feature comparable functional effects as rs1360780 in humans, where the AT- vs. CG-allele is more strongly induced by glucocorticoids and linked to the aetiology of psychiatric disorders. To enable studying in more detail the mechanistic impact of the human SNP on stress physiology and the aetiology of psychiatric disorders, *Fkbp5*-humanised mouse lines carrying either the AT- or CG-allele of this SNP were generated. Characterisation of the *Fkbp5* × ELA mouse model showed mechanistic and face validity with aspects of psychiatric disorders. Female AT- vs. CG-allele carriers after ELA showed attenuated diurnal rhythmicity of glucocorticoids, lower activity, and less responsiveness to novel environments. On a molecular level, reduced expression of genes related to circadian entrainment and synaptic communication as well as increased expression of genes related to mitochondrial respiration between AT- vs. CG-allele carriers imply a genetic predisposition of their psycho-immune-neuro-endocrine system to allostatic changes reported in mild chronic stress settings. Since ELA lead to decreased circadian entrainment in the hippocampus, which in turn influences the circadian entrainment in the hypothalamus, the combination of ELA and *Fkbp5* SNPs could synergistically modify the HPA axis to respond less to stimuli. Given that dynamic variability in glucocorticoid levels and plasticity are required for adaptation to challenges, this predisposition increases the risk of an unsuccessful resolution of allostatic loads and thus elevates the risk of developing stress-related disorders. In combination with severe or chronic stress exposure, the observed *Fkbp5* × ELA interactions likely contribute to the aetiology of stress-related pathology. First indication of the transcriptomic findings in mice being translatable

to man were obtained using hiPSCs differentiated into astrocytes and neurons but require further investigation due to the small sample size available. Taken together, we are confident that this novel animal model will contribute to more comprehensive analyses of *FKBP5*-induced alterations in the stress response network that causally lead to the development of pathology.

DATA AVAILABILITY

Raw and aggregated data will be made available upon request. The hiPSC data sets will be made available upon request. The accession code for the murine NGS data set on NCBI's Sequence Read Archive is PRJNA743189.

CODE AVAILABILITY

The R code to process, analyse, and visualise the data will be made available upon request.

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AUTHOR CONTRIBUTIONS

Allers: conceptualization, maternal separation, data interpretation, revision. Blasius: T maze. Del Prete: hiPSCs cultivation, FACS, and NGS. Harris: support RNA isolation. Hengerer: conceptualization, revision. Kolassa: data interpretation, revision. Koros: support with maternal separation. Nold: conceptualization and execution, sample and data analysis, manuscript. Peleh: support social chamber test. Portenhauser: support RNA isolation, corticosterone assay, social chamber test. Slezak: conceptualization hiPSC experiments, revision

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COMPETING INTERESTS

IH and I-TK declare no conflict of interest. KAA, AB, BH, EK, VN, TP and MP are employees at Boehringer Ingelheim Pharma GmbH & Co KG. MS and DDP were employees at BioMedX during preparation of data used in this publication.

ETHICS APPROVAL

Experiments were performed under the allowance of the regional council for animal welfare (Regierungspräsidium Tübingen, Baden-Württemberg, Germany) and adhere to ARRIVE guidelines.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41380-022-01549-z.

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2.3. Novel Blood-Based Biomarkers of Cognition, Stress, and Physical or Cognitive Training in Older Adults at Risk of Dementia: Preliminary Evidence for a Role of BDNF, Irisin, and the Kynurenine Pathway

2.3.1. Summary Study III

Given its interactions with several components of the PINE network, the catabolism of tryptophan via the kynurenine pathway is thought to link stress signalling, energetic demands and inflammation to excitation, neural networking and cognition [37, 224, 225]. Extending this hypothesis, we assumed that alterations in the profiles of TRYCATs would be likely to indicate changes in the PINE network and could serve as diagnostic biomarkers. Since lack of social [444], cognitive [186], and physical [646, 30] activity is thought to convey a risk for cognitive decline and previous studies report positive effects of physical training on cognition [4, 106, 574], we furthermore hypothesized that TRYCAT profiles could help to monitor the success of cognitive and physical training.

These hypotheses were tested in 47 older adults at risk for dementia that were enrolled in a longitudinal control-intervention trial (ClinicalTrials.gov, Identifier NCT01061489, registered February 2, 2010). Inclusion criteria comprised an age of $\geq$ 55 years, subjective and/or objective memory impairment, fluency in German language, independent living, and a Mini Mental State Examination (MMSE) score $\geq$ 20. Exclusion criteria were moderate to severe neurocognitive disorders, a history of other neurological or psychiatric disorders (except mild to moderate depression [652]), stable anti-dementive or antidepressant medication as well as severe hearing, visual or physical impairment. Cognitive impairments in episodic and working memory as well as attention and executive functions were assessed for each participant [326]. In addition, regular physical, cognitive, and social activities were determined [573]. To assess potential risk factors and previous stress exposure, the Clinician-Administered PTSD Scale Life Events Checklist [527] was applied. The named assessments as well as blood collections were carried out at pre- and post-test, with a 10-week intervention period starting 1-4 weeks after the pre-test and ending up to 4 weeks before the post-test. The cohort was divided into three, representing the cognitive training (n = 18, description [370]), physical training (n = 21, description [595]) and waiting list (n = 25) group that were matched with respect to age, education, gender, and cognitive status. Profiling of TRYCATs was performed via HPLC/MS-MS analytic in serum.

Correlation analyses of TRYCATs with cognitive abilities revealed a negative association of QUIN with executive functions. The correlation analyses between TRYCATs and the risk and protective factors at baseline indicated a positive linkage between negative life events and 3-HK, the precursor of QUIN. After physical training, 3-HK levels decreased on trend level. Cognitive training lead to a decrease in 3-HK and KYNA. Despite the changes in TRYCATs, no significant effects of the training interventions on cognition were detected.

Taken together, **study III** showed that levels of TRYCATs can have an indicative function for life time stressful events and cognitive performance. Moreover, the findings suggest that psychosocial stress and cognitive training may impact the kynurenine pathway. The absolute and relative TRYCAT levels may be altered by therapeutic intervention and therefore could hold a potential to be used as biomarkers to monitor treatment effects. Larger scale trials are needed to replicate and extend these findings.

2.3.2. Reprint of the Original Research Article

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Novel Blood-Based Biomarkers of Cognition, Stress, and Physical or Cognitive Training in Older Adults at Risk of Dementia: Preliminary Evidence for a Role of BDNF, Irisin, and the Kynurenine Pathway

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Abstract. Psychosocial stress and physical, cognitive, and social activity predict the risk of cognitive decline and dementia. The aim of this study was to elucidate brain-derived neurotrophic factor (BDNF), irisin, and the kynurenine pathway (KP) as potential underlying biological correlates. We evaluated associations of irisin and the KP with BDNF in serum and with cognition, stress, and activities. Furthermore, changes in serum concentrations of BDNF, irisin, and KP metabolites were investigated after physical or cognitive training. Forty-seven older adults at risk of dementia were assigned to 10 weeks of physical training, cognitive training, or a wait-list control condition. Previous physical, cognitive, and social activities and stressful life events were recorded; global cognition, episodic memory, and executive functions were assessed. Serum levels of L-kynurenine, kynurenic acid, 3-hydroxykynurenine (3-HK), and quinolinic acid (QUIN) were determined by validated assays based on liquid chromatography coupled to tandem mass spectrometry. BDNF and irisin serum levels were determined with enzyme-linked immunosorbent assays. BDNF and irisin correlated positively with global cognition and episodic memory, while the neurotoxic metabolite QUIN correlated negatively with executive functions. Stressful life events

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were associated with reduced BDNF and increased 3-HK. 3-HK decreased after cognitive training, while BDNF tended to increase after physical training. This suggests that psychosocial stress as well as cognitive and physical training may impact BDNF serum levels and the KP. Irisin and QUIN may constitute novel serum biomarkers of cognitive impairment, in addition to BDNF. Larger scale trials are needed to replicate and extend these novel findings.

Keywords: Brain-derived neurotrophic factor, cognitive function, dementia, exercise training, kynurenine, lifestyle

INTRODUCTION

A number of lifestyle factors influence the risk of cognitive decline and dementia in old age. While a high amount of physical, cognitive, and social activities lowers the risk of cognitive decline and dementia [1], psychosocial stress can increase the risk [2, 3]. In recent years, an increasing number of studies focused on unravelling the underlying biological mechanisms of lifestyle effects, with the aim of identifying novel treatment strategies for an increasing number of older adults with neurodegenerative diseases.

The neurotrophin brain-derived neurotrophic factor (BDNF) is a probable mediator of lifestyle effects on cognition. BDNF plays a major role in neuroplasticity [4] and is linked to learning and memory processes [5, 6]. Reduced peripheral BDNF concentrations have been reported in patients with Alzheimer's disease (AD) [7, 8] and in individuals with mild cognitive impairment (MCI) [9, 10], but results are mixed [11]. Experimental studies demonstrated an involvement of BDNF in the positive effects of physical exercise on cognition [12]. Some studies also reported BDNF enhancements in serum after cognitive interventions [13–15]. In contrast, serum BDNF seems to be reduced in individuals with high stress loads at work [16] or a higher number of stressful life events [17]. Recently, exercise-induced BDNF alterations have been linked to the myokine irisin and the kynurenine pathway (KP) in animal studies.

The discovery of the "exercise hormone" irisin attracted a great deal of attention [18]. Irisin was identified as a communicator between the skeletal muscle and adipocytes, and thus a potential bearer of positive effects of physical exercise on other target organs outside the muscle [19]. Boström et al. [18] demonstrated that irisin is cleaved from the transmembrane receptor fibronectin type III domain containing 5 (FNDC5) in skeletal muscle and secreted into the periphery as a myokine, from where it acts on adipocytes. Ten weeks of physical training resulted in increased levels of irisin in plasma of humans [18]. Subsequent studies confirmed altered irisin levels after acute bouts of exercise [20, 21]. Results were, however, more equivocal with respect to physical training interventions over several weeks [22–25].

The observation that irisin administration increased the proliferation of hippocampal cells *in vitro* [26] and FNDC5 expression resulted in elevated irisin concentrations and BDNF gene expression in cell-culture [27] suggested that irisin might also constitute a potential therapeutic target in neurodegenerative disorders [28–30]. Despite that, associations between cognition and irisin in older adults or patients with neurocognitive disorders have not yet been investigated. Two studies reported correlations between cognition and irisin in serum in younger healthy adults, with one finding positive associations of irisin with the Mini-Mental State Examination (MMSE) [31], the other finding negative associations with measures of executive functions [32].

Another link between lifestyle factors, cognition and neuroplasticity constitutes the KP, the major route of tryptophan (TRP) metabolism. The essential amino acid TRP is metabolized into L-kynurenine (KYN). The KP itself consists of two branches with neuroactive metabolites as key products: One branch is initiated by kynurenine 3-monooxygenase (KMO)—an enzyme that catalyzes compounds with rather neurotoxic characteristics, including 3-hydroxykynurenine (3-HK) [33] and quinolinic acid (QUIN) [34]. The other branch of the KP leads to the formation of kynurenic acid (KYNA) through kynurenine aminotransferases (KAT I-IV). Depending on its concentration, KYNA can have neuroprotective effects and is able to counteract QUIN-induced neurotoxicity in the brain [35]. Furthermore, KYNA is not able to pass the blood-brain barrier, in contrast to KYN [36]. Thus, the catabolism of KYN to KYNA in the periphery may also reduce the amount of (deleterious) KYN passing to the brain.

Altered levels in kynurenine metabolites have been detected in neurodegenerative disorders [37, 38].

In AD, an increased activation of the KP and a shift toward the neurotoxic metabolites with higher levels of 3-HK and QUIN was found in the hippocampus [39, 40] as well as in peripheral tissue [41–43]. Cognitive performance was negatively associated with QUIN and positively associated with KYNA concentrations in plasma [41]. Agudelo and colleagues [36] recently demonstrated in an animal study that stress increased the activity of KMO in plasma and reduced BDNF in the hippocampus. Both actions could be prevented by overexpression of the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) in the muscle, as a model for physical exercise. PGC-1α overexpression as well as physical exercise increased metabolism along the KYNA branch, demonstrated by an increased gene expression of KATs in the muscle and corresponding increases of KYNA in plasma. Similarly, 3 weeks of physical training in humans were also associated with increases in KATs [36].

In sum, BDNF has been related to effects of stress, physical and cognitive training, and an activity-enriched environment on cognition, although evidence in humans is mixed. Irisin and the KP may be further underlying biological mechanisms of lifestyle effects, including physical and cognitive activity, on cognition and may be linked to BDNF increases in the brain.

This study investigated older adults at risk of AD, who completed an elaborate assessment of lifestyle parameters as well as neuropsychological tests at baseline, and received a 10-week period of either physical training (PT), cognitive training (CT), or a wait-list control (WLC) condition. We aimed at evaluating BDNF, irisin, and kynurenine metabolites as potential underlying biological mechanisms of associations between lifestyle risk and protective factors of dementia and cognition and of training effects on cognition. We therefore tested the hypotheses that 1) Irisin and the KP metabolites are associated with BDNF in serum; 2) BDNF, irisin, and the neuroprotective KP metabolite KYNA are associated with better cognitive performance, while the neurotoxic KP metabolites (3-HK and QUIN) are associated with poorer cognitive performance at baseline; 3) BDNF is negatively and the neurotoxic KYN metabolites are positively associated with stress; and 4) BDNF, irisin and KYNA are positively associated, while the neurotoxic KP metabolites are negatively associated with the amount of physical, cognitive, and social activities. We further evaluated 5) whether a short physical or cognitive training program altered serum levels of irisin, the KP metabolites and BDNF as early biomarkers.

MATERIALS AND METHODS

Study design

The results reported here are part of a clinical trial (ClinicalTrials.gov Identifier NCT01061489, registered February 2, 2010), of which we previously reported cognitive outcomes [44] and diffusion tensor imaging data [45]. Here, we report associations and training-induced alterations of blood-based biomarkers.

Participants

The study population has previously been described in detail [44]. In brief, subjects were recruited in the Memory Clinic of the University Hospital Ulm, Germany and the Center for Psychiatry Reichenau, Germany or via public advertisements. Inclusion criteria were age of 55 years or older, subjective memory complaints and either objective (German version of the California Verbal Learning Test [46]: average of learning and long-delayed free recall trials below –1 *SD* of the age norm) or clinically apparent memory impairment (e.g., increased difficulty in relocating objects, keeping appointments, remembering conversations or events), and fluency in the German language. Exclusion criteria were any psychiatric or neurologic disorders, severe hearing or visual impairment, physical impairment which would have prevented participation in the PT program, changes in antidementive or antidepressive medication before study initiation, and moderate or severe dementia (MMSE <20; see Fig. 1).

Procedure

The study was approved by the Ethics Committees of the Universities of Konstanz and Ulm, Germany. Written informed consent was obtained from participants prior to study participation. Cognitive tests, a diagnostic interview with self-report questionnaires, and the collection of blood were usually performed on two appointments for each, the pre- and the post-test. One to 4 weeks after the pre-test, the 10-week intervention period started, followed by the post-test up to 4 weeks after the last training session.

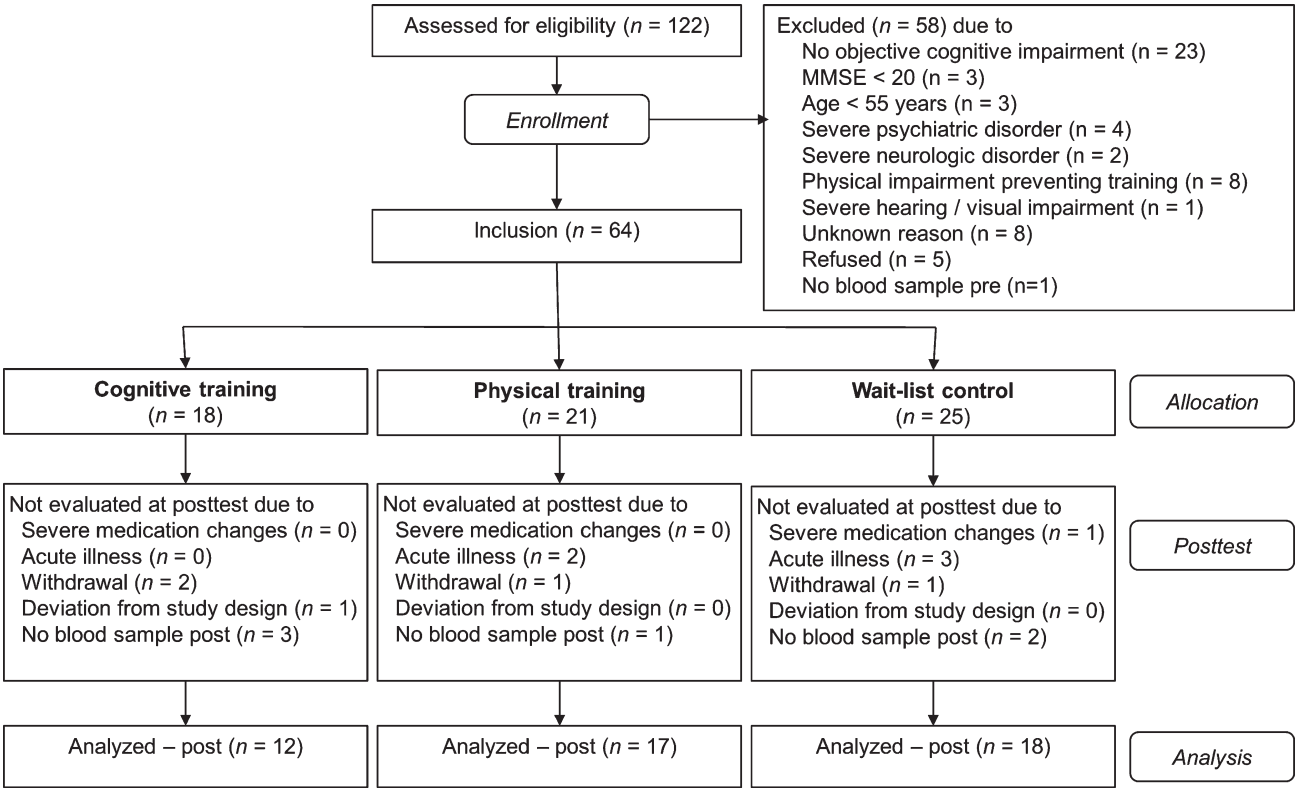

Fig. 1. Flow of participants within the cognitive training, physical training, and wait-list control group.

Groups were matched with respect to age, education, gender, and cognitive status (MMSE) using a minimization approach, in order to avoid a selection bias. The investigators who conducted the neuropsychological assessments at post-test and who performed the BDNF and irisin measurements were blinded to the subjects' group assignments. Investigators conducting the KP measurements were not blinded to group allocation in order to evenly distribute the samples of the three groups on the measurement plates to avoid a measurement bias.

Assessment of biological parameters in serum

Blood collection, sample pre-processing and storage

Venipuncture was performed between 8 : 30 and 11 : 00 a.m., prior to which participants were asked to refrain from physically demanding activities and were seated for a resting period of at least 5 min before venipuncture. Fasting was not mandatory. Blood collection time at pre- and post-test was kept constant for each participant. Blood was collected in 7.5 ml serum tubes (Sarstedt, Nümbrecht, Germany) containing beads coated with a clotting activator (silcate). Blood samples were centrifuged 2.5 h (± 30 min) post collection for 4 min at 2700 g and 4°C. Serum samples were frozen at –80° C until analysis.

BDNF

Serum levels of total BDNF were measured using the Enzyme-linked Immunosorbent Assay (ELISA) kit BDNF Emax ImmunoAssay Systems (Cat #: G7610, Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. All samples of each participant and each time-point (pre, post) were assayed in duplicate on each plate, in order to test the intra-assay variation. Serum samples were diluted 1 : 100 in blocking buffer and acidified before used in the system. A BDNF standard was used on each plate to generate a linear standard curve ranging from 7.8–500 pg/ml. The absorbance at 450 nm was recorded within each well using an automated microplate reader (Biotek). The samples were processed on 4 plates in total. Samples, which were to be compared, i.e., pre- and posttest samples of each participant, were processed on the same plate. Four samples, in which the absorbance of the duplicates differed by 100 pg/ml or more, were excluded from the analyses.

Irisin

Irisin concentrations in serum were determined using a competitive, commercially available ELISA kit (Phoenix, EK-067-52) according to the manufacturer's instructions. Samples were measured in duplicate; given values are averages. To determine irisin concentrations, absorbance at 450 nm wavelength was measured using a spectrophotometer (Thermo Scientific, Multiskan FC).

Kynurenine metabolites

Measurements were conducted at Boehringer Ingelheim Pharma GmbH & Co. KG, DMPK Germany, Biberach an der Riss, Germany. Quantification of serum levels of TRP and its catabolites KYN, KYNA, 3-HK, and QUIN was performed by validated assays based on liquid chromatography tandem mass spectrometry. TRP, KYNA, 3-HK, and QUIN concentration levels in serum samples were quantified together in one assay, while KYN serum levels were quantified separately. The assays comprised sample clean-up by protein precipitation followed by reversed-phase chromatography and mass spectrometric detection in the positive ion multiple reaction monitoring mode using the deuterated analogues of the analytes, namely [D_5] tryptophan, [D_4] kynurenine, [D_5] kynurenic acid, [D_3] 3-hydroxykynurenine, and [D_3] quinolinic acid as internal standards. The lower limits of quantification in serum were 2000 nM for TRP, 770 nM for KYN, 5 nM for KYNA, 20 nM for 3-HK, and 50 nM for QUIN. Assay accuracy (in terms of relative deviation, dev., from nominal concentrations) and precision (in terms of coefficient of variability, CV, of multiple measurements) were determined for each analyte by the fourfold analysis of quality control samples at four concentration levels. Assay accuracy and precision were <6.1% (dev.) and <8.7% (CV) for TRP;<6.7% (dev.) and <14.1% (CV) for 3-HK;<23.2% (dev.) and 5.3% (CV) for KYN; <11.3% (dev.) and 20.1% (CV) for KYNA; and <14.1% (dev.) and <8.6% (CV) for QUIN.

Cognitive assessment

Global cognition, memory functions, and attentional and executive functions were assessed with an extensive neuropsychological test battery. Principal component analysis served to construct these three component scores (see [44]). All variables were *z*-standardized by using the baseline data. For each participant the two component scores (memory functions and attention/executive functions) were built as the weighted average of the standardized variables with loadings of at least $a_{ij} = 0.40$ on the respective component. The global cognition score represents the average of the two component scores.

The neuropsychological assessment included German versions of the MMSE [47], subtests of the test battery of the Consortium to Establish a Registry for Alzheimer's Disease [48], the Alzheimer's Disease Assessment Scale – Cognitive Subscale [49], and an adapted German version of the California Verbal Learning Test [46] as well as the digit-span and digit-symbol-coding test of the Wechsler Adult Intelligence Scale [50] and the working memory subtest of the Everyday Cognition Battery [51].

Assessment of lifestyle protective and risk factors

Physical, cognitive, and social activities

The Community Healthy Activities Model Program for Seniors Physical Activity Questionnaire for Older Adults [52] was applied to assess regular physical, cognitive, and social activities of the participants. The questionnaire assesses the frequency and duration of 40 activities in a typical week within the previous 4 weeks. The activities were categorized into physical, cognitive, and social activity domains, as reported previously [44]. A score for each activity domain was built, reflecting the percentage of performed activities in relation to the possible number of activities in this domain. Then, an overall activity score was built by averaging the three domain scores.

Stressful life events

The Clinician-Administered PTSD Scale Life Events Checklist [53] was applied to assess the number of potentially traumatic life events. An event (personally experienced or witnessed) is considered traumatic if it poses a potential threat to life or physical integrity (criterion A1 for posttraumatic stress disorder) and is accompanied by the experience of intensive anxiety, helplessness, or horror (criterion A2). Regarding 19 event types, participants were asked whether they had experienced or witnessed the event at least once in their life and to shortly describe it including the experienced emotions. Two sum-scores were built, one comprising all events fulfilling criterion A1 (*critical life events*) and one including only those events which also fulfilled criterion A2 (*traumatic life events*).

Depressive symptoms

The Geriatric Depression Scale-15 [54] was used to determine depressive symptoms. Scores over 5 are indicative of mild to moderate depression; scores over 10 indicate severe depression.

Other potential influencing factors

We assessed age, education in years, the number of cigarettes smoked per day, and the number of glasses of alcohol consumed per day in a standardized interview and measured height and weight to calculate the body mass index.

Training interventions

Cognitive training

The CT group was asked to complete 1-h computer-based training sessions five times per week for a total of 10 weeks (i.e., 50 sessions in total), which were carried out individually at the participants' homes. The training was an adapted and translated German version of a program developed by the Posit Science Corporation, San Francisco, CA and focuses on the training of auditory discrimination and working memory (for detailed descriptions see [44, 55]).

Physical training

The PT group was asked to attend 1-h sessions twice a week in groups of five to ten participants and three 20-min sessions per week at home for 10 weeks (i.e., 20 group- and 30 home-based sessions in total). The multimodal PT program included endurance, coordination, balance, flexibility, and strengthening components, embedded into an imaginary journey. A similar program with the same structure yielded beneficial effect on cognition in frail nursing-home residents [56].

Wait-list control group

Participants of the WLC group were asked to continue their daily routine as usual and were offered to take part in one of the training programs after their study participation.

Statistical analyses

Statistical analyses were carried out with R version 3.1.2 [57]. Baseline group differences in continuous variables were evaluated with one-way analyses of variance. Baseline differences in categorical variables were analyzed with χ^2-tests.

Associations of irisin and the KP measures (KYN, KYNA, 3-HK, QUIN) with cognition and with risk and protective lifestyle factors were calculated as Pearson's product-moment correlations. As BDNF values were not normally distributed, all associations with BDNF were calculated with Spearman rank correlations (see also [58]). Significant associations with measures of cognition are also reported after statistically accounting for influencing covariates, by including the covariates into a multiple regression analysis (for irisin and KP measures) or calculating partial correlations after partialling out the covariates (for BDNF).

To evaluate effects of training on irisin and KP measures, linear mixed effects models were conducted with Group (PT, CT, WLC) and Time (pre, post) as fixed effects and Subject as a random intercept, using the nlme package 3.1–119 in R. Significant Group × Time interactions indicated effects of training on the biological measures. Changes from pre- to post-test within each group were analyzed with paired *t*-tests. Effects of training on BDNF were analyzed with Wilcoxon tests within each group.

RESULTS

Subject characteristics

The study population consisted of 20 male and 27 female participants with a mean age of 71.2 years ($SD = 6.0$, range 60–88 years), a mean education time of 14.1 years ($SD = 3.4$), and a mean MMSE score of 28.0 ($SD = 1.9$). The sample included mostly individuals with MCI ($n = 32$), 11 participants with no objective memory impairment, and four participants with probable beginning dementia. Three participants ($n = 1$ in each group) had Geriatric Depression Scale scores indicative of mild to moderate depression (6–8 points). Self-reported leisure activity was high (6–20 regular activities, $M = 13.77$, $SD = 3.70$) considering the age of the sample. The participants reported 0 to 11 critical life events ($M = 3.74$, $SD = 2.49$), of which 0 to 5 were traumatic ($M = 1.23$, $SD = 1.35$). The three groups (CT, PT, and WLC) did not differ in any demographic characteristics, cognitive status, lifestyle, or biomarker concentrations (see Table 1). Thirty-four participants reported current medical conditions, most of them with one ($n = 10$) or two ($n = 14$) diagnoses, the most frequent being hypertension ($n = 14$) and arthrosis ($n = 9$). Thirty-six subjects reported current medication intake, most frequently antihypertensives ($n = 15$) or thyroid

Table 1
Baseline characteristics for each of the three intervention groups

	CT ($n = 12$)	PT ($n = 17$)	WLC ($n = 18$)	Statistic	p
Demographic data					
Age: *M* (*SD*)	70.50 (6.14)	73.18 (5.99)	69.89 (5.70)	$F_{2,44} = 1.47$	0.24
Gender: male/female	5/7	6/11	9/9	$\chi^2(2) = 0.78$	0.68
Education in years: *M* (*SD*)	12.79 (4.06)	14.06 (3.00)	15.06 (3.28)	$F_{2,44} = 1.61$	0.21
Biomarker data					
BDNF: *Mdn* (IQR)	339 (221)	291 (284)	388 (385)	$\chi^2(2) = 1.27$	0.53
Irisin: *M* (*SD*)	55.2 (9.9)	57.9 (10.6)	56.4 (14.1)	$F_{2,39} = 0.16$	0.85
KYN: *M* (*SD*)	1983 (457)	2090 (479)	1902 (419)	$F_{2,38} = 0.68$	0.51
KYNA: *M* (*SD*)	45.7 (14.1)	40.8 (10.2)	41.3 (18.2)	$F_{2,38} = 0.37$	0.69
3-HK: *M* (*SD*)	53.5 (11.3)	52.7 (16.3)	49.9 (12.9)	$F_{2,37} = 0.26$	0.77
QUIN: *M* (*SD*)	681 (277)	560 (201)	560 (295)	$F_{2,38} = 0.80$	0.46
Cognitive data					
Global cognition: *M* (*SD*)	0.16 (0.60)	0.04 (0.62)	–0.14 (0.85)	$F_{2,44} = 0.67$	0.52
MMSE: *M* (*SD*)	28.08 (1.68)	27.88 (1.69)	28.06 (2.29)	$F_{2,44} = 0.05$	0.95
Lifestyle data					
Overall activity: *M* (*SD*)	0.28 (0.13)	0.28 (0.09)	0.31 (0.07)	$F_{2,44} = 0.77$	0.47
CAPS critical life events: *M* (*SD*)	3.58 (2.15)	4.06 (2.66)	3.56 (2.64)	$F_{2,44} = 0.21$	0.82
GDS-15: *M* (*SD*)	2.33 (2.23)	2.59 (1.73)	2.28 (1.69)	$F_{2,44} = 0.14$	0.87

KYN, KYNA, 3-HK, and QUIN are measured in nM, irisin is measured in pg/ml, and BDNF is measured in ng/ml. 3-HK, 3-hydroxykynurenine; BDNF, brain-derived neurotrophic factor; CAPS, Clinician-Administered PTSD Scale; CT, cognitive training group; GDS-15, Geriatric Depression Scale-15; KYN, L-kynurenine; KYNA, kynurenic acid; MMSE, Mini-Mental State Examination; PT, physical training group; QUIN, quinolinic acid; WLC, wait-list control group.

hormones ($n = 9$). Six participants took antidementive, two participants antidepressive medication.

Irisin measurements were missing for five participants, KP metabolite measurements were missing for six participants, as there were not enough serum samples available. BDNF measurements were missing for five subjects at baseline. For pre-post comparisons, the BDNF data of a further 6 subjects were excluded, as post-data were missing. Test-retest reliability between pre- and post-measurements was reasonable for all biomarkers (Irisin: $r = 0.67$; BDNF: $\rho = 0.70$; KYN: $r = 0.68$; KYNA: $r = 0.55$; 3-HK: $r = 0.72$; QUIN: $r = 0.83$).

Irisin but not the kynurenine metabolites are associated with BDNF

To evaluate proposed links of irisin and the KP with BDNF, baseline associations between the biomarkers were calculated. Irisin levels correlated positively with BDNF levels ($\rho = 0.32$, $p = 0.05$), while the kynurenine metabolites were not associated with BDNF ($ps \geq 0.45$).

Irisin, QUIN, and BDNF levels correlate with measures of cognition

To test the hypothesis whether irisin, KP metabolites, and BDNF are associated with cognitive performance, correlations with global cognition as well as with the composite scores were calculated at baseline (see Table 2). Global cognition was significantly associated with irisin serum concentrations ($r = 0.37$, $p = 0.02$) as well as a with BDNF serum concentrations ($\rho = 0.33$, $p = 0.04$), but not with any of the KP metabolites ($ps \geq 0.14$). Within the two cognitive component scores, memory correlated significantly with irisin ($r = 0.45$, $p = 0.003$) and BDNF ($\rho = 0.36$, $p = 0.02$), while the component of attention/executive functions was inversely correlated with QUIN ($r = –0.31$, $p = 0.05$; see Fig. 2).

Table 2
Baseline associations of biological parameters with measures of cognition

	Global Cognition	Memory	Attention/EF
BDNF	0.33*	0.36*	0.21
Irisin	0.37*	0.45**	0.20
KYN	0.001	0.05	–0.05
KYNA	0.02	–0.04	0.09
3-HK	–0.14	–0.17	–0.08
QUIN	–0.24	–0.13	–0.31*

Associations with BDNF are Spearman rank correlations, all other associations are Pearson product-moment correlations. 3-HK, 3-hydroxykynurenine; Attention/EF, attention/executive functions; BDNF, brain-derived neurotrophic factor; KYN, L-kynurenine; KYNA, kynurenic acid; QUIN, quinolinic acid. $^{**}p < 0.01$, $^{*}p < 0.05$.

After statistically accounting for age and education, the associations of global cognition with BDNF

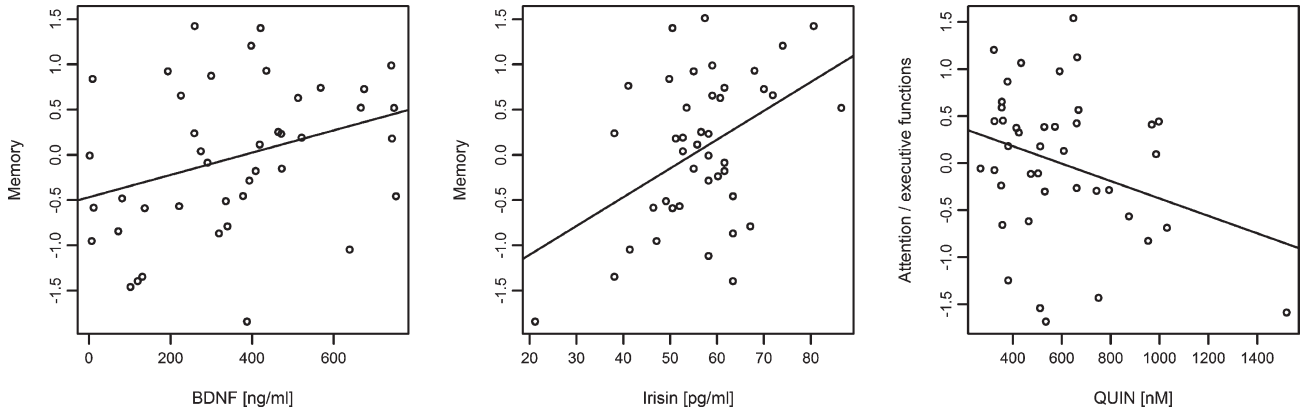

Fig. 2. Baseline associations of biomarkers with composite measures of cognition. Higher brain-derived neurotrophic factor (BDNF) and irisin serum levels were significantly associated with better memory performance ($\rho = 0.36$, $p = 0.02$; and $r = 0.45$, $p = 0.003$, respectively). Higher levels of quinolinic acid (QUIN) in serum were associated with poorer performance in a composite score of attention and executive functions ($r = –0.31$, $p = 0.05$).

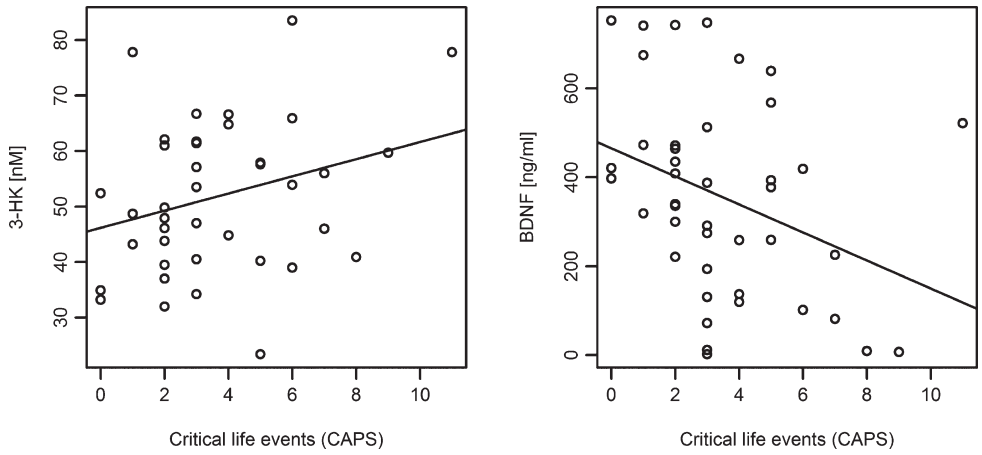

Fig. 3. Baseline associations of critical life events with 3-HK and BDNF serum levels. The number of critical life events was assessed with the Life Events Checklist of the Clinician-Administered PTSD Scale (CAPS), as a measure of lifetime psychosocial stress. There was a trend for a positive correlation of critical life events with 3-hydroxykynurenine (3-HK) serum levels ($r = 0.29$, $p = 0.07$) and a significant negative correlation with brain-derived neurotrophic factor (BDNF) serum levels ($\rho = –0.40$, $p = 0.009$).

($p < 0.01$) as well as of memory with irisin ($p = 0.046$) and BDNF ($p < 0.01$) remained significant, while the associations of global cognition with irisin was no longer significant ($p = 0.21$). The association between attention/executive functions and QUIN remained marginally significant after accounting for either age and education ($p = 0.07$) or alcohol consumption ($p = 0.10$).

Lifetime stress is associated with lower BDNF and higher 3-HK levels

To assess associations of the biomarkers with lifetime stress as a risk factor of AD we used the Clinician-Administered PTSD Scale Life Events Checklist. The number of experienced critical life events, which pose a threat to life or physical integrity, was negatively associated with BDNF ($\rho = –0.40$, $p = 0.009$) and tended to positively correlate with 3-HK levels ($r = 0.29$, $p = 0.07$, see Fig. 3 and Table 3). Associations with the number of traumatic life events which were also connected with the experience of extreme fear or helplessness, were not significant.

No association of self-reported activity with the investigated biomarkers

The amount of regular physical, cognitive, and social activities before study participation was assessed with a questionnaire. The amount of activity was not associated with serum levels of irisin, KP metabolites, or BDNF at baseline (see Table 3).

Table 3
Baseline associations of biological parameters with lifestyle risk and protective factors and covariates

	Irisin	BDNF	KYN	KYNA	3-HK	QUIN
Age	–0.42**	–0.25	–0.04	–0.01	0.09	0.08
Years of education	0.12	0.26	0.13	0.26#	–0.07	–0.09
Body mass index	0.02	0.00	0.16	–0.09	0.06	0.06
Overall activity	0.05	0.25	0.09	0.22	0.03	–0.03
CAPS critical life events	–0.16	–0.40**	0.21	0.02	0.29#	0.14
CAPS traumatic life events	–0.05	0.01	0.12	0.05	0.13	–0.12
GDS-15	–0.03	–0.09	0.05	–0.07	0.15	–0.09
Alcohol consumption	–0.04	–0.06	–0.27#	0.28#	–0.16	–0.40**
Cigarette consumption	0.02	–0.09	–0.05	–0.07	–0.10	–0.04

Associations with BDNF are Spearman rank correlations, all other associations are Pearson product-moment correlations. 3-HK, 3-hydroxykynurenine; BDNF, brain-derived neurotrophic factor; CAPS, Clinician-Administered PTSD Scale; GDS-15, Geriatric Depression Scale-15; KYN, L-kynurenine; KYNA, kynurenic acid; QUIN, quinolinic acid. $^{**}p<0.01$, $^{*}p<0.05$, $^{\#}p<0.10$.

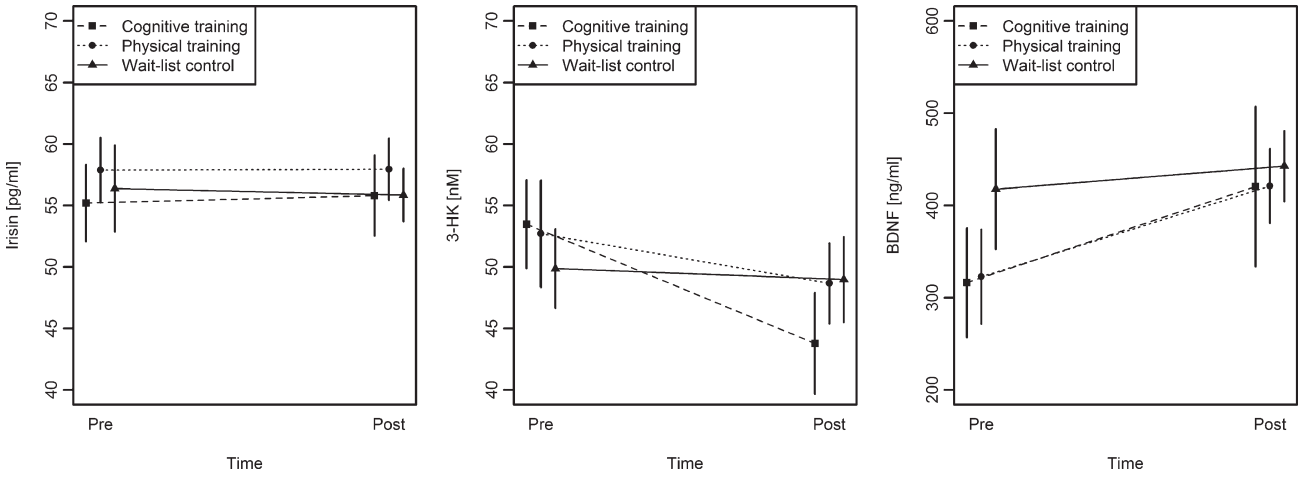

Fig. 4. Pre to post changes in irisin, 3-HK and BDNF serum levels for the three groups. Irisin levels remained unchanged in all three groups. 3-HK levels decreased significantly from pre to post within the cognitive training group in comparison to the wait-list control group. BDNF levels tended to increase within the physical training group, while levels remained unchanged in the wait-list control group. Error bars depict standard errors of the mean.

Training-induced alterations of BDNF and 3-HK levels

There was a trend for an increase in BDNF levels from pre to post in the PT group (Wilcoxon $V=25$, $p=0.09$). BDNF also increased in the CT group, but the difference did not reach significance ($V=11$, $p=0.11$; see Fig. 4 and Table 4). When taking the two training groups (CT and PT) together, the BDNF serum levels significantly increased from pre- to post-test ($V=66$, $p=0.02$), while there was no alteration in the WLC group.

We found a significant Group × Time interaction on 3-HK, $F(2,37)=3.25$, $p=0.05$ (see Fig. 4). 3-HK decreased significantly in the CT group, $t(9)=3.17$, $p=0.01$, and tended to decrease in the PT group, $t(13)=1.76$, $p=0.10$, while it remained unchanged in the WLC group, $t(15)=-0.11$, $p=0.91$. KYNA decreased significantly within the CT group, $t(9)=2.23$, $p=0.05$, but a Group × Time interaction was not significant, $F(2,38)=0.36$, $p=0.70$.

There were no other significant Group × Time interactions for modelling kynurenine metabolites ($ps>0.18$). There was also no significant Group × Time interaction on irisin levels, $F(2,39)=0.05$, $p=0.95$.

DISCUSSION

We aimed to evaluate potential neurobiological correlates of lifestyle- and training-related associations with cognition in a sample of older adults at risk of dementia. We found significant associations

Table 4
Comparisons of biological parameters in the three intervention groups between pre- and post-test

Variable	*n*	Pre-test *M (SD)*	Post-test *M (SD)*	Statistic	*p*
KYN					
CT	10	1983 (457)	1802 (405)	$t = 1.73$	0.12
PT	14	2090 (479)	2043 (519)	$t = 0.55$	0.59
WLC	17	1902 (410)	1978 (379)	$t = -0.83$	0.42
KYNA					
CT	10	45.7 (14.1)	40.4 (15.4)	$t = 2.23$	0.05
PT	14	40.8 (10.2)	40.1 (11.4)	$t = 0.45$	0.66
WLC	17	41.3 (18.2)	39.0 (10.6)	$t = 0.51$	0.61
3-HK					
CT	10	53.5 (11.3)	43.8 (13.1)	$t = 3.17$	0.01
PT	14	52.7 (16.3)	48.7 (12.2)	$t = 1.76$	0.10
WLC	16	49.9 (12.9)	49.0 (14.3)	$t = -0.11$	0.91
QUIN					
CT	10	681 (277)	595 (301)	$t = 1.52$	0.16
PT	14	560 (201)	524 (185)	$t = 0.90$	0.38
WLC	17	560 (295)	551 (258)	$t = 0.30$	0.77
Irisin					
CT	10	55.2 (9.9)	55.8 (10.4)	$t = -0.88$	0.40
PT	16	57.9 (10.6)	58.0 (10.0)	$t = -0.06$	0.95
WLC	16	56.4 (14.1)	55.9 (8.6)	$t = 0.16$	0.88
BDNF[a]					
CT	10	339 (221)	427 (231)	$V = 11$	0.11
PT	14	291 (284)	469 (123)	$V = 25$	0.09
WLC	12	388 (385)	444 (110)	$V = 35$	0.79

KYN, KYNA, 3-HK, and QUIN are measured in nM, irisin is measured in pg/ml, and BDNF is measured in ng/ml. 3-HK, 3-hydroxykynurenine; BDNF, brain-derived neurotrophic factor; CT, cognitive training group; KYN, L-kynurenine; KYNA, kynurenic acid; QUIN, quinolinic acid; PT, physical training group; WLC, wait-list control group. [a]descriptive statistics for pre- and post-test expressed as median and interquartile range.

of BDNF, irisin, and the neuroactive kynurenine metabolite QUIN in serum with measures of cognition. Lifetime psychosocial stress, as a risk factor of cognitive decline and dementia, correlated with BDNF and 3-HK. In the interventional part of the study, we found significant decreases of the neurotoxic 3-HK after 10 weeks of cognitive training in comparison to a wait-list control group and a trend for an increase of BDNF after physical training.

Involvement of Irisin and the kynurenine pathway in neuroplasticity and cognition

Corroborating our hypotheses, we found that irisin levels were positively associated with BDNF in serum, a marker of neuroplasticity, and with cognition, especially with hippocampus-related memory functions. This adds to evidence of animal and *in vitro* studies, which suggested connections of irisin and its progenitor FNDC5 with BDNF and neuroplasticity in the central nervous system, as part of a pathway between physical exercise and cognition [26, 27, 59]. In accordance with our results, a positive association of serum irisin levels with a screening measure of cognition as well as with BDNF had been demonstrated in young adults [31]. The herein observed associations between irisin, BDNF, and cognition might indicate BDNF as a mediator between irisin and cognition, as suggested by previous research in animals [27]. However, other mechanisms by which irisin is connected to cognition are also plausible, for instance by an influence of mitochondrial processes. In adipocytes, FNDC5 enhanced mitochondrial density [18]; and in rat cardiomyoblasts, irisin upregulated mitochondrial metabolism [60].

In our study, higher QUIN levels were associated with poorer performance in attentional and executive functions. Our results are in line with studies demonstrating an increased QUIN concentration in brain [40] and serum [41] of AD patients, with a negative association between QUIN serum levels and the clock-drawing test, a cognitive screening tool for dementia.

Associations of the kynurenine pathway and BDNF with lifetime psychosocial stress

In line with the hypothesis, we found reduced BDNF serum levels and higher 3-HK serum levels in individuals who reported stressful life events. This adds evidence to results of animal studies, which demonstrated stress-related changes in tryptophan metabolism, with a shift toward the KP and an increase of neuroactive KP metabolites in plasma and brain [36, 61, 62], including increases of 3-HK [61]. The stress-related increases of 3-HK and reductions of BDNF observed here might contribute to the increased risk of cognitive deficits and dementia in stressed individuals [63, 64]. The number of experienced stressful life events was low, as expected within a non-psychiatric sample of this age [65]. Future studies should examine the associations with 3-HK and BDNF in samples with higher exposure to stress.

Biological alterations with physical or cognitive training

We observed increases in BDNF levels and reductions of 3-HK after 10 weeks of physical and cognitive training, respectively. Physical exercise-induced changes in BDNF concentrations have been

reported in numerous animal studies, mainly within the hippocampus [66, 67]. Effects of physical training on peripheral BDNF in humans, including older adults [68, 69] and patients with MCI [70] were less consistent [71]. Changes in BDNF concentrations after 10 weeks of cognitive training did not reach significance in this study. Cognitive training with the BrainFitness program (as applied in this study) yielded BDNF enhancements in other patient populations [14, 15], but have not been reported in the context of neurodegenerative disease. Further research with larger sample sizes is needed to elucidate the underlying biological mechanisms of cognitive training, particularly in individuals with neurocognitive disorders.

We observed a decrease of the neurotoxic metabolite 3-HK in the cognitive training group and a tendency for a decrease in the physical training group, in comparison to the control group. Little literature exists with respect to training-induced alterations in kynurenine metabolism. Changes in kynurenine metabolism have been demonstrated after a longer period of physical training in mice and humans, namely an increase of KATs in skeletal muscle which convert KYN to KYNA [36]. However, other studies [72]—like ours—failed to find effects of physical activity on kynurenine metabolites in serum of humans. As KP alterations after cognitive training have not been reported before, the 3-HK reductions observed here were not expected and need further confirmation.

Irisin levels remained unchanged after physical training. This is in accordance with previous reports [25], although other studies reported effects of physical training regimens with similar duration as ours [18, 23]. It has been hypothesized that irisin levels only rise after acute bouts of physical exercise but are not chronically elevated in response to physical training programs [73]. This notion is supported by reports of only transient irisin enhancements after a bout of exercise with a subsequent return to initial levels within 24 h [20, 74]. Another exciting hypothesis is that irisin is only increased in states of energy need [20, 29], that is when exercise constitutes a challenge to present energy resources, for example, in untrained, sedentary individuals. The participants in our study were quite active before taking part in the physical training. Thus, energy demands of the training program may have been too low in these participants to yield elevations of irisin.

Notably, we did not find any significant training-related effects on cognition in this sample [44]. This seems contrary to the alterations in BDNF and 3-HK on the molecular level reported here. In contrast to alterations on the biomarker level, training-induced changes in cognition might have been masked by substantial retest-effects in all groups. Furthermore, alterations on the biomarker level might have been too small to transfer to improvements in cognition. Cognitive improvements during the same time-period were, however, correlated to the self-reported activity level of the participants' lifestyles. Considering both the training-related alterations on the biomarker level and the lifestyle-related changes in cognition, another possibility is that changes at the molecular level are more likely to be influenced by short-term measures, such as the 10-week training programs, while changes at the cognitive level rather occur after longer term activity, as with an active lifestyle.

Limitations and outlook

By nature of the human study population, biomarker measurements were obtained in the periphery. Thus, we cannot infer that the observed alterations in biomarker concentrations in serum reflect those in the brain. However, regarding BDNF, close relationships between brain and serum concentrations of BDNF have been demonstrated in different species [75–77]. Furthermore, BDNF is able to pass the blood-brain barrier in both directions [78] and an interesting study recently demonstrated that the brain is the main source of exercise-induced BDNF elevations in the blood [79].

The validity of irisin ELISA measurements, in particular the identification of irisin in serum as a cleaved product of the transmembrane receptor FNDC5, has been doubted [80]. However, the immunoblot identification of irisin has recently been validated by mass spectrometry, which implicates ELISA validity [81]. BDNF measurements faced challenges due to a large variance. For this reason, statistical analyses including the BDNF measures were performed with non-parametrical, rank-based tests, as recommended by Ziegenhorn and colleagues [58]. This may have constrained the power to detect changes, e.g., after cognitive training. Unfortunately, no international recommendations or standard operating procedures with respect to BDNF measurements in serum exist and measurements may be influenced by the applied commercial assay [82]. Standardized procedures and validation for the assays for the determination of BDNF in human material are needed to improve the comparability of study results. The catabolite 3-HK

turned out to be quite instable during measurements, which might imply influences of clotting time on the concentration. However, venipuncture and centrifugation procedures were kept constant and did not differ between groups. Furthermore, a high correlation between pre- and post-measurements indicated reasonably reliable measures. Clotting time was fairly long (2-3 h, but compare [82]), but was kept constant for all probes. Pre-analytic handling of the probes may influence the measurements of the biomarkers [82], which is why a standardized procedure is of utmost importance. The validity of the results in this study might be improved by a different pre-analytic procedure.

Other demographic or lifestyle factors, such as age, education, or the body mass index, may influence biomarker levels or their associations with cognition. We found associations of age with irisin and of alcohol consumption with some of the kynurenine metabolites. After statistically accounting for these variables most of the reported associations between biomarker concentrations and cognitive performance remained (at least marginally) significant. However, the association between irisin and global cognition lost significance after accounting for age, indicating that this association may be influenced by associations with age. Apart from that, serum concentrations of BDNF, irisin, and the kynurenine metabolites are probably not specifically associated to cognition, psychosocial stress, or training, as described here, but also to other diseases (e.g., adiposity [74] or depression [83]) or treatments (e.g., antidepressant medication [83]).

The limited sample size likely impeded the detection of small effects. Further research is needed to confirm our results and investigate the potential role of BDNF, irisin, and the KP in lifestyle- and training-effects on cognition in the context of dementia and other neurodegenerative disorders. In addition, other potential mediators of effects of physical and cognitive training on neuroplasticity and cognition should be examined. Understanding the biological mechanisms which connect an "active lifestyle" to enhanced brain health will strengthen the support for lifestyle changes and also open possibilities for the development of further treatment strategies, including pharmacological ones.

Conclusion

Associations of irisin and metabolites of the KP with BDNF and cognition on the one hand, and with psychosocial stress as well as cognitive or physical training on the other hand, indicate that these biological measures may constitute candidate mediators of lifestyle influences on cognition and dementia in old age. Further research in larger scale studies is necessary to confirm the results and elucidate the potential role of irisin and kynurenine metabolites in prevention strategies of neurodegenerative diseases.

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2.4. Astrocytes as Mediators of Fkbp5 Polymorphism-Associated Risk for Pathology – First Insights from Novel Humanized Mouse Strains

2.4.1. Summary Study IV

A bigger proportion of the HPA axis regulation occurs in the CNS than in the periphery [253], so we hypothesized that understanding the negative feedback mechanisms in the brain holds a great potential to identify intervention points to normalize stress response signalling. Within each cell, the interference of the chaperone FKBP51 with the maturation of the glucocorticoid receptor complex leads to a functional inhibition of glucocorticoid signaling [358, 196, 167, 220]. Since the encoding gene *FKBP5* is itself a target of glucocorticoid-mediated transcription, an ultra-short intra-cellular feedback loop is formed [266]. This regulatory loop may influence the negative feedback to the HPA axis and thus the duration of the stress response. In PMBCs from human carriers of the single nucleotide polymorphism rs1360780, a higher induction of the *FKBP5* gene and a prolonged secretion of glucocorticoids upon stress exposure was reported for the AT- vs. CG-allele [309].

The contribution of CNS cell types to this *FKBP5*-mediated disinhibition of the HPA axis remained elusive and the influence of both *FKBP5* variants on the stress responsiveness of different cell types in the CNS had not been clarified so far. The overarching research question adressed in **study IV** was thus whether the single nucleotide polymorphism rs1360780 would have an influence on the central regulation of the stress response in *Fkbp5*-humanized mice. Based on the inhibitory potential of *Fkbp5* [192, 142, 465], we hypothesized that CNS cell types with a high baseline expression of *Fkbp5* would display a lower responsiveness to glucocorticoid stimulation (1), which would be further modulated by the baseline expression levels of the glucocorticoid receptor (2). Since CNS cell types respond different to stress [362, 479, 487], we assumed that the basal expression levels of *Fkbp5* and the glucocorticoid receptor (*Nr3c1*) as part of the cellular identity would differ between CNS cell types (3) and mediate their differential responsiveness (4). Assuming that characteristics of the human SNP rs1360780, like higher glucocorticoid-mediated induction of the AT- vs. CG-allele [309], could be transferred to the *Fkbp5*-humanized mice, we expected cells carrying the AT-allele to respond with a stronger induction of *Fkbp5* compared to CG-allele carrying cells (5).

To test these hypotheses, homozygous animals that carry the AT- or the CG-allele were bred from the novel *Fkbp5*-humanized mice C57BL/6NTac-Fkbp5tm4570 (FKBP5) Tac and C57BL/6NTac-Fkbp5tm4571 (FKBP5) Tac, respectively. Primary astrocytes, microglia and neurons of their offspring as well as of homozygous wild type mice as background control were cultured and the expression of *Fkbp5* and *Nr3c1* at baseline was investigated. Moreover, the expression of selected glucocorticoid response element harbouring genes after stimulation with endogenous (corticosterone) or synthetic glucocorticoids (dexamethasone, prednisolone) was investigated. Neurons were stimulated on day 12 *in vitro* (when a full arborization of the neurites was present), microglia were exposed to glucocorticoids 1 day after harvest from the shake flasks (when the cells reverted to a less amoebic shape), and astrocytes were stimulated 15 days after plating (when full confluency and end of replication was reached). Besides the four glucocorticoid doses (with final concentrations of 0.8, 4, 20 or 100nM for dexamethasone and corticosterone or 5.6, 28, 140 and 700nM for the less active prednisolone),

a vehicle control containing the same concentration of solvent like the glucocorticoid stimulations (0.005% dimethyl sulfoxide), and a half medium exchange as handling control were performed. For each set of experiments, all stimulations were carried out in triplicates during the morning and samples were collected four hours later at noon. In total, from CG-allele mice 19 astrocyte, 4 microglia and 19 neuron preparations; from AT-allele mice 9 astrocyte, 11 microglia and 5 neuron preparations and from wild type mice 16 astrocyte, 7 wild type and 38 neuron preparations were analysed. No sample had to be excluded from analyses due to diminished viability after stimulation, as assessed by visual inspection and occasional lactate-dehydrogenase-based viability assays, nor due to low quality of the extracted RNA. To ensure comparability, the input RNA concentrations were normalized and data was set in relation to the vehicle control and to the expression levels of succinate dehydrogenase complex subunit A (Sdha), which we previously determined as stable house keeping gene for all three cell types. To externally validate whether other glucocorticoid response element harbouring genes that were not genetically modified during the humanization process of *Fkbp5* would be differentially induced by glucocorticoids *in vitro*, fold changes of glucocorticoid-induced leucine zipper (*Tsc22d3*), nuclear factor-κ-B-inhibitor α (*NFkBia*), period 1 (*Per1*), sestrin 1 (*Sesn1*) and serum and glucocorticoid regulated kinase 1 (*Sgk1*) expression were analyzed in addition to the stimulated expression levels of *Nr3c1* and the human and murine *Fkbp5*.

As hypothesized, cell type dependent expression differences were present at baseline with astrocytes displaying the lowest *Fkbp5* levels, followed by microglia and neurons. The same observations regarding relative expression between cell types were made for cells from wild type and *Fkbp5*-humanized mice. However, the humanized gene was found to be higher expressed than the murine *Fkbp5* in astrocytes. Western Blot analyses of homogenates from prefrontal cortex, amygdala, and hippocampus 24 hours after subcutaneous injection of dexamethasone qualitatively confirmed that the humanized protein FKBP51 nevertheless becomes expressed in transgenic mice at detectable levels. When comparing baseline gene expression between both humanized strains, no difference within each cell type was observed. Regarding *Nr3c1*, both glial cell types showed the highest expression, while neurons displayed the lowest expression levels. In the stimulation experiments, *Fkbp5* expression levels as well as the relative expression of *Fkbp5* to *Nr3c1* were negatively correlated with cellular glucocorticoid responsiveness. Regarding the humanized mice, the data from the stimulation experiments confirmed the functional responsivity of the humanized *Fkbp5* to different glucocorticoids. The AT-allele showed a stronger increase in expression levels after glucocorticoid exposure than the CG-allele. Moreover, an interaction of cell type and dose was observed with astrocytes showing the highest fold changes in *Fkbp5* expression after stimulation with dexamethasone for 4 hours, followed by microglia, while in neurons no significant change was observed (Fig. 2.7, revised graphical abstract of [429]). Similar findings were obtained with corticosterone and prednisolone, indicating that the observed effects were not specific to dexamethasone but a general response to glucocorticoids. With respect to other glucocorticoid responsive genes, in astrocytes a positive fold change regarding the expression of *Tsc22d3, Per1, Nfκbia* and *Sgk1* and a trend for suppressive effects on *Nr3c1* was observed, while microglia and neurons again showed less or no response, respectively.

In summary, the findings of **study IV** are a first indication that *Fkbp5* expression levels as well as availability of the glucocorticoid receptor shape the responsiveness to glucocorticoids and presumably stress signalling in different CNS cell types. More-

over, the differential induction of *Fkbp5*-variants in the humanized mice confirmed the transgenic mice as a valid tool to causally investigate the molecular mechanisms how the human SNP rs1360780 exerts an impact on stress responsiveness. Evidences from primary CNS cell types of wild type and *Fkbp5*-humanized mice emphasize prominent effects of glucocorticoids on the metabolism in the CNS, since astrocytes showed the strongest responses to glucocorticoids compared to microglia and neurons. Astrocytes might thus mediate *Fkbp5* genotype dependent effects on the cerebral energy supply and coping with stress. The data imply astrocytes as promising cellular target for further research and novel therapies in the context of stress-associated pathology.

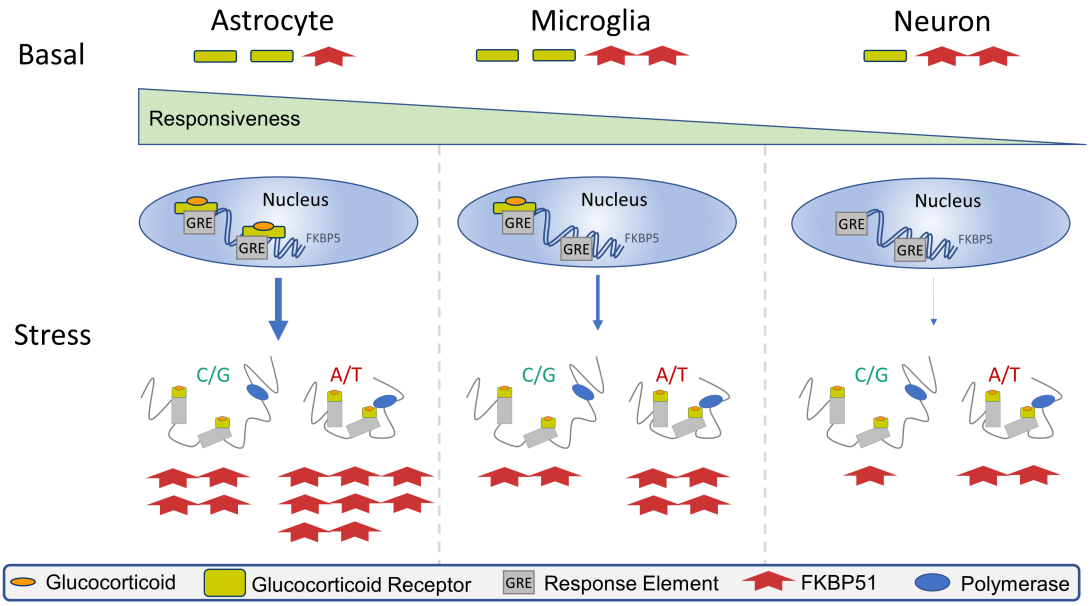

Figure 2.7.
Cell Type Specific Basal Expression of FKBP51 and the Glucocorticoid Receptor Interact with the Humanized *Fkbp5*-Variants to Modulate Responsiveness to Glucocorticoids
Following the acute exposure to glucocorticoids, the cellular levels of *Fkbp5* are changed and thus the responsiveness to a subsequent stimulation with glucocorticoids will deviate from the initial one dependent on timing.

2.4.2. Reprint of the Original Research Article

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RESEARCH REPORT

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FKBP5 polymorphisms induce differential glucocorticoid responsiveness in primary CNS cells – First insights from novel humanized mice

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Abstract

The brain is a central hub for integration of internal and external conditions and, thus, a regulator of the stress response. Glucocorticoids are the essential communicators of this response. Aberrations in glucocorticoid signaling are a common symptom in patients with psychiatric disorders. The gene *FKBP5* encodes a chaperone protein that functionally inhibits glucocorticoid signaling and, thus, contributes to the regulation of stress. In the context of childhood trauma, differential expression of *FKBP5* has been found in psychiatric patients compared to controls. These variations in expression levels of *FKBP5* were reported to be associated with differences in stress responsiveness in human carriers of the single nucleotide polymorphism (SNP) rs1360780. Understanding the mechanisms underlying *FKBP5* polymorphism-associated glucocorticoid responsiveness in the CNS will lead to a better understanding of stress regulation or associated pathology. To study these mechanisms, two novel humanized mouse lines were generated. The lines carried either the risk (A/T) allele or the resilient (C/G) allele of rs1360780. Primary cells from CNS (astrocytes, microglia, and neurons) were analyzed for their basal expression levels of *FKBP5* and their responsiveness to glucocorticoids. Differential expression of *FKBP5* was found for these cell types and negatively correlated with the cellular glucocorticoid responsiveness. Astrocytes revealed the strongest transcriptional response, followed by microglia and neurons. Furthermore, the risk allele (A/T) was associated with greater induction of *FKBP5* than the resilience allele. Novel *FKBP5*-humanized mice display differential glucocorticoid responsiveness due to a single intronic SNP. The vulnerability to stress signaling in the shape of glucocorticoids in the brain correlated with *FKBP5* expression levels. The strong responsiveness of astrocytes to glucocorticoids implies astrocytes play a prominent role in the stress response, and in *FKBP5*-related differences in glucocorticoid signaling. The novel humanized mouse

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lines will allow for further study of the role that *FKBP5* SNPs have in risk and resilience to stress pathology.

KEYWORDS

astrocyte, CNS cell types, *Fkbp5*, glucocorticoid responsiveness, psychiatric disorders

1 | INTRODUCTION

Glucocorticoids are secreted following the diurnal rhythmicity of the hypothalamus-pituitary-adrenal (HPA) axis, and on demand during challenging situations that require allostatic adjustment. Glucocorticoids influence a plethora of physiological processes via transactivation or transrepression of gene expression. A high demand on the stress response system, such as with chronic exposure to stress, can lead to allostatic over-load and stress-related pathologies (Juster et al., 2010; McEwen, 2004; Nold et al., 2019). In stress-associated disorders like post-traumatic stress disorder (PTSD) or depression, dysregulation of the HPA axis is a common shared pathology, although the specifics of this dysregulation can range from hypo- to hyper-sensitivity (Pariante, (2009); Perrin et al., 2019). As chronic stress has a direct impact on HPA-axis function, it is considered to be an environmental risk factor for the development of many disorders (Chandola et al., 2006; Cohen et al., 2012; Lagraauw et al., 2015; Machado et al., 2014). The brain is a central hub for integration of psychological stress perception and consequent physiological stress responses. This can be demonstrated, for example, at the circuit level by combining neuroimaging with paradigms meant to induce stress (Rauch et al., 2006). Of course, these circuit-level responses are not only purely driven by neuronal activity but also have a glial cell contribution. It is known that at the cellular level, either diurnal circulating or stress-induced glucocorticoids impact neurons, microglia, and astrocytes to a different extent (Lucassen et al., 2014; Radley et al., 2015; Ressler & Smoller, 2016). Hence, the sensitivity to glucocorticoids, and regulators of such sensitivity, may be addressed at the cellular level. The chaperone FK506-binding protein 51 (FKBP51), which is encoded by the *FKBP5* gene, is a potent negative regulator of glucocorticoid function. FKBP51 binds to heat-shock protein 90 (Hsp90) complexes, and works within this complex to aid in folding or stabilizing a number of 'client' proteins (Lorenz et al., 2014). When the client protein is the glucocorticoid receptor, the receptor is expected to be in a low-affinity state. Release of FKBP51 from this complex configuration is necessary for the glucocorticoid receptor to be active (Fries et al., 2017; Pirkl & Buchner, 2001; Scammell et al., 2003; Wochnik et al., 2005). As *FKBP5* is a target of glucocorticoid-mediated transcription, activation of the glucocorticoid receptor with its ligand induces *FKBP5* and, subsequently, its protein FKBP51, again providing functional inhibition. Therefore, *FKBP5*/FKBP51 represents an ultra-short negative feedback loop within the cell to terminate glucocorticoid signaling (Chun et al., 2011; Jaaskelainen et al., 2011; Yeo et al., 2017). Expression levels of *FKBP5* are, therefore, a critical setscrew for glucocorticoid sensitivity.

In the human population, there is a wide variation in perception of stressful challenges, and variation in physiological expression of stress. Single nucleotide polymorphisms (SNPs) within the *FKBP5* gene locus have been reported to impact stress responsivity, and risk or resilience to psychiatric disorders (Appel et al., 2011; Binder, 2009; Criado-Marrero et al., 2019; Liebermann et al., 2017; Wilker et al., 2014). Differential expression levels of *FKBP5* have preclinically been shown to impact on stress-coping behavior and the SNP rs1360780 was reported to profoundly influence stress coping, suggesting *FKBP5* genotypes as one source of human variation (Ising et al., 2008; Touma et al., 2011). In combination with early-life adversity, demethylation of *FKBP5* has been reported (Hohne et al., 2014; Klengel et al., 2013), which could lead to a higher expression of *FKBP5* rendering the affected individual more prone to glucocorticoid resistance and psychiatric disorders (Binder et al., 2008; Zimmermann et al., 2011). The ability of the A/T or C/G rs1360780 allele to impart differential stress responses could contribute to the wide variation in stress responses observed in the human population.

These SNPs do not exist in laboratory rodents. Hence, in order to study the effect of these human gene variants preclinically, two novel humanized mouse lines carrying the risk-associated rs1360780-A/T or the resiliency-associated rs1360780-C/G allele of the human *FKBP5* were developed at Taconic Biosciences. Hereafter, these lines are referred to as risk (A/T) and resilience (C/G), respectively. There were three goals in these first studies: (1) establish that the two mouse lines, carrying only a single nucleotide difference, responded to glucocorticoid stimulation, therefore, validating both the functional polymorphism and intact glucocorticoid signaling following humanization; (2) determine if, in primary cell culture, differential responses between risk (A/T) and resilience (C/G) could be observed; and (3) assess relative expression across cell types and association with glucocorticoid responsiveness. In these novel strains, we investigated whether the human risk or resiliency version of *FKBP5* would influence acute glucocorticoid responsiveness

of primary astrocytes, microglia, and neurons. Based on the inhibitory capacity of *FKBP5* on glucocorticoid signaling, we hypothesized that high basal expression level of *FKBP5* would be associated with a decreased responsiveness to glucocorticoids and that the risk (A/T) allele of *FKBP5* should show a higher reactivity to glucocorticoids than the resiliency allele. A more far reaching goal is for these mice to serve as a unique tool to further study the influence of the human *FKBP5* gene variants on the risk and resilience to stress-related disorders.

2 | MATERIALS AND METHODS

2.1 | Generation of FKBP5 – Transgenic mice

Taconic Biosciences was commissioned to generate two novel transgenic mouse models carrying either the cytosine (C)/guanidine (G) variant at position 3622 in the human FKBP5 gene (Ensembl gene ID: ENSMUSG00000024222; NCBI gene ID: 14229) or the risk-associated adenine (A)/thymidine (T) version of rs1360780 (Insertion gene identifier: ENSG00000096060 [Ensembl gene ID]; 2289 [NCBI gene ID]). This process was performed under the scientific guidance of Dr. Elisabeth Binder from the Max Planck Institute for Psychiatry in Munich. The targeting strategy for the constitutively humanized FKBP5 gene was based on the NCBI transcript NM_010220_4 (Ensembl transcript ENSMUST00000079413, Fkbp5_001) for the mouse and the transcript NM_001145775_2 (ENSMUST00000536438, Fkbp5_201) for the human gene. Using BAC clones from the mouse C57BL/6J RPCI-23 and human RPCI-11 BAC and/or CalTechD libraries, a targeting vector was generated. The mouse genomic region between exon 2, containing the translation initiation codon, and exon 11, containing the termination codon, has been replaced with the human counterpart containing a neomycin resistance (NeoR, flanked by FRT, exon 3) and a puromycin resistance (PuroR, flanked by F3, exon 11) for positive selection of clones. A schematic map of the targeting vector (Figure SA1) and the sequence (Supplementary Material Text 1) is provided. The linearized DNA targeting vector was transfected via electroporation into the Taconic Biosciences C57BL/6N Tac ES cell line, incorporated via homologous recombination, and recombinant clones were isolated based on double-positive (NeoR and PuroR) and negative (Thymidine kinase) selections. Single integration and homologous recombination at the 5′ and 3′ side were assessed via digestion with restriction enzymes (BauI, MfeI, KpnI, SpeI, EcoRI, BmtI, ScaI, EcoRV, and PacI) and southern blotting as well as PCR-based sequencing of the 5′ and 3′ junction between human and murine regions. The selected heterozygote-targeted ES cells were transiently transfected with the circular vector pCAG-Flpe-pA (3465) containing a Flp recombinase targeting the F3, F5, or FRT sites as well as the vectors pCAG-Cre for loxP sites and phiC31 for attB/attP sites, via nucleofection for in vitro removal of the selection markers. Recovery of the humanized allele was confirmed using PCRs and tested for sensitivity to the respective antibiotics. The selected ES cells containing the humanized allele were transferred into blastocysts and the resulting chimeras were used for breeding with C57BL/6NTac mice and founding the novel mouse strain C57BL/6NTac-Fkbp5tm4570 (FKBP5) Tac, carrying the risk-associated rs1360780-A/T or the mouse-line C57BL/6NTac-Fkbp5tm4571 (FKBP5) Tac carrying the resiliency version, respectively. Breeding of the homozygote humanized mice and their wild types was performed in house. The ownership of these animals was transferred to Taconic to make them publicly available.

2.2 | Primary cell cultures

Protocols were validated in house for optimal yield and development of cell type-specific features and experiments were performed under the allowance of the regional council for animal welfare (Regierungspräsidium Tübingen, Baden-Württemberg, Germany).

To obtain single cell suspensions for neuronal cultures, cortex and hippocampus of embryos at E16.5 were dissected, enzymatically digested, and mechanically dissociated. If viability was above 90%, cells were seeded into PDL-Laminin–coated plates in a density of 100,000 cells per 24-well and incubated at 37°C and 5% CO_2. Half exchanges of the serum-free culture medium (5 ml GlutaMax, 10 ml SM1-Supplement [#05711; Stemcell Technologies, Köln, Germany], 5 ml HEPES 1 M [#83264-100Ml-F; Sigma Aldrich, Taufkirchen, Germany], and 500 ml Neurobasal [#12348017; Invitrogen]) were performed in intervals of 3–4 days.

Cell suspensions for glial cultures were obtained by isolating cells from the cortices of neonates via enzymatic digestion with DNase (#LS002139; Worthington, NJ, USA) and 2.5% trypsin (#15090046; Invitrogen) followed by mechanical homogenization in flask medium (10% FCS, 5 ml Penicillin/Streptomycin, 5 ml HEPES, and 500 ml advanced DMEM [#12491015; Invitrogen]). Cells were cultured in precoated flask (75 cm^2 flasks, Poly-L-Ornithin Hydrobromid [MW: ≤30,000–70,000 Dalton, #P3655; Sigma]) at 37°C with 5% CO_2. Every 3–4 days, the flasks were washed with PBS (#14040174; Invitrogen) and refilled with flask medium. Microglia were harvested in 3–4 days and plated at a density of 150,000 cells per 24-well of uncoated PRIMARIA plates (#353847; Corning, Germany).

For plating of astrocytes, remaining microglia were shaken off, flasks were washed, and the astrocyte layer was

detached using 0.05% trypsin-EDTA solution (#25300054; Invitrogen). Astrocytes were suspended in 50-ml advanced DMEM containing 10% FCS to stop trypsination per flask and 1 ml of this suspension was used per 24-well of a PRIMARIA plate. On the next day, a full medium exchange was performed. On post-plating day (PPD) 8, a confluent astrocyte layer was obtained and cells were exposed to AraC medium (#251010; Cytosine Arabinoside, Sigma, 8 μM) for 4 days. On PPD11, the medium was exchanged to LME medium (L-leucine methyl esters, #L1002; Sigma, 50 mM) for 1 hr and astrocytes were subsequently washed three times with medium. On PPD14, the medium was exchanged to serum-free medium and the assay was performed the next day.

2.3 | Stimulation with glucocorticoids

Stimulation was performed between 08:00 a.m. to 10:00 a.m. with neuronal cultures being stimulated on day in vitro 12, while microglia were stimulated 1 day and astrocytes 15 days after plating, respectively. Stocks of glucocorticoids in dimethyl sulfoxide (DMSO) were freshly diluted 1:200 in the respective warmed culture medium and cells were stimulated by replacing 0.5 ml of the medium in the well with the obtained agonist solutions so that final concentrations of 0.8, 4, 20, or 100 nM for dexamethasone and corticosterone or 5.6, 28, 140, and 700 nM for prednisolone were obtained. To control for manipulation of vehicle effects, a half medium exchange was performed or cells were treated with medium containing 0.005% DMSO, respectively. To investigate transcriptional responses to an acute challenge, after 4 hr of incubation cells were lysed in 250 μl RLT buffer (#79216; Qiagen, Hilden, Germany) containing 1% beta-mercapto-ethanol (#M3148-100Ml; Sigma), and frozen at −20°C prior to RNA isolation. No sample had to be excluded from analyses due to diminished viability after stimulation, as assessed by visual inspection and occasional lactate-dehydrogenase–based viability assays.

2.4 | Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated using RNeasy Plus kit (#74192; Qiagen) following the manufacturer's recommendations. Prior to reverse transcription of the RNA, integrity was confirmed to be above a RNA integrity number of 8 on occasion using the Fragment analyzer (Thermo Fisher Scientific, Langenselbold, Germany) and the obtained yield was determined spectrophotometrically (QIAxpert, Qiagen) to allow normalization of the input RNA concentration to 500 ng. Reverse transcription of the total mRNA to complementary DNA was performed using the high-capacity cDNA kit (#4368813; Qiagen). All TaqMan gene expression assays were labeled with FAM (#4352042; Thermo Fisher) and used in conjunction with the fast universal PCR Master Mix (#4351368; Thermo Fisher). The used primers were as follows: succinate dehydrogenase complex subunit A (Sdha, Mm01352366_m1), human FKBP5 (Hs01561006_m1), murine Fkbp5 (Mm00487403_m1), FK506 binding protein 4 (Fkbp4, Mm00487391_m1), glucocorticoid receptor (Nr3c1, Mm00433832_m1), mineralocorticoid receptor (Nr3c2, Mm01241596_m1), glucocorticoid-induced leucine zipper (Tsc22d3, Mm01306210_g1), nuclear factor-κ-B-inhibitor α (NFkBia, Mm00477798_m1), period 1 (Per1, Mm00501813_m1), sestrin 1 (Sesn1, Mm01185732_m1), and serum and GC-regulated kinase 1 (Sgk1, Mm00441380_m1). Samples were analyzed in technical triplicates on a QuantStudio 6 (Thermo Fisher). All gene expression levels were normalized relative to the cycle thresholds measured for Sdha and relative to DMSO-treated cells within the same cell type, genotype, and mouse strain for stimulation experiments.

2.5 | Statistics

Technical replicates of stimulations, RNA isolations, cDNA, and TaqMan were summarized per sample so that each individual data point shown and used for analysis represent one individual biological sample. Thus, the number of data points represents the replicates per group. All data visualization and analysis were performed using R version 3.6.1. Distribution of data points and model residuals were tested for departure from normality and homogeneity of variances using a Shapiro-Wilk test, visual inspection, as well as the Bartlett's test or Levene's test, respectively. One-way ANOVA of type II for baseline values of Fkbp5 in wild-type cells was used to compare between untreated (native) and DMSO-treated cells. A Kruskal–Wallis ranked ANOVA was used for comparing the dCT data of the glucocorticoid receptor expression due to not normally distributed residuals. A two-way ANOVA of type I was used for the (ranked) fold-change data with cell type and glucocorticoid dose as model factors. In case of significant findings in the ANOVA, the Tukey Honest Significant Difference test or Wilcoxon test was used post hoc to determine which groups differed and to adjust *p*-values for multiple comparisons. The contrasts of interest were defined a priori. Rank-based tests were applied when required. All tests were performed in a two-sided manner.

3 | RESULTS

3.1 | Cell type-specific expression of *Fkbp5* and *Nr3c1* in the CNS

In primary murine wild-type (WT) astrocytes, microglia, and neurons, the basal mRNA expression of the glucocorticoid

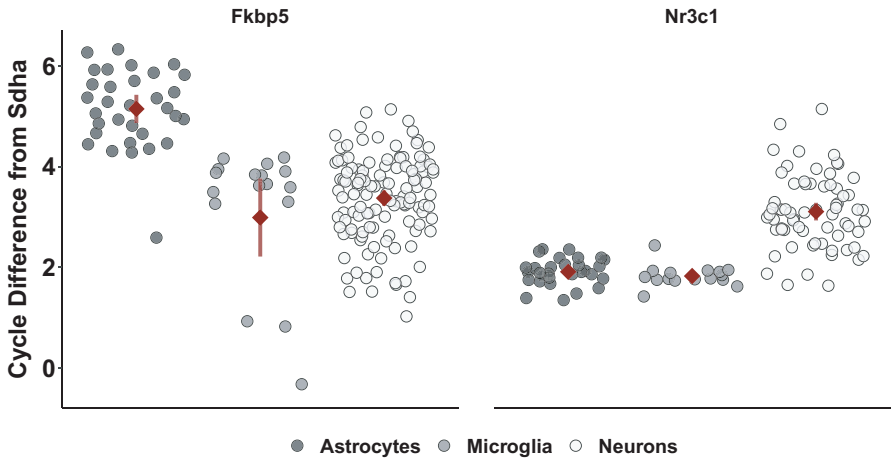

FIGURE 1 *Sdha*-normalized mRNA expression levels of *Fkbp5* (left panel) and *Nr3c1* (right panel) of primary murine wild-type astrocytes (dark grey, left), microglia (grey, middle), and neurons (ecru, right). Individual data points are shown alongside with their mean ± 95% CI (red). High values in the PCR cycles needed to reach the set threshold represent low amounts of the targeted mRNA and, hence, a low expression of the gene while low cycle numbers indicate higher expression

receptor (*Nr3c1*) and its functional inhibitor FKBP51 (*Fkbp5*) was examined and is visualized as qPCR cycle number difference from the housekeeper *Sdha* (Figure 1). A one-way ANOVA revealed that cell types differed in the expression of *Fkbp5* ($F(3, 168) = 33.54$; $p < 0.0001$) and a Tukey Honest Significance *Post Hoc* Test showed that astrocytes had the lowest expression of the inhibitory chaperone *Fkbp5* (5.14 ± 0.78) compared to microglia (2.99 ± 1.56, $p < 0.0001$) and neurons (3.37 ± 0.85, $p < 0.0001$). *Nr3c1* was also differentially expressed between the cell types (Kruskal–Wallis rank-sum test $\chi^2(3) = 64.07$, $p < 0.0001$) with the highest expression in both glial cell types (astrocytes = 1.91 ± 0.26, microglia = 1.83 ± 0.22) compared to neurons (3.11 ± 0.72, $p < 0.0001$).

3.2 | Induction of glucocorticoid response element containing genes differs across cell types

To determine responsiveness to glucocorticoids, the different cell types were stimulated with increasing doses of the synthetic glucocorticoid dexamethasone. In response, prominent fold changes in the expression of glucocorticoid-responsive genes were observed (Table 1). A ranked ANOVA revealed an interaction of cell type and dose, $F(8, 309) = 95.9$, $p < 0.0001$, on the induction of *Fkbp5*. Subsequent analysis indicated that astrocytes (21.01 ± 10.52) differed from microglia (7.81 ± 8.09) and neurons (2.13 ± 1.76). Furthermore, all dexamethasone doses (10.85 ± 11.11) were different from vehicle (1.25 ± 1.28). For the glucocorticoid receptor, the ANOVA of ranks suggested differences between cell types, $F(2, 237) = 37.2$, $p < 0.00001$, and *post hoc* testing revealed astrocytes (0.72 ± 0.20) to have a reduced expression of *Nr3c1* after stimulation. For *Tsc22d3*, the ANOVA of ranks suggested a highly significant interaction of cell type and dose, $F(8, 237) = 38.0$, $p < 0.0001$, which was attributable *post hoc* to both glial cell types differing from neurons (2.73 ± 2.77). Astrocytes (16.92 ± 7.95) were more responsive than microglia (9.90 ± 10.31), and stimulated (11.09 ± 9.08) were different from vehicle-treated (1.33 ± 0.96) cells. Fold changes of *Per1* showed a significant interaction of cell types and dexamethasone dose, $F(8, 238) = 21.9$, $p < 0.0001$, in the ANOVA of ranks that originated from stronger responses in astrocytes (4.42 ± 1.80) than in microglia (2.19 ± 1.36) and neurons (1.18 ± 0.54). The difference between vehicle-treated (1.11 ± 0.50) and dexamethasone-treated (5.07 ± 1.08) astrocytes was also demonstrated. Ranked fold changes of *Nfκbia* were found to vary between cell types and dexamethasone stimulation, $F(8, 238) = 12.5$, $p < 0.0001$, which was attributed to differences between astrocytes (3.12 ± 1.25) and microglia (2.03 ± 1.53) and differences between all glial cells and neurons (1.3 ± 0.47). Differences were also demonstrated between stimulated (2.32 ± 1.29) and vehicle-treated (1.11 ± 0.73) cells. For the ranked fold changes of *Sgk1*, the same interaction was observed, $F(8, 238) = 9.67$, $p < 0.00001$, and confirmed *post hoc* to be based on astrocytic responsiveness to dexamethasone (3.50 ± 1.06) compared to vehicle-treated astrocytes (1.04 ± 0.30).

3.3 | Expression levels of *Fkbp5* correlate with cellular glucocorticoid responsiveness

Correlation analyses of basal expression levels of *Fkbp5* and *Nr3c1* with induction of glucocorticoid response element containing genes indicated a strong relation (Table 2). As measure of glucocorticoid responsiveness, the cycle difference from

TABLE 1 Dexamethasone-induced fold changes of glucocorticoid response element containing genes in primary cell types of the central nervous system

Cell type	Astrocytes					Microglia					Neurons				
Dexamethasone (nM)	**0**	**0.8**	**4**	**20**	**100**	**0**	**0.8**	**4**	**20**	**100**	**0**	**0.8**	**4**	**20**	**100**
Fkbp5 304 *df*															
N	15	18	19	20	18	9	5	7	7	6	54	12	43	51	40
Mean	1.14	27.35	24.36	23.90	24.49	2.04	7.31	7.86	10.34	13.86	1.15	2.14	1.84	2.81	2.89
SD	0.84	5.95	6.86	6.11	5.04	3.21	4.14	5.28	9.45	12.16	0.71	0.62	1.44	2.36	1.73
LCL	−1.83	24.64	21.72	21.32	21.77	−1.80	2.16	3.51	5.98	9.16	−0.42	−1.19	0.09	1.20	1.07
UCL	4.12	30.07	27.00	26.47	27.20	5.88	12.46	12.22	14.69	18.57	2.71	5.46	3.60	4.43	4.71
Nr3c1 237 *df*															
N	14	18	18	19	18	9	5	7	7	7	33	12	27	31	27
Mean	1.01	0.70	0.69	0.67	0.62	1.02	0.83	0.91	0.90	0.85	1.39	1.16	1.41	1.43	1.16
SD	0.17	0.18	0.13	0.17	0.10	0.18	0.08	0.31	0.29	0.22	0.75	0.24	0.82	0.80	0.74
LCL	0.57	0.31	0.31	0.29	0.23	0.47	0.10	0.29	0.28	0.23	1.10	0.69	1.10	1.13	0.84
UCL	1.45	1.08	1.08	1.04	1.01	1.56	1.57	1.53	1.52	1.47	1.67	1.64	1.73	1.72	1.48
Nfkbia 238 *df*															
N	14	18	18	19	18	9	5	7	7	7	34	12	27	31	27
Mean	1.01	3.64	3.58	3.54	3.33	1.47	1.75	2.19	2.35	2.44	1.06	1.46	1.24	1.41	1.44
SD	0.14	0.87	0.98	0.92	0.94	1.73	0.51	1.45	1.74	1.77	0.36	0.46	0.47	0.48	0.51
LCL	0.36	3.07	3.01	2.98	2.76	0.66	0.66	1.27	1.44	1.53	0.64	0.76	0.78	0.97	0.98
UCL	1.66	4.21	4.15	4.09	3.90	2.28	2.83	3.11	3.27	3.36	1.48	2.16	1.71	1.84	1.91
Per1 238 *df*															
N	14	18	18	19	18	9	5	7	7	7	34	12	27	31	27
Mean	1.04	5.29	5.38	4.87	4.74	1.24	2.49	2.56	2.55	2.45	1.10	1.30	1.13	1.14	1.33
SD	0.3	1.13	0.94	1.17	1.08	0.81	1.03	1.22	1.90	1.41	0.47	0.43	0.54	0.48	0.70
LCL	0.37	4.69	4.79	4.30	4.15	0.40	1.37	1.61	1.60	1.50	0.67	0.57	0.65	0.69	0.84
UCL	1.71	5.88	5.97	5.45	5.33	2.08	3.61	3.51	3.50	3.40	1.53	2.02	1.62	1.59	1.81
Sgk1 238 *df*															
N	14	18	18	19	18	9	5	7	7	7	34	12	27	31	27
Mean	1.04	3.41	3.43	3.24	3.93	1.24	2.17	1.98	2.31	2.69	1.13	1.08	1.36	1.34	1.46
SD	0.3	0.92	0.88	0.85	1.61	1.01	1.19	1.36	1.95	2.46	0.61	0.64	0.89	0.73	0.86
LCL	0.24	2.70	2.73	2.55	3.22	0.25	0.84	0.86	1.18	1.57	0.62	0.21	0.78	0.81	0.88
UCL	1.84	4.11	4.14	3.92	4.63	2.24	3.51	3.11	3.44	3.82	1.64	1.94	1.93	1.88	2.03

(Continues)

TABLE 1 (Continued)

Cell type	Astrocytes					Microglia					Neurons				
Dexamethasone (nM)	**0**	**0.8**	**4**	**20**	**100**	**0**	**0.8**	**4**	**20**	**100**	**0**	**0.8**	**4**	**20**	**100**
Tsc22d3 237 *df*															
N	14	18	18	19	18	9	5	7	7	7	33	12	27	31	27
Mean	1.04	20.55	20.06	20.36	18.87	1.06	20.75	11.25	10.37	11.71	1.52	4.52	2.70	3.56	2.51
SD	0.35	3.68	3.10	4.75	4.82	0.32	10.43	9.90	10.29	10.20	1.18	1.49	2.79	4.03	2.19
LCL	−2.35	17.56	17.07	17.45	15.88	−3.17	15.08	6.45	5.57	6.91	−0.69	0.86	0.26	1.28	0.07
UCL	4.44	23.54	23.05	23.27	21.86	5.29	26.43	16.04	15.17	16.51	3.73	8.19	5.14	5.84	4.95

Abbreviations: nM, nano-molar; *N*, number of observations; *SD*, standard deviation; *df*, degrees of freedom; LCL, lower 95% confidence level; ULC, upper 95% confidence level.

housekeeper assessed after exposure to 100nM dexamethasone was used. The cycle difference after stimulation negatively correlated with the basal expression of *Fkbp5* for the genes *Nfkbia* ($\beta = -0.46$, $p = 0.006$), *Tsc22d3* ($\beta = -0.42$, $p = 0.01$), and *Sgk1* ($\beta = -0.34$, $p = 0.05$) and was marginally significant for *Nr3c1*, *Sesn1*, and *Fkbp5*. Including the basal levels of *Nr3c1* resulted in significant correlations of the differences from *Fkbp5* to *Nr3c1* with all tested glucocorticoid-responsive genes. The difference of *Fkbp5* to *Nr3c1* expression levels at baseline strongly correlated with cell type ($\rho = 0.8$, $p < 0.00001$).

3.4 | FKBP5 is expressed in CNS cells of humanized mice

In primary astrocytes, microglia and neurons derived from the *FKBP5*-humanized mouse lines carrying either the risk (A/T) allele or the resiliency (C/G) allele the, same cell type-specific expression differences of *FKBP5*, as observed for WT cells, were found (Figure SA2). An ANOVA of ranks suggested a significant difference in basal *FKBP5* expression, $F(2, 101) = 33.80$, $p < 0.0001$, and a trend of the transgenic strains expressing less *FKBP5* than wild type ($F(1, 101) = 3.36$, $p = 0.07$). The difference between cell types was *post hoc* found to originate from astrocytes (5.10 ± 1.35) differing from microglia (3.73 ± 0.62) and neurons (3.43 ± 0.60). To confirm that the humanized FKBP51 protein is expressed in these mice, Western Blot analyses were performed. An exemplary blot of prefrontal cortex, amygdala, and hippocampus homogenate from a humanized mouse and a wild-type mouse 24 hr after stimulation with 0.1 mg/kg subcutaneous dexamethasone is provided (Figure SA3) together with a description of the method.

3.5 | Differential responsiveness of human FKBP5 rs1360780 variants

The responsiveness of these primary cell cultures to glucocorticoids was investigated by measuring the fold change of *FKBP5* expression after stimulation with dexamethasone (Figure 2). In astrocytes and microglia, a dose-responsive increase in *FKBP5* mRNA expression was detected, which was stronger in cells carrying the risk (A/T) than the resilience (C/G) allele or the murine *Fkbp5*. In the ANOVA of ranks, an interaction between strains and dose was found in astrocytes, $F(8, 260) = 14.1$, $p < 0.0001$. *Post hoc* testing showed this was attributable to a significant difference between the risk (A/T) allele carrying astrocytes that were treated with 100 nM or 20 nM compared to wild-type or resilience (C/G) allele carrying astrocytes. With respect to dose, only the risk (A/T) allele carrying astrocytes showed a dose-responsive effect to dexamethasone (55.3 ± 28.6 at 100 nM versus 1.2 ± 0.7 in vehicle-treated

TABLE 2 Correlation of glucocorticoid response element containing gene induction after stimulation with 100 nM dexamethasone to basal *Fkbp5* and *Nr3c1* expression

	Basal *Fkbp5*					Basal difference *Fkbp5-Nr3c1*				
Gene	**Method**	**S/*t***	***df***	***p*-value**	**β**	**Method**	**S/*t***	***df***	***p*-value**	**β**
Fkbp5	Spearman	9,434		0.06	−0.32	Spearman	11,000		0.001	−0.54
Nfkbia	Spearman	10,424		0.006	−0.46	Spearman	12,486		<0.00001	−0.75
Nr3c1	Pearson	−1.99	33	0.06	−0.33	Pearson	−5.96	33	<0.00001	−0.72
Sesn1	Pearson	−1.93	33	0.06	−0.32	Pearson	−6.04	33	<0.00001	−0.72
Sgk1	Pearson	−2.09	33	0.05	−0.34	Pearson	−3.44	33	0.002	−0.51
Tsc22d3	Spearman	10,122		0.01	−0.42	Spearman	11,114		0.0006	−0.56

Abbreviations: nM, nano-molar; S, Spearman's rank correlation statistic sum of squared rank differences; *t*, Pearson's statistic *t*-value; *df*, degrees of freedom; β, correlation estimate.

astrocytes). In resilience (C/G) allele carrying astrocytes or wild types, a dose-responsive induction was visible but not of statistical significance and both strains did not differ from each other. In microglia, the interaction of strain and dexamethasone was significant ($F(8, 123) = 7.0$, $p < 0.0001$). This was due to differences in microglia carrying the risk (A/T) allele between vehicle-treated (1.1 ± 0.5) and stimulated cells (100 nM dexamethasone: 10.5 ± 4.7; 20 nM dexamethasone: 7.3 ± 4.6). For neurons, no significant changes were observed.

3.6 | Human SNP rs1360780 modifies cellular glucocorticoid responsiveness

To rule out dexamethasone-specific effects, the cellular responsiveness to other glucocorticoids was assessed. Stimulation with corticosterone or prednisolone resulted in a dose-responsive increase in *FKBP5* expression in astrocytes with risk (A/T) allele carrying cells trending to respond more than resiliency (C/G) allele carriers (Figure 3). The ANOVA of ranks suggested a significant dose effect in astrocytes, $F(4, 252) = 53.8$, $p < 0.0001$, which was caused by differences between astrocytes stimulated with 100 nM corticosterone (7.8 ± 4.8) and astrocytes treated with lower amounts (20 nM: 3.7 ± 2.7; 4 nM: 1.7 ± 1.6; 0.8 nM: 2.0 ± 3.8) or vehicle (2.7 ± 4.5). Furthermore, the effect of strain was significant, $F(2, 252) = 3.4$, $p < 0.04$, originating from differences between wild types (3.4 ± 3.8) and risk (A/T) allele carriers (4.6 ± 5.8; $t(690) = -3.3$, $p < 0.04$). In microglia, the ANOVA of ranks suggested a significant interaction of corticosterone and strain, $F(8, 110) = 13.0$, $p < 0.0001$, based on differences between microglia treated with 100 nM corticosterone

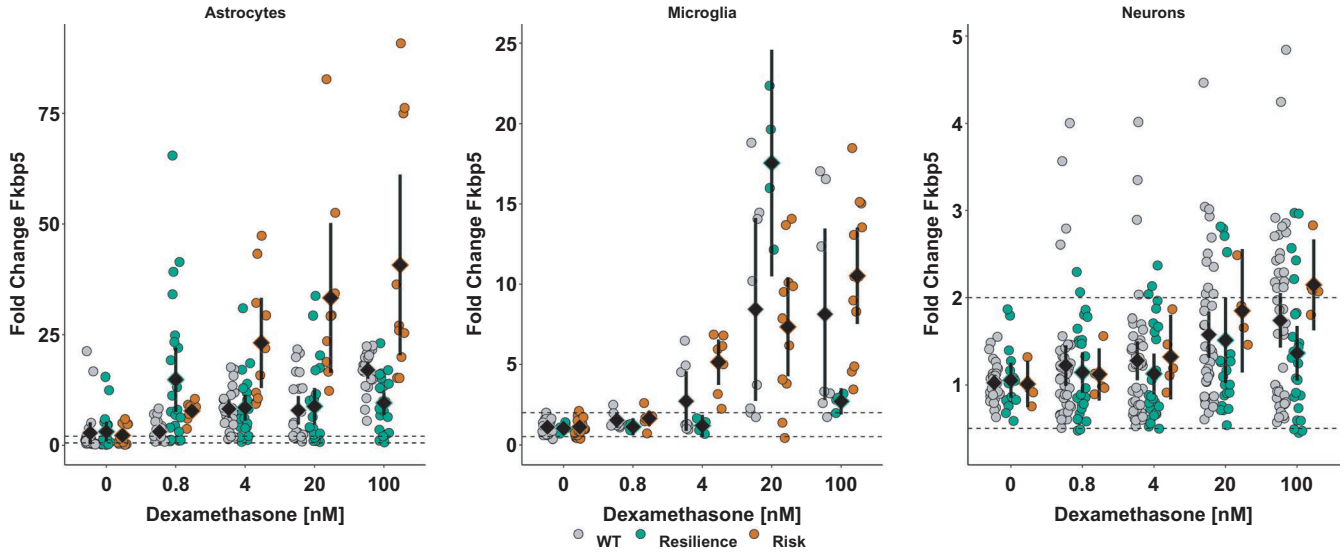

FIGURE 2 Dose-responsive increase in *FKBP5* expression in primary murine astrocytes (left), microglia (middle), and neurons (right) after 4 hr stimulation with dexamethasone relative to expression of the housekeeper *Sdha* and *FKBP5* expression in DMSO-only treated cells. Cells carrying the risk version of the human FKBP5 are depicted in orange while cells carrying the rs1360780-C/G are depicted in green and cells derived from wild-type mice are visualized in grey. Data points are shown alongside with their mean ± 95% CI. Dashed lines at 0.5 and 2 delimit the area of technically unclear change, which is based on the variability in *Sdha* expression levels

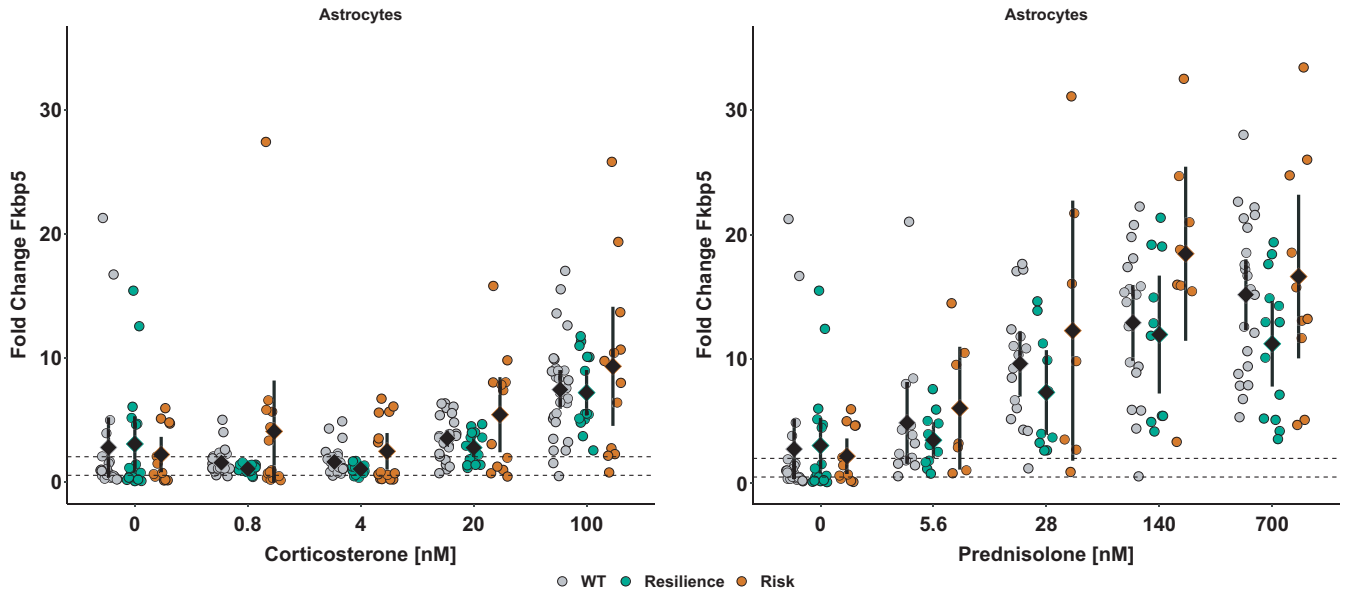

FIGURE 3 Astrocytes respond to a 4 hr stimulation with corticosterone (left) and prednisolone (right) in a dose-responsive manner. Cells derived from humanized mice carrying rs1360780-A/T (orange) trend to have higher fold changes in *FKBP5* mRNA expression than astrocytes carrying the resilience version with the C/G-allele (green) or wild-type astrocytes (grey). Data points are visualized alongside with their mean ± 95% CI. Dashed lines at 0.5 and 2 delimit the area of technically irrelevant change, as determined by variability of *Sdha* expression, as this gene was used as a house keeper to normalize for potential variation in utilized RNA concentrations

(8.6 ± 5.4) and lower doses (20 nM: 4.0 ± 2.0; 4 and 0.8 nM: 1.6 ± 0.4) or vehicle (1.1 ± 0.4) that varied between strains. In neurons, no changes bigger than technical resolution were found. For prednisolone, the ANOVA of ranks suggested significant influence of strain, $F(2, 181) = 5.2$, $p < 0.007$, with risk (A/T) allele carrying astrocytes showing significant responses to prednisolone at concentration of 700 nM (16.6 ± 9.2) and 140 nM (18.5 ± 8.4) compared to vehicle-treated (2.2 ± 2.2) but not in resilience (C/G) allele carrying astrocytes.

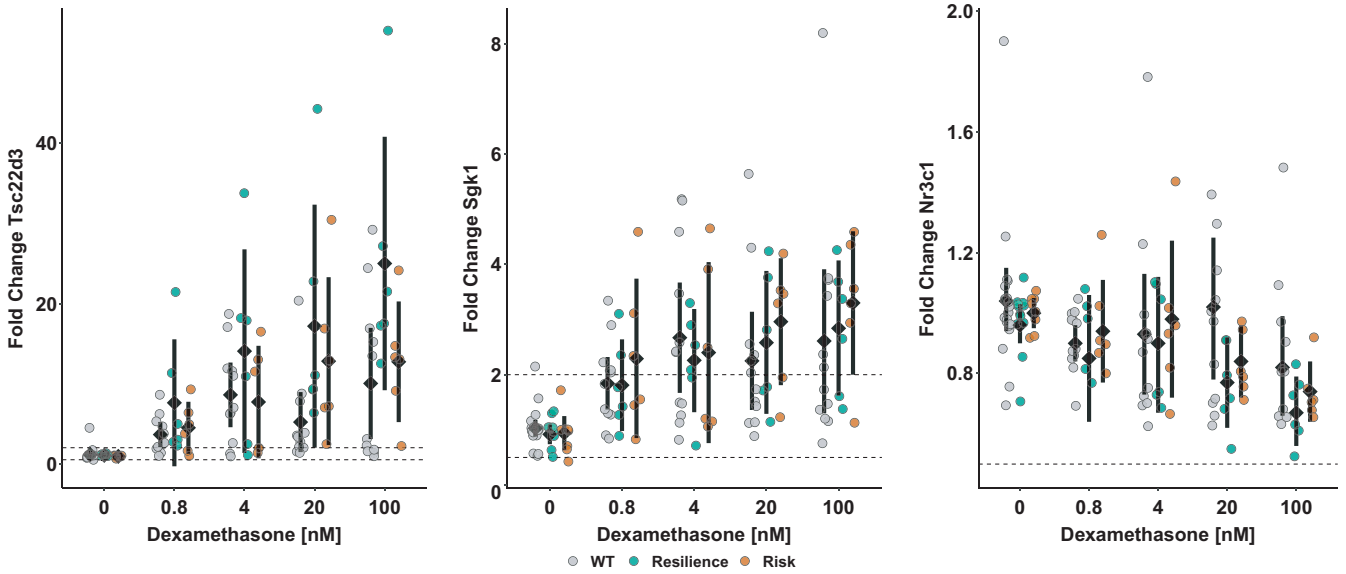

FIGURE 4 Astrocytes respond to a 4 hr stimulation with dexamethasone in a dose-responsive manner with changes in gene expression of *Tsc22d3* (left), *Sgk1* (middle), and *Nr3c1* (right). Cells derived from humanized mice carrying rs1360780-C/G (green) have higher fold changes in *Tsc22d3* and by trend *Nr3c1* mRNA expression than astrocytes carrying the risk version with the A/T-allele (orange) or wild-type astrocytes (grey). Data points are visualized alongside with their mean ± 95% CI. Dashed lines at 0.5 and 2 delimit the area of technically irrelevant change, as determined by variability of *Sdha* expression, as this gene was used as a housekeeper to normalize for potential variation in utilized RNA concentrations

3.7 | Functional induction of other glucocorticoid response element harboring genes

To address whether the humanization of the murine *Fkbp5* locus would interfere with the induction or suppression of other glucocorticoid response element harboring genes, *Tsc22d3*, *Nr3c1*, and *Sgk1* expression was analyzed (Figure 4). For *Tsc22d3*, the ANOVA suggested a significant interaction of strain and dexamethasone dose, $F(8, 119) = 2.1$, $p = 0.04$, for which *post hoc* was attributed to resiliency (C/G) allele carrying astrocytes after dexamethasone treatment differing from vehicle-treated cells (1.1 ± 0.1) and resiliency (C/G) allele astrocytes exposed to 100 nM dexamethasone (25 ± 15.1) differing from wild-type cells exposed to the same dose (10 ± 10.3). For *Sgk1* expression, only the main effect of dose was significant in the ANOVA, $F(4, 123) = 13.5$, $p < 0.00001$, which was due to astrocytes treated with 100 nM dexamethasone (2.8 ± 1.7) differing from vehicle-treated cells (1 ± 0.3). With respect to *Nr3c1*, the ANOVA suggested a statistically significant effect of dose, $F(4, 123) = 5.1$, $p = 0.0008$, stemming from differences between vehicle-treated (1 ± 0.2) and 100 nM dexamethasone-treated cells (0.8 ± 0.2). Furthermore, a trend for strain, $F(2, 123) = 2.8$, $p = 0.07$, attributable to differences between wild-type astrocytes (1 ± 0.3) and resiliency (C/G) allele carriers (0.9 ± 0.2) was found for *Nr3c1*. However, the reported changes for *Nr3c1* were within the range of technical variability.

4 | DISCUSSION

We demonstrated that astrocytes, microglia, and neurons show a differential responsiveness to glucocorticoids based on the induction of RNA transcripts from numerous glucocorticoid-responsive genes. In addition, we demonstrated that astrocytes, microglia, and neurons derived from mice carrying the human rs1360780 polymorphism display a differential responsiveness to glucocorticoids. Astrocytes derived from the risk (A/T) strain are more responsive than astrocytes derived from the resiliency (C/G) strain. As the SNP-associated differences were most notable in astrocytes, our findings imply astrocytes may be a functional mediator of *FKBP5* polymorphism-associated variations in stress responses in the CNS.

The brain is a central hub for the integration of changing internal and external conditions and, thus, plays a pivotal role in the initiation and termination of the stress response. Within this response, glucocorticoids elicit immediate transcriptional changes in their target cells. Various cells types in the brain will, dependent on their function, differently respond to glucocorticoid stimulation (Lucassen et al., 2014; Radley et al., 2015; Ressler & Smoller, 2016). Regulators of glucocorticoid signaling, such as FKBP51, will necessarily contribute to cell type-specific responsiveness. Previous studies found a correlation of *Fkbp5* expression levels and stress responses in peripheral blood mononuclear cells and immortalized lympohblastoid cell lines (Chun et al., 2011; Yeo et al., 2017). These cell types are informative on peripheral responses of the immune system to stress. A critical missing factor in the literature was whether *Fkbp5* expression levels in the brain might affect cellular stress responsiveness. Data on human-induced pluripotent stem cells that were differentiated into forebrain-lineage neural cultures and exposed to a high dose of dexamethasone did not indicate a neuronal response to glucocorticoids (Liebermann et al., 2017). This is in line with our data, where neurons appeared to be protected from glucocorticoid signaling and only showed a minor induction of glucocorticoid-responsive genes. In conformity with the common understanding of *Fkbp5* acting as potent negative regulator of glucocorticoid signaling, this low responsiveness of neurons to glucocorticoids was correlated with a high expression of *Fkbp5* at baseline in neurons. The previous remark that forebrain-lineage neurons may not be an optimal neural cell type to examine relationships between glucocorticoid receptor activation and *Fkbp5* expression (Liebermann et al., 2017) could, thus, be extended to the hypothesis that neurons, in general, are not the primary target cells of glucocorticoid signaling. As at individual cell level the responsiveness of microglia and especially astrocytes to glucocorticoids was much stronger than in neurons, our data support the need for further study on the impact of glial FKBP51 signaling on central stress responses.

The observed prominent role of astrocytes fits with transcriptomic studies where an astrocyte-specific gene up-regulation after glucocorticoid exposure was reported to occur prior to adaptations in other cell types (Carter et al., 2012). This may be necessary to prevent neuronal loss by ensuring metabolic support for example via shuttling lactate (Genc et al., 2011; Ma et al., 2020) to circumvent the glucose-restricting effects of glucocorticoids (Homer et al., 1990; Virgin et al., 1991). Our data provide insights that astrocytes could fulfill this function by promptly up-regulating *Fkbp5* to inhibit glucocorticoid signaling and *Sgk1*, a kinase favoring cellular glucose uptake from the circulation (Boini et al., 2006). In addition, astrocytes responded to glucocorticoids by induction of *Tsc22d3*, which drives metabolism toward oxidative phosphorylation (Andre et al., 2017). This might be a means to provide astrocytes with more energy for clearance of the synaptic cleft. Notably, the combined exposure to corticosterone and the excitatory neurotransmitter glutamate was reported to deprive cells from energy in the shape of adenosine-tri-phosphate (Tombaugh & Sapolsky, 1992). This simultaneous presence is likely to occur during the stress response and would threat neuronal energy supply. Enhanced

clearance of the synaptic cleft from glutamate by astrocytes could, therefore, be another way to safeguard neurons from shortcomings in energy.

Besides their supportive role in terms of metabolism, astrocytes were shown to influence information processing and cognition by integrating local sensory information and behavioral state (Hallmann et al., 2017; Lima et al., 2014; Oksanen et al., 2019; Peteri et al., 2019; Slezak et al., 2019). In response to glucocorticoids, resetting of the circadian clock via non-canonical pathways (Suchmanova et al., 2019) was reported. Our data on the up-regulation of *Per1* following stimulation with glucocorticoids suggest an astrocytic involvement in the regulation of the circadian rhythm. Furthermore, astrocytic responses to glucocorticoids were reported to directly influence (emotional) learning by structurally reorganizing neuronal networks via an increased expression of *Sgk1* (Slezak et al., 2013) or micro-RNAs, described to influence neurogenesis (Luarte et al., 2017). Astrocytes might, hence, play an important role in shaping plasticity in response to emotional stress and set the stage for future stressful encounters (Bender et al., 2016; Tertil et al., 2018). Taken together, the observed astrocytic responses would be appropriately placed to at least partially mediate the known interaction of FKBP5 and early-life trauma.

Very little is known about how genetic risk factors for stress vulnerability might affect astrocyte functions. By humanizing the murine *Fkbp5*-locus with either the risk (A/T) or the resiliency (C/G) version of rs1360780, we sought to create a unique model that would allow further mechanistic studies into how these SNPs can alter the trajectory of pathology due to environmental stressors. In astrocytes, we found the risk (A/T) allele to stronger respond to glucocorticoid exposure than the resiliency (C/G) allele. The associated inherent higher reactivity of astrocytes carrying the risk rs1360780-A/T gene variant could lead to excessive inhibition of further GC signaling, or faster inhibition via the fast intracellular feedback. These data are supported by a preliminary, albeit un-quantified, observation that after stimulation with dexamethasone, the FKBP51 protein is also translated in the humanized mice. Taken together, this is the first demonstration that human risk and resilience *FKBP5* polymorphisms can be transferred successfully to mice.

There are technical considerations relevant to production of such humanized mouse lines. It is technically difficult to humanize a large (>16,000 bp) gene, hence, only truncated versions were inserted in place of the murine *Fkbp5*. The observed responsiveness suggests that humanized alleles can interact normally with murine gene regulator elements. Nonetheless, our results on cellular glucocorticoid responsiveness are limited to mRNA expression and quantification of cell type–specific effects on protein expression remains challenging. The SNP is only known to affect chromatin folding, not the structure or function of the protein. However, it would be important to establish normal FKBP51 interactions with the glucocorticoid receptor, such as the ligand binding affinity. Additionally, in vitro experiments can only reflect certain in vivo aspects and, hence, further studies are needed to draw conclusions as to whether the risk (A/T) allele would have functional influence on global glucocorticoid responsiveness in these mice and whether these could be used to model disorders. These mice have been made commercially available by Taconic Biosciences to aid in future studies by all researchers.

FKBP5 SNPs have been shown to impart variability in HPA-axis responses in the healthy population, but more importantly to impart differential risk for pathology when combined with environmental risk factors such as early life adversity. Early life adversity has been discussed to prime the stress response systems to pathological processes (Ganguly & Brenhouse, 2015; Jens & Zakreski, 2015; Kompier et al., 2019; Lesuis et al., 2018, 2019; Vaiserman & Koliada, 2017) and astrocytes were reported to be involved in the early programming of stress responsivity (Abbink et al., 2019; Gunn et al., 2013; Saavedra et al., 2017). During neuronal development, demethylation of the *FKBP5* locus as consequence of glucocorticoid exposure was reported (Provençal et al., 2019) and if the same holds true for astrocytes, allele-specific methylation changes in *FKBP5* could exacerbate the observed *FKBP5*-SNP-associated differences in glucocorticoid reactivity. These data provide a first indication that differential stress sensitivity via astrocytic signaling could play a role in pathology linked to *FKBP5* SNPs.

In sum, cell type-specific expression of *FKBP5* regulates responsiveness to glucocorticoids in the CNS with astrocytes being a promising cellular target for advancing the current knowledge on how metabolic processes and astrocytic functioning can shape the termination of the central stress response. These novel *FKBP5*-humanized mouse lines are a unique tool to advance the current knowledge on how *FKBP5* variants interact with glucocorticoid sensitivity to influence physiology and we hope that their use in future studies will benefit patients.

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CONFLICTS OF INTEREST

Nadine Richter, Bastian Hengerer, and Kelly Allers are employees of Boehringer Ingelheim Pharma GmbH & Co KG. Verena Nold and Iris-Tatjana Kolassa have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

V Nold – conception, experimental design, acquisition, analysis and interpretation of data; writing of the manuscript. N Richter – acquisition of data. B Hengerer – conception, revision of the manuscript. IT Kolassa – data interpretation, revision of the manuscript. KA Allers – conception, data interpretation, revision of manuscript.

DATA AVAILABILITY STATEMENT

All raw data files, summary data frames, and the R code written to analyze and visualize the herein contained data will be made available by the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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3. Discussion

3.1. Bringing Physiologic and Pathologic Stress Responsiveness into Context

'The only universal constant in life is change' (Heraclitus of Ephesus)

Throughout life, everyone faces several changes in the requirements of the surrounding environment and needs to adjust physiology and behaviour accordingly. These reactions to new situations are part of the so-called 'stress response' and manifest in alterations of the PINE network [397]. All challenges during lifetime contribute to the personal development and are an opportunity to grow [197]. However, unsuccessful attempts to deal with novel situations may result in negative feelings like uncertainty regarding one's abilities, anxiety towards the unfamiliar, and avoidance of changes [627, 601, 503]. The way individuals respond to stress shapes their development and at the same time influences how future situations are dealt with [198, 350, 609]. In supportive environments, coping strategies from family and peers that aid in finding a solution to fulfil new requirements may be learned and negative effects of suboptimal stress responses may be buffered. In competitive environments, negative experiences may reinforce themselves and climax in pathology [108, 323, 144, 177]. It is thus not only the number of challenges and the dose of potentially associated stress but also the personal history and context that decide whether a challenge is perceived and handled as opportunity or seen as load that threatens well-being.

Chronically high stress levels as result of unsuccessful coping with stressors, or of frequent exposure to highly demanding situations, or both, is viewed as common risk factor in the aetiology of many somatic and psychiatric disorders [602, 90, 577, 55, 402, 436, 568, 642]. The individual responsiveness to stress is therefore an important modulator of the risk to develop pathologic symptoms. A better understanding of the factors that in turn regulate the responsiveness to stress will promote targeted interventions for individuals at risk or patients suffering from pathology.

This doctoral thesis focused on the prodromal state to further elucidate the complex interplay of physiological adjustments in the stress response system that occur prior to pathology. Even though the stress responsiveness is highly individual, we hypothesized that common underlying mechanisms may exist since excessive stress converges to shared symptoms in multiple disorders. Based on similarities during the evolution of rodents and man, we further hypothesized that these underlying mechanisms might be preserved across species and that valid animal models would therefore enable the clarification of causal relationships between stress responses in the PINE network and the human transition to disorders.

Given that repeated stress exposure and social isolation are factors shown to influence stress coping and to be linked to a higher likelihood of disorders, transient exposure to stressors and lack of social support during different stages of development were selected as common experimental factors in both *in vivo* animal studies of this thesis. **Study I** was centred around the consequences of chronic mild stress on the

PINE network in rats, while **study II** aimed at molecular and behavioural differences between *Fkbp5*-humanized mice carrying risk- or resilience-associated variants. With a confirmed list of risk or resilience factors, screening campaigns could be carried out to identify individuals that are likely to develop disorders given their exposure to these factors. However, a high complexity of pathological transition states could arise from the combinatory potential of individual risk and resilience factors. This could result in the identification of a high number of persons that might need help to maintain health. Offering a broad range of preventive measures to all of them could be impractical. Moreover, some preventive measures might be mostly effective for certain individuals dependent on their overall personal history and environment.

From a diagnostic and treatment perspective, refining risk factor based screenings with efficient diagnostic tools to further stratify the target group is needed for application in the clinic. Having a tool that jointly indicates several pathological symptoms like latent inflammatory processes and reduced cognitive abilities, or that is sensitive to detect negative transitions early on would be desired. Along these lines, insights into the translational potential of TRYCATs as dynamic biomarkers for lifetime vs. recent events such as therapeutic interventions were investigated in **study III** in elderly human subjects.

Regarding the discovery of novel treatment approaches, common alterations in the PINE network that were investigated in the *in vivo* studies of this thesis are a promising starting point to launch interventions. Since the brain was confirmed to be a central hub for the regulation of stress and an interesting target organ, **study IV** focussed on the cerebral responses to acute stress signalling in the shape of glucocorticoids. To potentially refine the targeting strategy on a cellular level, the glucocorticoid responsiveness of different CNS cell types was investigated in the context of genetic predisposition through *Fkbp5* variants.

3.2. The Environment Impacts PINE Network Functioning Throughout Lifetime

While the timely limited exposure to stressors was observed to unleash energy that can be used to resolve the triggering challenge, excessive stress and energy turnover were linked to the development of diverse medical conditions [27, 49]. What is less clearly defined is the middle of this spectrum, namely which effects mid-term exposure to mild stress elicits. By applying different types of mild stressors, a prodromal state was induced with the set of experiments carried out within **study I**. Since social context is expected to modulate the effects of stress, social stressors like JSH and hostile encounters with peers were included in addition to the mild physical stressors. This strategy of using various stress doses and exposure times allowed to search for putative biomarkers that could indicate early and later changes in the PINE network. Moreover, the hypothesized cumulative or interacting effects of different stressors could be investigated and their potential as risk factors could be determined.

The absence of pathological alterations regarding the diurnal rhythmicity of corticosterone could be viewed as confirmation that the mild stressors did not trigger a transition to disorders [430]. However, the JSH-associated shifting of the diurnal peak towards the afternoon at an age of 10 weeks instead of 15 weeks as seen in controls [430] could indicate that the functioning of the HPA axis is modulated by negative experiences during adolescence in a way that resembles quicker ageing. This interpre-

tation would extend previous publications discussing the effects of stress on accelerated ageing [44, 232] by suggesting that stress during adolescence also accelerates ageing. In aged mice, chronic social stress during adolescence was shown to induce cognitive impairments [572] and post-adolescence treatment with the selective serotonin reuptake inhibitor paroxetine was shown to rescue a stress-induced change in the HPA axes of aged mice [523]. Given that witnessing JSH appeared to similarly affect the HPA axis like experiencing JSH, our data suggest that even mild stressors hold the potential to alter stress coping and to precipitate in age-associated aberrations. However, further investigation of adolescent stress exposure on accelerated ageing are needed. Since the observations of stress effects made in humans mainly focussed on reproduction strategy [655] and inflammaging [424, 170], further research regarding the effects of social isolation during adolescence on premature ageing of these systems would be helpful.

In addition to accelerated ageing, alternative interpretations could explain the observations regarding diurnal corticosterone rhythmicity. Given that the levels of corticosterone were assessed in faeces, changes in food intake, physical activity, or gut motility could interfere with the measured levels and the interpretability of the data [636, 497, 318]. Difficulties to reliably measure glucocorticoid levels in different biospecimens and to compare them between studies using different sampling schemes is also a common problem in human studies, resulting in contradictory findings [461, 476, 522]. This illustrates that harmonized protocols and supplementary measures to better elucidate kinetics and regulation of HPA axis functioning are needed to improve interpretability.

For the first time it was shown that the kynurenine pathway can be activated by JSH. Moreover, the kynurenine pathway was activated in a similar way by short- to medium-term exposure to uCMS. With both stressors, the catabolism of tryptophan was elevated in such a way that more of the anti-oxidative and anti-excitatory KYNA was formed, while less of the NMDAR-stimulating and oxidative QUIN was produced [430]. These findings are suggestive for reduced neural excitation propensity [458, 532, 519] and could be a preventive measure against excitotoxicity during the first phase of chronic stress exposure. In addition, the alterations in the kynurenine pathway may be protective against unnecessary adjustments to non-persistent stressors by limiting neural excitation. In patients with ongoing inflammatory processes, treatment-resistant depression, and anxiety disorders, a bias of tryptophan catabolism towards QUIN was reported [504, 159, 194]. In addition to this potential as diagnostic biomarker for pathology, the data of this thesis suggest that profiling of TRYCATs could serve as prognostic tool to detect early signs of stress-induced alterations. If stressors were to persist, limiting the neural activity needed to adapt to them could turn out to be an undesirable coping strategy.

An additional response to uCMS was the increased presence of immune cells such as monocytes, granulocytes, and lymphocytes [430]. This could indicate preparations for fighting of pathogens and wound healing in case of injury [94, 605, 272]. Such stress-induced alterations in blood counts and in the composition of the innate and adaptive immune system are in line with previous observations [149, 582, 348] and could, if temporally limited, represent an evolutionary advantage in the aftermath of physical battles. If persistent, such changes might cause extra expenses that exceed the capabilities of the PINE network and would even lead to the opposite effect, namely delayed healing under chronic stress exposure [613, 67, 218]. Interestingly, chronic latent inflammation is a frequently observed co-morbid symptom in subgroups of depressive patients

[329], so the observed stress-induced changes in the immune system could be a first step in the trajectory from life-saving adaptation to pathomechanism. Following medium-term stress exposure, the levels of cytokines were not significantly changed and the kynurenine pathway did not indicate a bias to its inflammation-driven arm [430], suggesting that no latent inflammatory processes were triggered yet [557, 80, 28]. Viewed together, alterations in blood composition appear to proceed a potential switching into pro-inflammatory states and could be used as early biomarker for stress-induced alterations in the immune system.

A further observed adaptation to uCMS was an increase of the mitochondrial density in the hippocampus. Only in the double-hit stress group, elevated routine respiration and an increased usage of the second complex of the ETC relative to complex I accompanied the increase in mitochondrial density [430]. These mitochondrial adjustments allow for sufficient energy supply of brain regions involved in stress processing without the risk of generating elevated levels of ROS [160, 223, 354]. Such adjustments within the ETC were not reported previously in the context of social isolation and, together with the anti-oxidative buffering effects of the altered kynurenine pathway activity [519, 533, 363], could represent a protection mechanism against oxidative stress. In newer literature, oxidative damage of DNA and cellular structures through an imbalance of excessive mitochondrial respiration and limited anti-oxidative potential is accepted as key mechanism during the development of stress-associated psychiatric disorders [56, 352]. A traumatic stress history was shown to affect mitochondrial function in synaptosomes [542] and was linked to the development of depression [387]. After treating patients suffering from major depressive disorder with selective serotonin reuptake inhibitors, an increase in ETC complex II activity was reported [182]. The data on consequences of uCMS suggest that the PINE network is prepared for intermediate exposure to stress without the risk to accumulate oxidative damage. However, cumulative stress overload e.g. in a chronic setting could exceed the adaptive capacities of the PINE network against elevated energetic demands [293].

An early sign of potential pathological alterations in reaction to uCMS during adolescence and early adulthood could be the observed reduced expression of immediate early genes in the PFC [430]. Their reduction could indicate reduced neural activity, which might be a measure to limit energy consumption [241, 612, 546]. This may contribute to the suggested beneficial effects of reduced immediate early gene expression for acute stress resiliency [627]. The parallel higher levels of KYNA might show synergism with this measure. Since the PFC is a highly connected brain region crucial for the regulation and cognitive processing of stress [140, 604, 210], the putative reduction in its activity could however precipitate in reduced plasticity and reduced adaptive capability for future challenges [410, 385, 466, 482, 267, 276, 443]. Impaired cognitive flexibility and working memory were shown to precede depressive-like symptoms in rats [375].

Impairments in the processing of stress in the brain however are thought to be a corner stone for later pathology [392, 394]. Indeed, a mild transcriptome signature in the hippocampus towards changes in cellular health was observed in the experimental group exposed to uCMS for a prolonged period of 14 weeks [430]. Given that the hippocampus receives inhibitory feedback from the PFC [15, 209], both NGS findings jointly suggest that lacking plasticity in the PFC could result in excessive activity, energy consumption and subsequently wear and tear of the hippocampus that on the long run might even climax in impairments of mitochondrial performance [293] resulting in lack of energy and hippocampal cell loss [543].

Besides a lack of energy, apathy, helplessness and anxiety are other common symptoms in depressed patients [378, 308, 176]. While apathy was only qualitatively observed during handling of the uCMS-exposed animals, the time spent immobile during the forced swim test can be used as quantifiable proxy for helplessness [82, 553, 647] and the latency to leave the center of the open field arena as measure for anxiety [317, 536]. After a prolonged exposure to uCMS, increased immobility in the forced swim test and an increased freezing behaviour in the open field test were observed [430]. This indicates that initial adjustments in the PINE network after a certain dose of stress are not sufficient to prevent from the development of behaviours commonly judged to be pathological.

In summary, **study I** generated new insights into allostatic mechanisms involved in a 'prodromal' phase of stress-induced pathology by investigating the effects of variable exposures to different mild stressors. The results clearly show synergistic and cumulative effects of different stressors on shaping the PINE network. Despite the time-lag, JSH modulated effects of uCMS during adulthood. This confirms that environmental stressors during different developmental phases interact with each other and that the individual stress history can have an impact on the stress response system. The data underscores that stress exposure during adolescence as well as chronic stress during adulthood are risk factors for decreased health.

Among the adjustments to short- and mid-term stress exposure, indications for an increased readiness of the immune and energy system but decreased perceptive alertness or situative engagement were observed. Reduced excitatory signalling may prevent from unnecessary energy consumption and adaptation to putatively transient demands, which appears to be acceptable in the context of a laboratory experiment where challenging situations do not persist. However in the real world, ignorance towards challenges or surrendering to them could come at the cost of survival in the event of life-threatening dangers. Even in less drastic situations, failure to adjust to chronic stressors could become costly, since the resulting persistent allostatic loads could eventually exceed the adaptive capabilities of the affected individual. Which degree of responsiveness to stressors is adequate thus should be assessed dependent on the context.

An additional learning from **study I** is that functioning of PINE network components need to be considered jointly, since the hypothesized complex interactions between the brain, immune system and energy metabolism as well as the stress-induced adjustments in them were confirmed. Together with literature on pathological states, the described consequences of uCMS on mitochondria suggest an inverted U-shaped relationship between stress and the regulation of energy supply and consumption. A longitudinal study of mitochondrial physiology under stress could yield empirical evidence of the shape of the regression curve. A holistic approach considering stress history, exposure, timing, and the state of PINE network components could help to spot and intervene against critical transitions in the stress response network in time.

3.3. Genetic Predisposition × Early Life Adversity Alter Functioning in Adulthood

Developing systems undergoing construction are thought to be less stable. Therefore, childhood as well as adolescence are considered to represent times of elevated vulnerability to pertubations [489, 171, 621, 21, 26, 526, 212]. In combination with negative life events during childhood, the genetic predisposition of carrying the rs1360780-AT-allele of *Fkbp5* was found to be linked to the development of disorders [52, 658]. By investigating this gene × environment interaction in humanized mice, **study II** assessed whether the *Fkbp5* × ELA mouse model would be valid to research causal relationships and molecular mechanisms playing a role in human pathology.

The analyses of behaviour in novel environments revealed that female AT- vs. CG-allele carriers after ELA showed less responsiveness to challenge [428]. This became manifest in alterations during dark-light box testing, locomotor habituation, and in the social chamber test and is in line with several animal models of stress where decreased behavioural flexibility was observed [362]. Differences in the responsiveness to environmental stimuli between AT- and CG-allele carriers were also demonstrated in humans [269]. The lower behavioural responses to novel environments of AT- vs. CG-allele carriers that experienced ELA imply that *Fkbp5* is part of a core molecular mechanism that regulates responsiveness to challenges and that the *Fkbp5*-genotype contributes to the variability seen in stress responsiveness.

Regarding social behaviour, female CG- and AT-allele carriers after ELA-exposure spent less time in direct interaction with a social stimulus [428]. Since decreased sociability was shown to be a negative modulator towards psychiatric pathology due to the lack of social support [441, 635] and its buffer capacities against stress [114, 199, 367], behavioural differences attributable to differences in stress responsiveness may reinforce themselves. It is therefore interesting to see that CG-allele carriers after ELA nevertheless spent more time in the surrounding proximity of the unfamiliar mouse, which may suggest that they maintained social interest but were more cautious than CG-allele carrying female controls. In contrast, ELA-exposed vs. control AT-allele carriers spent less time in the surrounding of a social stimulus, suggesting that they lost their social interest after ELA.

The behavioural read outs in CG-allele carrying females with ELA compared to controls furthermore indicated increased activity during the murine active phase and in novel environments [428]. Individuals carrying the AT-allele that experienced ELA were indistinguishable from controls [428]. These observations might indicate that higher locomotion during the murine day is a stress coping strategy [639, 32, 134, 273] which CG-allele carriers adopt after ELA exposure. This higher locomotion in CG-allele carriers with ELA experience decreased after sunrise, which points towards a distinct activity pattern that follows a diurnal rhythmicity.

The analyses of diurnal HPA axis activity revealed that female AT- vs. CG-allele carriers after ELA showed attenuated diurnal rhythmicity of glucocorticoids with elevated nadir levels in the morning and no statistically significant increase of corticosterone towards the evening, the time of murine awakening [428]. Given that the cortisol awakening response is thought to influence performance during the upcoming day in humans [192], differences in HPA axis activity are likely to explain why AT- vs. CG-allele carrying female mice showed less responsiveness to novel environments. From a clinical

perspective, a blunted awakening response is a frequently observed symptom in depression and its measurement is an established part of clinical practice [620, 264]. Moreover, elevated nadir levels of corticosterone in parallel to elevated *Fkbp5* levels were proposed as female-specific biomarker of prolonged cortisol load and the associated risk of psychiatric disorders [334, 469]. Decreased diurnal rhythmicity of the HPA axis in the *Fkbp5* × ELA model demonstrates face validity with human symptomatology.

Alike the cortisol awakening response, pronounced fluctuations in glucocorticoid levels during the day were reported to be essential for normal responsivity to emotional and cognitive challenges in humans [285]. The absence of a diurnal rhythm in female AT-allele carriers with and without ELA [428] could therefore contribute to a lower behavioural responsiveness to challenge and indicate alterations in the dynamics of glucocorticoids throughout the day. Alterations of their ultradian HPA axis activity [623], originating from disturbed negative feedback to the HPA axis [207] in the presence of longer inhibition of glucocorticoid signaling [465, 521, 142] due to the higher levels of FKBP51 which may originate from the stronger induction of the AT- vs. CG-allele of *Fkbp5* [54, 429, 309] could be an additional reason for the differences in the responsiveness to mild stress challenges between AT- vs. CG-allele carriers.

First evidence for SNP-dependent differences in the kinetics of glucocoticoid signaling and its regulation may lie in the expression levels of *Fkbp5* in the morning. Even though the morning corticosterone levels were higher in AT- than CG-allele carriers, their hippocampal expression of *Fkbp5* mRNA was lower [428]. This could indicate that AT-allele carriers had higher levels of the FKBP51 protein than CG-allele carriers in the morning, which reduced *Fkbp5* mRNA induction by inhibiting glucocorticoid signaling and thus contribute to less pronounced ultradian oscillations. Further investigation including quantification of the FKBP51 protein and *Fkbp5* mRNA with higher measurement frequency combined with repeated exposure to glucocorticoids, and the analysis of other glucocoticoid-responsive genes would be needed to address this hypothesis.

General analyses of gene expression in the hypothalamus and hippocampus revealed that in the subset of ELA-exposed mice compared to controls more genes differed between AT- and CG-allele carriers [428]. This is a confirmation for the proposed gene × environment interaction reported in psychiatric patients [658]. Matching to the behavioural findings that AT- vs. CG-allele carriers already showed differences only due to their genotype, the effects of ELA vs. control measured by the count of differentially expressed genes was lower in the AT- vs. CG-allele carriers [428]. Viewed together, the counts of differentially expressed genes could be a first indication of cumulative effects or interactions between both hits, the genetic predisposition and ELA.

Reduced expression of genes related to synaptic communication in the hippocampus and hypothalamus of AT- vs. CG-allele carriers were detected on a pathway level [428]. These observations are in line with findings obtained in the PFC of *Fkbp5*-knockout mice were the expression of genes related to CNS development and intracellular signal transduction were found to differ [101]. Dynamic neural activity in distributed brain regions like the PFC, amygdala, hippocampus, and hypothalamus was shown to be required for resilient coping with stress signals [552]. However in a chronic stress setting, several animal models indicated that this dynamic neural activity vanishes and is replaced by reduced synaptic communication and plasticity [597, 430, 164, 324, 479].

This literature on chronic stress and neural activity is extended by the findings of **study II** linking synaptic actvitiy to the regulation of the HPA axis. Given that neural activity and plasticity was shown to be decisive for shaping the HPA axis in early life [180] and that these brain regions regulate glucocorticoid signaling [140] as well as the cortisol awakening response in anticipation of challenge [192], the observed lower expression of genes related to synaptic communication in AT- vs. CG-allele carriers are likely to converge in alterations of the HPA axis activity. Indeed, the differentially expressed genes related to circadian entrainment in the hypothalamus of AT- vs. CG-allele carriers and in the hippocampus of ELA-exposed vs. control mice are suggestive of an altered HPA axis activity [428]. Since the hippocampus influences the circadian entrainment in the hypothalamus [192], the combination of ELA and *Fkbp5* SNPs could synergistically modify the HPA axis to decrease circadian entrainment and to respond less to stimuli. The NGS data thus provide additional hints why AT-allele carriers displayed a flattened glucocorticoid rhythm and were less responding to changes in their environment than CG-allele carriers with ELA exposure.

In addition to the differences regarding behaviour, circadian rhythmicity, and neural activity, parallels regarding cerebral energy metabolism are present between the *Fkbp5* × ELA model and chronic stress models of psychiatric disorders. The AT- vs. CG-allele was associated with higher expression of genes related to metabolism and OXPHOS [428], which resembles the observed activation of mitochondrial respiration after mid-term exposure to mild stressors [430]. Carrying the AT-allele might therefore be beneficial to ensure energy provision in acute stress settings. However, elevated mitochondrial activity is associated with elevated production of reactive oxygen species that may introduce damage and cell death which could demark the transition into a pathological state [463, 464, 374].

Taken together, the data of **study II** regarding the reduced expression of genes related to synaptic communication and the higher expression of genes related to metabolism imply that AT- vs. CG-allele carriers may be genetically predisposed to changes in the PINE network that mimic allostatic changes in mild chronic stress settings. In the presence of occasional stress challenges, these genetically motivated adjustments of the PINE network may introduce an evolutionary advantage of AT- vs. CG-allele carriers, since these are already in allostasis. However, in a severe and repeated stress context, these initial synergies may reinforce themselves and cause irreversible changes that accelerate pathological transitions. By combining *Fkbp5* genotypes with ELA and a third hit like chronic or severe stress, the *Fkbp5*-humanized mice are a unique tool to elucidate the causal sequence of events leading from altered stress responsiveness to psychiatric pathology.

3.4. Biomarkers for Cognitive Capabilities in the Context of Negative Life-Time Events and Therapeutic Interventions

The catabolism of tryptophan along the kynurenine pathway is thought to link stress signalling, energetic demands, and inflammation to neural excitation and cognition [37, 224, 225]. Thus, profiling of TRYCATs was proposed as biomarker in depression and anxiety disorders [85, 504, 159, 194]. Given the neuroactive effects of KYNA and QUIN [458, 532], the role of relative TRYCAT levels in neurocognitive disorders was further analyzed within **study III**. Based on the proposed linkage between stress and

cognitive functioning [377, 453, 280], lifetime stressful events were assessed in parallel. Since positive effects of physical training on inflammatory processes and cognition were reported previously [41, 106, 574], the potential of TRYCAT profiling to monitor treatment success was additionally investigated. Moreover, cognitive training was included as alternative therapeutic intervention due to its application in the clinic [110, 289] and the observation that lack of cognitive activity may convey a risk for cognitive decline [186].

Correlation analyses of TRYCATs with cognitive abilities revealed a negative correlation of QUIN with executive functions [327]. This finding is congruent with previous observations where QUIN was found to be negatively correlated with cognitive performance [225]. It furthermore is in line with the ameliorating effects of IDO-inhibition on cognition in a mouse model of Alzheimer's disease [654]. Our data suggests that serum levels of QUIN might constitute a biomarker of cognitive impairment. Meanwhile, this suggestion was confirmed in different medical conditions, e.g. after stroke [113, 313] or in schizophrenic patients [91].

Aetiologywise, stressful life events showed a positive association with 3-HK, the precursor of QUIN [327]. Given that increased 3-HK serum concentrations were reported to differentiate Alzheimer's disease patients from controls [534], this finding indicates that experiencing stressful life events could contribute to cognitive impairments. Mechanistically, the previously shown stress-induced increase in the activity of the IDO enzyme [194, 411], which converts TRP to KYN, and the enzyme KMO [4], which further converts KYN to 3-HK, could explain this observation. Both enzymes are furthermore inducible by inflammatory signalling. Thus, increased 3-HK levels could be suggestive for long-lasting effects of the lifetime stress load that contribute to a latent shifting towards a pro-inflammatory milieu in stress-associated pathologies and ageing [85].

After physical training, 3-HK levels decreased on trend level [327]. This could be owing to the potential of physical activity to decrease inflammatory processes [41, 106, 574] that occur in parallel to ageing [512, 183, 72], which eventually would normalize inflammation-induced expression levels of IDO and KMO. As consequence, the catabolism of tryptophan would be expected to decrease with levels of QUIN and 3-HK being more likely to decrease than the levels of KYN and KYNA, since the first two are KMO- and IDO-dependent while the latter two depend on IDO and KATs [557, 80, 28]. The absence of a statistically significant effect of physical training on TRYCAT levels might be explained by the composition of our study cohort where persons at risk of, but not diagnosed with, neurocognitive deficits were included. This might have limited the presence of inflammatory processes and the potential of physical training to reduce them and 3-HK. Alternatively, the trend to reduced 3-HK levels could be mediated by the previously reported increase of peroxisome proliferator-activated receptor gamma coactivator 1 alpha in the muscle after exercise [4]. This coactivator was found to increase the expression of KATs and thus could increase the conversion of KYN to KYNA rather than 3-HK.

For cognitive training, both decreased 3-HK and decreased KYNA levels relative to baseline were observed [327]. This is the first empirical data showing a linkage between cognitive intervention and tryptophan catabolism. In pre-clinical studies elevated exposure to KYNA was associated with learning and memory impairment [617, 6] and pharmacological reduction of KYNA ameliorated cognitive performance [316]. Based on these findings, the reduction in KYNA observed in **study III** appears to be a ben-

eficial response to the cognitive training intervention. Since 3-HK and KYNA were reduced, the data could indicate that a molecular change at the level of KYN underlies this mechanism and that KATs likely play a minor role.

With respect to outcomes of both training types on cognition, no significant training-related effects were observed [327]. This could indicate that longer interventions might be needed to overcome the effects of negative lifetime events like stress or to be differentiable from the positive effects of maintaining a healthy, active lifestyle [326]. Alternatively, the ratio between excitatory and inhibitory neuroactive catabolites did not change significantly despite the nominal changes in tryptophan catabolites. Thus, the influence of tryptophan catabolites on excitation propensity might not have been changed by the interventions.

Summarized, the findings of **study III** suggest that changes on molecular level can manifest rather short-term while changes in cognition occur on a long-term basis. Profiling of TRYCATs may serve as early biomarker for intervention effects, but further investigations are needed to clarify whether and after which amount of time beneficial effects of training on cognition would follow the observed changes in TRYCATs.

3.5. Astrocytes as Cellular Target to Integrate Fkbp5-Variants, Stress Signalling, and Brain Homeostasiss

Given the universal role of glucocorticoids in communicating the stress response, aberrations in the HPA axis regulation are associated with a plethora of symptoms [602, 55, 144, 402, 436, 568, 642]. Since the brain is a fundamental organ in orchestrating the HPA axis, a deeper understanding of the cerebral responses and negative feedback loops to glucocorticoids is needed [253, 392]. Within each cell, *Fkbp5* contributes to the regulation of glucocorticoid signalling [266, 142, 465]. Recent stress exposure and genetic variants influence the expression of *Fkbp5* [309]. By analysing basal expression levels in the context of these *Fkbp5*-variants and transcriptional changes after glucocorticoid stimulation at CNS cell type level, **study IV** contributes to the elucidation of cerebral responsiveness to acute stress.

At baseline, astrocytes displayed the lowest *Fkbp5* levels, followed by microglia and neurons. At the same time, astrocytes and microglia expressed the highest levels of *Nr3c1* [429]. Considering the functions of both genes, these findings suggest that astrocytes would be most responsive to glucocorticoids, while microglia would show medium responsiveness, and neurons would be expected to respond the least. Since these expression differences between the investigated CNS cell types were observed at baseline, they suggest that regulation of the responsiveness to glucocorticoids is anchored in the cellular identity and might be associated with cell type specific functions. From a practical point of view, the naturally occurring differences allowed for testing the impact of expression levels on glucocorticoid responsiveness without the need to artificially modulate gene expression prior to stimulation.

While neurons were protected from responding to glucocorticoids, microglia showed medium responsiveness whereas astrocytes responded the most [429]. The absence of transcriptional responses in neurons is in line with findings in human-induced pluripotent stem cells that were differentiated into neural cultures and exposed to high doses

of dexamethasone [344]. These matching observations in transcriptomic data combined with functional activity data could indicate that, in order to respond to acute glucocorticoid exposure, neurons rely on synaptic signaling via established brain circuits rather than on modifications in their gene expression and metabolism [178, 656, 477, 11].

Given that glucocorticoids can restrict the availability of glucose [261] and may deprive cells from ATP when paired with excitatory neural signalling [596], mechanisms need to be in place to allow continued or even elevated neuronal firing under glucocorticoid exposure to prevent excitotoxicity. Known astrocyte functions like the shuttling of lactate to neurons [200] or the clearance of the synaptic cleft from glutamate and the shuttling of glutamine back to neurons [68] represent crucial mechanisms to promote neuronal survival. The observed pronounced responsiveness of astrocytes to stimulation with glucocorticoids might therefore be a measure to ensure the astrocytic support for neurons. In favor of this hypothesis, astrocyte-specific gene up-regulation after glucocorticoid exposure was reported to occur prior to adaptations in other cell types [89], which would allow astrocytes to support neurons before these get compromised in their functioning. Moreover, the GR-dependent release of ATP from astrocytes in the medial PFC was shown to mediate stress vulnerability [360].

Since glucocorticoids would limit glucose as well as ATP in astrocytes [614], the observed induction of *Sgk1* and *Tsc22d3* after glucocorticoid exposure could serve astrocytes to cover their energetic requirements by favoring glucose uptake from the circulation [59] and promoting OXPHOS [16], respectively. Besides their supportive role in terms of metabolism, astrocytes have been shown to influence current and future stress responses by interacting with information processing [460, 554], cognition [230, 347, 507], and emotional learning [555, 592, 46, 1, 226]. The observed induction of *Per1* in astrocytes following glucocorticoid stimulation suggests that the astrocytic circadian clock was altered. This mechanism has previously been reported [579]. Considering that astrocytes are more than metabolic supporters for neurons, alterations in their circadian rhythmicity could modulate overall awakeness and energy expenditure.

Combined with the data on basal expression of *Fkbp5* and *Nr3c1*, the data from the stimulation experiments confirmed the expected correlation between basal expression of glucocorticoid signalling regulators and transcriptional responsiveness to glucocorticoids [429]. This finding is congruent with observations made in immortalized lymphoblastoid cell lines [651], PBMCs [105], and adipose tissue [457]. It suggests shared, *Fkbp5*-dependent mechanisms of central and peripheral regulation of the stress response with implications for the brain, immune system and metabolism. Due to the importance of cerebral regulation of the stress response [392, 394] and the finding that astrocytes are most responsive to stress signalling in the shape of glucocorticoids [429], it could be hypothesized that by targeting *Fkbp5* expression in astrocytes an overall regulation of glucocorticoid and stress signalling may be achieved.

Regarding the humanized variants of *Fkbp5*, analyses of their baseline expression levels of *Fkbp5* indicated that cell type specific differences between astrocytes, microglia and neurons were successfully transfered to the humanized mice [429]. Interestingly, both humanized *Fkbp5* variants were more abundant than the murine wild type version in astrocytes. This indicates that the CG-allele carrying mice are a more appropriate reference for potential effects of the AT-allele than wild type mice and underscores that the genetic background of animal models should be considered during analyses [483, 157, 586]

In the stimulation experiments, the AT-allele showed a stronger increase in expression levels after glucocorticoid exposure than the CG-allele [429]. Given that the FKBP51 protein was detectable after stimulation with glucocorticoids in different brain regions, these findings provide a validation that the humanized *Fkbp5* gene is functional. Moreover, the differential induction of both alleles is in line with observations made in human PBMCs [309]. This indicates that the different functionality of the human SNP was successfully transferred to the murine context. However, the induction of other glucocorticoid response element harbouring genes was not influenced by the humanized versions. This could be attributable to the absence of basal expression differences within cell types between the humanized strains and the design of the experiment where only a single stimulation with glucocorticoids was performed. Repeated exposure to glucocorticoids and more sampling time points might reveal potentially different dynamics between the humanized *Fkbp5* variants which could eventually have an impact on glucocorticoid-mediated responses. Ultimately, it remains to be confirmed in future studies that the differences on RNA level translate into differences on protein level.

Viewed as a whole, the results of **study IV** indicate a primary role of astrocytes in the cerebral responses to stress signalling in the shape of glucocorticoids. Since astrocytes are important regulators of the brain energy homeostasis, the observed differential induction of *Fkbp5* in astrocytes carrying the AT- vs. CG-allele might constitute an acute benefit through quicker inhibition of the metabolically challenging effects of glucocorticoids. It furthermore implies that *FKBP5*-related differences in glucocorticoid signaling may manifest in differences in the stress response through astrocytes; suggesting astrocytes as interesting cellular target for therapeutic interventions against stress-related neuropathologies. Further research is needed to clarify this potential and to address in more detail the spatial and temporal dynamics underlying *Fkbp5*-dependent mechanisms of stress regulation.

3.6. Flexibility of Stress Responses in a Preserved Network

'All models are wrong, some are useful.' (George Box)

An efficient network of key physiological functions to respond to challenging situations by adaptation developed under the evolutionary pressure 'survival of the fittest' [129, 501]. This stress response network was preserved across species resulting in general mechanisms like immediate adrenergic signalling and the release of glucocorticoids being observable in different studies [353, 128]. While adrenergic signalling is essential for the acute stress responses that manifest in behaviour like freezing, flight, or fight [380, 296], glucocorticoids are associated with the mid- to longterm responses to stress and play a role in adjusting the stress response system to future challenges [185, 229].

As a main communicator of the stress response through their mutual effects on metabolism, the immune system, and the CNS, glucocorticoids are involved in allocating energy for vital processes and influence learning [96, 305, 86, 644]. Despite the general response of releasing glucocorticoids, specificity is reached in the target organs dependent on dose, timing and context [516, 124, 189]. Models of the linkage between glucocorticoids and physiologic responses suggested a non-linear, inverted U-shaped relationship [35, 281, 382, 575, 145, 7]. The data on increased mitochondrial energy production capabilities after uCMS [430] or under genetic predisposition [428], in contrast to impaired OXPHOS in elderly psychiatric patients [293] is in line with this

notion. Studies with higher temporal resolution are required for a final conclusion. In addition to the likely influence of time, a dose-dependency was observed with additional stress experience during adolescence boosting the potential to generate energy. These additive effects are in line with the common observation of cumulative loads in stress research.

Regarding the putative decrease in neural activity suggested in both *in vivo* studies in parallel to the activation in the energy regulation despite the same cumulative load and duration of exposure, the relevance of considering the physiologic context becomes apparent. The seemingly contradictory findings from CNS and metabolism match with the commonly accepted dose-, timing-, and context-dependency of stress effects in human studies. These multivariate dependencies imply that different kinetics and responses are to be expected from the different physiologic functions [135, 147, 310, 294, 296, 249]. In turn, the response scales of the PINE network components need to be considered jointly to obtain a holistic impression of the health status of the investigated subject or to monitor effectiveness of treatments. In addition, treatments that account for imbalance in several components of the PINE network would be expected to be more effective [472].

Given that the preclinical data of this thesis matches the central hypotheses in human stress research, the animal models used and characterized during this thesis hold great potential to be valid models for aspects of human pathology [641, 45, 584]. Likewise, they may be valid for finding underlying pathological mechanisms shared between animals and humans that otherwise could not be investigated [634, 584]. The purpose of these animal models to identify and test novel preventive or curative treatments for persons at risk or patients seems to be fulfilled. Whether more criteria to ensure translatability between rodents and humans are met needs to be clarified in future studies. In the following paragraphs, the preventive and therapeutic potential of key findings resulting from a joint interpretation of the presented studies shall be discussed.

3.7. Energy Regulation Facilitates or Limits Choices to Cope with Stress

In the presence of a stressful challenge, several behavioural responses as well as adaptations to physiology would theoretically be imaginable, but not all of them might be eligible for the stressed individual. Besides the diversity of innate behavioural strategies and acquired behaviours, the energy level could be a limiting factor [559, 239]. In yeast, a convergence of different stress responses on mitochondrial processes has been reported, as well as a switch in behaviour from OXPHOS to respirofermentative metabolism in case respiration could not provide sufficient ATP [328]. Also in eukaryotic cells, the integrated stress response was shown to depend on mitochondria, energy levels, and the state of the cell [407]. *In vitro*, it was shown that tumour cells even developed ways to alter the mitochondrial capacities of T-cells to evade the immune defense [509]. Under oxidative stress, morphological and metabolic changes in mitochondria were described to support the first-line cellular defense and compensate for energy loss [502].

The prime role of regulating energy consumption and provision during acute stress was furthermore suggested in ***in vitro* study**. Given that astrocytes play a crucial role in the metabolic support of neurons and the observation that, among the tested cell types, astrocytes showed the strongest responsiveness to acute glucocorticoid stim-

ulation, the findings imply that the regulation of energy is a primary goal of cerebral stress response signalling [429]. The effectiveness of this mechanism has implications for brain functioning, since the energy status is critical for neural activity. Constraints in the shuttling of glucose and lactate to neurons via astrocyte networks was shown to compromise long-term potentiation and reveals how astrocyte bioenergetics can gate synaptic plasticity and control experience-dependent information [581, 418]. Moreover, astrocytes facilitate network integration that is involved in sexual maturation [455] and play a role in emotional learning [46, 660]. In light of this central role of astrocytes, the stronger astrocytic induction of *Fkbp5* in AT- vs. CG-allele carrying mice observed in this thesis [429] and the potential consequences of this on subsequent stress responsiveness suggest astrocytes as promising CNS target cell type for studying the differential effects of the human SNP rs1360780 on stress vulnerability. More insights into the translatability between human and murine astrocyte functions are needed to fully leverage this potential [340].

Another indication of the role of energy regulation on brain functioning is that the location and activity of mitochondria in neurons was shown to modulate synaptic plasticity [342, 93, 118, 544]. Mechanistically, the importance of mitochondria in the brain could be explained by the fact that neurons rely more on mitochondria than glycolysis to produce ATP [241] and that the calcium-regulating activities of mitochondria are essential for signal transduction [390, 292, 51] as well as for the regulation of apoptosis [238] or OXPHOS [162, 661]. This body of evidence suggests that the differential expression of genes related to mitochondrial metabolism and synaptic communication seen in AT- vs. CG-allele carrying *Fkbp5*-humanized mice [428] could mitigate the functional outcomes of each other. The presumable elevation of mitochondrial activity could buffer the presumable reduction in the neurotransmitter-associated signalling machinery. This illustrates that alterations on gene expression level need to be assessed for their impact on functional level.

The findings of higher mitochondrial density and elevated respirometric performance with a lower propensity to generate ROS after JSH and uCMS in rats could indicate that processes associated with a medium dose of stress signalling indeed translate into functional differences [430]. Since both *in vivo* studies of this thesis indicated adjustments towards increased capabilities of providing energy through OXPHOS in response to genetic predisposition or mild stress, the conclusion that mitochondria are masters of adaptation that are capable of positively withstanding stress for extended durations could be drawn [464]. However, misdirected or excessive use of energy resources could come at the cost of increased production of ROS leading to a rebound effect that ultimately could compromise mitochondrial functioning and overall health [293, 415, 278]. In **study I**, the possible increase in energy provision capacity appeared to not have been fully utilised [430]. Based on the framework between energy metabolism and synaptic activity outlined above, an elevation of neural communication would be expected in rats subjected to uCMS. However, the reduced expression levels of immediate early genes, which can serve as markers for neural activity, in the PFC in parallel to the findings in mitochondria is indicative of the opposite [430]. Following a prolonged exposure to uCMS, a similar observation was made in the hippocampus [430]. Since this brain region is connetected to the PFC, functional changes in the stress-processing circuit between PFC and hippocampus are suggestive but need to be verified in future experiments.

Given that the brain is trained on effectively allocating energy, actions that are evaluated to be most cost-effective might be chosen over other functions that can not simultaneously be permitted [211, 12, 598, 70]. Since the mild stressors used in **study I** were only transient, letting the situation go and not changing the *status quo* could have been a sufficient reaction. Given that the scheduled stressors ended without the possibility to modulate their duration, this positive feedback could have reinforced this strategy of ignorance, increasing the likelihood to again choose and train this approach. In contrast, it is an active, energy-requiring process to change the current setting, suppress undesirable thoughts, forget negative memories by overwriting them with new information, and to learn alternative coping strategies [539, 508]. To be able to pick an adequate response and to allow for higher flexibility in the response to novel environmental challenges, increasing the richness of the behavioural repertoire through cognitive and behavioural therapy could be a way forward.

A possible indicator of behavioural diversity could be the individual activity. In humans, activity can provide information on arousal and engagement in a situation. The same holds true for laboratory animals. Alterations in activity were one parallel between the *in vivo* studies of this thesis, with reduced and passive behaviour beeing associated with findings that are suggestive for a negative transition. Rats that experienced uCMS over a period of five weeks were more lethargic (observation not quantified) and showed depression-like behaviour including more floating in the forced swim test, decreased sucrose preference, and more freezing in the center of the open field after 14 weeks of uCMS [430]. In the *Fkbp5*-humanized mice that experienced ELA, carriers of the risk-associated AT-allele, showed less social interest. Moreover, AT-allele carrying females that experienced ELA were less active in response to novel environments, while in female CG-allele carriers, ELA was associated with increased locomotion not only during the murine active phase but also in behavioural tests conducted during the day [428].

These differences between the SNP variants were reflected in higher body weights in AT- vs. CG-allele carriers. This weight difference is an interessting feature of the *Fkbp5*-humanized mouse model, since not many animal models capture a weight gain following psychologic stress [242]. Weight is gained if the pro-obesogenic effects of stress, mediated via glucocorticoids and neuropeptide Y signalling, outweigh anorexigenic effects via CRH and catecholamine signalling [478]. It matches to the observation that knockout or inhibition of *Fkbp5* protects mice from high-fat diet-induced weight gain, improves glucose tolerance, and increases insulin signaling in skeletal muscles [36]. The effects of *Fkbp5* on body weight could be explained by the linkage of *Fkbp5* with the mammalian target of rapamycin (mTOR) pathway and the regulation of autophagy [248], which have been discussed to play a role in the effectiveness of antidepressants [484] and neurodegenerative disorders [34].

Given that the CG-allele of *Fkbp5* is thought to convey resilience to stress, the leaner phenotype and higher activity in carriers after ELA suggest that physical activity could be a healthy coping strategy to face stress and to relieve from its negative symptoms. This hypothesis would be in line with treatment strategies that include physical exercise as modulator of stress [496, 307, 273]. Mechanistically, a beneficial modulation of *FKBP5* via aerobic exercise was proposed [511] and exercising was reported to promote changes in mitochondrial volume, structure, and capacity that improve mitochondrial health [403] and allow for a lower production of ROS during daily activities [332], while bed rest leads to opposite mitochondrial adaptation [74].

Synergistic to the effects of physical exercise on mitochondrial fitness, a bi-directional relationship between physical activity and the circadian rhythm exists [373, 633, 17, 585, 653]. In healthy individuals, glucocorticoid levels are lowest during the resting phase and peak sharply towards awakening followed by a gradual decrease during the active phase. This natural rhythm is paced by the cycle of day and night and represents a measure to allocate energy expenditure and regulate activity dependent on the current requirements of the environment. On a molecular level, the connection between energy metabolism and circadian rhythmicity is reflected in the expression and activity of kinases, phosphatases, and nuclear receptors, which follow a circadian rhythm and alter circadian regulation and metabolism *vice versa* [47, 485, 587]. Activity and circadian rhythm can modulate awakeness and alertness and thus have an influence on the current individual abilities to engage with the environment and to respond to challenges [154, 158, 513].

3.8. The Choice of the Stress Response Depends on Cognitive Processing

Strong variability of the stress responses between individuals can be observed in the real world as well as in an experimental setting [274, 123, 510, 337]. Besides the impact of timing on energetic state and alertness as outlined in 3.7, a big share of the individual differences in the stress response can be attributed to differences in the relative contribution of instincts versus free will on the choice of behavioural responses [413, 506, 580]. This makes the brain an attractive target organ for interventions, since it is responsible for the first processing of stress signals before further downstream cascades are initiated [392]. Already at exposure, a diverging perception of a potential stressor as not being worth to be considered or as eminent threat can contribute to the high variability of the stress response. Together with the context, the individual connectivity of brain circuits will modulate this judgement and further processing of the stressor [251, 421]. Immediate adjustments of autonomic signalling via a glutamate-dependent communication from the PFC to the hypothalamus [296] are followed by learning processes.

Elevated expression of immediate early genes, could serve as indicators of ongoing neural activity and learning processes. The reduced expression of these genes in the rat PFC and hippocampus after uCMS however suggests that less neural activity was taking place [430]. Since the expression of genes related to neurotransmitter signalling is a prerequisite for neural activity, the lower expression of genes related to synaptic communication in the hypothalamus and hippocampus of AT- vs. CG-allele carrying mice indicates that genetic factors could synergize with the effects of uCMS to limit neural activity and learning capacity [428]. While activation of GR signalling was shown to decrease excitatory neurotransmission in the PFC, low levels of *Fkbp5* were proposed to distort the excitatory/inhibitory balance in this brain region [505]. Real-time analyses like fMRI, EEG or electrophysiological measurements would help to consolidate the implications of the gene expression data regarding decreased synaptic communication in genetically predisposed or chronically stressed individuals with insights on functional outcomes. Moreover, a high resolution regarding excitatory and inhibitory neuronal signalling should be aimed for, since the imbalance of these is a pathological aberration observed in psychiatric disorders [169, 537, 414, 562, 79] that may be exacerbated by stress [493, 520].

Additional hints for reduced neural activity under chronic stress are provided by the analyses of TRYCAT levels. Profiling of these neuroactive substances in uCMS rats suggested that the kynurenine pathway is activated by a mild level of stress in an anti-excitatory manner, since the plasma and CSF concentrations of KYNA increased while the levels of QUIN, decreased [430]. The resulting lower stimulation of excitatory receptors might be an additional mechanism to limit neural signalling and could explain the decreased expression of immediate early genes in the brains of uCMS-exposed individuals. In the clinical trial including elderly humans at risk to develop neuro cognitive disorders of which **study III** was part of, a positive correlation between lifetime stress and cognitive performance was observed [326]. This supports the findings in rats by suggesting that stress contributes to decreased cognition in humans. In contrast to lower levels of QUIN in young adult rats after uCMS [430], serum levels of QUIN were increased in participants with elevated lifetime stress [327]. While the outcome of stress on neural activity and cognition appears the same in both studies, the underlying mechanisms differ. The findings suggest that the effects of QUIN on cognition seem to follow an inverted U-shaped curve: Too low levels of QUIN contribute to a lack of stimulation, while too high levels reduce cognitive performance through excessive oxidative and excitatory signalling that may compromise cellular health or even cause neurotoxicity. Further studies at peak QUIN concentrations are needed to define the middle part of the putative curvature.

Cognitive training resulted in decreased levels of 3-HK and KYNA, which indicates bidirectional relations between TRYCATs and neural activity. However, the training interventions did not improve cognitive performance [327]. This absence of improvement suggests that changes in the PINE network like regulation of the kynurenine pathway occur faster than changes in neural wiring and cognition. Regarding the consequences of stress exposure, this may imply that adjustments in the PINE network are transient and can be reverted after the end of the triggering challenge, preserving a high flexibility of the stress response system and resilience of the exposed individual. Regarding the treatment of medical conditions, it could suggest that once critical transition points are crossed and brain circuitry is altered, the therapy required to turn back adverse changes over a lifetime needs to simultaneously address several modifications of the PINE network or be of longer duration, too. As alternative hypothesis that could explain the absence of measurable treatment effects on cognition, the relative reduction of KYNA might not have been enough to overcome the described negative effects of accumulated KYNA on learning and memory impairment associated with ageing [617]. Since the pharmacological reduction of KYNA through administration of a KAT inhibitors was shown to ameliorate cognitive performance [316], additional therapeutic approaches that limit formation of KYNA might improve the effects of physical and cognitive training on cognition. Dependent on the source of cognitive impairment being either excessive anti-excitatory signalling via KYNA or cellular damage via QUIN, presumably different routes of intervention should be chosen to obtain a benefit for the patient.

Since pressure and stress is thought to negatively interfere with learning performance in the classroom [615, 302] and as uCMS was shown to trigger an activation of the kynurenine pathway in an anti-excitatory manner [430] which may hinder formation of persisting memories [660], strategies to decrease stress signalling could be beneficial to improve cognition and learning. However, the need for a certain level of arousal reflected by low to moderate increases in cortisol to reach top performance has been shown [525, 192], as well as a dependency of anxiety treatment effects and cortisol states [637].

This observation is in line with the postulated inverted U-shaped relationship between neural activity and glucocorticoids [151, 451, 304, 8] and illustrates that finding the right level of glucocorticoid signalling during interventions might boost the effectiveness of therapeutic interventions. To benefit from this knowledge, either the provocation of an increase in cortisol [71], as happening during exposure therapy [201, 432], or making use of the natural rhythm of glucocorticoids by scheduling the intervention in a permissive window of the diurnal rhythm [405, 331] could be considered as modulators of treatment success in future studies.

3.9. Circadian Rhythmicity as Lynchpin for Flexible Stress Responses

In living organisms, no steady state or equilibrium of physicochemical reactions is present but systems are open and oscillate [618, 569]. This dynamic behaviour allows for adaptation to changing external or internal inputs and is thereby essential for normal functioning [468, 629, 286]. In the field of psychology, the diurnal and ultradian rhythmicity of glucocorticoids plays a central role since disruption of their oscillation has been linked to pathology and compromised well-being [606, 229, 191, 632]. On a molecular level, diurnal rhythms of gene expression have been described [247, 121] and a loss of these rhythms was associated with altered metabolic function [491], impaired sleep and memory [63], vulnerability to helplessness [470], and psychosis [299]. Furthermore, ageing was associated with altered expression patterns of so called 'clock genes' which could contribute to reduced memory and elevated risk for depression in the elderly [98].

This body of literature emphasizes that loss of circadian entrainment is an important aspect of psychiatric disorders, which valid animal models should capture. Previously, the clock system has been targeted to elicit symptoms like anxiety, depression, and mania in mice [417, 389, 388]. The uCMS rat model and the *Fkbp5*-humanized mice characterized within this dissertation represent another route to generate valid models. The first model uses stress as known trigger for the development of disorders and it was assessed whether alterations in glucocorticoid signalling would be captured as well. In the second model, versions of the *FKBP5* gene that are known to modulate disease risk were used and their effects on different aspects of psychiatric disorders were investigated.

Matching the overall interpretation of **study I** that the medium stress exposure was associated with allostatic adaptation, diurnal rhythmicity of faecal glucocorticoid levels was observed in stressed and control rats. Nevertheless, the premature shift in the timing of the glucocorticoid peak in animals experiencing or witnessing JSH compared to controls could suggest that the manipulation is capable of altering the HPA axis regulation of glucocorticoids. Other studies using uCMS found that the diurnal rhythmicity of locomotion [217], corticosterone [103], and gene expression is altered [355, 630]. The absence of this finding in **study I** could be due to differences regarding study design including the duration and intensitiy of the stressors. Further, different read-outs and associated methodology contribute to diverging observations.

In line with the concept that the risk-allele of *FKBP5* should be linked to aberrations seen in a pathologic state, the AT-allele was associated with a flattened diurnal corticosterone amplitude in females [428]. In male mice, the diurnal corticosterone amplitude was lower than in females [428]. This reduced diurnal amplitude would be

expected to modulate the activity of the affected individuals and might reduce their adaptive capacity towards novel situations. Carriers of the AT-allele were less responsive to novel environments as assessed during behavioural testing. In addition, males were less active than females, with the genotype-based differences in behaviour being less pronounced [428]. Aberrant responses to environmental cues in parallel to a disrupted circadian rhythm have previously been reported [18].

Given that all animals were exposed to the same lighting conditions, the differences in the diurnal HPA axis rhythmicity need to be attributable to internal adjustments of overall activity in response to stress or genetic predisposition. Pathway enrichment analyses of differentially expressed genes between the *Fkbp5*-humanized mice confirmed that genes related to circadian entrainment in the hypothalamus were lower expressed in AT- vs. CG-allele carriers [428]. Early life adversity led to an additional reduction in expression of those genes in the hippocampus [428], which is known to co-regulate the HPA axis to adjust performance during the upcoming day in the event of anticipated challenges [192].

Changes in the expression of genes involved in regulating dopaminergic signalling were an additional finding in the *Fkbp5*-humanized mice [428]. Dopamine was shown to correlate with the stress response in the healthy situation but in chronically stressed individuals less responsiveness of the dopaminergic system to stressors was reported [57]. Moreover, dopamine is a key player in reward neurophysiology and synaptic plasticity [448]. It was brought into context with circadian rhythmicity, since changes in brain regions related to dopamine were shown to express clock-genes and genes required for dopamine production were shown to follow a diurnal rhythm [299, 357]. Additionally, altered firing of dopaminergic neurons was linked to mood cycling and altered circadian rhythmicity in clock-mutant mice [117, 551]. Given that dopamine receptors activate signalling molecules that are linked to metabolism [40], this further contributes to the tight relation of energy regulation, neural signalling, and diurnal rhythmicities. It could furthermore suggest that altered dopamine signalling and circadian entrainment, via the mechanisms outlined above, modulate the choice of the stress response in AT- vs. CG-allele carriers.

Current and recent levels of glucocorticoids influence the responsiveness to acute stress [490, 42] and a robust stress response, combined with strong negative feedback, was shown to be important for persisting in rapidly changing environments [663]. Therefore, maintaining or re-establishing the dynamic flexibility of the HPA axis is a prominent goal in the therapy of stress-associated disorders. A strict sleep control and behavioural therapy was shown to stabilize mood [246]. Moreover, the effects of lithium on mood have been shown to rely on regulation of the circadian clock [339] via a similar mechanism like the mode of action of valproate [649]. In addition, valproate was suggested to increase rhythm amplitude and to help synchronising the circadian clock through its actions on dopamine and the regulation of energy [29, 356]. Nobiletin, which was shown to influence liver metabolism via coordination of cholesterol and bile acid, is also investigated for its effects on circadian rhythm [97, 426]. The effectiveness of directly targeting casein kinase 1 $\epsilon\delta$, a player in the clock gene machinery, using the compound CK01 is investigated in a clinical trial [24]. If the current environment, lifetime history, or genetic predisposition lead to elevated induction of *FKBP5* and thus to a presumably slower oscillation of glucocorticoids, bringing the expression or activity of *FKBP5* to lower levels could be an alternative treatment goal to improve the flexibility of the stress response.

3.10. Overall Conclusion

The results of my thesis confirm the hypothesis that common mechanisms underlying the stress response exist across species, and that animal models may be a valid tool to investigate causal relationships between exposure to stressors at different developmental stages and their impact on health in later life. The data is in line with a broad body of literature describing close interactions of the brain, immune system, and metabolism which involve endocrine signalling that is fine-tuned by time, dose, and context in a non-linear manner. The inverted U-shaped relationships between stress exposure and functioning of components of the PINE network as well as the number of interactions within the network illustrate its high complexity, which could be an explanation for the observed high variability in response to stressors. This individual component makes the finding of generalisable patterns a challenging task in stress research where the main effect sizes may be small. Nevertheless, the multitude of interactions between subtle changes can add up to substantial differences in the coping with stress, allowing for flexibility of the stress response system and promoting resilience to different kinds of stressors up to a certain dose or frequency.

Many of the stress-induced changes observed during this dissertation appear to be adaptive to the transient, non-life-threatening challenges the animals were exposed to. The findings indicate energy regulation as an important function of the PINE network in the early responses to stress, with astrocytes as the main players in the brain. Reduced behavioural responsiveness, circadian HPA axis rhythmicity, and neural activity in chronically stressed or AT-allele carrying individuals could potentially be a measure to save energy by limiting adjustments to the new situations. This advantage could be an explanation for the relatively high abundance of the AT-allele in humans.

In persistently changed environments, this mechanism could lead to maladaptation, which may cause allostatic loads that could lead to wear and tear over time. Since chronic stress and the AT-allele in combination with ELA were identified to induce similar changes in the PINE network despite the different backgrounds and designs, their effects on the responsiveness to stress might synergize. This could explain the higher prevalence of psychiatric disorders in AT-allele carriers growing up and potentially still living in aversive environments. Investigations of a triple hit model that combines these genetic, developmental, and environmental factors are needed to further address this derived hypothesis. Moreover, this animal model can be used to further investigate risk and resilience factors, to better elucidate causal pathomechanisms, and to develop a stratification strategy for therapeutic interventions that considers the individual combination of external and internal factors and thus could pave the way to a more personalised medicine.

Based on the findings of this thesis, a higher flexibility of the stress response system might be protective against the negative consequences of stress and re-establishing this flexibility appeares to be a promising therapeutic goal. Approaches that increase the repertoire of coping strategies by e.g., ensuring social support during development, training alternative interpretation options through cognition exercises, and learning different reaction schemes in behavioural therapy could promote this. If the energy and glucocorticoid level is considered, the success of the intervention sessions may be improved. Beneficial modulation of the energy and glucocorticoid levels might be obtained by physical training, the intake of healthy nutrition, and sufficient sleep since those help to structurize the day and lead to a better entrainment of circadian rhythmicity. Many of these suggestions are already used in clinical practice, but on their own

failed to cure the patients. Using the synergies between them by intervening against more than one of the pathological drivers in a multimodal approach might be a key to offer efficacious treatments to patients that are considered treatment resistant at the moment.

To boost efficacy, scheduling of the treatment sessions in permissive windows would be beneficial. The additional determination of glucocorticoid and *FKBP5* dynamics could help to find the optimal timing. Moreover, these measures could indicate when additional, pharmacological approaches might be sensible: In genetically or environmentally burdened individuals where on average more *FKBP5* than normal is present, its inhibitory effects may limit the flexibility of the PINE network by slowing down glucocorticoid dynamics, which could manifests in a dampened diurnal rhythmicity around an elevated level. This could in turn hinder adequate stress responses, resulting in more allostatic load, and a reinforcement of altered glucocorticoid signalling. To overcome this deadlock, pharmacological inhibition of *FKBP5* might help to improve treatment efficacy and ultimately well-being. To optimize the timing of such interventions, the *Fkbp5*-humanized mice models could be a valid tool to resolve the kinetics of cellular and brain circuit responses to stress and pharmacological treatments in more detail.

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C. Statutory Declaration

I hereby declare that I wrote the present dissertation with the title

Consequences of Stress on the Psycho-Immune-Neuro-Energy Network - On the Search for Novel Biomarkers and Protective Factors

independently and used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works. I also hereby declare that I carried out my scientific work according to the principles of good scientific practice in accordance with the current 'Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis' (Rules of Ulm University for Assuring Good Scientific Practice). All study procedures encompassed in this thesis have been previously approved by the ethics committees of the responsible authorities.

Ulm, December 2021 _____