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Clustering Schizophrenia Genes by Their Temporal Expression Patterns Aids Functional Interpretation

Dennis van der Meer[*,1,2], Weiqiu Cheng[1], Jaroslav Rokicki[1,3], Sara Fernandez-Cabello[1], Alexey Shadrin[1], Olav B. Smeland[1], Friederike Ehrhart[2], Sinan Gülöksüz[2,4], Lotta-Katrin Pries[2], Bochao Lin[2], Bart P. F. Rutten[2] Jim van Os[2,5], Michael O'Donovan[6], Alexander L. Richards[6], Nils Eiel Steen[1], Srdjan Djurovic[7,8], Lars T. Westlye[1,3,9], Ole A. Andreassen[1,9], and Tobias Kaufmann[1,10,11] on behalf of Genetic Risk and Outcome of Psychosis investigators (GROUP)[12] and on behalf of European Network of National Schizophrenia Networks Studying Gene-Environment Interactions Work Package 6 (EU-GEI WP6)[12]

[1]Division of Mental Health and Addiction, Norwegian Centre for Mental Disorders Research (NORMENT), Oslo University Hospital & Institute of Clinical Medicine, University of Oslo, Oslo, Norway; [2]Faculty of Health, Medicine and Life Sciences, School of Mental Health and Neuroscience, Maastricht University, Maastricht, The Netherlands; [3]Department of Psychology, University of Oslo, Oslo, Norway; [4]Department of Psychiatry, Yale University School of Medicine, New Haven, CT; [5]Department of Psychiatry, Utrecht University Medical Centre, Utrecht, The Netherlands; [6]Division of Psychological Medicine and Clinical Neurosciences, Cardiff University Centre for Neuropsychiatric Genetics and Genomics, School of Medicine, Cardiff University, Cardiff, UK; [7]Department of Medical Genetics, Oslo University Hospital, Oslo, Norway; [8]Department of Clinical Science, Norwegian Centre for Mental Disorders Research, University of Bergen, Bergen, Norway; [9]K.G. Jebsen Centre for Neurodevelopmental Disorders, University of Oslo, Oslo, Norway; [10]Department of Psychiatry and Psychotherapy, Tübingen Center for Mental Health, University of Tübingen, Tübingen, Germany; [11]German Center for Mental Health (DZPG), Tübingen, Germany

12Full list of consortium authors and affiliations given in the acknowledgements.

*To whom correspondence should be addressed; Kirkeveien 166, 0450, Oslo, Norway; tel: +31638210393, e-mail: d.v.d.meer@medisin.uio.no

***Background*: Schizophrenia is a highly heritable brain disorder with a typical symptom onset in early adulthood. The 2-hit hypothesis posits that schizophrenia results from differential early neurodevelopment, predisposing an individual, followed by a disruption of later brain maturational processes that trigger the onset of symptoms. *Study design*: We applied hierarchical clustering to transcription levels of 345 genes previously linked to schizophrenia, derived from cortical tissue samples from 56 donors across the lifespan. We subsequently calculated clustered-specific polygenic risk scores for 743 individuals with schizophrenia and 743 sex- and age-matched healthy controls. *Study results*: Clustering revealed a set of 183 genes that was significantly upregulated prenatally and downregulated postnatally and 162 genes that showed the opposite pattern. The prenatally upregulated set of genes was functionally annotated to fundamental cell cycle processes, while the postnatally upregulated set was associated with the immune system and neuronal communication. We found an interaction between the 2 scores; higher prenatal polygenic risk showed a stronger association with schizophrenia diagnosis at higher levels of postnatal polygenic risk. Importantly, this finding was replicated in an independent clinical cohort of 3233 individuals. *Conclusions*: We provide genetics-based evidence that schizophrenia is shaped by disruptions of separable biological processes acting at distinct phases of neurodevelopment. The modeling of genetic risk factors that moderate each other's effect, informed by the timing of their expression, will aid in a better understanding of the development of schizophrenia.**

Key words: schizophrenia/gene expression/2-hit hypothesis/polygenic risk score/cortical tissue/neurodevelopment

Introduction

Schizophrenia is a severe disorder that is highly heritable,[1–3] determined by a large number of interacting genetic and environmental factors.[4] Its clinical onset is typically in early adulthood, yet there are behavioral and biological indicators present many years before onset,[5] with deviations of neurodevelopmental trajectories early in life.[3]

Schizophrenia is a dynamic and heterogeneous disorder, and the early development and clinical course of the illness reflects many biological processes interacting over time. The classic 2-hit hypothesis of schizophrenia

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posits that the clinical phenotype stems from a combination of early-acting risk factors that predispose an individual, followed by a second "hit" at a later stage of development that leads to the onset of symptoms and subsequent diagnosis.[6] The first, priming hit is thought to disrupt neurogenesis and differentiation in early neurodevelopmental phases, while the second hit may involve processes more related to neuroplasticity.[7] Inflammatory processes have been implicated in this chain of events, with several lines of evidence indicating a link between the immune system and the development of schizophrenia,[8] albeit an enigmatic one.[9] Given the complex etiology and heterogeneous clinical manifestation of schizophrenia, the 2-hit hypothesis is undoubtedly an oversimplification that bins numerous biological processes and their waxing and waning effects over time. Yet, the underlying notion that risk factors act primarily at different stages of development, moderating each other's influence on neurodevelopmental trajectories, represents an often-overlooked developmental dynamic dimension, which can explain some of the observed etiological and clinical heterogeneity of schizophrenia.

Genome-wide association studies (GWAS) of schizophrenia have discovered hundreds of associated common genetic variants, mapped onto genes expressed primarily in the brain that are important for synaptic functioning.[10] The effects of genetic variants on brain morphology have been shown to be age dependent,[11] as also suggested by strong changes in gene expression levels over the lifespan.[12] Furthermore, genes that are expressed together are more likely than random pairs of genes to participate in the same functional processes.[13] This indicates that sets of genes defined by their temporal patterns of expression might reflect biological processes relevant for the risk and clinical course of schizophrenia at distinct phases of disease development. Here, we clustered genes previously found to be significantly associated with schizophrenia into 2 groups based on their age-associated expression patterns, allowing us to infer dynamic processes involved in schizophrenia.

Methods

All data processing and statistical analyses were carried out through R v4.1.0,[14] unless specified otherwise, with code available via https://github.com/norment/open-science.

Selection of Risk Genes

We used the summary statistics from the Psychiatric Genomics Consortium, wave 3 (PGC3) schizophrenia GWAS[10] to identify schizophrenia risk genes, restricted to the European cohorts. We selected a version of the meta-analyzed summary statistics excluding the Thematically Organised Psychosis (TOP) sample, preventing sample overlap. This version contained 13 025 668 single nucleotide polymorphisms (SNPs) for 50 965 individuals with schizophrenia and 68 049 controls.

We selected all genes that showed a significant association with schizophrenia based on the GWAS summary statistics, as determined by gene-based tests using MAGMA v1.08 with default settings.[15] This entails the application of a SNP-wide mean model and use of the 1000 Genomes Phase 3 EUR reference panel. For schizophrenia, 508 genes had a *P*-value smaller than 0.05/19 047, surviving multiple comparisons correction for the total number of protein-coding genes tested. Of these, 431 had a probe in the brain expression dataset, and of those 345 were protein-coding genes with evidence of being expressed in the brain, as summarized by the human protein atlas project (www.proteinatlas.org).[16] We chose gene-based tests over locus mapping as the downstream analyses were all gene- rather than variant-centric, with these tests thereby aggregating a greater amount of information for the purpose of identifying the most relevant genes. Integration of information on expression quantitative trait loci (eQTLs) and chromatin interactions may allow for further improvements in the identification of genes involved based on GWAS data. However, recently introduced tools that enable this, such as e-MAGMA[17] or H-MAGMA,[18] work with annotation files based on, eg, fetal or adult tissue. This age-specificity precludes their use in this study, as they would bias the results.

For Alzheimer's disease,[19] attention deficit hyperactivity disorder (ADHD),[20] major depressive disorder (MDD),[21] autism spectrum disorder (ASD),[22] and bipolar disorder[23] we made use of the latest GWAS summary statistics, as described in the references. For bipolar disorder, we also ensured to use data with the TOP sample excluded. We used the same steps to select significant genes as described for schizophrenia.

Gene Expression Data Processing

We made use of gene expression data derived from brain tissue from 55 clinically unremarkable donors ranging in age from 8 weeks postconception to 82 years.[24] We took the transcriptome data as preprocessed by Kang *et al.*, downloadable via the Gene Expression Omnibus public archive under accession number GSE25219. This is genome-wide exon-level RNA data, obtained via an Affymetrix GeneChip Human Exon 1.0 ST array, which has undergone extensive quality control, outlier detection, and normalization procedures,[24] leaving $\log_2$-scaled signal intensity levels. For each gene, we selected the probe with the highest differential stability by calculating the mean Spearman's correlation coefficient across pairs of donors and regions,[25] leaving n = 16 660 probes. Click or tap here to enter text. Given the relatively high homogeneity of expression patterns across

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cortical brain samples,[26] we subsequently averaged over 11 cortical regions (A1C, IPC, M1C, S1C, V1C, DFC, ITC, STC, MFC, OFC, and VFC), within donor, and scaled the expression values, within probe, across donors, to a range between 0 (lowest observed value) and 100 (highest observed value). For visualization, we converted the age of the donors into days since conception and applied a $\log_{10}$ scaling, as well as using the developmental stage categorization originally proposed by Kang *et al.*[24]

Clustering of the Expression Data

We first checked for the optimal number of components among the scaled expression data of the schizophrenia gene set, using the NbClust R package with distance set to Euclidean and method set to ward.D2.[27] Across a range of indices, there was most support for a 2-component solution,[7] followed by a four[6] and three[5] component solution, see Supplementary figure 1. In line with our aim to test the 2-hit hypothesis of schizophrenia and in accordance with the majority rule, we selected the 2-component solution. We then applied hierarchical clustering, through the *hclust* function, to the Euclidian distance matrix of this expression data, with the ward.D2 method.

Functional Annotation

Functional annotation of the clusters was achieved by uploading the gene lists to Reactome (https://reactome.org/), and running the over-representation analysis with default settings. This entails hypergeometric tests, checking for the enrichment of the schizophrenia risk genes, clustered based on cortical tissue expression trajectories, among all Reactome pathways. These pathways are formed based on information about biological interactions between molecules and curated by experts. A list of all enriched pathways, significant after multiple comparisons correction through false discovery rate, together with the number of molecules matched compared with the total number of molecules, is provided in Supplementary table 2. Over-representation analyses through hypergeometric tests were further carried out with the *goana* and *topGO* functions, part of the *limma* R package, checking for enrichment among all Entrez gene IDs associated with at least one of 22.749 Gene Ontology processes, as listed in the Molecular Signatures Database (MsigdB; v7.1).

Plotting of the mean expression over time per gene set was done with *ggplot2* in R v4.0.3., with geom_smooth(method="gam") using default settings.

To determine the association between the schizophrenia gene clusters and other brain disorders, we employed competitive gene-set analysis through MAGMA, applied to the respective GWAS summary statistics. This is a linear regression using genes as data points, with the *P*-value resulting from a test whether the mean association of the genes in the set with the outcome is greater than that of genes not in the set.[15]

Polygenic Scoring Cohort Descriptions

We selected data from individuals with European ancestry admixture, from the TOP clinical cohort, for the polygenic scoring analyses. We had complete genetic and covariate data available for 743 individuals with schizophrenia (mean age 32.76 years, *SD* = 13.29; 42.80% female) and 1074 healthy individuals. The healthy individuals were then matched to those with schizophrenia on age and sex, using default settings of the MatchIt package, keeping 743 healthy individuals (mean age 31.66 years, *SD* = 11.04; 42.66% female). DNA samples obtained from blood or saliva were sent for genotyping at deCODE Genetics, Reykjavik, Iceland using Illumina Infinium genotyping arrays. Quality-controlled genotypes were phased using Eagle, and missing variants were imputed with MaCH using Haplotype Reference Consortium (HRC) reference panel. Conventional genetic QC procedures were carried out in PLINK and as described in detail previously.[28] European ancestry admixture was based on self-report. Diagnostic and Statistical Manual (DSM)-IV diagnosis of schizophrenia was determined based on the Structured Clinical Interview for DSM-IV Axis I Disorders, carried out by trained physicians or clinical psychologists. Controls were individuals without brain damage or a lifetime history of a severe psychiatric disorder themselves or in first-degree relatives. Each sample was collected with the participants' written informed consent. The Oslo Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate gave ethical approval for this study.

Data for replication of the polygenic scoring analyses was provided by work package 6 of the European Network of National Networks studying Gene-Environment Interactions in Schizophrenia (EUGEI)[29] and the Genetic Risk and Outcome of Psychosis (GROUP) study within the EUGEI.[30] Data were collected between 2010 and 2015 in the Netherlands, Turkey, Spain, and Serbia. We had complete genetic and covariate data available for 1689 individuals with European ancestry admixture with schizophrenia (mean age 31.54 years, *SD* = 8.96; 29.78% female) and 1544 unrelated healthy individuals (mean age 33.43 years, *SD* = 10.62; 50.65% female). Samples of all individuals were genotyped at Cardiff University Institute of Psychological Medicine and Clinical Neurology, using custom Illumina HumanCoreExome-24 BeadChip genotyping arrays. Genotypes were imputed via the Michigan Imputation Server using the HRC reference panel. Pre- and post-imputation quality control procedures were conducted in PLINK, as described in detail previously.[29] European ancestry admixture was based on self-report. DSM-IV-TR diagnosis of a schizophrenia spectrum disorder was determined. The diagnosis was

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later confirmed by the Operational Criteria Checklist for Psychotic and Affective Illness in the EUGEI WP6, and the Schedules for Clinical Assessment in Neuropsychiatry or the Comprehensive Assessment of Symptoms and History in GROUP. Controls were unrelated individuals without a lifetime history of a severe psychiatric disorder. Participants were excluded if they had a diagnosis of psychotic disorder due to another medical condition, a history of head injury with loss of consciousness, or an intelligence quotient <70. Each sample was collected with the participants' written informed consent. The projects were approved by the medical ethics committees of all participating sites and conducted in accordance with the Declaration of Helsinki.

Polygenic Score Analyses

We used the PRSet functionality of PRSice-2 to make set-specific polygenic scores.[31,32] These scores were based on the effect sizes of lead SNPs within the genomic boundaries of the genes that made up the 2 sets, with these boundaries being based on genome build GTCh37. We included a minor allele frequency filter of 0.05.

To analyze the association between the set-specific polygenic scores and schizophrenia, we used logistic regression, with diagnosis as the outcome measure and the 2 polygenic scores, as well as age, sex, and 20 genetic principal components as the predictors. We also included an interaction term between the 2 cluster-specific polygenic scores. See Supplementary table 4 for the full model. This model was the same for the replication cohort, with the addition of a covariate for the acquisition site. We meta-analyzed the results from the discovery and replication analyses through the "metaphor" R package,[33] inputting the log odds and standard errors, and applying default settings.

Results

First, we sought to identify genes associated with schizophrenia, regardless of the timing of their expression. To this end, we used gene-based tests[34] aggregating effects of variants across each gene, applied to the European-specific PGC schizophrenia GWAS wave 3 summary statistics.[10] Out of the 508 significant genes identified through this procedure, 345 protein-coding genes had brain expression data available from 55 clinically unremarkable donors ranging from 56 days postconception up to 82 years in age.[12,25] The mean expression across these 345 genes and overall cortical brain samples was highest prenatally, in line with the framing of schizophrenia as a neurodevelopmental disorder.[3,35] For comparison, we also calculated the mean expression across 82 genes associated with Alzheimer's disease,[19] ie, a neurodegenerative disease affecting older people, as well as across all 16 660 genes with available expression data, both of which showed a distinct pattern with higher peaks of expression postnatally (figure 1a).

We applied hierarchical clustering to the data, grouping the putative schizophrenia genes by similarity of the scaled expression patterns over time, through Ward clustering[36] based on Euclidian distances. We selected a 2-cluster solution, in line with the 2-hit hypothesis and as deemed optimal by clustering indices (Supplementary figure 1; the list of genes per cluster is provided in Supplementary table 1). The distinction between the 2 clusters of genes, based on their mean expression over time, can be most clearly characterized as being predominantly expressed prenatally (peak early in pregnancy) vs postnatally (peak in adulthood) (figure 1b). This pattern remained identical when the numbers of genes in both clusters was equalized (Supplementary figure 2). Analyses of differential expression of the genes through the FUMA pipeline[37]

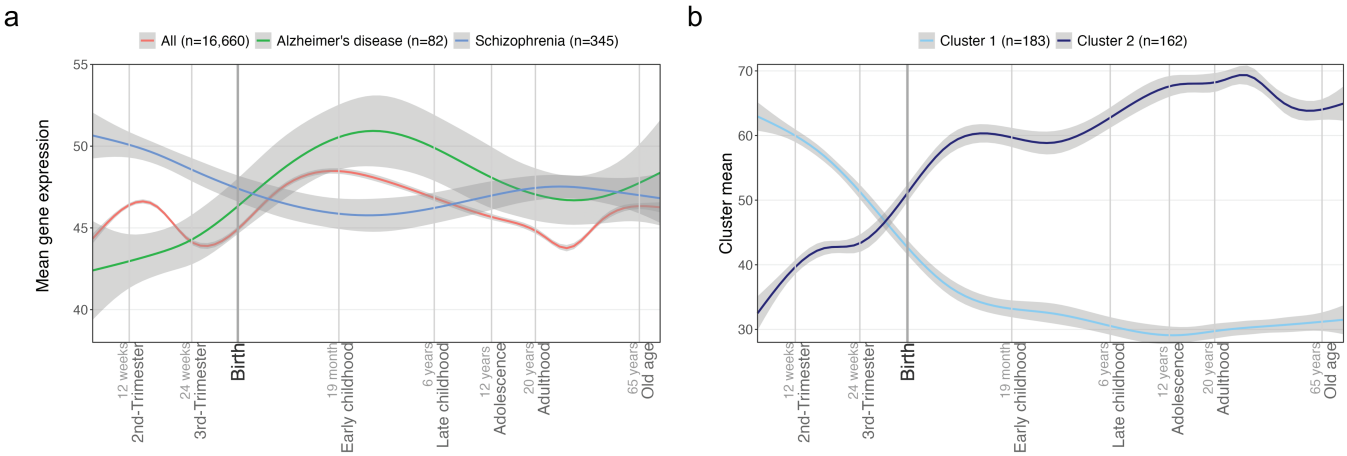

Fig. 1. Mean cortical gene expression over the lifespan. (a) Mean expression (*y*-axis) over time (*x*-axis) for genes significantly associated with schizophrenia (SCZ), Alzheimer's disease (AD), or all genes, as indicated in the legend. (b) Mean expression over time, for both of the 2 sets of genes identified through hierarchical clustering applied to the expression of the schizophrenia genes. Lines were fitted through generalized additive modeling, with the gray shading reflecting the standard error 0.95 confidence interval.

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recapitulated these findings, indicating highly significant (minimum $P = 8.4 \times 10^{-10}$) upregulation in prenatal brain tissue and downregulation postnatally for the first cluster, and vice versa (minimum $P = 1.1 \times 10^{-11}$) for the second cluster, see Supplementary figure 3. As such, this result indicates that gene sets can be formed and distinguished from each other based on their cortical expression trajectories. We will refer to the 2 gene sets as the pre- and postnatal cluster.

The 2 clusters of schizophrenia risk genes expressed in brain tissue were associated with distinct biological processes, as indicated by over-representation analyses among all Reactome pathways[38] and Gene Ontology terms.[39] On the highest level of hierarchy of Reactome, the prenatal cluster was significantly associated with chromatin organization ($P = 5.0 \times 10^{-7}$), reproduction ($P = 3.3 \times 10^{-6}$), DNA replication (6.0×10^{-5}), DNA repair ($P = 2.4 \times 10^{-4}$), cell cycle ($P = 3.2 \times 10^{-4}$), and cellular responses to stimuli (7.7×10^{-4}). The postnatal cluster on the other hand was associated with the immune system ($P = 2.3 \times 10^{-4}$), disease ($P = 2.7 \times 10^{-4}$), and the neuronal system ($P = 4.6 \times 10^{-4}$). This is visualized in Supplementary figure 4, with the full list of enriched terms at lower levels of the hierarchy provided in Supplementary table 2. In agreement with this, the most significant enrichment among 22 749 individual Gene Ontology terms were "regulation of DNA-templated transcription" ($P = 1.2 \times 10^{-8}$) for the prenatal cluster and "neuron projection" ($P = 5.2 \times 10^{-12}$) for the postnatal cluster, see Supplementary table 3 for a list of the most enriched terms per cluster. In order to check whether the clustering truly aided functional interpretation, we randomly selected 5000 subsets of genes of the same size as the 2 clusters ($n = 183$ and $n = 162$) from either all protein-coding genes ($N = 19\,427$) or from the set of schizophrenia risk genes ($N = 345$). We then performed the same gene-set enrichment analyses on each of these random sets and recorded the smallest observed P-value for each of the 5000 runs. The results, displayed in figure 2, indicated that the enrichment reached for the pre- and postnatal cluster were far above the chance level in either case. Therefore, clustering schizophrenia risk genes based on their age-associated expression patterns aided in identifying distinct biological processes.

Using competitive gene-set analyses through MAGMA, applied to the latest GWAS summary statistics, we checked the association of the 2 gene clusters with other brain disorders. As shown in figure 3, these clusters of schizophrenia risk genes were significantly associated with several of the brain disorders, albeit to differing degrees. Most notable is that MDD was only highly significantly associated with the postnatal cluster, not the prenatal, suggesting that its genetic overlap with schizophrenia[40,41] is mostly driven by genes expressed postnatally. In contrast, the data from the ASD GWAS revealed

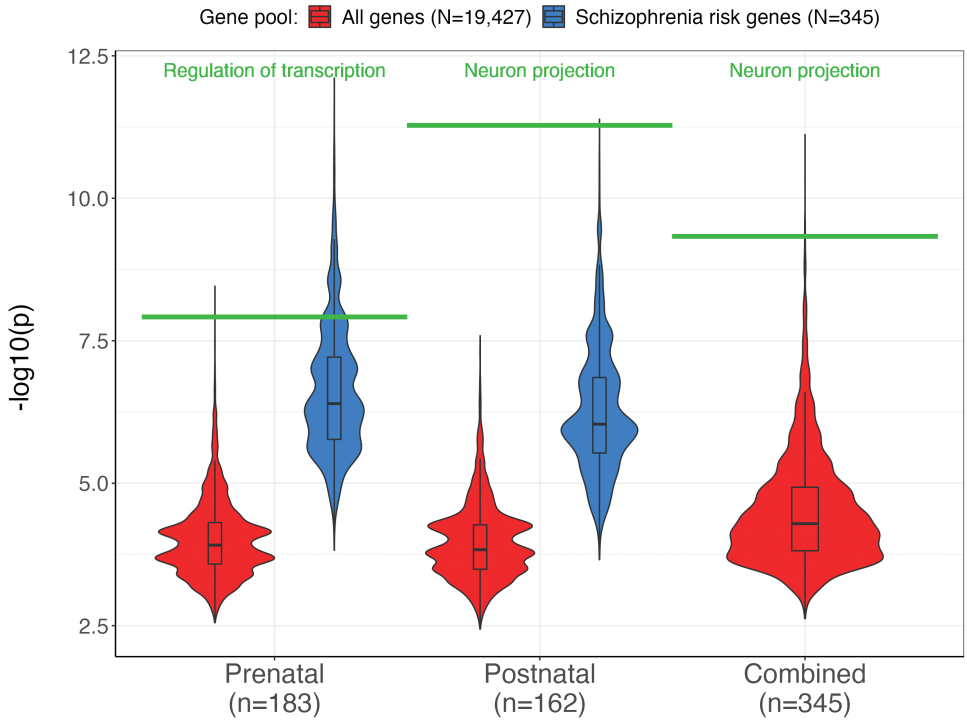

Fig. 2. Functional annotation of the 2 gene clusters. Significance (y-axis) of the most significant Gene Ontology pathway for each of 5000 randomly drawn sets of genes of the same size as either the prenatal, postnatal, or both combined (x-axis). For each of the groupings, left violins show the $-\log_{10}$(P-value) distribution for random gene sets when drawn out of the entirety of protein-coding genes, and right violins show this distribution for random gene sets when drawn out of the smaller pool of schizophrenia genes. The green horizontal line indicates the $-\log_{10}$(P-value) of the most significant pathway, listed at the top, for the true pre- and postnatal gene sets.

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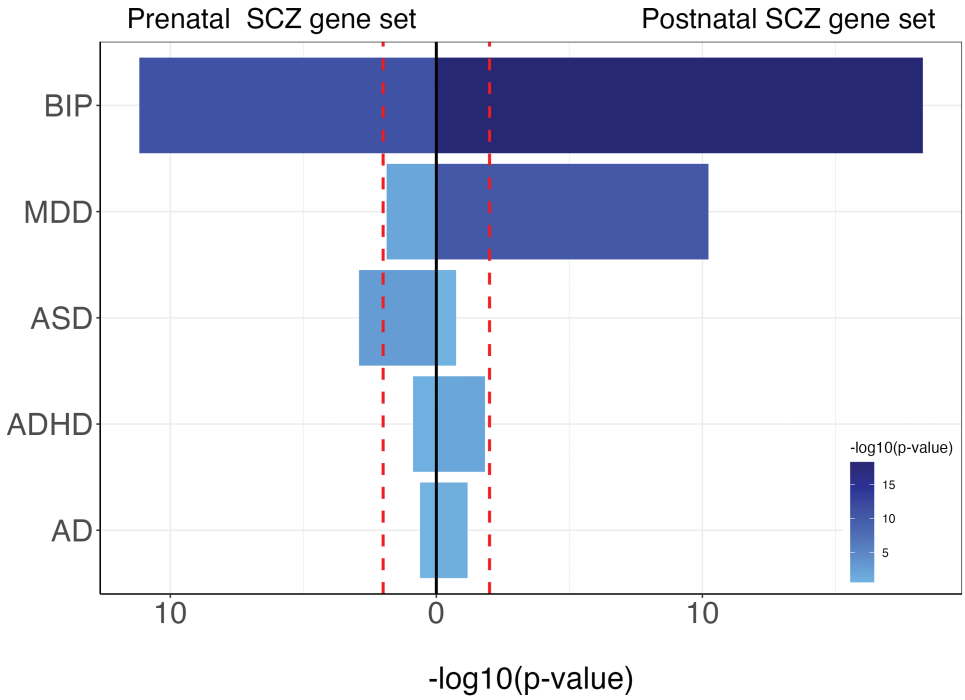

Fig. 3. Association of brain disorders (*y*-axis) with the sets of schizophrenia genes expressed prenatally (left) or postnatally (right), with the *x*-axis indicating significance in $-\log_{10}$(*P*-value) of the gene-set analyses. The fill also reflects significance, as shown in the legend, with the vertical dotted lines indicating the Bonferroni significance threshold at $P = .01$. *Note*: AD, Alzheimer's disease; ADHD, attention deficit hyperactivity disorder; ASD, Autism spectrum disorder; BIP, bipolar disorder; MDD, major depressive disorder; SCZ, schizophrenia.

the opposite pattern, ie, ASD was associated with the prenatal gene cluster but not the postnatal cluster, in line with its early onset.

Next, we constructed cluster-specific schizophrenia polygenic scores by clumping only variants within genes belonging to either cluster, using PRSet.[32] We tested for associations with both cluster scores in data from 743 individuals with European ancestry admixture with schizophrenia and 743 age- and sex-matched healthy controls using logistic regression, with diagnosis as an outcome measure and the 2 mean-centered polygenic scores, as well as mean-centered age, sex, and 20 genetic principal components as the predictors. We included an interaction term between the 2 cluster-specific polygenic scores to test the 2-hit hypothesis, that the occurrence of schizophrenia can be explained by the joint effect of 2 temporally distinct biological processes. We found that there was indeed a significant interaction effect ($\beta = 0.11$, $P = .02$), as visualized in figure 4a, such that the association of the prenatal cluster score (conditional $\beta = 0.16$, $P = .004$) with schizophrenia diagnosis was positively moderated by the postnatal score (conditional $\beta = 0.17$, $P = .003$). The full output of the model is listed in Supplementary table 4. Supplementary figure 4 shows the normal distribution of scores split by diagnosis. The goal of this analysis was not to optimize prediction, but rather to provide evidence for the presence of interacting sets of genes, ie, gain mechanistic insight. Nagelkerke R^2 for the prenatal set of genes was 1.0%, when the postnatal gene set was added to the model this increased to 1.7% variance explained beyond the null model, and when the interaction term was added, this was 2.2% ($P = 1.8 \times 10^{-5}$). Figure 4b further shows the odds ratio for individuals with different genetic risk loads, by dividing them into quintiles based on their polygenic risk scores for either the pre- or postnatal set or their product.

To enhance confidence in the finding, we sought out an independent clinical cohort for replication, consisting of 1649 individuals with a schizophrenia diagnosis and 1533 control subjects, with European ancestry admixture. After calculating the 2 set-specific polygenic scores in the exact same manner as for the discovery analysis, we ran a logistic regression controlling for the same covariates, as well as for acquisition site, on schizophrenia diagnosis. The pattern of results matched that of the discovery sample, with both the interaction and conditional effects being significant and in the same direction, see figure 4b. The full output of this replication is provided in Supplementary table 3. To further establish the pattern of results, we meta-analyzed the results across both studies. The outcome of this is summarized in figure 4c.

As a final check of robustness, we repeated the analyses after excluding all genes in the major histocompatibility complex (MHC) region, given that the high linkage disequilibrium (LD) across this region impedes precise estimation of the source of genetic signal. The pattern of results

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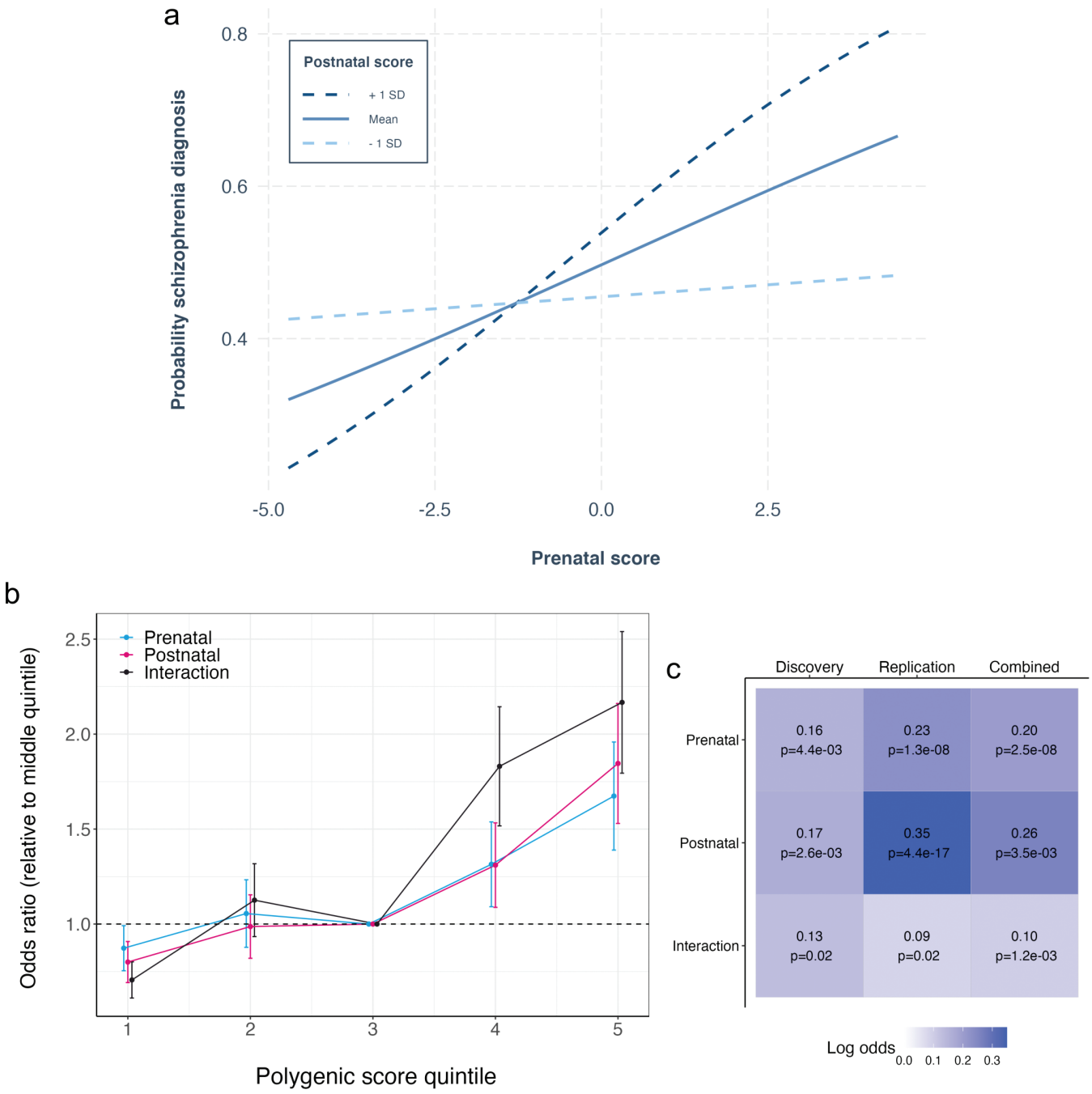

Fig. 4. Interaction between the set-specific polygenic scores. (a) Association between the prenatal polygenic score (*x*-axis) and schizophrenia diagnosis (*y*-axis), moderated by the postnatal polygenic score (line type and color). SD, standard deviation. (b) Visualization of the schizophrenia odds ratios (*y*-axis) as a function of polygenic score quintiles (*x*-axis) for the prenatal and postnatal sets of genes, as well as the product of these scores (lines), relative to the middle quintile. Vertical bars indicate standard error. (c) Summary of the log odds and significance of the terms (*y*-axis) per analysis (*x*-axis).

following the removal of this region was highly similar to that described above, with a clear pre- and postnatal division of genes (Supplementary figure 6) and a significant interaction between the set-specific polygenic scores on schizophrenia diagnosis in both the discovery and replication samples (Supplementary figure 7). Removal of MHC genes did lead to a more specific annotation of the postnatal set of genes with neuronal communication, as the coupling with immune system processes was no longer present (Supplementary figure 8).

Discussion

The 2-hit hypothesis posits that the risk and clinical course of schizophrenia are dependent on the joint effects of temporally distinct biological processes occurring

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during different phases of development. Here, we clustered genes associated with schizophrenia based on their age-dependent expression patterns, and showed that the genes belonging to the resulting 2 clusters are transcribed predominantly prenatally vs postnatally. Through functional annotation, we discovered that such a division contributes to the characterization of the biological processes involved in schizophrenia. We additionally provide evidence that variation in postnatally expressed genes moderated the effects of variation in prenatally expressed genes on schizophrenia, a finding that we replicated in an independent sample. These results illustrate the importance of integrating temporal dynamics of schizophrenia into genetic research.

We found that putative schizophrenia risk genes implicated by common genetic variants are expressed above average early in life, in line with the neurodevelopmental conceptualization of the disorder,[3,35] and corroborating findings of other gene expression studies.[42] Our clustering approach further divided schizophrenia genes into sets that are predominantly expressed prenatally or postnatally. Importantly, for both sets, functional annotation reached greater significance than when taking random subsets of genes of the same size from the total set of schizophrenia genes. This shows that the division based on estimated age-associated expression patterns succeeded in capturing distinct processes. The prenatally expressed set of genes was functionally annotated to fundamental cell cycle processes. Fitting the hypothesized first hit, perturbations of such early processes are likely to affect neurodevelopmental trajectories, increasing the likelihood of dysfunctional neural circuits that mature in adolescence, which may underlie the onset of symptoms.[43] Annotation of the postnatally expressed set of genes indicated they are primarily involved in neuronal communication, corroborating most recent findings and knowledge about the important role for synaptic pruning in the etiology of schizophrenia, taking place during childhood and adolescence.[44,45] This also fits with the identification of other significant pathways that are in line with the longstanding characterization of schizophrenia as a disorder of synaptic functioning and dysconnectivity.[10,46] We note hereby that enrichment analyses tend to identify larger gene sets with limited specificity, impeding insight into the molecular mechanisms involved. Future studies may complement current approaches to gene set analyses with novel techniques that account for pathway size.[47]

Comorbidity of schizophrenia with a range of other disorders is likely to be partly due to the central role of the biological processes captured by the 2 sets of genes in neurodevelopment. While bipolar disorder was strongly associated with both sets, ASD was only associated with the prenatal cluster, and MDD only with the postnatal cluster, which may be informative with regard to characterizing their comorbidity with schizophrenia. The observed pattern of associations of these gene sets with disorders that vary in their onset is thereby in line with the notion that schizophrenia results from a combination of dysfunction in both early and later-in-life brain maturation processes.

By combining information from GWAS with expression data from clinically unremarkable donors across the lifespan, this study complements work focusing on genes with differential expression between cases and controls,[48] as those may overlook the effects of genes acting early, before diagnosis, and be confounded by secondary disease processes. Indeed, previous work has found that gene expression differs between individuals in different clinical stages of schizophrenia.[49] Our results are thereby in accordance with the 2-hit hypothesis of schizophrenia, that differential brain maturation exacerbates earlier insults to the system to bring about the disorder. We provide genetics-based evidence of this hypothesis, captured by an interaction term between the set-specific polygenic scores; while the "hits" are generally assumed to be environmental due to the temporal dimension, they may also be partly genetic, considering that genes exert their effects at specific times depending on when they are expressed. The amount of expression, and its impact on molecular processes downstream, can thereby be moderated both by environmental influences and genetic variation. The set-specific polygenic scores used in this study thereby provide a way to aggregate weak individual genetic effects acting on specific pathways and at specific time points, which may enable us to capture the understudied role of genetic interactions in schizophrenia and how these contribute to neurodevelopment.

Overall, this study provides a proof of concept that information on the timing of gene expression aids the modeling of genetic effects on schizophrenia, albeit with several notable limitations. Given the complexity of schizophrenia and the continuous nature of the biological processes underlying it, the 2-hit hypothesis is less likely to adequately describe the etiology of schizophrenia than a multi-hit model or a continuum.[50] Nonetheless, we were able to capture this through our interaction analyses and replicate the finding in an independent cohort with a similar composition, enhancing confidence in our findings. It will be of interest to investigate potential clinical and demographic modifiers of this effect. Future studies could further look into the moderating effects of exposure to environmental factors during specific phases, investigation of specific symptom domains or other clinical characteristics as outcome measures, and brain regional specificity of the identified effects. We also note the limited number of donors of brain gene expression data, additional samples would enhance confidence in the findings. The approach used to map genetic variation to genes may further be developed; in the current context, gene-based tests offer advantages over locus mapping by aggregating more information. Information on eQTLs and chromatin interactions may further improve the identification of the

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genes involved, yet currently available annotations for common gene-based tests are specific to a developmental phase, which would bias our results.

To conclude, the modeling of genetic risk factors that moderate each other's effect, informed by the timing of their expression or occurrence, will aid in a better understanding of the development of schizophrenia.

Supplementary Material

Supplementary material is available at https://academic.oup.com/schizophreniabulletin/.

Collaborators

European Network of National Schizophrenia Networks Studying Gene-Environment Interactions Work Package 6 (EU-GEI WP6) investigators

Eduardo J. Aguilar[1,2], Berna Akdede[3], Köksal Alptekin[3,4], Vesile Altınyazar[5], Silvia Amoretti[1,6,7], Sanja Andric-Petrovic[8,9], Celso Arango[1,10] Manuel Arrojo[11], Cem Atbaşoğlu[12], Miguel Bernardo[1,6,7], Tolga Binbay[3], Julio Bobes[1,13–15], Eylem Şahin Cankurtaran[16], Angel Carracedo[17,18], Burçin Cihan[19], Philippe Delespaul[20,21], Maria Paz García-Portilla[1,13–15], Javier González-Peñas[10], Sinan Guloksuz[20,22], Güvem Gümüş-Akay[23–25], Estela Jiménez-López[1,26], Semra Ulusoy Kaymak[27], Gunter Kenis[20], Bochao D. Lin[20,28], Gonzalo López[1,10], Jurjen J. Luykx[28–30], Nadja P. Maric[9,31], Gisela Mezquida[6,7], Marina M. Mihaljevic[31,32], Tijana Mirjanic[32], Mara Parellada[1,10], Lotta-Katrin Pries[20], Olga Rivero[1,33,34], Bart P. F. Rutten[20], Pilar A. Saiz[1,13–15], Meram Can Saka[12], Julio Sanjuan[1,2], José Luis Santos[1,35], Haldun Soygür[36], Alp Üçok[37], Halis Ulaş[38], Jim van Os[20,29,39], Berna Yalınçetin[4]

[1]Biomedical Research Networking Centre in Mental Health (CIBERSAM), Spain
[2]Department of Psychiatry, Hospital Clínico Universitario de Valencia, School of Medicine, Universidad de Valencia, Valencia, Spain
[3]Department of Psychiatry, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey
[4]Department of Neuroscience, Graduate School of Health Sciences, Dokuz Eylul University, Izmir, Turkey
[5]Department of Psychiatry, Faculty of Medicine, Adnan Menderes University, Aydin, Turkey
[6]Barcelona Clinic Schizophrenia Unit, Neuroscience Institute, Hospital Clinic of Barcelona, University of Barcelona, Barcelona, Spain
[7]Institut d'Investigacions Biomèdiques August Pi I Sunyer, Barcelona, Spain
[8]University of Belgrade, Faculty of Medicine, Belgrade, Serbia
[9]Institute of Mental Health, Belgrade, Serbia
[10]Department of Child and Adolescent Psychiatry, Institute of Psychiatry and Mental Health, Hospital General Universitario Gregorio Marañón, IiSGM, School of Medicine, Universidad Complutense, Madrid, Spain
[11]Department of Psychiatry, Instituto de Investigación Sanitaria, Complejo Hospitalario Universitario de Santiago de Compostela, Santiago de Compostela, Spain
[12]Department of Psychiatry, School of Medicine, Ankara University, Ankara, Turkey
[13]Department of Psychiatry, School of Medicine, Universidad de Oviedo, Oviedo, Spain
[14]Instituto de Investigación Sanitaria del Principado de Asturias (ISPA), Oviedo, Spain
[15]Mental Health Services of Principado de Asturias (SESPA), Oviedo, Spain
[16]Güven Çayyolu Healthcare Campus, Ankara, Turkey
[17]Grupo de Medicina Genómica, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER) Universidad de Santiago de Compostela, Santiago de Compostela, Spain
[18]Fundación Pública Galega de Medicina Xenómica (SERGAS), IDIS, Santiago de Compostela
[19]Department of Psychology, Middle East Technical University, Ankara, Turkey
[20]Department of Psychiatry and Neuropsychology, School for Mental Health and Neuroscience, Maastricht University Medical Centre, Maastricht, The Netherlands
[21]Mondriaan Mental Health Centre, Maastricht/Heerlen, The Netherlands
[22]Department of Psychiatry, Yale School of Medicine, New Haven, CT, USA
[23]Department of Physiology, School of Medicine, Ankara University, Ankara, Turkey
[24]Brain Research Center, Ankara University, Ankara, Turkey
[25]Neuroscience and Neurotechnology Center of Excellence (NÖROM), Ankara, Turkey
[26]Health and Social Research Center, Universidad de Castilla-La Mancha, Cuenca, Spain
[27]Atatürk Research and Training Hospital Psychiatry Clinic, Ankara, Turkey
[28]Department of Translational Neuroscience, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[29]Department of Psychiatry, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[30]GGNet Mental Health, Apeldoorn, The Netherlands
[31]Faculty of Medicine, University of Belgrade, Belgrade, Serbia
[32]Special Hospital for Psychiatric Disorders Kovin, Kovin, Serbia
[33]INCLIVA Biomedical Research Institute, Fundación Investigación Hospital Clínico de Valencia, Valencia, Spain
[34]Department of Genetics, Universitat de València, Valencia, España
[35]Department of Psychiatry, Hospital Virgen de la Luz, Cuenca, Spain

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[36]Turkish Federation of Schizophrenia Associations, Ankara, Turkey
[37]Department of Psychiatry, Faculty of Medicine, Istanbul University, Istanbul, Turkey
[38]Department of Psychiatry, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey (Discharged by statutory decree No:701 at 8th July of 2018 because of signing "Peace Petition")
[39]Department of Psychosis Studies, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK

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Genetic Risk and Outcome of Psychosis (GROUP) Investigators
Behrooz Z. Alizadeh[1,2], Therese van Amelsvoort[3], Wiepke Cahn[4,5], Lieuwe de Haan[6,7], Frederike Schirmbeck[6,7], Jim van Os[8,9], Wim Veling[1]
[1]Department of Psychiatry, Rijksuniversiteit Groningen, University Medical Center Groningen, Groningen, The Netherlands
[2]University Medical Center Groningen, Department of Epidemiology, Groningen, The Netherlands
[3]Maastricht University Medical Center, Department of Psychiatry and Neuropsychology, School for Mental Health and Neuroscience, Maastricht, The Netherlands
[4]University Medical Center Utrecht, Department of Psychiatry, Brain Centre Rudolf Magnus, Utrecht University, Utrecht, The Netherlands
[5]Altrecht, General Mental Health Care, Utrecht, The Netherlands
[6]Amsterdam UMC, University of Amsterdam, Department of Psychiatry, Amsterdam, The Netherlands
[7]Arkin, Institute for Mental Health, Amsterdam, The Netherlands
[8]University Medical Center Utrecht, Department of Translational Neuroscience, Brain Center Rudolf Magnus, Utrecht, The Netherlands

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[9]King's College London, King's Health Partners, Department of Psychosis Studies, Institute of Psychiatry, London, UK

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Author contributions

D.v.d.M. and T.K. conceived the study; D.v.d.M., J.R., L-K.P., B.L., and T.K. preprocessed the data. D.v.d.M. performed all analyses, with conceptual input from T.K.; All authors contributed to the interpretation of results; D.v.d.M. drafted the manuscript and all authors contributed to and approved the final manuscript.

Data availability

The data incorporated in this work were gathered from public resources. The code is available via https://github.com/norment/open-science [published upon acceptance]. Correspondence and requests for materials should be addressed to d.v.d.meer@medisin.uio.no.

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