Aus dem Institut für Psychiatrische Phänomik und Genomik
Klinikum der Universität München

Understanding the Involvement of Environmental Exposures, Genetic Risk, and Epigenetic Mechanisms in the Course of Severe Mental Illness

Dissertation
zum Erwerb des Doctor of Philosophy (Ph.D.)
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
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aus
Burlington, Vermont, USA

Jahr
2020

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Date of oral defense: July 8, 2020

Abbreviations

CNV	copy number variant
CpG	cytosine-phosphate-guanine
DSM	Diagnostic and Statistical Manual of Mental Disorders
DST	Differential Susceptibility Theory
EWAS	epigenome-wide association study
G × E	gene-by-environment
GWAS	genome-wide association studies
HiTOP	Hierarchical Taxonomy of Psychopathology
ICD	International Classification of Diseases
iPSYCH	Lundbeck Foundation Initiative for Integrative Psychiatric Research
LD	linkage disequilibrium
PGC	Psychiatric Genomics Consortium
PRS	polygenic risk score
RDoC	Research Domain Criteria
SNP	single nucleotide polymorphism

List of gene names

BDNF	Brain-derived neurotrophic factor
FKBP5	FKBP Prolyl Isomerase 5
IL-6	Interleukin 6
OXTR	Oxytocin Receptor
POU6F2	POU Class 6 Homeobox 2
SLC6A4	Solute Carrier Family 6 Member 4

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1 Introductory summary

> *It is now necessary to turn away from arranging illness in orderly well-defined groups, and to set ourselves the undoubtedly higher and more satisfying goal of understanding their essential structure.*
>
> – EMIL KRAEPELIN

1.1 Context of the study

Psychiatric disorders like major depression, schizophrenia, and bipolar disorder affect a significant proportion of the global population, accounting for a third of disability worldwide (World Health Organization, 2008). The enormous personal, societal and economic burden of these disorders demands a better understanding of their etiology (Collins et al., 2011). Since the start of the 20th century, psychiatric nosology has been defined by clinical observations of categorical syndromes based on signs and symptoms. However, despite the clinical utility of current classification systems, our understanding of mental disorders through observable behaviors and self-reported feelings and thoughts alone has reached its limits (Clark, Cuthbert, Lewis-Fernandez, Narrow, & Reed, 2017).

With little evidence to support natural boundaries of many mental disorders, the validity of the current Diagnostic and Statistical Manual of Mental Disorders (DSM) and International Classification of Diseases (ICD), is undoubtedly limited (Jablensky, 2016). This is especially true given the arbitrary thresholds used to define psychopathology versus normality, ambiguous boundaries between distinct diagnoses and excessive comorbidity between putatively independent disorders, together with heterogeneity within and instability of psychiatric diagnoses (Clark et al., 2017; Hengartner & Lehmann, 2017; Van Os, 2015). Moreover, the genetic architecture of most common psychiatric disorders does not support diagnostic categories as discrete entities, especially considering their cross-disorder heritability (Lee et al., 2013; Smoller et al., 2019). Needless to say, the current diagnostic system lacks etiological and pathophysiological justification for these clinically and genetically heterogenous disorders.

With hopes of circumventing these caveats, efforts continue to be made towards a psychiatric nosology informed by disease mechanisms. Unprecedented progress in psychiatric genetics has been made in the last decade with the help of improved molecular technologies and cooperation

among psychiatric researchers (Sullivan, Daly, & O'Donovan, 2012). Genome-wide association studies (GWAS) alongside innovative disciplines like systems biology and functional genomics have begun reporting robust and replicable findings (Howard et al., 2019; Pardiñas et al., 2018; Stahl et al., 2019; Sullivan et al., 2012) which hold potential towards converting these disorders into pathophysiologically-defined diseases. Additionally, new research frameworks using dimensional models of psychopathology have emerged with hopes of better understanding the nature of psychopathology. The Hierarchical Taxonomy of Psychopathology (HiTOP) (Kotov et al., 2017) and Research Domain Criteria (RDoC) (Insel et al., 2010) are just two examples.

Moving forward, continued optimization of existing methodologies in psychiatric genetics and complementary fields like epigenetics have potential to inform disease mechanisms and provide clues to the complex etiology of psychiatric disease. As first recognized by the pivotal work of Emil Kreapelin more than a century ago, longitudinal measures are a key element of psychiatric investigations and should continue to be paired with other approaches (Kraepelin, 1921; McInnis & Greden, 2016). Detailed accounts of environment and longitudinal moderators of disease course are particularly important to better understand the complex relationship between environmental influences and biology (Craddock et al., 2009). Such findings could supplement clinical ratings to form biologically-valid diagnostic criteria and lead to improved diagnostic, personalized therapeutic, and preventative measures. This thesis presents two approaches in alignment with these efforts.

1.2 An introduction to psychiatric genetics

Following the success of linkage studies for the identification of Mendelian disorders, early studies of common psychiatric disorders hoped to discover single genetic variants with large effects (Moreno-De-Luca, Ross, & Ross, 2018). Yet, despite early family and twin studies supporting their strong genetic basis and high heritability (Shih, Belmonte, & Zandi, 2004), empirical data did not support this monogenic theory proving candidate gene studies to be insufficient (Schulze, Fangerau, & Propping, 2004). Rather, it became evident that the genetic structure of these disorders was more likely polygenic (involving many genetic loci) and heterogenous (affected individuals do not all share the same combination of risk alleles) (Fullerton & Nurnberger, 2019). At the turn of the millennium, GWAS became possible for the identification of common single nucleotide polymorphisms (SNPs) on a genome-wide scale, moving the field away from unsuccessful candidate gene studies. With initial GWAS bearing little fruit as a result of their limited sample sizes, it was quickly realized that due to the very small effect sizes of

individual common SNPs and inherent multiple testing burden of GWAS, huge studies and mega analyses were required. This paradigm shift encouraged the formation of large consortia like the international Psychiatric Genomics Consortium (PGC; https://www.med.unc.edu/pgc/) and the Lundbeck Foundation Initiative for Integrative Psychiatric Research (iPSYCH; https://ipsych.dk/en/) with hopes of leveraging data from thousands of individuals to perform powerful analyses. Today, microarrays and array-based technologies along with rapid expansion in methodological approaches have supported this effort. Through unprecedented technological advancements in the last decade, we are now able to genotype millions of SNPs across the genome in large samples at a quick pace and affordable price. Thereby, the complex polygenic and genetically heterogenous architecture of major psychiatric disorders have been uncovered. Accordingly, new methodologies continue to be developed to analyze the vast amounts of data coming from GWAS, especially towards a better understanding of the complex interplay between genetic variants.

1.3 A look at genome-wide polygenic scores

While evidence supports the substantial contribution of common SNPs towards risk for disease, overall these variants account for only a fraction of genetic risk and a remarkable part of heritability has yet to be explained. While GWAS have identified robustly associated genome-wide significant variants for bipolar disorder (30 loci) (Stahl et al., 2019), schizophrenia (145 loci) (Pardiñas et al., 2018) and major depression (102 loci) (Howard et al., 2019), alone they explain only a small fraction of liability and hold little predictive accuracy. For example, the genome-wide significant loci for schizophrenia explain just 1.1% of variance on the liability scale (Pardiñas et al., 2018). Much debate has revolved around the reason for this missing heritability, with hypotheses pointing towards unaccounted for small effect variants, gene-gene interactions, as well as rare copy number variants (CNVs), *de novo* mutations, and epigenetic variations (Avramopoulos, 2010; Maher, 2008; Manolio et al., 2009; McCarthy & Hirschhorn, 2008).

In 2007 and 2009, two seminal papers demonstrated that a substantial polygenic component of psychiatric disorders is not found in the strongly associated loci, but rather in the thousands of common alleles that individually do not achieve genome-wide significance (Purcell et al., 2009; Wray, Goddard, & Visscher, 2007). Accordingly, they introduced an approach to capture the cumulative effects of these genetic loci as a single quantitative metric and thus introduced polygenic risk scores (PRSs) to psychiatric genetics. PRSs are calculated as the sum of risk alleles carried by an individual weighted by corresponding genotype effect sizes, derived from summary

statistics from an independent GWAS. These scores are calculated across many *p*-value thresholds to determine, for example, the optimal threshold for case discrimination, i.e., the threshold with greatest predictive power (Fullerton & Nurnberger, 2019). PRSs have already shown success in real-world health care settings, with a recent study using a schizophrenia PRS to detect risk for schizophrenia and psychosis using health record data (Zheutlin et al., 2019).

In the last years, PRSs have been widely used for a range of applications including the investigation of pleiotropic effects, i.e., the genetic overlap between disorders, evaluating the predictive power of genetic data, differentiation of cases and controls, and to perform experiments comparing individuals at extremes of the PRS distribution (Wray et al., 2014). Another use of PRSs proving to be valuable is their application to the genetics of endophenotypes, also known as intermediate or proxy phenotypes. Endophenotypes, as introduced to psychiatry by Gottesman and Shields, are measurable constructs that lie on the path between phenotypic expression and genes (Gottesman & Shields, 1972; Walters & Owen, 2007). This concept assumes that variation in an endophenotype will be associated with a simpler genetic architecture than the complex disorder as a whole. By definition, endophenotypes are associated with illness in the population, heritable, primarily state-independent, co-segregate with the illness within families, and are found in unaffected relatives at higher rates than in the general population (Gottesman & Gould, 2003). One broadly studied presumed endophenotype is cognitive deficits in patients with severe mental illness (Bora, Yucel, & Pantelis, 2009; Burdick, Goldberg, Harrow, Faull, & Malhotra, 2006; Ivleva et al., 2012).

1.4 An application of PRSs: Introduction to Original Article 1

Comes, A.L., Senner, F., Budde, M., […], Falkai, P., Schulze, T.G., Papiol, S. (2019). The genetic relationship between educational attainment and cognitive performance in major psychiatric disorders. *Transl Psychiatry*, 9(1), 210. doi:0.1038/s41398-019-0547-x.

Individuals with severe mental illness experience broad cognitive deficits that are often treatment resistant and persistent throughout the lifetime (Sheffield, Karcher, & Barch, 2018). The onset and severity of these cognitive symptoms are highly predictive of patients' long-term prognoses (Bowie & Harvey, 2006; Green, 1996). Given that cognitive deficits are a core feature of severe mental illness, understanding these impairments could have major implications for offering better insight into the psychiatric disorder itself and for targeting effective therapies. Given the characteristics of these deficits, i.e., heritability, presence in unaffected first-degree relatives and

in affected individuals even during periods of remission, they have been named a noteworthy endophenotype for psychotic disorders (Bora et al., 2009; Snitz, Macdonald, & Carter, 2006).

While GWAS for social-science genetics is often limited, proxy-phenotypes can be used to test for associations with well-measured endophenotypes such as cognitive functioning. Educational attainment is strongly correlated with cognition both phenotypically (0.50) and genetically (0.65) (Rietveld et al., 2014). In 2018, a GWAS on educational attainment was conducted in 1.1 million individuals from the general population (Lee et al., 2018). Using these summary statistics, the authors were able to conduct a multi-phenotype analysis generating polygenic scores that explained up to 10% of variance in cognitive performance.

The aim of Original Article 1 was to explore this association across several cognitive domains in a subset of patients from the longitudinal PsyCourse study (Budde et al., 2019). Considering the positive genetic correlations between education and risk for both schizophrenia and bipolar disorder (Hill et al., 2018), we also investigated the association of cognitive performance and PRSs for both disorders. Overall, we hoped this work would allow us to gain insight into the genetic underpinnings of distinct cognitive domains in patients with severe mental illness.

In our sample of patients with known cognitive deficits, educational attainment polygenic scores explained up to 2% of variance in cognitive domains related to learning and working memory. Specifically, the educational attainment polygenic score could explain variance in the number of correctly recalled words of the Verbal Learning and Memory Test (Helmstaedter, Lendt, & Lux, 2001), the backwards task of the Verbal Digit Span (Aster, Neubauer, & Horn, 2006), and crystallized intelligence measured with the Mehrfachwahl-Wortschatz-Intelligenztest (Lehrl, 2005). These findings were particularly interesting considering these cognitive domains are among the most impaired in patients with severe mental illness (Barbosa et al., 2018). Post hoc analyses showed these findings were robust to the effects of diagnosis and current medications. Considering rather inconsistent findings in the literature, it was not completely surprising that no significant effects were observed when investigating the associations between disorder specific PRSs and cognitive outcomes (Schaupp, Schulze, & Budde, 2018). To some extent, these findings support the notion that genetic determinants of cognitive variation within these disorders are at least partly independent from those common SNPs that predispose an individual to a schizophrenia or bipolar diagnosis, however, more evidence is needed.

Findings such as these could have important implications for identifying subgroups of patients with higher risk for a more burdened course of disease and could contribute towards predictive models for more personalized interventions. Further investigation of these associations longitudinally could help identify critical periods of genetic influence on cognitive abilities (Mistry, Harrison, Smith, Escott-Price, & Zammit, 2018). Furthermore, future studies could optimize the polygenic scores used to further reveal the genetic architecture of these deficits, for example, by taking into consideration intricate pleiotropic effects or using hypothesis-based polygenic scores (Lam et al., 2019). Lastly, this study supports the continued investigation of quantitative endophenotypes given their potential to inform the biological pathways lying between genetic variants and fuzzy, qualitative diagnoses. Given the prevalence of endophenotype deficits across many heritable domains, studies aimed at a better understanding of their nature are in alignment with efforts like RDoC with hopes to 'bridge genomic complexity and disorder heterogeneity' (Braff & Tamminga, 2016; Insel & Cuthbert, 2009).

1.5 Beyond traditional genetic data: The role of epigenetics

The fact that current PRS estimates explain only a small percentage of risk for psychiatric disorders, e.g., up to 5% in schizophrenia using the optimal *p*-value threshold of 0.05 (Pardiñas et al., 2018), is not unexpected given the major proportion of risk that environmental factors contribute to psychiatric disorders. Not only do distinct psychiatric disorders share some genetic risk factors, they also share environmental risks with factors like early life adversities playing a major role in risk for multiple psychiatric syndromes (Green et al., 2010; Kessler et al., 2010). Gene-by-environment (G × E) interaction studies have therefore been an integral component to understanding complex psychiatric disorders. Such studies examine the effects of environmental and genetic determinants on a phenotype and further explore their joint effects. Traditionally, the Diathesis-Stress Model has been the guiding conceptual framework for G × E studies (Monroe & Simons, 1991), which assumes that one's genetic predisposition makes them vulnerable to the development of a psychiatric disorder when exposed to a certain environmental adversity (Assary, Vincent, Keers, & Pluess, 2018). An opposing view, the Differential Susceptibility Theory (DST), however, proposes individuals vary more generally in their developmental plasticity thus in their susceptibility to both positive and negative environmental influences (Belsky, Bakermans-Kranenburg, & van Ijzendoorn, 2007; Belsky & Pluess, 2009; Ellis, Boyce, Belsky, Bakermans-Kranenburg, & Van IJzendoorn, 2011). Despite their value, G × E studies have faced many limitations such as poor replication rates and high false discovery rates, therefore complementary

approaches have been suggested to further uncover the underlying biological mechanisms of these interactions (Halldorsdottir & Binder, 2017).

One approach to explain more variability in psychiatric phenotypes is to investigate the potential molecular mechanisms underlying G × E interactions, for example, through the study of epigenetics (Heim & Binder, 2012). Coined by Conrad Waddington in the early 40's, the term 'epigenetics' was first used to describe the involvement of gene-environment interaction during development. The literal meaning of epigenetics is 'on top of the genes' and refers to stable and heritable changes in gene activity or expression without change in the DNA sequence (Jiang, Bressler, & Beaudet, 2004). Epigenetic modifications, acquired throughout the lifetime, include DNA methylation and hydroxymethylation of cytosines, post-translational histone modifications, and expression of non-coding RNAs (Jaenisch & Bird, 2003; Ptak & Petronis, 2010). The epigenotype encompasses the signature of these epigenetic marks across the genome. DNA methylation, that is the addition of a methyl group to a cytosine primarily at a cytosine-phosphate-guanine (CpG) dinucleotide, is thus far the most accessible and widely studied epigenotype (Liu, Faraone, & Glatt, 2019). In general, DNA methylation disrupts binding of transcription factors and leads to transcriptional repression (Murgatroyd, Wu, Bockmuhl, & Spengler, 2010).

In the last decade, different approaches have been used to explore DNA methylation in relation to environmental exposures in patients with severe mental illness through global methylation, candidate gene, and epigenome-wide studies (Pishva et al., 2014). These studies have supported the role of DNA methylation as an adaptive mechanism by which environment, especially early life adversities, can become "embedded" in the genome and have lasting effects that can become maladaptive and lead to psychopathology in adulthood (Matosin, Cruceanu, & Binder, 2017; Nöthling, Malan-Müller, Abrahams, Hemmings, & Seedat, 2019; Szyf, 2011; Szyf & Bick, 2013; Vinkers et al., 2015). However, considering the high cost of epigenotyping, limited studies have conducted hypothesis-free, epigenome-wide investigations, and few have investigated methylation signatures longitudinally. Moreover, few studies have focused specifically on DNA methylation signatures in individuals with bipolar disorder, especially in relation to non-traumatic stressful life events. Considering that epigenetic modifications are sensitive to environment, stable, and reversible, they hold promise to better understand and treat psychiatric disorders (Kular & Kular, 2018).

1.6 Exploring DNA methylation in the course of bipolar disorder: Introduction to Original Article 2

Comes, A.L., Czamara, D., Adorjan, K., […], Falkai, P., Schulze, T.G., Heilbronner, U. (2020). The role of environmental stress and DNA methylation in the longitudinal course of bipolar disorder. *Int J Bipolar Disord*, 8(1), 9. doi:10.1186/s40345-019-0176-6.

Evidence indicates an important association between stressful life events and age of onset and the clinical course of affective disorders (Aldinger & Schulze, 2017). However, there is still much to be understood regarding the underlying mechanisms associated with these consequences. While studies have focused on DNA methylation as a trait and state marker of bipolar disorder, as previously reviewed in detail (Ludwig & Dwivedi, 2016), little has been done to investigate the effects of environment on methylation in bipolar individuals (Perroud et al., 2016).

The aim of Original Article 2 was to explore DNA methylation in association with self-reported stressful life events in a sample of bipolar patients from the PsyCourse study (Budde et al., 2019). Specifically, we determined the associations between DNA methylation and childhood trauma according to the Childhood Trauma Screener (Bernstein, Ahluvalia, Pogge, & Handelsman, 1997; Bernstein et al., 2003; Grabe et al., 2012), and impact ratings of positive and negative life events experienced in the last 6 months according to the Life Events Questionnaire (Norbeck, 1984; Sarason, Johnson, & Siegel, 1978). Whole blood samples from two time-points (1 year apart) were used to measure DNA methylation using the Infinium MethylationEPIC BeadChips from Illumina. First, we conducted a targeted analysis by interrogating CpG sites in the vicinity of candidate genes with established roles in the stress response, namely *BDNF*, *OXTR*, *IL6*, *SLC6A4*, and *FKBP5*. We then conducted an exploratory epigenome-wide association study (EWAS), taking into consideration the limited sample size of our study and therefore reducing our analyses to only the most variable CpG sites across the genome. Lastly, we explored epigenetic age-related measures (Horvath, 2013) in association with change in stress and symptom measures over time.

To the best of our knowledge, our study was the first to describe epigenome-wide methylation signatures of bipolar patients over time and in association with non-traumatic stress. Although not a single locus withstood correction for multiple testing, methylation at a single CpG site was suggestively associated with stressful life events. This CpG falls in proximity to the *POU6F2* gene, which has been associated with several psychiatric traits (Anney et al., 2010; Goes et al., 2015; Koshimizu et al., 2019; Nagel, Watanabe, Stringer, Posthuma, & van der Sluis, 2018) as well as educational attainment (Lee et al., 2018; Okbay et al., 2016), and intelligence (Davies et

al., 2018; Hill et al., 2018). The overlap in these findings highlights the importance of integrating and exploring data transdiagnostically to potentially uncover trends and perhaps shared mechanisms across different phenotypes. Additionally, DNA methylation in blood at this CpG site is correlated with methylation in the brain, and *POU6F2* is highly expressed in brain tissue. At the current sample size, however, our study provided limited evidence of the role of DNA methylation in the course of bipolar disorder. Larger, longitudinal studies of well-characterized bipolar patients are warranted to better understand the role of epigenetics in the course of mood disorders.

1.7 Where do we go from here?

Our work took advantage of the unique longitudinal features of the PsyCourse cohort (www.PsyCourse.de) to explore the role of genetics, epigenetics, and environment in the course of severe mental illness.

Our findings from Original Article 1 support the use of educational attainment polygenic scores to identify individuals who might be at greater risk of more severe cognitive impairments and consequently lower levels of functioning. Such information could one day be used to target earlier and perhaps more effective care for these individuals. This work also supports transdiagnostic approaches, showing that the genetic determinants of cognitive functioning were at least partially independent of disorder-specific risks.

As samples continue to grow and become more diverse with the help of large collaborations like the PGC, PRSs will continue to be optimized allowing for more comprehensive genomic risk prediction profiles. Moreover, continued discovery of common SNPs with smaller effects will allow for PRSs that explain greater variance with increased precision (Liu et al., 2019). Future PRSs could also consider weighting and calibration, for example, incorporating linkage disequilibrium (LD) structure, and inclusion of other contributing factors like low-frequency and high-penetrance genetic variants, rare CNVs, epigenetics, and epistatic relationships between variants. Efforts are already being made towards improved PRSs (Ge, Chen, Ni, Feng, & Smoller, 2019). With continued refinement of PRSs, they have potential to become a powerful tool with considerable clinical utility. Especially when paired with other data they could be used to assess the long-term prognosis of patients, subtype disease, and inform treatment response (Fullerton & Nurnberger, 2019). PRSs have already shown clinical utility in multimodal prediction models. For example, in the case of coronary heart disease, the addition of a PRS has been shown to

improve risk prediction relative to clinical risk predictors (Abraham et al., 2016). Furthermore, they have also proved to be useful for prediction in the case of type 1 diabetes, where a PRS could predict progression to type 1 diabetes in children with a family history of the disease (Redondo et al., 2018). Lastly, future studies should continue exploring endophenotypes with PRSs to uncover the level and functional significance of the genetic contributions of psychiatric disorders (Braff & Tamminga, 2016).

Considering the important role of environment in risk for severe mental illness, epigenetic mechanisms are likely to be an important puzzle piece towards precision psychiatry. In Original Article 2, we conducted the first, longitudinal epigenome-wide profiling of DNA methylation in patients with bipolar disorder. Despite the clear limitation of sample size, our study took into consideration critical confounding factors like methylation-based smoking estimates that are often unaccounted for and lead to spurious findings in methylation studies. To gain power, beyond increasing sample size, future studies should reduce the noise of confounding factors, for example, by taking into consideration the disorder-specific, sex, age and genotype-dependent, and tissue-specific nature of DNA methylation (Boks et al., 2015; Marzi et al., 2018; Mehta et al., 2017; Smith et al., 2011; Uddin et al., 2010; Vinkers et al., 2015).

In the future, molecular phenotypes like epigenetics will continue to contribute to our understanding of disease mechanisms, especially when paired with other laboratory and clinical endpoints. In particular, functional research should be paired with epigenetic studies to better understand the role of epigenetic modifications in psychiatric disorders (Mill & Heijmans, 2013). Integrating -omics data like genomics, epigenomics, transcriptomics, and proteomics could lead to the "identification of novel 'multidimensional' markers and [to] reveal novel insight in the classification of complex diseases" (Comes et al., 2018).

1.8 Conclusion

Translational studies which integrate multiple levels of data, for example into biopsychosocial models, have potential to reveal novel insight into the etiology and inheritance of severe mental illness. Moreover, such studies have potential to eventually lead to a more valid diagnostic framework of psychiatric disease which could ultimately improve patient prognosis, diagnosis, and treatment. This thesis used approaches to identify mechanisms related to a worse course of disease *within* patients with severe mental illness. In doing so, we showed a genome-wide polygenic score for educational attainment could be used to explain variance within some of the

most impaired cognitive domains associated with psychiatric illness. We also reported longitudinal, epigenome-wide measures of methylation related to stressful life events in bipolar patients, which, to the best of our knowledge, is the first study of its kind. With improved methodology and the integration of data from genetic, epigenetic, and environmental studies, we will continue to pave the way towards precision medicine. Taking advantage of unique resources like the transdiagnostic, longitudinal, PsyCourse study will continue to be invaluable towards this cause.

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2 Original Article 1

2.1 Individual contributions and reference

The study "The genetic relationship between educational attainment and cognitive performance in major psychiatric disorders" was published in *Translational Psychiatry* in 2019. It was conducted under the supervision of T.G.S. and S.P. The research was designed by A.L.C. in consultation with F.S. and M.B. S.P. supported A.L.C in the calculation of polygenic risk scores. A.L.C performed the statistical analysis, wrote the manuscript, and accompanied the publication process as corresponding author. All co-authors critically revised and approved the manuscript.

Comes, A.L., Senner, F., Budde, M., […], Falkai, P., Schulze, T.G., Papiol, S. (2019). The genetic relationship between educational attainment and cognitive performance in major psychiatric disorders. *Transl Psychiatry*, 9(1), 210. doi:0.1038/s41398-019-0547-x.

Comes et al. *Translational Psychiatry* (2019)9:210
https://doi.org/10.1038/s41398-019-0547-x

Translational Psychiatry

ARTICLE Open Access

The genetic relationship between educational attainment and cognitive performance in major psychiatric disorders

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Abstract

Cognitive deficits are a core feature of psychiatric disorders like schizophrenia and bipolar disorder. Evidence supports a genome-wide polygenic score (GPS) for educational attainment (GPS_{EDU}) can be used to explain variability in cognitive performance. We aimed to identify different cognitive domains associated with GPS_{EDU} in a transdiagnostic clinical cohort of chronic psychiatric patients with known cognitive deficits. Bipolar and schizophrenia patients from the PsyCourse cohort ($N = 730$; 43% female) were used. Likewise, we tested whether GPSs for schizophrenia (GPS_{SZ}) and bipolar disorder (GPS_{BD}) were associated with cognitive outcomes. GPS_{EDU} explained 1.5% of variance in the backward verbal digit span, 1.9% in the number of correctly recalled words of the Verbal Learning and Memory Test, and 1.1% in crystallized intelligence. These effects were robust to the influences of treatment and diagnosis. No significant associations between GPS_{SZ} or GPS_{BD} with cognitive outcomes were found. Furthermore, these risk scores did not confound the effect of GPS_{EDU} on cognitive outcomes. GPS_{EDU} explains a small fraction of cognitive performance in adults with psychiatric disorders, specifically for domains related to linguistic learning and working memory. Investigating such a proxy-phenotype longitudinally, could give intriguing insight into the disease course, highlighting at what time genes play a more influential role on cognitive performance. Better understanding the origin of these deficits might help identify those patients at risk for lower levels of functioning and poor social outcomes. Polygenic estimates may in the future be part of predictive models for more personalized interventions.

Introduction

Cognitive deficits are a core and robust feature of psychiatric disorders like bipolar disorder and schizophrenia, present even during periods of remission[1–3]. These deficits are key predictors of long-term functional and social outcomes and are difficult to treat with current pharmaceutical options or behavioral interventions[4–6].

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Considering the associated psychosocial burden and high prevalence of these deficits among patients, psychiatric researchers have put considerable effort towards understanding their underlying mechanisms. Thus far, genome-wide association studies (GWAS) have provided evidence supporting the polygenic architecture and remarkable heritability of cognitive performance in population-based cohorts[7–10]. Furthermore, evidence supports the phenotypic and genetic stability of individual cognitive differences across the lifetime in domains including executive functioning, attention, and verbal memory[11–14]. As studies have shown evidence of impairments even in unaffected first-degree relatives of individuals with psychiatric disorders, cognitive deficits have been hypothesized as a valuable endophenotype of interest for better understanding the genetic risk factors of psychiatric disorders[15–17].

Intelligence, encompassing cognition, is highly heritable and an imperative predictor of occupational and health outcomes[18]. Despite high heritability estimates of intelligence, indicated to be up to 80% in adulthood[19], unraveling the underlying genetic contribution of intelligence differences using GWAS has been challenging and thus far little of the observed heritability has been explained[11,20]. To date, studies on intelligence have been limited by insufficient sample sizes and further complicated by the challenge of precise and reliable measurements for this complex phenotype[7–10,20]. The latest GWAS on intelligence identified 205 genomic loci implicating up to 1016 genes, which explained approximately five percent of the variance in intelligence[21]. Another large study reported a genome-wide polygenic score (GPS) that could explain 4.3% of variance in general cognitive function[11].

Educational attainment is moderately heritable and has been obtained as a demographic item in countless medical datasets and for cohorts of which genetic data is available[22]. In the last decade, educational attainment has been proposed as a proxy-phenotype for cognition, as it is highly associated with intelligence both on a phenotypic (0.50) and genetic level (0.65)[18,20,23–26]. Notably, GPS based on GWAS summary statistics for years of education predict more variance in intelligence than the phenotype years of education per se[18,25], reflecting the substantial genetic correlation between both phenotypes. The largest GWAS of educational attainment published to date, based on 1.1 million individuals, identified 1271 lead single nucleotide polymorphisms (SNPs)[22]. Through a multi-phenotype analysis of educational attainment and three cognitive phenotypes, the authors were able to generate a GPS which explained 7–10% of variance in cognitive performance in the general population. The SNPs identified implicated genes involved in neurodevelopmental processes and neuron-to-neuron communication[22]. The authors showed that the use of educational attainment as a proxy-phenotype could uncover genetic variants to be used as a set of "empirically-based candidate genes" for future studies, for example testing associations with important endophenotypes like cognition[27].

Studies have already demonstrated an important association of educational attainment GPS (GPS_{EDU}) with cognitive performance, showing that, in a general population, a higher GPS is associated with higher performance on neurocognitive tests[28]. However, limited evidence exists supporting this association in patients with known cognitive deficits[29,30], and there remains a need to investigate this association across different cognitive domains. Here we analyzed whether GPS_{EDU} could be used to explain variability in different cognitive domains in chronic patients with schizophrenia and bipolar disorder from the PsyCourse cohort[31]. This transdiagnostic approach aligns with the growing evidence for dimensional models that cut across diagnostic categories in psychiatry and is supported by the large cognitive, clinical and genetic overlaps between both disorders[32,33]. Particularly, the genetic overlap between both disorders has been firmly established by heritability estimates derived from population-based multi-generation registers[34] and by recent molecular studies that have reported an outstanding genetic correlation ($r_g = 0.70 \pm 0.02$)[35].

Considering the positive genetic correlations reported between education and both schizophrenia ($r_g = 0.10$) and bipolar risk ($r_g = 0.28$)[36], we further assessed how GPSs for both schizophrenia (GPS_{SZ}) and bipolar disorder (GPS_{BD}) were associated with cognitive performance in our sample.

Materials & methods

Participants

Data were used from the multicenter, PsyCourse study in Germany and Austria, consisting of participants of European ancestry (www.PsyCourse.de)[31]. Participants were phenotyped using a comprehensive battery including data on socio-demographics, psychopathology, cognition, and functioning assessed at each of four visits (6-month intervals). Recruitment strategies and characterization of all participants has been previously described in detail[31]. The sample selected for this project comprised a total of 730 participants with a DSM-IV[37] diagnosis of schizophrenia, schizoaffective disorder, or bipolar disorder (type I or II). Additionally, cognitive data available from 320 nonclinical (control) participants was used to give an orientation to the range of phenotypic data available in the PsyCourse cohort and to confirm general, well-replicated findings of lower cognitive performance in patients with psychiatric disorders compared to healthy controls. The study was approved by the local ethics committee for each study center and was carried out

following the rules of the Declaration of Helsinki. All individuals provided written informed consent as previously described[31].

Psychopathology psychometric instrument

The Positive and Negative Syndrome Scale (PANSS) is a clinical instrument used to measure symptom severity in schizophrenia and routinely used to assess a variety of disorders including bipolar disorder[38]. A continuous, total score of the three subscales, i.e., positive (e.g., hallucinations and delusions), negative (e.g., emotional withdrawal and blunted affect), and general symptoms (e.g., somatic concern and poor attention) was used as an indication of disease severity at the time of testing.

Cognitive performance psychometric instruments

Cognitive tests were administered at each study visit. The Verbal Learning and Memory Test (VLMT) was introduced at visit 2. For all other cognitive measures, scores from visit 1 were used for analyses.

Crystallized intelligence

The MWT–B (Mehrfachwahl–Wortschatz–Intelligenz test) was used to measure crystallized intelligence[39,40]. In this test, subjects were presented with 37 sets of five words arranged according to the level of difficulty. Four words of each set were fictitious constructions of known vernacular (i.e., they do not exist in the German language), while one word really exists. Subjects were asked to cross out the word they know to exist. The total number of correctly marked lines was used as a score[40].

Trail-Making-Test (TMT)

The TMT is a measure of visual attention and task switching and is one of several executive functioning measures. The test consists of two parts, part A assesses psychomotor speed of the participant, and part B assesses switching between two automated tasks (counting and reciting the alphabet). The time taken to complete each part of the test was measured and the difference in time needed (part B-part A) was used, as it is considered a more accurate measure of the divided attention and alternating sequencing tasks tested in part B[41–43]. In this case, a higher score meant worse cognitive performance.

Verbal digit span

The verbal digit span, from the Wechsler Adult Intelligence Scale, assesses short-term (forward digit-span) and working memory capacity (backward digit-span). Briefly, participants were asked to recall verbally a sequence of digits, with increasingly longer sequences in each trial. For each correctly recalled string of digits, one point was given. The test was ended when the participant was unable to correctly repeat two presented strings of the same length. The difference between the forward and backward task is that the latter involves mental manipulation as the participant is required to repeat the digits in backward order[44]. A score for each task was considered.

Digit-Symbol-Test (DST)

The DST is a subset of the Wechsler Adult Intelligence Scale[45] and measures processing speed, working memory, visuospatial processing and attention. In this test, the participant was asked to use a key of numbers 1–9 with coinciding symbols to draw the appropriate symbol that matched the number given. The participant was given 120 s to fill in as many corresponding symbols as possible. In the end, the correct number of symbols drawn was totaled to get an overall score.

Verbal Learning and Memory Test (VLMT)

The VLMT is the German version of the Auditory Verbal Learning Test[46]. This word-list learning paradigm assesses several memory parameters through serial list learning with subsequent distraction, retrieval after distraction and half-hour time delay, and through a recognition task. The test consists of two different word lists which are each 15 independent words and a recognition list which includes 30 words from the two lists and 20 similar distractor words. Four VLMT scores were rated, the first for the number of correctly recalled words from the first list, a second score for the number of words lost after distraction, a third score of words lost after a time interval, and a fourth score of correctly recalled words from the recognition list[47].

Biological samples

Peripheral blood samples were used for DNA extraction using standard techniques. DNA samples were then used to genotype patients for calculation of GPSs. Genotype data for controls was not available at the time of this investigation and they have not been used for GPS analyses.

GPS estimation

DNA samples were genotyped using the Infinium PsychArray Beadchip (Psychip, Illumina, San Diego, CA, USA). Following standard quality control procedures, imputation was performed using the 1000 Genomes Phase 3 reference panel as previously described in detail[31,48]. GPSs were calculated for all individuals using PLINK 1.90b5.3[49]. Summary statistics for educational attainment were obtained from the Social Science Genetic Association Consortium (https://www.thessgac.org/data)[22]. These summary stats are derived from analyses excluding 23andMe samples. Summary statistics from the most recent Psychiatric Genomics Consortium GWASs for schizophrenia[50] and bipolar disorder[51] were used. All

GPSs were calculated based on summary statistics from the discovery datasets, excluding low quality imputed variants (info score < 80%) in the test dataset, rare SNPs (minor allele frequency < 0.05), and ambiguous markers (A/T and C/G). Following the methodology of previous studies[52], SNPs in the extended major histocompatibility complex region (chromosome 6: 25–34 Mbp) were completely excluded for the calculation of GPS_{EDU} while only the top-associated SNP in this region was included for the calculations of GPS_{SZ} and GPS_{BD}. Data was clumped in windows of 500 kbp, discarding variants in LD ($R^2 > .1$) with another more significantly associated marker.

GPSs were then calculated by multiplying the imputation dosage for each risk allele by the log(OR) of each genetic variant. The resulting values were summed to obtain an individual estimate of the genetic burden in each individual across different SNP *p*-value thresholds (p_T). Scores for GPS_{SZ} and GPS_{BD} were calculated based on best discrimination thresholds according to previous findings, i.e., $p_T < 0.05$[50] and $p_T < 0.01$[51], respectively. GPS_{EDU} was calculated at four different *p*-value thresholds, from including only genome-wide significant SNPs to inclusion of all SNPs: $p_T < 5 \times 10^{-8}$, 0.05, 0.1, and 1. All GPSs were approximately normally distributed and standardized via z-score transformation.

Statistical analyses

Sample characteristics

As proof of concept, the effect of case status on cognitive performance was investigated using participants from the PsyCourse cohort. Visual inspection of boxplots comparing case versus control scores was performed and the effect of case status on cognitive domains was further determined through linear regression models, adjusting for age and sex. Socio-demographic and clinical characteristics were tested for between-group differences using the independent sample t-test for continuous data and Pearson's chi-squared tests for categorical variables. As an additional validation analysis, we investigated the relationship between GPS_{EDU} and educational attainment in our sample using ordinal logistic regression, adjusting for age, sex, the interaction between age and sex, and the first 10 PCs, according to previous work[22]. All analyses were performed using R statistical software version 3.4.0[53]. An initial examination of the distributions of raw cognitive scores was performed to identify and exclude outliers based on Tukey's definition (removal of values beyond 3× the interquartile range)[54].

GPS analyses of cognitive performance

The effect of GPS_{EDU} on cognitive performance of cases was explored. Blockwise linear regression models were used to estimate the amount of variation in cognitive performance explained by the z-standardized GPS_{EDU} at the four thresholds previously described. For each cognitive outcome, all base models were adjusted for confounding variables measured at the time of testing, i.e., age, age^2, sex, in/outpatient status, study center, and PANSS sum scores. Although our participants are chronic patients, duration of illness was considered an important covariate which could confound our results. However, as duration of illness proved to be well correlated with age ($r = 0.53$), ultimately only age was kept in the models. To guard against population stratification, the first 20 ancestry principal components (PCs) were included in our models and selected for each cognitive outcome tested using backward model selection ($p < 0.05$)[55]. The significant PCs were as follows: PCs 12 and 17 for the Verbal digit span (forward task); PC 7 for the DST; PCs 1 and 18 for the MWT-B; PCs 1 and 5 for the VLMT- loss of words after time; and PC 16 for the VLMT- correctly recognized words. No significant PCs were found for the other cognitive outcomes. For each cognitive outcome of interest, we measured the incremental adjusted-R^2, that is the gain in the coefficient of determination when the GPS_{EDU} was added as covariate to the regression model for each phenotype (cognition score) on a set of baseline covariates. Multiple testing was corrected for using the False Discovery Rate (FDR) method correcting for the polygenic profiles at all four thresholds and for all phenotypes investigated. Visual inspection of the residuals for each model was performed to be sure the requirement of normally distributed model residuals had been fulfilled.

GPS analyses of schizophrenia and bipolar disorder

Using blockwise linear regression models as described above, we tested whether polygenic scores for schizophrenia and bipolar disorder influenced cognitive outcomes. This was tested for both the GPS_{SZ} and GPS_{BD} separately. We then determined how the genetic risk for schizophrenia and bipolar disorder influenced the effect of GPS_{EDU} on cognitive outcomes. Both scores were included (separately) in those models in which GPS_{EDU} was significantly associated with the cognitive outcome tested.

Additional analyses

Post hoc analyses were performed to determine the robustness of our findings when correcting for diagnosis (bipolar-I disorder, bipolar-II disorder, schizophrenia, or schizoaffective disorder) and medication (number of antipsychotics, antidepressants, mood stabilizers, and tranquilizers at time of assessment). Furthermore, taking into consideration the significant correlation between memory and crystallized intelligence[56], we performed a mediation analysis introducing the DST and VLMT (number of correctly recalled words) as covariates in our model testing the association between GPS_{EDU} ($p_T < 1$)

and crystallized intelligence. Multicollinearity diagnostics were performed.

Results

A description of socio-demographic variables for participants is presented in Table 1. Seven-hundred and thirty patients with schizophrenia and bipolar disorder were used for analyses. The mean age of these participants was 43.19 years, the majority of which were male. The majority of cases (46.2%) were diagnosed with schizophrenia, 10.0% were schizoaffective, 35.1% were bipolar-I patients and 8.7% were bipolar-II patients. During baseline visits, 47.7% of patients were being treated as day/inpatients.

The correlations between cognitive domains were assessed (Supplementary Fig. 1). Boxplots depicting case versus control performance across all cognitive domains are shown in Supplementary Fig. 2. Investigation of linear models to test the effect of case status on cognitive performance, after adjusting for age and sex, showed a significant effect in the direction expected, i.e., a decreased performance for cases (Supplementary Table 1). Educational attainment was significantly associated with GPS_{EDU} in the direction expected (Supplementary Table 2).

Our investigation of the effect of GPS_{EDU} ($p_T < 1$) on cognitive performance in patients resulted in a significant increase in Nagelkerke's R^2 of 1.5% for the verbal digit span (backward; Fig. 1a), 1.9% for the VLMT number of

Table 1 Sample characteristics

	Cases ($n = 730$)[b]	Controls ($n = 320$)[b]	Test statistic	Degrees of freedom (df)	*p*-value
Age at baseline	43.19 (13.01)	37.53 (15.83)	5.62	516.11	<0.001
Sex			24.22	1	<0.001
Male	414 (56.7)	128 (40.0)			
Diagnosis		N/A	N/A	N/A	N/A
Schizophrenia	337 (46.2)				
Schizoaffective	73 (10.0)				
Bipolar-I disorder	256 (35.1)				
Bipolar-II disorder	64 (8.7)				
Education[a]			154.11	6	<0.001
0	10 (1.4)	0 (0.0)			
1	46 (6.3)	2 (0.6)			
2	146 (20.0)	8 (2.5)			
3	179 (24.5)	98 (30.6)			
4	130 (17.8)	31 (9.7)			
5	87 (11.9)	35 (10.9)			
6	114 (15.6)	142 (44.4)			
Missing	18 (2.5)	4 (1.3)			
Duration of illness	12.93 (10.81)	N/A	N/A	N/A	N/A
Baseline treatment		N/A	N/A	N/A	N/A
None	23 (3.2)				
Outpatient	355 (48.6)				
Day patient	38 (5.2)				
Inpatient	310 (42.5)				
Missing	4 (0.5)				

[a]The PsyCourse study measures status in the German educational system in detail. In order to make the German educational system comparable to English-speaking systems information on specialized schools, high school and professional education in Germany have been combined to form an ordinal educational scale with "6" being the highest level of education obtained

[b]Age and duration of illness have been reported as mean (standard deviation), while all other categorical variables have been reported as *n* (%). A *t*-test was used for comparison of mean age and X^2-tests were used for all categorical comparisons

Socio-demographic information of participants

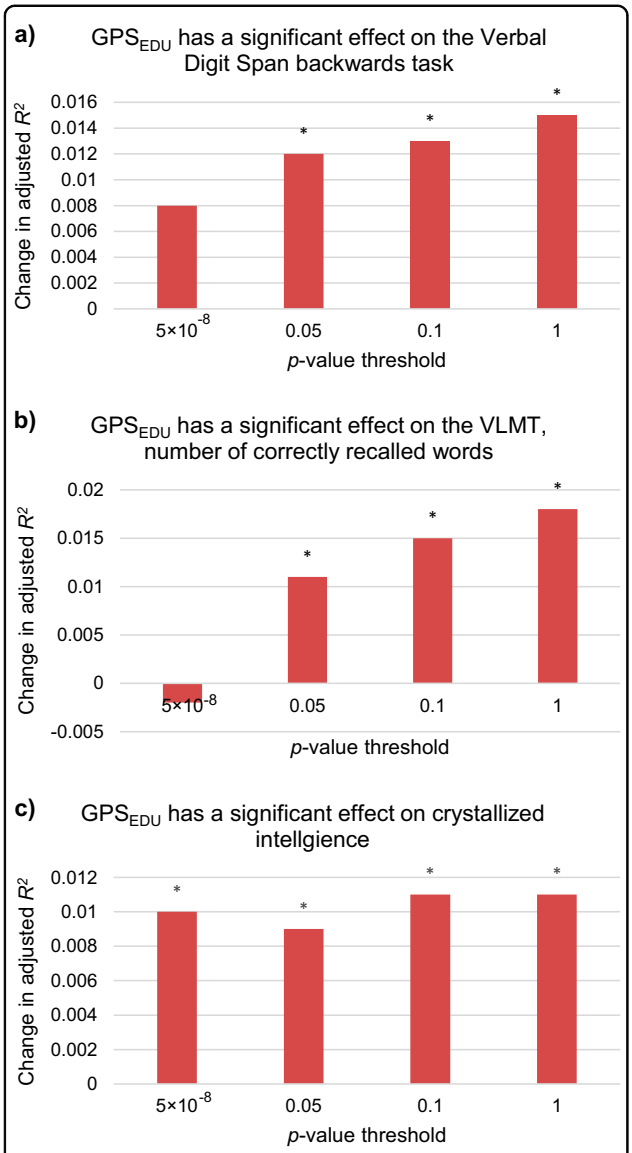

Fig. 1 Effect of genome-wide polygenic risk scores for educational attainment (GPS$_{EDU}$) on cognitive performance (Significant *p*-value thresholds were labeled with an Asterix (* $p < 0.05$)). **a** Change in adjusted-R^2 after inclusion of GPS$_{EDU}$ in the verbal digit span (backward) model. Baseline model: Adjusted-R^2 0.109; FDR-corrected *p*-values for *p*-value threshold $p_T < 5 \times 10^{-8}$ to 1: 0.050, 0.021, 0.021, 0.021. **b** Change in adjusted-R^2 after inclusion of GPS$_{EDU}$ in VLMT (number of correctly recalled words) model. Baseline model: Adjusted-R^2 0.224; FDR-corrected *p*-values for *p*-value threshold $p_T < 5 \times 10^{-8}$ to 1: 0.653, 0.045, 0.025, 0.021. **c** Change in adjusted-R^2 after inclusion of GPS$_{EDU}$ in crystallized intelligence (MWT-B) model. Baseline model: Adjusted-R^2 0.214; FDR-corrected *p*-values for *p*-value threshold $p_T < 5 \times 10^{-8}$ to 1: 0.030, 0.031, 0.021, 0.021

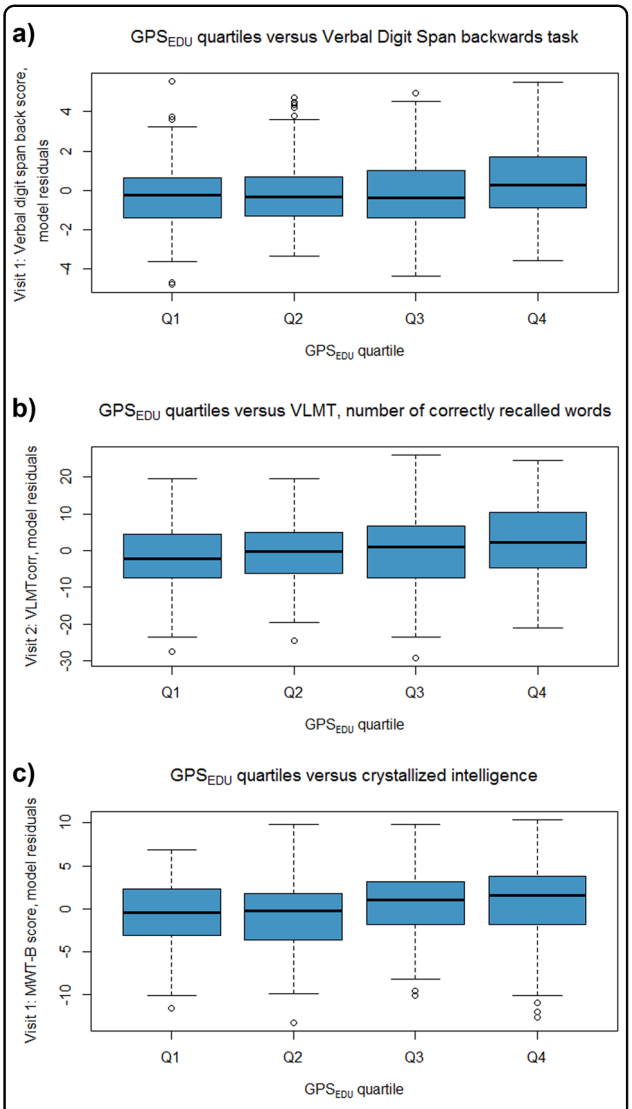

Fig. 2 Cognitive performance model residuals plotted against quartiles of genome-wide polygenic risk score for educational attainment (GPS$_{EDU}$). **a** A trend is seen for increased verbal digit span performance with increased load of GPS$_{EDU}$. **b** A trend is seen for increased VLMT performance with increased load of GPS$_{EDU}$. **c** A trend is seen for increased MWT-B performance with increased load of GPS$_{EDU}$

correctly recalled words (Fig. 1b) and 1.1% for crystallized intelligence (Fig. 1c). With more stringent *p*-value thresholds used, i.e., the inclusion of less SNPs, the change in adjusted-R^2 decreased. For the verbal digit span (backward) and the VLMT, the GPS$_{EDU}$ based on the *p*-value thresholds $p_T < 0.05$, 0.1, and <1 were significant (FDR-adjusted $p < 0.05$; Supplementary Table 3). The score was significant at all *p*-value thresholds for crystallized intelligence (FDR-adjusted $p < 0.05$). The examination of model residuals via quantile–quantile (QQ) plots did not show any extreme deviation from normality (Supplementary Figs. 3–5). Further inspection of model residuals against GPS$_{EDU}$ quartiles showed evidence of increased performance on all three domains with increased GPS$_{EDU}$ scores (Fig. 2). Our results remained robust after correcting for medication (Supplementary

Table 4) and diagnosis (Supplementary Table 5). Furthermore, our mediation analysis supports a robust association between GPS_{EDU} and crystallized intelligence that is not mediated by memory parameters (GPS_{EDU} $p < 0.05$; change in adjusted-$R^2 = 0.0091$).

No significant associations between cognitive outcomes and polygenic scores for schizophrenia or bipolar disorder were observed (Supplementary Table 6). Furthermore, neither risk score influenced the significant effects of GPS_{EDU} on the three cognitive domains reported above (Supplementary Table 7). Multicollinearity diagnostics showed no issues of collinearity in our regression analysis (variance inflation factor <5 for all independent variables).

Discussion

Our study aimed to identify the influence of GPS_{EDU} on several cognitive domains in a transdiagnostic cohort of psychiatric patients. Confirming results of previous research, patients with bipolar disorder and schizophrenia in the PsyCourse cohort performed worse on tests of neurocognitive functioning in comparison to nonclinical controls. In patients, we observed a significant improvement in prediction of cognitive performance with inclusion of GPS_{EDU} for the backward verbal digit span, VLMT (correctly recalled words), and for crystallized intelligence. These findings confirm the ability of GPS_{EDU} to explain variability in linguistic cognitive performance related to working memory and learning in patients with known cognitive deficits. Furthermore, our findings show that cognitive performance measured for these domains were associated with the genetic underpinnings of GPS_{EDU} and not confounded by or associated with GPS_{SZ} or GPS_{BD}.

Previous studies have investigated the association between cognitive performance and GPS_{EDU} using summary statistics from an earlier GWAS on educational attainment by Okbay et al.[24]. Our findings compliment earlier evidence supporting an association between cognitive performance and educational attainment, but not schizophrenia genetic risk, in clinical patients. For example, a study by Shafee et al. compared the effect of GPS_{SZ} on three cognitive phenotypes i.e. general cognitive function, premorbid intellectual potential, and years of education completed[30]. The authors found that among healthy individuals, GPS_{SZ} was significantly associated with lower general cognitive functioning, however, found no association between GPS_{SZ} with any cognitive phenotype in patients with psychosis. Furthermore, the authors found significant positive correlations between GPS_{EDU} and both educational attainment and premorbid intelligence in patients with and without psychosis. Another study by Bansal et al. showed GPS_{EDU} could predict 2.09% of variance in premorbid IQ in a large schizophrenia sample[29]. Our findings support earlier suggestions that different cognitive phenotypes vary in their etiologic relationship with schizophrenia and in their genetic overlap with educational attainment[30]. Furthermore, our findings are in line with evidence from the first educational attainment GWAS of 126,559 individuals which identified variants which implicated genes (including *BSN, GBX2, LRRN2, and PIK3C2B*) linked to processes such as learning and long-term memory[27]. These findings are especially interesting given that learning and working memory are among some of the most impaired cognitive process for patients with psychiatric disorders[57].

While a polygenic score for educational attainment in the general population explained 7–10% of variance in cognitive performance, the score explained at most ~2% in our transdiagnostic cohort[22]. It is difficult to determine whether the smaller effect in our cohort was the result of a different phenotype being measured, i.e., specific cognitive domains and not a composite score, or whether this might reflect the cognitive performance of this unique, transdiagnostic sample being related to other complex genetic-environmental factors. Clearly, future investigations looking at other measures of cognition in large cohorts are warranted. Confounding variables such as acute symptoms may also contribute to the lack of variability explained in this case, although we have tried to capture this by controlling for current in/outpatient status and symptom severity. Furthermore, although based on big samples, polygenic scores "may not be sufficiently powerful to capture signs of disrupted neurodevelopment" in these patients as they exclude rare copy number variations and deleterious exonic mutations which may have important consequences[52].

On both a phenotypic and genetic level, intelligence has been associated with psychiatric disorders. For example, individuals with a level of intelligence one standard deviation below the mean, have ~60% higher risk of hospitalization for schizophrenia[58]. There is also evidence supporting an association between poorer school performance and higher risk for schizophrenia[52]. In addition, several longitudinal studies have linked deficits in premorbid IQ with subsequent schizophrenia development, which was also shown for mood disorders[52,59]. The evidence, however, linking intelligence and affective disorders has been more inconsistent. For example, bipolar disorder has been associated with higher childhood IQ and an increased genetic risk of bipolar disorder has been associated with creativity and higher education[60–62]. However, no such associations have been reported by studies of adolescent or adult IQ[60]. Nevertheless, there are known genetic variants influencing both intelligence and psychiatric disorders which, in part, explain the phenotypic link between intelligence and these disorders[58].

We investigated the potential influence of GPS_{SZ} and GPS_{BD} on cognitive performance. These relationships

seem to be complex and while the genetic overlap between schizophrenia susceptibility with cognitive performance has been widely investigated in the literature with conflicting findings, less has been done in bipolar disorder[8,63–65]. The lack of an association observed between either the GPS_{SZ} or GPS_{BD} with cognitive performance in our study emphasizes several issues inherent to these types of investigations. The first is that GPS_{EDU} is based on a much larger discovery sample than GPS_{SZ} and GPS_{BD}, meaning GPS_{EDU} had higher statistical power to capture smaller effect sizes and more accurate estimates for single SNPs of which the score is based on. Presuming the most optimistic estimate for variance explained in cognitive performance by the GPS_{SZ} of 1.6% that has previously been reported[8], a sample of ~500 participants would be required to drive the effect of schizophrenia genetic risk scores on cognitive performance. However, given a more conservative estimate of 0.3%[66] variance explained, a sample size of over 2 600 participants would be required, suggesting that power may indeed be an issue in our study (Supplementary Fig. 6). This is also true with regards to GPS_{BD} in which genetic effects are likely to be at least as subtle. This again highlights the value of analyzing a proxy-phenotype such as educational attainment.

The second issue is in relation to the cognitive domains that were analyzed. As studies often use different cognitive tests from the wide variety that are available, it could be that the genetic risk of schizophrenia and bipolar disorder are more closely linked to domains that went unmeasured in our study. Perhaps if we had used a composite score across all domains or different neurocognitive tests in general, a significant effect would have been observed. Unfortunately, due to the longitudinal nature of our study which led to missing data across the different cognitive outcomes tested, a composite score analysis with adequate power was not feasible.

Our findings should be considered in light of a few limitations. The first is that our patients represent a chronic sample of heterogeneously treated patients. As these patients have been prescribed a wide range of medications at different dosages, correcting for the possible influence of medication is not an easy task. Not knowing how different drugs might interact with or influence cognition throughout the course of the disorder is a limitation that always must be considered in psychiatric research, and this problem has yet to have a perfect solution. A second limitation of our study is generalizability considering we investigated raw scores for several cognitive domains. As mentioned above, one of the major issues in the field at this time is the complexity in measuring this phenotype and with a plethora of tests that can be used, it is difficult to say how generalizable our findings are to other cognitive tests within the same cognitive domain in different cohorts. For example, while crystallized intelligence was measured, our study failed to consider fluid intelligence which has a higher heritability component than crystallized intelligence[67]. It is also important to note that while executive functions are related, they are also diverse[68]. While the TMT used in our study is a measure of task switching, other executive functions like the updating process of working memory and inhibition should be explored. Lastly, we must acknowledge that our study has only assessed linguistic memory and not visuospatial memory. As these are two unique types of memory[69], future investigations are warranted to determine how the two might differ in association with GPS_{EDU}.

Although remarkable heterogeneity of cognitive deficits exists among individuals with psychiatric disorders, in general these deficits are, by a moderate degree, less severe in chronic bipolar patients in comparison to chronic schizophrenia patients. Furthermore, the trajectories of these impairments are quite different[70]. Often, cognitive deficits are apparent before the onset of disease in individuals with schizophrenia[71]. Approximately 70% of bipolar patients exhibit cognitive deficits, especially related to verbal memory and attention[57], which often manifest in young adults[60]. Despite these known differences for bipolar disorder and schizophrenia, we did not observe a significant effect of diagnosis on the effect of GPS_{EDU} related to cognitive performance. These diagnostic differences were most likely captured by the PANSS sum scores included in our models, which was highly significant. Evidence also supports an increase in the heritable component of intelligence with age[72]. Considering this knowledge, future studies, longitudinal in design, would be highly beneficial. It would be intriguing to see how the polygenic score for educational attainment can explain variability in cognitive performance throughout the course of the disorder. While our sample consisted of chronic mid-aged patients in which cognitive performance was rather stable across visits, it would be valuable to investigate younger cohorts of patients, even before the onset of disease, to determine how instability in cognitive performance throughout the disease course might influence the association between GPS_{EDU} and cognition. This would help determine at which points the underlying genetic components are most influential and help identify at which periods environmental influences might be more prominent in determining cognitive abilities.

Conclusions

Identifying a genetic component related to distinct neurocognitive profiles has potential to identify a more burdened subgroup of patients that in turn might be at risk for lower levels of functioning and poor social outcomes. This sort of information targets patients for more personalized interventions[73,74]. Here we have explained

only a small fraction of variance in cognitive performance in patients with psychiatric disorders using the genetic variants associated with educational attainment. These findings highlight the importance of other uncaptured environmental exposures that have major influences on cognitive abilities and ultimately levels of functioning in these patients. Future studies, over the course of the disorder, would be informative to determine how this association changes over time, and at which periods environment may play the most influential role[60]. Furthermore, future studies should factor in the complex pleiotropic relationships between these traits to generate enhanced polygenic scores to further clarify their genetic architecture[75]. Moreover, hypothesis-based polygenic scores could help uncover biological pathways related to cognitive performance.

Acknowledgements
Thomas G. Schulze and Peter Falkai are supported by the German Research Foundation (Deutsche Forschungsgemeinschaft; DFG) within the framework of the projects www.kfo241.de and www.PsyCourse.de (SCHU 1603/4-1, 5-1, 7-1; FA241/16-1). The genotyping was in part funded by the German Federal Ministry of Education and Research (BMBF) through the Integrated Network IntegraMent (Integrated Understanding of Causes and Mechanisms in Mental Disorders), under the auspices of the e:Med Program with grants awarded to Thomas G. Schulze (01ZX1614K), Marcella Rietschel (01ZX1614G), and Markus M. Nöthen (01ZX1614A). Thomas G. Schulze received additional support from the German Federal Ministry of Education and Research (BMBF) within the framework of the BipoLife network (01EE1404H) and the Dr. Lisa Oehler Foundation (Kassel, Germany). Sergi Papiol was supported by a 2016 NARSAD Young Investigator Grant (25015) from the Brain and Behavior Research Foundation. Marcella Rietschel and Markus M. Nöthen received funding from the German Research Foundation (DFG, grant FOR2107; RI 908/11-2 to Marcella Rietschel, NO 246/10-2 to Markus M. Nöthen). Franziska Degenhardt received support from the BONFOR Programme of the University of Bonn, Germany. Jens Wiltfang is supported by an Ilídio Pinho professorship and iBiMED (UID/BIM/04501/2013), at the University of Aveiro. Udo Dannlowski was funded by the German Research Foundation (DFG, grant FOR2107 DA1151/5-1; SFB-TRR58, Project C09) and the Interdisciplinary Center for Clinical Research (IZKF) of the medical faculty of Münster (grant Dan3/012/17 to Udo Dannlowski). Volker Arolt was funded by the European Union FP-7 Research Grant MOODINFLAME. We would like to express our profound gratitude to all study participants without whom this work would not have been possible.

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Conflict of interest
Jens Reimer received honoraria from Otsuka-Lundbeck for participation in a speakers' bureau. The remaining authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (https://doi.org/10.1038/s41398-019-0547-x).

Received: 13 March 2019 Revised: 3 June 2019 Accepted: 17 July 2019
Published online: 28 August 2019

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2.2 Original Article 1: Supplementary material

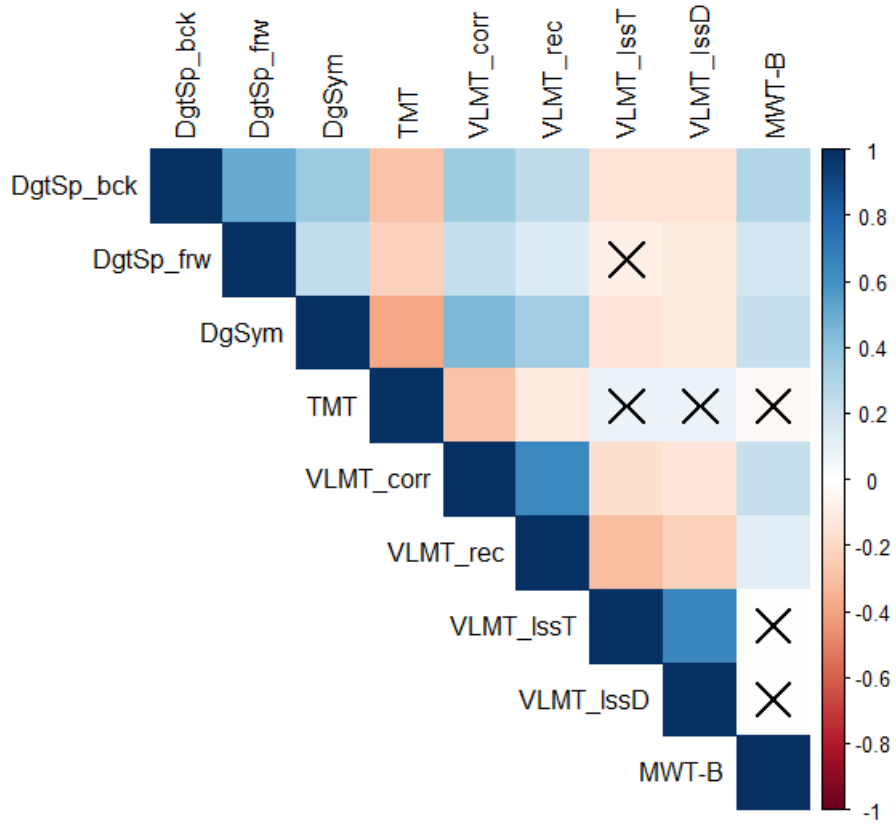

Figure S1. Pairwise correlations between cognitive outcomes in cases only. Shown are Pearson correlation coefficients, insignificant correlations labeled with "X" (uncorrected $p > 0.05$). *Note*: DgtSp_bck – Verbal digit span (backwards task); DgtSp_frw – Verbal digit span (forwards task); DgSym – Digit symbol test; TMT – TMT reaction time difference; VLMT_corr – VLMT number of correctly recalled words; VLMT_lssT – VLMT loss of recalled words after time; VLMT_lssD – VLMT loss of recalled words after distraction; VLMT_rec – VLMT number of correctly recognized words

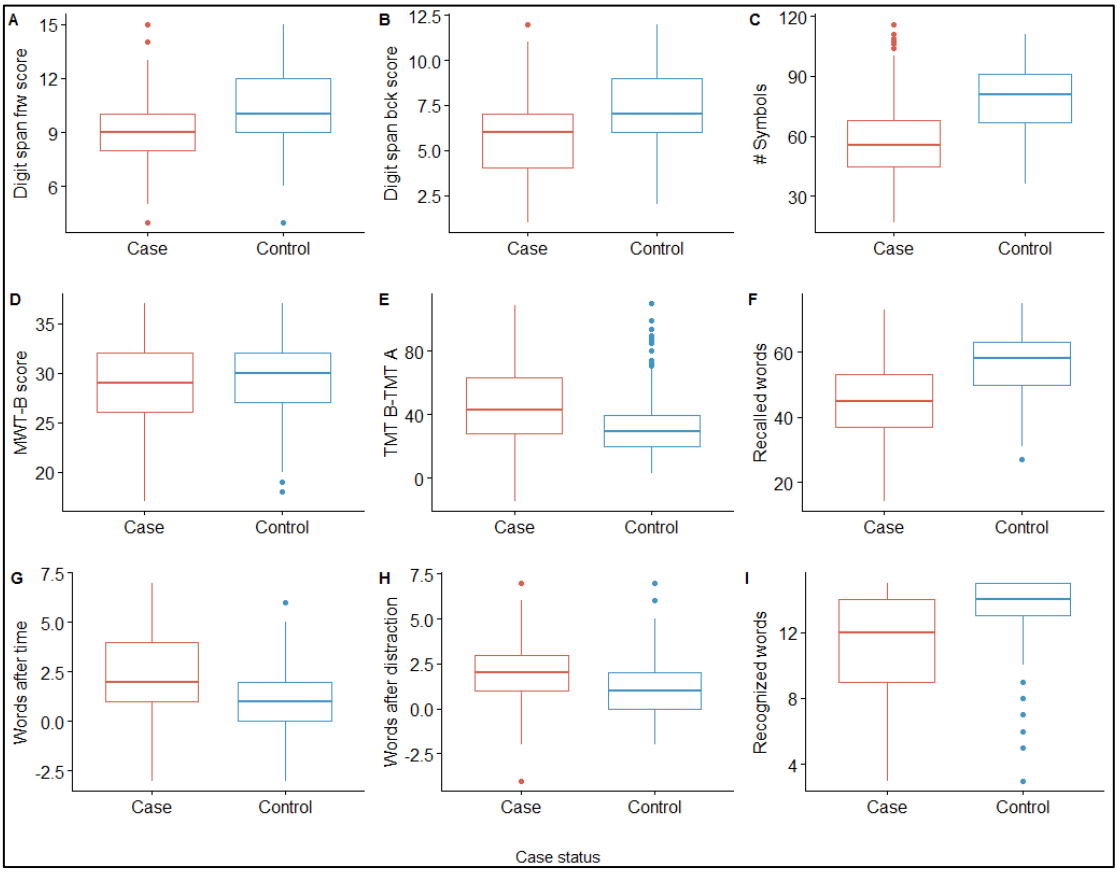

Figure S2. Boxplots depicting the distribution of cognitive performance for cases and controls across all cognitive domains**. A.** Verbal digit span, forward task score **B.** Verbal digit span, backward task score **C.** Digit symbol test, number of correct symbols **D.** MWT-B score **E.** Trail-Making-Test (TMT), difference in reaction time (TMT B-TMT A) **F.** Verbal Learning and Memory Test (VLMT): number of correctly recalled words **G.** VLMT: number of words lost after time **H.** VLMT: number of words lost after distraction **I.** VLMT: number of correctly recognized word

Table S1. The effect of case status on cognitive performance

Outcome	Cases mean (sd)	Controls mean (sd)	Estimate	Std. Error	T-value	*p*-value
Visit 1						
TMT reaction time difference	47.78 (24.80)	32.10 (17.91)	-12.55	1.49	-8.43	$< 2.00\times10^{-16}$ ***
Digit symbol test	56.37 (15.79)	79.03 (15.91)	18.39	1.03	17.86	$< 2.00\times10^{-16}$ ***
Verbal digit span backwards task	5.72 (1.95)	7.25 (2.14)	1.40	0.14	9.87	$< 2.00\times10^{-16}$ ***
Verbal digit span forwards task	9.01 (1.94)	10.32 (2.06)	1.18	0.15	8.15	1.13×10^{-15} ***
MWT-B (crystallized intelligence)	28.34 (4.56)	29.63 (3.61)	1.67	0.33	5.05	5.90×10^{-7} ***
Visit 2						
VLMT: correctly recalled words	44.99 (11.57)	56.35 (9.24)	8.87	0.83	10.70	$< 2\times10^{-16}$ ***
VLMT: loss of words after time	2.21 (2.01)	1.22 (1.77)	-0.80	0.16	-5.02	6.81×10^{-07} ***
VLMT: loss of words after distraction	2.07 (1.82)	1.26 (1.65)	-0.66	0.15	-4.41	1.21×10^{-05} ***
VLMT: correctly recognized words	11.28 (3.46)	13.44 (2.13)	1.45	0.23	6.20	1.06×10^{-09} ***

Note: **Effect estimates and associated standard errors, T-values and *p*-values adjusted for age and sex**

Table S2. The effect of GPS_{EDU} quartile on level of educational attainment in cases, adjusting for age, sex, the interaction between age and sex, and the first 10 principle components

	Proportional odds ratio	**95% CI**	***p*-value**
GPS_{EDU} quartile 2	1.478	1.013 - 2.159	0.043*
GPS_{EDU} quartile 3	1.794	1.237 - 2.606	0.002*
GPS_{EDU} quartile 4	2.495	1.706 - 3.657	2.56×10^{-6}***
Age (years)	0.866	0.695 - 1.078	0.200
Sex (male)	0.728	0.555 - 0.956	0.022*
PC1	1.138	1.002 - 1.303	0.046*
PC2	1.137	0.995 - 1.300	0.057
PC3	0.910	0.787 - 1.048	0.192
PC4	1.005	0.857 - 1.174	0.952
PC4	0.957	0.810 - 1.110	0.571
PC6	1.101	0.960 - 1.263	0.169
PC7	0.970	0.850 - 1.107	0.653
PC8	0.896	0.785 - 1.022	0.102
PC9	0.981	0.859 - 1.120	0.778
PC10	1.003	0.882 - 1.141	0.959
Age (years):Sex (M)	1.952	1.473 - 2.590	3.36×10^{-6}***

* $p < 0.05$ ** $p < 0.005$ *** $p < 0.005$

Table S3. Effect of GPS_{EDU} on cognitive performance models adjusted for age, age^2, sex, in/outpatient status, center, PANSS sum scores, principle components

Model	GPS *p*-value threshold	*p*-value	FDR corrected *p*-value	Base model adjusted R^2	Adjusted R^2 after GPS_{EDU} inclusion
Visit 1					
V1: Verbal digit span backwards task				0.109	
	1	0.001	0.021		0.124
	0.1	0.002	0.021		0.122
	0.05	0.004	0.021		0.121
	5×10^{-8}	0.015	0.050		0.117
V1: Verbal digit span forwards task				0.066	
	1	0.477	0.553		0.066
	0.1	0.313	0.470		0.066
	0.05	0.384	0.522		0.065
	5×10^{-8}	0.435	0.553		0.065
V1: Digit symbol test				0.292	
	1	0.206	0.417		0.293
	0.1	0.241	0.429		0.293
	0.05	0.469	0.553		0.292
	5×10^{-8}	0.765	0.787		0.291
V1: MWT-B				0.214	
	1	0.003	0.021		0.225
	0.1	0.003	0.021		0.225
	0.05	0.008	0.030		0.223
	5×10^{-8}	0.007	0.030		0.224
V1: TMT B-TMT A				0.091	
	1	0.454	0.464		0.091
	0.1	0.611	0.417		0.090
	0.05	0.560	0.417		0.090
	5×10^{-8}	0.391	0.417		0.091
V2: VLMT correctly recalled words				0.224	
	1	0.002	0.021		0.243
	0.1	0.005	0.025		0.239
	0.05	0.013	0.045		0.235
	5×10^{-8}	0.617	0.652		0.222
V2: VLMT loss of words after time				0.050	
	1	0.283	0.463		0.050
	0.1	0.3075	0.522		0.049
	0.05	0.296	0.464		0.050
	5×10^{-8}	0.193	0.417		0.052
V2: VLMT loss of words after distraction				0.015	
	1	0.199	0.417		0.017
	0.1	0.220	0.417		0.017
	0.05	0.187	0.417		0.017
	5×10^{-8}	0.035	0.104		0.025
V2: VLMT correctly recognized words				0.142	
	1	0.199	0.417		0.144
	0.1	0.210	0.417		0.144
	0.05	0.250	0.429		0.143
	5×10^{-8}	0.936	0.936		0.139

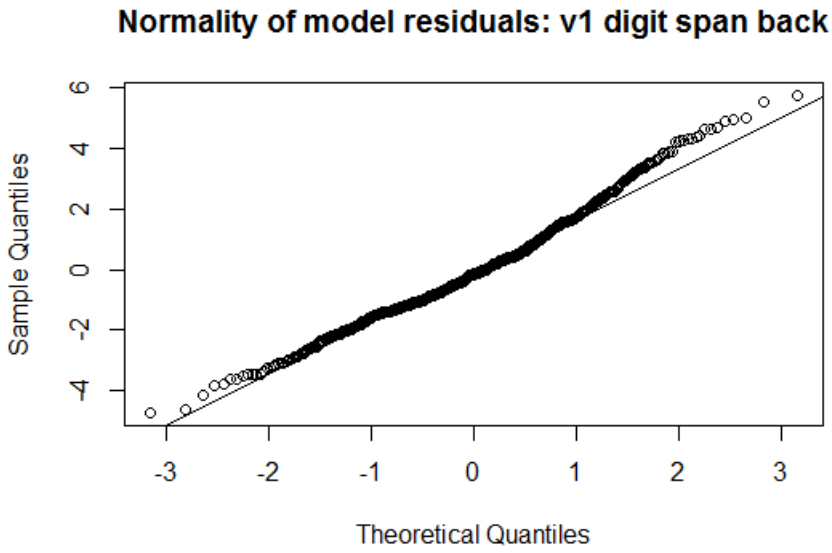

Figure S3. QQ plot for visual inspection of normality of model residuals – Visit 1 verbal digit span backward model

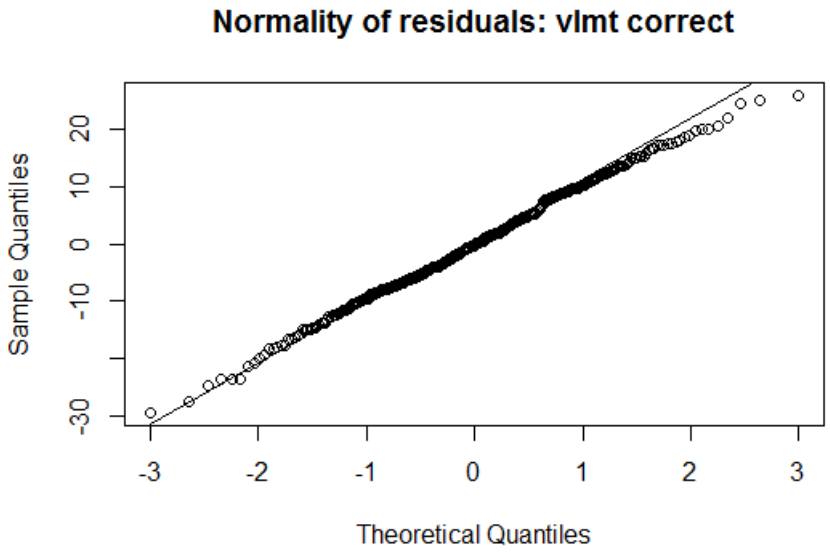

Figure S4. QQ plot for visual inspection of normality of residuals- Visit 2 VLMT correctly recalled words model

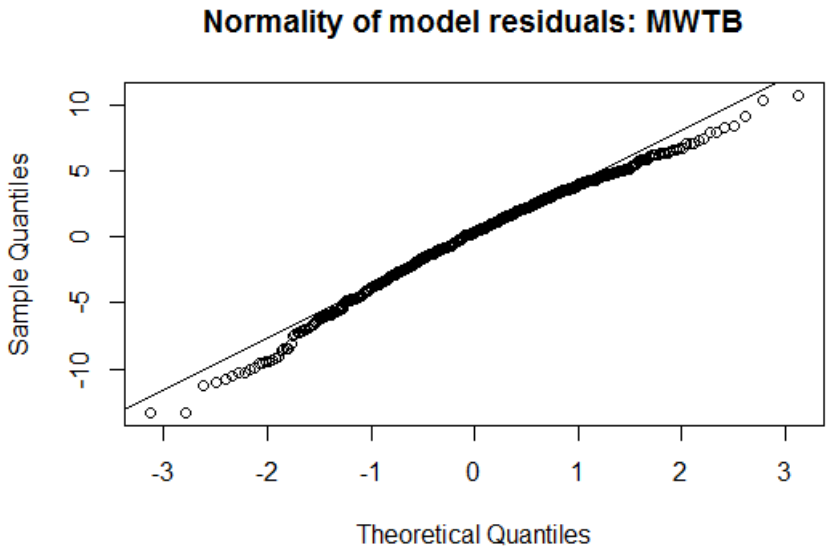

Figure S5. QQ plot for visual inspection of normality of residuals- Visit 1 MWT-B (crystallized intelligence) model

Table S4. Effect of GPS_{EDU} on cognitive performance models adjusted for age, age^2, sex, in/outpatient status, center, PANSS sum scores, principle components, and **medication**

Model	GPS *p*-value threshold	*p*-value	FDR corrected *p*-value	Base model adjusted R^2	Adjusted R^2 after GPS_{EDU} inclusion
V1: Verbal digit span backwards task				0.109	
	1	0.001	0.010		0.124
	0.1	0.002	0.010		0.123
	0.05	0.003	0.014		0.121
	5×10^{-8}	0.012	0.041		0.117
V1: Verbal digit span forwards task				0.066	
	1	0.387	0.478		0.066
	0.1	0.242	0.396		0.067
	0.05	0.300	0.446		0.066
	5×10^{-8}	0.390	0.478		0.066
V1: Digit symbol test				0.301	
	1	0.184	0.346		0.302
	0.1	0.202	0.346		0.302
	0.05	0.398	0.478		0.301
	5×10^{-8}	0.747	0.767		0.300
V1: MWT-B				0.213	
	1	0.001	0.010		0.226
	0.1	0.002	0.010		0.226
	0.05	0.004	0.015		0.224
	5×10^{-8}	0.006	0.020		0.223
V1: TMT B-TMT A				0.098	
	1	0.526	0.592		0.097
	0.1	0.680	0.720		0.097
	0.05	0.623	0.680		0.097
	5×10^{-8}	0.388	0.478		0.098
V2: VLMT correctly recalled words				0.253	
	1	0.001	0.010		0.276
	0.1	0.001	0.010		0.273
	0.05	0.004	0.015		0.269
	5×10^{-8}	0.465	0.540		0.252
V2: VLMT loss of words after time				0.046	
	1	0.290	0.446		0.047
	0.1	0.391	0.478		0.046
	0.05	0.310	0.446		0.046
	5×10^{-8}	0.141	0.338		0.050
V2: VLMT loss of words after distraction				0.014	
	1	0.182	0.346		0.016
	0.1	0.196	0.346		0.016
	0.05	0.166	0.346		0.017
	5×10^{-8}	0.022	0.066		0.027
V2: VLMT correctly recognized words				0.148	
	1	0.125	0.321		0.152
	0.1	0.125	0.321		0.152
	0.05	0.151	0.340		0.151
	5×10^{-8}	0.859	0.859		0.146

Table S5. Effect of GPS_{EDU} on cognitive performance models adjusted for age, age^2, sex, in/outpatient status, center, PANSS sum scores, principle components, and **diagnosis**

Model	GPS *p*-value threshold	*p*-value	FDR corrected *p*-value	Base model adjusted R^2	Adjusted R^2 after GPS_{EDU} inclusion
V1: Verbal digit span backwards task				0.106	
	1	0.001	0.018		0.121
	0.1	0.002	0.018		0.119
	0.05	0.003	0.018		0.118
	5×10^{-8}	0.015	0.050		0.114
V1: Verbal digit span forwards task				0.071	
	1	0.419	0.525		0.070
	0.1	0.262	0.450		0.071
	0.05	0.342	0.492		0.071
	5×10^{-8}	0.365	0.506		0.071
V1: Digit symbol test				0.302	
	1	0.233	0.441		0.302
	0.1	0.290	0.474		0.302
	0.05	0.548	0.616		0.302
	5×10^{-8}	0.725	0.746		0.302
V1: MWT-B				0.214	
	1	0.003	0.018		0.226
	0.1	0.003	0.018		0.226
	0.05	0.006	0.026		0.224
	5×10^{-8}	0.006	0.026		0.224
V1: TMT B-TMT A				0.102	
	1	0.448	0.537		0.101
	0.1	0.603	0.638		0.100
	0.05	0.564	0.616		0.100
	5×10^{-8}	0.409	0.525		0.101
V2: VLMT correctly recalled words				0.226	
	1	0.002	0.018		0.246
	0.1	0.004	0.019		0.242
	0.05	0.011	0.039		0.238
	5×10^{-8}	0.559	0.616		0.224
V2: VLMT loss of words after time				0.054	
	1	0.316	0.492		0.054
	0.1	0.423	0.525		0.053
	0.05	0.331	0.492		0.054
	5×10^{-8}	0.192	0.441		0.056
V2: VLMT loss of words after distraction				0.015	
	1	0.204	0.441		0.017
	0.1	0.231	0.441		0.017
	0.05	0.216	0.441		0.017
	5×10^{-8}	0.037	0.111		0.025
V2: VLMT correctly recognized words				0.138	
	1	0.207	0.441		0.140
	0.1	0.222	0.441		0.140
	0.05	0.261	0.450		0.139
	5×10^{-8}	0.893	0.893		0.135

Table S6. Effect of GPS_{BD} and GPS_{SZ} on cognitive performance, adjusted for age, age^2, sex, in/outpatient status, center, PANSS sum scores, and principle components

Model	Base model adjusted- R^2	*p*-value GPS_{SZ}	Adjusted R^2 after GPS_{SZ} inclusion	*p*-value GPS_{BD}	Adjusted-R^2 after GPS_{BD} inclusion
V1: Verbal digit span backwards task	0.109	0.268	0.109	0.312	0.109
V1: Verbal digit span forwards task	0.067	0.373	0.067	0.399	0.067
V1: Digit symbol test	0.291	0.838	0.290	0.786	0.290
V1: MWT-B	0.214	0.436	0.214	0.498	0.214
V1: TMT B-TMT A	0.093	0.174	0.094	0.208	0.094
V2: VLMT correctly recalled words	0.223	0.363	0.222	0.531	0.221
V2: VLMT words lost after time	0.038	0.399	0.037	0.050	0.046
V2: VLMT words lost after distraction	0.019	0.826	0.016	0.456	0.018
V2: VLMT correctly recognized words	0.143	0.60	0.150	0.965	0.140

Table S7. Effect of GPS_{BD} and GPS_{SZ} on the association between GPS_{EDU} and cognitive performance

Model	*p*-value GPS_{EDU} after adjusting for GPS_{SZ}[a]	Change in adjusted-R^2 after GPS_{EDU} inclusion[a]	*p*-value GPS_{EDU} after adjusting for GPS_{BD}[b]	Change in adjusted-R^2 after GPS_{EDU} inclusion[b]
V1: Verbal digit span backwards task	0.002	0.013	0.001	0.014
V1: MWT-B	0.004	0.011	0.003	0.011
V2: VLMT correctly recalled words	0.002	0.020	0.002	0.019

[a] Adjusting for age, age^2, sex, in/outpatient status, center, PANSS sum scores, principle components and GPS_{SZ}

[b] Base model including age, age^2, sex, in/outpatient status, center, PANSS sum scores, principle components and GPS_{BD}

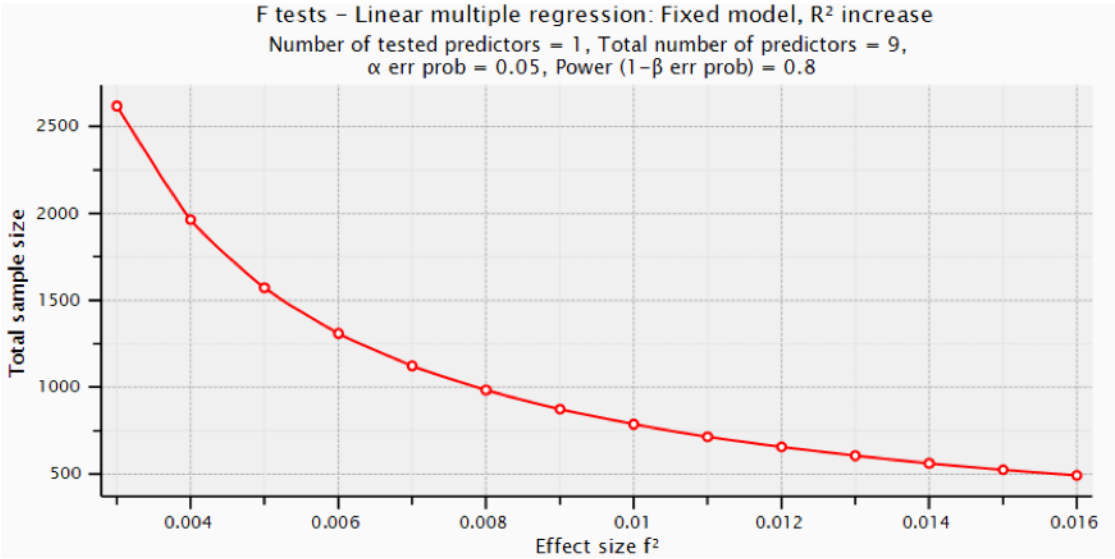

Figure S6. Post-hoc power calculation using the freely available *G* Power 3.1*(Faul, Erdfelder, Buchner, & Lang, 2009; Faul, Erdfelder, Lang, & Buchner, 2007). Plot depicting sample size required to drive the effect of GPS_{SZ} on cognitive performance to significance according to a range of effect sizes (change in adjusted R^2 between a model including only covariates and a model including covariates and GPS_{SZ}). *Note*: Alpha level 0.05, 80% power, number of tested predictors = 1, total number of predictors = 9.

Original Article 1: Supplementary references

Faul, F., Erdfelder, E., Buchner, A., & Lang, A.-G. (2009). Statistical power analyses using g* power 3.1: Tests for correlation and regression analyses. *Behavior Research Methods, 41*(4), 1149-1160.

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G*power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods, 39*(2), 175-191. doi:10.3758/bf03193146

3 Original Article 2

3.1 Individual contributions and reference

The study "The role of environmental stress and DNA methylation in the longitudinal course of bipolar disorder" was published in the *International Journal of Bipolar Disorders* in 2020. It was conducted under the supervision of T.G.S. and U.H. The research was designed by T.G.S. and A.L.C. Methylation data quality control and pre-processing was performed by A.L.C. with support from S.S. Data analysis was performed by A.L.C. with statistical guidance from U.H., D.C., and T.A. A.L.C. wrote the manuscript and accompanied the publication process as corresponding author. All co-authors critically revised and approved the manuscript.

Comes, A.L., Czamara, D., Adorjan, K., […], Falkai, P., Schulze, T.G., Heilbronner, U. (2020). The role of environmental stress and DNA methylation in the longitudinal course of bipolar disorder. *Int J Bipolar Disord*, 8(1), 9. doi:10.1186/s40345-019-0176-6.

Comes *et al. Int J Bipolar Disord* (2020) 8:9
https://doi.org/10.1186/s40345-019-0176-6

International Journal of Bipolar Disorders

RESEARCH Open Access

The role of environmental stress and DNA methylation in the longitudinal course of bipolar disorder

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Abstract

Background: Stressful life events influence the course of affective disorders, however, the mechanisms by which they bring about phenotypic change are not entirely known.

Methods: We explored the role of DNA methylation in response to recent stressful life events in a cohort of bipolar patients from the longitudinal PsyCourse study ($n = 96$). Peripheral blood DNA methylomes were profiled at two time points for over 850,000 methylation sites. The association between impact ratings of stressful life events and DNA methylation was assessed, first by interrogating methylation sites in the vicinity of candidate genes previously implicated in the stress response and, second, by conducting an exploratory epigenome-wide association analysis. Third, the association between epigenetic aging and change in stress and symptom measures over time was investigated.

Results: Investigation of methylation signatures over time revealed just over half of the CpG sites tested had an absolute difference in methylation of at least 1% over a 1-year period. Although not a single CpG site withstood correction for multiple testing, methylation at one site (cg15212455) was suggestively associated with stressful life events ($p < 1.0 \times 10^{-5}$). Epigenetic aging over a 1-year period was not associated with changes in stress or symptom measures.

Conclusions: To the best of our knowledge, our study is the first to investigate epigenome-wide methylation across time in bipolar patients and in relation to recent, non-traumatic stressful life events. Limited and inconclusive evidence warrants future longitudinal investigations in larger samples of well-characterized bipolar patients to give a complete picture regarding the role of DNA methylation in the course of bipolar disorder.

Keywords: DNA methylation, Bipolar disorder, Stressful life events, Longitudinal, Epigenomics, Epigenetic aging

Background

Bipolar disorder (BD) remains an interesting candidate for neurobiological analyses owing to its heterogenous presentation and both genetic and environmental risk factors (Ludwig and Dwivedi 2016). While genome-wide

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association studies (GWAS) in BD have identified dozens of associated variants, they have explained only a small fraction of overall disease liability (Stahl et al. 2019). Therefore, the last decade has seen a shift towards investigating the complex interplay between genetic and environmental risk factors (Sharma et al. 2016). Advances in technologies have supported high-throughput investigations of biological markers representative of environmental modulation of the genome. These biomarkers hold promise for stratifying symptom-based phenotypes and assessing the prognosis of individual patients (Kobeissy et al. 2012). Moreover, these biomarkers could contribute to a more accurate multi-level diagnostic framework which relies on biological measures to supplement clinical ratings of symptoms (Meana and Mollinedo-Gajate 2017).

BD is a chronic, disabling, and severe mental illness characterized by recurrent depressive and manic episodes, somatic and psychiatric comorbidities, and functional impairments (Goodwin and Jamison 2007). Considering the high global burden and lifetime prevalence of bipolar spectrum disorders, estimated at approximately 2.4% (Rowland and Marwaha 2018), there is a need to better understand the factors affecting its onset and course. The significance of environment, especially childhood trauma and stressful life events on the trajectories of affective disorders, including vulnerability, onset, relapse and occurrence, has been well established (Aldinger and Schulze 2017; Lex et al. 2017; Johnson 2005; Alloy et al. 2005; Paykel 2003). However, little is known about the mechanisms involved in the consequences of such life events.

Recently, emphasis has been placed on the potential role of epigenetic variation in the etiopathogenesis of BD (Li et al. 2015). Epigenetics is an adaptive mechanism which can modulate the stress response through subtle gene expression modifications (Aas et al. 2016). In particular, DNA methylation (DNAm), the addition of a methyl group to DNA, primarily at cytosine-guanine dinucleotides (CpG), may pose a "mechanism by which life-experiences become 'embedded' in the genome" (Marzi et al. 2018).

Increasing evidence from both animal and human data supports the epigenetic programming of genes in response to trauma and chronic stress. Consistent findings have linked prenatal (Monk et al. 2012; Weaver et al. 2004) and early-life adversities to epigenetic modifications of genes, especially those involved in the hypothalamic–pituitary–adrenal (HPA) axis (Kular and Kular 2018; McGowan et al. 2009; Vinkers et al. 2015; Jaworska-Andryszewska and Rybakowski 2019). While several studies have shown methylation changes associated with trauma during the adult period, few studies have investigated non-traumatic chronic stress (Matosin et al. 2017) or acute stressful life events. Candidate gene approaches in the general population have reported differential methylation of CpGs in the vicinity of *SLC6A4* (Alasaari et al. 2012), *TH* (Myaki et al. 2015), and *BDNF* (Song et al. 2014) in association with sustained work-related stress. One study, which examined *LINE-1* as a proxy for global methylation, found no signification associations with chronic lifestyle stress (Duman and Canli 2015). To the best of our knowledge, not a single study has explored epigenome-wide signatures of DNAm in relation to acute, non-traumatic stress in humans. With regards to BD, studies have investigated methylation differences as both trait and state markers of the disorder in several promoter regions including *SLC6A4*, *PPIEL31*, *BDNF*, *HCG9*, *KCNQ3*, *5HTR1A* and *GPR24* (Ludwig and Dwivedi 2016; Fries et al. 2016; Pishva et al. 2014). Interestingly, evidence supports altered DNAm profiles for high-risk affected and even unaffected offspring of individuals with BD in comparison to low risk controls. Moreover, there seems to be a unique rate of change in DNAm over time for high risk individuals (Duffy et al. 2019). However, despite findings of differential epigenetic profiles, results have been inconsistent and there remains a need for genome-wide methylation studies, especially ones longitudinal in design.

This study aims to gain a better understanding of the role of epigenetic modifications, specifically DNAm, in relation to stress during the course of BD. Using repeated measures over a 1-year period, we explored the relationship between DNAm and stressful life events in chronic BD patients. We took a two-pronged approach, first by interrogating CpGs in the vicinity of candidate genes previously implicated in the stress response and, second, by conducting an exploratory epigenome-wide analysis. Furthermore, we determined whether changes in symptom and stress measures over time were associated with a DNAm-based age estimate and epigenetic aging.

Methods

Study sample

The study was conducted using data from the longitudinal PsyCourse cohort. PsyCourse has been described in detail (Budde et al. 2019). Briefly, PsyCourse is a multisite, naturalistic study, based in the German and Austrian population. Psychopathology, pharmacological treatment, childhood trauma and current stressful life events were among other variables assessed at each of four visits (6-month intervals). Likewise, peripheral blood samples were collected at each visit, paving the way for a detailed analysis of the longitudinal correlation between disease status and peripheral biomarkers. For the purpose of this study, a subset of PsyCourse participants ($n = 96$) was

selected according to a DSM-IV diagnosis (American Psychiatric Association 2002) of type I or II BD, availability of genotype data and biomaterial, and completed childhood trauma and stressful life events questionnaires. Demographic and clinical characteristics of these patients are reported in Table 1. The study was approved by the local ethics committee for each study center and was carried out following the rules of the Declaration of Helsinki. All individuals provided written informed consent.

Measures

Stressful life events

Current stressful life events were assessed with the Life Events Questionnaire (LEQ), a 79-item self-report instrument that has been described in detail (Norbeck 1984; Sarason et al. 1978). The LEQ covers a wide range of stressor exposure related to health, work, school, residence, love and marriage, family and friends, parenting, the personal sphere or social environment, finances, crime and legal matters. At each visit, participants reported whether they experienced any of the listed events in the last 6 months. When the patient experienced a specific event, they rated: (1) the nature of the event (good/bad) and (2) the impact of the event on his/her life (0–3). At each time point, adverse life events were summed to yield a stress score that reflects the impact ratings of all "bad" events. The same was done for the impact ratings of "good" events. A total score was also summed including impact ratings of both "bad" and "good" events. These three LEQ scores were used as outcome measures in our association analyses.

Table 1 Sample demographic and clinical characteristics

	Baseline ($n=96$)	1-year follow-up ($n=95$)	*p*-value
Sex			
Female	50	50	
Age, mean ± SD	45.2 ± 12.4	46.17 ± 12.4	
Duration of illness, mean ± SD	13.52 ± 11.8	14.66 ± 11.8	
DSM-IV diagnosis			
BD-I	79	78	
BD-II	17	17	
Medication			
Combo therapy	81	75	
Monotherapy	14	16	
No meds	1	4	
Childhood trauma (yes)	48	48	
LEQ scores, mean ± SD			
Bad events	10.2 ± 13.8	6.3 ± 6.6	0.004[b]
Good events	9.7 ± 10.2	8.4 ± 7.6	0.191[b]
Total events	19.9 ± 18.4	14.1 ± 10.7	0.001[b]
Symptom ratings			
GAF, mean ± SD	61.5 ± 12.6	65.8 ± 12.4	0.032[a]
YMRS sum, mean ± SD	3.9 ± 5.8	2.4 ± 3.7	0.216[b]
IDS-C_{30}, mean ± SD	13.7 ± 11.0	10.6 ± 9.7	0.124[b]
PANSS sum, mean ± SD	42.8 ± 11.8	39.2 ± 9.6	0.063[b]

[a] Paired sample t-test

[b] Wilcoxon signed rank test

Childhood trauma

The Childhood Trauma Screener (CTS) is a German, short version of the Childhood Trauma Questionnaire (Bernstein et al. 1997, 2003; Grabe et al. 2012). The screener includes five questions to assess sexual, physical and emotional abuse, as well as emotional and physical neglect. Validated threshold values (Glaesmer et al. 2013) were used to transform ratings for each item into a dichotomous scale in order to identify individuals with reported childhood trauma (yes/no). Details on reported childhood trauma and thresholds used can be found in Additional file 1: Table S1.

Symptom ratings

The Positive and Negative Syndrome Scale (PANSS) was used as a measure of psychopathology at the time of testing (Kay et al. 1987). A continuous total score of the three subscales, i.e. positive, negative, and general symptoms was used. The Global Assessment of Functioning (GAF) score was used as a measure of psychosocial functioning (Luborsky 1962; Endicott et al. 1976). The Young Mania Rating Scale (YMRS) was used as a measure of manic symptoms in the last 48 h (Young et al. 1978). Lastly, the Inventory of Depressive Symptomatology (IDS-C_{30}), a 30-item rating scale, was used to assess the severity of depressive symptoms (Trivedi et al. 2004).

Analysis of DNA methylation

DNA samples

Genomic DNA was extracted from whole blood using the PerkinElmer Chemagen Kit (chemagic DNA Blood10k prefilling VD120419.che) and all samples were subsequently stored in a Hamilton Bios M system at − 80 °C. DNA quality was assessed using the QIAxcl® system. DNA samples from baseline and 1-year follow-up visits were used to obtain methylation data. Prior to downstream analyses, potential population stratification was evaluated, and an initial step to remove European population outliers was taken (Budde et al. 2019). Thus, our sample consists of an ethnically homogenous population of Caucasians of European descent.

Illumina EPIC chip processing

Bisulfide conversion of DNA and processing of methylation arrays was accomplished in collaboration with the Institute of Human Genetics, University of Bonn, Germany. Whole-blood genomic DNA diluted with water (50 ng/µl) was treated with sodium bisulfite using the EpiTect® Bisulfite Kit from QIAGEN® following the manufacturer's protocol. DNAm was assessed using the Illumina Infinium Human MethylationEPIC BeadChip array (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. To minimize batch effects during DNAm measurement, an algorithm for sample randomization was used for positioning samples onto 96-well plates according to exposures of interest and confounding variables (see Additional file 1).

Quality control and normalization

Quality control

The Bioconductor R package *minfi* was used to read raw intensity data files (.idat files) into R and for the subsequent quality control and normalization of methylation data (Aryee et al. 2014). Concordance between methylation-predicted and reported sex was confirmed. Filtering of poor-performing samples and probes was performed (see Additional file 1: Table S2). Probes with low detection p-values (>0.05 in $>10\%$ of samples) were excluded. Using the function dropLociWithSnps(), SNPs inside the probe body and at the nucleotide extension were removed according to a minor allele frequency $\geq 5\%$ based on dbSNP. To prevent a possible gender effect, X and Y chromosomes were removed. According to a list previously published (Chen et al. 2013), non-specific probes i.e. probes on the EPIC array that co-hybridize to alternate genomic sequences, were removed. Lastly, probes with a bead count <3 were removed.

Normalization

Data were normalized using functional normalization (FunNorm), an extension of quantile normalization. FunNorm uses internal control probes present on the array to infer between-array technical variation, by default using the first two principal components of the control probes (Fortin et al. 2014). Density plots were used to evaluate the distribution of M-values before and after functional normalization (see Additional file 1: Fig. S1).

Technical batch effects were then identified using linear regressions to inspect the association of principal components of the methylation values with possible technical batches. Additionally, the R package *shinyMethyl* was used for visual inspection of principle component analysis (PCA) plots. Identified batch effects (i.e., array and slide) were removed using the Empirical Bayes' method *ComBat* (Johnson et al. 2007). Batch corrected M-values after *ComBat* were used for downstream analyses (see Additional file 1: Fig. S2). According to inspection of PCA plots, a single sample remained an outlier after batch correction and was excluded.

Confounders

Considering cell-type composition is a confounding factor in epigenome-wide association studies (EWAS), the *minfi* function estimateCellcounts() was used to estimate the cell type composition for our samples. This function uses a modified version of the Houseman algorithm to obtain a cell counts vector for the six cell-types (i.e., CD4T, CD8T, NK, B cells, monocytes, and granulocytes) for each sample (Houseman et al. 2012).

Active smoking is another established modifier of DNA methylation (Lee and Pausova 2013). Methylation-based smoking scores were calculated based on the methylation profile of the 187 CpG sites identified in Zeilinger et al. (2013). First, raw beta values were normalized using the Teschendorff et al. beta-mixture quantile dilation (BMIQ) strategy (Teschendorff et al. 2013). Adjusted beta-values were then used for calculation of methylation-based smoking scores using methods previously described (Elliott et al. 2014). The correlation between self-reported number of cigarettes smoked yearly and methylation-based smoking scores was assessed (Spearman's $\rho = 0.64$; $p < 0.001$).

To rule out possible confounding effects of medication, 5 samples were excluded in sensitivity analyses. These samples were participants who were not taking psychotropic drugs at the time of testing. All other participants were taking at least one (monotherapy) or a combination (combo therapy) of the following (1) antidepressants, (2) antipsychotics, (3) mood stabilizers, (4) tranquilizers, or (5) other psychiatric medications.

Statistical analyses

All statistical analyses were performed in R version 3.4.4 (http://www.r-project.org/) (R Core Team 2014).

Change in methylation over time

The general "stability" of methylation over time was investigated. First, the absolute change in methylation β-values between baseline and 1-year follow-up visits were calculated across all CpG sites. To determine whether differential methylation between visits remained significant after adjusting for known confounders, the package *lme4* (Bates et al. 2015) was used to fit a linear mixed-effects model (LMM) with the dependent variable "M-value" and the independent variable "time", adjusting for age, sex, DNAm smoking scores, and cell composition estimates. Patient ID was included as the random effect term.

Candidate gene analysis

The association between LEQ scores and the interaction between CT and total LEQ scores with DNAm was assessed via LMMs, adjusting for covariates as described above. We interrogated DNAm in the vicinity of genes previously implicated in the HPA-axis (i.e. *BDNF*, *FKBP5*, *IL6*, *SLC6A4*, and *OXTR*). All probes on the EPIC array annotated to each of these five genes were identified. The number of probes per gene ranged from 22 to 124. We corrected for multiple testing on a gene-level by applying the false discovery (FDR) correction (Benjamini and Hochberg 1995) per gene, with FDR-corrected p-values ≤ 0.05 deemed significant. Afterwards, Bonferroni-correction was used to correct overall for the number of candidate-genes tested.

Exploratory EWAS

An exploratory EWAS was conducted. As a means of noise reduction, the top 10% of the most variable CpGs of the normalized, batch corrected M-values were extracted according to median absolute deviation (MAD) scores i.e. the median of the absolute deviations from the data's median. Associations between the most variable sites and LEQ scores and the interaction between childhood trauma and total LEQ scores were then tested using LMMs, adjusting for covariates as described above.

Epigenetic aging

DNAm-based age prediction was performed using the Horvath age estimation algorithm (Horvath 2013) with a freely available online tool (https://dnamage.genetics.ucla.edu/home) which predicts DNAm-age based on the methylation of 353 CpGs using an elastic net penalized regression model. The difference between the estimated epigenetic age and chronological age (Δage) and a measure of epigenetic age acceleration (AA), i.e., the residual from regressing DNAm age on chronological age, were calculated. LMMs were used to determine the effect of LEQ scores on Δage, adjusting for chronological age, sex, DNAm smoking scores, cell composition estimates, and technical batch effects (sample slide and array). Additionally, the difference in symptom ratings and stress scores between visits were calculated. The association between the change in symptoms and LEQ scores between baseline and 1-year follow-up with AA at 1-year follow-up was determined via linear regression models, again controlling for chronological age, sex, DNAm smoking scores, cell composition estimates and technical batch effects.

Additional analyses

Nominally significant CpGs (unadjusted $p < 0.05$) associated with total LEQ scores were used for gene-based enrichment analysis using the *GOmeth* function from the Bioconductor package *missMethyl*. *GOmeth* maps a vector of CpG sites to Entrez Gene IDs, and tests for gene ontology (GO) term pathway enrichment using a hypergeometric test (Geeleher et al. 2013). Additionally, the correlation between DNAm in blood and four brain regions was explored for the most suggestive CpGs associated with total LEQ scores (see Additional file 1).

Results

Change in methylation over time

The mean absolute difference in methylation (β) between visits 1 and 3 (|Δβ|) was calculated across all samples for all CpG sites (Fig. 1). Over the 1-year period, |Δβ| ranged from < 0.001 to 0.299 with an average change of 0.014. Of 753,251 CpG sites, only 68 had an |Δβ| of 0.10 or more, while 8454 sites differed by at least 0.05 between visits. Just over half of the sites (428,610) showed an absolute difference in methylation of at least 1%. Investigation of the functional genomic distribution of the least stable CpGs over time ($|\Delta\beta| \geq 0.10$) revealed the majority of CpGs fell within Open Seas, while 12 fell within CpG Islands, and the remaining in CpG Shores and Shelves (Fig. 2). In summary, 34,776 CpG sites showed a nominally significant difference over time (unadjusted p-value < 0.05), after correcting for age, sex, smoking and cell composition estimates. However, not a single locus withstood correction for multiple testing (FDR-corrected p-value < 0.05).

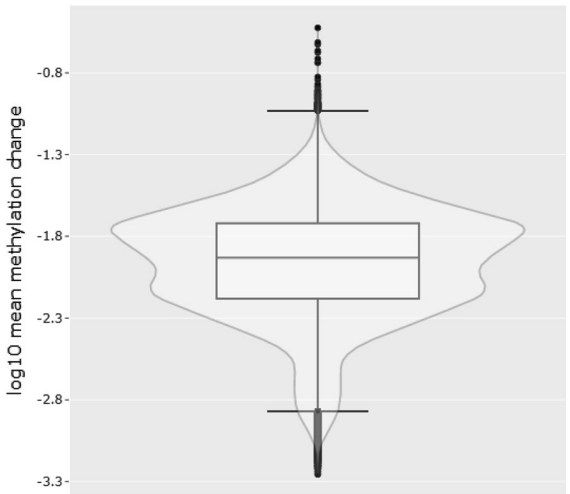

Fig. 1 Boxplot depicting the log10 mean change in methylation (β) between baseline and 1-year follow-up visit

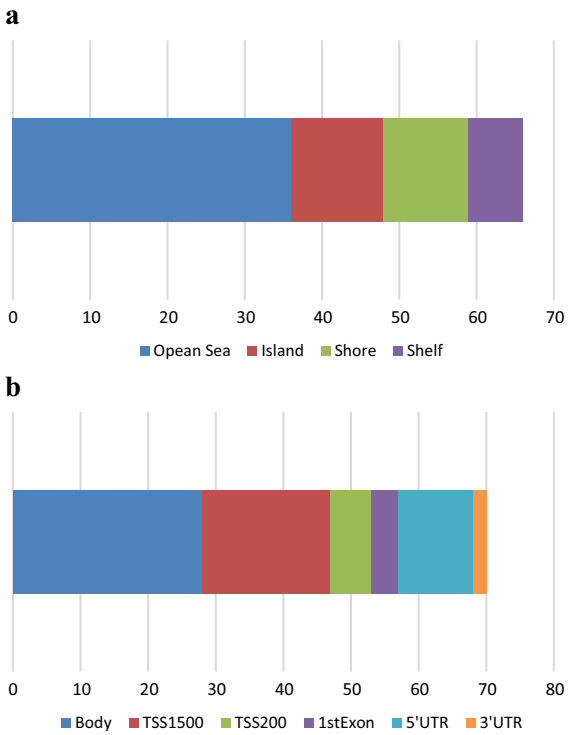

Fig. 2 Functional genomic distribution for the least stable CpG sites over 1-year period ($|\Delta\beta| \geq 0.10$). **a** Depicts the distribution of probes that fell within CpG Islands (12/66), Shelves (7/66), Shores (11/66) and the Open Sea (36/66). **b** Depicts the distribution of probes that fell within the gene body (28), 5′ UTR (11), 3′ UTR (2), 1st Exon (4), TSS 1500 (19) and TSS 200 (6)

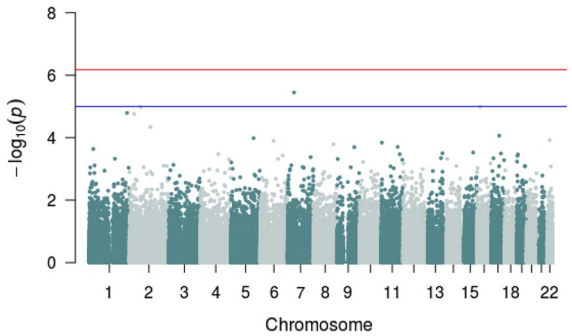

Fig. 3 Manhattan plot for association between DNA methylation and total LEQ scores. The horizontal red line represents the epigenome-wide significant threshold for this study ($p < 6.6 \times 10^{-7}$) and the blue line represents a suggestive significance threshold ($p < 1.0 \times 10^{-5}$)

Methylation association analysis

We performed an exploratory analysis looking for associations between LEQ scores and DNAm in individual CpG probes in the vicinity of candidate genes previously implicated in the stress response and in the most variable CpG sites across the epigenome. Methylation at a single CpG site (cg15212455; *POU6F2*; "POU class 6 homeobox 2"; chr 7) was associated with impact ratings of total LEQ scores with a suggestive significance of $p < 1.0 \times 10^{-5}$, although not a single locus withstood correction for multiple testing (FDR-corrected $p > 0.05$ for all comparisons). Figure 3 shows the Manhattan plot depicting all analyzed CpG sites with their calculated *p*-values for the association between DNAm and total LEQ scores. Table 2 lists the top 20 loci associated at nominal significance with total LEQ scores. Inspection of quantile–quantile (QQ) plots did not show evidence for inflation or bias (Fig. 4; Lambda factor = 0.98). Manhattan plots and associated QQ plots for additional association analyses can be found in Additional file 1: Fig. S3–S8. The sensitivity analysis, excluding subjects who did not take psychotropic drugs at the time of testing, did not yield signification associations. These results, specific to modeling the association between DNAm and total LEQ scores, are presented in Additional file 1: Figs. S9 and S10.

Epigenetic aging

As expected, there was a strong positive correlation between individuals' DNAm age and chronological age ($r = 0.941$, $p < 0.001$; see Additional file 1: Fig. S11). According to Horvath's estimate, the mean (SD, range) AA was − 0.23 years (3.71, range − 9.94 to 9.86 years) at baseline and 0.25 years (3.95, range − 8.12 to 9.43 years) at the 1-year follow-up. Between visits, the mean (SD, range) change in AA was 0.50 years (4.97, range − 10.72 to 13.85 years). Overall, no statistically significant associations between epigenetic aging and symptom or stress measures were detected.

Additional analyses

We included genes mapped by the top CpG sites (unadjusted $p < 0.05$) associated with total LEQ scores in an enrichment analysis. No biological processes survived FDR correction (see Additional file 1: Table S3). Blood brain correlation coefficients for methylation of the top 20 loci associated with total LEQ scores (overlapping with the 450 K Beadchip array) are presented in Table 3. Eight of the top 20 most differentially methylated loci associated with total LEQ scores showed a significant correlation between methylation in the blood and methylation in at least one brain region. Methylation of the CpG site that was most strongly associated with total LEQ scores was significantly correlated with methylation in all four brain regions ($p < 0.001$; see Additional file 1: Fig. S12).

Table 2 Top 20 CpG sites associated with total LEQ scores

Probe	t value	*p*-value	FDR-corrected *p*-value	Chr	Relation to island	Annotated gene
cg15212455	− 4.87	3.56E−06	0.263	chr7	Open Sea	*POU6F2*
cg05335886	− 4.55	1.02E−05	0.263	chr16	Island	*TMC5*
cg09725915	4.54	1.05E−05	0.263	chr2	Island	
cg24511004	4.43	1.62E−05	0.263	chr1	Open Sea	
cg18110277	− 4.50	1.74E−05	0.263	chr2	Open Sea	
cg21516302	− 4.24	4.58E−05	0.575	chr2	Open Sea	
cg05180443	− 4.04	8.61E−05	0.927	chr17	Island	*CHAD; ACSF2*
cg01440452	− 3.97	1.03E−04	0.946	chr5	N Shore	*PURA*
cg26730347	− 4.00	1.20E−04	0.946	chr22	N Shore	*SLC5A1*
cg15869582	3.94	1.26E−04	0.946	chr6	S Shore	*IBTK*
cg05919744	3.95	1.44E−04	0.977	chr11	S Shore	*SLC22A18AS;SLC22A18*
cg26822318	3.86	1.61E−04	0.977	chr8	Open Sea	*FER1L6*
cg27296293	− 3.80	1.98E−04	0.977	chr11	Island	*RP11-748H22.1; TRPC6*
cg06334363	3.82	2.01E−04	0.977	chr9	S Shore	*RP11-235C23.5; FKTN*
cg00356897	− 3.79	2.30E−04	0.977	chr1	Open Sea	*RP4-594I10.2*
cg24795825	3.72	2.98E−04	0.977	chr15	N Shore	*MORF4L1*
cg17984201	3.69	3.17E−04	0.977	chr13	Open Sea	
cg18002447	− 3.67	3.20E−04	0.977	chr17	Island	
cg07349208	− 3.66	3.36E−04	0.977	chr4	Island	*RP11-380D23.2*
cg05705044	3.65	3.38E−04	0.977	chr11	S Shore	*RBM7*

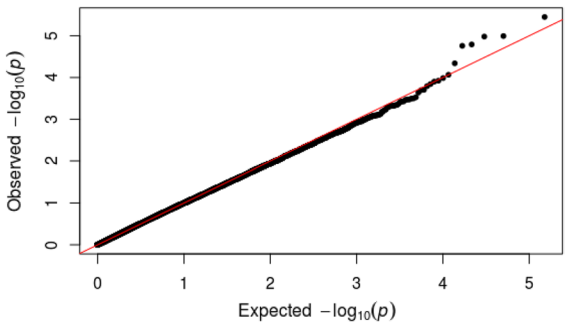

Fig. 4 QQ plot. The plot shows no evidence for inflation or bias in the association analysis of DNA methylation with total LEQ scores (Lambda = 1.04)

Discussion

To the best of our knowledge, our study is the first to investigate epigenome-wide methylation changes over time in BD patients. Moreover, it is the first to explore methylation changes related to non-traumatic stressful life events on an epigenome-wide scale. Although no locus withstood correction for multiple testing, our suggestive findings and secondary analyses provide limited evidence supporting a role of DNAm in association with non-traumatic life events in chronic BD patients.

We identified a single, suggestively significant, CpG site associated with total LEQ scores, mapping to *POU6F2*, which has been associated with several psychiatric traits as well as intelligence and educational attainment. More specifically, genome-wide association studies have identified *POU6F2* risk variants associated with psychological distress (Koshimizu et al. 2019), feeling emotionally hurt (Nagel et al. 2018), schizophrenia (Goes et al. 2015), autism (Anney et al. 2010), educational attainment (Lee et al. 2018; Okbay et al. 2016) and intelligence (Hill et al. 2018; Davies et al. 2018). Additionally, in a longitudinal investigation of DNAm changes preceding adolescent psychotic experiences, DNAm of the CpG site cg11604728 (*POU6F2*) measured at age 15–17 was among the top 20 CpG sites indicative of psychotic experiences at age 18 (Roberts et al. 2019). Furthermore, *POU6F2* is highly expressed in the brain with the highest expression found in the frontal cortex (Additional file 1: Fig. S13) and methylation of our suggestive CpG site in blood is correlated with methylation in brain tissue across multiple brain regions. Interestingly, another of our top 20 CpG sites (cg26822318) falls in proximity to the *FER1L6* gene, of which a variant (rs4870888) has been associated with suicide attempts in a meta-analysis of major depressive disorder, schizophrenia and BD (Mullins et al. 2019). Furthermore, another GWAS reported a *FER1L6*

Table 3 Blood-brain methylation correlation for top differentially methylated CpGs associated with total LEQ scores

Probe	Blood-PFC	*p*-value	Blood-EC	*p*-value	Blood-STG	*p*-value	Blood-CER	*p*-value
cg15212455	*0.721*	*4.16E−13*	*0.731*	*4.64E−13*	*0.747*	*1.48E−14*	*0.631*	*3.71E−09*
cg05335886	− 0.086	0.467	− 0.101	0.404	− 0.145	0.213	− 0.155	0.196
cg09725915	*0.576*	*7.86E−08*	*0.532*	*1.76E−06*	*0.626*	*1.96E−09*	*0.489*	*1.49E−05*
cg21516302	*0.373*	*0.001*	*0.522*	*3.01E−06*	*0.507*	*3.46E−06*	*0.307*	*0.009*
cg05180443	0.204	0.081	*0.336*	*0.004*	*0.298*	*0.009*	0.175	0.144
cg01440452	− 0.097	0.413	0.062	0.606	− 0.119	0.309	0.020	0.870
cg26730347	*0.499*	*6.05E−06*	*0.568*	*2.33E−07*	*0.562*	*1.51E−07*	*0.493*	*1.27E−05*
cg27296293	0.131	0.265	− 0.214	0.073	0.037	0.755	*0.236*	*0.048*
cg24795825	0.016	0.894	*0.299*	*0.011*	*0.247*	*0.033*	0.121	0.317
cg18002447	0.038	0.749	0.034	0.776	0.042	0.720	*− 0.262*	*0.028*
cg07349208	0.167	0.156	0.095	0.431	− 0.112	0.338	0.068	0.571

PFC prefrontal cortex, *EC* entorhinal cortex, *STG* superior temporal gyrus, *CER* cerebellum

Significant correlations in italics

variant (rs10481151) suggestively associated with cognitive performance (Need et al. 2009).

At the current sample size, our study provides only minimal evidence supporting an association between methylation of individual CpGs and non-traumatic, recent stressful life events in BD. These findings, however, corroborate other reports of a limited role of DNAm with non-traumatic stress (Marzi et al. 2018). Noteworthy, a recent study reported hypermethylation of *KITLG* associated with childhood trauma in healthy controls ($n=91$) but not in bipolar patients ($n=50$) (He et al. 2018). Although the mechanistic role of DNAm in the phenotypic expression of early life adversities is well established in the literature, other mechanisms may be responsible in adulthood and in association with subsequent events. This notion aligns with theories such as Post's kindling hypothesis and the decay model which suggest a higher impact of life events on first episode than on subsequent episodes in BD (Aldinger and Schulze 2017; Kemner et al. 2015; Hillegers et al. 2004). Furthermore, it must be considered whether positive epigenetic associations with life events could be disorder-specific, genotype-dependent, associated with specific trauma exposure, age groups, sex and/or tissues measured (Marzi et al. 2018; Vinkers et al. 2015; Uddin et al. 2010; Boks et al. 2015; Smith et al. 2011; Mehta et al. 2017). While there is no gold standard for life stress measurements, differences in how to quantify stress may also have a major effect on findings (Johnson 2005; Bender and Alloy 2011; Monroe 2008; Dohrenwend 2010; Brown and Harris 2012).

The main strength of our study is its longitudinal design, allowing for repeated measures within individuals and to investigate methylation changes over time and in relation to symptomatology and stressful life events. To the best of our knowledge, this is the first study to collect repeated epigenome-wide methylation measures in bipolar patients. Furthermore, our study paid attention to critical confounding factors which often lead to spurious findings. For example, the use of methylation-based smoking scores better controls for the extent of smoking throughout the lifetime than the use of self-reported smoking measures (Elliott et al. 2014; Shenker et al. 2013). Finally, in contrast to most other studies, we have included an exploratory epigenome-wide approach.

Despite the strengths of our study, several limitations need to be addressed. First, our study was limited by our small sample size which makes identifying subtle differences in methylation difficult. Taking power into consideration, and as an attempt to address the inherent multiple testing problem associated with EWAS, we limited our EWAS to only the most variable CpG sites according to MAD scores. While the fact that not a single site-specific association in DNAm survived correction for multiple testing could reflect the limited statistical power of our small sample, it may also be related to an overly conservative multiple testing correction considering the lack of variability in methylation at many CpGs and spatial correlation of methylation with nearby sites (Walker et al. 2016; Lunnon et al. 2015). A recent study estimated there are approximately 530,000 independent tests in a whole blood EPIC array DNAm study. Accordingly, they proposed a corrected significance threshold of 9.42×10^{-8} to be used as a standard threshold for future EWAS based on the EPIC array (Mansell et al. 2019). Furthermore, the study introduced a freely available online tool which allows users to perform power calculations to guide sample sizes, accounting for the individual properties of each DNAm site and using their empirically derived significance threshold. According to their tool, an effect size of just

1% difference between cases and controls would require a sample of 1000 participants, for only a third of methylation sites to have > 80% power. We observed an effect size below 5% in our study (based on median split) for our most significantly associated site, indicating that our study is nevertheless underpowered. Future studies should take advantage of this tool to assess, a priori, required sample sizes according to their expected effect sizes. Furthermore, complementary systems biology approaches such as weighted gene co-methylation network analysis (WGCNA) could be beneficial for studies with limited sample sizes, providing more insight into the functional role of altered DNAm (Langfelder and Horvath 2008).

Another limitation is in relation to the fact that our sample represents a cohort of chronic BD patients which likely influenced our investigation of epigenetic aging related to symptom ratings over time. The chronicity of patients may also confound our findings with regards to the heterogenous treatments patients have received over the years. To acknowledge this critical factor, we conducted a sensitivity analysis excluding those subjects not taking psychotropic drugs at the time of testing, however, this also did not lead to significant results. One must also consider the possible recall and desirability biases associated with self-rating questionnaires like the LEQ and CTS. Lastly, little is known about the temporal stability of epigenetic markers (Byun et al. 2012; Talens et al. 2010). We cannot be sure whether the time interval of 1 year was too long or short to observe dramatic methylation changes or at what time window following exposure to stressful life events one might observe changed methylation profiles.

Conclusions

BD is a multifactorial psychiatric illness, and for many patients full interepisodic remission never occurs (Sam et al. 2019). Stressful life events have been associated with a worse course of BD (Aldinger and Schulze 2017) and there remains a need to better understand the mechanisms which allow these stressors to bring about phenotypic change. Our study provides limited evidence supporting an association between DNAm and recent, non-traumatic stressful life events in BD patients. As findings in clinical populations have been inconsistent, there is still much to be understood especially with regards to the temporal nature of environmentally induced DNA modifications. Future larger studies of well-characterized patients, longitudinal in design, are warranted.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40345-019-0176-6.

Additional file 1. Supporting methods, Figures S1-S13, and Tables S1-S3.

Abbreviations

BD: bipolar disorder; GWAS: genome-wide association studies; DNAm: DNA methylation; CpG: cytosine-guanine dinucleotides; HPA: hypothalamic–pituitary–adrenal; LEQ: Life Events Questionnaire; CTS: Childhood Trauma Screener; PANSS: Positive and Negative Syndrome Scale; GAF: Global Assessment of Functioning; YMRS: Young Mania Rating Scale; IDS-C_{30}: Inventory of Depressive Symptomatology; FunNorm: functional normalization; EWAS: epigenome-wide association analysis; BMIQ: beta-mixture quantile dilation; LMM: linear mixed-effects model; FDR: false discovery rate; MAD: median absolute deviation; AA: age acceleration; GO: gene ontology; QQ: quantile-quantile; WGCNA: weighted gene co-methylation network analysis.

Acknowledgements

The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the gene tissue expression analysis described in the this manuscript and reported in Additional file 1 were obtained from the GTEx portal: GTEx Analysis Release V8 (dbGaP Accession phs000424.v8.p2) on 09/24/19.

Authors' contributions

All authors made substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. Authors ALC and TGS designed the study. Authors TGS and UH provided supervision. Author ALC conducted the analysis in consultation with UH, SS, DC and TA. Author ALC wrote the first draft of the manuscript. All authors contributed to the manuscript. All authors read and approved the final manuscript.

Funding

Thomas G. Schulze and Peter Falkai are supported by the German Research Foundation (Deutsche Forschungsgemeinschaft; DFG) within the framework of the projects http://www.kfo241.de and http://www.PsyCourse.de (SCHU 1603/4-1, 5-1, 7-1; FA241/16-1). The genotyping was in part funded by the German Federal Ministry of Education and Research (BMBF) through the Integrated Network IntegraMent (Integrated Understanding of Causes and Mechanisms in Mental Disorders), under the auspices of the e:Med Program with grants awarded to Thomas G. Schulze (01ZX1614K), Marcella Rietschel (01ZX1614G), and Markus M. Nöthen (01ZX1614A). Thomas G. Schulze received additional support from the German Federal Ministry of Education and Research (BMBF) within the framework of the BipoLife network (01EE1404H) and the Dr. Lisa Oehler Foundation (Kassel, Germany). Sergi Papiol was supported by a 2016 NARSAD Young Investigator Grant (25015) from the Brain and Behavior Research Foundation. Marcella Rietschel and Markus M. Nöthen received funding from the German Research Foundation (DFG, grant FOR2107; RI 908/11-2 to Marcella Rietschel, NO 246/10-2 to Markus M. Nöthen). Franziska Degenhardt received support from the BONFOR Programme of the University of Bonn, Germany. Jens Wiltfang is supported by an Ilídio Pinho professorship and iBiMED (UID/BIM/04501/2013), at the University of Aveiro.

Availability of data and materials

Participants of the PsyCourse study have consented to the sharing of their pseudonymized data with other researchers and research consortia. Thus, PsyCourse data will be made available to bona fide researchers collaborating with us given a mutually agreed written memorandum of understanding has been signed.

Ethics approval and consent to participate

The study was approved by the local ethics committee for each study center and was carried out following the rules of the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
Dr. Jens Reimer received honoraria from Otsuka-Lundbeck for participation in a speakers' bureau. Dr. Max Schmauß received honoraria form Otsuka-Lundbeck, Neuraxpharm, Aristo and Janssen for participation in the speakers's bureau or advisory boards. All other authors declare that they have no competing interests.

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Received: 25 September 2019 Accepted: 18 December 2019
Published online: 12 February 2020

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Publisher's Note

3.2 Original Article 2: Supplementary material

Supplementary methods

Batch effects. We used R version 3.4.4 to randomly draw samples from selected individuals stratified by groups (age, sex, and exposures of interest) and to assign them to batches (e.g. different microarray chips). Standard statistical tests were then used to assess if the putative technical batches (e.g. plates, microarray chips, chip rows/columns) were significantly associated with age, gender or exposure of interest. This procedure was repeated for up to 10000 permutations and the combination of samples that produced the least significant associations was selected. As within-individual variability over time was explored, baseline and visit 3 samples for each individual were loaded onto the same chip. Post hoc tests were then run on the selected combination of samples to confirm no significant associations with other variables, namely diagnosis (Bipolar I/II) or disease severity.

Blood-brain methylation correlation. The freely available Blood Brain DNA Methylation Comparison Tool (http://epigenetics.iop.kcl.ac.uk/bloodbrain/) (Hannon, Lunnon, Schalkwyk, & Mill, 2015) was used to determine the likely correlation between DNAm in blood with four different brain regions (prefrontal cortex, entorhinal cortex, superior temporal gyrus and cerebellum) for the most suggestive CpGs associated with total LEQ scores.

Table S1. Reported childhood trauma type

Childhood trauma type		n (%)
Abuse only		22 (45.8)
Neglect only		6 (12.5)
Number of traumas		
1		22 (45.8)
2		11 (22.9)
3		8 (16.7)
4		1 (2.1)
5		1 (2.1)
Missing complete CTS		5 (10.4)
CTS single items	Trauma threshold	
". . . I had the feeling to be loved"	"Seldom" or "Not at all"	19 (39.6)
". . . persons in my family hit me so hard that I bruised"	"Sometimes" to "Very often"	16 (33.3)
". . . I had the feeling someone in my family hated me"	"Sometimes" to "Very often"	24 (59.0)
". . . someone harassed me sexually"	"Seldom" to "Very often"	15 (31.2)
". . . there was someone who took me to the doctor when I needed it"	"Seldom" or "Not at all"	10 (20.8)

Table S2. Probe filtering

CpGs probes EPIC	**866,238**
Low detection p-values	6529
Bead count < 3	17315
X and Y probes	19096
CpGs with SNPs	29310
Cross-reactive probes	37968
Non-specific probes	2769
CpGs after filtering	**753,251**

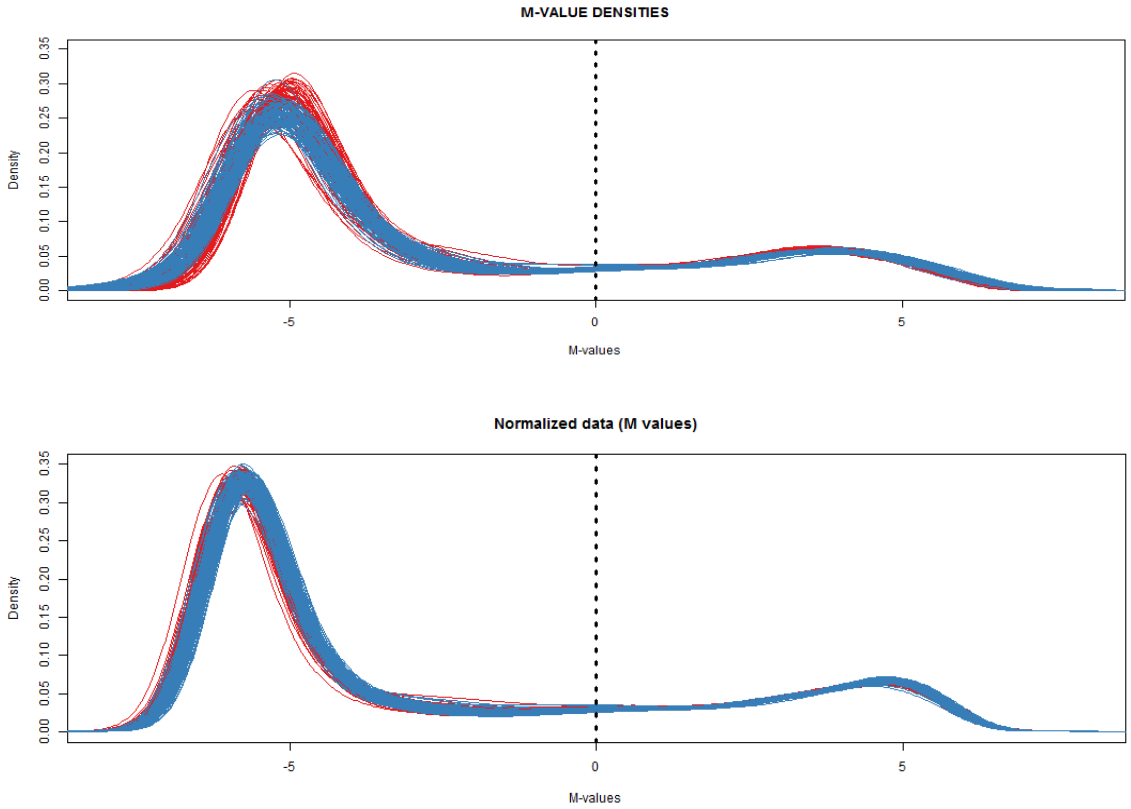

Figure S1. M-Value densities before and after functional normalization.

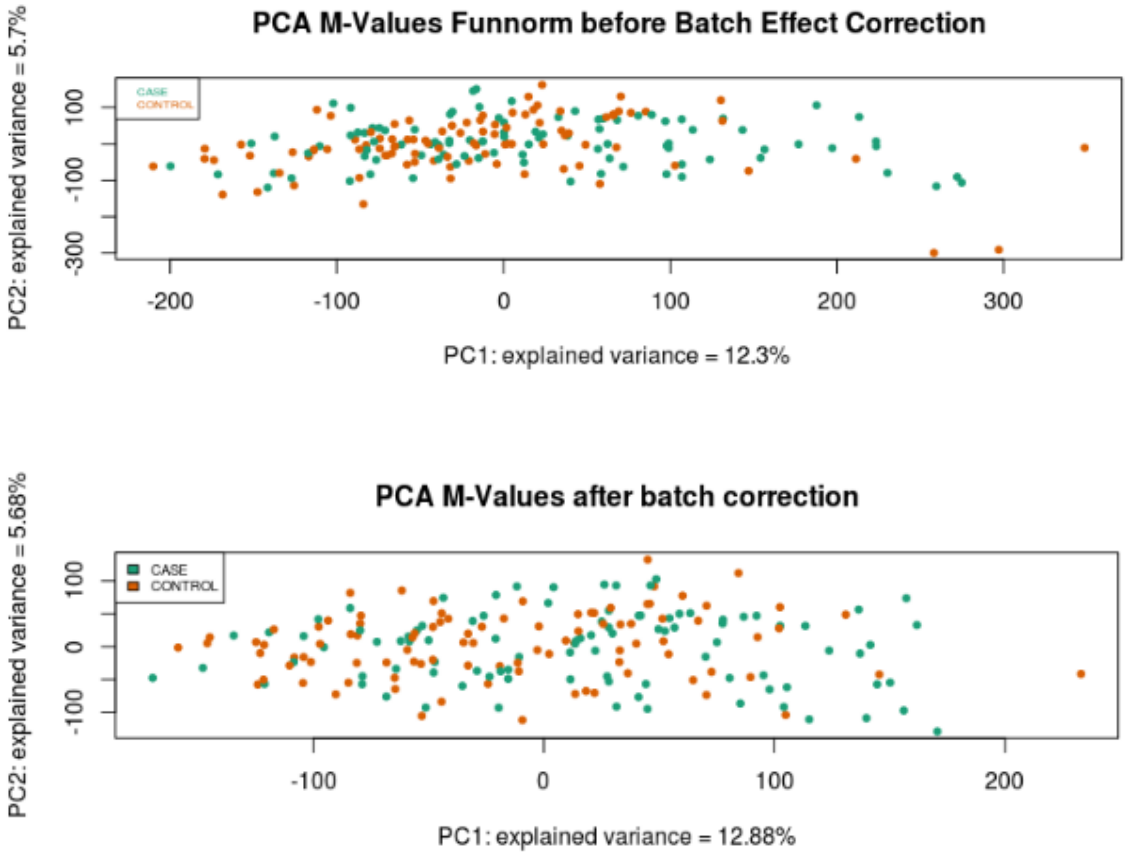

Figure S2. Inspection of batch effects through principle component analysis plots before and after batch correction with *ComBat*

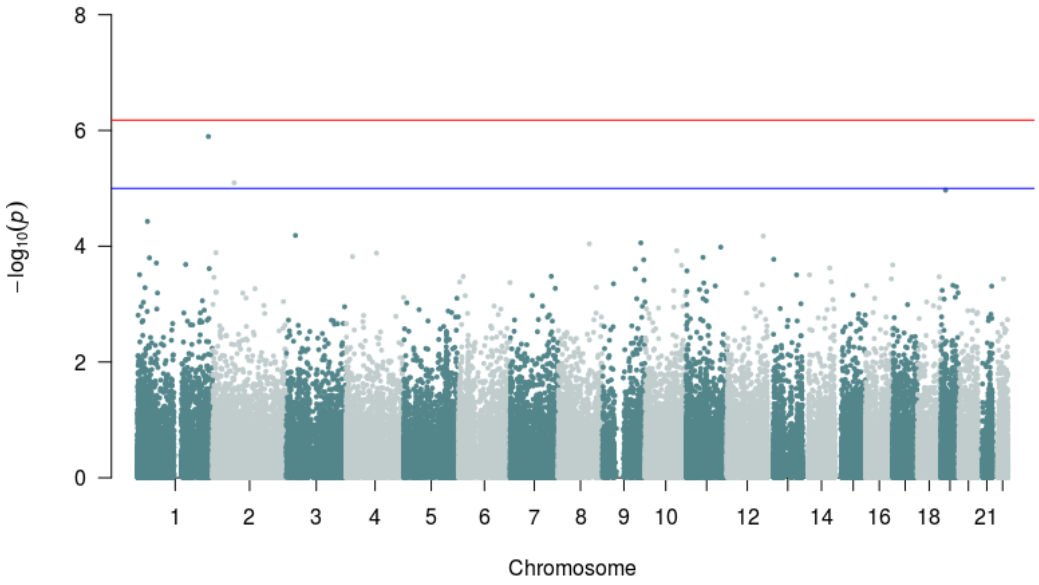

Figure S3. Manhattan plot. The Manhattan plot depicts the association between DNA methylation and LEQ "good" scores (n = 191). The horizontal red line represents the epigenome-wide significant threshold for this study ($p < 6.6\times10^{-7}$) and the blue line represents a suggestive significance threshold ($p < 1.0\times10^{-5}$).

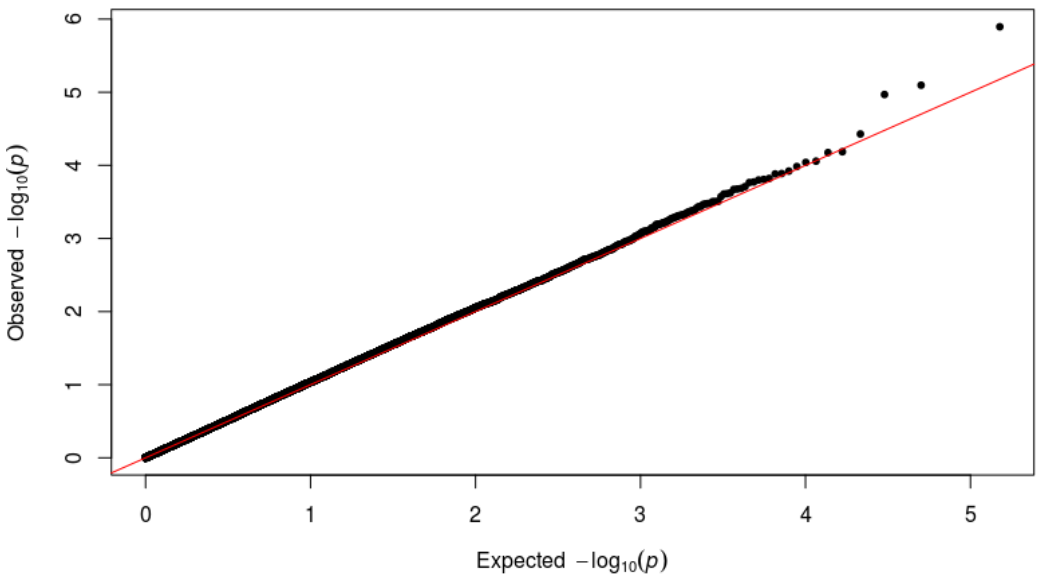

Figure S4. QQ plot. The QQ plot shows no evidence for inflation or bias in the association analysis of DNA methylation with "good" LEQ scores (Lambda = 1.04).

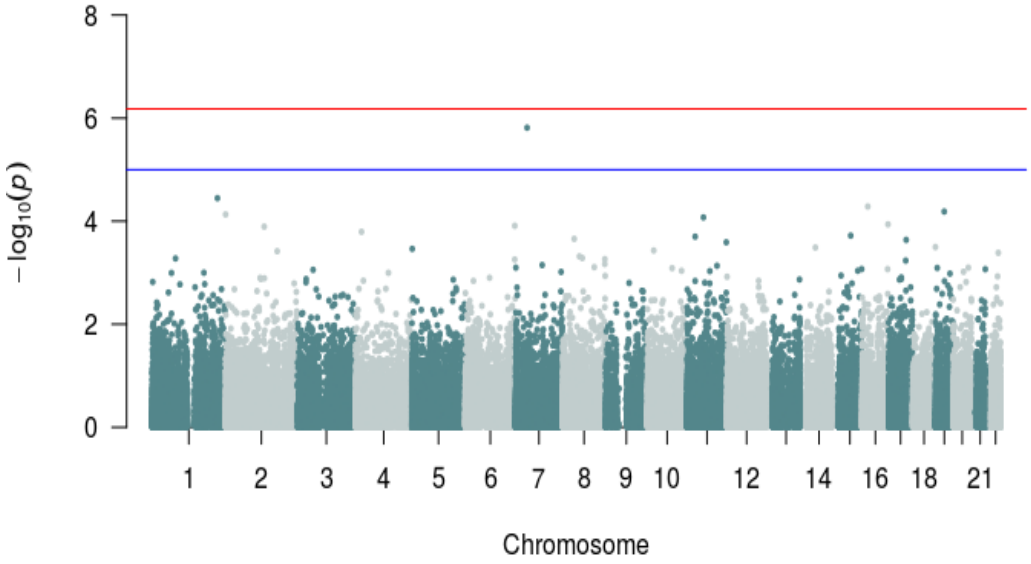

Figure S5. Manhattan plot. The Manhattan plot depicts the association between DNA methylation and "bad" LEQ scores ($n = 191$). The horizontal red line represents the epigenome-wide significant threshold for this study ($p < 6.6\times10^{-7}$) and the blue line represents a suggestive significance threshold ($p < 1.0\times10^{-5}$).

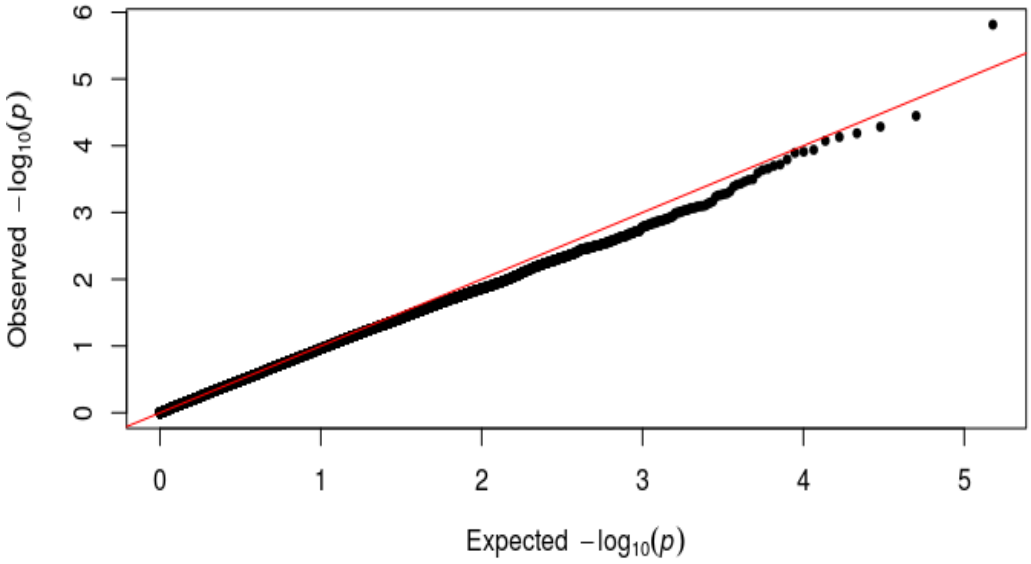

Figure S6. QQ plot. The QQ plot shows no evidence for inflation or bias in the association analysis of DNA methylation with "bad" LEQ scores (Lambda = 0.96).

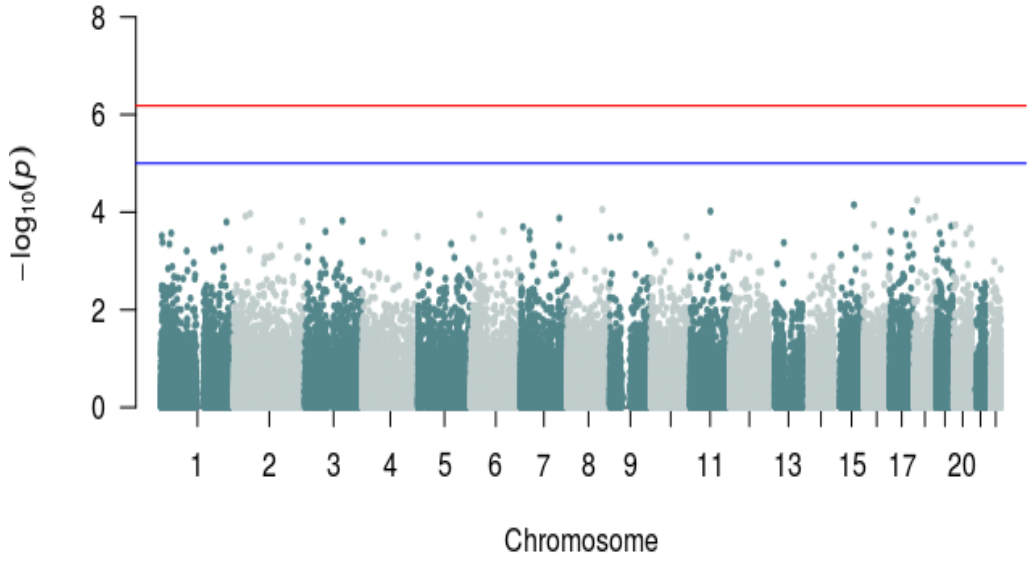

Figure S7. Manhattan plot. The Manhattan plot depicts the association between DNA methylation and the interaction between childhood trauma and total LEQ scores (n = 191). The horizontal red line represents the epigenome-wide significant threshold for this study ($p < 6.6\times10^{-7}$) and the blue line represents the suggestive significance threshold ($p< 1.0\times10^{-5}$).

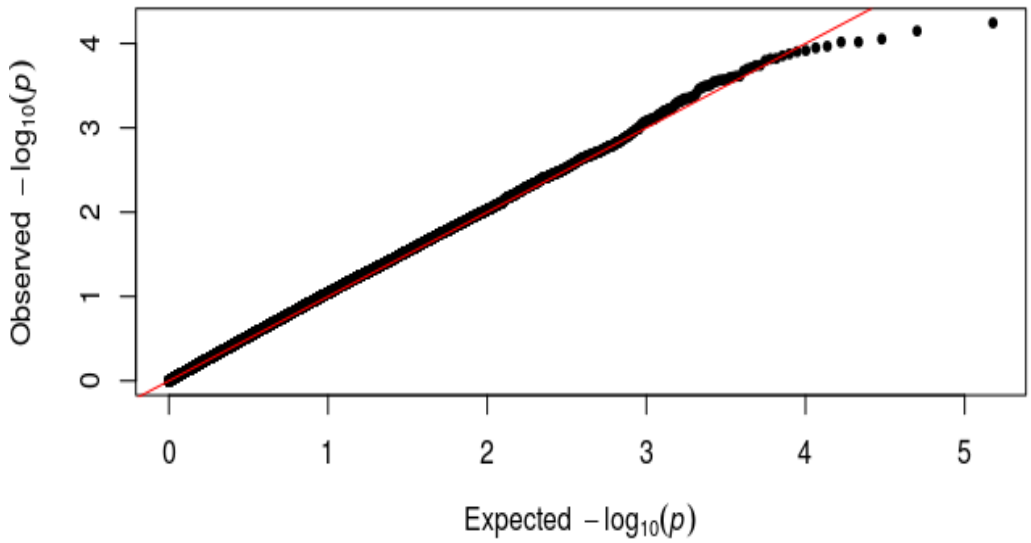

Figure S8. QQ plot. The QQ plot shows no evidence for inflation or bias in the association analysis of DNA methylation with the interaction between childhood trauma and total LEQ scores (Lambda = 1.10).

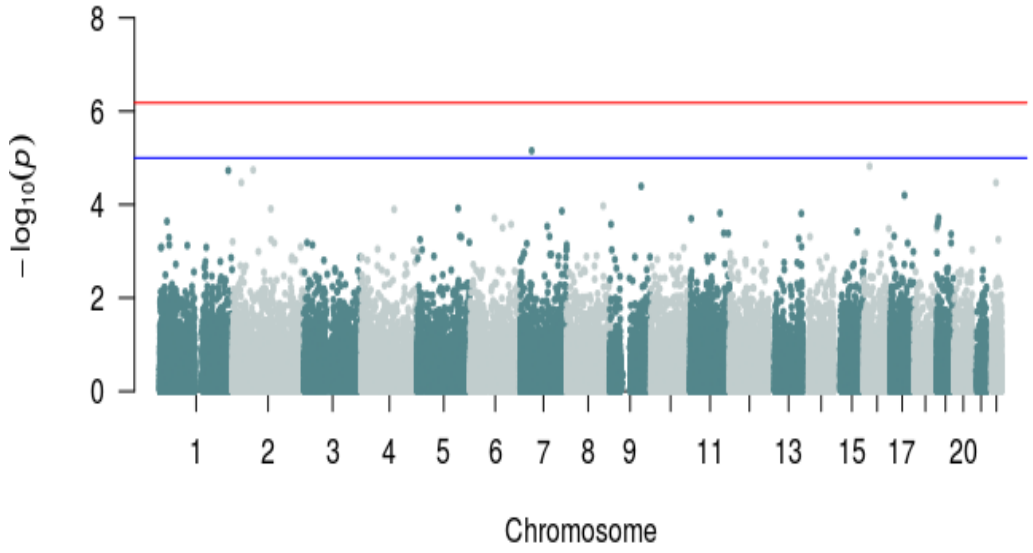

Figure S9. Manhattan plot. The Manhattan plot depicts a sensitivity analysis testing the association between DNA methylation and total LEQ scores after removing patients not taking psychotropic drugs at the time of testing ($n = 186$). The horizontal red line represents the epigenome-wide significant threshold for this study ($p < 6.6\times10^{-7}$) and the blue line represents the suggestive significance threshold ($p < 1.0\times10^{-5}$).

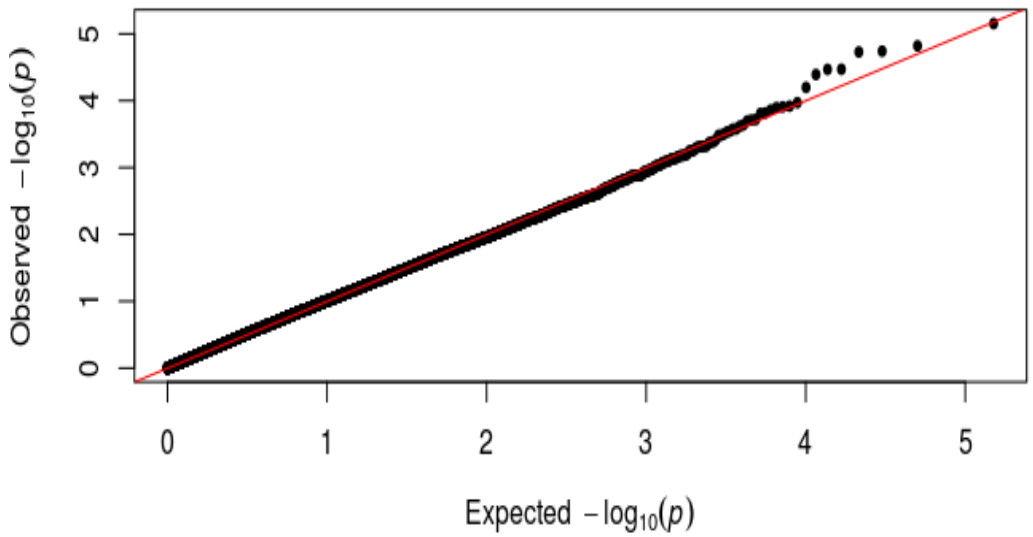

Figure S10. QQ plot. The QQ plot shows no evidence for inflation or bias in the association analysis of DNA methylation with total LEQ scores after removal of patients not on taking psychotropic drugs at the time of testing (Lambda = 1.06).

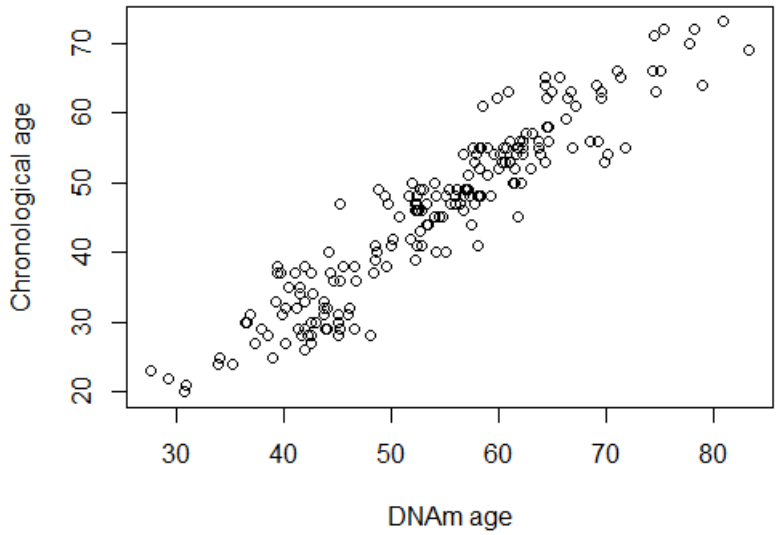

Figure S11. Scatterplot. The scatterplot illustrates the significant and positive correlation between DNA methylation age (DNAm age in years, calculated based on the Horvath algorithm) and chronological age (years) at baseline (Spearman's ρ = 0.94).

Table S3. GOmeth enrichment analysis

Biological process	N	DE	P.DE	FDR
homophilic cell adhesion via plasma membrane adhesion molecules	136	54	7.28E-05	1
spindle midzone assembly	8	6	0.001	1
cell-cell adhesion via plasma-membrane adhesion molecules	199	64	0.001	1
leukotriene biosynthetic process	10	6	0.002	1
exploration behavior	22	11	0.003	1
maintenance of unfolded protein	3	3	0.003	1
maintenance of unfolded protein involved in ERAD pathway	3	3	0.003	1
regulation of epithelial cell migration	154	41	0.004	1
myelination	87	26	0.004	1
vesicle targeting, trans-Golgi to endosome	2	2	0.004	1
regulation of endothelial cell migration	110	30	0.005	1
negative regulation of osteoclast development	5	4	0.006	1
ensheathment of neurons	90	26	0.006	1
axon ensheathment	90	26	0.006	1
positive regulation by host of viral transcription	9	5	0.007	1
cellular response to arsenic-containing substance	12	5	0.007	1
germinal center B cell differentiation	2	2	0.007	1
hormone-mediated apoptotic signaling pathway	3	3	0.007	1
positive regulation of axon extension	26	11	0.007	1
nervous system development	1747	369	0.008	1

Abbreviations: N – number of genes in the GO; DE – number of genes that are differentially methylated; P.DE – *p*-value for the over-representation of the GO term

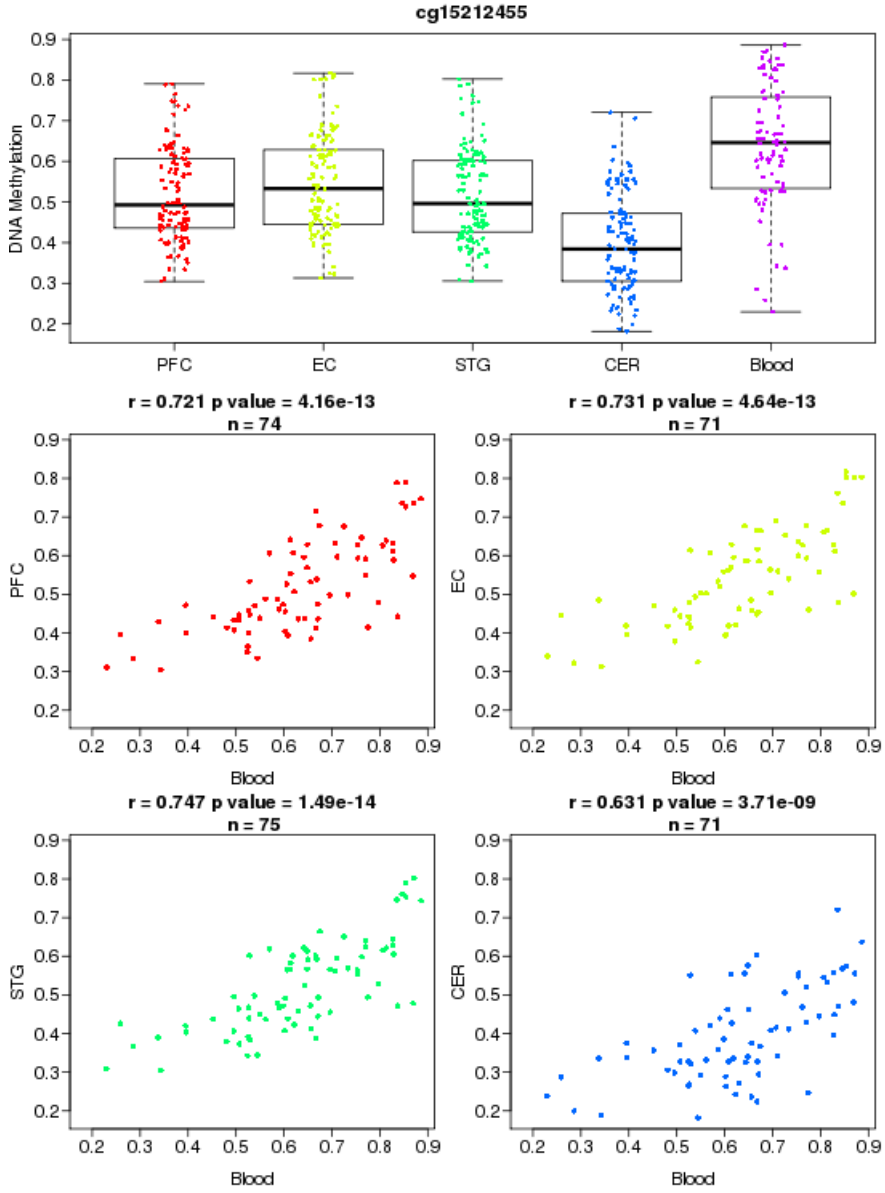

Figure S12. Blood-brain methylation correlation. Publicly available data from Hannon et al. was used to assess cg15212455 DNA methylation patterns across blood, the prefrontal cortex (PFC), entorhinal cortex (EC), superior temporal gyrus (STG), and cerebellum (CER).

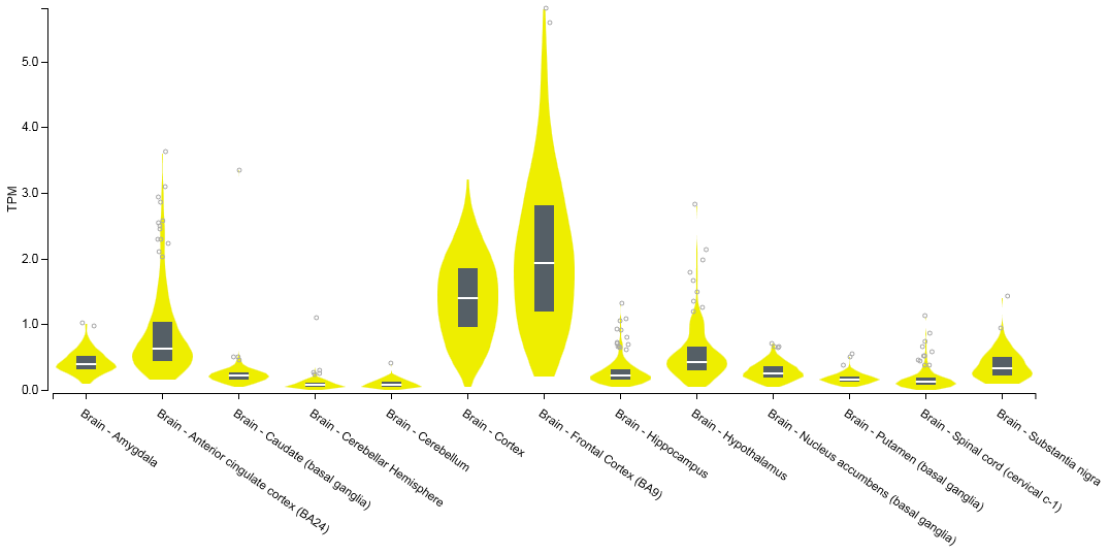

Figure S13. Gene expression patterns of *POU6F2* across multiple brain regions. The freely available Genotype-Tissue Expression (GTEx) Project Database portal (www.gtexportal.org) was used to determine the expression patterns of *POU6F2* across multiple tissues. The figure shows expression levels across multiple brain regions, showing the highest expression of *POU6F2* in the frontal cortex.

Original Article 2: Supplementary references

Hannon, E., Lunnon, K., Schalkwyk, L., & Mill, J. (2015). Interindividual methylomic variation across blood, cortex, and cerebellum: Implications for epigenetic studies of neurological and neuropsychiatric phenotypes. *Epigenetics, 10*(11), 1024-1032. doi:10.1080/15592294.2015.1100786

Acknowledgements

This thesis would not have been possible without the guidance and support from many individuals. I would like to express my sincerest gratitude to everyone who helped me in this endeavor.

To my supervisor, *Prof. Dr. Thomas G. Schulze*, for his continuous support of my PhD study and related research. His infectious enthusiasm and optimistic spirit motivated me to continue pushing forward. I am also grateful for the many opportunities he provided me to attend international conferences and workshops which greatly enriched my PhD work.

To *Prof. Dr. Dr. Elisabeth Binder* for her contributions as a member of my thesis advisory committee (TAC). I am appreciative of the expertise she shared and recommendations she made which allowed me to widen the scope of my PhD project. I am especially thankful for her invitation to attend a weekly data and journal club which allowed me to broaden my understanding of translational research in psychiatry and obtain valuable feedback from an interdisciplinary team. Thank you to the other members of my TAC, *Prof. Dr. Peter Falkai* and *Prof. Dr. Bertram Müller-Myhsok*, for their insightful suggestions and encouragement.

To the *IPPG team* for supporting me in many ways throughout the entirety of my PhD work, especially *Monika Budde*, *Dr. Urs Heilbronner*, and *Dr. Sergi Papiol* for their mentorship and critical feedback. Also, thank you to the PsyCourse study participants without whom this work would not have been possible.

To *Dr. Darina Czamara* for sharing her expertise on DNA methylation analyses. Her guidance was instrumental in conducting my first EWAS.

I am grateful to have taken part in the structured *International Max Planck Research School for Translational Psychiatry (IMPRS-TP)*. The infrastructure provided by IMPRS-TP ensured the successful completion of this PhD work.

Thank you to my *friends*, for picking up the phone when I needed it most and always knowing the right thing to say to support me on this journey. To my *family*, both near and far, for your love, reassurance, and never-ending faith in my abilities. And to *Sven*, for providing me the encouragement, confidence, and motivation I needed to finish what I started.

Affidavit

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I hereby declare, that the submitted thesis entitled

Understanding the Involvement of Environmental Exposures, Genetic Risk, and Epigenetic Mechanisms in the Course of Severe Mental Illness

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

München, 22.07.2020	Ashley Lynn Lütticke
Place, Date	Signature doctoral candidate

Confimation of congruency between printed and electronic version of the doctoral thesis

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I hereby declare that the electronic version of the submitted thesis, entitled

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is congruent with the printed version both in content and format.

München, 22.07.2020 — Ashley Lynn Lütticke

Place, Date — Signature doctoral candidate

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