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Stress-related tissue-specific regulation of DNA methylation in non-coding elements of FKBP5 in brain and blood of mice, humanized mice and humans

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List of abbreviations

BA	Brodmann area
BD	bipolar disorder
BDNF	brain-derived neurotrophic factor
CORT	corticosterone
CpG	cytosine-phosphate-guanine dinucleotide
CTCF	CCCTC-binding factor
DEX	dexamethasone
DNAm	DNA methylation
ELS	early-life stress
FANS	fluorescence-activated nuclei sorting
FC	frontal cortex
FKBP5	FK506-binding protein 5
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid-responsive element
GxE	gene by environment
HAM-TBS	high accuracy DNA methylation measurement via targeted bisulfite sequencing
HIP	hippocampus
HPA	hypothalamic-pituitary-adrenal
HSP90	heat shock protein 90
LBN	limited nesting and bedding
MDD	major depressive disorder
PFC	prefrontal cortex
PTSD	posttraumatic stress disorder
SCZ	schizophrenia
SNP	single nucleotide polymorphism
T-DMR	tissue-specific differentially methylated region
TAD	topologically associating domain
UTR	untranslated region

List of publications

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Contribution to the publications

Contribution to paper I

Paper I (Yusupov et al., 2023) was published in *The European journal of neuroscience* in 2023. I contributed to study conception, pre-processed, analyzed and interpreted the data, prepared and edited tables and figures, wrote the initial draft and revised the manuscript. LvD contributed to data acquisition and interpretation, wrote the initial draft and revised the manuscript. SR contributed to study conception, supervised pre-processing. TW contributed to study conception and methodology. MK, SS and MRH contributed to data acquisition. MVS and EBB acquired funding, contributed to study conception, analysis and interpretation of the data and supervised the project. All authors contributed to and have approved the final manuscript.

Contribution to paper II

Paper II (Yusupov et al., 2024) was published in *Molecular Psychiatry* in 2024. I contributed to study conception, methodology and data acquisition, pre-processed, analyzed and interpreted the data, prepared and edited tables and figures, wrote the initial draft and revised the manuscript. SR contributed to study conception, contributed to and supervised pre-processing. LSE contributed to study conception, methodology and data acquisition. SC, SL, LUT, SS and MK contributed to data acquisition. ASF contributed to data acquisition and preparation. NM contributed to study conception and data preparation. DC contributed to methodology and data analysis. JMD and EBB acquired funding, contributed to study conception and methodology, analysis and interpretation of the data and supervised the project. Funding was acquired by EBB. All authors contributed to and have approved the final manuscript.

Contribution to paper III (Appendix A)

Paper III (Martins et al., 2022) was published in *Pharmacology Biochemistry & Behavior* in 2022. NY, JM, EBB, DZ and TMB performed a narrative literature review, wrote the initial draft and revised the manuscript. DZ and TMB additionally supervised the project.

1. Introductory summary

1.1 Theoretical background

1.1.1 Gene by environment interaction hypothesis in psychiatry

Psychiatric disorders are common diseases contributing greatly to the global disease burden without evident reduction in the last three decades (GBD Mental Disorders Collaborators, 2022). Across disorders, resistance to current treatments affects up to 60% of patients (Howes et al., 2022). Despite considerable efforts, only small advances were made in the development of mechanistically novel psychiatric drugs (Howes & Baxter, 2023). Among possible reasons, methodological challenges, clinical heterogeneity of patients and limited understanding of disease-underlying biological mechanisms were postulated (Machado-Vieira, 2012; Paul & Potter, 2024). Attempts to understand the genetic etiology of psychiatric disorders (G) revealed a complex polygenic architecture, but were unable to solely explain the variance in phenotype, *e.g.*, observed traits (Andreassen et al., 2023; Sullivan et al., 2018). Similarly, exposure to environmental factors (E) such as stress and adverse life events, especially early-life stress (ELS) during biologically sensitive developmental periods of the brain (Danese & McEwen, 2012; Shonkoff et al., 2009), was linked to the risk of developing psychiatric disorders (Lippard & Nemeroff, 2023; Smith & Pollak, 2020). Environment alone could also only partly explain phenotypic variance (Kessler et al., 2010). The acknowledgment of complex genetic, non-genetic and interacting influences on psychiatric traits (Grotzinger, 2021), led to an integrative approach, which explored interactive effects of both genetics and environment (GxE) in order to better understand psychiatric disorders (Assary et al., 2018). At heart, this approach is in line with the diathesis-stress model of psychopathology, according to which individuals with a genetic predisposition (high genetic susceptibility) are more prone than others (low genetic susceptibility) to the development of psychiatric disorders once exposed to certain environmental factors such as stress (Broerman, 2020).

1.1.2 *FKBP5* involvement in gene by environment interactions

One of the important target genes found to present GxE effects in psychiatric research was FK506-binding protein 5 (*FKBP5*). The gene encodes for FKBP51 or FKBP5, a co-chaperone (accessory protein) of the heat shock protein 90 (HSP90) and part of the glucocorticoid (GC) receptor (GR) heterocomplex (Pratt & Toft, 1997). In the recovery process from stress, the FKBP5 protein functions as a negative regulator of GR-induced transcriptional activation by modulating GR affinity to GCs and delaying the translocation to the nucleus (Denny et al., 2000; Scammell et al., 2001; Wochnik et al., 2005). The *FKBP5* gene itself contains GC-responsive elements (GREs), that allow an intracellular ultra-short negative feedback loop (Vermeer et al., 2003). The chronic overexpression of *FKBP5* may lead to a disrupted negative feedback loop with reduced GC sensitivity or "GC resistance" of the GR and elevated cortisol levels (Denny et al., 2000; Reynolds et al., 1999; Scammell et al., 2001; Wochnik et al., 2005).

Following the discovery that intragenic single nucleotide polymorphisms (SNPs) in *FKBP5* are associated with psychiatric disorders (Binder et al., 2004), Binder et al. described their interaction effects with reported abuse in childhood to predict the severity of posttraumatic stress disorder (PTSD) symptoms later in adulthood (Binder et al., 2008). Studies that followed this discovery provided further evidence (Wang et al., 2018 for meta-analysis). Possibly, the risk allele of rs1360780 SNP creates a TATA-box binding site enhancing a three-dimensional chromatin loop formation, that results in a closer contact with the transcription start site and the RNA polymerase II (Klengel et al., 2013), leading to increased mRNA transcription upon GC exposure. The emerging importance of *FKBP5* in the regulation of the hypothalamic-pituitary-adrenal (HPA)-axis via GR sensitivity (Zannas et al., 2016) fit well to the established hypothesis of insufficient regulation of the response to stressful stimuli in the development of psychiatric disorders (Tafet & Nemeroff, 2016). Specifically, alterations in the termination of the HPA-axis-related stress response were suggested as a possible pathological mechanism involving *FKBP5* as an intracellular negative regulator of GR activity (Vermeer et al., 2003). Such alterations were hypothesized to occur due to chronic and/or strong activation of the HPA-axis in early periods of development

and were supported by a vast literature of animal and human studies (Lupien et al., 2009).

1.1.3 DNA methylation linked to gene by environment interactions and stress-related psychopathology

Epigenetic mechanisms, *i.e.*, molecular processes that regulate genetic information and result in transcriptional changes without changing the DNA sequence itself, were pointed out as biological processes that are influenced by both early experiences (Szyf & Bick, 2013) and genetic variation (Villicana & Bell, 2021). Importantly, these mechanisms were shown to be dynamic and have the capability to induce long-term changes following environmental stimuli in postmitotic tissue, making them suitable to mediate changes into later adult life (Szyf et al., 2008). A growing body of evidence from studies in mice and humans pointed towards the involvement of DNA methylation (DNAm), a chemical modification occurring mainly at cytosine-phosphate-guanine dinucleotides (CpGs) in mammals (Moore et al., 2013), in the response to trauma and stress (Matosin et al., 2017). According to current knowledge, DNAm may mediate long-term effects of exposure to ELS on genes involved in the HPA-axis and is believed to shape the variability of the stress response (Reshetnikov et al., 2018; Silberman et al., 2016). This can result in a dysregulation of the HPA-axis and an increased risk of psychopathology in various psychiatric disorders (Murphy et al., 2022).

1.1.4 *FKBP5* as biomarker and drug target in psychiatric disease

The human *FKBP5* locus, located on chromosome 6, contains 13 exons, 12 introns and multiple functional SNPs, with rs1360780 (in intron 2) as the most relevant for psychiatric research (Binder et al., 2008; Binder et al., 2004; Ellsworth et al., 2013; Jääskeläinen et al., 2011). GREs are located upstream of the promoter and in intronic elements throughout the locus and can enhance *FKBP5* transcription by chromatin conformation induced interactions (Paakinaho et al., 2010). Current literature suggests the following allele-specific environmentally

shaped mechanism: Upon stress and subsequent trans-regulatory binding of a GR homodimer as a transcription factor, individuals with a genetic “risk” variant (minor A/T-allele) of the rs1360780 SNP of *FKBP5* (which leads to higher transcriptional activation with GC) and a prior exposure to childhood maltreatment, are more susceptible to demethylation of DNA in GREs, which then leads to an enhanced *FKBP5* expression and a prolonged cortisol response (Matosin et al., 2018) which go beyond the effects observed with the risk genotype alone. In this process, DNAm is considered an important mechanism to facilitate GxE effects and to last into adulthood as shown in both human blood and neuronal cells (Klengel et al., 2013). In fact, the genotype alone does not seem to be strongly associated with psychiatric disease risk, this association is only seen in the context of early adversity (Matosin et al., 2018). This has led to the proposition that genetic and epigenetic disinhibition of FKBP5 are needed to increase risk.

While epigenetic mechanisms are generally tissue-specific, environmental effects might influence different tissues similarly (Provençal et al., 2012). Effects on DNAm in the brain can lead to psychopathology and similar alterations in easily obtained tissues such as peripheral blood could be used as a biomarker for the detection of patients with altered GR sensitivity. DNAm at specific sites of *FKBP5* was suggested as a biomarker for the transdiagnostic identification of a subgroup of patients, that could possibly profit from *FKBP5*-targeted therapies (Matosin et al., 2018) given that low DNAm levels at sensitive GREs are associated with higher FKBP51 expression. Selective inhibition of FKBP51 using SAFit2 *in vivo* provided promising results for modulation of the HPA-axis regulation and reduction of anxiety-like behavior in rodents (Gaali et al., 2015; Hartmann et al., 2015). Moreover, chronic application of SAFit2 promotes stress resilience and changes in hippocampal neurogenesis (Codagnone et al., 2022). Importantly, until now there is no evidence against the use of FKBP5 as a drug target. Especially as *FKBP5* knockout in a whole mouse neither shortened duration of life, nor did it alter glucose tolerance, blood cell type composition, cytokine profiles or locomotion (O'Leary et al., 2011; Sabbagh et al., 2014). Yet, to achieve progress in blood biomarker and new therapeutic strategy development, it is important to explore GxE mechanisms in both blood and brain tissue. A possible translational solution for the limited applicability of environmental interventions in the human brain, is the study of model organisms, an approach also known as “reverse translation”

(Figure 1). Hence, along anatomical, physiological, genetical proximity and other practical reasons (Rosenthal & Brown, 2007), an important motivation for the use of mouse models in biomedical research is the ability of genetic and/or environmental manipulation or drugs administration and subsequent investigation of multiple, otherwise in humans not accessible, tissues. Moreover, "reverse translation" could be further enhanced by using humanized mice, *i.e.*, genetically manipulated mice with exchanged orthologue human gene sequences (Figure 1). However, the study of non-coding elements as in this work, which are considered to be less conserved across species, requires a prior exploration of the epigenetic mechanisms involved to ensure translational appropriateness (as discussed in detail in section 1.2.3).

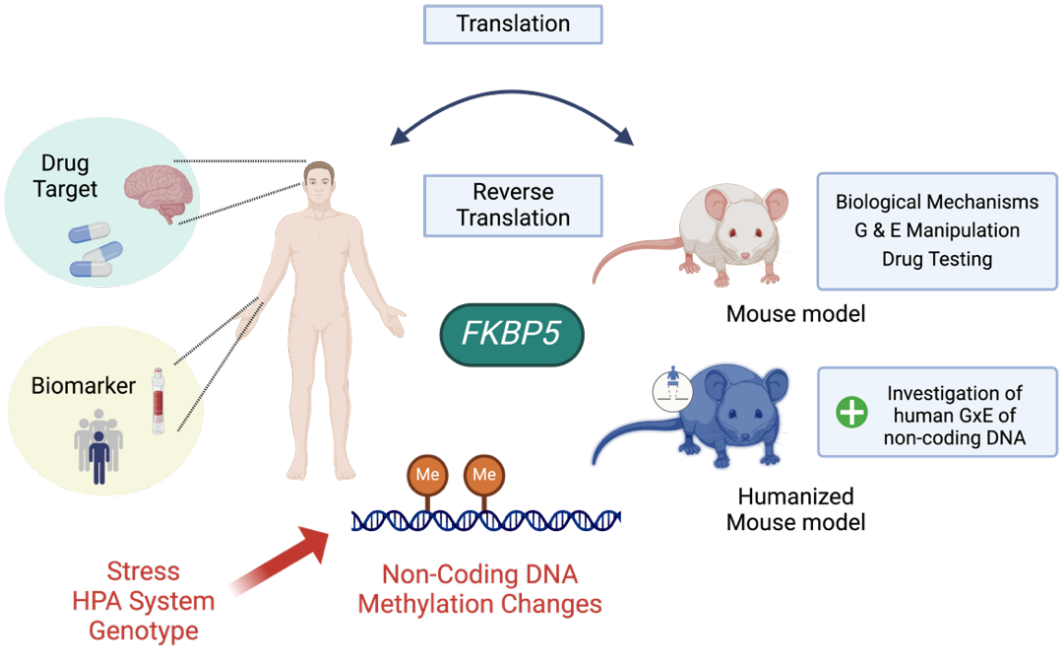

Figure 1. Translational approaches towards *FKBP5* as drug target and biomarker. On the left, two applications derived from human research are listed. On the right, advantages of "reverse translation" to a mouse model (upper part) and additional advantages of humanized mouse models (lower part) are presented. Figure created with BioRender.com.

1.1.5 Prefrontal cortex and hippocampus as regions of interest

Many different cortical and subcortical brain structures are involved in the functional processing of perceived environmental stress (Herman et al., 2016; Lupien et al., 2018). This thesis focuses on the prefrontal cortex (PFC) and the hippocampus (HIP). Areas in the PFC, such as the orbitofrontal cortex and the medial prefrontal cortex, transport integrated sensory information to the hypothalamus and are involved in the adaptive stress response and regulation of the HPA-axis (Sullivan & Gratton, 2002). Additionally, the PFC was suggested to carry long-lasting changes in the HPA-axis feedback mechanism (Mizoguchi et al., 2003). The HIP is involved in contextual integration later projected to the amygdala and can negatively regulate the HPA-axis (Cole et al., 2022; Herman et al., 2016; Sapolsky et al., 1984). Importantly, these two brain regions were consistently altered across psychiatric disorders (McTeague et al., 2020), and involved in stress-associated *FKBP5* biology (Criado-Marrero et al., 2019; Criado-Marrero et al., 2017; Criado-Marrero et al., 2020; Kwon et al., 2019; Ryu et al., 2021; Schmidt et al., 2015; Sinclair et al., 2013; Touma et al., 2011). Several studies showed higher expression of *FKBP5* in the frontal cortex (FC): in the dorsolateral PFC in schizophrenia (SCZ) and bipolar disorder (BD) (Sinclair et al., 2013), in the middle frontal gyrus in autism spectrum disorder (Patel et al., 2016) and in a smaller sample also in major depression (MDD, Mamdani et al., 2015). Evidence also exists for changes in neuronal morphology in relation to increased *FKBP5* expression, revealing reduced dendritic spine density in the medial orbitofrontal cortex (Brodmann area, BA 11) of patients with PTSD (Young et al., 2015) and of other psychiatric patients with stressful life events (Kaul et al., 2020). A comprehensive study by Matosin et al., found higher expression of FKBP5 in the dorsolateral prefrontal, medial orbitofrontal and ventral anterior cingulate cortex (BA 9, 11 and 24/24a) of patients with SCZ and MDD, most consistently in superficial layer excitatory neurons in BA 11. This was also associated with lower levels of brain-derived neurotrophic factor (BDNF) and reduced dendritic mushroom spine density (Matosin et al., 2023). According to data from adult mice, different basal expression levels of *Fkbp5* are present in these areas, with high expression in HIP and low expression in the FC (Scharf et al., 2011). Notably, regions with low basal expression presented a higher activation upon induction (Scharf et al.,

2011). Finally, in both medial PFC and HIP, *FKBP5* deficiency led to functional alterations of neurotransmission, most importantly the reduction of GR-mediated effects (Qiu et al., 2019; Ryu et al., 2021; Zhang et al., 2022).

The knowledge summarized above and the remaining gaps discussed in detail in the next chapter lay the conceptual ground for this thesis.

1.2 Knowledge gaps addressed in this thesis

1.2.1 DNA methylation of *Fkbp5* in mouse models

As our knowledge about stress-related *FKBP5* DNAm changes in human tissues is mounting (Mendonça et al., 2021), much less is known in rodents. So far, a limited number of studies investigated DNAm of *Fbkp5*, the murine homologue on chromosome 17 (Cox et al., 2021; Ewald et al., 2014; Lee et al., 2010; Sabbagh et al., 2014; Sawamura et al., 2016; Seifuddin et al., 2017). Moreover, up to this point, only a small number of CpG sites was characterized, mainly due to technical limitations related to sequencing length of pyrosequencing (Šestáková et al., 2019). A broader mapping is essential to understand whether CpGs around other possible functional non-coding elements of the DNA are important and can be used as biomarkers. Existing literature is further limited by lower accuracy of pyrosequencing as DNAm quantification technique, which is relevant in psychiatric research due to expected sizes of effects (Roeh et al., 2018). Finally, in the studies mentioned above, investigation of DNAm was performed upon treatment with corticosterone (CORT), which might not resemble biological effects of psychosocial stress. To enable future exploration of *Fkbp5*-related GxE mechanisms and the involvement of DNAm in their regulation in mice, a more extensive and accurate exploration of DNAm in potentially regulatory elements of the gene under a natural stress paradigm was sought. In this work, high accuracy DNAm measurement via targeted bisulfite sequencing (HAM-TBS) was applied, a quantification technique for very accurate in-depth investigation of candidate genes in mixed tissues (Moser et al., 2020; Roeh et al., 2018).

1.2.2 Brain-specific DNA methylation of *FKBP5* locus

The brain is etiologically the most relevant tissue in psychiatric research. So far, our understanding of *FKBP5* DNAm in human brain tissue is rather limited. The exploration of these mechanisms in brain tissue is essential for a possible future development of targeted treatments. Studies in humans, widely investigated the DNAm within this gene in peripheral tissues such as blood and saliva (Klengel et al., 2013; Parade et al., 2017; Piyasena et al., 2016; Tyrka et al., 2015; Wiechmann et al., 2019; Yehuda et al., 2016; Zannas et al., 2019). For murine brain tissue, few studies are available for *Fkbp5* DNAm in a limited number of CpGs, as discussed in detail in section 1.2.1. As to human postmortem brain tissue, differences in *FKBP5* DNAm were explored in the medial temporal gyrus of an Alzheimer's disease sample (Blair et al., 2013). In the context of psychiatric disorders, studies so far focused mainly on gene expression (as described in detail in section 1.1.5). Although the use of postmortem brain tissue to study biological mechanisms in the brain is valuable (McCullumsmith & Meador-Woodruff, 2011), only retrospective environmental exposure can be examined. This issue is especially relevant in GxE research, which requires exposures. Since this cannot be performed in postmortem tissue, mechanisms can be explored in mice or humanized mice brain. With this approach the involvement of DNAm in GxE mechanisms can be explored within the complexity of an organism.

1.2.3 Translational implications of non-coding DNA conservation

Generally, as an important adaptive neuroendocrine stress system, the HPA-axis is highly conserved among vertebrates (Denver, 2009). From a genetical perspective, humans and mice are very similar and present over 99% of homologue gene pairs (Mouse Genome Sequencing et al., 2002). The degree of conservation is, however, much lower in non-coding regions of the genome such as introns, 5′ and 3′ untranslated regions (UTRs) and vary across different genes (Mouse Genome Sequencing et al., 2002). Accordingly, simple assumptions related to CpGs lying in non-coding regions of the genome cannot be made from humans to mice and vice versa. Moreover, GxE mechanisms are often mediated

by effects of genetic variation on regulatory elements within non-coding areas (Starnawska & Demontis, 2021). Addressing this translational gap is important to allow GxE investigation upon manipulation and/or to different time points over the life span. Humanized mouse models were suggested to bridge this gap and allow investigation of human GREs in model organisms (Ye & Chen, 2022). In our case, the humanized *FKBP5* mouse model, generated from C57BL/6NTac mice by replacement of the murine *Fkbp5* gene sequence by the human *FKBP5* (exon 3 to 12, including translation initiation and termination codons, see Nold et al., 2021 for full technical details) might be a solution since the replaced sequence contains relevant intronic GREs. However, DNAm, a core biological mechanism of *FKBP5* function in the context of stress, has not yet been investigated in this model.

1.2.4 DNA methylation of *FKBP5* as a biomarker

Peripheral blood is easily accessible and is routinely obtained in clinical practice. It is therefore suitable for the development of DNAm-based biomarkers. However, the extent of concordance between DNAm signatures across tissues is unclear. Using Illumina's DNAm arrays or methylated DNA immunoprecipitation and sequencing (MeDIP-seq), previous investigations of global within-individual blood and brain DNAm in humans found various extents of correlation across tissues (Davies et al., 2012; Edgar et al., 2017; Hannon et al., 2015; Walton et al., 2016). However, extrapolation from these studies to *FKBP5* is limited due to the scarce representation of GC-responsive CpGs within key enhancer regions of the *FKBP5* relevant for psychiatric GxE research on the arrays (Roeh et al., 2018). Most likely, differences in DNAm between tissues are not only gene-specific, but present a much more complex biology such as variability between different functional areas of a gene (Davies et al., 2012; Hannon et al., 2015) and even a CpG-specific variability (Edgar et al., 2017). DNAm might also change in a tissue-specific manner upon intervention. For example, low overlap of differentially methylated regions was found between brain and blood upon GC-treatment in mice (Seifuddin et al., 2017). Interestingly, over 50% of differentially methylated regions of both tissues were in intronic regions, highlighting these non-coding regions as susceptible for tissue-specific effects (Seifuddin et al.,

2017). Prior studies in mice found correlations of DNAm in blood and brain of different *Fkbp5* introns (Ewald et al., 2014; Lee et al., 2010). Such tissue-specific variation in the location of stress-responsive GREs was suggested before (Seifuddin et al., 2017). Others suggested that some CpGs can be GC-responsive across tissues (Klengel et al., 2013; Provencal et al., 2020). To date, the degree of DNAm correlation between brain and blood in enhancer regions of *FKBP5* in both humans and rodents remains largely unknown. In order to establish *FKBP5* as a blood-based DNAm biomarker, assessing the degree of similarity is important. While DNAm is generally highly tissue and cell-type-specific (Loyfer et al., 2023), GC-responsive elements were enriched in regions with active enhancer function across many different tissue (Penner-Goeke et al., 2023). It is possible that enhancer GREs are cross-tissue reactive, and even connected by a back-effect from the peripheral immune system to the brain.

Beyond the exploration of *FKBP5* DNAm as a biomarker, it was also suggested as a possible future drug target (Malekpour et al., 2023; Menke, 2024). To determine whether the humanized *FKBP5* mouse model is suitable for further exploration of DNAm-related mechanisms it is important to evaluate the resemblance of DNAm in the brain of humanized mice and humans. This highlights the need of a comprehensive analysis of DNAm patterns in key enhancer elements of the gene in mice, humanized mice and humans. To my knowledge, DNAm in the brain of humanized mice used in psychiatric research have never been studied before. Therefore, the question remains, whether DNAm patterns in relevant regulatory elements would replicate in the humanized *FKBP5* model.

1.3 Aims and results of the thesis

The previous sections emphasized the potential and the necessity of “reverse translation” as an important approach for further mechanistic exploration of *FKBP5* in future psychiatric biomarker and/or drug target development, but also presented the lack in knowledge and the current challenges related to the translational investigation of epigenetic mechanisms in mouse models.

The thesis consists of two parts with the joint purpose of exploring the DNAm of the *Fkbp5*/*FKBP5* gene, a central epigenetic mechanism proposed as a biomarker of stress-related GxE response and a possible drug target of psychiatric disorders. For this purpose, experiments were performed in two different mouse model organisms. One represents a widely used model (C57BL6/n mouse), while the other is a recently generated humanized model (two humanized *FKBP5* lines differing in rs1360780 alleles: “risk”-associated A/T vs. “resilience”-associated C/G). The latter increases the translational value due to the ability to investigate human sequences. The assessment of DNAm in peripheral blood and two stress-related brain regions was performed in parallel using the HAM-TBS method in the context of ELS or pharmacological manipulation of the HPA-axis with close comparison to corresponding human tissues and prior findings.

Paper III (Appendix A) includes a detailed introduction of the GxE paradigm in psychiatry and reviews the supporting literature focusing on evidence for the role of *FKBP5* in GxE across different intermediate phenotypes.

The first study (paper I) pursued the exploration of DNAm in a C57BL6/n mouse model at previously described (intron 1 and 5) and at new potentially significant regulatory regions of the *Fkbp5* gene (intron 8, transcriptional start site, proximal enhancer and CCCTC-binding factor (CTCF)-binding sites of topologically associated domains (TADs) within the 5'UTR) in adulthood following a naturalistic ELS paradigm (limited nesting and bedding, LBN). The results showed complex and heterogenous DNAm patterns in untreated animals, with various degrees of similarity across tissues, brain regions, functional genomic regions, and even single CpGs. Long-term changes associated with moderate ELS exposure were discovered in blood (intron 1 and 5) and FC (intron 5 and proximal enhancer) and overlapped partially with previously described alteration after chronic CORT treatment.

The second study (paper II) aimed at the exploration of DNAm in blood and brain of the humanized model organism at baseline, in regard to genotype (rs1360780 SNP) and following administration of dexamethasone (DEX). Included regions comprised three introns (intron 2, 5 and 7) of *FKBP5*, known to contain important GREs. Additionally, DNAm was quantified in human blood and postmortem human brain PFC to allow for comparison and further evaluation of

the relevance of the model to humans. Overall, the results indicated a recapitulation of human DNAm patterns in blood and more so in brain in the humanized mouse model. Substantial tissue-specific DNAm was discovered across the three tissues/brain regions with low correlations between DNAm in blood and brain of the model organism and humans (using publicly available data from Edgar et al., 2017; Hannon et al., 2015). Effects of DEX in blood (for most sites no effects were detected in brain tissues), and to a lower degree, of genotype on DNAm were similar to humans.

1.4 Conclusion

Taken together, the studies included in my thesis extend our knowledge of *FKBP5* DNAm in brain and blood of mice, humanized mice and humans and their involvement in the molecular response to stress. The work demonstrates the benefit of significant explorative expansion of key regulatory sites involved in epigenomic regulation to identify new target CpGs affected by ELS in mice that can be explored both as biomarkers and as possible drug targets. While detected DNAm changes after ELS in blood are in line with the suggested utility of *FKBP5* as a biomarker, long-lasting changes in the brain reinforce prior work highlighting areas in the FC as important for *FKBP5*-related psychopathology. Most importantly, the thesis provides the first evidence that DNAm patterns are recapitulated in cardinal non-coding regions of *FKBP5* in the brain (PFC) of a humanized mouse model. Further, genotype-dependent differential DNAm and similarity in the DEX response (primarily in blood) indicate that the humanized mouse model could assist in reverse translation in psychiatry and should be further evaluated. The data also suggest that responsivity might be present as a DNAm signature, proposing a shift from a “single CpGs” towards a “DNAm pattern” approach for *FKBP5* as a DNAm-related biomarker. Moreover, as indicated by prior findings (Ewald et al., 2014; Lee et al., 2010), the results show that differences in DNAm absent at baseline might appear only upon stimulation, with no relation between baseline DNAm and the extent of change once stimulated. These observations and the relation of DNAm to mRNA response suggest a detectable, not yet fully understood, stress-related stimulatory “epigenetic memory” by *FKBP5*. It is

possible, that DNAm in *FKBP5* has the ability to change future responsiveness due to previous environmental experiences.

The findings also hint towards brain region-specific long-term effects on DNAm related to ELS in the FC (despite similar DNAm across brain regions at baseline) and a possible stressor-specific effect on *FKBP5* DNAm after LBN as opposed to prior findings after chronic CORT. Such differences in effects on *FKBP5* due to various stress paradigms were suggested before (Schmidt et al., 2015) and further emphasize the importance of applying naturalistic stress. Curiously, most sites with differential DNAm after ELS were found in regions with substantial variability in baseline DNAm across tissues, so called tissue-specific differentially methylated regions (T-DMRs). The observations are in line with prior assumptions, postulating these regions as especially dynamic and highly relevant for transcription factor-related regulation and responses to environmental cues (Davies et al., 2012; Wan et al., 2015; Ziller et al., 2013). Effects on DNAm in the FC might be related to higher FKBP5 expression, lower BDNF and alterations in neuronal structure such as reduced mushroom spine density (Matosin et al., 2023; Young et al., 2015) proposing a suppressed GC-related synaptic plasticity (Bennett & Lagopoulos, 2014) and a reduced top-down control of the FC due to alterations in *FKBP5*-related regulation of the HPA-axis.

Finally, this work presents the potential and the need of a more extensive mapping of epigenetic effects, especially DNAm, of *FKBP5* in different tissues and brain regions. Stress sensitivity often observed in psychiatric patients and the current knowledge about the regulatory function of *FKBP5* in stress responsivity makes it an interesting target protein for the development of novel intervention strategies to the treatment of stress-related disorders. Although many important questions such as timing of treatment, target brain region and/or cell type remain, the mouse and possibly to a greater extent the humanized *FKBP5* mouse model might help uncover the full mechanism and answer these questions in the future.

1.5 Limitations and future directions

This work presents several limitations, that need to be considered as well as challenges in the field, that need to be addressed in future research. The focus of the analyses was DNAm. Although changes in DNAm are often taken as a proxy for changes in gene expression, a combination with expression analysis, and more so with transcriptomic techniques and reporter gene assays, can shed light upon possible consequences of the detected changes in DNAm. Moreover, future research should include additional epigenetic mechanisms for a more comprehensive analysis of the epigenetic regulation.

An additional limitation is the use of bulk tissue. It was, therefore, not possible to determine whether a specific cell type was driving or diluting the observed effects. Theoretically, the detection of GC-induced effects on DNAm in brain bulk tissue might be easier, since proportions of cells are not expected to change significantly following a GC treatment (Seifuddin et al., 2017). The observed lack of DNAm response in the humanized mouse brain after DEX, might also be due to an active removal of DEX by the blood brain barrier and/or suppression of the endogenous HPA-axis activation (De Kloet, 1997). This can be addressed by future use of CORT, higher dexamethasone dose or naturalistic stress paradigms. Cell-type-specific findings in astrocytes showing a GC-related *FKBP5* upregulation (Carter et al., 2013; Carter et al., 2012; Nold et al., 2021) and the recently detected clear cell-type-specific differences of *FKBP5* expression in postmortem human brain (highest expression in excitatory neurons, microglia and astrocytes (Matosin et al., 2023)), stress the importance of future assessment of DNAm in a cell type-specific manner. This could be achieved by using fluorescence-activated nuclei sorting (FANS) or better single cell sequencing approaches for a more comprehensive analysis. Furthermore, a potential influence of changes in cell type composition due to glucocorticoids or stress cannot be excluded for blood (Dhabhar et al., 2012; Ohkaru et al., 2010; Seifuddin et al., 2017) and to a lesser extent for brain (Anacker et al., 2013), potentially even masking ELS-dependent effects (Etzel et al., 2022). Measurement of cell-type-specific DNAm after GC-stimulation in the murine cortex have yielded a clearer demethylation

signal as opposed to bulk tissue (Seifuddin et al., 2017). Therefore, future studies should focus on cell-type specific DNAm changes.

A further limitation is the exclusive use of male mice, which did not allow a sex-specific interpretation. The importance of sex-related differences in the human brain stress system was discussed before (see Brivio et al., 2020 for a review) and differences for *Fkbp5*/*FKBP5* in mice and humans were also recently demonstrated (Kuznetsova et al., 2022; Nold et al., 2022; van Doeselaar et al., 2023). Intriguingly, a recent study showed not only sex-specific but even opposing effects following *Fkbp5* deletion in GABAergic vs. glutamatergic neurons of the forebrain (van Doeselaar et al., 2023). Hence, a combination of sex-specific with cell-type-specific exploration would be important for future drug targeting.

As to the brain tissue, the focus lied on two highly relevant brain regions and demonstrated a complex biology, presenting not only tissue-, but also brain region-specific effects on DNAm. It is possible that *FKBP5* regulatory role varies across the brain, which could explain pleiotropic effects (Zannas & Binder, 2014). Electrophysiological experiments hint towards opposing changes in excitatory-inhibitory balance after *Fkbp5* deletion, with an increase in the HIP but a decrease in the mPFC (Ryu et al., 2021; Zhang et al., 2022). Interestingly, disorder-specific differential *FKBP5* expression might be present even within defined regions. For example, a reduced expression was shown in the ventromedial prefrontal cortex and anterior cingulate (BA 25) of patients with PTSD (Holmes et al., 2017). Accordingly, *FKBP5* function and epigenetic regulation should be explored in more brain regions in future.

As to the behavioral characterization of the humanized mouse model, only few experiments have already been performed (Nold et al., 2022), but a more intensive behavioral evaluation of the model under baseline and stress conditions in a sex-specific manner is needed and could include new deep learning-based behavioral analysis approaches such as DeepOF (Bordes et al., 2023). The translational value could be further increased if combined with *in vivo* structural magnetic resonance imaging and diffusion tensor imaging to explore brain connectivity and structure (Engelhardt et al., 2021).

Finally, a more intensive investigation of the regulatory role of *FKBP5* in metabolic and immune-related processes is needed as these tissues show the

highest *FKBP5* expression across all human tissues (Smedlund et al., 2021). *FKBP5* is involved in processes of metabolic dysfunction (Smedlund et al., 2021) and inflammation (Park et al., 2007; Zannas et al., 2019). This is an important and a promising line of research, since these two complex processes are considered to be altered in psychiatric disorders, especially in MDD (Chávez-Castillo et al., 2020). Model organisms could assist by allowing investigation of many tissues beyond blood and brain upon interventions and with it assist to even more holistic understanding of *FKBP5* effects in health and disease.

2. Paper I

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RESEARCH REPORT

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Extensive evaluation of DNA methylation of functional elements in the murine *Fkbp5* locus using high-accuracy DNA methylation measurement via targeted bisulfite sequencing

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Abstract

FKBP5 is an important stress-regulatory gene implicated in stress-related psychiatric diseases. Single nucleotide polymorphisms of the *FKBP5* gene were shown to interact with early life stress to alter the glucocorticoid-related stress response and moderate disease risk. Demethylation of cytosine-phosphate-guanine-dinucleotides (CpGs) in regulatory glucocorticoid-responsive elements was suggested to be the mediating epigenetic mechanism for long-term stress effects, but studies on *Fkbp5* DNA methylation (DNAm) in rodents are so far limited. We evaluated the applicability of high-accuracy DNA methylation measurement via targeted bisulfite sequencing (HAM-TBS), a next-generation sequencing-based technology, to allow a more in-depth characterisation of the DNA methylation of the murine *Fkbp5* locus in three different tissues (blood, frontal cortex and hippocampus). In this study, we not only increased the number of evaluated sites in previously described regulatory regions (in introns 1 and 5), but also extended the evaluation to novel, possibly relevant regulatory regions of the gene (in intron 8, the transcriptional start site, the proximal enhancer and CTCF-binding sites within the 5'UTR). We here describe the assessment of HAM-TBS assays for a panel of 157 CpGs with possible functional relevance in the murine *Fkbp5* gene. DNAm profiles were tissue-specific, with lesser differences between the two brain regions than

Abbreviations: CpG, cytosine-phosphate-guanine-dinucleotide; CTCF, CCCTC-binding factor; DNAm, DNA methylation; ELS, early life stres; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; GxE, gene by environment; HAM-TBS, high accuracy DNA methylation measurement via targeted bisulfite sequencing; Hi-C, high-throughput chromatin conformation capture; IVC, individually ventilated cages; LBN, limited bedding and nesting material; MDD, major depressive disorder; P, postnatal day; PTSD, post-traumatic stress syndrome; SD, standard deviation; SNP, single nucleotide polymorphism; TADs, topologically associating domains; WHO, World Health Organization.

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between the brain and blood. Moreover, we identified DNAm changes in the *Fkbp5* locus after early life stress exposure in the frontal cortex and blood. Our findings indicate that HAM-TBS is a valuable tool for broader exploration of the DNAm of the murine *Fkbp5* locus and its involvement in the stress response.

KEYWORDS
DNA methylation, early life stress, FKBP5, HAM-TBS, mouse model

1 | INTRODUCTION

Mental health disorders, such as major depressive disorder (MDD), anxiety disorders or post-traumatic stress disorders (PTSD), are a growing global health threat that is of rising concern (World Health Organization (WHO), 2017), and the need for valuable biomarkers and treatments is essential. Genetic and environmental factors, as well as their interplay (GxE), are considered important for the development of psychiatric diseases (Martins et al., 2022). In the past decades, an increasing number of biological targets have been identified that interact with adverse environmental exposure (Elbau et al., 2019; Risch et al., 2009). One psychiatric risk candidate gene that has received an expanding amount of attention in light of gene x environment interactions is the *FKBP5* gene, which encodes the glucocorticoid receptor (GR) co-chaperone FKBP51 (Criado-Marrero et al., 2018; Matosin et al., 2018). By binding to the GR, FKBP51 can decrease the GR's sensitivity to circulating glucocorticoids and thereby play a vital role in controlling endocrine responses to stress. In the presence of glucocorticoids, GR translocates to the nucleus, where it can bind so-called glucocorticoid-responsive elements (GREs), specific binding site sequences that are present in a variety of genes, and subsequently induce or inhibit gene transcription (Beato et al., 1996; Walters, 1985; Wochnik et al., 2005). It was found that GREs are also located throughout the *FKBP5* gene itself (Mendonça et al., 2021; Paakinaho et al., 2010), resulting in an ultra-short feedback loop following GR activation (Matosin et al., 2018).

It has become evident from human studies that the *FKBP5* gene variants particularly interact with exposure to early life adversity. Several studies revealed that single nucleotide polymorphisms (SNPs) of the *FKBP5* gene interact with early life stress (ELS) exposure to increase the risk for the development of various psychiatric disorders (Matosin et al., 2018). Ever since, there have been increasing efforts to unravel the exact mechanisms behind this interactive process (Criado-Marrero et al., 2019, 2020; Klengel et al., 2013; Klengel & Binder, 2015), and data from humans and mice have already suggested the involvement of epigenetics (Cox et al., 2021; Klengel et al., 2013; Lee et al., 2010; Matosin et al., 2018; Wiechmann et al., 2019; Womersley et al., 2022; Zannas et al., 2016). Klengel and colleagues demonstrated that individuals carrying the risk allele of the rs1360780 SNP of the *FKBP5* gene, who were also exposed to childhood trauma, showed a demethylation at cytosine-phosphate-guanine-dinucleotides (CpGs) near GREs in intron 7, leading to an increased *FKBP5* mRNA induction by GR (Klengel et al., 2013). It is thus believed that the convergence of genetic and environmental risk leads to changes in DNA methylation (DNAm) at regulatory elements that contribute to further disinhibition of *FKBP5* and result in a chain of molecular and cellular changes that alter activation in specific brain circuits and associate with altered endocrine regulation, behaviour and risk for psychiatric disorders (Elbau et al., 2019; Matosin et al., 2018).

While there is an expanding knowledge on the epigenetic involvement in the consequences of ELS exposure on the human *FKBP5* gene, only little is known about epigenetic changes in the mouse *Fkbp5* gene. The rodent model is an indispensable tool in studying the neurobiological underpinnings of stress susceptibility and resilience because it allows not only for genetic manipulations but also for investigating environmental exposures in a regulated setting. Although stress endocrine processes such as GR activation and HPA-axis regulation are considered to be conserved between species (Mouse Genome Sequencing Consortium, 2002), intronic elements are much less conserved, and the exact location of relevant regulatory *Fkbp5* CpG sites in the mouse model remains largely unmapped. To further investigate the biological mechanism underlying the suggested GxE process, it is highly important to make a broader characterisation of DNAm of CpG sites near GREs at the mouse *Fkbp5* gene, particularly with respect to ELS exposure. Previous studies have described the relationship between pharmacological GR activation and DNAm changes in the mouse *Fkbp5* gene in various tissues (Cox et al., 2021; Ewald et al., 2014; Lee et al., 2010; Sawamura

et al., 2016). So far, glucocorticoid-induced differential DNAm profiles were observed in intron 1 of the *Fkbp5* locus in the blood and in intron 5 in brain regions such as the hippocampus and the medial prefrontal cortex (Cox et al., 2021; Lee et al., 2010). However, none of these studies addressed the consequences of adverse early life exposures. Moreover, previous research only covered a limited number of CpGs in putative regulatory regions of the murine *Fkbp5* gene (GREs in introns 1 and 5 and the promoter region).

In this study, we extended the evaluation of DNAm in these regions of the mouse *Fkbp5* gene by using high-accuracy DNAm measurement via targeted bisulfite sequencing (HAM-TBS), a next-generation sequencing method for the assessment of DNAm. We also added further potentially relevant regulatory regions of the *Fkbp5* locus (GRE-binding and CCCTC-binding factor [CTCF]-binding sites in topologically associating domains, TADs). The final panel spanned over 157 CpGs in multiple intronic enhancers (introns 1, 5 and 8), the transcription start site (TSS), the proximal enhancer and the 5'UTR. The HAM-TBS method is a valuable tool to study DNAm at a more detailed level (Roeh et al., 2018; Wiechmann et al., 2019), as it allows wider coverage than other targeted methods combined with high accuracy, allowing to reliably detect differences in DNAm of less than 1%. To our knowledge, this is the first time that this technique is used to describe DNAm at the murine *Fkbp5* locus. In addition, we detected differential methylation profiles upon exposure to moderate ELS. Our findings help to better understand the DNAm landscape of important regulatory elements of the *Fkbp5* gene.

2 | MATERIALS AND METHODS

2.1 | Animals and housing conditions

All animals were held in the animal facility of the Max Planck Institute of Psychiatry (Munich, Germany) in individually ventilated cages (IVC; 30 cm × 16 cm × 16 cm) serviced by a central airflow system (Tecniplast, IVC Green Line—GM500) under stably controlled, standard housing conditions (12 h:12 h light/dark cycle, temperature of 23 ± 2°C, humidity of 55%). Sufficient bedding and nesting material was provided to the animals, unless specifically stated otherwise, and mice were offered water and food (standard research diet by Altromin 1318, Altromin GmbH, Germany) ad libitum. All experiments and protocols were performed in accordance with the European Communities' Council Directive 2010/63/EU and were approved by the committee for the care and use of laboratory animals of the Government of Upper Bavaria. All efforts were made to minimise the suffering of the animals throughout the experiments.

2.2 | ELS paradigm and experimental set up

Three- to five-month-old female C57BL6/n mice were paired with male C57BL6/n mice, and after a 2-week breeding period, they were single-housed and monitored daily throughout pregnancy for the birth of a litter. The day of the birth of pups was defined as postnatal day 0 (P0), and dams and their offspring were then randomly assigned to a control or an ELS condition (Figure 1). The ELS condition was based on the limited bedding and nesting (LBN) material paradigm, as previously described by Rice and colleagues (Rice et al., 2008) and was previously used by our research group (Kohl et al., 2015; Santarelli et al., 2017; Wang, Labermaier, et al., 2012). In short, at P2, dams and offspring of the control condition were placed in an IVC with a standard amount of bedding and Nestlet material (Ancare, Bellmore, NY, USA; two full pieces), whereas dams and pups assigned to the ELS condition were put in an IVC with a metal grid at the bottom of the cage. Furthermore, dams and pups with the ELS condition were provided only with a reduced amount of Nestlet material (one half piece). Dams and their offspring were then left undisturbed until P9, at which point they were weighed. Subsequently, animals in both conditions were put in fresh cages under standard housing conditions. Between P25 and P27, mice were weaned and group-housed in groups of 4–5 mice of the same sex. Finally, at 3 months of age, male offspring were selected ($n = 16$ control and $n = 16$ ELS) and sacrificed.

2.3 | Tissue sampling

Tail vein blood (80 μL) was collected in 1.5 mL EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Nümbrecht, DE) that were kept on ice. Blood was then immediately transferred into Eppendorf tubes that were prefilled with 220 μL of the solution from the PAXgene® Blood RNA tube (BD Biosciences, Franklin Lakes, NJ, US) at room temperature (ratio 1: 2.76). Subsequently, animals were anaesthetised with a lethal dose of isofluorane, followed by decapitation. Brains were extracted, and the olfactory bulb was removed. Following this, the frontal cortex and the hippocampus were individually dissected and put in 1.5 mL Eppendorf tubes that were immediately saved on dry ice and stored at −80°C.

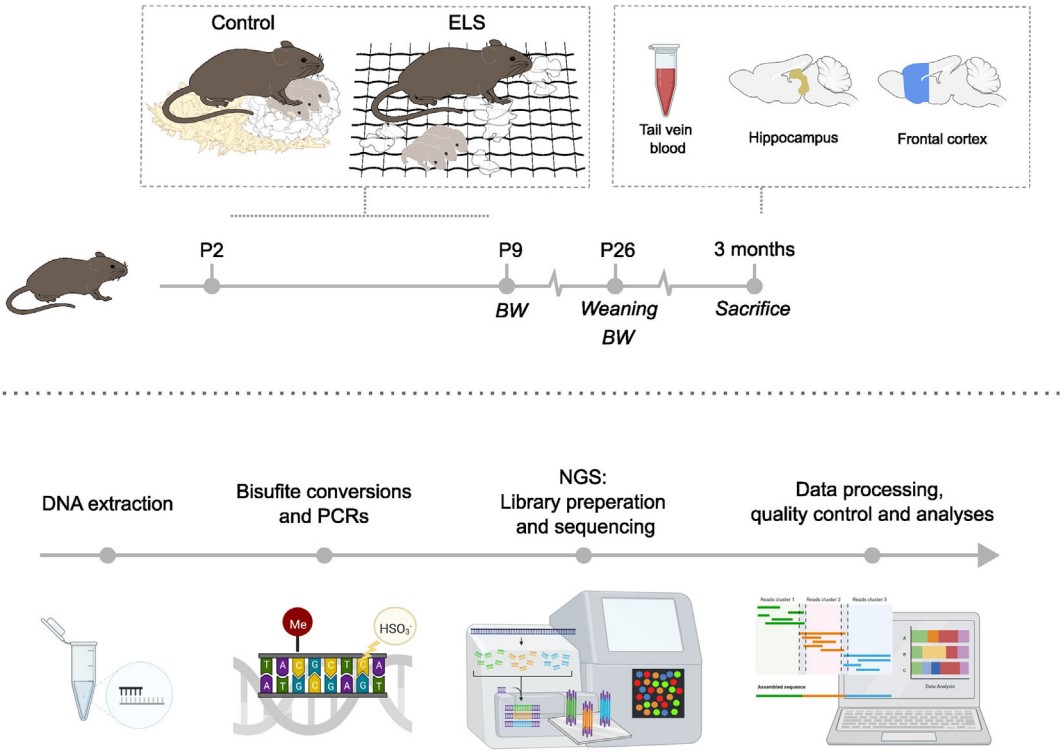


FIGURE 1 Experimental set-up and targeted bisulfite sequencing (TBS) workflow. C57BL6/n pups and dams were exposed to a limited bedding and nestling (LBN) early life stress (ELS) paradigm from postnatal day (P)2 to P9. At 3 months of age, tail vein blood was collected from male offspring, after which they were sacrificed. Brains were collected, and the frontal cortex (FC) and hippocampus (HIP) were dissected separately. Subsequently, DNA was extracted from the peripheral blood and the two brain regions. DNA methylation (DNAm) assessment at the murine *Fkbp5* locus was then performed using high-accuracy DNAm of TBS (HAM-TBS), including bisulfite conversions, PCRs, library preparation and sequencing. Finally, the data were processed, quality control was done, and final analyses were performed. Created with the help of BioRender.com.

2.4 | DNA extraction

Genomic DNA was extracted from frozen tissue (−80°C) of the hippocampus, frontal cortex and tail vein blood of all samples using the Quick-DNA/RNA MagBead Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. Prior to DNA extraction, samples of each tissue were randomised (as separate blocks) into one 96-well plate with regards to treatment condition using the Omixer R package (Sinke et al., 2021). One sample (hippocampus, control group) was excluded due to an insufficient DNA amount (final $n = 95$ samples; see Table S1).

2.5 | Primer selection

A panel of bisulfite-specific primers for the murine *Fkbp5* gene on chromosome 17 (NCBI37/mm9 assembly, 28,536,040–28,654,469) was generated to include previously assessed CpG sites in the murine *Fkbp5* locus (GREs in introns 1 and 5) (Lee et al., 2010), but also to extend CpG sites within these regions as well as to additional potential regions of interest (see Figure 2 for a detailed map of the CpGs covered within the experiment). Regions were chosen to assess CpGs near GREs according to available ChiP-Sequencing data for the GR (NCBI GEO accession GSE61877 and GSM788650) (Jubb et al., 2016; Uhlenhaut et al., 2013). Further, CpGs within genomic locations involved in the 3D conformation of chromatin were included. Selection for these sites was based on available ChiP-seqencing data from the ENCODE project (ENCODE Project Consortium, 2012) at the UCSC browser (https://genome.ucsc.edu/ENCODE) for the CTCF for murine whole brain tissue (wgEncodeEM002595, GEO: GSM918730, laboratory of Ren, Ludwig Institute for Cancer Research, San Diego, California) and B-cell lymphoma cell line (CH12, wgEncodeEM001922, GEO: GSM923568, laboratory of Ross Hardison, Pennsylvania State University, University Park, Pennsylvania). Selected CTCF-binding sites that are shared across blood and brain tissue were then further narrowed down by high-throughput chromatin conformation capture (Hi-C) data in a lymphoblastoid cell

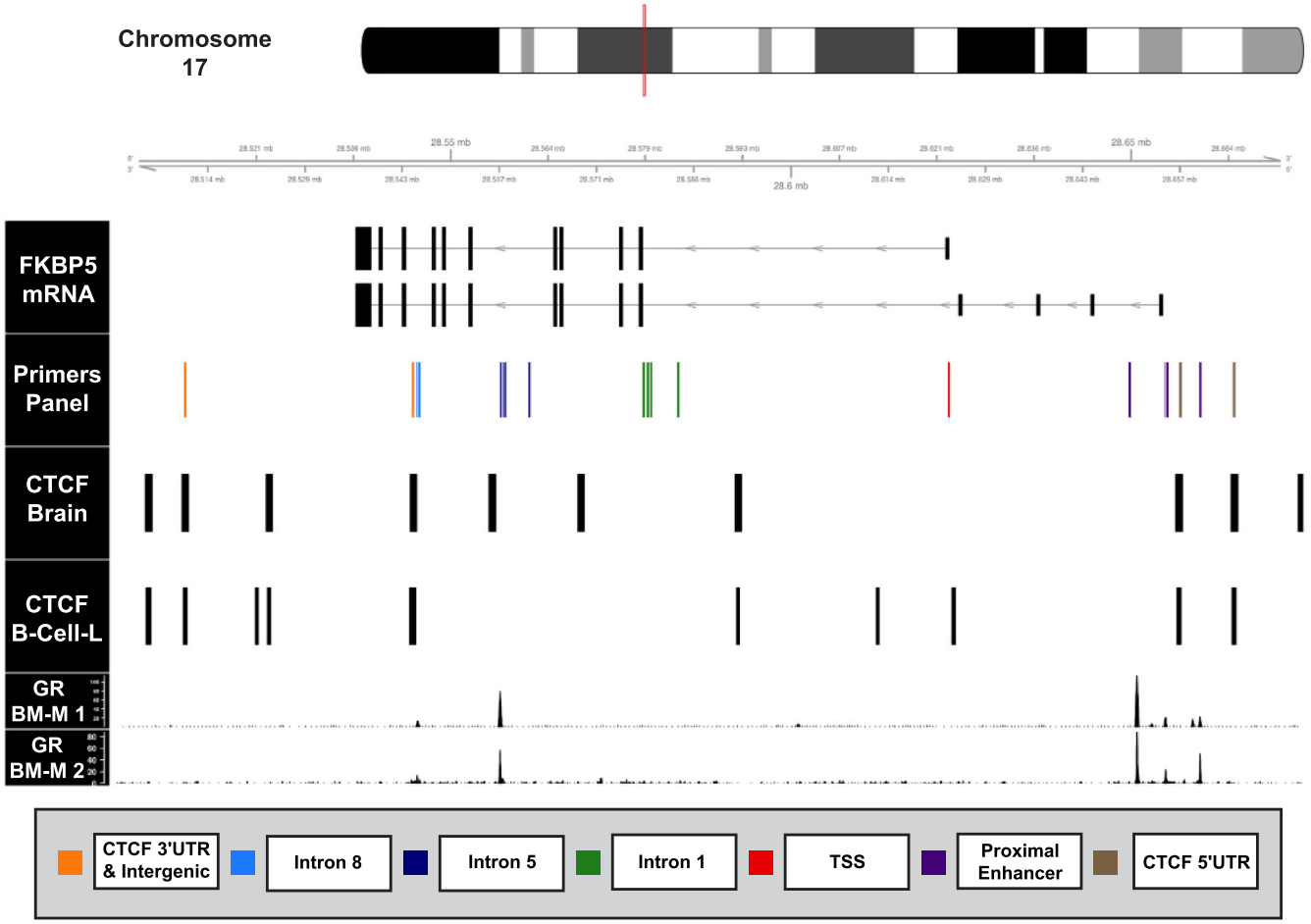


FIGURE 2 The murine *Fkbp5* locus and the primer panel. Depicted are the murine *Fkbp5* gene locus on chromosome 17 (NCBI37/mm9 assembly, 28,536,040–28,654,469), the common splicing variants of the gene, the genomic locations of the bisulfite-specific primers used in the experiment (colours represent following functional regulatory regions—orange: the CCCTC-binding factor [CTCF]-binding sites at 3UTR and intergenic; light blue: intronic enhancer 8; dark blue: intronic enhancer 5; green: intronic enhancer 1; red: transcription start site; violet: proximal enhancer; brown: CTCF-binding sites at 5'UTR), ChiP-Seqencing (chromatin immunoprecipitation) data from the ENCODE project at the UCSC browser (https://genome.ucsc.edu/ENCODE) for the CTCF for murine whole brain tissue (wgEncodeEM002595, GEO: GSM918730, laboratory of Ren, Ludwig Institute for cancer research, San Diego, California) and B-cell lymphoma cell line (CH12, wgEncodeEM001922, GEO: GSM923568, laboratory of Ross Hardison, Pennsylvania State University, University Park, Pennsylvania). Finally, ChiP-seqencing data of dexamethasone-treated mouse bone marrow-derived macrophages (BM-M) for the glucocorticoid receptor (GR) is depicted (NCBI GEO accession GSE61877 and GSM788650).

line (GM12878), revealing TADs, which are considered to enable regulatory interactions between cis-regulatory elements and promoters of genes (Bonev & Cavalli, 2016; Rao et al., 2014). The final panel included 157 CpGs (see Figure 2 as well as Table S2 for primers and Table S3 for amplicons).

2.6 | TBS

DNAm assessment at the murine *Fkbp5* locus was performed using HAM-TBS, a next-generation sequencing method for medium-throughput detection of DNAm in specific regions that has been previously described in detail for human blood (Roeh et al., 2018). Briefly, triplicates of samples (200–500 ng DNA) were treated with bisulfite using the EZ DNAm kit (Zymo Research, Irvine, CA). Amplification of target sequences was performed using the TaKaRa EpiTaq HS Polymerase (Clontech, Mountain View, CA; final concentration: 0.025 U/l). Afterwards, amplicons were quantified using the Agilent 4200 TapeStation (Agilent Technologies, Waldbronn, Germany) and pooled by the Hamilton pipetting robot. After speed vacuum and resuspension in 50 µL, a double-size selection using Agencourt AMPure XP beads (Beckman Coulter GmbH, Krefeld, Germany) was performed in order to remove excess primers and genomic DNA. Next, PCR-free libraries were prepared with the

Illumina TruSeq DNA PCR-Free HT Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's standard protocol (500 ng of starting material). Qubit 1.0 (Thermo Fisher Scientific Inc., Schwerte, Germany) was used for the quantification of each library before equimolar pooling. Agilent's 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and KAPA Library Quantification Kit on LC480 (Roche, Mannheim) were used for the quality check of the final pooled library. Finally, libraries were sequenced on an Illumina MiSeq machine with Reagent Kit v3 (Illumina, San Diego, CA; 600 cycles, 12 pM library) in paired-end mode, with 15% PhiX added.

2.7 | Data processing

The raw data underwent multiple preprocessing steps. After quality assessment of reads with FastQC (Andrews, 2010), reads were trimmed with cutadapt v1.11 (Martin, 2011), setting the minimal read length to 100 bp. Next, reads were mapped to a restricted reference comprised of the amplicon sequences, including 50 bp padding on each side, with Bismark v0.18.2 (Krueger & Andrews, 2011). The removal of overlapping ends of reads was performed symmetrically to avoid a sequence quality drop towards the end of each read. Alignment efficiency was similar between all investigated tissues (blood: mean 75.6%, SD 2.8%; frontal cortex: mean 77.4%, SD 2.0%; hippocampus: mean 77.9%, SD 1.1% [Figure S1]). Further preprocessing steps were carried out in R v4.0.4 (R Core Team, 2021), including the following steps: (1) exclusion of PCR artefacts (no significant differences across evaluated tissues, $p = 0.13$); (2) exclusion of samples with low median coverage (low sequencing depth) within an amplicon (total read number < 1000, $n = 39$ in all amplicons); (3) exclusion of samples with low bisulfite conversion rates (<95%, none were excluded); and (4) failed amplicons. All amplicons, except for 15_fk5_ctcf_interg and 16_ctcf_3UTR, achieved sufficient coverage in more than 50% of the samples and were therefore included in further downstream analysis. Raw methylation calling and bisulfite conversion assessment were performed by the methylKit R package v1.6.3. (Akalin et al., 2012), setting the minimum Phred quality score to 30 (base call accuracy of 99.9%). After quality control, a total of 157 CpGs were available for subsequent analysis in the following regions: introns 8, 5 and 1, TSS, proximal enhancer and 5'UTR (see Table S4 for a list of CpGs with their genomic locations). Next, technical outlier samples per amplicon were excluded (DNAm < [1. quartile − 2xIQR] or > [3. quartile + 2xIQR] in over 50% of CpGs [blood: $n = 7$; hippocampus: $n = 5$; frontal cortex: $n = 7$]). To exclude major sources of variation explained by technical batch effects, a principal component analysis was performed separately per tissue after imputation using the missMDA R package v1.18 (Josse & Husson, 2016). Using ANOVA of linear models of the first five principal components, no technical batch effects were identified.

2.8 | Statistical analysis

Mean and standard deviations (SD) were used for the comparison of the percentage of DNAm in the different regions. To evaluate the effects of ELS on DNAm, multiple linear regressions were performed on M transformed values (M = log2(Beta/[1-Beta]) (Du et al., 2010). Prior to regression modelling, non-variable CpGs within blood or brain tissues (interquartile range, IQR < 1%) of non-treated animals were removed (final number of CpGs for blood: 60; frontal cortex: 68; hippocampus: 60 [Figure S2]). The following linear model was used separately in the blood and both brain tissues and for each of the individual CpGs: DNAm ~ ELS exposure. *P*-values were FDR-corrected for multiple testing. *P*-values, q-values, beta estimates, standard error and F-statistic are reported. All statistical analyses were conducted in R version 4.0.4 (R Core Team, 2021).

3 | RESULTS

3.1 | Comparison of mean DNAm levels in blood, frontal cortex and hippocampus

The hippocampus and frontal cortex regions were selected for *Fkbp5* DNAm analysis as they have been shown to be important brain regions in the context of (early) life stress vulnerability, express high levels of FKBP51 (Scharf et al., 2011), are sensitive to *Fkbp5* epigenetic changes upon GR activation (Lee et al., 2010) and have repeatedly been implicated in the pathophysiology of stress-related disorders (Campbell & Macqueen, 2004; Sapolsky, 2000; Wellman et al., 2020). In addition, blood was collected to increase the translational value of the characterisation, as stress-induced differential DNAm in the *Fkbp5* gene has previously been observed in blood samples (Ewald et al., 2014; Lee et al., 2010; Wiechmann et al., 2019). To ensure a broader characterisation of the DNAm in the selected tissues within the *Fkbp5* locus and near TADs, we assessed DNAm levels of 157 CpGs near GREs or CTCF-binding sites in six functional regulatory regions (GREs in introns 8, 5 and 1, TSS, proximal enhancer and CTCF-binding regions at the 5'UTR

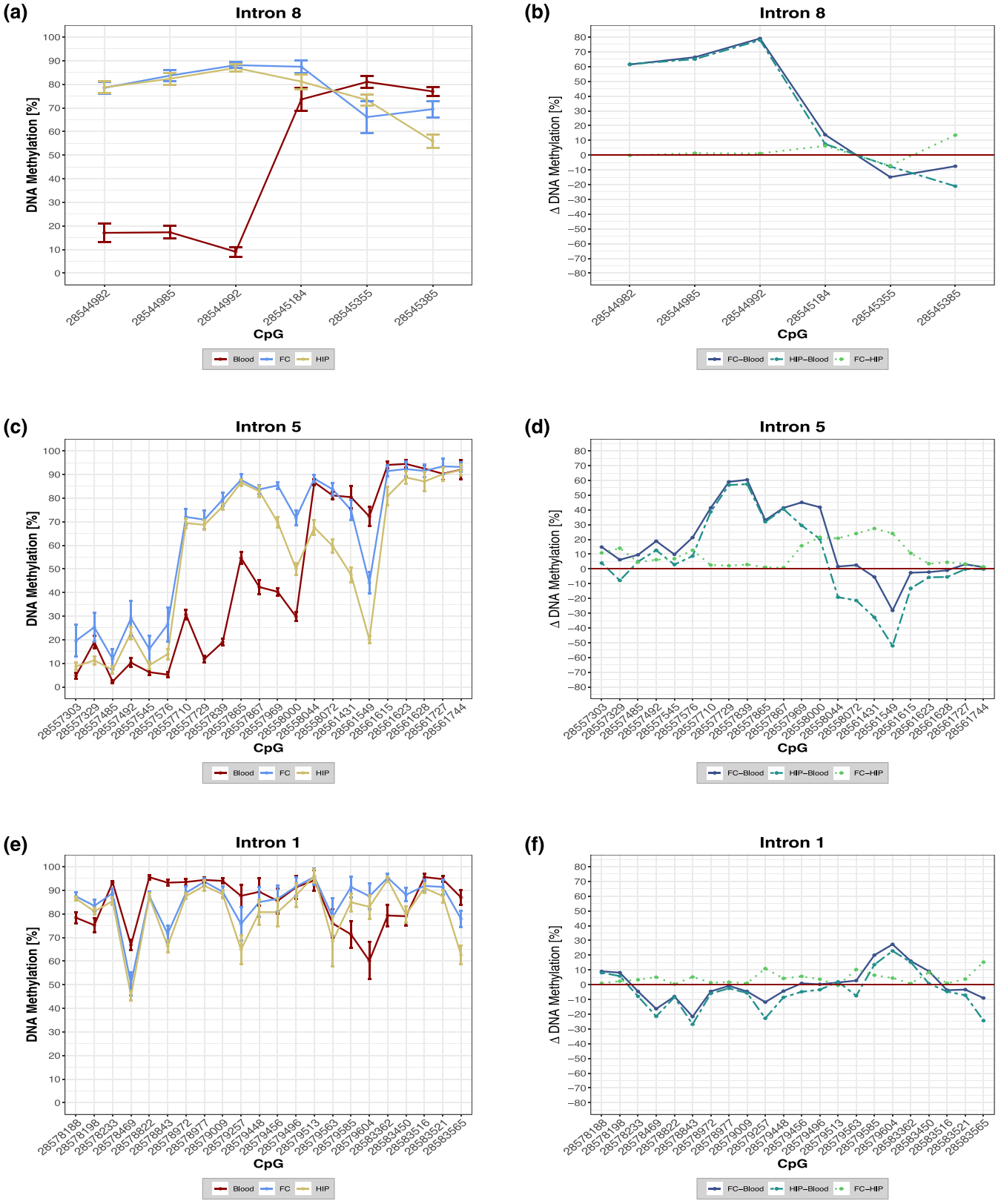


FIGURE 3 Legend on next page.

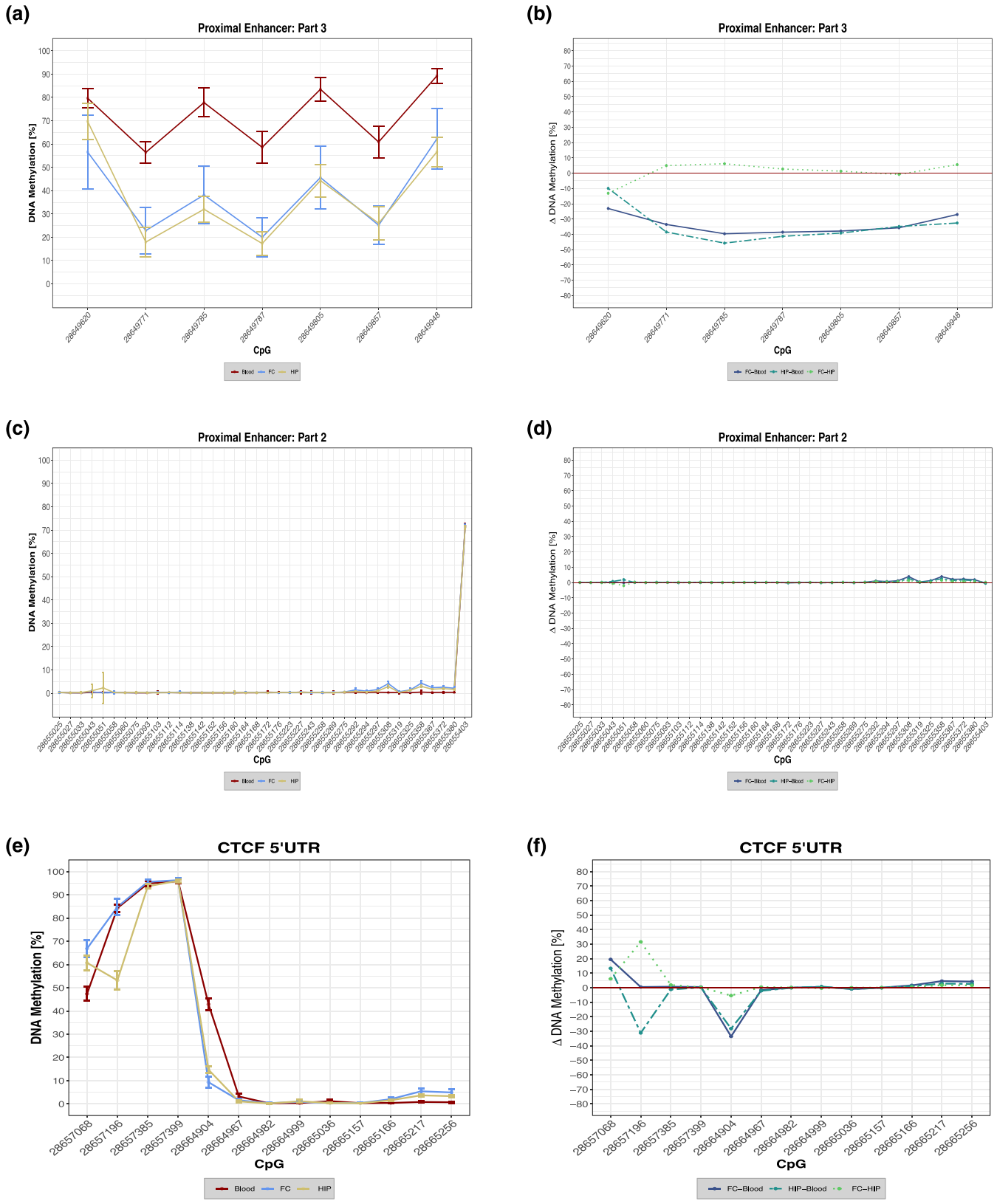
[Figure 2]). In control animals, unique DNAm patterns were observed across the different regulatory regions (Figures 3 and 4 and Table S5 for mean and SD of DNAm percentage of each CpG for each tissue) and among the three tissues/brain regions (blood, frontal cortex and hippocampus; Figures 3 and 4). For all three intronic enhancer regions, the two different brain regions presented a high similarity of DNAm patterns at the majority of CpGs and had higher levels of DNAm at many CpGs as compared with blood. The highest cross-tissue differences between the two brain regions and the blood were observed in introns 8 and 5. For example, the difference in mean DNAm or delta DNAm in comparison to blood at CpG 28557729 in the GRE in intron 5 was 59% for frontal cortex and 57% for hippocampus; for CpG 28649771 and 28649785 in the proximal enhancer: −34% and −40% for frontal cortex or −38% and −46% for hippocampus, respectively (see Figures 3 and 4 and Table S6 for delta mean percentage of DNAm). While the largest differences were observed between the blood and the two brain regions, specific CpGs did show differences in DNAm levels between the frontal cortex and the hippocampus. This was most notable in the GRE of intron 5 (highest values of delta DNAm 27.4% for CpG 28561431 in the GRE of intron 5, Table S6).

In addition to the three intronic regions, the proximal enhancer and CTCF-binding region also showed differential DNAm patterns, with unique DNAm levels often drastically different (close to 0% to 100% DNAm) for different CpGs within each region (Figure 4). Similar to the intronic enhancers, the brain tissues showed more comparable DNAm levels in both the proximal enhancer and the CTCF-binding regions as compared with the blood (see delta mean graphs in Figure 4b,d,f). The only exception was CpG 28657196, where a higher delta mean value could be observed (31.6% [Figure 4f]). DNAm was close to 0% in CpGs within the TSS and distal part of the proximal enhancer (part 1) of all investigated tissues. Our results in control animals largely overlapped with DNAm levels that were detected in previous studies, which investigated DNAm at the *Fkbp5* locus using the pyrosequencing technique with a Pearson correlation of r = 0.92 (p-value < 2.2e-16) across the two methods (Cox et al., 2021; Ewald et al., 2014; Lee et al., 2010; see Table S7 for comparison and Figure S3 for correlation plot).

3.2 | Effects of ELS exposure on DNAm levels in blood, frontal cortex and hippocampus

We further explored the long-term epigenetic consequences of moderate ELS exposure on the *Fkbp5* gene. ELS resulted in a significantly reduced body weight at postnatal day 9 ($t(30) = 6.263$, $p < 0.001$; ELS: mean = 3.213, SD = 0.534; control: mean = 4.350, SD = 0.493), which is an indication that the stress paradigm was effective. Using linear regression models, we compared changes in DNAm at the *Fkbp5* locus in different tissues of mice that were exposed to ELS vs. control mice. Differences between ELS and control mice that remained significant after correction for multiple tests were detected in the blood and the frontal cortex (Figure 5). Within the blood, ELS leads to sustained hypermethylation of CpG 28557969 in the enhancer in intron 5 ($\beta = 0.113$, SE = 0.036, t = 3.093, $p = 0.004$, q = 0.008, R2-adj. = 0.228) and demethylation of CpG 28579496 in the enhancer in intron 1 ($\beta = -0.758$, SE = 0.267, t = −2.833, $p = 0.008$, q = 0.016, R2-adj. = 0.185) (Figure 5a,b). Nominal differences ($p < 0.05$) in blood tissue were found in introns 8 and 5 (see Table S8 for the full results). In the frontal cortex, significant demethylation of CpG 28557729 in the enhancer in intron 5 ($\beta = -0.213$, SE = 0.083, t = −2.574, $p = 0.015$, q = 0.031, R2-adj. = 0.154 [Figure 5c]) as well as demethylation of CpG 28649771 ($\beta = -0.642$, SE = 0.250, t = −2.562, $p = 0.016$, q = 0.032, R2-adj. = 0.156 [Figure 5d]) and 28649785 ($\beta = -0.618$, SE = 0.231, t = −2.670, $p = 0.012$, q = 0.025, R2-adj. = 0.170 [Figure 5e]) in the proximal enhancer was observed upon ELS exposure. Nominal differences ($p < 0.05$) were also found in the frontal cortex in introns 5 and 1 and the proximal enhancer (see Table S9 for the full results). Finally, no differences withstanding correction for multiple testing were found in CpGs in the hippocampal tissue between ELS vs. control mice. Only nominal differences ($p < 0.05$) were found in CpG 28657196 ($\beta = 0.183$, SE = 0.084, t = 2.166, $p = 0.0387$, q = 0.076, R2-adj. = 0.110 [Figure 5f]) and CpG 28657385 ($\beta = 0.204$, SE = 0.088, t = 2.321, $p = 0.0275$, q = 0.056, R2-adj. = 0.1276 [Figure 5g]) in the CTCF-5'UTR region (see Table S10 for the full results). We did not find any

FIGURE 3 Mean and delta mean of DNA methylation (DNAm) across tissues in the intronic enhancer elements 8, 5 and 1 of the *Fkbp5* locus. Depicted are the mean and standard deviation (SD) (in percent) of control DNAm levels of cytosine-phosphate-guanine-dinucleotides (CpGs) (genomic location) within the intronic enhancers 8 (a), 5 (c) and 1 (e) in the *Fkbp5* locus. To demonstrate the differences in DNAm between tissues/brain regions, line plots (b,d,f) of delta mean DNAm (in percent) between the brain tissues (frontal cortex [FC] and hippocampus [HIP]) and peripheral blood (FCmean – Bloodmean and HIPmean – Bloodmean) and both brain regions (FCmean – HIPmean) are depicted. Red line of the delta mean percentage of DNAm is drown at zero as a reference.



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overlap between the CpGs with altered DNAm following ELS exposure and the CpGs that were changed upon chronic corticosterone treatment (4 weeks in drinking water) in adolescence, as described before (Cox et al., 2021; Ewald et al., 2014; Lee et al., 2010).

4 | DISCUSSION

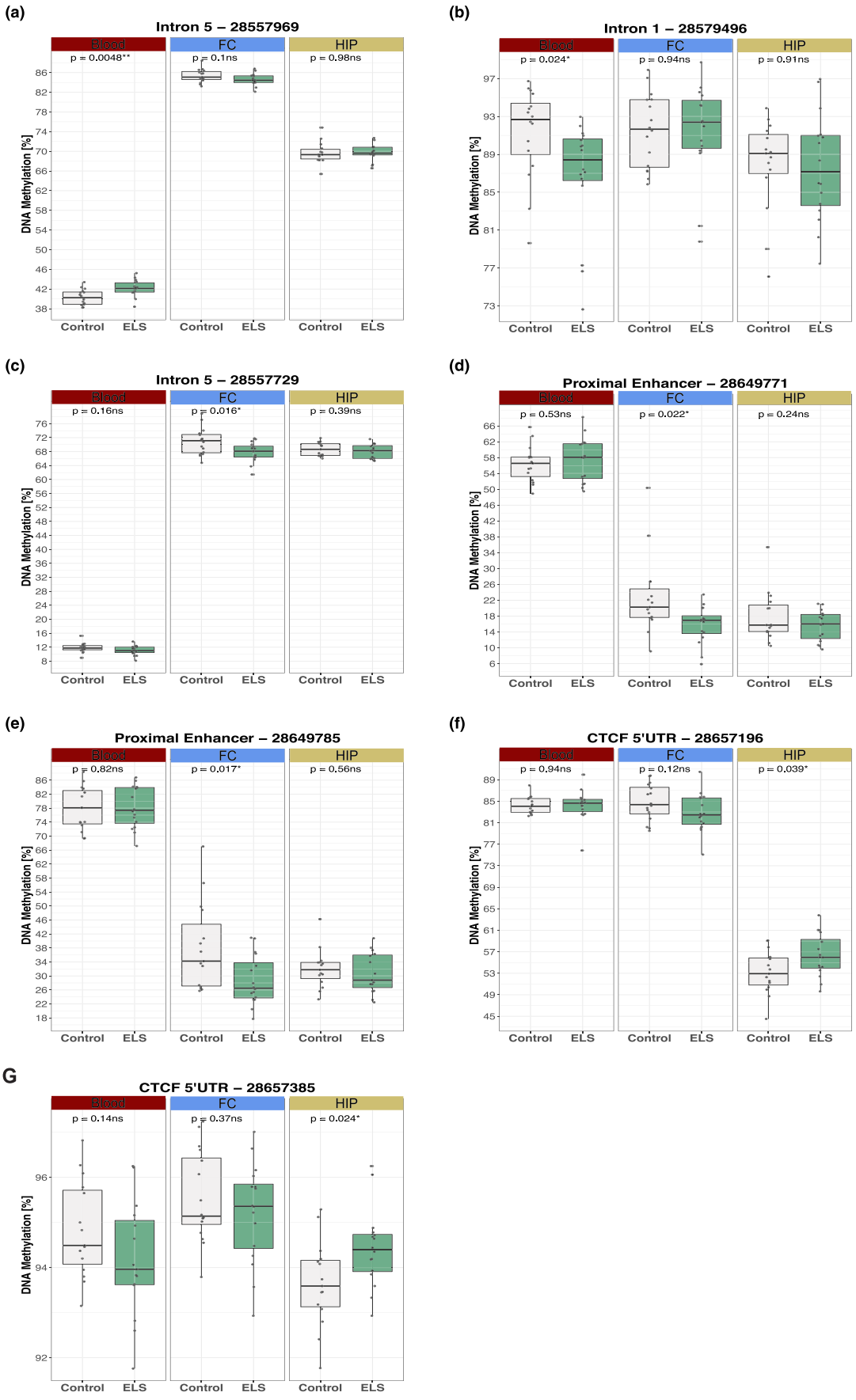
This study was conducted to evaluate the feasibility and potential of assessing the DNAm of the murine *Fkbp5* gene in blood and brain tissue using the HAM-TBS technique (Roeh et al., 2018; Wiechmann et al., 2019). Our assessment showed a high quality of the generated data across all investigated tissues (blood, frontal cortex and hippocampus). DNAm of regions in the *Fkbp5* locus have been investigated before using the pyrosequencing method (Cox et al., 2021; Ewald et al., 2014; Lee et al., 2010; Sawamura et al., 2016); however, using the HAM-TBS method, we not only significantly extended the evaluation of important, previously described regulatory regions of the gene but also explored DNAm in additional, potentially relevant CpGs that have not yet been investigated, including those within mapped GREs and CTCF-binding sites. We used this method to also explore long-lasting changes in DNAm at all of these CpGs in relationship to ELS exposure.

Here, we evaluated a total of 157 CpGs, far beyond the 6 to 16 CpG sites for which DNAm had been investigated in prior publications (Cox et al., 2021; Ewald et al., 2014; Lee et al., 2010; Sawamura et al., 2016). In addition to covering the regulatory elements of the locus more extensively, we also explored the long-term impact of ELS in an animal model across tissues. Prior studies have used pyrosequencing to measure DNAm, but this technique is limited to shorter sequencing lengths with a relatively high cost per base and is therefore mainly used to investigate restricted amounts of CpGs (Šestáková et al., 2019). HAM-TBS, on the other hand, allows us to analyse several hundred CpGs from a large number of samples within a single experiment. In addition, this method has a high accuracy for detecting small differences in DNAm, which are expected with complex stressor in heterogeneous tissues (Masser et al., 2013; Roeh et al., 2018).

Our assessment of DNAm with HAM-TBS in control animals of CpGs previously investigated in introns 1 and 5 using pyrosequencing showed positively correlated levels of DNAm across all CpGs. As mentioned above, previous studies have investigated CpGs located in introns 1 and 5 as well as in the promoter region. The fact that the correlation across the two types of studies is not perfect (see Table S7 and Figure S3) likely stems from differences in measurement accuracy as well as differences in how tissue was collected and brain regions were dissected, as cell type composition is among the strongest influences on DNAm variance (Jaffe & Irizarry, 2014). In addition, we also investigated CpGs in intron 8, the proximal enhancer and CTCF-binding sites within the 5'UTR, which have not been evaluated before. These CpGs lie in and around GREs and CTCF-binding sites and are therefore potentially functionally relevant in regulating responses to (environmental) stress. CTCF was shown to be involved in multiple genomic functions in a complex manner that is mechanistically not yet fully understood (Özdemir & Gambetta, 2019). DNAm was proposed as an epigenetic mechanism regulating CTCF binding (Bell & Felsenfeld, 2000; Wang, Maurano, et al., 2012) and was suggested to interfere with loop formation of DNA when introduced via epigenetic editing at CTCF-binding sites (Liu et al., 2016). Even though the observed DNAm changes in this region of the hippocampus were only nominal, they could still indicate potential regulatory effects.

Cross-tissue comparisons revealed a higher similarity of DNAm profiles between the two brain regions (frontal cortex and hippocampus) than between the two with blood. The strongest differences in DNAm were observed in introns 8 and 5 and in the proximal enhancer, with differences in average DNAm up close to 80%. Other regions, such as intron 1, only showed minimal cross-tissue differences. Several studies have suggested that CpGs with differential DNAm across tissues (i.e., tissue-specific differentially methylated sites [T-DMRs]) might be functionally relevant for response to environmental cues (Davies et al., 2012; Varley et al., 2013; Wan et al., 2015; Ziller et al., 2013). They could thus present interesting candidates for exploration in future studies. In fact, these tissue-specific CpGs were also among those that were found to be reactive to GR activation via

FIGURE 4 Mean and delta mean of DNA methylation (DNAm) across tissues in the proximal enhancer and CTCF-binding sites at the 5'UTR of the *Fkbp5* locus. Depicted are the mean and standard deviation (SD) (in percent) of control DNAm levels of CpGs (genomic location) within the proximal enhancer (a,c) and the CTCF-binding sites (e) in the *Fkbp5* locus. To demonstrate the differences in DNAm between tissues/brain regions, line plots (b,d,f) of delta mean DNAm (in percent) between the brain tissues (frontal cortex [FC] and hippocampus [HIP]) and peripheral blood (FCmean − Bloodmean and HIPmean − Bloodmean) and both brain regions (FCmean − HIPmean) are depicted. Red line of the delta mean percentage of DNAm is drown at zero as a reference.



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corticosterone administration (Cox et al., 2021; Ewald et al., 2014) and to ELS exposure in our study. CpGs in introns 8 and 5 and in the proximal enhancer that were differentially methylated upon ELS exposure had large DNAm differences between the tissues in control animals.

In addition to providing a broader characterisation of the DNAm of relevant regulatory elements in the *Fkbp5* gene, we used our technique to assess DNAm changes in adulthood following exposure to a model for ELS, the LBN paradigm, in the three different tissues/brain regions. We found DNAm to be altered in all tissues/brain regions following ELS exposures, but in different functional regions and to a varying extent. The mean significant differences ranged from 1.9% to 9.5% and included novel regions such as the proximal enhancer. Especially DNAm differences on the lower end of the range are better resolved with HAM-TBS than other methods that have lower accuracy, including pyrosequencing, mass spectrometry or whole genome bisulfite sequencing (Li & Tollefsbol, 2021; Tost, 2003). In the frontal cortex, ELS exposure led to changes in DNAm levels primarily in the GREs of intron 5 and in the proximal enhancer. In blood, DNAm of CpGs was also affected in intron 5 but additionally in intron 1. The hippocampus did not show differences surviving correction for multiple tests. This may indicate that hippocampal tissue is less sensitive to the effects of ELS than frontal cortex tissue, at least at the *Fkbp5* locus. Interestingly, the murine hippocampus is characteristic for a very high baseline expression of *Fkbp5* (Scharf et al., 2011), which could be related to the differential DNAm in certain regions (e.g., intron 5) and a lower DNAm variability. As alluded to above, cross-tissue variability may indicate higher relevance for regulatory activity in the context of environmental challenges. Notably, four out of the five CpGs that significantly differed in DNAm levels following ELS exposure also showed strong tissue-specific differences in DNAm in controls. Previous work has also investigated the long-term effects of chronic administration of the stress hormone corticosterone over a four-week period on *Fkbp5* DNAm in adolescent mice (Cox et al., 2021; Ewald et al., 2014; Lee et al., 2010). Matching our results, they found the largest stress-induced DNAm changes in blood tissue in intron 1, but also some changes in intron 5 (Ewald et al., 2014; Lee et al., 2010). The greatest differences in DNAm were found in CpGs in intron 5 for the hippocampus and the prefrontal cortex, only the latter being in line with our findings. Nevertheless, none of the CpGs that were found to be affected by chronic corticosterone administration overlapped with the CpGs affected by our ELS model. Moreover, we mostly detected smaller fold DNAm changes than reported in these previous studies. This might be due to the superphysiological concentrations of glucocorticoids used in these studies (direct corticosterone administration via mini-pump) as opposed to our natural stress paradigm. These results underline the importance of the nature and timing of stress exposure on epigenetic regulation.

Our study was primarily conceived to establish DNAm assays for additional regulatory regions in the murine *Fkbp5* locus. Thus, there are limitations to our study. Sex differences are an important factor that needs to be considered in stress research, and sex-specific DNAm changes have already been reported, including in *FKBP5* (Jessen & Auger, 2011; Wiechmann et al., 2019). Additional experiments in female mice need to be performed to map potential sex differences in control animals as well as following ELS. Also, in this experiment, we did not have the possibility of relating the epigenetic changes following ELS to behavioural consequences. This needs to be the subject of subsequent investigations, also in larger cohorts and including both sexes. Finally, we used bulk tissues in this study, but since DNAm can vary in a cell-type-specific manner (Jaffe & Irizarry, 2014), future studies need to investigate DNAm in specific cell types in stress-related brain regions.

5 | CONCLUSION

Our findings offer established, high-accuracy DNAm assays for a large range of regulatory regions in the murine *Fkbp5* locus that can now be used to study this regulation in a number of different stress models, different developmental context and additional brain regions and tissues as well as cell types.

FIGURE 5 Changes in DNA methylation (DNAm) in the *Fkbp5* locus after early life stress (ELS). DNAm levels (in percent) are depicted for mice that received ELS (green) vs. controls (white) for three tissues/brain regions: peripheral blood, frontal cortex (FC) and hippocampus (HIP). Hypermethylation in intronic enhancer 5 (a) and demethylation in intronic enhancer 1 (B) were detected in peripheral blood. Demethylation in intronic enhancer 5 (c) and the proximal enhancer (d,e) was detected in the FC. Hypermethylation was detected in CCCTC-binding factor (CTCF)-binding sites at the 5'UTR (f,g) in the HIP. *P*-values are displayed for each cytosine-phosphate-guanine-dinucleotide (CpG). **** $p \leq 0.0001$; *** $p \leq .001$; ** $p \leq .01$; * $p \leq .05$; ns = not significant.

AUTHOR CONTRIBUTIONS

Natan Yusupov: Conceptualization; methodology; software; data curation; formal analysis; writing—original draft preparation; writing—review and editing. **Lotte van Doeselaar**: Investigation; formal analysis; writing—original draft preparation; writing—review and editing. **Simone Röh**: Conceptualization; software, data curation. **Tobias Wiechmann**: Conceptualization; methodology. **Maik Ködel**: Investigation. **Susann Sauer**: Investigation. **Mathias V. Schmidt**: Funding Acquisition; conceptualization; project administration; supervision; methodology; writing—review and editing. **Elisabeth B. Binder**: Funding acquisition; conceptualization; project administration; supervision; methodology; writing—review and editing. All authors contributed to and have approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ejn.16078.

DATA AVAILABILITY STATEMENT

The raw data generated in the experiment was uploaded to the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra, BioProject accession number PRJNA972177). Processed data and main analysis code in R are available in a public repository on Github Enterprise (https://github.molgen.mpg.de/mpip/Fkbp5_DNAm_HAMTBS_mouse).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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3. Paper II

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ARTICLE OPEN

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DNA methylation patterns of *FKBP5* regulatory regions in brain and blood of humanized mice and humans

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Humanized mouse models can be used to explore human gene regulatory elements (REs), which frequently lie in non-coding and less conserved genomic regions. Epigenetic modifications of gene REs, also in the context of gene x environment interactions, have not yet been explored in humanized mouse models. We applied high-accuracy measurement of DNA methylation (DNAm) via targeted bisulfite sequencing (HAM-TBS) to investigate DNAm in three tissues/brain regions (blood, prefrontal cortex and hippocampus) of mice carrying the human FK506-binding protein 5 (*FKBP5*) gene, an important candidate gene associated with stress-related psychiatric disorders. We explored DNAm in three functional intronic glucocorticoid-responsive elements (at introns 2, 5, and 7) of *FKBP5* at baseline, in cases of differing genotype (rs1360780 single nucleotide polymorphism), and following application of the synthetic glucocorticoid dexamethasone. We compared DNAm patterns in the humanized mouse ($N = 58$) to those in human peripheral blood ($N = 447$ and $N = 89$) and human postmortem brain prefrontal cortex ($N = 86$). Overall, DNAm patterns in the humanized mouse model seem to recapitulate DNAm patterns observed in human tissue. At baseline, this was to a higher extent in brain tissue. The animal model also recapitulated effects of dexamethasone on DNAm, especially in peripheral blood and to a lesser extent effects of genotype on DNAm. The humanized mouse model could thus assist in reverse translation of human findings in psychiatry that involve genetic and epigenetic regulation in non-coding elements.

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INTRODUCTION

Genetic loci associated with risk for psychiatric disorders frequently lie in non-coding regions such as cis-regulatory DNA elements [1, 2]. These gene regulatory elements (REs) have been shown to be enriched for disease-associated genetic variants, but are also targets for epigenetic alterations related to environmental risk exposures [3]. While increasing numbers of risk-associated loci are being cataloged, there remains a large gap in our understanding of their functional consequences on multiple levels, from molecular, to cellular to systems. Human induced pluripotent stem cell- (iPSC) derived systems in combination with gene editing are tools that now allow to assess the functional consequences of both genetic and epigenetic alterations in disease-related gene REs, but they still lack the complexity of an intact organism. This level of exploration requires model organisms, such as rodents, which are established for investigating disease-related neurobiological mechanisms and preclinical testing of pharmacological targets [4]. However, these models are of limited use for the exploration of gene REs relevant to disease due to the lack of sequence similarity with humans in intergenic and gene regulatory regions [5, 6]. While genomic conservation across human and mouse genomes is high overall, only about 40% alignment can be reached at the nucleotide level [6]. The lack of conservation is especially apparent in non-coding regions [6, 7]. To overcome this limitation and allow further mechanistic exploration of disease-relevant human gene REs in whole organisms, genetically engineered humanized mouse models are an option. "Humanized mouse models" refer to mice that carry human genetic sequences, where the mouse gene is substituted by its human orthologue [8–10]. Such models can allow to explore human gene REs that are not conserved in rodents in an intact behaving organism and across all tissues and cell types.

Several humanized mouse models have been applied in neuropsychiatric research. Most of these have targeted coding regions [11–21], while some inserted full-length genes with a potential to model genetic differences lying in non-coding regions [22–30]. However, epigenetic modification of gene REs, also in the context of gene x environment interactions, have not yet been explored in such models. This is the aim of this study, focusing on a widely investigated candidate gene in psychiatric stress research, *FKBP5*, as an example. *FKBP5* encodes a co-chaperone molecule, the FK506-binding protein 5 (FKBP5), a strongly stress-responsive protein that modulates the hypothalamic–pituitary–adrenal (HPA) axis among other targets [31]. Genetic variants in this locus are mainly tagged by the intronic single nucleotide polymorphism (SNP) rs1360780. This

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SNP has been repeatedly associated with increased risk for a range of psychiatric disorders, mainly in the context of exposure to early adversity [32]. The current mechanistic model derived from human and animal studies proposes that disease risk is mediated by enhanced FKBP5 levels through genetic and epigenetic mechanisms, with downstream, tissue-specific consequences on its many interaction partners [32, 33]. Induction of *FKBP5* transcription by stress/glucocorticoids (GCs) is triggered by binding of the activated glucocorticoid receptors (GR) to specific DNA sequences, so-called glucocorticoid-responsive elements (GREs) [34]. This GC-related induction is moderated by the functional SNP rs1360780 (C/T) which is located close to a GRE in intron 2 of the gene. The minor T allele induces a stronger *FKBP5* expression by generation of an additional TATA-Box binding element to loop back to the transcription start site and is associated with a prolonged systemic cortisol response likely through the effects of FKBP51 on HPA-axis regulation [32, 35]. While this genetic variant has been associated with increased risk for psychiatric disorders, associations mainly occur in the context of early adversity and it has been proposed that the regulatory effects of the SNP need to be accompanied by additional epigenetic changes in other GREs of the *FKBP5* locus that are induced by adversity and stress hormone activation. Demethylation of DNA in cytosine-phosphate-guanine dinucleotides (CpGs) near GREs in introns 2, 5, and 7 of the *FKBP5* gene have been reported following exposure to environmental stressors such as childhood abuse [35] and is likely mediated by direct binding of the GR to GREs [36]. Demethylation of GREs has been associated with subsequent increased transcriptional responsivity of *FKBP5* to GC-stimulation [35]. In summary, it appears that the minor allele of the rs1360780 SNP and the demethylation of DNA at and around GREs are both necessary to increase FKBP5 expression above a disease-relevant threshold ([32] for review). In animal models, increased *FKBP5* activity in specific, mainly limbic, brain regions has been associated with behaviors indicative of increased anxiety and reduced stress coping [37], while blocking of endogenous FKBP51 resulted in opposite behavioral effects [38–40]. In postmortem brain, *FKBP5* expression is higher in patients with schizophrenia and depression, especially in upper layer excitatory neurons [41] and FKBP51 has been proposed as an interesting drug target for a subset of patients [42]. To follow-up on this it would be critical to better understand the epigenetic and genetic regulation of the locus in the context of an organism, which would also improve biomarker development of central *FKBP5* hyperactivity.

Recently, two humanized mouse lines were generated by substituting the murine *Fkbp5* gene by the human *FKBP5* gene differing only in the intronic rs1360780 SNP allele [43]. Nold et al. confirmed that human *FKBP5* is expressed in CNS cells of these mice and that the risk-associated genotype leads to a greater induction of the gene by GCs [44]. It is unclear, however, whether DNA methylation (DNAm) profiles in the relevant intronic GREs would also be recapitulated in the humanized *FKBP5* locus and respond to GR activation in a SNP-dependent way as shown for human cells [36] and how such effects would correlate between brain and blood.

Applying high-accuracy DNAm measurement via targeted bisulfite sequencing (HAM-TBS [45]), we aimed to explore DNAm patterns of CpGs located within three functional intronic GREs of *FKBP5* in three tissues/brain regions: blood, prefrontal cortex (PFC) and hippocampus (HIP) in the humanized *FKBP5* mouse model. We compared DNAm patterns in blood and PFC of the humanized model with data from human cohorts of psychiatric patients and healthy controls (two for blood and one for postmortem brain). Finally, we investigated effects of genotype and of GC-stimulation on DNAm using the GC-analog dexamethasone in the different humanized mouse tissues/brain regions and compared them to effects on DNAm in humans.

MATERIALS AND METHODS

Samples

Humanized FKBP5 *mouse.* All animal experiments were conducted with the approval of and in accordance with the Guide of the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany. Mice were group-housed under standard lab conditions (22 ± 1 °C, 55 ± 5% humidity) and maintained under a 12 h light-dark cycle with food and water *ad libitum*. All experiments were conducted with adult male mice (age: 2–4 months). Generated mice carried either the risk A/T (RiA, C57BL/6NTac-*Fkbp5*$^{tm4570(FKBP5)Tac}$) or the resilient C/G (ReG, C57BL/6NTac-*Fkbp5*$^{tm4571(*FKBP5)Tac}$) allele of rs1360780 SNP of the *FKBP5* gene (N_{RiA} = 28, N_{ReG} = 30; Fig. 1 and Supplementary Table 1). In these mouse lines the murine *Fkbp5* gene on chromosome 17 (from the start to the stop codon, i.e, exon 2-12 including interspersed introns of ENSMUST00000079413) was substituted by the homologous segment of the human *FKBP5* gene (exon 3–12 of ENST00000536438) (see [43] for a detailed description).

Human blood tissue

Study 1: We included 447 subjects with and without current (past 4 weeks) psychiatric disorders who consented for the Max Planck Institute of Psychiatry (MPIP) and were recruited in Munich, Germany as participants of two studies: the Biological Classification of Mental Disorders study (BeCOME, registered on ClinicalTrials.gov, TRN: NCT03984084, *N* = 319) [46] and a subset of patients recruited for major depression from a clinical psychotherapy study (OPTIMA, registered on ClinicalTrials.gov, TRN: NCT03287362; *N* = 128) [47] who agreed to participate in an additional biobanking project (see Table 1 and supplementary methods for detailed cohort description). All participants provided written informed consent. The studies and all procedures as well as a specific withdrawal request from the MPIP Biobank were approved by the Ludwig Maximilian University Ethics Committee (application 338-15).

Study 2: We used previously acquired data of DNAm and genotyping for 89 Caucasian participants at our institute (see [36], Table 1 and supplementary methods for detailed cohort description). DNAm levels were determined using the same method (HAM-TBS) as in all cohorts of our study but were available only for introns 5 and 7.

Human postmortem brain tissue: study 3. Postmortem brain tissues from the orbitofrontal cortex (BA 11) for 86 subjects were obtained from the NSW Brain Tissue Resource Centre (University of Sydney, Australia). Tissue was dissected from the 3rd 8–10 mm coronal slice of each fresh-frozen hemisphere (see [41], Table 1 and supplementary methods for detailed cohort description). Informed consent for brain autopsy was provided by the donors or their next of kin. The study was approved by the Ludwig Maximilian University Ethics Committee (project 17-085, application 22-0523).

Experimental design

Mice were treated either with vehicle or 2 mg/kg body weight dexamethasone intraperitoneally and were assessed after four and 24 h (Fig. 1 and Supplementary Table 1 for details of the experimental design). Five mice of each genotype remained untreated and were sacrificed at t0. Three tissues/brain regions of each mouse (blood, PFC, and HIP) were harvested upon sacrifice and stored at −80 °C for further processing.

Extraction of DNA

Humanized mouse. Genomic DNA was extracted from frozen tissue (−80 °C) of the HIP, PFC and submandibular vein blood of all samples except one blood sample (due to blood clot). Prior to DNA extraction, samples of each tissue were randomized (as separate blocks) into two 96-well plates with regards to genotype, time point and treatment using the *Omixer* R package [48].

Human blood. Genomic DNA was extracted from blood draw according to standard procedures [36]. Prior to DNA extraction, samples from study 1 were randomized into five 96-well plates with regards to sex, age, childhood maltreatment, and self-reported case-control status using the *Omixer* R package [48].

Human postmortem brain tissue. Genomic DNA was extracted from approximately 10 mg fresh-frozen tissue using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instruction

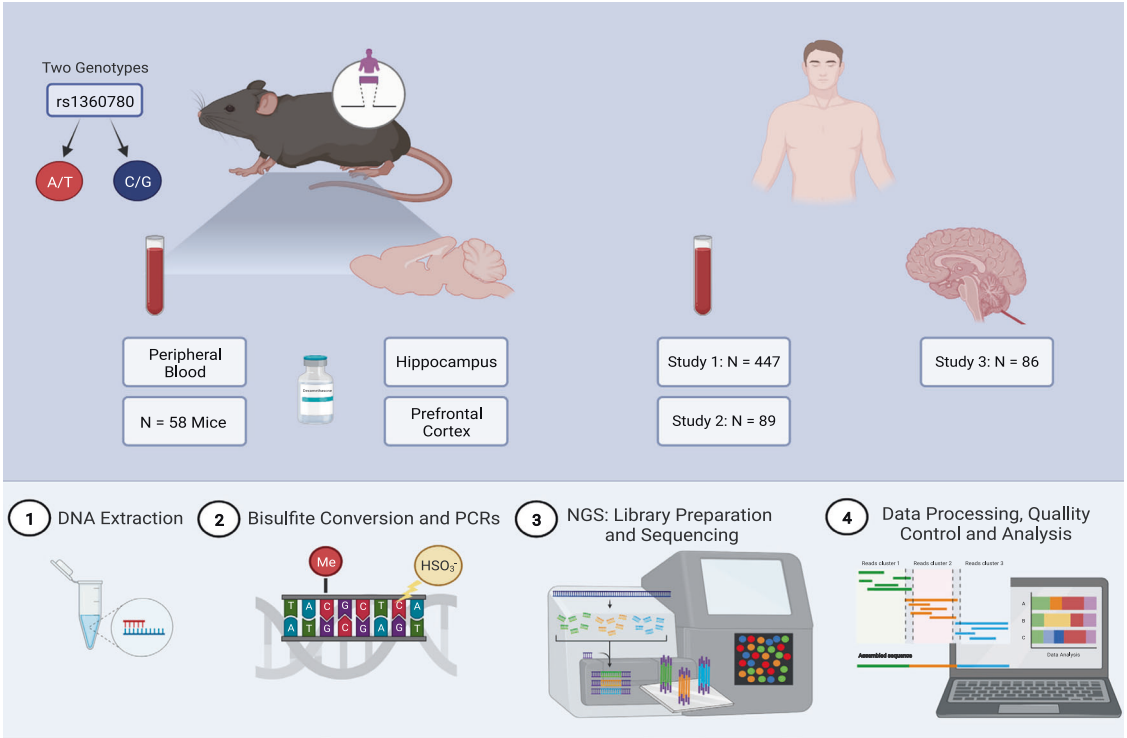

Fig. 1 Summary of study design and cohorts. Upper part: Human DNA methylation (DNAm) data was available/generated for three cohorts of different tissues/brain regions: two cohorts of peripheral blood and one of postmortem prefrontal cortex tissue (orbitofrontal cortex, BA 11). Humanized Mouse DNAm data of three tissues: blood, prefrontal cortex, and hippocampus, was available for two humanized mouse lines (carrying different alleles of the rs1360780 SNP) for the *FKBP5* gene. Mice were treated with 2 mg/kg body weight dexamethasone or vehicle and tissue harvested at three time points (baseline, after four and 24 h). Lower part: brief description of high-accuracy measurement of DNAm via targeted bisulfite sequencing (HAM-TBS) preparation and analysis workflow including: DNA extraction, bisulfite conversion of DNA, targeted PCRs amplification and library preparation followed by new generation sequencing, data processing with quality control and subsequent analysis. Created with BioRender.com.

Table 1. Demographic details for human cohorts.

Tissue	Study 1 Peripheral blood	Study 2 Peripheral blood	Study 3 Postmortem brain
Final N	440	89	84
Age (years)			
Mean (SD)	37.6 (13.0)	41.6 (14.0)	52.7 (14.1)
Median [Min, Max]	34 [19, 74]	42 [12, 75]	54 [22, 84]
Sex			
Female N (%)	271 (61.6%)	22 (24.7%)	31 (36.9%)
Current Psychiatric Diagnosis			
Yes N (%)	264 (60%)	59 (66.3%)	51 (60.7%)
Missing N (%)	7 (1.6%)	0 (0%)	0 (0%)
rs1360780 SNP			
CC N (%)	189 (42.9%)	50 (56.2%)	42 (50%)
TC N (%)	154 (35%)	30 (33.7%)	24 (28.6%)
TT N (%)	39 (8.9%)	9 (10.1%)	9 (10.7%)
Missing N (%)	58 (13.2%)	0 (0%)	9 (10.7%)

protocol. DNA samples were concentrated using the DNA Clean & Concentrator-5 (Zymo Research, Irvine, CA). DNA concentration was measured using Qubit™ dsDNA BR-Assay (Invitrogen, Carlsbad, California, USA).

DNA methylation analysis

DNAm at the *FKBP5* locus was assessed with high-accuracy DNAm measurement via targeted bisulfite sequencing (HAM-TBS), a next-generation sequencing method for detection of DNAm changes in specific regions, as described in detail by Roeh et al. [45]. Briefly, triplicates of samples (200-500 ng DNA each processed according to the manufacturer's instructions) were treated with bisulfite using EZ DNA Methylation Kit (Zymo Research, Irvine, CA). Amplification of target sequences (Supplementary Table 2: primers list; Supplementary Table 3: amplicons list) was performed using TaKaRa EpiTaq HS Polymerase (Clontech, Mountain View, CA; final concentration: 0.025 U/l). Selected bisulfite-specific primers originated from a validated panel of regulatory regions within the *FKBP5* locus (details in [45]). Amplicons were quantified using the Agilent 4200 TapeStation (Agilent Technologies, Waldbronn, Germany) and pooled by Hamilton pipetting robot. To remove excess of primers and genomic DNA, after speed-vacuum and resuspension in 50 µl, a double-size selection using Agencourt AMPure XP beads (Beckman Coulter GmbH, Krefeld, Germany) was performed. Next, PCR-free libraries were prepared with Illumina TruSeq DNA PCR-Free HT Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's protocol (500 ng of starting material). Qubit 1.0 (Thermo Fisher Scientific Inc., Schwerte, Germany) was used for quantification of libraries prior to equimolar pooling. Quality assessment of final pooled library was performed with Agilent's 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and Kapa Library quantification kit on LightCycler480 (Roche, Mannheim, Germany). Sequencing of libraries was conducted on an Illumina MiSeq with Reagent Kit v3 (Illumina, San Diego, CA; 600 cycles, 12pM Library, paired-end mode, 15% PhiX).

Proportions of blood cell types were calculated from Illumina Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA, USA) data for study 1 ($N = 436$, see details in [49]) and the Illumina Infinium HumanMethylation450 BeadChip for study 2 ($N = 89$, see details in [36]) as suggested by Houseman et al. [50].

Data processing

The following preprocessing was applied separately to all generated data of humanized mouse, human blood and human postmortem samples. Quality of reads was assessed with *FastQC* [51]. Reads were trimmed with *cutadapt* v1.11 [52], setting the minimal read length to 100 bp. Reads were mapped with *Bismark* v0.18.2 [53] to a restricted reference comprised of the amplicon sequences, including 50 bp padding on each side. Overlapping ends of reads were removed symmetrically to avoid sequence quality dropping towards the end of each read. Data was inserted to R v4.0.4 [54] and underwent further preprocessing steps: (1) exclusion of PCR artifacts, (2) exclusion of samples with low median coverage (low sequencing depth) in every amplicon (total read number <1000, humanized mouse: $N = 3$; human blood: $N = 30$, human postmortem brain: none), (3) exclusion of samples with low rates of bisulfite conversion (<95%, none), and (4) failed amplicons: sufficient coverage in more than 50% of the samples in all amplicons. Raw methylation calling and bisulfite conversion assessment were performed by *methylKit* R package v1.6.3 [55], with minimum Phred quality score of 30 (99.9% base call accuracy). After QC, a total of 20 CpGs in introns 2, 5, and 7 of the *FKBP5* locus shared by the three data sets remained. CpGs were named after their positions on chromosome 6 of the human reference genome hg19 (Supplementary Table 4: list of CpGs with genomics locations). Next, we excluded technical outlier samples per amplicon (DNAm < (1. quartile − 2xIQR) or > (3. quartile + 2xIQR) in over 50% of CpGs; humanized mouse: PFC: $N = 1$, Blood: $N = 1$ and HIP: $N = 5$; human blood: $N = 24$; human postmortem brain: $N = 6$). One animal was excluded due to hydrocephalus, leaving 57 subjects in the final cohort. Seven human blood and two human postmortem samples were removed due to technical issues (blood: two failed library preparation, one pipetting error, four had missing CpGs > 20%; brain: missing CpGs > 20%, outlier in DNA isolation batch). Final samples comprised 440 and 84 subjects for human blood and postmortem brain, respectively. To exclude major sources of variation explained by technical batch effects, DNAm data of each data set (in the humanized mouse each tissue separately) was dimensionality reduced via principal component analysis after imputation using the *missMDA* R package v1.18 [56]. Subsequently principal components were tested with ANOVA of linear models for possible batch effects (humanized mouse: row, column, plate and dissector (in brain tissues); human blood: row, column, plate and isolation batch; human postmortem brain: row, column, isolation batch, hemisphere, brain pH, postmortem interval and storage time). Batch effects of column and dissector were detected in brain tissue of the humanized mouse and included as covariates in all statistical models. The same procedure revealed batch effects of storage time and brain pH in human postmortem brain as well as isolation batch and plate in human blood. The covariates were included in all statistical models. Batch-corrected data was used for visualized comparison of means between tissues/brain regions and species as well as correlation analysis (corrected with ComBat of the sva R package v3.38.0 [57]).

Genotyping of human postmortem prefrontal cortex

Genotyping was conducted using Illumina global screening arrays (GSA-24v3-0, Illumina, San Diego, CA, US) excluding SNPs with low call rate (98%), a minor allele frequency <1% or deviation from Hardy-Weinberg-Equilibrium (p-value < 1×10^{-05}). Individuals with call rates <98% were excluded. Only unrelated individuals were included for further analysis. After LD-pruning, outliers on multi-dimensional scaling components of the genotypes IBS matrix (>3 standard deviations (SD) from the mean on any of the first 10 axes) and heterozygosity outliers (>3 SD from mean heterozygosity) were removed. Allelic information for the rs1360780 SNP was retrieved. Complete data with genotype and DNA methylation was available for 75 subjects.

Statistical analysis

Mean and SD (in percent) were used for comparison and Spearman correlations were used to analyze similarities in DNAm levels between tissues/brain regions. To evaluate genotype and dexamethasone treatment effects on DNAm of the humanized mouse, multiple linear regressions were performed on M transformed values (M = log2(Beta/(1-Beta)) [58]). Normality of values was evaluated using quantile-quantile plots. Prior to regression modeling, non-variable CpGs (interquartile range, IQR < 1% methylation) within a tissue were removed (Blood and PFC: $N = 2$; HIP: $N = 4$; Supplementary Fig. 1). The following linear model was used: CpG Methylation ~ significant batch covariates (if present) + genotype + treatment + genotype × treatment. *P*-values were FDR corrected for multiple testing. *P*-values, *q*-values, beta estimates, standard error and F-statistic are reported. Even considering removed samples, a power analysis (G*Power version 3.1.9.6 [59]) showed a sufficient power (>0.8) for detecting medium main effects (Cohen's $f^2 = 0.2$) at a significance criterion of $\alpha = 0.05$ in the multiple linear regression. All statistical analyses were conducted in R version 4.0.4 [54].

RESULTS

Similarity of DNA methylation levels in relevant intronic *FKBP5* GREs in blood, prefrontal cortex and hippocampus of humanized *FKBP5* mice

Twenty CpGs (named according to positions on the human reference genome hg19) within six amplicons of the humanized *FKBP5* locus, covering the three main intronic GREs (Fig. 2), were investigated in 57 animals. CpGs of intron 7 showed similar DNAm patterns between both brain regions (5% average of delta mean DNAm) and these differed strongly from blood (−59% for PFC and −53% for HIP average of delta mean DNAm). CpGs in intron 2, however, were similar across all analyzed tissues (5% average of delta mean DNAm; Fig. 2; Supplementary Table 5: baseline mean

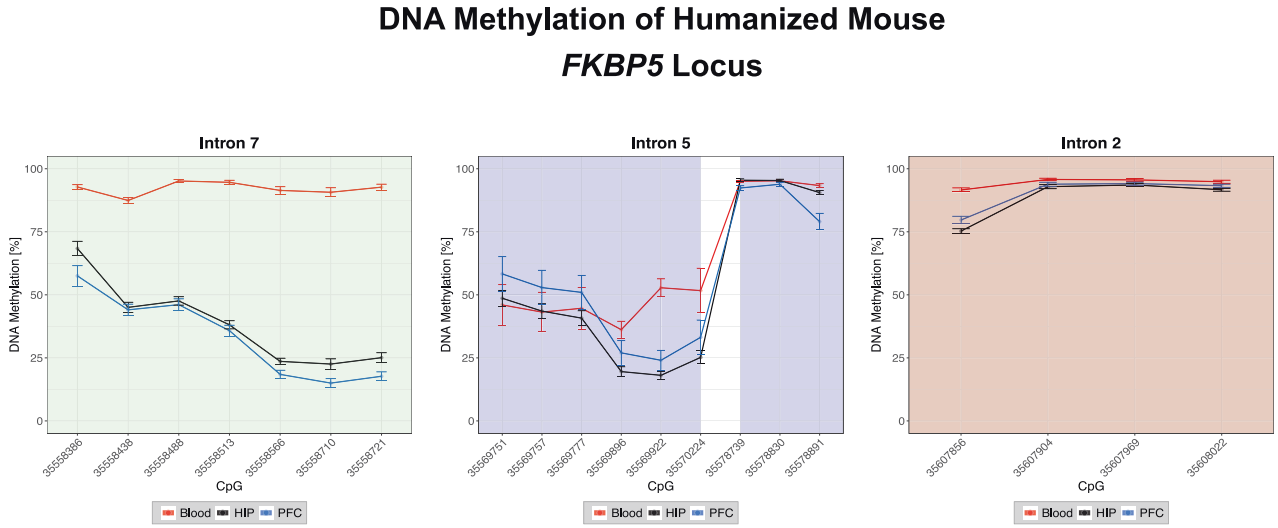

Fig. 2 DNA methylation levels in peripheral blood, prefrontal cortex and hippocampus of humanized mouse. Depicted are DNAm levels of 20 CpGs (mean and standard deviation in percent) of three introns of the humanized *FKBP5* gene (green: intron 7, blue: intron 5, red: intron 2) across three tissues at baseline ($N = 10$). CpGs are named according to their positions on the human reference genome hg19.

and SD of DNAm; Supplementary Table 6: delta mean DNAm across tissues/brain regions). Intron 5 showed more diverse DNAm patterns with CpGs where DNAm in blood was similar to HIP and PFC (delta mean DNAm in 35569751, 35569757, 35569777, 35578739 and 35578830 of <4% for HIP and <12% for PFC) and CpGs with different DNAm levels in all three tissues (35569896, 35569922, 35570224, 35578891; Fig. 2; Supplementary Table 5, 6). Overall, we observed low correlations of DNAm levels across CpGs in brain and blood of the humanized mouse with only very few correlations reaching significance (Supplementary Fig. 2; Supplementary Table 7). This was also reflected in different inter-CpG correlation structure of blood and the two brain regions, with the latter being more similar (but still showing relevant differences in correlation structure as compared to blood (Supplementary Fig. 3)).

To compare brain-blood correlations of CpGs from our humanized mice to correlations previously reported in humans, we used two publicly available tools from studies that have evaluated correlations of CpG DNAm between blood and several brain regions in humans using a genome-wide DNAm array (Illumina Infinium HumanMethylation450 BeadChip): Blood Brain DNA Methylation Comparison Tool (https://epigenetics.essex.ac.uk/bloodbrain/ [60]) and Blood-Brain Epigenetic Concordance (BECon, https://redgar598.shinyapps.io/BECon/ [61]). However, due to the limited representation of our CpGs within the *FKBP5* gene on the array, only one CpG from our panel was present: 35570224 in intron 5 (cg14284211). Similar to the humanized mouse model (Blood-PFC: $r_s = 0.09$, $N = 30$; Blood-HIP: $r_s = 0.1$, $N = 29$), there were low correlations between blood and different brain regions in the Blood Brain DNA Methylation Comparison Tool (blood-PFC: $r_s = -0.009$, $N = 74$; blood-entorhinal cortex: $r_s = -0.006$, $N = 71$; blood-superior temporal gyrus: $r_s = -0.11$, $N = 75$ and blood-cerebellum: $r_s = -0.027$, $N = 71$) but higher correlations were reported in BECon (blood-BA10: $r_s = 0.41$; blood-BA20: $r_s = 0.15$; blood-BA7: $r_s = 0.27$, $N = 16$). Overall, 31 CpGs within the *FKBP5* locus have been assessed in the Blood Brain DNA Methylation Comparison Tool but only two showed significant correlations (cg06087101 and cg08915438). Our analysis in humanized *FKBP5* animals with tissues ascertained at the same time thus supports low correlation between DNAm of blood and brain tissue in this locus, especially considering that DNAm seems to be well recapitulated in humanized mice as described below.

Comparison of DNA methylation levels in blood and prefrontal cortex of humanized *FKBP5* mouse and humans

We next compared baseline DNAm levels of the ascertained REs (introns 2, 5, and 7) at the *FKBP5* locus between humanized mouse and human tissue. We used previously generated DNAm data of peripheral blood ([36] for details, study 2) and two newly generated human data sets (study 1 for blood and study 3 for PFC) using HAM-TBS technology. Study 2 did not include CpGs of intron 2, but was otherwise comparable. Only non- or vehicle-treated animals were considered for this analysis ($N = 31$). In the two human datasets with peripheral blood, DNAm did not differ significantly between datasets after regressing out effects of age, sex, and cell type proportions, indicating consistent DNAm pattern in this locus in the same tissue across different cohorts and measurement batches (Supplementary Table 8: comparison of means; Supplementary Table 9: mean and SD of DNAm).

Overall, we observed similar DNAm patterns between humanized mouse and humans in blood in all CpGs of intron 2 (<3% delta mean DNAm), and some within introns 5 (35578739, 35578830, 35578891; <5% delta mean DNAm) and 7 (35558386, 35558488, 35558513; <9% delta mean DNAm), but stronger divergence in the other CpGs of intron 5 and 7 (delta mean DNAm range of 35-51% and 14-35% respectively; Fig. 3; Supplementary Table 10: delta mean DNAm of blood). We did not find any significant differences in DNAm pattern in human blood (cohort 1) or in brain due to current disease status after regressing out age, sex and calculated cell-types from both blood and brain (Supplementary Fig. 4). In fact, small differences dependent on depression status were only seen in cohort 2 for one CpGs site also tested in the humanized mouse model but with less than 2.2% DNAm difference at baseline [36]. DNAm patterns in human postmortem PFC showed higher similarity to DNAm of humanized mouse PFC (Fig. 3; Supplementary Table 11: baseline DNAm mean and SD; Supplementary Table 12: delta mean DNAm of PFC). Introns 7 and 2 presented highly similar patterns (mean and SD of delta mean DNAm: intron 7: mean 2%, SD 4%; intron 2: mean 4%, SD 4%). While intron 5 presented somewhat lower similarity at the DNAm levels (mean and SD of delta mean DNAm: part 1: mean 14%, SD 4%; part 2: mean −7%, SD 7%), the pattern of DNAm was highly similar (Fig. 3). Moreover, if comparing to a similar human age group (20–29 years [62]), even more similar DNAm levels were observed in the PFC (Fig. 4 and Supplementary Table 13).

Genotype and dexamethasone effects on DNA methylation in humanized *FKBP5* mouse

To investigate genotype, dexamethasone, and genotype-specific glucocorticoid-induced effects on DNAm in the different tissues/brain regions (blood, PFC, and HIP), we administered 2 mg/kg dexamethasone (or vehicle) intraperitoneally to the two humanized mouse models and measured DNAm levels after four and 24 h. After removing CpGs with low variability within each tissue/brain region (IQR < 1%), we performed multiple linear regression models with 18 CpGs in blood and PFC and 16 CpGs in HIP.

Regarding genotype, the risk-associated allele of rs1360780 SNP was associated with significantly lower DNAm levels at ten CpGs after FDR correction in blood at baseline (Supplementary Table 14). The strongest effects were observed in four CpGs of both introns 5 and 7 (Supplementary Fig. 5A). In the human data, decreased DNAm with the T allele was also observed at a number of CpGs in cohort 2. The extent of genotype-related effects, however, was not always matching the humanized mouse model, with strongest effects observed in the human data in intron 7 but not intron 5 (Supplementary Fig. 5B). In the humanized mouse, no significant genotype effects were detected in PFC and HIP (Supplementary Table 15 and 16). However, univariate effects of risk allele homozygosity on DNAm levels were observed in the human PFC primarily in intron 5 but also in intron 7 (Supplementary Fig. 6). Nonetheless, the three CpGs in intron 5 with the strongest reduction in DNAm with the TT genotype in human PFC were also those with the largest effect sizes in the humanized mouse model (Supplementary Fig. 6).

As to dexamethasone effects, in the humanized mouse model, administration of dexamethasone was associated with significantly decreased DNAm after FDR correction in most blood CpGs, which returned to baseline after 24 h (Fig. 5A; Supplementary Fig. 5C; Supplementary Table 14). The strongest effects were seen in intron 5 (35569777, 35569757, 35569751). While the effects sizes very closely matched results from cohort 2 that had explored the effects of 1.5 mg dexamethasone orally in whole blood after 3 and 23 h in intron 7, the large effect sizes observed intron 5 of the humanized mice were not observed in cohort 2 (Supplementary Fig. 7). For the two brain regions, no human data was available for direct comparison of dexamethasone effects. The PFC showed no dexamethasone treatment effects (Supplementary Table 15). In the HIP, most intron 5 CpGs showed an increase in DNAm four h post-dexamethasone (35569922, 35569896, 35569751, 35569757 and 35569777; Supplementary Fig. 8), which was reversed after 24 h (Supplementary Table 16). Interaction effects of dexamethasone treatment with the risk allele were only nominal ($p < 0.05$) and did not survive correction for multiple testing. The strongest interaction was observed in intron 5 after 24 h in HIP in 35570224 (Supplementary Fig. 9; Supplementary Table 16).

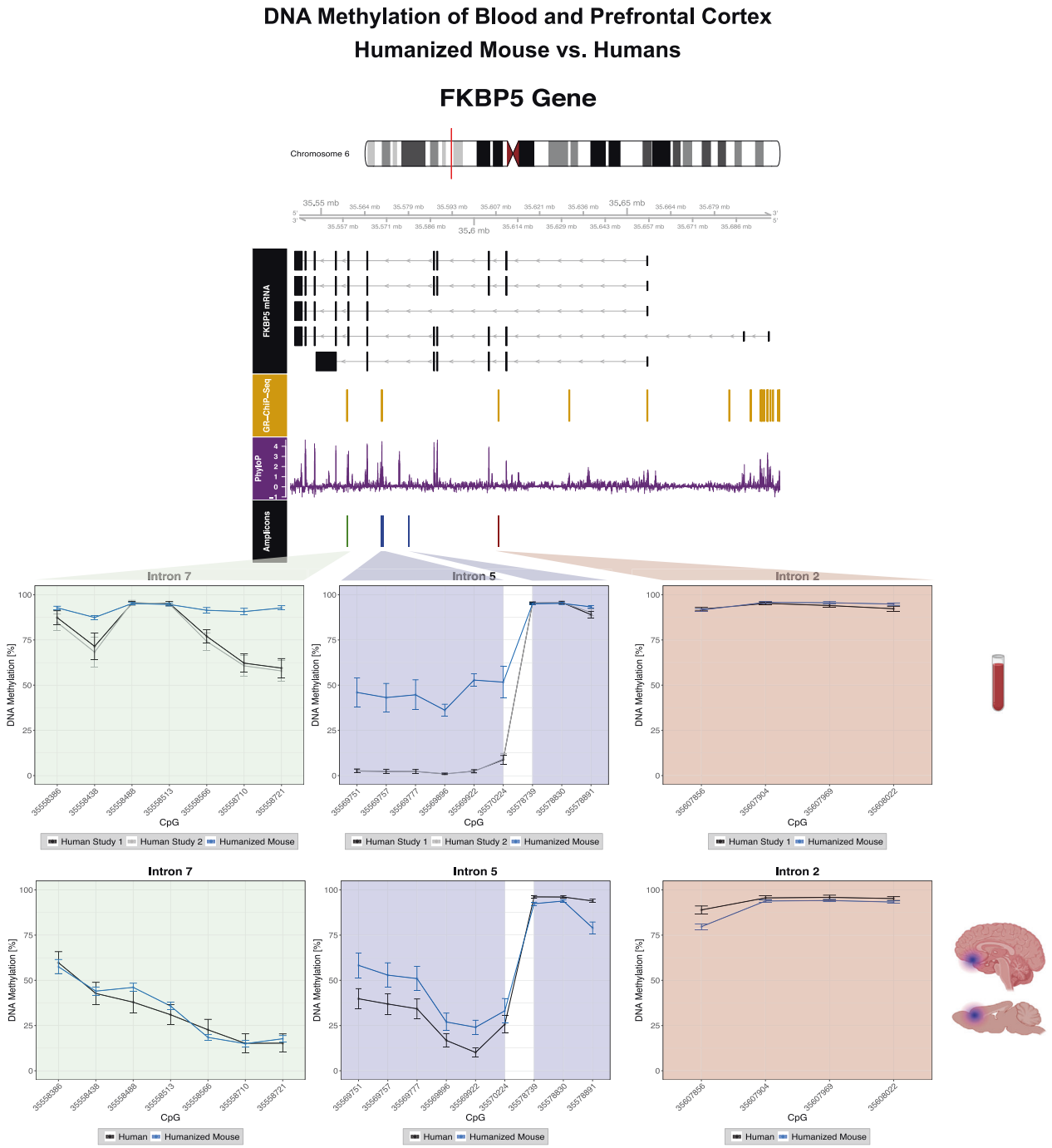

Fig. 3 Comparison of DNA methylation patterns in peripheral blood and prefrontal cortex between humanized mouse and human. Depicted are the *FKBP5* human locus on chromosome 6 (hg19, 35541362-35656719), the common human splicing variants of the gene, the genomic locations of glucocorticoid-responsive elements, regions with transcription factor binding derived from ChiP-Sequencing experiments in two cell lines (A549, ECC-1) from the ENCODE project available at the UCSC browser (https://genome.ucsc.edu/, laboratory of Richard Myers, HAIB, Huntsville, Alabama) and PhyloP basewise conservation score available at the UCSC browser (https://genome.ucsc.edu/). Finally, genomic locations (named according to their positions on the human reference genome hg19) and DNAm levels of 20 CpGs in three introns of the *FKBP5* gene (green: intron 7, blue: intron 5, red: intron 2) are displayed as mean and standard deviation (in percent) in three cohorts for blood (upper part; study 1, study 2 and the humanized mouse) and in two cohorts for prefrontal cortex (lower part; study 3 and the humanized mouse). Blood DNAm data was not available for CpGs in intron 2 in study 2, but was otherwise comparable. Regions further away from each other in intron 5 are separated by a white space. GR-ChIP = glucocorticoid-receptor chromatin immunoprecipitation, CpG = cytosine-phosphate-guanine-dinucleotides. Symbols were created with BioRender.com.

DISCUSSION

In this study, we investigated DNAm patterns in functional intronic GREs of the *FKBP5* gene in brain and blood of a humanized *FKBP5* mouse model. This allowed cross tissue comparisons of human *FKBP5* GRE DNAm at baseline, in the context of a functional intronic variant and following GC-stimulation as well as comparisons with DNAm patterns of *FKBP5* in human tissues.

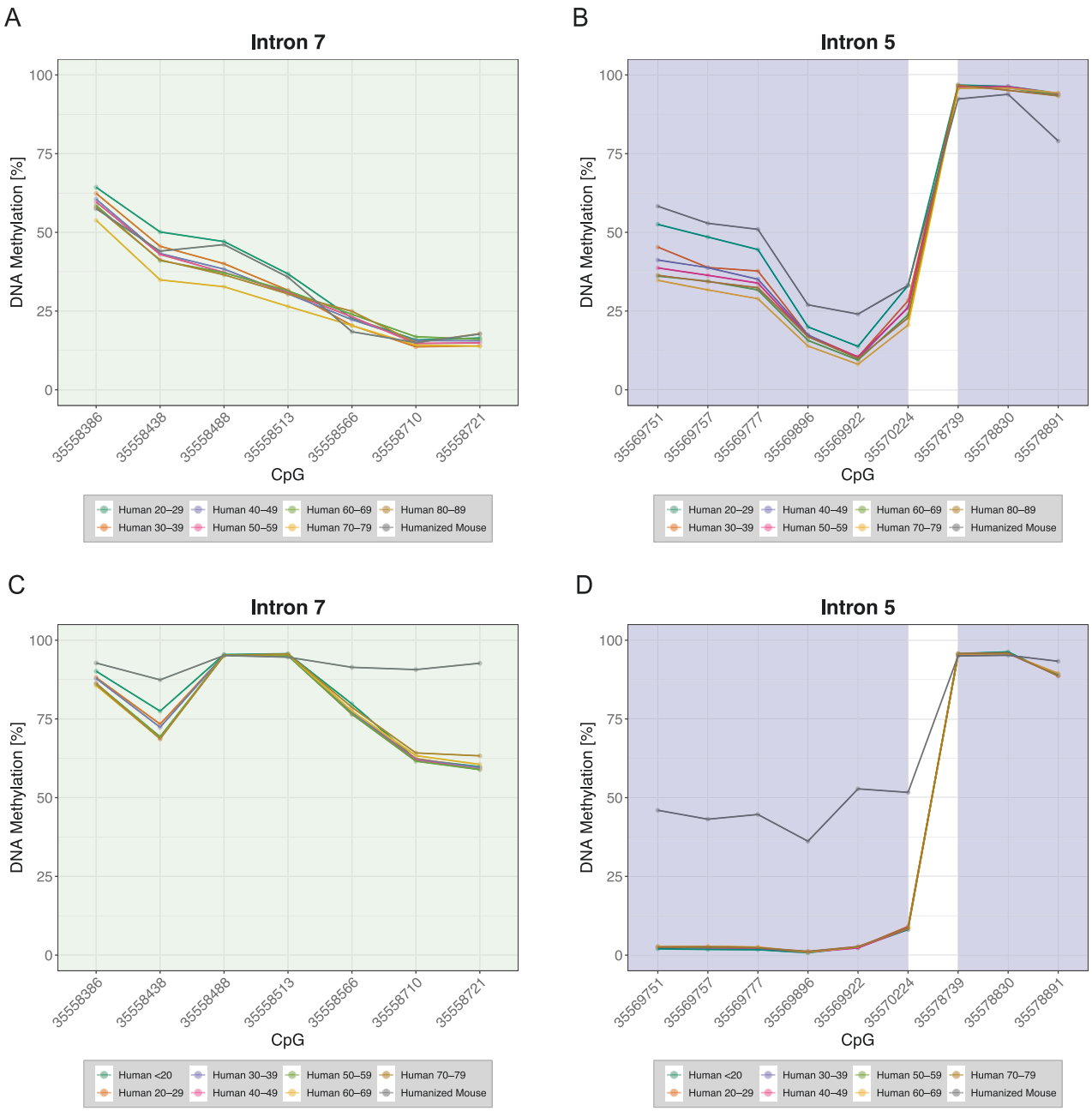

Fig. 4 Age-dependent DNA methylation patterns in peripheral blood and prefrontal cortex between humanized mouse and human. Depicted are mean DNAm levels of 16 CpGs (in percent) of two introns of the *FKBP5* gene (green: intron 7, blue: intron 5) for prefrontal cortex (**A**, **B**; study 3 and the humanized mouse) and for blood (**C**, **D**; study 1 and the humanized mouse). Human subjects are categorized into age bins in years (N of subjects in each bin for study 1: <20: 6, 20–29: 158, 30–39: 103, 40–49: 63, 50–59: 84, 60–69: 22, 70–79: 4; N of subjects in each bin for study 3: 20–29: 3, 30–39: 12, 40–49: 17, 50–59: 27, 60–69: 16, 70–79: 5, 80–89: 4). CpGs are named according to their positions on the human reference genome hg19.

Overall, DNAm patterns of the humanized mouse model seemed to recapitulate DNAm patterns observed in native human tissue. This is likely attributable to the fact that the DNA sequence itself is one of the main drivers of local DNAm [63]. This was present to a higher extent in brain tissue, where DNAm patterns of relevant GREs within the *FKBP5* gene (introns 2, 5, and 7) of the humanized mouse model were highly similar to postmortem human tissue PFC. In blood, the least convergence was observed in intron 5, but with overall conserved pattern on DNAm. Differences in DNAm levels in blood could be related to differences in immune cell composition (*e.g.*, neutrophils and lymphocytes balance) between mice and humans [64].

Beyond baseline levels, the humanized mouse model also mostly recapitulated effects of dexamethasone and, to a lesser extent, of genotype on DNAm. In fact, dexamethasone led to a reversible DNA demethylation in peripheral blood in all intronic GREs, very similar to previously reported effects in humans ([36], see Fig. 5). However, at CpGs with higher baseline methylation levels in humanized mice than humans such as in intron 5, effects sizes of dexamethasone-associated demethylation were larger in the animals, possibly suggesting effects of baseline DNAm on reactivity. While dexamethasone effects were observed in blood, most CpGs did not show altered DNAm in brain tissue. This could be due to lower GC-responsivity of specific regions such as the

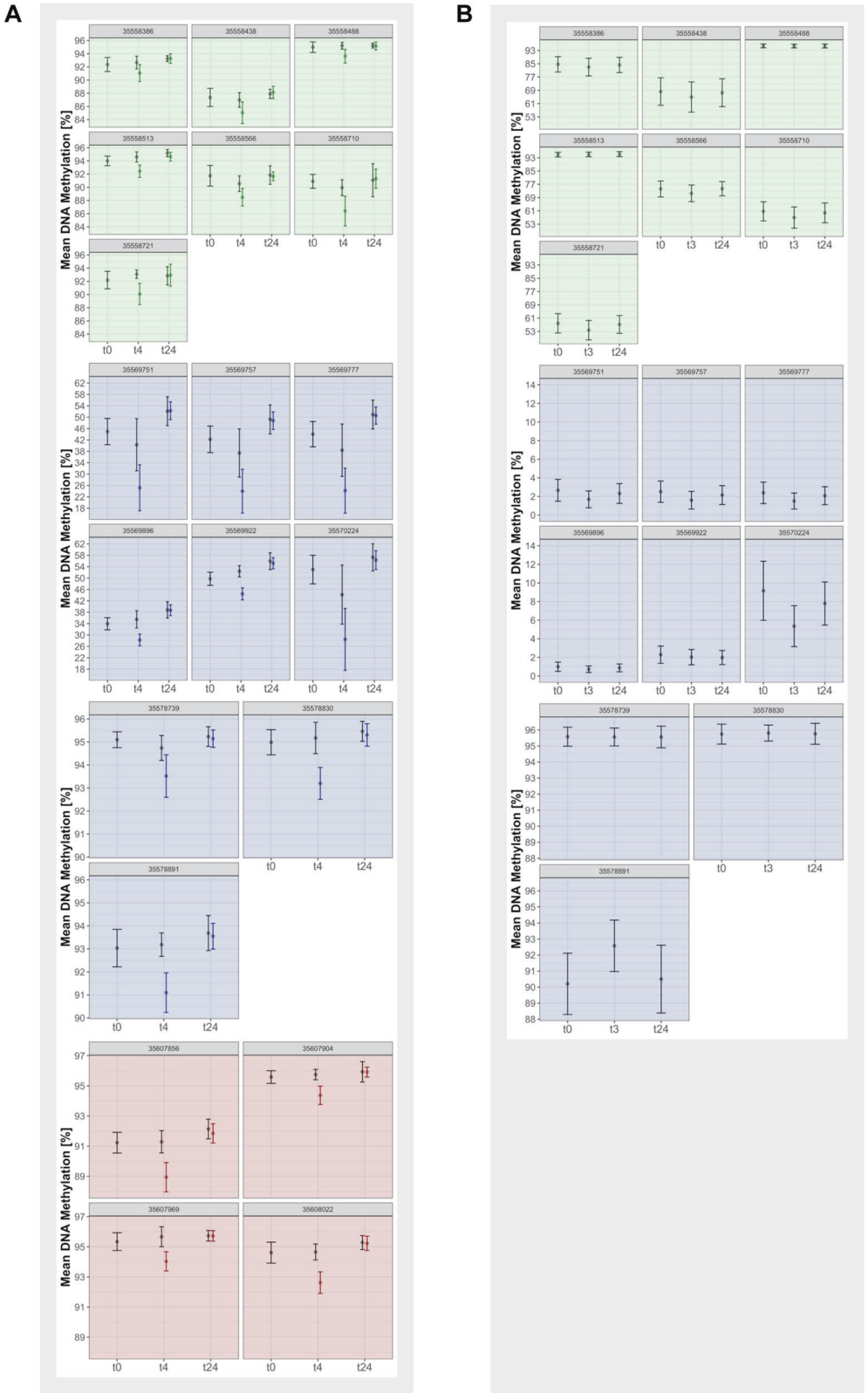

Fig. 5 Dexamethasone effects on DNA methylation of each CpG in humanized *FKBP5* mouse and human blood. Depicted are mean and standard deviation of DNA methylation (in percent) of each CpG within introns of *FKBP5* (red: intron 2, blue: intron 5 and green: intron 7). **A** Sample group at baseline, 4 h, and 24 h after dexamethasone treatment in the humanized *FKBP5* mouse blood. **B** Samples at baseline, 3 h and 24 h after dexamethasone treatment in human blood (data from [36] was used).

HIP, as previously suggested [65], but could also be attributed to a lower dose of dexamethasone and thus reduced intracerebral levels as compared to blood levels due to active extrusion at the blood brain barrier [66], differences in cell type heterogeneity or longer temporal dynamics of the GC-induced DNAm response in the brain. It is interesting to note that the CpG in location 35570224 in intron 5 showed the strongest DNAm change (25.4%) with dexamethasone in blood and a nominal interaction effect between dexamethasone treatment and genotype after 24 h in the HIP. Thus, following dexamethasone, only mice with the risk allele (A/T) showed a reduction in DNAm after 24 h. In a similar fine mapping of effects of dexamethasone on DNAm in the *FKBP5* locus of a human hippocampal progenitor cell line, the same CpG showed the strongest long-lasting effects of DNAm (−20.1% [67]).

While genotype effects were mostly recapitulated this was to a lesser extent than the dexamethasone effects and could be related to differences in genetic background and haplotype structure in humans as opposed to the animal model that only differed in this one SNP and limited power.

Our evaluation of DNAm patterns also revealed substantial tissue-specific DNAm across the three tissues/brain regions, an aspect previously reported for regions responsive to environmental stimuli [68–70]. While DNAm patterns in the humanized *FKBP5* mouse were similar between the two investigated brain regions (in mean DNAm and correlation structure), blood and brain concordance was low. Furthermore, there were also very little similarities of dexamethasone and genotype effects across brain and blood in the animal model. This is concordant with data from humans and confirms that differences are not due to differences in the timing of tissue extraction as in the case for postmortem brain studies, with brains sampled after death and blood often collected before. Our data therefore emphasizes the importance of tissue-specific DNAm levels, also with regard to genotype-associations and responsivity to environmental challenges.

Our study provides encouraging results regarding future use of humanized mouse models in the functional investigation of complex GxE that involve genetic and epigenetic regulation in non-coding elements. For example, Codagnone et al. have suggested that chronic selective inhibition of FKBP51 with the selective antagonist SAFit2 can induce stress resilience and change hippocampal neurogenesis in a chronic stress mouse model [39]. The humanized mouse model could elucidate whether such effects would be potentiated in a genotype-specific manner and/or are mediated by DNAm as a regulatory epigenetic mechanism, and could thus support genotype-guided treatment in patients.

Our results have several important limitations. First, the investigation was performed on a tissue-level, meaning an average across heterogeneous cells in each sample. Since DNAm is cell-type-specific, changes in DNAm of the whole tissue might not indicate effects on single cell types [71, 72]. Nold et al. showed that astrocytes derived from these humanized *FKBP5* mice have the strongest induction of *FKBP5*, yet the epigenetic correlates remain unknown [43]. Future studies thus need to evaluate cell-type-specific DNAm. Second, the analysis was limited to male mice. A recent study in the same model investigated sex-specific effects on the HPA axis and behavior after ELS modeled by prolonged maternal separation [44]. Females demonstrated higher corticosterone levels and more pronounced reduction after administration of dexamethasone. While slight genotype- and/or ELS-dependent behavioral differences were present in females, males were generally less affected behaviorally by genotype and ELS. Since sex-specific effects are plausible [73, 74], and evidence is expending for *FKBP5* [36, 37, 75], future studies should include the investigation of sex-specific effects. Third, results from humanized mouse models should be interpretated with caution due to differences in the immune system [64], the genetic background and the complex environment between the species. Finally, the interaction analysis of dexamethasone and genotype effects was likely underpowered due to the size of each individual group and results should be replicated in a larger cohort.

In conclusion, our analysis suggests that DNAm in GREs in the humanized *FKBP5* mouse model are similar to humans, especially in the PFC. Furthermore, we highlight the difficulties using peripheral blood as a proxy for changes in the brain. Given the necessity of exploring the molecular underpinnings of GxE interplay in psychiatric disorders, the recently engineered mice could present a powerful tool for studying the effects of human *FKBP5* polymorphism-related glucocorticoid response in disease-relevant tissues. Combined with naturalistic stress paradigms, behavioral tests, and/or neuroepigenetic editing, the humanized mouse can support mechanistic biological investigation of stress-related, *FKBP5*-induced psychopathology and enhance reverse translation of human findings.

DATA AVAILABILITY

The raw data generated in the TBS experiments was uploaded to the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra, BioProject accession numbers PRJNA1042684, PRJNA1042690, PRJNA1042916). Processed data and main analysis code in R are available in a public repository on Github Enterprise (https://github.molgen.mpg.de/mpip/Fkbp5_DNAm_HAMTBS_humanized_mouse_humans). Clinical data can be obtained upon a reasonable request.

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AUTHOR CONTRIBUTIONS

NY: Conceptualization, Methodology, Software, Data Curation, Formal Analysis, Writing—Original Draft Preparation, Writing—Review & Editing. SR: Conceptualization, Software, Data Curation. LSE: Conceptualization, Investigation. SC: Investigation. SL: Investigation. LUT: Investigation. ASF: Investigation, Data Curation. SS: Investigation. MK: Investigation. NM: Conceptualization, Data Curation. DC: Methodology, Formal Analysis. JMD: Conceptualization, Project Administration, Supervision, Methodology, Writing—Review & Editing. EBB: Funding Acquisition, Conceptualization, Project Administration, Supervision, Methodology, Writing—Review & Editing. All authors contributed to and have approved the final manuscript.

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Appendix A: Paper III

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Early adversity as the prototype gene × environment interaction in mental disorders?

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ABSTRACT

Childhood adversity (CA) as a significant stressor has consistently been associated with the development of mental disorders. The interaction between CA and genetic variants has been proposed to play a substantial role in disease etiology.

In this review, we focus on the gene by environment (GxE) paradigm, its background and interpretation and stress the necessity of its implementation in psychiatric research. Further, we discuss the findings supporting GxCA interactions, ranging from candidate gene studies to polygenic and genome-wide approaches, their strengths and limitations. To illustrate potential underlying epigenetic mechanisms by which GxE effects are translated, we focus on results from *FKBP5* × CA studies and discuss how molecular evidence can supplement previous GxE findings.

In conclusion, while GxE studies constitute a valuable line of investigation, more harmonized GxE studies in large, deep-phenotyped, longitudinal cohorts, and across different developmental stages are necessary to further substantiate and understand reported GxE findings.

1. Background of the GxE paradigm with regards to early adversity

The study of gene-by-environment (GxE) interactions is well embedded in psychiatry's long-held theory within the diathesis-stress or vulnerability-stress model, which states that mental disorders are caused by interactions between dispositional (diathesis) and environmental factors (stress) (Broerman, 2020). It roots in the observation that neither dispositional (e.g., genetic, G) nor environmental factors (E) alone are sufficient for explaining the development of psychiatric disorders. Among the environmental factors studied in psychiatry, stress and adverse life events are the most prominent risk factors, especially when occurring early in life.

Childhood/early adversity (CA; EA), also referred to as early-life stress (ELS) or adverse childhood experiences (ACE) (see Table 1 for a clarification of terms), is one of the most-studied and evidenced environmental risk factors for the development of mental health problems later in life (Bellis et al., 2019). Therefore, we will focus on CA within the context of GxE interactions in this review.

Although the question of what exactly constitutes CA has been handled differently in the literature, the broad and not uniformly defined concept of CA almost always includes the category "child maltreatment" (CM, see Table 1), which consists of more clearly defined abuse and neglect subtypes (Zeanah and Humphreys, 2018). Convincing evidence for a strong association of CA (including child abuse and neglect) with onset and persistence of mental disorders comes from several large-scale and population-based national and global mental health surveys (Benjet et al., 2010; Green et al., 2010; Kessler et al., 2010; McLaughlin et al., 2010). These studies showed that the effect of CA on first occurrence of mental disorders is not limited to childhood and adolescence but persists into adulthood, confirming that CA has rather long-lasting effects on mental health and also accounts for adult-onset disorders. The estimated proportion of mental disorders attributable to CA ranges from approximately 40% in childhood to 19% in adulthood (Kessler et al., 2010).

The concept of CA has not only been criticized for being too vague

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Table 1
Childhood adversity terms.

Term	Definition
Childhood adversity (CA)	Broad and not clearly defined umbrella term combining many different types of events and circumstances that are assumed to cause harm and distress to the child (e.g., domestic violence, poverty/socio-economic status, bullying, natural disasters, housing conditions, maltreatment, serious accident/injury or illness, parental substance use and mental health problems, parental criminality, interpersonal loss events etc.).
Adverse childhood experiences (ACE)	Originally devised by the Adverse Childhood Experience (ACE) Study (Felitti et al., 1998), it includes the following seven categories of adverse childhood experiences: psychological abuse, physical abuse, sexual abuse, violence against mother; substance abuse in household members, mental illness/suicide attempt in household member, imprisonment of household members.
Childhood maltreatment (CM)	Term summarizing various forms of childhood abuse (sexual, physical, emotional) and neglect (physical, emotional).

but also for its "*lumping together*" approach, which might cloud specific influences of certain adversity subtypes (K. E. Smith and Pollak, 2021). However, when looking into specific associations of certain adversity types with different classes of mental disorders, the above-mentioned epidemiological (e.g. Green et al., 2010; Kessler et al., 2010) as well as other non-population-based studies (Carr et al., 2013) did not observe a large degree of specificity. Different CAs were found to be associated with the entire spectrum of mental disorders including mood, anxiety, substance use and behavioral disorders (Green et al., 2010; Kessler et al., 2010). Other studies, which are beyond the scope of this review, indicate that CA, especially CM, is also related to poorer physical health such as cardiovascular disease (Hughes et al., 2017), underlining again a broad and system-wide effect of CA. Since different types of CAs tend to co-occur and are highly correlated (Dong et al., 2004; Green et al., 2010), the isolation of specific effects of certain types of CA is often impossible. This complexity also impacts GxE studies, as detailed below.

The mechanisms by which CA exerts its broad and long-lasting effects on mental health are not yet entirely understood. It is assumed that exposure to adverse events during childhood, when the maturation and shaping of biological systems takes place, has the potential to alter the functioning of these systems in a lasting manner and thus, becomes biologically embedded (Berens et al., 2017). This view is supported by a growing number of studies finding neurobiological effects of CA on the function of major biological systems, for instance the hypothalamic–pituitary–adrenal (HPA) axis (Kuhlman et al., 2017) or brain structure and function (see McCrory et al., 2012 for a review).

In the context of the diathesis/vulnerability-stress model, various attempts have been made to identify genetic contributions to mental disorders in order to shed light on any pre-existing vulnerability for mental disorders. Several heritability studies have been conducted (Federenko et al., 2006; Sartor et al., 2012; Woo et al., 2017) providing information about the extent of genetic influence on a specific disorder. However, in this type of study, no specific genes nor underlying mechanisms can be detected. The broad-sense heritability (H^2), namely the proportion of phenotypic variance that can be attributed to genetic variation, is estimated to range from 30 to 80% for most psychiatric disorders (Kendler, 2013). These estimates suggest that a rather high proportion of phenotypic variance cannot be explained by genetics alone. Importantly, such models assume that genotype does not correlate or interact with the environment and hence ignore GxE interactions. Going beyond broad-sense heritability, genome-wide association studies (GWAS) have confirmed the important role of genetic risk factors and identified numerous genetic loci that influence the risk of developing psychiatric disorders such as major depressive disorder (MDD) (Clements et al., 2021; Wray et al., 2018) and schizophrenia (Lam et al., 2019; Schizophrenia Working Group of the Psychiatric Genomics, 2014). These studies have shown the highly polygenic architecture of psychiatric disorders, often with shared genetic risk factors across phenotypes, but so far, it cannot fully explain phenotype variance. In fact, variances explained in the most recent GWAS for psychiatric disorders range from just a few to over 20% (Lam et al., 2019; Wray et al., 2018).

From the heritability as well as molecular genetics studies, we can conclude that common genetic variants alone cannot explain the full variability of mental disorders. However, CA alone cannot convey the whole picture either, as not everyone who experienced CA develops a mental disorder later in life (Lippard and Nemeroff, 2020). Hence, the investigation of only genetic variation or only environmental exposure cannot explain all phenotypic variance and their interaction needs to be taken into account.

The theory of GxE interactions postulate that differences in long-lasting effects between subjects are influenced by both individual genetic background (G) and environmental stimuli (E). GxE studies assess environmental effects on a phenotype that differs depending on the genetic background or, in other words, genetic effects on phenotype that depend on exposure to certain environmental factors. From a statistical point of view, a GxE interaction is present if the risk for the disorder, when carrying the risk variant (G) and being exposed to the risk environment (E), differs from the sum (additive model) or the product (multiplicative model) of the risks of only G and only E (Sharma et al., 2016). A statistically significant GxE, therefore indicates that the joint occurrence of two factors (G and E) produces synergistic (greater) or antagonistic (lesser) effects on an outcome that go beyond the addition or multiplication of single effects. This implies that the effect of the same gene on a certain outcome can go in different directions depending on environment. One of the first studies of GxE in the field of psychiatry was performed by Caspi et al. (2003). It identified a polymorphism of the serotonin transporter (5-HTT) moderating the effect of CM and later stressful life events on the risk of depression and this effect was absent when no stress exposure had occurred. Specifically, the example of the 5-HTT gene and stress resulting in modified risk of depression has shown inconsistent results (Winham and Biernacka, 2013) underscoring a major concern related to the findings of GxE studies, i.e., the lack of reproducibility. Conflicting results might also arise due to heterogeneity in outcome and environmental phenotypes as well as in cohorts and statistical analyses. Nevertheless, inconsistent findings led researchers to question, whether "true" interaction effects exist.

In this review, we present and critically discuss types of GxE studies across different investigation approaches (candidate genes, polygenic, genome-wide and/or accounting for different environments) and show how the investigation of biological processes can help to substantiate GxE findings.

2. Genome-wide approaches to GxE

2.1. From candidate genes to genome-wide approaches

In alignment with the GxE framework and after the landmark study by Caspi et al. (2003), research efforts in the last two decades have focused on the investigation of genetically driven, long-lasting effects of CA with regards to various psychiatric disorders, with many studies investigating hypothesis-driven candidate genes related to the HPA-axis (Normann and Buttenschon, 2020).

These studies (Ceruso et al., 2020; Hosseini-Kamkar et al., 2021) linked candidate genes to subsequent individual vulnerability to stress and stress-related disorders such as MDD and post-traumatic stress disorder (PTSD) later in life. While some of the findings in candidate genes could be replicated, others have shown conflicting results and inconsistencies between GxE studies (Border et al., 2019). A large collaborative meta-analysis including almost 39,000 individuals (Culverhouse et al., 2018) also found no strong evidence for the original

finding by Caspi et al., which put the true existence of GxE into question. Several methodological challenges of GxE studies can contribute to inconsistencies in findings. For example, the power necessary to reliably identify GxE associations is quite high, with at least a four-fold larger sample required for interaction as compared to a main effect of comparable magnitude (Smith and Day, 1984). Failure to correct for all covariate interactions (Keller, 2014) and potential GxE correlation bias (Dai et al., 2012) can result in spurious interactions. Furthermore, the number of factors per model result in an exponential increase in the computational complexity and number of hypotheses tested (Mehta and Binder, 2012). Due to this strong increase of factors per model and the resulting sample size requirements, large-scale population-based biobanks with detailed phenotypic and environmental information, such as UK-Biobank, represent great resources for identifying GxE effects. A number of methods (see Box 1) try to leverage these resources to reliably identify GxE interactions, however, while biobanks provide useful resources for the detection of GxE, one drawback remains the validity of the shallow phenotyping as compared to phenotypes obtained through clinical interviews and guided structured questionnaires (Coleman, 2021). Slight variations in the definition or in measurement scales of outcomes and environmental factors (e.g., categorical vs. dimensional variables) can change the statistical significance of GxE results. If not carefully conducted, starting from the selection of genes and a thorough, predefined, analysis plan, GxE studies are prone to produce a high rate of false positive findings.

In order to investigate potential false positives, a recent study by Border et al. examined 18 empirically identified candidate genes for depression and attempted to validate the previous findings by conducting a series of analyses including main effects of the polymorphisms, polymorphism-by-environment interactions and gene-level effects across multiple polymorphisms (Border et al., 2019). Despite high statistical power (sample sizes ranged between 62,000 and 443,000 individuals), the authors found no clear evidence for interaction effects of genetic polymorphisms and traumatic events on depression phenotypes for any of the studied candidate genes. Accordingly, they concluded that it was time to move away from historic candidate gene and candidate gene-by-environment interaction hypotheses. Indeed, given the fact that all these disorders are highly polygenic in nature, it is not clear, why we should expect strong GxE with single candidate genes.

2.2. Polygenic approaches

Acknowledging the polygenic nature of complex diseases, the question arises whether a combination of disease-relevant risk variants derived from GWAS could be more effective in the assessment of disease risk as compared to single genetic variants. Polygenic risk scores (PRS) summarize the estimated effect of many genetic variants on an individual's phenotype and are usually calculated as a weighted sum of trait-associated alleles. Using this technique, 1–2% of variation in depression and anxiety can be explained (Howard et al., 2019; Levey et al., 2020).

Consequently, the combination of environmental phenotypes and PRS (PRSxE) evolved, (Mullins et al., 2016; Peyrot et al., 2014), testing a possible increase or decrease of PRS effects if a specific environmental risk factor is present. Although theoretically, GxE effects are more likely to be found if polygenic information is included, a meta-analysis of 5765 individuals with depression did not show interaction effects of PRS and childhood trauma (Peyrot et al., 2018). Coleman et al., performed a PRSxE study using UK Biobank data, and thus substantially more samples (final sample size range: 24,094-92,957), and identified a significant additive effect of the MDD PRS and self-reported trauma exposure on risk of MDD (Coleman et al., 2020). Both findings (Coleman, Peyrot) might be, by the very definition of PRS, modelling additive effects of genetic variants, which does not include the interaction of every genetic variant with the environment. Furthermore, the current use of GWAS to derive PRS for GxE studies is limited as most GWAS are based on a case-control comparison for a specific disorder (e.g., depression) and do not specifically account for environmental risk factors with often unknown proportion of exposed subjects. Since PRS are built on genetic variants that are directly associated with the disorder phenotype, a lack of interactions with PRS does not generally contradict the existence of GxE interactions considering the assumption of the diathesis-stress model that genetic effects may only become apparent when exposed to certain environments.

An unbiased alternative to understand how the interaction of environmental factors and the genetic predisposition influences complex traits are genome-wide gene-by-environment interaction studies (GEWISs). However, to date, only few GEWIS have been published. A major reason for this is the lack of large suitable cohorts with homogenous ethnic background and comparable assessment of phenotypic and

Box 1
Methods to detect GxE.

Methods to model GxE interactions include traditional approaches such as comparing linear or logistic regression models (depending on the outcome variable) as well as more complex machine learning approaches. Traditional approaches usually use an analysis of variance (ANOVA) to decide which model fits the data best. In this case, while the first model includes both factors alone (referred to as “main effects”), the second contains additionally an interaction term between the G and E variables of choice as well. The F-statistic is then used to decide, whether including GxE term significantly improves the model. In regression models, additional covariates can be included to correct for confounders such as age, sex or ethnicity. Instead of using one genetic variant, also haplotype or PRS can be used as a G term in a similar manner. The state-of-the-art whole-genome methods for estimating GxE include genotype-covariate interaction genomic restricted maximum likelihood (GREML) and random regression GREML (Robinson et al., 2017). The main drawback of GREML-based methods, however, is the requirement of individual-level genotypes and its computational cost. Two approaches aiming to reduce these costs are SPAGE (SaddlePoint Approximation implementation of GxE analysis) (Bi et al., 2019) and GxEsum (Shin and Lee, 2021).
Non-parametric methods, including multifactor dimensional reduction (MDR), are based on testing for an association with the collapsed contingency table of G and E (Hahn et al., 2003). More complex machine learning approaches to model GxE interactions can be divided into three main categories: decision tree-based methods (e.g. multivariate adaptive regression splines (MARS, Cook et al., 2004)), random forests (Lunetta et al., 2004), classification and regression trees (CART, Strobl et al., 2009), data reduction approaches, e.g. focused interaction testing framework (Millstein et al., 2006), combinatorial partitioning method (Nelson et al., 2001), restricted partitioning method (R. Culverhouse et al., 2004), and pattern recognition or data mining, e.g. support vector machines (SVM, Chen et al., 2008), penalized regression (M. Y. Park and Hastie, 2008) and Bayesian methods (Zhang and Liu, 2007). Recently, GxE approaches especially suited for large biobank data have also been developed (Bi et al., 2019; Shin and Lee, 2021).

environmental outcomes for meta-analysis. Two recent GEWIS investigated genetic and environmental determinants of depressive symptoms incorporating stressful life events (SLEs) during adulthood. While both studies identified novel candidate loci, these findings could not be validated in replication cohorts (Arnau-Soler et al., 2019; Dunn et al., 2016). However, the study by Arnau-Soler et al. showed that adding GxE information to the model improved PRS-based predictions of depressive symptom scores. The variability of measures and sample sizes make it difficult to contrast findings between cohorts with a trade-off between deep phenotyping and sample size. For example, none of these GEWISs included CA or CM as an environmental factor probably due to the fact that this information was not available in the adult cohorts sampled with the aim of maximizing the *N* for genetic analysis at the cost of deep phenotyping. It is also questionable if SLEs occurring in adulthood have the same potential for getting biologically embedded as environmental events at an early life stage. Therefore, GxE findings may not be comparable across GEWIS due to differences in both phenotypic measures and stressors investigated. Finally, the role of gene-environment correlation (rGE) and interaction (GxE) need to be explored in more detail, as has been done for educational attainment, where the authors report widespread rGE and unsystematic GxE contributions to these phenotypes (Allegrini et al., 2020).

3. Molecular mechanisms as evidence supporting GxE

The studies published so far, looking into results of candidate genes as well as genome-wide approaches, leave some uncertainty regarding the question of whether GxE effects truly exist or if these effects are just false-positive results. While some studies demonstrated positive replication of previous findings across studies, the interpretation of interaction studies is not straight forward. There are valid concerns about statistically significant gene-environment interactions where none exists and cases where "true" interactions remain undetected (Dick, 2011). Therefore, it is crucial to substantiate a significant GxE finding with functional data. Once an association between a clinical phenotype (e.g., diagnosis of MDD) and a GxE with potential etiological role for the development of the clinical phenotype has been established, further studies are needed to look at the deeper layers of the same phenomenon in order to uncover the mechanisms underlying this GxE effect (Boyce et al., 2021). In particular, studies examining the effect of GxE on outcome measures that are closer to the underlying biology, rather than those using self-reported data from questionnaires or interviews, can contribute to a better understanding of the biological relevance.

A replication of a significant phenotypic GxE finding at a molecular or biological level could help to separate true GxE from false positive findings. One molecular process, which has been suggested as possible mediator of how environmental exposure could be embedded in the genome, is epigenetics and DNA methylation (DNAm) in particular (Szyf and Bick, 2013). It has not only been suggested that DNAm is dynamic due to environmental cues (Provencal and Binder, 2015; Szyf and Bick, 2013) but also that variability of DNAm is mainly shaped by GxE effects (Czamara et al., 2019; Czamara et al., 2021). Hence, looking into GxE not only with regards to disease outcome, but also on the molecular level of DNAm could add important knowledge to possible biological relevant processes underlying GxE findings.

One gene, for which GxE interactions with regards to DNAm have been identified in various studies (Klengel et al., 2013; Matosin et al., 2021; Tozzi et al., 2018), is *FKBP5*, a key modulator of the stress response. *FKBP5* was shown to confer genetic risk for stress-related disorders, specifically in the event of ELS (as reviewed by Criado-Marrero et al., 2018). This was confirmed in a meta-analysis, which investigated the robustness of the three most studied *FKBP5* single nucleotide polymorphisms (SNPs; rs1370860, rs3800373 and rs9470080) across 14 studies with a pooled total sample-size of 15,109 participants (Wang et al., 2018). In a systematic review investigating GxCM of 51 SNPs of HPA axis genes across 21 studies, *FKBP5* was among the most prevalent GxE genes (Normann and Buttenschon, 2019).

4. Effects of *FKBP5* × CA on epigenetic regulation as an example

Epigenetic regulation serves as an additional layer of alteration mediating the relationship between genotype and internal and external environmental factors by fine-tuning gene expression levels (Mohtat and Susztak, 2010). Importantly, DNAm changes have been demonstrated to be allele-specific suggesting that the genetic context of epigenetic changes induced by stressors needs to be considered (Meaburn et al., 2010).

DNAm changes at the *FKBP5* locus have been reported for various mental disorders such as MDD (Park et al., 2019) and PTSD (Grasso et al., 2020) and could be attributed to CA (Parade et al., 2021). Demethylation following CA has been shown in regulatory elements in intron 2 and 7 of *FKBP5*, consequently increasing gene expression following glucocorticoid receptor (GR) signaling and impeding the negative feedback mechanism of the HPA axis (Klengel et al., 2013; Misiak et al., 2020). Interestingly, the interaction with the genotype and adversity seems to be restricted to ACEs, as no relationship between *FKBP5* DNAm and adversity has been reported in adults (Alexander et al., 2020). This suggests the existence of a vulnerable period during childhood in which adverse events can influence the stress response in adulthood, affecting risk for stress-related phenotypes (Dunn et al., 2019).

To date, most studies have focused on adults and only a few have investigated DNAm changes in children following CA. One study by Parade et al. demonstrated demethylation of *FKBP5* (measured in saliva) over the course of 6 months following CM in preschoolers (Parade et al., 2017). DNAm changes over time in intron 7 were associated with CM, but only when children were exposed to high levels of contextual exposures. This finding suggests that epigenetic changes following adversity depend on the type and severity of ELS. Another study by Mulder et al. investigated the joint effects of genotype and DNAm on child outcome. They presented elevated cortisol reactivity following the Strange Situation procedure in 14-month-old infants who carried the stress sensitive T-allele of the *FKBP5* SNP rs1360780 and presented with *FKBP5* demethylation (Mulder et al., 2017).

Expression level differences in response to GR signaling were linked to the epigenetic regulation of the *FKBP5* locus by Klengel et al. (2013). In this study, our group showed an association between CM and genotype-dependent demethylation of a distal enhancer in *FKBP5* resulting in increased *FKBP5* expression. An additional cell line experiment helped to gain more insight into the mechanism of the GxE at the cellular level. It showed that the increase in gene expression was driven by allele-specific differences in DNA 3D conformation (see Box 2).

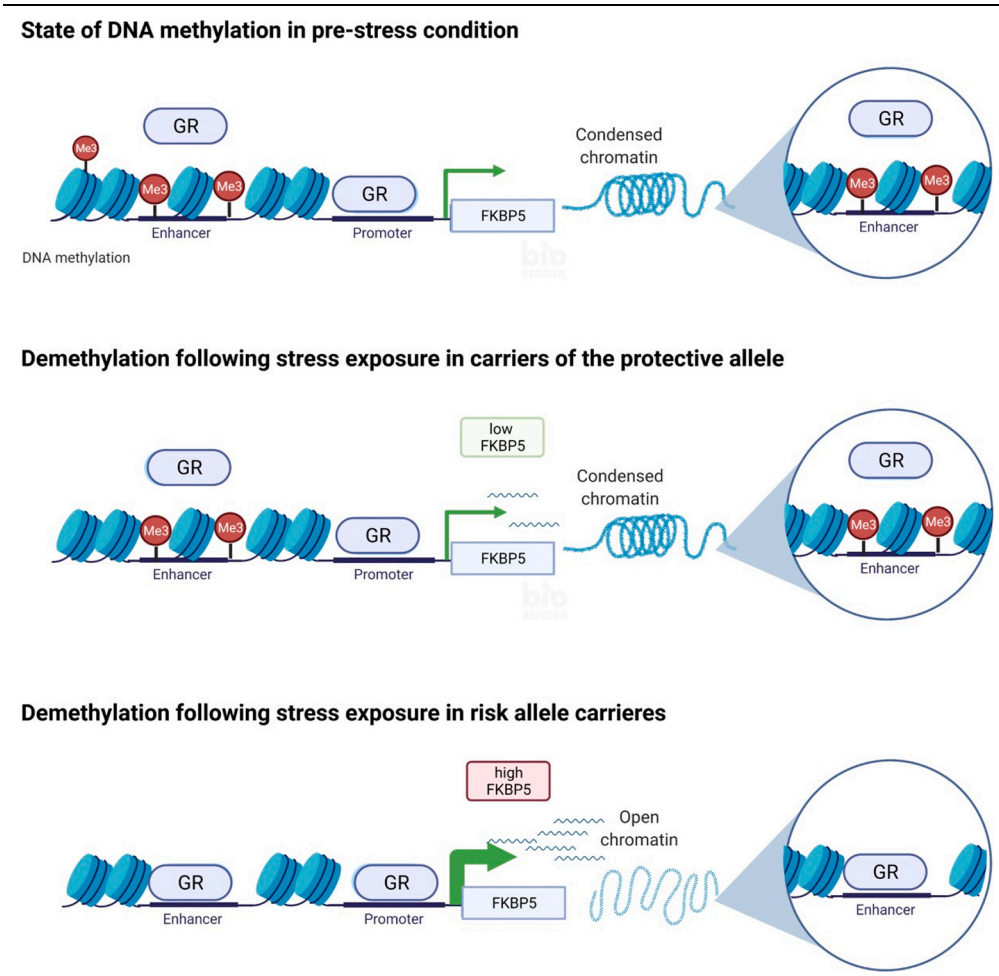
All of these FKBP5 epigenetic studies focused on DNAm derived from peripheral tissues (e.g., cord blood, whole blood, saliva), however brain tissue would be considered as more relevant for mental health. To date there is only one study looking at the *FKBP5* dynamics in the human brain. Matosin et al. reported consistently heightened mRNA and protein levels in post-mortem samples from patients with severe psychopathology and showed moderation effects by genotype, age and DNAm in key enhancers of the *FKBP5* gene (Matosin et al., 2021), matching the findings in peripheral tissues.

GxE in *FKBP5* has been studied across different molecular layers and intermediate phenotypes. Supporting evidence for a potential underlying biological mechanism that integrates genetic and environmental influences meanwhile comes from multiple studies at different investigation levels (see Box 3).

5. Timing of childhood adversity

Studies comparing the characteristics of CM events on adult depression and post-traumatic stress symptoms found that the severity and timing of CM were the greatest predictors (Jaye Capretto, 2020).

Box 2
Allele-dependent epigenetic regulation of the FKBP5 locus.



FKBP5 is expressed across multiple tissues and species and shows robust induction by GR, but shows significant variation in expression patterns. The SNP rs1360780 with the risk allele T is located within an enhancer region of intron 2 of the *FKBP5* gene and was repeatedly shown to be the functional variant conferring risk in the haplotype. Epigenetic modifications such as DNAm constitute an additional layer of regulation of gene expression the relationship between genotype and environmental factors. The regulatory regions of FKBP5 can be altered by CA via allele-specific demethylation in and around glucocorticoid response elements resulting in *FKBP5* demethylation risk-allele carriers which leads to enhanced transcription, decreased GR signaling, and an increased risk for developing stress-related psychiatric disorders.

Few studies have investigated the differential impact of maltreatment events across early and late exposure periods. For example, using data from the National Longitudinal Study of Adolescents, Dunn et al. (Dunn et al., 2013) reported that exposure at 3–5 years of age was more strongly associated with depression and suicidality than exposure in other developmental periods (0–2 or 6–8 years). In a later study, Dunn et al. (2019) also found that developmental timing of adversity explained more variability in DNAm than the accumulation or recency of exposure. In support of the sensitive period of the exposure model, vulnerability was augmented by type and timing of ACE, in particular emphasizing pre-school (age 4–5) and pre-adolescent (8–9) ages as sensitive periods for the impact of physical and emotional neglect (Schalinski et al., 2016).

Neuroimaging studies suggest that sensitive periods for the effects of maltreatment may be brief and that both early and late windows of vulnerability exist, as seen most clearly in studies on hippocampal volume (Andersen et al., 2008; Pechtel et al., 2014). These results were related to the fact that the childhood brain is highly plastic and under constant modification and therefore has specific temporal sensitivity for each brain region in terms of both structure and function (Andersen and Teicher, 2008; Teicher and Samson, 2013). The findings on the impact of timing of CM suggest that the simplified comparison of "exposed" versus "unexposed" children may obscure potential within-group differences related to the age at onset of maltreatment events.

6. Discussion

Studies of only genetic variation (G) and only environmental

Box 3
Supporting evidence for FKBP5 GxE across multiple layers.

Layer (intermediate phenotype)	Findings related to GxE	GxE
Epigenetic modifications	Demethylation of intron 2 and 7 following CA in adults (Klengel et al., 2013; Misiak et al., 2020) and in children (Mulder et al., 2017; Parade et al., 2017)	G × CA (measured by CTQ) G × CM
Gene expression & Protein abundance	Increased mRNA induction in individuals exposed to ELS in peripheral tissues (Appel et al., 2011; Zannas et al., 2016)	G × ELS
	Increased expression linked to methylation and genotype (Klengel et al., 2013)	G × ELS
Brain structure & function	Threat related amygdala activity with FKBP5 × ELS interaction (White et al., 2012)	G × ELS
	Reduced grey matter volume in exposed risk-allele carriers (Grabe et al., 2016)	G × CM
	Lower fractional anisotropy (Tozzi et al., 2016) Resting state functional connectivity between centromedial amygdala and right posterior insula (Wesarg et al., 2021)	G × CA G × CA

Note: This box exclusively lists studies with significant GxE findings. In all of the findings listed above, G is represented by the SNP rs1360780. Many other studies report significant main effects at each layer of regulation (Gene expression: Matosin et al., 2021); (Brain structure: Fani et al., 2014; Holz et al., 2015).

exposure (E) led to many important findings with regards to disease susceptibility, but they failed to explain all phenotypic variance. To overcome this issue, GxE interaction studies have evolved to look into combined effects of G and E. Genome-wide approaches including PRSxE and GEWISs as well as candidate gene approaches have identified several GxE effects. However, it still remains unclear if GxE truly exist or if additive effects of G and E perform better (Halldorsdottir et al., 2019).

GxE studies present with limitations and face critique. Looking more closely into these issues reveals potential improvements. One main criticism of GxE studies is that they often cannot be replicated. This might be due to the usually small sample sizes of GxE studies, which can lead to false-positive results and be affected by the "*winners curse*" phenomenon (Xiao and Boehnke, 2009). Furthermore, due to limited power, in general, larger sample sizes are needed in GxE studies as compared to studies looking at G or E alone. Also, heterogeneity with regards to genetic as well as phenotypic data, is a big confounder. For example, certain interactions might only present in individuals of the same ethnic origin and with a very specific phenotype. Often, different information on exposure to CA is gathered, ranging from prospectively to retrospectively assessed questionnaires and from information from official registries to self-reports. Subjective measures of CM do not always agree with objectively documented cases of maltreatment but show stronger relationships with psychopathology than only objectively ascertained cases of maltreatment that are not subjectively recalled as maltreatment (Danese and Widom, 2020). This underlines the importance of cognitive appraisal (Campbell et al., 2013) in the perception and personal interpretation of environmental experiences as being stressful. Although it is justified to criticize subjective reports for being imprecise and biased by current mental health status (Reuben et al., 2016), they still provide valuable data for understanding the link between adversity exposure and mental health and should not be disregarded in future research. Subjective measures not only reflect the occurrence of adversities in a yes/no manner but also if ACE have been incorporated in the view of oneself and others. When trying to understand the mechanisms underlying the association of CA with mental disorders, many GxE studies have examined the biological embedding of CA and looked at biological outcomes (see Sections 3 and 4). The question of how ACE are embedded in one's view of the self and the world and how much GxE contribute to the shaping of these personal views has received less attention. Many psychotherapies, such as cognitive behavioral therapy, rest on the assumption that a distorted view of oneself and others grows out of ACE and predisposes the individual to the development and maintenance of mental disorders. Introducing self-concept or other outcomes related to the self as another layer of GxE research might add to the understanding of mechanisms linking CA with psychopathology.

Further, harmonization of the assessment of CA across studies is greatly needed but firstly requires a uniform definition of CA. The question of what exactly counts as CA varies from study to study and is more often determined by the availability and accessibility of certain measures rather than a thorough consideration of what would constitute a good construct for neurobiological research (Smith and Pollak, 2021). Many environmental measures of CA comprise adversities with a genetic component such as mental illness and substance abuse in parents and might be correlated with the genes of interest. Since some interactions only occur in a specific time-window, they might be overlooked when embedding multiple time points of exposure to environmental stress (such as in utero, childhood, adolescence and adulthood) into one framework. As such, it is important to assess *when* or *at what ages* the exposure took place. In sum, well-defined environmental measures are needed that not only assess in a simplistic yes/no manner whether CA has occurred but additionally include dimensions of severity, timing and subjective impact.

Another criticism of GxE studies is that statistical interaction does not necessarily reflect biological relevance or lacks the potential for clinical application. We need to go beyond classical association approaches to better understand which biological processes are mirrored in an identified GxE result but also to be able to sub-stratify patients. Therefore, not only large sample-sizes but also data across all layers are needed. The regulation of the *FKBP5* gene involves interactions between genetic variants, environmental stressors, and epigenetic modifications of glucocorticoid response elements. These interactions can prompt the disinhibition of *FKBP5* and lead to phenotypic deviations in humans as well in rodent models. This *FKBP5* disinhibition could have clinical utility for patient stratification across diagnoses (Matosin et al., 2018; Ressler and Smoller, 2016). Further, combining additional levels of investigation such as genotype, DNAm, structural and functional MRI in a multi-level approach such as demonstrated by Tozzi et al. is a promising way forward. They integrated interactive effects of CA, *FKBP5* genotype (rs1360780 allele), DNAm as well as structural and functional MRI data in MDD patients and healthy controls (Tozzi et al., 2016). In concordance with earlier findings (Klengel et al., 2013), lower

methylation of *FKBP5* was predicted by CA in MDD patients with the T allele of rs1360780.

In accordance with the assumption of the vulnerability-stress model of mental disorders that genetic effects only become apparent in the face of adversity, most GxE studies focused on negative environmental events. However, as pointed out by Belsky and Pluess (2009), genes might not only be associated with an increased vulnerability but with a general heightened sensitivity to both negative and positive environmental stimuli. Positive environmental factors such as social support can compensate for ACEs and, when overlooked, can reduce the amount of phenotypic variation explained by GxCA. As outlined above, CA contributes to the development of psychiatric disorders and represents the prototypic environmental factor in GxE studies related to mental health outcomes. However, most mental disorders develop without any exposure to CA. Future GxE studies, therefore, need to examine a wider range of environmental factors such as positive environments, life-style factors and milder environmental stressors. The repeated sampling of environmental experiences in real-time by the use of digital devices (e.g., smart phones) could enrich GxE studies (Shiffman et al., 2008). A shift away of GxE studies from the classical diathesis-stress model towards positive environmental experiences and desirable phenotypic outcomes such as resilience could further increase our understanding of the GxE interplay in influencing mental health. GxE approaches, especially on a genome-wide level, have become more prevalent in the last years and pinpointing down specific biologically relevant GxE combinations could help to unravel and understand etiology of disease risk better. At the current stage, however, the detection of robust GxE findings still remains elusive.

The studies highlighted in this review combine and extend known findings from GxE research. The molecular findings, especially on the epigenetic level, yield a possible mechanism explaining the underlying process of differential gene expression following GR activation. Allele-dependent alterations in gene expression across cell types and brain regions following ELS have been mainly examined in animal models. Spatial and temporal dynamics of gene expression may help to explain the observations of GxE effects on functional readouts and other intermediate phenotypes. However, more evidence is needed in order to establish a link between the molecular findings via observations on circuit-level to phenotypes and behavior. Large-scale, single-cell or at least regional investigations of the brain (post-mortem human or in animal models) are required to draw conclusions on the spatial dynamics of gene expression. Longitudinal studies in healthy and clinical cohorts with larger sample sizes will be necessary in order to elucidate the temporal dynamics of the embedding of E.

As a conclusion, findings from GxE studies can help to identify subgroups in the general population, which have an increased susceptibility for psychiatric disorder and can be specifically targeted by early intervention. Further, these findings can be used to stratify clinical samples into subgroups that might benefit from specific treatment strategies. While GxE are a promising area of future research, more harmonized GxE studies in large, deep-phenotyped, longitudinal cohorts, and across different developmental stages are needed; firstly, to replicate published findings and secondly to substantiate robust findings with molecular experiments to assess if the identified statistical interactions are of biological relevance. These could be facilitated if adverse but also positive life events were standardly assessed in cohort studies, hence automatically enlarging available sample sizes.

Finally, computational methods might extend GxE research possibilities in the future. The use of algorithms, often referred to as artificial intelligence, in biology is a promising field and can be beneficial in solving complex biological issues as was recently shown for the prediction of three-dimensional (3D) protein structures (Jumper et al., 2021). Furthermore, machine learning models were suggested to facilitate the integration of different data types e.g. clinical data, genetics, epigenetics and others used in GxE research (Lin and Tsai, 2019). Yet, since machine learning methods learn to recognize patterns based on empirical data that is already present, usually addressed to as training data (Lin and Lane, 2017), improving the issues mentioned above to achieve better quality and quantity of data bases are fundamental prerequisites for future application of such tools in GxE interaction research.

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